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ORIGINAL PAPER

Proliferation of the hyperthermophilic archaeon *Pyrobaculum islandicum* by cell fission

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Abstract *Pyrobaculum islandicum* is a hyperthermophilic archaeon. *P. islandicum* cells have been suggested to multiply by constriction, budding and branching, as no septa were observed in cells by phase-contrast light microscopy. In this study, we observed the cells using transmission electron microscopy, scanning electron microscopy, and light microscopy with dark-field image analyses, and we report binary fission via septum formation to be the main mode of *P. islandicum*'s proliferation. "Long cells" reported previously were found to comprise several cylindrical cells that align in tandem.

Keywords Archaeon · Hyperthermophile · Proliferation · Cell fission · Septum

Abbreviations

TEM	Transmission electron microscopy
SEM	Scanning electron microscopy
LMD	Light microscopy with dark-field image analyses

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Introduction

Pyrobaculum islandicum is an anaerobic crenarchaeon belonging to the order *Thermoproteales*, and it grows optimally at 100°C (Huber et al. 1987). Its entire genome has been reported recently (NC_008701, CP000504). Cell division in the Bacteria and the Euryarchaeota is mediated by tubulin-like FtsZ protein, which forms a contractile Z ring (Vaughan et al. 2004; Beuria et al. 2009), and all crenarchaeal orders except for the *Thermoproteales* possess a *cdv* operon that produces Cdv proteins during constriction (Lindås et al. 2008). However, a cell-division-related protein or gene is yet to be identified in *Thermoproteales*. *P. islandicum* cells have been suggested to multiply by constriction, budding, and branching (Huber et al. 1987), as no septa have been observed in the cells using phase-contrast light microscopy. Likewise, septum formation has not been reported in other genera of this order, including *Thermocladium* (Itoh et al. 1998), *Caldivirga* (Itoh et al. 1999), and *Thermofilum* (Stetter and Zillig 1985), while cell division by septum formation has been documented in *Thermoproteus tenax* (Wildhaber and Baumeister 1987), and no constriction was observed in *P. aerophilum* (Lundgren et al. 2008). Although we observed bud- or branch-shaped *P. islandicum* cells, the fraction of these cells was less than 1% under normal culture conditions, and cells in the process of division were rarely observed. In order to clarify the mode of *P. islandicum* cell proliferation, we examined cell division using transmission electron microscopy (TEM), scanning electron microscopy (SEM), and light microscopy with dark-field image analysis (LMD). We also report that the "long cell" of *P. islandicum* described in previous studies is composed of several cells aligned in tandem.

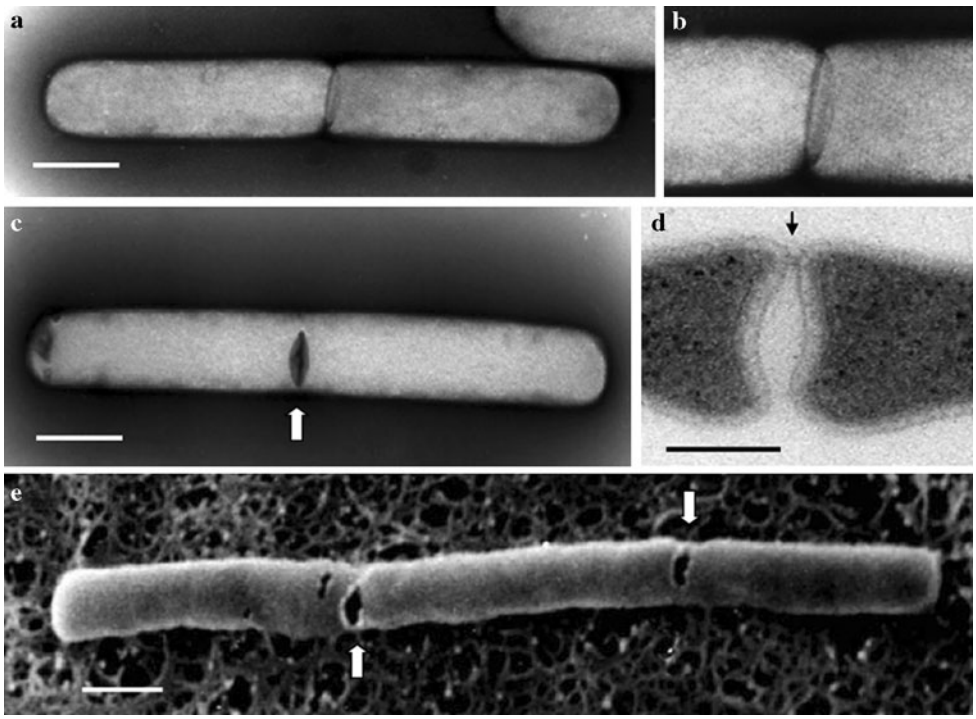

Fig. 1 **a**, **b** A *Pyrobaculum islandicum* filament with mature septum. The outermost S-layers at the septum of a negatively stained filament are shown. The same filament was observed with different magnifications: **a** ×12,000, **b** ×30,000. *Scale bar* 0.5 μm. **c–e** Fission of *P. islandicum* filaments. **c** Negatively stained filament. *Scale bar* 0.5 μm. **d** TEM micrograph of an ultrathin grazing section of a negatively stained filament that has almost finished fission. An intervening structure is visible between the cells (*arrow*). *Scale bar* 0.25 μm. **e** SEM micrograph. *Scale bar* 0.5 μm. *Thick white arrows* (**c**, **e**) indicate the fission of mature septa

Materials and methods

P. islandicum DSM 4184 culture was grown anaerobically in a 20-ml screw-capped tube at 95°C in the medium described by Huber et al. (1987), modified slightly to reduce black FeS sediments. In the modified method, 20.0 g/l sulfur and 0.5 g/l $Na_2S{\cdot}9H_2O$ were replaced by 0.5 g/l L-cystine and 2.0 g/l $Na_2S_2O_3{\cdot}5H_2O$, respectively, and $FeCl_3{\cdot}6H_2O$ was not included. We used L-cystine because it costs only one-fourth that of L-cysteine. The culture was transferred every 24 h with 2 ml of the culture being seeded into 18 ml of the fresh culture medium pre-incubated overnight at 95°C.

Whole cells were stained with 2% (v/v) uranyl acetate. For ultrathin section, cells were fixed with 2.5% (v/v) glutaraldehyde and postfixed with 2% (v/v) osmium tetraoxide. The cells were embedded in epoxy resin after dehydration with ethanol dilution series. Ultrathin sections thus prepared were stained with 2% (v/v) uranyl acetate and lead citrate. The negative-stained whole cells and the ultrathin sections were examined by TEM using an electron microscope (1200 EX II; JEOL, Tokyo). For SEM, cells were transferred onto a small agar block and fixed with 1% (v/v) glutaraldehyde. The fixed cells were dehydrated with acetone dilution series and finally added with isoamyl acetate. The samples were dried in a HCP-1 critical point dryer (Hitachi Koki, Tokyo) and coated with gold–palladium using a cool sputter coater (E-5150, Polaron, Hertfordshire, UK). Further, the cells were examined using a light microscope (Optiphot; Nikon, Tokyo) equipped with a dark-field condenser/1.43–1.20 oil (Nikon), and objective lenses, 40×/0.65 oil (Nikon), UVFL 40× PL/1.30 oil (Olympus, Tokyo), and DApo 100×/1.30 oil (Olympus). Optical images from the light microscope were obtained using a C2847 Super eye video camera (Hamamatsu Photonics, Hamamatsu, Japan), and they were further enhanced using a real-time image processor (Image Σ-II; Japan Abionics, Yokohama, Japan) (Kamitsubo and Kikuyama 1992). A U-matic videocassette recorder (Sony, Tokyo) was used for recording and data analysis. Of the real-time images, several scenes are presented in this report.

Results

Binary fissions of *P. islandicum* cells are indicated in Fig. 1. As septum formation proceeded, the septa became clearly visible by negative staining (Fig. 1a, b). The outer S-layers of the cells were mutually separated at the septa. In Fig. 1c and d, the filaments seem to have initiated rupture at one side of the S-layer (arrows). A septum such as that shown in Fig. 1e was tentatively termed "mature septum", and the term "immature septum" was given to a thin septum (Fig. 2b), which appears to comprise 2 tightly attached S-layers with thin spacers (Baumeister and Lembcke 1992). The septum thickness might have increased to a comparable thickness with other parts of the cell envelope in the mature septum. Figure 2a presents the "cells" longer than the ellipsoidal single and short cells (approximately 0.5 μm wide and 0.6–1.5 μm long) shown

in Fig. 2c. The 2 "long cells" in Fig. 2d and e, obtained using differently focused objectives, each multiplied from a single cell thereby producing a longer "cell" than before. In Fig. 2b, the immature septum is shown. Such septa may also be found in cells as shown in Fig. 2c (arrows). Immature septa observed by LMD are outlined in Figs. 2d, e, and 3a (arrows); they were not visible by SEM (Fig. 2a). The cells in Fig. 2c–e seem differently shaped than the fixed cells observed by SEM or TEM. Figure 3 shows incomplete septa that appear to be in the process of formation.

The short cells were usually observed in a normal culture transferred consistently every 24 h. In cultures left for a few days without transfer, various lengths of elongated cells containing no septa appeared under LMD observations (not shown). The longest cell was 10 μm. In this report, a "long cell" composed of more than 2 cells was referred to as a filament. The relation between the formation of filaments (cells) longer than 10 μm and culture temperature was examined using phase-contrast light microscopy (Fig. 4). At 95°C, the fraction of long filaments was low, at approximately 1% of the culture despite the large number of filaments. The growth rate was reduced by lowering the temperature; at 85°C, the culture reached a stationary phase 48 h after transfer, when many longer filaments appeared. The fraction of longer filaments was highest in the 85°C culture at the 48th and 72nd hours among the 4 different growth temperatures tested. At temperatures below 75°C, proliferation or cell fission of filaments was inhibited, as indicated by the small cell or filament numbers in Fig. 4.

Synchronized septum formation is shown in Fig. 5a. The cells on both sides of the new septa (arrows) appear to be tightly connected. After cell fission, a few percent of short cells or filaments remain loosely connected, making a V-form (Fig. 5b, c). The two filaments in Fig. 5b were not separated by shaking, suggesting the presence of a cementing substance or structure between the 2 cells.

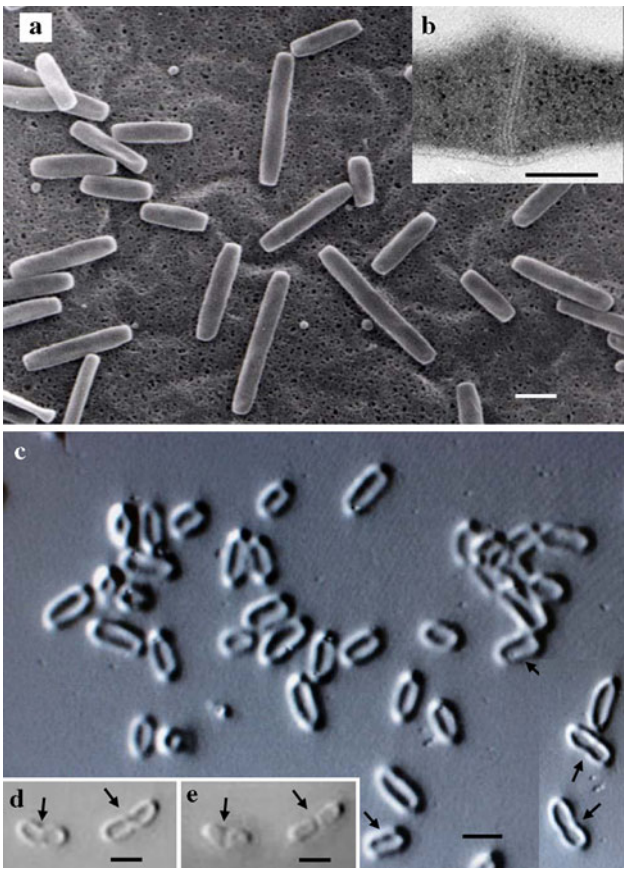

Fig. 2 **a** Short filaments of *P. islandicum* seen in the SEM micrograph. *Scale bar* 1.0 μm. **b** TEM micrograph of a grazing section of an immature septum that was negatively stained. *Scale bar* 0.25 μm. **c** LMD micrograph of single cells and short filaments with immature septa of *P. islandicum*. Immature septa are indicated with *arrows*. *Scale bar* 1.0 μm. **d**, **e** The same filaments observed with different focuses. *Scale bars* 1.0 μm

Discussion

The domain Bacteria and the phylum Euryarchaeota possess FtsZ-related cell-division machineries (Vaughan et al. 2004; Beuria et al. 2009). A *cdv*-gene-encoded division machinery was recently identified (Lindås et al. 2008; Ettema and Bernander 2009) in all known Crenarchaeota other than *Thermoproteales*. The Cdv proteins exhibit a distribution complementary to FtsZ-based archaeal division systems. However, no such gene or protein related to cell division has been found in *Thermoproteales*. In the present study, we observed binary fission by septum formation in *P. islandicum* cells. The septa might be formed by centripetal growth of the membrane and S-layer, as incomplete septa perpendicularly attached to the cell envelopes were observed (Fig. 3). The septa may initially exist as a ring attached to the inside of the envelopes. Centripetal septum formation has been reported in several filamentous organisms over different phylogenic groups: the *Thermoproteales*, *T. tenax* (Wildhaber and Baumeister 1987); the

Fig. 3 LMD micrographs of *P. islandicum* filaments with septa in the process of formation. *Scale bars* 0.5 μm

euryarchaeon, *Methanothermobacter soehngenii* (Zehnder et al. 1980); a coryneform bacterium, *Arthrobacter crystallopoietes* (Krulwich and Pate 1971); and a filamentous bacterium that attaches to the small intestine of mice (Ferguson and Birch-Andersen 1979). These organisms may have FtsZ or Cdv, and it seems that septum formation in *P. islandicum* may proceed with division machinery other than that involving FtsZ or Cdv proteins.

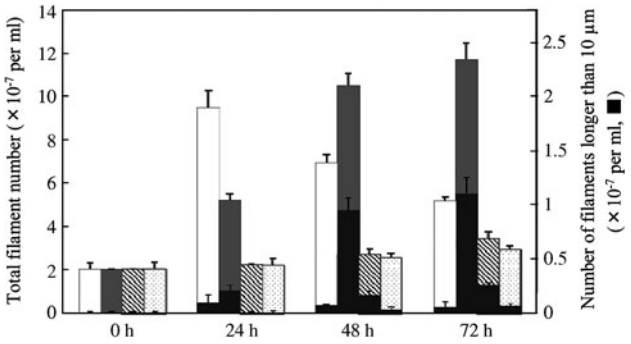

Fig. 4 Temperature dependency of *P. islandicum* growth and proportion of long filaments. The culture was carried out at 95°C (*open bars*), 85°C (*grey bars*), 75°C (*hatched bars*), and 60°C (*dotted bars*), and the cell number was counted every 24 h using a Thoma counting chamber. Filament numbers are reported as mean ± SD for 5 counts in different regions of the Thoma counting chamber. The number of filaments longer than 10 µm is shown by *solid bars*

Previously described "long cells" were found to comprise more than 2 cells. At the lower temperature of 85°C, the cells were able to divide, but unable to separate from each other (Fig. 4). Maturation of the septa seems to be a prerequisite for fission completion: septation between the 2 cells was accomplished by complete separation of the S-layer. Archaeal cells are enveloped by S-layers containing a spacer, an interspace between the plasma membrane and porous outer canopy of the S-layer (Wildhaber and Baumeister 1987; Phipps et al. 1990; Baumeister and Lembcke 1992; Völkl et al. 1993). Therefore, maturation of the septa may imply an increase in the spacer thickness. Taken together, we present a hypothetical scheme (Fig. 6) for the mode of *P. islandicum* cell fission.

After the completion of septum formation and the binary fission, some fractions of the cells were still connected forming a V-shape by the structure outlined in Fig. 1e (arrow) or by the unknown substance shown in Fig. 5c (arrow). The connective structures or substances between the 2 cells have been suggested to be thin fibrils (Phipps et al. 1991), cytoplasmic strings (Zillig et al. 1990; Rieger et al. 1997), or some other materials in the compartment

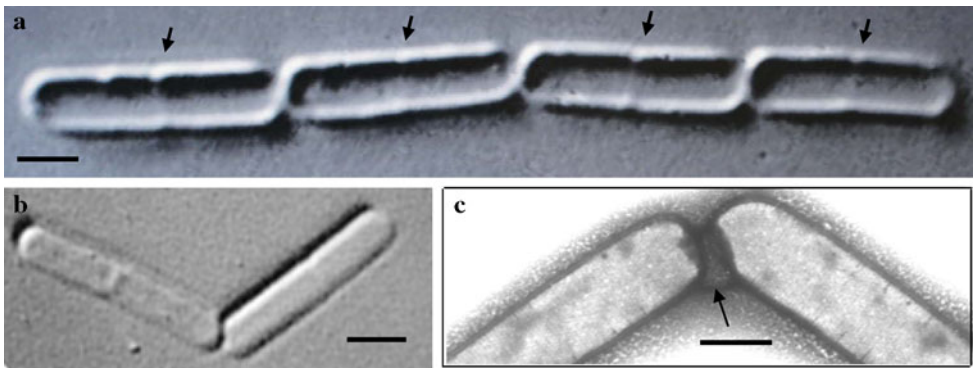

Fig. 5 *P. islandicum* filaments with loosely connected cells. **a** An LMD micrograph of a filament comprising 8 cells with possibly synchronously formed septa. Immature septa are indicated by *arrows*. **b** Two filaments arranged in a V-shape shown by LMD micrograph. **c** A negatively stained TEM micrograph of material intervening the 2 separated cells. *Scale bars* 0.5 µm

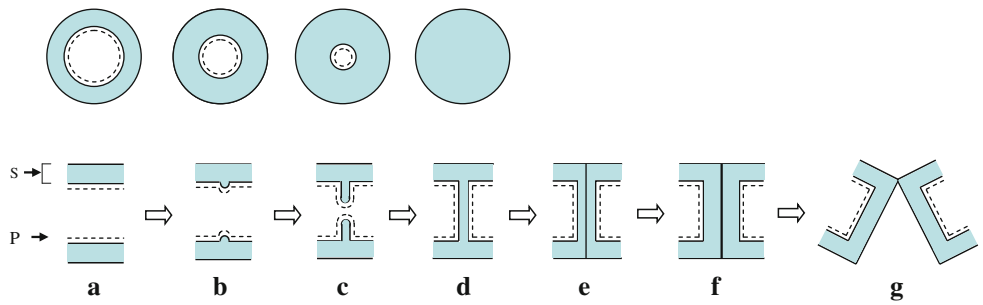

Fig. 6 A schematic diagram for the mode of binary fission of *P. islandicum* cells. *Upper layer* Cross-sectional images at the septum; *lower layer* longitudinal images near the septum. *S* S-layer, *P* (*dotted line*) plasma membrane. **a** A cell before septum formation. **b** A ring of S-layer is formed attached to the inside of the cell, enveloped by S-layer. **c** The ring-shaped S-layer grows inward. **d** The ring-shaped S-layer combines at the center of the cell and forms a septum. **e** The septum divides into 2 S-layers. **f** Each of the septum S-layers thickens. **g** The 2 cells rupture and separate from each other

space of *T. tenax* filaments (Wildhaber and Baumeister 1987) were suggested.

P. islandicum resembles *T. tenax* in that they are segmented filaments. However, the *P. islandicum* filaments possess no compartment space between the 2 cells as seen in the *T. tenax* filament (Wildhaber and Baumeister 1987). Cells arranged in a V-shape have been observed with filamentous archaea, *P. islandicum* (Huber et al. 1987), *P. aerophilum* (Völkl et al. 1993; Lundgren et al. 2008), *T. tenax* (Horn et al. 1999), and *M. thermautotrophicus* (Majerník et al. 2005). In cultures of coryneform bacteria such as *A. crystallopoietes*, V-forms of cells were also commonly observed (Krulwich and Pate 1971).

In this study, *P. islandicum* was found to proliferate by binary fission via centripetal septum formation, and the involvement of a new division machinery other than the one involving FtsZ or Cdv was suggested. The "long cell", a filament in this report, was found to comprise more than 2 connected cells whose septa had not grown completely.

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