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The cell division genes \textit{ftsQ} and \textit{ftsZ}, but not the three downstream open reading frames YFIH, ORF5 and ORF6, are essential for growth and viability in \textit{Brevibacterium lactofermentum} ATCC 13869

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Abstract The three ORFs (YFIH, ORF5 and ORF6) located downstream of the cell division genes \textit{ftsQ} and \textit{ftsZ} in \textit{Brevibacterium lactofermentum} were disrupted by single homologous recombination events between internal fragments of the corresponding genes and the chromosomal sequences. The phenotypes of the disrupted mutants were similar to that of the wild type, suggesting that these genes are dispensable for growth and viability. However, using different plasmid constructs, it was not possible to obtain disrupted \textit{ftsZ} or \textit{ftsQ} mutants by single crossover events. When the \textit{ftsZ} or \textit{ftsQ} gene sequence was disrupted in vitro and used to replace the homologous chromosomal gene by double recombination, only single recombination events took place, and therefore no disruptants were obtained. It may be concluded therefore that, as in \textit{Escherichia coli}, the cell division genes \textit{ftsQ} and \textit{ftsZ} are indispensable for growth and viability of \textit{B. lactofermentum}. Northern hybridisation analyses performed using internal fragments of the genes coding for YFIH, ORF5 and ORF6 allowed us to dissect their transcriptional organization and to confirm the disruption of these genes.

Keywords \textit{dew} cluster · \textit{FtsZ} · \textit{FtsQ} · Corynebacteria · Cell division genes

Introduction

Part of the \textit{dew} (division-cell wall biosynthesis) gene cluster of coryneform bacteria has previously been cloned from \textit{Brevibacterium lactofermentum} (Honrubia et al. 1998) and \textit{B. flavum} (Kobayashi et al. 1997; Wachi et al. 1999). Sequence analysis revealed the presence of six ORFs (MurC, FtsQ, FtsZ, YFIH, ORF5 and ORF6) that share homologies with various proteins in the data banks. All of them are transcribed in the same direction (Honrubia et al. 1998). The genomic organization of the cluster is similar to that found in \textit{Mycobacterium tuberculosis} (Cole et al. 1998).

\textit{FtsZ} is a tubulin-like protein (Erickson 1995; Nogales et al. 1998) that acts at an early stage of cell division, polymerising into a ring at the site of septum formation (Addinall and Lutkenhaus, 1996a, 1996b; Ma et al. 1996) that presumably serves as a scaffold for the assembly of the cell division apparatus. The \textit{ftsZ} gene is essential for cell division in bacteria that divide by binary fission, such as \textit{Escherichia coli} and \textit{Bacillus subtilis} (Beall and Lutkenhaus 1991; Dai and Lutkenhaus 1991), whereas in \textit{Streptomyces} \textit{ftsZ} is required only for separation during sporulation (McCormick et al. 1994). In \textit{Bacillus subtilis}, the gene is not only required for vegetative cell division but also for asymmetric division during sporulation (Beall and Lutkenhaus 1991).

\textit{FtsQ} and DivIB are membrane proteins with a single transmembrane domain, characterised in \textit{E. coli} and \textit{B. subtilis}, respectively. Whereas in \textit{B. subtilis} DivIB is present at a concentration of 5000 molecules per cell (Rowland et al. 1997), in \textit{E. coli} FtsQ is present in only about 25 molecules per cell (Carson et al. 1991). The function of FtsQ/DivIB in the process of septum formation is not very well understood, although it has been proposed to play some role in shape determination in bacteria (Pucci et al. 1997). In \textit{E. coli}, FtsQ is essential for cell growth and is required throughout the formation of the cell septum (Barondess et al. 1991; Carson et al. 1991). In \textit{B. subtilis} a divIB null mutant was found to be extremely temperature sensitive and was unable to form colonies at 37°C (Beall and Lutkenhaus 1989). In \textit{S. coelicolor}, FtsQ is required for efficient sporulation but not for growth and viability (McCormick and Losick 1996).

The three ORFs (YFIH, ORF5 and ORF6) located downstream from \textit{ftsZ} in \textit{B. lactofermentum} (Honrubia...
et al. 1998) are homologous to three ORFs of unknown function also found downstream from fisZ in the Gram-positive bacteria *B. subtilis* (Kunst et al. 1997) and *M. tuberculosis* (Cole et al. 1998). Because the gene coding for YFIH in *B. subtilis, M. tuberculosis* and *Streptomyces* (Dharmatlake and Kendrich 1994) is located close to fisZ, it has been proposed that YFIH may be involved in cell division or cell-wall biogenesis in these organisms. In *E. coli* and *Shigella flexneri* YFIH may participate in the biosynthesis of the enterobacterial common antigen (ECA) (Macpherson et al. 1994; Marolda and Valvano 1995).

*B. lactofermentum* ORF5 also shows similarities with the hypothetical proteins YPII of *Vibrio alginoliiticus* and YPT5 of *Pseudomonas aeruginosa*. The genes coding for both hypothetical proteins are located in the pilT region of the chromosome (within the twitching motility region; Whitchurch et al. 1991) and are probably involved in proline biosynthesis.

Comparison of the *B. lactofermentum* ORF6 with sequences in the databases, using the FASTA or BLITZ programs, revealed significant homologies only with hypothetical proteins from *M. tuberculosis* (YAK1), *B. subtilis* (YLMF) and *Synechocystis* sp.

The aim of this work was to determine whether fisZ and fisQ are essential genes in *B. lactofermentum*, as in *E. coli*, or whether they are dispensable for growth, as is *Streptomyces*. In addition, because the function of none of the three ORFs located downstream from fisZ is known in any microorganism, we decided to perform disruption experiments in the hope of understanding the function of these ORFs in *B. lactofermentum*.

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**Materials and methods**

**Bacterial strains and plasmids**

The bacterial strains and plasmids used are described in Table 1. *E. coli* cells were grown in L broth (LB) at 37°C. *B. lactofermentum* cells were grown in TSB, TSA (TSB containing 2% agar) or MM (Kaneko and Sakaguchi 1979) at 30°C. When necessary, cultures were supplemented with kanamycin (Km; 50 μg/ml for *E. coli* and 25 μg/ml for *B. lactofermentum*) and ampicillin (Ap, 100 μg/ml for *E. coli*).

**DNA isolation and manipulation**

Plasmid DNA was isolated from *E. coli* and from corynebacteria as described by Kieser (1984). *E. coli* cells were transformed by the method of Hanahan (1983) and corynebacteria by electroporation (Duncan and Schivnan 1989). The plasmids to be transferred by conjugation from *E. coli* to *B. lactofermentum* were introduced by transformation into the donor strain *E. coli* S17-1 (Table 1). *B. lactofermentum* R31 was used as the recipient strain. Conjugation between *E. coli* and *B. lactofermentum* was performed according to Fernández-González et al. (1996).

Purification of DNA fragments was carried out using Qiaex II (Qiagen). Restriction enzymes were purchased from Promega and Amersham.

Total DNA was isolated from corynebacteria using the method described for *Streptomyces* (Hopwood et al. 1985), except that the cells were treated with lysozyme for 3 h at 30°C. Total DNA from *B. lactofermentum* transformants or transconjugants was digested with various restriction enzymes and hybridised with the kan gene or with internal fragments of the fisZ, fisQ, YFIH, ORF5 and ORF6 genes, labelled with digoxigenin according to the manufacturer’s (Boehringer Mannheim) instructions.

Amplification of internal fragments of the genes fisQ, YFIH, and ORF5 of *B. lactofermentum*

The primers used for PCR amplification of a fragment of fisQ were fisQ-A (5’-ACGGCGACAGTCCGGATCAA-3’) and fisQ-B (5’-CAGCTTCCCCGATCCA-3’). Amplification of YFIH and ORF5 was carried out with the primer pairs designed to introduce EcoRI sites at the ends: YFIH-A (5’-CTAGAATTCGATTATTTTCTGATCCTG-3’) and YFIH-B (5’-CTAGAATTCGGAGGATCCGTGAGATCTTGACACAGT-3’), and ORF5-A (5’-CTAGAATTTGACCTCAGAGAGAT-3’) and ORF5-B (5’-CTAGAATTTGACCTCAGAGAGAT-3’).

PCR assays were performed on a Perkin-Elmer programmable thermal reactor in 0.1 ml reactions containing AmpliTaq buffer, dNTPs at 2 mM, primers at 20 μM, 0.1–1 μg of DNA template, and 2.5 U of AmpliTaq DNA polymerase. Each of the 30 amplification cycles consisted of denaturation for 45 s at 95°C, annealing for 60 s at 50–60°C (depending on the primer pair used), and polymerisation for 45 s at 72°C. The PCR products were separated by electrophoresis on 2% agarose gels in TRES-acetate-EDTA, excised, and purified by extraction with phenol-chloroform and ethanol precipitation. The purified PCR fragments were used as probes in Northern experiments, or digested with EcoRI and cloned in the plasmids pK18mob and pULM170 (Table 1).

**Northern hybridization and primer extension analysis**

DNA from the *B. lactofermentum* strains ATCC 13869 and R31, and disrupted mutants, were isolated after culture in TSB medium for various times, using the RNeasy kit (Qiagen). For Northern experiments, 20 μg aliquots of total RNA were loaded into a 1.5% formaldehyde-agarose gel, electrophoresed, and transferred onto a nylon membrane. Filters were hybridised with an internal fragment of YFIH (517 bp PCR fragment), ORF5 (496 bp PCR fragment) or ORF6 (409 bp EcoRI-SacI fragment) from *B. lactofermentum*, labelled by nick translation.

**Microscopy**

Cell morphology was examined by microscopy as described previously (Honrubia et al. 1998).

**Results and discussion**

Attempts to disrupt fisQ and fisZ in *B. lactofermentum* using internal fragments of the genes

In order to determine whether fisQ and fisZ are necessary for the viability of *B. lactofermentum* we attempted to disrupt the genes using the technique initially described by Shortle et al. (1982). Due to the