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EXPERIMENTAL ARTICLES

Susceptibility of Archaea to the Antibiotic Effect of the Parasporal Inclusion Proteins from Different *Bacillus thuringiensis* subspecies

T. G. Yudina, A. L. Bryukhanov, and A. I. Netrusov

Biological Faculty, Moscow State University, Vorob'evy gory, Moscow, 119899 Russia

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Abstract—The proteins of parasporal inclusions from three *Bacillus thuringiensis* subspecies (*kurstaki*, *amagiensis*, and *monterrey*) inhibited growth of methanogenic archaea of two species belonging to two genera, *Methanobrevibacter arboriphilus* and *Methanosarcina barkeri*. The minimal inhibitory concentrations of these proteins were 20 to 50 μg/ml. Lysozyme exhibited similar bactericidal effect on archaea. The perspective of comparative studies on the effect of polyfunctional proteins on bacteria and archaea is discussed.

Key words: proteins, parasporal inclusions, *Methanobrevibacter.*

Proteins of parasporal inclusions (Cry-proteins) from various subspecies of *Bacillus thuringiensis* are known to affect susceptible insects and some other invertebrates by destroying the cytoplasmic membrane (CM) of their intestinal epithelium [1]. Cry-proteins are polyfunctional: they exhibit both lectin-like [2] and antimicrobial [3, 4] activities. Their effect on prokaryotic cells is less specific than on the cells of susceptible invertebrates [5].

The studies on the mechanism of action of the aforementioned unique inclusion proteins on prokaryotes are still in their infancy [6]. The antibiotic effect of these proteins on archaea remains unstudied, although this item is of special interest, since the structure of archaeal CM has some distinctive features and their cell walls are characterized by great diversity [7].

We studied the antibiotic activity of the crystal proteins from three *B. thuringiensis* subspecies against two species of methanogenic archaea. The reasons for choosing these subspecies of entomopathogenic bacilli are as follows: the crystal proteins (Cry1Aa, Cry1Ab, Cry1Ac, Cry2A, and Cry2B) from several strains of *B. thuringiensis* subsp. *kurstaki* killing lepidopterous insects are well studied [1]; the solutions of crystals from the *amagiensis* subspecies kill certain termites [8]; Cry-proteins from the *monterrey* subspecies kill several cockroach species [9]. Termites and cockroaches live in symbiosis with numerous microorganisms represented mainly by anaerobes (archaea and bacteria), which make cellulose-containing substrates utilizable and allow the successful development of insects [10, 11]. The study of an *M. arboriphilus* strain isolated from termite gut and *M. barkeri*, which is common in anaerobic marine sediments, lake silts, and methane tanks [12], allows a comparison to be made of the antibiotic effects of the crystal proteins from *B. thuringiensis* on termite-symbiotic and free-living archaea. As mentioned above, some termites were shown to be affected by the solutions of crystals from *B. thuringiensis* subsp. *amagiensis* [8].

The aim of this work was a comparative study of the antibiotic effects of the parasporal inclusion proteins from different subspecies of *B. thuringiensis* on two species of anaerobic archaea, *M. arboriphilus* and *M. barkeri*.

MATERIALS AND METHODS

The bacterial strains *Bacillus thuringiensis* subsp. *kurstaki* MDMSU 7, *B. thuringiensis* subsp. *amagiensis* MDMSU 29, and *B. thuringiensis* subsp. *monterrey* MDMSU 28 from the Pasteur Institute were kindly provided by L.I. Burtseva (Institute of Systematics and Ecology of Animals, Siberian Division, Russian Academy of Sciences) and are now stored at the Microbial Culture Collection of the Microbiology Department, Moscow State University. Bacteria were grown on potato agar at 30°C for 7 days until complete cell lysis and the release of spores and crystals occurred.

The archaeal strains *M. barkeri* Fusaro (DSMZ 804) and *M. arboriphilus* AZ (DSMZ 744) from the DSMZ Collection (Braunschweig, Germany) were grown in 5 ml of the recommended media [13, 14] at 37°C under anaerobic conditions according to the modified Hungate method with the use of 10% inoculum.

The mixtures of spores and crystals of three *B. thuringiensis* subspecies were removed from agar medium, washed three times with distilled water, and separated by treatment with *n*-xylene [15]. The amount of crystals in the precipitates was no less than 95%. The

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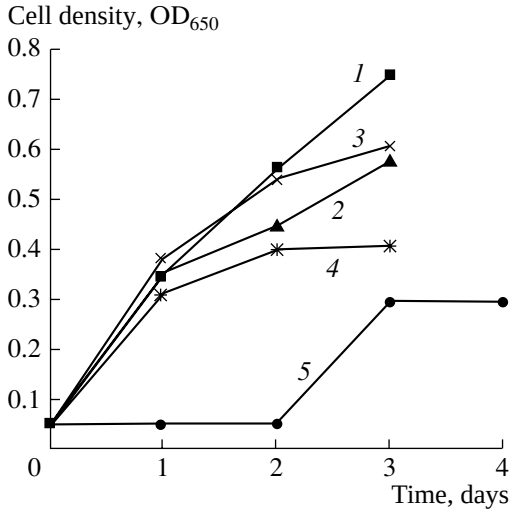

Time course of *M. arboriphilus* growth in (*1*) control medium supplemented with 0.05 M phosphate buffer, pH 7.8, and in the presence of Cry-proteins from *B. thuringiensis* subspecies (*2*) *kurstaki*, 20 μg/ml; (*3*) *amagiensis*, 20 μg/ml; (*4*) *amagiensis*, 30 μg/ml; and (*5*) *monterrey*, 36 μg/ml.

crystals were washed three times with distilled water to remove *n*-xylene, centrifuged (30000 *g*, 15 min), and resuspended in an acetone–water mixture (1 : 1) for desorption of possible impurities from the crystal surfaces. Then, the crystals were separated by centrifugation, washed three times with sterile distilled water, incubated in 1 M NaCl at room temperature for 2 h, and washed 3–5 times with water.

The washed crystals were dissolved in 0.02 N NaOH on a shaker (220 rpm) at room temperature for 1 h; the precipitate was removed by centrifugation (30000 *g*, 20 min). The supernatant was kept overnight at room temperature to allow the formation of activated toxins from protoxins by bacterial proteinases associated with the crystal lattice of parasporal crystals [16]. The crystal proteins were precipitated by treatment of the supernatant with glacial acetic acid at pH 4–6 and centrifuged (30000 *g*, 30 min, 2–4°C). The precipitate was immediately resuspended in 0.05 M phosphate buffer (pH 7.8). Protein concentration in the obtained solution was determined spectrophotometrically at 280 nm assuming that the Cry-protein solutions at a concentration of 1 mg/ml have an ε_{280} value equal to 1.6 [1].

The solution of the crystal proteins from *B. thuringiensis* in 0.05 M phosphate buffer (pH 7.8) was added anaerobically in the amount of 0.3 ml to the media for cultivation of archaeal strains. To ensure anaerobic conditions, the air from media and solutions was evacuated three times by means of a water-jet pump with subsequent flushing with nitrogen gas. At the last stage, nitrogen was pumped out, and the inoculum or the appropriate solutions were injected anaerobically into the media with a syringe. The media contained reducing agents. Antibiotic activities of the crystal protein solutions were estimated by measuring the cell density (OD_{650}), gas mixture uptake (atm), and methane production (mmol methane/l culture liquid). The minimal inhibitory concentrations (MIC) of the parasporal inclusion proteins were determined by a standard method [15] by measuring antibiotic activities of serial protein dilutions and were expressed as the minimal protein concentration in the incubation medium (μg/ml) that inhibited archaeal growth. We also determined the antibiotic activity of Egg White Lysozyme (Sigma, USA) dissolved in 0.05 M phosphate buffer (pH 7.8).

Experiments were carried out in triplicate; the results of typical experiments are presented in this work.

RESULTS AND DISCUSSION

The crystal proteins (σ-endotoxins) from *B. thuringiensins* inhibited the growth of archaea isolated from termite gut at concentrations equal to or greater than 20 μg/ml (figure, Table 1). The inhibitory effect on gas uptake was more pronounced than the effect on cell growth. The inhibition of archaeal growth was accompanied by the formation of flocks consisting of the

Table 1. Effect of the crystal proteins from *B. thuringiensis* on the uptake of a hydrogen–carbon dioxide mixture by *M. arboriphilus*

Variant	Gas pressure, atm;			
	1 day	2 days	3 days	4 days
Control	0.5	0.4	0	–
+ Cry-proteins from the *kurstaki* subspecies (20 μg/ml)	0.9	0.8	0.8	–
+ Cry-proteins from the *amagiensis* subspecies (20 μg/ml)	0.7	0.6	0.6	–
+ Cry-proteins from the *amagiensis* subspecies (30 μg/ml)	0.8	0.8	0.7	–
+ Cry-proteins from the *amagiensis* subspecies (50 μg/ml)	1.0	1.0	1.0	–
+ Cry-proteins from the *monterrey* subspecies (36 μg/ml)	1.0	1.0	0.8	0.8
+ Cry-proteins from the *monterrey* subspecies (40 μg/ml)	1.0	1.0	1.0	1.0

Note: Control is medium supplemented with 0.05 M phosphate buffer (pH 7.8); "–" stands for "not determined."

adhered cells; this may be due to cell surface impairment or to slime formation as a result of defense reactions of the cells against the inhibitory effect of σ-endotoxins. The archaea grew actively in the control medium supplemented with 0.05 M phosphate buffer (pH 7.8) instead of crystal proteins; the cell density OD_{650} reached a value of 0.75 by the 3rd day of cultivation; the hydrogen–carbon dioxide mixture was completely consumed after two days of cultivation; minor amounts of flocks were formed on the first day; then, their quantity diminished. The addition of crystal proteins from *B. thuringiensis* subspp. *amagiensis* and *kurstaki* at a concentration of 20 μg/ml considerably inhibited the uptake of hydrogen and carbon dioxide by the growing cells, especially in the case of the σ-endotoxins from the *kurstaki* subspecies (gas uptake decreased 5- to 6-fold compared to the control). Biomass accumulation was one-fourth less than in the control; flock formation was insignificant.

The solution of crystals from *B. thuringiensis* subsp. *amagiensis* showed a less pronounced inhibitory effect on the growth of the archaea isolated from termite gut than the crystal solution of the same concentration (20 μg/ml) from the *kurstaki* subspecies (figure). In the presence of these proteins, gas uptake was about twofold less than in the control; flock formation was considerably promoted. An increase in the concentration of the crystal proteins from the *amagiensis* subspecies to 30 μg/ml resulted in an about twofold decrease in cell density compared to the control; flock formation was insignificant. In this case, gas uptake was negligible; the gas pressure only decreased from 1.0 to 0.8 atm on the first day of incubation; on the second day, no gas consumption was observed (Table 1). Further increase in the concentration of the crystal proteins from the *amagiensis* subspecies to 50 μg/ml completely inhibited the growth of *M. arboriphilus* and gas uptake; therefore, this concentration represents the MIC for the archaeon under consideration.

The solution of crystals from *B. thuringiensis* subsp. *monterrey*, which are known to affect some cockroaches, showed a more pronounced inhibitory effect on the archaea isolated from termite gut than the crystal proteins from the *amagiensis* subspecies, which affect certain termites (figure). The concentration of the solution of crystals from the *monterrey* subspecies equal to 40 μg/ml represents the MIC for *M. arboriphilus*. At a crystal protein concentration of 36 μg/ml, no cell growth or gas uptake were observed in the first 2 days of cultivation; by the 3rd day of cultivation, cell density increased slightly (to 0.3 OD units); the gas pressure lowered only from 1.0 to 0.8 atm; flocks were virtually absent. No changes in the archaeal growth were observed by the 4th day of incubation (figure). The crystal proteins from the *monterrey* subspecies at a concentration of 40 μg/ml completely inhibited the archaeal growth. Thus, the crystal proteins from the *amagiensis* subspecies, which are known to affect some termites, exhibited the same inhibitory effect on the growth of the termite-symbiotic *M. arboriphilus* as did the crystal proteins from the other *B. thuringiensis* subspecies, to which termites were insusceptible. This is further proof that different mechanisms are involved in the Cry-protein action against prokaryotes and the midgut cells of susceptible insects; specific binding of Cry-proteins to midgut receptors is well known [1, 5].

It should be mentioned that the inclusion proteins from different bacteria, much like other antibiotics, are active against particular prokaryotes and characterized by different spectra of antibiotic activities. Earlier, this was inferred from a study of bacterial susceptibility to the inclusion proteins from representatives of the genera *Bacillus* and *Xenorhabdus* [4, 5]. This conclusion is also valid for the effect of Cry-proteins on archaea. The inhibitory effects of Cry-proteins on the growth of *M. arboriphilus* were different and proportional to their concentrations.

The Cry-proteins from *B. thuringiensis* also inhibited the growth of the other archaeon, *M. barkeri.* Table 2 shows the data of two typical experiments. In experiment I, the medium was inoculated with cells not subjected to preliminary treatment, whereas in experiment II, the cells were washed prior to inoculation. In experiment I, cell growth in the control continued for 3 days. The crystal proteins from the *kurstaki* subspecies at a concentration of 21 μg/ml stopped the growth of *M. barkeri* on the first day of cultivation, when the biomass density was twofold less than in the control; at a later time, no cell growth or methane production was observed. The Cry-proteins from the *amagiensis* subspecies added at the same concentration (21 μg/ml) showed a less pronounced inhibitory effect on the growth of *M. barkeri* than the Cry-proteins from the *kurstaki* subspecies: on the first day of cultivation, the cell density also comprised one half of its level in the control; however, both cell growth and methane production continued for 3 days. Therefore, the inhibitory effect of Cry-proteins from the *kurstaki* subspecies on both archaea, *M. barkeri* and *M. arboriphilus*, was stronger than that of Cry-proteins from the *amagiensis* subspecies.

The inhibitory effect of Cry-proteins from the *monterrey* subspecies on *M. barkeri* was less pronounced than its effect on *M. arboriphilus* (figure, Tables 1, 2). At a Cry-protein concentration of 36 μg/ml, a slight growth of *M. barkeri* was observed (cell density was twofold less than in the control), whereas the growth of *M. arboriphilus* was completely inhibited within 2 days of incubation. In experiment II, the biomass of *M. barkeri* reached its maximum after 1 day of incubation at 30°C.

A comparison of the antibiotic effects of two close concentrations of Cry-proteins from the *amagiensis* subspecies (21 and 24 μg/ml in experiments I and II, respectively) on the growth of *M. barkeri* revealed a decrease in the level of biomass compared to the control by 2- and 3.3-fold, respectively. In experiment II, Cry-

Table 2. Effect of the crystal proteins from *B. thuringiensis* and lysozyme on the development of *M. barkeri*

Variant	OD_{650}			Methane production, mmol/l		
	1 day	2 days	3 days	1 day	2 days	3 days
Experiment I						
Control	0.40	0.70	0.90	27.5	35.0	37.5
+ lysozyme (20 μg/ml)	0.30	0.50	0.60	22.5	15.0	5.0
+ Cry-proteins from the *amagiensis* subspecies (21 μg/ml)	0.21	0.30	0.45	20.0	12.5	7.5
+ Cry-proteins from the *kurstaki* subspecies (21 μg/ml)	0.20	0.20	0.20	10.0	5.0	0
+ Cry-proteins from the *monterrey* subspecies (36 μg/ml)	0.41	0.50	0.50	18.8	10.0	7.5
Experiment II						
Control	1.0	–	–	35.0	–	–
+ Cry-proteins from the *kurstaki* subspecies (18 μg/ml)	0.30	0.34	0.35	8.8	5.5	0
+ Cry-proteins from the *amagiensis* subspecies (24 μg/ml)	0.30	0.40	0.45	5.0	2.3	1.0
+ Cry-proteins from the *monterrey* subspecies (26 μg/ml)	0.85	1.0	1.0	27.5	32.5	35.0
(52 μg/ml)	0.30	0.30	0.30	6.3	2.5	0

Note: In experiment I, the cells were not washed prior to inoculation of the growth medium; in experiment II, the cells were washed prior to inoculation; control is medium supplemented with 0.05 M phosphate buffer (pH 7.8); "–" stands for "not determined" (the cell growth reached its maximum on the first day of cultivation).

proteins from the *kurstaki* subspecies strongly inhibited *M. barkeri* growth, whereas Cry-proteins from the *monterrey* subspecies at a concentration of 26 μg/ml virtually did not affect cell growth and methane production. An increased concentration of Cry-proteins from the *monterrey* subspecies (36 μg/ml) inhibited growth of *M. barkeri* to a lesser extent than the growth of *M. arboriphilus* (figure, Tables 1, 2).

Thus, the crystal proteins from *B. thuringiensis* subspp. *amagiensis*, *kurstaki*, and *monterrey* exhibited different antibiotic effects on the growth of the two methanogenic archaea, *M. arboriphilus* and *M. barkeri.*

We also found (in collaboration with I.A. Zalunin, L.P. Revina, G.G. Chestukhina, and L.I. Kostina) that chromatographically pure Cry11A, CytA, and other crystal proteins from *B. thuringiensis* subsp. *israelensis* exhibited an antibiotic effect on the archaea under study (data not shown).

Thus, the crystal proteins from three subspecies of *B. thuringiensis* that affected representatives of different insect orders showed different antibiotic effects on methanogenic archaea, *M. arboriphilus* and *M. barkeri*.

In view of the life strategy of *B. thuringiensis* cultures, the ability of their parasporal inclusion proteins to inhibit the development of microorganisms is quite important, since microorganisms may interfere with the germination of *B. thuringiensis* spores in the insects affected by Cry-proteins (due to the highly specific effect of Cry-proteins on the epithelium CM in the insect midgut). That is why the crystal proteins from *B. thuringiensis* exhibit an antibiotic effect on some bacteria penetrating into the insect gut with feed, such as erwinias, micrococci, streptomycetes, and others, as well as prokaryotic insect symbionts. Cry-proteins also exert a bacteriocin-like effect on some other subspecies of *B. thuringiensis* [3, 5].

The results obtained in this work demonstrate the antimicrobial activity of the parasporal crystal proteins against microflora consisting of both bacteria and archaea, which are able to compete with the *B. thuringiensis* development in infected insects. The death of termites, cockroaches, and other insects appears to occur due to the action of Cry-proteins on the CM of the intestinal epithelium; this process is accompanied by the inhibition of symbiotic microflora utilizing the cellulose-containing feed of termites, as well as of cockroaches, for which symbiotic prokaryotes are of great importance [11].

The ability of Cry-proteins from *B. thuringiensis* to affect some anaerobic archaea is of special interest. The cell walls of the archaea under study differ considerably in their composition: e.g., the cells of *M. arboriphilus*, unlike cells of *M. barkeri*, are covered with pseudomurein [12]. The results obtained allow the assumption that the crystal proteins from *B. thuringiensis* affect the archaeal CM rather than the cell walls. This conclusion is confirmed by our experiments with lysozyme. It is known that lysozymes catalyze the hydrolysis of β-1,4-bonds of *N*-acetylmuramic acid, which is a part of proteoglycans and glucosamineglycans in murein-containing bacterial cell walls [17]. It was found that lysozyme at a concentration equal to that of crystal proteins from *B. thuringiensis* (20 μg/ml) caused a 1.5-fold inhibition of the growth of *M. barkeri* and suppressed methane production (Table 2). Since *M. barkeri* cells contain no pseudomurein, a constituent of the *M. arboriphilus* cell walls, the enzymatic activity of lysozyme towards *M. barkeri* cannot be realized because of the substrate

absence. The mechanism of the antibiotic effect of lysozyme on the cells of *M. barkeri* remains unclear. The dual biological activity of lysozyme (its ability to exert antibiotic effect on various bacteria after the loss of enzymatic activity) has recently been reported. In recent years, both enzymatic and nonenzymatic antimicrobial activities of lysozymes have been actively studied [17]. The ability of Cry-proteins, toxins that specifically affected invertebrates, to exert antimicrobial effect as well has long been known [3–5]. At present, studies on the polyfunctional properties of various proteins attract increased interest. It is becoming evident that a particular protein may exert activities of different specificities due to the operation of different sites in its molecule [18]. The studies on the mechanisms of the antibiotic effect of proteins, in particular, the inclusion proteins from *B. thuringiensis* on prokaryotes, are still in their infancy. In this connection, the comparative studies of the effect of Cry-proteins on the cell walls of bacteria and archaea, which are considerably different in their structure, are most promising.

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