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Gluconate 5-dehydrogenase (Ga5DH) participates in *Streptococcus suis* cell division

Zhongyu Shi1,2, Chunling Xuan1,2, Huiming Han1, Xia Cheng1,3, Jundong Wang3, Youjun Feng4, Swaminath Srinivas5, Guangwen Lu1, George F. Gao1,2,6,7

1 CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China
2 University of Chinese Academy of Sciences, Beijing 100049, China
3 College of Animal Science and Technology, Shanxi Agricultural University, Taigu 030801, China
4 Department of Microbiology, University of Illinois, Urbana, IL 61801, USA
5 Department of Biochemistry, University of Illinois, Urbana, IL 61801, USA
6 Laboratory of Protein Engineering and Vaccines, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China
7 Research Network of Immunity and Health (RNIH), Beijing Institutes of Life Science, Chinese Academy of Sciences, Beijing 100101, China

Correspondence: gaof@im.ac.cn (G. F. Gao)

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ABSTRACT

Bacterial cell division is strictly regulated in the formation of equal daughter cells. This process is governed by a series of spatial and temporal regulators, and several new factors of interest to the field have recently been identified. Here, we report the requirement of gluconate 5-dehydrogenase (Ga5DH) in cell division of the zoonotic pathogen *Streptococcus suis*. Ga5DH catalyzes the reversible reduction of 5-ketogluconate to D-gluconate and was localized to the site of cell division. The deletion of Ga5DH in *S. suis* resulted in a plump morphology with aberrant septa joining the progeny. A significant increase was also observed in cell length. These defects were determined to be the consequence of Ga5DH deprivation in *S. suis* causing FtsZ delocalization. In addition, the interaction of FtsZ with Ga5DH in vitro was confirmed by protein interaction assays. These results indicate that Ga5DH may function to prevent the formation of ectopic Z rings during *S. suis* cell division.

KEYWORDS  *Streptococcus suis*, Ga5DH, cell shape, cell division, FtsZ localization

INTRODUCTION

*Streptococcus suis* (*S. suis*) is an emerging zoonotic pathogen that causes life-threatening diseases, including septicemia, meningitis, and endocarditis (Kay et al., 1995; Mazokopakis et al., 2005), with more than 750 reported human cases of infection worldwide (Feng et al., 2010). Amongst the 35 known serotypes of *S. suis*, serotype 2 (SS2) is considered to be the most pathogenic and prevalent form in both pigs and humans (Nghia et al., 2008). In addition to sporadic cases of human SS2 infections (Feng et al., 2009; Feng et al., 2010), two large scale outbreaks of human SS2 endemics, with an unprecedented high rate of morbidity and mortality in China, were reported in 1998 and 2005 (Tang et al., 2006). Potent inhibitors and therapeutic agents to effectively control *S. suis* infection are required to address the re-emergence of SS2 as a zoonotic pathogen in humans and the rapid increase of antibiotic-resistant strains among clinical isolates.

D-gluconate, an important carbon source for many microorganisms, is required for *Escherichia coli* (*E. coli*) to colonize the streptomycin-treated mouse large intestine (Sweeney et al., 1996), suggesting that gluconate might play an important role in both bacterial survival and virulence. Membrane-bound gluconate 5-dehydrogenase (Ga5DH) catalyzes the inter-conversion of D-gluconate and 5-keto-D-gluconate, while simultaneously generating NADPH, which acts as a hydrogen donor for many biosynthetic processes. Cell cycle proteins have traditionally been an attractive target for antibacterial agents, but with our increasing understanding in this area, Ga5DH also seems like an effective...
target for the development of novel potent antibacterial agents. In our previous work, *S. suis* Ga5DH was characterized both structurally and enzymatically (Zhang et al., 2009).

Cell division is initiated by the formation of a cytokinetic ring at the prospective division site (Bi & Lutkenhaus, 1991), which leads to the production of two identical daughter cells. Ellipsoid-shaped bacteria such as *S. suis* divide at the mid-point of the cell along successive parallel planes perpendicular to the long axis. The first known event in bacterial cytokinesis is the polymerization of the tubulin homologue GTPase FtsZ into a ring structure at the prospective site of division (Bi and Lutkenhaus, 1991). The polymerization of FtsZ into the Z ring at the future division site is critical for cell division because it guides septum synthesis, location, and shape (Addinall & Lutkenhaus, 1996). Diverse cell division components interact with FtsZ to regulate FtsZ assembly. In *E. coli*, two division proteins, ZipA and FtsA, directly interact with FtsZ and cooperate in anchoring FtsZ to the membrane (Pichoff & Lutkenhaus, 2002).

Bacteria display a wide variety of cell shapes. Although some divisome proteins such as FtsZ are conserved among nearly all bacteria, other components of the cell division machinery diverge significantly to reflect the diversity of bacterial shapes. For example, the Min proteins are required for preventing Z ring assembly at cell poles in *Bacillus subtilis* and *E. coli* but are missing from some cocci, such as *Staphylococcus*, *Enterococcus*, and *Streptococcus* (Zapun et al., 2008).

The mechanism of FtsZ localization and the regulators that affect Z ring assembly have been extensively studied in the rod-shaped laboratory workhorses *E. coli* and *B. subtilis*. In this study, we report for the first time that Ga5DH plays a role in the cell growth and cell division of *S. suis*. In the absence of Ga5DH, cells exhibited a reduced growth rate and plump sausage-like shape with non-constricted septa joining the progeny. We found that the cells lacking Ga5DH display aberrant formation of FtsZ rings. Furthermore, protein interaction studies revealed that Ga5DH is capable of binding to FtsZ in vitro. These results suggest that Ga5DH is involved in maintaining correct cell shape and in cell division.

**RESULTS**

**Construction of Δga5dh in S. suis 05ZYH33**

SS2 05ZYH33 was isolated from patients with streptococcal toxic shock syndrome (STSS) in Sichuan Province, China (Tang et al., 2006). To further investigate the function of Ga5DH in the bacterial physiology of SS2, we constructed a homologous suicide plasmid, pUC::ga5dh, with a spcR cassette. The suicide plasmid was then transformed into *S. suis*, and positive transformants were screened on THY agar plates with the selective antibiotic spectinomycin. Successful construction of the Δga5dh strain was confirmed by multiplex-PCR analysis. In the Δga5dh mutant, the entire ga5dh gene was replaced with a spectinomycin cassette (Fig. 1A). Western blot assays confirmed the absence of Ga5DH in the deletion mutant and the return of Ga5DH expression in the complemented strain (Fig. 1B).

**Growth phenotype of the Δga5dh mutant**

We first characterized the growth kinetics of the mutant strain. The wild type (WT) and Δga5dh strains were grown overnight and then inoculated into fresh nutrient-rich THY medium at 37°C. A reduced growth rate was observed for the Ga5DH-deficient cells compared to the WT strain, indicating that Ga5DH is critical for *S. suis* growth (Fig. 2A). Indeed, complementation of ga5dh (Cga5dh) partially restored the growth defect. It is noteworthy that in THY medium, where gluconate is not utilized as the main carbon source, the Δga5dh growth defect phenotype was still dramatic.

We further examined the cell growth of the WT, deletion mutant, and complemented strains in THY medium with extra carbon source by supplementation with 2% (w/v) gluconate sodium (Fig. 2B). Similar growth profiles to those obtained in THY medium were recorded. Based on these observations, we believe it is unlikely that the impaired cell growth in Δga5dh was due to an energy deficiency or exhaustion. Therefore, we hypothesized that Ga5DH might function to affect *S. suis* cell division, in addition to its traditional role as a gluconate metabolic enzyme.

**Deletion of the ga5dh gene affects cell morphology and division**

Scanning electron microscopy (SEM) was employed to study the morphology and division pattern of the WT and mutant *S. suis* strains. WT cells grew as diplococci or short chains showing a characteristic ellipsoid shape and normal division pattern. The Δga5dh strain, however, developed into plump, sausage-shaped cells that were less ovoid and significantly longer than the WT cells. In addition, multiple indentations appeared on the cell surface of the Δga5dh strain (Fig. 3A). A quantitative analysis of the cell length was further performed to analyze the morphological phenotypes of the ga5dh deletion mutant (Fig. 3B and 3C). Single membrane-stained cells were randomly selected, and their longitudinal length was measured. The statistics of cell length for the three *S. suis* strains (WT, Δga5dh, and complemented) all revealed a typical Gaussian distribution pattern. Nevertheless, the WT and the complemented bacteria concentrate mainly in a length interval of 1.0–1.7 μm, whereas the majority of the Δga5dh cells were distributed in a region with a cell length of 1.5–2.3 μm (Fig. 3B). On average, the calculated cell length along the long axis in the deletion strain was 1.95 μm (n = 200), compared to 1.42 μm in the WT cells (n = 200) and 1.55 μm in the Ga5DH-complemented cells.
Cell width in Δga5dh cells was also slightly greater than that of the WT cells (Fig. 3C). Additionally, we used transmission electron microscopy (TEM) and FM4-64 (a styryl membrane-specific dye) staining to gain an exact view of the cell shape and cell septa of both the WT and the mutant cells in details. In contrast to the WT cells with their typical ovoid shape, Δga5dh cells were enlarged, and their cell poles were round (Fig. 4A and 4B). Further, by staining with FM4-64, severe defects in morphology were observed in the Δga5dh cells. For example, the mutant cells were significantly longer and presented multiple aberrant septa. Cell constriction was also impaired, indicating that the septa might have no or abnormal functions. In addition, the Δga5dh cells exhibited severe division defects, including abnormal septum position (Fig. 4A) and asymmetrical divisions (Fig. 4B). For the WT strain, however, the dividing cells displayed the normal tight coordination of septum formation and constriction, showing characteristics of a symmetrical division. As expected, complementation of the ga5dh gene largely restored a normal cell-dividing phenotype in S. suis. These data indicate that Ga5DH is involved in S. suis cell division and suggest that Ga5DH might play a role in septum constriction.

Distribution of Ga5DH at mid-cell sites

We further tested the subcellular localization of Ga5DH in exponentially growing cells by fluorescence microscopy using polyclonal antibodies directed against Ga5DH. As expected, no signal was detected in the Δga5dh cells (Fig. 5). However, the Ga5DH protein in both the WT and the complemented cells was mainly distributed as bands at the cell division septum (Fig. 5).

Ga5DH interacts with FtsZ in vitro

The subcellular localization of Ga5DH at the cell division septum implied a potential interaction of Ga5DH with FtsZ, an important cell division initiation factor that also localizes at this site. A variety of assays were performed to evaluate the physical interactions between the Ga5DH and FtsZ proteins of S. suis in vitro. First, FtsZ- or BSA-coated microtiter enzyme-linked immunosorbent assay (ELISA) plates were incubated with varying concentrations of Ga5DH. As shown in Fig. 6A, Ga5DH bound to the FtsZ-coated wells but not to the wells coated with BSA.
Next, GST pull-down assays were performed to further confirm the interaction between Ga5DH and FtsZ. FtsZ was N-terminally fused to GST, expressed in *E. coli*, and purified to homogeneity (Fig. 6B). To determine whether Ga5DH and FtsZ could form a stable complex, equimolar amounts of GST-FtsZ or GST were mixed with *E. coli* extracts containing Ga5DH and loaded onto a column packed with glutathione resin. Following washing, the bound proteins were eluted with glutathione. Bound Ga5DH, which was identified by Western blotting, was detected only for GST-FtsZ but not for the control protein of purified GST (Fig. 6C). Therefore, we provide solid evidence that Ga5DH directly interacts with FtsZ.

Deletion of Ga5DH results in Z ring delocalization

The *in vitro* observation of a direct Ga5DH/FtsZ interaction urged us to further analyze the effect of Ga5DH deletion on FtsZ localization *in vivo*. Immunofluorescence assays of septal FtsZ ring morphology in the WT and Δga5dh cells were performed using a polyclonal antibody against FtsZ. As expected, the majority of WT cells displayed FtsZ protein that was regularly distributed as a line at mid-cell (Fig. 7). The FtsZ localization in the Δga5dh cells, however, was dramatically altered compared to that in the WT cells. For the Ga5DH deletion mutant, FtsZ was present in aberrant singlets or doublets, distributed along the length of the cell. Moreover, in the absence of Ga5DH, some FtsZ failed to localize to the potential division sites. As expected, complementation of the ga5dh gene faithfully restored a normal FtsZ localization pattern (Fig. 7). This suggests that in addition to its enzymatic activity, Ga5DH may play a physical role in *S. suis* cell division for Z ring localization.

**DISCUSSION**

Tight regulation of the cell wall synthesis and degradation machinery is required to maintain the proper shape and size of bacterial cells. Septal cell wall formation during division must be coordinated with other processes of cell division, such as
Ga5DH participates in cell division

Figure 4. Micrographs of the WT, Δga5dh, and Cga5dh strains. (A) FM4-64 staining; scale bar, 5 µm. (B) Transmission electron micrographs; scale bar, 0.5 µm. Green arrows highlight the correct septum placement in WT cells. Red arrows mark the aberrant septum placement in Δga5dh cells. Cell morphology of the WT strain revealed the characteristic ellipsoid shape and normal division pattern of S. suis, with correct septum placement and symmetric daughter cells. The mutant cells displayed an increased length and multiple aberrant septa with incorrect placement.

Figure 5. Subcellular localization of Ga5DH. DNA was visualized with DAPI (blue), Ga5DH was visualized using an anti-Ga5DH polyclonal antibody and an anti-mouse IgG secondary antibody coupled to Alexa Fluor 488 (green). The merged pictures show the overlay of Ga5DH and DAPI staining. Scale bar, 1 µm.

The primary role of Ga5DH is metabolic, catalyzing the inter-conversion of D-gluconate and 5-keto-D-gluconate. In previous studies, other metabolic proteins such as ManA and UgtP have been reported to affect cellular structures and cell division in B. subtilis (Elbaz & Ben-Yehuda, 2010; Weart et al., 2007). In B. subtilis, nutrient availability has a dramatic effect on UDP-glucose accumulation. Under conditions in which UDP-glucose levels are high, UDP-glucose directly interacts with the diacylglycerol glucosyltransferase UgtP to inhibit FtsZ assembly and delay maturation of the Z ring. It is possible that the metabolic enzyme Ga5DH operates as a sensor that synchronizes cell division with metabolite availability through its role in coordinating FtsZ localization.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions

Strains and plasmids used in this study are listed in Table 1. Streptococcus suis strains were grown in Todd-Hewitt broth (THB) (Difco Laboratories, Detroit, MI, USA) supplemented with 2% yeast extract (THY) or plated on THY agar at 37°C (Feng et al., 2007; Feng et al., 2008). THY medium and THY medium supplemented with 2% glucose were used for growth curve measurements. Experiments were performed in triplicate.

E. coli strains DH5α and BL21 were incubated in Luria-Bertani (LB) medium or plated on LB agar. When required, antibiotics were routinely added at the following concentrations: spectinomycin, 100 mg/mL for both S. suis and E. coli; erythromycin, 1 mg/mL for S. suis and 250 mg/mL for E. coli. 50 mg/mL of ampicillin was always present to screen the transformants of E. coli.

Cloning, mutagenesis, and genetic complementation

To delete ga5dh, the two flanking sequences of ga5dh were amplified from the chromosomal DNA of S. suis 05ZYH33 (Feng et al., 2008;
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1982). And then the resulting plasmid pVAΔ by electroporation into the E. coli digested and its promoter region were generated by PCR using specific primers were cloned into a pUC18 vector to generate the recombinant procedures (Feng et al., 2008; Li et al., 2008).

Table 2. For cloning ga5dh homologous regions, two pairs of specific primers (LA-P1/RA-P2, carrying EcoRI/BamHI and PstI/HindIII restriction enzyme sites) were used, respectively. After digestion with the corresponding restriction enzymes, the DNA fragments were cloned into a pUC18 vector to generate the recombinant plasmid pUC::ga5dhLR. Then, the spcR gene cassette was inserted into BamHI/PstI digested plasmid pUC::ga5dhLR to generate the ga5dh knockout plasmid pUC::ga5dh. To obtain the isogenic mutant Δga5dh, S. suis 05ZYH33 was transformed by electroporation of the resulting plasmid pUC::ga5dh following previously described procedures (Feng et al., 2008; Li et al., 2008).

To construct the complement strain, a DNA fragment encoding ga5dh and its promoter region were generated by PCR using specific primers (Cga5dh-F/Cga5dh-R) and inserted into the digested E. coli-S. suis shuttle vector pVA838 (Macrina et al., 1982). And then the resulting plasmid pVA::ga5dh was transformed by electroporation into the Δga5dh mutant. Transformants were screened on THY plates with selection for spectinomycin and erythromycin resistance.

Overexpression and purification of FtsZ and Ga5DH

The ftsZ gene was amplified by PCR using the chromosomal DNA of S. suis 05ZYH33 as template and specific primers described in Table 2. The FtsZ PCR product was digested by enzymes NdeI and XhoI and then inserted into the digested pET30a vector to generate the recombinant plasmid pET30-ftsZ. For expression of FtsZ with a fused GST tag, ftsZ gene was cloned into the pGEX-6P-1 vector via the BamHI and EcoRI sites. For expression of His-tagged Ga5DH, the ga5dh PCR product was inserted into the pET28b vector via the Ndel and XhoI restriction sites (Zhang et al., 2009). The resulting recombinant plasmid was termed pET28b-ga5dh. In each case, the recombinant proteins were over-expressed in E. coli BL21 (DE3) in Luria Broth medium.

Cells from overnight liquid were thawed to an OD600 of 0.4 and then induced by addition of 0.5 mmol/L IPTG. The cells were harvested by centrifugation and resuspended in a buffer consisting of 20 mmol/L Tris-Cl pH 8.0 and 50 mmol/L NaCl and sonicated. After centrifugation, the supernatant was applied to a 5-mL column of HisTrap FF resin or of Glutathione Sepharose FF resin (GE Healthcare). Following extensive washing with the binding buffer (20 mmol/L Tris-Cl pH 8.0, 50 mmol/L NaCl), samples were eluted with either buffer A (20 mmol/L Tris-Cl pH 8.0, 50 mmol/L NaCl, 300 mmol/L imidazole) for His-tagged proteins or buffer B (20 mmol/L Tris-Cl pH 8.0, 50 mmol/L NaCl, 20 mmol/L glutathione) for GST-tagged proteins. Peak fractions were pooled. Proteins were further purified by gel filtration chromatography using a Superdex-200 10/300 GL column (GE Healthcare) with 20 mmol/L Tris-HCl and 50 mmol/L NaCl, pH 8.0 as running buffer and then stored at −80°C. The His-tagged FtsZ and Ga5DH were individually used to immunize mice to produce polyclonal antibodies.

Li et al., 2008; Li et al., 2011). All primers used in this study are listed in Table 2. For cloning ga5dh homologous regions, two pairs of specific primers (LA-P1/RA-P2) carrying EcoRI/BamHI and PstI/HindIII restriction enzyme sites were used, respectively. After digestion with the corresponding restriction enzymes, the DNA fragments were cloned into a pUC18 vector to generate the recombinant plasmid pUC::ga5dhLR. Then, the spcR gene cassette was inserted into BamHI/PstI digested plasmid pUC::ga5dhLR to generate the ga5dh knockout plasmid pUC::ga5dh. To obtain the isogenic mutant Δga5dh, S. suis 05ZYH33 was transformed by electroporation of the resulting plasmid pUC::ga5dh following previously described procedures (Feng et al., 2008; Li et al., 2008).

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Solid phase binding assay

Five hundred micrograms of purified FtsZ or BSA were coated in a buffer containing 14 mmol/L Na₂CO₃ and 36 mmol/L NaHCO₃ overnight at 4°C onto the enzyme-linked immunosorbent assay plates. After adsorption, the wells were washed three times with phosphate-buffer saline containing 0.5% Tween-20 (PBST) to remove the excess unbound proteins. Nonspecific binding sites of the wells were blocked with 10% goat serum in PBS for 2 h at 37°C. This was followed by incubation for 2 h at 37°C with different concentrations of Ga5DH protein in PBS. The plates were then rinsed with PBST three times. Finally, the interaction of Ga5DH with FtsZ was detected with anti-Ga5DH antibodies by an enzyme-linked immunosorbent assay. The anti-Ga5DH antibodies used for the immunosorbent assay were pretreated with immobilized His-tagged enolase protein (Lu et al., 2012) to fish out anti-His antibodies. Wells coated with BSA served as negative controls. All assays were performed in triplicate.

GST pull down assay

The pull down assay was performed with the purified GST-FtsZ or GST proteins by utilizing a modified method (Dziedzic et al., 2010). In brief, equal-molar amounts of GST-FtsZ or GST in PBS were mixed and incubated at 4°C with E. coli lysates containing His-tagged Ga5DH for about 4 h. Then the mixtures were allowed to bind to glutathione-Sepharose. The slurries were washed thoroughly with PBS. The bound proteins were eluted with glutathione. The samples were boiled in SDS-PAGE loading buffer for 10 min. The separated proteins were subjected to SDS-PAGE and immunoblotted. Samples were probed with an anti-His monoclonal antibody (MBL).

Immunofluorescence microscopy

Immunofluorescence was performed as previously described with small modifications (Foulquier et al., 2011). Cells from overnight culture were diluted 100-fold into 3 mL fresh THY medium and grown overnight at 37°C. The underlined sequences represent the restriction sites.

Table 1. Bacterial strains and plasmids used in this study

| Strains/plasmids | Characteristics | Origins |
|------------------|----------------|---------|
| **Bacterial strains** | | |
| 05ZYH33 | A virulent Chinese isolate of S. suis serotype 2 | Feng et al. (2008); Tang et al. (2006) |
| 05ZYH33 Δga5dh | 05ZYH33 derivative with the ga5dh gene replaced by a spcR gene cassette | This work |
| Cga5dh | Complemented strain of 05ZYH33 Δga5dh | This work |
| DH5α | An E. coli cloning host | Lab stock |
| BL21 | An E. coli expression host | Lab stock |
| **Plasmids** | | |
| pUC-18 | Cloning vector | TaKaRa |
| pET28b | His-tag fusion expression vector | Novagen |
| pET30a | His-tag fusion expression vector | Novagen |
| pGEX-6P-1 | GST-tag fusion expression vector | Novagen |
| pVA-838 | E. coli - S. suis shuttle vector | Lab stock |

Table 2. Primers used in this study

| Primers | Sequence (5′-3′) | Function |
|---------|-----------------|----------|
| LU | TTCCGGCTGTTTACCAATGT | PCR detection |
| RD | CTAAGGGAATAATTGCTTTG | |
| LA-P1 (EcoRI) | CCGGAATTCTAGTCTTCAAACTAGACTGG | Mutant strain construction |
| LA-P2 (BamHI) | CGCGGATCCTAGTATAGCCTATATAAAAGTG | |
| RA-P1 (PstI) | AACAGCAATCTGCACTCTTGAATTGTTT | Mutant strain construction |
| RA-P2 (HindIII) | CCCAAGCTTTGTGGTTTCAAGACTTTTG | |
| Cga5dh-F (BamHI) | CGCGGATCCTAGTATAGCCTATATAAAAGTG | ga5dh cloning and complementation |
| Cga5dh-R (SalI) | GCCATGGACCAATACGTATATA | |
| spc-F (BamHI) | GCAGGATCCGCTTCTGAATACATTTATA | spc cloning and PCR detection |
| spc-R(PstI) | GGGCTGAGGTTTCTAATAAATTGAT | |
| ftsZ-F (Ndel) | GGAATTCCATATGGCCTTTCATTGGTGAAGCA | ftsZ cloning into pET30a and protein expression |
| ftsZ-R (Xhol) | CCCTCGAGGCGAGCTTACGGAAGAATG | |
| ftsZ-GST-F (BamHI) | CGCGGATCCTAGTCTTCACTTGGAGAAGC | ftsZ cloning into pGEX-6P-1 and protein expression |
| ftsZ-GST-R (EcoRI) | CCCTCGAGGCTTACGGAAGAATG | |
| ga5dh-F (Ndel) | GGAATTCCATATGGCCTTTCATTGGTGAAGCA | ga5dh cloning in pET28b and protein expression (Zhang et al., 2009) |
| ga5dh-R (Xhol) | GCCATGGACCAATACGTATATA | |

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at 37°C to exponential phase. The cells (1 mL) were then harvested and resuspended in 10 mL of ice-cold 80% methanol and incubated for 1 h at room temperature. The suspension was then concentrated, resuspended in 200 μL of freshly prepared 16% formaldehyde and incubated for 5 min at room temperature. Then samples were centrifuged and washed once with 1 mL of ice-cold 80% methanol. After centrifugation, cells were permeabilized at 37°C for 10 min in 20 mmol/L sodium phosphate buffer pH 6.2, 50 mmol/L sucrose, 500 mg/ml lysozyme. Cells were then washed with PBST, saturated with 200 μL of PBST-10% goat serum containing anti-Ga5DH or anti-FtsZ antibodies (dilution 1/100) and incubated overnight at 4°C. The samples were washed twice with PBST and incubated with a 1:300 dilution of the secondary goat anti-mouse antibody coupled with Alexa Fluor 488 (Santa Cruz) in the dark. DNA was visualized by treatment with the DNA fluorescent stain DAPI. For FM4-64 staining, all cells were harvested and incubated in 5 μmol/L FM4-64 in dark for 20 min. The excess dye was washed with THY medium. Finally, cells were mounted directly onto microscope slides covered with a thin film of 1.2% agar in water. Fluorescent images were acquired by laser scanning confocal microscope (Leica TCS SP2).

Transmission electron microscopy

All samples were harvested at an OD600 of 0.8 and fixed with 2.5% glutaraldehyde (1 mL) followed by washing with PBS. The cells were treated with 1% osmium tetroxide for 2 h in dark. Then the subsequent dehydration steps with ethanol were carried out as follows: 50% for 15 min, 70% for 15 min, 95% for 15 min, 100% for 20 min. The samples were embedded in Spurr’s plastic and sectioned. Cell morphology was then visualized using a JEM-1400 (JEOL) transmission electron microscope.

Scanning electron microscopy

All samples were grown in THY broth and harvested at an OD600 of 0.8. Cells were spotted onto polylysine coverslips followed by washing with PBS. The cells were fixed in 0.18 mol/L cacodylate buffer (pH 7.6) containing 2% glutaraldehyde. Then the subsequent dehydration steps with ethanol were carried out, passage in HMDS buffer (pH 7.6) containing 2% glutaraldehyde (1 mL) followed by washing with PBS. The cells were treated with 1% osmium tetroxide for 2 h in dark. Then the subsequent dehydration steps with ethanol were carried out, passage in HMDS buffer (pH 7.6) containing 2% glutaraldehyde (1 mL) followed by washing with PBS. The cells were treated with 1% osmium tetroxide for 2 h in dark. Then the subsequent dehydration steps with ethanol were carried out, passage in HMDS buffer (pH 7.6) containing 2% glutaraldehyde (1 mL) followed by washing with PBS. The cells were treated with 1% osmium tetroxide for 2 h in dark. Then the subsequent dehydration steps with ethanol were carried out, passage in HMDS buffer (pH 7.6) containing 2% glutaraldehyde (1 mL) followed by washing with PBS. The cells were treated with 1% osmium tetroxide for 2 h in dark. Then the subsequent dehydration steps with ethanol were carried out, passage in HMDS buffer (pH 7.6) containing 2% glutaraldehyde (1 mL) followed by washing with PBS.

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COMPLIANCE WITH ETHICS GUIDELINES

Zhongyu Shi, Chunling Xuan, Huiming Han, Xia Cheng, Jundong Wang, Youjun Feng, Swaminath Srinivas, Guangwen Lu, and George F. Gao declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by the any of the authors.

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