African Journal of Microbiology Research Vol. 4(5), pp. 317-322, 4 March, 2010
Available online http://www.academicjournals.org/ajmr
ISSN 1996-0808 ©2010 Academic Journals

Full Length Research Paper

Study on the function of a plasmid harbored by a newly isolated *Bacillus subtilis*

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Accepted 8 January, 2010

With a view to exploring whether there was a correlation between a novel antibiotic and a plasmid, the plasmid was eliminated by SDS. Because the elimination of the plasmid had no effect on the bacterium's producing antibiotic, it was determined that the plasmid had nothing to do with the antibiotic. However, with the plasmid being eliminated, the ability of the bacterium to secrete a kind of adhesive substance was lost, and the phenotype of the bacterium was also changed. This indicated that the plasmid was closely related to this adhesive substance and that the adhesive substance determined the bacterium's phenotype. In addition, losing the plasmid meant losing life if the bacterium was under adverse circumstances like cold condition. It meant that the function of the plasmid (or the adhensive substance) was to protect the bacterium.

Key words: Plasmid, phenotype, antibiotic, viability, bacterium.

INTRODUCTION

In the suburban district of Hangzhou City, Zhejiang Province, P.R. China, from the eggplant leaf, we screened a strain of *Bacillus subtilis* (named BS). BS could secrete an antibiotic (named BS antibiotic). Our previous work had determined that the molecular formula of BS antibiotic was $C_{27}H_{46}O$. The molecular formula, the infrared spectrum and the ultraviolet spectrum indicated that BS antibiotic was a novel antibiotic (PhD dissertation, unpublished), the chemical structure of which is in progress. Considering that researchers had paid more and more attention to finding more safe bio-pesticides such as microorganisms and/or their secondary metabolites (Singh et al., 2009; Wu et al., 2007; Zheng Wet et al., 2009; Copping and Duke, 2007), we are very interested in the finding that both BS and BS antibiotic can antagonize different kinds of plant pathogens such as many bacteria and fungi (Xao-xi Chen et al., 2000, 2002, 2003).

We found that BS harbored a plasmid named BS plasmid plasmid. In some antibiotic-producing bacteria, antibiotic-related genes are located in a plasmid (Ceotto et al., 2009; Vincent and Morero, 2009; Mashentseva et al., 2009), and therefore, we wondered if genes controlling synthesis of the BS antibiotic existed in BS plasmid. If such conjecture were confirmed BS plasmid could find some application in bio-control research.

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MATERIALS AND METHODS

The microorganism and the culture medium

The BS was isolated from the eggplant in the suburban district of Hangzhou City, Zhejiang Province, P. R. China. *Rhizoctonia solani* (fungus), *Escherichia coli* and *B. subtilis were* gifts from Zhejiang University, P. R. China. *Staphylococcus Epidermidi, Klebsiela Pneumoniae, Salmonella Typhi, Streptococcus viridans* and *Staphylococcus aureas 25923* were from Zhejiang Chinese medicine University, P. R. China.

The formula for Potato - dextrose agar (PDA) medium and LB (Luria-Bertani) medium is described by the reference (Bin Zhao and Shaojiang, 2002). PDA medium was used to culture BS. LB medium was used to culture all bacteria except BS. PDA plate, which was prepared by adding 15 g/L of agar to PDA, was used to culture *R. solani* (fungus). PDA medium consists of: potato extract, 230 ml, glucose, 20 g, distilled water, 770 ml, potato extraction was prepared by adding 100 g potato (peeled and sliced in a minicer) to 300 ml distilled, it was left overnight at 4℃, and filled through cloth. LB medium consists of yeast extract (Oxoid), 5 g, Peptone (BD microbiology system), 10 g, NaCl, 10 g, distilled water 800 ml. pH

was adjusted to 7.3 with 1 mol NaOH, and the volume was adjusted to 1000 ml by adding distilled water.

The plasmid elimination and the plasmid extraction

SDS (sodium dodecyl sulphate) curing and heat treatment were applied to the wild BS in different experimental setups: (i) different concentrations of SDA (0.1, 0.05 and 0.025 μg/ml) in PDA medium were used; (ii) logarithmic growth and then 3, 7 and 14 day's stationary phase treatment was applied; and (iii) growing at high temperatures with agitation (at 37℃ during logarithmic growth and at 40°C in stationary phase) was applied. Every day, the cultures were respectively diluted and spread on the PDA medium plates (15% agar) and then incubated at 37°C to check if there appeared any bacterium whose phenotype (e.g. activity against *R. solani* and/or *Escherichia coli*) had changed. If any phenotype change was observed, 100 clones, including the wild BS and its phenotype-changed offspring, were selected at random, and then the percentage of phenotype-change was counted. Two clones of the phenotype-changed offspring were incubated in PDA medium with agitation at 37°C respectively through five passages. For every passage, the culture was diluted and spread on the PDA medium plates (15% agar) and then incubated at 37°C to check whether there appeared clones whose phenotype returned into that of the ancestry(that is the wild BS).

The presence/absence of BS plasmid was monitored as follows: the wild BS colonies and the phenotype-changed BS colonies were respectively picked and grown in 50 ml PDA media overnight at 37°C. They were subjected to a standard alkaline lysis preparation with minimum modification (Sambrook et al., 1989). The plasmid solution was estimated by agarose gel electrophoresis (0.7%, con-taining Ethidium Bromide). In addition, the presence/absence of the plasmid was further confirmed as follows: the wild BS's DNA and the phenotype-changed BS's DNA were respectively extracted from 500 ml culture as described by the reference with minimum modification 8. Both DNAs were purified by CsC_{l2}-Ethidium Bromide Gradients, and then the centrifugal tubes that contained the DNA were respectively and immediately put under ultraviolet rays to check DNA bands (the tubes were see-through).

The second method to eliminate BS plasmid was as follows: EB (Ethidium Bromide) curing and heat treatment (at 40°C) were applied to the wild BS. The EB concentrations used were 20, 30, 40 and 50 µg/ml. Other procedures used were the same as that of SDS, as described above.

The third method to eliminate BS plasmid was as follows: only heat treatment was applied to the wild BS, that is, the wild BS was respectively grown at 42, 48, 58°C for one week. Other procedures used were the same as that of SDS, as described above.

The extraction of BS antibiotic

The wild BS and the phenotype-changed BS were respectively cultured on PDA plates (15% agar) at 37°C for one week (20 liter of PDA was used). Then the BS antibiotic was extracted by ether. The ether was evaporated at room temperature (25 - 30°C). The remainder was chromatographed on silica gel TLC, with gradient elution (from 100% benzene: 0% ether to 0% benzene: 100% ether). The activity of BS antibiotic was evaluated by inhibition zone diameter of *R. solani* /*E. coli*.

The extraction of the adhesive substance that piled up outside the wild BS

Two methods were used. (1) the wild BS and the phenotype-changed BS were respectively cultured at 37°C on PDA plates (15% agar) for 4 days, then the adhesive substance, which piled up outside wild BS, was collected by a scraper; (2) the wild BS and the phenotype-changed BS were respectively cultured in 500 ml PDA medium with agitation at 37°C for 4 days. 30% (W/V) ammonium sulfate was added respectively into both cultures with agitation. Both cultures were left at 4°C overnight, and then they were centri-fuged at 5000 rpm for 20 min at 4°C. The sediment (that is, the adhesive substance) was collected. The ability of the wild BS/the phenotype-changed BD to produce BS antibiotic

Tenfold serial dilutions (from 100 to 1 µg/ml) of BS antibiotic, which was derived from the wild BS or the phenotype-changed, were made in 1 ml tubes of LB medium. Each tube was then inocu-lated from an overnight culture and incubated with shaking at 37°C. The growth was monitored by a microscope. The activities were expressed as the MICs, the concentration at which no growth was observed. Trials were performed in triplicate.

The ability of the wild BS and the phenotype-changed BS to stand cold treatment.

The wild BS and the phenotype-changed BS were respectively grown to an OD_{600} of 2 (the optical density at 600 nm) at 37°C in 100 mL PDA medium. 0.1 ml of the two cultures was respectively spread on two PDA medium plates (15% agar). For cold treatment, the two plates were left in 4°C refrigerator for 4 days and for 4 weeks respectively. Afterwards, the two plates were respectively washed three times with PDA medium. The washed bacteria were respectively combined together and the volume was respectively adjusted to 100 ml by adding PDA medium. Both cultures were respectively incubated on shaker at 85 rpm at 37°C. When cell density came to OD_{600}, 2.0, 0.1 ml culture was respectively inoclated into two new flasks that contained 100 mL PDA medium. Then the two flasks were again respectively incubated on shaker at 80rpm at 37°C. In above operations, the growth of the cultures was moni-tored by measuring the OD_{600} at different time, and the growth time that was required for OD_{600} of 2.0 was used as an indicator to evaluate the viability of the bacteria.

The control was the wild BS that was always living in favorable condition (37°C, PDA medium, agitation). Procedures for the control were the same as the wild BS, as described above. All above experiments were performed in triplicate.

RESULTS

The elimination of BS plasmid

All concentrations of SDS (0.1%, 0.0 and 0.025 g/ml) could cause a portion of wild BS to alter their phenotype. The percentage of phenotype-change was reciprocally proportional to SDS concentration (Table 1). In addition, while the bacteria were respectively grown in SDS-contained PDA medium, the growth speed was also reciprocally proportional to SDS concentrations (data not shown, (Table 1). The shape of the wild bacterium was smooth–type) whereas the shape of the phenotype-changed BS was rough-type (Figure 1).

Two clones of the phenotype-changed BS had been incubated respectively for 5 consecutive passages and no offspring's phenotype had been returned into that of its ancestry (the wild phenotype or the smooth-type). This indicated that the phenotype-change was genetically stable. Besides, the combination of EB curing and heat treatment (at 40℃), as well as the heat treatment (42, 48 and 58℃ respectively), did not cause any individual bacterium of the wild BS to alter its phenotype.

Table 1. A portion of wild BS changed their phenotype after treatment of SDS at 40℃.

		0.1 µg/ml SDS treatment at 40℃	**0.05 µg/ml SDS treatment at 40℃**	**0.025 µg/ml SDS treatment at 40℃**	
SDA at 40℃		Bacteria grown slowly	Bacteria grown less fast	Bacteria grown faster	
	Wild BS was cultured 3 days	100% wild type	100% wild type	100% wild type	
	Wild BS was cultured 7 days	100% wild type	About 20% became rough type	About 60% became rough type	
	Wild BS was cultured 14 days	About 10% became rough type			
		20 µg/ml EB treatment at 40℃	**30 µg/ml EB treatment at 40℃**	**40 µg/ml EB treatment at 40℃**	**50 µg/ml EB treatment at 40℃**
EB at 40℃		Bacteria grown slowly			
	Wild BS was cultured 3 days	100% wild type	100% wild type	100% wild type	100% wild type
	Wild BS was cultured 7 days	100% wild type	100% wild type	100% wild type	100% wild type
	Wild BS was cultured 14 days	100% wild type	100% wild type	100% wild type	100%s wild type
		40℃	42℃	48℃	58℃
Heat treatment		Bacteria grown fast			
	Wild BS was cultured 7 days	100% wild	100% wild type	100% wild type	100% wild type

The extraction of the adhesive substance that pilled up outside the wild BS

A kind of adhesive substance could be collected from the wild BS that had been cultured on PDA plates for four days, whereas no same adhesive substance could be obtained from the phenotype-changed BS that had also been cultured under same conditions and for same period of time. In addition, via the ammonium sulfate precipitation, a kind of adhesive substance could be prepared from the wild BS culture that had been grown in PDA medium for 4 days, while no same kind of adhesive substance could be obtained from the phenotype-changed BS culture that also had been grown under the same conditions for same period of time. This indicated that the wild BS could secret the adhesive substance and that the phenotype-changed BS had lost this ability.

The extraction of BS plasmid

The agarose gel electrophoresis shown that a plasmid could be extracted from wild BS, whereas no plasmid could be extracted from the phenotype-change BS. The molecular weight of the plasmid was about 20 KD (Figure 2). In addition, under ultraviolet rays, within centrifugal tubes containing the wild BS DNA, which was purified with $CsCl_2$ -ethidium bromide gradients, two bands could be observed: the above band was plasmid DNA, and the below band was chromosome DNA. Under same conditions, within centrifugal tubes containing phenotype-changed BS DNA, which was prepared in the same way, no plasmid band could be seen, that is to say, only one band, which was equivalent to chromosome DNA, could be observed.

The antibacterial activities of the wild BS/the phenotype-changed BS

In terms of the antibacterial activity, there seemed to be no difference between the wild BS and the phenotype-changed BS. That is, in concentrations of 100, 10 and 1 µg/ml, both BS antibiotic, from

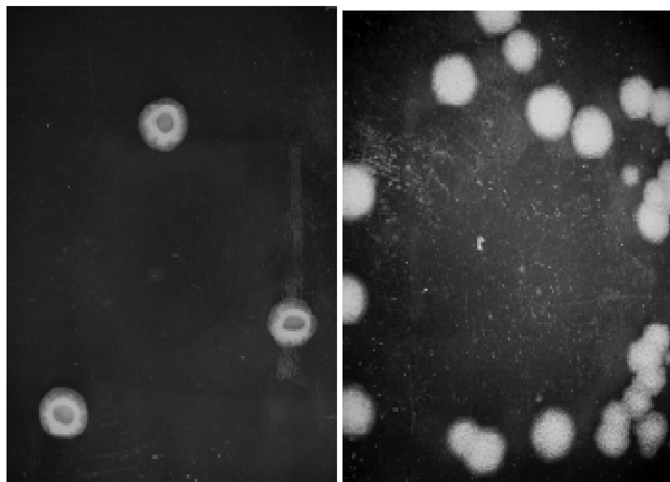

Figure 1. A portion of the wild BS had been changed their phenotype after the treatment of SDS at 40℃, A: The wild BS, whose phenotype was smooth-type, B The phenotype-changed BS whose phenotype was rough-type.

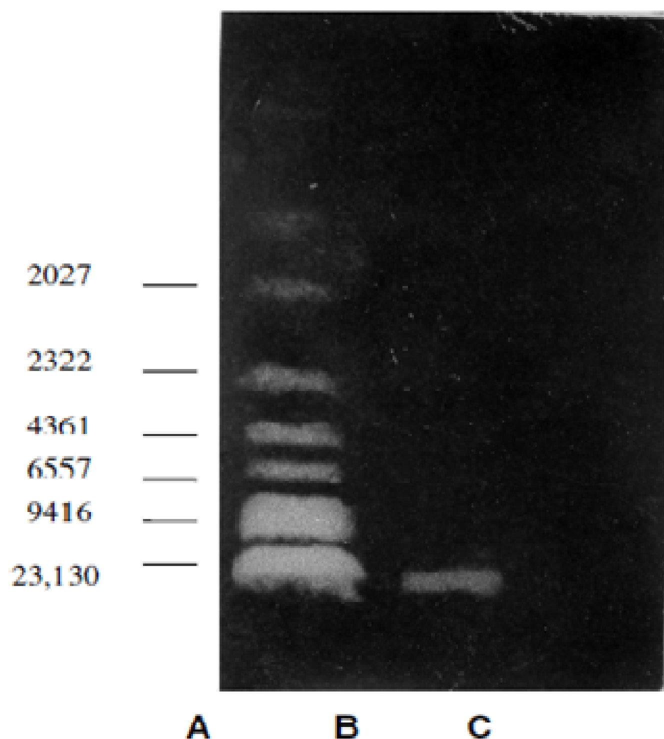

Figure 2. Phenotype change was accompanied with the lose of the plasmid. A: Marker, B: A plasmid could be extracted from wild BS; C: When wild BS changed its phenotype, the bacterium lost its plasmid.

the wild BS and (from the phenotype-changed BS) could inhibit all six bacteria's growth. For these six bacteria, MIC ≤ 1 µg/ml (Table 2).

The ability of the wild BS and the phenotype-changed BS to stand cold treatment

There were three samples: (1) the wild BS that had been left in 4 at refrigerator for 4 days (rather than 4 weeks); (2) the wild BS that was always living in favorable condition (used as control); and (3) the phenotype-changed BS that had been left in 4 at refrigerator for 4 weeks (rather than 4 days). When same number of above bacteria was respectively incubated under same conditions, growth situation between these different samples was very different. When the phenotype-changed BS was growing in PDA medium, individual bacterium was combined together to form particles, which were look like willow catkin there were numerous catkin-like particles in the PDA culture medium). If penicillin (0.15 µg/mL) was added to the phenotype-changed BS culture, which had grown overnight, such catkin-like particles became larger. On the other hand, if the wild BS was cultured in same conditions, no similar catkin-like particles was observed. That is, the individual wild BS was not combined together (photograph concerning this happening was not clear and distinguishable. So the results not shown). It was possible that such happening was another way in which the phenotype-changed BS's phenotype was different from that of the wild BS.

The growth time that was required for OD_{600} of 2.0 was used as an indicator to evaluate bacterium's viability. The OD_{600} of 2.0 for different BS samples was as follows: (1) 38 h and 32 min for the phenotype-changed BS that had been left in 4℃ refrigerator for 4 days; (2) 8 h and 30min for the wild BS that had been left in 4℃ refrigerator for 4 days; (3) 4 h and 30 min for the wild BS that had never been treated with cold. However, when the wild BS and the phenotype-changed BS, both of which were once in 4℃ refrigerator for 4 days, had been activated (that is when OD_{600} value had come to 2.0) and then incubated respectively under same conditions (37℃, PDA medium, agitation), in terms of the growth time that was required for OD_{600} of 2.0, there was no a notable difference between the wild BS and the phenotype-changed BS, that is, the OD_{600} value of 2 was 4 h and 30 min for the wild BS and 4h and 50min for the phenotype-changed BS respectively.

The phenotype-changed BS that had been left in 4℃ refrigerator for 4 weeks (rather than 4 days) could not been incubated again at all. On the other hand, the wild BS that also had been left in 4℃ refrigerator for 4 weeks could be incubated; although the growth time that was required for OD_{600} value of 2.0 was extended to 16 -19 h. For the control, the growth time that was required for OD_{600} value of 2.0 was only about 5 h. All above data were the average of three independent experiments. The results were summarized in Figure 3. These results indicated that the ability of the phenotype-changed BS to put up with cold condition was much less than that of the wild BS.

Table 2. Antagonistic activities against different kinds of bacteria.

Bacteria	Wild BS (µg/ml)	Phenotype-changed BS (µg/ml)
S. epidermidis	MIC ≤ 1	MIC ≤1
E. aerogenes	MIC ≤ 1	MIC ≤1
K. pneumoniae	MIC ≤ 1	MIC ≤1
S. typhi	MIC ≤ 1	MIC ≤1
S. viridans	MIC ≤ 1	MIC ≤1
Escherichia coli	MIC ≤ 1	MIC ≤ 1
S. aureas 25923	MIC ≤ 1	MIC ≤ 1
Bacillus subtilis	MIC ≤ 1	MIC ≤ 1

For all six bacteria, MIC ≤ 1 µg/ml, it is because in concentration of 1 µg/mL, all bacteria could not grow at all.

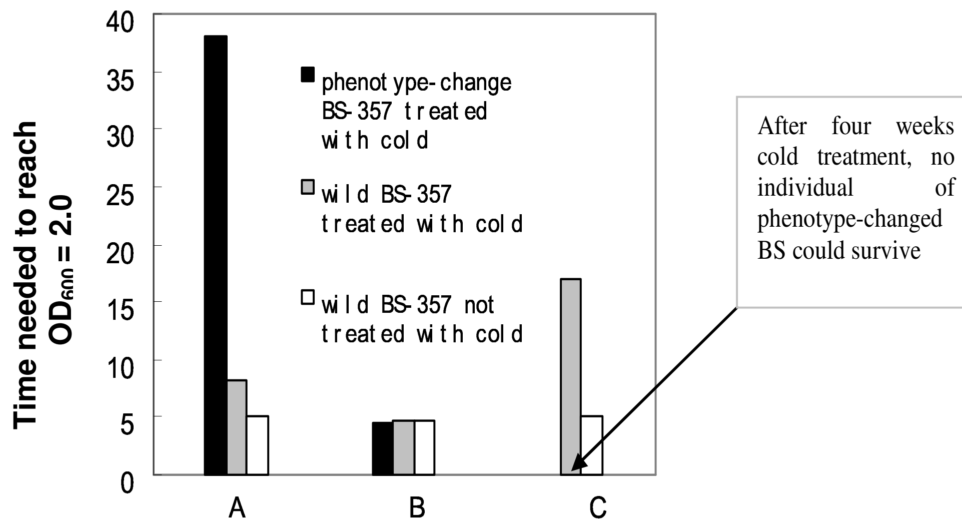

Figure 3. Phenotype-changed BS reduces its viability or even died after cold treatment
A. after 4 days cold treatment, it took much longer time for phenotype-changed BS to reach growing density of OD_{600} than wild BS-357; B. After activated, for both BS, time to reach growing density of OD_{600} was almost the same; C. after 4 weeks cold treatment, phenotype-changed BS could be cultured at all (all bacteria) had died.

DISCUSSION

In some antibiotic-producing bacteria, antibiotic production is controlled by a plasmid, because among these bacteria, antibiotic-related genes are located in the plasmid (Ceotto et al., 2009; Vincent and Morero, 2009; Mashentseva et al., 2009). The wild BS harbored a plasmid, and therefore we hoped that the antibiotic-related genes were within the plasmid so that this plasmid might find application in bio-control research. However, this paper’s work had confirmed that this plasmid had nothing to do with antibiotic production.

After the wild BS lost its plasmid, its phenotype was changed from the smooth type (that is the wild type) into the rough type. Obviously, the smooth type was determined by the adhesive substance that existed outside the bacterium. The loss of the plasmid was always accompanied with the loss of the adhesive substance, and therefore it was determined that the synthesis of the adhesive substance was controlled by the plasmid.

It was certain that producing secondary metabolites like BS antibiotic and the adhesive substance constituted a burden upon the wild BS, because it consumed energy as well as nutrition. Evolution seldom brought worthless substance and the function of BS antibiotic was clear, that is, increasing the bacterium’s capacity to fight against other microorganisms. What was the function of the adhesive substance? We inferred that the function of the adhesive substance was to protect BS. The phenotype-changed BS’ viability became much less because it had lost the protection of the adhesive substance.

Actually, the plasmid-losing BS was not a nature

creating microorganism that had undergone natural selection, If this bacterium came to the natural environment, where cold or other adversities were usual occurrences, its chance to pass down to next generation should not be so optimistic.

The wild BS and the phenotype-changed BS had many different features, including the viability, the phenotype and the existence of the plasmid. Thus, a question might be come up with: so called phenotype-changed BS might not belong to the progeny of the wild BS, that is to say, it might be a contaminating germ that had been accidentally introduced during the plasmid-eliminating process. Such possibility was excluded as follows: except the viability, the phenotype and the plasmid, every feature of the phenotype-changed BS we had observed was identical to that of the wild BS. These features include the antagonistic activities, special smell and the silica gel TLC analysis results by using substances secreted by the wild BS/the phenotype-changed BS (only the antagonistic activities is shown in this paper). Moreover, independent plasmid–eliminating operation always produced same kind of phenotype-changed BS.

There existed the following possibility: BS's phenotype - change might be caused by SDS-related mutation rather than by the ose of the plasmid. Such possibility was excluded as follows:

(1) The percentage of the phenotype-change was as high as 60%, and mutation frequency was impossible to be so high (Yao Li-bo, 2008);
(2) The existence of the plasmid was strictly relevant to the shape of the wild BS. The fact that the shape of the wild BS was strictly related to the existence of BS plasmid could also rule out the following possibility: BS might belong to pleomorphic bacteria that had potential to assume several shapes, and therefore it might be the environmental conditions rather than the lose of the plasmid that altered the shape of the wild BS.

ACKNOWLEDGEMENTS

This work was supported by (1) Nature Science Fund Project of Zhejiang province, P.R. China (Project code number: Y205362); (2) Medical Science Research Fund Project of Health Bureau of Zhejiang Province P. R. China (Project code number: 2004A062), (3) Medical Science Research Fund Project of Health Bureau of Zhejiang Province, P. R. China (Project code number: 2002A070) and testing Fund Project of Zhejiang province, P. R. China (Project code number: 04041). Most of this work was conducted in Institute of Biotechnology of Zhejiang University.

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