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# Altered morphology produced by *ftsZ* expression in *Corynebacterium glutamicum* ATCC 13869

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*Corynebacterium glutamicum* is a Gram-positive bacterium that lacks the cell division FtsA protein and actin-like MreB proteins responsible for determining cylindrical cell shape. When the cell division *ftsZ* gene from *C. glutamicum* (*ftsZ*$_{Cg}$) was cloned in different multicopy plasmids, the resulting constructions could not be introduced into *C. glutamicum*; it was assumed that elevated levels of FtsZ$_{Cg}$ result in lethality. The presence of a truncated *ftsZ*$_{Cg}$ and a complete *ftsZ*$_{Cg}$ under the control of P*lac* led to a fourfold reduction in the intracellular levels of FtsZ, generating aberrant cells displaying buds, branches and knots, but no filaments. A 20-fold reduction of the FtsZ level by transformation with a plasmid carrying the *Escherichia coli lacI* gene dramatically reduced the growth rate of *C. glutamicum*, and the cells were larger and club-shaped. Immunofluorescence microscopy of FtsZ$_{Cg}$ or visualization of FtsZ$_{Cg}$–GFP in *C. glutamicum* revealed that most cells showed one fluorescent band, most likely a ring, at the mid-cell, and some cells showed two fluorescent bands (septa of future daughter cells). When FtsZ$_{Cg}$–GFP was expressed from P*lac*, FtsZ rings at mid-cell, or spirals, were also clearly visible in the aberrant cells; however, this morphology was not entirely due to GFP but also to the reduced levels of FtsZ expressed from P*lac*. Localization of FtsZ at the septum is not negatively regulated by the nucleoid, and therefore the well-known occlusion mechanism seems not to operate in *C. glutamicum*.

## INTRODUCTION

FtsZ is one of the most conserved cell division proteins and it have been found in all bacteria examined [except *Chlamydia trachomatis*, *Ureaplasma urealyticum* (Dziadek *et al.*, 2002), and crenarchaea (Margolin, 2000)], chloroplasts (Osteryoung & Vierling, 1995) and certain mitochondria (Osteryoung, 2001). It has been suggested that the primary role of FtsZ is to cause invagination of the cytoplasmic membrane. This hypothesis is supported by the facts that FtsA (Ma *et al.*, 1996) and ZipA (Hale & de Boer, 1997, 2002) bind directly to FtsZ polymers at the future division site, followed by the sequential addition of FtsK, FtsQ, FtsL, FtsW, FtsI and FtsN.

Heterologous expression of the *ftsZ* gene from different micro-organisms in *Escherichia coli* leads to filamentation, probably because heterologous FtsZ interferes with the resident FtsZ (Honrubia *et al.*, 1998; Margolin *et al.*, 1991; Salimnia *et al.*, 2000; Yaoi *et al.*, 2000). This was confirmed by the introduction of FtsZ–GFP from *Rhizobium* (*Sinorhizobium*) *meliloti* into *E. coli* cells, which resulted in the formation of ring structures, suggesting co-localization with the *E. coli* FtsZ within non-functional division rings (Ma *et al.*, 1996). However, when the level of *ftsZ* expression was low, heterologous FtsZ co-localized with endogenous FtsZ, forming a functional FtsZ ring, and the cells divided normally.

The homologous expression of *ftsZ* genes has mainly been studied in *E. coli*, where it has been shown that a two- to sevenfold increase in the level of the FtsZ protein causes increasing numbers of additional septa to form near the cell poles, producing minicells, and that an increase in the level of FtsZ beyond this range results in complete inhibition of cell division (Ward & Lutkenhaus, 1985). Plasmid pZAQ, carrying the complete *ftsQ*, *ftsA* and *ftsZ* genes from *E. coli*, increased FtsZ levels by about sevenfold and increased the frequency of both polar and centrally located septa (Begg *et al.*, 1998; Bi & Lutkenhaus, 1990). The increase in both types of septa was originally attributed to increased FtsZ

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Abbreviation: DAPI, 4′,6-diamino-2-phenylindole.

level, but it was later shown that an increase in both FtsZ and FtsA is required to cause early central division (Begg *et al.*, 1998).

Homologous overexpression of FtsZ has been also studied in *Neisseria gonorrhoeae* (Salimnia *et al.*, 2000), *Halobacterium salinarum* (Margolin *et al.*, 1996) and *Mycobacterium tuberculosis* (Dziadek *et al.*, 2002), among others. In *N. gonorrhoeae*, the expression of FtsZ resulted in abnormal cell division in some cells, characterized by the presence of multiple and atypically arranged cell division sites. *H. salinarium* transformed with a plasmid carrying its own *ftsZ* gene yielded few large but many small transformant colonies. Cells from small colonies contained the original plasmid, whereas the plasmid isolated from large colonies had undergone an internal deletion, leading to an inactivated *ftsZ* gene; this indicated that *ftsZ* expression was deleterious. Furthermore, cells from large colonies were morphologically of the wild-type (containing only the chromosomal *ftsZ* gene), whereas cells from small colonies were pleomorphic (Margolin *et al.*, 1996). In *M. tuberculosis*, unregulated expression of *ftsZ* from constitutive promoters resulted in lethality, and when a small number of transformants was obtained, plasmids isolated from those transformants displayed deletions in the *ftsZ* coding region (Dziadek *et al.*, 2002).

When we attempted to study the homologous expression of *ftsZ* in *Corynebacterium glutamicum* using different high-copy-number vectors and different transformation methods we consistently failed to obtain transformants. Because our goal was to manipulate cell division genes in this industrially important micro-organism, we constructed a strain carrying a truncated *ftsZ* gene and a complete *ftsZ* under the control of a known promoter. Here we describe the morphological changes observed.

## METHODS

**Bacterial strains and plasmids.** All strains and plasmids used are listed in Table 1.

**DNA isolation and manipulation.** Plasmid DNA was isolated from *E. coli* and from corynebacteria using the alkaline lysis method described for *Streptomyces* (Kieser, 1984) but treating corynebacterial cells with lysozyme for 2–3 h at 30 °C.

Total DNA from corynebacteria was isolated using the Kirby method described for *Streptomyces* (Kieser *et al.*, 2000), but cells were treated with lysozyme for 4 h at 30 °C.

Samples of total DNA from different *C. glutamicum* transconjugants were digested with *Eco*RI and hybridized separately with a 789 bp *Nde*I–*Bam*HI fragment from the *C. glutamicum ftsZ* gene ($ftsZ_{Cg}$) isolated from plasmid pPHEZ4 and with a 1·4 kb *Hin*cII fragment from plasmid pULMJ8 carrying *kan*. Both fragments were labelled with digoxigenin according to the manufacturer's (Boehringer Mannheim) instructions.

RNA from different *C. glutamicum* strains was isolated at different culture times in TSB media using the RNeasy kit (Qiagen). For Northern experiments, 20 μg RNA was loaded into a 1·5 % formaldehyde-agarose gel and transferred to nylon membranes. Filters were hybridized with an internal fragment of $ftsZ_{Cg}$ (513 bp *Hin*cII fragment) from *C. glutamicum* radiolabelled by nick-translation.

**Plasmid constructions.** To express $ftsZ_{Cg}$ in *C. glutamicum*, a 2·1 kb *Bgl*II fragment from the *C. glutamicum* chromosome (obtained from plasmid pPHEZQ1; see Table 1) containing the whole *ftsZ* gene and upstream (358 nt) and downstream (433 nt) sequences was cloned into the unique *Bgl*II site of plasmid pUL880M (Adham *et al.*, 2001b) or into the high-copy-number conjugative bifunctional plasmid pECM2 (Jager *et al.*, 1992), creating plasmids pBZ81 and pECZ1 respectively (Table 1). These plasmids were constructed in *E. coli* and transferred to *C. glutamicum* by electroporation or conjugation respectively. The copy number of plasmid pECM2 and derivatives was estimated as 30–40 copies per cell by densitometry as described previously (Santamaria *et al.*, 1984).

To introduce a second copy of $ftsZ_{Cg}$ into the chromosome of *C. glutamicum*, a 3 kb *Eco*RV–*Ecl*136II fragment from plasmid pPHEZQ1 (carrying *ftsQ*, *ftsZ* and the 5′-end of *yfiH*) (Honrubia *et al.*, 2001) was cloned into pK18mob (Schafer *et al.*, 1994) digested with *Nde*I (Klenow filled), creating plasmid pIZ1 (Table 1).

Plasmid pKZLac, used to disrupt the chromosomal copy of $ftsZ_{Cg}$ and to introduce a $ftsZ_{Cg}$ gene under the control of P*lac*, was constructed as follows: a 789 bp *Nde*I (Klenow-filled)–*Bam*HI fragment from plasmid pPHEZ4 (Table 1) encoding the first 263 amino acids from FtsZ was cloned into the *Sma*I–*Bam*HI sites of pK18mob (Table 1).

Plasmid pK18-3ΔZ was constructed by cloning a 1146 bp *Bam*HI (Klenow-filled) fragment from plasmid pPHEZ1 (Table 1) into *Nde*I-digested and Klenow-filled plasmid pK18-3 to integrate, by single recombination, the deleted *ftsZ* into one of the three ORFs located downstream from *ftsZ*.

To make a *ftsZ–gfp* translational fusion, the 3′ end of *ftsZ* was amplified by PCR using primers Zgfp-1 (5′-GCAACCATGGACGGCGCAACTGGCGTCCTG-3′) and Zgfp-3 (5′-CCCTTAAGCATATGGAGGAAGCTGGGTACATCCAGGTCG-3′). These primers were designed to replace the two final codons of *ftsZ* [CAG (Gln) and TAA (stop)] by an *Nde*I site (CAT ATG) (His and Met). All PCR reactions were performed as described previously (Adham *et al.*, 2003) and the amplified fragment was digested with *Bam*HI (present in the amplified fragment) + *Nde*I (CATATG) and cloned together with *gfp* (as a *Nde*I–*Xba*I fragment) into plasmid pET28a, creating pETGFP (Table 1). Therefore the last amino acid of $FtsZ_{Cg}$ will be His instead of Gln, and fused to GFP. The *gfp* gene used was *egfp2* (Enhanced green fluorescent protein) from Clontech, including the V163A and S175G mutations introduced by Siemering *et al.* (1996). The in-frame fused *′ftsZ–gfp* gene was isolated from pETGFP as a *Bam*HI–*Xba*I fragment, sequenced (see later), and cloned into plasmid pK18mob (digested with *Bam*HI and *Xba*I), to give plasmid pKZGFP (Table 1), which was introduced by conjugation into *C. glutamicum* and integrated into its chromosome by single recombination.

Plasmid pK18-3ZG was constructed by cloning a 1·2 kb *Eco*RV–*Bam*HI fragment from pPHEZQ1 (carrying *ftsQ*, *ftsZ′*) and the *′ftsZ–gfp* gene isolated from pETGFP as a *Bam*HI–*Xba*I (Klenow filled) fragment into *Nde*I-digested and Klenow-filled plasmid pK18-3 to integrate, by single recombination, *ftsZ–gfp* into one of the three ORFs located downstream from *ftsZ*.

**DNA sequence determination.** When necessary, both strands of the plasmids constructed were sequenced either manually or with an ALF automated sequencing apparatus (Pharmacia), using specific primers.

**Table 1.** Bacterial strains and plasmids

| Strain or plasmid | Relevant genotype or description | Source or reference |
|---|---|---|
| ***E. coli*** | | |
| DH5α | $r^- m^-$; used for general cloning | Hanahan (1983) |
| JM109 (DE3) | JM109 derivative containing a chromosomal copy of the gene for T7 RNA polymerase | Promega |
| S17-1 | Mobilizing donor strain, *pro recA*; has an RP4 derivative integrated into the chromosome | Schafer *et al.* (1990) |
| ***C. glutamicum*** | | |
| 13869 | Wild-type | ATCC* |
| R31 | 13869 derivative used as host for transformation, electroporation or conjugation | Santamaria *et al.* (1985) |
| AR1 | R31 derivative containing two copies of *ftsZ* by stable integration of pIZ1 | This work (Fig. 1) |
| AR2 | R31 derivative containing an incomplete copy of *ftsZ* under its own promoter and a complete copy of *ftsZ* under P*lac* obtained by integration of pKZLac | This work (Fig. 1) |
| AR2A | R31 derivative containing an incomplete copy of *ftsZ* under its own promoter and a complete copy of *ftsZ* under P*lac* obtained by integration of pOJZLac | This work |
| AR12 | R31 derivative containing an incomplete copy of *ftsZ* and a complete copy of *ftsZ* under their own promoters obtained by integration of pK18-3ΔZ | This work (Fig. 1) |
| AR20 | *C. glutamicum* AR2 derivative containing pECKX99E | This work |
| AR5 | R31 derivative containing an incomplete copy of *ftsZ* and a complete copy of *ftsZ–gfp* under their own promoters obtained by stable integration of pKZGFP | This work (Fig. 1) |
| AR50 | *C. glutamicum* AR2 derivative containing an incomplete copy of *ftsZ* and a complete copy of *ftsZ–gfp* under P*lac* obtained by stable integration of pOJZ-GFP | This work (Fig. 1) |
| ML13 | R31 derivative containing a complete copy of *ftsZ–gfp* and a complete copy of *ftsZ* under their own promoters obtained by integration of pK18-3ZG | This work (Fig. 1) |
| **Plasmids** | | |
| pBS KS/SK | *E. coli* vectors containing *bla*, *lacZ*, *orif1* | Stratagene |
| pULMJ8 | pBR322 derivative containing the *kan* gene from Tn*5* and used as source of *kan* | Fernandez-Gonzalez *et al.* (1994) |
| pPHEZQ1 | 2·9 kb *Xho*I–*Sac*I fragment (*ftsZ*) subcloned in pBSK+ | Honrubia *et al.* (2001) |
| pECM2 | Mobilizable *E. coli*/*C. glutamicum* bifunctional plasmid containing *kan* and *cat* | Jager *et al.* (1992) |
| pUL880M | Bifunctional *E. coli*/*C. glutamicum* promoter-probe vector with *bla* and *hyg* genes as selective markers and the promoterless *kan* gene as a reporter gene | Adham *et al.* (2001b) |
| pECZ1 | pECM2 derivative containing a 2·1 kb *Bgl*II fragment from pPHEZQ1 carrying *ftsQ* (incomplete), *ftsZ* and *yfih* (incomplete) from *C. glutamicum* | This work |
| pBZ81 | pUL880M derivative containing a 2·1 kb *Bgl*II fragment from pPHEZQ1 carrying *ftsQ* (incomplete), *ftsZ* and *yfih* (incomplete) from *C. glutamicum* | This work |
| pK18mob | Mobilizable plasmid containing an *E. coli* origin of replication and *kan* | Schafer *et al.* (1994) |
| pIZ1 | pK18mob derivative carrying a 3 kb *Eco*RV–*Ecl*136II fragment from *C. glutamicum* containing *ftsQ*, *ftsZ* and the 5′-end of *yfih* | This work |
| pT7.7 | *E. coli* vector containing *bla* and promoter $\phi$10 | Tabor & Richardson (1985) |
| pPHEZ4 | pT7.7 derivative containing *ftsZ* from *C. glutamicum* without upstream sequences | This work |
| pKZLac | pK18mob carrying a 789 bp *Nde*I (Klenow filled)–*Bam*HI fragment from pPHEZ4 encoding the first 263 amino acids of FtsZ | This work |
| pOJ260 | Mobilizable plasmid containing an *E. coli* origin of replication and the apramycin resistance gene | Bierman *et al.* (1992) |
| pOJZLac | pOJ260 carrying a 789 bp *Nde*I (Klenow filled)–*Bam*HI fragment from pPHEZ4 encoding the first 263 amino acids of FtsZ | This work |
| pK18-3 | Mobilizable plasmid containing an *E. coli* origin of replication, *kan* and the three *C. glutamicum* ORFs of unknown function located downstream from *ftsZ* | Adham *et al.* (2001a) |
| pK18-3ΔZ | pK18-3 derivative containing a 1146 bp *Bam*HI (Klenow-filled) fragment from pPHEZ1/EZQ1 encoding the first 263 amino acids of FtsZ and upstream sequences | This work |
| pECKX99E | Bifunctional *E. coli*/*C. glutamicum* plasmid containing *lacI*$^q$ | Amann *et al.* (1988) |
| pET-28a(+) | *E. coli* vector containing *kan*, *lacI*, *orif1*, N-terminal and C-terminal His-tag | Novagen |
| pETGFP | pET28a derivative containing the in-frame fused *′ftsZ–gfp* gene | This work |
| pKZGFP | pK18mob derivative containing the in-frame fused *′ftsZ–gfp* gene | This work |
| pOJZ-GFP | pOJ260 derivative containing the in-frame fused *′ftsZ–gfp* gene | This work |
| pK18-3ZG | pK18-3 derivative containing *ftsZ–gfp* and upstream sequences | This work |

*ATCC, American Type Culture Collection.

**Preparation of cell-free extracts, PAGE and Western blotting.** Cell-free extracts of *C. glutamicum* were prepared by sonication as described previously (Ramos *et al.*, 2003b). SDS-PAGE was carried out essentially as described by Laemmli (1970). After electrophoresis, proteins were stained with Coomassie blue or electroblotted to PVDF membranes (Millipore) and immunostained with anti-FtsZ polyclonal antibodies raised against purified His-tagged FtsZ from *C. glutamicum* (Honrubia *et al.*, 2005). The dilution of the anti-FtsZ antibodies used was 1 : 10 000.

**Microscopic techniques.** Scanning electron microscopy and fluorescence microscopy of *C. glutamicum* cells were performed according to protocols described previously (Ramos *et al.*, 2003b). Staining with DAPI (4′,6-diamino-2-phenylindole) and immunofluorescence microscopy was performed as described by Daniel *et al.* (2000), except that *C. glutamicum* cells were permeabilized overnight at 30 °C with lysozyme at a final concentration of 10 mg $ml^{-1}$. Permeabilized cells were incubated overnight at 4 °C with 1 : 10 000 dilution of the above-mentioned anti-$FtsZ_{Cg}$ antiserum. Anti-rabbit fluorescein isothiocyanate conjugate (Sigma) at 1 : 10 000 dilution was used as secondary antiserum.

## RESULTS

### Overexpression of *ftsZ*$_{Cg}$ is lethal in *C. glutamicum*, as in *E. coli*

It has been described that expression of *ftsZ*$_{Cg}$ in *E. coli* is lethal and leads to filamentation (Honrubia *et al.*, 1998) whereas *ftsZ*$_{Cg}$ overexpression in *E. coli* leads to the creation of deleted and innocuous variants of $FtsZ_{Cg}$ (Honrubia *et al.*, 2005). In order to study the effect of multiple copies of *ftsZ*$_{Cg}$ in *C. glutamicum*, the bifunctional plasmid pBZ81 [a pUL880M derivative containing the whole *ftsZ* gene and upstream (358 nt) and downstream (433 nt) sequences] was introduced into *C. glutamicum* by electroporation or by using protoplasts in the presence of PEG; after several transformation and electroporation experiments, no transformants were obtained in any case. Control experiments using pUL880M were always successful ($10^5$ transformants per μg DNA). Occasionally, a small number (< 10 transformants per μg DNA) of hygromycin-resistant transformants was obtained, but these transformants bore plasmids that had undergone rearrangements or deletions. Similar results were obtained by Dziadek *et al.* (2002) when expressing *M. tuberculosis* FtsZ in *M. tuberculosis* under the control of different constitutive promoters.

Because, at least in our hands when using *C. glutamicum* R31, conjugation was a more efficient method than transformation or electroporation for introducing plasmid DNA into *C. glutamicum*, plasmid pECZ1 [a pECM2 derivative containing the whole *ftsZ* gene and upstream (358 nt) and downstream (433 nt) sequences] was introduced into *C. glutamicum* cells by conjugation. No *C. glutamicum* transconjugants were obtained, indicating the lethality of the *ftsZ* gene in *C. glutamicum* when cloned in high-copy-number vectors. As above, control experiments using pECM2 were always successful ($10^3$–$10^4$ transconjugants per $10^7$ donor *E. coli* cells).

### Introduction of a second copy of *ftsZ* into the chromosome of *C. glutamicum*

pUL880M and pECM2 are multicopy plasmids (approx. 30 copies per cell) derived from the endogenous plasmids pBL1 (Santamaria *et al.*, 1984) and pCG1 (Jager *et al.*, 1992) respectively. Therefore, the high level of FtsZ produced might be responsible for the lethality of FtsZ as described previously (Margolin *et al.*, 1996). Because of the lack of low-copy-number vectors for corynebacteria, we introduced a second copy of *ftsZ* into the genome of *C. glutamicum* by homologous recombination. The conjugative suicide plasmid pIZ1 (Table 1) was introduced into *C. glutamicum* by conjugation. Kanamycin-resistant transconjugants were morphologically wild-type, and after Southern blot analysis (not shown) it was confirmed that a second copy of *ftsZ* was present in their genome. One of these transconjugants was named *C. glutamicum* AR1 (Table 1, Fig. 1). We therefore conclude that two copies of *ftsZ* under the control of their own promoters do not negatively affect the morphology or the viability of *C. glutamicum*.

### Construction of *C. glutamicum* strains carrying a unique complete copy of *ftsZ* under the control of P*lac*

To confirm the lethality of high levels of *ftsZ*$_{Cg}$ expression in *C. glutamicum*, we disrupted the chromosomal copy of *ftsZ* and replaced it by an *ftsZ*$_{Cg}$ gene under the control of the *lac* promoter (P*lac*) from *E. coli*. This promoter was chosen because it has been described as an efficient promoter in *C. glutamicum* (Tsuchiya & Morinaga, 1988).

Plasmid pKZLac was introduced by conjugation into *C. glutamicum* and transconjugants were selected by resistance to kanamycin. Southern analysis of the transconjugants showed the expected DNA pattern, which confirmed the integration of pKZLac by single recombination. The resulting strain has a partial *ftsZ* gene (capable of expressing a C-terminally truncated product of 263 amino acids) in the original chromosomal position and a copy of *ftsZ* (438 amino acids) under the control of P*lac*. One of these transconjugants was named *C. glutamicum* AR2 (Fig. 1). We assumed that the partial *ftsZ* would encode a non-functional FtsZ, whereas P*lac* would direct the expression of the complete *ftsZ* gene. The transconjugants showed a slower growth rate than that of *C. glutamicum* R31, and an aberrant morphology was observed by phase-contrast microscopy (Fig. 2a) and scanning electron microscopy (Fig. 2b).

To rule out the possibility that this aberrant morphology might be due to the expression of the partial *ftsZ* gene, still present in the chromosome of *C. glutamicum* AR2 (see Fig. 1), plasmid pK18-3ΔZ (Table 1) was introduced by conjugation into *C. glutamicum* to insert the partial *ftsZ* gene in a non-essential chromosomal region using a strategy designed to introduce any gene into the *C. glutamicum* chromosome (Adham *et al.*, 2001a). The new strain, *C. glutamicum* AR12, contained the partial *ftsZ* and the original

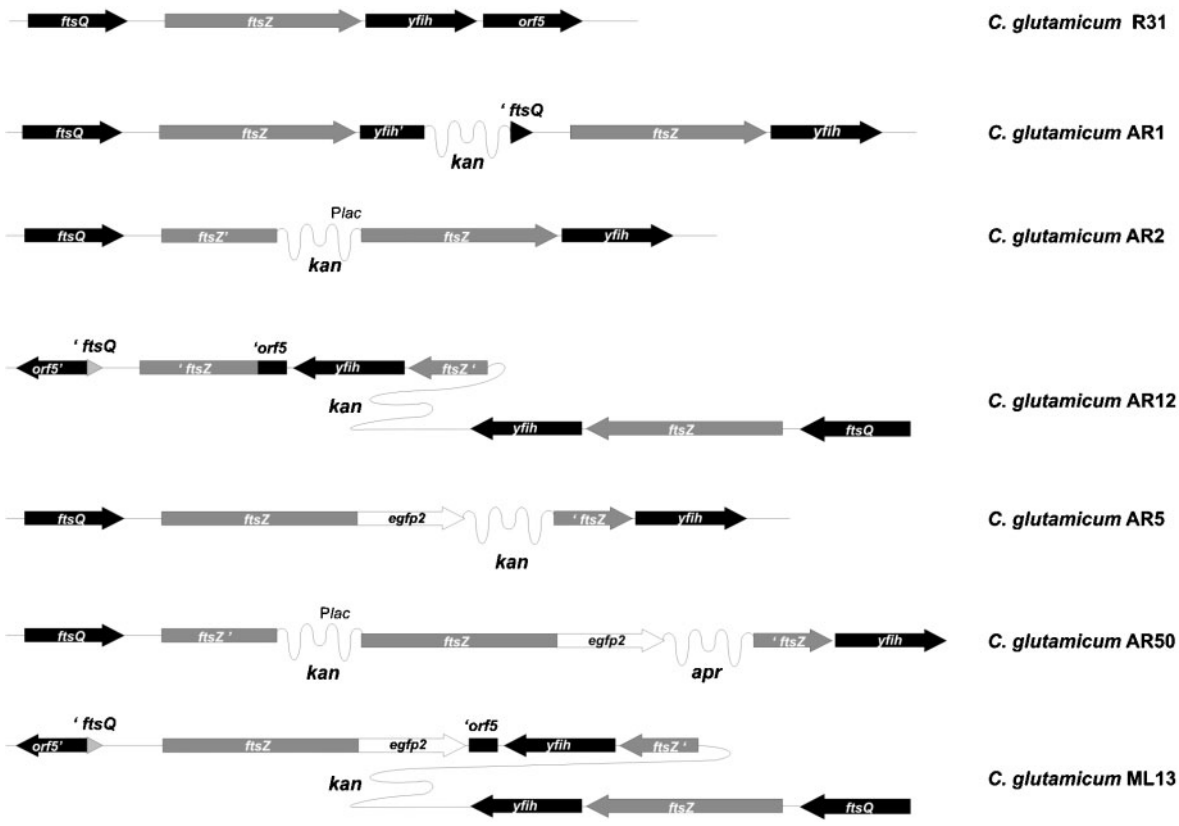


**Fig. 1.** Schematic representation of the chromosomal DNA regions around the *ftsZ* gene in the different *C. glutamicum* strains constructed in this work. Arrows represents ORFs. *'gen* and *gen'* represent a gene lacking the 5′-end or the 3′-end respectively. *egfp2* is the gene encoding the GFP used in this work. P*lac* is the promoter of the *E. coli lac* operon. Wavy lines represent a plasmid inserted in the chromosomal DNA carrying the kanamycin (*kan*) or the apramycin (*apr*) resistance gene.

*ftsZ* under the control of their endogenous promoters (Fig. 1). This strain was morphologically wild-type and we therefore concluded that the aberrant morphology of *C. glutamicum* AR2 must be due to the expression of *ftsZ* under the control of P*lac*.

Because P*lac* has been described to be an inducible promoter in *C. glutamicum* (Tsuchiya & Morinaga, 1988), and because there is no *lacI*, nor *lac* operon, in the genome of *C. glutamicum* (A. Ramos, unpublished), we assumed that *ftsZ* was being overexpressed and that the aberrant morphology observed was due to high levels of $FtsZ_{Cg}$. To confirm this hypothesis, RNA was isolated from exponentially growing cells of *C. glutamicum* R31 and AR2 and hybridized with an internal fragment of $ftsZ_{Cg}$. The amount of specific mRNA for *ftsZ* in *C. glutamicum* R31 (measured by densitometry) was roughly 1·5–2 times higher than the level of mRNA for *ftsZ* in *C. glutamicum* AR2 (data not shown). Western blot experiments with polyclonal anti-$FtsZ_{Cg}$ antibodies gave similar results (four times more FtsZ protein in *C. glutamicum* R31 than in *C. glutamicum* AR2 measured by densitometry) (Fig. 2c). In sum, filamentous cells containing buds, knots and branch-like outgrowths are obtained at FtsZ concentrations fourfold below physiological levels.

To see the phenotypic effect of much more reduced $ftsZ_{Cg}$ expression from P*lac*, the $lacI^q$ gene from *E. coli* present in plasmid pECKX99E (30 copies per cell) (Table 1) was introduced into *C. glutamicum* AR2A (Table 1). The resulting strain (*C. glutamicum* AR20) showed a more reduced level of FtsZ (20 times less FtsZ than *C. glutamicum* R31) (Fig. 2c), a marked globular morphology (Fig. 2a, b) and a much slower growth rate than *C. glutamicum* AR2 (Fig. 3, Table 2).

## Visualization of $FtsZ_{Cg}$ in *C. glutamicum*

Taking into account previous results concerning the lethality of $FtsZ_{Cg}$ overexpression in *C. glutamicum*, in order to visualize FtsZ in living cells we constructed a strain [*C. glutamicum* AR5 (Fig. 1)] bearing *ftsZ–gfp* as a single copy in the chromosome, using the conjugative suicide plasmid pKZ-GFP (Table 1). The localization of $FtsZ_{Cg}$–GFP was characterized in live *C. glutamicum* AR5 (Fig. 4) cells from exponential-phase liquid cultures ($OD_{600}$ 1). Phase-contrast microscopy revealed that cells were twice as large as the wild-type, as has been described for *E. coli* (Sun & Margolin, 1998). Fluorescence microscopy revealed that most of the cells showed a fluorescent band,

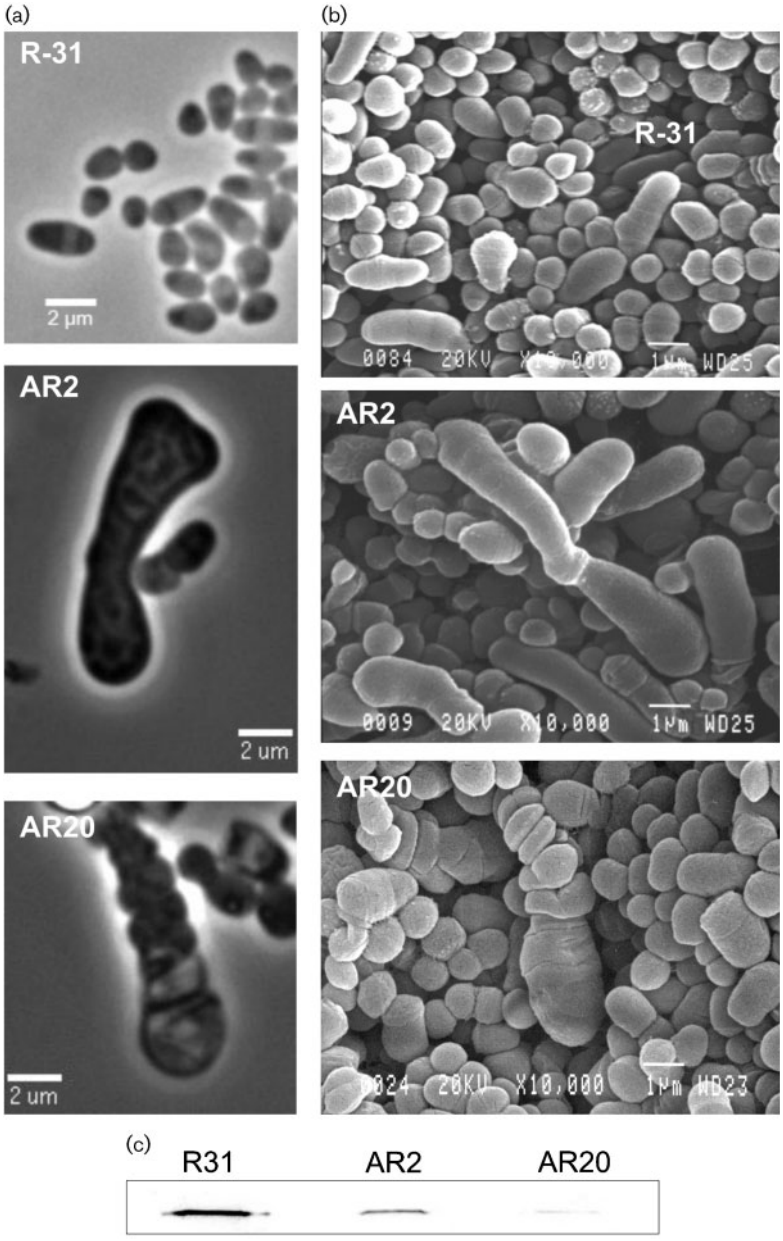


**Fig. 2.** (a, b) Phase-contrast microscopy (a) and scanning electron microscopy (b) of *C. glutamicum* strains R31, AR2 and AR20. (c) Western blot analysis of total protein extracts obtained from these three strains probed with polyclonal anti-$FtsZ_{Cg}$ antibody. The amount of protein loaded was 1 μg and it was obtained from cultures at an $OD_{600}$ of 1.

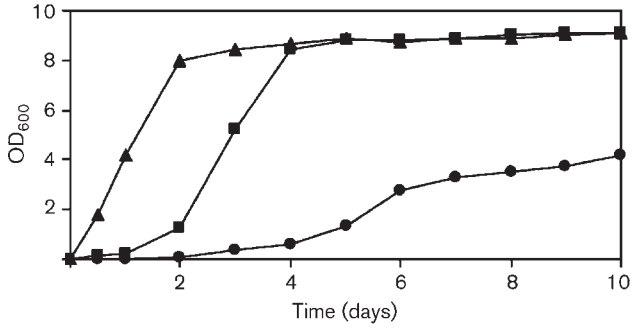


**Fig. 3.** Growth kinetics of *C. glutamicum* strains R31 (▲), AR2 (■) and AR20 (●) in TSA medium (Difco) without kanamycin (for R31) or with 25 μg kanamycin $ml^{-1}$ (for AR2 and AR20).

**Table 2.** Viable counts of three *C. glutamicum* strains at various $OD_{600}$ values

| $OD_{600}$ | Viable count (cells $ml^{-1}$) | | |
|---|---|---|---|
| | R31 | AR2 | AR20 |
| 1·2 | $1{\cdot}5 \times 10^8$ | $3{\cdot}3 \times 10^6$ | $8{\cdot}1 \times 10^6$ |
| 3·1 | $4{\cdot}1 \times 10^8$ | $7{\cdot}2 \times 10^6$ | $9{\cdot}5 \times 10^7$ |
| 6·2 | $7{\cdot}2 \times 10^8$ | $2{\cdot}2 \times 10^7$ | –* |
| 8·4 | $4{\cdot}1 \times 10^9$ | $2{\cdot}9 \times 10^8$ | –* |

*Strain AR20 did not reach these $OD_{600}$ values (see Fig. 3).

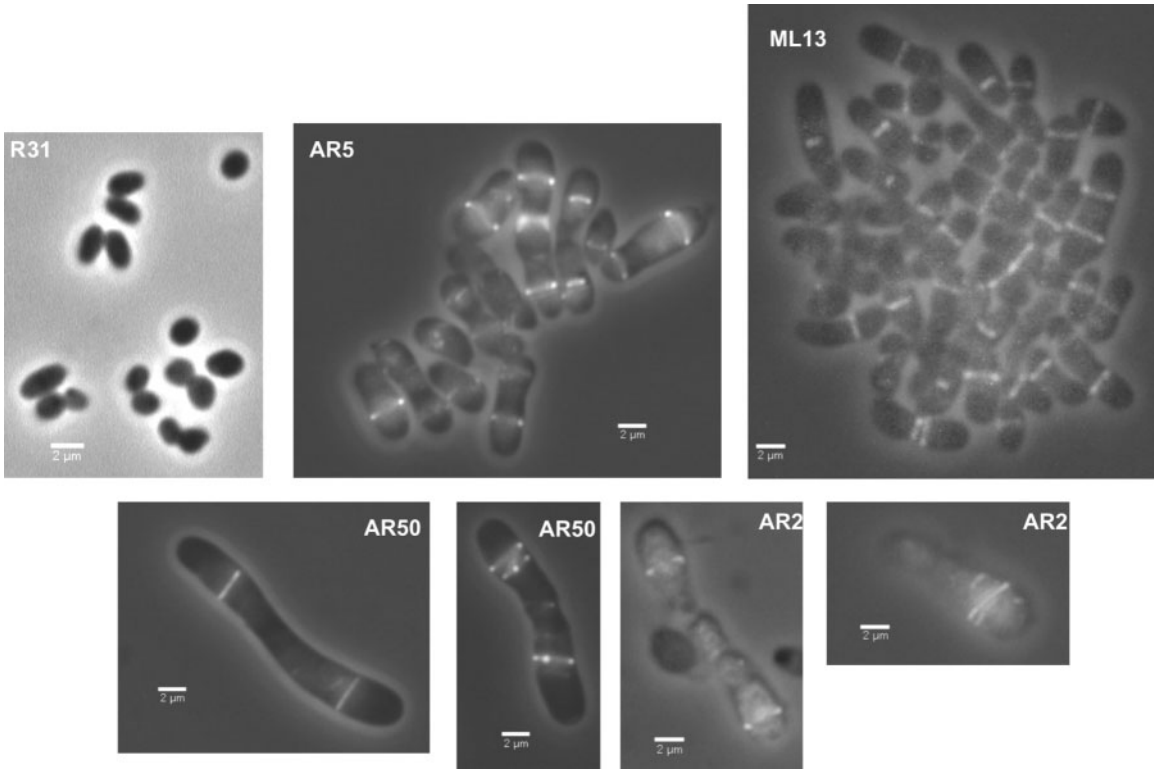


**Fig. 4.** FtsZ distribution in *C. glutamicum* strains AR5 (*ftsZ–gfp* as a single copy in the chromosome), AR50 (*Plac–ftsZ–gfp* as a single copy in the chromosome), ML13 (merodiploid) and AR2. Note that the pictures of *C. glutamicum* AR5, AR50 and ML13 were taken by fluorescence microscopy (FtsZ–GFP) whereas those of *C. glutamicum* AR2 were taken by immunofluorescence microscopy. Phase-contrast microscopy of *C. glutamicum* R31 was included as control. Scale bars, 2 μm.

probably a ring, at the mid-cell; these bands were not detected by phase-contrast microscopy, indicating that they were not insoluble inclusions but true cytoskeletal structures. These cells were probably in division, suggesting that FtsZ–GFP polymerizes and permits septation. It was also possible to observe larger cells containing two FtsZ rings in the mid-cell of the future daughter cells. It may be concluded that FtsZ–GFP is mostly functional because it can completely replace wild-type $FtsZ_{Cg}$ with no significant morphological problems. The apparent off-centre localization of the FtsZ ring is not unexpected because *C. glutamicum* does not divide symmetrically (M. Letek, unpublished).

## Visualization of $FtsZ_{Cg}$ in *C. glutamicum* carrying *ftsZ* under the control of P*lac*

Plasmid pOJZ-GFP (a suicide conjugative plasmid containing an apramycin resistance gene and the in-frame fused ′*ftsZ–gfp* gene; Table 1) was introduced by conjugation from *E. coli* into *C. glutamicum* AR2 and transconjugants were selected by resistance to kanamycin and apramycin. Two types of transconjugants were anticipated: those that would arise by single recombination between pKZlac previously integrated into the chromosome of *C. glutamicum* AR2 and pOJZ-GFP, and those integrated at the 3′-end of $ftsZ_{Cg}$ under the control of P*lac*. Thirty-four kanamycin- and apramycin-resistant transconjugants were obtained, and two of these showed fluorescence when observed under the fluorescence microscope. Total DNA was isolated from these transconjugants, and both of them exhibited the expected Southern hybridization pattern (not shown). One of these transconjugants was named *C. glutamicum* AR50 and its genetic structure around *ftsZ* was determined (Fig. 1).

Fluorescence microscopy revealed that most of the *C. glutamicum* AR50 cells showed one or two fluorescent bands, probably rings, at the mid-cell, and these bands were true cytoskeletal structures (Fig. 4). Spirals or multiple FtsZ septa were also present in some aberrant cells.

Because in *C. glutamicum* AR5 and *C. glutamicum* AR50 the only functional copy of FtsZ is fused to GFP, we constructed a merodiploid strain (*C. glutamicum* ML13) carrying a functional *ftsZ* allele in addition to the *ftsZ–gfp* fusion. In such contexts, the fusions often interfere less with division or growth and may give more reliable localization data. Fluorescence microscopy of *C. glutamicum* ML13 revealed that FtsZ–GFP localizes mainly in the septum, and because the growth rate of this strain is normal, we can conclude

that $FtsZ_{Cg}$–GFP copolymerizes with $FtsZ_{Cg}$ and permits septation.

### The nucleoid does not inhibit the localization of FtsZ at mid-cell in *C. glutamicum*

To confirm the localization data of FtsZ obtained using the merodiploid strain *C. glutamicum* ML13, immunofluorescence microscopy was used to determine the localization of FtsZ in exponentially growing cells from *C. glutamicum* strains R31 and AR2, the parental strains of C. *glutamicum* AR5 and *C. glutamicum* AR50 respectively (see Fig. 1). Immunofluorescence microscopy of *C. glutamicum* R31 revealed that most of the cells showed a fluorescence band at the mid-cell overlapping with the nucleoid (Fig. 5, vertical arrows). These results might indicate that the assembly of the FtsZ ring at the cell division site in *C. glutamicum* is not negatively regulated by the nucleoid as in *E. coli* (Sun & Margolin, 2004) or *Bacillus subtilis* (Wu & Errington, 2004). Two FtsZ rings can be observed after nucleoid segregation (Fig. 5, horizontal arrows) and a reduced polar accumulation of FtsZ in some small, probably just replicated, cells which is interpreted as a remnant of the previous FtsZ ring.

Immunofluorescence microscopy of C. *glutamicum* AR2 (Fig. 4) confirmed the presence of FtsZ rings or spirals when the expression of FtsZ takes place from P*lac*.

## DISCUSSION

It is becoming clear that bacteria have a cytoskeleton composed of structural homologues of tubulin and actin. This cytoskeleton is responsible for cell growth, division and shape. FtsZ is the structural homologue of tubulin, and FtsA/MreB/MreB-like protein (Mbl) are the structural homologues of actin. FtsZ and FtsA are required for cell division, whereas MreB and Mbl are involved in cell shape and localize as a helical filament that guides dispersed cell wall biosynthesis (Daniel & Errington, 2003; Margolin, 2003).

Corynebacteria are Gram-positive micro-organisms that lack both the FtsA and MreB systems and therefore lack the structural homologues of actin. How, then, do corynebacteria control cell shape, division and growth in the absence of a typical actin homologue?

*C. glutamicum* formed elongated, club-shaped or dumbbell-shaped rods, but not filaments, in the presence of antibiotics that inhibit septation or DNA synthesis (Kijima *et al.*, 1998), and similar cells were obtained when a single copy of $ftsZ_{Cg}$ was expressed under the control of P*lac* in *C. glutamicum* AR2 (see Fig. 2). The presence of *lacI* (*C. glutamicum* AR20) led to a reduced growth rate and to the presence of more cells with a club-shaped morphology. This phenotype is very similar to that obtained when $divIVA_{Cg}$ is overexpressed in *C. glutamicum* (Ramos *et al.*, 2003b); the main difference between the two phenotypes is the inhibition of cell division when the expression of FtsZ is diminished. *C. glutamicum*

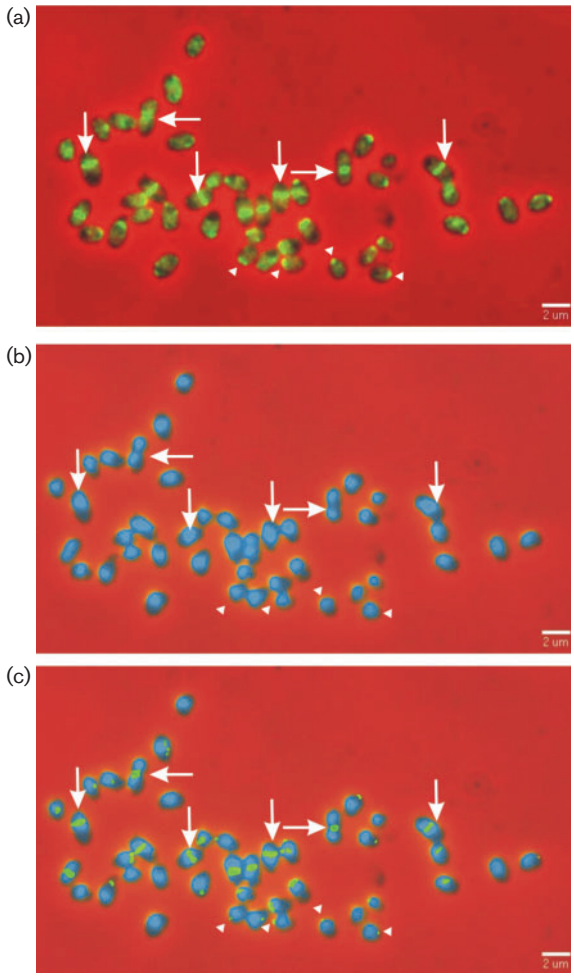


**Fig. 5.** FtsZ and nucleoid distribution in *C. glutamicum* R31. (a) FtsZ immunofluorescence staining. (b) DAPI staining. (c) Overlay of (a) and (b). Vertical arrows indicate single FtsZ rings (in panel a) and DAPI-stained nucleoid (in b). The horizontal arrow marks a dividing cell with two possible FtsZ rings and two separated nucleoids. Note the presence of apical FtsZ foci at one end of some just divided cells (arrowheads in a).

AR2 and *C. glutamicum* R31 reached the same optical density after 96 h of growth, but the number of viable cells was one or two orders of magnitude higher in R31 than in AR2 for the same $OD_{600}$ (Table 2).

Our results concerning the expression of $ftsZ_{Cg}$ under P*lac* are in partial agreement with the results obtained in *Rhizobium* (*Sinorhizobium*) *meliloti* (another rod-shaped bacterium that lacks MreB homologues) (Latch & Margolin, 1997) and *Mycobacterium tuberculosis* (Dziadek *et al.*, 2002). It may be concluded that in rod-shaped micro-organisms lacking MreB, branching and swelling are default pathways for increasing mass when cell division is blocked either by FtsZ overproduction (Latch & Margolin, 1997) or by partial depletion of FtsZ (this work).

Daniel & Errington (2003) used fluorescent vancomycin to determine the spatial pattern of peptidoglycan biosynthesis in *C. glutamicum*, and observed that cell division septa and cell poles were labelled by vancomycin. The labelling of cell division septa confirms the notion that peptidoglycan synthesis takes place at the septum, but the labelling of cell poles implies that peptidoglycan biosynthesis occurs mainly at the poles in the absence of the helical scaffold MreB. This type of apical growth and peptidoglycan biosynthesis at the cell poles has been described previously for *Corynebacterium diphtheriae* (Umeda & Amako, 1983). Two different proteins have been found localized at the cell poles of *C. glutamicum*: an inorganic pyrophosphatase (PPase) and DivIVA$_{Cg}$ (Ramos *et al.*, 2003a, b). Pyrophosphate is a by-product in the biosynthesis of UDP-*N*-acetylglucosamine by UDP-*N*-acetylglucosamine pyrophosphorylase (EC 2.7.7.23) and hydrolysis of pyrophosphate by PPase is essential for cell wall biosynthesis (Ramos *et al.*, 2003a). DivIVA$_{Cg}$ might form oligomeric structures (perhaps through its coiled-coil regions) with a possible structural function at *C. glutamicum* growing cell poles, and probably accumulates there through interaction with proteins involved in peptidoglycan biosynthesis. Is DivIVA$_{Cg}$ the scaffold for peptidoglycan biosynthesis in MreB-lacking rod-shaped corynebacteria? DivIVA$_{Cg}$ seems to be an essential protein, and therefore it was not possible to disrupt *divIVA*$_{Cg}$ (Ramos *et al.*, 2003b). Further experiments will be needed to deplete DivIVA$_{Cg}$ in order to study its possible effect on cell morphology and peptidoglycan biosynthesis by vancomycin staining.

Microscopic studies of *C. glutamicum* carrying a chromosomal *ftsZ*$_{Cg}$*–gfp* gene fusion under the control of the *ftsZ*$_{Cg}$ endogenous promoters clearly indicated that these cells were probably in division, suggesting that FtsZ$_{Cg}$–GFP polymerizes and permits septation. Spirals of FtsZ have been observed in *E. coli* overexpressing FtsZ–GFP (Ma *et al.*, 1996) as well as in *B. subtilis* in the switch from medial to asymmetric division prior to sporulation (Ben Yehuda & Losick, 2002). In contrast, spirals were observed in *C. glutamicum* when *ftsZ* was expressed at low levels.

It was also possible to see small polar aggregates of FtsZ in *C. glutamicum* R31 by immunofluorescence microscopy that may represent FtsZ left from the previous division or a polar Z ring involved in the next division cycle, because *C. glutamicum* seems to grow in a polar fashion (Daniel & Errington, 2003).

In contrast to *E. coli* or *B. subtilis*, the localization of the FtsZ ring at the mid-cell in *C. glutamicum* is not negatively regulated by nucleoid occlusion (see Fig. 5), nor by the MinCD system, since no homologue of MinCD has been detected in the *C. glutamicum* genome (Ramos *et al.*, 2003b). It is becoming clear that corynebacteria have a mechanism of cell division and peptidoglycan synthesis different from that in other classical rod-shaped bacteria and well-known model organisms; therefore the study of cell division in corynebacteria may reveal a different model of cell division and peptidoglycan biosynthesis.

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