Antimicrobial activity of protein inclusions from bacteria symbiotic with entomopathogenic nematodes

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Accepted for publication 10 November 2011

Summary. Proteins of inclusions in bacteria symbiotic to entomopathogenic nematodes *Xenorhabdus* spp. and *Photorhabdus* spp. have a pronounced antibiotic effect suppressing growth of different bacteria on agar media. The toxicity of proteins from *Photorhabdus luminescens* and entomopathogenic bacteria *Bacillus thuringiensis* inclusions towards *Escherichia coli* has been compared. Inclusion proteins from *Xenorhabdus bovienii* inhibit the growth of two assayed subspecies of *B. thuringiensis*: *kurstaki* and *israelensis*. The vegetative cells of *X. bovienii,* isolated from *Steinernema feltiae,* proved to be sensitive to the *B. thuringiensis* ssp. *israelensis* Cyt1A crystal protein.

Key words: antibiotic effect, entomopathogenic nematodes, inclusion proteins, symbiotic bacteria, *Xenorhabdus*.

Xenorhabdus and *Photorhabdus* bacteria colonise the intestines of the infective soil-dwelling stage of entomopathogenic nematodes (EPN) of the genera *Steinernema* and *Heterorhabditis,* respectively. Infective juveniles infect susceptible insect larvae and release the bacteria into the insect haemocoel. The bacteria kill the insect larvae and convert the cadaver into a food source suitable for nematode growth and development (Goodrich-Blair & Clarke, 2007). Two forms or phases of both *Photorhabdus* and *Xenorhabdus* are reported, with the first phase mainly found in nematode infective juveniles, and the second phase arising inside insect cadavers at the late stages of nematode development. It is the first phase which produce the main part of insecticidal toxins, antibiotics and other biological active components (Banerjee *et al*., 2006, Koppenhöfer, 2007;), including intracellular protein inclusions, which can account for 40% of the total protein content of cells (Bintrim & Ensign, 1998; Bowen & Ensign, 2001). It was also presumed that these proteins contribute to the nutrition of the host nematode. *Photorhabdus* has two distinct types of crystals formed by small (10 kDa), hydrophobic proteins encoded by *cipA* and *cipB* genes (Bintrim & Ensign, 1998). *Xenorhabdus nematophila* also produced two crystal proteins, IP1 (26 kDa), and IP2, (22 kDa), but only the gene encoding IP1, *pixA*, has been identified (Couche & Gregson, 1987; Goetsch *et al*., 2006). Consistent with a potential role for crystal proteins in nutrition, when expressed in *Escherichia coli*, *Photorhabdus cipA* or *cipB* can promote nematode development (Joyce *et al*., 2006; You *et al*., 2006).

Unlike δ-endotoxins from inclusion proteins of the entomopathogenic bacterium *Bacillus thuringiensis,* the inclusion proteins of EPN symbiotic bacteria are not insecticidal (Koppenhöfer, 2007). Some δ-endotoxins (Cry proteins of classes 5, 6, 12, 13) are active against nematodes (Soberon *et al*., 2010). The δ-endotoxins are divided into two families: the invertebrate-specific Cry toxins and cytolytic Cyt toxins. Their cytolytic effect does not require binding to a membrane receptor, as in case of Cry proteins, but rather is mediated by direct binding to the lipid (Bravo *et al*., 2007; Li *et al*., 2009). Also, Cyt proteins can be the receptors for Cry proteins

Table. 1. Antibacterial activities of inclusion proteins from bacterial symbionts of entomopathogenic nematodes.

Test microorganism	Specific antibacterial activities of inclusion proteins, U (mm mkg^{-1}):		
	Xenorhabdus bovienii str. T 319	***Xenorhabdus bovienii*** from ***Steinernema intermedium.***	***Photorhabdus luminescens*** str.ZM1
Micrococcus luteus strain 140	67.5 ± 7.3	39.8 ± 5.0	47.5 ± 4.9
M. luteus 137	77.1 ± 8.7	42.5 ± 4.7	52.7 ± 5.5
M. aurantiacus 131	64.1 ± 6.1	45.8 ± 4.7	57.1 ± 6.1
Rhodococcus erytropolis 119	12.9 ± 1.5	11.7 ± 1.3	21.0 ± 2.7
Rh. rubroperctinctus 117	–	7.8 ± 0.9	19.7 ± 2.1
Brevibacterium citreum 213	5.2 ± 0.5	7.1 ± 0.8	15.3 ± 1.4
Lactococcus lactis 163	21.1 ± 2.5	19.5 ± 1.8	–
Nocardia calcarea 215	16.5 ± 1.9	–	15.0 ± 1.7
Streptomyces rimosus 267	19.2 ± 2.0	13.3 ± 1.4	17.0 ± 1.8
Streptomyces chrysomallus 257	11.7 ± 1.3	10.5 ± 1.3	21.7 ± 2.3
Bacillus subtilis 9	9.5 ± 0.9	3.7 ± 0.5	Trace activity
B. thuringiensis ssp. *kurstaki* Z-52	4.5 ± 0.6	2.7 ± 0.4	7.0 ± 0.8
B. thuringiensis ssp. *israelensis* B-2395	3,5 ± 0,3	–	–
Bacillus megaterium 11	Trace activity	0	–
Zoogloea ramigera 1	-	–	6.1 ± 0.5
Pseudomonas fluorescens 70	18.9 ± 2.1	16.8 ± 1.3	11.9 ± 1.3
Pseudomonas aeruginosa 47	10.7 ± 1.3	8.5 ± 1.1	3.9 ± 0.5
Escherichia coli 52	11.8 ± 1.5	13.7 ± 1.5	12.5 ± 1.5
Erwinia carotovora 35	Trace activity	–	0

Note: Trace activity – if the bacteria-free zone around the well ≤ 1-2 mm. '–' - no values.

(Canton *et al*., 2011). δ-Endotoxins from parasporal crystals of *B. thuringiensis* also display an antibiotic effect on some microorganisms (Yudina & Egorov, 1996; Yudina *et al*., 2003, 2007; Cahan *et al*., 2008).

Previously we reported the antimicrobial activity of inclusion proteins in EPN symbiotic bacteria (Yudina & Ivanova, 1996; Yudina & Egorov, 1996; Yudina & Spiridonov, 1997). The comparison of antibacterial effects of inclusion proteins produced by *Xenorhabdus* and *Photorhabdus* in comparison with those of the *B. thuringiensis* is presented here.

MATERIAL AND METHODS

Inclusion proteins were isolated from the *X. bovienii* (T319) donated by Dr Ray Akhurst. *Xenorhabdus bovienii* was also isolated from infective juveniles of *Steinernema intermedium* and *Steinernema feltiae. Photorhabdus luminescens* strain ZM1 was isolated from Moldavian *Heterorhabditis bacteriophora*. For isolation, symbiotic bacteria of nematodes were grown on the NBTA medium (Koppenhöfer, 2007). Isolated symbiotic bacteria were cultures on NBTA or in liquid YS media (Koppenhoefer, 2007). The microorganisms studied as test cultures (Table 1) were obtained from the collection of the Microbiology Department of Lomonosov Moscow State University (MDMSU).

To determine the antibiotic activity of inclusion proteins of *Xenorhabdus* and *Photorhabdus* bacteria, the test microorganisms were grown on solid media, as described previously (Yudina *et al*., 2003). The methods of *B. thuringiensis* cultivation, δ-endotoxins isolation and antibacterial activity have also been described (Yudina *et al*., 2003). Protein inclusions were separated after cell lysis by centrifugation. The washed inclusions were dissolved at pH of 11.5 for 1 h at 35°C, and the crystal proteins were precipitated by glacial acetic acid at pH values close to pI of the proteins and then separated from the supernatant by centrifugation and redissolved in 0.05 M tris-HCl buffer pH 8.0 with 0.2M NaCl or in 0.05 M phosphate buffer, pH 7.8. The obtained dilutions were then immediately subjected

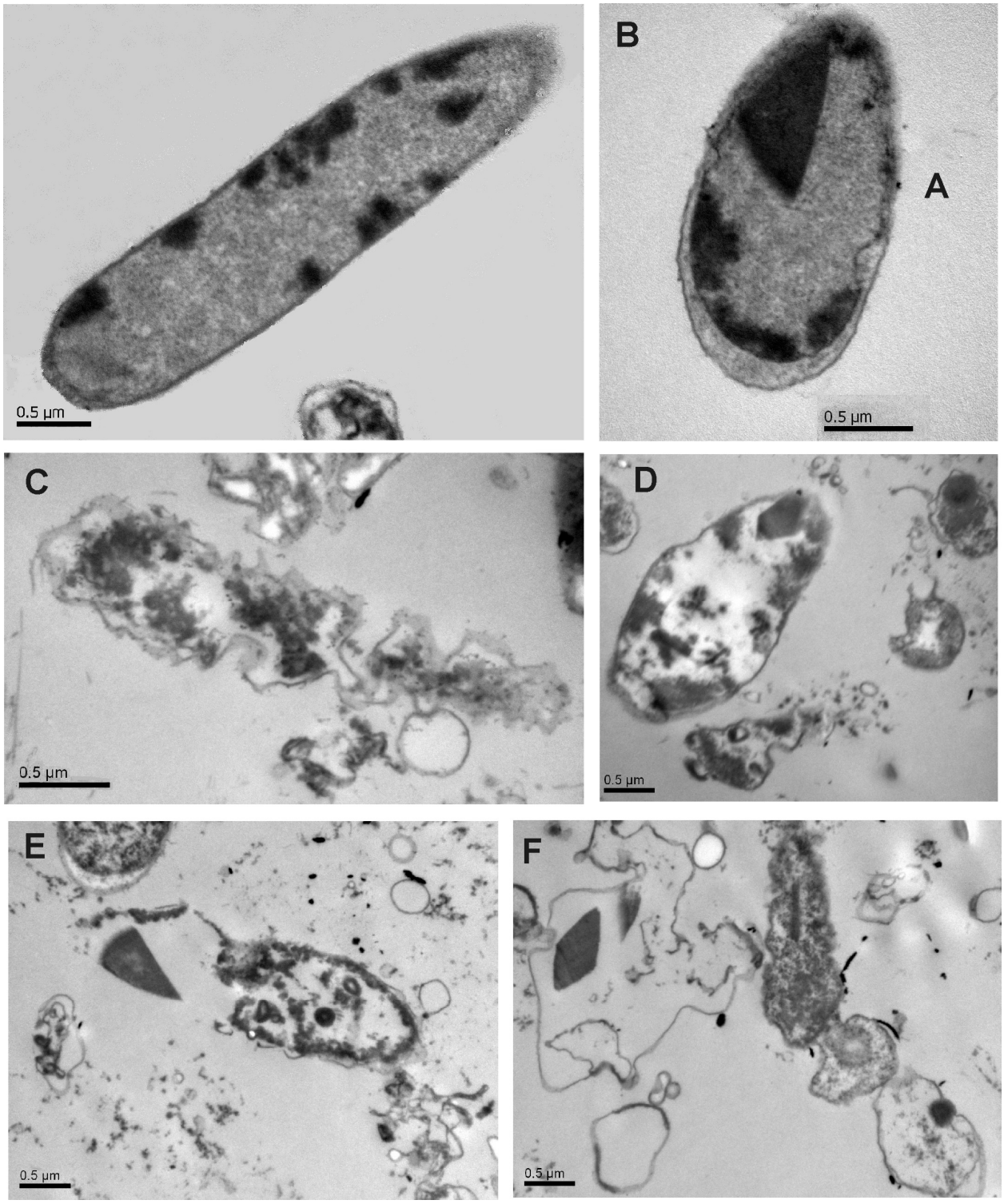

Fig. 1. TEM microphotographs of *Xenorhabdus bovienii* cells sections undergoing lysis after treatment with Cyt1A protein from *Bacillus thuringiensis* ssp. *israelensis*. A: control, formation of protein crystals inside cell; B : slightly swollen cell, cell coating mildly damaged; C, D: vacuolisation, cell swelling, cytoplasm clarification due to disruption of membrane permeability and wall destruction, formation of ring membrane structures from residues of cytoplasmic membrane; E, F: vacuolisation, cell lysis, cell walls rupture and emergence of protein crystals. Outer membrane ripple, and rupture and residues of membranes from lysed cell seen.

to analysis of the antimicrobial activity using an agar diffusion method. The antibiotic activity of Cyt1A against cells of *X .bovienii* was determined in liquid YS medium ((Couche & Gregson, 1987; Lancini & Parenti, 1982; Yudina *et al*., 2003).

The ratio of the growth inhibition zone (width mm) to the introduced protein amount (mkg) obtained in the proportional region of the dose-response curve was taken as the specific antibiotic activity (U). The minimal concentration of the protein solution resulting in the growth inhibition was taken as the minimal inhibiting concentration (MIC), as it previously reported (Lancini & Parenti, 1982; Yudina *et al*., 2003, 2007).

The dilutions of the inclusion proteins from EPN symbiotic bacteria and those from *B. thuringiensis* subsp. *monterrey* were immediately subjected to analysis of the toxicity. The experiments were carried out with genetically engineered bioluminescent *Escherichia coli* TG1 (pXen7) strain (the principal component of the *Ecolum* test system, see Strakhovskaya *et al*., 2002).

Ultrathin sections of EPN symbiotic bacteria were studied using a transmission electron microscope JEM 1011, Jeol Ltd (Japan) according to standard techniques. *Xenorhabdus bovienii* cells from *S. feltiae* were contrasted with 2% aqueous solution of uranyl acetate (Gerhardt, 1981; Yudina *et al*., 2003).

RESULTS

Inclusion proteins **(**in 0.05 M tris-HCl buffer pH 8.0 with 0.2M NaCl) from three strains of EPN symbionts suppressed the growth of the different test-bacteria (Table1).

Antibacterial action of inclusion proteins from *X. bovienii* T319 on micrococci (*Micrococcus luteus, M. aurantiacus*) was characterised by values of the specific antibiotic activity (U) at 40 – 80 mm mkg^{-1} (Table 1). The MIC of the inclusion protein solution from *X. bovienii* when used against *M. luteus* 137 was 5 – 7 mkg ml^{-1}.

Nocardia calcarea, *Streptomyces rimosus*, *S. chrysomallus*, *Brevibacterium citreum* and *Lactococcus lactis* were significantly affected by EPN inclusion proteins (see Table 1). An effect of *P. luminescens* inclusion proteins on rhodococci, *S. chrysomallus* and *B. citreum* was at least twice as stronger as that of *Xenorhabdus* inclusion proteins. The bacteriostatic action of *P. luminescens* on *Zoogloea ramigera* (U = 6) was observed. The growth of the vegetative cells *B. thuringiensis* subspp. *kurstaki* and *israelensis* was suppressed by *Photorhabdus* and *Xenorhabdus* inclusion proteins (U = 2.7 – 7). Weak action or only traces of it were reported for *Bacillus megaterium* (Table 1). Among Gram-negative bacteria, both species of *Pseudomonas* were found to be the most susceptible to antimicrobial activity of EPN inclusion proteins. The effect of *Xenorhabdus* inclusion proteins on *Pseudomonas* spp. was 2-5 times higher than that of *P. luminescens*. *Erwinia carotovora* cells were not affected by any inclusion proteins in the concentrations used. *Escherichia coli* susceptibility to *X. bovienii* inclusion proteins was two times lower than *P. fluorescens,* whereas *P. luminescens* effect on both *Pseudomonas* spp. was nearly identical (Table 1). Inclusion proteins from *B. thuringiensis* were also found to be bactericidal for *M. luteus* 140, but U values were lower than those of EPN-symbiotic bacteria: 7.5, 28.8 and 42 for *B. thuringiensis* subspp. *kurstaki, israelensis, monterrey,* respectively, and 67.5, 39.8 and 47.5 U for *X. bovienii* 319, *X. bovienii* from *S. intermedium,* and *P. luminescens* ZM1, respectively.

A bacteriostatic effect of inclusion proteins on *E. coli* bacteria was observed after 24 - 48 h of bacteria growth on solid media at 30°C or 37°C. After an additional 24 - 48 h growth at 30°C or 37°C, the bacteriostatic zones usually disappeared.

The susceptibility of cells *E. coli* in liquid media containing an inserted *lux* operon and a gene for ampicillin resistance for toxicological action of inclusion proteins *P. luminescens* and *B. thuringiensis* ssp. *monterrey* was examined using an Ecolum test system.

The primary control of the *P. luminescens* inclusion proteins toxicity for *E. coli* showed that these proteins rapidly associate with *E. coli* cells significantly reducing their bioluminescence (as the result of disruption in the cell membrane permeability and subsequent cell death). After 5 min of incubation, *P. luminescens* inclusion proteins (240 mkg ml^{-1}) were already bound to the cells in the suspension and inhibited its bioluminescence by about 40% of the control value. The proteins of *B. thuringiensis* ssp. *monterrey* crystals (260 mkg /ml^{-1}) inhibited its luminescence by about 80% of the control value only after 15 min of incubation and up to about 50% at the end of treatment, *i.e*., after 60 min of incubation. About a quarter of all the *E. coli* cells were viable after the exposure to *P. luminescens* inclusion proteins and demonstrated growth on LB agar medium, whereas there were only about 40% of viable *E. coli* cells after exposure to δ-endotoxins of *B. thuringiensis*.

The MIC value of the activity of Cyt1A of *B. thuringiensis* ssp. *israelensis* for the cells of *X. bovienii* isolated from *S. feltiae* and cultured on

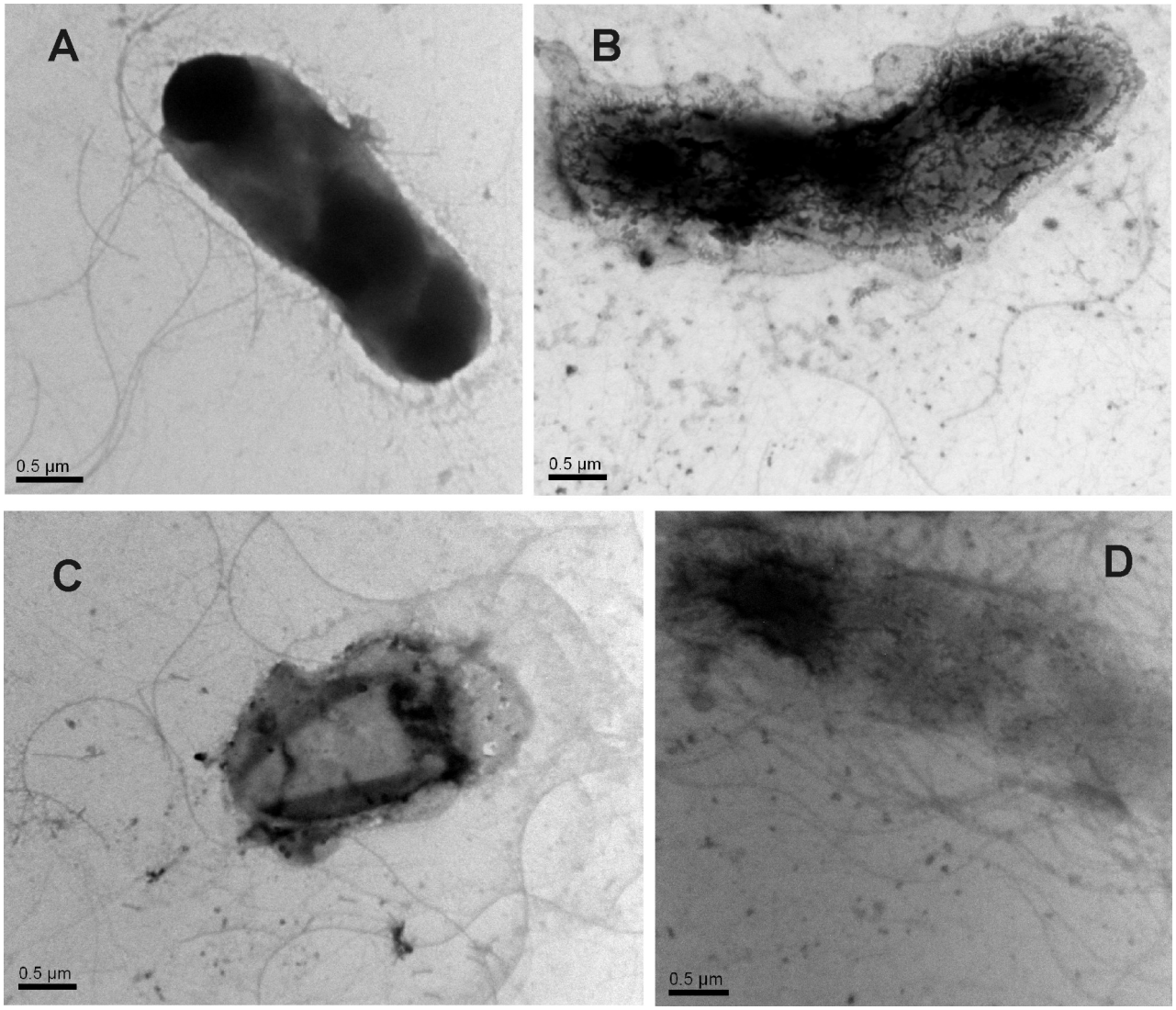

Fig. 2. TEM microphotographs of contrasted *Xenorhabdus bovienii* cells. A: control, logarithmic phase cell with flagella, fimbriae; B, C: swollen cells with damaged membrane and clarified cytoplasm, inclusions present; D: cell partially destroyed; inclusion seen on intact part.

liquid YS medium was estimated as 12.5 mkg ml^{-1}. The TEM studies on morphology of the phase 1 *X. bovienii* cells after the treatment with Cyt1A protein (50 mkg ml^{-1}, 1 h treatment under 20°C and then incubated in liquid YS medium 1 h under 30°C, 200 rpm) demonstrated damaged cell surface and cytoplasmic membrane, malformations and cell lysis (Fig. 1). In controls, late logarithmic phase cells did not undergo lysis, proving that cell lysis was caused by the Cyt1A protein treatment. Numerous fimbriae were discernible on the surface of *Xenorhabdus* cells after the contrasting treatment with uranylacetate. It means that cells are on the exponential phase of their growth and their lysis is a result of the action of Cyt1A protein (Fig.2).

DISCUSSION

Using the agar diffusion method, we have shown that inclusion proteins of EPN bacterial symbionts demonstrate antimicrobial activity against some Gram-positive (*Micrococcus*, *Lactococcus, Rhodococcus, Nocardia, Streptomyces, Brevibacterium* and *Bacillus* spp.) and Gram-negative (*Pseudomonas, Escherichia* spp.) aerobic bacteria. Yudina *et al*. (2003) reported that the manifestation of antimicrobial activity of inclusion proteins strongly depends on salt composition and the presence of reduced compounds in the growth medium for test-culture. The phenomenon that antibacterial agents are growth-rate-dependent and

nutrition-dependent was described in numerous reports (*e.g*., Hadas *et al.*, 1995).

For some test organisms the activity of inclusion proteins from EPN symbionts and *B. thuringiensis* differs significantly. Thus, U-values of *Xenorhabdus* and *Photorhabdus* proteins against *Micrococcus* spp. were 2-30 times higher than that of parasporal crystals from different subspecies *B. thuringiensis*. No antibacterial effect on rhodococci or brevibacteria was demonstrated for *B. thuringiensis* prior to our study.

The determination of bioluminescence intensity was proved in our study to be a reliable, rapid and sensitive method of detection of antimicrobial activity of inclusion proteins. The disruption in the cell membrane permeability subsequently causes cell death and cessation of bioluminescence. It was shown that parameters of bioluminescence and colony-forming ability of TG1 (pXen7) *E. coli* were correlated (Strakhovskaya *et al*., 2002; Zarubina *et al*., 2009).

Inclusion proteins of *P. luminescens* were more toxic to *E. coli* with *lux*-operone than *B. thuringiensis*, which was demonstrated by colony count of *E. coli* retaining the colony-forming ability after treatment with inclusion proteins. At the same time, the Gram-negative *S. feltiae* bacterial symbionts of *X. bovienii* were found to be susceptible to the antibacterial activity of Cyt1A of *B. thuringiensis.* The TEM studies have shown that Cyt1A action cause malfunction of cytoplasmic membrane permeability and destruction of cell wall followed by lysis. Similar effects were observed on different microorganisms treated by crystal proteins of *B. thuringiensis* (Yudina *et al*., 2003, 2007; Revina *et al*., 2005). As it is seen from microphotographs (Fig. 2), the contrasted *X. bovienii* cells are in exponential phase of their growth (having peritrichous flagella and fimbriae) and being lysed due to Cyt1A protein action. Bacterial symbionts of EPN are motile only when in Phase 1, they then have peritrichous flagella, which is characteristic for the exponential phase of growth (Koppenhöfer, 2007). The antibacterial effect of different inclusion proteins on Gram-negative bacteria is significant since the outer membrane usually enables the bacteria to withstand antibacterial molecules by excluding them or reducing their penetration into the cells.

It is also important to note that *B. thuringiensis* ssp. *israelensis* producing Cyt1a protein is susceptible to the antibacterial action of *X. bovienii* inclusion proteins (Table 1). The inclusion proteins of EPN symbiotic bacteria are similar to the *B. thuringiensis* Cyt proteins by having relatively low molecular masses of 10−28 kD. By contrast to the Cry proteins, their cytolytic affect is less specific. The MIC values on micrococci of the proteins studied were also similar: 5-7 mkg ml^{-1} for *X. bovienii* and 6-7 mkg ml^{-1} for Cyt1A (Yudina *et al*., 2003).

ACKNOWLEDGEMENT

The authors are grateful to Dr Ray Akhurst, who presented *Xenorhabdus bovienii* strain T319. The work of S.E. Spiridonov and N. S. Shepeleva was supported by RFBR grant 11-04-00590a.

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Юдина, Т. Г., И. А. Залунин, А. П., Зарубина, Даньян Го, Н. С. Шепелева, С. Э. Спиридонов. Антимикробное действие протеиновых включений симбиотических бактерий, ассоциированных с энтомопатогенными нематодами.

Резюме. Протеины из внутриклеточных включений ассоциированных с энтомопатогенными нематодами бактерий *Xenorhabdus* spp. и *Photorhabdus* spp., проявляют выраженное антибиотическое действие, подавляя рост различных бактерий на агаризованных средах. Проведено сравнение токсичности для *Escherichia coli* протеинов включений из *Photorhabdus luminescens* с таковой из энтомопатогенных бактерий *Bacillus thuringiensis*. Протеиновые включения из *Xenorhabdus bovienii* также подавляют рост двух исследованных подвидов *B. thuringiensis*: *kurstaki* и *israelensis*. В то же время вегетативные клетки *X. bovienii,* изолированные из *Steinernema feltiae,* оказались чувствительными к действию кристаллических протеинов Cyt1A, полученных из кристаллов *B. thuringiensis* ssp. *israelensis*.