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Effect of Antibiotics from a Strain of *Micromonospora carbonacea* on Cell Morphology and Protein Synthesis of *Xanthomonas oryzae* pv. *oryzae*

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Xanthomonas oryzae* pv. *Oryzae* is the most devastating pathogen to rice and has been shown to cause bacterial blight, which has been reported as one of the most destructive diseases of rice. The effect of antibiotics from a strain of *Micromonospora carbonacea* on the morphology and protein synthesis of *X. oryzae* pv. *Oryzae* were studied in this paper, and the objective of the study is to reveal the functional mechanism of the antibiotics against *X. oryzae* pv. *Oryzae*. The results showed that, In the role of antibiotics from *M. carbonacea*, the cell Gram staining reaction remained unchanged, but the cell morphology changed from the rod into long filamentous, and the cell protein content of *X. oryzae* pv. *Oryzae* decreased with the increase the concentration of antibiotics, combined with that the protein synthesis be inhibited by antibiotics in cell-free protein expression system, the antibacterial mechanism for antibiotics from *M. carbonacea* against *X. oryzae pv. oryzae*, can be deduced to inhibit the synthesis of cell protein, and then the cell division. The study may lay a theoretical foundation for development of a new agricultural antibiotics against *X. oryzae* pv. *Oryzae.

Key words: *Micromonospora carbonacea*; Antibiotics; *Xanthomonas oryzae* pv. *oryzae;* Antibacterial mechanism.

Bacterial blight disease of rice caused by *Xanthomonas oryzae* pv *oryzae* (*Xoo*) is one of the most important diseases of rice in most of the rice growing countries and has been reported to result in a severe loss in yield and quality [1].

Bacterial blight of rice has high epidemic potential and destructiveness to high-yielding cultivars in both temperate and tropical regions, especially in Asia. Its occurrence in the 70s in Africa and the Americas has added a new dimension to concerns about its transmission and dissemination. In addition, bacterial blight has been a serious problem in hybrid rice cultivation areas in China, Vietnam, Myanmar and so on [2].

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Research on bacterial blight of rice was commenced as early as in 1901, and the efforts were focused mainly on ecological studies and chemical control. Now, bismerthiazol is the main chemical for prevention and control of rice bacterial blight, but there is lower sensitivity of *X. oryzae* pv *oryzae* to bismerthiazol with the increase of usage, meanwhile the resistant mutants has been showed up [3].

Thus, it is necessary to develop safe natural bioactive substances that can be used as alternatives to artificial pesticides. Biological control using microorganisms for inhibition of plant disease holds promise as a robust and long-lasting alternative to synthetic chemicals [4].

A strain of *Micromonospora carbonacea* with broad-spectrum antimicrobial activity, which was termed JXNU-1, was obtained in our former study[5], and a new type of antibiotics, characterized

by nucleoside antibiotics, was isolated from the broth of *M. carbonacea* JXNU-1[6]. This antibiotics can effectively inhibit the growth of *X. oryzae* pv. *Oryzae*[7]. It is not clear about the mechanism for the antibiotics against *X. oryzae* pv. *oryzae*.

The effect of antibiotics from *M. carbonacea* **JXNU-1** on cell morphology and protein synthesis of *X. oryzae* pv. *oryzae* was studied in this paper, the purpose of this study is to reveal the functional mechanism of the antibiotic, which may lay a theoretical foundation for development and use of a new agricultural antibiotics.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals or reagents used in this study were of analytical grade, all biochemicals were purchased from Sangon Biotech (Shanghai) Co. Ltd, and Tiangen Biotech (Beijing) Co. Ltd.

Microorganisms

M. carbonacea JXNU–1 was isolated from soil samples and was stored in Gause's No.1 medium slant at 4 °C. *X. oryzae* pv. *Oryzae* was obtained from Microbiology laboratory of College of Life Sciences, Jiangxi Normal University, and was stored in nutrient agar slant at 4 °C.

Fermentation of *M. carbonacea*

The *M. carbonacea* JXNU-1 was first spread onto the Gause's No. 1 medium slopes and incubated at 28 °C for 7 days. Then the slopes were washed with sterile water, and about 1×10^6 spores were inoculated into 50 ml seed culture medium (Containing 22.5 g Sucrose, 22.5 g soybean, 1 g NaCl, 0.2 g KH_2PO_4, 0.1 g Na_2SO_4, 2 g $CaCO_3$, 0.01 g $FeSO_4\cdot7H_2O$ in 1000 ml distilled water, pH 7.2, sterilize in autoclave at 121 °C for 20 min) in a 250 ml shake flask. After incubation at 28 °C and 200 rev/min for 72 h, the seed cultures were inoculated into 20 ml fermentation medium (Containing 20 g starch, 13 g sugar, 35 g peanut meal, 0.2 g KH_2PO_4, 0.1 g Na_2SO_4 1 g NaCl, 0.01 g $FeSO_4\cdot7H_2O$, 3 g $CaCO_3$ in 1000 ml distilled water, pH 7.7, sterilize in autoclave at 115 °C for 30 min) in a 250 ml shake flask at the inoculation ratio of 6%(v/v). For the production of antibiotics, the fermentation was also carried out at 28 °C and 200 rev/min for 108 h. The fermentation broth was collected for further study.

Preparation of antibiotics solution

The antibiotics solution was prepared as previously described[6]. Since the fact was proved that there is only a single component of antibiotics in fermentation broth with broad-spectrum, the process for preparation of antibiotics solution was simplified as follow: The supernatant was collected by the centrifugation at 4000 rev/min for 20 min, then the small hypha and floating oil in the upper layer were removed when the supernatant was filtered through Xinhua filter paper. The filtrate was added by twice the volume of dehydrous ethanol and was precipitated at 4 °C for 24 h. The supernatant adjusted to pH 7.0 was collected by the centrifugation at 4000 rev/min for 15 min. The alcohol in the supernatant was removed under vacuum at 45 °C. The supernatant was added to the original volume by distilled water and was sterilized at 121 °C for 20 min.

Preparation of cell suspension of *X. oryzae* pv. *Oryzae*

Xanthomonas oryzae pv. *Oryzae* were spread onto the beef extract peptone medium slopes and incubated at 37 °C for 24 h, then a loop of cultures were inoculated into 50 ml MH medium in a 250 ml shake flask. After incubation at 37 °C with orbital shaking at 200 rev/min, the cultures were collected when the growth stage of microorganisms was at logarithmic phase. The concentration of cell suspension was diluted to be about 10^6 cfu/ml for further study.

Minimum inhibition concentration

The minimum inhibition concentration (MIC) was detected by liquid dilution method[8]. Serial two-fold dilutions of antibiotic were prepared in MH medium, and inoculated with the bacterium suspension to give a final concentration of 10^5 cfu/ml. The broth were incubated at 37 °C for 24 h, and the lowest concentration of antibiotic which inhibited the growth of bacterium, as determined by naked eye examination, was taken as the MIC. Each experiment was performed in triplicate.

Effect of antibiotics on the cell morphology

The bacterium suspension was inoculated into the MH medium containing antibiotics, to give the final concentration of antibiotics of MIC and the final bacterium concentration of approximately 10^5 cfu/ml. The broth was incubated at 37 °C with orbital shaking at 200 rev/min for 12-24 h. The cell

morphology was observed by the gram staining method.

Effect of antibiotics on cell protein content

The bacterium suspension was inoculated into the MH medium containing antibiotics, to give the final bacterium concentration of approximately 10^5 cfu/ml and the final concentration of antibiotics of a series of MIC. The broth was incubated at 37 ^oC with orbital shaking at 200 rev/min for 12-24 h. The cultures, with growth stage of the control cell at the logarithmic phase, were collected by the centrifugation at 4000 rev/min for 20 min. The supernatant was abandoned and the wet weight of bacteria was measured after the water had been completely removed. The known volume of distilled water was added, and the cell wall was damaged through ultrasonic wave. The supernatant was collected by the centrifugation at 12,000 rev/min for 10 min. The protein concentration in supernatant was determined, and the protein content bacteria was calculated.

Effect of antibiotics on GFP synthesis by cell-free protein synthesis system

The RTS 100 *E.coli* HY Kit system (5 PRIME) was used to detect the effect of antibiotics on expression of positive control protein—green fluorescent protein (GFP) in kit system, the designed reaction systems were shown in table 1, and the specific operation procedures and requirements were followed by the kit manuals. The reaction system A without antibiotics was used as a negative control, and the reaction system B without GFP vector was used as blank control.

Determination of protein concentration

The protein concentration was determined by Coomassie brilliant blue dry-binding method [9].

RESULTS

Effect of antibiotics on cell morphology

The changes of cell morphology, treated with the antibiotics at the concentration of MIC, were observed. The results indicated that, the gram staining characteristics of *X. oryzae* pv. *Oryzae* treated by antibiotics were constant for 7d, but the number of bacteria in the test tube was less than that in control, as the test tube was clear and the control tube was turbid. The length of cell increased obviously, some changed from rod to filament (Fig. 1).

The Morphology are showed for *X. oryzae* pv.*oryzae* treated with the antibiotics at the concentration of MIC (a), and the control (b). All cells are grown in MH broth.

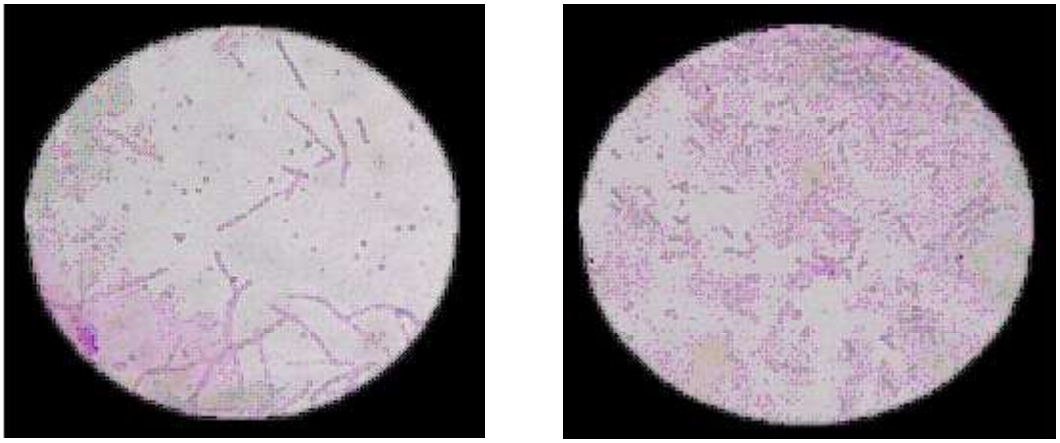
Effect of antibiotics on cell protein contents

The cell protein content of *X. oryzae* pv. *Oryzae*, treated with different antibiotic concentration, was determined, and the results showed in figure 2. The Figure 2 showed that, in a certain range of antibiotic concentration, the cell protein content was decreased with the increase of antibiotic concentration, and a straight line can be obtained by plotting of the cell protein contents versus the antibiotic concentration.

The sample loading volume for SDS-PAGE is 1ul at lane A1, 3 ul at lane A2 and 5 ul at other (Line A Negative control) Line B: Blank control; Line C-E: Reaction system with 2ul, 4ul and 6ul antibiotics respectively)

Table 1. Reaction system for GFP synthesis by the cell-free protein synthesis system

Reaction systemReaction components	A	B	C	D	E
E.coli lysate (µl)	12	12	12	12	12
Reaction mix (µl)	10	10	10	10	10
Amino acids (µl)	12	12	12	12	12
Methionine (µl)	1	1	1	1	1
Reconstitution buffer (µl)	5	5	5	5	5
sterile DNase- and RNase-free water (µl)	9	10	7	5	3
Antibiotics solution (µl)			2	4	6
GFP vector (µl)	1		1	1	1
Total volume (µl)	50	50	50	50	50



a) b)

Fig. 1. Effect of the antibiotic from *M. carbonacea* JXNU-1 on the morphology of *X. oryzae* pv. *oryzae.* The Morphology are showed for X. oryzaepv.oryzae treated with the antibiotics at the concentration of MIC (a), and the control (b). All cells are grown in MH broth.

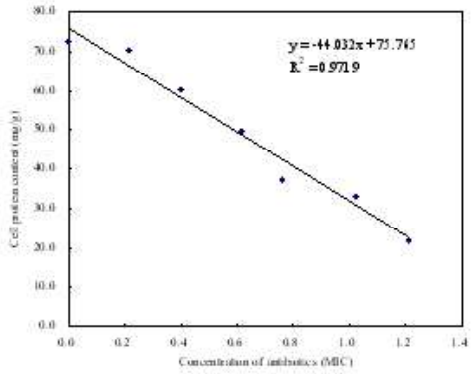


Fig.2. Effect of the antibiotic concentration on cell protein content of *X. oryzae* pv. *oryzae*

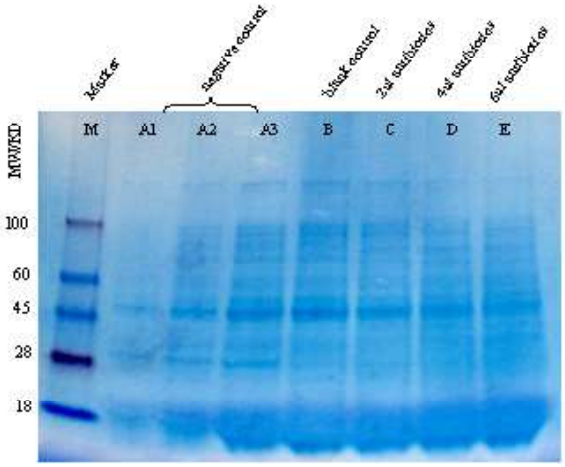


Fig. 3. Effect of the antibiotic from *M. carbonacea* JXNU-1 on GFP synthesis by the cell-free protein synthesis system The sample loading volume for SDS-PAGE is 1ul at lane A1, 3 ul at lane A2 and 5 ul at other (Line A Negative control?Line B: Blank control; Line C-E: Reaction system with 2ul, 4ul and 6ul antibiotics respectively)

DISCUSSION

Expounding the antimicrobial mechanism of antibiotics is the precondition of reasonable use of the antibiotics. The antibiotics from *M. carbonacea* has been proved to be characterized by the physical and chemical properties of nucleoside antibiotic[6], and the antibacterial mechanism of nucleoside antibiotics is closely related to their structural features, for example, simple nucleoside analogs often act as inhibitors of nucleic acid synthesis[10], however, acyl or glycosyl nucleosides may often inhibit the synthesis of protein[11], cell wall glycan[12] and other biological macromolecules, and then affect cell division[13].

From the point of the results in this study, The cell morphology of *X. oryzae* pv. Oryzae showed some different characteristics when treated with the antibiotics from *M. carbonacea* JXNU-1, that is, the Gram's staining reaction stayed the same, and cell form varied form rods into filamentous. These results indicated that antibiotics from *M. carbonacea* JXNU-1 do not affect the cell wall structure, but inhibited cell division of *X. oryzae* pv. *Oryzae.*

There is a variety of reasons for inhibition of cell division, for example, biosynthetic inhibition of nucleic acid, protein, or other biological macromolecules so on. From the fact that the cell protein content was decreased with the increase of antibiotic concentration, we can deduce that the protein synthesis of *X. oryzae* pv. *Oryzae* was obviously inhibited by antibiotics, this assumption was confirmed by that antibiotics can inhibit protein synthesis in cell-free protein synthesis system. All these results indicated that the antibiotics, from *M. carbonacea* JXNU-1 do not affect the structure of cell wall, but inhibit the cell protein synthesis, and then inhibit the cell division of *X. oryzae* pv. *Oryzae*. Of course, the specific molecular mechanism for bacterial protein synthesis inhibition has yet to be further research.

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