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• BASIC RESEARCH •

Identification of *anrF* gene, a homology of *admM* of andrimid biosynthetic gene cluster related to the antagonistic activity of *Enterobacter cloacae* B8

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Abstract

AIM: To identify the gene (s) related to the antagonistic activity of *Enterobacter cloacae* B8 and to elucidate its antagonistic mechanism.

METHODS: Transposon-mediated mutagenesis and tagging method and cassette PCR-based chromosomal walking method were adopted to isolate the mutant strain (s) of B8 that lost the antagonistic activity and to clone DNA fragments around Tn5 insertion site. Sequence compiling and open reading frame (ORF) finding were done with DNAStar program and homologous sequence and conserved domain searches were performed with BlastN or BlastP programs at www.ncbi.nlm.nih.gov. To verify the gene involved in the antagonistic activity, complementation of a full-length clone of the *anrF* gene to the mutant B8F strain was used.

RESULTS: A 3 321 bp contig around the Tn5 insertion site was obtained and an ORF of 2 634 bp in length designated as *anrF* gene encoding for a 877 aa polyketide synthase-like protein was identified. It had a homology of 83% at the nucleotide level and 79% ID/87% SIM at the protein level, to the *admM* gene of *Pantoea agglomerans* andrimid biosynthetic gene cluster (AY192157). The Tn5 was inserted at 2 420 bp of the gene corresponding to the COG3319 (the thioesterase domain of type I polyketide synthase) coding region on B8F. The antagonistic activity against *Xanthomonas oryzae pv. oryzae* was resumed with complementation of the full-length *anrF* gene to the mutant B8F.

CONCLUSION: The *anrF* gene obtained is related to the antagonistic activity of B8, and the antagonistic substances produced by B8 are andrimid and/or its analogs.

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Key words: *Enterobacter cloacae* B8; Antagonistic mechanism; *anrF* gene; Andrimid biosynthetic gene cluster

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INTRODUCTION

Enterobacter cloacae strain B8 isolated from rice leaves was first recognized as an antagonistic bacterium of *Xanthomonas oryzae pv. oryzae*[1]. Its biological characteristics, antagonistic activity, and substances have been reported[2,3]. Satisfactory results have been obtained on anti-rice bacterial leaf brilliant in net house and in field[4]. Additionally, *E. cloacae* B8 is also antagonistic to many other plant bacterial pathogens such as *Pseudomonas*, *Agrobacterium*[3]. It has been reported that substances purified from *E. cloacae* B8 inhibit human (animal) bacterial pathogens such as MRSA[5,6].

Few but an *E. cloacae* strain EcCT-501R3 was reported to have antagonistic activity, which inhibits *Pythium ultimum* sporangium germination[7,8]. A competitive exclusion mechanism, competitive utilization of long-chain fatty acids of seed exudates, has been discovered and verified with transposon-mediated mutagenesis and tagging[9]. Previous researches on *E. cloacae* B8, however, showed that it produces antagonistic substances and differs from EcCT-501R3[1,3].

To elucidate the antagonistic mechanism of *E. cloacae* B8, transposon tagging[9-12] strategy was employed to clone DNA fragment related to its antagonistic activity. After a DNA fragment was cloned, cassette PCR-based chromosomal

walking[13-15] was used to get the flanked unknown DNA. We have previously reported the cloning and analysis of an antagonistic-related DNA fragment[16,17] and the verification of loss of antagonistic activity of mutant strains, by Southern blotting analysis, as a result of the insertion of a single copy of Tn5[18]. Here, we report the cloning and identification of the full-length *anrF* gene and the verification of its participation in the antagonism of B8.

MATERIALS AND METHODS

Bacterial strains, plasmids and chemicals

E. cloacae B8 (rif^r) is the antagonistic bacterium isolated from rice leaves in our laboratory[3,19]. *X. oryzae pv. oryzae* strain Xcom3104 (rif^r kan^r) was used as an indicator bacterium of antagonistic activity. The suicide plasmid pZJ25 (cm^r kan^r mob::Tn5) and its host *E.coli* S17-1 (thi pro hsdR$^-$ hsdM$^+$ recA RP4)[20] were gifts from Professor Jin-Sheng Wang of Nanjing Agricultural University, China. The cloning vector pBluescript SK+ (pBS) and its host *E. coli* DH5a were purchased from Stratagene. pFastBac^{TM}HTA was purchased from Invitrogen. pMD18-T vector was a product of Takara. Restriction enzymes, T4 DNA ligase, CIAP, *Taq* DNA polymerase, dNTPs, DNA marker DL2000, marker 3 kb, PCR product purification kit, etc., were supplied by Promega, Takara, Sangon and/or Zeheng. Oligonucleotides and primers used in this research were synthesized in Bioasia Biotech.

Transposon-mediated mutagenesis

The transfer of suicide plasmid pZJ25 from *E. coli* host S17-1 (donor) to *E. cloacae* B8 (receptor, rif^r) was mediated with conjugation[21]. Tranposonated B8 clones (rif^r kan^r cm^s) were selected on plates with rifampicin and kanamycin. The antagonistic characteristics were then checked using Xcom 3104 (rif^r kan^r) as an indicator[19].

Cloning of F fragment from B8F with tagging method

Genomic DNA of B8F, an antagonistic mutant strain of B8, was isolated, cut with *Bam*HI and ligated to *Bam*HI cut and dephosphorylated pBS. Recombinant clones were selected on plates with both kanamycin and ampicillin. Only one DNA fragment on the left of the insertion site was obtained with this step, as *Bam*HI cut Tn5 into two fragments and the intact *Kan^r* gene was in the 2.7 kb left fragment. The F fragment was a DNA fragment on the left of inserted Tn5 on B8F genome (Figure 1).

Cloning of DNA on the right of insertion site with chromosomal walking

Two sets of cassettes and primers were adopted in our chromosomal walking experiments. The *Pst*I cassette and its related CP primer were synthesized as previously described[12]. The *Sau*3AI cassette and its related CS primer and FS2, F1815 and F2000, the specific primers corresponding to known sequences, were newly designed and synthesized. They were as follows:

*Pst*I cassette:
5' HO-AGATTCGGTGCGTGCTTGACTGCA-OH 3'
3' HO-TCTAAGCCACGCACGAACTG-OH 5'
*Sau*3AI cassette:
5' HO-CTGTGGTGGTTCCGATGTATG-OH 3'
3' HO-ACCACCAAGGCTACATACCTAG-OH 5'
Cassette primers:
CP primer: AGATTCGGTGCGTGCTTGAC
CS primer: CTGTGGTGGTTCCGATGTAT
Specific primers:
FS2: GTTTACTGGCCAGTTATTGTTG
F1815: CCACAGAACGTCTTGTCAT
F2000: CGGAATGAAGAGGGTAAGG

Genomic DNA was extracted from B8 and partially digested with *Sau*3AI or completely digested with *Pst*I. DNA of 2-10 kb in length was recovered from gel and ligated to *Sau*3AI or *Pst*I cassette to construct cassette genomic libraries. Related cassette primer (CS or CP) and specific primer were used to amplify target DNA of unknown region from the libraries. The PCR conditions were as follows: pre-denaturation for 5 min at 94 ℃, and denaturation at 94 ℃ for 30 s, annealing at 52 ℃ for 60 s and extension at 72 ℃ for 5 min for 30 cycles, and a final extension at 72 ℃ for 10 min. Three rounds of PCR-based chromosomal walking, using *Pst*I and *Sau*3AI cassettes and related primers by turns, were performed to cover the full length of *anrF* gene (Figure 1).The full-length *anrF* gene was shown on the top (gray single line). The direction of the gene (narrow horizontal arrow, at right) and the Tn5 insertion site (thick vertical arrow) were marked. The long double line underneath stood for the F contig obtained in this research. The dark part on

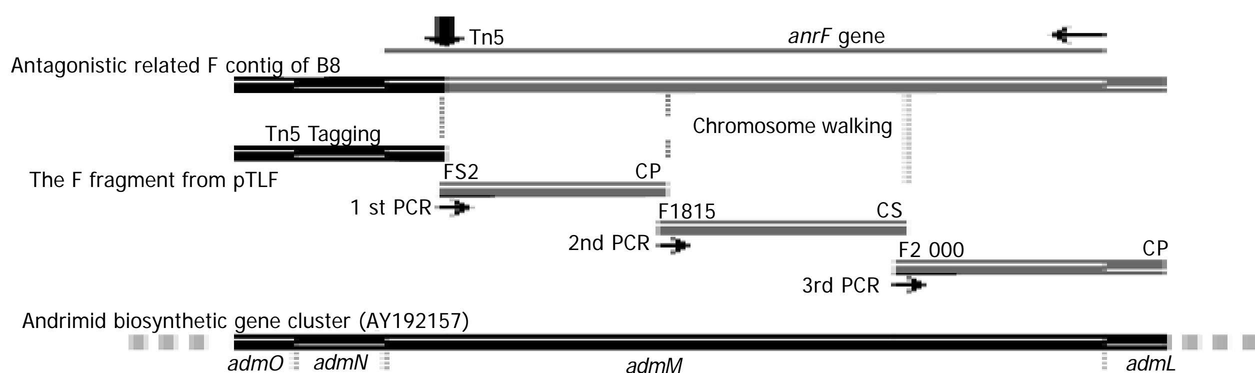

Figure 1 Framework of the cloning of full-length *anrF* gene with transposon tagging and chromosome walking.

the left of Tn5 insertion site (the F fragment) was obtained with transposon tagging. The gray part on the right was obtained with chromosomal walking. The four separate lines below were four steps (one tagging and three rounds of PCR) to get the contig. Primers were marked on top of the three gray lines. The black double line at the bottom showed the corresponding genes of *P. agglomerans* andrimid biosynthetic gene cluster (AY192157).

DNA sequencing and data analysis

The F fragment was sequenced, after subcloning of pTLF from M13 universal primer and P1 primer (CATGGAAG-TCAGATCCTGGA), a primer designed according to the sequence of the left edge of Tn5. PCR products of chromosomal walking were cloned into T-vector and sequenced using M13 universal primer. The DNA sequencing was done in Bioasia Biotech, Shanghai. DNA sequences obtained were edited, compiled, translated, and compared using DNAStar program. Homologous sequence and putative-conserved domain searches were done with BlastN and/or tBlastX and/or BlastP program at www.ncbi.nlm.nih.gov.

Continuity analysis of anrF gene in original B8 and its mutant B8F strain

Two primers were designed to amplify the full-length *anrF* gene. The upstream primer F21 (TTTTGTTGATGGAA-AGTCGCA) was located 144-126 bp up of the start codon. The downstream primer F2600 (CACCCAGCTGA-TGAAGTAAT) was located on opposite strand 73-56 bp down of the stop codon. The DNA fragments between the primers in the genomes of B8 and B8F were about 2 851 bp and 8.3 kb respectively. Genomic DNA of B8 or B8F was used as template. After 30 cycles of amplification, 6 μL of PCR products were applied to agarose gel electrophoresis to check the amplification. The amplified products were cloned and sequenced.

Verification of anrF gene participation in antagonism of B8

The full-length *anrF* gene amplified from B8 was cloned into pMD18-T vector and the resulting recombinant plasmid was designated as pMD-F. Gentamicin-resistance gene (*Gen^r^*) was amplified with two primers (acc1-up: CGTGGAAACG-GATGAAG, acc1-down: ACCTGGCGGCGTTGTGACA) from pFastBac^{TM}HTA and cloned into pMD18-T too. The resulting recombinant plasmid was designated as pMD-G. The *Gen^r* gene was recovered from pMD-G with *Hin*dIII and *Bam*HI double cut and filled with klenow and inserted into *Xba*I cut and klenow filled pMD-F. The target plasmid with *Gen^r* and the full-length *anrF* gene was designated as pMD-FG.

Mutant *E. cloacae* B8F was cultured to an A_{600} about 0.6 in LB medium, and washed thrice in cold glycerol buffer. Plasmid pMD-FG was transformed into B8F with electroporation. The transformed bacteria were screened on plates with both ampicillin and gentamicin and verified by PCR amplification of *anrF* and *Gen^r* genes. The pMD-FG transformed strains were then inoculated on Wakimoto agar and cultured at 30 ℃ for 48 h, the resume of antagonistic characteristics was checked with the indication of *X. oryzae pv. oryzae* strain Xcom3104.

RESULTS

Transposon-mediated mutagenesis

The suicide plasmid pZJ25 was transferred into *E. cloacae* B8 by conjugation with the help of donor *E. coli* S17-1. The conjugation and transposition ratio was about 5×10^{-6}. A total of 1 500 transposed colonies (Rif^r Kan^r Cm^s) were obtained and two antagonistic mutant strains, one of which was designated as B8F, were selected.

Cloning of F fragment from B8F with tagging method

A recombinant plasmid designated as pTLF, a plasmid with intact *Kan^r* gene of Tn5 and a DNA fragment on the left of the insertion site (designated as F fragment), was isolated. The pTLF had two foreign *Bam*HI fragments, one of which was about 3.5 kb in length and contained the target F fragment and a fragment of Tn5 (with the *Kan^r* gene, about 2.7 kb in length). After subcloning, the 735 bp sequence of the F fragment was obtained with M13 universal sequencing primer and P1 primer.

Cloning of DNA fragments on the right of Tn5 insertion site with chromosomal walking

Only one DNA fragment on the left of insertion site was obtained with transposon tagging method. In order to know the full-length gene interrupted by Tn5 insertion, cassette PCR-based chromosomal walking method was adopted to clone DNA fragments on the right of insertion site. The framework of the cloning of the full-length *anrF* gene with transposon tagging and chromosomal walking was shown (Figure 1). With the use of specific primers (FS2, F1815, or F2000) and cassette primers (CP or CS) by turns, DNA fragments of unknown region were amplified from related cassette (*Pst*I or *Sau*3AI) libraries. The PCR products from three different rounds of chromosomal walking were obtained (Figure 2). They were about 1.2 kb in *Pst*I library (1st walking), about 950 bp in *Sau*3AI library (2nd walking), and about 900 bp in *Pst*I library (3rd walking), respectively. The total sequence obtained with chromosomal walking was 2 586 bp.

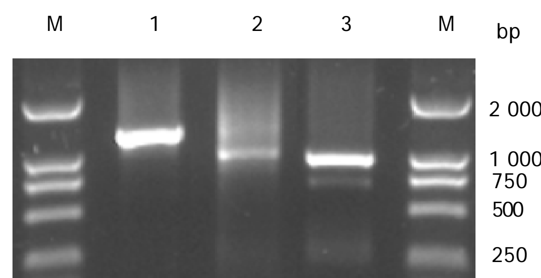

Figure 2 PCR products from three rounds of chromosomal walking. 1: PCR products, 1 209 bp in size, amplified from B8/*Pst*I-cassette library with FS2 and CP primers; 2: PCR products, 962 bp in size, amplified from B8/*Sau*3AI cassette library with F1815 and CS; 3: PCR products, mainly 900 bp in size, amplified from B8/*Pst*I cassette library with F2000 and CP; M: marker DL2000.

Sequence analysis of full-length anrF gene

A contig of 3 321 bp in length was obtained by transposon tagging and three rounds of chromosomal walking. Four ORFs, two complete and two partial were identified. One ORF, 2 634 bp in length, disrupted by the insertion of Tn5

in B8F was designated as *anrF* gene (accession no. AY633625) encoding for a 877 aa polyketide synthase-like protein. The Tn5 insertion site was at 2 420 bp of the *anrF* gene, and was at 214 bp before the stop codon.

BlastN search showed that the nucleotide sequence of *anrF* gene had a homology of 83% to that of *admM* gene of *P. agglomerans* andrimid biosynthetic gene cluster (AY192157). The alignment of *anrF* and *admM* genes is shown in Figure 3.

BlastP search showed that the deduced amino acid sequence encoded by the *anrF* gene was homologous to AdmM (79%

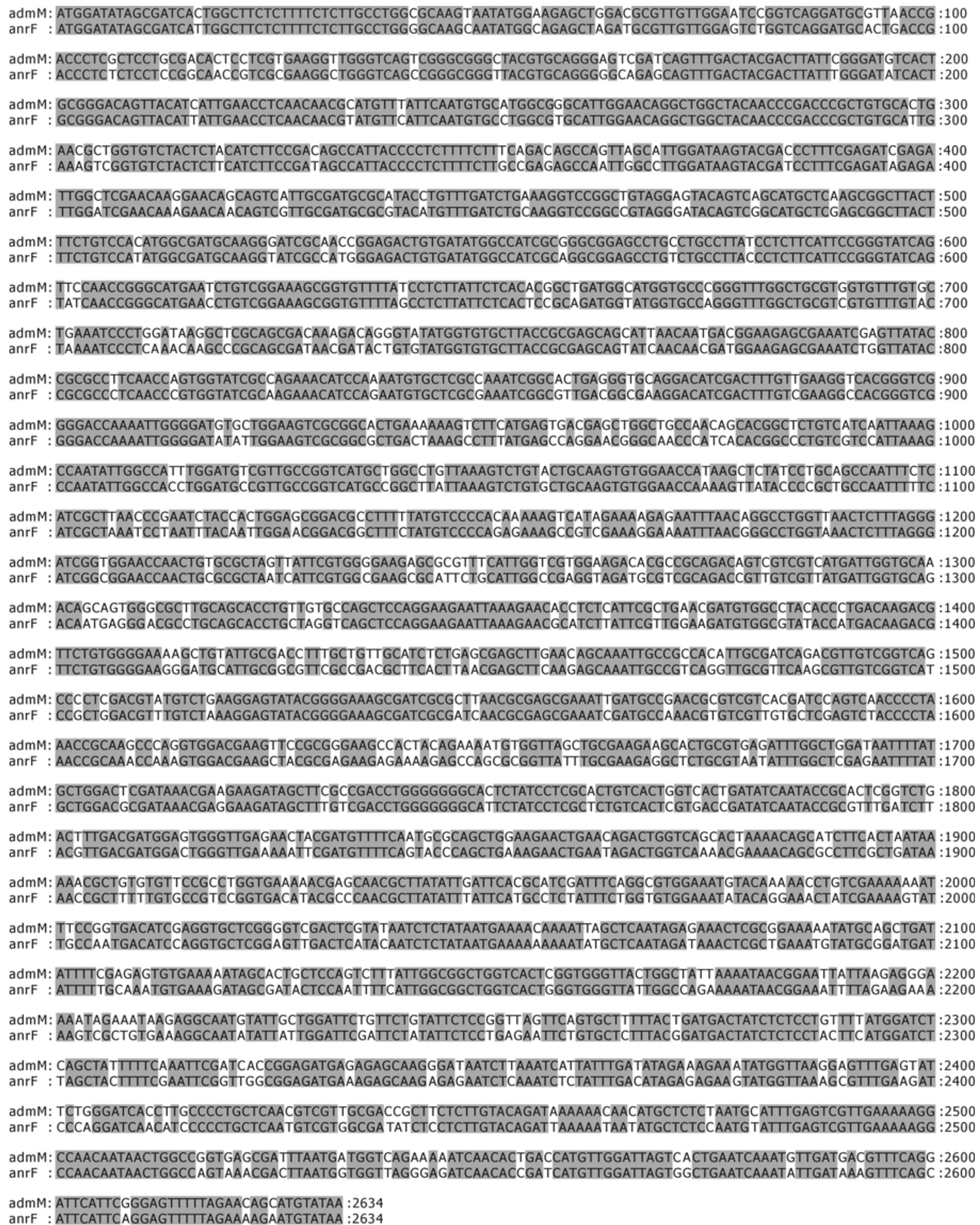

Figure 3 Alignment of nucleotide sequences of *anrF* gene and *admM* gene of *P. agglomerans* andrimid biosynthetic gene cluster (AY192157). The names of the genes are typed at the left and the number of nucleotides is at the right. The identical nucleotides between two genes are marked with gray background.

ID/87% SIM) and many other proteins of polyketide/non-ribosomal peptide synthetase or polyketide synthase (type I) modules (about 28-38% ID/45-58% SIM). Conserved domain search showed that the N-terminal of the protein (about 1-410 aa) encoded for a polyketide synthase module (COG3321) or animal-type fatty-acid synthase (KOG1202) or ketoacyl synthase (pfam00109 and pfam02801) or 3-oxoacyl-(acyl-carrier-protein) synthase (FabB or COG0304, and KOG1394)-like domain. The middle sequence (about 550-610 aa) encoded for a phosphopantetheine attachment site (pfam00550)-like domain. The C terminal (about 640-877 aa) encoded for a thioesterase of type I polyketide synthase (COG3319, pfam00975, and COG3208)-like domain, which was disrupted by the insertion of Tn5 in B8F.

Continuity analysis of anrF gene in original B8 and its mutant B8F strain

With F21 and F2600 as primers, no band was amplified in the reaction mixture using B8F genomic DNA as template, while a PCR product 2.8 kb in length, as expected, was observed in that of B8 (Figure 4). The 2.8 kb PCR product of B8 was further cloned and sequenced. The sequence result conformed the continuity of the *anrF* gene in B8.

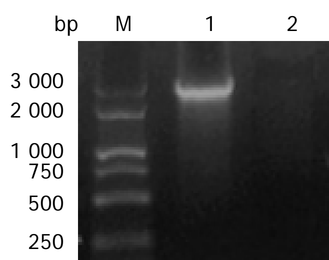

Figure 4 PCR amplification of full-length *anrF* gene. 1: PCR products amplified from B8; 2: PCR products amplified from B8F; M: marker 3 kb.

Verification of anrF gene participation in antagonism of B8

The pMD-FG, a plasmid with the full-length *anrF* and *Gen*^{r} genes, was constructed and transformed into the mutant *E. cloacae* B8F. The antagonistic activity of pMD-FG transformed B8F against *X. oryzae pv. oryzae* was then resumed, though the antagonistic ring was a little bit small and ambiguous (Figure 5).

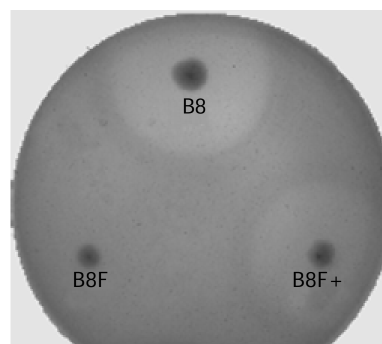

Figure 5 Antagonistic activity of B8, B8F and B8F/pMD-FG. B8F+: pMD-FG transformed B8F.

DISCUSSION

E. cloacae B8 is an antagonistic bacterium isolated from rice leaves, using *X. oryzae pv. oryzae* Xcom3104 as an indicator[1]. It has a strong antagonistic activity against *X. oryzae pv. oryzae* and satisfactory results have been obtained on anti-rice bacterial leaf brilliant in net house and in field[4]. Further researches showed that *E. cloacae* B8 and its antagonistic substances are also antagonistic to many other plant bacterial pathogens such as *Pseudomonas*, *Agrobacterium*[3], and human (animal) bacterial pathogens such as MRSA[5,6]. *E. cloacae* B8 and its antagonistic substances can be potentially used for the control of the rice bacterial leaf brilliant and many other bacterial diseases of plants, animals and humans.

It was reported that *E. cloacae* EcCT-501R3 has an antagonistic activity[7,8]. Two antagonistic-related genes for linoleic acid utilization, *fadB* and *fadL*, have been cloned using transposon-mediated mutagenesis and tagging. Further researches have verified that *E. cloacae* EcCT-501R3 inhibits *Pythium ultimum* sporangium germination by a competitive exclusion mechanism, the competitive utilization of long-chain fatty acids of seed exudates[9].

Our previous work, however, showed that B8 has an antagonistic mechanism different from what has been reported[1,3]. To elucidate the antagonistic mechanism and to clarify the antagonistic substances of *E. cloacae* B8, transposon tagging strategy[9-12] was employed to clone genes related to its antagonistic activity. With suicide plasmid pZJ25 carrying Tn5, two antagonistic mutant strains, one of which was named B8F, were selected. Tagging with *Kan*^{r} gene of Tn5, the F fragment on the left of Tn5 insertion site was cloned in pTLF. Then, cassette PCR[13-15]-based chromosomal walking was applied to the amplification of unknown DNA fragments on the right of Tn5 insertion site. A contig of 3 321 bp in length was obtained after three rounds of chromosomal walking together with the F fragment from transposon tagging. One complete ORF of 2 634 bp in length, disrupted by the Tn5 insertion in B8F, was identified in the contig. It encoded for a 877 aa polyketide synthase-like protein and was designated as *anrF* gene. The Tn5 insertion site was at 2 420 bp of the *anrF* gene, and was at 214 bp before the stop codon, suggesting that the loss of antagonistic activity of mutant strain B8F was a result of the knock-out of *anrF* gene by Tn5.

To confirm this theory, the continuity of the *anrF* gene in the original B8 and its mutant B8F strains was checked. The complete *anrF* gene 2.8 kb in length was amplified from the B8 genome. The sequence result further confirmed the continuity of *anrF* gene in the original B8. The length of the DNA fragment between the primers in the genome of B8F was about 8.3 kb. However, no band was amplified from B8F. As the parameters set for amplification were suitable for amplification fragments 2.8 kb or less in length but not for fragments up to 8.3 kb.

In addition, plasmid pMD-FG with full-length *anrF* and *Gen*^{r} genes was constructed. After pMD-FG was transformed into B8F, a mutant strain of B8 knocked out by Tn5-mediated mutagenesis, the antagonistic

characteristics were resumed, indicating that the *anrF* gene participated in the production of the antagonistic activity of B8.

BlastN search showed that the nucleotide sequence of the *anrF* gene had a homology of 83% to that of the *admM* gene of *P. agglomerans* andrimid biosynthetic gene cluster (AY192157). BlastP search showed the deduced amino acid sequence encoded by the *anrF* gene was homologous to the AdmM (79% ID/87% SIM) and many other proteins of polyketide synthase/non-ribosomal peptide synthetase (about 28-38% ID/45-58% SIM). Domain search showed that the N-terminal of the protein encoded for a polyketide synthase module (COG3321) or animal-type fatty-acid synthase (KOG1202)-like domain. The middle sequence encoded for a phosphopantetheine attachment site (pfam00550)-like domain. And the C terminal encoded for a thioesterase of type I polyketide synthase (COG3319)-like domain, which was disrupted by the insertion of Tn5. It was this insertion that led to the loss of antagonistic activity of B8F, suggesting that the protein encoded by *anrF* gene is a polyketide synthase similar to AdmM of *P. agglomerans*, and that the antagonistic substances produced by B8 are andrimid and/or analogs of andrimid. This result is consistent with our early observations[1,3].

Andrimid and its analogs are antibiotic substances of the polyketide (pyrrolidinedione) family[22]. Only three different strains of bacteria, other than B8, have been reported to produce these substances so far. Frenenhagen *et al*.[23], first reported the identification of andrimid, a new peptide antibiotic produced by an intracellular bacterial symbiont isolated from a brown planthopper. It has been reported that andrimid exhibits a moderate activity against *Bacillus sp*. and a very good activity against *X. campestris*, an early name of the pathogen of bacterial blight in rice plants[23]. Needham *et al*.[24], reported that andrimid and Moiramides A-C are produced in culture by a marine isolate of *Pseudomonas fluorescens*. Singh *et al*.[25], further showed that andrimid secreted from this strain exhibits antibacterial activity against both Gram-positive and Gram-negative bacteria. Oclarit *et al*.[26], found that anti-*Bacillus* peptide antibiotic, an andrimid in the marine sponge, *Hyatella* species, is produced by an associated *Vibrio* species bacterium. Freiberg *et al*.[22], reported that andrimid and its analogs act as selective inhibitors of bacterial acetyl-CoA carboxylase.

However, no genes related to the production of andrimid are published so far, except for the andrimid biosynthetic gene cluster of *P. agglomerans* (AY192157). The complete genome of AY192157 is 28 485 bp in length and encodes for 21 genes, from *admA*, *admB* to *admU*. The *anrF* gene cloned in this research has a homology of 83% to *admM*. Furthermore, the arrangement and the sequences of other ORFs or non-coding regions around the *anrF* gene have shown that the F contig obtained in this research is an equivalent of a part of AY192157 (data not shown). *P. agglomerans* (formerly *Erwinia herbicola*) is a closely related but nonpathogenic bacterium of *E. amylovora*[27]. It usually accompanies *E. amylovora* in the wild and produces a family of antibiotics[28-30] to inhibit fire blight, a devastating disease of rosaceous plants such as apple and pear caused by the latter bacterium. *E. cloacae* B8 has been isolated from rice leaves as an antagonistic bacterium of *X. oryzae pv. oryzae*. However, both *E. cloacae* and *P. agglomerans* are members of the *Enterobacteriacea* family. Whether this is the reason why the *anrF* and *admM* genes have a high similarity, or whether the equivalents of andrimid biosynthetic genes are similar or identical in bacteria other than *Enterobacteriacea* family, including *Pseudomonas* and *Vibrio* remains to be studied.

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