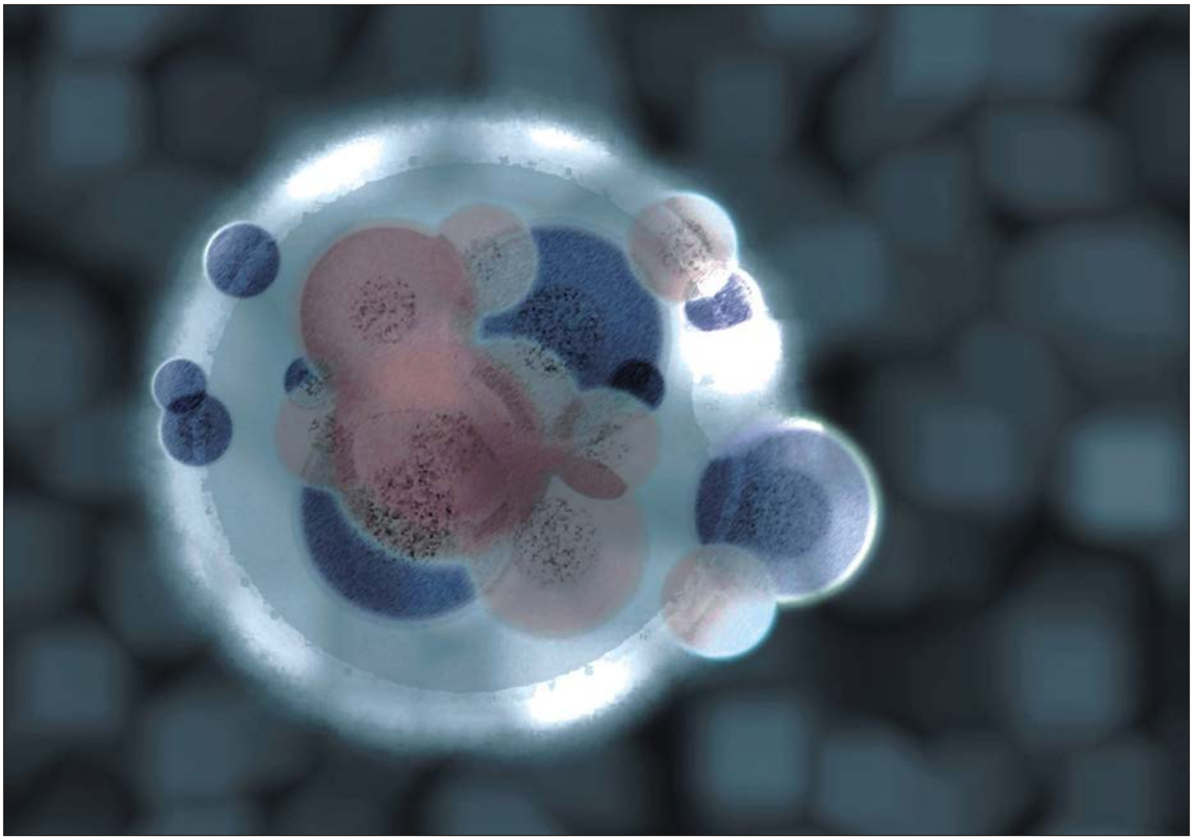

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Effect of High Concentrations of Sodium Azide on the Isolated Thermophilic *Bacillus* Phages in Different Temperatures and pH-values

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Abstract: Thirteen thermotolerant *Bacillus* phages were isolated from Jordanian hot springs. These phages were tested for their resistance to the presence of high concentrations of sodium azide at different temperatures and pH-values. At 43°C and pH 6.5, almost all phages were still active in the presence of 1% sodium azide but inactive moderately or immediately when treated with 3 or 5% sodium azide, respectively. The exception was for the phage 18 that showed resistance to 3% sodium azide and slight growth in the presence of 5% sodium azide. The phage 18, treated with 3% sodium azide, was able to resist the high temperatures 103 and 113°C and showed moderate growth when assayed at 43°C. Different observations were also seen at different pH-values for *Bacillus* phages treated with 1% sodium azide, phage 16 was unable to grow in alkaline condition (pH = 11) on the other hand, only three phages (18, 20 and 30) were able to grow in acidic condition (pH = 2). For the phage 18 treated with 3% sodium azide was able to grow at pH values 6, 8 and 9, respectively. These results may reflect their high resistance of their protein components.

Key words: *Bacillus,* phages, sodium azide, temperature, pH-value

INTRODUCTION

The use of sodium azide as a selective inhibitor for Gram-negative and most Gram-positive bacteria was reported in various studies (Edwards, 1938; Mallmann, 1940; Snyder and Litchstein, 1940). Rikhvanov *et al.* (2000) showed that pretreatment of yeast with sodium azide was found to induce thermotolerance yeast, whereas sodium azide in combination with heat shock enhanced the thermotolerance of *Saccharomyces cervisae*.

Bacteriophages have a restricted host range, being limited to a single bacterial species. However, phages are very common in the environment (Jiang and Paul, 1998) and are relatively stable, being protected by the protein coat. Phages are more compact and thus more diffusible than naked DNA (Kuttler, 1997). They may continue to coexist with the bacteria in the form of lysogenes and be liberated in some distant future, in response to environmental factors, such as heavy metals and toxic chemicals (Dunn, 1996; Jiang and Paul, 1998).

Until now, no one showed the effect of toxic chemicals on the bacteriophages, except for three studies. One study showed the effect of chloroform and ether in the growth of coliphages (Price and Rooyen, 2001); the other study showed resistance of thermotolerant *Bacillus* phages to chloroform and ethanol (Hazem, 2002). Archbold *et al.* (2002) showed sensitivity of filamentous bacteriophages to sodium azide.

The results presented in this research reflect the resistance of isolated thermotolerant *Bacillus* phages to sodium azide at different concentrations, temperatures and pH-values.

MATERIALS AND METHODS

In our lab, at the Hashemite University, the water samples from Jordanian hot springs were filtered through a 0.22 μm filter. A 100 μL of filtered water was plated together with 1000 μL of isolated and known strains of thermotolerant *Bacillus* cultures on nutrient agar plates. All plates and cultures were incubated at optimum temperature 43°C. Only those *Bacillus* strains showed plaque morphologies were used for further tests.

Dilutions of 10^{-2}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} of the sample and then plated 100 μL each of the dilution and its specific *Bacillus* strain onto nutrient agar plates. These were incubated overnight at 43°C, after which the phage titer could be determined by counting the number of plaque forming unit (pfu) for each dilution. It was also determined at this stage which dilution factor gave the best countable number of plaques. This dilution factor was then used for all other experiments.

One hundred microliter of the optimal phage dilution, diluted using saline, was added to 900 μL for each used concentrations (1, 3 and 5%) of sodium azide. After 24 h had elapsed 100 μL was removed from

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this tube and added to sloppy agar, which had been previously inoculated with its specific *Bacillus* host culture. This was then plated on nutrient agar and incubated overnight at 43°C. Only those phages showed resistance to sodium azide used for further experiments. The resistant phages were analyzed at different temperatures and pH-values after treatment with the sodium azide for 24 h.

RESULTS

Recently, we isolated *Bacillus* phages from Jordanian hot springs. They showed countable plaques at temperatures ranges between 33 to 83°C. The largest growth was centered on acidic, neutral and alkaline pH range 4 to 10 (Hazem, 2002).

Effect of different concentrations of sodium azide on *Bacillus* phages: The resistance of thirteen isolated thermophilic *Bacillus* phages against sodium azide is shown in Table 1. Six phages (14, 16, 20, 46, 55 and 81) were slightly resistant and only isolated phage 18 showed complete resistance when treated with 3% sodium azide. Their presence in one-percent sodium azide was completely resistance, except four phages (7, 53, 80 and 81) were moderately resistances. Further work was carried out only with phages showed a complete resistance to sodium azide.

Effect of temperatures on the growth of *Bacillus* phages treated with 1% sodium azide: Nine isolated *Bacillus* phages out of thirteen were used for further test due to their resistance to 1% sodium azide. All tested *Bacillus* phages treated with 1% sodium azide, incubated overnight at 43°C and assayed at different temperatures (Table 2a). All the tested phages grew well at 53, 63 and 73°C, respectively. Three phages (16, 46 and 55) were unable to grow at 28 and 37°C, respectively. The only two phages (18 and 35) were able to grow at 28°C.

All tested *Bacillus* phages treated with 1% sodium azide, incubated overnight at different temperatures, and assayed at 43°C (Table 2b). Five phages (14,16, 18, 20 and 26) out of nine phages grew well at 103°C; on the other hand, only two phages (14 and 18) showed ability to grow at 113°C.

The effect of temperature on the growth of the phage 18 treated with 3% sodium azide for 24 h at 43°C presented in (Table 3a). No reduction in the growth of the isolated phage 18 was observed. Similar results observed when the phage 18 treated with 3% sodium azide at higher temperatures and assayed at 43°C (Table 3b). The surprise was the ability of the phage to grow moderately at 103 and 113°C, respectively.

Table 1: Effect of different concentrations of sodium azide on the growth of the isolated thermophilic *Bacillus* phages incubated overnight and assayed at 43°C

	Resistance of *Bacillus* phages (pfu mL^{-1})		
	Concentration of sodium azide (%)		
Bacillus phages	1	3	5
7	20	4	NPF
14	NTC	40	20
16	NTC	20	20
18	NTC	NTC	5
20	NTC	20	10
23	NTC	NPF	NPF
26	NTC	NPF	NPF
35	NTC	5	NPF
46	NTC	30	20
53	7	2	NPF
55	NTC	40	15
80	40	2	NPF
81	40	25	15

Where, NTC = Not Countable; NPF = No Plaque Forming

Table 2: Effect of temperatures on the growth of nine *Bacillus* phages treated with 1% sodium azide

	Resistance of *Bacillus* phages (pfu mL^{-1})										
	Temperatures (°C)[a]					Temperatures (°C)[b]					
Bacillus phages	28	37	53	63	73	53	63	73	83	103	113
7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
14	NPF	300	NTC	NTC	NTC	NTC	NTC	NTC	NTC	260	20
16	NPF	NPF	NTC	NTC	NTC	NTC	NTC	NTC	360	100	NPF
18	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	50
20	NPF	300	NTC	350	250	NTC	NTC	NTC	350	250	NPF
23	NPF	250	NTC	250	NPF	NTC	NTC	150	100	NPF	NPF
26	NPF	150	NTC	250	100	NTC	300	300	250	100	NPF
35	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NPF	NPF
46	NPF	NPF	NTC	100	100	NTC	100	100	50	NPF	NPF
53	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
55	NPF	NPF	NTC	NTC	NTC	NTC	NTC	NTC	350	NPF	NPF
80	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
81	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Where, NTC= Not Countable; NPF= No Plaque Forming; ND = Not Determined. [a] *Bacillus* phages treated with 1% sodium azide and incubated overnight at 43°C and assayed at different temperatures; [b] *Bacillus* phages treated with 1% sodium azide and incubated overnight at different temperatures and assayed at 43°C

Table 3: Effect of temperatures on the growth of *Bacillus* phage 18 treated with 3% sodium azide

	Resistance of *Bacillus* phages (pfu mL^{-1})										
	Temperatures (°C)[a]					Temperatures (°C)[b]					
Bacillus phage	28	37	53	63	73	53	63	73	83	103	113
18	NTC	NTC	250	250	50	NTC	NTC	NTC	250	120	20

Where, NTC= not countable; [a] *Bacillus* phage treated with 3% sodium azide and incubated overnight at 43°C and assayed at different temperatures; [b] *Bacillus* phage treated with 3% sodium azide and incubated overnight at different temperatures and assayed at 43°C

Effect of pH-values on the growth of *Bacillus* phages treated with sodium azide: The *Bacillus* phage 16 was unable to grow in alkaline condition (pH = 11). All tested phages showed maximum growth at pH-values (6, 8 and 9). In acidic condition (pH 2), only three phages

Table 4: Effect of pH-values on the growth of *Bacillus* phages treated with sodium azide

Bacillus phages	Resistance of Bacillus phages (pfu mL^{-1}) pH-values[a]						pH-values[b]					
	2	4	6	8	9	11	2	4	6	8	9	11
7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
14	NPF	NPF	250	NTC	NTC	100	ND	ND	ND	ND	ND	ND
16	NPF	150	NTC	360	360	NPF	ND	ND	ND	ND	ND	ND
18	50	120	320	NTC	NTC	280	20	50	NTC	320	120	20
20	50	180	NTC	NTC	NTC	NTC	ND	ND	ND	ND	ND	ND
23	80	200	NTC	NTC	200	150	ND	ND	ND	ND	ND	ND
26	NPF	NPF	350	NTC	NTC	NTC	ND	ND	ND	ND	ND	ND
35	NPF	NPF	NTC	NTC	NTC	250	ND	ND	ND	ND	ND	ND
46	NPF	100	360	NTC	250	170	ND	ND	ND	ND	ND	ND
53	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
55	NPF	200	360	NTC	NTC	NTC	ND	ND	ND	ND	ND	ND
80	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
81	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Where, NTC= Not Countable; NPF= No Plaque Forming; ND= Not Determined; [a] *Bacillus* phages treated with 1% sodium azide and incubated overnight and assayed at 43°C; [b] *Bacillus* phages treated with 3% sodium azide and incubated overnight and assayed at 43°C

(18, 20 and 13) showed moderate growth, while the others did not grow at that pH-value (Table 4a).

The effect of different pH-values on the treated phage 18 with 3% sodium azide was also observed (Table 4b). At pH-values (6,8 and 9), it was completely resistant to 3% sodium azide, but was moderately resistant to acidic pH-values (2 and 4) and alkaline pH 11.

DISCUSSION

Cellular response to starvation is complicated if the nutrient in question is essential but also toxic. The unique redox properties of azide lead to a central role in numerous respiratory and metabolic enzymes, especially those that utilize dioxygen or reactive oxygen species as substrates. The azide toxicity led to widespread use of azide compounds as antifungal and antimicrobial agricultural applications. The mechanisms of cytotoxicity of sodium azide are not yet established.

The present results indicate that each isolate responded differently to the physical conditions imposed upon it; each had different plaque forming unit, which survived the period of testing. The data further confirmed that these phages were different phages.

The isolated *Bacillus* phages, treated with different concentrations of sodium azide, were found to be still infective but varied from one phage to another (Table 1).

Solvents are capable of denaturing proteins by disrupting the hydrophobic interaction between proteins, which can also lead to a loss of viability. These effects are especially true for strong solvents like sodium azide. Head structures or receptor binding could have been affected by protein alterations induced by toxic sodium azide. These effects observed in filamentous phages (Rapora and Robert, 1993) but not in most of our isolated thermophilic *Bacillus* phages.

The tested *Bacillus* phage 18 showed resistance to the presence of either 1% or 3% sodium azide: that may be due to the very high resistance of its protein components compared with the other tested phages. On the other hand, The thermophilic *Bacillus* phages 14, 16 and 46 showed moderate resistance to the presence of high concentrations of sodium azide: that may be due to the high resistance of most of their protein components. The treatment with 3% and 5% sodium azide showed total inhibition growth for the tested phages 7, 23, 26, 35, 53 and 80. These results may be due to protein alterations of the head structures by toxic chemical sodium azide at very high concentrations. Almost all tested phages were unaffected when treated with 1% sodium azide, similar results observed in previous study (Hazem, 2002) when seven out of thirteen isolated phages treated with either chloroform or 75% ethanol.

Different observations were also seen at different pH-values for *Bacillus* phages treated with 1% sodium azide, phage 16 was unable to grow in alkaline condition (pH = 11); on the other hand, only three phages (18, 20 and 30) were able to grow in acidic condition (pH = 2). For the phage 18 treated with 3% sodium azide was able to grow at pH values 6, 8 and 9, respectively.

The study of resistance-isolated phages to the presence of sodium azide is important for further identification and characterization of isolated thermophilic *Bacillus* phages. The toxicity effect of sodium azide needs to be studied in order to understand the mechanisms of resistance of the isolated phages.

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