*J. Microbiol. Biotechnol.* (1999), **9**(3), 352–357

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY



# Molecular Cloning and Expression of Genes Related to Antifungal Activities from *Enterobacter* sp. B54 Antagonistic to *Phytophthora capsici*

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Received: July 28, 1998

**Abstract** *Enterobacter* sp. B54 inhibited growth of the fungus *Phytophthora capsici* on potato dextrose agar (PDA). Three mutants with antifungal activities (denoted M54-47, M54-113, and M54-329) which were lost or increased, through P1::Tn*5 lac* mutagenesis, were used to isolate genes responsible for fungal inhibition on PDA. Two clones were selected from the partially *Eco*R1-digested genomic library of the wild-type strain by probing with genomic flanking sequences of each mutant. We have isolated a 20-kb *Eco*R1 genomic DNA fragment from this strain that contains genes involved in hyphal growth inhibition of *P. capsici* on PDA. Subcloning and expression analysis of the above DNA fragment identified a 8-kb region which was necessary for antifungal activities. A 8-kb *Hin*dIII DNA fragment covers three genomic loci inserted by Tn*5 lac* in each mutant. This suggested that all genes which are related to antifungal activities might be clustered in simple forms of at least 5-8 kb sizes.

**Key words:** Antagonistic genes, Antifungal activities, *Enterobacter*, *Phytophthora capsici*, Tn*5 lac* mutagenesis

Bacteria which were shown or thought to have antagonistic ability by the production of antifungal substances occur in many genera, including *Agrobacterium, Bacillus, Enterobacter, Pseudomonas, Serratia,* and *Streptomyces* [1, 3, 7, 9, 11, 16].

Fungal damage to plants can be reduced by specific antagonistic microoganisms that inhibit the growth of one or more phytopathogenic fungi. Biological control of plant pathogens, widely known as biocontrol, is strongly correlated with the production of antifungal factors by the bacterial such as antibiotics, hydrolytic enzymes, and siderophores [4, 9, 14, 18]. A number of antagonistic microoganisms that protect plants from fungal disease also produce antibiotics on agar media.

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Some strains of *Enterobacter* are known to be potential biocontrol agents for different fungal pathogens [2, 7, 10, 15, 18]. Various traits, including the production of antibiotics, are involved in their biocontrol mechanism. In general, however, the antifungal ability of *Enterobacter* strains has not been as widely investigated as that of *Pseudomonas*. Cloning and expression of antifungal genes from *Enterobacter* spp. antagonistic to phytopathogenic fungi are also not well studied. Because most substances related to antifungal activities, except hydrolytic enzymes, result from the concerted action of many genes, it is not easy to clone for and reveal the expression of these genes. However, studies of cloned genes for antifungal substances are now providing much information that should allow the application of a combination of traditional and recombinant DNA methodologies, to develop a novel agricultural microorganism exhibiting antagonistic activities against various plant pathogenic fungi. Such clones can now also be used to test the idea that novel antibiotics could arise through the transfer of biosynthetic genes between other microorganisms producing different antibiotics [17].

In a previous paper, three mutants of *Enterobacter* sp. B54, which inhibits the growth of *Phytophthora capsici* on potato dextrose agar medium, had been reported. Two mutants with loss of antifungal activities and the other mutant with increase of activities have been selected by *in vitro* antagonistic assay after induction of Tn*5 lac* mutagenesis [20]. In this study, we report the successful cloning of a set of genes related to antifungal activities in *Enterobacter* sp. B54 with using these mutants and the expression of these genes in *E. coli*.

## Bacterial Strains and Plasmids

Bacterial strains and plasmids used in this study are summarized in Table 1. *E. coli* MC1061 was used as a host in the cloning experiment and the plasmid pBR322 (Amp$^r$, Tet$^r$) was used as a vector. *E. coli* strains and *Enterobacter* sp. B54 were grown on Luria-Bertani medium (pH 7.5) containing trypton (10 g), yeast extract (5 g), and NaCl (10 g) per liter at 38°C. *P. capsici* was grown on potato dextrose agar

**Table 1.** Bacterial strains and plasmids used in this study.

| Bacterid Strains and plasmids | Genetic characteristic | Reference or sources |
|---|---|---|
| Bacterial strains | | |
| *Enterobacter* sp. B54 | Antagonistic bacterium to *Phytophthora capsici* | RDA collection |
| *Escherichia coli* MC1061 | *hadR. haaM*$^+$, *ara*DB9(*ara-leu*) 7679(lac), 74 *galU*, *galK*, *rpsL* (str$^r$) | RDA collection |
| T413&T414 | Transformants carrying pAFY413 and pAFY414 | This study |
| T100 | Transformants carrying pAFY100 | " |
| T81 | Transformant carrying pAFY81 | " |
| *Phytophthora capsici* | Phytopathogenic fungus causing *Phytophthora* blight in red-pepper | RDA collection |
| Plasmids | | |
| pBR 322 | ColEl Amp$^r$ Tet$^r$ | RDA collection |
| pAFY 1135 | pBR322 containing flanking sequences with Tn*5 lac* in M54-47 | This study |
| pAFY 1135 | pBR322 containing flanking sequences with Tn*5 lac* in M54-113 | " |
| pAFY 3294 | pBR322 containing flanking sequences with Tn*5 lac* in M54-329 | " |
| pAFY 413 & 414 | pBR322 carrying 20-kb chromosomal DNA fragment related to antagonists of *Enterobacter* sp. B54 | " |
| pAFY 100 | pBR322 carrying 10-kb *Bam*HI DNA fragment from pAFY413 | " |
| pAFY 81 | pBR322 carrying & 8.1 kb *Hin*dIII DNA fragment from pAFY413 | " |

medium (Difco Co. Detroit, U.S.A.) at 28°C. Antibiotics were used at the following concentrations, unless described otherwise: ampicillin (Amp), 50 μg/ml; kanamycin (Km), 50 μg/ml; tetracycline (Tet), 12.5 μg/ml; chloramphenicol (Cat), 25 μg/ml; and streptomycin (Sm), 25 μg/ml.

## Fungal Inhibition Assays

Fungal inhibition assays were performed as described previously [20].

## Cloning of Flanking Sequences Concerned with Alterations of Antifungal Activities in Three Mutants

Although it was impossible to clone simultaneously bidirectional sequences flanked by the insertion sites of Tn*5 lac* on chromosomal DNA of mutants, because Tn*5 lac* sequences have a unique *Eco*R1 site, cloning of flanking sequences including an 8-kb Tn*5 lac* DNA fragment was very easy owing to a selectable kanamycin resistant marker. Each recombinant plasmid contained 14-kb insert DNA fragments in M54-47, and 17-kb insert DNA fragments in M54-113 and M54-329. This was consistent with the previous result that three *Eco*RI genomic DNA fragments carrying Tn*5 lac* of 14 kb, 17 kb, and 17 kb in sizes had been found in the hybridization with *Eco*RI-digested chromosomal DNAs of M54-47, M54-113, and M54-329, respectively [20]. These recombinant plasmids were designated as pAFY470, pAFY1135, and pAFY3294. This result suggests that mutant M54-47 contains the insertion of Tn*5 lac* next to the 6-kb *Eco*RI chromosomal DNA fragment, and the other two mutants, M54-113 and M54-329, have insertions of Tn*5 lac* next to two different 9-kb *Eco*RI chromosomal DNA fragments.

Physical mapping of these plasmids was performed by digestions with various restriction enzymes and analysis of the resulting fragments by agarose gel electrophoresis. Figure 1 shows that the intact flanking sequence excluding the 8-kb Tn*5 lac* fragment was a 6-kb chromosomal DNA fragment in pAFY470, and two different 9-kb chromosomal DNA fragments in pAFY1135 and pAFY3294. Because the 9-kb DNA fragments of pAFY1135 also include all 6-kb DNA fragments of pAFY470, the Tn*5 lac* of M54-113 must be located 3 kb downstream from the Tn*5 lac* insertion site of M54-47, and the orientation of the Tn*5 lac* insertion in M54-47 and M54-113 is identical. However, because the digestive patterns by the same restriction enzymes were different between the 9-kb *Eco*R1 DNA fragment of pAFY1135 and the 9-kb *Eco*R1 DNA fragment of pAFY3294, it is suggested that the Tn*5 lac* insertional orientation of M54-113 and M54-329 on chromosomal DNA may be opposite to each other, or the distance between the Tn*5 lac* insertional sites of the two mutants must be separated by at least a further 9 kb if insertional orientation is identical.

## Selection of Antifungal Genes from the Genomic DNA Library

To identify antifungal genes from chromosomal DNA fragments digested with various restriction enzymes, Southern hybridization was performed (Fig. 2). 5.4 kb of the $\alpha$-$^{32}$P labelled *Bgl*II DNA fragment excluding lactose-coding ORF sequences in pAFY3294 was hybridized with *Bam*HI, *Eco*RI, and *Kpn*1-digested chromosomal DNA fragments of *Enterobacter* sp. B54 because *Enterobacter* sp. B54 has lactose-utilizing ability originally. Antagonistic gene fragments showing homologies with the 5.4-kb *Bgl*II

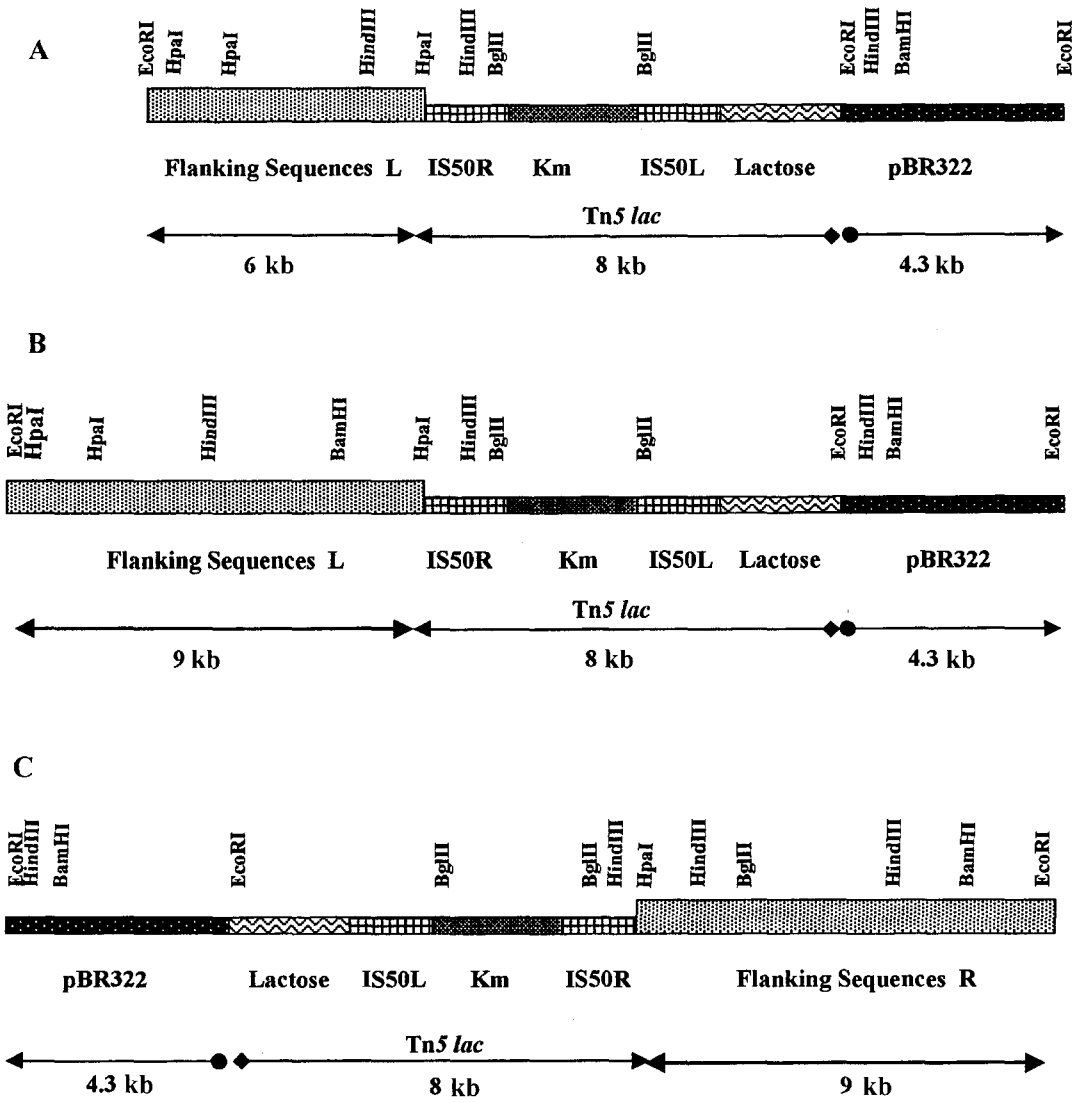


**Fig. 1.** Physical maps of pAFY470 (A), pAFY1135 (B), and pAFY3294 (C).
pAFY470: pBR322 carrying the *Eco*RI genomic loci flanked by an 8-kb Tn*5 lac* fragment from the M54-47 mutant of which fungal inhibition was more strengthened by Tn*5 lac* insertion. pAFY1135: pBR322 carrying the *Eco*RI genomic loci flanked by an 8-kb Tn*5 lac* fragment from the M54-113 mutant of which fungal inhibition was lost by Tn*5 lac* insertion. pAFY3294: pBR322 carrying the *Eco*RI genomic loci flanked by an 8-kb Tn*5 lac* fragment from the M54-329 mutant of which fungal inhibition was lost by Tn*5 lac* insertion.

flanking sequences are located at 10 kb in the case of *Bam*HI digestion and at 20 kb in the case of *Eco*RI and *Kpn*I digestions on agarose gel. As shown in lane 5 of Fig. 2, the location of antifungal genes was not in cryptic plasmid, but on chromosomal DNA of *Enterobacter* sp. B54. The pBR322-derived partial genomic library was constructed by cloning 9-22 kb chromosomal DNA fragments into a linear pBR322 vector with cohesive ends corresponding to *Eco*RI according to short-gun methods. Such pBR322-derived partial genomic library allowed selections of clones carrying antagonistic genes to high efficiency. Positive clones, which were hybridized with $\alpha$-$^{32}$P labelled on two different flanking sequences using a 4.0-kb *Hpa*I DNA fragment of pAFY470 and a 5.4 kb *Bgl*II fragment of pAFY3294, were selected from the *Eco*RI-genomic library by colony hybridization. Southern analysis of recombinant plasmids of selected clones indicates that they all contain the same 20-kb *Eco*RI chromosomal DNA insert although its insertional direction is opposite to each other (Fig. 3). they were designated as pAFY413 and pAFY414 according to orientation of the insert 20-kb DNA fragment.

Restriction enzyme maps were constructed to verify that the cloned sequences in pAFY413 and pAFY414 were homologous to the genomic loci bearing Tn*5 lac* insertions in M54-47, M54-113, and M54-329 (Fig. 4). When the physical map of pAFY413 was colinear with a physical map of M54-47, M54-113, and M54-329 deduced from Southern blot analysis, the alignment of restriction enzyme

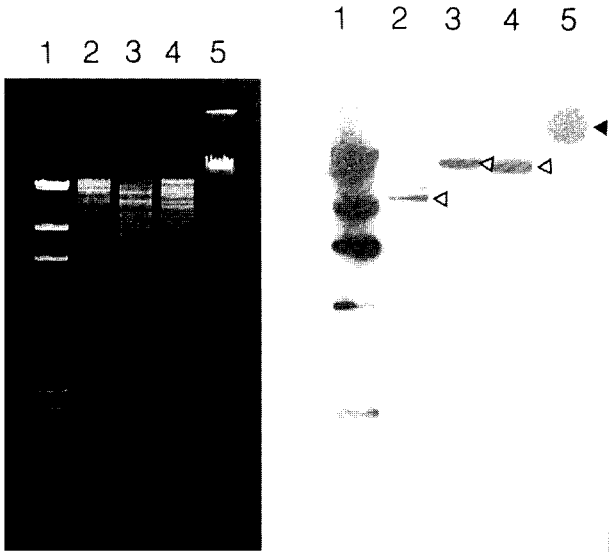


**Fig. 2.** Southern hybridization analysis of the antagonistic gene on chromosomal DNAs of *Enterobacter* sp. B54 digested with restriction enzymes.
The used probe is the 5.4-kb *Bgl*II DNA fragment of pAFY3294, which contains part of Tn*5* *lac* in the chromosomal DNA fragment related with the production of antifungal substances. 1: λ/*Hin*dIII, 2: B54/*Bam*HI, 3: B54/*Eco*RI, 4: B54/*Kpn*I, 5: B54.

sites in the genomic flanking sequences of the Tn*5* *lac* insertion in the three mutants indicated that two flanking 9-kb *Hpa*I/*Eco*RI fragments of pAFY1135 and pAFY3294 were contiguous in the genomic DNA as well as in the cloned parental DNA. A 20-kb *Eco*RI DNA fragment of pAFY413 included all flanking sequences of pAFY470, pAFY1135, and pAFY3294. Therefore, it is suggested that this 20-kb DNA fragment contains all or most of the genes involved in the biosynthesis of substance inhibiting the *in vitro* growth of *P. capsici* causing blight in red pepper.

### Location of the Tn*5* *lac* Insertion

It is important for the estimation of essential DNA regions responsible for antifungal activity to determine the Tn*5* *lac* insertion sites and the direction of the three mutants on the 20-kb *Eco*R1 chromosomal DNA fragment. Reciprocal comparative analysis for physical maps of pAFY470, pAFY1135, pAFY3294, and pAFY413 allowed to localize Tn*5* *lac* insertions on chromosomal DNA as shown in Fig. 5. Tn*5* *lac* insertion sites of M54-113 and M54-329, showing no inhibitory effects against *P. capsici* on PDA media, are located far downstream, 3 kb and 5 kb from the Tn*5* *lac* insertion site of M54-47, with stronger antifungal activity than that of the wild-type strain. This suggests that the size of essential genes related to changes of antifungal activities might be at least above 5 kb. Because most antifungal substances of secondary metabolites result from the concerted action of many genes, only a few cases of expression of the whole set of genes for the biosynthetic pathway have been reported [5, 6, 17, 19]. But, in recent years, Hammer *et al.* (1997) reported that the nucleotide sequences of the 6.2-kb region from *Pseudomonas fluorescens* contained a cluster of four genes responsible for the production of pyrrolnitrin, and also demonstrated that the transfer of the four gene clusters into *E. coli* resulted in the production of pyrrolnitrin [8].

### Subcloning and Expression of Antifungal Genes

pAFY100, pAFY81, and pAFY62 were constructed by subcloning on the basis of physical maps of pAFY413. *In vitro* fungal inhibition assays for five transformants, in

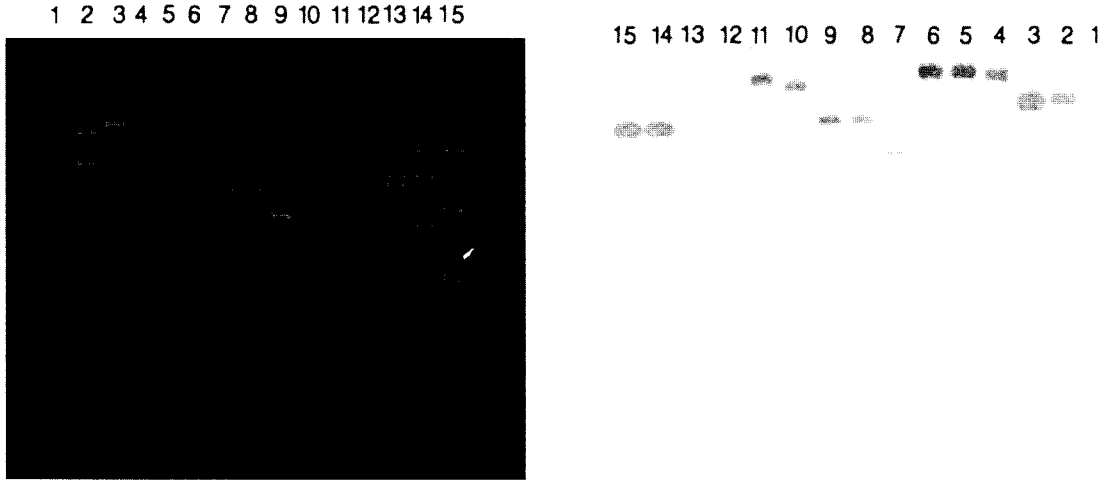


**Fig. 3.** Southern hybridization of pAFY413 and pAFY414 digested with various restriction enzymes to analyze regions of flanking sequences (probe: 5.4-kb *Bgl*II DNA fragment).
1: pAFY1135/*Bam*HI, 2: pAFY413/*Bam*HI, 3: pAFY414/*Bam*HI, 4: pAFY1135/*Eco*RI, 5: pAFY413/*Eco*RI, 6: pAFY414/*Eco*RI, 7: pAFY1135/*Hin*dIII, 8: pAFY413/*Hin*dIII, 9: pAFY414/*Hin*dIII, 10: pAFY1135/*Hpa*I, 11: pAFY413/*Hpa*I, 12: pAFY414/*Hpa*I, 13: pAFY1135/*Pst*I, 14: pAFY413/*Pst*I,15: pAFY414/*Pst*I.

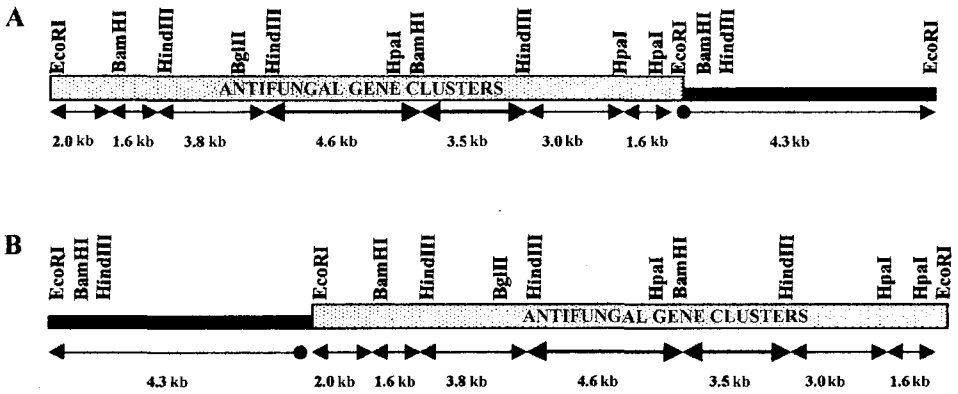


**Fig. 4.** Physical maps of pAFY413 (A) and pAFY414 (B), which contain gene clusters for the production of antifungal substances. *Kpn*I, *Xba*, and *Sma*I do not have recognition sites in the 20 kb antifungal gene clusters.

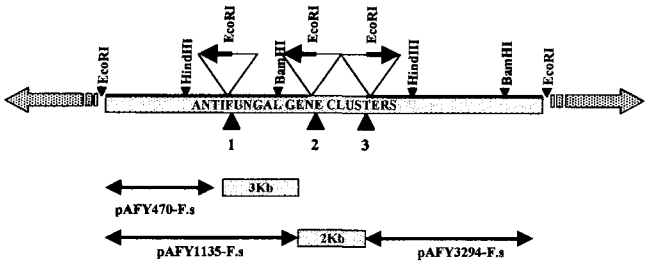


**Fig. 5.** Location and orientation of the Tn*5 lac* insertion on chromosomal DNA of M54-47 (1), M54-113 (2), and M54-329 (3) that showed changes of antagonistic activity to *Phytophthora capsici*.
*F.s: Flanking sequences.

which these recombinant plasmids were transformed into *E. coli* MC1061, were performed. The results are shown in Table 2 and Fig. 6. T413 and T414, which were transformed with pAFY413 and pAFY414, showed stronger antagonistic activity than that of the wild-type *Enterobacter* sp. B54. The reason for the above results might be due to the presence of an endogenous promoter in the 20 kb *Eco*RI DNA fragment and an increase of copy number. T81, obtained by subcloning, had maintained *in vitro* fungal inhibitory activity for long periods (at least 3 weeks), although it exhibited narrower fungal inhibition zones than *Enterobacter* sp. B54. This might be due to the partial absence of an endogenous promoter or operator in the 8.1 kb *Hin*dIII DNA fragment. *Enterobacter* promoters, especially those involved in secondary or unique metabolic pathways, might be recognized in *E. coli* because of the similarity of either a specific alternate sigma factor or another positive-activating factor. In the case of T100 and T62 for *in vitro* fungal inhibition assay, non-inhibitory activities of two transformants indicate that right-end regions of the 20 kb *Eco*RI DNA fragment might be nonessential for the production of antifungal substances. The endogenous promoter for the production of antifungal substances presumably is located near the left end of the 8.1 kb *Hin*dIII fragment of pAFY81 including some structural genes. At the moment, we do not have enough information on which genes are essential for the production of antifungal substances within pAFY81 or pAFY413, and how they are regulated. Therefore, structural analysis of antifungal metabolites produced by pAFY413 is required for

**Table 2.** Antagonistic activity assay of subclones with each recombinant plasmid.

| Recombinant Plasmid | Insert Fragment | Antagonistic Activity to *P. capsici* |
|---|---|---|
| pAFY414 | EcoRI HpaI HpaI HindIII BamHI HpaI HindIII BglII HindIII BamHI EcoRI | +++ |
| pAFY100 | BamHI HindIII BamHI | - |
| pAFY81 | HindIII HindIII | + |
| pAFY62 | BamHI BglII | - |

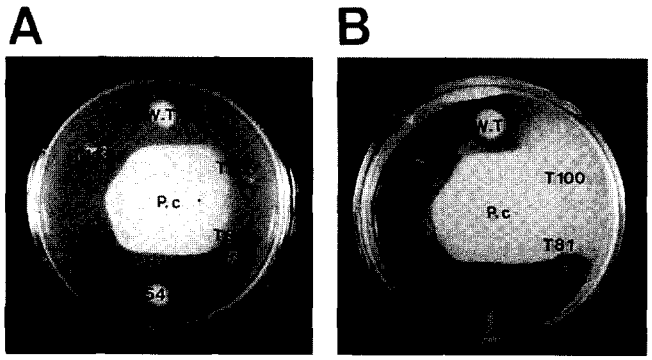


**Fig. 6.** *In vitro* fungal inhibition assays of *E. coli* MC1061 strains transformed by each recombinant plasmids.
A: One day after co-cultivation of both *Phytophthora capsici* and *E. coli* transformants on the same potato dextrose agar media. B: Three days after co-cultivation of both *P. capsici* and *E. coli* transformants on the same potato dextrose agar media.

identification of antifungal genes from *Enterobacter* sp. B54.

So far, numerous secondary metabolites have been isolated from bacteria, and the importance of antibiotic production as a mechanism of biological control has long been assumed [4]. Cloning of genes involved in antifungal metabolites biosynthesis has opened the possibility of designing superior biocontrol agents by incorporating desirable traits from several strains into a novel strain. Therefore, we believe that pAFY81 will provide a valuable gene sources for the development of a novel microorganism with various antagonistic spectra. Identification of primary structures of 8,100 bp of pAFY81, which is expected to include gene clusters essential for the production of antifungal substances, and the regulatory mechanism of genes for its metabolic pathway need to be unveiled in the near future.

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