

## Can Frozen *Burkholderia pseudomallei* Come Back to Life?

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### Abstract

*Burkholderia pseudomallei* is known to survive harsh climatic conditions and adverse environments. However, the potential of *B. pseudomallei* to survive at low temperatures is currently still not well understood. The aim of the study was to investigate the viability and sustainability of *B. pseudomallei* at low temperatures, including negative. The ability of 3 *B. pseudomallei* strains of different origin to survive during the 90-day incubation with a gradual decrease in temperature ranging from plus 15 to 18 degrees below zero was tested. The strains gradually adapted to the cold survived both under continuous exposure to temperature minus 18°C and at least after five freeze-thaw cycles for at least 25 days. A different degree of reduction in CFU/ml was observed for all three strains. The colony morphology of strains after a cold exposure was different from initial isolates in all cases. It was concluded that tolerance of *B. pseudomallei* to low temperature differs from strain to strain. The range of survival at low temperatures, evidently, is essential to understanding the climatic limits of the potential distribution of *B. pseudomallei*.

**Keywords:** *Burkholderia pseudomallei*; Cold Temperatures; Survival After Freezing; Colony Morphology

### Abbreviations

TSA: Tryptone Soya Agar; CFU/ml: Colony-Forming Units Per Milliliter; ST: Sequence Type

### Introduction

*Burkholderia pseudomallei* is an environmental saprophyte and the aetiological agent of melioidosis, a life-threatening infection. Case reports of melioidosis and predictive modelling studies suggest that it is probably widely present in many countries across the tropics and subtropics [1]. The ability of *B. pseudomallei* to persist and even multiply across a wide range of environmental conditions is extraordinary: the microbe survived during a sixteen-year incubation in distilled water [2], can remain viable for a long time in dry conditions [3], can endure nutrient-depleted environments as well as a wide range of pH, salt concentrations [4] and under oxygen-limited conditions [5]. *B. pseudomallei* can grow and survive in a broad range of temperatures [6,7]. Facts of local distribution and long-term persistence of *B. pseudomallei* in temperate regions are known [8,9]. However, the potential of *B. pseudomallei* to survive at low temperatures is currently still not well understood. Here we demonstrate that *B. pseudomallei* can long-term survive at low temperatures, including negative.

### Materials and Methods

All manipulations of *B. pseudomallei* were carried out in the Department of Tropical Medicine, Vietnamese-Russian Tropical Research Center, Hanoi, Vietnam. Three morphologically distinctive *B. pseudomallei* strains of three sequence types (ST 46 - soil isolate, ST 70, and ST 85 - clinical isolates) have been investigated. The bacteria were subcultured on Tryptone Soya Agar (TSA) (HiMedia, Mumbai, India)

for 48 hours at 37°C. Bacterial cultures were suspended in 10 ml of sterile saline solution (0.45% NaCl solution, pH 4.5 - 7.0, CareFusion, Mexico) to a concentration of approximately  $1.0 \times 10^{10}$  CFU/ml. Serial tenfold dilutions to calculate the exact number of CFU/ml of the stock suspensions were used. The survival of *B. pseudomallei* strains was controlled during the 90-day incubation with a gradual decrease in temperature ranging from plus 15 to 18 degrees below zero. Incubation conditions are shown in table 1. Control inoculations were made on Ashdown agar. Colonies were counted after four days of incubation at 37°C.

Statistical analysis were conducted with a paired two-sample t-test using Microsoft Excel 2016.

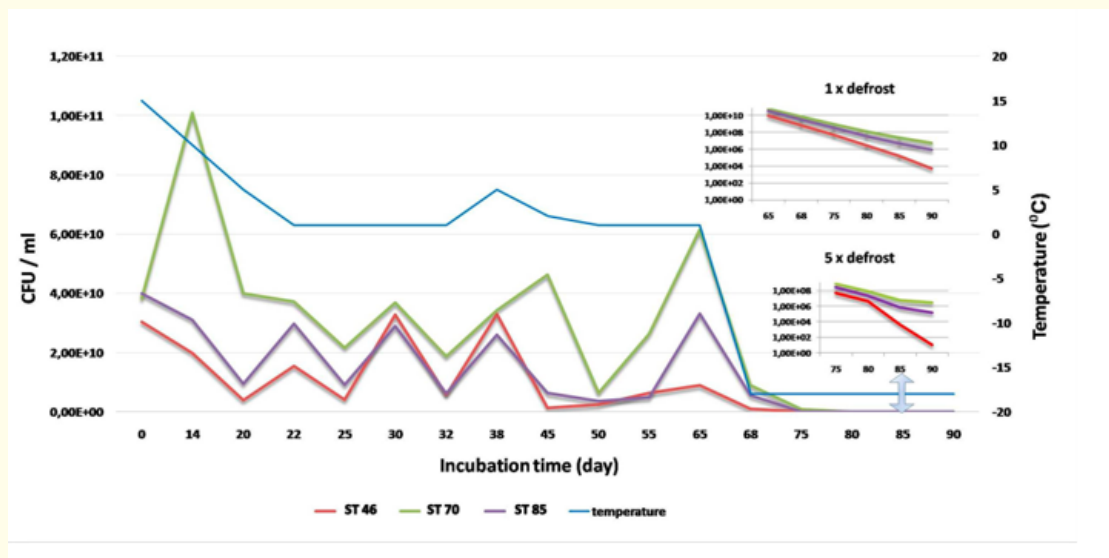
### Results and Discussion

All three strains survived after 20 days of gradual temperature decrease from 15°C to 1°C and at 1°C over 40 days. There was found a slight decrease in the number of viable bacteria for two strains, and an increase of CFU/ml was noted for the third strain (Table 1). Statistical analyses indicated no significant differences between baseline and post-incubation CFU/ml values ( $p > 0.05$ ). Nevertheless, fluctuations in CFU/ml values in the zone of positive temperatures were statistically significant ( $p \leq 0.02$ ) (Figure 1). Interestingly, the fluctuation in the number of bacteria does not depend on temperature changes in the range from 15°C to 1°C. Previous studies have indicated that *B. pseudomallei* can survive in sterile distilled water at 2°C and tryptic soy broth at 5°C for at least 28 and 100 days, respectively. However, there was a significant decrease in the recovered viable count [8,10]. It can be assumed that the differences in the results obtained earlier and in this work are due to differences in the design of the experiment. In particular, in the cited studies, the bacteria were incubated immediately at 2 - 5°C, that is, they were subjected to cold shock. In this study, we studied the survival of *B. pseudomallei* by gradually lowering the temperature to simulate the cold season conditions in the temperate zone and adapt the bacteria to low temperatures. Nevertheless, despite the distinct differences, in the main thing - *B. pseudomallei* survives at temperatures close to zero and the level of resistance differs from strain to strain - our results are identical with the data obtained earlier.

Temperature (°C)	Test sowing day number	Incubation period (day)	Strains	CFU/ml	No. of defrosts
25	0*	0	<i>B. pseudomallei</i> ST 46	3,03E+10	
			<i>B. pseudomallei</i> ST 85	3,99E+10	0
			<i>B. pseudomallei</i> ST 70	3,82E+10	
15	10	10	<i>B. pseudomallei</i> ST 46	ni	
			<i>B. pseudomallei</i> ST 85	ni	0
			<i>B. pseudomallei</i> ST 70	ni	
10	15	5	<i>B. pseudomallei</i> ST 46	1,98E+10	
			<i>B. pseudomallei</i> ST 85	3,10E+10	0
			<i>B. pseudomallei</i> ST 70	1,01E+11	
5	20	5	<i>B. pseudomallei</i> ST 46	3,84E+09	
			<i>B. pseudomallei</i> ST 85	9,42E+09	0
			<i>B. pseudomallei</i> ST 70	4,00E+10	
1	22	2	<i>B. pseudomallei</i> ST 46	1,54E+10	
			<i>B. pseudomallei</i> ST 85	2,96E+10	0
			<i>B. pseudomallei</i> ST 70	3,72E+10	
1	25	3	<i>B. pseudomallei</i> ST 46	4,02E+09	
			<i>B. pseudomallei</i> ST 85	9,03E+09	0
			<i>B. pseudomallei</i> ST 70	2,17E+10	
1	30	5	<i>B. pseudomallei</i> ST 46	3,27E+10	
			<i>B. pseudomallei</i> ST 85	2,89E+10	0
			<i>B. pseudomallei</i> ST 70	3,69E+10	

1	32	2	<i>B. pseudomallei</i> ST 46	5,64E+09	
			<i>B. pseudomallei</i> ST 85	6,08E+09	0
			<i>B. pseudomallei</i> ST 70	1,87E+10	
5	38	6	<i>B. pseudomallei</i> ST 46	3,29E+10	
			<i>B. pseudomallei</i> ST 85	2,60E+10	0
			<i>B. pseudomallei</i> ST 70	3,43E+10	
2	45	7	<i>B. pseudomallei</i> ST 46	1,32E+09	
			<i>B. pseudomallei</i> ST 85	6,25E+09	0
			<i>B. pseudomallei</i> ST 70	4,63E+10	
1	50	5	<i>B. pseudomallei</i> ST 46	2,33E+09	
			<i>B. pseudomallei</i> ST 85	3,56E+09	0
			<i>B. pseudomallei</i> ST 70	6,39E+09	
1	55	5	<i>B. pseudomallei</i> ST 46	6,20E+09	
			<i>B. pseudomallei</i> ST 85	4,78E+09	0
			<i>B. pseudomallei</i> ST 70	2,63E+10	
1	65	5	<i>B. pseudomallei</i> ST 46	8,83E+09	
			<i>B. pseudomallei</i> ST 85	3,31E+10	0
			<i>B. pseudomallei</i> ST 70	6,16E+10	
-18	68	3	<i>B. pseudomallei</i> ST 46	9,23E+08	
			<i>B. pseudomallei</i> ST 85	5,43E+09	1
			<i>B. pseudomallei</i> ST 70	8,88E+09	
-18	75	7	<i>B. pseudomallei</i> ST 46	4,67E+07	
			<i>B. pseudomallei</i> ST 85	2,60E+08	2
			<i>B. pseudomallei</i> ST 70	7,84E+08	
-18	80	5	<i>B. pseudomallei</i> ST 46	4,34E+06	
			<i>B. pseudomallei</i> ST 85	1,95E+07	3
			<i>B. pseudomallei</i> ST 70	8,52E+07	
-18	85	5	<i>B. pseudomallei</i> ST 46	4,18E+03	
			<i>B. pseudomallei</i> ST 85	6,44E+05	4
			<i>B. pseudomallei</i> ST 70	5,54E+06	
-18	90	5	<i>B. pseudomallei</i> ST 46	1,00E+01	
			<i>B. pseudomallei</i> ST 85	1,35E+05	5
			<i>B. pseudomallei</i> ST 70	2,68E+06	
-18	90	ni	<i>B. pseudomallei</i> ST 46	4,82E+03	
			<i>B. pseudomallei</i> ST 85	8,32E+05	1**
			<i>B. pseudomallei</i> ST 70	5,54E+06	
*: Source culture					
**: Samples of cultures were frozen for 25 days					
ni: Not Investigated					

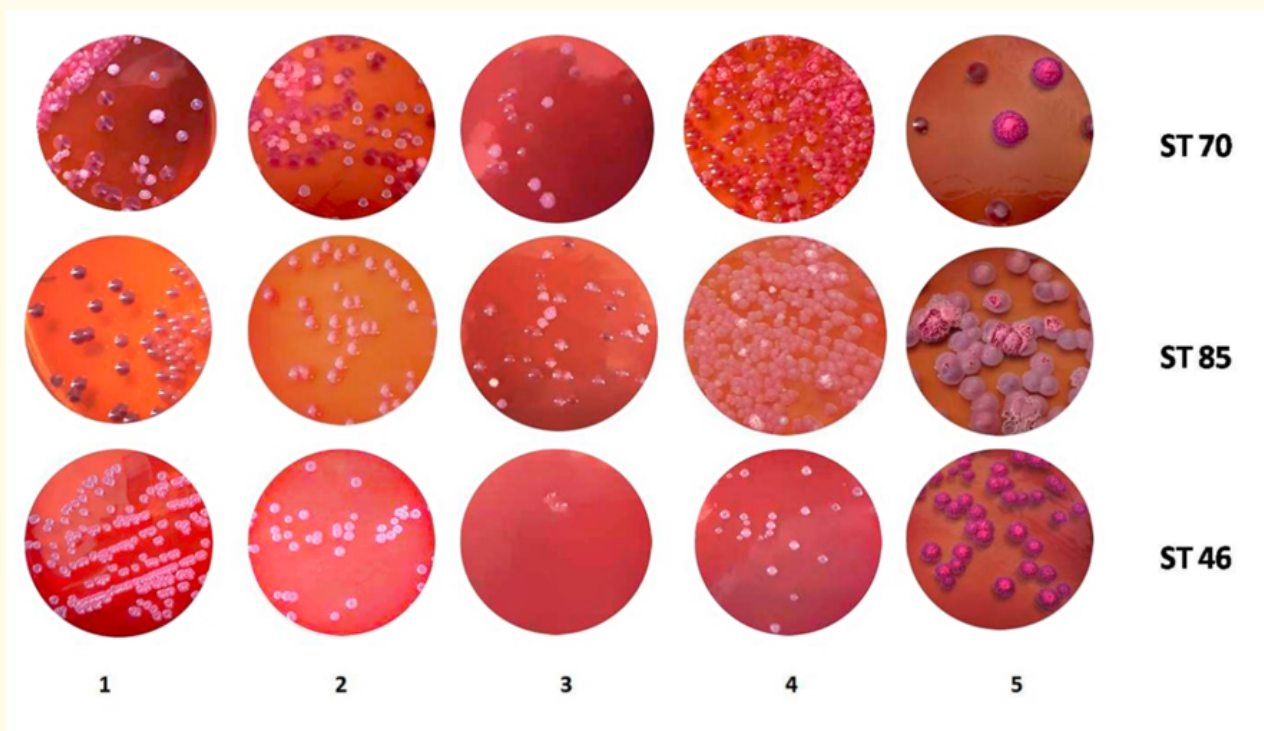
**Table 1:** *B. pseudomallei* survival rates at low temperatures.



**Figure 1:** Survival of three strains of *B. pseudomallei* over a 90-day temperature decreasing from 15 to -18°C.

Aliquots of the cold-adapted bacteria were frozen at minus 18°C without cryoprotectants. Then one of the aliquots of each strain was five times thawed at room temperature, and control inoculations were made, then the sample was frozen again. The second aliquot was kept frozen for 25 days and was inoculated at the end of the observation period.

The strains gradually adapted to the cold survived both under continuous exposure to temperature minus 18°C and at least five rounds of freeze and thaw for at least 25 days. However, there was a significant ( $1.4 \times 10^4$  to  $10^9$ -fold) decrease CFU/ml for all three strains after five freeze-thaw cycles and at  $4.8 \times 10^3$  -  $6.3 \times 10^6$ -fold after a single defrosting. The strain of *B. pseudomallei* most diverse in colony morphology (ST 70) showed the best survival. The strain of ST 85 that initially grew as microcolonies on Ashdown’s agar had a medium survival rate, and strain of ST 46 (one dominant and one minor colony morphotype) had the lowest (Figure 1). The colony morphology of strains after a cold exposure was different from initial isolates in all cases (Figure 2).



**Figure 2:** Dynamics of changes the morphology of *B. pseudomallei* strains under the influence of low temperatures. The cultures shown in lines 1 - 4 were grown at 37°C for 4 days. Lines: 1 - initial cultures, 2 - after ten days of incubation at 1°C, 3 - after 25 days at minus 18°C and five defrosts, 4 - after 25 days at minus 18°C and a single thaw, 5 - cultures of line 4 after additional incubation (5 days) at room (about 25°C) temperature.

Most *B. pseudomallei* strains are capable of morphotypic changes, which is one of the mechanisms of adaptation of the pathogen to specific stress conditions [11-14]. In the process of adaptation to low temperatures, all morphotypes showed a decrease in the color saturation of the colonies. Also, there was a trend towards the transformation of colonies with a roller around the circumference, and a center in the form of one or more tubercles or flat ("buttons"), into smooth volumetric colonies similar in shape to erythrocytes. After the second thawing, most of the grown colonies were in the S-form. And after the fifth thawing, the R-forms appeared in the ST 70 and ST 85 strains, while earlier in the ST 85 strain, the colonies were uniform. Button-like colonies disappeared by the end of the observation period. Possibly, the zigzag dynamics of *B. pseudomallei* abundance during incubation at low positive temperatures are associated with the selection of cold-adapted clones.

### Limitation of the Study

The obtained data are not exhaustive, since we only calculated cultivated forms, while *B. pseudomallei* is known to form a significant persistent subpopulation of viable but uncultured cells highly tolerant to adverse factors. It is also impossible to completely simulate natural conditions under which the pathogen avoids a sharp change in temperature, moving into the deep layers of the soil.

### Conclusion

As early as 1993, E. Yabuuchi and colleagues called for a revision of the view that *B. pseudomallei* is an exclusively tropical inhabitant [10]. The significance of the present study is primarily to confirm the ability of *B. pseudomallei* to adapt and survive in conditions of prolonged exposure to low temperatures, including negative.

Cold tolerance can allow *B. pseudomallei* to survive outside the tropics, which explains known facts of local distribution and persistence of *B. pseudomallei* in temperate areas for several decades [8,9]. Quite possibly, the area of environmental suitability for the persistence of *B. pseudomallei* is much broader than previously predicted. Since melioidosis is often fatal to humans, its possible persistence in temperate climates is a cause of concern.

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