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RESISTANCE TO HIGH TEMPERATURES OF SPORES FROM BACILLI OF SHALLOW HYDROTHERMAL VENTS ORIGINS

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ABSTRACT. Thermophiles from shallow hydrothermal vents (SHV) are ideal candidates to extend our knowledge for understanding the environmental limits for terrestrial life, which are also relevant in the field of astrobiology. The spore resistance of two thermophilic marine strains, *Geobacillus vulcani* DSM 13174 and *Bacillus licheniformis* T14, isolated from two SHV (Eolian Islands, Italy), to wet- and dry- heat was compared to their close phylogenetic relatives (*G. stearothermophilus* DSM 22^T and *B. licheniformis* DSM 13^T), and to the biodosimetry and space microbiology model strain *B. subtilis* 168. To determine the heat resistance, spore suspensions (10^7/ml) of each strain were exposed at wet-heat (95°C for 60 min) and dry-heat (130°C for 90 min) conditions. The spores viability was determined plating aliquots of each treated sample onto Tryptone Soy Agar plates, and finally the resistance of spores to both wet- and dry-heat treatments was expressed as LD_{90}. The highest degree of spore resistance was observed for *G. stearothermophilus*, with similar level of resistance for *G. vulcani*. Spores from *B. licheniformis* T14 were more resistant than those of the closely related *B. licheniformis* DSM 13^T, and also than those of *B. subtilis* 168. Spores of the two thermophilic marine strains were more resistant to heat stresses than *B. subtilis* 168, which may reflect their own adaptation to the severe environmental vents conditions. Due to their thermal resistance, the two bacilli of shallow hydrothermal vents origins may have a novel use as bacterial model organisms for further investigation into the spore responses to environment stressors, also simulating space conditions.

1. Introduction

Microorganisms able to tolerate environmental extremes, or extremophiles, are ideal candidates to extend our knowledge of the limits for terrestrial life, including sporicidal treatments, and also on their ability to survive at conditions mimicking space environments.

Thermophilic bacteria are able to grow at or above 60 °C, showing optimal growth temperatures in the range 45–70°C (Nazina et al. 2001). Bacilli are widely distributed around the world in hot environments, and they have been isolated from hot springs, solfataras or geothermally heated soils, and shallow hydrothermal vents or from man-made thermal systems (hot water pipelines, heat exchangers, waste treatment plants, etc.).

Members of *Bacillus* genus are able to produce spores that represent dormant and resistant forms to several environmental and laboratory stresses, including sterilization techniques, such as chemical oxidizing agents, extreme desiccation, wet- and dry-heat, ultra violet and gamma irradiations (Setlow 2006). For these reasons they have been acknowledged as the hardiest known form of life on Earth (Nicholson *et al.* 2000). On the other hand, bacterial spores also have an enormous impact on many areas of human activity, since spores of bacilli represent a major problem for the dairy, pharmaceutical and food industries, because of their potential role in quality deterioration and alterations of products (Sadiq *et al.* 2016). The heat treatments represent common methods used in human industry and also in astrobiology to avoid microbial contamination and to simulate space conditions.

Until the 1980s, *Geobacillus stearothermophilus* was regarded as the only known obligate thermophile belonging to bacilli (Zarilla and Perry 1987). Several studies have been carried out on the thermal resistance of *G. stearothermophilus*, allowing to consider it as an industrial thermal sterilization indicator (Donk 1920) and model for spores' resistance studies. Due to their high resistance to temperature, the spores of *G. stearothermophilus* and *Bacillus subtilis* were indicated as biological indicators to confirm the inactivation of pathogens in medical wastes (Slavic *et al.* 1998).

However, sterilization treatments and approaches are often compromised by the innate resistance of spores to high temperatures. Spores from *G. stearothermophilus* were demonstrated able to survive incinerator environment (Wood *et al.* 2008), whereas those from *B. sporothermodurans* were resistant to pasteurization process (Scheldeman *et al.* 2006), and therefore these findings pose a great concern in ensuring the biosecurity of products. Although several studies were carried out to determine the resistance in fluid or wet-heat treatments (Spotts Whitney *et al.* 2003; Montville *et al.* 2005), few data have been reported until now in the dry-heat resistance of spores (Wood *et al.* 2010).

Shallow hydrothermal vent areas off the Eolian Islands (Italy) provide easily accessible sampling locations to study microorganisms inhabiting extreme marine ecosystems for the purpose of gaining new insights into microbial diversity and to isolate novel thermophiles (Maugeri *et al.* 2002; Gugliandolo *et al.* 2012; Spanò *et al.* 2013) *Geobacillus vulcani* DSM 13174 (Caccamo *et al.* 2000) and *Bacillus licheniformis* T14 (Spanò *et al.* 2013) were isolated from two submarine hydrothermal vents (Eolian Islands, Italy) and they were reported to possess interesting physiological characteristics and biotechnological applications. However, the resistance of spores from these two strains to high temperatures was never investigated until now.

In this study the thermal resistance of spores from two thermophilic marine strains, *Geobacillus vulcani* DSM 13174 and *Bacillus licheniformis* T14, evaluated in wet- and dry-heat conditions, was compared to two close phylogenetic relatives (*G. stearothermophilus* DSM 22^T and *B. licheniformis* DSM 13^T), and the biodosimetry and space microbiology model strain *B. subtilis* 168.

2. Material and Methods

2.1. Bacterial strains. *Bacillus licheniformis* T14 was isolated from a thermal fluid sample collected at 8-m depth by SCUBA divers from Bottaro vent (Lat. 38° 38'31"N-Long. 15°06'597"E), located off the eastern coast of Panarea Island (Italy) (Spanò *et al.* 2013) At the sampling site of the thermal fluid, the temperature was 50°C, the pH was 5.42 and the conductivity was 42.90 mS cm^{-1}. *Geobacillus vulcani* was isolated from sediment

Strain	Reference
B. licheniformis DSM^T 13	Skerman *et al.* 1980
B. licheniformis T14	Spanò *et al.* 2013
B. subtilis 168	Nicholson *et al.* 2000
Geobacillus stearothermophilus DSM 22^T	Donk 1920
Geobacillus vulcani DSM 13174	Caccamo *et al.* 2000

TABLE 1. *Bacillus* and *Geobacillus* strains used in the study

of a shallow hydrothermal vent at Vulcano Island (Italy), where the registered temperature was 49°C. *B. licheniformis* DSM 13^T, the closest phylogenetic relative strain to *B. licheniformis* T14, *B. subtilis* 168, and *G. stearothermophilus* DSM 22^T were used in this study for comparison. The strains used in this study are listed in 1.

2.2. Spore production. To produce spores the strains were plated onto Schaeffer agarized medium (containing 0.1% KCl, 0.012% $MgCl_2$, 0.5 mM_2, 0.01 mM $MnCl_2$, 0.001 mM $FeSO_4$, and 8 g/l nutrient broth) and modified by adding NaCl (1%). The plates were incubated at each strain optimal growth temperature: *B. licheniformis* DSM 13^T (optimal T=30°C), *B. subtilis* 168 (optimal T=37°C), *B. licheniformis* T14 (optimal T=50°C), *G. vulcani* DSM 13174 and *G. stearothermophilus* DSM 22 (Donk 1920)(optimal T=60°C). To verify the presence of spores, each culture was observed under phase contrast microscopy (1000×).

The morphology and distribution of spores were determined using scanning electron microscopy (SEM) (Zeiss, Sigma). Before SEM observations, spores from each strain suspension were posed onto aluminum stubs and dried at room temperature for 24h and then coated with a homogeneous layer (18 ± 0.2 nm) of Au–Pd alloy, using a coating device (MED 020, Ba Tec AG, Tucson, AZ, USA).

2.3. Spore thermal resistance assays. Wet-heat resistance. The level of spore resistance to wet-heat was evaluated as described by Moeller et al. (2012). Briefly, the suspensions of 10^7 spores/ml were heated at 95°C for 10, 20, 40 and 60 min.

Dry-heat resistance. The resistance of spores to dry-heat was determined using the method described by Moeller et al. (2007). Briefly, air-dried spore monolayers (10^6 spores/ml) immobilized on 7-mm-diameter steel discs were exposed at 120°C for 10, 30, 60 and 90 min.

Spore survival was determined by plating serial dilutions, prepared in sterile distilled water, on solid Tryptone Soy Agar (TSA) medium. After overnight incubation, at the optimal temperature of growth for each strain, colonies were counted and expressed as Colony Forming Units (CFUs). The surviving fraction was determined from the quotient N/N_0, with N being the number of CFUs of the treated sample and N_0 the CFUs of the non-treated controls. By plotting the logarithm of N/N_0 as a function of each treatment, survival curves were obtained, and LD_{90} values (*i.e.*, times reducing spore survival to 10% of the initial spore culture) were calculated for each strain. Inactivation kinetics of the spores were determined in response to the respective treatment and the best-fit curves were used to calculate LD_{90} values for statistical comparison. Each experiment was repeated at least three times and results were expressed as averages ± standard deviations.

3. Results

Spore preparations were essentially free of vegetative cells and consist of >99% phase-bright spores by phase-contrast microscopy. Morphology and distribution of spores were also observed by SEM (Figure 1), depicting spores from *B. licheniformis* T14, *G. vulcani* DSM 13174 and*G. stearothermophilus* DSM 22^T immersed into an extraneous layer connecting the spores.

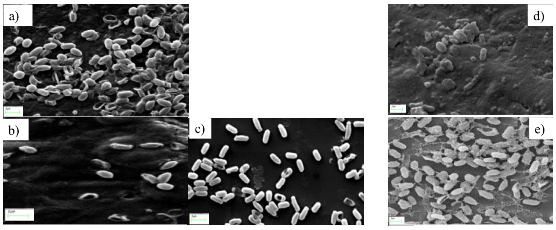

FIGURE 1. Scanning electron-micrographs (bar 2μm) of spores from a) *B. licheniformis* T14 and d) *G. vulcani* DSM 13174 in comparison with those from c), *B. subtilis* 168, e) *G. stearothermophilus* DSM 22^T and b) *B. licheniformis* DSM 13^T.

The spore resistance to wet-heat treatment are showed in Figure 2. Spores of strain *G. vulcani* were reduced of only 1 log in 30 min of treatment and they were more resistant (LD_{90}=34.8 min) than those of *B. licheniformis*T14 (LD_{90}=31.2). The spores from *B. licheniformis* T14 were more resistant (P<0.01) than those of *B. licheniformis* DSM 13^T and of *B. subtilis* 168.

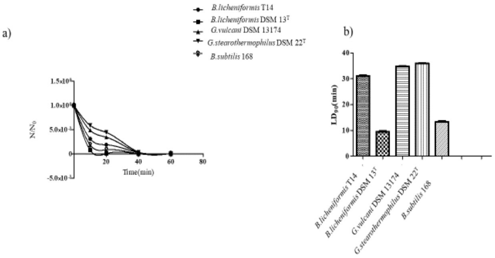

FIGURE 2. Resistance to wet-heat (95°C) of spores from *B.licheniformis* T14, *B. licheniformis* DSM 13^T *G. vulcani* DSM 13174 , *G. stearothermophilus* DSM 22^T and *B. subtilis* 168. a) The surviving curves of spores from each strain, and b) Bar graphs displaying the LD_{90} values (*i.e.*, times reducing spore survival to 10% of the initial spore culture) of the different tested strains.

Differently to the other strains, the mesophilic *B. licheniformis* DSM 13^T lost 1 log of vital spores in 10 min (Figure 3a). Spores from *G. vulcani* DSM 13174 showed similar LD_{90} level (16.8 min) than the *G. stearothermophilus* DSM 22^T (18.5min) ($P>0.05$) after exposition to dry heat (Figure 3 b). The spores of *B. licheniformis* T14 (11.75 min) were more resistant ($P<0.05$) than those of its closest strain *B. licheniformis* DSM 13^T (8.6 min).

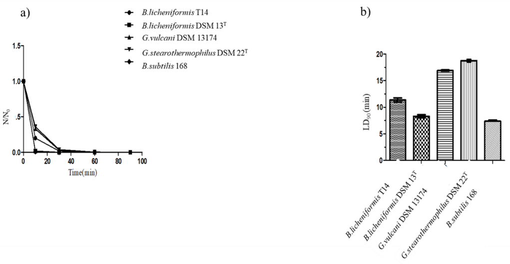

FIGURE 3. a) Resistance to dry-heat (130°C) of spores from *B. licheniformis* T14, *B. licheniformis* DSM 13^T, *G. vulcani* DSM 13174, *G. stearothermophilus* DSM 22^T and *B. subtilis* 168. a) Surviving curves of spores from each strain, and b) Bar graphs displaying the LD_{90} values (*i.e.*, the applied treatment time leading to a spore survival of 10%) of the different tested strains.

4. Discussion

The heat resistance is considered the hallmark property of bacterial spores, which represents the main issue for microbial contamination in different human activities and in planetary protection field. In the aerospace industry, microorganisms could have uniquely disastrous effects, since spacecraft could contaminate other cosmic bodies with terrestrial microbes if the space-bound vehicles are not sufficiently decontaminated before launch (Carlson *et al.* 2018)

In this study, the spore resistance of two thermophilic marine strains, *Geobacillus vulcani* DSM 13174 and *Bacillus licheniformis* T14, isolated from two Eolian shallow hydrothermal vents (SHV), to wet- and dry- heat was compared to their close phylogenetic relatives (*G. stearothermophilus* DSM 22^T and *B. licheniformis* DSM 13^T), and also to the biodosimetry and space microbiology model strain *B. subtilis 168*. The thermal treatments here used to investigate the dry- and wet-heat spore resistance were previously used in the decontamination processes in space missions, to avoid the forward contamination of celestial bodies with terrestrial organisms, which represents one of the main issues of astrobiology (Crawford 2005; Nicholson *et al.* 2005). Previous studies reported that spores of thermophiles invariably were more resistant to heat than those of mesophiles (Nicholson *et al.* 2000; Zammuto *et al.* 2018). Spores from *G. vulcani* and *G. stearothermophilus* DSM 22^T were more resistant to both heat stresses than those from *Bacillus licheniformis* T14, confirming that members belonging to *Geobacillus* genus are more resistant to high temperature than *Bacillus*. Nevertheless, spores of *B. atropheus* ATCC 9372, indicated as the official bioindicator in dry-heat sterilization processes (Unit State Phamacopoeia, 2011), were reported to possess similar dry thermal resistance of *G. stearothermophilus* (Wood *et al.* 2010), used to validate steam sterilization procedures. Spores from *B. licheniformis* T14 were more heat resistant than those from its closest phylogenetically related strain *B. licheniformis* DSM 13^T, suggesting that the genetic make-up at species level is not sufficient to determine the degree of resistance. The adaptation to high temperatures of spore's structures, such as the lipid composition of membranes and the high stability of proteins and DNA, could allow thermophiles to resist to extreme environmental conditions registered at SHV. The mechanisms involved in the wet-heat resistance were previously reported to prevent the denaturation processes of spore protein (Setlow 2014). Among them, the high concentration of mineral ions in the spore core was associated with the greater wet-heat resistance of the spores (Melly *et al.* 2002; Setlow 2014). Spores of *G. vulcani* and *B. licheniformis* T14 were more resistant to heat stresses than the biodosimetry strain and space microbiology model organism *B. subtilis*168, which may reflect their own adaptation to the severe environmental conditions. As recently reported by Zammuto *et al.* (2018), the marine strain *B. horneckiae* SBP3, isolated from a shallow hydrothermal vent off Panarea Island (Eolian Islands, Italy), showed higher resistance to dry-heat in comparison with *B. subtilis* 168, suggesting that Eolian SHV represent a rich source of bacteria more thermal resistant than their terrestrial counterparts. Differently from wet-heat, the dry-heat stress could kill the spores through oxidation processes (Russell 2001) and DNA disruption (Setlow 2006). Spores from thermophilic strains able to resist to dry heat were also resistant to desiccation (Nicholson *et al.* 2000; Mastascusa *et al.* 2014; Di Donato *et al.* 2018). As reported for *B. subtilis* 168 spores, the mechanisms involved in dry heat and desiccation stress conditions are related to the α/β type small acid soluble proteins (SASP) (Setlow 2006). Several thermophilic and thermotolerant bacilli strains isolated from Eolian shallow vents were able to produce exopolymers, such as exopolysaccharides and poly-γ- glutamic acids, with physico-chemical and biological properties that could be involved in the heat resistance (Gugliandolo *et al.* 2012; Spanò *et al.* 2016; Caccamo *et al.* 2018).. The exopolysaccharides could play a key role in the water retention, maintaining hydrated microenvironment (Nwodo *et al.* 2012), and therefore against the damages related to desiccation and dry-heat. As showed in Figure 1, the external matrix to the spores of *G. vulcani* DSM 13174 and *B. licheniformis* T14, could represent a barrier against the dehydration of spores and the damage induced by heat stresses. This work encourages future researches especially on the mechanisms involved in the spore resistance to extreme conditions, which are almost unknown for spores of environmental strains isolated from shallow hydrothermal vents. Due to their thermal resistance, *B. licheniformis* T14 and *G. vulcani* DSM 13174 may have a novel use as bacterial model organisms for further investigation into the spore responses to environment stressors, also simulating space conditions.

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