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研究报告

Nutrition utilization of entomopathogenic nematode *Steinernema* sp. SY-5 on recombinant crystalline inclusion proteins

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Abstract: [Objective] Phase I cells of *Photorhabdus luminescens* bacteria, symbiotically associated with entomopathogenic *Heterorhabditis* nematode, produce two types of intracellular crystalline inclusions, CipA and CipB, to support the symbiont. This study aimed to investigate the possible influence of Cip proteins on non-symbiotic *Steinernema* nematode. **[Methods]** Based on constructed *Escherichia coli* expression system of Cip proteins, co-culture system of recombinant *E. coli* bacteria and *Steinernema* sp. SY-5 nematode was set up. **[Results]** The Cip proteins significantly promoted the development of SY-5 nematode to make high adult rate of 65%−82%, gravid rate of 80%−95%, 30−50 eggs per nematode, and low mortalities. **[Conclusion]** As the nutrient reserves for *Heterorhabditis* nematode, Cip proteins can be accepted and utilized by *Steinernema* nematode. The progress on these proteins will provide insights into the mechanisms governing bacteria-nematode symbiosis.

Keywords: Crystalline inclusion protein, *Steinernema*, Nutrition utilization

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昆虫病原斯氏线虫 SY-5 对重组 Cip 晶体蛋白的营养利用

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摘 要: 【目的】初生型 *Photorhabdus luminescens* 细菌产生两种胞内晶体蛋白 CipA 和 CipB, 为其共生的昆虫病原异小杆线虫提供营养。探索非共生的斯氏线虫对 Cip 蛋白的营养利用情况。【方法】在已构建重组 Cip 蛋白大肠杆菌表达体系的基础上, 建立重组菌细胞与无菌斯氏 SY-5 线虫共培养系统, 检测线虫的生长发育情况。【结果】Cip 蛋白对目标线虫生长有显著支持作用: 发育为成虫的比例达到 65%−82%, 雌虫的怀卵率为 80%−95%, 平均怀卵量为 30−50 粒, 并显著降低各虫态的死亡率。【结论】Cip 蛋白不仅为共生的异小杆线虫提供营养, 亦能为斯氏线虫所利用。

关键词: 胞内晶体蛋白, 斯氏线虫, 营养利用

1 Introduction

Entomopathogenic nematodes (EPN) *Steinernema* and *Heterorhabditis*, mutualistically associated with bacteria *Xenorhabdus* and *Photorhabdus* respectively, are strongly virulent towards a wide range of insect lavae[1]. As a potential biocontrol agent, EPN are applied popularly against insect pests of horticulture, agriculture, home and garden[2−3]. Carrying bacteria in the gut, infective juveniles (IJs), a free-living form of the nematode, seek out and gain entrance to insect larvae in the soil. The symbiotic bacteria are released and proliferate. Working together, nematodes and their bacterial symbiont kill the insect within 48 h[4−7]. According to the life cycle of infection, the nematode takes advantage of the pathogenic potential of the bacteria to kill the insect host. The bacteria also supply the nutrient for the growth of the nematode.

Two types of proteinaceous crystalline inclusions, designated CipA and CipB (11.6 kD and 11.3 kD), are found in the phase I cells of *Photorhabdus* spp.. The Cip proteins, whose amino acid composition and content are similar to specific nutrient requirement of entomopathogenic nematodes, can't be utilized by the bacterial cells[8−9]. And *cipA*$^-$ or *cipB*$^-$ mutant of *P. luminescens* maintained its pathogenicity to insect but failed to support growth and development of associated nematode[10−11]. These findings indicate that Cip proteins as a kind of nutrient reserves are involved in the mutualistic relationship between the bacteria and the nematode[8−9].

As a rich supply of essential amino acids, whether Cip proteins have a universal function of biology in the field of nematode? Both two bacteria-nematode associations have high-degree specificity that one species of nematode retains one species of bacteria. This specificity for *Heterorhabditis-Photorhabdus* is generally more restrictive than

for the *Steinernema-Xenorhabdus* symbiosis. That is, *Steinernema* nematodes can accept the nutrients not only from the symbiotic bacteria, but also from other sources. To explore the possible utilization of Cip proteins by non-symbiotic *Steinernema* nematode, the recombinant cells expressing CipA or CipB were used to feed target nematodes. In the complex interaction of bacteria-nematode, it is inevitable that Cip proteins perform their biological significances in the presence of other metabolites produced by the bacteria. So the extracellular metabolites of SY-5 bacterium symbiont were also considered in the study. The differential development of target nematodes will be helpful to better understand the symbiosis of bacteria-nematode, inspire and spread a new thought based on Cip proteins for the industrialization of EPN.

2 Materials and Methods

2.1 Bacteria, nematode and insect host

E. coli BL21(DE3)/pET-15b-cipA, BL21(DE3)/pET-15b-cipB, BL21(DE3)/pET-15b were previously constructed by Guangdong Entomological Institute[12] and grown at 37 °C in Luria-Bertani (LB) broth (1% Tryptone, 0.5% Yeast extract, 1% NaCl, pH 7.2) or on LB agar (1.2% Bacto-agar) containing 50 mg/L carbenicillin disodium (Sigma chemical Co.).

Entomopathogenic nematode *Steinernema* sp. strain SY-5, carrying the symbiotic bacteria, *Xenorhabdus* sp. SY-5, was originally collected from Hainan province and maintained in Integrated Agricultural Pest Management Key Laboratory of Guangdong Province. *G. mellonella* larvae were artificially feed according to Poinar[13] by Guangdong Entomological Institute.

Bacterial colonies of *Xenorhabdus* sp. SY-5 were obtained from the haemolymph of *Galleria mellonella* larvae after infection with *Steinernema* sp. SY-5 nematodes. The primary form of SY-5 bacterium was obtained by selecting green or blue-green colonies on NBTA medium (0.5% Beef extract, 1% Peptone, 0.5% NaCl, 2% Nutrient agar, 0.002 5% Bromothymol blue, 0.004% Triphenyltetrazolium chloride) and cultured in PW broth (1% Peptone, 0.5% NaCl) at 25 °C on a shaker.

2.2 Culture and induction expression of BL21(DE3) recombinants

To prepare the culture of BL21(DE3) recombinants, a few microliters of the frozen stocks were respectively scraped and streaked on LB agar plates containing 50 mg/L carbenicillin. After a 24-hour-incubation, a single bacterial colony of at least 1mm in diameter was picked and inoculated in 3 mL of LB medium (50 mg/L Carbenicillin) at 37 °C, 200 r/min for 2.5 hours. 0.9 mL of the resulting culture were added in 30 mL fresh LB medium (50 mg/L Carbenicillin) and incubated at 37 °C with shaking to mid-logarithmic growth phase (an OD_{600} of approximately 0.8−1.0). Prior to induction, the culture was split into 2×15 mL aliquots, followed by one of them with 1 mmoL/L isopropyl-β-D-thiogalactopyranoside (IPTG, MD Bio) added. Both aliquots were incubated at 37 °C, 200 r/min for 8 hours. Before biological assays, the cells of different cultures were collected by centrifugation at 6 000 r/min and resuspended in fresh LB broth (Carbenicillin), diluted to the equal OD_{600} value with LB (Carbenicillin) to make the same cell density. The expression of recombinant Cip proteins was examined with Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.3 Preparation of sterile J1 nematodes

The test nematodes were feed *in vivo* in *G. mellonella* larvae, followed by co-cultured *in vitro* with symbiotic bacteria associated [14] and kept in sponge medium.

To obtain enough J1 larvae of target nematode, the infection model of nematode-host must be set up at first. Axenic (bacteria-free) J1 juveniles were prepared with a modified method according to Lunau et al[15]. Several sponges containing the infective juveniles (IJs) of target nematode were placed in 10−20 mL of axenic H_2O in a sterile plate. After several minutes, the IJs climbed out from sponges and dispersed in H_2O. Two pieces of sterile qualitative filter paper were placed on another sterile plate. Then 5−10 *G. mellonella* larvae and 1−2 mL IJ suspension were added on the paper to establish infec-

tion combination and incubated at 28 °C. When most of the females carry eggs inside, the gravid females were harvested from infected insects and rinsed in sterile M9 buffer (0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.5% NaCl, 0.025% $MgSO_4·7H_2O$). After surface-sterilized for 30 min at 25 °C, the gravids were dissected to collect the eggs. After rinsing twice in sterile M9 buffer, these eggs were collected in sterile LB broth. The resulting suspension of eggs was placed on 24-well cell plates (Corning, New York, USA), hatched out to be J1 larvae at room temperature, and checked their axenicity for 48 h. If LB broth remained clear, the J1 larvae were confirmed to be axenic and ready for the following biological assay.

2.4 Bioassay of the nematode development

2.4.1 Co-culture system of *E. coli* cells and nematodes

Recombinant *E. coli* cells induced and uninduced were used to set up co-culture liquid system with sterile J1 nematodes of isolate SY-5 as follows. Axenic J1 nematodes were collected by centrifugation and diluted to 10 000−20 000 per mL with LB medium. In 96-well cell plates, 50 μL cell suspension of BL21(DE3)/pET-15b-cipA, BL21(DE3)/pET-15b-cipB and BL21(DE3)/pET-15b, IPTG-induced and uninduced, were added respectively. Sterile LB medium (Carbenicillin) were used as blank control. Then 5 μL of J1 suspension were added in each well. Three replicates were established for each treatment. The plates were incubated on a slow shaker at room temperature.

2.4.2 Co-culture system of *E. coli* cells, extracellular productions of symbiotic bacteria and nematodes

A single colony of primary form of *Xenorhabdus* sp. SY-5 was picked from a freshly streaked NBTA plate into 5 mL of PW broth to grow overnight at 28 °C, 120 r/min. The starter culture were added into 50 mL of PW broth and incubated with shaking at 28 °C to reach stationary phase. To obtain the extracellular productions, the supernatant of the culture was collected by centrifugation at 14 000×*g*, 4 °C for 10 min and went through 0.2 μm-pore-size filter.

In the cell plates, 20 μL culture filtrate of *Xenorhabdus* sp. SY-5, 30 μL recombinant *E. coli* cells suspension induced and uninduced were used to set up another co-culture system with 5 μL sterile J1 nematodes as the above treatments described in 2.4.1.

2.5 Microscopical examination and statistical analysis

On inverted microscope and sterecscope, development of juveniles, sex ratio of the adult nematodes, gravid status of the females, mortalities of nematodes in different stages, and appearance of the next generation were observed every day.

Data presented as percentages were subjected to arcsine square root transformation and analyzed by ANOVA. The significance between treatments was evaluated by Duncan's multiple range tests (DMRT) with SPSS 10.0 software. Differences between means were significant at the 5% level.

3 Results and Analysis

IPTG (1 mmol/L) and carbenicillin (50 mg/L) were confirmed to be harmless to the target nematode in previous experiments. Sterile J1 of SY-5 nematode can live but can't grow or multiply in sterile LB liquid containing 50 mg/L carbenicillin or/and 1 mmol/L IPTG. So the sterile LB liquid (50 mg/L Carbenicillin) was used as the background and the control in liquid co-culture experiments.

In the blank control, the J1 nematodes were active and kept the status to die off, which weren't affected by the addition of the supernatant of SY-5 bacterial culture.

In the LB liquid with recombinant *E. coli* cells, the J1 nematodes feed those bacterial cells non-selectively and grew up. Some distinguishing differences of growth existed in the different co-culture combinations.

While applied to bioassays, *E. coli* cells of different cultures were sampled and analyzed with SDS-15% PAGE. After induced with IPTG, CipA and CipB expressed constituted about 30% and 33% of the total bacterial protein, respectively (Fig. 1).

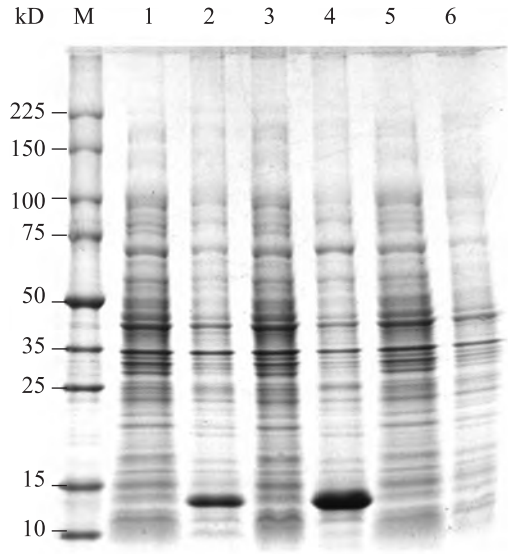

Fig. 1 SDS-PAGE analyses of *E. coli* whole-cell lysates in different liquid co-culture combinations

图 1 不同液体共培养组合中菌体蛋白的 SDS-PAGE 分析

Note: M: Broad range protein molecular weight markers; 1: BL21(DE3)/pET-15b-cipA uninduced; 2: BL21(DE3)/pET-15b-cipA induced with IPTG; 3: BL21(DE3)/pET-15b-cipB uninduced; 4: BL21(DE3)/pET-15b-cipB induced with IPTG; 5: BL21(DE3)/pET-15b uninduced; 6: BL21(DE3)/pET-15b induced with IPTG.

3.1 Formation of adult nematodes

Entomopathogenic nematodes have a simple lifecycle including egg stage, juvenile stage 1−4 and the adult stage. In different co-culture combinations, the J1 nematodes fed on *E. coli* cells could develop through juvenile stage to the adults in different proportions of 30%−80%. Grown on the cells expressing at least one of Cip proteins, much more Juveniles, over 65% of the total nematodes, reached the adult stage, while this data decreased to be 30%−50% in those combinations without Cip expressed. To add the culture filtrate of symbiotic SY-5 bacterium supported more juveniles to be the adults except of those in LB control. Especially in Composition 2, 4, and 6, the *E. coli* cells carrying *cip* gene can't supply Cip protein for the target nematodes without inducement of IPTG. However, the adult rate of nematodes in these compositions were raised from 30% to 50% because of the culture filtrate of symbiotic bacteria added (Fig. 2).

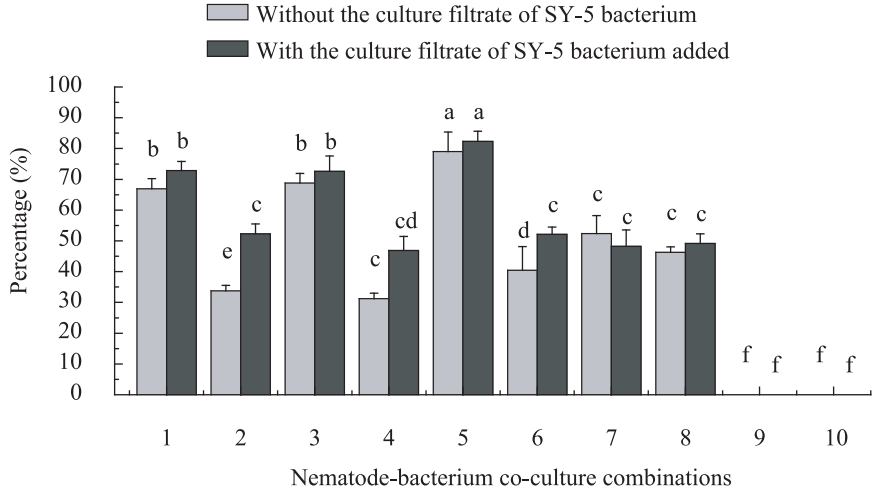

Fig. 2 Percentages of the adult nematodes in different liquid co-culture combinations

图 2 不同液体共培养组合中成虫的百分比

Note: Means followed by the same letter are not significantly different from each other at 5% level according to Duncan Test. 1: J1 nematodes + BL21(DE3)/pET-15b-cipA cells induced with IPTG; 2: J1 nematodes + BL21(DE3)/pET-15b-cipA cells uninduced; 3: J1 nematodes + BL21(DE3)/pET-15b-cipB cells induced with IPTG; 4: J1 nematodes + BL21(DE3)/pET-15b- cipB cells uninduced; 5: J1 nematodes + BL21(DE3)/pET-15b-cipA cells induced + BL21(DE3)/pET-15b-cipB cells induced; 6: J1 nematodes+ BL21(DE3)/pET-15b-cipA cells uninduced + BL21(DE3)/pET-15b-cipB cells uninduced; 7: J1 nematodes + BL21(DE3)/pET-15b induced with IPTG; 8: J1 nematodes + BL21(DE3)/pET-15b uninduced; 9: J1 nematodes + sterile LB (50 mg/L Carbenicillin, 1 mmol/L IPTG)medium; 10: J1 nematodes + sterile LB (50 mg/L Carbenicillin) medium.

The development of the adult stage showed no noteable differences in those combinations without Cip expressed, which contained the cells with uninduced *cip* gene, or with pET blank vector. Noticeably, on the *E. coli* culture producing both Cip proteins (Combination 5), more juveniles grew up to be the adults than those on the cultures with either CipA or CipB (Fig. 2).

3.2 Sex ratio of adult nematodes

Once beyond juvenile stage, *Steinernema* nematodes develop into amphimictic male or female adults. The female with protuberant copulation orifice in the middle body is larger than the male with bending copulatory spicule in the tail.

In the adult nematodes, the males took a larger proportion. And the ratio of the female to the male was about 1:1−1:1.5. This data was sensitive to the presence of Cip proteins in the culture well. Fed on either one of Cip proteins or both, the target nematodes with similar level of development showed no distinct difference of sex ratio. To add the supernatant of SY-5 bacterial culture did not change the ratio, while brought more the males and the females in most test combinations (Table 1).

Table 1 Percentages of the male and female nematodes in different liquid co-culture combinations
表 1 不同液体培养组合中成虫的性别分布

Combination 组合	The female 雌虫 (%)	The female^{+S} 雌虫$^{+S}$ (%)	The male 雄虫 (%)	The male^{+S} 雄虫$^{+S}$ (%)
1	29.2±3.7d	32.8±2.0d	37.7±0.5f	40.1±1.0fg
2	16.8±1.2b	20.4±2.2bc	17.0±2.4b	31.9±3.4e
3	30.7±2.3d	33.0±3.2d	38.1±0.8f	39.6±1.8fg
4	16.4±1.6b	19.4±4.8bc	14.8±0.3b	27.4±2.1d
5	33.9±3.1de	39.4±2.7e	45.1±3.2h	42.9±1.3gh
6	17.8±4.7bc	22.8±2.5c	22.6±3.1c	29.3±1.7de
7	20.6±6.0bc	19.7±4.0bc	31.8±1.5e	28.6±1.5de
8	16.2±0.8b	20.3±2.1bc	30.1±1.7de	28.8±1.5de
9	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a
10	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a

Note: Means followed by the same letter are not significantly different from each other at 5% level according to Duncan Test. Numbers represent the same treatments as in Fig. 2. $^{+S}$: With the culture filtrate of SY-5 bacterium added.

The stable sexual constitution of SY-5 nematodes showed that some special factor(s) deciding the sex of the adult should not be intracellular crystalline inclusions or extracellular products of the bacterium in symbiotic association with this nematode.

3.3 Gravid statuses of the female and the next generation

Not all the female will be gravid to produce the next generation. The gravid rate of the females and the amount of eggs per gravid nematode reflect the availability of nutrient supply for nematode reproduction.

After 4-day incubation, the gravid female was observed. In all the treatments except of LB control, most of the females had more than 10 eggs. Even feeding on the cells without Cip proteins, the females made a gravid rate of over 40% and carried 10−20 eggs per nematode. If the *E. coli* cells supplied at least one of Cip proteins, the gravid status of the target nematodes was further improved to have more than 80% gravid females with 30−50 eggs per nematode. Especially in the combination with CipB & CipA, almost all the females (about 95%) had eggs. Interestingly, in Combination 1, 3 and 5, every gravid nematode made very close eggs to each other and gave a higher average value (Fig. 3, 4).

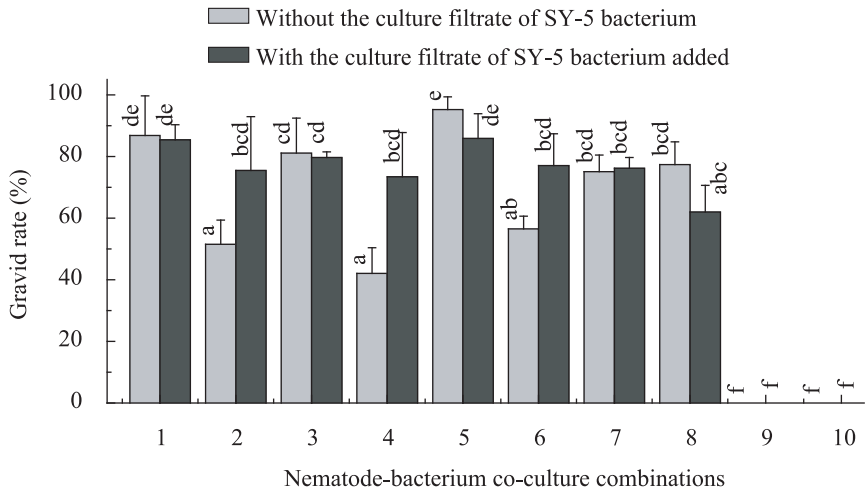

Fig. 3 Gravid rate of the female nematodes in different liquid co-culture combinations

图 3 不同液体共培养组合中雌虫的怀卵率

Note: Means followed by the same letter are not significantly different from each other at 5% level according to Duncan Test. Numbers represent the same treatments as in Fig. 2.

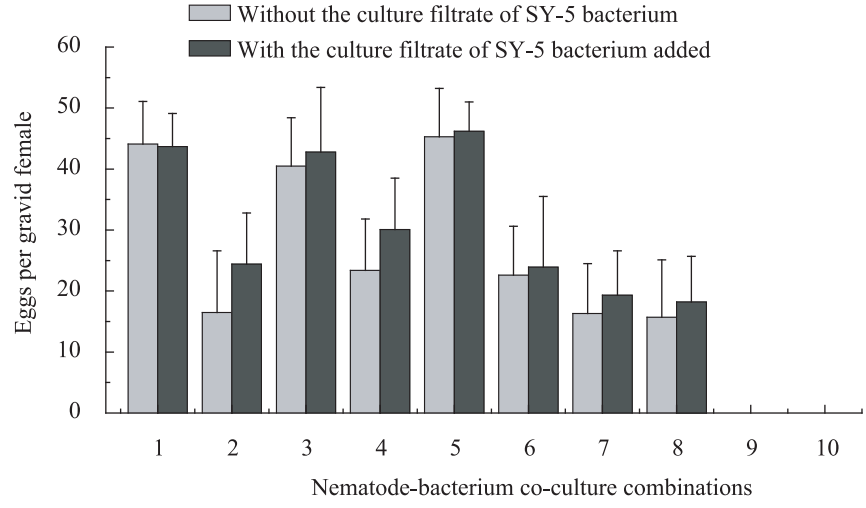

Fig. 4 Quantity of eggs inside the gravid in different liquid co-culture combinations

图 4 不同液体共培养组合中单条雌虫怀卵量

Note: Numbers represent the same treatments as in Fig. 2.

The cell-free supernatant of SY-5 bacterial culture made no distinct effect on the gravid status in the presence of CipA and/or CipB, but significantly raised the gravid rates and the amount of eggs in Combination 2, 4, 6, with *cip* gene but uninduced (Fig. 3, 4).

After 6 days, new J1 began to crawl out from the large females. Almost all the eggs can be hatched out. The babies supported with Cip were more active than those without Cips, which were not changed by the addition of the culture filtrate of SY-5 bacterium symbiont.

3.4 Death status of nematodes

When the gravid female appeared in the wells of cell plate, the nematodes in the culture combinations present to be four states of development: the gravid female, unconceived female, the male and non-adult. In the whole incubation, all the four

kinds of nematodes always had a population in different proportions that can't survive to the next stage of growth. Generally speaking, started from J1 juveniles, if supported by the cells expressing CipA or/and CipB, most of nematodes in all the states were active and made low mortalities. Especially, the number of dead females was very close to zero and no gravid females died before the next J1 were hatched and crawled out. However, if feeding the cells without Cip proteins, one third of the gravid females would be dead after 14-day-incubation though only 10%−20% of the females can't go through. The males were more sensitive to Cip proteins. It seemed high survival rate of the male came from high expression of Cip (Fig. 5, 6).

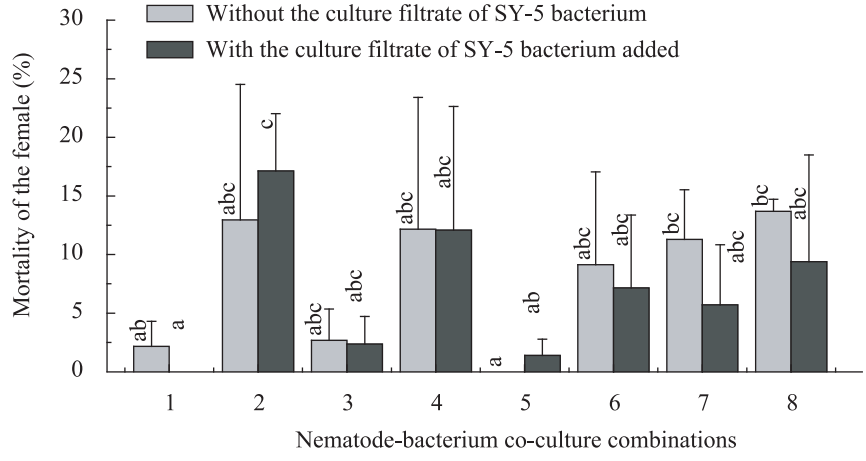

Fig. 5 Mortalities of the female nematodes in liquid co-culture combinations

图 5 不同液体共培养组合中雌虫的死亡率

Note: Means followed by the same letter are not significantly different from each other at 5% level according to Duncan Test. Numbers represent the same treatments as in Fig. 2.

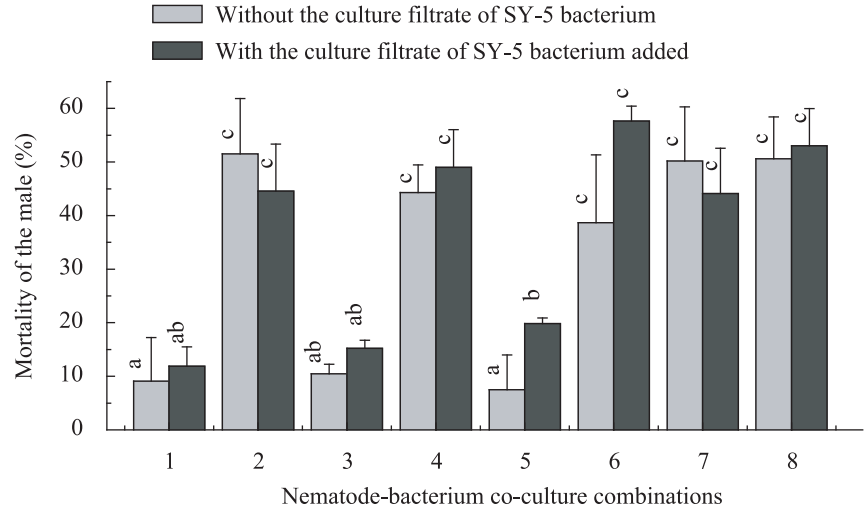

Fig. 6 Mortalities of the male nematodes in liquid co-culture combinations

图 6 不同液体共培养组合中雄虫的死亡率

Note: Means followed by the same letter are not significantly different from each other at 5% level according to Duncan Test. Numbers represent the same treatments as in Fig. 2.

The culture filtrate of SY-5 bacterium had no obvious improvement on the survival of nematodes in different states except of the males in the test combinations (Fig. 5, 6).

Except of those in the culture with expressed CipA, 25%−40% of juveniles in the test cultures failed to live through J3 or J4 stage. The extracellular supernatant of SY-5 bacteria made very limited support for the survival rate of juveniles (Fig. 7).

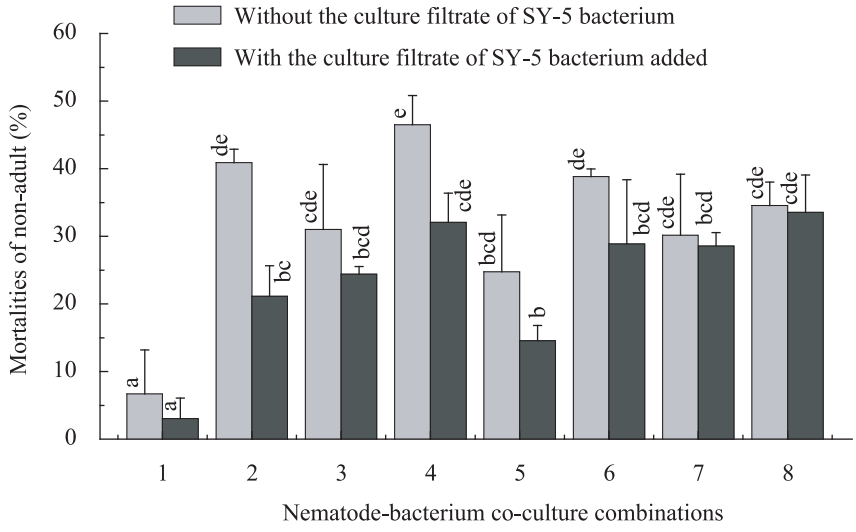

Fig. 7 Mortalities of the non-adult nematodes in liquid co-culture combinations
图 7 不同液体共培养组合中非成虫的死亡率

Note: Means followed by the same letter are not significantly different from each other at 5% level according to Duncan Test. Numbers represent the same treatments as in Fig. 2.

It seems that crystalline proteins provided by *E. coli* can influence but can't decide the death level of the target nematodes. It may come from different nutrient requirements of the nematodes in different states of development, specific food signals, population effect of nematode isolate, and even individual differences of nematodes.

4 Discussion

As anybody knows, bacteria *Bacillus thuringiensis* and *Bacillus sphaericus*, two kinds of biopesticide, both produce toxic intracellular crystals. However, from *P. luminescens*, the high-pathogenic bacterium, crystalline inclusion proteins (Cip) which constitute up to 40% of the total cell proteins make no contribution to its pathogenity[10]. And *P. luminescens* cells in hungry can't consume their Cip proteins inside. It is believed that these inclusion proteins as energy reserves for the associated nematodes were involved in the symbiosis of nematode-bacterium.

"Nematode-bacterium" is a classic symbiosis model in nature. Of which, the bacterium produces rich metabolites to perform its pathogenic effect on insects and symbiotic effect on nematodes. To exclude other metabolites, it is necessary to establish a biological assay system out of symbiotic relationship. Surprisingly, the nematodes can't accept the nature Cips separated from bacterial cells unless the proteins were carried by live cells. Two facts as followed took *E. coli* into our attention. *Steinernema* nematodes can utilize *E. coli* cells to develop and reproduce. And recombinant *E. coli* cells usually express foreign genes in high level to inclusion body. These made it available that the Cip proteins transferred by *E. coli* cells should be accepted

by *Steinernema* nematode.

To choose juveniles in stage 1 in bioassay test were based on two reasons. Preparation method of sterile J1 juveniles established successfully would supply enough target nematodes without the interruption of symbiotic bacteria. More importantly, J1 needed the most fully and basic resources. The life phase from this stage would show all the nutrient requirements of the whole lifecycle.

In liquid culture, *E. coli* cells expressing recombinant CipA or/and CipB made SY-5 J1 grow to more adults with a stable sex ratio, more gravid females with more eggs and more active state with lower mortalities. These facts confirm that Cip proteins not only match the specific nutrient requirement of symbiotic *Heterorhabditis* nematode, but also are available resources for the development and reproduction of non-symbiotic *Steinernema* nematodes. In addition, the development time and juvenile amount of SY-5 nematodes on different Cip proteins showed no distinct difference between CipA and CipB. Extracellular productions of SY-5 strain performed an assistant role in the nematode-bacterium system. To a limited extent, the supernatant of SY-5 bacterium improved the growth status of nematodes in different stages especially those feeding the cells with *cip* gene uninduced.

The findings in this paper are consistent with the view that Cip proteins are specific nutrient resource for nematode symbiont because of the growth-advantaged status in the presence of Cip. On the other hand, intracellular Cip inclusions are the major factor in the growth of nematodes, but not the only effect, extracellular metabolites, whose exact functions still remain unclear, are also involved in nematode development. It was evaluated that symbiotic bacteria might secret some development signals in the different stages of nematodes. Furthermore, this study also proposed some new questions: Are there separate functional attributions of CipA and CipB in the development of nematodes? What kind of signal materials symbiotic bacteria will secret in the specific growth stage of nematodes? …To answer these will make us further understand the symbiosis mechanism of nematode-bacterium and specific metabolite pathways of nematodes.

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稿件书写规范

专论与综述论文的撰写要点

专论与综述是本刊重要栏目之一，主要反映国内外微生物学及相关领域学科研究最新成果和进展，其内容要求新颖丰富，观点明确，论述恰当，应包含作者自己的工作内容和见解。因此，作者在动笔之前必须明确选题，一般原则上应选择在理论和实践中具有重要意义的学科专题进行论述。围绕专题所涉及的各个方面，在综合分析和评价已有资料基础上提出其演变规律和趋势，即掌握其内在的精髓，深入到专题研究的本质，论述其发展前景。作者通过回顾、观察和展望，提出合乎逻辑并具有启迪性的看法和建议。另外，作者也可以采用以汇集文献资料为主的写作方法，辅以注释，客观而有少量评述，使读者对该专题的过去、现在和将来有一个全面、足够的认识。

需要特别说明的是：在专论与综述中引用的文献应该主要是近 5 年国内外正式发表的研究论文，引用文献数量不限。