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

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 (ftsZCg) was cloned in different multicopy plasmids, the resulting constructions could not be introduced into C. glutamicum; it was assumed that elevated levels of FtsZCg result in lethality. The presence of a truncated ftsZCg and a complete ftsZCg under the control of Plac 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 FtsZCg or visualization of FtsZCg–GFP in C. glutamicum revealed that most cells showed one fluorescent band, most likely a ring, at the mid-cell, and some showed two fluorescent bands (septa of future daughter cells). When FtsZCg–GFP was expressed from Plac, 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 Plac. 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.

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 (Salminia et al., 2000), Halobacterium salinarium (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 EcoRI and hybridized separately with a 789 bp Ndel–BamHI fragment from the C. glutamicum ftsZ gene (ftsZCg) isolated from plasmid pPHEZ1 and with a 1·4 kb HinII fragment from plasmid pUL880M 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 RNAspar KI (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 ftsZCg (513 bp HinII fragment) from C. glutamicum radiolabelled by nick-translation.

**Plasmid constructions.** To express ftsZCg in C. glutamicum, a 2·1 kb BglII 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 BglII 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 (Santamaría et al., 1984).

To introduce a second copy of ftsZCg into the chromosome of C. glutamicum, a 3 kb EcoRV–EcoI fragment from plasmid pPHEZQ1 (carrying ftsQ, ftsZ and the 5′-end of yHiH) (Honrubia et al., 2001) was cloned into pK18mob (Schafer et al., 1994) digested with Ndel (Klenow filled), creating plasmid pIZ1 (Table 1).

Plasmid pKZLac, used to disrupt the chromosomal copy of ftsZCg and to introduce a ftsZCg gene under the control of Plac, was constructed as follows: a 789 bp Ndel (Klenow-filled)–BamHI fragment from plasmid pPHEZ4 (Table 1) encoding the first 263 amino acids from FtsZ was cloned into the Smal–BamHI sites of pK18mob (Table 1).

Plasmid pK18-3AZ was constructed by cloning a 1146 bp BamHI (Klenow-filled) fragment from plasmid pPHEZ1 (Table 1) into Ndel-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′-GCAACCATGGAGGCGACAC-TGGCTGCTGTG-3′) and Zgfp-3 (5′-CCCTTAAGCATATGAGGAGAG-CTGGTACATCCAGGTCG-3′). These primers were designed to replace the two final codons of ftsZ [CAG (Gln) and TAA (stop)] by an Ndel site (CAT ATG) (His and Met). All PCR reactions were performed as described previously (Adham et al., 2003) and the amplified fragment was ligated with BamHI (present in the amplified fragment) and Ndel (CATATG) and cloned together with gfp (as a Ndel–XbaI fragment) into plasmid pET28a, creating pETGFP (Table 1). Therefore the last amino acid of FtsZ 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 BamHI–XhoI fragment, sequenced (see later), and cloned into plasmid pK18mob (digested with BamHI and XhoI), 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 EcoRV–BamHI fragment from plasmid pPHEZQ1 (carrying ftsQ, ftsZ) and the ‘ftsZ–gfp’ gene isolated from pETGFP as a BamHI–XhoI (Klenow filled) fragment into Ndel-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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or description</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>r− m−; used for general cloning</td>
<td>Hanahan (1983)</td>
</tr>
<tr>
<td>JM109 (DE3)</td>
<td>JM109 derivative containing a chromosomal copy of the gene for T7 RNA polymerase</td>
<td>Promega</td>
</tr>
<tr>
<td>S17-1</td>
<td>Mobilizing donor strain, pro recA; has an RP4 derivative integrated into the chromosome</td>
<td>Schafer et al. (1990)</td>
</tr>
<tr>
<td><strong>C. glutamicum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13869</td>
<td>Wild-type</td>
<td>ATCC*</td>
</tr>
<tr>
<td>R31</td>
<td>13869 derivative used as host for transformation, electroporation or conjugation</td>
<td>Santamaria et al. (1985)</td>
</tr>
<tr>
<td>AR1</td>
<td>R31 derivative containing two copies of ftsZ by stable integration of pIZ1</td>
<td>This work (Fig. 1)</td>
</tr>
<tr>
<td>AR2</td>
<td>R31 derivative containing an incomplete copy of ftsZ under its own promoter and a complete copy of ftsZ under Plac obtained by integration of pKZLac</td>
<td>This work (Fig. 1)</td>
</tr>
<tr>
<td>AR2A</td>
<td>R31 derivative containing an incomplete copy of ftsZ under its own promoter and a complete copy of ftsZ under Plac obtained by integration of pOJZLac</td>
<td>This work</td>
</tr>
<tr>
<td>AR12</td>
<td>R31 derivative containing an incomplete copy of ftsZ and a complete copy of ftsZ under their own promoters obtained by integration of pK18-3ΔZ</td>
<td>This work (Fig. 1)</td>
</tr>
<tr>
<td>AR20</td>
<td>C. glutamicum AR2 derivative containing pEKX99E</td>
<td>This work</td>
</tr>
<tr>
<td>AR5</td>
<td>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</td>
<td>This work (Fig. 1)</td>
</tr>
<tr>
<td>AR50</td>
<td>C. glutamicum AR2 derivative containing an incomplete copy of ftsZ and a complete copy of ftsZ–gfp under Plac obtained by stable integration of pOJZ-GFP</td>
<td>This work (Fig. 1)</td>
</tr>
<tr>
<td>ML13</td>
<td>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</td>
<td>This work (Fig. 1)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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</tr>
<tr>
<td>pBS KS/KS</td>
<td>E. coli vectors containing bla, lacZ, orif1</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pULM183</td>
<td>pBR322 derivative containing the kan gene from Tn5 and used as source of kan</td>
<td>Fernandez-Gonzalez et al. (1994)</td>
</tr>
<tr>
<td>pPHEZQ1</td>
<td>2.9 kb XhoI–SacI fragment (ftsZ) subcloned in pBSK+</td>
<td>Horrubia et al. (2001)</td>
</tr>
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<td>pECM2</td>
<td>Mobilizable E. coli/C. glutamicum bifunctional plasmid containing kan and cat</td>
<td>Jager et al. (1992)</td>
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<tr>
<td>pUL880M</td>
<td>Bifunctional E. coli/C. glutamicum promoter-probe vector with bla and lacI genes as selective markers and the promoterless kan gene as a reporter gene</td>
<td>Adham et al. (2001b)</td>
</tr>
<tr>
<td>pECZ1</td>
<td>pECM2 derivative containing a 2.1 kb BglII fragment from pPHEZQ1 carrying ftsQ (incomplete), ftsZ and yfh (incomplete) from C. glutamicum</td>
<td>This work</td>
</tr>
<tr>
<td>pBZ81</td>
<td>pUL880M derivative containing a 2.1 kb BglII fragment from pPHEZQ1 carrying ftsQ (incomplete), ftsZ and yfh (incomplete) from C. glutamicum</td>
<td>This work</td>
</tr>
<tr>
<td>pK18mob</td>
<td>Mobilizable plasmid containing an E. coli origin of replication and kan</td>
<td>Schafer et al. (1994)</td>
</tr>
<tr>
<td>pIZ1</td>
<td>pK18mob derivative carrying a 3 kb EcoRV–Ecl136II fragment from C. glutamicum containing ftsQ, ftsZ and the 5′-end of yfh</td>
<td>This work</td>
</tr>
<tr>
<td>pT7.7</td>
<td>E. coli vector containing bla and promoter φ10</td>
<td>Tabor &amp; Richardson (1985)</td>
</tr>
<tr>
<td>pPHEZ4</td>
<td>pT7.7 derivative containing ftsZ from C. glutamicum without upstream sequences</td>
<td>This work</td>
</tr>
<tr>
<td>pKZLac</td>
<td>pK18mob carrying a 789 bp Ndel (Klenow filled)–BamHI fragment from pPHEZ4 encoding the first 263 amino acids of FtsZ</td>
<td>This work</td>
</tr>
<tr>
<td>pOJ260</td>
<td>Mobilizable plasmid containing an E. coli origin of replication and the apramycin resistance gene</td>
<td>Bierman et al. (1992)</td>
</tr>
<tr>
<td>pOJ260Lac</td>
<td>pOJ260 carrying a 789 bp Ndel (Klenow filled)–BamHI fragment from pPHEZ4 encoding the first 263 amino acids of FtsZ</td>
<td>This work</td>
</tr>
<tr>
<td>pK18-3</td>
<td>Mobilizable plasmid containing an E. coli origin of replication, kan and the three C. glutamicum ORFs of unknown function located downstream from ftsZ</td>
<td>Adham et al. (2001a)</td>
</tr>
<tr>
<td>pK18-3ΔZ</td>
<td>pK18-3 derivative containing a 1146 bp BamHI (Klenow-filled) fragment from pPHEZ1/EZQ1 encoding the first 263 amino acids of FtsZ and upstream sequences</td>
<td>This work</td>
</tr>
<tr>
<td>pECKX99E</td>
<td>Bifunctional E. coli/C. glutamicum plasmid containing lacI</td>
<td>Amann et al. (1988)</td>
</tr>
<tr>
<td>pET-28α (+)</td>
<td>E. coli vector containing kan, lac, orif1, N-terminal and C-terminal His-tag</td>
<td>Novagen</td>
</tr>
<tr>
<td>pETGFP</td>
<td>pET28a derivative containing the in-frame fused ’ftsZ–gfp gene</td>
<td>This work</td>
</tr>
<tr>
<td>pKZGFP</td>
<td>pK18mob derivative containing the in-frame fused ’ftsZ–gfp gene</td>
<td>This work</td>
</tr>
<tr>
<td>pOJ260GFP</td>
<td>pOJ260 derivative containing the in-frame fused ’ftsZ–gfp gene</td>
<td>This work</td>
</tr>
<tr>
<td>pK18-3ZG</td>
<td>pK18-3 derivative containing ftsZ–gfp and upstream sequences</td>
<td>This work</td>
</tr>
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</table>

*ATCC, American Type Culture Collection.*
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 conjugal 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 Plac

To confirm the lethality of high levels of ftsZCop expression in C. glutamicum, we disrupted the chromosomal copy of ftsZ and replaced it by an ftsZCop gene under the control of the lac promoter (Plac) 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 Plac. One of these transconjugants was named C. glutamicum AR2 (Fig. 1). We assumed that the partial ftsZ would encode a non-functional FtsZ, whereas Plac 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...
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* 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<sub>Cg</sub> antibody. The amount of protein loaded was 1 μg and it was obtained from cultures at an OD<sub>600</sub> 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<sup>-1</sup> (for AR2 and AR20).

Table 2. Viable counts of three C. glutamicum strains at various OD<sub>600</sub> values

<table>
<thead>
<tr>
<th>OD&lt;sub&gt;600&lt;/sub&gt;</th>
<th>R31 (cells ml&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>AR2 (cells ml&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>AR20 (cells ml&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2</td>
<td>1.5 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>3.3 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>8.1 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>3.1</td>
<td>4.1 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>7.2 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
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<td>7.2 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>2.2 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>–*</td>
</tr>
<tr>
<td>8.4</td>
<td>4.1 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>2.9 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>–*</td>
</tr>
</tbody>
</table>

*Strain AR20 did not reach these OD<sub>600</sub> values (see Fig. 3).
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<sup>Cg</sup> with no significant morphological problems. The apparent off-centre localization of the FtsZ ring is not unexpected because <i>C. glutamicum</i> does not divide symmetrically (M. Letek, unpublished).

**Visualization of FtsZ<sub>Cg</sub> in C. glutamicum carrying ftsZ under the control of Plac**

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 <i>E. coli</i> into <i>C. glutamicum</i> 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 <i>C. glutamicum</i> AR2 and pOJZ-GFP, and those integrated at the 3′-end of ftsZ<sub>Cg</sub> under the control of Plac. 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 <i>C. glutamicum</i> AR50 and its genetic structure around ftsZ was determined (Fig. 1).

Fluorescence microscopy revealed that most of the <i>C. glutamicum</i> 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 <i>C. glutamicum</i> AR5 and <i>C. glutamicum</i> AR50 the only functional copy of FtsZ is fused to GFP, we constructed a merodiploid strain (<i>C. glutamicum</i> 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 <i>C. glutamicum</i> ML13 revealed that FtsZ–GFP localizes mainly in the septum, and because the growth rate of this strain is normal, we can conclude

![Image](http://mic.sgmjournals.org)
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 Plac.

**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 Plac 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 Plac 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 diptheriae (Umeda & Amako, 1983). Two different proteins have been found localized at the cell poles of C. glutamicum: an inorganic pyrophosphatase (PPase) and DivIVA<sub>Cg</sub> (Ramos et al., 2003a, b). Pyrophosphate is a by-product in the biosynthesis of UDP-N-acetylmuramylpentapeptide by UDP-N-acetylmuramylpentapeptide pyrophosphorylase (EC 2.7.7.23) and hydrolysis of pyrophosphate by PPase is essential for cell wall biosynthesis (Ramos et al., 2003a). DivIVA<sub>Cg</sub> 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<sub>Cg</sub> the scaffold for peptidoglycan biosynthesis in MrEB-lacking rod-shaped corynebacteria? DivIVA<sub>Cg</sub> seems to be an essential protein, and therefore it was not possible to disrupt divIVA<sub>Cg</sub> (Ramos et al., 2003b). Further experiments will be needed to deplete DivIVA<sub>Cg</sub> 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<sub>Cg</sub>-gfp gene fusion under the control of the ftsZ<sub>Cg</sub> endogenous promoters clearly indicated that these cells were probably in division, suggesting that FtsZ<sub>Cg</sub>–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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