Structure and organization of the *rrnD* operon of '*Brevibacterium lactofermentum*': analysis of the 16S rRNA gene

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Five rRNA operons (*rrn*) were found by hybridization in the genome of '*Brevibacterium lactofermentum*' ATCC 13869 and *Corynebacterium glutamicum* ATCC 13032. '*B. lactofermentum*' DSM 20412 differed from the other corynebacteria tested in showing six hybridizing *Bam*HI bands. Two of the *rrn* operons (*rrnD* and *rrnE*) were located in a single cosmid. Sequencing of the *rrnD* operon showed that it contains a complete 16S rRNA–23S RNA–5S rRNA gene cluster. Phylogenetic studies using the complete 16S rRNA sequence showed that '*B. lactofermentum*' is closely related to several species of the genus *Corynebacterium* but only distantly related to the type species *Brevibacterium linens* and the authors suggest that it should be reclassified as *Corynebacterium lactofermentum*. The 5' end of mature 16S rRNA was identified by primer extension. Sequence elements similar to those of mycobacteria implicated in transcription antitermination (Boxes A, B, C) and in processing of the pre-rRNA to 16S rRNA were identified. An open reading frame encoding an *rpoD*-like sigma factor (named SigC) different from the previously reported SigA and SigB proteins was found upstream of *rrnD* in the opposite orientation. Both *rpoD* and *sigC* seem to be expressed from a bidirectional promoter region.

Keywords: corynebacteria, rRNA, clusters, expression, sigma factor

INTRODUCTION

'*Brevibacterium lactofermentum*' belongs to the 'glutamic acid' group of non-pathogenic corynebacteria, so called because of their well-known amino acid overproducing capability (Eikmanns *et al.*, 1993). Knowledge on the molecular biology of this group of bacteria has advanced considerably in the last decade, including studies on codon usage (Malumbres *et al.*, 1993), sigma factors (Oguiza *et al.*, 1996), promoters (Pátek *et al.*, 1996), terminators and regulatory sequences (Martín, 1989; Guerrero *et al.*, 1994; Mateos *et al.*, 1994), insertion sequences (Bonamy *et al.*, 1994; Correia *et al.*, 1996; Vertès *et al.*, 1994b), transposon mutagenesis (Vertès *et al.*, 1994a) and genome organization (Bathe *et al.*, 1996; Correia *et al.*, 1994). Several amino acids are accumulated extracellularly at millimolar levels in cultures of '*B. lactofermentum*' or *Corynebacterium glutamicum* following growth limi-

tations that impair protein synthesis. However, in spite of their industrial importance little is known about the molecular mechanisms that control growth and amino acid production (Malumbres & Martín, 1996).

The control of rRNA and protein synthesis is a complex and strictly regulated process; the first level of this regulation occurs at the initiation of transcription of rRNA and is a key to understanding the control of ribosome synthesis and, therefore, to elucidating growth control and amino-acid producing ability.

rRNA (*rrn*) operons are transcribed as single units giving pre-rRNAs, which are cleaved by RNases to pre-16S rRNA, pre-23S rRNA and pre-5S rRNA. Further RNase processing produces mature rRNA species. There are numerous bacterial rRNA sequences available (mainly used for phylogenetic studies), including some partial sequences from corynebacteria deposited in GenBank; however, no information was available on the upstream regulatory sequences or about transcription of *rrn* operons in corynebacteria. It was, therefore, of great interest to study the *rrn* operons of amino acid

The GenBank accession number for the sequence reported in this paper is Y12792.

producing corynebacteria as a way to understand growth regulation and the mechanism that triggers amino acid accumulation under nutritional starvation.

C. glutamicum and '*B. lactofermentum*' were initially proposed to belong to two different genera (Yamada & Komagata, 1970; Abbe & Takayama, 1972). '*B. lactofermentum*' was separated from other *Brevibacterium* species by its ability to ferment lactose, producing acid in litmus milk (Abbe & Takayama, 1972). However Liebl *et al.* (1991) proposed to transfer '*B. lactofermentum*' to the genus *Corynebacterium*, as *C. glutamicum*. The cloning of the rRNA operons (*rrn*) provides a very useful instrument to settle the polemic about the taxonomic relatedness of these two glutamic acid producing corynebacteria. In this article we describe the characterization of an rRNA operon from '*B. lactofermentum*', including the complete sequence of the 16S rRNA gene and upstream regulatory region and a transcription analysis. Analysis of the *rrn* operons of *C. glutamicum* and '*B. lactofermentum*' shows that these two species are closely related but not identical.

METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown at 37 °C on Luria-Bertani medium supplemented with ampicillin (50 µg ml⁻¹) when needed. Corynebacteria were grown on trypticase soy broth (TSB) or trypticase soy agar (TSA) (trypticase soy broth with 2% agar).

DNA hybridization. Chromosomal DNA from corynebacterial strains was digested with *Bam*HI and *Eco*RI. DNA fragments were resolved by electrophoresis and transferred to nylon membranes by standard methods (Sambrook *et al.*, 1989). Specific probes were labelled with [³²P]dCTP (Amersham) by nick-translation (Promega). Blots were hybridized at high stringency (50% formamide, 42 °C) as described by Sambrook *et al.* (1989).

DNA sequencing. Subclones of the cloned DNA fragments were sequenced on both strands by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) using a Pharmacia ALF automatic sequencer.

Primer extension assays. Primer extension assays were performed with AMV reverse transcriptase (Pharmacia Biotech) by standard methods (Sambrook *et al.*, 1989). Primers O-1 (5' CCACAAAAACAATAAAAAAGA 3') and O-2 (5' TCG-CCCACGTGTTACTCACCCG 3') were used for extension. Total cellular RNA was prepared by ultracentrifugation using the RNA extraction system of Pharmacia Biotech according to the manufacturer's instructions.

Phylogenetic analysis. This was performed using the MegAlign Program from DNASTAR. The sequences used for comparative studies were obtained from EMBL/GenBank.

RESULTS

Corynebacterial rRNA gene clusters: cloning of a '*B. lactofermentum*' ribosomal operon

Total DNA from (i) '*B. lactofermentum*' ATCC 13869 and two derivatives (R31 and a *recA* mutant), (ii) '*B. lactofermentum*' DSM 20412 and (iii) *C. glutamicum*

Table 1. Strains and plasmids used

Strain or plasmid	Description*	Source or reference
Bacteria		
<i>E. coli</i> XL1Blue	Standard strain for DNA manipulations	Stratagene
<i>E. coli</i> WK6mutS	Δ(<i>lac-proAB</i>) <i>galE strA mu</i> :S215 Tn10/F' <i>lacIZΔM15 proAE</i> ⁺	R. Zell, Jena, Germany
' <i>B. lactofermentum</i> ' ATCC 13869	Type strain	ATCC†
' <i>B. lactofermentum</i> ' R31	Melys ^r Aec ^r ; restriction-deficient mutant of ATCC 13869	Santamaría <i>et al.</i> (1985)
' <i>B. lactofermentum</i> ' <i>recA</i>	Recombination-deficient mutant of ATCC 13869	K. Dunican, University of Galway, Ireland
' <i>B. lactofermentum</i> ' DSM 20412		Liebl <i>et al.</i> (1991)
<i>C. glutamicum</i> ATCC 13032	Type strain	ATCC†
Plasmid, phage and cosmid		
M13K07	Helper phage	Mead & Kemper (1988)
pBluescript KS(+)	Phagemid derived from pUC; Ap ^r	Stratagene
pBA2	Plasmid with an insert of 16S rRNA from <i>Bacillus subtilis</i>	Iglesias <i>et al.</i> (1983)

* Melys^r, resistance to *N*-methyllysine; Aec^r, resistance to *S*-aminoethylcysteine.

† American Type Culture Collection.

Fig. 1. '*B. lactofermentum*' rRNA gene clusters. Southern blots of *Bam*HI (left) or *Eco*RI (right) digests were hybridized with a 2.3 kb *Hind*III DNA fragment from pBA2 encoding the 16S rRNA of *Bacillus subtilis*. Strains used were: Lanes 1 and 6, '*B. lactofermentum*' DSM 20412; lanes 2 and 7, *C. glutamicum* ATCC 13032; lanes 3 and 8, '*B. lactofermentum*' ATCC 13869; lanes 4 and 9, '*B. lactofermentum*' *recA*; lanes 5 and 10, '*B. lactofermentum*' R31. Sizes of the bands for ATCC 13869 are indicated on the left.

ATCC 13032 was digested with *Bam*HI, *Eco*RI and other enzymes (see below) and probed with a 2.3 kb *Hind*III fragment from pBA2 (Iglesias *et al.*, 1983) containing part of a *Bacillus subtilis* ribosomal operon. Five *Bam*HI hybridizing bands were observed for all strains used except one, '*B. lactofermentum*' DSM 20412 (see below), with sizes of 14.5, 10.7, 8.9, 8.7 and 8.0 kb for '*B. lactofermentum*' ATCC 13869 (Fig. 1). Other enzymes such as *Kpn*I, *Sac*I, *Eco*RV, *Pst*I, *Sal*I or *Hind*III produced more hybridization signals, some of them with about half the intensity, suggesting that conserved restriction sites for these enzymes occur in some *rrn* clusters of the different strains.

The different corynebacterial strains used showed simi-

lar *rrn* patterns but with some differences. In *Eco*RI digests the smallest *C. glutamicum* *rrn* band migrated close to the next and the slowest-migrating band was larger than the corresponding bands present in the '*B. lactofermentum*' ATCC strains. '*B. lactofermentum*' DSM 20412 showed a different *rrn* hybridization pattern with an extra band (9.2 kb) in *Bam*HI digests, suggesting the possible existence of six *rrn* clusters (Fig. 1); however, only five *Eco*RI bands were seen in this strain, with an identical pattern to that of '*B. lactofermentum*' ATCC 13869 except for the slowest-migrating band, which showed a smaller size than the corresponding bands of '*B. lactofermentum*' ATCC strains. Therefore, we cannot exclude the possibility that the sixth *Bam*HI band in '*B. lactofermentum*' DSM 20412 is originated by an internal *Bam*HI site in one of the *rrn* operons that does not occur in the other '*B. lactofermentum*' strains.

When the '*B. lactofermentum*' ATCC 13869 cosmid bank (Correia *et al.*, 1996) was probed with the *Bacillus subtilis* 2.3 kb *Hind*III fragment, 60 out of 480 recombinant cosmids gave a positive hybridization signal. Twenty of them were selected and digested with *Bam*HI and the bands were blotted and reprobed. Some of the cosmids contained two positive *Bam*HI bands, of 8.0 and 8.7 kb, indicating that at least two of the five operons are linked in a 40 kb DNA region (data not shown), an arrangement that also occurs in other bacteria. Bathe *et al.* (1996) reported a scattered location of four *rrn* loci in the *C. glutamicum* chromosome but admitted that one of the *rrn* loci may contain two *rrn* clusters.

Assuming that each of the five *Bam*HI genomic bands of '*B. lactofermentum*' ATCC 13869 that gave a positive signal contains an *rrn* operon we designated them by letters A to E in decreasing size order; the rRNA operons located in the 8.7 and 8.0 kb bands subcloned from the same cosmid (see Fig. 2) corresponded to the hybridizing bands D and E, respectively. A region of approximately 5.5 kb internal to both *rrnD* and *rrnE* operons is boxed

Fig. 2. Restriction map of two '*B. lactofermentum*' rRNA operons. *Bam*HI fragments of 8.7 kb (*rrnD*) and 8.0 kb (*rrnE*) from the same cosmid were cloned in pBluescript. The regions outlined with a dotted box show a similar restriction pattern. A, *Apa*I; B, *Bam*HI; Bs, *Bst*XI; C, *Cla*I; E, *Eco*RI; Ev, *Eco*RV; H, *Hind*III; N, *Not*I; S, *Sal*I; Sc, *Sac*I; Sp, *Spe*I; P, *Pst*I; X, *Xba*I; Xh, *Xho*I. The three arrows correspond to the 16S, 23S and 5S genes found in *rrnD*. The 1.2 kb *Bam*HI-*Kpn*I fragment containing the upstream region of operon *rrnD* and the ORF1 is indicated by a thin line below. The 2.7 kb probe of *Bacillus subtilis* is indicated by a solid bar.

Fig. 3. Model of the secondary structure of '*B. lactofermentum*' 16S rRNA based on that of *E. coli* (Noller, 1984) and *M. tuberculosis* (Kempell et al., 1992). The domains and motifs in the sequence are indicated. V1, V2, etc., are variable regions.

Fig. 4. Phylogenetic tree of '*B. lactofermentum*' and some other Gram-positive bacteria (particularly coryneform bacteria) according to 16S RNA sequence. The tree was constructed using the CLUSTAL method (MegAlign, DNASTAR). Accession numbers: '*C. fastidiosum*' X84245, '*C. segmentosum*' X84437, *C. accolens* X80500; *C. pseudotuberculosis* D38577, *C. afermentans* X81874, *C. renale* X81909, *C. mycetoides* X84241, *C. cystitidis* X84252, *C. diphtheriae* X84248, *C. ammoniagenes* X84440, *C. striatum* X81910, *C. variabilis* X53185, *C. glutamicum* X84257, *C. callunae* X84251, *C. xerosis* X84446, *M. bovis* X55589, *M. tuberculosis* X52917, *M. leprae* X53999, *M. gordonae* X52923, *R. fascians* X53204, *B. linens* X77451, *Frankia* sp. U72717, *S. hygrosopicus* X79853, *Bacillus subtilis* X60646, '*B. lactofermentum*' Y12792 (*B.*, *Brevibacterium*; *C.*, *Corynebacterium*; *M.*, *Mycobacterium*; *R.*, *Rhodococcus*; *S.*, *Streptomyces*). Note the low degree of homology between *B. linens* (the type species of the genus *Brevibacterium*) and '*B. lactofermentum*'.

	Box B	BoxC	RNAse	BoxA
<i>B. lactofermentum</i>	CCCCGAA ^{CA} GGG	TGTTT ^{TTTT}	AACTAA	GTGT ^{GTGG}
<i>M. tuberculosis</i>	CCCCGAA ^{GCG} GGG	TGTTT ^{TTT}	AACTAA	GTGT ^{TGG}
Similarity (%)	83.3	77.7	100	87.5

Fig. 5. Alignment of the antiterminator motifs (Boxes B, C and A) and the RNase III cleavage site of *M. tuberculosis* with similar motifs of '*B. lactofermentum*'. Similar motifs occur in all mycobacteria and other Gram-positive bacteria. The similarity is given as percentage of identical nucleotides in the motif.

in Fig. 2. Band D was confirmed to contain one rRNA operon by total sequencing of the 16S gene and partial sequencing of the 23S and 5S genes and comparison with known rRNA sequences deposited in GenBank. Analysis of these subclones confirmed the 16S–23S–5S gene arrangement present in the majority of bacteria. No evidence of the presence of any tRNA gene in the gene cluster was obtained.

Primary and secondary structure of the 16S rRNA

A 2.7 kb *Hind*III fragment as well as a 1.2 kb *Bam*HI–*Kpn*I fragment from the *rrnD* operon were subcloned in

pBluescript SK(+) (both orientations) and used for sequencing the entire 16S RNA gene and the 5' regulatory region. The putative 5' end of the mature 16S rRNA was assigned at nucleotide position 1171 numbered from the left *Bam*HI site of the sequence and the 3' end was located at nucleotide position 2664 by comparison with the 16S rRNA genes of *Mycobacterium* spp. (Ji *et al.*, 1994a, b, c) and *Streptomyces coelicolor* (Condon *et al.*, 1995). Therefore, the '*B. lactofermentum*' 16S rRNA gene is 1494 nt long, slightly larger than those described for *Streptomyces* or *Myco*-*bacterium* species.

The anti-Shine–Dalgarno sequence comprising a tract of

Fig. 6. Nucleotide sequence and transcriptional features of the bidirectional promoter region between *rrnD* and ORF1. Sequence elements implicated in the control of transcription and in processing to form mature 16S rRNA were identified by comparison with elements of known function. Shaded boxes show the putative antitermination elements (Box B, Box A and Box C). The potential RNase III cleavage site is shown in boldface letters. The 5' end(s) of mature 16S rRNA are indicated by vertical arrows. The two primers O-1 and O-2 used in primer extension studies are shown in boldface italics. The putative 5' end of precursor rRNA (or start of transcription) is indicated by an asterisk at position 758. The GC-rich stringent control element is underlined. ORF1, showing extensive homology with known sigma factors, is indicated in the opposite direction to *rrnD*. The 3' end of the 16S rRNA gene corresponding to the anti-Shine-Dalgarno sequence is labelled 'Anti-SD'.

Fig. 7. Primer extension analysis of the 16S rRNA gene. Primer extension with reverse transcriptase was performed as described by Sambrook *et al.* (1989) using 100 ng RNA. Primers O-1 and O-2 were labelled as described in the text. The bands (lane P) corresponding to the sites of RNA initiation or processing (see text) are indicated by arrows. Experiments were carried out twice with both primers. The nucleotide sequence of the DNA region corresponding to the product of the primer extension reaction is shown on the right; the two Gs at the 5' end of mature rRNA are indicated by asterisks.

bacterium tuberculosis by Kempell *et al.* (1992); therefore, the hypothetical Shine-Dalgarno mRNA sequence complementary to the anti-Shine-Dalgarno of '*B. lactofermentum*' is 5' AGAAAGGAGG (see Fig. 3).

Since the sequences of bacterial 16S rRNAs are highly conserved they have been used for phylogenetic studies. When the sequence for structural 16S rRNA was compared with those present in the GenBank/EMBL databases an optimal alignment was found with other corynebacterial sequences. The homology was in the range 90–96% when compared with all sequences belonging to the genus *Corynebacterium* (95.6% with *C. glutamicum*); however, the homology was significantly lower (82%) with *Brevibacterium linens* ATCC 9172, the type species of the genus. Fig. 4 shows a phylogenetic tree of some representative eubacteria with special reference to corynebacteria (see Discussion).

rRNAs are known to fold into several stem and loop structures by internal pairing. The '*B. lactofermentum*' 16S rRNA sequence was aligned and folded into a secondary structure (Fig. 3) based on the folding model for rRNAs proposed by Noller (1984) for *E. coli*. The secondary structure obtained for '*B. lactofermentum*' is highly similar to that observed in *Mycobacterium tuberculosis* (Kempell *et al.*, 1992). The sequence conservation is higher in loop regions than in paired regions and only some motifs are different, especially in helix 5 and 6 (region V1). The sequence of helix 5 is a characteristic feature of a particular 16S rRNA (Ji *et al.*, 1994a). Also significant differences were seen in the variable regions V2 (helix 8 and 10), V3 (helix 18) and V8 (helix 43 and 44) as expected. The remaining differences were mainly nucleotide substitutions.

10 pyrimidines with the sequence 5' CCUCCUUUCU' at the 3' end of the '*B. lactofermentum*' 16S rRNA showed total identity with the sequence described for *Myco-*

Fig. 8. Alignment of the deduced amino acid sequence of ORF1 with the sequence of SigA of '*B. lactofermentum*'. Regions 1.1 and 1.2 of *rpoD*-like (sigma-70) factors are overlined. Amino acids which are identical in both sequences are shown in reverse typeface.

Transcription of the '*B. lactofermentum*' *rrnD* operon and processing of the transcript

The 5' end of mature 16S rRNA and sequence elements implicated in processing of the pre-rRNA to form 16S rRNA were identified by primer extension and by comparison with elements with the same function recognized in a wide range of bacteria (King *et al.*, 1986). Antitermination of transcription occurs in the *E. coli* *rrn* operons; therefore, we searched for possible anti-termination elements present in the leader region of *rrnD* similar to those described for species of *Mycobacterium* (González-y-Merchand *et al.*, 1996; Ji *et al.*, 1994a, b). Three elements similar to Box B, Box A and Box C from *M. tuberculosis* were identified with 83.3, 87.5 and 77.7% identical nucleotides respectively (Fig. 5). Three nucleotides upstream of Box B there is a G (position 758 in Fig. 6) that is a putative site for the transcription initiation of the pre-rRNA that also occurs in *M. tuberculosis*.

Pre-16S rRNA derives from pre-rRNA by the action of RNase III. One RNase III cleavage site was located within the leader region (sequence 5' AACTAA) showing total identity with the cleavage site described in *Streptomyces coelicolor* and *Mycobacterium* species. In addition, a palindrome, presumably forming a loop around the RNase III site (AACAAATACTAATTTATTGTT) was found. A GC-rich sequence known as the discriminator, close to the +1 RNA transcription start site, has been noted as a common feature of promoters regulated by the stringent response (Condon *et al.*, 1995). A putative sequence with such a function (GTTGCCCC) was found partially overlapping Box B.

Two oligonucleotides, O-1 and O-2 (in bold face in Fig. 6), were used to locate by primer extension the RNA 5' ends resulting from either transcription initiation or processing in the relatively short sequence between the nucleotide +1 corresponding to the mature 16S rRNA and the upstream open reading frame. Results of the primer extension are shown in Fig. 7. Four signals were observed: two strong ones corresponding to consecutive Gs (nucleotides 1169 and 1170 in Fig. 6) appeared repeatedly using oligonucleotide O-2 and two very faint

ones were observed with O-1 as primer. The strong signals correspond to the processing sites for the 16S mature rRNA. Sites for transcription initiation and processing are, therefore, located in a short stretch of 500 nt upstream of the sites that we found by primer extension and the upstream open reading frame. Preliminary evidence indicates that a short DNA fragment upstream of Box B showed promoter activity when coupled to a promoter probe vector (E. Amador, J. M. Castro & J. F. Martín, unpublished results).

An *rpoD*-like sigma factor gene is located upstream of *rrnD*

Upstream from the Box B motif of the *rrnD* operon, in the same DNA strand but in the opposite orientation, ORF1 (224 residues) was identified starting at nucleotide 672 with a GTG codon that is truncated in the initial *Bam*HI site (Fig. 6). Computer analysis gave a significant homology with SigA sigma factor of '*B. lactofermentum*' described by Oguiza *et al.* (1996) (Fig. 8). The protein encoded by ORF1 has an 87% amino acid identity with SigA specially in a region of 110 amino acids corresponding to the 1.1 domain of *rpoD*-like sigma factors. The homology with SigB was much lower since SigB does not contain the 1.1 region. We have named this *rpoD*-like gene *sigC*.

DISCUSSION

There is a broad correlation between the number of ribosomes and the growth rate of different bacteria (Bremer & Dennis, 1987). The number of ribosomes in the cell depends on the availability of ribosomal proteins and rRNA, which is determined by the number of *rrn* operons, the strength of their promoters and the efficiency with which the operons are transcribed.

Five *rrn* gene clusters have been found in '*B. lactofermentum*' ATCC 13869. There are seven *rrn* operons in *E. coli* (Condon *et al.*, 1992), six in *Streptomyces coelicolor* (van Wezel *et al.*, 1994), four in *Streptomyces ambofaciens* (Pernodet *et al.*, 1989), two in *Myco-*

bacterium phlei and *Mycobacterium smegmatis* (Bercovier *et al.*, 1986; Ji *et al.*, 1994c) but only one in the slow-growing *Mycobacterium tuberculosis* and *Mycobacterium leprae* (Bercovier *et al.*, 1986). Corynebacteria of the 'glutamic acid' group contain more *rrn* operons than mycobacteria, which is consistent with their faster growth rate.

Sequencing of the 16S rRNA has allowed us to identify an anti-Shine-Dalgarno sequence for corynebacteria. There were no previous studies about the degree of complementarity between the sequence of the 3' end of the 16S rRNA and the sequence of the mRNA 5' to the translational start codon (Martín, 1989). In a few cases the putative Shine-Dalgarno sequence is located 2–10 nt (2–11 nt in *E. coli*) upstream from the AUG or GUG start codon and has the potential of forming four to eight base-pairs with the 3' end of 16S rRNA. *E. coli* has a shorter anti-Shine-Dalgarno sequence (consensus AGGA). Sequences that show good complementarity to the anti-Shine-Dalgarno sequence described in this article have been found preceding the coding sequences of many genes cloned from corynebacteria (see review by Martín, 1989).

Analysis of the *rrnD* sequence has provided information on the phylogeny of '*B. lactofermentum*' and on the molecular control of macromolecule gene expression.

There has been controversy about the relationship between the two corynebacteria used routinely as amino acid producing strains, '*B. lactofermentum*' and *C. glutamicum* (Liebl *et al.*, 1991; Correia *et al.*, 1994). Phylogenetic studies of coryneform bacteria by analysis of 5S rRNA sequences suggested that they should be clustered together with *Nocardia*, *Streptomyces* and *Micrococcus* species and indicated that *Brevibacterium* species are phylogenetically distant from other coryneform bacteria (Park *et al.*, 1987). Liebl *et al.* (1991) proposed to transfer '*B. lactofermentum*' to the genus *Corynebacterium* (as *C. glutamicum*) based on DNA-DNA hybridization, chemotaxonomic and biochemical studies. However, a restriction fragment length polymorphism analysis in which oligonucleotides targeted against conserved regions of 16S and 23S rRNA genes were used as probes showed that although there is a relatively high degree of similarity there were enough clear differences to allow separation of '*B. lactofermentum*' from *C. glutamicum*. The overall restriction map as shown by PFGE (Correia *et al.*, 1994) showed significant differences that confirmed earlier reports on the difference between these two organisms based on their morphological characteristics.

One conclusion of our 16S rRNA analysis is that *B. lactofermentum* ATCC 13869 should no longer be considered as a member of the genus *Brevibacterium* and that it should be transferred to the genus *Corynebacterium*, in agreement with the proposal of Liebl *et al.* (1991). However, the divergence shown in Fig. 4 between '*B. lactofermentum*' and *C. glutamicum* is higher than that between different species of the genus *Coryne-*

bacterium, e.g. *C. fastidiosum*, *C. segmentosum*, *C. accolens* and *C. pseudotuberculosis*, which indicates that '*B. lactofermentum*' is a different species of the genus *Corynebacterium*. We suggest, therefore, that '*B. lactofermentum*' should be changed to *Corynebacterium lactofermentum*, maintaining the name of the species. '*B. lactofermentum*' was clearly different from the glutamic acid producer *C. ammoniagenes* (Fig. 4). It would be interesting to study if similar conclusions apply to other glutamic acid producing corynebacteria such as '*C. melassecola*' and '*B. flavum*', but no data are available on their *rrn* genes.

Four *rpoD*-like genes were found by hybridization with an *rpoD* probe in the genome of '*B. lactofermentum*'. Two of these genes, *sigA* and *sigB*, were sequenced (Oguiza *et al.*, 1996). ORF1, extending upstream from *rrnD*, encodes a gene similar to but different from *sigA* and *sigB*, that has been named *sigC*. Although this gene is truncated in the cloned fragment, the presence of 1.1 and part of the 1.2 regions of *rpoD*-like genes (Lonetto *et al.*, 1992) suggests that the gene encoded by ORF1 is indeed an additional *sig* gene.

The presence of an *rpoD*-like gene in the opposite orientation to that of *rrnD* that must be expressed from a promoter located in the bidirectional promoter region is intriguing. Clustering of genes for macromolecule (rRNA) biosynthesis and sigma factors suggests that a common control mechanism (e.g. stringent control: Condon *et al.*, 1995; Wehmeier *et al.*, 1998) exerted on the bidirectional promoter region may affect sigma factor formation and rRNA synthesis.

The two strong signals found by primer extension differed by one nucleotide and correspond to the 5' end of the mature 16S rRNA. However we could not clearly observe the 5' end corresponding to the pre-16S rRNA although the RNase cleavage sequence AACUAA identical to that of mycobacteria and *Bacillus subtilis* (Ji *et al.*, 1994a, c; Ogasawara *et al.*, 1983) is conserved in the upstream region. It is likely that processing of pre-rRNA and pre-16S into 16S rRNA is very efficient and therefore we cannot detect the pre-rRNA. The similarity of elements A, B and C present in the non-coding region of *rrnD* with those of mycobacteria suggests that an antitermination mechanism of control of transcription similar to that reported in other species (Berg *et al.*, 1989; Ji *et al.*, 1994a) also occurs in corynebacteria. In *E. coli* the Box A sequence of the pre-rRNA forms a complex with the transcriptional factor *nusB* and protein S10, which then attach to the RNA polymerase, allowing transcription to proceed (Nodwell & Greenblatt, 1993).

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