Effect of antibiotics treatment of *Coptotermes formosanus* on cellulase secretion

By

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Abstract

Studies of physical and biochemical associations among termites, flagellates and prokaryotes are very important in understanding the dual cellulolitic systems of lower termites. The effect of antibiotics on cellulase secretions and on the structure of the symbiotic intestinal microbial community of *Coptotermes formosanus* was studied. It was apparent that inhibition of the protists symbiotic bacterial community could lead to the regular changes in secretion of cellulases secretion of *C. formosanus*. *Pseudotrichonympha grassii*, a major kind of intestinal protist, played an important role in the intestinal symbiotic cellulolitic system. We supposed antibiotic could induce the growth of *P. grassii* via a function of then symbiotic bacteria. Furthermore, the results of the present study suggest that endo-β-1, 4-glucanase and exo-β-1, 4-cellobiohydrolase had more associated interactions between every two among the three kinds of cellulase. The findings above led us to hypothesize that intestinal symbiotic prokaryotes possess a stress-response pathway via energy metabolism to connect the dual cellulolitic system of *C. formosanus*.

Key words: antibiotics; cellulase; *Coptotermes formosanus*

Introduction

C. formosanus, a typical wood-feeding lower termite, is arguably the most destructive termite species in the world andwas found to have a more effective cellulose hydrolysis system when compared to all other termites(Tokuda et al., 2004).

Usually, the cellulases of termites are comprised of the following enzymes: endo-β-1, 4-glucanase (EC; EG. 3.2.1.4), exo-β-1, 4-cellobiohydrolase (CBH; EC. 3.2.1.91) and β-glucosidase (BG; EC. 3.2.1.21) for hydrolysis of cellulose (Willis et al., 2010; Arakawa et al., 2009). So far, in *C. formosanus*, different EG and BG mRNAs in salivary glands, the midgut and the hindgut were confirmed by RT-PCR, whereas the expression of CBH can only be detected in hindgut protists (Nakashima et al., 2002).

Indeed, the *C. formosanus* possess two lignocellulolitic systems: one locateds in the salivary glands and the midgut where cellulose is digested by endogenous cellulases, and the other locateds in the hindgut where symbiotic fauna provide exogenous cellulases (Xie et al., 2012; Nakashima et al., 2002). Large numbers of flagellated protists densely packed in their hindgut are considered to

play an irreplaceable role in cellulose decomposition inof lower termites (Ohkuma et al., 2000). In addition to these eukaryotic symbiota, numerous prokaryotic microbes (bacteria and archae) are found either attaching to the surface (ectosymbiont) or living within the protists cells (endosymbiont), developing an intimate relationship with the protists. Newly researches suggested that these prokaryotic organisms are actively involved in H_2 metabolism, CO_2-reductive acetogenesis, nitrogen fixation and amino acid synthesis, excluding their contribution ton ligocellulose decomposition (Xie et al., 2012; Ohkuma, 2008; Warnecke et al., 2007)

Recently, although many researches on the host-symbiont digestion system of lower termites using a through metatranscriptome approach provided abundant genomic resources which revealed a lot of cellulolitic bacteria hydrolases (Xie et al., 2012; Tartar et al., 2009; Warnecke et al., 2007), it is not clear what are the to what the details of thised host-symbiont cooperative mechanism. Lower termites establish symbiotic associations not only with the intestinal flagellated protist but also with other protist- associated prokaryotes symbionts. In this study, we used took qualitative methods, including molecular techniques to analyses the effect of antibiotics treatment of *C. formosanus* on cellulases secretions. The aim of this work was to explore whether protists associated with the prokaryotes symbionts can affect the endogenous cellulose system if the bacterial community changes.

Materials and methods

Termites and antibiotic treatment Termites used in this study were *C. formosanus* which were maintained in the lab. Workers were selected for pre-treatment hunger for 3 days at 28℃ and 60% relative humidity (RH). Then, these termites were divided into two groups: one wais the negative control, feeding on distilled water soaked filter paper; the other wais the sample of antibiotic treatment, feeding on ampicillin and kanamycin soaked filter paper. Termites of each group were collected separately at 6-hours, 12-hours, 24-hours, 48-hours and 72-hours after feeding on the with different diets. Three biological replicates were provided for each group.

Cellulase activity assays For cellulase activity assays, termites collected from every replicate were immobilized on ice and dissected into two regions: (i) salivary gland /foregut& midgut (ii) hindgut. Each set of dissected gut regions were homogenized in ice-cold SAB buffer (0.1 M sodium acetate, pH 5.6). After homogenization, gut region and preparations were centrifuged. The resulting supernatants were used in the assays for EG, CBH and BG activity. Each assay for every biological replicates was performed in triplicate.

Total RNA isolation, reverse transcription and quantitative PCR The expression level of two genes: endogenous EG and protist CBH of *C. formosanus* were determined by quantitative real-time RT-PCR (qRT-PCR). Relative expression levels for specific genes were normalized to the

reference gene Heat shock 70 kDa protein (HSP70) and determined by the $2^{-\Delta\Delta CT}$ method. Three independent replicates were performed, each conducted in triplicate.

Microscopic examination of flagellates of hindguts The disintegrated hindgut pieces were macerated gently in the SAB buffer to facilitate the release of the protozoa. Then the protozoa and bacteria were identified by using a light microscope.

Results

Effects of antibiotics diets on the lignocellulase activity and distribution through the *C. formosanus* gut

Activity and distribution of three different cellulases were assayed for the negative control and antibiotic treated samples simultaneously. Total activitiyes of EG and CBH in antibiotic treated samples were higher than that in the negative control, excepted for the BG activity. The related highest CBH activities (285.6% of negative control) in the hindgut were detected at 6 hours after antibiotic treatment. Interestingly, only at 12hours after treatment, did the CBH activity of foregut/salivary gland & midgut exhibited an big increase (284.8% of negative control), so did its distribution percentage, and meanwhile CBH activity in hindgut showedgot a big reduction (Fig.1c). EG and BG activity variation results were showed more complicated. Relative endogenous EG activity was 245.8% of the control and had a maximum increase at 6 hours after treatment. In addition, at this time, diversity of the EG activity ration [of (foregut/salivary gland & midgut) / hindgut] between samples and the control achieved a maximum (33.85% for control and 60.44% for sample). Whereas, relative EG activity of the hindgut had a maximum increase (170.9% of control) at 24 hours after treatment (Fig. 1a). There was a little difference in endogenous BG activity level between samples and controls . By contrast, BG activity of the hindgut soared to 388.2% of the controls after 12 hours and then decreased sharply to only 59.6% of the controls after 24 hours. Meanwhile, 12 hours after comparative feeding , the difference of BG activity ration [of (foregut/salivary gland & midgut) / hindgut] between samples and controls achieved maximum (116.26% for control and 24.45% for sample). Interestingly, that ratio difference got a reversed at collected timing of 48 hours (363.44%control and 760.75% for sample), which meant the hindgut symbiotic fauna of antibiotic treated sample had a stronger reboundnounce in BG activity level (Fig. 1b).

Effects of antibiotics diets on the cellulase gene expression through the *C. formosanus* gut

WeIn an attempted to correlate two different sources ofd celluloases activityies (EG and CBH), relative to expression levels of two genes (endougenous *eg* and exogenous *cbh*) were measured by qRT-PCR. In this study, termites feeding onwith ddH_2O soaked filter paper were used as a control to normalize gene expression respectively at different phases.

Endogenous *eg* exhibited a 1.7~2.0-fold expression relative to controls from 6 hours to 12 hours after antibiotic treatment , whereas, its expression level decreased sharply to 0.31- fold relative to the controls 12 hours later (p<0.05). Exogenous *cbh* exhibited 0.3~0.4-fold expression relative to controls from 6 hours to 24 hours after antibiotic treatment, and recovered to 1.11- fold after 48 hours antibiotic treatment (p<0.05, Fig. 2).

Effects of antibiotic treatment on the intestinal protozoa community

Microscopic observation result of *C. formosanus* starved hungered for 3 days showed that the intestinal protozoa community displayedgot a significant reductionlose in numbers and species. Six hours after feeding on with filter paper, the negative controls showed an more increase in protist numbers compared to the antibiotic treated sample, and the major species weare *Holomastigotoides mirabile* and *Spirotrichonympha leidyi*. Numbers and species of protists followed presented closely between control and treated samples, and *P. grassii* could be observed when 12 hours after comparative feeding. Interestingly, *P.grassii* number sharply increased in the antibiotic treatment samples after 24 hours feeding. The intestinal protozoa community of the negative control approached to that of the antibiotic sample until feeding for 48 hours. Then numbers and species of protists between the two treatmentermites groups exhibited basically the same numbers after 72 hours of comparative feeding (Fig.3).

Conclusion

The combination of the two separate cellulolytic systems (the endogenous and symbiotic cellulases) in the lower termite may be considered as a kind of synergism (Nakashima et al., 2002). It is still unclear what are the key factors that associate the two cellulolitic systems in lower termites. The results of this study suggested that inhibition of the protists symbiotic bacterial community could lead to regular changes in theof cellulases secretedion in C. *formosanus*. So far, cellulases in the hindgut of lower termites are considered to be contributed exclusively by symbiotic protists (Brune and Ohkuma, 2011). These results suggested *P. grassii* played an important role in the symbiotic cellulolitic system of *C. formosanus*, and the antibiotics induced the growth of *P. grassii* via a function on the intestinal prokaryotes. The findings above led us to hypothesized that intestinal symbiotic prokaryote possess a stress-response pathway via energy metabolism to connect the dual cellulolitic system of *C. formosanus***.** It is consistent with the statement that low termite's endogenous glucose production rate is insufficient to meet their metabolic needs (Nakashima et al., 2002). In addition, total activity levels of three cellulases in the antibiotic treated sample weare roughly higher than the negative control. Metatranscriptome analyses of host and symbiont genes in lower termites uncovered many cellulose hydrolysis related genes which belonged to bacteria(Xie et al., 2012; Tartar et al., 2009). So, we supposed that there are bacterial cellulase genes in the hindgut of *C. formosanus*, Furthermore, the results also presented that EG and CBH had more associated

interactions between every two of the among three kinds of cellulase. The reason for this result may be is that BG is responsible for the last step in digesting cellulose to glucose.

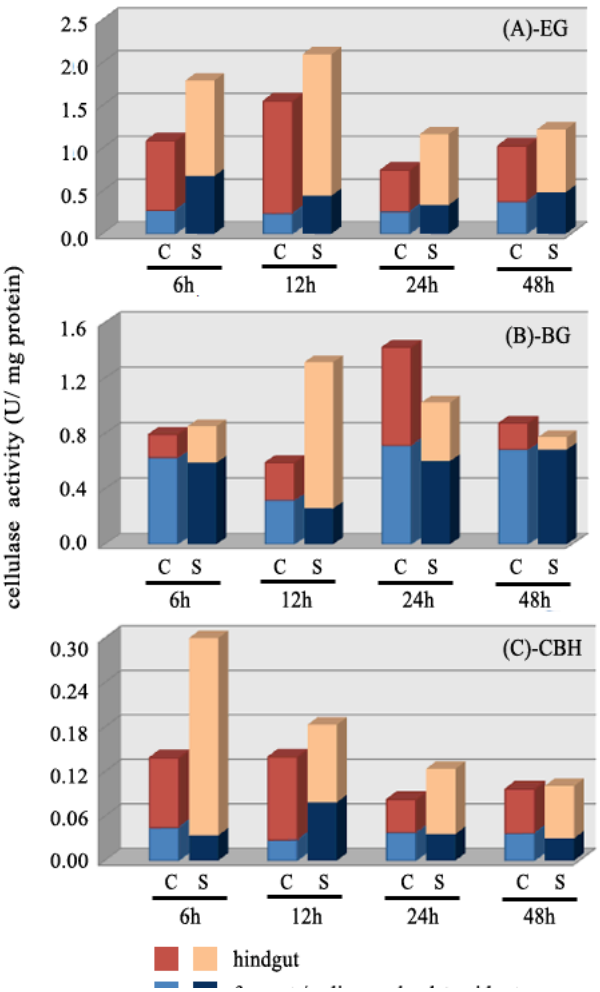

Fig.1 Effect of antibiotics on cellulase activity levels and distribution in *C. formosanus*. (A) endo-β-1, 4-glucanase activity. (B) β-glucosidase activity. (C) exo-β-1, 4-cellobiohydrolase activity. The columns indicate the mean of three of measurements. Cellulase activityies detected from negative control and antibiotic treated samples are presented as "C" and "S" specifically.

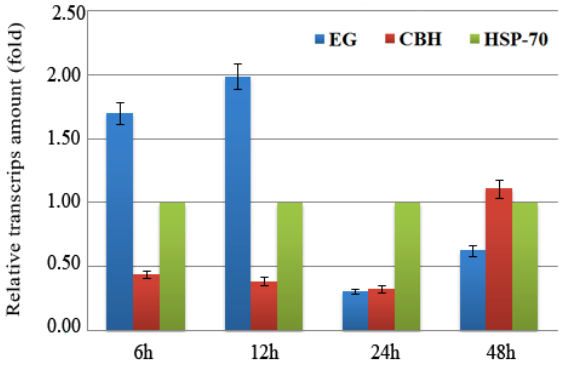

Fig. 2 Relative transcript level of cellulase genes determined by real-time quantitative PCR. All data indicated the mean ± SD of three experiments.

Reference List

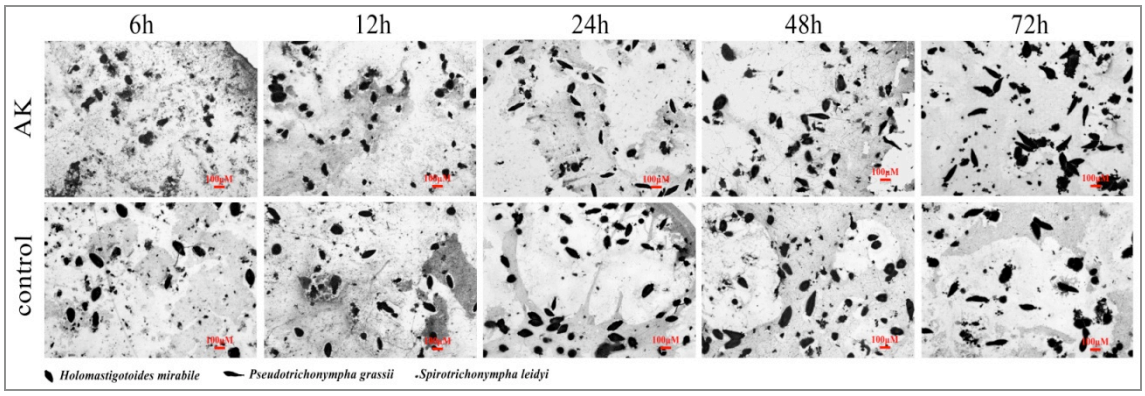

Fig.3 Photographs of three species of protozoa in the hindgut of *C. formosanus* specifically fed with antibiotics and distilled water soaked filter paper. AK indicates ampicillin and kanamycin treated sample, control indicates negative control.

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