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Development of a Monitoring Vector for *Leuconostoc mesenteroides* Using the Green Fluorescent Protein Gene

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Abstract The vector pCW5 with plasmid pC7, originally isolated in *Lactobacillus paraplantarum* C7 derived from *kimchi*, was constructed using a p32 strong promoter, the pC7 replicon, and green fluorescent protein (GFP) as the reporter. The constructed vector was transformed into *E. coli* and *Leuconostoc mesenteroides*, and GFP expression detected using a Western blot analysis. GFP fluorescence was recognized in *E. coli* and *Leuconostoc mesenteroides* using a confocal microscope. In addition, GFP fluorescence was also clearly detected in several industrially important lactic acid bacteria (LAB), including *Lactobacillus bulgaricus*, *Lactobacillus paraplantarum*, and *Lactobacillus plantarum*. Thus, pCW5 was shown to be effective for *Leuconostoc mesenteroides* when using GFP as the reporter, and it can also be used as a broad-host-range vector for other lactic acid bacteria.

Keywords: GFP, monitoring system, *Leuconostoc mesenteroides*, plasmid pC7

Among traditional Korean fermented foods, *kimchi* is the most well-known and spontaneously fermented by lactic acid bacteria (LAB), which are recognized for their health-promoting effects, such as the nonspecific enhancement of the immune system, protection against intestinal infection, lowering of cholesterol levels, and therapeutic effects for allergic diseases [7, 16, 23]. The regulation and control of LAB for *kimchi* fermentation has already been noted [8, 11, 12], and among this group of LAB, the genera *Leuconostoc* and *Lactobacillus* are believed to be co-determinants or promoters of taste, overripening, and acidification [14, 18]. In particular, *Leu. mesenteroides* would seem to play an important role in commencing *kimchi* fermentation [11]. Moreover, the genus *Leuconostoc* is utilized in starter cultures and for flavor development and food preservation worldwide, plus its importance in lactic fermentation is considered to be due to its manufacture of CO_2 and C4 aroma compounds through lactose heterofermentation and citrate utilization [2].

As *kimchi* contains numerous microorganisms and diverse bacteria-fermented products [4, 10], it is important to monitor certain key LAB, such as *Leu. mesenteroides* and *L. plantarum*, which are abundant in *kimchi*. Various molecular methods, including a polymerase chain reaction (PCR), have already been developed to classify microorganisms from diverse species. Specific primers, however, are required to detect individual bacteria with PCR methods [1, 15, 20]. Although several reporter genes, such as the chloramphenicol acetyl transferase gene, *Escherichia coli* β-glucoronidase gene, and β-galactosidase gene, have been used to monitor microorganisms, the use of these genes has limitations, as they all require an additional substrate. Thus, the green fluorescent protein (GFP) gene, which does not require any additional substrate, was used to detect LAB, such as *Lactococcus lactis* [21]. Gory *et al.* [5] also used GFP to monitor *Lactobacillus sakei* in fermented products. The expression of GFP does not alter bacterial growth and is

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detectable using epifluorescence microscopy, allowing the detection and monitoring of the development of GFP+ specific bacterial strains under laboratory growth conditions. However, it would seem that no system using this gene has yet been developed to detect *Leu. mesenteroides*.

Recently, we have reported on a bacteriocin-producing *L. paraplantarum* isolated from kimchi, and then characterized a cryptic plasmid pC7 from the strain [17]. When constructing a cloning vector for lactic acid bacteria on the basis of the pC7 replicon, it was found that several species of *Lactobacillus* and *Leuconostoc* could be transformed using the resultant vector [19].

Accordingly, this study developed a monitoring vector using the pC7 replicon and GFP as the reporter for *Leu. mesenteroides*. In addition, the broad-host-range of the constructed vector was tested for several LAB, such as *L. bulgaricus*, *L. paraplantarum*, and *L. plantarum*. Under the control of the constitutive p32 promoter, GFP expression was recognized using a confocal microscope.

The strains used in this study were *E. coli* XL-1 Blue, *Leu. mesenteroides* KFRI 218, *L. paraplantarum* R5 (cured *L. paraplantarum* C7), *L. bulgaricus* KCTC1121,

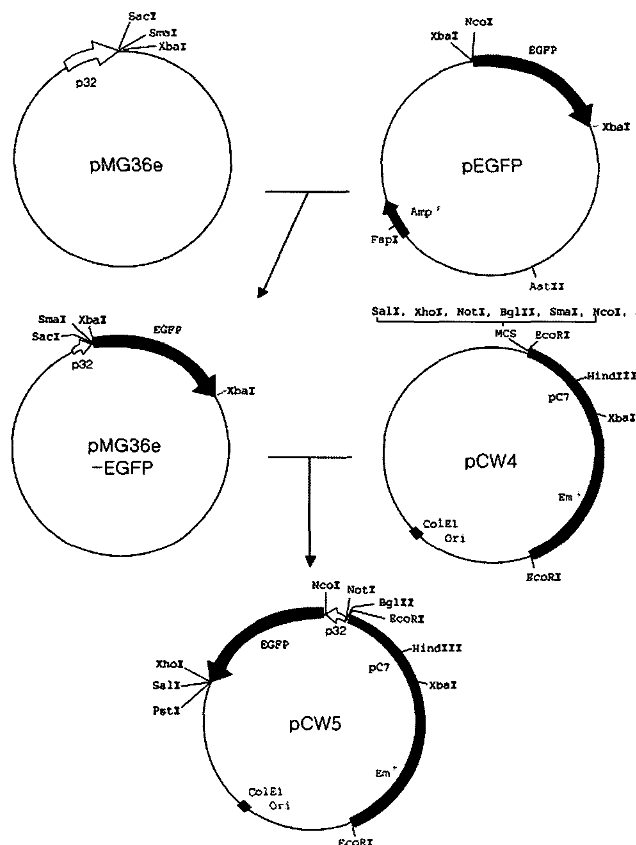

Fig. 1. Construction of pCW5, including EGFP.
EGFP, enhanced green fluorescent protein from pEGFP; p32, promoter32 from pMG36e; Em^R, erythromycin-resistant gene from pCW4; and ColE1 ori, origin of Gram-negative bacteria from pCW4.

and *L. plantarum* KCTC1048. Competent *E. coli* and LAB cells were prepared and transformed through electrophoration using a Gene Pulser apparatus (Bio-Rad), as previously described by Holo and Nes [9]. Plasmid pCW5 was constructed for the purpose of GFP expression, whereas plasmid pCW4, which consisted of the erythromycin (Em) resistance gene, ColE1 gene for replication in *E. coli*, and repA gene for replication in several LAB, was based on the construction of pCW5. The EGFP gene was isolated from plasmid pEGFP (Clontech) and cloned into the XbaI restriction enzyme site of pMG36e [6], resulting in pMG36e-EGFP. The promoter p32-EGFP gene fragment was digested with EcoRI and NotI, and then inserted into the NotI restriction enzyme site of pCW4 [19] through blunt-end ligation to construct plasmid pCW5 (Fig. 1). The orientation of the fragment was confirmed through sequence analysis.

Plasmid pCW5 with the EGFP (Enhanced Green Fluorescent Protein) gene was transformed into *E. coli*. After comparing the GFP expression of pEGFP and pCW5 on plates, it was found that the GFP production by pCW5 was higher than that by pEGFP. Even though the p32 promoter is a LAB promoter, it seemed to accelerate the GFP expression in *E. coli*. The strong expression of GFP was visible to the naked eye during the amplification of pCW5 in *E. coli* (data not shown). Like the p32 promoter, certain LAB promoters are used in *E. coli* to express carried proteins. Chen and Steele [3] previously reported that the *L. helveticus* promoter works in *E. coli* as well as other LAB. However, no colonies of *Leu. mesenteroides* were observed to display fluorescence.

To determine whether the EGFP gene had been expressed in *Leu. mesenteroides*, a Western blot analysis was conducted according to the method described by Laemmli [13] with minor modifications. The bacterial cells containing EGFP were disrupted with glass beads, and then the LAB protein concentrations were determined using a protein assay kit (Bio-Rad). After transferring the proteins to a nitrocellulose membrane (Amersham Pharmacia Biotech), the membrane was allowed to react with a primary antibody (anti-GFP from rabbit, Clontech), followed by a 2nd antibody (anti-rabbit IgG, Amersham Pharmacia Biotech), and then exposed using an ECL kit (Amersham Pharmacia Biotech) according to the supplier's instructions. The EGFP gene was found to express a 27 kDa green fluorescent protein. As shown in Fig. 2, GFP was detected in the *Leu. mesenteroides* extracts harboring pCW5, yet the amount of GFP in the *E. coli* seemed to be several hundred times that in the LAB, which would explain why only fluorescent *E. coli* was visible to the naked eye. Moreover, GFP was also recognized in the *L. paraplantarum* extracts, *L. bulgaricus* extracts, and *L. plantarum* extracts harboring pCW5. Although the amount of expressed GFP differed slightly according to the kind of LAB, the differences were negligible relative to that between the *E. coli* and the LAB.

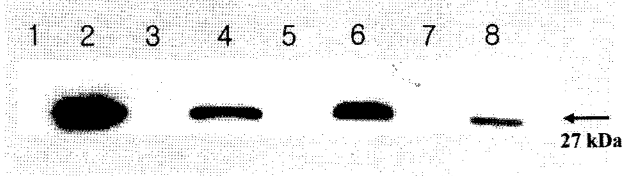

Fig. 2. Western blot analysis of GFP in *Leu. mesenteroides* and other LAB.

Lane 1, *L. paraplantarum* C7; lane 2, *L. paraplantarum* C7, harboring pCW5; lane 3, *Leu. mesenteroides*; lane 4, *Leu. mesenteroides*, harboring pCW5; lane 5, *L. bulgaricus*; lane 6, *L. bulgaricus*, harboring pCW5; lane 7, *L. plantarum*; lane 8, *L. plantarum*, harboring pCW5. GFP is indicated by an arrow.

The *E. coli* and *Leu. mesenteroides* with pCW5, including the EGFP gene, were recognized using a confocal microscope (Zeiss LSM at 488 nm). Briefly, the bacterial cells were attached to cover slips by centrifugation and fixed with 4% paraformaldehyde. The cells were then washed with PBS, mounted, and examined using a confocal microscope. In addition, *L. paraplantarum*, *L. bulgaricus*, and *L. plantarum* with pCW5 were also easily detected (Fig. 3). Moreover, the growth phase of the strains was found to affect the degree of fluorescence, where the closer the growth phase of the bacteria to the stationary phase, the easier it was to recognize. This may have been due to the slow photobleaching of the GFP and total number of cells related to the intensity of the fluorescence [22]. Furthermore, the samples washed 3 times with PBS were compared with unwashed samples to determine whether washing with PBS (phosphate-buffered saline) affected the degree of fluorescence. The samples washed with PBS showed more fluorescence.

In summary, pCW5 was constructed using a p32 strong promoter, the pC7 replicon, and GFP as the reporter. As a result, pCW5 is seemingly the first monitoring vector for *Leu. mesenteroides* using GFP. The monitoring system constructed in this study can also be used to detect several other LAB, such as *L. paraplantarum*, *L. bulgaricus*, and *L. plantarum*, in diverse *in-vitro* and *in-vivo* experiments. Because of the broad-host-range of pC7, plasmid pCW5 can be used as a broad-host-range monitoring system for several LAB.

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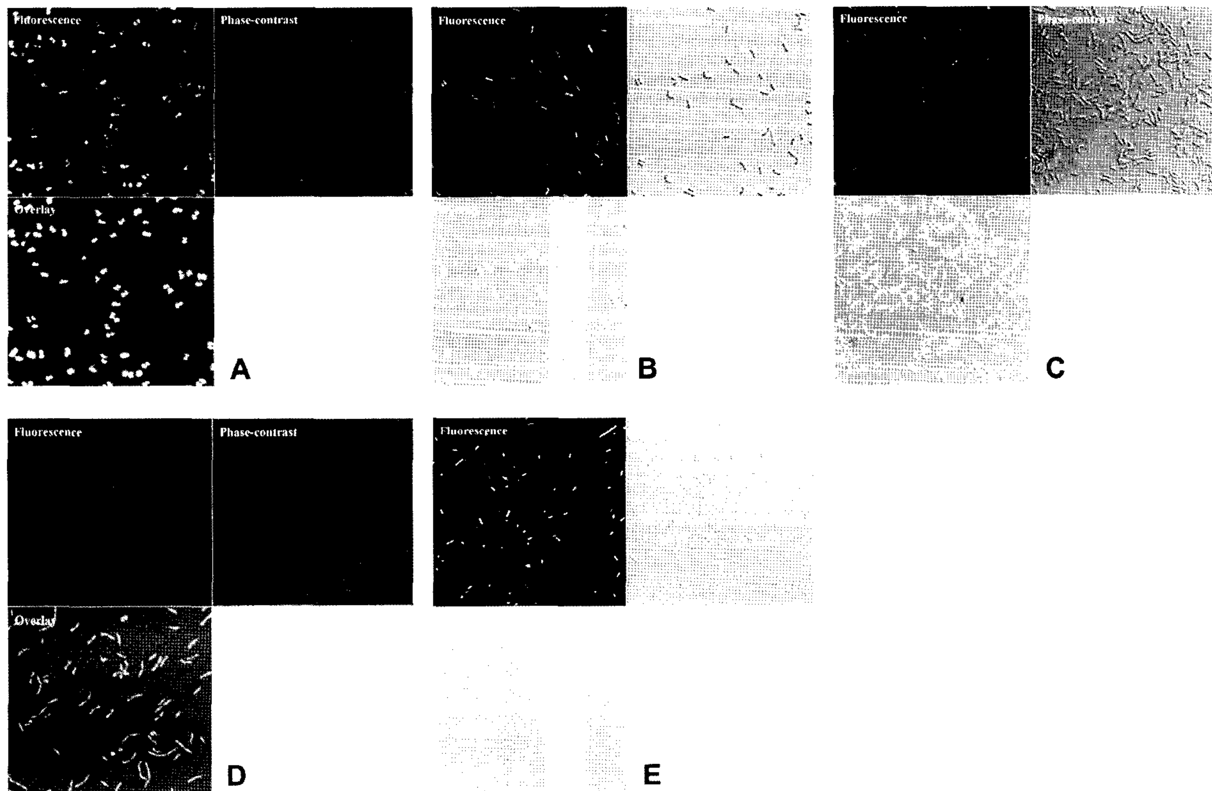

Fig. 3. Detection of GFP expression using confocal microscopy.

A. *Leu. mesenteroides*, harboring pCW5; **B.** *L. paraplantarum* R5, harboring pCW5; **C.** *L. bulgaricus*, harboring pCW5; **D.** *L. plantarum*, harboring pCW5; and **E.** *E. coli*, harboring pCW5. Each picture consists of three images: Phase-contrast, Fluorescence, and Overlay.

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