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SHORT COMMUNICATION

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Culturability and survival of an extreme thermophile isolated from deep-sea hydrothermal vents

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Abstract The culturability of a strictly anaerobic, extremely thermophilic archaeon, *Thermococcus peptonophilus* (optimal growth temperature: 85° C), was studied during survival stages at various temperatures (98, 85, 70, and 4° C). Total cell number (determined by DAPI staining), active cells (rhodamine-stained cells), and culturable cells (using most-probable-number) were counted over time. The number of culturable cells decreased under each condition tested. The total number of cells significantly decreased only at temperatures close to the maximum for growth (98° C); at this temperature, the cells spontaneously lysed. Our results suggested that survival at 4° C in oxygenated waters might be a mechanism for the dispersion of extreme thermophiles in the ocean. In addition, we proved the existence of *T. peptonophilus* cells in several physiological states: culturable cells, active non-culturable cells, inactive non-culturable cells, and dead cells. Cell death was caused by cellular lysis.

Key words *Thermococcus peptonophilus* · Deep-sea · Hydrothermal vents · Survival · Extreme thermophiles · Culturability · Active cells · Mortality · Dead cells

Introduction

Survival strategies aimed to resist adverse environmental conditions have been found in every microorganism tested (Roszak and Colwell 1987a). Since microorganisms live in changing environments, they adapt in order to survive under adverse circumstances. Several strategies have been observed. Spore formation by microorganisms is one example of a clearly differentiated behavior for surviving extreme conditions. Non-spore-forming microorganisms are usually able to enter dormant (non-culturable) physiological states, allowing them to resist long terms under adverse conditions (Roszak and Colwell 1987a). In nature, the largest proportion of cells are in a dormant state (Roszak and Colwell 1987a) waiting for better conditions to come; thus, most of the cells present in natural environments cannot be cultured by common culturing techniques. In general, less than 0.1% of these microorganisms in natural samples can be cultured (Hobbie et al. 1977; Roszak and Colwell 1987a).

It has not yet been reported whether extreme thermophilic archaea exist in different physiological states of survival, such as those found for bacteria (Roszak and Colwell 1987a and b). The objective of this study was to characterize the cells of an extremely thermophilic archaeon, *Thermococcus peptonophilus*, in various physiological states of survival.

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Materials and methods

Strain and growth conditions

Thermococcus peptonophilus, strain OG1 (JCM 9653) (González et al. 1995), was used in this study and grown as described by González et al. (1995). Duplicate experiments were carried out at 98, 85, 70, and 4° C. Experiments were started at the beginning of the stationary phase of growth, once a plateau of cell numbers was observed in the growth curve. *T. peptonophilus* was grown at the selected temperature, except for the experiments performed at 4° C, where the cells were grown at 85° C. The effect of oxygen on cells incubated at 4° C was also tested; these cultures were grown at 85° C, cooled down on ice, and aerated for 10 min with air pumped through a 0.2-µm-pore-diameter filter.

Cell counting and staining

Samples for total counts were immediately fixed with formalin (1% final concentration) and kept on ice. Cells were stained with

4′, 6′-diamidino-2-phenylindole (DAPI; 1 μg/ml final concentration) for 10 min, and collected on a 0.2-μm-pore-diameter black polycarbonate filter.

Rhodamine staining is dependent on its active incorporation by the cells (Yu and McFeters 1994); thus, rhodamine-stained cells can be considered as those showing a detectable active metabolism. The technique proposed by Yu and McFeters (1994) was adapted for use with *Thermococcus peptonophilus*. An EDTA solution [50 μl; 3% (w/v) NaCl, 5 mM EDTA, and 50 mM Tris-HCl (pH 8.0)] was added to 0.5 ml of sample and mixed. After a 5-min incubation at room temperature, rhodamine 123 solution (5 μg/ml final concentration) was added, and the sample was incubated at 85° C for 2 h, and then cooled down in ice-water. Processed sample (100 μl) was added to a tube containing DAPI solution [10 μl DAPI (0.1 mg/ml) and 1 ml rhodamine 123 solution (5 μg/ml)], and the sample was mixed and incubated for 5 min on ice. The stained sample was filtered to collect the cells on a 0.2-μm black polycarbonate filter, and the filter was rinsed once with 1 ml of rhodamine 123 solution (5 μg/ml). The cells were observed with a Nikon Optiphot microscope equipped with the standard UV and blue filter sets.

Culturable cell number was obtained by the most probable number (MPN) method (Koch 1994). Dilutions (10-, 100-, and 1,000-fold) with three tubes per dilution were used. The medium described by González et al. (1995) was used, and incubations were carried out at 85° C. Dilutions were made in anoxic saline solution containing NaCl, resazurin, and Na_2S at the same concentrations as employed in the culture medium (González et al. 1995).

Several physiological states of survival were differentiated. The fraction of cells in each physiological state were estimated as summarized in Table 1.

Results and discussion

One of the responses of *T. peptonophilus* to unfavorable conditions was a reduction of the number of culturable cells, but not necessarily a reduction of the total cell number. This led to a progressive formation of nonculturable cells during growth. Among these nonculturable cells, some were metabolically active, whereas others were inactive (Table 1). At 85, 70, and 4° C, an increase in the fraction of active, non-culturable cells was observed together with a decrease in the fraction of culturable cells (Fig. 1). Throughout our experiments, the fraction of inactive, non-culturable cells represented only a small portion of the total cell number present at these temperatures (Fig. 1); the total cell number did not decrease under these conditions (Fig. 1).

At 98° C, in addition to a progressive decrease in the fraction of culturable cells, we observed a decrease in the total cell number. Thus, the number of dead cells (Table 1) increased during the experiments (Fig. 1). These dead cells or "ghosts" could be directly visualized as lysed cells in these samples. To our knowledge, this is the first study showing several physiological states of a strictly anaerobic, extreme thermophilic archaeon (Fig. 1).

The concept of bacterial mortality has attracted the attention of microbiologists for a long time (Roszak and Colwell 1987a). Similarly, this concept should be extended to include Archaea. The classic concept of bacterial mortality as stated by Postgate (1977), i.e., a loss in the ability to show growth on a suitable culture medium, is no longer valid for Bacteria and Archaea. Archaea (this study), like Bacteria (Roszak and Colwell 1987b), are able to progressively enter non-culturable survival states; these non-culturable states can be detected as being either metabolically active or inactive (no detectable activity) states. As a consequence, the current concept of dead bacteria (Servais et al. 1985; Roszak and Colwell 1987b; González et al. 1992) can be expanded by considering a dead prokaryote (either Bacteria or Archaea) to be one that has lost its morphological integrity. Thus, dead prokaryotes will only be detected by decreases of total cell counts over time.

The temperature of 4° C, under either anoxic or oxygenated conditions, appeared to be an adequate temperature for short-term storage of *T. peptonophilus* cells, as reported for other extreme thermophiles (Huber et al. 1990). Our experiments revealed that *T. peptonophilus* can survive at 4° C, maintaining a high proportion of active cells and an important fraction of culturable cells (Fig. 1). In addition, it appears that the presence of oxygen does not have a significant effect on the proportion of survivors of *T. peptonophilus* at 4° C (Fig. 1). Since the temperature of the deep-sea is about 2–4° C, *T. peptonophilus* may survive for relatively long periods in deep-sea waters. We suggest that this finding represents a possible mechanism for the dispersion of extreme thermophiles in marine waters.

Table 1 Physiological states of survival identified by using several counting procedures (*DAPI* 4′, 6′-diamidino-2-phenylindole, *MPN* most probable number)

Counting procedures[a]	Physiological state
MPN	Culturable cells
Rhodamine staining – MPN	Active, nonculturable cells
Rhodamine staining	Active cells
DAPI – rhodamine staining	Inactive, nonculturable cells
DAPI staining	Total cells (viviform population)
DAPI staining – MPN	Nonculturable cells
DAPI staining, decrease over time[b]	Dead cells

[a] Minus sign indicates that the latter count was subtractedfrom the former. The first procedure listed generally gave higher counts
[b] Maximum total cell number minus total cell number at a determined time

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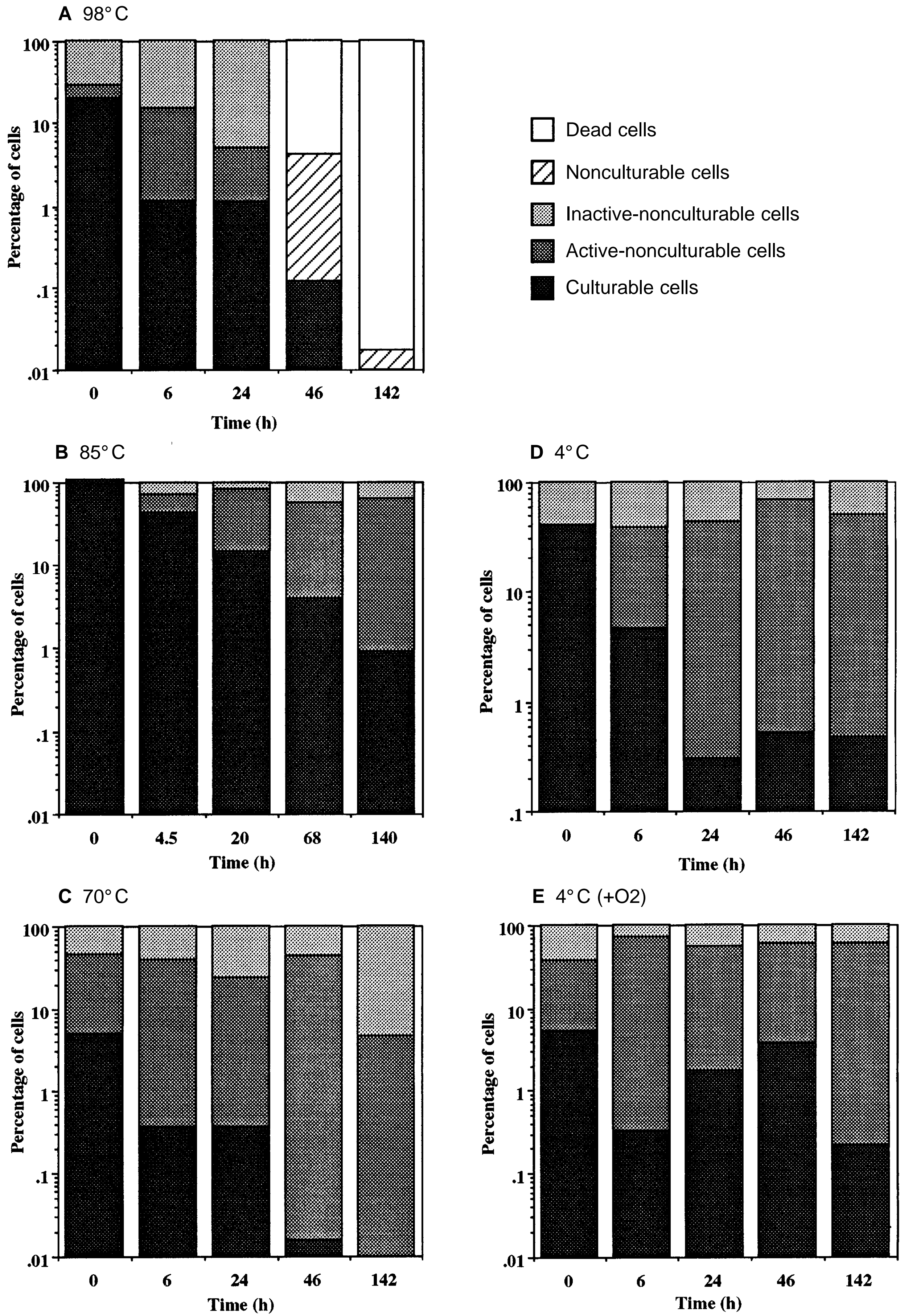
Fig. 1A–E Characterization of *Thermococcus. peptonophilus* cells ▶ in several physiological states of survival during our experiments. Culturable, active nonculturable, inactive nonculturable, and dead cells are shown as percentages of the initial total cell number. Experiments performed at **A** 98° C, **B** 85° C, **C** 70° C, **D** 4° C, and **E** 4° C (oxygenated sample) are shown. At 98° C, no differentiation of non-culturable cells into active and inactive cells was made after 24-h incubation


A 98° C
Percentage of cells
100
10
1
.1
.01
0
6
24
46
142
Time (h)
Dead cells
Nonculturable cells
Inactive-nonculturable cells
Active-nonculturable cells
Culturable cells
B 85° C
0
4.5
20
68
140
D 4° C
C 70° C
E 4° C (+O2)

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