# Describiendo la respuesta al choque térmico de *Bacillus* spp. bajo condiciones de inactivación isotérmicas

# Describing the heat shock response of *Bacillus* spp. under isothermal inactivation conditions

L. Georgalis*, P.S. Fernández, A. Garre

Departamento de Ingeniería Agronómica. Universidad Politécnica de Cartagena. Pº Alfonso XIII, 48, 30203, Cartagena, Murcia, España.

*leonidas.georgalis@upct.es

***Resumen***

**El estrés ambiental y los métodos de procesamiento de alimentos, como el calentamiento, la acidez, son responsables de provocar respuestas adaptativas a las bacterias. La respuesta general al estrés en la mayoría de las bacterias Gram positivas, incluidas *B. subtilis*, *L. monocytogenes*, está regulada por el factor sigma alternativo σB que induce la transcripción de genes capaces de proporcionar a las células vegetativas resistencia al estrés. En este estudio, se analizó la resistencia al calor de las células vegetativas de *B. subtilis* bajo calentamiento isotérmico, así como la influencia de la ausencia del gen sigB en la resistencia al calor bacteriano. Los experimentos isotérmicos se llevaron a cabo en agua peptonada (pH 7) a 51, 52,5, 55 y 57,5°C y mostraron que ambas cepas eran bastante sensibles al calor. El mutante sigB presentó mayor inactivación a 51 y 52.5°C.**

**Palabras clave:** seguridad alimentaria; evaluación de riesgos; factor sigma; respuesta al calor.

***Abstract***

**Adaptive responses to bacteria are triggered by environmental conditions and food processing processes such as heating and acidity. In the majority of Gram positive bacteria, including *B. subtilis* and *L. monocytogenes*, the overall stress response is governed by the alternative sigma factor σB (sigB), which stimulates the transcription of genes that provide resistance to stress to the vegetative cells. The heat resistance of *B. subtilis* vegetative cells was investigated under isothermal heating, as well as the effect of the sigB gene absence on bacterial heat resistance. Isothermal studies at 51, 52.5, 55, and 57.5°C in peptone water (pH 7) demonstrated that both strains were extremely heat sensitive. At 51 and 52.5°C, the sigB mutant presented more inactivation.**

**Keywords**: food safety; risk assessment; sigma factor; heat response.

## 1. INTRODUCTION

Heat stress can modify numerous morphological and phenotypical characteristics of the cell, including changes in protein homeostasis and nuclear activities. For example, a rapid increase in temperature results in protein unfolding and the formation of protein aggregates. Cellular functions such as growth and proliferation are hindered, and the cumulative effects can culminate in cell death. Nonetheless, when heat stress is not lethal, it can result in increased resistance to progressively severe pressures. This could be because of the development of so-called heat shock proteins (HSPs), which can also elicit cross-protection in response to other stimuli (1).

The heat shock response is a mechanism by which cells maintain homeostasis and remove accumulated proteins. Chaperones are a significant class of HSPs. Chaperones bind to unfolded

proteins and allow them to refold to their original state. This prevents the creation of massive aggregates, which would otherwise result in cell death (2). In general, sigma factor B plays a critical role in the control of the stress response in bacteria such as *Bacillus*, *Listeria monocytogenes*, and *Staphylococcus aureus* (3). Stress is perceived and then signalled, activating the sigma factor, which binds to RNA polymerase and induces transcription of the B regulon (-regulated genes) (4).

Understanding the vegetative cells' heat adaptive response can also provide light on the spores' great heat resistance. According to Periago *et al.* (5), minor heat stress on *B. subtilis* vegetative cells may result in the production of more heat resistant spores. The purpose of this study was to determine the behaviour of *B. subtilis* 168 wild type and its sigB mutant when subjected to isothermal heating.

## 2. MATERIALES Y MÉTODOS

### 2.1 Bacterial culture and media

Experiments were performed using the *Bacillus subtilis* strains 168-wild type and the mutant BY47 supplied by NIZO (https://www.nizo.com), the Netherlands. The wild type possesses the sigB gene, a regulator that controls about 300 genes, whereas in the BY47 it is deleted, meaning that the cells have lost an important regulator that helps them adapt to stress. However, the mutant has other heat regulon regulators that are still active.

To prepare the growth culture, 100 µL of working stock was transferred to 10 mL Luria-Bertani (LB) broth medium (Scharlab Chemie S.L., Spain) and then incubated at 30°C for less than 12 hours, with constant stirring at 200 rpm. After the overnight incubation, a washing step was followed. The culture was centrifuged for 10 min at 4°C and at 3600 rpm and then, the supernatant was removed, and 1ml peptone water (Scharlab Chemie S.L., Spain) was added to the sediment. This procedure was followed for both strains.

### 2.1 Thermal treatment and survival enumeration

Thermal treatments were carried out using a Mastia thermoresistometer (6). Before starting the treatment, the vessel was filled with 400 mL of peptone water (10 g/L peptone from casein (Scharlau Chemie) and 5 g/L NaCl (Scharlau Chemie) as the heating medium. In order to achieve a homogeneous temperature distribution, the vessel of the thermoresistometer was constantly stirred during the treatment. The heating medium was inoculated with 0.2 mL of the bacterial suspension in order to achieve approximately $10^6$ CFU/mL

Isothermal experiments were performed at 51, 52.5, 55, 57.5°C, keeping the temperature constant during the treatment. Once the temperature in the vessel was stable, the bacterial suspension was inoculated. The heating medium was adjusted to pH 7.0 for both strains during treatments.

### 2.2 Modelling of microbial inactivation

The models were fitted to the data obtained under isothermal conditions using the one-step approach with the *bioinactivation* package for R (7).

## 3. RESULTADOS Y DISCUSIÓN

Isothermal experiments in peptone water pH 7 demonstrated that the vegetative cells of *B. subtilis* behave similarly at 55 and 57.5°C, with high inactivation rates, implying that *B. subtilis* is extremely heat sensitive (Fig. 1). This is also evident in the predicted heat resistance parameter obtained from fitting the data to the Weibull inactivation model. At 52.5°C, the wild type has a $\delta_{52.5}$=0.63min, whereas the mutant has a $\delta_{52.5}$=0.25 min (Table 1).

Additionally, we observed differences in the delta values of the wild type and mutant at temperatures of 51 and 52.5°C, which can be explained by the mutant lacking the sigB gene, making it more heat sensitive, whereas there is no significant difference in the delta values at 55 and 57.5°C, a temperature that can cause severe damage to *B. subtilis* vegetative cells without recovery.

Similarly, Somolinos et al. (4) observed that deletion of the sigB gene resulted in increased heat sensitivity when *L. monocytogenes* was heated in peptone water with a pH of 7 at 60°C, demonstrating that the heat inactivation rate of *Listeria* is sigB dependent. Additionally, Cebrián et al. (8) examined the effect of sigB deletion in Staphylococcus aureus and demonstrated that mild heat shock increased heat tolerance even in the absence of sigB. They did note, however, that wild type cells were always more resistant to heat than sigB mutant cells, indicating that this protein is essential for full heat tolerance to develop during a heat shock. Additionally, they noted that *S. aureus* activates the sigma b factor at a slower rate than *B. subtilis*.

## 4. CONCLUSIONES

It is well established that when bacteria survive a stress, they typically gain increased resistance to that stress and/or subsequent pressures, with *B. subtilis* being one of them. It would also be difficult to evaluate the effect on heat resistance of pre-exposure of *B. subtilis* wild type and sigB mutant to other stresses such as acid, ethanol, or even mild heat stress.

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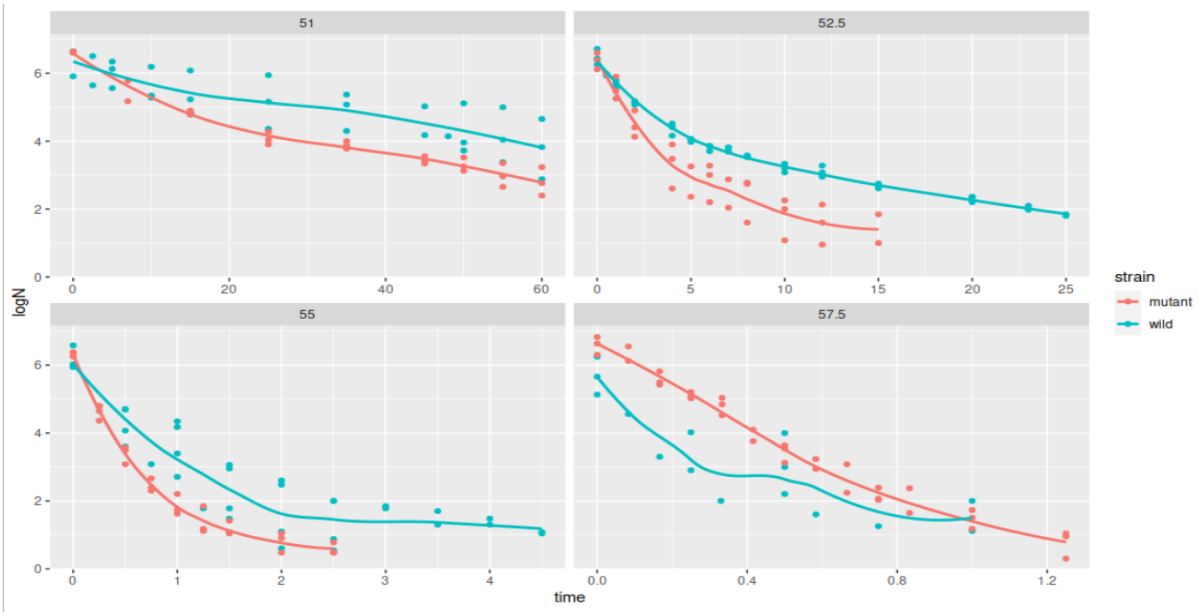


**Figure 1.** Comparison of isothermal inactivation of *B. subtilis* 168 (wild type) and BY47 (mutant) at 51, 52.5, 55, and 57.5°C.

**Table 1.** Estimated parameters of the Weibull model using Bioinactivation, obtained in peptone water under isothermal conditions, for *B. subtilis* 168 and the mutant BY47.

| Strain | Temperature (°C) | δ-value (min) | logδ (log min) | SE δ-value | p | SE p | z-value (°C) |
|---|---|---|---|---|---|---|---|
| ***B. subtilis*** **wild type 168** | 51 | 19.08 | 1.28 | 9.02 | 0.76 | 0.25 | 2.87 |
| | 52.5 | 0.63 | −0.20 | 0.12 | 0.43 | 0.02 | |
| | 55 | 0.21 | −0.67 | 0.12 | 0.53 | 0.08 | |
| | 57.5 | 0.06 | −1.23 | 0.07 | 0.37 | 0.11 | |
| ***B. subtilis*** **BY47 mutant** | 51 | 7.14 | 0.85 | 1.65 | 0.62 | 0.06 | 3.45 |
| | 52.5 | 0.25 | −0.60 | 0.16 | 0.39 | 0.05 | |
| | 55 | 0.12 | −0.91 | 0.03 | 0.66 | 0.06 | |
| | 57.5 | 0.05 | −1.30 | 0.02 | 0.55 | 0.05 | |