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Control Activity and Antibiotic Gene Detection of Endophytic Bacteria in Suppressing Cocoa Black Pod Disease (*Phytophthora palmivora* Butl.)

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ABSTRACT

Background: Black pod rot disease of cocoa caused by (*Phytophthora palmivora* Butl.) is one of the major diseases on cocoa plantations worldwide. Many attempts have been made to prevent or reduce the infection of pathogens, but they have not provided optimum results. This study aims to detect antibiotic genes in endophytic bacteria that can suppress cocoa black pod disease.

Methods: Eight endophytic bacteria were isolated from healthy cocoa pods and twigs that showed potentials in suppressing *P. palmivora* growth *in vitro* were used in suppressing of black pod rot disease *in vivo* tests. Antibiotic biosynthesis-related genes from eight endophytic bacterial isolates were confirmed by using PCR method, which includes phenazine-1-carboxylic (PCA), pyrrolnitrin (PRN), phenazine-1-carboxamide acid (PCN), pyoluteorin (PLT) and 2,4-diacetylphloroglucinol (DPAG/Phl).

Result: The endophytic bacteria, 4RSI, 5BR B3 and 2RW B2 isolates showed the highest disease suppressing index to black pod rot disease *in vivo*, *i.e.*, 70.27%, 70.08% and 56.64%. The isolates 5BR B3 and 2RW B2 DNA yielded PCR product by using PCA primers (1400 bp), PRN primers (700 bp) and DAPG primers (1600 bp), while the 5RSI isolate yield PCR product using PRN primers only. Endophytic bacterial isolates 5BRB3 and 2RW B2 provided partial disease suppression to pod rot disease by inhibit pathogen growth and antibiotic compounds production.

Key words: Antibiotic genes, Black pod disease, Cocoa, Endophytic bacteria.

INTRODUCTION

(*Phytophthora palmivora* Butl.) which causes pod rot is one of the important pathogens in cacao plants. This disease can reduce world cocoa production by 20-30% every year (Guest, 2007), it can even reduce the yield by 90% (Bowers *et al*., 2001). Various control techniques such as garden sanitation and use of resistant clones, as well as chemicals have been carried out, but this disease remains a major problem in cacao growing worldwide.

Endophytic bacterial is one of the biological agents that has the potential to be developed as a controlling agent for black pod rot disease in cocoa, which is effectively inhibits the development of pathogens and is safe for the environment and consumers (Marwan *et al*., 2011; Nguyen *et al*., 2016; Ramli *et al*., 2016). Endophytic bacteria live in plant tissue without causing disturbance and damage to plant tissue (Munif *et al*., 2013; Miliute *et al*., 2015; Kandel *et al*., 2017). The presence of endophytic bacteria in plant tissue can provide several benefits, one of which can play a role in plant pathogen biocontrol agents, such as: controlling blood diseases in banana plants (Marwan *et al*., 2011), inhibiting the growth of *Fusarium oxysporum* f. sp. *vanillae* causes stems rots of Vanilla (Suniti 2015) and *Sclerotium* sp. in peanut sprouts (Arios *et al*., 2014), suppressing the development of *Xanthomonas axonopodis* pv. *glycines* in soybean plants (Habazar *et al*., 2015), *Ganoderma boninense* in palm trees (Buana *et al*., 2014), *Fusarium oxysporum* and *Meloidogyne incognita* in pepper plants (Wiratno *et al*. 2019), as well as inhibiting the development of *Oncobasidium theobromae* causes of *vascular streak dieback* (VSD) in cocoa (Zubir *et al*., 2019).

Khaeruni *et al*. (2019a) reported that eight isolates of endophytic bacteria from healthy cacao plants had an inhibitory ability of >50% *in vitro* against 3 different *P. palmivora*

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isolates. A number of endophytic bacteria having the ability to inhibit the development of plant pathogens are reported to produce antimicrobial compounds such as *pyrrolnitrin* and *phenazines* (Kirner *et al*. 1998), 2,4-*diecetylphloroglucinol* (2,4-DAPG) and *pyoluteorin* (Subagio and Foster 2003). Genes related to the production of antimicrobial compounds from biocontrol agents can be detected by Polymerase Chain Reaction (PCR) techniques using gene specific primers that encodes phenazine-1-carboxylic acid, 2,4-DAPG, pyoluteorin, phenazine-1-c and pyrrolnitrin from the genomes of *Pseudomonas* and *Burkholderia* (Bakthavatchalu *et al*., 2013). With this background the study was taken up to determine the potential of endophytic bacteria from healthy cocoa plants as a biological controlling agents for control cocoa black pod rot disease *in vivo* and detect the presence of genes that encode antimicrobial compounds in these endophytic bacterial isolates.

MATERIALS AND METHODS

Source of endophytic bacterial isolates

The isolates used for the study include eight endophytic bacterial isolates from healthy cocoa pods and branches that had *in vitro* inhibition against *P. palmivora* >50%, namely: isolates 4RSI, 1RSI3, 2RWB2, 5BRB3, 5RSI, 3BTO1, 3BAE, 4RS (Khaeruni *et al*., 2019a), isolate *P. palmivora* KS01 collected from of Plant Protection Laboratory, Faculty of Agriculture, Halu Oleo University Kendari.

Fruit inoculation in living plants with endophytic bacteria

Inoculation of endophytic bacteria on fruit of cocoa in the tree was carried out at the Field Station of Plantation and Horticulture Office, Southeast Sulawesi Province, 8 endophytic bacteria were selected for the study that had inhibitory tests *in vitro* ≥50%. Selected endophytic bacteria were multiplied in (Trypticase Soy Agar) TSA medium and incubated at room temperature. After 2 days the bacteria were suspended in sterile distilled water until the cell density reached 10^8 CFU/mL. When treated in the field, bacterial suspension was mixed with 0.1% Tween 80 and sprayed on selected fruit with a relatively uniform age and size (length +10 cm). For each endophytic bacteria, spraying was carried out on three trees of five fruits per tree. A mixture of Tween 80 and sterile aquades was used as a control. After two weeks, the endophytic bacteria treated fruits were picked and then inoculated with *P. palmivora* in the laboratory.

Phytophthora palmivora inoculation on endophytic bacteria treated cocoa fruits

The *P. palmivora* isolate KS01, obtained from a single purified zoospora. The making of *P. palmivora* inoculum suspension was carried out in accordance with AVRDC Mycology (2000) method, The isolates were grown in V8 agar medium under continuous irradiation at 28°C for 4-5 days. Furthermore, isolates were divided into four, each part was transferred to a new petri dish and cut into 5 mm^2 pieces. The pieces were immersed in water for one hour, discarded and flooded again and incubated under continuous irradiation for 24 hours. Then the isolates were transferred to a room at 4°C for 2 hours and 28°C for 1-2 hours. While the method of pathogen inoculation in fruit was carried out according to state by Tondok *et al*. (2012), harvested zoospores were diluted to 5×10^4 mL^{-1}. Before use, zoospores were stored at 4°C for 3 days to germinate. Sterile circular filter paper 10 mm in diameter was dropped with 1 mL of zoospores, two papers were placed upside down on the surface of the cocoa pods inoculated by endophytic bacterial isolates.

Inoculated cocoa pods were then placed in plastic containers, five pods per container. Plastic containers were wrapped with thin plastic and then placed in wet tissue paper. The plastic container was then placed in an incubation rack arranged according to the experiments used. Disease severity was observed at two, four and six days after inoculation with a score: 1= x≤5; 2= 5<x≤15; 3= 15<x≤25; 4= 25<x≤35; 5= 35<x≤45 and 6= x>45, where x = range of symptoms (%) (Tondok *et al*. 2012).

The area under disease progress curve (AUDPC) value and disease Suppression Index (DSI) of each endophytic bacteria is calculated from the severity of the disease in cocoa pods. AUDPC was calculated by the formula proposed by Khaeruni *et al.* (2018) and DSI was calculated by the formula proposed by Wijayanto *et al.* (2017).

Antifungal encoding gene detection

(a) DNA extraction of endophytic antagonistic bacteria

Bacterial isolates were grown in the Yeast Peptone Agar (YPA) medium which was incubated for 24-48 hours. Furthermore, DNA extraction carried out according to the method of Schaad *et al*. (2001) with some modification. One loop of bacterial culture was suspended into 1000 µL sterile *aquadest* at Eppendorf tube 1.5 ml, then centrifuged at 10000 rpm for 10 minutes and supernatant removed. Next 500 mL TE buffer and 30 µL 10% SDS were added and carefully vortexed, then incubated at 37°C using water bath for an hour. After incubation, 100 µL 5 M NaCl and 10 µL CTAB/NaCl were added first to the above mixture followed by 750 µL Chloroform: Isoamyl-Alcohol, 24:1 (CIAA) and it was shaken until homogeneous for ± 2 minutes, continued with centrifugation at 14000 rpm for 5 minute. The top layer was then transferred to a new Eppendorf tube and added with 600 µL Phenol chloroform: isoamyl alcohol, 24:1 (PCIAA), shaken and centrifuged at 14000 rpm for 5 minutes.

As much as ±500 µL of the upper liquid phase was transferred to the new Eppendorf tube, then ± 500 µl of isopropanol was added and incubated at -20°C for an hour. After incubation, the DNA suspension was centrifuged at a speed of 14000 rpm for 5 minutes and then supernatant was carefully disposed. DNA pellets were washed by adding 1 mL of 70% ethanol gollowed by vortex and centrifuge at 14000 rpm for 5 minutes. Then, supernatant was removed

and dried the pellet in the Laminar air flow cabinet for ±24 hours. DNA pellets were suspended in 20-40 µL TE buffer and stored in a refrigerator at -20°C.

(b) Detection of antimicrobial encoding genes of endophytic antagonistic bacterial isolates

Detection of antimicrobial encoding genes in endophytic bacteria was carried out using PCR method. Specific primers that encoded the antibiotic compounds such as phenazine-1-carboxylic (PCA), pyrrolnitrin (PRN), phenazine-1-carboxamide (PCN), pyoluteorin (PLT) and 2,4-diacetylphloroglucinol (DPAG/Phl) genes were used according to the procedure of Bakthavatchalu *et al.* (2013) (Table 1). The PCR results of each genes were in electrophoreses with 1% *agarose gel* and visualized using *Etidium Bromide (EtBr) via* gel doc transilluminator.

Statistical analysis

In the testing phase of *P. palmivora* inoculation on fruit that has been picked from the cacao tree, it was arranged based on a completely randomized design (CRD) with eight treatments of endophytic bacterial isolates plus control. Each treatment was repeated three times, bringing the total of the union units to 21 units. Variance analysis was carried out to determine the potential of endophytic bacteria in inhibiting the use of rotten cacao fruit *in vivo*. Based on the effect, proceeded with Duncan's multiple range test (DMRT) at a 95% significance level.

RESULTS AND DISCUSSION

Disease severity, area under disease progress curve and disease suppressing index

All endophytic bacteria tested have the ability to inhibit the development of cocoa pod rot, this is evidenced by the disease severity which is always lower than the control (Table 2). At sixth DAI (day after inoculation), the endophytic bacterial isolates 2RWB2 and 4RSI treatments showed the lowest disease severity value of 25.55%, which is significantly different from control (70% disease severity). The suppression of disease progression by 2RWB2 and 4RSI endophytic bacteria was also evident from the lower AUDPC values compared to other treatments, recorded 11.12 and 11.11 units respectively (Table 2).

The results of the analysis of the disease suppression index showed that eight endophytic bacterial isolates tested, 5 isolates were obtained, namely 4RSI, 5BRB3, 2RWB2; 3BAE and 1RSI3 which have disease suppression index> 50%, two of which endophytic bacteria 4RSI and 5BRB3 have disease suppression index above 70% (Table 3).

The PHZ1 and PHZ2 successfully amplified 1400 bp DNA fragment from 2RWB2, 5RSI and 5BRB3 isolates. The Prncf and Prncr were successfully amplified 700 bp size DNA fragment from the endophytic isolates of 2RW B2, 5RSI and 5BR B3, while the main pair of Prncf and Prncr successfully amplified DNA fragment of 700 bp in size from endophytic bacterial isolates of 2RW B2, 5RSI and 5BRB3. Succeeded in amplifying 1600 bp DNA fragment of DPAG/Phl gene in 2RW B2 and 5BRB3 isolates (Fig 1, 2 and 3).

The main pair of PhzH-up and PhzH-low and the main pair of PLTC1 and PLTC2 did not succeed in amplifying the DNA fragment of all tested isolates. These results indicate that endophytic bacterial isolates 2RW B2 and 5BRB3 contain three antimicrobial encoding genes namely pyrrolnitrin, phenazine-1-carboxamide and 2,4-diacetylphloroglucinol. Isolate 5RSI contain antimicrobial encoding genes such as pyrrolnitrin, while five other isolates

Table 1: Primer and amplification of gene encoding antimicrobial genes in endophytic bacteria.

Antibiotic	Primers	Primer sequence (5′ to 3′)
PCA	PHZ1	GCGACATGGTCAACGG
	PHZ2	CGGCTGGCGGCGTATTC
PCN	PhzH-up	CGCACGGATCCTTTCAGAATGTTC
	PhzH-low	GCCACGCCAAGCTTCACGCTCA
DPAG/Phl	Phl2a	GAGGACGTCGAAGACCACCCA
	Phl2b	ACCGCAGCATCGTGTATGAG
PRN	Prncf	CCACAAGCCCGGCCGGAGC
	Prncr	GAGAAGAGCGGGTCGTGAAGCC
PLT	PLTC1	ACAGATCGCCCCGGTACAGAACG
	PLTC2	GGCCCGGACACTCAAGAAACTCG

Table 2: Disease severity of cocoa fruit rot treated with endophytic bacteria.

Isolates	Disease severity (%) on…day after inoculation		
treatment	2	4	6
A (4RSI)	8.89bcd	14.44de	25.55c
B (1RSI3)	7.78bcd	9.98ef	32.22c
C (2RW B2)	1.11e	8.89f	25.55c
D (5BR B3)	13.33ab	18.89d	30.11c
E (5RSI)	12.22abc	24.22b	65.55a
F (3BTO1)	6.66cde	21.11bc	46.33b
G (3BAE)	11.11abc	17.78c	36.66bc
H (4RS)	4.44de	12.21ef	38.89bc
K (control)	15.55a	37.78a	70.00a

Table 3: AUDPC of fruit rot disease on cocoa treated with endophytic bacteria.

Isolates	AUDPC value, % day (unit)		Disease suppressing
treatment	2-4 DAI	4-6 DAI	index (%)
A (4RSI)	5.56	11.11	70.27
B (1RSI3)	2.22	25.56	55.35
C (2RW B2)	7.78	16.67	56.64
D (5BR B3)	5.56	11.22	70.08
E (5RSI)	11.33	42.00	9.33
F (3BT O1)	14.44	25.22	37.81
G (3BAE)	6.67	18.89	55.70
H (4RS)	7.77	26.68	41.14
K (Control)	22.23	32.22	0.00

contain none of the antimicrobial encoding genes that have tested (Table 4).

Phytophthora palmivora is an important pathogen and can attack all parts of cacao plant (Azis *et al*., 2013), especially on immature fruits are the most detrimental (Rubiyo and Amaria 2013). Cocoa fruit inoculation in the field with endophytic bacteria followed by inoculation of *P. palmivora* in the laboratory gave different responses to the severity of the disease in cocoa. Endophytic bacterial inoculation in cocoa can inhibit the development of fruit rot disease, this is evidenced by the disease severity which is always lower at all times of observation in all the treatments of endophytic bacteria compared to control (Table 1).

At the end of the observation (sixth day after inoculation (DAI) the severity of the disease in the treatment of endophytic bacteria ranged from 25.55%-65.55%, while the control severity reached 70%. Endophytic bacterial treatment of 4RSI and 2RW B2 isolates showed the lowest disease severity of 25.55% and it was significantly different from control at 5% level. The lower disease severity in the treatment of endophytic bacteria 4RSI and 2RW B2 was recorded with followin by AUDPC values which were also relatively lower, respectively 11.11% and 11.22%, while the control reached 32.22%. Endophytic bacteria used in this study have the ability to inhibit the development of *P. palmivora* *in vitro* >50%. Of the 8 isolates tested, there were 5 isolates, namely: 4RSI, 5BRB3, 2RWB2, 1RSI3 and 3BAE, consistently showed an index of suppression to the development of *P. palmivora in vivo* >50%, even 4RSI3 and 2RWB2 isolates were able to suppress the development of cocoa black pod rot disease to 70.27% and 70.08% respectively (Table 2).

Previous researchers reported that the ability of endophytic bacteria as biological control agents against phytopathogens in cacao plants in both *in vitro* and *in vivo*. *In vitro*, endophytic bacteria LKM-UL is able to inhibit the development of *P. palmivora* from cacao plants (Hamzah *et al*. 2017), endophytic bacteria 2RWB2 and 5BPR1 *in vitro* are able to inhibit the development of *Colletotrichum gloeosporioides* causing anthracnose in cacao each by 69% and 62% (Khaeruni *et al*. 2019b). *In vivo* BT8 endophytic bacteria were able to inhibit the development of *P. capsici* inoculated on cacao leaves (Melnick *et al*. 2008) and LKM-BL endophytic bacteria at the age of 30 days after inoculation

Table 4: Summary of PCR products of antibiotic genes from endophytic bacterial isolates.

Endophytic bacterial isolates	Antimicrobial genes amplified				
	PCA	PRN	2,4 DAPG/Phl	PCN	PLT
4RSI	-	-	-	-	-
1RSI3	-	-	-	-	-
2RW B2	+	+	+	-	-
5BR B3	+	+	+	-	-
5RSI	-	+	-	-	-
3BTO1	-	-	-	-	-
4RS	-	-	-	-	-
3BAE	-	-	-	-	-

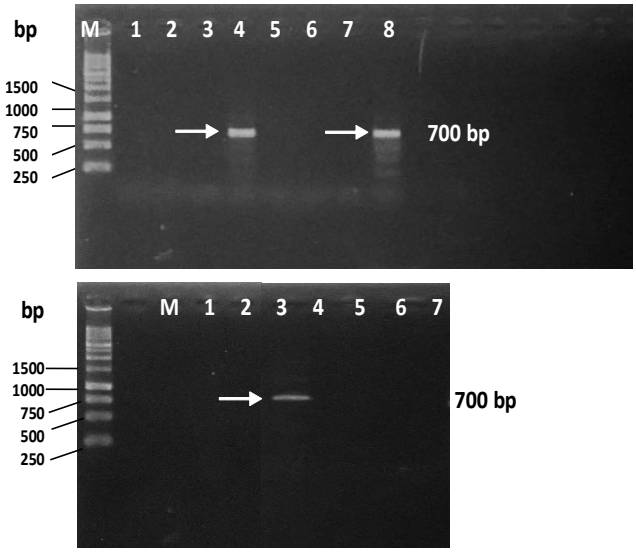

Fig 2: PCR product of pyrrolnitrin (PRN) gene amplification by Prncf and Prncr primers. Line M=marker1 kb; 1=3BAE; 2=4RS; 3=4RSI; 4=2RW B2; 5=5RSI; 6=3BT O1; 7=1RSI3; 8=5BRB3.

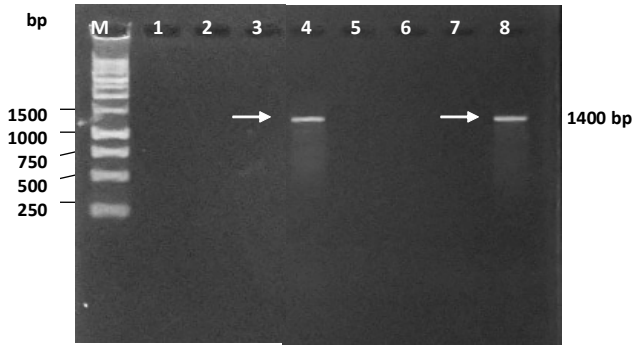

Fig 1: PCR product of phenazine-1-carboxylic acid (PCA) gene amplification by PHZ1 and PHZ2 primers. Line M=marker 1 kb; 1=3BAE; 2=4RS; 3=4RSI; 4=2RW B2; 5=5RSI; 6=3BTO1; 7=1RSI3; 8=5BR B3.

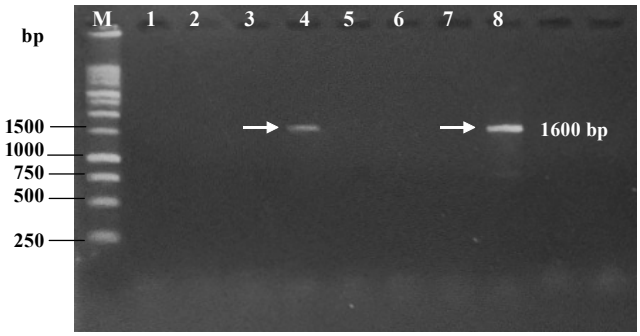

Fig 3: PCR product of 2,4-diacetylphloroglucinol(DPAG/Phl) gene amplification by Phl2a and Phl2b primers. Line M=marker 1 kb; 1=3BAE; 2=4RS; 3=4RSI, 4=2RW B2; 5=5RSI; 6=3BTO1; 7=1RSI3; 8=5BR B3.

were able to inhibit the development of VSD (Vascular Streak Dieback) in the cacao nursery by 87.90% (Zubir *et al*. 2019). The ability of endophytic bacteria to inhibit the development of pathogens and increase is related to various antagonistic mechanisms, including synthesizing antibiotic compounds, produce of hydrolytic enzymes, nutrient limitation and by priming plant defenses (Gao *et al*. 2010; Afzalab *et al*. 2019). The result of previous studies showed that some endophytic bacterial isolates used in this study, including isolates 5BRB3, 3BAE, were able to produce cellulase and protease hydrolytic enzymes (Khaeruni *et al.* 2019a), 2RWB2 isolate able tolyse the cell wall of *C. gloesporioides*, thought to be related its ability to cellulase produce the enzyme chitinase (Khaeruni *et al.* 2019b).

The results of antibiotic-encoding genes detection by PCR technique using specific primers showed that 2RWB2 and 5BRB3 DNA isolates are able to amplified PCA encoding genes of 1400 bp size, PRN encoding genes of 700 bp and DPAG encoding genes of 1600 bp, this indicating that both isolates have the potential to produce three antibiotic compounds namely phenazine-1-carboxylic acid, pyrrolnitrin and 2,4-diacetylphloroglucinol, whereas from the endophytic bacterium 5RSI isolate amplified PRN encoding gene size of 700 bp indicated the potential for producing pyrrolnitrin antibiotic compounds. DNA amplification measurements obtained from each antibiotic-coding gene primer were used in this study by accordance with the results of Bakthavatchalu *et al.* (2013). Pyrrolnitrin encoding gene was successfully amplified in three different isolates, this indicates that the pyrrolnitrin compound is one of the antibiotic compounds that play a role in controlling plant pathogens by antagonistic bacteria. Endophytic Bacteria, *Burkholderia* sp. which has antagonistic activity against *G. anodermaboninense* has the potential to produce pyrrolnitrin antibiotic compounds (Buana *et al.* 2014), *Enterobacter agglomerans* which have strong antagonistic activity against various types of pathogens detected to produce the enzyme chitinase and pyrrolnitrin compounds (Chernin *et al.,* 1996). Pyrollnitrin is a chlorinated phenypyrrole antibiotic first isolated from *Burkholderia pyrrocinia* (Kloepper and Ryu, 2006). Pyrollnitrin is synthesized by four proteins encoded by 4 genes, prnA, prnB, prnC and prnD. The prnD gene is the final protein to form an active pyrrolnitrin compound. The prnD catalyzes the oxidation of the amino group of aminopyrrolnitrin to a nitro group to the pyrrolnitrin form (Kirner *et al*. 1998).

CONCLUSION

It was concluded that the ability of endophytic bacterial isolates 2RW B2 and 5BR B3 to produce antibiotic compounds such as pyrrolnitrin, phenazine-1-carboxylic and 2,4-diacetylphloroglucinol suspected to be one of the antagonistic mechanisms play an important role in inhibiting *P. palmivora* infections in cocoa fruit, as evidenced by the value of 2,4-diacetylphloroglucinol. Disease Suppression Index (DSI) of the two isolates were quite high at 56.64% for 2RWB2 and 5BRB3 at 70.08%, witnessing the potential to develop as a biological control agent against cocoa pod rot. In addition, the 5RSI isolate also has the potential to be developed as a biological agent because it has a DSI of 70.27% cocoa pod rot disease, the highest of all the isolates tested, although no gene was detected for encoding the antibiotic compound tested, but perhaps the ability of the antagonist through other antibiotic encoding genes or another antagonistic mechanism.

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