Full Paper

A life cycle of branched aerial mycelium- and multiple budding spore-forming bacterium *Thermosporothrix hazakensis* belonging to the phylum *Chloroflexi*

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As far as known, sporulation modes in prokaryotes include formation of endospores exemplified by *Firmicutes* bacteria, myxospores by myxobacteria and arthrospores by actinomycetes. Here we describe *Thermosporothrix hazakensis* strain SK20-1^T belonging to the phylum *Chloroflexi* with a life cycle including a novel prokaryotic sporulation mode. Microscopic observations showed that strain SK20-1^T formed multiple exospores per mother cell by budding in branched aerial mycelia. Although branched aerial mycelia are characteristic of actinomycetes, multiple budding sporulation has not been previously described in prokaryotes. The strain SK20-1^T could be a model microbe for cellular differentiation with multiple budding spore formation.

Key Words—*Chloroflexi*; compost; multiple budding sporulation mode; *Thermosporothrix hazakensis*

Introduction

Thermosporothrix hazakensis strain SK20-1^T was isolated from compost by incubation at 50°C on plates (Yabe et al., 2009a). The compost was produced by a field-scale composter, which had been used for treatment of livestock excrement. The details of this system were previously described (Yabe et al., 2009b). Strain SK20-1^T formed a branched substrate and aerial mycelia with spores; thus, the colony morphology was very similar to aerial mycelium-forming actinomycetes.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SK20-1^T is AB500145.

Thermosporothrix hazakensis strain SK20-1^T (=JCM 16142^T =ATCC BAA-1881^T).

The cultural characteristics of this strain are that it is fast growing, gram-positive and thermophilic (optimum, 50°C). Phylogenetic analysis based on 16S rRNA gene sequences showed that the strain appears in a cluster of the phylum *Chloroflexi*. The closest relative of strain SK20-1^T with a validly published name is *Ktedonobacter racemifer* (Cavaletti et al., 2006) (88.5% sequence similarity). We have separately described the details of the taxonomic position of strain SK20-1^T (Yabe et al., 2009a). All species belonging to the phylum *Chloroflexi* described so far do not form spores and branched mycelia, except *K. racemifer* described by Cavaletti et al. (2006). However, there are no studies concerning the mode of sporulation in *K. racemifer*. Therefore, we studied the life cycle including the sporulation mode in *T. hazakensis*.

Materials and Methods

For scanning electron microscopy (JSM6700F, JEOL, Inc.), a suitable agar block including a colony grown at

50°C for 2, 3, 4, 7 or 14 days on ISP 3 (Shirling and Gottlieb, 1966) agar was cut out with a scalpel, mounted on a glass slide, fixed for 24 h in a sealed bin containing 2% osmium acid and freeze dried for 4 h. The dried sample was mounted on a specimen support with carbon tape, covered with gold film by sputter coating under a vacuum, and observed in high-vacuum mode at 3 kV. For transmission electron microscopy (TEM), a suitable agar block including a colony grown at 50°C for 4, 7, or 14 days on ISP 3 agar was fixed overnight by steeping with 2% glutaraldehyde buffered with 0.1 M sodium cacodylate. After washing with the same buffer, the block was post-fixed for 90 min with 1% permanganic acid potassium salt and washed with distilled water. The TEM preparations were carried out at 4°C. The block then was dehydrated in an acetone series at room temperature and embedded in Spurr's resin at 60°C for 24 h. It was cut by ultramicrotome into 80–90 nm slices, stained with tannic acid, uranyl acetate and lead citrate and observed and photographed on a JEOL JEM1200EX at an acceleration voltage of 80 kV.

Results and Discussion

Each life cycle stage of strain SK20-1^T was observed using scanning and transmission electron microscopy. The life cycle might consist of spore-germination, substrate mycelial growth, aerial mycelial development, budding sporulation and spore maturation and dispersal (Fig. 1).

Spore-germination and substrate mycelia formation

Fig. 1. Schematic representation of the life cycle of strain *Thermosporothrix hazakensis* strain SK20-1^T.

were observed using microscopy after a 1–2 day incubation (data not shown). Spore-germination occurred on one side of spores. The substrate mycelium was 0.3–0.8 μm in width and formed septa at irregular spaces (0.7–4.0 μm in length).

Aerial mycelia developed after the substrate mycelium was grown within and on the surface of the agar medium (after a 2–3-day incubation), and the mycelia were 0.8–1.5 μm in width. Mycelia at the round stage were smooth with rounded ends (Fig. 2A), and aerial mycelia formed septa at irregular spaces (0.5–5.5 μm in length). Figure 2 (B and C) shows the beginning of spore formation from mother cells, which was initiated by projecting a cell membrane outside the cell wall (Fig. 2C-a). Subsequently, young spore cells were formed from swellings and were separated from aerial mycelial cells by septa (Fig. 2C-b) after a 4-day incubation. Multidirectional swellings of the young spores were clearly smaller in width than aerial mycelia; therefore, the sporulation might be caused by budding. Furthermore, multiple spores (four or more per cell) budded from one mother cell surrounded by septa (Fig. 2D and E). The size of each spore in a mother cell was different (Fig. 2B); minimum spore size of young spore cells separated by septa from the mycelia cell was 0.4 μm long and 0.3 μm wide (Fig. 2C-b). Therefore, there must be differences in the time and speed of budding formation. On the other hand, substrate mycelium did not form spores. The strain SK20-1^T spore shapes resembled those of *Saccharomonospora* sp. (Greiner-Mai et al., 1988; Runmao et al., 1988) and *Actinomadura formosensis* (Hasegawa et al., 1986) belonging to actinomycetes, but fundamentally differed in their sporulation modes. Actinomycetes spores are produced by differentiation of fragmented mycelial cells into spores; thus, its mode is arthrospore formation. Spore formation by budding is known to occur in several bacteria. *Pseudonocardia spinosa* (Henssen et al., 1983) of actinomycetes forms budding aerial hyphae resulting in spore formation. *Micromonospora chalicea* (Luedemann and Casmer, 1973) and *Kineosporia* sp. (Pagani and Parenti, 1978) of actinomycetes were also described as showing budding sporulation; however, photographs of TEM in each description indicated that those spores are formed by differentiation from the cell at the end of branched mycelia, and therefore those sporulation modes may be arthrospore formation such as *Saccharomonospora* and *Actinomadura formosensis*, not a budding mode. *Planctomyces*

Fig. 2. Scanning and transmission electron micrographs of strain SK20-1^T on ISP 3 medium after incubation at 50°C for 2, 4 and 14 days.

(A) SEM observation of the early stage after 2 days. Bar, 1 μ m. (B) SEM observation of the budding sporulation stage after a 4-day incubation. Bar, 1 μ m. (C), (D) and (E) TEM observation of the budding sporulation stage after a 4-day incubation. Bar, 500 nm. Arrowed line (a) is the budded spore before septa formation, arrowed line (b) is the spore after septa formation, arrowed line (c) and (d) are septa. (F) SEM observation of germination or secondary sporulation formation from spores that remained attached to an aerial mycelium after a 14-day incubation. Bar, 1 μ m.

sp. (Hirsch, 1972) is known as a budding spore-forming bacterium; however, the budding cells are daughter cells, not spores (Angert, 2005). In either case, the above budding bacteria produce one spore or cell per mother cell. Multiple spore formation occurs in *Metabacterium polyspora* (Angert and Losick, 1998) and *Anaerobacter polyendosporus* (Duda et al., 1987); however, these produce endospores. These observations indicate that the sporulation mode of strain SK20-1^T differs from other prokaryotic sporulation modes. To produce multiple spores per mother cell, the mother cell must synthesize and supply many genome copies

to the spore cells. Consequently, the genetic mechanism for the multiple budding sporulation mode in the life cycle of strain SK20-1^T is probably highly controlled. This sporulation mode is similar to eukaryotes, such as *Mixia osmundae* (Nishida et al., 1995) in Basidiomycota, rather than to prokaryotes.

The aerial mycelia of strain SK20-1^T budded more spores after an incubation of over 7 days. The morphology of strain SK20-1^T at this stage was described previously (Yabe et al., 2009a). At this stage, there was infrequent spore germination or secondary sporulation from spores attached to aerial mycelia (Fig. 2F).

The spore dispersal stage of strain SK20-1^T is difficult to observe. Because the cell cytoplasm of the aerial mycelia after spores matured was almost empty, it is probably difficult for the aerial mycelia to support mature spores. Therefore, it is likely that the spores have a dispersal stage in their life cycle.

As models, numerous studies have examined the sporulation modes of *Bacillus subtilis* (endospore), *Myxococcus xanthus* (myxospore) and *Streptomyces coelicolor* (arthrospore). The sporulation mode in strain SK20-1^T in which multiple budding spores are formed per mother cell during a complex life cycle like a typical actinomycete is the first report in prokaryotes, indicating that strain SK20-1^T has genetic systems that are highly controlled and programmed for cell differentiation. This is a microbiologically important finding, and strain SK20-1^T would be useful as a model microbe for cell differentiation studies. The life cycle of strain SK20-1^T is similar to that of typical actinomycetes and *Thermoactinomyces* sp. (Yoon et al., 2005) except for the sporulation stage. Branched aerial mycelia forming *Thermoactinomyces* sp. belonging to the phylum *Firmicutes* is also known as a bacterium morphologically differentiated like typical actinomycetes in spite of being distantly related to the phylum *Actinobacteria*. Strain SK20-1^T and *Thermoactinomyces* acquired the actinomycete-like properties, perhaps during a process of convergent evolution. Incidentally, Cavaletti et al. (2006) isolated 11 spore- and branched mycelium-forming strains including *K. racemifer* SOSP1-21^T (strain SOSP series) in the class *Ktedonobacteria* from soil. Stott et al. (2008) also isolated 4 spore- and branched mycelium-forming strains (isolate P359, T81, P352, T104) from geothermal soils. However, those isolates are not proposed with valid names except *K. racemifer*, and the sporulation mode of those has not been described. The sporulation mode of bacteria belonging to the class *Ktedonobacteria* may be the multiple budding sporulation mode.

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