Nucleotide sequence of a *Lactococcus lactis* gene cluster encoding adenylate kinase, initiation factor 1 and ribosomal proteins

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We have previously isolated a putative promoter from the *Lactococcus lactis* subsp. *lactis* chromosome. We now report the sequence of the promoter fragment and its extension in the 5'-direction. The region contains several open-reading frames which correspond to ribosomal protein L15, SecY, adenylate kinase, initiation factor 1 and ribosomal proteins B and S13. The order of the genes, *rplO* (L15), *secY*, *adk*, *infA*, *rpmJ* (B) and *rpsM* (S13), is similar to that in the *spc* and α operon region of *Bacillus subtilis*, with the exception of the *map* gene, coding for methionine amino peptidase, which is located between *adk* and *infA* in *B. subtilis*. The putative promoter is located between *adk* and *infA*.

Introduction

The biochemistry and genetics of ribosomal proteins have been extensively studied in *Escherichia coli*, which has over 50 ribosomal protein genes (Lindahl & Zengel, 1986; Bachmann, 1990). Several of the proteins have been purified and characterized, and most of the genes have been isolated and sequenced. The genes are scattered around the chromosome. Nevertheless, approximately half of them map at 73 min on the *E. coli* chromosome, at the *str*-*spc* locus, which is organized into the four transcription units *str*, S10, *spc* and α . In *Bacillus subtilis*, an extensively studied Gram-positive bacterium, about 40 ribosomal genes have been localized (Piggot *et al.*, 1990). Similarities between the ribosomal operons of *E. coli* and *B. subtilis* have been found in the *spc* and α regions (Boylan *et al.*, 1989; Henkin *et al.*, 1989).

The *spc* and α operons contain genes for ribosomal proteins but also some other genes. In *E. coli*, this region contains a gene for the α subunit of the DNA-dependent RNA polymerase, and a gene for SecY, a protein involved in protein secretion (Cerretti *et al.*, 1983; Bedwell *et al.*, 1985). These genes are also included in the *spc*- α region of *B. subtilis*, which, in addition, includes genes for adenylate kinase (Adk), methionine amino peptidase (Map) and initiation factor 1 (IF1) (Boylan *et al.*, 1989; Nakamura *et al.*, 1990; Suh *et al.*, 1990). Adk

catalyses the reaction $\text{ATP} + \text{AMP} \rightarrow 2 \text{ADP}$ (Schulz *et al.*, 1986) and Map removes the amino-terminal formyl-methionine from cellular proteins (Ben-Bassat *et al.*, 1987). IF1 participates in protein synthesis by forming the initiation complex with IF2 and IF3, although the exact role of IF1 in this process is not known (Pon *et al.*, 1979).

Lactococci are important micro-organisms in food and feed fermentations; this has resulted in considerable efforts to understand their molecular biology. However, there are no data on the ribosomal proteins or their genes in lactococci. We have previously isolated chromosomal promoter fragments from *L. lactis* subsp. *lactis* (Koivula *et al.*, 1991*b*). One of the cloned DNA fragments contained three open-reading frames (ORFs) downstream from the promoter. The ORFs corresponded to IF1 and to the ribosomal proteins B and S13. In this paper we report a nucleotide sequence for the region of the *L. lactis* chromosome which closely resembles the end of the *spc* operon and the beginning of the α operon of *E. coli* and *B. subtilis*.

Methods

Bacterial strains, plasmids and culture conditions. *L. lactis* subsp. *lactis* MG1614 (Gasson, 1983) was the source of chromosomal DNA. *E. coli* DH5 α F' (Hanahan, 1983) was used as a cloning host for plasmid constructions based on pUC18 and pUC19 (Yanisch-Perron *et al.*, 1985). *B. subtilis* IH6064 (Palva, 1982) was the source of chromosomal DNA used as a control in hybridization. The plasmid pKTH1816 has been described previously (Koivula *et al.*, 1991*b*).

The nucleotide sequence data reported in this paper have been submitted to EMBL and have been assigned the accession number X59250.

E. coli and *B. subtilis* cells were grown in Luria broth (Maniatis *et al.*, 1982) supplemented with ampicillin ($100 \mu\text{g ml}^{-1}$) as necessary. *L. lactis* cells were grown in M17/glucose (Terzaghi & Sandine, 1975) medium supplemented with chloramphenicol ($5 \mu\text{g ml}^{-1}$) as needed.

DNA methods. *E. coli* cells were transformed by the method of Hanahan (1983). Plasmids were isolated from *E. coli* and *L. lactis* cells by the alkaline method (Maniatis *et al.*, 1982) except that mutanolysin (5 to 50 U ml^{-1}) was used with lysozyme at 37°C for 30 min to disrupt the cell wall of *L. lactis*. The chromosomal DNA of *L. lactis* and *B. subtilis* was isolated according to Marmur (1961). Restriction enzyme digestions, ligations, gel electrophoresis and Southern blotting were done as described by Maniatis *et al.* (1982). Hybridization conditions were similar to those described previously (Koivula *et al.*, 1991b). The same conditions were used for nick-translated probes and for terminally labelled oligonucleotide probes except that the hybridization was done at $T_m - 10^\circ\text{C}$ (10°C below the melting temperature of the probe) for 30 min and at $T_m - 20^\circ\text{C}$ for 30 min when oligonucleotide probes were used. Polymerase chain reactions (PCRs) were done as recommended by the supplier of the AmpliTaq enzyme (Perkin-Elmer/Cetus).

DNA sequencing and sequence analysis. For DNA sequencing, the plasmids were denatured by alkali (Koivula *et al.*, 1991b) and sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using [α - ^{35}S]dATP deoxyadenosine 5'- α -[^{35}S]thiotriphosphate [$>1000 \text{ Ci mmol}^{-1}$ ($>37 \text{ TBq mmol}^{-1}$); Amersham] as the labelled nucleotide. The DNA polymerase used was Sequenase (USB) and the protocol recommended by the manufacturer was followed. Universal primers were used to sequence the inserts in pUC18 and pUC19 together with specific primers made on an Applied Biosystems DNA synthesizer model 381A. Both strands of the DNA were sequenced.

The sequence was analysed with the GCG package (Devereux *et al.*, 1984) and the PCGENE set of programs (Intelligenetics). The protein sequence data banks were searched with the FASTA program (Pearson & Lipman, 1988).

Results and Discussion

Cloning of the ribosomal operon fragment

We have previously isolated promoter fragments from *L. lactis* (Koivula *et al.*, 1991b). Plasmid pKTH1816 is one of the constructions that was obtained. It contains 2.8 kb of *L. lactis* chromosomal DNA in front of a promoterless chloramphenicol acetyltransferase gene (*cat*) in a promoter probe vector pKTH1750. The promoter (P1) was first localized by sequencing. The promoter P1 was initially identified by sequencing. Its localization was supported by primer extension mapping of the 5'-end of the *cat*-specific transcript produced by the plasmid construct in *L. lactis* (Koivula *et al.*, 1991b). Downstream of the promoter, we found three ORFs, which were identified as initiation factor IF1 and ribosomal proteins B and S13 (Table 1).

The entire chromosomal fragment was sequenced to determine the organization of this region in *L. lactis* in more detail. According to the sequence data, the insert of pKTH1816 was 2848 bp long. It was composed of three *Sau3AI* fragments (A, 1470 bp; B, 234 bp; C, 1144 bp;

see Fig. 1a), which was unexpected since the chromosomal fragments originated from a complete *Sau3AI* digestion. The size of the insert was determined previously by restriction enzyme digestions using *EcoRI*, *PvuII*, *BclI* and *PstI*, but not *Sau3AI*, since a single *Sau3AI* fragment was expected.

Southern hybridization was used to analyse whether the three *Sau3AI* fragments were adjacent on the chromosome. *L. lactis* chromosomal DNA was digested with *EcoRI*, *HindIII* and *Sau3AI*, and with the enzyme combinations *EcoRI*–*HindIII* and *HaeII*–*EcoRI*. The digestions were hybridized with [γ - ^{32}P]rATP-labelled oligonucleotides 466 (5'-TCTGTAGAATAATGCCC-3') and 468 (5'-GTGATAAGTTGCTCCAC-3'), which were used for sequencing the fragments A and C, respectively. The results indicated that the fragments A and C were not colinear on the *L. lactis* chromosome (data not shown). There was no suitable probe for the small B-fragment, but the hybridization results with oligonucleotide 468 suggested that it is not colinear with the C-fragment.

The C-fragment (Fig. 1a), which was the *Sau3AI* fragment located closest to the *cat* reporter gene, contained the promoter P1 and the ORFs coding for IF1, B and S13. Upstream from the promoter another ORF was identified, which coded for a carboxy-terminal fragment of a protein homologous to Adk. This ORF continued beyond the *Sau3AI* site between fragments B and C. However, the B-fragment had no homology to Adk. This is a further indication that fragments B and C are not colinear on the *L. lactis* chromosome.

Because the entire *adk* gene was not in the C-fragment, we decided to clone an additional DNA fragment to complete the *adk* sequence. The cloned region of *L. lactis* had an organization similar to the *B. subtilis* *spc- α* region, except for the absence of the *map* gene between *adk* and *infA*. In *B. subtilis*, the *secY* gene is localized immediately upstream of *adk* (Nakamura *et al.*, 1990), and so we assumed that in *L. lactis* *secY* may also be located upstream from the *adk* gene. The sequences of the SecY proteins of both *E. coli* (Cerretti *et al.*, 1983) and *B. subtilis* (Nakamura *et al.*, 1990; Suh *et al.*, 1990) are known and they contain several nearly identical amino acid stretches. This allowed us to design an oligonucleotide (465) to clone the missing 5'-region of *adk* and the 3'-region of *secY* by using the PCR. The oligonucleotide 465 was synthesized with an inosine (I), or a mixture of A and T, or T and C, at the third nucleotide of most codons to allow binding irrespective of the identity of the third nucleotide in the codon; for serine a codon IIA/T was used (Fig. 1b).

First, Southern hybridization was done to confirm that oligonucleotide 465 binds efficiently to the chromosomal DNA of *L. lactis*. Oligonucleotide 465 was labelled with

Fig. 1. (a) Plasmid pKTH1816 contains three *Sau*3AI (S) fragments – A, B, and C – in the insert. The C-fragment contains the 3'-end of the *adk* gene, the 5'-end of the *rpsM* (S13) gene and the *infA* and *rpmJ* (B) genes. The sequence of the C-fragment was extended in the 5'-direction by cloning and sequencing a PCR fragment, which contained the 5'-region of *adk* and the 3'-region of *secY*. The primers used in PCR are indicated as 465 and 468. (b) Oligonucleotide 465 was based on an identical region between the SecY proteins from *E. coli* (Cerretti *et al.*, 1983) and *B. subtilis* (Suh *et al.*, 1990). The amino acid residues indicating the location of the fragments in the SecY proteins are indicated. Restriction enzyme abbreviations: A, *Acc*I; H, *Hind*III; P, *Pst*I; Pv, *Pvu*II; S, *Sau*3AI.

Table 1. Comparison of *L. lactis* proteins to related proteins
 aa, amino acid.

<i>L. lactis</i> protein (size)	Identical amino acids	Homologous protein	Reference
Adk (215 aa)	60%	<i>B. subtilis</i> (217 aa)	Nakamura <i>et al.</i> (1990)
	46%	<i>E. coli</i> (214 aa)	Brune <i>et al.</i> (1985)
	42%	<i>Paracoccus denitrificans</i> (217 aa)*	Spürigin <i>et al.</i> (1989)
	38%	<i>Saccharomyces cerevisiae</i> (222 aa)	Proba <i>et al.</i> (1987)
	30%	<i>Mycoplasma capricolum</i> (213 aa)	Ohkubo <i>et al.</i> (1987)
IF1 (72 aa)	81%	<i>B. subtilis</i> (72 aa)	Boylan <i>et al.</i> (1989)
	70%	<i>E. coli</i> (71 aa)*	Pon <i>et al.</i> (1979); Sands <i>et al.</i> (1987)
	70%	<i>Marchantia polymorpha</i> (78 aa)†	Ohyama <i>et al.</i> (1986)
B (38 aa)	86%	<i>B. subtilis</i> (37 aa)	Boylan <i>et al.</i> (1989)
	87%	<i>B. stearothermophilus</i> (37 aa)*	Tanaka <i>et al.</i> (1984)
	62%	<i>E. coli</i> (38 aa)	Cerretti <i>et al.</i> (1983)
S13 (51 aa; N-terminus)	65%	<i>E. coli</i> (121 aa)	Boylan <i>et al.</i> (1989)
	58%	<i>B. stearothermophilus</i> (119 aa)*	Brockmöller & Kamp (1988)
	48%	<i>E. coli</i> (118 aa)	Bedwell <i>et al.</i> (1985)

* Sequences determined by peptide sequencing; others were deduced from the nucleic acid sequence.

† Liverwort chloroplast protein.

SecY
 1410 1420 1430 1440 1450 1460 1470 1480 1490
 TATTTACTTAAACGTAATATGCAGGATTTATGGATAATCCACTTGAACAAAATAAAATCTTGTCATTAGACAAGGTTTTCTTTATTT
 Y L L K R K Y A G F M D N P L E T K -
 1500 1510 1520 1530 1540 1550 1560 1570 1580
 TTCAGTAAAAATACGCTAAATAGTGTAGGTAAAAGACAGTGTACATCTGCATTTTACTGACAGAGCTGTCAGTAATTTATAAAATCAAA
 Adk
 1590 1600 1610 1620 1630 1640 1650 1660 1670
 ATCAGGAGAAAAACAAATGAATTTATTAATCATGGGACTTCCAGGAGCTGGTAAAGGAACTCAAGCCGAATTTATCGTTAAAAATTATGG
 M N L L I M G L P G A G K G T Q A E F I V K N Y G
 1680 1690 1700 1710 1720 1730 1740 1750 1760
 TGTAACCATATTTCAACAGGAGATATGTTCCGTGCAGCGATGAAAAATGAAACAGAAATGGGTAAACTTGCTAAATCTTTTATTGATAA
 V N H I S T G D M F R A A M K N E T E M G K L A K S F I D K
 1770 1780 1790 1800 1810 1820 1830 1840 1850
 AGGTGAACCTCGTTCCTGATGAAGTAACCAATGGTATTGTCAAAGAACGTTTAGCACAAGATGATATCAAAGCTTCAGGTTTCTTGCTTGA
 G E L V P D E V T N G I V K E R L A Q D D I K A S G F L L D
 1860 1870 1880 1890 1900 1910 1920 1930 1940
 TGGATACCCACGTACGATTGACCAAGCGCATGCTCTTGATACAATGCTTGAAGAAGCTTGGCATTAACTTGATGCTGTTGTAATATTGT
 G Y P R T I D Q A H A L D T M L E E L G I K L D A V V N I V
 1950 1960 1970 1980 1990 2000 2010 2020 2030
 TGTTAATCCAAATATTTTGGTTGATCGTTTAAAGTGGTGGTACATCTGCCGTAATTTGGGAGCAACTTATCACAAAATTTCAACCCAAC
 V N P N I L V D R L S G R Y I C R N C G A T Y H K I F N P T
 2040 2050 2060 2070 2080 2090 2100 2110 2120
 TAAAGTTGAAGGAACTTGTGACGTTTGTGGTAGCCATGACCTTTACCAACGTGCTGATGATGTACCAGAAACAGTTAAAAACCGTTTAGA
 K V E G T C D V C G S H D L Y Q R A D D V P E T V K N R L D
 2130 2140 2150 2160 2170 2180 2190 2200 2210
 TGTCACATCAAAGAATCTGCGCCAATTATTGAACATTACACTGAAGTGGACTTGTAAAAATATCGAAGGCGAACAAGAAATTAGTCA
 V N I K E S A P I I E H Y T E L G L V K N I E G E Q E I S Q
 2220 2230 2240 2250 2260 2270 2280 2290 2300
 AGTGACAGATGATATTAATAAAGTTTTAGGCTAATAAATCATAAAACCTCTGTGACAGGTTTTTTATTTTAAATATGAAAAATGAAAGAT
 V T D D I K K V L G - → ←
 2310 2320 2330 2340 2350 2360 2370 2380 2390
 AAAATTTACTGACAGAAAAGTCAACAAGCTTAAAAATAAAAGAAACACCCGAAAGCATTGCCATAGGTACTCTTATCAGATAATCTGAA
 P1
 2400 2410 2420 2430 2440 2450 2460 2470 *
 AATAAAATGGACTCAGGCTAGAAAAATAAAGGCTTTTATGAAAGAAAGACTTGCATTTGTTGTTGAAAAATGCTAAAAATACATAAGTCC
 IF1
 2490 2500 2510 2520 2530 2540 2550 2560 2570
 GACTTTTTAGATATATTTAAATTTGATTTATATCTTTGCGGAAATTTTTAAGGAGGTACTTTTGTGCTGGCAAAAGATGATGTAATTGAA
 M A K D D V I E
 2580 2590 2600 2610 2620 2630 2640 2650 2660
 GTAGACGGCAAAGTCGTTGACACCATGCCGAATGCGATGTTCACTGTTGAAGTGAAGTGGTACCAAGTCTTGGCGACGATTTCAAGGA
 V D G K V V D T M P N A M F T V E L E N G H Q V L A T I S G
 2670 2680 2690 2700 2710 2720 2730 2740 2750
 AAAATTCGTAAGAATTATATCCGTATCTTGCCTGGAGACAAGGTTCAAGTTGAAGTCTCACCATACGATTTGACACGCGGACGAATTACT
 K I R K N Y I R I L P G D K V Q V E L S P Y D L T R G R I T
 B
 2760 2770 2780 2790 2800 2810 2820 2830 2840
 TACCGATTTAAGTAATCCGACATTCATTGAGTGCATGATGCACAGTAACCATAGAAAGGAAGACACAATGAAAGTAAGACCATCAGTTA
 Y R F K - M K V R P S V
 2850 2860 2870 2880 2890 2900 2910 2920 2930
 AACCAATCTGCGAATACTGTAAAGTATTGCTCGTAACGGTCGTGTAATGGTAATTTGCCCTGCCAATCCAAACACAAACACGTCAGG
 K P I C E Y C K V I R R N G R V M V I C P A N P K H K Q R Q
 S13
 2940 2950 2960 2970 2980 2990 3000 3010 3020
 GATAGAAAGGAAATAGAAAAATATGGCTCGTTTGTGGAGTTGATATTCCAACGAAAAACGCATTGTTATTTATTAAACATACGTATT
 G - M A R F A G V D I P N E K R I V I S L T Y V F
 3030 3040 3050 3060 3070 3080 3090 3100
 CGGTGTTGGAAGTCAACATCTAAGAAAGTACTTGCAGCTGCAGGTGTTTCAGAAGATATCCGTACTAAAGATTGACATCAGATC
 G V G L Q T S K K V L A A A G V S E D I R T K D L T S D

[γ - 32 P]rATP and used as a hybridization probe. This probe identified a 1.7 kb *Hind*III and a 1.6 kb *Hind*III–*Eco*RI fragment from the chromosomal DNA of *L. lactis* (data not shown). The probe also hybridized to a 6 kb *Eco*RI fragment of chromosomal DNA of *B. subtilis* IH6064, which was used as a control.

Oligonucleotides 465 and 468 were used in the PCR to clone the 5'-end of the *adk* gene from the chromosomal DNA of *L. lactis* (Fig. 1a). PCR yielded a 1.3 kb fragment which was cloned into the *Sma*I site of pUC19 and sequenced. Since the AmpliTaq polymerase used in the PCR makes errors during DNA synthesis, the PCR fragment was sequenced from three independent clones. In case of mismatches the nucleotide that was the same in two out of three clones was chosen. The sequence of the fragment was 1347 bp long, and contained the 3'-region of *secY* and the 5'-region of *adk* which overlapped with the 3'-region of the C-fragment (Fig. 1). There were 10 differences between the three clones: six of these were T→C changes but there were also two A→G, one A→C and one T→G change.

Organization of the ribosomal operon fragment

The nucleotide sequence of the region including the *adk*, *infA*, *rpmJ* (B) and *rpsM* (S13) (5'-region) genes is shown in Fig. 2. The sequences for the 3'-end region of *rplO* (L15) and *secY* have already been published (Koivula *et al.* 1991a). The genes begin with the initiation codon ATG except for *infA*, which begins with TTG. In front of the putative initiation codon of each gene there is a nucleotide sequence which shows a close complementarity to the 3'-end of the 16S rRNA of *L. lactis* DSM20250 (Ludwig *et al.*, 1985) and which resembles ribosomal-binding sites (RBSs) previously isolated from lactococci (de Vos, 1987). The calculated free energies (ΔG) (Tinoco *et al.*, 1973) for the binding of the putative RBSs were –11.6 kcal (–48 kJ) for *adk*, –18.1 kcal (–76 kJ) for *infA*, –18.0 kcal (–75 kJ) for *rpmJ* and –18.0 kcal (–75 kJ) for *rpsM*. The difference between the ΔG values of *adk* and the genes involved in protein synthesis may indicate a more efficient translation of the latter genes in accordance with studies in *B. subtilis* (Sharp *et al.*, 1990).

The previously isolated promoter P1 is located in the region between *adk* and *infA*. The promoter was isolated with a *cat*-based promoter probe plasmid by

chloramphenicol selection in *L. lactis* (Koivula *et al.*, 1991b), and the 5'-end of the transcript was determined from a plasmid construction. It is not known how this promoter functions when located in the chromosome, but it is notable that promoter P1 was found in a search for strong chromosomal promoters. In *E. coli* there is a promoter (P_{α}) located between the B and S13 genes (Post *et al.*, 1980), but the majority of the transcripts for the α region originate upstream from the *spc* operon (Cerretti *et al.*, 1983). In *B. subtilis*, there is a weak promoter activity in the region containing IF1, B and S13, but the major promoter encoding the α -region lies well upstream from this area (Boylan *et al.*, 1989).

The initiation factor IF1 and the ribosomal proteins B and S13 of *L. lactis* show a high degree of similarity to their counterparts in *E. coli* and *B. subtilis* (Table 1). This may reflect the functional roles of these proteins in ribosomes. Ribosomal subunits interact closely with each other which reduces the acceptable mutations in the proteins. In contrast, *L. lactis* adenylate kinase has a significantly lower degree of similarity to the same enzyme of other species, as is typical for globular enzymes. Only the catalytic residues and the inner core domain are highly conserved while the residues facing outside may change quite freely, allowing much divergence between different species (Chothia & Lesk, 1987).

Conclusions

The organization of the lactococcal genes in the chromosomal DNA fragment sequenced during this work is quite similar to the gene order in the corresponding region in *B. subtilis*. The major difference is the absence of the *map* gene from *Lactococcus*. The organization of the *spc* and α operons in the Gram-negative *E. coli* is essentially similar to that in *B. subtilis* except for the absence of *adk*, *map* and *infA* in *E. coli*. Another Gram-negative bacterium, *Mycoplasma capricolum*, contains the *adk* gene downstream from the *secY* gene, but there is no sequence further downstream (Ohkubo *et al.*, 1987). Thus, there appears to be species-specific differences even though the organization is quite similar in the regions that have been determined.

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Fig. 2. Nucleotide sequence for the *L. lactis* gene cluster encoding Adk, IF1 and ribosomal proteins. Promoter P1 is underlined and the 5'-end of the transcript, as determined by S1 mapping (Koivula *et al.*, 1991b), is marked by an asterisk at 2477 bp. The RBSs are underlined twice. The deduced amino acid sequences are given below the nucleotide sequence. An inverted repeat structure is indicated by arrows. The genes are identified by the name of the protein close to the RBS. The C-fragment starts at 1963 bp and the PCR fragment ends at 2013 bp. The numbering is consistent with the upstream region containing the 3'-end of *rplO* (L15) and *secY*, which has been published previously (Koivula *et al.* 1991a). The nucleotide sequence has been submitted to EMBL and given the accession number X59250.

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