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Generation of a Constitutive Green Fluorescent Protein Expression Construct to Mark Biocontrol Bacteria Using P43 Promoter from *Bacillus subtilis*

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Marking biocontrol bacteria is an essential step to monitor bacterial behavior in natural environments before application in agricultural ecosystem. In this study, we presented the simple green fluorescent protein (GFP) reporter system driven by the promoter active in *Bacillus* species for tagging of the biocontrol bacteria. A constitutive promoter P43 from *Bacillus subtilis* was fused to an enhanced promoterless *gfp* gene by overlap extension PCR. The GFP expression was demonstrated by the high fluorescence intensity detected in *B. subtilis* and *Escherichia coli* transformed with the P43-*gfp* fusion construct, respectively. The GFP reporter system was further investigated in two bacterial biocontrol strains *B. licheniformis* and *Pseudomonas fluorescens*. When the reconstructed plasmid pWH34G was introduced into *B. licheniformis*, GFP level measured with the fluorescence intensity in *B. licheniformis* was almost equivalent to that in *B. subtilis*. However, GFP expression level was extremely low in other biocontrol bacteria *P. fluorescens* by transposon based stable insertion of the P43-*gfp* construct into the bacterial chromosome. This study provides information regarding to the efficient biomarker P43-*gfp* fusion construct for biocontrol *Bacillus* species.

Keywords : *Bacillus*, biocontrol, GFP, P43

Biological control of plant disease using soil bacteria has received much attention as an alternative to chemical control of plant disease (Cook, 1993). Development of biopesticides using the soil bacteria allowed practical application of a large number of single species of bacterium in agricultural ecosystem (Fravel et al., 1998). However, bacterial behavior needs to be monitored in natural environment before commercialization to environment friendly and effective plant disease management. Several biomarkers are available to monitor bacterial ecology in native ecosystem. Those biomarker genes include *lacZ* (Mo and Gross, 1991), *lux* (O'Kane et al., 1988), *inaZ* (Loper and Lindow, 1994), *xylE* (Buell and Anderson, 1993), and *gus* (Jefferson, 1989).

The green fluorescent protein (GFP) and the corresponding gene from the bioluminescent jellyfish could be used as a reporter for various organisms (Chalfie et al., 1994; Prasher et al., 1992; Prendergast and Mann, 1978). Although the gene was isolated from eukaryotic organism, its utility as a reporter gene was not limited to eukaryotic system. Its biotechnological utility as a biomarker and biosensor was extensively demonstrated in various expression systems not only with original GFP but also with various mutant GFPs (March et al., 2003; Stepanenko et al., 2008). One of the mutated GFPs has higher protein solubility and red-shifted excitation wavelength, which exhibited improved fluorescence (Cormack et al., 1996; Heim et al., 1995). Furthermore, the improved promoterless *gfp* gene with a translational enhancer is available in pGreenTIR (Miller and Lindow, 1997). Therefore, the *gfp* gene could be used for various purposes, when it is expressed by different promoter.

Overlap extension PCR (OE-PCR) was originally developed to generate various mutations on target gene by employing three-step PCR (Higuichi et al., 1988) and it was further utilized to create chimeric genes (Horton et al., 1989). Therefore, by adopting OE-PCR, it is possible to make a fusion between a promoter element and a promoterless *gfp*. A constitutive promoter P43 was initially identified from *Bacillus subtilis* (Wang and Doi, 1984) and its utility as a strong expression promoter has been demonstrated in *B. subtilis* (Zhang et al., 2005). The promoter contained two overlapping promoter elements and may be recognized by other bacterial sigma factors. Therefore, it is plausible to examine if this promoter could be functional in other bacterial system.

Here, we used the improved *gfp* gene from pGreenTIR to express the GFP using P43 promoter from *B. subtilis* in other bacterial systems such as soilborne biocontrol strains including *B. licheniformis* (Lee et al., 2006) and *Pseudomonas fluorescens* (Choi et al., 2006). Through this study, we developed a constitutive GFP expression system that could be used to mark soilborne biocontrol bacteria.

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Materials and Methods

Bacterial strains and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown at 37°C on Luria-Bertani (LB) agar or in LB broth supplemented with the appropriate antibiotics. *B. subtilis* strain 168 and *B. licheniformis* N1 (Lee et al., 2006) were routinely grown at 30°C on nutrient agar (NA) or in nutrient broth (NB) containing appropriate antibiotics. *P. fluorescens* strain pc78 (Choi et al., 2006) cultures were routinely grown at 28°C on mannitol-glutamate medium (MG) (Keane et al., 1970) supplemented with yeast extract (0.25 g/liter) (MGY) or in MGY broth. The following antibiotic concentrations were used for *E. coli* strains, *Bacillus* strains and *P. fluorescens* strains: tetracycline, 15 μg/ml; ampicillin, 100 μg/ml; and kanamycin, 25 μg/ml.

Recombinant DNA technology, overlap extension PCR and plasmid construction. Plasmid preparation, restriction endonuclease digestion, DNA ligation, plasmid DNA transformation, agarose gel electrophoresis, and other standard recombinant DNA techniques were carried out following standard methods (Sambrook et al., 1989). DNA sequencing and primer synthesis were performed commercially at the DNA sequencing facility of GenoTech Corp. (Daejeon, Korea).

To construct a fusion between P43 promoter of *B. subtilis* 168 and a promoterless *gfp* gene, we performed an OE-PCR (Higuchi et al., 1988) by the following procedure. DNA amplification by PCR reactions in this study was carried out in a 50 μl (total volume) reaction mixture which contained *Taq* DNA polymerase buffer (Promega Corp, USA), each deoxynucleotide triphosphate at a concentration of 200 μM, 1.5 mM $MgCl_2$, each primer at a concentration of 1 μM and 2.5 Unit of *Taq* DNA polymerase. The template DNA added was 200 ng for bacterial genomic DNA or 10-100 pg for plasmid DNA.

P43 promoter was first amplified from *B. subtilis* 168 by PCR using two primers, P43-1 (5'-TATACTAGTTGATAGGTGGTATGTTTTCGC-3') and P43GFP-1 (5'-CCTCCTTATAAAGTTAACTATAATGGTACCGCTAT-3'). The PCR was conducted by using the following program: an initial DNA template denaturation step at 95°C for 3 min; 30 cycles consisting of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s; and a final extension step at 72°C for 5 min. The amplified 285 bp fragment was cloned into pGEM-Teasy to produce pEZ43 and its identity was confirmed by DNA sequence analysis. The second PCR to amplify *gfp* gene from pGreenTIR was performed by using two primers, P43GFP-2 (5'-ATAGCGGTACCATTATAGTTAACTTTATAAGGAGG-3') and Gfp-3 (5'-GGTGCATGCCTCGAATTCCTATTTGTATAG-3'). The underlined sequences of P43GFP-1 and P43GFP-2 are complementary each other to anneal for overlap extension. PCR program to amplify GFP gene was identical to the previous one except extension time for 1 min. Both amplified PCR product carrying P43 promoter and *gfp* gene was purified and mixed at the equivalent molar ratio to perform third PCR for fusion reaction without any primer. This fusion reaction contained all PCR components except primer. The third fusion PCR was conducted by using the following program: an initial DNA template denaturation step at 95°C for 3 min; 10 cycles consisting of denaturation at 95°C for 1 min, annealing at 50°C for 30 s, and extension at 72°C for 1 min; and a final extension step at 72°C for 5 min. The fusion product was subsequently amplified by adding primers P43-1 and Gfp-3 and proceeding for PCR at the following condition: an initial DNA template denaturation step at 95°C for 3 min; 20 cycles consisting of denaturation at 95°C for 1 min, annealing at 50°C for 30 s, and extension at 72°C for 1 min; and a final extension step at 72°C for 5 min. The PCR products were separated and cloned into pGEM-Teasy to generate pEZ43G.

Transformation of bacterial strains. To introduce pWH1520 and pWH43G (Table 1) into *B. subtilis* 168 and *B. licheniformis* N1, electroporation was performed as previously described (Xue et al., 1999). Briefly, electrocompetent cells were prepared as follows. Both strains were grown in LB broth supplemented with 0.5 M sorbitol at 37°C until absorbance at 600 nm (A_{600}) reached to 0.85-0.95. The grown cells were extensively washed with ice-cold electroporation medium (0.5 M sorbitol, 0.5 M mannitol and 10% glycerol) and subsequently resuspended with the same medium to have 1-1.3 × 10^{10} cfu/ml. Approximately 100 ng of purified plasmids were mixed with 60 μl of the prepared electrocompetent cells in an ice-cold cuvette. Electro-transformation protocol was basically same as described previously (Xue et al., 1999), except 18 KV/cm of field strength.

In order to test if our P43-*gfp* construct could be expressed in Gram-negative bacteria, biocontrol bacterium *P. fluorescens* pc78 was marked with transposon insertion into bacterial chromosome. One of plasposon vectors was modified to generate pTnEZG1-1 (Table 1) by inserting *Spe*I fragment of pEZ43G into pTn*Mod*-OKm. The plasmid pTn*Mod*-OKm was kindly provided by Gerben J. Zylstra from Rutgers University, New Jersey, USA. The constructed transposon was introduced into *P. fluorescens* pc78 by triparental mating with pRK2013 as a helper plasmid. Transconjugants carrying transposon insertion were selected on MG supplemented with kanamycin. Since the MG medium is a kind of minimal medium, *E. coli* donor or helper strains, which are auxotrophs, would not grow on the

Table 1. Bacterial strains and plasmids used in this work

Strains or plasmids	Relevant characteristics[a]	Source or reference
E. coli strains		
DH5α	F⁻ *recA1* Δ(*lacZYA-argF*) U169 *hsdR17 thi-1 gyra66 supE44 endA1 relA1* Φ80Δ(*lacZ*) M15	Sambrook et al., 1989
HB101	F⁻ *hsdS20* (r- m-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 (Str^r) xyl-5 mtl-1 supE44* λ⁻	Boyer and Roulland-Dussoix, 1969
B. subtilis 168	*trpC2* (X-ray mutagenesis of *B. subtilis* Marburg)	Burkholder and Giles, 1947
B. licheniformis N1	Biocontrol strain	Lee et al., 2006
P. fluorescens pc78	Biocontrol strain	Choi et al., 2006
Plasmids		
pGEM-Teasy	Ap^r; TA cloning vector	Promega, USA
pTn*Mod*-OKm	Km^r: plasposon	Dennis and Zylstra, 1998
pGreenTIR	A plasmid carrying an promoterless improved GFP gene	Miller and Lindow, 1997
pWH1520	Tc^r: expression plasmid for *B. megaterium*	Mo Bi Tec, Germany
pRK2013	Km^r: mobilization helper	Figurski and Helinski, 1979
pEZ43	Ap^r: PCR product of P43 promoter from *B. subtilis* 168 cloned in pGEM-Teasy	This study
pEZ43G	Ap^r: P43-*gfp* fusion overlap extension PCR product cloned in pGEM-Teasy	This study
pTnEZG1-1	Km^r : 1.05 kb *Not*I fragment carrying P43-*gfp* fusion from pEZ43G cloned in pTn*Mod*-OKm	This study
pWH43G	Tc^r: 1.05 kb *Spe*I fragment carrying P43-*gfp* fusion from pEZ43G cloned in pWH1520	This study

[a]*Str^r*, chromosomal streptomycin resistance; Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance.

selection medium.

Microscopic visualization of GFP expression. Expression of GFP in *B. licheniformis* N1 with pWH43G was visualized in bacterial cells grown exponentially in nutrient broth by using a model LSM510 confocal laser scanning microscope (CLSM, Carl Zeiss, Germany). The light source to excite GFP was a laser that provided an excitation wavelength of 488 nm (Argon). Fluorescence signal from GFP were detected with the filter set for fluorescein isothiocyanate (FITC; BP 505-530 green).

Measurement of the fluorescence. Expression of green fluorescent protein was determined by measuring fluorescence of bacterial strains carrying the P43-*gfp* fusion construct. The bacterial strains were grown in appropriate liquid medium until mid exponential phase and washed with sterile saline twice to remove any background fluorescence, especially for *P. fluorescens* pc78 strains which produces fluorescent siderophore. Bacterial cells were resuspended in sterile saline to adjust bacterial cell density to 0.8 of A_{600} (approximately 5×10^8 cells/ml). The fluorescence was measured on a SpectraMax Gemini XPS spectrofluorometer (Molecular Device, USA) by excitation at 490 nm and detection of emission at 510 nm. Mean emission intensity was determined from three replications.

Results and Discussion

Fusion of *gfp* with P43 promoter. In order to construct a constitutive expression of green fluorescence protein, we made the GFP fusion construct driven by P43 promoter using OE-PCR (Zhang et al., 2005). The amplified P43-*gfp* fusion was cloned to generate pEZ43G and the correct fusion was confirmed by DNA sequence analysis. Furthermore, *E. coli* cells carrying pEZ43G turned pale green and fluoresce strongly under UV illumination. This implied that P43 promoter is active enough to induce *gfp* expression in *E. coli*. Fluorescence intensity was measured with *E. coli* DH5α carrying various plasmids. Background fluorescence by *E. coli* carrying pWH1520 or pTn*Mod*-OKm was ignorable, while *E. coli* carrying either pWH43G or pTnEZG1-1 fluoresces strongly (Fig. 1). This result indicated that P43 promoter from *Bacillus subtilis* is also active in *E. coli*. The high copy number of pTnEZG1-1 might contribute to the higher fluorescens compared to pWH43G in *E. coli*.

The enhanced *gfp* gene in pGreenTIR contained several advantages to allow the enhanced expression in bacterial system, such as higher excitation wavelength, increased solubility, a translation enhancer, a consensus ribosome binding site with an optimized spacer region (Miller and Lindow, 1997). It was previously demonstrated that P43 promoter was active both at exponential growth phase and at stationary phase in *Bacillus* species (Wang and Doi,

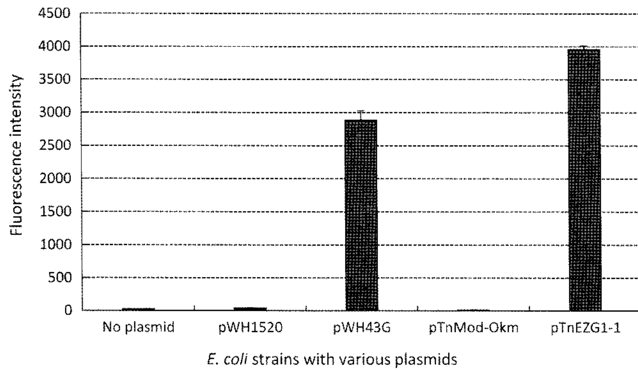

Fig. 1. GFP expression measured by fluorescence intensity in *E. coli* strains with the P43-*gfp* construct. The plasmids pWH1520 and pTnMod-Okm were used as a control for pWH43G and pTnEZG1-1. Error bars represent the standard deviation of three replications.

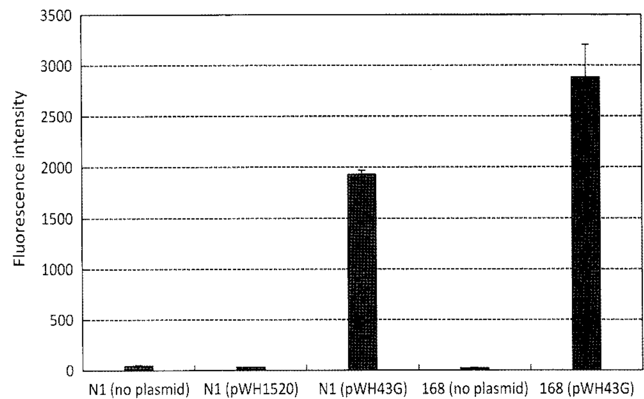

Fig. 2. GFP expression measured by fluorescence intensity in *Bacillus* strains with the P43-*gfp* construct. N1 and 168 represent *B. licheniformis* N1 strain and *B. subtilis* 168 strain, respectively. Error bars represent the standard deviation of three replications.

1984). The previous result implies that P43 promoter has an advantage to express *gfp* in bacterial strains in natural ecosystem, where many bacteria are under starvation or stationary phase. Therefore, a constitutive strong *Bacillus* promoter P43 fusion with the enhanced *gfp* gene could be used as a constitutive biomarker for bacterial strains both *in vitro* and in situ. We further investigated if P43 fused with GFP could be used to mark other biocontrol strains such as *Bacillus* species and *Pseudomonas* strain.

GFP expression in *Bacillus* strains. GFP expression under P43 promoter was investigated in two *Bacillus* species by introducing pWH43G. The plasmid pWH43G was derived from pWH1520 (Table 1), which is the stable expression plasmid in *B. megaterium*. Using the previously described methods (Xue et al., 1999), we could obtain efficient transformation rate from *B. subtilis* 168 strain and *B. licheniformis* N1 (Lee et al., 2006) with pWH43G (data not shown). When the plasmid pWH43G was introduced into *B. subtilis* 168 or *B. licheniformis* N1, the green fluorescence intensity from the transformants were significantly increased compared to the same strain carrying pWH1520 or the strain without any plasmid (Fig. 2). Especially, the fluorescence in *B. subtilis* 168 with pWH43G was equivalent to that in *E. coli* carrying pWH43G (Fig. 1). This result indicated that P43-*gfp* fusion can be used as a reporter system and needs to be tested in other bacterial strains. A biocontrol bacterium *B. licheniformis* N1 carrying pWH43G also exhibited the elevated fluorescence expression, although, the GFP expression was slightly lower than that in *B. subtilis* 168 strain carrying pWH43G (Fig. 2). Since P43 was isolated from *B. subtilis* 168, it is likely that P43 promoter in *B. licheniformis* N1 would be less active than in *B. subtilis* 168. GFP expression in N1 strain carrying pWH43G was also visualized by CLSM. The

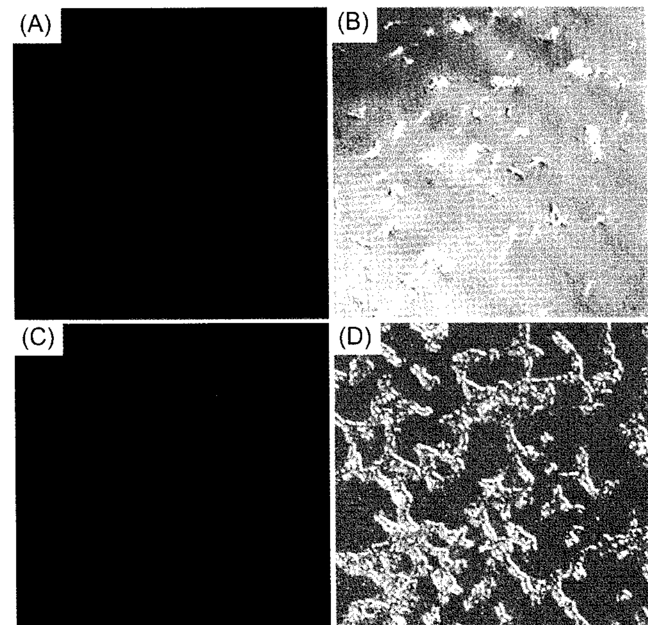

Fig. 3. Photographs of microscopic images (800X magnification) of *B. licheniformis* N1 carrying pWH1520 or pWH43G taken from confocal laser scanning microscope. *B. licheniformis* N1 cells carrying pWH1520 were visualized under fluorescence (A) and visible light (B). *B. licheniformis* N1 cells carrying pWH43G were visualized under fluorescence (C) and visible light (D).

green fluorescence was apparent from *B. licheniformis* N1 with pWH43G compared to the N1 strain without the plasmid (Fig. 3). However, it seems likely that the fluorescence intensity of *B. licheniformis* N1 with pWH43G was not uniform in the transformed bacterial cells (Fig. 3C). Expression of *gfp* gene in N1 strain may still carry some variation at individual cell level. This was also observed by the presence of bacterial colonies of *B. licheniformis* N1 carrying pWH43G but no fluorescence under UV lights, while majority of transformants exhibited fluorescence (data

not shown). The variable fluorescence from *B. licheniformis* N1 carrying pWH43G may be due to plasmid stability. However, when we applied the N1 strain carrying pWH43G on plant surface, the GFP expression was relatively stable (data not shown), suggesting that plasmid-based construct of P43-*gfp* should be still valid to monitor bacterial distribution in natural ecosystem. If the construct could be delivered into bacterial chromosome as a stable integration, the expression variation might be minimized.

GFP expression by P43 promoter in *B. licheniformis* N1 could be used to study bacterial ecology in agricultural ecosystem when the biocontrol bacteria were applied to protect crops from plant diseases (Kim et al., 2007: Lee et al., 2006). Since a number of *Bacillus* species are effective biocontrol agents with diverse traits (Handelsman and Stabb, 1996; Kim et al., 2009; Park et al., 2007), the successful expression of P43-*gfp* fusion in *Bacillus* strains would be valuable to monitor bacterial ecology in natural ecosystem. GFP expression was successfully used in *B. subtilis* to investigate the growth stage specific expression (Webb et al., 1995) and endophytic *B. mojavensis* was successfully transformed with fluorescent proteins (Olubajo and Bacon, 2008). However, this study may provide the GFP reporter construct driven by constitutive P43 promoter, which could be potentially used as a biomarker system in other *Bacillus* strains.

GFP expression in a biocontrol bacterium *Pseudomonas fluorescens*. We further investigated if GFP could be expressed by P43 promoter in a gram-negative biocontrol bacterium. *P. fluorescens* pc78 was subjected to transposon insertion tagging by pTnEZG1-1 (Table 1). A modified plasposon pTnEZG1-1 contained P43-*gfp* fusion at the downstream of kanamycin resistance gene in the same direction. Throughout transposon insertion, we randomly selected two mutants, pc78-46 and pc78-26, showing different phenotypes in antifungal activity (data not shown). The transposon insertion was confirmed by PCR with *gfp* gene primers and Southern blot with kanamycin resistance gene probe. Southern blot result revealed that P43-*gfp* fusion within transposon was inserted as a single copy (data not shown). While the fluorescence intensity from one strain pc78-26 was over 5-fold increased compared to non-transformed control wild type strain pc78, that from pc78-48 was not significantly different from wild type (Fig. 4). Fluorescence intensity from pc78 strains were much lower compared to that in *Bacillus* species. It is likely that P43 promoter is not functionally active in our tested *P. fluorescens*. Therefore, our transposon based construct could not be used as a marker in a biocontrol pc78 strain. However, the original transposon pTn*Mod*-Okm was designed to make it possible to identify the transposon insertion site

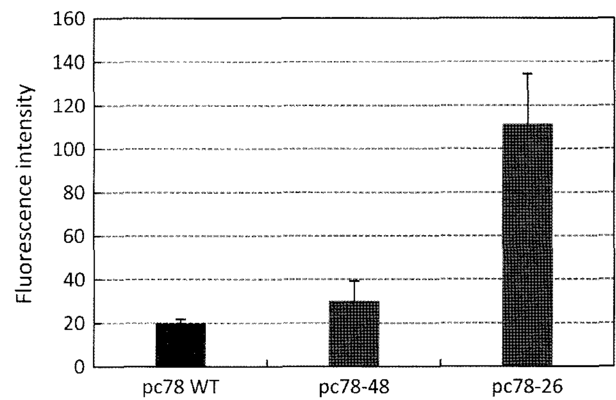

Fig. 4. GFP expression measured by fluorescence intensity in *P. fluorescens* pc78 strains. pc78WT represents *P. fluorescens* pc78 wild type which does not contain the P43-*gfp* construct. The pc78-26 and pc78-48 indicated two derivatives from pc78 wild type by transposon insertion carrying the P43-*gfp* construct. Error bars represent the standard deviation of three replications.

rapidly in bacterial chromosome (Dennis and Zylstra, 1998). Our modified transposon retained the same features with P43-*gfp* fusion. Therefore, real time quantitative PCR would be plausible using a pair of primer from transposon insertion strain to investigate bacterial ecology in agricultural ecosystem. One primer could be designed from *gfp* gene which is located near transposon flanking sequences and the other primer from transposon inserted chromosomal location. Two primers would be only specific for the constructed strain.

Conclusively, we generated a new fusion *gfp* marker with P43 promoter to express GFP genes in biocontrol bacterial strains and to tag other bacterial strains. Utility of the P43-*gfp* fusion in biocontrol bacteria was shown in *B. licheniformis* in this study.

Acknowledgments

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