

The Mutation of the *rpoS* Gene, the Central Regulator of Stationary Phase, Affects the Cell Division in *Flexibacter chinensis*

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Abstract

A one kb portion of the *rpoS* gene from *Flexibacter chinensis* was isolated by PCR, sequenced and compared to the *rpoS* gene of a variety of other organisms. The gene was found to be 98% similar to previously sequenced genes. Mutation of the *rpoS* gene with tri-parental mating produced strain JR101 and the growth rate of the mutant was compared with that of the wild-type. The mutant grew slower, and produced longer cells in the stationary phase. The cell size of the mutant was not reduced during the stationary phase, suggesting that cell division was inhibited during the stationary phase. The mutant also showed a much reduced survival under starvation conditions compared to the wild-type strain.

Keywords: *Flexibacter chinensis*; *rpoS*; SOS response

Introduction

The *rpoS* gene encodes a global regulatory protein of central importance (named sigma factor σ^S) for the control of many genes whose expression is stimulated by starvation or during entry into the stationary phase [1]. During starvation RpoS protein levels increase dramatically and RNA polymerase containing RpoS transcribes over 70 genes involved in stress resistance and protection [2].

Its sequence turned out to have strong similarity to the sequence of other bacterial sigma factors. *rpoS* is located at 58.9 min on the genetic linkage map of the *E. coli* genome. *rpoS* is a promoter-distal gene in an operon together with *nlpD* which encodes a lipoprotein involved in cell wall formation. While the two *nlpD*

promoters contribute to *rpoS* expression during exponential phase, the major *rpoS* transcript starts at a position within the *nlpD* gene. There are two start codons separated by 11 codons at the 5' end of *rpoS* and the second of these is the initiation codon. *rpoS* encodes a protein of 330 amino acids with a molecular mass of 37.9 kDa [3]. The control of cell division by the central regulatory system gene, σ^S , has been reported by Dewar *et al.* [4].

Lange and Aronis [5] reported that *rpoS* mutant cells are bigger than wild type cells in *E. coli* and remained rod shaped in the stationary phase. They reported that the *rpoS* gene product was required for the stationary phase induction of *bolA* (a growth rate-dependant gene). One of the main aims of this work was to investigate the molecular effects of starvation on the cell size of *F.*

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chinensis. Through the generation of mutants in this organism deficient in the expression of central regulatory system gene, σ^S , we attempted to investigate the effects of starvation of the entry into the stationary phase on the cell size of *F. chinensis*.

Materials and Methods

Bacterial Strains and Plasmids

The bacterial strains used were, the *Flexibacter chinensis* strains (Dr. K. Flint, University of Warwick) and the *Escherichia coli* strains including ML30 (Dr. K. Flint, University of Warwick), TG1 [6], S17-1 [7], and the used plasmids, were listed in Table 1.

Bacterial Growth Media and Conditions

All bacterial strains were routinely grown in Luria Broth (10 g/l Bacto tryptone, 5 g/l yeast extract, 5 g/l NaCl, pH 7.2) or on Luria Agar (10 g/l bacto tryptone, 5 g/l yeast extract, 5 g/l NaCl, 15 g/l Agar).

Plasmid Mobilization

The method have been described [13,14] but were used with slight modification; *E. coli* S17-1 was used as the donor for the transfer of the recombinant plasmid, pLRPOS38 into *F. chinensis*. The plasmid *RP4* is an *incP* type plasmid which is integrated in the chromosome of *E. coli* S17-1 and the plasmid,

pLYLO3, contains an *oriT* (transfer origin) from *PK2*, an *incP1* plasmid, which is recognized by *IncPa* plasmids, such as *RP4*, but not by *IncPβ* plasmids. The recombinant plasmid, pLRPOS38, was transferred into the *E. coli* S17-1 by transformation and ampicillin resistant cells were isolated on LB plates containing 100 mg/ml ampicillin. The donor strain, *E. coli* S17-1, were grown to mid exponential phase in LB containing ampicillin as a selective agent for construct (Erythromycin resistance is not expressed in *E. coli* strains) at 37°C and the recipient kanamycin as a selective agent at 30°C. Both donor and recipient strains were harvested by centrifugation, mixed together (1:1 ratio), and approximately 10^9 cells were spotted onto LB agar plates without antibiotics. After incubation for 18 h at 30°C, the cells were scraped off the plates, diluted in LB and plated onto LB agar containing 200 µg Erythromycin to select for transconjugants. The plates were incubated for 2 to 3 days at 30°C.

Sequencing

The dideoxy chain termination DNA sequencing method was carried out. Samples were prepared and sent to MWG-Biotech Co. (Germany).

Computer Analysis of Sequence

After reading the sequence, the data were further analysed and aligned using the GCG programme (Wisconsin package, Version 8) [15].

Table 1. Plasmids used in this study

Plasmid	Characterisation	Reference
pBluescriptII KS+	High copy cloning vector, Ap ^r	Stratagene
pIC19H	High copy cloning vector, Ap ^r	[8]
pKNG101	Suicide vector, SacB, Suc ⁸ , Sm ^r , Ap ^r	[9]
pKRP10	Cassette carrier vector, Cm ^r , Ap ^r	[10]
pLYLO3	Suicide vector, Err, Ap ^r	[11]
pMOS Blue	PCR cloning vector, Ap ^r	Amersham International (PLC)
JC2926	Conjugational plasmid, Tc ^r , Cm ^r	Personal Communication (Dr. P. Wan)
RP4	conjugational Plasmid, Ap ^r , Km ^r , Tc ^r	[12]
pMRPOS38	1.003 kb PCR product of <i>rpoS</i> gene cloned into pMOS Blue vector	This study
pBRPOS38	0.996 kb <i>EcoRI/BamHI</i> subclone of pMRPOS3 into pBluescriptII KS ⁺	This study
pICRPOS38	0.996 kb <i>EcoRI/BamHI</i> subclone of pBRPOS38 into pIC19H	This study
pKRPOS38 Ω	1.800 kb <i>BamHI/BgIII</i> subclone of pICRPOS38 Ω into pKNG101	This study
pLRPOS38	0.603 kb <i>Bam HI/PstI</i> subclone of pICRPOS38 Ω into pLYLO3	This study

Southern Blot Protocol

The DNA was digested and separated on a 0.7% agarose gel, then incubated in 0.25 M HCl at 20°C for 20 min, in denaturation solution (50 ml 10 M NaOH and 87.66 g/l NaCl in 1 L distilled water) for 45 min and in neutralization solution (121.14 g/l Tris and 87.66 g/l NaCl in 1 L distilled water) for 30 min. The transfer buffer (20 x SSC) contained 175.3 g/l NaCl and 88.2 g/l Tri-sodium citrate in 1 L distilled water.

The southern transfer method was carried out following the procedure in Maniatis *et al.* (16). A glass plate which was wider than the gel size was placed on top of a barrier in a tray. The tray was filled with transfer buffer, and a platform of 3 mm Whatman filter paper was soaked in the transfer buffer so that both ends of the paper platform reached the bottom of the tray. The gel was inverted and placed on top of Whatman paper. A Hybond-N⁺ nylon membrane (Amersham) was cut slightly wider than the gel, washed in the transfer buffer and placed on the gels avoiding air bubbles between the gel and the membrane. 3 pieces of 3 mm Whatman filter paper were cut to the same size as the gel and placed on top of the membrane. A pile of tissues, the same size as the gel were prepared and placed on top of the 3 mm Whatman paper. A glass plate was placed on top of the tissues with a 0.5 kg weight on the plate. The blot was left overnight to allow the complete transfer of DNA to the membrane. The membrane was washed with SSC buffer and the DNA cross-linked using a UV illuminator for 2 to 5 min. The hybridization was performed at 68°C for 18 h in Hybridization oven. A commercially available DIG DNA-labeling & detection kit (Boehringer Mannheim) was used to label the DNA and the sub clone of pLRPOS38 was used as the probe.

Polymerase Chain Reaction Technique (PCR)

The PCR technique was used to manipulate the genes, using the primers, (forward) 5': GGGGGA TCCCGTCAAGGGATCACGGGTAGGAGCCAC :3', and (reverse) 5': GGGGAATTCTTCAACCTGA ATCTGGCGAACACGTTTC :3'. A total of 35 cycles consisted of 94°C for 45 sec, 65°C for 45 sec or 1 min and 72°C for 30 sec was repeated.

Viable Count

The viable count was determined using a surface spread plate technique. Samples (1 ml) were taken from the flasks and serial dilutions prepared as a 10-fold serial dilution in Quarter-strength Ringers solution (2.25 g/l

NaCl, 0.12 g/l CaCl₂, 0.05 g/l NaHPO₄ and 0.105 g/l KCl in 1 L Distilled water). 100 µl of the diluted samples was spread on duplicate plates. The plates were incubated at 30°C for at least 48 h. Plates were counted manually by using an illuminated colony counter. The results were expressed as cfu/ml.

Total Counts

The total count was determined using a Coulter counter ZM (Coulter Euro Diagnostics GMBH). The data were analyzed using Coulter channelyzer software to estimate the size distribution. The samples were diluted in an isotonic buffer containing 0.4% (v/v) glutaraldehyde to fix the cells. Total count was expressed as total particles/ml. The software also determines mean cell size and volume.

Transmission Electron Microscopy

Transmission electron microscopy was used after samples were negatively stained with phosphotungstic acid. Samples were placed onto a Formvar-coated copper grid (100 segment mesh; Agar scientific) for 30 sec. After drying, the grid was negatively stained with one drop of 1% (w/v) phosphotungstic acid. The samples were examined using the Jeol JEM-100S transmission electron microscope with an 80 kV accelerating voltage. Photographs were taken using Kodak Panasonic film, which was developed in Kodak D-19 developer at 20°C for 3 min and fixed in Kodak fixer. Final pictures were printed on Kodak Veribrom paper.

Results

Construction of a Vector Carrying *rpoS* Gene

A 1 kb fragment of *rpoS* gene of *F. chinensis* amplified using PCR and cloned into the the *EcoRI/BamHI* site of pBluescript KS⁺. This construct was named pBRPOS38.

The restriction digestion of pBRPOS38 with *EcoRI* and *BamHI* resulted in two bands, one band of 2.9 kb size corresponding to the initial vector and one band of 1 kb size which was the *rpoS* gene of *F. chinensis* (Fig. 1). This 1kb portion which represented the *rpoS* region of the *F. chinensis* chromosome was sequenced. Two parts of the sequences, one from upstream and the other from downstream, which were very important in the recognition of the open reading frame of the *rpoS* gene were compared to similar sequences in other organisms. Alignment analysis showed that in *rpoS* both the upstream and down stream regions were highly

conserved and about 98 percent similar to the *rpoS* genes of other organisms (data not shown).

Construction of *rpoS* Mutant Strain

The 5.97 kb pLYLO3 suicide vector was digested with *Bam*HI and *Pst*I sites and a 0.603 kb *Bam*HI fragment subclone of pICRPOS38 Ω (description given in Table 1) was cloned into linearized pLYLO3 digested with *Bam*HI-*Pst*I. This construct was named pLRPOS38.

A biparental conjugation method was used for the introduction of the construct, pLRPOS38, into *F. chinensis*. One of the parents was *E. coli* S17-1 which contains a derivative of RP4 (RP4-2::MuKm::Tn7Tc^r) integrated into the chromosome [7]. The construct pLRPOS38 contains oriT to allow conjugative transfer form *E. coli* S17-1. The other parent was *F. chinensis* as a recipient strain. The construct containing oriT carried the specific mob site which is recognized by incPa plasmids, such as RP4, and facilitates transfer of the construct from *E. coli* S17-1. Fifteen erythromycin resistant colonies, which were also ampicillin resistant, were single recombinants since the target gene was disrupted at one site. The recombinant clone carrying pLRPOS38 was named *F. chinensis* JR101 (Fig. 2).

Southern blot analysis of *F. chinensis* strain JR101 was carried out to confirm the interruption of the *rpoS* gene in the chromosome by a single cross-over event between the disrupted internal part of the *rpoS* gene cloned in pLYLO3 and the intact *rpoS* gene in the chromosome. Chromosomal DNA was purified from the wild type and the mutant strain, and both were digested with *Eco*RV.

Southern blot analysis showed that the probe was hybridized in strain JR101 to a fragment approximately 5.97 kb bigger than in the wild-type (Fig. 3). With the wild-type chromosomal DNA digested with *Eco*RV, the probe was hybridized to a fragment slightly larger than 3.054 kb, while there was no hybridization with JR101 strain. The probe was hybridized with a fragment from the mutant slightly larger than 9.162 kb as expected. The result also proved that there is no wild-type copy of the *rpoS* gene in JR101 due to the result of single cross-over recombination. Non-specific bands appeared, probably due to the high conservation and evolution relationship between sigma (σ) factors [17].

Growth Characteristics of Mutant Strain

The growth rate of *F. chinensis* wild-type and strain JR101 were determined at 20°C, 30°C and 37°C (Data not shown). The growth rate in the wild-type was better at 30°C than at 20°C, and at 37°C there was no growth

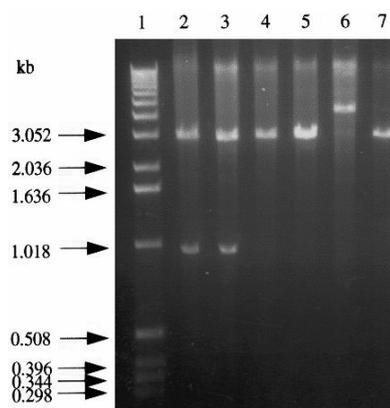


Figure 1. Restriction digestion of pBRPOS38. Lane 1; λ ladder of 1 kb DNA as a molecular size marker. Lanes 2 and 3; pBRPOS38 digested with *Eco*RI and *Bam*HI. Lanes 4, 5, 6 and 7 do not contain the clone.

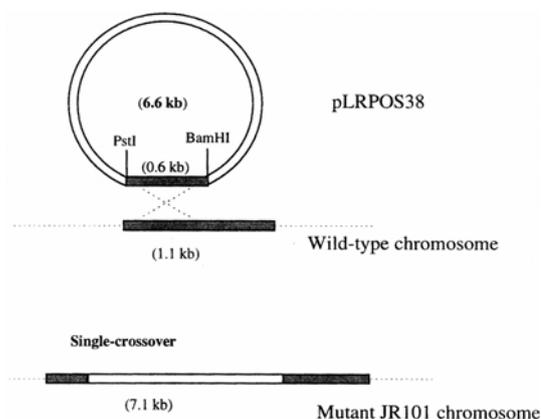


Figure 2. Integration of pLRPOS38 in the *F. chinensis* wild type chromosome. The integration of pLRPOS38 into the chromosome by a single crossover event is schematically represented. The size of the *Bam*HI/*Pst*I fragment and the vector are shown.

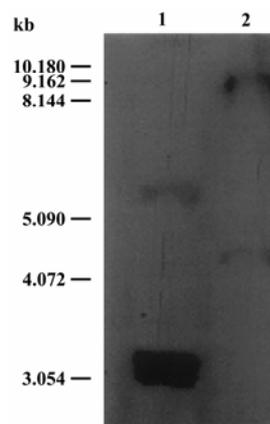


Figure 3. Southern blot analysis of wild-type and single recombinant Chromosomal DNA was digested with *Eco*RV. Lane 1; wild-type. Lane 2; JR101.

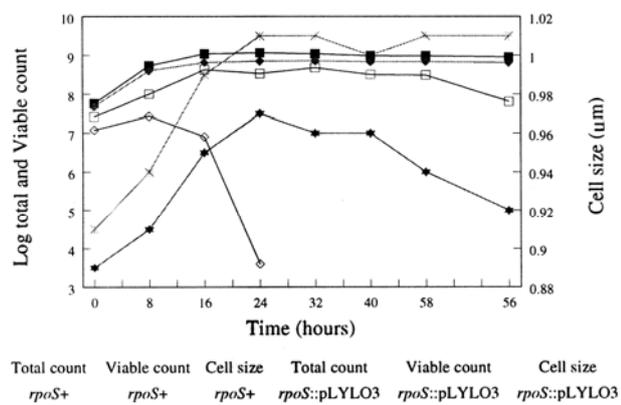


Figure 4. The viable count and cell size of the wild-type and JR101. The wild type strain and JR101 were grown overnight at 30°C in nutrient broth. The cells were harvested, washed and resuspended in sterile fresh nutrient broth. The fresh microcosms were inoculated to give an initial viable count of 10⁷ cfu/ml, and incubated with shaking at 30°C. Every 8 h, viable counts were determined on nutrient agar plates. The cell size was determined using a CellFacts machine after dilution of the samples in the appropriate electrolyte solution to which 0.4% (v/v) glutaraldehyde had been added to fix the cells.

over a 12 h incubation period. At 20°C and 30°C growth was slower for JR101 than for the wild type and there was no growth at 37°C even after 12 h of incubation. The cultures incubated at 37°C were transferred to a lower temperature after 12 h of incubation. The wild type then began to grow showing that it was not killed by the higher incubation temperature. JR101 did not begin to grow even after a further 12 h incubation period. The total count, viable count and cell size of the wild type and JR101 were measured in nutrient broth over a period of 64 h (Fig. 4). The maintenance of cell viability of wild-type and JR101 in a rich medium was investigated. JR101 showed no viability at 30°C (as determined by viable count) after 26 h of incubation, while the wild-type cells were still viable even after 64 h. The loss of cell viability in JR101 must be due to the irregularity of the stationary phase in these cells.

For preparing photomicrographs, samples of the wild type and JR101 were taken at different incubation times (Fig. 5). After 16 h, there was no difference between the cell size of the wild-type and JR101 and After 24 h incubation, the cells were different in the size, so that in JR101 the cells were longer than the wild-type cells. The cell size of JR101 increased for the first 26 h of incubation and remained constant unlike the wild-type which began to decrease after 26 h of incubation. These changes in cell size were more obvious after 48 h incubation.

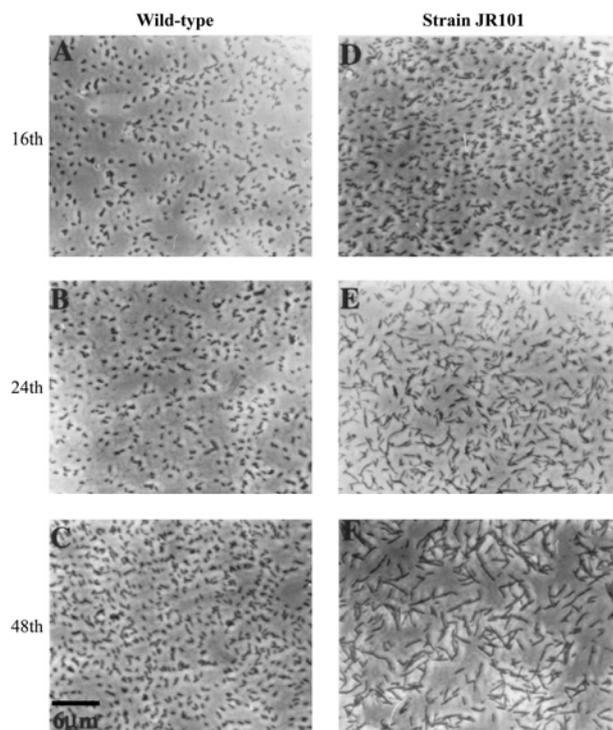


Figure 5. Microphotography of the wild-type and JR101. Cultures were grown in nutrient broth at 30°C. At different optical densities, samples were directly applied to slides, fixed, gram stained and photographed.

Discussion

rpoS encodes the sigma (σ) factor which has a central role in many stationary phase and starvation response genes [1,2]. In *E. coli*, the primary response to the limitation of a specific nutrient is due to activation of certain sets of genes to allow high-affinity uptake of the nutrient in low concentration. This system includes the cAMP receptor protein (CRP) regulon for the use of alternative carbon sources, the Ntrb, NtrC, σ^{54} regulon for nitrogen limitation and the *PhoB/PhoR* regulon that is induced under phosphate limiting conditions. If an alternative nutrient source is present in the growth medium, the cells will continue to grow and divide. However, if the environment is totally exhausted for the essential nutrient, the cells have to enter the stationary phase or die. The results shown here demonstrate that the *rpoS* mutant of *F. chinensis* do not survive in starvation medium which is exhausted of all nutrient sources either in the short term such as the stationary phase or for a long term starvation period. Any attempt to recover the mutant cells from a starvation medium failed.

Microphotography of the wild-type and JR101 strain at different incubation time during the exponential and

stationary phases, revealed that there was no difference in cell size and shape during the exponential phase but during the stationary phase the cells of JR101 strain were longer than those of the wild-type. These results are paralleled by those obtained from the CellFacs analysis. The cell size for wild type showed a decline after 26 h of incubation, whilst that of JR101 remained constant. These all suggest that the decrease in cell size seen as *F. chinensis* enters the starvation phase is due to cell division.

Lange and Aronis [5] reported that in *E. coli* the strains with a mutation in the *rpoS* gene exhibited a lower ratio of cell number to cell mass than those with the wild type gene indicating that the mutant had a larger cell volume. This phenotype is particularly pronounced in stationary phase cells. Studying single cells under the light microscope revealed that, in *E. coli*, the *rpoS* gene must be involved in the determination of the size of the cells. They also reported that during exponential growth in rich media, wild-type and *rpoS* mutant cells exhibited the classical rod-shape morphology, but *rpoS*-deficient cells appeared slightly elongated. A dramatic difference in cells shape was observed during the stationary phase. They reported that, while the wild-type cells were almost spherical, *rpoS*-deficient cells exhibited a wide range of cell lengths from filamentous cells to short rod-shaped cells.

The effects of starvation on wild type cells and JR101 at 30°C were examined. The viable cells of JR101 were not detectable after 10 days in the starvation medium, while the wild-type was culturable for up to 30 days. The total cell counts on both cases remained constant. The cell size of the wild-type declined over the first 60 days of starvation while that of JR101 remained constant. Any attempt to recover non culturable JR101 cells was unsuccessful, but wild-type cells recovered their culturability after incubation in a recovery medium. The cell size in wild-type during recovery also increased, while that of JR101 remained constant. It is possible that JR101 cells after starvation are viable but not culturable by the applied methods here. However it is more likely that JR101 cannot respond to starvation conditions as the wild-type does and is unable to enter the starvation survival mode essential for survival under the condition of long term starvation stress.

McCann *et al.* [18] demonstrated that a *rpoS* mutant of *E. coli* survived carbon and nitrogen starvation poorly, as many of the σ^S regulated starvation proteins are common to those induced by other stresses such that there is cross-protection to osmotic, oxidative and heat stresses *rpoS* mutants of *E. coli* die off rapidly under starvation condition and do not develop the resistance to

multiple stresses characteristic for stationary phase [19]. σ^S influence on the maintenance of the culturability of *E. coli* and *S. typhimurium* in sea water closely depends upon the conditions under which cells are grown before they are transferred to sea water. Therefore the growth states of the cells are important as the protective effect of *rpoS* was observed only in stationary phase cells grown at low osmolarity [20]. However, *RpoS* in *V. cholerae* is not critical for *in vivo* survival, as determined by an infant mouse intestinal competition assay [21].

The cell morphology was also studied in both the wild-type and JR101 using transmission electron microscopy. Cells of both strains from the exponential phase of growth could not be differentiated. Electron microscopy of starved wild-type cells compared to starved JR101 showed the changes in the cytoplasm of the wild-type cells which were not observed in JR101. The cytoplasm of starved wild-type cells became clear and shrunk leaving cells which had an odd shape and appearance. In JR101 the cells had a quite different shape and no shrinkage in the cytoplasm was observed. Shrinking of the cytoplasm of starved *E. coli* has been reported by Ozkanka [22] and in *A. hydrophila* by Lim [23]. The results presented here suggest that the shrinkage of the cytoplasm observed during prolonged starvation is due to the miniaturization of the cell during starvation. In JR101 the mutation of the *rpoS* gene prevents the cell responding normally to starvation conditions and the cells remain almost normal.

Analysis of cell size by both the CellFacs analyzer and electron microscopy demonstrated that starvation of *F. chinensis* resulted in a reduction in size and cell volume to the minimal possible size to prevent energy wastage and maintain cell viability. In starvation media, this could occur in two ways either through cell division in the starvation medium or by the reduction in cell size through miniaturization brought about by the utilization of cellular constituents. In these experiments, it has been shown that wild-type cells divide in the stationary phase and in a starvation medium but this cell division was inhibited in JR101 through mutation of the *rpoS* gene. Miniaturization of starved cells of the *rpoS* mutant was also inhibited. At the end our results showed, σ^S , the gene product of *rpoS* gene, have an important role in general in the stationary phase and under starvation conditions and in particular a major role in cell division and cell morphology under these conditions.

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