Physiological and genetic regulation of rRNA synthesis in *Lactococcus*

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The macromolecular composition of *Lactococcus* was regulated by growth rate in the same general way as that of less fastidious bacteria such as *Escherichia coli* and *Salmonella typhimurium*. The ratios of RNA:DNA and RNA:protein increased approximately threefold over a 13.5-fold increase in growth rate, whereas the ratio of DNA:protein remained approximately constant. Using reporter genes fused to a DNA fragment of a cloned lactococcal rRNA operon, promoter activity was located upstream of the 16S rRNA structural gene. This DNA fragment had some characteristics typical of a *rrn* promoter in *E. coli*. Two consensus promoter sequences P1 and P2 were located 296 and 157 bp, respectively, upstream of the start of the 16S rRNA gene. Between P2 and the start of the 16S rRNA gene, sequences were identified with typical anti-termination motifs characteristic of *E. coli* *rrn* promoter regions. A putative transcription terminator sequence was identified downstream of the 5S rRNA gene and putative primary RNA transcript processing sites at both ends of the lactococcal rRNA operon were also noted.

Introduction

Lactic acid bacteria play a very important role in the preservation of dairy, meat and plant food products. In dairy products they are used primarily to convert lactose to lactic acid but in addition they contribute to the flavours characteristic of those products (Daly, 1983). With the development of molecular biology techniques a significant effort is being made, in several research laboratories, to understand the physiology and genetics of these industrially important bacteria. In the forefront of this work are studies on lactococci, formerly recognized as constituent members of the streptococci (Schleifer *et al.*, 1985). Most of the initial genetic analyses have focussed on those traits associated with plasmids (de Vos, 1987), due both to their accessibility and the

industrially important traits which they encode. However, if greater practical manipulation of these bacteria is to be achieved, a more complete understanding of their physiology and growth regulatory mechanisms must be obtained. In this context we have initiated work on macromolecular changes in cell composition affected by growth rate of lactococci.

Compared to *Escherichia coli*, lactococci have a greatly curtailed biosynthetic ability. Unlike *E. coli* the growth of lactococci is not possible in a glucose–ammonia–salts medium as several amino acids and vitamins are essential nutrients. Furthermore, in environments such as milk, growth of lactococci is dependent on their abilities to hydrolyse proteins and peptides (Reiter & Oram, 1962; Marshall & Law, 1984). We decided to examine if the models generally accepted for growth-rate-associated changes in cell macromolecular composition established with enteric bacteria can be confidently applied to the much more fastidious lactococci.

According to these models bacteria adjust their macromolecular composition according to nutrition-imposed growth rate; as the growth rate increases the cell biomass becomes richer in ribosomes and slightly poorer in protein and DNA. These changes allow the cells to grow at the maximum rate that the particular medium is capable of supporting (Schaechter *et al.*, 1958). The increase in ribosome content is due to increased transcription of rRNA operons and an increase in

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Abbreviations: Cm, chloramphenicol; CAT, chloramphenicol acetyltransferase; Em, erythromycin; Tc, tetracycline.

The nucleotide sequences of the 16S RNA gene promoter region and the 5S RNA gene region have been assigned EMBL accession numbers X65713 and X65712, respectively.

Table 1. *Bacterial strains and plasmids used in this study*

Strain or plasmid	Relevant feature	Reference
<i>L. lactis</i> subsp. <i>lactis</i> NCDO 712	Parent strain	Gasson (1983)
<i>L. lactis</i> subsp. <i>lactis</i> MG1363	Plasmid-free strain of NCDO 712	Gasson (1983)
<i>E. coli</i> HB101	Transformation strain	Maniatis <i>et al.</i> (1982)
pTB201	Lactococcal <i>rrn</i> genes in pACYC184	Beresford & Condon (1991)
pMP220	Tc ^R , promoterless <i>lacZ</i> gene	Spaink <i>et al.</i> (1987)
pGKV210	Em ^R , promoterless Cm ^R gene	Van der Vossen & Venema (1985)
pTB301	pMP220 containing <i>L. lactis</i> subsp. <i>lactis</i> rRNA promoter region	This study
pTB401	pGKV210 containing <i>L. lactis</i> subsp. <i>lactis</i> rRNA promoter region	This study

ribosomal protein synthesis due mainly to an increased rate of translation of mRNA from ribosomal (r-) protein/protein mRNA (Bremer & Dennis, 1987; Jinks-Robertson & Nomura, 1987). *E. coli* has seven *rrn* operons (Ellwood & Nomura, 1982) with approximately the same organization: tandem promoters P1 and P2–16S–spacer–23S–5S genes–terminator region (Jinks-Robertson & Nomura, 1987). Each promoter region is subject to at least three control systems. In the first, *rrn* expression from P1 is regulated by the growth rate (Nomura *et al.*, 1984). The second, known as the stringent response (Stent & Brenner, 1961) is characterized by the inhibition of expression from P1, on cessation of protein synthesis, indicated by the absence of charged amino acyl tRNA (Cashel & Rudd, 1987). The third control is characterized by a stimulation of *rrn* expression from P1 following binding of a protein known as the Fis protein to regions upstream of P1 (Ross *et al.*, 1990). It is thought that expression from *rrn* P2 is constitutive and designed to ensure a continuance of a low level of expression when expression from *rrn* P1 is blocked (Sarmientos & Cashel, 1983). In addition expression of *E. coli* *rrn* operons is affected by anti-termination sequences located between P2 and the 16S rRNA structural gene. These sequences are similar to *nut* anti-termination sequences of bacteriophage λ (Gourse *et al.*, 1986; Berg *et al.*, 1989).

This report is concerned with measurement of RNA, DNA and protein of a lactococcal strain growing over a wide range of growth rates and examination of a recently cloned lactococcal *rrn* operon (Beresford & Condon, 1991) for transcription regulation sequences.

Methods

Bacteria strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1.

Growth and macromolecular composition. *E. coli* strains were routinely grown and maintained in LB medium (Maniatis *et al.*, 1982) with antibiotics as appropriate. The concentrations were as follows: tetracycline (Tc), 10 $\mu\text{g ml}^{-1}$; ampicillin (Ap), 25 $\mu\text{g ml}^{-1}$; and erythromycin (Em), 500 $\mu\text{g ml}^{-1}$. When required, X-gal was added at 50 $\mu\text{g ml}^{-1}$. *L. lactis* subsp. *lactis* strains were grown routinely on M17 medium (Terzaghi & Sandine, 1975) with glucose as the carbon source (GM17). Em was added at 5 $\mu\text{g ml}^{-1}$ and chloramphenicol (Cm) was used as indicated.

Growth of *L. lactis* subsp. *lactis* NCDO 712 in batch cultures for assay of growth rate and macromolecular composition was in either Thomas *et al.* (1979) chemically defined medium (TEL) or in modifications of this medium. Modified TEL (MTEL) contained the amino acids; Arg, Cys, Glu, His, Ile, Leu, Lys, Met, Phe, Pro and Val at the concentrations present in TEL. MTEL+T and MTEL+YE were made by adding tryptone or yeast extract, respectively, to MTEL at 0.5% (w/v). All batch cultures were incubated at 30 °C without aeration. Growth was monitored as OD₆₀₀ on a Phillips model Pye Unicam PU8600 UV/Vis spectrophotometer. The growth rate (k , h⁻¹) of batch cultures was calculated from semi-log graphs of OD₆₀₀ against time using the formula:

$$k = \ln X_2 - \ln X_1 / t_2 - t_1$$

where X_2 and X_1 were exponential phase OD₆₀₀ values of the cultures at times t_2 and t_1 , respectively.

In continuous chemostat cultures TYG medium containing tryptone (17 g l⁻¹), yeast extract (3 g l⁻¹) glucose (5 g l⁻¹), Na₂HPO₄ (2.28 g l⁻¹), KH₂PO₄ (3.27 g l⁻¹) was used in a BioFlo chemostat (New Brunswick Scientific). Cultures were incubated at 30 °C under a head of nitrogen and were agitated at 200 r.p.m. The pH in the fermenter vessel was maintained at approximately 6.7 (6.52–6.82) by continuous addition of 4 M-NaOH.

Biochemical assay of macromolecular composition was as follows. Protein was extracted from cells in 1 M-NaOH at 90 °C for 10 min and measured by the Lowry method. Bovine serum albumin was used as a standard. RNA was measured by the orcinol method as described by Griswold *et al.* (1951) with ribose as the standard. Total cellular DNA was assayed by the method of Burton (1957) with herring sperm DNA, degraded free acid, as the standard.

DNA manipulations. Plasmid DNA was isolated from *E. coli* by the method of Birnboim & Doly (1979). Plasmid DNA was extracted from *L. lactis* subsp. *lactis* by the method of Anderson & McKay (1983). DNA required for cloning and sequencing was further purified by CsCl

gradient density centrifugation (Maniatis *et al.*, 1982). Endonuclease enzymes, alkaline phosphatase and DNA ligase were obtained from Boehringer Mannheim and were used according to the manufacturer's instructions. Transformation of *E. coli* was according to Mandel & Higa (1970). Electroporation of *L. lactis* subsp. *lactis* was achieved by the method of Holo & Nes (1989).

The nucleotide sequence of the promoter region and the 3' end of the *rrn* operon was determined in both directions by the chain termination method of Sanger *et al.* (1977) with a T7 polymerase sequencing kit obtained from Promega and [α - 35 S]ATP from Amersham. Sequencing templates were alkali-denatured double-stranded DNA pTB201 molecules (Beresford & Condon, 1991).

Cloning of a putative lactococcal *rrn* promoter. The starting material was a plasmid pTB201 containing a complete lactococcal *rrn* operon on a 5.4 kb DNA fragment. The *rrn* operon contained a 1.4 kb *EcoRI* fragment which included part of the 16S RNA structural gene (0.6 kb) and the region (0.8 kb) immediately upstream. The 1.4 kb *E. coli* fragment was fused to a promoterless *lacZ* gene of the promoter probe vector pMP220 and transformed into *E. coli* HB101. A number of transformants were selected using X-gal as an indicator and checked to confirm the presence of 1.4 kb inserts in the original (pTB201) orientation. One of these clones, pTB301, was selected for further study.

Since pTB301 does not replicate in lactococci the 1.4 kb lactococcal DNA insert from pTB301 was cloned as a *Bgl*/II/*Pst*I fragment into the shuttle promoter probe vector pGKV210. This vector has a promoterless *Cm* acetyltransferase (*CAT*) gene and a selectable *Em* resistance (*Em*^R) gene. The ligation mix was transformed into *E. coli* HB101 and plated on LB with *Cm* and *Em*. One resultant clone, pTB401, was analysed to confirm the presence of the 1.4 kb lactococcal insert in its original (pTB201) orientation.

Promoter activity. β -Galactosidase activity was measured according to the method of Miller (1972). *CAT* activity was determined by the method of Shaw (1975). Minimal inhibitory concentrations of chloramphenicol on *L. lactis* subsp. *lactis* strains were determined in GM17 medium after overnight incubation at 30 °C.

Results

Effect of growth rate on the RNA, DNA and protein composition of L. lactis

Two approaches were taken to determine whether *L. lactis* NCDO 712 varied its macromolecular composition according to its rate of growth. The first approach was to measure RNA, DNA and protein in exponentially growing batch cultures. To vary the growth rate the composition of the growth medium was varied. The chemically defined TEL medium contains 20 amino acids. A modified version of this medium (MTEL) was devised based on a previous report (Reiter & Oram, 1962), which indicated that all 20 amino acids were not required for growth of NCDO 712. MTEL contained 11 amino acids. Two further modifications of MTEL were made; one was MTEL plus tryptone (+T) and the other MTEL plus yeast extract (+YE). *L. lactis* NCDO 712 grew 4.9-times faster in MTEL+YE, the medium supporting the fastest growth rate, than in MTEL which supported the slowest growth rate. The DNA:protein

ratio remained relatively constant over this range of growth rate. However, the RNA content was growth-rate-dependent; the RNA:protein and the RNA:DNA ratios increased by factors of 1.9 and 2.2, respectively, as the growth rate increased 4.9-fold.

The second approach to varying the growth rate was to vary the availability of the energy source by growing the cultures in a chemostat at different dilution rates. The chemostat was operated with a complex medium, TYG, in which glucose was limiting and the growth rate was altered by changing the flow rate of the incoming medium. In this manner a 6.25-fold variation in growth rate was obtained. Under these growth conditions the DNA:protein ratio again remained constant. The RNA:protein ratio increased 1.76-fold over this range of growth rates and the RNA:DNA ratio varied by 1.73-fold.

Combining both sets of data indicates that there is an approximately linear relationship between the specific growth rate and the RNA:protein and RNA:DNA ratios of *L. lactis* subsp. *lactis* NCDO 712 cells, over the range of growth rates monitored (Fig. 1*a* and *b*, respectively). A 13.5-fold range in specific growth rate was achieved, over which RNA:protein varied 2.5-fold and RNA:DNA varied 3.1-fold. A positive intercept was

Fig. 1. (a) Graph showing the relationship between the growth rate [k (see Methods) or D (dilution rate)] and the ratio of RNA:protein, measured in both batch ($\square$) and chemostat ($\blacklozenge$) cultures. (b) Graph showing the relationship between the growth rate and the ratio of RNA:DNA, measured in both batch ($\square$) and chemostat ($\blacklozenge$) cultures.

noted on the ordinate of both graphs indicating a minimum stable RNA content at zero growth rate. These data indicate that the stable RNA content of the lactococcal cell is regulated by growth rate.

Identification of promoter activity associated with the 5' end of the rrn operon

The correlation of RNA content with growth rate suggested that expression of *rrn* operons in lactococci responds to growth rate stimulation and that promoters of *rrn* operons might have some structural features similar to those identified in *E. coli*. In a previous paper (Beresford & Condon, 1991) the genes for one of the rRNA operons of NCDO 712 were cloned on a plasmid, pTB201, which contains 5.4 kb of the lactococcal chromosome. The start of the 16S rRNA gene was identified on a 1.4 kb *EcoRI* fragment which also contained 0.8 kb of DNA upstream of the structural 16S rRNA gene. This 0.8 kb was a likely location for the promoter of the rRNA operon. To test for promoter activity, the 1.4 kb *EcoRI* fragment of pTB201 was fused to a promoterless *lacZ* gene of a promoter probe vector, pMP220. One of the resultant constructs, pTB301, was assayed for promoter activity by assaying β -galactosidase activity in exponential phase *E. coli* HB101 cells. The lactococcal insert in pTB301 caused a 300-fold increase in β -galactosidase activity, compared to that in cells containing the vector pMP220 alone. This confirmed that even in a heterologous host, substantial promoter activity was associated with the 1.4 kb *EcoRI* fragment of pTB201.

The plasmid pTB301 was unable to replicate in *L. lactis* subsp. *lactis*. To prove that the promoter activity noted in *E. coli* was also present in a lactococcal background, the insert from pTB301 was cloned into the shuttle promoter probe vector pGKV210 which has a promoterless CAT (Cm^R) gene. A resultant plasmid, pTB401, was transformed by electroporation into *L. lactis* subsp. *lactis* MG1363 to determine if the lactococcal insert was capable of promoting the expression of the CAT gene in its homologous background. Transformants were initially selected on GM17 with Em ($5 \mu\text{g ml}^{-1}$) and clones were then spotted onto GM17 plates containing Em ($5 \mu\text{g ml}^{-1}$) with a Cm concentration of $1\text{--}4 \mu\text{g ml}^{-1}$. Controls of *L. lactis* subsp. *lactis* MG1363 containing the plasmid pGKV210 were also spotted onto the plates. All clones containing pTB401 were able to grow in the presence of Cm at $4 \mu\text{g ml}^{-1}$, whereas the controls were not. The ability of pTB401 to promote expression of the CAT gene was measured by assaying the biomass generated after 24 h growth in GM17 broth containing increasing amounts of Cm. The results (Fig. 2) indicate that strains carrying the vector

Fig. 2. Growth measured by OD₆₀₀ after 24 h at 30 °C in GM17 medium with various concentrations of Cm, of *L. lactis* MG1363 with either pGKV210 (□) or its derivative pTB401 (◆). pGKV210 has a promoterless CAT gene; pTB401 has 1.4 kb of the 5' side of the *rrn* operon from NCDO 712 fused to the CAT gene of pGKV210.

alone were very sensitive to Cm and did not grow well at concentrations greater than $2 \mu\text{g ml}^{-1}$, whereas those carrying pTB401 grew well in the presence of at least $25 \mu\text{g Cm ml}^{-1}$. This confirms that the 1.4 kb *EcoRI* fragment contains DNA with strong promoter activity when expressed in *L. lactis* subsp. *lactis*.

DNA sequence analysis of the putative rrn promoter region of L. lactis

The base sequence of the *rrn* promoter region of *L. lactis* NCDO 712 was determined. The starting material was pTB201 which has a full *rrn* operon and about 0.8 kb DNA upstream of the start of the structural genes. The 5' end of the sequence was initiated from an oligonucleotide primer complementary to a known vector sequence just outside the insert DNA and the 3' end was generated from an oligonucleotide complementary to a conserved region (Collins *et al.*, 1989) about 100 bp downstream of the 16S rRNA sequence start point. The DNA sequenced is 833 bp long (Fig. 3) and its GC content is 35.4%, which is within the range expected for lactococcal DNA (Stackebrandt & Teuber, 1988). The sequence included 25 bp corresponding to the 5' end of the 16S rRNA sequence as determined by alignment to *E. coli* (Brosius *et al.*, 1981) and 16S rRNA sequences from other lactococci (Collins *et al.*, 1989). An *EcoRI* site was located at the extreme 5' end of the insert and *XhoI* and *HindIII* sites were located internally. The *XhoI* site was 452 bp downstream of the *EcoRI* site and the *HindIII* was a further 80 bp downstream. These locations for the restriction sites were in good agreement with earlier data, determined by restriction analysis (Beresford & Condon, 1991).

E 60
GAATTCGAAGGTTTATTTCTGGAATGAAACCAATCATCAGAAAATGATGAAACAAATT
GluPheGluGlyLeuPheSerGlyMetLysProIleIleArgLysLeuMetLysGlnIle

120
CGAATCAAAGCATGGGACATAGAGGATTATTTATCAAGAAGGAATGATTATTTTGCATCAC
ArgIleLysAlaTrpAspIleGluAspTyrTyrGlnGluGlyMetIleIleLeuHisHis

180
CTTTTGAAGAAAATCACCCATCCACTAATATTTATACAAAGTTCAAAGTAAATATCAT
LeuLeuGluGluAsnHisProSerThrAsnIleTyrThrLysPheLysValLysTyrHis

240
CAACATTGATTGATGAACACGCCATAGCTACGCCAAAAACGGCTTCATGACCATTTT
GlnHisLeuIleAspGluLeuArgHisSerTyrAlaLysLysArgLeuHisAspHisPhe

300
GTAGGCTGACACATTTATGAATGTTCGGACTGGATAGATGCAGGAGGAAGTACCCCTGAA
ValGlyLeuAspIleTyrGluCysSerAspTrpIleAspAlaGlyGlySerThrProGlu

360
AGCGAGCTTGTGTTCAATCATCTTTTAGCAGAAGTTTGAAGGATTAGCGCCCATCTAT
SerGluLeuValPheAsnHisLeuLeuAlaGluValTyrGluGlyLeuSerAlaHisTyr

420
CAGGAATTACTCGTGGCTCAAATGAGAGGAGAAGAACTCAGCGAATGGAACGCTATCGG
GlnGluLeuLeuValArgGlnMetArgGlyGluGluLeuThrArgMetGluArgTyrArg

X 480
CTAAGAGAAAAAATCAAAAATATACTATTTCTCGAGATGATGATTAAAAAAGCTCCCT
LeuArgGluLysIleLysAsnIleLeuPheSerArgAspAspAsp

P1 H 540
TATCTTTGATAGGGAGTTATTTATTTTAAAAATAAATAAAAAAACAAGAAAAGCTTCAA
-35 -10

600
GCGTTTGCTAGCCTAAGTTAAACGGATACATAAGTAGATTAAACGAACAAAGGGGAA

P2 660
GAAAACCTAAAAATGACAGTTAATTCCTGACAGGGAGAGATAGGTTTGATAGGAATATAA
-35 -10

* 720
TAGTTGTGACAGAGAGAGAGAGAGCTGAAGCGGTGACAAAGCTAGACCATGAAAACTGAATAA

BoxB BoxA 780
AGAAGAATGACTCAATGTGATGTAGCAATACATCAAAAATCTGTCAATCAATTAATGAAAGA

BoxC 838
CAAGCCAGTCACTTCGGTGAATTAATCTTTATtttgagagtttgatcctggtcagg

Fig. 3. Sequence of the entire promoter region from the *L. lactis* subsp. *lactis* rRNA operon. The upstream open reading frame is indicated with the derived amino acid sequence. Restriction sites are denoted by: E, *EcoRI*; X, *XhoI*; H, *HindIII*. The -10 and -35 sites of the two putative promoters P1 and P2 are underlined. The BoxB anti-termination region is highlighted by arrows. BoxA and C motifs are denoted by over- and underlining. The probable transcriptional start point of P2 as determined by Van der Vossen *et al.* (1985) is indicated by *. The putative processing site is indicated by the overline which partially overlaps BoxA. The 5' end of the mature 16S rRNA, as determined by sequence alignment to reported 16S rRNA sequences (Brosius *et al.*, 1981; Collins *et al.*, 1989), is shown in lower case italicized lettering.

Extending downstream from the *EcoRI* site there is an open reading frame which encodes part of a protein of 155 amino acids. No significant homology was observed between the amino acid sequence and those previously stored in the databanks. No attempt was made to determine whether or not this protein is synthesized *in vivo*.

By comparison with published lactococcal promoter sequences (see Discussion) two putative tandem

CTCGAGGACT 15 20 30 40 50
X TTACCAAAAG AGTCAAATAT AAAATGCTTA TGGTTTAGAA
60 70 80 90 100
GTTAGAAGAA TTGTTATTTA GTTTTGAATG TTCAAAGTAA CATTTAATGA
110 120 130 140 150
TTTGGTCATC ATTGCGATGG AGATACACCT GTTCCCATGT CGAACACAGA
160 170 180 190 200
AGTTAAGTCC ATCTACGGCG GAAGTACTTG GGGGTTGCC CTTGGGAGAT
S
210 220 230 240 250
ATGCGAGTGG CCAAGTGAAC CAAAGGAGCT AAGGCTCCTT TTTTGTGTT
B
260 270 280 290 300
CTTGCTCCTT TATTTCTCGA TATGTTAAAA GCCCTAAGGG CTGTTTTTAT
310 317
CTTTGGAGAA TGAATTC
E

Fig. 4. Sequence of the *XhoI/EcoRI* fragment of pTB201. The sequence of the 5S rRNA gene is underlined. The 5' and 3' ends of the 5S rRNA gene were determined by sequence comparison to that obtained for *L. lactis* subsp. *cremoris* (Neimark *et al.*, 1983). The positions of restriction sites are indicated by: X, *XhoI*; S, *SacI*; B, *BalI*; E, *EcoRI*. The arrows indicate the inverted repeat putative transcription termination sequences. A putative primary processing site is indicated by an overline.

promoters were identified 296 and 157 bp from the 5' start of the mature 16S rRNA sequence. These were designated P1 and P2 (Fig. 3), consistent with the notation used for *E. coli* *rrn* promoters (Young & Steitz, 1979). Sequences with characteristics of anti-termination motifs in *E. coli* (see Discussion) were located between P2 and the start of the 16S rRNA gene.

Putative *rrn* operon termination

Previous work established that the plasmid pTB201 contained the structural genes for the 16S and 23S rRNAs and that both genes were tightly linked (Beresford & Condon, 1991). A small 0.3 kb *XhoI/EcoRI* DNA fragment adjacent to the 3' end of the 23S rRNA gene was also identified in that work. The complete nucleotide sequence of the *XhoI/EcoRI* fragment was obtained and by comparison with a previously determined *L. lactis* subsp. *cremoris* sequence (Neimark *et al.*, 1983), this region was shown to encode a 5S rRNA gene of 117 bp. Examination of the sequence region immediately downstream of the 5S rRNA gene revealed a putative transcription terminator, a perfect inverted repeat sequence which forms an 8 bp stem with an unpaired 4 base loop followed by a sequence rich in T residues (Fig. 4).

Identification of putative rRNA processing sites

On the basis of comparison with other Gram-positive bacteria (see Discussion) two putative processing sites have been identified in the *rrn* operon of *L. lactis* NCDO

712. The first overlaps the BoxA region between P2 and the 16S rRNA sequence (Fig. 3) and the second which is complementary to the first is located in the spacer region between the 23S and the 5S rRNA genes (Fig. 4).

Discussion

In both batch and continuous chemostat cultures of *L. lactis* NCDO 712 an increase in the growth rate correlated to an increased RNA content of biomass relative to either protein or DNA. Above a threshold value, corresponding to the macromolecular composition at zero growth rate, the RNA content increased directly in proportion to growth rate increases (Fig. 1). Since the rate of increase of RNA with growth rate was the same in batch cultures, where the energy source was in excess, and in chemostat cultures, where the energy source was limiting (Fig. 1), it is unlikely that the RNA content is controlled by the nutritional status of the medium. It is more likely that the RNA content is regulated by the growth rate *per se*, as has been observed with enteric bacteria and other microbes (Nierlich, 1978).

The RNA measured here is stable RNA which consists mainly of rRNA. In an earlier report (Beresford & Condon, 1991) we showed that the *L. lactis* NCDO 712 genome has six sets of rRNA genes. One set was cloned and was found to be organized in a manner similar to that in *E. coli*, in that genes for 16S, 23S and 5S RNA were closely linked within a DNA fragment of approximately 5.4 kb. The start of the 16S rRNA gene resided about 0.8 kb inside the 5' edge of the cloned fragment. This 0.8 kb piece was an ideal sequence in which to search for regulatory sites.

The nucleotide sequence revealed an organization similar in outline to that in *E. coli*. First, two tandem promoter-like sequences, P1 and P2, were identified, 296 and 157 bp upstream of the 16S rRNA gene (Fig. 3), respectively. The distal putative promoter, P1, has both -10 and -35 sites that conform well to the lactococcal consensus sequences. Five of the six nucleotides in the -35 motif (5' TTGATA 3') are present in the consensus sequence (5' TTGACA 3'). In his review, de Vos (1987) noted that the C at position 5 was conserved in only five of ten *Lactococcus* promoters examined, whilst a T was present in three of the remainder. Five of the six consensus nucleotides (5' TATAAT 3') are again present in the -10 sites (5' AATAAT 3'). The 5' T is a highly conserved nucleotide, occurring in nine of the ten promoters examined. However, the exception also has an A in this position (de Vos, 1987). The 20 bp spacing between the putative -10 and -35 sites is bigger than the normal spacing, which is 15–18 bp (de Vos, 1987). However, Koivula *et al.* (1991) have also identified promoters in *L. lactis* subsp. *lactis* which have unusual

lengths between their -10 and -35 sites; one of the promoters which they characterized had a 20 bp sequence between the two sites. The large distance between the -10 and -35 sites suggests that P1 may be a weak promoter. However, between -35 and -80 the sequence is 71% AT compared to the genomic average of 61% (Stackebrandt & Teuber, 1988); this is characteristic of very active promoters. AT-rich DNA can form unusual secondary structures referred to as bent DNA, which is thought to be involved in elevated rates of expression of promoters containing it (Gourse *et al.*, 1986). This may compensate for any reduced activity associated with the large spacing between the -10 and -35 sites. Occupying the first and fourth positions downstream of the -10 sequence, two adenine residues are located which is also characteristic of lactococcal promoters.

The -35 motif of the P2 promoter conforms perfectly to the proposed lactococcal canonical sequence. The -10 motif (TAGAAT) has one mismatch in the 6 bp sequence. In the ten promoters examined by de Vos (1987) only one had a G located at position 3; it is a variable site, however, with only four of the promoters examined conforming to the consensus at this site. The distance between the -10 and -35 sites was 17 bp which is typical of lactococcal promoters (de Vos, 1987) and again an AT-rich region was identified immediately upstream of the -35 region.

Comparison of the sequences within and downstream of the P2 promoter indicated that it was highly homologous to promoter P59, one of several promoters identified by Van der Vossen *et al.* (1985) who used a promoter probe vector to clone promoter fragments from a *L. lactis* subsp. *cremoris* strain. In the 98 bp downstream of the -35 site of P2, only three mismatches were noted with P59. In the first 19 bp upstream from the -35 site in these two promoters the sequence identity was also good at 84% but then it dropped off rapidly. Anti-termination BoxB and BoxA motifs are also evident in the P59 sequence. This evidence supports the suggestion that P59 is an rRNA promoter from *L. lactis* subsp. *cremoris*. Van der Vossen *et al.* (1985) identified the transcriptional start of promoter P59. As the sequence of the *L. lactis* subsp. *lactis* rRNA promoter reported here is identical to P59 in this region, the transcriptional start of the *L. lactis* subsp. *lactis* rRNA P2 promoter is probably the A at position 662 (Fig. 3).

A second major similarity in organization between *E. coli* *rrn* promoter regions and the lactococcal DNA upstream of the 16S rRNA gene concerns anti-termination sequences. Uncoupling of translation from transcription in bacteria leads to premature termination of transcription (Adhya & Gottesman, 1978). This potential problem for transcription of *rrn* operons is avoided by the presence of anti-termination sequences

which permit readthrough of *rrn* structural genes by RNA polymerase. Anti-termination sequences in *E. coli* consist of three sequence motifs known as BoxA, B and C which are very similar to bacteriophage λ anti-termination sequences. BoxB is a region of hyphenated dyad symmetry whereas BoxA and BoxC are specific sequences (Berg *et al.*, 1989). Sequence motifs similar to those of BoxA, B and C of *E. coli rrn* promoter regions were identified in the lactococcal *rrn* promoter region (Fig. 3). Furthermore the location and orientation of the three motifs between P2 and the start of the 16S rRNA gene was similar in both organisms. Putative anti-termination sequences have been identified in the promoter regions of *rrn* operons in many bacteria besides *E. coli* (Berg *et al.*, 1989). Similar motifs have also been noted in the spacer regions between 16S and 23S rRNA genes. The BoxA sequences were found either abutting or within the primary transcript processing sites (Pernodet *et al.*, 1989; Berg *et al.*, 1989).

The circumstantial evidence obtained by sequence analysis and comparison, that the sequence upstream of the 16S rRNA gene should be capable of promoter activity was corroborated from studies with fusions of the relevant lactococcal DNA fragment with promoterless reporter genes. A 1.4 kb fragment consisting of the first 600 kb of the 16S rRNA gene together with the 0.8 kb immediately upstream, promoted expression of the *lacZ* gene in *E. coli* and the CAT gene in *E. coli* and *L. lactis*. No effect of growth rate on the expression of the *lacZ* gene from the fusion plasmid in *E. coli* was noted (data not shown) but a growth rate effect was observed on expression of CAT activity in *L. lactis*. Expression of CAT activity from pTB401 in *L. lactis* MG1363 increased approximately twofold for a 33% increase in growth rate (data not shown). This could be explained if the copy number of pTB401 was greater in the faster growing cells but no appreciable difference in plasmid content between the two cultures was noted. Although the evidence is insufficient, the positive response to growth rate is encouraging and worthy of further experimentation.

The sequence of the region on the 3' side of the 23S gene indicates that it contains a typical 5S RNA gene sequence (Fig. 4) which is identical to a published sequence from *L. lactis* subsp. *cremoris* (Neimark *et al.*, 1983) except that the latter has a T in the first position instead of an A in the NCDO 712 gene. The 117 bp length of the 5S rRNA is the expected length for a Gram-positive eubacterium (Hori & Osawa, 1979). Unlike *E. coli rrn* operons, typical tRNA sequences were not noted in the 101 bp downstream nor in the 99 bp upstream of the 5S rRNA gene.

Just 5 bases after the 3' end of the 5S rRNA gene sequence there is a perfect inverted repeat sequence

which forms a structure typical of ρ -independent transcriptional terminators and is in the right position to terminate a complete transcript of the *rrn* operon (Young, 1979).

The 16S, 23S and 5S rRNAs are formed by processing of the primary transcript (Birenbaum *et al.*, 1978; King & Schlesinger, 1987). The primary (RNase III) processing sites are located in sequences on either side of the 16S and 23S rRNAs; these sequences form double-stranded stalks with the 16S and 23S rRNAs as extensive loops (King & Schlesinger, 1987). Pernodet *et al.* (1989) demonstrated a remarkable degree of conservation in 21 base sequences associated with primary processing sites of Gram-positive bacteria; furthermore these sites shared extensive homology with BoxA anti-termination sequences of *E. coli*. By comparison with the Pernodet *et al.* (1989) sequences two putative processing sites have been identified in the *L. lactis* NCDO 712 *rrn* operon. The first, which includes the putative BoxA sequence is located in the leader sequence between the 16S rRNA gene (Fig. 3). The lactococcal sequence shares 12 of 14 highly conserved bases with other Gram-positive processing sequences. A second sequence was identified in the 23S–5S rRNA spacer region (Fig. 4) which is highly complementary to the 16S rRNA leader sequence. This is the likely processing site expected at the 3' side of the 23S rRNA gene according to the current model for primary transcript processing.

In conclusion, the observations made in this report indicate that rRNA synthesis in *Lactococcus* is regulated according to the classic model generated from work mainly with *E. coli*. Previously (Beresford & Condon, 1991) we showed that *Lactococcus* has six *rrn* operons compared to seven in *E. coli* (Ellwood & Nomura, 1982) and that the lactococcal *rrn* operons conformed to the classical 16S–23S–5S rRNA structural gene sequence. In the present study we have shown that the sequence upstream of the 16S rRNA gene has several characteristics of the model *rrn* promoter region such as tandem consensus promoter sequences and putative anti-termination sequences. Furthermore, this putative lactococcal promoter turns on promoterless reporter genes in both homologous and heterologous hosts.

The classic correlation between RNA content of cell biomass and growth rate is evident in lactococcal cultures. We have very preliminary data which suggests that the cloned putative promoter may respond to growth rate stimulation. We have not yet looked for evidence of a stringent response or of a response similar to the Fis protein stimulation observed in *E. coli* (Ross *et al.* 1990). Finally, according to the classic model a ρ -independent terminator should exist at the end of the 5S RNA structural gene (Young, 1979) and RNase III processing sites should be located at either side of the 16S

and 23S RNA transcripts (Birenbaum *et al.*, 1978). We have noted characteristic sequences which indicate that termination and processing of rRNA transcripts in lactococci are in accord with the classic model.

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