浙 江 农 业 大 学 学 报 18(4): 115～119, 1992
Acta Agriculturae Universitatis Zhejiangensis

A Drug Resistant Mutant of Antagonistic Bacterium *Enterobacter cloacae B8*①

Xu Ping, Chen Weiliang, Zhu Weiguang, Li Debao
(Institute of Biotechnology, Zhejiang Agricultural University, Hangzhou China 310029)

Abstract To study colonization and antagonistic action of *Enterobacter cloacae* B8 on rice leaves in field, a drug resistant mutant BX8 from B8 was obtained by natural selection and Tn5 mutagenesis. BX8 was able to grow in LB medium containing rifampicin up to 400μg/ml and kanamycin up to 300μg/ml. Our experiment results showed that the generation of drug resistance did not decrease its antagonistic activity. The drug resistance of BX8 was stable within at least 60 generations when it was cultured in Rf and Km free medium.

Key words antagonisn, drug resistance; Tn5 mutagenesis; *Enterobacter cloacae* B8; *Xanthomonas oryzae pv. oryzae.*

徐 平、陈卫良、朱伟光、李德葆(浙江农业大学生物技术研究所,杭州 310029)
拮抗细菌 *Enterobacter cloacae* B8 的一株抗菌素抗性突变体. 浙江农业大学学报, 1992,18(4):115～119.

摘 要 为了研究拮抗细菌 *Enterobacter cloacae* B8 在野外水稻叶面上的定殖情况及拮抗作用,由自然选择和 Tn5 诱变从 B8 产生了一株抗菌素抗性突变体 BX8. BX8 能在含利福平达 400 μg/ml 或卡那霉素达 300 μg/ml 的 LB 培养基中生长. 实验表明该抗菌素抗性的产生没有降低 BX8 的拮抗活性. 该抗性比较稳定,在无抗菌素培养基中培养,BX8 至少分裂生长 60 代内抗性不变.

关 键 词 拮抗作用; 抗菌素抗性; 定殖; Tn 5; *Enterobacter cloacae* B8; *Xanthomonas oryzae* pv. *oryzae*
中图分类 Q 933

The use of antagonistic microorganisms is a potential method for biological control of plant diseases. Several reports have shown that this method can work well for different diseases[1]. Much more attention has been paid since it is able to minimize pesticide residue and environment pollution now.

Transposable elements that encode drug resistance are becoming important tools in biotechnological research. The conventional chemical or radiation mutagenesis results in a nonselectable phenotype and is not able to transfer foreign genes. But transposable elements can be used to orientate genes, elucidate gene functions and transfer foreign genes into bacteria[2]

Received: 1991-11-21
①国家 863 课题资助

Many antagonistic bacteria from rhizosphere, phyllosphere, atmosphere and other places have been isolated in this laboratory. To determine antagonistic activity of certain bacteria on rice leaves in field and to study antagonistic gene functions and foreign gene transformations, *E. cloacae* B8, one of the strongest antagonistic bacteria isolated, was selected for drug resistant mutants by natural selection and Tn5 mutagenesis.

1 Materials and Methods

1.1 Organisms and plasmids

An antagonistic bacterium *E. cloacae* B8 was isolated from rice leaves in this laboratory and cultured at 28℃. The indicator strains *Xanthomonas oryzae* pv. *oryzae* were from Professor Huang Danian (CNRRI, Hangzhou). *Escherichia coli* S17-1(RP4-2-Tc::Mu-Km::Tn7) and plasmid pSUP1011(pACYC184::Tn5 $Km^{[r]}$ $Cm^{[r]}$ were a kind gift of professor Hong Menmin , (Institute of Plant Physiology, Academic Sinica, Shanghai). *E. coli* was cultured at 37℃ in the experiment. The viable count of bacteria was performed as described by Schlegal (1986)[3].

1.2 Selection of B8 mutant for Rf resistance and bacterial matings

B8 was cultured in LB broth (from Sigma) containing Rifampicin(Rf). After successive culture with increasing concentration of Rf, B8 was spread on LB agar plates with gradient Rf[4] from 0 to 100μg/ml. The colonies that appeared near the location of high level Rf were isolated. They were then cultured in LB medium over night and spread on LB agar plates containing Rf(100μg/ml). The steps were repeated several times until the resistance to Rf was stable .

Conjugative transfer of plasmid pSUP1011 from *E. coli* to B8 was performed as described by Chatterjee(1980)[5]. All matings were carried out on sterile 0.45μ cellulose filters.

1.3 Determination of the drug resistance and its stability

Bacteria with or without drug resistance were cultured over night in LB with or without Rf (100μg/ml) and Km(50μg/ml), respectively. To make the amount of inoculation equivalent for comparing with each other, both cultures were measured for OD_{600} and diluted with fresh LB to make them equal. Then 10 ml LB media with different concentrations of Rf or Km were inoculated and incubated in shaker at 28℃. The viable bacteria were counted and the number of divisions(n) were determined by n=(lgN-lgNo)/lg2(3).

To determine the stability of the drug resistance, 10ml LB without drugs was ino-culated with 10μl of the mutant culture and was cultured for 12 hrs. Then the number of divisioms was determined and the culture was inoculated to fresh LB for further 12 hrs growth. These steps were repeated several times. Finally , the culture was diluted and spread on LB agar plates with or without 100μg/ml Rf and 50 μg/ml Km. The colonies on the plates were counted after 24 hrs.

1.4 Antagonistic test

The test bacteria were inoculated on PSA[6] plates and incubated at 28℃ for 24 hrs. They were then killed by exposure to chloroform vapor for 1 hr. After chloroform evaporated completely, indicator strain culture which had been cultured for 2 days in PS medium was spread over the plates. The plates were incubated at 28℃ for further 24 hrs and the diameters of inhibition zones were measured.

2 Results

2.1 Selection of Rf resistant mutant of B8

Rf resistant mutants were obtained after successive culture of B8 in medium containing Rf. Their antagonistic activities against *X. oryzae* pv. *oryzae* were tested and a colony which produced the biggest inhibition zone was used as recipient for mating. After conjugal transfer for 10 hrs, the bacteria were scraped from the filters and spread with dilution on LB agar plates containing Rf and Km. The frequency of $Km^{[r]}$ transconjugant was about 2×10^{-7} per input recipient cell. The survived colonies were streaked on LB agar plates with Rf and Km several times to eliminate *E. coli*. The obtained colonies were tasted for their antagonistic activity and a colony (named BX8)producing the biggest inhibition zone was used for further tests.

2.2 Comparison of antagonistic activity between B8 and BX8

To determine the lovel of the antagonistic activity of BX8, B8 and BX8 were inoculated on PSA plates and incubated at 28℃ for 12 hrs . Then the indicator strain was spread on plates as described above and the diameters of the inhibition zones were measured. The diameters of the inhibition zones were no signficant difference between B8 and BX8 by test to (table 1), which suggested that the drug resistant mutant retained the antagonistic activity.

Table 1　The diameters of the inhibition zones of B8 and BX8

No.	1	2	3	4	5	6	7	8	9	10	$\bar{X}$	$\bar{X}$
B8	21.0	23.0	20.0	27.5	32.5	31.0	35.0	32.0	32.0	30.0	28.5	a
BX8	32.0	28.0	27.5	24.5	25.0	20.0	32.0	32.0	21.0	23.0	26.5	a

$t<t_{0.50}$

2.3 Growth of BX8 in drugs

To compare the tolerance to Rf and Km, B8 and BX8 were cultured over night in LB with or without Rf(100μg/ml) and Km (50μg/ml), respectively. Then 10 ml LB media with different concentrations of Rf or Km were inoculated. BX8 could still grow in LB containing 400μg/ml Rf or 300μg/ml Km, but B8 could not grow at 50μg/ml Rf or 50μg/ml Km. The number of divisions of BX8 was decreased as increasing of drug concentrations after 8 hrs. All cultures of BX8 reached stationary phase after 24 hrs(table 2).

2.4 Stability of BX8 in drug resistance

Since the antagonistic study will be carried out in field, it is important to check the drug re-

sistant stability. The drug resistant stability of BX8 was determined when the number of divisions reached about 60 in successive culture. The culture was diluted and spread on LB agar plates with or without 100μg /ml Rf and 50μg/ml Km. There was no significant difference between the numbers of colonies growing on LB agar plates with and without drugs by t test (table 3), which suggested that the drug resistance of BX8 was stable within at least 60 generations.

Table 2 Division numbers of B8 and BX8 in different concentrations of drugs

	drug conc. (μg/ml)	B8 after cultured 8 hrs	BX8 after cultured 8 hrs	B8 after cultured 24 hrs	BX8 after cultered 24 hrs
Rf	0	17.54	18.42	20.24	19.61
	50	0.00	14.70	0.00	19.09
	100	0.00	12.99	0.00	18.24
	200	0.00	11.65	0.00	19.07
	400	0.00	10.45	0.00	19.42
Km	0	17.54	18.42	20.24	19.61
	25	/	15.36	/	20.10
	50	0.00	14.20	0.00	21.02
	100	0.00	13.29	0.00	19.40
	300	0.00	9.64	0.00	20.36

Table 3 The colony numbers of BX8 on plates with or without Rf and Km

No.	1	2	3	4	5	6	7	8	9	$\bar{x}$	
LB	249	194	249	238	262	172	222	201	199	220.67	a
Km Rf	214	236	280	193	212	211	213	264	226	227.67	a

$t < t_{0.50}$

3 Discussion

Rice bacterial leaf blight is one of the most serious rice diseases in the world. It decreases about 10～20 percent of the rice production. Since the pathogenic bacteria invade wounded rice easily and spreads rapidly in rainstorm season, it is difficult to use chemical pesticides to control it by now. The utilize of antagonistic bacteria may be a potential way to protect the rice plant from bacterial leaf blight. Some antagonistic bacteria we obtainedare screened out from rhizosphere and phyllosphere, it is possible to use them directly in practice. By molecular genetics methods, it is also possible to transfer some useful foreign genes into predominant bacteria on plants to get genetic engineered bacteria, then use such recombinants directly. *E. cloacae* B8 which is screened out from rice leaves is one of the promising candidates for this purpose.

Transposable element Tn5 is a powerful tool to study gene functions. It can insert into genome randomly and cause mutation in which the mutant allele is linked to the selectable drug resistant marker. Additionally, the use of Tn5 allows to isolate genes, construct dele-

tions and transfer foreign genes[2]. In the experiment, some colonies of B8 lost the antagonistic activity after Tn5 treatment and might be used to study the properties of antagonistic protein genes. Since the Km resistance of BX8 is stable, it is also possible to introduce other foreign genes to improve it for agricultural research.

The colonization and antagonistic action of BX8 on rice leaves are studying in our laboratory. Since BX8 can grow in media with Rf or Km up to 400μg or 300μg respectively, it is easy to distinguish it from other microorganisms. But a few of other microorganisms have Km resistance in nature(data not show). So if only the low concentration of Km is used as inhibitor, we should be careful to analyze the results. However, there is hardly problem when other drugs(for example Rf) are used together with Km.

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An expedient and reliable method to identify gene constructs in *Agrobacterium* vectors

Ping Xu, Dae-Jin Yun, Paul M. Hasegawa and Ray A. Bressan
Center for Plant Environmental Stress Physiology, Horticulture Building, Purdue University, West Lafayette IN 47907-1165, USA

Abstract

We report a procedure for detection of presence, size and orientation of insert DNA in recombinant plasmids harbored in individual *Agrobacterium tumefaciens* colonies. The procedure utilizes polymerase chain reaction (PCR) amplification of insert DNA directly after isolation of individual colonies without the necessity of separate procedures for DNA isolation and purification. We have demonstrated the applicability of this technique for detection of DNA inserts encoding either a pathogenesis-related (PR-1) protein gene or a cysteine proteinase inhibitor gene constructed into *Agrobacterium* binary vectors.

Introduction

In recent years remarkable progress has been made in the application of genetic transformation methodology for crop improvement. *Agrobacterium*-mediated DNA delivery is the most common and successful transformation method used, particularly for dicotyledonous species (Klee *et al.*, 1987). Numerous binary vector expression cassettes are available that facilitate construction of plant gene expression vectors for transformation after mobilization to *Agrobacterium*. One limiting step in the procedure of constructing plant gene expression vectors and *Agrobacterium* transformation is the verification process that confirms the integrity of the binary vector while it is harbored in the *Agrobacterium* strain. It is imperative that no structural alterations have occurred that would mitigate the function of the vector for transformation (An *et al.*, 1988). Isolation of plasmid DNA from *Agrobacterium* and restriction analysis, or retransformation of *Escherichia coli* are laborious procedures. Recently, techniques have been developed to detect the presence, size and orientation of inserts in recombinant plasmids by polymerase chain reaction (PCR) amplification of DNA isolated from individual colonies of *E. coli* (Gussow and Clackson, 1989; Pampfer, 1993) and *Agrobacterium* (Haas *et al.*, 1995). We have simplified the procedures for template isolation from *Agrobacterium*. Principally, the technique was abridged by elimination of boiling and centrifugation steps and minimized the cross contaminating among different samples.

Materials and Methods

Culture of bacteria and bacterial colony template preparation

Cells of *A. tumefaciens* (strain LBA4404) were grown in solid or liquid YEP medium (10 g/l tryptone, 10 g/l yeast extract and 5 g/l NaCl) supplemented without or with 30 mg/l rifampicin and 50 mg/l kanamycin at 28°C. LBA4404 cells were transformed with a binary vector by the direct transfer method (Herrera-Estrella and Simpson, 1988). After *Agrobacterium* colonies had grown sufficiently to be visible on the agar medium (1–2 mm), a sample from each colony was obtained using a sterile wooden toothpick. The majority of the sample was directly placed into 10 μl of PCR mixture contained in a microfuge tube. Also, a bacterial sample from each toothpick was inoculated onto agar medium for subsequent recovery of the colony. One drop of mineral oil was added into each tube. The tubes were centrifuged in a microfuge for 2 s at 12,000 g to sediment all of the reaction mixture below the mineral oil.

PCR reaction

The primers 5′-TCTAGACATGGGATTCTTAACAACAATAG-3′ and 5′-GTCGACTTAAAAGGGACGTTGTCCTATAAA-3′ were used to amplify PR-1 cDNA (EMBL X14065) and 5′-GCCGCTCTAGAGAAAGTG-3′ and 5′-CTATTGGGTGCTAGAAAC-3′ were used to amplify CysPI N2 (GenBank U51854). Each PCR reaction mixture was 10 μl and contained 1X PCR buffer, 200 μM dNTPs, 2.0 mM $MgCl_2$, 1 μM of each primer and 0.5 units of *Taq* DNA polymerase (Promega). In the first PCR cycle, samples were heated to 94°C and 5 min. This was followed by 40 cycles of 94°C for 45 s, 55°C for 1 min and 72°C for 1 min using a DNA thermal cycler (Perkin Elmer Cetus). In the final cycle, the reaction period at 72°C was extended to 10 min.

Southern hybridization

For Southern analysis of *Agrobacterium*, DNA plasmids from 3 ml of 2-day-old cultures were isolated with Wizard™ DNA purification kit (Promega) and one-fourth of the DNA was digested with restriction enzymes. DNA was separated on 2.0% (w/v) agarose gels. Southern transferring and hybridization were carried out as described (Sambrook *et al.*, 1989).

Results and Discussion

Since PCR amplification is very sensitive, it is imperative that sources of cross contaminating DNA, including extraneous microbes, be avoided. All solutions and supplies used for PCR were autoclave sterilized. Two constructs were tested in our experiment; one contained the open reading frame of pathogenesis-related (PR)-1 protein gene from tobacco (Payne *et al.*, 1989) inserted into pBTEX (derivative of pBI121), pXBP, and the other contained soybean cysteine proteinase inhibitor (CysPI N2) coding sequence (Zhao *et al.*, 1996) in pKYLX71:35S^2 (gift from Dr. A. Hunt, University of Kentucky), pXKN. LBA4404 cells were transformed with pXBP

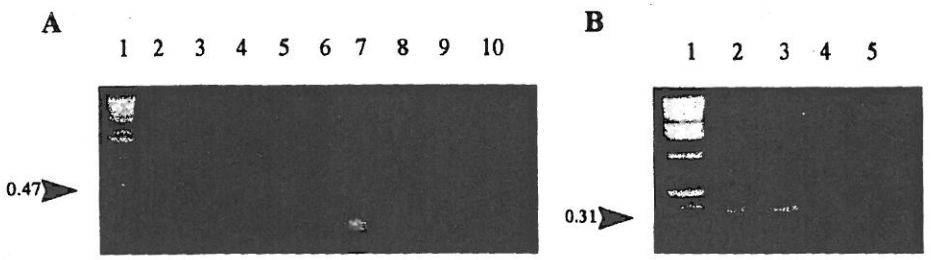

Fig. 1. PCR detection of insert DNA in recombinant plasmids harbored in *Agrobacteria*. A. PCR products from *Agrobacterium* colonies containing recombinant plasmid with PR-1 insert. Lane 1 is DNA markers; lanes 2–6 are from colonies harboring pXBP (PR-1 insert); lanes 7–9 are colonies containing vector without insert; lane 10 is the result of PCR without bacterial colony. Arrow indicates PR-1 insert. B. PCR products from *Agrobacterium* colonies containing recombinant plasmid with CysPI N2 insert. Lane 1 is DNA markers; lanes 2 and 3 are colonies harboring pXKN (CysPI insert); lane 4 is a colony harboring vector without insert; lane 5 is the result of PCR without bacterial colony. Arrow indicates CysPI N2 insert.

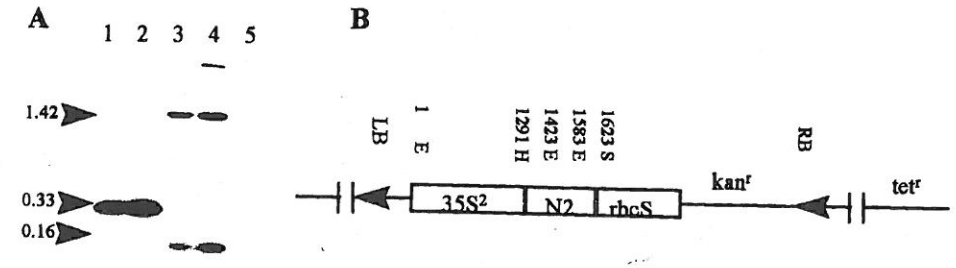

Fig. 2. Southern blot analysis. A. Southern blot of DNA isolated from *Agrobacterium* colonies containing pXKN digested with *Hin*dIII and *Sac*I (lanes 1 and 2), pXKN digested with *Eco*RI (lanes 3 and 4) and *Agrobacterium* containing vector without CysPI insert (lane 5) using CysPI N2 probe. Colonies represented in lanes 1–4 correspond to these illustrated in B. B. Construct of pXKN. The expression cassette contains a double CaMV 35S promoter, CysPI N2 coding region, the rbc S poly(A) termination sequence and the T-DNA left and right border, respectively. E, *Eco*RI; H, *Hin*dIII; S, *Sac*I.

or pBTEX (vector control) and pXKN or pKYLX721:35S^2 (vector control), respectively. The PR-1 or CysPI N2 encoding sequences were detected after PCR amplification from the appropriate colonies (Figs. 1A, 1B). To confirm that PCR amplified the appropriate insert, DNA was isolated from PCR positive *Agrobacterium* colonies containing the CysPI N2 fragment and digested with *Hin*dIII/*Sac*I or *Eco*RI. Since the DNA quantity was too low for visualization under UV light with ethidium bromide staining, the DNA was evaluated by Southern blot analysis using CysPI N2 probe (Fig. 2A). It was confirmed that the bacterial colonies which produced PCR products in Fig. 1B contained the CysPI N2 insert.

In our experience, the most reliable results were obtained if small quantities of cells and fresh bacterial colonies (less than 2 weeks after inoculation) were analyzed using this PCR method. Bacterial cells were deposited into the microfuge tube by scraping the toothpick along the tube wall facilitated efficient cell recovery. A 10°C lower temperature than *Tm* was used for annealing and 40 PCR cycles of amplification were used to ensure detection of the signal. It may be possible to improve the sensitivity of the PCR by increasing the annealing temperature or by reducing PCR cycles if non-specific amplification is a problem. This method can be used routinely to evaluate insert sequences constructed into vectors harbored in individual bacterial colonies. This method substantially reduces time and effort required to evaluate the authenticity of inserts in *Agrobacterium* binary vectors.

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