

Multiple Sigma Factor Genes in *Brevibacterium lactofermentum*: Characterization of *sigA* and *sigB*

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Four *rpoD* hybridizing signals have been identified in the chromosome of *Brevibacterium lactofermentum*. Two *rpoD*-like genes, *sigA* and *sigB*, have been cloned and sequenced, and they encode principal sigma factors of the RNA polymerase. The deduced amino acid sequences of SigA and SigB showed very high similarities to those of *Mycobacterium smegmatis* MysA and MysB proteins, respectively, and also to those of HrdB proteins from different *Streptomyces* species. SigA and SigB maintain the conserved motifs of σ^{70} -like principal sigma factors. *sigB* is closely linked to the *dtxR* gene (encoding a repressor of iron-regulated promoters homologous to the diphtheria toxin repressor from *Corynebacterium diphtheriae*).

The corynebacteria *Brevibacterium lactofermentum* and *Corynebacterium glutamicum* are gram-positive nonpathogenic soil bacteria used widely for the production of amino acids (12). There is great interest in the amino acid biosynthetic pathways and the molecular mechanisms of control of these pathways because of the economic importance of these microorganisms. However, little is known about the control mechanisms of these pathways at the transcription level. It is necessary, therefore, to achieve an understanding of the transcription initiation mechanisms that govern promoter efficiency in corynebacteria. At least two classes of promoters of corynebacteria have been identified. Type I promoters (corynebacterial *Escherichia coli*-like promoters) are used in both *E. coli* and corynebacteria and show standard -10 and -35 consensus sequences recognized by the principal sigma factor of *E. coli* and *Bacillus subtilis*. Type II promoters (corynebacterial specific promoters) are recognized in corynebacteria but not in *E. coli* and lack the consensus -10 and -35 sequences (13).

In procaryotic organisms, the sigma subunit of RNA polymerase confers specificity to the process of transcription initiation through recognition of specific promoter sequences of genes and operons (3). Under normal growth conditions, eubacteria use an RNA polymerase holoenzyme containing the principal sigma factor (σ^{70} in *E. coli*), which is responsible for promoter recognition of housekeeping genes (7). The characterization of the corynebacterial RNA polymerase holoenzyme, in particular that of its major sigma factors in addition to any minor sigma subunits, is essential to understanding the molecular mechanism of differential transcription initiation in corynebacteria. In this article, we report the identification and characterization of two *rpoD*-like genes from *B. lactofermentum*, *sigA* and *sigB*, which are homologous to *Streptomyces hrdB* encoding sigma subunits of the DNA-dependent RNA polymerase.

Cloning of *rpoD*-like genes from *B. lactofermentum*. To clone genes for the principal sigma factors of *B. lactofermentum*, DNA fragments from the *hrdB* (1.16-kb *SalI*-*ApaI* fragment) and *hrdT* (1.0-kb *ApaI* fragment) genes of *Streptomyces griseus* IMRU 3570, which encode sigma factor-like proteins (11), and

the “*rpoD* box” oligonucleotide (19) were used as hybridization probes with total DNA isolated from *B. lactofermentum* digested with different restriction enzymes. Two hybridizing bands were present in chromosomal DNA of *B. lactofermentum* digested with different restriction enzymes in hybridizations performed with the *hrdB* and *hrdT* probes (Fig. 1A). When the “*rpoD* box” oligonucleotide (corresponding to a conserved sequence in the principal sigma factors of *E. coli* and *B. subtilis* [19]) was used as the probe, four different *rpoD* hybridizing signals were detected (Fig. 1A). Two of these *rpoD* hybridizing bands corresponded to those previously obtained for the hybridizations with the *S. griseus hrdB* and *hrdT* probes, but the other two *rpoD* signals were completely different. These results indicated that the *B. lactofermentum* genome can contain four possible *rpoD* homologous genes and that two of them were closely related to the *hrdB* and *hrdT* genes from *S. griseus*. Two hybridizing signals were also observed when *C. glutamicum* total DNA digested with the same restriction enzymes was hybridized with *S. griseus hrdB* and *hrdT* probes, but in some digestions, the sizes of the hybridizing bands were different from those obtained with *B. lactofermentum* (data not shown).

A library of genomic *B. lactofermentum* DNA constructed in cosmid Cos4 (5) was hybridized with the same probes as discussed above, allowing the isolation of a series of positive clones. Cosmid pULAC5A2 was selected because in hybridizations with the *hrdB*, *hrdT*, and “*rpoD* box” probes, it showed 1.6- and 2.4-kb *HindIII* hybridizing bands in all cases. These two bands corresponded to the 1.6- and 2.4-kb *HindIII* fragments observed in the *B. lactofermentum* total DNA (Fig. 1A), showing that there was no DNA rearrangement in this clone. It is interesting that two *B. lactofermentum* genes homologous to *hrdB* from *S. griseus* were present in the same clone, pULAC5A2. The arrangement of two sigma genes closely linked in the chromosome is not restricted to corynebacteria; the *mysA* and *mysB* sigma factor genes of mycobacteria are also linked but separated by a 3.5-kb intergenic region in *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* (17) and by a 3.8-kb region in *Mycobacterium leprae* (18a).

Other cosmid clones that hybridized only with the “*rpoD* box” probe showed a single *HindIII* hybridizing band with a size of 3.5 or 10 kb (data not shown), which is in agreement with the sizes of the *HindIII* fragments observed in the hybridizations of total DNA with this probe (Fig. 1A). These results

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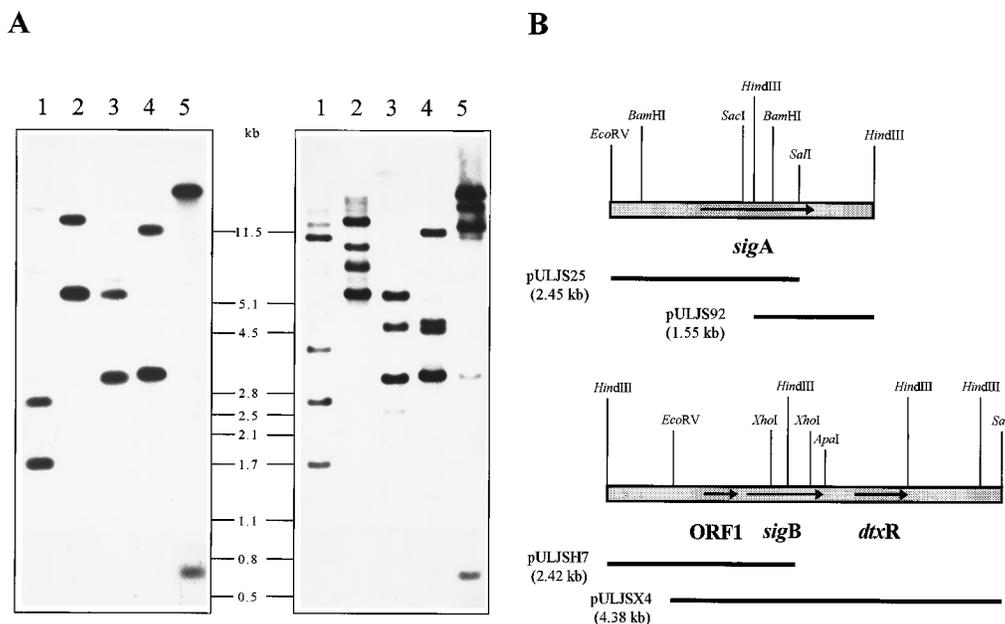


FIG. 1. (A) Hybridizations of total DNA from *B. lactofermentum* cleaved with *Hind*III (lane 1), *Eco*RV (lane 2), *Sal*I (lane 3), *Pvu*II (lane 4), and *Xho*I (lane 5), with the *S. griseus hrdB* (or *hrdT*) gene (left panel) and the "rpoD box" oligonucleotide (right panel) being used as probes. The numbers between the panels indicate the sizes of standard DNA fragments in kilobases. (B) Restriction maps of the DNA fragments containing *sigA* and ORF1-*sigB*-*dtxR*. Arrows indicate the positions of the open reading frames. Lines correspond to the inserts cloned into every plasmid.

indicate that there are two other possible *rpoD*-like genes in addition to *sigA* and *sigB* in different regions of the chromosome of *B. lactofermentum*.

Identification of the *B. lactofermentum* principal sigma factor gene, *sigA*. The hybridizing 1.6-kb *Hind*III restriction fragment from cosmid pULAC5A2 was subcloned in pUC119, giving rise to plasmid pULJS92 (Fig. 1B). The nucleotide sequence revealed the presence of a truncated open reading frame, with significant homology to the principal sigma factors. To cover entirely this open reading frame, a 2.5-kb *Eco*RV-*Sal*I fragment from cosmid pULAC5A2 that overlapped with pULJS92 was subcloned into pBluescript KS(+), giving rise to plasmid pULJS25 (Fig. 1B). The 3.5-kb *Eco*RV-*Hind*III DNA fragment located in plasmids pULJS25 and pULJS92 was completely sequenced in both strands, and a 1,494-bp open reading frame encoding a 497-amino-acid protein with a deduced molecular mass of 54.8 kDa was found. Sequences closely related to the consensus *E. coli* -35 and -10 regions and to a potential ribosome binding site were found upstream of the potential GTG start codon. A search of the EMBL, GenBank, and DDJB databases revealed that the deduced amino acid sequence of this open reading frame has high similarity with those of the principal sigma factors of related microorganisms: MysA (69.9% identical amino acids) and MysB (57.4%) of *M. smegmatis* (17); HrdB from *S. coelicolor* (60.9%) (4), *Streptomyces aureofaciens* (58.2%) (9), and *S. griseus* (57.9%) (11); and HrdT of *S. griseus* (51.9%) (11). Alignment of the sequence of this *B. lactofermentum* sigma factor with those of *S. coelicolor* HrdB and *M. smegmatis* MysA (Fig. 2) showed the conserved regions of the σ^{70} family (numbers 1.1 to 4.2) (10), which suggested that these factors play similar functions. We have designated this gene encoding a *B. lactofermentum* sigma factor *sigA*, following the terminology employed with other sigma factor genes of gram-positive microorganisms.

Identification of the *B. lactofermentum* ORF1-*sigB*-*dtxR* region. The hybridizing 2.4-kb *Hind*III fragment from cosmid

pULAC5A2 was also subcloned in plasmid pBluescript KS(+), resulting in pULJSH7 (Fig. 1B). The open reading frame contained in this plasmid was incomplete, and it was necessary to reclone an overlapping 4.4-kb *Eco*RV-*Sal*I fragment from cosmid pULAC5A2 into pBluescript KS(+), resulting in plasmid pULJSX4 (Fig. 1B). Three open reading frames, ORF1, ORF2, and ORF3 (Fig. 1B), were identified when the nucleotide sequence of the 4.0-kb *Hind*III DNA fragment located in pULJSH7 and pULJSX4 was sequenced in both orientations. ORF3 (687 bp) corresponded to the *B. lactofermentum dtxR* gene located in the 1.1-kb *Apa*I-*Hind*III fragment of plasmid pULJSX4. This gene encodes the repressor protein of iron-regulated genes, analogous to the diphtheria toxin repressor of *Corynebacterium diphtheriae* (15). The *B. lactofermentum dtxR* gene is located 417 bp away from ORF2, which extends for 1,089 bp and is preceded by a potential ribosome binding site upstream of an in-frame ATG triplet. The ORF2 product consists of a 331-amino-acid protein with a deduced molecular mass of 37.5 kDa that is related to the MysB protein from *M. smegmatis* (73.3% identical amino acid residues) (17) (Fig. 2) and to the 143-amino-acid protein (92.3% identical residues) from an incomplete open reading frame located upstream of *C. diphtheriae dtxR* (2) (and that corresponds, therefore, to a sigma factor). ORF2 protein also showed good similarity values with MysA from *M. smegmatis* (55.6%) (17) and with HrdB from *S. coelicolor* (54.8%) (4), *S. aureofaciens* (54.5%) (9), and *S. griseus* (53.4%) (11). From these results, we concluded that ORF2 encoded another *B. lactofermentum* sigma factor, and we designated this gene *sigB*. The similarity of *B. lactofermentum* SigA and SigB was high (57.9% identical amino acids), although insufficiently high to indicate that they are duplicate copies of the same gene.

Upstream of *sigB*, the 435-bp ORF1 encoding a 144-amino-acid protein of unknown function was identified. A putative ribosome binding site was found upstream from the start codon of ORF1, suggesting that the gene is probably translated

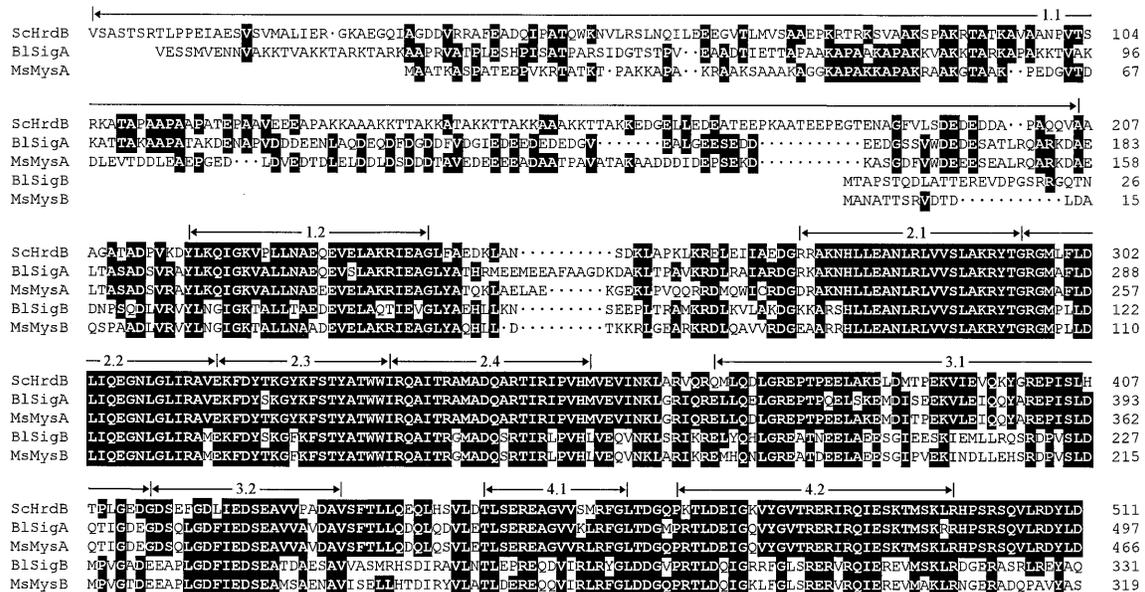


FIG. 2. Comparison of the deduced amino acid sequences of *S. coelicolor* HrdB (ScHrdB) (4), *B. lactofermentum* SigA (B1SigA) and SigB (B1SigB), and *M. smegmatis* MysA (MsMysA) and MysB (MsMysB) (17). Identical amino acids are in reverse type. The regions of the sigma factors are indicated above the sequences (10). Dots indicate gaps in the alignment, and the numbers on the right of each sequence correspond to amino acid positions relative to the start of each protein sequence.

into a protein. The deduced protein sequence showed significant homology to the sequences of two proteins with unknown functions: the *E. coli* o145 protein (34.2% identical amino acids) coded for by a gene that maps in the genome between 87.2 and 89.2 min (16) and the ORF1 protein (41.1% identical amino acids) in the streptokinase region of *Streptococcus equisimilis* H46A (14). Three inverted repeated sequences were found in the region between ORF1 and the *sigB* genes; these possible secondary structures in the mRNA might act as transcriptional attenuators or terminators, as described previously for *Streptomyces* species (6, 8).

Functional domains of *B. lactofermentum* SigA and SigB sigma factors. The identification, characterization, and sequence analysis of sigma factors have revealed that they fall into two broad classes: the σ^{54} and σ^{70} families (10). Alignment of the sequences of SigA and SigB sigma factor proteins with those of other sigma factors shows high sequence similarity in the four consensus regions of the σ^{70} family (Fig. 2) (7, 10). Region 1, composed of subregions 1.1 and 1.2, is the most variable between SigA and SigB. The length of subregion 1.1 of SigA is intermediate between those of *S. coelicolor* HrdB and *M. smegmatis* MysA, with few residues conserved in all of them. Several repetitions of a short peptide sequence starting with two basic amino acids (KK or KR) are present in SigA, as described previously for this region in *M. smegmatis* MysA (17). SigB subregion 1.1 is very short, but it is longer than that from *M. smegmatis* MysB. Subregion 1.2 is very highly conserved in all the compared proteins (Fig. 2), suggesting that it must have structural and functional importance.

Region 2 and its corresponding subregions, 2.1, 2.2, 2.3, and 2.4, are also highly conserved in SigA and SigB, as they are in other principal sigma factors (Fig. 2). All the changes that occur in subregion 2.4 are conservative, except for the Gly-161 residue of SigB that is also present in *M. smegmatis* MysB. This high conservation of subregion 2.4, which is implicated in the recognition of the -10 region of promoters, suggests that the compared proteins can recognize the same -10 hexamer.

Region 3 (divided into subregions 3.1 and 3.2) and region 4 (composed of subregions 4.1 and 4.2) are separated by a short spacer and are also highly conserved. Subregion 4.2, which participates in the recognition of -35 promoter sequences by a helix-turn-helix DNA-binding motif (10), is particularly well conserved in SigA but not so well conserved in SigB.

Organization of *sigA* and *sigB* genes in the chromosome of *B. lactofermentum*. The organization of the *sigA* and *sigB* genes is similar to that observed in a *Mycobacterium* sp. for *mysA* and *mysB* genes (17), with the genes mapping relatively close to each other in the chromosome, but different from that of *S. coelicolor* (4), in which the *hrdA*, *hrdB*, *hrdC*, and *hrdD* genes are located in different regions of the chromosome (21). The *mysA* and *mysB* mycobacterial sigma factor genes are closely linked in the genome on a 10-kb *EcoRI* fragment (17), and a similar arrangement has been observed for *B. lactofermentum*, in which the *sigA* and *sigB* genes are located in cosmid pULAC5A2 (the distance between them is less than 20 kb) (data not shown).

Another point of interest is the conservation of the close linkage of the *sigB* and *dtxR* genes in *B. lactofermentum* and *C. diphtheriae*. This organization is not restricted to corynebacteria and occurs also in mycobacteria (18a). The conservation of the *dtxR* gene (encoding the diphtheria toxin repressor [20]) linked to the *sigB* gene in corynebacteria and also in mycobacteria (*mysB-dtxR*) suggests that these two genes can form a unit that has evolved together in related gram-positive bacteria.

In gram-negative bacteria, the gene encoding the principal sigma factor (*rpoD*) is part of a macromolecule synthesis operon which contains three essential genes, *rpsU*, *dnaG*, and *rpoD*, whose products, ribosomal protein S21, DNA primase, and the principal sigma factor, are necessary for the initiation of protein, DNA, and RNA synthesis, respectively (22). In some gram-positive species, the macromolecule synthesis operon appears to lack *rpsU* but maintains *dnaG* next to *rpoD* (22), as has been shown for *B. subtilis* (23), *Clostridium acetobutylicum* (18), and *Lactococcus lactis* (1). No evidence for a

DNA primase gene forming an operon with the *B. lactofermentum* principal sigma factor gene, *sigA*, or with the *sigB* gene was obtained. These different organizations of the principal sigma factor have also been found for other actinomycetes. The *S. coelicolor hrdB* gene is not associated with *dnaG* or *rpsU*, and these genes are also separated from *hrdA*, *hrdC*, and *hrdD* (21). Similar observations have been reported for the *S. griseus hrdB*, *hrdD*, and *hrdT* genes (11) and the *M. smegmatis mysA* and *mysB* genes (17).

Nucleotide sequence accession numbers. The EMBL accession numbers for the *B. lactofermentum sigA*, *dtxR*, and ORF1-*sigB* sequences are Z49822, L35906, and Z49824, respectively.

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REFERENCES

- Araya, T., N. Ishibashi, S. Shimamura, K. Tanaka, and H. Takahashi. 1993. Genetic and molecular analysis of the *rpoD* gene from *Lactococcus lactis*. *Biosci. Biotechnol. Biochem.* **50**:88–92.
- Boyd, J. M., K. C. Hall, and J. R. Murphy. 1990. Molecular cloning and DNA sequence analysis of a diphtheria *tox* iron-dependent regulatory element (*dtxR*) from *Corynebacterium diphtheriae*. *Proc. Natl. Acad. Sci. USA* **87**:5968–5972.
- Burgess, R. R., A. A. Travers, J. J. Dunn, and E. R. F. Bautz. 1969. Factor stimulating transcription by RNA polymerase. *Nature (London)* **221**:43–46.
- Buttner, M. J., K. F. Chater, and M. J. Bibb. 1990. Cloning, disruption, and transcriptional analysis of three RNA polymerase sigma factor genes of *Streptomyces coelicolor* A3(2). *J. Bacteriol.* **172**:3367–3378.
- Correia, A., A. Pisabarro, J. M. Castro, and J. F. Martín. Cloning and characterization of an IS-like element present in the genome of *Brevibacterium lactofermentum* ATCC 13869. Gene in press.
- Deng, Z., T. Kieser, and D. A. Hopwood. 1987. Activity of a *Streptomyces* transcriptional terminator in *E. coli*. *Nucleic Acids Res.* **15**:2665–2675.
- Helmann, J. D., and M. J. Chamberlin. 1988. Structure and function of bacterial sigma factors. *Annu. Rev. Biochem.* **57**:839–879.
- Ingham, C. J., I. S. Hunter, and C. M. Smith. 1995. Rho-independent terminators without 3' poly-U tails from the early region of actinophage ϕ C31. *Nucleic Acids Res.* **23**:370–376.
- Kormanec, J., M. Farkasovsky, and L. Potuckova. 1992. Four genes in *Streptomyces aureofaciens* containing a domain characteristic of principal sigma factors. *Gene* **122**:63–70.
- Lonetto, M., M. Gribkov, and C. A. Gross. 1992. The σ^{70} family: sequence conservation and evolutionary relationships. *J. Bacteriol.* **174**:3843–3849.
- Marcos, A. T., S. Gutiérrez, B. Díez, F. J. Fernández, J. A. Oguiza, and J. F. Martín. 1995. Three genes *hrdB*, *hrdD* and *hrdT* of *Streptomyces griseus* IMRU 3570, encoding sigma factor-like proteins, are differentially expressed under specific nutritional conditions. *Gene* **153**:41–48.
- Martín, J. F. 1989. Molecular genetics of amino-acid producing corynebacteria, p. 25–59. In S. Baumberg, I. Hunter, and M. Rhodes (ed.), *Microbial products: new approaches*. Cambridge University Press, Cambridge.
- Martín, J. F., R. F. Cadenas, M. Malumbres, L. M. Mateos, C. Guerrero, and J. A. Gil. 1990. Construction of promoter-probe and expression vectors in corynebacteria. Characterization of corynebacterial promoters, p. 283–292. In H. Heslot, J. Davies, J. Florent, L. Bobichon, G. Durand, and L. Penasse (ed.), *Proceedings of the 6th International Symposium on Genetics of Industrial Microorganisms*. Société Française de Microbiologie, Strasbourg, France.
- Mechold, U., K. Steiner, S. Vettermann, and H. Malke. 1993. Genetic organization of the streptokinase region of the *Streptococcus equisimilis* H46A chromosome. *Mol. Gen. Genet.* **241**:129–140.
- Oguiza, J. A., X. Tao, A. T. Marcos, J. F. Martín, and J. R. Murphy. 1995. Molecular cloning, DNA sequence analysis, and characterization of the *Corynebacterium diphtheriae dtxR* homolog from *Brevibacterium lactofermentum*. *J. Bacteriol.* **177**:465–467.
- Plunkett, G., III, V. Burland, D. L. Daniels, and F. R. Blattner. 1993. Analysis of the *Escherichia coli* genome. III. DNA sequence of the region from 87.2 to 89.2 minutes. *Nucleic Acids Res.* **21**:3391–3398.
- Predich, M., L. Doukhan, G. Nair, and I. Smith. 1995. Characterization of RNA polymerase and two sigma-factor genes from *Mycobacterium smegmatis*. *Mol. Microbiol.* **15**:355–366.
- Sauer, U., A. Treuner, M. Buchholz, J. D. Santangelo, and P. Dürre. 1994. Sporulation and primary sigma factor homologous genes in *Clostridium acetobutylicum*. *J. Bacteriol.* **176**:6572–6582.
- Smith, I. Personal communication.
- Tanaka, K., T. Shiina, and H. Takahashi. 1988. Multiple principal sigma factor homologs in eubacteria: identification of the "rpoD box." *Science* **242**:1040–1042.
- Tao, X., N. Schiering, H. Y. Zeng, D. Ringe, and J. R. Murphy. 1994. Iron, DtxR and the regulation of diphtheria toxin expression. *Mol. Microbiol.* **14**:191–197.
- van Wezel, G. P., M. J. Buttner, E. Vijgenboom, L. Bosch, D. A. Hopwood, and H. M. Kieser. 1995. Mapping of genes involved in macromolecular synthesis on the chromosome of *Streptomyces coelicolor* A3(2). *J. Bacteriol.* **177**:473–476.
- Versalovic, J., T. Koeuth, R. Britton, K. Geszvain, and J. R. Lupski. 1993. Conservation and evolution of the *rpsU-dnaG-rpoD* macromolecular synthesis operon in bacteria. *Mol. Microbiol.* **8**:343–355.
- Wang, L. F., and R. H. Doi. 1986. Nucleotide sequence and organization of *Bacillus subtilis* RNA polymerase major sigma operon. *Nucleic Acids Res.* **14**:4293–4307.