The Journal of Microbiology, September 2004, p.243-247 Vol. 42, No. 3

NOTE

Green Fluorescent Protein as a Marker for Monitoring a Pentachlorophenol Degrader *Sphingomonas chlorophenolica* ATCC39723

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(Received May 18, 2004 / Accepted June 17, 2004)

***Sphingomonas chlorophenolica* ATCC39723 was successfully labeled with the *gfp* (green fluorescent protein) gene inserted into the *pcpB* gene by homologous recombination. As the *gfp* recombinant was easily distinguished from other indigenous organisms, the population of *gfp* recombinant was monitored after being released into the soil microcosms. Their population density dropped from 10^8 to 10^6 (cfu/ml) in the non-sterilized soil microcosms during the first 6 days. Moreover, the *gfp* recombinant was not detected even at lower dilution rates after a certain time period. The recombinant, however, survived for at least 28 days in the sterilized soil microcosms. Although the *gfp* recombinant did not degrade pentachlorophenol (PCP), this experiment showed the possibility of using *gfp* as a monitoring reporter system for *S. chlorophenolica* ATCC39723 and potentially other species of *Sphingomonas*.**

Key words: *gfp*, microcosm, monitoring, pentachlorophenol (PCP), *Sphingomonas chlorophenolica*

Pentachlorophenol (PCP) is a polychlorinated aromatic chemical that is mainly used as a wood preservative in lumber industries. Other uses of PCP include the following: a herbicide, an insecticide, and a fungicide (McAllister, *et al.*, 1996). Cleanup of PCP is necessary in contaminated soils and other environments because of its high toxicity; furthermore, bioremediation is a feasible alternative to conventional physical and chemical technologies of soil reclamation (Blackburn and Hafker, 1993; McAllister, *et al.*, 1996).

Sphingomonas chlorophenolica ATCC39723, a Gram-negative bacterium, can mineralize pentachlorophenol (PCP) and *p*-nitrophenol (PNP) at the high concentrations of 100 to 200 ppm (Saber and Crawford, 1985; Leung *et al.*, 1999). It has been known that 4-monooxygenase encoded by the *pcpB* gene in this bacterium, leads to the primary hydroxylation of PCP (Cai and Xun, 2002).

Because removal of PCP from the environment by physical and chemical technologies is less cost-effective, many researchers have studied bioremediation as an alternative technology that employs PCP degraders such as *Arthrobacter* (Edgehill and Finn, 1982) and *Sphingomonas* (Lange *et al.*, 1996) as well as degraders of fungal species (Mileski *et al.*, 1988).

The development of an efficient monitoring system for organisms that can degrade toxic chemicals, including PCP, will be necessary to track their behaviors in the bioremediation of these chemicals in the environment. The *gfp* gene, which originated from *Aequarea victoria,* is used as a marker gene because it is stably and successfully expressed in prokaryotic and eukaryotic cells and it is independent of their metabolism. In addition, it is easily detected by the use of standard microscopes equipped with commonly available fluorescent filter sets (Cubitt *et al.*, 1995; Bloemberg *et al.*, 1997). Another advantage of using the *gfp* gene as a reporter gene is that it does not require the fixing and staining processes that are necessary when using a light and electron microscope (Bloemberg *et al.*, 1997) and the colonies of the host cells labeled with the *gfp* gene are easily distinguished by a hand UV system. We have orchestrated research investigating the bioremediation of hazardous synthetic chemicals such as polychlorinated biphenyls (PCBs) using the PCBs-degraders *Ralstonia, Pseudomonas*, and *Rhodococcus* sp., as well as in the development of an *in situ* monitoring sys-

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tem using the *gfp* gene as a reporter (Jung *et al.*, 2001; Kim *et al.*, 2003; Oh *et al.*, 2003). In the current study, we attempted to label a known PCP degrader *S. chlorophenolica* ATCC39723 with the *gfp* gene and to monitor *in situ* the organism in the soil. The *gfp* labeled *S. chlrophenolica* ATCC39723 was successfully monitored in terms of population dynamics in soil microcosms.

S. chlorophenolica ATCC39723 was maintained in tryptic soy broth (TSB) and mineral salt medium (MS) containing pentachlorophenol (PCP; 50 μg/ml) to determine the degradation activity of PCP at 28°C (Chanama and Crawford, 1997). *Esherichia coli* DH5α, a host for plasmids, and *E. coli* JB122 harboring pJBA29 were cultured in Luria Bertani (LB) medium at 37°C (Andersen *et al.*, 1998; Sambrook *et al.*, 2001). *S. chlorophenolica* ATCC39723 and *E. coli* electrocompetent cells were prepared and transformed as previously described (Bloemberg *et al.*, 1997; Sambrook *et al.*, 2001). A polymerase chain reaction (PCR) was used to confirm the *gfp* recombinant of *S. chlorophenolica* ATCC39723. Nucleotide positions were based on the sequences of the *gfp* gene (forward, 5'-AAG GAA AAA AGC GGC CGC ATG AGT AAA GGA GAA GA-3'; reverse, 5'-AAG GAA AAA ACG CCG GCG GCT ATT TGT ATA GTT CA-3') (Chalfie *et al.*, 1994) and the *pcpB* gene (forward, 5'-GAG AGA TTG TTA TTA TGT CGA CCT A-3'; reverse, 5'-TTT GTC ATC GCA CGG GTC TCC TCA G-3') (Errampalli *et al.*, 1998; Leung *et al.*, 1999). The PCR was performed in a thermal cycler (Techne, U.S.A.) that was programmed for a denaturation step at 94°C for 5 min, 30 cycles of 94°C for 1 min, 55°C for 1 min (*gfp* gene) or 4 min (*pcpB* gene), and 72°C for 1 min, and finally 72°C for 10 min. Initially, electrotransformation was attempted with the use of a mini-transposon of pJBA29 for *gfp* gene labeling in *S. chlorophenolica* ATCC39723. However, we could not obtain *gfp* labeled cells. Hence, *S. chlorophenolica* ATCC 39723 was labeled with *gfp* by homologous recombination, which specifically induced single crossover recombination in the cell. The plasmid pCO222, which contains the intact *pcpB* gene on a 2.3 kb *Bam*HI to *Eco*RI fragment in pBluescript, was cut with *Sph*I restriction endonuclease to interrupt the open reading frame of *pcpB*. It was then treated with a Klenow fragment to make a blunt end of the DNA fragment (Lange *et al.*, 1996). This fragment was then ligated into the Klenow filled-in *gfp* gene cut from pJBA29 which was harboring the mini-Tn*5* transposon (Fig. 1). This plasmid was used for homologous recombination by electrotransformation as described by Oh *et al.* (2003). Approximately 0.5 μg of plasmid DNA

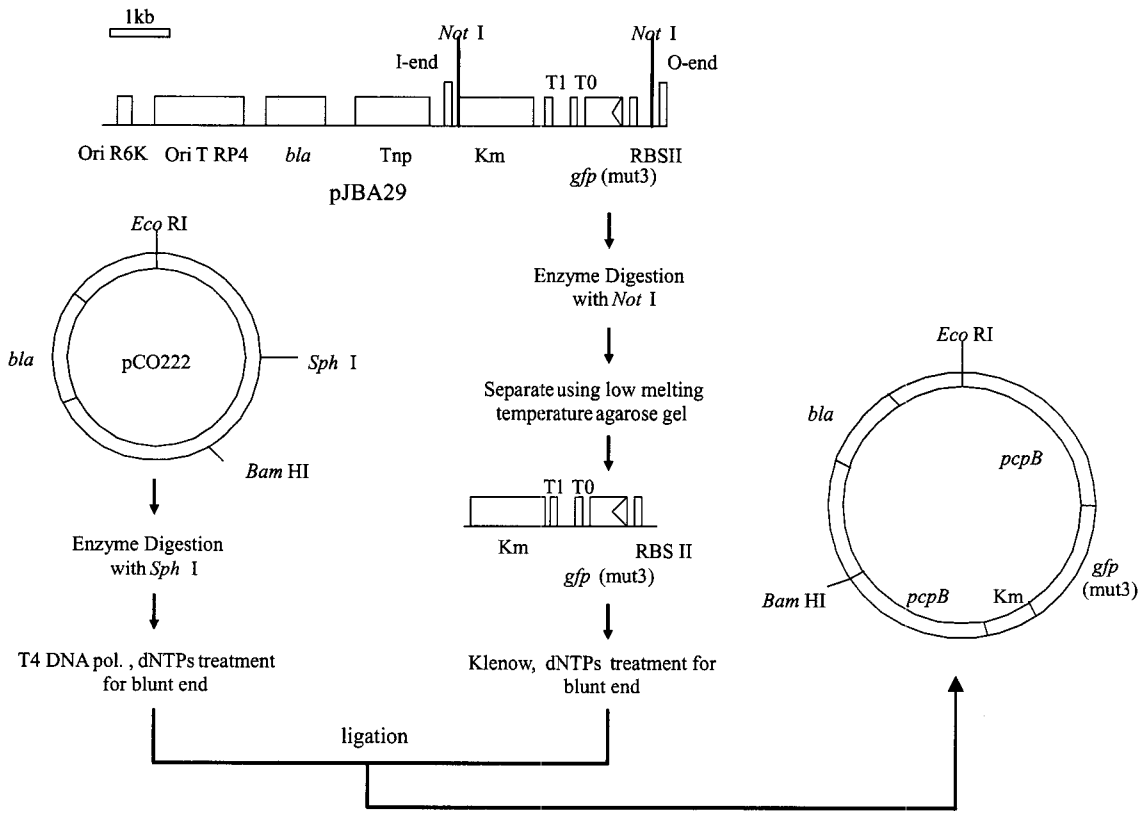

Fig. 1. Construction of *pcpB*::*gfp* plasmid *bla*, a gene coding β-lactamase (ampicillin resistance gene); Km, kanamycin resistance gene; Tnp, transposase; *gfp* (mut3), mutated *gfp* gene; RBSII, ribosome binding site of phage T5; T0, terminator from phage lambda; T1, terminator from the *rrnB* operon of *E. coli*; *pcpB*, a 4-monooxygenase gene.

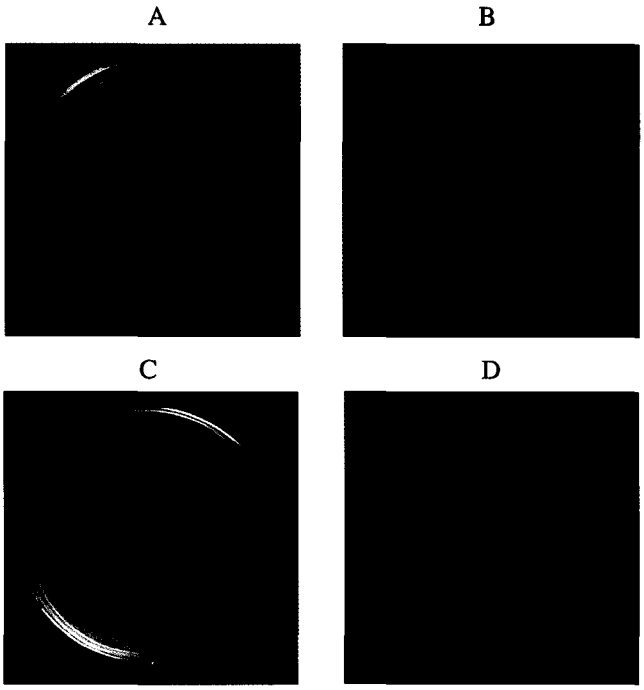

Fig. 2. Selection of the *S. chlorophenolica* ATCC39723 *gfp* recombinant. A, M9 agar plate under visible light: B, M9 agar plate under UV light: C, M9 agar plate supplemented with ampicillin (100 μg/ml) and kanamycin (50 μg/ml) under visible light: D, M9 agar plate supplemented with ampicillin (100 μg/ml) and kanamycin (50 μg/ml) under UV light. Left and right streaks of the plates indicate colonies of the wild type and the *gfp* recombinant, respectively.

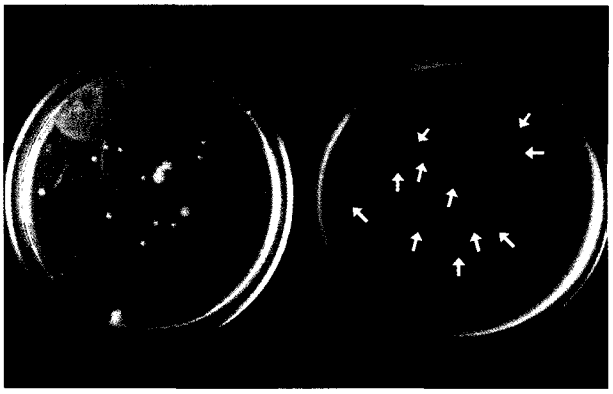

Fig. 3. Detection of the *gfp* recombinant of *S. chlorophenolica* ATCC39723 with a background of the indigenous microorganisms. Left, under visible light; right, under U.V. light; arrows indicate the *gfp* recombinant.

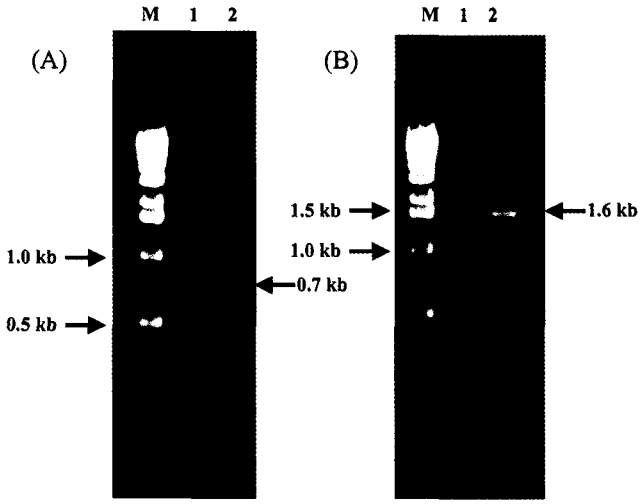

Fig. 4. PCR amplification of total genomic DNA as a template prepared from the wild type (lane 1) and the *gfp* recombinant (lane 2) of *S. chlorophenolica* ATCC39723 using the *gfp* primer set (panel A) and *pcpB* gene primer set (panel B). M, 1 kb DNA ladder.

was mixed with 60 μl of electrocompetent cells, added to a 2 mm gap electroporation cuvette, and electroporated at 1.8 kV, 25 mA, and 4.5 ms with the Gene Pulser II apparatus (Invitrogen, USA). The electroporated cells were diluted into 500 μl of TSB medium and grown for 2 h before being plated on TSA supplemented with kanamycin (final concentration: 50 μg/ml). An epifluorescent microscope (Leica, Germany) equipped with a digital camera (Nikon, U.S.A.) was used to visualize and select the fluorescent bacterial colonies on tryptic soy agar (TSA).

As shown in Fig. 2, the *gfp* labeled *S. chlorophenolica* ATCC39723 was easily detected on TSA and MS agar (M9 agar) plates with appropriate antibiotics (ampicillin, 100 μg/ml; kanamycin, 50 μg/ml). Positive clones containing the *pcpB*::*gfp* gene were identified by kanamycin resistance and green fluorescence. When mixed with other indigenous organisms, labeled cells were easily distinguished and consistently showed the *gfp* phenotype (Fig. 3). To analyze the *gfp* recombinant, total genomic DNA of both the wild type and *gfp* recombinant were prepared as described by Sambrook *et al.* (2001). In the case of *gfp* gene amplification by PCR, there was no amplified product in the DNA of wild type, while the DNA of the recombinant allowed an amplified fragment. The size of the amplified product was about 0.7 kb which was the same as that of *gfp* (Fig. 4A). For the *pcpB* gene amplification using a *pcpB* primer set, the amplified products from the total genomic DNA were analyzed by gel electrophoresis. The product size was about 1.6 kb in both the wild type and the recombinant (Fig. 4B). This indicates that the homologous recombination did not occur through a double crossover homologous recombination. With regards to the double crossover homologous recombination, there should be two PCR product sizes of 1.6 kb and 3.6 kb. However, we were only able to detect a 1.6 kb fragment (Fig. 4B). This could be explained by the fact that the PCR condition that we employed in this study did not allow the amplification of the 3.6 kb larger fragment. Concurrently, the *gfp* recombinant was not able to degrade PCP (data not shown). From these results, it was

assumed that the *pcpB* gene was somewhat deleted during a single crossover event and that the *pcpB* gene which was interrupted by the *gfp* gene (*km*^{r} gene) was not sufficiently amplified by PCR. Although further study is needed to clarify this result, it was demonstrated that *S. chlorophenolica* ATCC39723 was successfully tagged with the Gfp protein.

For the monitoring study, *gfp* recombinants of *S. chlorophenolica* ATCC39723 were inoculated into a test tube containing 5 g of soil from a lawn ground at Inha University (Incheon, Korea). Sampling was performed in triplicate. To sterilize the soil, half of the soil was autoclaved three times at 121°C for 30 min with an interval of 1 day between the autoclaving cycles, and the other half was stored at room temperature (no longer than 5 days) and used without autoclaving. Five grams of the autoclaved and non-autoclaved soils were poured into test tubes. Two milliliters of the *gfp* recombinants of *S. chlorophenolica* ATCC39723 (5.1×10^8 cfu/ml) were inoculated into the test tubes and incubated at 28°C for 1 month. To monitor the population dynamics of the organisms in the microcosms, five milliliters of sterilized water were added to each test tube and then vortexed vigorously for 1 min. Then the mixtures were allowed to stand for 2 min before being vortexed again for 1 min. One milliliter of the soil supernatant was then carefully taken, serially diluted and spread on M9 and TSA plates. The inoculated plates were then incubated at 28°C for 3 days and the target colonies were visualized under UV light and counted. As shown in Fig. 5, the population dynamics of the labeled cells in the microcosms were measured for one month. The labeled target strains were easily distinguished from others, thus indicating *gfp* can be used as a reporter gene in a monitoring study. There was no significant difference in density between the two media used for the plate counting. When some microbes are released into the environment, they may be affected by indigenous microbes that compete for the available nutrients, toxic chemicals, and predators such as bacteriophages and protozoa (Galli *et al.*, 1996). Hence, protozoan and indigenous microbial populations may have suppressed the competition ability of the *gfp* recombinant, and this may have caused the rapid decrease of the released recombinant population for the first 6 days (Fig. 5A) in the non-sterilized soil microcosms. However, the recombinant was not detected after 6 days at lower dilution rates. In the autoclaved soil, the population dynamics data showed that the *gfp* recombinant could be detected up to 28 days when observed by counting on TSA and MS plates (Fig. 5B). The decreasing pattern of the released *gfp* recombinant population was similar to that in a monitoring study of *gfp* labeled *E. coli* in an aquatic system (Leff and Leff, 1996). This study clearly demonstrates the possibility of using *gfp* as a monitoring reporter system in *S. chlorophenolica* ATCC39723 which can biodegrade PCP in a soil system and other

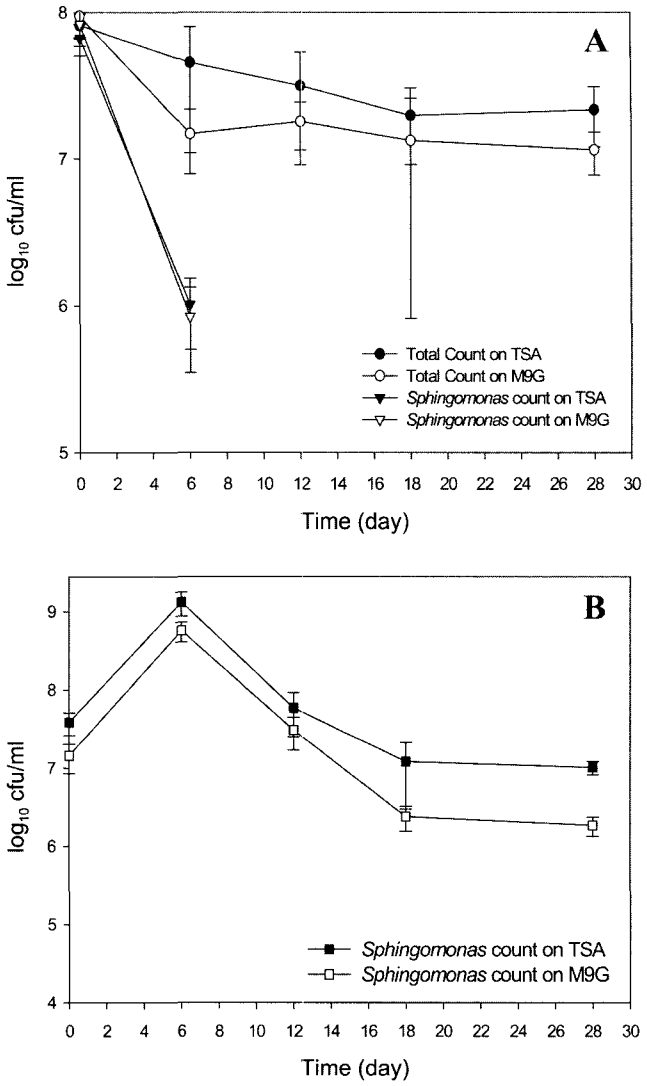

Fig. 5. Population dynamics of the *gfp* recombinant of *S. chlorophenolica* ATCC39723 with indigenous microorganisms in non-autoclaved soil microcosms (panel A) and in autoclaved soil microcosms (panel B) as observed by different counting media.

environmental conditions despite the lack of a PCP degradation capability in the *gfp* recombinant. To the best of our knowledge, this gene tagging system is the first trial for the *Sphingomonas* species and will be highly helpful in developing a similar monitoring system for other species of *Sphingomonas* that may be involved in the degradation of other xenobiotic compounds. However, a construction of a reporter using transcriptional fusion (*pcp* gene::*gfp*) will also be useful to specifically monitor the population of *Sphingomonas* species which is able to degrade PCP in the environment.

Acknowledgment

This work was supported by a grant (KRF 2001-015-

DP0443) from the Korea Research Foundation.

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