Occurrence of *Bacillus thuringiensis* and their phages in Yemen soil

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Abstract *Bacillus thuringiensis* (Bt) isolates were found in all samples of soil in nine Governorates of Yemen. From 384 isolates of *Bacillus* recovered from these soil samples after acetate selection, 104 isolates (27.1 %) were Bt. Five isolates of Bt were selected and insecticidal activity was tested against *Culex pipiens*, *Callosobruchus maculatus* and *Spodoptera littoralis*. The Bt isolate YH18 gave toxicity to all tested insects larvae. This study extended to isolate phages active against the selected Bt isolates. Five phages were isolated and classified into two groups of tailed phages. Four phages with long non-contractile tails and hexagonal heads (Siphoviridae) and one phage with very short tail and isometric head (Podoviridae). Susceptibility of selected Bt to infect by these phages was studied by spot-test technique. Also the Bt isolate no YH18 was resistant to all tested phages. This is the first report illustrates the diversity and the abundance of Bt and Bt phage in Yemen soil.

Keywords *Bacillus thuringiensis* · Phages · *Culex pipiens* · *Callosobruchus maculatus*

Introduction

Bacillus thuringiensis (Bt) is Gram-positive, naturally occurring soil bacterium that produces proteinaceous endotoxins, known as crystal inclusion (cry) protein, which

are highly toxic to insects and pests [17, 37, 47]. More than 60 cry genes, with toxicity spectra extending over several orders of insects and other pests, have been reported from natural Bt strains [17]. Bt strains have been isolated from diverse habitats such as, soils and aquatic environments [24], plants [28] insects [14], animal faeces [25] and arid environments [10]. The natural occurrence of Bt in the environment and its insecticidal activity has been reported from various countries in Europe [8, 36], North America [29, 35], South America [9, 23, 29, 35], Asia [13, 20, 21, 29, 34, 49], and Africa [2, 29, 33, 38]. In the Middle East, the natural occurrence of *B. thuringiensis* in soil environments was reported from Egypt [31] and Jordan [39]. One of the efficient producers of biological insecticides is Bt which is used in industrial application by many Government and Companies [46]. However, sensitivity of Bt to lysis by bacteriophages is the most one of complicated to such industry. Lysis can either be caused by exogenous phage infection or be due to strain lysogeny. About 83 % of Bt subspecies contain lysogenic phage [5]. During Bt fermentation, failures caused by lysogenic phages account up to 15–30 % of the total, and sometimes up to 50–80 %. At worst, lysogenic phage may result in a complete loss of yield [52]. On the other hand, Som and Chowdhury [42] examined the occurrence of Bt in sample of soil, mud and duping water collected from 105 different regions of west Bengal, India using phages P1, P2 and P3. They isolated six colonies affected by phages and these colonies were effective against *Bombyx mari* larva.

In Yemen very little information is known about the presence of Bt and its phages in soil habitats [10]. Thus the aim of this study was to isolate and investigate the natural occurrence of Bt strains and their phages in soil samples collected from various Governorates in Yemen.

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Materials and methods

Isolation of Bt from soil

Twelve samples of cultivated clay soil were collected from different localities, belonging to nine Governorates in Yemen during November 2009. Samples were collected from 2 to 5 cm below the surface with a shovel. From each location four samples were taken at random and thoroughly mixed to one sample. Each soil sample (0.5 kg) was placed in plastic bag at ambient temperature (25–30 °C). Bt strains were isolated from 1 g of soil after addition to 10 ml of a acetate buffered L-broth. This soil suspension was shaken at 250 rpm for 4 h at 30 °C, and then 1 ml of each supernatant was heated at 90 °C for 3 min to kill most non-spore forming organisms. Samples were plated in duplicate on L-agar, and incubated overnight at 30 °C. The colonies with a pale white, matt morphology were picked up to T3 agar medium prepared in the form of agar dots (32 dots/plate) and allowed to grow and sporulated after 72 h at 30 °C [30, 44]. Bacillus that growing on T3 agar medium were microscopically examined using oil immersion at 100× magnification for morphology features of vegetative cells, spores and presence of parasporal inclusions. Further insecticidal activity was used as confirmatory test.

Insect toxicity assays

Cultures that exhibited highly presence of parasporal inclusions were selected and prepared as stock cultures on T3 agar medium, then were grown at 30 °C in 3 ml of T3 broth for 72 h. Concentrated suspensions of sporulated cultures (10^7 spore/ml) were prepared in T3 broth for qualitative oral toxicity test against the third and early fourth instar for larval of *Culex pipiens*; first instar larvae of *Spodoptera littoralis* and cowpea seeds with egg of *Callosobruchus maculatus*. For screening of Bt against mosquito larvae colony of *C. pipiens* was maintained at the mosquito rearing facilities in the insect laboratory, Faculty of Science, Sana'a University. Rearing of mosquitoes was achieved according to the techniques described by Chapman and Barr [15]. From spore stock culture, an amount of 1 ml was added to 50 ml of distilled water in 100 ml plastic cup. Twenty larvae of third and early fourth instar laboratory reared *C. pipiens* were placed in this water and provided with suitable amount of larvae feed, consisted of ground dried bread and dried brewers yeast (2:1), similar cups served as untreated control. Another laboratory colony of the cotton leaf worm, *S. littoralis* was reared and detected at laboratory of plant protection of Ministry of Agriculture in Sharkia Government, Egypt by Dr. Ali Meshref. Larvae were fed on semi artificial diet. 1 ml of spore suspension was spreading on the surface of solidified

formaldehyde free diet (20 ml in 250 ml plastic cups). Twenty-first instar larva's of *S. littoralis* were placed in each cup by means of a fine camel-hair brush. Cups were covered with perforated plastic lids, then kept in an inverted position at laboratory temp (25–27 °C). For screening the activity of the isolated Bt against *C. maculatus*. Culture of this insect was obtained by transfer of a large number of males and females to cowpea seeds in glass jars and allows them laying their eggs on seeds. Healthy seeds were previously incubated for 48 h in freezer to kill any larval stage. Jars with seeds and insects were left for 7 h to allow females getting mated. To determine the effect of Bt on this insect, seeds with eggs (10 eggs/seed) were collected and immersed in Bt suspension (1.2×10^7 spore/ml) for 1 or 2 min then, transferred to boxes and incubated for 8 days at relative humidity of 50 % and temperature of 25 °C. Another set were prepared without bacteria and used as control. The percentage of liberated insects was determined in comparison with control. In all tested insects each treatment was made in triplicate and mortality percentages were usually corrected by Abbott formula [1].

Isolation and purification of Bt phages

From the same location used for isolation of *B. thuringiensis*, 100 g soil were taken for phage(s) isolation. Soil samples were suspended in 100 ml of 0.1 M phosphate buffer (pH 7.2), kept on an incubator shaker at 28 °C for 1 h, and filtered through cotton cloth. The filtrate was centrifugated at 3,000 rpm for 30 min, and passed through 0.45 µm. Phage activity was assayed by spotted on lawn bacteria. Soil suspension which gave positive lytic area, was plaque assayed by the double layer agar-plate technique [7]. To isolate spontaneously released phages from bacteria, cultures were grown in L-broth at 30 °C with shaking (300 rpm) for 19 h. Cells were discarded by centrifugation by two cycles of low, (3,000 rpm for 30 min) and high (20,000 rpm for 10 min), speed centrifugation at 4 °C. The supernatant was filtered through 0.45 µm membrane filter and assayed for phage by the above method. After five successive isolations of single plaque, phages were propagated in plate to give confluent lysis, from which a high-titer phage stock was obtained.

Electron microscopy

Fifty millilitre of filtered suspension from each phage were precipitated at 20,000 rpm in a cooling centrifuge (4 °C) for 3 h. Pelleted phage was suspended in 1 ml of 0.1 M phosphate buffer (pH 7.2), and then negatively stained using 2 % aqueous uranyl acetate (pH 4.8). The stained preparation was then dropped onto formvar coated grids,

Table 1 Distribution of *B. thuringiensis* in Yemen

Governorate	Location	Crop	Total bacterial count $\times 10^5$ (cfu/g soil)	Spore count of BT $\times 10^3$ (cfu/g soil)	B.T. index	No. and shape of crystal
Sanaa	Sapeen Hospital	<i>M. sativa</i> L.	5.7 ± 1.5	7.9 ± 1.5	0.31 (10)	9a 1c
Sanaa	Gaismy filed	<i>M. piperita</i>	6.0 ± 1.2	9.6 ± 1.8	0.25 (8)	6a 2c
Sanaa	Sanna	<i>L. sativa</i> L.	23.1 ± 2.1	31.8 ± 2.1	0.31 (10)	4a 5b
Ibb	Alnajed Al Ahamer	<i>C. edulis</i> Forsk	2.0 ± 1.5	7.3 ± 1.5	0.18 (6)	3a 2b 1c
Aljawf	Bragish	<i>L. sativa</i> L.	13.5 ± 1.8	10.8 ± 1.4	0.06 (2)	1a 1b
Albaydaa	Radaa	<i>C. edulis</i> Forsk	40.0 ± 2.5	6.2 ± 1.3	0.22 (7)	6a 1b
Al Hudyadah	Al mansoryah	<i>M. sativa</i> L.	40.0 ± 2.3	14.6 ± 1.4	0.47 (15)	12a 2b 1c
Al Dhala	Gobn	<i>C. edulis</i> Forsk	10.1 ± 1.2	27.3 ± 1.6	0.38 (12)	6a 5b 1c
Sada	Hidan	<i>C. edulis</i> Forsk	12.6 ± 1.5	4.5 ± 1.1	0.41 (13)	9a 3b 1c
Taiz	Alsamaa	<i>M. sativa</i> L.	23.4 ± 2.1	25.3 ± 1.8	0.28 (9)	7a 2b
Taiz	Al Rawna	<i>F. carica</i> L.	12.2 ± 1.3	10.0 ± 1.2	0.18 (6)	2a 4b
Dhamar	Gaharan	<i>C. edulis</i> Forsk	9.3 ± 1.5	21.2 ± 1.1	0.18 (6)	2a 2b 1c
Total	9		197.9 ± 3.8	176.5 ± 2.8	(104) 3.23	68a 27b 9c

Numbers in parenthesis represent total number from 32 colonies taken from total spore count/g soil of BT

a Bipyramidal, b spherical/cuboidal, c various

and examined with electron microscope (JEOL 100 CX) at 80 kV and magnification of 100.000, 80.000 and 27.000 $\times$ (Electron microscope unit, Zagazig University, Egypt). The size of phage particles was measured directly from negatives using SL-PI ultrastructure size calculator (Made in USA and the original slide guide by Dunn and Riedman Copywrite 1971, A.E.G. Punnl).

Host-range of isolated phages

Plates seeded with young cultures (19 h) of 5 selected Bt isolates (YS 4, YI15, YD10, YS18 or YH18) and 3 standard isolates: Bt Kurstaki (DI), Bt Kurstaki HDI (AB) or Bt Egyptii, Taizz, Sanaa (C18) which obtained from Dr. El-lazik (Prof. of microbiology at Botany Department, Faculty of Science, Al Mansoura University) were spotted by high stock titer of isolated phages. The phages were assayed to detect their ability to infect the different isolates of Bt.

Results

Isolation of BT from Yemen soil

The overall microbial activity determined from count of total bacterial and heat resistant (spore-forming) colonies varied significantly among the 12 tested soil samples. The highest total counts (40.0×10^5 cfu/g soil) were observed with samples no. 6 and 7 which were taken from soil in Al Bayda and Al Hudyadah soils cultivated by *Catha edulis* and *Medicago sativa* L. respectively (Table 1). The highest spore counts were observed with samples no. 3 (31.8×10^3 cfu/g

soil) and $8(27.3 \times 10^3$ cfu/g soil) which were taken from Sana'a and Al Dhala soil cultivated by *Lactuca sativa* L. and *C. edulis* respectively. After incubation period cells from *Bacillus* colonies were examined for morphological features of vegetative cells, spores, and the presence of parasporal inclusions, under light microscope. Bt isolates were recovered from all tested samples based on the microscopic examination of crystals. A total of 104 Bt were recovered from 12 soil samples. Al Hudyadah and Sada soils recorded the highest Bt index while, the lowest Bt index showed with soil obtained from Aljawf Governorate. In most samples of BT isolate gave spore and crystal with bipyramidal (68), cuboidal (27) and irregular (9) shape of colonies.

Larvicidal activity of isolated Bt

Isolates with highest percentage of parasporal inclusions (five isolates) were selected and tested for larvicidal activity against *C. pipiens*, *C. maculatus* and *S. littoralis*. Pesticidal activity of the all 5 isolates exhibited high activity with mortality percentage reached to 90 with isolates no YI15 and YD10 against *C. pipiens* and *C. maculatus* only while, isolates no YH18 gave mortality of about 50 % or up when tested against *C. pipiens*, *C. maculatus* and *S. littoralis* (Table 2).

Isolation and morphology of Bt phages

To isolate phages, bacteria or soil suspensions were freed by centrifugation (220 rpm) for 30 min and filtration then, tested for the presence of phages by spot-test method. Results given in Tables 3 and 4 showed that, suspension of

Table 2 Larvicidal effect of selected five isolates of *B. thuringiensis* isolated from Yemen soils against *C. pipiens*, *C. maculatus* and *S. littoralis*

Bt isolates no.	Concentration (cfu/ml) $\times 10^5$	Mortality % of ^a		
		<i>C. pipiens</i>	<i>C. maculatus</i>	<i>S. littoralis</i>
YS4	13.1	70.00	87.33	00.00
YI15	12.0	90.00	90.00	00.00
YD10	14.0	90.00	87.333	26.60
YS18	12.6	40.00	70.74	00.00
YH18	12.0	48.00	76.73	52.00

cfu/ml mean colony forming unit of BT

^a Mean corrected mortality according to Abbott [1]

bacterial isolate YS18 gave lytic area on a lawn test culture of isolate YD10. Also soil suspensions of Ibb and Al Hudaydah gave a lytic area with the same lawn bacteria. Suspensions, which gave positive lytic area was plaque assayed and plaques morphology, were used to distinguish between obtained phages. Some phages produced plaques of three types such as Ibb soil suspension gave large (2 mm) clear plaque (phage YII), small (1 mm) clear center and turbid area (phage YIS2) and small (<1 mm) turbid plaques (phage YIS1). Bacterial suspension of isolate no YS18 gave small (<1 mm) turbid plaques (Phage YLS18) while, Al Hudaydah soil suspension gave small (1 mm) and clear plaques (phage YHS). Electron microscopic studies (Fig. 1) showed that, the isolated phage had an isometric heads and long non-contractile or very short tail, thus corresponding to Bradley [12] basic morphological type B and C and to Siphoviridae and Podoviridae of phages with tails. Heads were icosahedral, as indicated by the presence of capsids with hexagonal outlines. The morphological structures of these phages are shown in Table 4 and Fig. 1. All of them were obtained from viewed the concentrated original stock in electron microscope (at 80 kV and magnification = 100.00 nm). Electron microscopic studies of phages YIL, YIS2, YIS1, YLS18 revealed that they have a polyhedral head and a tail, which appeared

more or less rigid solid tail with line cross-striations (Fig. 1c). These phages were belonged to the group B of Bradley's classifications (Siphoviridae). Phage YIS2 was the largest phage where it head measured 100.00 nm and tail length and diameter measured 187.5×37.5 nm respectively. On the other hand, phage YHS was the smallest one and this phage had very short tail and hexagonal head. This phage belonged to the group C of Bradley's classification (Podoviridae).

Host range of phages

To determine the host range of isolated phages, high titer of each phage was spotted on isolated and standard strains of Bt (Table 5). Bt isolate YH18 appeared with little or no susceptibility to be infected by any one of isolated phages, while isolates YS4 and Bt kurstaki (DI) have the same pattern of moderate susceptibility to phages. Bacteriophages YIS1, YIL, YHS and YIS2 produced opec lytic area when spotted on isolated bacterial YS18 or slandered strains Bt Egyptii, Taizz, Sanaa (C18) and kurstaki HDI (AB) while, phage YLS18 did not infected the latter strains. Phages isolated from the soil were wide host-range where they have the ability to infect the most of tested bacteria (infected 6 from 8) while, phage YLS18 which isolated from bacteria possessed the ability to infect only 3 from 8 of tested bacteria.

Discussion

In this study twelve soil samples were collected from eight Governorates in Yemen (Sana'a, Ibb, Aljawf, Al Bayda, Al Dhala, Sada, Taiz and Dhamar). The examined soil samples were agricultural soils cultivated by the following crops: *M. sativa* (3 samples); *Mentha piperita* (one sample); *L. sativa* L. (two samples); *Ficus carica* (one sample) and *C. edulis* (five samples). The results showed that Bt isolates were found in all tested soil samples. (Table 1). These results are in accordance with previous results in this

Table 3 Formation of lytic area by spot-test method for different bacteria or soil suspensions

Tested bacterial isolates no.	Formation of lytic area from bacterial or soil suspensions bacterial suspension											
	Bacterial suspensions of				Ibb soil suspensions				Al Hudaydah soil suspension			
	YS4	YD10	YS18	YH18	A	B	C	D	A	B	C	D
YS4	-	-	-	-	-	-	-	-	-	-	-	-
YI15	-	-	-	-	-	-	-	-	-	-	-	-
YD10	-	-	+++	-	-	+	-	+	-	+	-	+
YS18	-	-	-	-	-	-	-	-	-	-	-	-
YH18	-	-	-	-	-	-	-	-	-	-	-	-

- No lytic area, + little susceptible or not, +++ highly susceptible

Table 4 Morphology and measurements of phages active against *B. thuringiensis* isolated from Yemen soil

Phage no.	Source	Plaque morphology (size and appearance)	Titer (PFU/ml) $\times 10^4$	Dimension (nm) ^a	
				Head	Tail length $\times$ dimension
YIS1	Ibb soil	Small (<1 mm) turbid plaques	35.0	70.00	100.0 $\times$ 30.00
YIL	Ibb soil	Large (2 mm) Clear plaques	7.0	72.00	180.0 $\times$ 24.00
YIS2	Ibb soil	Small (1 mm) clear center and turbid area	201.0	77.00	187.5 $\times$ 37.5
YHS	Al Hudaydah soil	Small (1 mm) clear plaques	210.0	50.00	40.0 $\times$ 20.00
YLS18	Bacterial suspensions of isolate no. YS18	Small (<1 mm) turbid plaques	70.0	74.10	111.12 $\times$ 37.00

The morphology of the plaque depends upon the phage, the host, and the growth conditions

^a At least ten measurements taken directly from negatives

respect which reported that the recovery of the bacterium is more frequently from agricultural soils than urban or desert soils [29, 36, 47]. This is probably explained by the variation of the biotic (other microorganisms) and a biotic (temperature; humidity and texture etc.) factors which effects on the distribution of *B. thuringiensis*. Also the high incidence of these bacteria in cultivated soils may be due to the presence of entomofauna associated with the crops being cultivated in this soil.

It is important to mention that all soil samples analyzed in this study were collected from areas with no previous history of using Bt commercial products to control insect pests. The incidence of Bt in Yemen soils seems to be more frequent than in soils of United States [18] and Saudi Arabia [10], where 17 and 8 % of their isolates were producing parasporal inclusions respectively. A total of 65 % of parasporal inclusions of identified Bt were bipyramidal in shape followed by 26 % of spherical shape crystals and 0.1 % only of various shaped of crystals. The diversity of varieties found in Yemen soils concur with the findings of Martin and Travers [29] who revealed that, many soil samples contain several varieties of Bt and that the distribution of these particular varieties was unique to these soil samples. In this investigation five isolates of Bt with high percentage of parasporal inclusions (YS4, YH18, YD10 YI15 and YS18) were selected to investigate their larvicidal activity against *C. pipiens*, *C. maculatus* and *S. littoralis* (Table 2). The results showed that all 5 isolates exhibited strong toxicity to *C. pipiens* and *C. maculatus* while, 2 isolates (YD10 and YH18) had larvicidal against *S. littoralis*. These results confirmed the results of microscopic examination of isolates no YD10 and YH18 which showed bipyramidal parasporal inclusions and exhibited strong toxicity to Lepidoptera [22, 32, 40, 45].

Bacteriophages, which attack Bt, may be isolated from lysogenic bacteria or soil. Results indicated that, phage active against Bt may be present in soil or integrated with host genome (lysogeny) or as independent linear replica within the cell. These results are in accordance with finding

of [19, 26, 27, 41, 43, 48]. Electron microscopic studies of the isolated phages (Fig. 1) showed that YIS1, YIL, YIS2 and YLS18 were morphologically similar and belonged to the B1 morphotype according to Ackermann's classification [3]. These phages had an isometric polyhedral capsid and a non-contractile long tail with transverse disks at the distal end of tail in phage YIS2. Morphologically, these four phage virtually did not differ from 8 phages described by Azizbekian et al. [11] and phage TB10 described by Ackermann et al. [4]. The tail and capsid measured here are totally different from *Bacillus cereus* phages measured by

Fig. 1 Electron micrograph of *B. thuringiensis* phages negatively stained with uranyl acetate: **a** phage YIS1, **b** phage YIL, **c** phage YIS2, **d** phage YHs and **e** phage YLS18

Table 5 Susceptibility of different isolates of *B. thuringiensis* to infect by isolated phages

Bt isolate	Phages isolate				
	YIS1	YIL	YHS	YIS2	YLS18
YS4	++*	++*	++*	+	⁰
YI15	⁰	⁰	⁰	⁰	⁰
YD10	+++*	+++*	+++*	+++*	+++*
YS18	++ ⁰	++ ⁰	++ ⁰	++ ⁰	++ ⁰
YH18	-	⁰	⁰	-	-
Kurstaki (DI)	+++*	+++*	+++*	+++*	+++*
Kurstaki HDI (AB)	++ ⁰	++ ⁰	++ ⁰	++ ⁰	-
Egyptii, Taizz, Sanaa (C18)	++ ⁰	++ ⁰	++ ⁰	++ ⁰	-

+ Formation of lytic area, - absence of lytic area, ⁰ turbid lytic area, +++ highly susceptible, ++ moderate susceptible, + little susceptible or not susceptible, * appearance of colonies inside the lytic area after increase of incubation period

[26] Meanwhile, Phage YHS was appeared with very short tail and isometric polyhedral capsid. This phage belonged to the group C1 morph. type of Ackermann's classification [3] and similar to phage ACG-C 40 infecting *E. coli*-associated urinary tract infections which described by [16]. Ackermann et al. [6] proposed two typing schemes-one for *Bacillus subtilis* and one for *B. thuringiensis*. Their *B. thuringiensis* scheme comprises 8 Phages and 25 strains of *B. thuringiensis* and can be applied to *B. cereus*. Bacterial isolates no YD10 and YH18 produced bipyramidal crystal and considered as *B. thuringiensis* kurstaki but they are totally different in their susceptibility to infect by tested phages where, BT Kurstaki (DI) and isolate YD10 are highly susceptible to all phages but isolate YH18 are resistant to these phages. Also isolates YS4 and YS18 which isolated from Sana'a and Sada respectively and produced spherical crystal and identified according to this crystal as Bt Israelensis. Susceptibility of these isolates to infect by tested phages are similar but appearance of lytic area are opec with isolate YS18 and clear with isolate YS4 which produced colonies by increase of incubation period. *B. thuringiensis* isolates YI15 which produced various shaped of crystal and identified as Egyptian. Susceptibility of this isolate to infect by tested phages are in highly similarity with stander isolate Bt Egyptii, Taizz, Sanaa (C18). These results are similar with finding of [50, 51]. Finally typing is suitable for subdividing *B. thuringiensis* kurstaki and useful in the identification of the purity of BT used in biotechnology and isolation of BT from soil [6, 52].

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