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Isolation of a strong promoter fragment from endophytic *Enterobacter cloacae* and verification of its promoter activity when its host strain colonizes banana plants

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Abstract To engineer endophytic *Enterobacter cloacae* as a biocontrol agent against banana fusarium wilt, a promoter-probe plasmid pUCK was constructed to identify a strong promoter to express disease resistance genes. Using a kanamycin resistance gene for selection, 10 fragments with strong promoter activity were identified from the genome of the *E. cloacae* KKWB-10 strain. The regions of these 10 fragments that were the primary contributors to the promoter function were identified, and their promoter activities were further evaluated using green fluorescent protein (GFP) as a reporter gene. Fragment 132a″ drove the highest level of GFP activity when the bacteria bearing the fragments were cultured in Luria–Bertani and banana stem extract media. The GFP-expressing strain harboring fragment 132a″ (K-pUCK7-132a″-GT) was then inoculated into banana plantlets (about 1×10^7 CFU per plant) to verify the activity of fragment 132a″ in planta. Ten days after inoculation, tissue sections of these banana plantlets were observed by laser confocal scanning microscope. Green fluorescence was observed in the tissues of banana plantlets inoculated with K-pUCK7-132a″-GT but not in uninoculated controls. These results suggest that fragment 132a″ possesses strong promoter activity when its host strain colonizes the banana plants and can be used to engineer endophytic *E. cloacae* KKWB-10 for biocontrol.

Keywords Endophyte · *Enterobacter cloacae* · Promoter cloning · GFP · Fluorescence observation

Introduction

Fusarium wilt of banana (Panama disease), which is caused by *Fusarium oxysporum* f. sp. *cubense* (Foc), is one of the most devastating diseases of bananas. Fusarium wilt is a major problem throughout most of the banana production regions of the world and influences the development of the banana industry. Foc is a soil-borne fungus that enters through the roots and blocks the vascular system, causing the plant to wilt and die. No effective chemical method exists to control it; however, alternative strategies include biological control, which is environmentally safe and economically profitable. One example is the use of endophytes as biocontrol agents for this disease.

Endophytes are microorganisms that colonize the internal tissue of the plant without producing external signs of infection or negative effects on their host (Holliday 1989; Schulz and Boyle 2006). In fact, endophytes exert beneficial effects for their host plant, such as promoting

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growth (Ting et al. 2008; Bae et al. 2009; Shi et al. 2009; Khan and Doty 2009), enhancing phytoremediation (van Aken et al. 2004; Weyens et al. 2009; Germaine et al. 2009), preventing disease and insect infestation (Akello et al. 2008; Shittu et al. 2009; Shi et al. 2010), inducing plant defense mechanisms (Waller et al. 2005; Vu et al. 2006; Harish et al. 2008), and nitrogen fixation (Iniguez et al. 2004; Miyamoto et al. 2004; Jha and Kumar 2007). These qualities make endophytes potential natural resources for the biological control of plant diseases. Genetically modified endophytic bacteria are excellent vectors for the introduction of heterologous-resistant genes into host plants. Thus, endophytic bacteria may confer new disease resistance or insect resistance characteristics to host plants without direct manipulation of the plant genome, which avoids generating transgenic plants. Turner and Lampel demonstrated that endophytic bacteria that introduced *Bacillus thuringiensis cryIA(c)* genes into corn improved insect resistance (Turner et al. 1991; Lampell et al. 1994). Similarly, endophytic *Pseudomonas fluorescens* containing the *chiA* gene from *Serratia marcescens* showed effective biocontrol activity against *Rhizoctonia solani* in bean seedlings under plant growth chamber conditions (Downing and Thomson 2000). However, to date no studies have reported the use of engineered endophytic bacteria as biological control against diseases in bananas.

To the best of our knowledge, endophytic bacteria, fungi, and *Actinomycete*s have all been isolated from banana plants (Cao et al. 2005; Lian et al. 2008; Ting et al. 2008; Thomas et al. 2008; Thomas and Soly 2009). Some endophyte isolates may be potential growth promoters of bananas (Weber et al. 2007; Ting et al. 2008; Chaves et al. 2009; Paparu et al. 2009), and some endophyte isolates have demonstrated effective antagonistic activity against Foc (Cao et al. 2005; Weber et al. 2007; Lian et al. 2008; Ting et al. 2008). Thus banana endophytes have shown potential as biocontrol agents for suppressing banana fusarium wilt. *Enterobacter cloacae* KKWB-10 was the predominant strain isolated from the corms of healthy banana plants (*Musa* AAA Giant Cavendish cv. Baxi) in our laboratory, and this strain inhibited the growth of Foc race 4 (Wang et al. 2010). *E. cloacae* has been reported to benefit host plants through nitrogen fixation (Yang et al. 1999), growth promotion (Madmony et al. 2005), and defense against disease (Hinton and Bacon 1995). Hence we proposed to genetically modify the KKWB-10 strain to express heterologous disease resistance proteins like polygalacturonase-inhibiting proteins and use the engineered KKWB-10 to prevent and control banana fusarium wilt.

To engineer the endophytic KKWB-10 in this way, we needed a promoter that has strong activity after the bacteria colonize the banana plants. However, little is known about *E. cloacae* promoters. Therefore, in the present study, we aimed to select and clone a suitable promoter from the KKWB-10 genome. Methods used to clone promoters include polymerase chain reaction (PCR), inverse PCR, panhandle PCR, sequence-specific primer PCR, thermal asymmetric interlaced PCR, the Y-shaped adaptor dependent extension method, and promoter-probe vectors. However, the promoter-probe vector, which has a promoterless reporter gene, is the only one of these methods that can be used to identify promoters without prior knowledge of the nucleotide sequence. Because the promoter sequences of KKWB-10 are not known, a promoter-probe vector was chosen to screen sequences and identify a strong promoter to engineer endophytic KKWB-10.

In the present study, we identified a promoter fragment exhibiting strong activity and used a green fluorescence protein (GFP) reporter to verify its promoter activity when its host bacterial strain resided in banana plants. Our findings have paved the way for the introduction of heterologous disease resistance genes in KKWB-10 for biocontrol in banana plants.

Materials and methods

Bacterial strains and plasmids

E. cloacae KKWB-10 (deposited in Agricultural Culture Collection of China as strain ACCC No. 05655) was isolated from healthy banana plants (*Musa* AAA Giant Cavendish cv. Baxi) in our laboratory. Bacterial strains and plasmids used in this study are shown in Table 1.

Media and culture conditions

Escherichia coli Top10 was cultured at 37°C in Luria–Bertani (LB) medium, which was supplemented with kanamycin at the appropriate concentration as necessary. *E. cloacae* KKWB-10 was cultured at 30°C in LB or banana stem extract (BSE) medium supplemented with kanamycin at the appropriate concentration as necessary. BSE medium was prepared as follows: the stem of a healthy banana plant was washed, cut, boiled with distilled water for 1 h, and then autoclaved at 121°C for 25 min.

Plant cultivation and growth conditions

Banana tissue culture plantlets were cultivated in sterile culture vessels sealed with film and then placed in a growth chamber with a 14-h day cycle at 22°C. There were three replicates per treatment, and each replicate consisted of three plantlets in one vessel. The soil mixtures for cultivation consisted of sand mixed with coconut coir in a 1:1 ratio by volume. Once mixed, the soil mixtures were autoclaved at 121°C for 1 h, and autoclaving was repeated three times.

Table 1 Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant properties	Source	Plasmids	Relevant properties	Source
Strains			pUCK35′-KT	Ap^r, Km^r	This work
Escherichia coli Top10		Our laboratory	pUCK52′-KT	Ap^r, Km^r	This work
Enterobacter cloacae	Ap^r	Our laboratory	pUCK54′-KT	Apr, Kmr	This work
KKWB-10			pUCK71′-KT	Ap^r, Km^r	This work
Plasmids			pUCK132′-KT	Ap^r, Km^r	This work
pUC19	Ap^r	Takara	pUCK1′-K′T	Ap^r, Km^r	This work
pET22b	Ap^r	Novagen	pUCK2′-K′T	Ap^r, Km^r	This work
pET30a	Km^r	Novagen	pUCK5′-K′T	Ap^r, Km^r	This work
pGFPuv	Ap^r	Clontech	pUCK7′-K′T	Ap^r, Km^r	This work
pUCK	Ap^r	This work	pUCK34′-K′T	Ap^r, Km^r	This work
pUCK1	Ap^r, Km^r	This work	pUCK35′-K′T	Ap^r, Km^r	This work
pUCK2	Ap^r, Km^r	This work	pUCK52′-K′T	Ap^r, Km^r	This work
pUCK5	Ap^r, Km^r	This work	pUCK54′-K′T	Ap^r, Km^r	This work
pUCK7	Ap^r, Km^r	This work	pUCK71′-K′T	Ap^r, Km^r	This work
pUCK34	Ap^r, Km^r	This work	pUCK132′-K′T	Ap^r, Km^r	This work
pUCK35	Ap^r, Km^r	This work	pUCK35″-KT	Ap^r, Km^r	This work
pUCK52	Ap^r, Km^r	This work	pUCK35″-K′T	Ap^r, Km^r	This work
pUCK54	Ap^r, Km^r	This work	pUCK132a″-KT	Ap^r, Km^r	This work
pUCK71	Ap^r, Km^r	This work	pUCK132a″-K′T	Ap^r, Km^r	This work
pUCK132	Ap^r, Km^r	This work	pUCK132b″-KT	Ap^r, Km^r	This work
pUCK1-5	Ap^r, Km^r	This work	pUCK132b″-K′T	Ap^r, Km^r	This work
pUCK2-2	Ap^r, Km^r	This work	pUCK7′	Ap^r, Km^r	This work
pUCK5-5	Ap^r, Km^r	This work	pET22b-gfp	Ap^r, gfp	This work
pUCK34-4	Ap^r, Km^r	This work	pUCK7-1′-GT	Ap^r, Km^r, gfp	This work
pUCK35-3	Ap^r, Km^r	This work	pUCK7-2′-GT	Ap^r, Km^r, gfp	This work
pUCK52-8	Ap^r, Km^r	This work	pUCK7-5′-GT	Ap^r, Km^r, gfp	This work
pUCK54-13	Ap^r, Km^r	This work	pUCK7-7′-GT	Ap^r, Km^r, gfp	This work
pUCK71-1	Ap^r, Km^r	This work	pUCK7-34′-GT	Ap^r, Km^r, gfp	This work
pUCK1′-KT	Ap^r, Km^r	This work	pUCK7-35″-GT	Ap^r, Km^r, gfp	This work
pUCK2′-KT	Ap^r, Km^r	This work	pUCK7-52′-GT	Ap^r, Km^r, gfp	This work
pUCK5′-KT	Ap^r, Km^r	This work	pUCK7-54′-GT	Ap^r, Km^r, gfp	This work
pUCK7′-KT	Ap^r, Km^r	This work	pUCK7-71′-GT	Ap^r, Km^r, gfp	This work
pUCK34′-KT	Ap^r, Km^r	This work	pUCK7-132a″-GT	Ap^r, Km^r, gfp	This work

Ap^r ampicillin resistance, Km^r kanamycin resistance, *gfp* green fluorescent protein gene

Primers

All primers used in this study are shown in Table 2.

General DNA manipulation

Plasmid preparation, restriction endonuclease digestion, genomic DNA preparation, DNA ligation, and other recombinant DNA techniques were carried out using standard methods (Sambrook et al. 1989). Plasmid DNA transformation of *E. coli* Top10 and *E. cloacae* KKWB-10 was carried out using the calcium chloride method (Sambrook et al. 1989). All enzymes were purchased from Fermentas China Co., Ltd. and TaKaRa Biotechnology (Dalian) Co., Ltd. The anti-GFP monoclonal antibody and goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) were purchased from Tiangen Biotech (Beijing) Co., Ltd.

Construction of promoter-probe plasmid

The promoter-probe plasmid pUCK was constructed as follows. The 1.1-kb DNA fragment KT containing a promoterless kanamycin resistance gene and T7 terminator derived from pET30a were amplified by PCR using primers K1, K2, and K3. The fragment was digested by *BamH*I and *Hind*III and inserted into the corresponding restriction sites

Table 2 Primers used in this study

Primer	Sequence
K1	5′-CGC GGA TCC CAA GGG GTG TTA TGA GCC ATA T-3′
K2	5′-CCC GTT TAG AGG CCC CAA GGG GTT ATG CTA TTG ATT TAT AAG GGA TTT TG-3
K3	5′-CCC AAG CTT CAA AAA ACC CCT CAA GAC CCG TTT AGA GGC CCC AAG-3′
pK1	5′-AAT TCG AGC TCG GTA CC-3′
pK2	5′-CCG TTG AAT ATG GCT C-3′
1f	5′-CCG GAA TTC ATT ACC TGC CTC GGC GCG-3′
1r	5′-CGC GGA TCC GAT CCG GAT TGT CTT CG-3
2f	5′-GCC GAG CTC AAT ACT GAA CGC CGG AAT TCA G-3
2r	5′-ATA ATC GATC GCC AAG AAG CTC AGC GGC-3
5f	5′-CCG GAA TTC AGC AAC TGC GCA ACA CGC TG-3′
5r	5′-TCC GATC TCG TTT CTG TCT CTT CAT GCC TCG-3′
34f	5′-CCG GAA TTC CCC GTG GTT GAT GAA AAG CAA C-3′
34r	5′-TCC GATC CCG GCG TTT TTA TAA CGC TCG-3′
35f	5′-CCG GAA TTC GTC AAT TAT GCC CCC GCT GTG CAA AT-3′
35r	5′-TCC GATC GCG AAA ATC CTT TTC TGC CTC-3
52f	5′-CCG GAA TTC TTT GAC ATT CCG CGT TGC AGC-3
52r	5′-TCC GATC GTC TTT AGT GGG AAA GTA GCG-3
54f	5′-TAT TAT CCG GAA TTC GAT CGC TGC CCA GAC GGC GTT-3′
54r	5′-TCC GATC GGA ACT CAT CAA TTT TTC TCC-3
71f	5′-CCG GAA TTC GGT ACC GTA AAA AGC GCC CCA CT-3′
71r	5′-TCC GATC GA GAT ATC CCT TAA TTA GCC GCA GGC-3′
Kf	5′-ATG AGC CAT ATT CAA CGG GAA ACG T-3′
Kr	5′-TCC TTT CGG GCT TTG TTA GAA AAA CTC ATC GAG C-3′
Tf	5′-GA GCT CTA CAA ATA ACA AAG CCC GAA AGG AAG-3′
Tr	5′-CGG GGT ACC CAA AAA ACC CCT CAA GAC C-3′
1′r1	5′-TTG AAT ATG GCT CAT CTT ATG CTC CCA TGA AT-3′
2′r1	5′-TTG AAT ATG GCT CAT CTC TTC GTT TGC CTC GT-3′
Tr1	5′-CGC GGA TCC CAA AAA ACC CCT CAA GAC CCG T-3′
5′r1	5′-TTG AAT ATG GCT CAT GCC TCG TTT CCC TCA TTC-3′
7f	5′-CCG GAA TTC GAT CAC CGA TTT ATG CCT-3′
7′r1	5′-CCG TTG AAT ATG GCT CAT AGT TGT CCT CAA TAA ACG CCG-3′
34′r1	5′-CCG TTG AAT ATG GCT CAT CGG TTT TGC CTA AAT GAA AC-3′
35′r1	5′-CCG TTG AAT ATG GCT CAT TGC CGG ACC TGA ACT GAA AT-3′
52′r1	5′-CCG TTG AAT ATG GCT CAT TAA ATT TTT CCC CCT GTC-3′
54′r1	5′-CCG TTG AAT ATG GCT CAT GAA AAG TGA GCT TGC TC-3′
71′r1	5′-CCG TTG AAT ATG GCT CAT GA GAT ATC CCT TAA TTA GCC-3′
132f	5′-CCG GAA TTC CGG TGT CAG ACC CTG ACC T-3′
132′r1	5′-TTG AAT ATG GCT CAT TGC TAA CCT CAT ACA ATC G-3′
1′r2	5′-TTC CCG TTG AAT ATG GCT CTT ATG CTC CCA TGA AT-3′
2′r2	5′-TTC CCG TTG AAT ATG GCT CTC TTC GTT TGC CTC GT-3′
5′r2	5′-TTC CCG TTG AAT ATG GCT GCC TCG TTT CCC TCA TTC-3′
7′r2	5′-TTC CCG TTG AAT ATG GCT AGT TGT CCT CAA TAA ACG CCG-3′
34′r2	5′-TTC CCG TTG AAT ATG GCT CGG TTT TGC CTA AAT GAA AC-3′
35′r2	5′-TTC CCG TTG AAT ATG GCT TGC CGG ACC TGA ACT GAA AT-3′
52′r2	5′-TTC CCG TTG AAT ATG GCT TAA ATT TTT CCC CCT GTC-3′
54′r2	5′-TTC CCG TTG AAT ATG GCT GAA AAG TGA GCT TGC TC-3′
71′r2	5′-TTC CCG TTG AAT ATG GCT GAG ATA TCC CTT AAT TAG CCG C-3′
132′r2	5′-TTC CCG TTG AAT ATG GCT TGC TAA CCT CAT ACA ATC G-3′
35″r1	5′-G TTG AAT ATG GCT CAT TTC CCC TCA CCA C-3′

Table 2 (continued)

Primer	Sequence
35″r2	5′-TTC CCG TTG AAT ATG GCT TTC CCC TCA CCA CGC T-3′
132a″r1	5′-G TTG AAT ATG GCT CAT ACA ATC GAC ATC GC-3′
132a″r2	5′-C CCG TTG AAT ATG GCT ACA ATC GAC ATC GCT T-3′
132b″r1	5′-CCG TTG AAT ATG GCT CAT TTA GGA CCA AAC AAT-3′
132b″r2	5′-C CCG TTG AAT ATG GCT TTA GGA CCA AAC AAT TGG-3′
Gf	5′-CCA AGG GTG TT ATG AGT AAA GGA GAA GAA CTT-3′
Gr	5′-CTT CCT TTC GGG CTT TGT TAT TTG TAG AGC TC-3′
Ka	5′-CTG GAA TGC TGT TTT ACC GGG GAT CGC AGT G-3′
Kb	5′-CCC GGT AAA ACAGCA TTC CAG GTA TT-3′
1′r3	5′-TTC TCC TTT ACT CAT CTT ATG CTC CCA TGA AT-3′
2′r3	5′-CTTC TCC TTT ACT CAT CTC TTC GTT TGC CTC GT-3′
Gr1	5′-CGGG CTT TGT TAT TTG TAG AGT TCA TCC ATG CCA TGT GTA ATC CC-3′
Tr2	5′-TCC CCC GGG CAA AAA ACC CCT CAA GAC CCG T-3′
5′r3	5′-CTTC TCC TTT ACT CAT GCC TCG TTT CCC TCA TTC-3′
7′r3	5′-CTTC TCC TTT ACT CAT AGT TGT CCT CAA TAA ACG CCG-3′
34′r3	5′-CTTC TCC TTT ACT CAT CGG TTT TGC CTA AAT GAA AC-3′
35″r3	5′-CTTC TCC TTT ACT CAT TTC CCC TCA CCA CGC T-3′
52′r3	5′-CTTC TCC TTT ACT CAT TAA ATT TTT CCC CCT GTC-3′
54′r3	5′-CTTC TCC TTT ACT CAT GAA AAG TGA GCT TGC TC-3′
71′r3	5′-CTTC TCC TTT ACT CAT GAG ATA TCC CTT AAT TAG CCG C-3′
132a″r3	5′-TTC TCC TTT ACT CAT ACA ATC GAC ATC GC-3′
G1	5′-CCGG GAA TTC CAT ATG AGT AAA GGA GAA G-3′
G2	5′-CG TTT CAT GTG ATC CGG ATA ACG GGA AAA G-3′
G3	5′-TAT CCG GAT CAC ATG AAA CGG CAT GAC TT-3′
G4	5′-CCC AAG CTT TTA TTT GTA GAG CTC ATC CAT GCC-3′

Restriction enzyme sites are indicated by a single underline

of pUC19, with the kanamycin resistance gene in reverse orientation with respect to the *lacZ* promoter. The recombinant plasmid was identified by digestion with *BamH*I and *Hind*III and sequenced by Invitrogen Biotechnology Co. Ltd. (Shanghai). The *E. coli* Top10 transformant harboring pUCK was cultured in LB broth and on an LB agar plate supplemented with 50 μg/ml kanamycin for 3 days at 37°C to confirm kanamycin resistance of the plasmid.

Promoter cloning of KKWB-10 chromosomal DNA

Genomic DNA of *E. cloacae* KKWB-10 was completely digested with *Bsp143*I, and DNA fragments ranging in size from 100 to 1,000 bp were recovered. These DNA fragments were ligated to pUCK, which had been digested by *BamH*I and treated with FastAP thermosensitive alkaline phosphatase. The ligation mixture was transformed into competent *E. coli* Top10 cells, and the cells were then spread onto LB agar containing 100 μg/ml ampicillin. Transformants-containing fragments with promoter function were screened on LB agar supplemented with 100 μg/ml kanamycin.

Screening of fragments with strong promoter function

Top10 transformants were cultured in 1 ml LB liquid medium with 100 μg/ml kanamycin overnight at 37°C, and clones that were growing well were subcultured in LB with increasing concentrations of kanamycin to determine their resistance to kanamycin (final concentrations: 200, 400, 800, 1,000, 1,500, 2,000, 2,500, 3,000, 3,500, 4,000, 4,500, and 5,000 μg/ml).

Recombinant plasmids were prepared from Top10 clones that were highly resistant to kanamycin and transformed into the *E. cloacae* KKWB-10 strain. The transformants were cultured on LB agar with increasing concentrations of kanamycin at 30°C to determine their kanamycin resistance (final concentrations: 3,000, 3,500, 4,000, 4,500, 5,000, 5,500, 6,000, 6,500, 7,000, and 7,500 μg/ml). KKWB-10 transformants were also cultured on BSE agar with increasing concentrations of kanamycin at 30°C to determine kanamycin resistance on BSE medium (final concentrations: 400, 500, 600, 700, 800, 900, 1,000, and 1,100 μg/ml).

Sequencing and promoter functional analysis of the DNA fragments

DNA fragments with strong promoter function were amplified by PCR using primers pK1 and pK2 and then sequenced by Invitrogen Biotechnology Co. Ltd. (Shanghai). Sequences were aligned with the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information website. Promoters were predicted with Neural Network Promoter Prediction (NNPP) v2.2 (http://www.fruitfly.org/index.html) and BPROM of SoftBerry (http://linux1.softberry.com/berry.phtml).

Identification of primary promoter functional fragments of complex cloned fragments

The results of BLAST analysis and promoter prediction were used to identify the DNA sequences likely to be primary contributors to the promoter activities of the eight complex fragments recovered from the KKWB-10 transformants. These short DNA sequences were amplified by PCR with the following primer pairs: 1f and 1r, 2f and 2r, 5f and 5r, 34f and 34r, 35f and 35r, 52f and 52r, 54f and 54r, and 71f and 71r. The PCR products were then digested with *EcoR*I and *Mbo*I (except fragment 2, which was digested with *Sac*I and *Mbo*I) and inserted upstream of the kanamycin resistance gene of pUCK. The recombinant plasmids were identified by double digestion and sequencing, and the levels of kanamycin resistance of these Top10 positive transformants were determined.

Identification of the optimal reading frame of promoter fragments

BLAST analysis of all 10 promoter fragments showed these fragments all appeared to contain partial coding sequences of genes, suggesting that the kanamycin resistance gene was expressed as a fusion protein with an extra peptide segment at the N-terminus. To eliminate the effects of these extra N-terminal residues on the kanamycin resistance gene and identify the optimal reading frame, the 3′ end coding sequences were removed from all promoter fragments. These fragments lacking the 3′ end coding sequences were then amplified by PCR with the following primer pairs: 1f and 1′r1, 2f and 2′r1, 5f and 5′r1, 7f and 7′r1, 34f and 34′r1, 35f and 35′r1, 52f and 52′r1, 54f and 54′r1, 71f and 71′r1, and 132f and 132′r1. The 10 PCR products (1′, 2′, 5′, 7′, 34′, 35′, 52′, 54′, 71′, and 132′) were ligated individually to the fragment KT, which contains a promoterless kanamycin resistance gene (primers Kf and Kr) and T7 terminator (primers Tf and Tr), producing 10 new fragments (1′-KT, 2′-KT, 5′-KT, 7′-KT, 34′-KT, 35′-KT, 52′-KT, 54′-KT, 71′-KT, and 132′-KT). These new fragments were digested with *EcoR*I and *Kpn*I (except fragment 2′-KT, which was digested with *Sac*I and *BamH*I) and inserted individually into the corresponding site of pUC19. The recombinant plasmids were identified by double digestion and sequencing, and the levels of kanamycin resistance of these Top10 transformants were determined.

Additional experiments were carried out to determine whether the kanamycin resistance genes of the Top10 transformants described above were primarily expressed directly from the promoter fragments and in the optimal reading frames. The 10 primary promoter fragments lacking the 3′ end coding sequences were amplified again with the following primer pairs: 1f and 1′r2, 2f and 2′r2, 5f and 5′r2, 7f and 7′r2, 34f and 34′r2, 35f and 35′r2, 52f and 52′r2, 54f and 54′r2, 71f and 71′r2, and 132f and 132′r2. The PCR products were individually ligated to the fragment K′T, from which the start codon (ATG) of the kanamycin resistance gene had been deleted. Then the 10 new fragments were inserted individually into the multiple cloning site of pUC19. The recombinant plasmids were identified by double digestion and sequencing, and the levels of kanamycin resistance of these Top10 transformants were determined. Low kanamycin resistance of a transformant indicated that the kanamycin resistance gene was expressed primarily from the promoter fragment and was in the optimal reading frame; high kanamycin resistance of a transformant indicated that the kanamycin resistance gene was likely expressed as a fusion protein, and the optimal reading frame was uncertain.

Functional analysis of the primary promoter fragments using a GFP reporter

The 10 primary promoter functional fragments without the 3′ end coding sequences were amplified with the following primer pairs: 1f and 1′r3, 2f and 2′r3, 5f and 5′r3, 7f and 7′r3, 34f and 34′r3, 35f and 35″r3, 52f and 52′r3, 54-f and 54′r3, 71f and 71′r3, and 132f and 132a″r3. The PCR products were ligated individually to the fragment GT containing the *gfpuv* gene (from the pGFPuv vector, primers Gf and Gr) and T7 terminator. The new fragments (1′-GT, 5′-GT, 7′-GT, 34′-GT, 35″-GT, 52′-GT, 54′-GT, 71′-GT, and 132a″-GT) were digested with *EcoR*I and *Kpn*I and inserted individually into the corresponding site of a highly kanamycin-resistant plasmid pUCK7, which had been obtained by screening pUCK. For fragment 2′, the PCR product was ligated to the fragment GT1 containing the *gfpuv* gene (from which the *Sac*I site was destroyed by nonsense mutation with primers Gf and Gr1) and T7 terminator (primers Tf and Tr2). The new fragment 2′-GT was digested with *Sac*I and *Sma*I and inserted into the corresponding site of plasmid pUCK7′, from which the *Sma*I site of the kanamycin resistance gene was destroyed by a nonsense mutation. The recombinant plasmids were identified by double digestion and sequencing and then transformed into Top10 and KKWB-10 bacteria. In addition, the plasmid pET22b-gfp was constructed as a positive control for Western blot analysis as follows. The fragment GFP with the *Nde*I site destroyed was amplified with primers G1, G2, G3, and G4. The PCR product was then digested with *Nde*I and *Hind*III and inserted into the corresponding site of pET22b to generate pET22b-gfp. The plasmid pET22b-gfp was transformed into *E. coli* BL21 (DE3) to express GFP directly.

The Top10 and KKWB-10 transformants were cultured overnight in LB with 100 μg/ml kanamycin, and KKWB-10 transformants were cultured in BSE under the same conditions. We then evaluated the fluorescence of Top10 and KKWB-10 strains that were cultured in LB or BSE medium with 50 μg/ml kanamycin for 12 h at 30°C. The cells were collected by centrifugation and resuspended in sterile water. The optical density of these bacterial cultures was measured at 600 nm (OD_{600}) and diluted to a value below 0.5 with sterile water. Fluorescence was measured by fluorescence spectrophotometer (Shimadzu RF-4500) with excitation at 395 nm and emission at 505 nm. Top10 and KKWB-10 bacteria without the *gfp* gene were used as controls. Fluorescence intensity was calculated as the quotient of fluorescence units (FU; mean value of four repetitions) and OD_{600} and expressed as relative fluorescence units ($RFU=FU/OD_{600}$). Standard fluorescence intensity was the RFU of test samples minus the RFU of controls. Western blot analysis was also used to compare GFP expression levels of these strains. Proteins were separated by SDS-PAGE using 12% (*w*/*v*) polyacrylamide gels and then transferred to a polyvinylidene fluoride membrane (Millipore). The membrane was blocked for 1 h in 5% skim milk powder solution (*w*/*v*) and then incubated for 1 h at room temperature with an anti-GFP monoclonal antibody (1:5,000 dilution in 50 mM Tris–HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.5 (TBST)). After washing with TBST, membranes were incubated with goat anti-mouse IgG/HRP at a 1:500 dilution for 1 h at room temperature. After washing again with TBST, proteins were detected by 3-amino-9-ethylcarbazole.

Fluorescence observation of the GFP-expressing strain colonizing the banana plants

Laser confocal scanning microscopy

Banana tissue culture plantlets were inoculated with a GFP-expressing KKWB-10 strain using the root dipping method (about 1×10^7 CFU per plantlet) and cultivated in sterile culture vessels sealed with film. Uninoculated plantlets were used as controls. After 10 days of growth, the plantlets were removed, washed, and surface sterilized. Then freehand sections of these plantlets were prepared for fluorescence observation by laser scanning confocal microscopy (LSCM; Olympus FluoView FV1000). Because plant tissues can emit strong autofluorescence when excited by the 405-nm laser line (GFPuv), we used the 488-nm laser line to excite GFP.

Isolation and identification of GFP-expressing bacterial strain colonizing banana plantlets

After fluorescence observation by LSCM, the remaining tissue sections of these plantlets were used to isolate the GFP-expressing bacterial strain. Tissue sections were sterilized by sequential immersion in 75% ethanol for 1 min and 0.12% mercuric chloride for 10 min. Then the samples were washed in sterile water three times to remove the sterilization agents. Each sample was divided into small fragments, plated on LB agar supplemented with 100 μg/ml kanamycin and 100 μg/ml ampicillin, and then incubated at 30°C for 3 days. The last set of washes used to rinse the tissues was cultured at 30°C as a control.

The strain growing on LB agar containing 100 μg/ml kanamycin and 100 μg/ml ampicillin was identified by PCR and fluorescence microscopy. The PCR assays were performed using primers 132f and Gr. Observations were performed by fluorescence microscope under UV excitation.

Nucleotide sequence accession numbers

Nucleotide sequences of the primary promoter functional fragments were deposited in the GenBank database. The accession numbers are HQ834304 (fragment 1-5), JN604501 (fragment 2-2), JN604502 (fragment 5-5), HQ834305 (fragment 7), JN604503 (fragment 34-4), JN604504 (fragment 35-3), JN604505 (fragment 52-8), JN604506 (fragment 54-13), JN604507 (fragment 71-1), and HQ834306 (fragment 132).

Results

Construction of promoter-probe plasmid

The promoter-probe plasmid pUCK was constructed and confirmed by double digestion and sequencing. Top10 cells harboring pUCK did not grow in LB liquid medium or LB agar plate supplemented with 100 μg/ml kanamycin, which indicated that the pUCK plasmid itself was not resistant to kanamycin and could be used to clone promoters from KKWB-10 using the kanamycin resistance gene as a selectable marker.

Selection of fragments with strong promoter function

About a thousand Top10 transformants isolated from LB agar plates with 100 μg/ml ampicillin were subcultured in

1 ml LB medium with 100 μg/ml kanamycin and grown overnight at 37°C; about 500 clones grew well. These 500 clones were cultured on LB agar with higher concentrations of kanamycin. The results of kanamycin resistance assays showed that about 300 clones grew well on LB with 400 μg/ml kanamycin and 189 clones grew well on LB with 800 μg/ml kanamycin. There were 147 clones that grew well on LB with 1,000 μg/ml kanamycin, which were denoted T-pUCK1 to T-pUCK147; the plasmids corresponding to these clones were denoted pUCK1 to pUCK147, and fragments corresponding to these plasmids were denoted fragment 1 to 147. With increasing concentrations of kanamycin, 39 clones grew well on LB with 2,000 μg/ml kanamycin, 17 clones grew well on LB with 3,000 μg/ml kanamycin, and 12 clones grew well on LB with 3,500 μg/ml kanamycin.

Plasmids isolated from the 12 clones resistant to 3,500 μg/ml kanamycin were transformed into KKWB-10. The kanamycin resistance of the KKWB-10 transformants was then determined in LB and BSE medium. As shown in Table 3, the kanamycin resistance of KKWB-10 transformants was higher than that of the Top10 transformants, which suggested that the promoter activities of these fragments were stronger in KKWB-10. The kanamycin resistance of these KKWB-10 clones cultured on BSE agar was lower than their resistance on LB agar. The transformant harboring pUCK1 and the transformant harboring pUCK132 showed the highest kanamycin resistance on LB and BSE.

BLAST analysis of promoter functional fragments

Twelve fragments were amplified by PCR using primers pK1 and pK2. The size varied from about 200 to 4,000 bp (Table 3). Sequencing of these fragments revealed that fragment 7 was identical to fragment 19, and fragment 34 was identical to fragment 42. The complete fragment sequences are shown in Electronic supplementary material. Sequence alignment of the 10 different fragments showed that nucleotide sequences of all fragments were most similar to three *Enterobacter* strains (*E. cloacae* subsp. *cloacae* ATCC 13047, *E. cloacae* subsp. *cloacae* NCTC 9394, and *Enterobacter* sp. 638), which indicated that these fragments originated from the *E. cloacae* KKWB-10 genome. Two were single DNA fragments (fragments 7 and 132), but each of the other eight fragments were complex fragments consisting of several distinct short fragments; some of these short fragments contained partial coding sequences of two adjacent genes and their intergenic spacer region, whereas others consisted of the partial coding sequence of a single gene (Fig. 1). Promoter prediction tools indicated that most promoter regions were located in the short fragments that contained partial coding sequences of two adjacent genes and their intergenic spacer region. Therefore, we concluded that the intergenic spacer regions were likely to contain the primary promoter fragments. We found that the primary promoter fragments of six cloned fragments (fragments 1, 5, 34, 35, 52, and 54) were the last short fragments (1-5, 5-5, 34-4, 35-3, 52-8, and 54-13), and the primary promoter fragments of fragments 2 and 71 were the second short fragment (2-2) and the first short fragment (71-1), respectively.

In some primary promoter fragments (1-5, 2-2, 5-5, 7-1, 35-3, 52-8, 54-13, and 132-1), the number of nucleotides between the start codon of the predicted gene and the start codon of the kanamycin resistance gene was a multiple of three, which suggested that the predicted gene and the kanamycin resistance gene were in the same reading frame; therefore the kanamycin resistance gene was likely to be

Table 3 Kanamycin resistance of the 12 original recombinant plasmids

Plasmid	Inserted fragment (bp)	Kanamycin resistance in *E. coli* Top10 in LB (μg/ml)	Kanamycin resistance in KKWB-10 in LB (μg/ml)	Kanamycin resistance in KKWB-10 in BSE (μg/ml)
pUCK1	1,334	4,500	7,000	1,000
pUCK2	597	4,000	7,000	800
pUCK5	1,749	3,500	6,500	800
pUCK7	199	4,000	5,500	900
pUCK19	199	4,000	5,500	900
pUCK34	662	4,000	5,500	600
pUCK35	708	4,000	5,500	700
pUCK42	662	4,000	5,500	600
pUCK52	1,503	3,500	5,000	500
pUCK54	3,894	4,000	7,000	900
pUCK71	770	3,500	5,500	500
pUCK132	611	4,500	7,000	1,000

E. coli Top10 clones were cultured on LB agar with increasing concentrations of kanamycin at 37°C for 24 h; *E. cloacae* KKWB-10 clones were cultured individually on LB and BSE agar with increasing concentrations of kanamycin at 30°C for 24 h

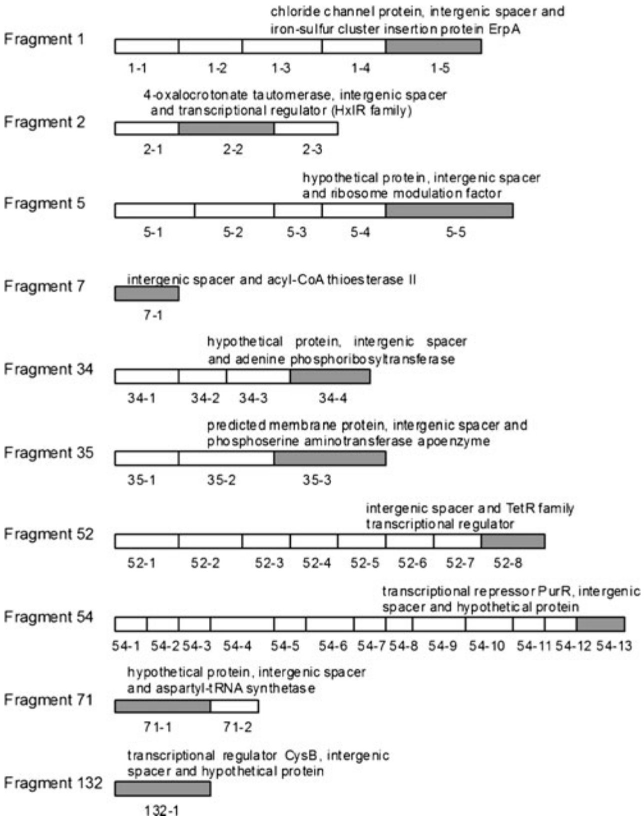

Fig. 1 Structures of 10 fragments isolated from the genome of *E. cloacae* KKWB-10. The predicted primary promoter fragments were shaded *gray*. The sequence characteristic were as follows: Fragment 1 consisted of five short fragments. 1-1: methyl-accepting chemotaxis sensory transducer; 1-2: asparaginyl-tRNA synthetase; 1-3: uncharacterized conserved protein; 1-4: anaerobic dimethyl sulfoxide reductase, A subunit; 1-5: chloride channel protein, iron–sulfur cluster insertion protein ErpA, and their intergenic spacer. Fragment 2 consisted of three short fragments. 2-1: peptide/nickel transport system ATP-binding protein; 2-2: 4-oxalocrotonate tautomerase, transcriptional regulator, HxlR family, and their intergenic spacer; 2-3: 1286-bp at 5′ end: transcriptional regulator, IclR family; 2,533 bp at 3′ end: Na/Pi-cotransporter. Fragment 5 consisted of five short fragments. 5-1: carboxypeptidase Taq; 5-2: flagellar basal body rod protein FlgF; 5-3: multidrug resistance protein MdtH; 5-4: high-affinity nickel transporter; 5-5: hypothetical protein, ribosome modulation factor, and their intergenic spacer. 7-1: intergenic spacer and acyl-CoA thioesterase II. Fragment 34 consisted of four short fragments. 34-1: Cl channel, voltage-gated family protein; 34-2: diguanylate cyclase/phosphodiesterase; 34-3: 997 bp at 5′ end: 5-(carboxyamino) imidazole ribonucleotide mutase; 355 bp at 3′ end: tRNA 2-selenouridine synthase; 34-4: hypothetical protein, adenine phosphoribosyltransferase, and their intergenic spacer. Fragment 35 consisted of three short fragments. 35-1: argininosuccinate synthase; 35-2: major facilitator superfamily MFS_1; 35-3: predicted membrane protein, phosphoserine aminotransferase apoenzyme, and their intergenic spacer. Fragment 52 consisted of eight short fragments. 52-1: putative U32 family peptidase; 52-2: MATE efflux family protein; 52-3: putative peptide/nickel transport system ATP-binding protein; 52-4: hypothetical protein; 52-5: DNA polymerase III subunit beta; 52-6: putative cation: proton antiport protein; 52-7: hypothetical protein; 52-8: intergenic spacer, and TetR family transcriptional regulator. Fragment 54 consisted of 13 short fragments. 54-1: fructose-6-phosphate aldolase; 54-2: hypothetical protein; 54-3: NADH dehydrogenase subunit M; 54-4: sensor histidine kinase, efflux transporter (MFP subunit) and their intergenic spacer; 54-5: ATP-dependent RNA helicase SrmB; 54-6: S-adenosylmethionine decarboxylase; 54-7: GntR family transcriptional regulator; 54-8: phosphoenolpyruvate carboxylase; 54-9: phospho-2-dehydro-3-deoxyheptonate aldolase, hypothetical protein, and their intergenic spacer; 54-10: citrate lyase, alpha subunit; 54-11: hypothetical protein; 54-12: cellulose synthase subunit BcsC; 54-13: transcriptional repressor PurR, hypothetical protein, and their intergenic spacer. Fragment 71 consisted of two short fragments. 71-1: hypothetical protein, aspartyl-tRNA synthetase, and their intergenic spacer; 71-2: hypothetical protein. 132-1: transcriptional regulator CysB, hypothetical protein, and their intergenic spacer

expressed as a fusion protein. But in the other two probable primary promoter fragments (34–4, 71–1), the number of nucleotides between the start codon of the predicted gene and the start codon of the kanamycin resistance gene was not a multiple of three, suggesting that the kanamycin resistance gene was likely to be expressed directly from the promoter.

Identification of primary promoter fragments of the eight complex fragments

The recombinant plasmids containing the eight short primary promoter fragments were denoted pUCK1-5, pUCK2-2, pUCK5-5, pUCK34-4, pUCK35-3, pUCK52-8, pUCK54-13, and pUCK71-1. The eight Top10 transform-

Table 4 Kanamycin resistance of 10 recombinant plasmids

Plasmid	Promoter fragment (bp)	Kanamycin resistance in *E. coli* Top10 in LB (μg/ml)	Kanamycin resistance in KKWB-10 in LB (μg/ml)	Kanamycin resistance in KKWB-10 in BSE (μg/ml)
pUCK1′-KT	207	4,000	6,500	1,000
pUCK2′-KT	199	4,000	6,000	800
pUCK5′-KT	570	5,000	7,500	1,200
pUCK7′-KT	118	3,500	5,000	700
pUCK34′-KT	187	3,500	5,500	700
pUCK35″-KT′	234	4,000	6,000	800
pUCK52′-KT	81	4,500	7,000	900
pUCK54′-KT	390	3,500	5,500	600
pUCK71′-KT	681	4,000	5,500	800
pUCK132a″-KT′	569	5,000	7,500	1,200

E. coli Top10 clones were cultured on LB agar plate with increasing concentrations of kanamycin at 37°C for 24 h; *E. cloacae* KKWB-10 clones were cultured individually on LB and BSE agar plate with increasing concentrations of kanamycin at 30°C for 24 h

ants (T-pUCK1-5, T-pUCK2-2, T-pUCK5-5, T-pUCK34-4, T-pUCK35-3, T-pUCK52-8, T-pUCK54-13, and T-pUCK71-1) were cultured on LB agar containing increasing concentrations of kanamycin to determine their resistance. The results showed that these eight Top10 transformants exhibited identical resistance to kanamycin with the original Top10 transformants (T-pUCK1, T-pUCK2, T-pUCK5, T-pUCK34, T-pUCK35, T-pUCK52, T-pUCK54, and T-pUCK71). These findings confirmed that the eight short fragments were the actual primary promoter fragments.

Identification of the optimal reading frame of the 10 promoter fragments

The 10 recombinant plasmids were denoted pUCK1′-KT, pUCK2′-KT, pUCK5′-KT, pUCK7′-KT, pUCK34′-KT, pUCK35′-KT, pUCK52′-KT, pUCK54′-KT, pUCK71′-KT, and pUCK132′-KT. After confirmation by sequencing, the Top10 and KKWB-10 transformants harboring these plasmids were individually cultured on LB or BSE agar containing increasing concentrations of kanamycin to determine their resistance (Table 4). We found that the promoter activity of the 10 fragments lacking the coding regions were not always consistent with that of the corresponding fragments harboring partial coding regions (Tables 3 and 4). The kanamycin resistance levels of some fragments increased slightly after removal of the partial coding sequence, perhaps because the extra peptide segment at the N-terminus suppressed function of the kanamycin resistance gene. The kanamycin resistance levels of other fragments decreased, perhaps because removal of the coding sequences also removed some promoter functional regions; however, the fragments without coding regions still possessed most of the promoter activity, suggesting that the kanamycin resistance gene was inserted into the optimal reading frame.

To further analyze these fragments, 10 recombinant plasmids harboring K′T (kanamycin resistance gene from which the start codon had been deleted) were constructed and denoted pUCK1′-K′T, pUCK2′-K′T, pUCK5′-K′T, pUCK7′-K′T, pUCK34′-K′T, pUCK35′-K′T, pUCK52′-K′T, pUCK54′-K′T, pUCK71′-K′T, and pUCK132′-K′T. After confirmation by sequencing, the kanamycin resistances of Top10 transformants harboring these plasmids were determined. The results showed that the kanamycin resistance levels of transformants harboring pUCK35′-K′T and pUCK132′-K′T were above 4,000 μg/ml kanamycin, but the resistance levels of the remaining eight strains were below 1,000 μg/ml kanamycin. This finding indicated that the kanamycin resistance gene in pUCK1′-KT, pUCK2′-KT, pUCK5′-KT, pUCK7′-KT, pUCK34′-KT, pUCK52′-KT, pUCK54′-KT, and pUCK71′-KT was primarily expressed directly from the promoter in the optimal reading frame, whereas the selectable marker in pUCK35′-KT and pUCK132′-KT did not appear to be expressed directly from the promoter in the optimal reading frame.

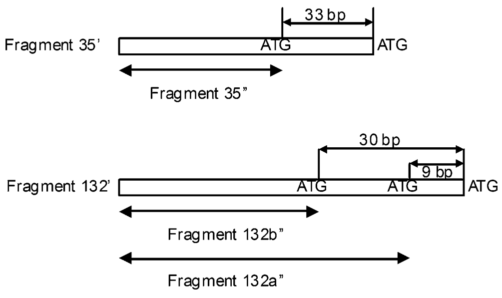

Fig. 2 Structures of fragment 35′ and fragment 132′

Fig. 3 The sequences of primary promoter functional fragments and their promoter domains, as predicated by BPROM and BDGP. Prediction result by BPROM: -10 regions are *underlined*, -35 regions are *double underlined*. Prediction result by NNPP: *gray shaded region*

1′:
GATCATTACCTGCCTCGGCGCGACACTATTAGCCCAGTTCCTGGGTGGAAAACCGCTATACTCCACCATCCTCGC
ACGTACCCTGGCGAAACAAGAGGCTGAACGGGCCGCCACGCAGAATACTTGAATGAATTACCAGGGTATTAGATA
ATGAGACAAAGAATTGGGTGAATTTTACCCAATAGCAGTATTCATGGGAGCATAAG
2′:
GATCAATACTGAACGCCGGAATTCAGGGTCGCCGTTTCCCTCAATTTCAACAATAAGGTCAGTCATACGACGTAGC
AGTTCTTTTTTTTGCTCCGAATTCATAATTCCATTAATGGTTTGCACATTTACGAAGGGCATTGTATTCTCCTTAAATA
ACTGAGTCTTTAAATCATACTCAGGTTACGAGGCAAACGAAGAG
5′:
GATCAGCAACTGCGCAACACGCTGGTGGCCAATCTGGGCAGTCAGCTTCCGGGCTGGGTTGTCGCGTCGCAGC
CGCTGGGTAACGACCAGGATACGCTTAACGTCACGGTAACGGGCTTCCACGGCCGTTATGACGGTGCGGTGGT
GATTAGCGGGGAGTGGTTGCTGAACCATCAGGGGCAGCTGATTAAGCGTCCTTTCCATCTGGAGCTGAAGCAGC
AGAAAGATGGCTATGACGAAATGGTGAAAGTCCTGGCTCAGGGTTGGGCACAGGAGTCGGCCAGCATCGCGAG
AGAAATTTCCCGGCTGCCATAAGTAAAATTCATCATTAAAAACCGCAGTTGGCCTCTCGCTGATTGCGGTTTTTTTT
CGTTTTCAGTTCAGTTTGTTACTTGTGCCACGTCACAATTTTTGTACAAATGAACTTCCAGCTAGCTCACAAATATG
ACACCCGTATGAATTTTGAACATTGACGATGCGACTGATTCGGGGTATTCGTTATCTGTGCCTGTGCATTTAGTGC
AGACACTGTTTTCTTTCCACCAGACAAAAGAATGAGGGAAACGAGGC
7′:
GATCACCGATTTATGCCTGAAAACGTGCCGATATTCAGATTATTCTACCCATCGGACCACTTTC*ATT*TCTGCGTTATA
CTCTGCCTATCTTTCGCTACGGCGTTTATTGAGGACAACT
34′:
GATCCCCGTGGTTGATGAAAAGCAACAAAAGCACTGAAGCCTTATCATGACTGTTGCAATTATTGCGCACAGCCA
GTAAATTCGACCGTTTTCGAGCACAGGTGCGCCTGGTCAAAGGTTAAACAATTGTTGCCTTGGCCGACTCGTTGC
GCGCTGTGAGTAACACTGTTTCATTTAGGCAAAACCG
35″:
GATCGTCAATTATGCCCCCGCTGTGCAAATCCGGAATGGACGAAGGTTAGCCGGGCCAAACGCATCACCTGACA
GCCATTTTGTCGGTTTTGTCTTAGCGAGGCGGCAGATTGTTTTGTGTGACGCGGGGCACATTTCACGGGTCATAA
GTTTTAGACATTGCGGCGCGTGTCACTGAATGATAAAACCGATATCCACAGTTATAACTTATGGCTTTTAGCGTGGT
GAGGGGAA
52′:
GATCTTTGACATTCCGCGTTGCAGCGTGTCAAAGCACGTGTTTCAATCAGGGTTTTTATAATTGACAGGGGGAAAA
ATTTA
54′:
GATCGCTGCCCAGACGGCGTTGCGCGTCTCTTCCGCCACAAAGCGGGTTTTGTTAATTACATGTGATACGGTTGT
AGTGGAAACGTTTGCGCGTTTTGCTACGTCTTTAATTGTTGCCATTAAATGTCACTCCAGACCATATCCTAAGCTCC
TGAAAAACTTGAAGGTAAACGTTTGCCTTCTCTCACCCTTATCTCGCAATGTTGAATTGCGGCACGCCGGGAAAA
CGACACGGGACGTCAGGAGGGGGTCAATGGCCGGTACGCTAATAAATTTAGCGTGGAATTTTGTCCTATCTTGAT
GAAAAGGGGAAGAGGTAAAGTGGTTATCAGTATAAATCCAGGAAGATTTTTGACGTAATCTGTGCAAAAATGAGCA
AGCTCACTTTTC
71′:
GATCGGTACCGTAAAAAGCGCCCCACTGGCGTTTGGTCACTTCAATATCGCTGTCGCGTTTGCCGAGTGCGGCC
GGGTACGTCCACCAGTTTTCCGGCAGGGCGCGTGCAGGAGCCTGGGCATCAACGGGCTGTTTTAACGCTTCGG
CGTAATCAGCGGACCAGCCGACTCGCACCATGACAACAGGCGCACCGCTGGCACGACATGTTTCCGCCAGGCG
AGCAGCGCGGTTGACCACATCATCCGCAGTATGCGGGCCACCGGCGAAGGGCAGAATGCCTTCCTGTAAATCAA
TCACAACAAGTGCGGTTTTACTGGCATCAAGTGTTAACATCGTTACTCCCGTTAAATGAATCACTTCCAGACACCAT
ACGACGTCATGGCCTGTTCCTGGTCGGATAATTTTGTTAATTTTTGTGAGAATGCGCAATAAGAGTGCAGCAAACG
ACCGCCTGGCCGTGGTTCGCTCTTATCGTCCGGCGGAATTTCCAGTATAATAGCCGCCTTTTTTCATCCAGTTGTG
ACATACAGAAAGCTGCGACATAGTAGCCTGCATACCAGGCGACATTTAGCCTGCGGCTAATTAAGGGATATCTC
132a″:
GATCCGGTGTCAGACCCTGACCTGGTGCGTCTTGATGCGCATGATGTTTTCAGTCATAGCACTACAAAGATTGGC
TTCCGTCGTAGCACCTTCCTCCGCAGCTATATGTATGATTTTATTCAGCGCTTTGCCCCTCATTTAACGCGTGACGT
GGTTGATACCGCCGTTGCATTACGCTCAAATGAAGATATTGAAGAGATGTTTAAAGACATCAAACTCCCCGAAAAAT
AATCACACCCGGTATCTTTCCCTTAGGGAAAGATACCGAAATAACGCCTCAAAGCTAAATTAGAATTATCATCATCTA
CGCATCCCCTGTCATATAATGTCATTATCCATTTAATCTGTAAGGTTATAATGTCGTGAATTGGCGACAAAAGTAGAA
ACTAATTTACGCCTTCGCAAATTTTTCTTTTCAATTATTTATTTCTGGTCAAAAGATTGAATATTTCATACCTTCCGGT
GCGTAAATTCACTGGCTTTTCGGCTAAAGTTTCTTTAGGATTTATCTCAACAGATGATTAATTGTACCAATTGTTTGG
TCCTAAATGATAAGCGATGTCGATTGT

Sequence analysis of fragment 35′ showed an ATG codon of the phosphoserine aminotransferase apoenzyme gene 33 bp upstream of the kanamycin resistance gene, and fragment 132′ contained two ATG codons of a hypothetical protein 9 and 30 bp upstream of the kanamycin resistance gene (Fig. 2). Because these ATG codons were in the same reading frame as the kanamycin resistance gene, they may have served as start codons to initiate translation of the selectable marker. Therefore the kanamycin resistance genes in pUCK35′-KT and pUCK132′-KT were likely expressed as fusion proteins. Additional experiments were carried out to further characterize the probable coding sequences and optimal reading frames of fragments 35′ and 132′. After deleting the probable coding sequences, the new fragments were denoted 35″, 132a″, and 132b″, respectively (Fig. 2). Six fragments were amplified with the primer pairs: 35-P1f and 35″r1, 35-P1f and 35″r2, 132f and 132a″r1, 132f and 132a″r2, 132f and 132b″r1, and 132f and 132b″r2. These fragments were individually ligated to fragment KT or K′T and inserted into pUC19 to generate six plasmids (pUCK35″-KT, pUCK35″-K′T, pUCK132a″-KT, pUCK132a″-K′T, pUCK132b″-KT, and pUCK132b″-K′T). The Top10 transformants were denoted T-pUCK35″-KT, T-pUCK35″-K′T, T-pUCK132a″-KT, T-pUCK132a″-K′T, T-pUCK132b″-KT, and T-pUCK132b″-K′T. After confirmation by sequencing, the kanamycin resistance of these Top10 transformants was determined as T-pUCK35″-KT (4,000 μg/ml), T-pUCK35″-K′T (500 μg/ml), T-pUCK132a″-KT (5,000 μg/ml), T-pUCK132a″-K′T (1,500 μg/ml), T-pUCK132b″-KT (1,500 μg/ml), and T-pUCK132b″-K′T (400 μg/ml) (Table 4). These results indicated that the kanamycin resistance gene in T-pUCK35″-KT and T-pUCK132a″-KT were primarily expressed directly from the promoter and in the optimal reading frame. The primary promoter sequences and predicted promoters of all 10 fragments lacking the 3′ end coding sequences are shown in Fig. 3.

Promoter activity testing of all fragments by GFP

The primary promoter fragments without the 3′ end coding sequences (1′, 2′, 5′, 7′, 34′, 35″, 52′, 54′, 71′, and 132a″) were individually ligated to a DNA fragment GT, and the recombinant fragments were individually inserted into plasmid pUCK7 to generate recombinant plasmids pUCK7-1′-GT pUCK7-2′-GT, pUCK7-5′-GT, pUCK7-7′-GT, pUCK7-34′-GT, pUCK7-35″-GT, pUCK7-52′-GT, pUCK7-54′-GT, pUCK7-71′-GT, and pUCK7-132a″-GT. These 10 plasmids were transformed into Top10 and KKWB-10. After culturing these strains harboring the recombinant plasmids in LB and BSE, GFP expression was assessed by Western blot and fluorescence was determined by fluorescence spectrophotometer. The Western blot result (Fig. 4) showed that GFP expression amounts of strains containing pUCK7-5′-GT and strains containing pUCK7-132a″-GT were higher than the others in both LB and BSE medium. The fluorescence intensity (Table 5) showed that fluorescence was strongest in Top10 and KKWB-10 strains containing pUCK7-132a″-GT in both LB and BSE medium and that was the second strongest in Top10 and KKWB-10 strains containing pUCK7-5′-GT, indicating that fragment 132a″ possessed the strongest promoter activity of all 10 fragments in vitro and fragment 5′ took second place.

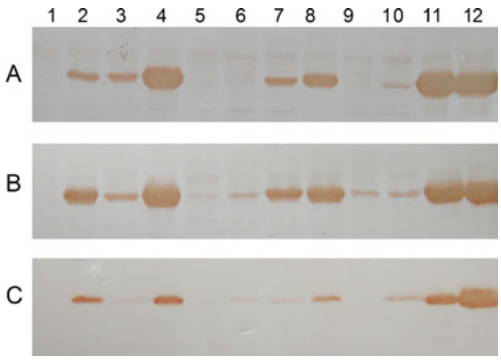

Fig. 4 Detection of the GFP reporter gene by Western blot. 1: pUCK7 (negative control); 2: pUCK7-1′-GT; 3: pUCK7-2′-GT; 4: pUCK7-5′-GT; 5: pUCK7-7′-GT; 6: pUCK7-34′-GT; 7: pUCK7-35″-GT; 8: pUCK7-52′-GT; 9: pUCK7-54′-GT; 10: pUCK7-71′-GT; 11: pUCK7-132a″-GT; 12: BL21-pET22b-gfp (positive control). *A*, plasmids transformed in *E. coli* Top10 and cultured in LB; *B*, plasmids transformed in KKWB-10 and cultured in LB; *C*, plasmids in *E. cloacae* KKWB-10 and cultured in BSE

Fluorescence observation of KKWB-10 harboring pUCK7-132a″-GT colonizing banana plants

Strain K-pUCK7-132a″-GT was chosen to inoculate the banana tissue culture plantlets. After 10 days of growth, freehand sections of the stems and roots were visualized by LSCM with laser excitation at 488 nm. Green fluorescence was observed in the sections of plantlets inoculated with K-pUCK7-132a″-GT (Fig. 5) but not in controls. To ensure that this observed strain was the *GFP*-expressing strain K-pUCK7-132a″-GT, the remaining tissue sections of these plantlets were used to isolate and identify strain K-pUCK7-132a″-GT. Bacteria isolated from the roots, corms, and pseudostems of these plantlets were cultured in LB and analyzed by PCR, which produced a 1,300-bp fragment confirmed as K-pUCK7-132a″-GT. In addition, the isolated strain emitted green fluorescence by UV excitation. These results indicated that fragment 132a″ had strong promoter activity when the host strain resided in banana plants and suggested its usefulness for engineering endophytic KKWB-10 bacteria.

Discussion

The engineering of bacteria requires well-characterized promoters. A number of papers have been published previously on *E. cloacae* promoters (Naas and Nordmann 1994; Deng and Shen 1995; Holguin and Glick 2001; Navarro-Lloréns et al. 2002; Martínez-García et al. 2003; Reisbig and Hanson 2004), and promoters from *E. coli* have been used in *E. cloacae*, such as the Km^{r} promoter (van Dijk and Nelson 2000), Tet^{r} promoter (Roberts et al. 2009), and *lac* promoter (Andreote et al. 2004). However, these promoters may not to be suitable for our purpose. We needed a promoter with strong activity in the *E. cloacae* KKWB-10 strain when it resides inside banana plants. Because such a promoter had not yet been reported, we screened the KKWB-10 genome to identify an appropriate promoter.

We used a promoter-probe plasmid to clone fragments from the KKWB-10 genome. Promoter-probe plasmids have been used to identify promoters from bacteria such as *Bacillus subtilis* (Donnelly and Sonenshein 1984), *Lactococcus lactis* (Jeong et al. 2006), *Corynebacterium*

Table 5 Standard fluorescence intensity of all strains expressing green fluorescent protein

Plasmid	Standard fluorescence intensity (RFU) in *E. coli* Top10 cultured in LB	Standard fluorescence intensity (RFU) in KKWB-10 cultured in LB	Standard fluorescence intensity (RFU) in KKWB-10 cultured in BSE
pUCK7-1′-GT	329±2.31	635±3.67	264±1.84
pUCK7-2′-GT	284±1.97	298±1.89	83±0.84
pUCK7-5′-GT	2238±5.83	2445±6.24	574±3.65
pUCK7-7′-GT	73±0.82	82±0.72	52±0.57
pUCK7-34′-GT	95±1.02	114±1.23	85±0.79
pUCK7-35″-GT	305±2.08	317±2.12	116±1.33
pUCK7-52′-GT	681±3.91	725±4.25	203±1.76
pUCK7-54′-GT	74±0.75	97±1.04	49±0.42
pUCK7-71′-GT	145±1.46	188±1.61	123±1.37
pUCK7-132a″-GT	3078±6.87	2672±6.43	652±3.82

All strains were cultured in LB and BSE medium with 50 μg/ml kanamycin for 12 h at 30°C. Results are expressed as the mean±standard deviation (n=10)

glutamicum (Patek et al. 1996), *Clavibacter xyli* subsp. *cynodontis* (Haapalainen et al. 1996), *Brevibacterium lactofermentum* (Cadenas et al. 1991), and *Pseudoalteromonas haloplanktis* (Duilio et al. 2004).

In the present study, the promoter-probe plasmid pUCK, which contains a promoterless kanamycin resistance gene, was constructed to identify strong promoters. *E. cloacae* KKWB-10 is sensitive to kanamycin; therefore, kanamycin could be used as a selective marker to identify strong promoters in this strain. Further, the screening of clones by gradually increasing the concentration of kanamycin in the agar plate is rapid and easy, whereas other reporter proteins require special devices or relatively complex procedures. However, the activity of an endophyte's promoters varies according to its environment. The nutrient level of the host banana plant is relatively poor compared with LB medium. Thus, promoter activity of KKWB-10 colonizing the host plant differs from that of KKWB-10 growing in LB medium. In this study, we used BSE medium to simulate the nutrient levels of banana plants, which simplified the screening of potential promoters. Promoter activity was lower in bacteria cultured in BSE medium than in bacteria cultured in LB medium, demonstrating the differential activity of endophyte promoters.

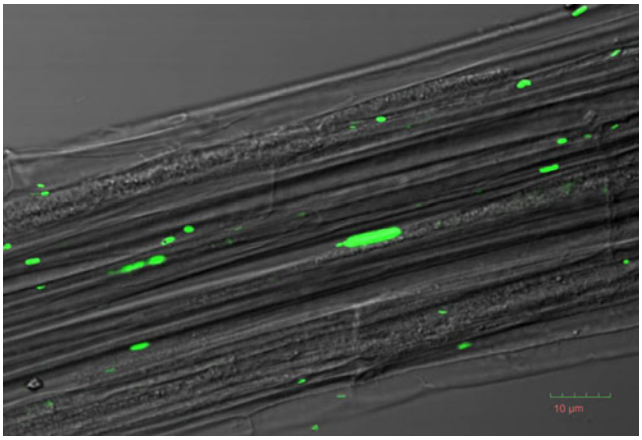

Fig. 5 Laser scanning confocal microscopy analysis of K-pUCK7-132a″-GT colonizing banana plants. Longitudinal section of a pseudostem; *arrows* point to K-pUCK7-132a″-GT cells. *Scale bar*=10 μm

In this study, 10 fragments exhibiting strong promoter activity were identified. However, promoter activity inside banana plants cannot be determined by kanamycin expression. Therefore, *gfp* was used as reporter gene in banana plants because its expression is easy to detect and quantify in plants (Dong et al. 2003; Iniguez et al. 2004; Compant et al. 2005). Analysis of fluorescence intensity and GFP expression in strains harboring these fragments indicated that fragment 132a″ had the strongest promoter function; therefore, strain K-pUCK7-132a″-GT was selected for the inoculation of banana plantlets. LSCM results verified that fragment 132a″ exhibited strong promoter activity in planta.

In conclusion, we constructed the promoter-probe plasmid pUCK to identify a strong promoter for biocontrol of banana fusarium wilt and isolated the promoter functional fragment 132a″ using the kanamycin resistance reporter gene in BSE medium. Fragment 132a″ exhibited strong promoter activity when the host bacterial strain KKWB-10 resided in banana plants, as assessed by LSCM. Therefore, fragment 132a″ appears to be suitable for expression of disease resistance genes in engineered endophytic KKWB-10 for the biocontrol of banana fusarium wilt. Furthermore, fragment 132a″ can be used as promoter in other engineered *E. cloacae* strains, and the GFP-expressing strain K-pUCK7-132a″-GT can be also used for the study of *E. cloacae* in host plants.

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