Involvement of DivIVA in the morphology of the rod-shaped actinomycete *Brevibacterium lactofermentum*

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In *Brevibacterium lactofermentum*, as in many Gram-positive bacteria, a *divIVA* gene is located downstream from the *dcw* cluster of cell-division- and cell-wall-related genes. This gene (*divIVA<sub>BL</sub>*) is mostly expressed during exponential growth, and the protein encoded, DivIVA<sub>BL</sub>, bears some sequence similarity to antigen 84 (Ag84) from mycobacteria and was detected with monoclonal antibodies against Ag84. Disruption experiments using an internal fragment of the *divIVA<sub>BL</sub>* gene or a disrupted *divIVA<sub>BL</sub>* cloned in a suicide conjugative plasmid were unsuccessful, suggesting that the *divIVA<sub>BL</sub>* gene is needed for cell viability in *Brev. lactofermentum*. Transformation of *Brev. lactofermentum* with a multicopy plasmid containing *divIVA<sub>BL</sub>* drastically altered the morphology of the corynebacterial cells, which became larger and bulkier, and a GFP fusion to DivIVA<sub>BL</sub> mainly localized to the ends of corynebacterial cells. This localization pattern, together with the overproduction phenotype, suggests that DivIVA may be important in regulating the apical growth of daughter cells.

INTRODUCTION

Corynebacteria are pleomorphic asporogenous Gram-positive bacteria widely distributed in nature. Some are plant and animal pathogens, and other non-pathogenic corynebacteria (*Brevibacterium lactofermentum* or *Corynebacterium glutamicum*) are used in the industrial production of amino acids and nucleotides (Gourdon & Lindley, 1999; Nakayama et al., 1978).

Hermann et al. (1998) identified the presence of a typical mycobacterial antigen (Antigen 84, Ag84) in *C. glutamicum* by protein microsequencing after two-dimensional gel electrophoresis (Hermann et al., 1998). The *Mycobacterium tuberculosis* gene encoding cytoplasmic Ag84 (*wag31*) (Cole et al., 1998) has been cloned (Hermans et al., 1995), and expressed as a 34 kDa protein in *Escherichia coli*, the recombinant protein corresponded to Ag84 in a crossed immunoelectrophoresis reference system. The homologous gene for Ag84 was also cloned from *Mycobacterium leprae* and its amino acid sequences showed 85% identity to the *M. tuberculosis* sequence, which indicates that Ag84 constitutes a group of conserved highly immunogenic mycobacterial antigens. The antibodies of almost 60% of lepromatous leprosy patients responded to Ag84 (Hermans et al., 1995).

Ag84 from *M. tuberculosis* is related to DivIVA, a protein encoded in the *dcw* cluster of several Gram-positive microorganisms (Massidda et al., 1998), including all Gram-positive bacteria sequenced to date. DivIVA has been extensively studied in *Bacillus subtilis*, in which inactivation of *divIVA* produces a minicell phenotype, whereas overproduction of DivIVA results in a filamentation phenotype (Cha & Stewart, 1997). Previous work has shown that in vegetatively growing *Bac. subtilis* cells, DivIVA is involved in cell division, and its role appears to be the sequestration of 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 *E. coli* MinE, which sequesters MinCD at the cell poles using a completely different mechanism (Marston et al., 1998). DivIVA has a second, quite separate role in sporulating cells of *Bac. subtilis*. Again, it acts at the cell pole but in this case it interacts with the chromosome segregation machinery to help position the oriC region of the chromosome at the cell pole, in preparation for the polar division event that initiates spore formation (Thomaides et al., 2001). More recently Harry & Lewis (2003), using a synchronous model system in *Bac. subtilis* (spore germination and outgrowth), found that DivIVA localizes to poles...
of germinated and outgrowing cells without the prior assembly of the division apparatus at this site, suggesting that its localization does not occur via a direct interaction with components of the division apparatus as proposed previously (Edwards et al., 2000). Later on DivIVA localizes, probably by interaction with a component of the cell division apparatus, to the division site before the final stages of Z-ring constriction (Harry & Lewis, 2003). Once the Z-ring constricts, DivIVA is attracted to the cell poles by an unidentified protein or by a physical property unique to the cell poles (Edwards et al., 2000; Harry & Lewis, 2003).

Here we show that the Brev. lactofermentum homologue of the wag31/divIVA is important in cell shape and morphology.

**METHODS**

**Bacterial strains, plasmids, and culture conditions.** Bacterial strains and plasmids are described in Table 1. E. coli cells were grown in Luria broth or Luria agar (Hanahan, 1983) at 37 °C with aeration. When necessary, kanamycin, hygromycin and ampicillin were added to a final concentration of 50 μg ml−1. *Brev. lactofermentum, Mycobacterium smegmatis, Bacillus subtilis* and *Streptomyces coelicolor* cells were grown in TSB (trypticase soy broth, Difco) or TSA (TSB containing 2 % agar) at 30 °C.

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

Plasmids to be transferred by conjugation from *E. coli* to *Brev. lactofermentum* were introduced by transformation into the donor strain *E. coli* S17-1. *Brev. lactofermentum* R31 was used as the recipient. The protocol for conjugation was a slight modification of the method developed by Schäfer et al. (1990).

DNA fragments were purified using the Gene Clean Kit (Bio 101). Restriction enzymes were purchased from Promega and New England Biolabs.

A library of *Brev. lactofermentum* ATCC 13869 DNA constructed in lambda gt11 (Honrubia et al., 1998) was checked for DNA–DNA hybridization screening using a 675 bp HindIII–XhoI fragment containing the 3’ end of ORF5, the intergenic region, and the ORF6 gene from *Brev. lactofermentum* present in plasmid pHFZ8 (probe A), and with a 600 bp BstXI–EcoRI fragment of the non-coding region (probe B) (Fig. 1). Two clones giving a positive signal with probe B and no signal with probe A were found, and one of them, which contained a 1.5 kb NolI insert, was subcloned into NolI-digested pBSK− and pBKs−, giving plasmids pARX2A and pARX2B.

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

Samples of total DNA from different *Brev. lactofermentum* trans-conjugants were digested with BamHI and hybridized with a 660 bp PvuII internal fragment (probe C, Fig. 1) of divIVA_R labelled with digoxigenin according to the manufacturer’s (Boehringer Mannheim) instructions.

RNA from *Brev. lactofermentum* strains containing plasmid pECM2 or plasmid pECAG1 was isolated at different culture times in TSB medium using the RNeasy commercial kit (Qiagen). For Northern experiments, 20 μg RNA was loaded onto a 1:5 % formaldehyde-agarose gel and transferred to nylon membranes. Filters were hybridized with an internal fragment of divIVA_R (513 bp HinII fragment; probe D; Fig. 1) from *Brev. lactofermentum* labelled by nick-translation.

**Plasmid constructions.** To disrupt divIVA_R by single recombination, two different constructions were made by cloning two internal overlapping DNA fragments separately into the conjugative suicide plasmid pK18mob (Table 1). A 500 bp internal BglII fragment (Klenow-filled) of divIVA_R (Fig. 1) was cloned into Smal-digested pK18mob and the resulting plasmid was named pKD8-1B (Table 1). A single reciprocal crossover event would create two interrupted DivIVA_R versions, one lacking 89 amino acids from its C-terminus, and the other lacking 100 amino acids from its N-terminus (see Fig. 2). The second plasmid (pKD8-1P) contained a 660 bp internal PvuII fragment (Fig. 1) cloned in Smal-digested pK18mob. The resulting truncated DivIVA_R proteins would lack 20 amino acids from the C-terminus or 150 amino acids from the N-terminus (Fig. 2; Table 1).

To disrupt the divIVA_R gene by double crossover recombination, a 1.4 kb PstI cassette (treated with mung bean nuclease) containing the hhy gene from *Streptomyces hygroscopicus* obtained from plasmid pUL880M (Table 1) was subcloned into the unique Ncol site (filled with Klenow) present in divIVA_R. The whole in vitro-disrupted divIVA_R gene was isolated by BstXI digestion, Klenow-filled, and subcloned into Smal-digested pK18mob, giving rise to pKD8-2 (Fig. 2).

To detect the presence of a functional promoter immediately upstream from divIVA_R, a 160 bp DNA fragment was amplified by PCR using the following primers: upper primer, 5'-GGGGTCTCTTGTGCTGGCTTGAAGTGTGCAAGG-3'; lower primer, 5'-GGGATTCCGATCGATTCCCTTGAGTTTAACGCTGGG-3'. Owing to the presence of a Ndel (CATATG) site in the lower primer and a single EcoRI in the amplified fragment, the PCR product was digested with EcoRI and Ndel and cloned into the promoter-probe vectors pECMcl-1 and pJMF24, both digested with EcoRI+ Ndel.

To overexpress divIVA_R in *Brev. lactofermentum*, a 1.3 kb EcoRI–BamHI fragment containing the entire divIVA_R gene (and upstream sequences) (Fig. 1) was cloned in the high-copy-number conjugative bifunctional plasmid pECM2 (Jager et al., 1992), giving pEAG1 (Table 1). The plasmid was then transferred to *Brev. lactofermentum* by conjugation.

Two different divIVA_R–gfp translational fusions were made. In both cases, the 3’ end of divIVA_R was amplified by PCR using the following primers: upper primer, 5'-ACGGGTTCAGCAGCTTTGAG-GCTGGTTCCGAATCAGGCTTTGCG-3'; lower primer, 5'-GGGAATTCCATATGGCACCAGATGCGTGGTGTGTTTG-3'. These primers were designed to replace the stop codon (TAA) of the divIVA_R gene (and upstream CATATG) by CAT (His), which after Ndel digestion and ligation with gfp will immediately be followed by the ATG start of gfp. Owing to the presence of restriction sites in the primers [SalI (GTCGAC) and Ndel (CATATG)] and a single Xhol in the amplified fragment, the SalI–Ndel-amplified fragment (480 nt) was cloned together with gfp (as a Ndel–XhoI fragment) in plasmid pET28a digested with SalI + Xbal. The gfp gene used was egfp2 from Clontech, and includes the mutations V163A and S175G introduced by Siemerling et al. (1996). The in-frame fused divIVA_R–gfp gene was isolated as a SalI–XhoI fragment, sequenced (see below), and cloned into plasmid pK18mob (digested with SalI and Xbal), to give pKAG1, which was introduced by conjugation into *Brev. lactofermentum*, and integrated into the chromosome by single recombination.
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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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td><em>E. coli</em> DH5α</td>
<td>r⁻ m⁻; used for general cloning</td>
<td>Hanahan (1983)</td>
</tr>
<tr>
<td><em>E. coli</em> S17-1</td>
<td>Mobilizing donor strain, <em>pro recA</em>, which has an RP4 derivative integrated into the chromosome</td>
<td>Schafer <em>et al.</em> (1990)</td>
</tr>
<tr>
<td><em>Brev. lactofermentum</em> ATCC 13869</td>
<td>Wild-type</td>
<td>American Type Culture Collection</td>
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<tr>
<td><em>Brev. lactofermentum</em> R31</td>
<td>13869 derivative used as host for transformation, electroporation or conjugation</td>
<td>Santamaria <em>et al.</em> (1985)</td>
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<tr>
<td><em>Brev. lactofermentum</em> AR20</td>
<td>R31 derivative containing a copy of divIVA–egfp in the chromosome</td>
<td>This work</td>
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<tr>
<td><em>Brev. lactofermentum</em> AR30</td>
<td>R31 transformed with plasmid pEAG2</td>
<td>This work</td>
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<tr>
<td><em>Mycobacterium smegmatis</em></td>
<td>Efficient plasmid transformation (Ept) strain</td>
<td>Snapper <em>et al.</em> (1990)</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> PY79</td>
<td>Wild-type</td>
<td>Youngman <em>et al.</em> (1984)</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pBKS/SK</td>
<td><em>E. coli</em> vectors containing bla, lacZ, orif1</td>
<td>Stratagene</td>
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<tr>
<td>pET28a</td>
<td><em>E. coli</em> vector containing kan, lacI, orif1</td>
<td>Novagen</td>
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<td>pPHFZ8</td>
<td>pBSK+ derivative containing a 3.3 kb NotI fragment from <em>Brev. lactofermentum</em> 13869 carrying the genes encoding YFIH, ORF5 and ORF6</td>
<td>Honrubia <em>et al.</em> (1998)</td>
</tr>
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<td>pARX2A</td>
<td>pBSK− derivative containing a 1.5 kb NotI fragment from <em>Brev. lactofermentum</em> 13869 carrying the genes encoding YlmG and DivIVA</td>
<td>This work</td>
</tr>
<tr>
<td>pARX2B</td>
<td>pBSK+ derivative containing a 1.5 kb NotI fragment from <em>Brev. lactofermentum</em> 13869 carrying the genes encoding YlmG and DivIVA</td>
<td>This work</td>
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<tr>
<td>pT7-5 and pT7-6</td>
<td><em>E. coli</em> vectors containing bla and promoter φ10</td>
<td>Tabor &amp; Richardson (1985)</td>
</tr>
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<td>pTAG1</td>
<td>pT7-5 derivative containing an 1.3 kb EcoRI–BamHI fragment containing the entire orf8 gene and upstream sequences</td>
<td>This work</td>
</tr>
<tr>
<td>pTAG2</td>
<td>pT7-6 derivative containing an 1.3 kb EcoRI–BamHI fragment containing the entire orf8 gene and upstream sequences</td>
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<td>pECMel-1</td>
<td>Bifunctional <em>E. coli–Brev. lactofermentum</em> promoter-probe vector containing kan as selective marker and the melC operon from <em>S. glaucescens</em> as reporter</td>
<td>Adham <em>et al.</em> (2003)</td>
</tr>
<tr>
<td>pJMFA24</td>
<td><em>E. coli</em> promoter-probe vector containing bla as selective marker and the promoterless kan gene as reporter</td>
<td>J. M. F. Abalos (unpublished)</td>
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<tr>
<td>pK18mob</td>
<td>Mobilizable plasmid containing an <em>E. coli</em> origin of replication and the kan resistance determinant</td>
<td>Schafer <em>et al.</em> (1994)</td>
</tr>
<tr>
<td>pK18-thrB1</td>
<td>pK18mob derivative containing a 750 bp EcoRV–PstI internal fragment of the <em>Brev. lactofermentum</em> 13869 thrB1 gene</td>
<td>Fernandez-Gonzalez <em>et al.</em> (1996)</td>
</tr>
<tr>
<td>pKD8-1B</td>
<td>pK18mob derivative containing a 500 bp BglII internal fragment of the <em>Brev. lactofermentum</em> 13869 divIVA gene</td>
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Table 1. cont.

<table>
<thead>
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<th>Strain or plasmid</th>
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<tr>
<td>pKD8-1P</td>
<td>pK18mob derivative containing a 660 bp PvuII internal fragment of the (13869) Brev. lactofermentum</td>
<td>This work</td>
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<tr>
<td>pUL880M</td>
<td>pUL880M</td>
<td>Adham et al. (2001)</td>
</tr>
<tr>
<td>pKD8-2</td>
<td>pK18mob derivative containing the (hyg) cassette from pUL880M inserted into the single NcoI site</td>
<td>This work</td>
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<tr>
<td>pECM2</td>
<td>Mobilizable plasmid capable of replicating in (Brev.) lactofermentum and containing the (divIVA) gene</td>
<td>Jager et al. (1992)</td>
</tr>
<tr>
<td>pEAG1</td>
<td>pECM2 derivative containing a 1 RI–BamHI fragment carrying the entire (divIVA) gene and (upstream) sequences</td>
<td>This work</td>
</tr>
<tr>
<td>pKAG1</td>
<td>pK18mob derivative containing the (D) gene (and upstream sequences) (Fig. 1)</td>
<td>This work</td>
</tr>
<tr>
<td>pEAG2</td>
<td>pTAG1 derivative containing the entire (divIVA) gene</td>
<td>This work</td>
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The \(\Delta divIVA_{BL}–gfp\) gene was also isolated from the pET28a derivative as an \(XhoI–XbaI\) fragment and cloned into plasmid pEAG1 (digested with \(XhoI\) and \(XbaI\)) to replace the 3' end of \(divIVA_{BL}\) (24 amino acids) by the 3' end of \(divIVA_{BL}\) fused to \(gfp\). The resulting plasmid, pEAG2, was constructed in \(E. coli\) and transferred by conjugation to \(Brev. lactofermentum\).

To express \(divIVA\) in \(E. coli\), a 1·3 kb EcoRI–BamHI fragment containing the entire \(divIVA\) gene (and upstream sequences) (Fig. 1) was cloned into plasmids pT7-5 and pT7-6 (both digested with EcoRI–BamHI), giving plasmids pTAG1 and pTAG2 respectively (Table 1).

**Sequencing and DNA analysis.** The 1·5 kb NotI fragments from plasmids pARX2A and pARX2B were trimmed down using the Erase A Base Kit (Promega). DNA sequencing was carried out by the dideoxy nucleotide chain termination method of Sanger. Computer analysis was performed with DNASTAR; database similarity searches were done at the BLAST and FASTA public servers (NCIB and EBI, Hinxton Hall, UK), and multiple alignments of sequences were accomplished using CLUSTAL W (EBI). The DNA sequence was deposited in the EMBL/GenBank database under accession number AJ242594. Plasmid constructions carrying \(\Delta divIVA_{BL}–gfp\) were confirmed to be correct by sequencing.

To localize the gene located downstream from \(divIVA\), we PCR amplified \(Brev. lactofermentum\) DNA using an upstream primer (5'-CCGCATCCACTCCTCTGCT-3') designed from \(divIVA\) and a downstream primer (5'-TCTGCACCTGGTCTGATGG-3') from the downstream gene (\(ileS\)) in the \(C. glutamicum\) genome. A DNA fragment of the expected size (1230 nt) was amplified and sequenced, and it was confirmed that \(ileS\) is the gene located downstream from \(divIVA\) in \(Brev. lactofermentum\), as in \(Streptomyces coelicolor\), \(Streptococcus pneumoniae\) and \(Corynebacterium diphtheriae\).

**Preparation of cell-free extracts, PAGE and Western blotting.** \(E. coli\) JM1109(DE3) cells transformed with pT7-5, pT7-6, pTAG1 or pTAG2 were grown at 37°C in LB broth with 100 \(\mu\)g ampicillin ml\(^{-1}\) until the OD\(_{600}\) reached 0·4. IPTG was added at a final concentration of 0·5 mM and the cultures were incubated for 3 h.

\(Brev. lactofermentum\), \(M. smegmatis\), \(S. coelicolor\) and \(Bac. subtilis\) cells were disrupted by sonication as follows. Cells (1 g wet weight) were suspended in 5 ml TES buffer (25 mM Tris/HCl, 25 mM EDTA, 10-3 % sucrose, pH 8). Sonication was carried out over periods of 30 s with 1 min intervals in an ice-cooled tube using a Branson sonifier (model B-12) at 75–100 W, until the cells had been completely disrupted as observed microscopically. Cell debris was removed by centrifugation, and the supernatants were used as cell extracts. \(E. coli\) cells were washed, resuspended in loading buffer, and boiled for 5 min. SDS-PAGE of cell extracts from the different micro-organisms 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 with monoclonal antibodies (FI26-2) raised against purified Ag84 from \(Mycobacterium kansasii\) provided by Professor A. H. J. Kolk (Royal Tropical Institute, Amsterdam, The Netherlands).

**Microscopic techniques.** \(Brev. lactofermentum\) cells containing constructions carrying GFP were observed under a Nikon E400 fluorescence microscope. Pictures were taken with a DN100 Nikon digital camera and assembled using Corel Draw.

For scanning electron microscopy, \(Brev. lactofermentum\) cells were collected by centrifugation, fixed for 2 h at room temperature in 2-5 %
glutaraldehyde in 100 mM cacodylate buffer (pH 7.4), rinsed three times in cacodylate buffer (pH 7.4), and postfixed for 2 h in 1 % osmium tetroxide. Cells were washed twice with cacodylate buffer and recovered by filtration in Millipore filters (0.20 μm diameter). After passage through 20 %, 50 %, 75 %, 95 %, and 100 % ethanol, filters containing the cells were dried using the critical-point method and finally coated with gold, giving a layer 40 nm thick. Cells were observed with a scanning electron microscope (JEOL JSM-6100) at an accelerating voltage of 20 kV.

**RESULTS**

Cloning of a DNA fragment of *Brev. lactofermentum* located downstream from the *dcw* cluster

Honrubia et al. (1998) cloned and sequenced a 6.5 kb fragment (Y08964) from the chromosomal DNA from *Brev.*
lactofermentum ATCC 13869 carrying part of the dcw cluster (genes involved in division and cell wall biosynthesis). In this 6.5 kb DNA fragment, they localized the genes encoding MurC, FtsQ, FtsZ, and three ORFs (YFIH, ORF5 and ORF6) of unknown function but not essential for the growth and viability of Brev. lactofermentum (Honrubia et al., 2001). In order to localize new genes from the dcw cluster, we cloned the DNA region located downstream from the 6.5 kb fragment.

A 1505 bp downstream sequence (GenBank accession no. AJ242594) contained two possible ORFs (ORF7 and ORF8). ORF7 encodes a small protein of 95 amino acids and showed similarities with ORF Rv2146c of M. tuberculosis (FASTA E value 2.6 × 10^{-26}), and with ORF SCO2078 from S. coelicolor A3(2) (FASTA E value 1 × 10^{-25}). These proteins belong to the group of YlmG proteins, a group of small (approx. 10 kDa) conserved hypothetical proteins with a transmembrane domain, localized downstream from ftsZ in most of the dcw clusters from Gram-positive bacteria analysed (Massidda et al., 1998).

ORF8 is located 488 bp downstream from ORF7, and encodes a protein of 365 amino acids with a predicted molecular mass of 38.7 kDa. A stem–loop-forming sequence \((1^{231}\text{GCTCCGCGTCGG}\cdots^{1266}\text{TCGGGATCCGGGAGCG})\) is located 28 nt downstream from the ORF8 gene and may be a transcriptional terminator. When the amino acid sequence of ORF8 was aligned with protein databases using BLAST (NCBI) it showed significant scores with Ag84 from M. tuberculosis \((E\ value 1 \times 10^{-21})\), M. leprae \((E\ value 7 \times 10^{-20})\) and ORF SCO2077 from S. coelicolor A3(2) \((E\ value 8 \times 10^{-8})\), all of which are encoded by genes located in the same position with respect to the dcw cluster. A very low score with Bac. subtilis DivIVA was found \((E\ value 1\text{-}8)\), and none with DivIVA from Streptococcus pneumoniae or Staphylococcus aureus. The gene was designated divIVA_{BL} like the S. coelicolor A3(2)
parologue (Flardh, 2003) to avoid misunderstanding. No similar gene was detected in the chromosome of *Brev. lactofermentum* on the basis of Southern analysis.

The N-terminal region of DivIVA homologues is highly conserved (Fig. 1B) and is predicted to adopt a coiled-coil conformation using the COILS algorithm (Lupas et al., 1991). Another coiled-coil domain is predicted at the C-terminus in all DivIVA proteins analysed. In *S. coelicolor* DivIVAs, the predicted coiled-coil regions are interrupted by a low-complexity region (from amino acid 69 to 201), mainly composed of glycine, proline and glutamic acid. DivIVA BL showed an alanine-rich region from amino acid 58 to 302 and two extra coiled-coil regions. It is also interesting to note that the DivIVA from *Brev. lactofermentum* has been included by InterPro within the 'Pollen allergen Poa PI signature' group of proteins (InterPro Entry IPR001778) and it has three clear domains characteristic of these proteins (Fig. 1B). These Poa signatures are absent in the Ag84 from *M. tuberculosis*, and in the DivIVA from *S. coelicolor* or *Bac. subtilis* (Fig. 1B).

Once the complete genome of *Corynebacterium glutamicum* had become available (accession no. AX114121), we analysed the DNA sequence located downstream from *divIVA* in *Brev. lactofermentum*. By PCR analysis we found that the next gene is oriented in the same direction, and encodes a putative tRNA synthetase (*ileS*), as in *S. coelicolor*, *Streptococcus pneumoniae*, *C. diphtheriae* and *C. glutamicum*.

Attempts to disrupt the *divIVA* gene in *Brev. lactofermentum* by single and double recombination

In order to see whether *divIVA* is necessary for the viability of *Brev. lactofermentum*, we attempted disruption experiments using internal fragments of *divIVA* (single crossover event, single recombination) or an *in vitro*-disrupted *divIVA* gene (double crossover event, double recombination).

The conjugative suicide plasmid pKD8-1B (containing an internal fragment of *divIVA*; Fig. 2A) and pK18-thrB1 (used as control) were introduced separately into *E. coli* S17-1 and mated with *Brev. lactofermentum* R31 cells. *Brev. lactofermentum* kanamycin-resistant transconjugants were obtained only after mating with *E. coli/pK18-thrB1*, suggesting the lethality of the *divIVA* gene disruption or the lethality of its polar effects. However, the comparatively small size of *divIVA* (500 bp, compared with the 750 bp *thrB1* fragment) might also have greatly reduced the frequency of integration events. Therefore, to further test whether *divIVA* is essential for the growth and viability of *Brev. lactofermentum* we attempted to replace the chromosomal *divIVA* by an *in vitro*-interrupted version of the gene. A knockout mutation (*ΔdivIVA*::hyg) conferring hygromycin resistance was created in plasmid pK18mob (containing *kan*), giving rise to pKD8-2. Since pKD8-2 cannot replicate in *Brev. lactofermentum*, hygromycin-resistant transconjugants were expected to arise by single or double recombination between the *Brev. lactofermentum* insert in the plasmid and homologous sequences in the chromosome (see Fig. 2B). However, all the transconjugants analysed contained the original *divIVA* gene and the Δ*divIVA*::hyg, suggesting that only a single crossover event had taken place. No hygromycin-resistant kanamycin-sensitive strains were found, even after several transfers on TSA lacking kanamycin and checking more than 10 000 hygromycin-resistant colonies. These results strongly suggest that *divIVA* is essential for the growth/viability of *Brev. lactofermentum*. Comparable results have been obtained in *Bac. subtilis* (Edwards & Errington, 1997) and in *S. coelicolor* A3(2) (Flardh, 2003).

Another plasmid (pKD8-1P) was constructed to interrupt *divIVA* by single recombination using an internal 660 bp *PvuII* fragment overlapping the 500 bp *BglII* fragment used above. pKD8-1P was introduced into *E. coli* S17-1 and mated with *Brev. lactofermentum* R31 cells. As can be observed in Figs 3 and 4, transconjugants from both solid and liquid media showed an altered morphology (rounder, large and swollen cells that tended to grow at the poles), indicating the possible participation of *divIVA* in the maintenance of cell morphology in *Brev. lactofermentum*. *Brev. lactofermentum* carrying the empty vector pECM2 never showed this kind of morphology (Fig. 3A and 4A). This aberrant morphology is obviously different from that observed in *M. tuberculosis* when overexpressing *ftsZ* (Dziadek et al., 2002), in *Rhizobium* (*Sinorhizobium*) *meliloti* overexpressing *ftsZEC* or *ftsZRM* (Latch & Margolin, 1997), or in *Brev. lactofermentum* overexpressing *ftsZBL* (A. Ramos, unpublished data); it was concluded that in rod-shaped micro-organisms branching and swelling are default pathways for increasing mass when cell division is blocked after *ftsZ* overexpression (Latch & Margolin, 1997).

To analyse whether the *divIVA* gene was overexpressed in *Brev. lactofermentum*/*pEAG1*, RNA was isolated at different points along the growth curve and compared with RNA from *Brev. lactofermentum*/*pECM2* cells. A clear delay was seen in the growth rate of *Brev. lactofermentum*/*pEAG1* as compared with *Brev. lactofermentum*/*pECM2* measured...
by optical density (Fig. 5A) or by counting the number of viable cells (Table 2), but at the stationary phase, both strains reached the same optical density and a similar number of viable cells. This result, together with the aberrant morphology of *Brev. lactofermentum*/pEAG1, suggests that there is no clear effect on the inhibition of cell division when *divIVABL* is overexpressed, and probably overexpression only affects the size and morphology of the cells.

A monocistronic transcript of 1.5 kb was observed at the beginning and during the exponential phase, clearly decreasing in the stationary phase in *Brev. lactofermentum*/pECM2, whereas an elevated amount of specific mRNA was detected in *Brev. lactofermentum*/pEAG1 (Fig. 5B). Using antibodies against Ag84 from *M. kansasii*, the level of DivIVA in *Brev. lactofermentum*/pEAG1 was 10-fold higher than in *Brev. lactofermentum*/pECM2 (Fig. 5C). Thus, overexpression of *divIVA* is not lethal.

**Fig. 3.** Phase-contrast microscopy of *Brev. lactofermentum*/pECM2 (A) and *Brev. lactofermentum*/pEAG1 (B1 and B2). These pictures were taken at the same magnification (× 400). (C) Digital enlargements of *Brev. lactofermentum*/pEAG1 cells.

**Fig. 4.** Scanning electron microscopy of *Brev. lactofermentum*/pECM2 (A) and *Brev. lactofermentum*/pEAG1 (B). These pictures were taken at the same magnification (× 10,000) and processed digitally using the same parameters. The bar represents 1 μm.
Table 2. Viable count (cells ml⁻¹) of *Brev. lactofermentum* at various OD₆₀₀ values

<table>
<thead>
<tr>
<th>OD₆₀₀</th>
<th><em>Brev. lactofermentum</em>/pECM2</th>
<th><em>Brev. lactofermentum</em>/pEAG1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1·6</td>
<td>2·1 × 10⁸</td>
<td>0·4 × 10⁸</td>
</tr>
<tr>
<td>3·6</td>
<td>6·2 × 10⁸</td>
<td>2·3 × 10⁸</td>
</tr>
<tr>
<td>6·3</td>
<td>9·1 × 10⁸</td>
<td>3·4 × 10⁸</td>
</tr>
<tr>
<td>7·2</td>
<td>1·4 × 10⁹</td>
<td>1·3 × 10⁹</td>
</tr>
</tbody>
</table>

**Construction of divIVA<sub>BL</sub>–gfp fusions**

Previous results in *Bac. subtilis* had suggested that DivIVA is recruited to mid-cell once the FtsZ ring has matured, and after cell division DivIVA and MinCD is localized to the cell poles (Harry & Lewis, 2003; Marston et al., 1998). To investigate a possible role of divIVA<sub>BL</sub> in corynebacterial morphogenesis, plasmids pKAG1 and pEAG2 were introduced by conjugation into *Brev. lactofermentum*, to give strains bearing a single copy of divIVA<sub>BL</sub>–gfp in the chromosome or multiple copies of divIVA<sub>BL</sub>–gfp, respectively.

Microscopical observation of both strains revealed that DivIVA–GFP was located mainly at the cell poles (Fig. 6), but in both cases there was a stronger accumulation at one pole than the other. This was especially noticeable in *Brev. lactofermentum* AR30 (multicopy fusion; Fig. 6B), where swollen and aberrant cells were formed. These accumulations were not detected by phase-contrast microscopy, indicating that they were not insoluble inclusions, but real structures. In those cells, and to a lesser extent in *Brev. lactofermentum* AR20 (single-copy fusion; Fig. 6A), after cell division the elongation of the daughter cells seemed to be asymmetrical, suggesting a nascent mycelial growth in this lower actinomycete. The size and intensity of the fluorescent region of DivIVA–GFP at the pole of the cell, especially in *Brev. lactofermentum* AR30, indicated that a large number of molecules was concentrated there. These observations were made clearer by the negligible fluorescence background seen in *Brev. lactofermentum* control strains, compared with the significant autofluorescence observed with some other bacteria (e.g. *S. coelicolor*). We assumed that the GFP–DivIVA<sub>BL</sub> fusions are functional because *Brev. lactofermentum*/pEAG1 showed comparable cell morphology to *Brev. lactofermentum*/pEAG2, and the morphology of *Brev. lactofermentum* AR20 is similar to that of *Brev. lactofermentum* R31 and *Brev. lactofermentum*/pECM2.

**Expression of divIVA<sub>BL</sub> in *E. coli* and detection using monoclonal antibodies**

To confirm that DivIVA<sub>BL</sub> is also an Ag84 homologue, we attempted to express the divIVA<sub>BL</sub> gene in a bacterial host lacking this gene, or encoding a homologue that is not recognized by F126-2 antibodies raised against Ag84 from *M. kansasii*. Using this antibody, we detected immunoreactive protein bands in cell-free extracts from *Brev. lactofermentum* ATCC 13869, *S. coelicolor* A3(2), *M. tuberculosis* and *M. smegmatis*, but not in those from *E. coli* or *Bac. subtilis* (Fig. 7). Therefore, *E. coli* seems to be an appropriate host to express divIVA<sub>BL</sub> and for detecting its gene product. As can also be observed in Fig. 7, an immunoreactive protein band of the expected size (38·7 kDa) was detected in cell-free extracts from *E. coli*/pTAG1 and *E. coli*/pTAG2, but not from *E. coli*/pT7-5 or *E. coli*/pT7-6. No morphological changes were observed in *E. coli*/pTAG1 or *E. coli*/pTAG2, and no apparent overexpression of the divIVA<sub>BL</sub> was observed after staining with Coomassie blue (Fig. 7).
Because in plasmid pTAG1 the 
*Brevis. lactofermentum*

divIVA<sub>BL</sub> gene is located downstream from the ϕ10 pro-
moter and in pTAG2 it is located in the opposite direction of
the ϕ10 promoter, divIVA<sub>BL</sub> should be expressed in *E. coli*
from its own promoter. To confirm this hypothesis, a 164 bp
DNA fragment upstream from divIVA<sub>BL</sub> was amplified
by PCR and cloned into two different promoter-probe
plasmids (pECMel-1 and pJMFA24). The 164 bp fragment
had promoter activity both in *E. coli* and in
*Brevis. lactofermentum* (data not shown), like many promoter sequences
isolated from *Brevis. lactofermentum* (Patek et al., 1996). In
conclusion, divIVA<sub>BL</sub> is expressed in *E. coli* from its own
promoter and its gene product is detected by antibodies
against Ag84 from *M. kansasii*.

**DISCUSSION**

The predicted products of divIVA-like genes in Gram-
positive bacteria show relatively poor primary amino acid
conservation but computer predictions strongly suggest
that they could form α-helical coiled-coils *in vivo*, which
might be involved in oligomerization (Muchova et al., 2002).
The extent of the divergence of DivIVA sequences suggests
that they might have evolved to serve organism-specific
purposes and their functions may also have diverged.

In *Brevis. lactofermentum*, divIVA<sub>BL</sub> seems to be essential for
viability, since no disruptants were obtained using different
strategies, and due to the fact that divIVA<sub>BL</sub> is expressed as a
monocistronic transcript of 1.5 kb during growth, no polar
effects are expected on the downstream essential ileS gene.
Owing to the lack of more effective systems to manipulate
*Brevis. lactofermentum*, it is difficult to introduce a second
copy of divIVA<sub>BL</sub> into the chromosome of *Brevis. lactofer-
mentum* and disrupt one of them. Nevertheless, the present
data strongly suggest that the gene is essential for viability.

In *Brevis. lactofermentum* overexpression of DivIVA is not

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![Fig. 6. Phase-contrast microscopy (PC), fluorescence microscopy (F) and phase-contrast microscopy + fluorescence microscopy (PC+F) of *Brevis. lactofermentum* AR20 (orf8–egfp as single-copy in the chromosome) (A) and *Brevis. lactofermentum* AR30 (orf8–egfp in the high-copy number plasmid pECM2) (B). Note that picture A-PC+F is a digital image overlay of pictures A-PC and A-F. All pictures were taken at the same magnification (×400) and enlarged digitally using the same parameters. The fluorescence pictures of *Brevis. lactofermentum* AR30 were exposed for 1/15 s, whereas that of *Brevis. lactofermentum* AR20 was exposed for 1 s.](image)

![Fig. 7. (A) SDS-PAGE of cell extracts from *E. coli* JM109(DE3) carrying plasmids pT7-5 (lane 1), pTAG1 (lane 2), pT7-6 (lane 3) and pTAG2 (lane 4) after IPTG induction, *Brevis. lactofermentum* ATCC 13869 (lane 6), *S. coelicolor* (lane 7), *M. tuberculosis* (lane 8), *Bac. subtilis* (lane 9) and *E. coli* (lane 10). Lane 5, molecular mass markers. (B) Western blot probed with the monoclonal antibody F126-2.](image)
lethal but it might partially affect cell division because cells containing pEAG1 are bigger, exhibit aberrant morphology and grew at a slower rate than cells carrying the empty vector pECM2. Therefore, the more visible effects of divIVA<sub>BL</sub> overexpression were on cell size and cell morphology. Moreover, we assume that DivIVA<sub>BL</sub> does not act through a MinCD system since no homologue of MinCD has been detected in the <i>C. glutamicum</i> genome (A. Ramos, unpublished results) nor in <i>S. coelicolor</i> A3(2) (Flardh, 2003). The above data therefore suggest that DivIVA<sub>BL</sub> is mainly important in the determination of cell shape, but we cannot exclude the possible role of DivIVA<sub>BL</sub> as a positive regulator of apical growth.

In <i>Bac. subtilis</i> DivIVA–GFP is targeted to a committed division site, and once daughter cells have become separated it remains at the cell poles, attracting MinD, and preventing these potential division sites from being used again, which would generate minicells (Marston et al., 1998). In corynebacteria, DivIVA–GFP is mainly localized to the cell poles, although to a lesser extent it can be found in the mid-cell area. The intensity of fluorescence, even when DivIVA–GFP is integrated as a single copy into the <i>Brev. lactofermentum</i> chromosome, suggests that DivIVA could form an oligomeric (perhaps through the coiled-coil regions) structure involved in the apical growth of corynebacterial cells. Thus, overexpression leads to dramatic changes in morphology. The asymmetric terminal location of the DivIVA protein may be widespread among actinomycetes, with different levels of morphological complexity, since a homologous protein in <i>S. coelicolor</i> A3(2) is located at the tips of the hyphae, where it appears to determine growth polarity (Flardh, 2003). On the other hand, staining of <i>S. coelicolor</i> mycelia with vancomycin labelled with fluorescein (Van-FL) gave bright staining at the ends of the hyphae and at intermediate sites corresponding to branching points (Daniel & Errington, 2003).

Staining of <i>Bac. subtilis</i> and <i>C. glutamicum</i> with Van-FL has clearly shown that using different growth strategies both Gram-positive bacteria achieve a rod-shaped morphology (Daniel & Errington, 2003). In <i>Bac. subtilis</i>, staining with Van-FL was mainly concentrated in the mid-cell but not at the cell poles, whereas in <i>C. glutamicum</i> Van-FL staining was observed at the mid-cell but mainly at the poles. It was concluded that in <i>C. glutamicum</i> cell elongation occurs from the new cell poles (Daniel & Errington, 2003) and therefore DivIVA<sub>BL</sub> is localized close to the sites of peptidoglycan assembly in <i>Brev. lactofermentum</i> as well as in <i>S. coelicolor</i> A3(2) (Flardh, 2003). The Van-FL staining of <i>C. glutamicum</i> is also in agreement with the cellular location of the inorganic pyrophosphatase from <i>Brev. lactofermentum</i> at the cell poles (Ramos et al., 2003). Because pyrophosphate is a by-product in the biosynthesis of UDP-N-acetylglucosamine by the UDP-N-acetylglucosamine pyrophosphorylase (EC 2.7.7.23), high levels of pyrophosphate should be formed during the biosynthesis of cell wall intermediates. Moreover, in the biosynthesis of the cell wall there is a strong requirement of ATP for the synthesis of UDP-N-acetylmuramic acid (UDP-MurNAc)-pentapeptide (Falk et al., 1996). Therefore hydrolysis of pyrophosphate is important for driving the above anabolic pathway in the direction of biosynthesis and replenishes the orthophosphate required for ATP biosynthesis (Perez-Castineira et al., 2002).

From all the data presented here it can be proposed that DivIVA is an essential protein with a possible structural function at <i>Brev. lactofermentum</i> growing cell poles, and probably accumulates there by an unknown affinity mechanism for curved surfaces (Harry & Lewis, 2003) or through interaction with proteins involved in peptidoglycan biosynthesis.

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