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Morphological changes and proteome response of *Corynebacterium glutamicum* to a partial depletion of FtsI

Noelia Valbuena,[1] Michal Letek,[1] Angelina Ramos,[1]† Juan Ayala,[2] Diana Nakunst,[3] Joern Kalinowski,[3] Luis M. Mateos[1] and José A. Gil[1]

Correspondence
José A. Gil
degjgs@unileon.es

[1]Departamento de Ecología, Genética y Microbiología, Área de Microbiología, Facultad de Biología, Universidad de León, 24071 León, Spain

[2]Centro Biología Molecular 'Severo Ochoa', Consejo Superior de Investigaciones Científicas, CSIC-UAM, Campus Universidad Autónoma, Cantoblanco, 28049 Madrid, Spain

[3]Institut fur Genomforschung, Universitat Bielefeld, Universitatsstrasse 25, D-33615 Bielefeld, Germany

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In *Corynebacterium glutamicum*, as in many Gram-positive bacteria, the cell division gene *ftsI* is located at the beginning of the *dcw* cluster, which comprises cell division- and cell wall-related genes. Transcriptional analysis of the cluster revealed that *ftsI* is transcribed as part of a polycistronic mRNA, which includes at least *mraZ*, *mraW*, *ftsL*, *ftsI* and *murE*, from a promoter that is located upstream of *mraZ*. *ftsI* appears also to be expressed from a minor promoter that is located in the intergenic *ftsL–ftsI* region. It is an essential gene in *C. glutamicum*, and a reduced expression of *ftsI* leads to the formation of larger and filamentous cells. A translational GFP-FtsI fusion protein was found to be functional and localized to the mid-cell of a growing bacterium, providing evidence of its role in cell division in *C. glutamicum*. This study involving proteomic analysis (using 2D SDS-PAGE) of a *C. glutamicum* strain that has partially depleted levels of FtsI reveals that at least 20 different proteins were overexpressed in the organism. Eight of these overexpressed proteins, which include DivIVA, were identified by MALDI-TOF. Overexpression of DivIVA was confirmed by Western blotting using anti-DivIVA antibodies, and also by fluorescence microscopy analysis of a *C. glutamicum* RESF1 strain expressing a chromosomal copy of a *divIVA-gfp* transcriptional fusion. Overexpression of DivIVA was not observed when FtsI was inhibited by cephalexin treatment or by partial depletion of FtsZ.

INTRODUCTION

Corynebacteria are Gram-positive, pleomorphic asporogenous bacteria that are widely distributed in nature. While some of them are pathogenic to plants, animals and humans, the non-pathogenic corynebacteria, such as *Corynebacterium glutamicum*, are widely used in the industrial production of amino acids and nucleotides (Gourdon & Lindley, 1999; Nakayama *et al.*, 1978).

In recent years, different approaches have been used to characterize cell division genes in corynebacteria, and the possible relationship between amino acid production and growth inhibition (Honrubia *et al.*, 1998; Kobayashi *et al.*, 1997; Ramos *et al.*, 2003b; Wachi *et al.*, 1999). Recent studies have shown that corynebacteria might follow an archaic pattern of mycelial growth that involves an asymmetric mechanism of division (Ramos *et al.*, 2005), with cell elongation occurring at the tip of the daughter cells (Daniel & Errington, 2003). As a member of the order *Actinomycetales*, corynebacteria show apical growth, similar to that seen in *Corynebacterium diphtheriae* (Umeda & Amako, 1983). The availability of the complete genome sequence of *C. glutamicum* (GenBank accession nos NC_003450 and BX927154) has enabled us to study the expression profile/regulation of the cell division gene *ftsI*, and perform proteomic studies in the organism.

FtsI homologues have been described in different bacteria, such as *Escherichia coli* (Begg *et al.*, 1992; Botta & Park, 1981) or *Bacillus subtilis* (Daniel *et al.*, 1996; Marston *et al.*, 1998). FtsI (also called penicillin-binding protein 3, PBP3) is a well-characterized protein that has been reported to be expressed in very low amounts in the cell (about 100

†Present Address: Departamento de Biología Funcional, Área de Microbiología, Facultad de Medicina, Universidad de Oviedo, 33006 Oviedo, Spain.

Abbreviations: Q-PCR, quantitative PCR; RACE, rapid amplification of cDNA ends.

molecules) (Dougherty *et al.*, 1996). The protein consists of a short cytoplasmic domain, a single membrane-spanning segment and a large periplasmic domain that encodes a transpeptidase activity that is involved in the biosynthesis of septal peptidoglycan. Immunofluorescence microscopy has shown that in *E. coli* during the later stages of cell growth FtsI localizes to the division site at the septum. The septal localization of FtsI, however, depends upon prior localization of the other cell division proteins, such as FtsZ, FtsA, FtsK, FtsQ, FtsL and FtsW (Mercer & Weiss, 2002; Weiss *et al.*, 1999), and therefore, it appears that FtsI is a late recruit to the division site. In *B. subtilis*, the septal localization of PBP3/FtsI is also delayed, but is needed for the localization of the division inhibitor MinC (Marston & Errington, 1999).

DivIVA is another cell division protein that has been extensively studied in *B. subtilis*. It enables cell division in the organism by sequestering the cell division inhibitors MinC and MinD at the cell poles (Cha & Stewart, 1997; Edwards & Errington, 1997; Marston *et al.*, 1998). In this respect, its role is similar to that of MinE of *E. coli*, which repels MinCD inhibitors at the cell poles (Marston *et al.*, 1998). DivIVA also acts at the cell pole by interacting with the chromosome segregation machinery, and it is involved in the correct localization of the *oriC* region at the cell pole, a step that precedes asymmetric division during sporulation (Thomaides *et al.*, 2001). More recently, Harry & Lewis (2003) found that DivIVA localizes at the poles of germinated and outgrown cells in *B. subtilis* without prior assembly of the division apparatus at this site, suggesting that its localization does not occur by direct interaction with components of the division machinery, as proposed by Edwards *et al.* (2000).

In *C. glutamicum*, as in many other bacteria, *divIVA* is located downstream from the *dcw* cluster. Its encoded product (DivIVA) appears to be an essential protein playing an important role at the cell poles in the organism (Ramos *et al.*, 2003b). Overexpression of DivIVA-GFP translational fusion in *C. glutamicum* leads to an altered morphology showing rounder larger and swollen cells, with the DivIVA-GFP product being preferentially localized at the cell poles. It has been previously suggested that DivIVA participates in the maintenance of cell morphology in *C. glutamicum* (Ramos *et al.*, 2003b).

In the present study we show that *ftsI* in *C. glutamicum*, as in *E. coli*, is an essential gene that is required for the maintenance of cell shape and morphology. We further show that a partial depletion of FtsI induces an increased concentration of DivIVA, opening new questions about the regulation of cell division and polar growth in this micro-organism.

METHODS

Bacterial strains, plasmids and culture conditions. All the bacterial strains and plasmids used in the study are described in Table 1. *E. coli* strains were grown at 37 °C in Luria–Bertani broth (Hanahan, 1983), supplemented with agar where appropriate. When necessary, the antibiotics kanamycin, apramycin and ampicillin were used at a final concentration of 50 μg ml^{-1}. *C. glutamicum* cells were grown in trypticase soy broth (TSB; Difco) or trypticase soy agar (TSA) (TSB containing 2 % agar) at 30 °C.

DNA manipulation. Plasmid DNA was isolated from *E. coli* according to the method of Holmes & Quigley (1981). *E. coli* cells (DH5α and S17-1) were transformed by the method of Hanahan (1983).

All the mobilizable plasmids (integrative and bifunctional) were introduced into the donor strain (*E. coli* S17-1) and then transferred to *C. glutamicum* RES167 (or additional recombinant strains) following the method described previously (Mateos *et al.*, 1996).

Purification of DNA fragments was carried out using a GENECLEAN kit (Bio 101). Restriction enzymes were purchased from Promega and New England Biolabs.

Total DNA from *C. glutamicum* was isolated using the Kirby method described for *Streptomyces* (Kieser *et al.*, 2000), except that the cells were treated with 5 mg lysozyme ml^{-1} for 4 h at 30 °C.

DNA probes for Southern blots were labelled with DIG-High Prime, according to the manufacturer's (Roche) instructions.

RNA isolation, RT-PCR analysis, Q-PCR and RACE-PCR. For total RNA isolation from *C. glutamicum*, cells were grown in TSB medium to OD_{600} 1·5. RNA was isolated using the RNeasy kit (Qiagen).

RT-PCR analysis of the total RNA preparation was carried out in order to detect the presence of a polycistronic transcript originating from the upstream *mraZ* that includes *ftsI*; 1 μg total RNA was used as the template to generate single strand cDNA using a first strand cDNA synthesis kit (Roche), essentially according to the manufacturer's recommendations. Primers F6, P2, P4, P6, P8 and P10 (Table 2), which were used to generate cDNA corresponding to the upstream regions of the *dcw* genes (Fig. 1b), were designed using Primer Express, v2.0 (Applied Biosystems). The generated cDNAs were used as templates for subsequent PCR amplification using the primer pairs P0/F6 (for the upstream *mraZ*), P1/P2 (for the intergenic *mraZ–mraW* region), P3/P4 (for the intergenic *mraW–ftsL* region), P5/P6 (for the intergenic *ftsL–ftsI* region), P7/P8 (for the intragenic *ftsI* region) and P9/P10 (for the intergenic *ftsI–murE* region) (Table 2, Fig. 1b). The PCR amplified products were analysed by electrophoresis on 2 % agarose gels. The absence of DNA contamination of the RNA samples was ascertained by PCR using appropriate primers as negative and positive controls.

For Q-PCR analysis of *ftsI*, the first RT-PCR to generate the cDNA was performed using only the forward primer (primer P11, Table 2). A 1/20 volume of the generated cDNA sample was then used as a template in the second step of the PCR in which both the forward and the reverse primers (P11 and P12, respectively; Table 2) were used in the reaction mixture, in a total volume of 25 μl. Reactions were performed using an ABI Prism 7000 sequence detection system (Applied Biosystems). Results are indicated relative to the Ct (cycle threshold) value. Ct is defined as the cycle at which fluorescence is determined to be statistically significant compared to the background, being inversely proportional to the log of the initial copy number; this value was calculated automatically by the ABI Prism 7000 SDS software.

RACE-PCR experiments were performed according to the 5′/3′ RACE kit, 2nd generation (Roche), protocol. In order to identify promoters that were located upstream from *mraZ* and *ftsI*, 2 μg total RNA preparation was used as a template to generate single strand cDNA using primers F5 and P11, respectively (Table 2). A homopolymeric A tail was added to the 3′ end of the purified cDNA preparation using terminal transferase, the dA-tailed cDNA that was obtained was used in two further PCR amplifications steps; the first one using the primer pair dT-primer/F5 (for *mraZ*) and dT-primer/F3 (for *ftsI*) (Table 2).

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or description*	Source or reference†
Strains		
E. coli DH5α	Strain used for general cloning	Hanahan (1983)
E. coli JM109 (DE3)	*E. coli* JM109 derivative containing a chromosomal copy of the gene for the T7 RNA polymerase	Promega
E. coli S17-1	Mobilizing donor strain, *pro recA*, which has an RP4 derivative integrated into the chromosome	Schäfer *et al.* (1990)
E. coli AX655	*ftsI* temperature-sensitive mutant	Walker *et al.* (1975)
C. glutamicum 13032	Wild-type strain	ATCC
C. glutamicum RES167	13032 derivative used as a host for transformation, electroporation or conjugation	Tauch *et al.* (2002)
C. glutamicum MAPF	RES167 derivative containing a complete copy of *ftsI* in its chromosomal position and a copy of *gfp-ftsI* under the control of P*kan* obtained by integration of plasmid pNV4A	This work (Fig. 2c)
C. glutamicum APF	RES167 derivative containing a deleted copy of *ftsI* in its chromosomal position and a copy of *gfp-ftsI* under the control of P*kan* obtained by integration of plasmid pNV5A	This work (Fig. 2b)
C. glutamicum RESF1	RES167 derivative containing a complete copy of *ftsI* under the control of P*lac* obtained by integration of plasmid pOJPB	This work (Fig. 3b)
C. glutamicum AR200	RES167 derivative carrying a deleted copy of *divIVA* and a copy of *divIVA-gfp* obtained by integration of plasmid pKAG1	This work (Fig. 3c)
C. glutamicum RESF12	RESF1 derivative carrying a deleted copy of *divIVA* and a copy of *divIVA-gfp* obtained by integration of plasmid pKAG1	This work (Fig. 3d)
C. glutamicum AR2	Strain containing an incomplete copy of *ftsZ* under its own promoter and a complete copy of *ftsZ* under the control of P*lac*	Ramos *et al.* (2005)
C. glutamicum AR20	AR2 derivative containing pECKX99E, a bifunctional *E. coli*/*C. glutamicum* plasmid containing the *lacI*^{q} gene	Ramos *et al.* (2005)
Plasmids		
pBS KS/SK	*E. coli* vectors containing *bla*, *lacZ*, *orif1*	Stratagene
pGEM-T Easy	*E. coli* vector used to clone PCR-amplified products using *Taq* enzyme	Promega
pFtsI	pGEM derivative containing a 1·9 kb fragment from *C. glutamicum* 13032 amplified by PCR and carrying the *ftsI* gene	This work
pEGFP	Bifunctional *E. coli*/*C. glutamicum* promoter-probe vector containing *kan* as a selective marker and the promoterless *gfp* gene as a reporter	Letek *et al.* (2006)
pEGFP-FtsI	pEGFP derivative containing *gfp* under the control of the 150 bp P*ftsI* (P*ftsI-gfp*)	This work
pEMel-1	Bifunctional *E. coli*/*C. glutamicum* promoter-probe vector containing *kan* as a selective marker and the *melC* operon from *Streptomyces glaucescens* as a reporter	Adham *et al.* (2003)
pEMel-FtsI	pEMel-1 derivative containing the *melC* operon under the control of the 150 bp P*ftsI* (P*ftsI-melC1*)	This work
pXHis1-Npro	*E. coli* vector containing the *xysA* gene from *Streptomyces halstedii* under the control of P*kan*	Adham *et al.* (2001)
pKFtsI	pXHis1-Npro derivative containing *ftsI* under the control of P*kan*	This work
pXEGFP2	Plasmid containing a *gfp* derivative gene under the control of *xysA* promoter (P*xys-gfp*)	Letek *et al.* (2006)
pNV3	pKFtsI derivative containing *gfp-ftsI* under the control of P*kan*	This work
pOJ260	*E. coli* mobilizable plasmid with *oriT*, *lacZ* and *apr* as a marker	Bierman *et al.* (1992)
pNV4A	pOJ260 derivative containing *gfp-ftsI* under the control of P*kan*	This work
pNV5A	pNV4A derivative containing *gfp-*Δ*ftsI* under the control of P*kan*	This work
pK18*mob*	*E. coli* mobilizable plasmid containing *oriT*, *lacZ* and *kan* as a marker	Schäfer *et al.* (1990)
pKInt1	pK18*mob* derivative containing a 340 bp *Bam*HI–*Sma*I internal fragment of the *C. glutamicum* 13032 *ftsI* gene	This work
pOJPB	pOJ260 carrying a 462 bp *Eco*RI–*Bam*HI fragment from the pFtsI plasmid encoding the first 154 amino acids from FtsI	This work

Table 1. cont.

Strain or plasmid	Relevant genotype or description*	Source or reference†
pECXK99E	Bifunctional *E. coli*/*C. glutamicum* vector containing *lacI*^{q}	Kirchner & Tauch (2003)
pBL1	*C. glutamicum* endogenous plasmid	Santamaria *et al.* (1984)
pABK	Mobilizable plasmid containing an *E. coli* origin of replication, pBL1 origin of replication and *kan* as a marker	Ana B. Campelo, unpublished‡
pALacI	pABK derivative containing the *lacI*^{q} gene from plasmid pECXK99E	This work
pKAG1	pK18*mob* derivative containing the Δ*divIVA-gfp* gene fusion	Ramos *et al.* (2003b)

**kan*, *apr* and *bla* are the genes for kanamycin, apramycin and ampicillin resistance, respectively; o*riT* is the transfer origin for mobilization.
†ATCC, American Type Culture Collection.
‡Departamento de Ecología, Genética y Microbiología, Universidad de León.

The amplified DNA product was used again in a second round PCR amplification using the primer pair dT-primer/F6 (for *mraZ*) and dT-primer/F4 (for *ftsI*) (Table 2). The amplified fragments were cloned into pGEM-T Easy vector (Table 1), utilizing a T–A cloning technique, and used to transform *E. coli* DH5α; five plasmids isolated from different clones (per assay) were sequenced.

Plasmid constructions. In order to subclone the complete *ftsI* gene from *C. glutamicum* using PCR-amplified DNA, primers F1 and F2 were designed (Table 2). These primers amplify *ftsI* including the second GTG (position 2 293 165) but not any upstream elements that are likely to contain the RBS and promoter. The 1·9 kb *Taq*-amplified PCR product was subcloned into pGEM-T Easy vector, creating plasmid pFtsI (Table 1). This plasmid was digested with *Nde*I and *Xho*I (target sites of which are present in F1 and F2 primers, respectively, Table 2), and the 1·9 kb fragment (corresponding to the *ftsI* gene) was used to replace the *xysA* gene from pXHis1-Npro, yielding plasmid pKFtsI (Table 1).

Table 2. Primers

Use	Name and sequence
Cloning of $ftsI_{CG}$	F1 5′-GGAATTCCATATGACCTACCGGCCTAAATCTTC-3′ (*Nde*I site underlined)
	F2 5′-CCGCTCGAGTTATTGAGCTTGAAGGATGATC-3′ (*Xho*I site underlined)
RACE P*ftsI*	F3 5′-ACATACGCGTTGCCTTCTTC-3′
	F4 5′-CAAACGCAAGGCCAAATCAG-3′
RACE P*mraZ*	F5 5′-TCCGCGCTTGCTGCAAGGTT-3′
	F6 5′-AACCGCGAGACTGTGGTCTT-3′
Cloning the *ftsI* promoter	F7 5′-CGGAATTCGTGACTTTCCCCAGCAATGG-3′ (*Eco*RI site underlined)
	F8 5′-GGAATTCCATATGTCCTTCTTCTTGGTTTACG-3′ (*Nde*I site underlined)
Fusion of *gfp-ftsI* genes	F9 5′-CGCGTTCGAACTGCCCGGCAA-3′
	F10 5′-GGGAATTCCATATGCTTGTACAGCTCGTCCATG-3′ (*Nde*I site underlined)
RT-PCR*	P0 5′-GTGTGGGTGACCTGCATGGA-3′
	P1 5′-TCGGTTCGGTGGATTT-3′
	P2 5′-TGTCTTGTACTTATGTTTGTGGAT-3′
	P3 5′-GAAAGAATCGGCAACAACTCAGGA-3′
	P4 5′-CATACCGTGGATCAGCGTCAGC-3′
	P5 5′-ATGCTGCAACCACCGGACAA-3′
	P6 5′-CCGGTAGGTCACTCCTTCTT-3′
	P7 5′-CGCACCACGGTGGATATGTT-3′
	P8 5′-TTCTGCGCCGTACCTGTCTT-3′
	P9 5′-CATGCTTGATGAGCCAGAAC-3′
	P10 5′-AGACCAATAGCCGCGATTGC-3′
Q-PCR of *ftsI**	P11 5′-GCCGTCCATGCTGATTCG-3′
	P12 5′-ATCCGCCAATACCCAAACG-3′
Cloning *lacI*^{q} from *E. coli*	L1 5′-CGGGATCCATTTTCTCCTTACGCATCTG-3′ (*Bam*HI site underlined)
	L2 5′-CGGGATCCTTCGCGCTAACTCACATTAATTGC-3′ (*Bam*HI site underlined)

*Primers F6 and P11 were also used for RT-PCR and P*ftsI* RACE assays, respectively.

In order to detect the presence of the promoter of *ftsI*, a 150 bp DNA fragment immediately upstream of the gene was PCR amplified using the primer pair F7/F8 (Table 2); the PCR product was digested with *Eco*RI and *Nde*I, the sites of which were included in the forward and reverse primers, respectively, and subcloned in the promoter probe vectors pEMel-1 and pEGFP, creating pEMel-FtsI and pEGFP-FtsI plasmids, respectively (Table 1).

To construct a GFP-FtsI translational fusion, we used a variant of GFP (Clontech) that includes the V163A and S175G mutations introduced by Siemering *et al.* (1996); this variant was found to be efficiently expressed in '*Brevibacterium lactofermentum*'/*C. glutamicum* (Ramos *et al.*, 2003a). The whole *gfp* gene was amplified from plasmid pXEGFP2 (Table 1) using primers F9 and F10 (Table 2). These primers were designed to replace the stop codon (TAA) of the *gfp* gene with CAT (His), which after *Nde*I digestion and ligation with the aforementioned *ftsI* gene will be immediately followed by the ATG start of *ftsI*. Because of the presence of two *Nde*I sites in the *gfp* gene, one at the primer F10 region and the other one at the start of the gene, the amplified fragment was digested with *Nde*I and cloned into *Nde*I-digested pKFtsI (see above) yielding plasmid pNV3 (Table 1), which contained the *gfp-ftsI* gene fusion flanked by *Bgl*II sites.

Plasmid pNV4A was obtained by cloning the *Bgl*II cassette from pNV3 (*Bgl*II-P*kan-gfp-ftsI-Bgl*II) into plasmid pOJ260 (*Bam*HI digested); pNV4A was then transferred by conjugation to *C. glutamicum* RES167, giving rise to the merodiploid strain MAPF (Table 1, Fig. 1), which carries the normal *ftsI* gene copy and an additional copy of the *gfp-ftsI* gene fusion under P*kan* on the chromosome.

Plasmid pNV4A was digested with *Stu*I and *Eco*RV (to remove the 3′ end of *ftsI*) and autoligated, affording plasmid pNV5A, which carries *gfp*-Δ*ftsI* under the control of P*kan* (Table 1). When pNV5A was transferred by conjugation to *C. glutamicum* RES167, the resulting transconjugant strain *C. glutamicum* APF contained an incomplete copy of *ftsI* and the fused *gfp-ftsI* under P*kan* on the chromosome (Table 1, Fig. 2b).

pKInt1 vector was designed to create an *ftsI*::null mutant of *C. glutamicum*. In this vector an internal fragment of *ftsI* that was obtained as a 340 bp *Bam*HI–*Sma*I fragment from pFtsI (Table 1, Fig. 1a) was subcloned at the *Sma*I and *Bam*HI sites of pK18*mob* (Table 1). A single crossover integration of pKInt1 in the chromosome at the *ftsI* locus would create two deleted FtsI versions: one lacking 385 amino acids from its C-terminus and the other lacking 154 amino acids from its N-terminus.

pOJPB vector was designed to disrupt the chromosomal copy of *ftsI* and place a second functional copy of *ftsI* under the control of the P*lac* promoter. This was achieved by subcloning the 462 bp *Eco*RI–*Bam*HI 5′ region of *ftsI* (which encodes the first 154 amino acids) from plasmid pFtsI into the *Eco*RI and *Bam*HI sites of pOJ260, downstream of the P*lac* promoter (Table 1).

Vector pALacI was constructed as follows: the *E. coli lacI*^{q} gene present in plasmid pECXK99E (Table 1) was PCR amplified using the primer pair L1/L2 (Table 2), digested with *Bam*HI and subcloned at the same site in pABK (Table 1).

All of the aforementioned plasmid constructs were confirmed by DNA sequencing.

DNA sequencing. DNA sequencing was carried out by the dideoxy nucleotide chain termination method of Sanger *et al.* (1977). Computer analysis was performed with DNASTAR (DNAstar); database similarity searches were carried out using the BLAST and FASTA public servers (National Center for Biotechnology Information, NCBI, and European Bioinformatics Institute, EBI), and multiple alignments of sequences were achieved using CLUSTAL W (EBI).

Preparation of cell-free extracts, SDS-PAGE, Western blotting and 2D electrophoresis. Cell-free extracts of *C. glutamicum* cells were disrupted by sonication as follows. One gram wet weight cells was suspended in 5 ml ice-cold TES buffer (25 mM Tris/HCl, 25 mM EDTA, 10·3 % sucrose, pH 8). Sonication was carried out for periods of 30 s with 1 min intervals in an ice-cooled tube using a Branson sonicator (model B-12) at 75–100 W, until the cells were completely disrupted, as observed microscopically. Cell debris was removed by centrifugation (8000 ***g***), and supernatants were used as cell extracts.

SDS-PAGE was carried out essentially as described by Laemmli (1970). Electrophoresis was performed at room temperature in a vertical slab gel (170 × 130 × 1·5 mm), using 10 % (w/v) polyacrylamide at 100 V and 60 mA. After electrophoresis, proteins were stained with Coomassie blue or electroblotted to PVDF membranes (Millipore), and immunostained using the following antibodies: mouse monoclonal antibodies (F126-2) raised against purified DivIVA/Ag84 from *Mycobacterium kansasii* (provided by Professor A. H. J. Kolk, Royal Tropical Institute, Amsterdam, The Netherlands) and goat anti-mouse IgG alkaline phosphatase-conjugated antibodies (Santa Cruz Biotechnology), or rabbit polyclonal anti-GFP and goat anti-rabbit IgG alkaline phosphatase-conjugated antibodies (both from Santa Cruz Biotechnology).

2D gel electrophoresis was performed as described by Vohradsky *et al.* (1997). The IEF of proteins in the first dimension was carried out using 24 cm Immobiline DryStrips, pH 4–7 (Amersham Pharmacia) followed by electrophoretic separation on 10 % SDS-PAGE gels in the second dimension. Preparative gels were loaded with 400 μg total protein and stained with Coomassie blue. Protein sizes and isoelectric point ranges of the 2D gels were determined using 2D-gel marker proteins (Bio-Rad). The 2D gels were matched and quantified by image analysis using the Z3 2D-gel analysis system (Compugen). Protein spots were identified by peptide mass fingerprinting after they were excised from Coomassie blue-stained preparative gels, destained, gel purified and digested with trypsin. The mass spectra of the peptides after proteolytic digestion were determined with a MALDI-TOF mass spectrometer (Bruker Biflex III).

Fluorescence microscopy. *C. glutamicum* cells expressing GFP-FtsI or DivIVA-GFP were observed under a Nikon E400 fluorescence microscope. Images were captured with a DN100 Nikon digital camera and assembled using Corel Draw, Adobe Photoshop and Metamorph.

RESULTS

Molecular analysis of the *ftsI* gene from *C. glutamicum*

Complete genome sequence information of two strains of *C. glutamicum* ATCC 13032 is available, NC_003450 (Ikeda & Nakagawa, 2003) and BX927154 (Kalinowski *et al.*, 2003), and the region of the *dcw* cluster is shown in Fig. 1(a). The structural similarity in the organization of the genes that are 5′ to the *dcw* cluster around the *ftsI* region in both strains suggests that *mraZ*, *mraW*, *ftsL*, *ftsI* and *murE* might be cotranscribed. In order to test this possibility, total RNA was extracted from *C. glutamicum* cells that were grown in TSB to exponential phase, and used in RT-PCR using

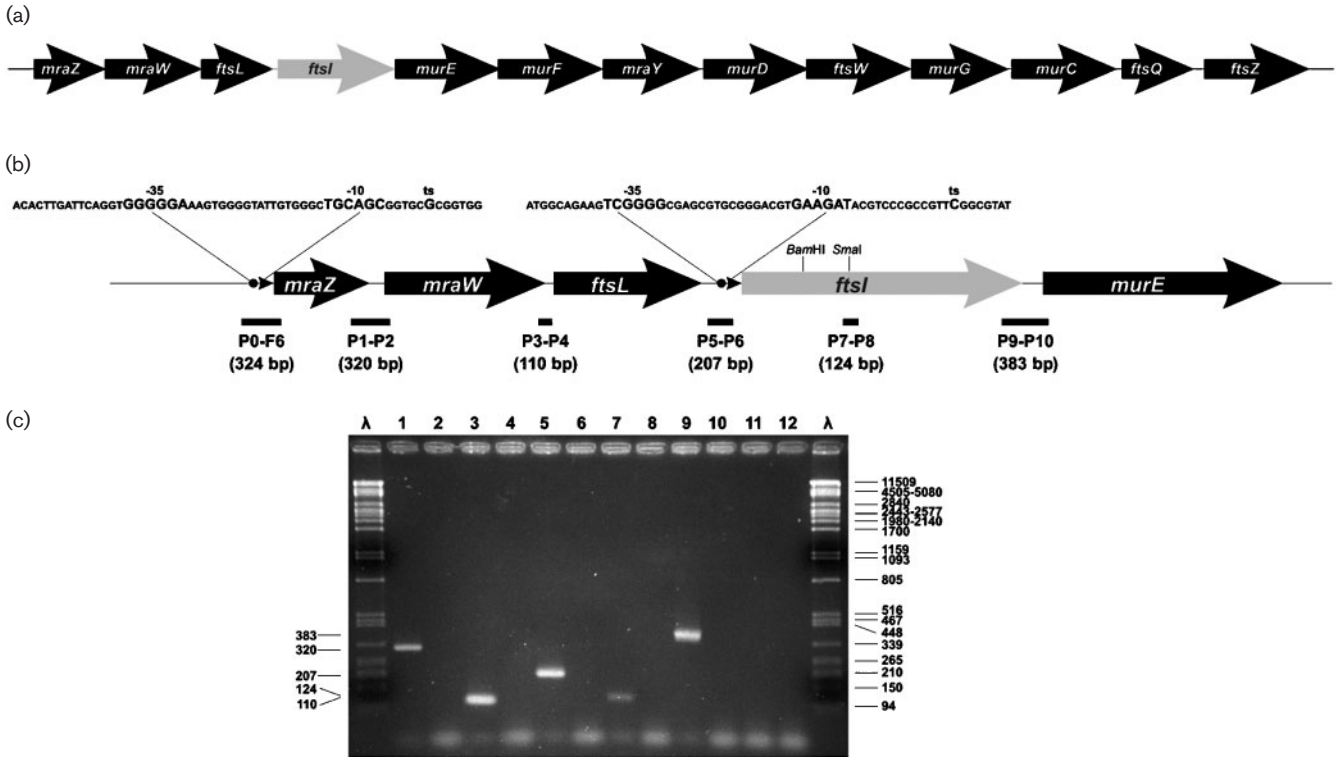

Fig. 1. (a) Physical map of the *dcw* cluster from *C. glutamicum* ATCC 13032. Arrows represent ORFs found in the sequenced DNA (accession no. NC_003450). (b) The relevant part of the *C. glutamicum* ATCC 13032 chromosome around *ftsI*. Black boxes represent the DNA amplified by RT-PCR using the indicated primer pairs. The upper sequences show the transcription start point (ts) (large G or C) and the hypothetical −10 and −35 boxes (large letters) of P*mra* and P*ftsI*. The *Bam*HI and *Sma*I sites used for *ftsI* gene disruption are indicated. (c) Agarose gel electrophoresis of different DNA fragments obtained after RT-PCR using *C. glutamicum* mRNA from a culture in exponential phase (odd lanes). Samples containing only mRNA were used as negative controls (even lanes). The primer pairs used for RT-PCR were as follows: lanes 1–2, primer pair P1/P2; lanes 3–4, primer pair P3/P4; lanes 5–6, primer pair P5/P6; lanes 7–8, primer pair P7/P8; lanes 9–10, primer pair P9/P10; lanes 11–12, primer pair P0/F6. λ, Lambda DNA digested with *Pst*I.

specific primers for the intergenic *mraZ–mraW*, *mraW–ftsL*, *ftsL–ftsI*, *ftsI–murE* regions, as well as for an internal region of *ftsI* (see Fig. 1b). RT-PCR products of the expected sizes were obtained in all cases, strongly suggesting that there is a transcriptional relationship between these five genes (Fig. 1c). No amplification band was seen when primer pair P0/F6 was used, indicating a lack of cotranscription with *mraZ* and upstream genes (Fig. 1c, lanes 11 and 12).

The main promoter of the *dcw* cluster was located by RACE PCR to be at the G residue, at position 2 295 904, which is 7 nucleotides downstream from the putative −10 promoter region TGCAGC (Fig. 1b) (Patek *et al.*, 2003) and 99 nt upstream from the *C. glutamicum mraZ* gene (at the beginning of the *mra/dcw* cluster of cell division and cell envelope biosynthesis genes) as seen in *E. coli* (Hara *et al.*, 1997; Mengin-Lecreulx *et al.*, 1998).

Two different initiation codons for *ftsI* were suggested for *C. glutamicum*; the first being a GTG codon at position 2 293 318, which would encode a 704 amino acid protein with a calculated molecular mass of 75·7 kDa (Wijayarathna *et al.*, 2001). The second being the other GTG at position 2 293 165, which would encode a 651 amino acid protein with a calculated molecular mass of 69·7 kDa (Kalinowski *et al.*, 2003). In order to determine which of the two protein products might be correct, and also to find a possible transcriptional start point, additional RACE-PCR experiments, using different primers upstream from *ftsI*, were performed. The results showed the presence of an mRNA starting at C (at position 2 293 257), which is located 13 nucleotides downstream from a putative −10 promoter region (GAAGAT) in the intergenic *ftsL–ftsI* region (Fig. 1a). A 150 bp fragment containing the above-mentioned region was PCR amplified using primers F7 and F8 (Table 2), and subcloned into the promoter probe vectors pEMel-1 and pEGFP (Table 1), creating pEMel-FtsI and pEGFP-FtsI, respectively. These vectors were mobilized into *C. glutamicum*, and the detection of melanin

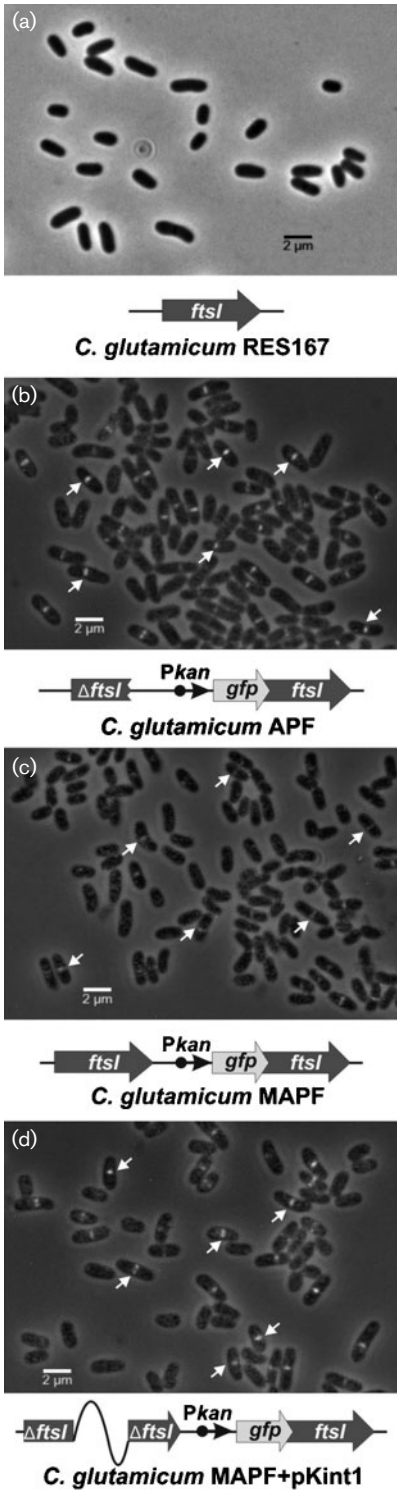

Fig. 2. Fluorescence microscopy images of *C. glutamicum* cells carrying *gfp-ftsI* under the control of P*kan*: (a) phase-contrast microscopy image of RES167, (b, c, d) overlays combining phase-contrast and fluorescence microscopy of the strains APF (b), MAPF (c) and MAPF transformed with pKInt1 (d).

production and GFP activity in the corynebacteria confirmed the presence of a promoter activity in that region. Therefore, it can be concluded that *ftsI* is mainly cotranscribed along with the *mraZ*, *mraW*, *ftsL* and *murE* genes of the operon probably from the P*mra* promoter, as described in *E. coli* and *B. subtilis* (Hara *et al.*, 1997), and probably also from a minor promoter (P*ftsI*) as in *B. subtilis* (Daniel *et al.*, 1996). This result also suggests that the second GTG (at position 2 293 165) most probably is the start codon of *ftsI*.

Taking into account the above results, a 1·9 kb fragment containing the gene encoding FtsI (Kalinowski *et al.*, 2003) was PCR amplified using specific primers, and ligated into the pGEM-T Easy *E. coli* vector using the A–T strategy, to create pFtsI (Table 1). However, all of the *E. coli* transformants that were tested for the recombinant plasmid were found to have the *ftsI* insert in the opposite orientation to the P*lac* promoter. This observation strongly suggests that overexpression of FtsI might be toxic in *E. coli*. Furthermore, we also observed that expression of *ftsI* was unable to complement the temperature-sensitive *ftsI*^{ts} *E. coli* AX655 strain, as has been described for similar complementation assays (Wijayarathna *et al.*, 2001).

Visualization of FtsI$_{CG}$-GFP fusions

In order to analyse the role of FtsI in cell division in *C. glutamicum*, various strains of the organism were constructed in which expression of the *gfp-ftsI* fusion was studied. The P*kan* promoter of the *kan* gene from Tn*5* is efficiently expressed in corynebacteria (Cadenas *et al.*, 1991). *C. glutamicum* transformed with pNV4A vector (MAPF strain) contains a wild-type copy of *ftsI* in addition to the *gfp-ftsI* cassette, which is under the control of the P*kan* promoter and inserted as a single copy in the chromosome (Fig. 2c); in *C. glutamicum* transformed with pNV5A vector (APF strain) *gfp-ftsI* expressed under the control of the P*kan* promoter is the only functional copy of the gene since this strain carries a truncated form of *ftsI* (Fig. 2b). Phase-contrast microscopy analysis of *C. glutamicum* APF (Fig. 2b) revealed typical corynebacterial cell morphology similar to the parental strain. Despite the chromosomal integration, no polar effects on the downstream genes were perceptible. The GFP-FtsI fluorescence signal was mainly located as foci in the mid-cell region (Fig. 2b, indicated by arrowheads). However, although the GFP signal in the merodiploid strain MAPF (Fig. 2c) clearly shows a reduced level of fluorescence, this is probably due to the competition between the original FtsI and GFP-FtsI. Nevertheless, the signal is well above the background autofluorescence that is seen in the parent *C. glutamicum* RES167 strain, which has no GFP fusion (not shown). Furthermore, the localization of GFP-FtsI at the mid-cell is strongly indicative of the involvement of FtsI in *C. glutamicum* cell division, most likely in the biosynthesis of septum peptidoglycan as described for other bacteria.

ftsI seems to be an essential gene in *C. glutamicum*

ftsI has been shown to be an essential gene in *E. coli* (Begg *et al.*, 1992) and in *B. subtilis* (Daniel *et al.*, 1996). Therefore, in order to determine whether *ftsI* was also necessary for the viability of *C. glutamicum*, we performed gene disruption experiments using the suicide plasmid pKInt1 (Table 1); all attempts to inactivate the *ftsI* gene using internal fragments were unsuccessful, similar to our earlier studies involving *murC*, another essential gene in *C. glutamicum* (Ramos *et al.*, 2004).

Disruption of *ftsI* was only possible in the merodiploid strain *C. glutamicum* MAPF using plasmid pKInt1. Cell morphology and growth rate of the transconjugants expressing the GFP-FtsI fusion (Fig. 2d) were found to be similar to the host MAPF strain. Disruption of the original chromosomal copy of *ftsI* was confirmed by Southern blotting using DNA isolated from ten fluorescent transconjugants (not shown). These results unambiguously substantiate that *ftsI* is essential in *C. glutamicum*, as it is in *E. coli* and *B. subtilis*.

Decreased expression of *ftsI* causes severe defects in cell morphology

It has been reported previously that the promoter of the lactose operon of *E. coli* (P*lac*) is not well recognized by the *C. glutamicum* RNA polymerase (Ramos *et al.*, 2005). As *ftsI* appears to be an essential gene in *C. glutamicum*, and since no *ftsI* null mutants could be obtained, the function of the FtsI was investigated by partial depletion of FtsI levels in the organism. To do this, *C. glutamicum* transformed with the plasmid pOJPB (Table 1) was used. Southern blotting of the transconjugant strain (*C. glutamicum* RESF1) revealed the pattern expected of Campbell integration of pOJPB at the *ftsI* locus (Fig. 3b). The strain carries a disrupted non-functional copy of *ftsI* and a functional copy under the control of P*lac*, and has a distinctive phenotype (see Fig. 3b). This may be due to a reduced expression of *ftsI* (2·9 times less than the wild-type strain as quantified by Q-PCR analysis); furthermore, cells were found to be irregularly shaped, swollen and larger than the parent *C. glutamicum* RES167 strain (Fig. 3a). However, the possibility exists that the aberrant phenotype may be due to the expression of the truncated *ftsI* or to polar effects

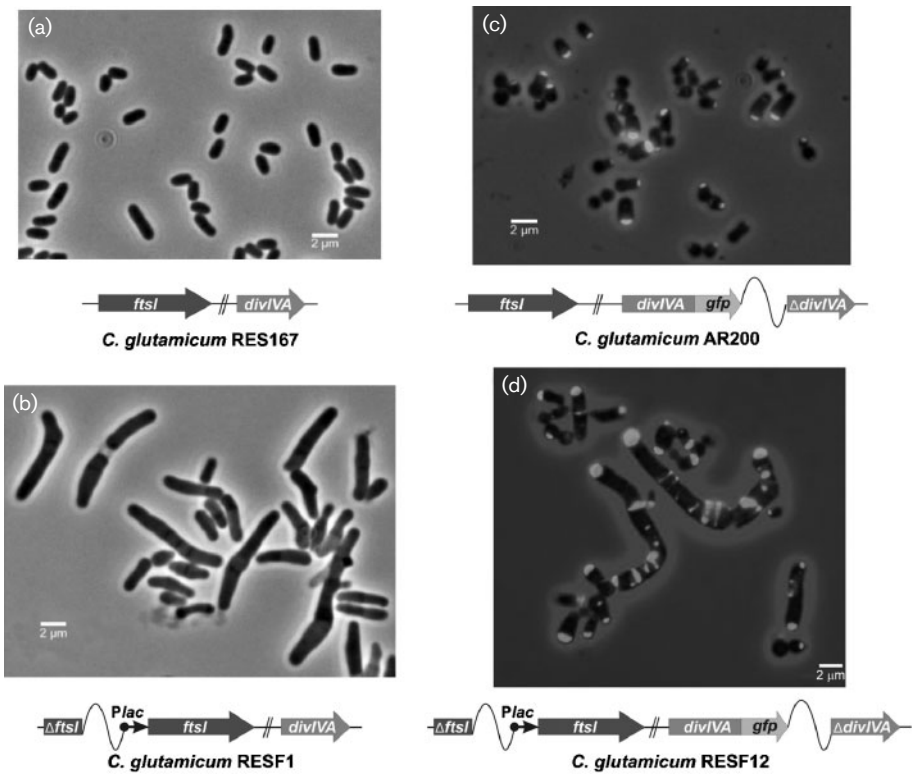

Fig. 3. Fluorescence microscopy images of *C. glutamicum* cells carrying the *ftsI* gene under the control of P*lac* and/or the gene fusion *divIVA-gfp*. The images are as follows: phase-contrast microscopy images of RES167 (a) and the partially depleted FtsI strain RESF1 (b), overlaid phase-contrast and fluorescence microscopy images of AR200 (*divIVA-gfp* as a single copy on the chromosome) (c) and RESF12 (d) obtained by integration of plasmid pKAG1 into the chromosome of RESF1.

on expression of other genes in the *dcw* cluster located downstream from *ftsI* (see Fig. 1a).

In order to further test the effect of a much reduced FtsI level in the organism, we carried out a similar experiment as used for the study of *ftsZ* levels in *C. glutamicum* (Ramos *et al.*, 2005). To reduce the levels of FtsI, we attempted to introduce the *lacI*^{q} gene of *E. coli*, which is present in the plasmid pALacI (Table 1), into *C. glutamicum* RESF1. All of our attempts to obtain viable kanamycin- and apramycin-resistant transconjugants using the vector pALacI were unsuccessful, while control plasmid pABK readily yielded transconjugants under the same conditions. These results seem to indicate that a more reduced *ftsI* expression due to the possible effect of the *lacZ* repressor (*lacI*^{q}) is lethal for *C. glutamicum.*

Decreased expression of *ftsI* induces the expression of several genes in *C. glutamicum*

Total cytoplasmic proteins synthesized by *C. glutamicum* RES167 (parent strain) and *C. glutamicum* RESF1 (partially depleted FtsI strain) were characterized by 2D gel electrophoresis (IEF SDS-PAGE). Representative gels depicting a consistent pattern of the protein profiles are shown in Fig. 4. Twenty-two proteins of various molecular sizes seem to be clearly overexpressed in *C. glutamicum* RESF1 compared to the parent RES167 strain. An attempt was made to identify these 22 proteins by the peptide mass mapping technique. Only eight proteins, namely the ribosomal protein L10, σ^{70} sigma factor, pyruvate carboxylase, enolase, arginine succinate synthase, *m*-diaminopimelate dehydrogenase, DivIVA and 6-phosphofructokinase, were positively identified. Except for DivIVA, which is a part of the cell division machinery, the identified proteins are known to be involved in central metabolic pathways. Therefore, these results indicate that partial depletion of FtsI in *C. glutamicum* induces the expression of several genes including *divIVA*, whose protein product is involved in the apical growth of corynebacterial cells (Ramos *et al.*, 2003b). The use of monoclonal antibodies raised against DivIVA/Ag84 of *M. kansasii* enabled us to measure the levels of DivIVA in *C. glutamicum* RES167 and *C. glutamicum* RESF1 strains, and we found 3–4 times more DivIVA protein in RESF1 than in RES167 (Fig. 5).

Overexpression of *divIVA* in *C. glutamicum* RESF1 strain was also ascertained by another experiment. The conjugative suicide plasmid pKAG1, which contains a Δ*divIVA-gfp* transcriptional fusion (Table 1), was introduced into *C. glutamicum* RESF1 strain and transconjugants were selected in TSA medium containing apramycin and kanamycin. Depending upon the region of integration two types of transconjugants were expected: (i) those that would arise by a single recombination event between plasmid pOJPB, which was previously integrated into the chromosome of *C. glutamicum* RESF1, and homologous sequences of the incorporated plasmid pKAG1, and (ii) those integrated at the chromosomal 3′ end of *divIVA*. Forty kanamycin- and

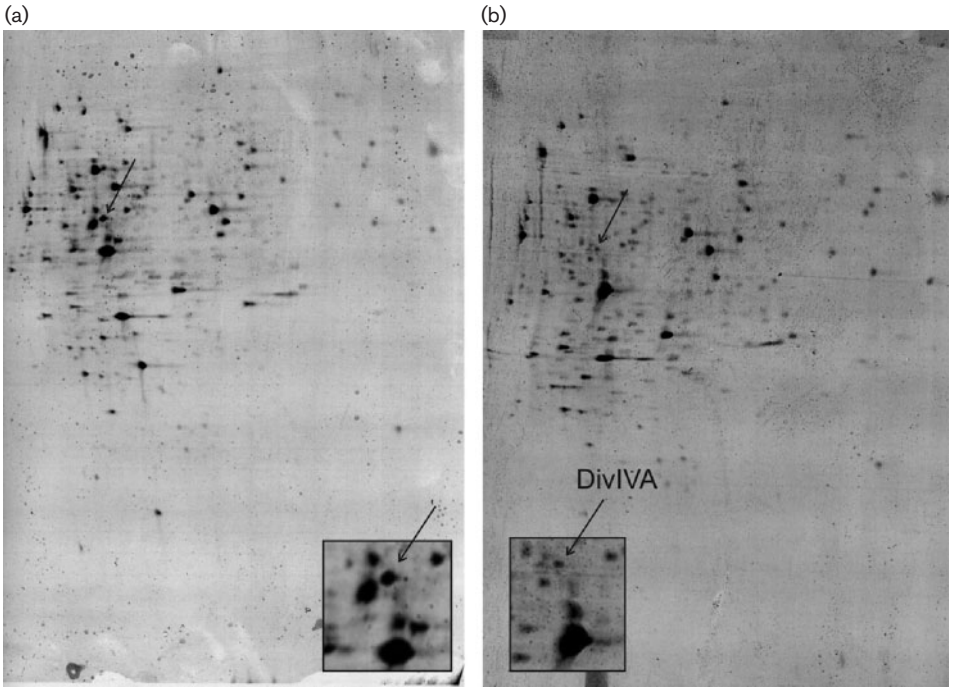

Fig. 4. 2D analysis of soluble proteins of *C. glutamicum* RESF1 (a) and RES167 (b). In each of the panels, the gels are orientated such that the acidic proteins are to the left and the high molecular mass proteins to the top. Insets show an enlarged region of the gel in the vicinity of DivVA (bottom).

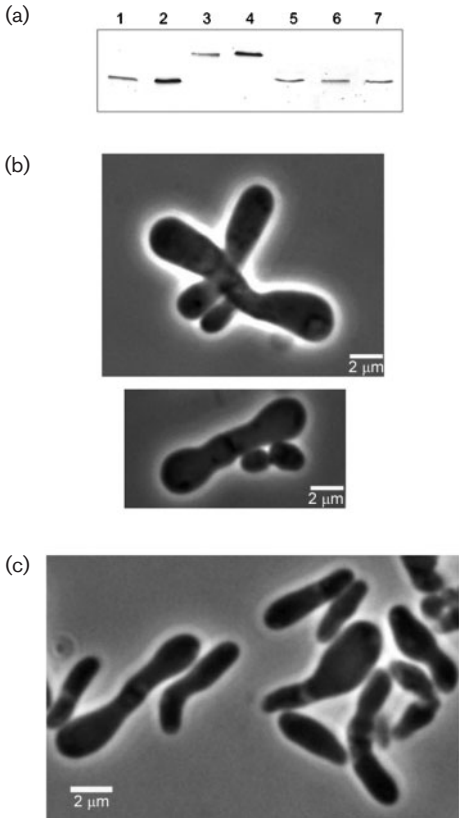

Fig. 5. (a) Detection of DivIVA (38·7 kDa) (lanes 1, 2, 5, 6, 7) or DivIVA-GFP (65·5 kDa) (lanes 3, 4) using anti-DivIVA antibodies after the SDS-PAGE of cell-free extract from *C. glutamicum* RES167 (lane 1), RESF1 (lane 2), AR200 (lane 3), RESF12 (lane 4), AR2 (lane 5), AR20 (lane 6) and RES167 grown in the presence of 0·6 μg cephalexin ml^{-1} (lane 7). The amount of protein loaded per lane was 1 μg. (b) Microscopy images of *C. glutamicum* AR2 cells carrying the *ftsZ* gene under the control of P*lac*. (c) Microscopy images of *C. glutamicum* RES167 in the presence of subinhibitory concentrations of cephalexin (0·6 μg ml^{-1}).

apramycin-resistant transconjugants were observed under the fluorescence microscope for the expression of DivIVA-GFP. Five clones among the forty obtained showed fluorescence, and these were then tested by Southern blot hybridization to confirm the integration of pKAG1 vector at the 3′ end of *divIVA* (data not shown). One among these transconjugants was named *C. glutamicum* RESF12 and was used in our studies. Its genetic structure is shown in Fig. 3(d).

The expression product of *divIVA-gfp* in *C. glutamicum* RESF12 strain was found to accumulate not only at the cell poles (Fig. 3d) but also at the mid-cell as previously shown (Ramos *et al.*, 2003b). The level of expression was comparable to that observed in a strain of *C. glutamicum*/pEAG2, which contains the *divIVA-gfp* fusion being expressed from a multicopy plasmid (Ramos *et al.*, 2003b), and higher than *C. glutamicum* AR200 strain in which *divIVA-gfp* was expressed as a single copy on the chromosome (Fig. 3c). Cell-free extracts from *C. glutamicum* AR200 and RESF12 were electrophoresed by SDS-PAGE, transferred and analysed by Western blotting, using anti-DivIVA (Fig. 5a) and anti-GFP antibodies (data not shown). In both cases, the level of DivIVA-GFP was 3–4 times higher in *C. glutamicum* RESF12 than in AR200 (Fig. 5a).

The β-lactam antibiotic cephalexin is a specific inhibitor for FtsI that blocks cell division but does not affect the level of FtsZ or FtsI in *E. coli* (Pogliano *et al.*, 1997). *C. glutamicum* cell division was blocked by growing cells in TSA media containing a subinhibitory concentration of cephalexin (0·6 μg ml^{-1}) (Fig. 5c) to see if the inactivation of FtsI by this drug would increase the level of DivIVA. As shown in Fig. 5(a), inhibition of FtsI by cephalexin did not increase the level of DivIVA.

It was recently described that *C. glutamicum* cell division was partially blocked when the chromosomal copy of *ftsZ* was expressed under the control of P*lac (C. glutamicum* AR2), and even more when the *lacI*^{q} repressor was introduced in AR2 (*C. glutamicum* AR20) (Fig. 5b); the resulting strains showed aberrant cells but no filaments (Ramos *et al.*, 2005). The level of DivIVA in *C. glutamicum* AR2 and AR20 is similar to that in the wild-type strain (Fig. 5a).

Therefore, it may be concluded that *divIVA* is being overexpressed when FtsI is partially depleted in *C. glutamicum* and not when cell division is blocked either by inactivation of FtsI by cephalexin treatment or by partial depletion of FtsZ.

DISCUSSION

This work is an extension of our earlier studies elucidating cell division and polar growth of coryneform bacteria lacking both the FtsA and MreB systems, the structural homologues of actin. The actin cytoskeleton acts as an internal scaffold that influences cell shape through correct localization of peptidoglycan biosynthetic enzymes (Young, 2003). FtsZ is also an important component of the internal cytoskeleton and its polymerization at the septum facilitates FtsI in redirecting peptidoglycan synthesis in *E. coli* or *B. subtilis* (Nanninga, 1998). Peptidoglycan synthesis in those model micro-organisms proceeds by diffuse intercalation of new material being synthesized along the length of each cell. Peptidoglycan at the cell poles is inert, not being recycled or only being recycled at an extremely low rate (de Pedro *et al.*, 1997).

The situation is different in *C. glutamicum*, where peptidoglycan synthesis takes place at the septum and also at the cell poles (Daniel & Errington, 2003). It has been suggested that cell elongation occurs from the new cell poles (Daniel & Errington, 2003) as was also described for *C. diphtheriae* (Umeda & Amako, 1983). Corynebacteria probably have an apical growth reminiscent of the characteristic apical growth of actinomycetes. The localization of GFP-FtsI in this

organism appears to be similar to that seen in *E. coli* (Weiss *et al.*, 1999) and *B. subtilis* (Daniel *et al.*, 2000), and this suggests the participation of FtsI in the biosynthesis of peptidoglycan for septum formation. Sometimes, and in a non-repetitive way, it was also possible to see accumulation of GFP-FtsI at the cell poles, which is reminiscent of previous septa or artefacts due to non-specific accumulation of GFP-FtsI as described in *E. coli* (Weiss *et al.*, 1999).

Our results suggest that *ftsI* is transcribed both from a minor promoter (P*ftsI*), as in *B. subtilis* (Daniel *et al.*, 1996), and also as a part of the polycistronic *mraZ*, *mraW*, *ftsL* and *murE* transcript from an upstream promoter (P*mra*), as described for *E. coli* and *B. subtilis* (Hara *et al.*, 1997; Mengin-Lecreulx *et al.*, 1998). The *ftsI* gene seems to be essential for the viability of *C. glutamicum* since gene disruption was possible only in the merodiploid strain *C. glutamicum* MAPF. No transformants were recovered when we attempted to elicit a stronger reduction in the expression of *ftsI* under the control of P*lac* in *C. glutamicum* RESF1 transformed with a plasmid carrying *lacI*^{q}.

When *ftsI* was expressed as a single copy in the chromosome under the control of P*lac* (strain *C. glutamicum* RESF1) morphologically abnormal cells (filamentous or branched filaments) were obtained. The shape of RESF1 cells contrasts with the bulky and elongated cells obtained when cell division is inhibited by cephalexin treatment or by partial FtsZ depletion (Fig. 5). These abnormal cells were considered as a general strategy by the bacteria for increasing cell mass when division is blocked in rod-shaped microorganisms that lack actin homologues as was suggested by Latch & Margolin (1997) and Ramos *et al.* (2005). The cephalexin treatment has no effect on the levels of *ftsI* or *ftsZ* (Pogliano *et al.*, 1997), suggesting that the characteristic shape of *C. glutamicum* RESF1, as a consequence of a severe reduction (2·9-fold) in the expression of *ftsI* (Fig. 3b), is not only due to a block in cell division. This possibility prompted us to compare the proteome of *C. glutamicum* RESF1 with that of the wild-type. It was noticeable from our results that the level of DivIVA, among other proteins, increases in the strains that are partially depleted for FtsI.

Several hypotheses can be proposed to explain why a reduction in the levels of FtsI could account for an increase in the levels of DivIVA, a cell division-associated protein with a possible structural function at *C. glutamicum* growing cell poles (Ramos *et al.*, 2003b). The first possibility is that the filamentous phenotype may be due to the expression of the truncated *ftsI* in *C. glutamicum* RESF1 (Fig. 3b), although no filaments were observed in *C. glutamicum* APF (having a truncated form of *ftsI* and a unique copy of *gfp-ftsI* under the control of P*kan*) (Fig. 2b). The observed effect of DivIVA overexpression could also be due to polar effects on the expression of other genes in the *dcw* cluster located downstream from *ftsI*, such as *murE* (encoding the tripeptide synthetase MurE) or *murF* (encoding pentapeptide synthase MurF) (Fig. 1a). It has been described for *E. coli* that the balance between pentapeptide and tripeptide precursors determines whether the cells will divide or elongate (Begg *et al.*, 1990). We cannot rule out this option, but no effect on the morphology was observed when different *C. glutamicum* strains were obtained by simple recombination in the chromosome (Fig. 2). A third possibility is that depletion of FtsI, and not the inhibition of FtsI by cephalexin, alters the turnover or stability of DivIVA, or even the expression of the *divIVA* gene. Because the amount of FtsI protein, and not the activity of FtsI, could be the start point of a mechanism leading to DivIVA overexpression, it is possible that a balance between FtsI and DivIVA would be needed for cell division in *C. glutamicum*. The requirement of a specific ratio between two proteins of the *dcw* cluster has been described by Dewar *et al.* (1992) and Flardh *et al.* (1998).

The filamentous phenotype observed when *ftsI* is under the control of P*lac* seems to be the result of a lack of FtsI to synthesize peptidoglycan at the septum and an increase in the concentration of enzymes involved in central metabolism and DivIVA, which might form a scaffold structure that guides cell wall biosynthesis and maintains the cell diameter in MreB-lacking rod-shaped corynebacteria.

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