The Role of *Escherichia coli* RNase E and RNase III in the Processing of the *citQRP* Operon mRNA from *Lactococcus lactis* biovar *diacetylactis*

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Abstract

Citrate transport in *Lactococcus lactis* biovar *diacetylactis* (*L. diacetylactis*) is catalyzed by citrate permease P (*citP*), which is encoded by the plasmidic *citP* gene. Two partial overlapping open reading frames *citQ* and *citR* are located upstream of *citP*. These two genes, together with *citP*, constitute the *citQRP* operon. In this report it was shown that in *L. diacetylactis* and *Escherichia coli*, *cit* mRNA is subject to the same specific cleavages at a complex secondary structure which includes the central region of *citQ* and the 5'-end of *citR*. The role of ribonucleases in the fate of the *cit* mRNA processing was investigated in *E. coli* RNase mutant strains. The results obtained indicate that both endoribonucleases RNase E and RNase III are involved in the generation of mRNA processed species. RNase E is responsible for the major cleavages detected within *citQ* and upstream of *citR*, whereas RNase III cleaves *citR* within its ribosomal binding site. Preliminary results indicate the existence of a RNaseII-like enzyme in *L. diacetylactis*. Based on these results, a model for the role of *cit* mRNA processing in the expression of *citP* is presented.

Introduction

In the dairy industry, lactic acid bacteria are important for their acidifying properties and their metabolisms. In addition, the use of citrate in milk leads to the production of aromatic and flavor products such as diacetyl and acetoin (reviewed by Hugenholtz, 1993). Many studies have been carried out to unravel the metabolic pathways involved in diacetyl biosynthesis (reviewed by Cocaign-Bousquet et al., 1996). However, the regulation and mechanism(s) of plasmid-mediated citrate utilization in *L. diacetylactis*, which is one of the main diacetyl producing bacteria in dairy products, is not completely understood. The citrate permease P encoded by the *citP* gene is the only essential requirement for the transport of citrate in this microorganism (Magni et al. 1994). Two partially overlapping open reading frames, *citQ* and *citR*, preceding the *citP* gene have been identified, and transcriptional analysis has shown that these three genes constitute an operon (López de Felipe et al., 1995). Transcription of the *citQRP* operon in *L. diacetylactis* is mainly driven by the P1 promoter, which is located 1 kb upstream of the *citQ* gene. The long untranslated leader region of the operon contains the insertion sequence IS982 (Magni et al., 1996). The introduction of this insertion sequence into the *citQRP* operon has provided the pCIT264 Cit⁺ plasmid with the P2' promoter, which is the only transcriptional signal for expression of the *citP* gene in *E. coli* (López de Felipe et al., 1996). In *L. lactis* and *E. coli*, the *citQRP* mRNA is subjected to specific cleavages at a complex secondary structure (López de Felipe et al., 1995; Drider et al., 1998), being the location of these endonucleolytic cleavages already established (Drider et al., 1998). We have proposed that expression of the *citQRP* operon is controlled at the posttranscriptional level by translational repression mediated by CitR. Moreover, we have postulated that *citQ* translation should permit synthesis of CitR by avoiding formation of the secondary structure and localized processing of *citQRP* mRNA (López de Felipe et al., 1995). In this report the involvement of the endoribonucleases RNase E and RNase III from *E. coli* in the processing of *cit* mRNA and ultimately in the regulation of the expression of this operon were further investigated.

Results

Processing of the Lactococcal *citQRP* mRNA in *E. coli*

We have previously shown that the *citQRP* operon is transcribed in *E. coli* to generate a mRNA whose 5'-end is positioned downstream of the P2' promoter and terminates within a ρ-independent terminator next to the 3'-end of *citP* (López de Felipe et al., 1996). A comparative analysis of the fate of *cit* mRNA in *L. diacetylactis* and *E. coli* RNase E, RNase III mutants and wild-type strains revealed that *cit* mRNA is processed in both hosts within a complex secondary structure (structure II in Figure 1) and indicated that RNase III had the greatest contribution for the decay of this transcript (Drider et al., 1998). To look more directly at the role of RNase III in *cit* mRNA decay, we took advantage of the existence of the *E. coli* SK5704 strain, which contains the *ams-1/rme1* (ts), *pnp7* and *mb500* (ts) mutated genes encoding deficiencies for the endoribonuclease RNase E and the 3'-5' exonucleases PNPase and RNase II, respectively. The aim was to obtain accumulation of specific processed RNA species (generated mainly by RNase III) by inactivation of the major *E. coli* RNases, other than RNase III, which are known to be implicated in mRNA decay. The proximity of the secondary structure to the 5'-end of the *cit* mRNA (111 nt) and the length of the transcript (2030 nt) did not allow discrimination between the
In order to overcome this problem, plasmid pDJD2 (Figure 1), in which the 5'-region of \textit{citR} was fused to the 3'-region of \textit{citP} and the transcriptional terminator, was constructed. Analysis of folding of the artificial \textit{cit} mRNA encoded by pDJD2 with the Fold program (Zuker and Stiegler, 1981) in the University of Wisconsin GCG software package (Devereux \textit{et al.}, 1984) predicted that the secondary structure II would be preserved in this transcript. Therefore, the fate of the \textit{cit} mRNA encoded by pDJD2 in the triple mutant strain (SK5704) (Figure 2B and 2D) and in the wild-type strain (MG1693) (Figure 2A and 2C) was investigated by Northern blot hybridization using a probe which encompasses the entire operon (see Figure 1). This analysis was performed with total RNAs extracted from MG1693[pDJD2] and SK5704[pDJD2], after stopping transcription and transferring the cultures to the non-permissive temperature of 44°C in order to inactivate...
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RNase E and RNase II in the SK5704 strain (see details in Experimental procedures). The expected full-length transcript of 1179 nt (band A) produced from pDJD2 and several processed species were detected at time 0 in RNA preparations from both strains (Figure 2). Two of them should correspond to RNA species of approximately 170 nt (band C) and 1000 nt (band B), which are the expected 5'- and 3'-RNA products of processing at structure II (see Figure 1 and Figure 3C) as previously confirmed by Northern analysis using differential probes (Drider et al., 1998). In addition, larger mRNAs were detected which should correspond to products of read-through transcription, since we have shown that the *cit* transcriptional terminator is not very efficient in *E. coli* (López de Felipe et al., 1996).

To further identify decay intermediates, total RNA was isolated from cultures of *E. coli* MG1693[pDJD2] and SK5704[pDJD2] and analyzed by primer extension (Figure 3). These RNAs were hybridized with primer A or primer B, which are complementary to the *cit* mRNA at locations proximal to either the 5'-end (Figure 3A) or the 3'-end of structure II (Figure 3B), respectively. The generated extended products were aligned with the dideoxy sequencing reaction generated with the same oligonucleotide used for primer extension. As expected from the results obtained by Northern blot, in the wild-type strain only RNA extracted at time 0 provided a substrate for the reverse transcriptase with both primers (Figure 3A, lane 1 and Figure 3B, lane 4). In turn, detection of extended products in the triple mutant was still possible even 60 min after transcription inhibition (Figure 3A, lane 6 and Figure 3B, lane 3). Extension of primer A revealed that in both strains, the mRNA encoded by pDJD2 starts at two A residues (Figure 3A, lane 1 and 4, nucleotides 1 and 3 in Figure 3C), as expected from utilization of the P2' promoter by the *E. coli* RNA polymerase (López de Felipe et al., 1996). In addition, four extra bands were also detected from RNA of both strains at time 0. These bands could correspond to artifacts due to early dissociation of the reverse transcriptase from the mRNA substrate at stop sites. It is known that the pattern and proportion of truncated DNA fragments generated by the enzyme will vary depending on the substrate and on the dissociation constant of the enzyme at each potential stop site. Thus, we should expect the same relative proportion of detected full-length and truncated extended fragments in all primer extensions performed with the same primer, and this was not the case in our experimental conditions. As a consequence, the short DNA species detected in Figure 2A should correspond to processed RNA species having their 5'-end at one A and three U residues (nucleotides 21, 26, 33 and 40 of the *cit* mRNA, see Figure 3C). All of these nucleotides are included in a small putative secondary structure (structure I in Figure 3C) predicted by the Fold program. This data suggested that the 5'-region of the *cit* mRNA was also subject to endonuclease cleavage at this location. Analysis of the decay of this mRNA in the wild-type and triple mutant with

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**Figure 3. Initiation of Transcription and Processing of *cit* mRNA in *E. coli* MG1693 (wild type) and SK5704 (*ams1/me1*(ts) *pnp7* *rne1*(ts) *rnb500*(ts)) Strains**

Exponential cultures of MG1693[pDJD2] and SK5704[pDJD2] (grown at 30 °C) were transferred to 44 °C and treated with rifampicin at time 0. Total RNA was prepared at the indicated times (in min), primer extensions were performed with primer A (A) or primer B (B). Panel C. Predicted structure of the 5'-region of *cit* mRNA. Location of major and common cleavage sites inferred from primer extension experiments are depicted. Symbols: closed and open arrowhead denote detection in both strains and in SK5704, respectively.
primer A (Figure 3A) showed stabilization of degradation products in the last strain. Furthermore, in the triple mutant only the RNA species having its 5'-end at nucleotide 40 had not decreased in levels 60 min after transcription inhibition. Utilization of primer B allowed detection of cit mRNA processing in both strains at time 0 (Figure 3B, lanes 1 and 4) at structure II, which includes part of the overlapping region of citQ and citR (see Figure 3C). Several extended products were detected from the SK5704[pDJD2] strain and, with the exception of the three longer ones, they should have been generated from RNA species having their 5'-end at the right arm of the structure II (Figure 3B, lane 1). Most of them remained stable upon inhibition of RNA synthesis (Figure 3B, lanes 2 and 3). Interestingly, the major band detected should be generated by cit mRNA cleavage at the ribosomal binding site of citR (between nucleotides 194 and 195), a cleavage which was previously observed in L. diacetylactis (Drider et al., 1998). By contrast, only two major (172 and 173) and one minor (203) extended products were generated from RNA extracted at time 0 from the wild-type strain (Figure 3B, lane 4). These bands, also previously detected in L. diacetylactis (Drider et al., 1998), were present in the E. coli triple mutant reaction (Figure 3B, lane 1) at different levels and decreased after shift to 44°C (Figure 3B, lanes 2 and 3), which should inactivate RNase E. These results indicated that the cit mRNA was subject to specific cleavages at structure II and this processing could be expected to interfere with translation of citQ and citR genes. Moreover, the processed species generated were presumably stabilized in the E. coli SK5704 strain due to the absence of RNase II and/or PNPase 3'-5' activities.

Role of RNase III and RNase E in Processing of cit mRNA

The aforementioned results suggested that in the absence of the endoribonuclease RNase E, the two major cleavages (172 and 173 in Figure 3C) detected in the E. coli wild-type strain did not take place, whereas processing at the ribosomal binding site of CitR (195 in Figure 3C) still occurred. To test these hypotheses processing of cit mRNA encoded by pDJD2 in E. coli SK5665 (ams1/rne1) upon inactivation of its thermosensitive RNase E was investigated. Cultures of E. coli MG1693[pDJD2] and SK5665[pDJD2] strains were grown until the middle of the exponential phase at 30°C. Then, cultures were further incubated during 30 min at 44°C and total RNA prepared. These RNAs and RNA extracted from an exponential phase culture of L. diacetylactis CRL264[pCIT264] were used for extension experiments utilizing primer B (Figure 4). Extended products corresponding to cleavages upstream and at the ribosomal binding site of citR were detected in all RNA preparations, confirming that these processing occurred in both L. diacetylactis (Figure 4, lane 3) as well as in E. coli (Figure 4, lanes 1 and 2). However, quantification of the 172 nt and 173 nt bands showed that they were 10.5±1 fold more abundant in the E. coli wild-type strain (Figure 4, lane 2) than in the RNase E mutant (Figure 4, lane 1). The differential behavior of the two strains could not be ascribed to a gene dosage effect, since determination of pDJD2 copy number showed that the copies per chromosome equivalent were 52±9 in the wild-type strain and 38±5 in the RNase E mutant. Moreover, the detection of similar levels for the processed species of 195 nt in both strains provided an internal control showing that this RNase E-independent processing was not affected by heat shock. These results suggested that cleavage at the ribosomal binding site of CitR could be catalyzed by RNase III. To test this idea, processing and fate of cit mRNA encoded by pDJD2 in E. coli SK5665 (ams1/rne1) and SK7622 (Δmrc-38::Km7) strains was investigated by primer extension utilizing primer B (Figure 5). The two major extended products generated with RNA from the wild-type strain (172 and 173 in Figure 3B, lane 4 and Figure 4, lane 2) were
the idea that RNase E could catalyze the cleavages upstream of citR. Therefore, the low levels of 172 nt and 173 nt processed species detected in the SK5665 strain, after inactivation of RNase E and in the absence of transcription inhibition, strongly suggested that RNase E cleaved cit mRNA upstream of citR.

**RNase III Activity and Expression of citP Gene**

We have previously proposed that CitR is a repressor, which controls expression of the citP gene (López de Felipe et al., 1995). The detection of a cleavage catalyzed by RNase III occurring at the ribosomal binding site of citR indicated that this processing could influence the translation of citP. To analyze whether this regulation takes place, plasmid pDJD100 (Figure 1) was constructed (see details in Experimental procedures) and transferred into the E. coli wild-type and RNase III deficient strains. This plasmid contains citQ, citR and a citP-cat gene fusion, in which the first AUG of cat was substituted by the start codon of citP and placed under the control of P2' promoter. Thus, levels of the citrate permease P in MG1693 and SK7622 could be measured by determination of the chloramphenicol acetyltransferase (CAT) activity encoded by pDJD100. Unfortunately, the thermosensitive nature of the RNase E mutant did not allow to test expression of citP in this host, since CAT synthesized at the permissive temperature would unmask the results obtained after heat shock. Comparison of the CAT activity detected in bacterial extracts from MG1693[pDJD100] (966±85 U/mg) and SK7622[pDJD100] (46±6 U/mg) strains revealed that the absence of RNase III resulted in a twenty-fold decrease of citrate permease P synthesis. The different levels of expression of the citP-cat fusion in the two hosts was not due to a gene dosage effect, since determination of the plasmid copy number, showed that 56±7 and 55±8 copies of pDJD100 plasmids were present per chromosome equivalent in the MG1693 and SK7622 strains respectively. The low level of CAT activity encoded by pDJD100 in the RNase III mutant thus correlated with lack of detection of processing of cit mRNA at the ribosomal binding site of citR (results not shown). The decrease of expression of citP in the mutant could be due to an increase in expression of citR, since this gene and not citP-cat is affected by the cleavage.

**Immunological Detection of RNase III-like Protein in L. diacetylactis CRL264[pCIT264]**

The lactococcal ribonucleases have not yet been identified. However, our results (Drider et al., 1998 and Figure 4, lane 3) suggested that an RNase III-like activity could be present in L. diacetylactis CRL264[pCIT264] strain. We performed Western blot hybridization using extracts of L. diacetylactis and E. coli wild-type and RNase III deficient strains and polyclonal antibodies raised against E. coli RNase III (Figure 6). Two bands were detected in the E. coli wild-type extracts. The lower band should correspond to RNase III, since it was detected in the purified RNase III preparation and it was not present in the mutant strain extracts. Its migration corresponds to a polypeptide of approximately 25 kDa, which is the molecular mass of the RNase III monomer. The E. coli RNase III acts as a dimer (Nicholson, 1995), composed of two identical subunits of 25.5 kDa (Nashimoto and Uchida, 1985) Thus, the upper band detected in the purified RNase III preparation could
correspond to dimeric forms of RNase III that were not denatured or renatured prior or during electrophoresis. In the extracts of _L. diacetylactis_ only one polypeptide of approximately 55 kDa cross-reacted with the antibodies (labeled as RNase III-like in Figure 6), and even after longer exposures no other polypeptides were detected (data not shown). Thus, our results show that _L. diacetylactis_ possesses a protein that cross-reacts with antibodies against _E. coli_ RNase III. This is the first indication that a RNase III-like enzyme could exist in _L. diacetylactis._

**Discussion**

Regulation of gene expression can be exerted at the posttranscriptional level. Besides the nature of the ribosomal binding site, translation coupled with mRNA structure and decay can also play a major role in this process (Klaft _et al._, 1996; Petersen, 1993). The structure of the _L. diacetylactis_ citQRP operon seems to be designed to provide a substrate for specific endonucleolytic cleavages, which result in posttranscriptional regulation of _citR_ gene expression. Since the _citR_ gene product is a repressor controlling citrate permease P levels in the cell, we propose that specific cleavages in the _citQRP_ mRNA are ultimately modulating citrate transport into the cell. In this report we have analyzed the synthesis and fate of the _cit_ mRNA in _E. coli_. Computer analysis of the 5′-region of the _cit_ mRNA predicts that the 5′-end of _citQ_ is located within two secondary structures (I and II in Figure 3C). Structure I is located at the 5′-end of the transcript synthesized in _E. coli_, and this location of secondary structures has been shown to stabilize several different downstream mRNA sequences (reviewed by Bechhofer, 1993). Interestingly, structure I was absent from the ancestral _citQRP_ operon (López de Felipe _et al._, 1996). Thus, the introduction of the insertion sequence IS982 between the lactococcal promoter P1 and the _citQ_ gene during evolution of _L. lactis_ provided not only a promoter that supports expression of the operon in _E. coli_, but also a 5′-stabilizer for the _cit_ mRNA in this organism. Structure II is subjected to the same specific endonucleolytic cleavages in _L. lactis_ (Drider _et al._, 1998 and this report) and _E. coli_ (Drider _et al._, 1998 and this report). This stem-loop structure includes the central region of the _citQ_ and the 5′-end of _citR_. Identification of the cleavage sites (Figure 3) revealed that a coordinated translation of both genes should be disrupted by the processing event. The translation of the _citQ_ gene should compete with folding and processing of _cit_ mRNA. Thus, the interplay of mRNA processing and the ribosomes translating _citQ_ could regulate the levels of synthesis of the CitR repressor. Analysis of the fate of _citQ_ mRNA in _E. coli_ MG1693 wild-type strain revealed that its half-life is less than 1 min (Figure 2). As a consequence, in _E. coli_ most of the _cit_ mRNA molecules seem to be processed at structure II instead of providing a substrate for translation of _citQ_ by the ribosomes.

We have previously shown by Northern blot hybridization that stabilization of the transcript without accumulation of processed species occurs in the RNase E single mutant, whereas processing plus stabilization of RNA species takes place in the absence of RNase III (Drider _et al._, 1998; Santos _et al._, 1997). In this report we present evidence supporting that in _E. coli_ the _cit_ mRNA is processed at the complex secondary structure II by RNase E and RNase III of _E. coli_. Our results indicate that RNase III catalyzes one endonucleolytic cleavage at the ribosomal binding site of _citR_ (195 in Figure 3C), and that this processing is located within an internal loop of structure II. This observation correlates with the fact that RNase III substrates with internal loops undergo single cleavage, whereas double stranded RNA substrates are subjected to coordinate double cleavages (Li and Nicholson, 1996). It is well documented that RNase III is involved in positive or negative control of gene expression: 1) directly when its processing activity itself alters the half-life of mRNAs and/or the accessibility of ribosomal binding sites; and 2) indirectly when its cleavage activity affects half-life of antisense RNAs (reviewed by Court, 1993). Furthermore, the cleavage sites of RNase III are located either in 5′ noncoding regions or in intercistronic regions. In the case of _citQRNA_, an RNase III target site is located in the overlapping region of _citQ_ and _citR_. Therefore, RNase III cleavage should impair expression of _citQ_ by disruption of the gene and it should knock out expression of _citR_ by elimination of its ribosomal binding site. In addition, RNase III action (presumably by avoiding synthesis of the CitR repressor) seems to play an important role in the expression of _citP_ gene, since we have detected that the absence of RNase III results in a twenty-fold increase of CAT activity encoded by a _citP-cat_ translational fusion. This, to our knowledge, is the first instance of a putative involvement of RNase III...
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In regulation of gene expression at the level of translation by disruption of translational signals and coding sequences.

In turn, RNase E seems to cleave *cit* mRNA (Figs. 4 and 5) within *citQ* and upstream of *citR* (172 and 173 in Figure 3C). Moreover, the location of the primer used for primer extension experiments (downstream of RNase III cleavage site) rules out the possibility that RNase E substrate had previously been cleaved by RNase III. The two detected cleavage sites are adjacent and located in a A+U rich region, which has been shown to be the preferred substrate for RNase E (Lin-Chao *et al.*, 1994; McDowall *et al.*, 1994). According to what has been reported so far, it seems that single strandness is a requirement for RNase E cleavage to occur (Cormack and Mackie, 1992). In agreement with these observations are the potential targets mapped in *cit* mRNA which are located at an internal loop present in structure II. It has also been proposed that flanking stem loops are not required for RNase E activity, but rather they can modulate RNase E cleavage of complex RNA substrates by providing access of the enzyme to specific susceptible sites (Alifano *et al.*, 1994; Cohen and McDowall, 1997). Thus, it is feasible that the upper stem of the secondary structure (containing 5 GC pairs, Figure 3C) located 5' from the RNase E cleavage sites in *cit* mRNA may serve to stabilize local structures, ensuring that only the specific target sites of RNase E remain single-stranded and available to the enzyme.

In this study, we have also observed that the 3'-RNA species generated by RNase E and RNase III are stabilized in the absence of both RNase II and PNPase (Figure 3). On these bases, the proposed model for processing and decay of *cit* mRNA in *E. coli* is that depicted in Figure 7. The transcript could be subject to endonucleolytic attack by either RNase E or RNase III generating 3'-processed species, which could be a substrate for synthesis of CitP. However, the end-result of the activity of the two enzymes in *citR* translation would be antagonistic. The internal cleavages of *cit* mRNA performed by RNase E would generate 3'-RNA products which could efficiently serve as substrates for translation of *citR* by the ribosomes. In contrast, RNase III should generate 3'-RNA species lacking the *citR* ribosomal binding site, thus being unsuitable for CitR translation. As a result, and in addition to *citQ* translation, the action of RNase E and RNase III could alter the levels of CitR in the cell, and as a consequence they would turn either on and off regulation of *citP* expression, depending upon which of the endonucleases cleaves the mRNA first. Our experimental data suggests that the detected mRNA species generated by RNase E cleavage have not yet been previously cleaved by RNase III, and its very unlikely that after cleaving RNase E originates a proper RNase III substrate. However, we have not ruled out the hypothesis that the two RNases could work in consonance, both on the same piece of mRNA. If this is the case, the RNA products containing *citP* will be exactly the same upon cleavages by RNase III or by RNase III and RNase E concertededly (lacking a functional *citR* gene) and different of those generated by RNase E alone. The model presented...
here is mainly based on our results obtained in the E. coli system, since the RNases from L. lactis have not been yet identified and RNase mutants have not been constructed. However, several instances indicate that indeed this model could also apply for L. lactis. The endonucleolytic cleaves at structure II, presumably catalyzed by RNase III and RNase E in E. coli, also take place at the exact same positions in L. lactis (Figure 5). RNase III seems to be highly homologous among bacteria (Mian, 1997) and a protein of L. Lactococci, since RNase E-like activity has been detected (Condon et al., 1997). However, no counterpart of the RNase III activity has been reported (Condon et al., 1997). However, no counterpart of the E. coli enzyme has been identified by homology although the entire genome of this microorganism has already been sequenced. This lack of homology could also apply for lactococci, since RNase E-like activity has been detected (this work and Parreira et al., 1996), and cross-reaction of lactococcal polypeptides with antibodies raised against E. coli RNase E was not observed (results not shown). These observations suggest that analogues but not homologues of E. coli RNase E could be included in the RNA degradosome of gram-positive bacteria. It would thus be feasible to propose in favor of a convergent evolution of filogenetically unrelated proteins taking place during evolution which would provide the various microorganisms with the RNA turnover mechanisms required for cell viability.

**Experimental Procedures**

**Bacterial Strains, Growth Media and Plasmids**

*Lactococcus lactis* biavar diacetylactis CRL264[pCit264] (Sesma et al., 1990) was grown in M17 medium (Gason, 1983) at 30°C. The E. coli bacteri- strains used in this work are listed in Table 1. JM109 strain was used for generation and maintenance of recombinant plasmids. Bacterial strains were grown at 30°C in Luria-Bertani (LB) medium (Sambrook et al., 1989) supplemented with thymine at 50 µg/ml, and with kanamycin at 50 µg/ml in the case of SK7622. Bacterial strains were transformed as described by Sambrook et al. (1989) and transformants were selected in agar medium containing ampicillin at 50 µg/ml. The plasmids used in this work were pCIT264 (Magni et al. 1994), pDJD1, pDJD2, pDJD10 and pDJD100 (this study).

**Construction of Derivatives of pCIT264**

To analyze the role of E. coli harboring pDJD2 were grown at 30°C to an absorbance of 0.4 to 600 nm. Cultures of *L. diacetylactis* CRL264[pCIT264] were grown at 30°C to an absorbance of 0.4 to 600 nm. Initiation of transcription was stopped by the concomitant addition of rifampicin (500 µM) and nalidixic acid (20 µg/ml) 20 seconds prior to shift to the non permissive temperature of 44°C (time 0). Samples were withdrawn at the indicated times (see Results). Total cellular RNAs were extracted by the procedure of Williams and Rogers (1987) and treated with RNase-free DNAse I (Boehringer-Mannheim) prior to use in Northern blot hybridization experiments. Total cellular RNAs were extracted. Total cellular RNAs were extracted as previously described (López et al., 1989) prior to use for primer extension experiments. The RNAs were checked for the integrity and yield of the rRNAs by analysis in agarose gels stained with ethidium bromide. The patterns of the RNAs were similar in the various preparations. In all experiments total RNA concentrations were determined by ultraviolet spectrophotometry.

**Northern Blot Hybridization**

For the Northern analysis, total RNA and molecular markers were denatured in deionized formamide containing xylene cyanol at 0.3%, bromo- phenol blue at 0.3% and fractionated on 5% denaturing polyacrylamide gel containing 7M urea. Staining of the gel with ethidium bromide allowed us to check the integrity of rRNAs after fractionation, and their proper transfer to the membrane after blotting. Nucleic acids were transferred to a nylon membrane by electroblotting using TAE 1X (0.4 M Tris-acetate, 1 mM EDTA) buffer at 20 V, 0°C during 2 h. The RNAs were fixed to the membrane by exposing the filters to UV light. The DNA probe used, which spans all the transcriptional units, was generated by digestion of pDJD2 with EcoRV and XbaI. The probe was purified by Gene-Clean (Bio 101, INC) and 32P-labelled using the rediprime DNA labeling system from Amersham. Hybridization’s were performed at 42°C in 50% formamide as described by Thomas (1983). For autoradiography blotted filters were exposed to Kodak X-Omat S films. The radioactivity present in the filter bands was directly quantified by means of the storage phosphor technology, with the aid of PhosphorImager ImageQuant equipment and software (Molecular Dynamics). The values obtained were corrected for variations due to sample loading into the gel. Quantification of the rRNAs bands was performed by scanning of the gels with the Molecular Analyser System (Bio-Rad Laboratories).

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**Table 1. E. coli Strains used in this Work**

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<th>Bacterial strains</th>
<th>Characteristics/genotype</th>
<th>Reference or source</th>
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<td>JM109</td>
<td>recA1 endA1 gyr96 thi hadR17 supE44 relA1 Δlac-proAB [F traD65 proA lacZDMD18]</td>
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<td>Arraiano et al. (1988)</td>
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Primer Extension Analysis and DNA Sequencing

Primer extension analysis was performed as previously described (De Felipe et al., 1995). Primer A (5'-CCCCAGTTGCTAAGTTAT-3') or primer B (5'-AGGGTTTTTGTATCTTGGT-3') (complementary to the cit mRNA from nucleotides 1135 to 1151 in GenBank sequence S77101) were used to determine either the 5'-end or the processing site of the cit mRNA. One pmol of either primer was annealed to 15 μg of total RNA obtained from E. coli or L. lactis cultures. Primer extension reactions were performed by incubation of the annealing mixtures with 20 U of AMV reverse transcriptase at 42°C for 30 min, and the reaction products were subjected to electrophoresis in 8% polyacrylamide gels containing 7 M urea. Bands labeled with 32P were detected by autoradiography on Kodak X-Omat S films, and were directly quantified with a PhosphorImager system (Molecular Dynamics). RNA samples from two different extracts of the same strain were run at least two times. DNA sequencing reactions used as standard were generated by the diodeoxy terminator method of Sanger et al. (1977) from pJD2 plasmid annealed to the same oligonucleotide as was used for primer extensions.

Determination of Plasmid Copy Number

E. coli strains harboring either pJD2 or pJD100 were grown as indicated in Results. Total DNA extracts containing chromosomal and plasmid DNA were prepared from 1.5 ml cultures essentially as previously described (Lacks et al., 1986). Total DNA was extracted by electrophoresis in 0.8% agarose gels. DNA bands were revealed by staining with ethidium bromide at 0.5 μg/ml. Quantification of the bands was performed by scanning of the gels with the Molecular Analyst System. Extreme precautions to ensure linearity of the determinations were taken. DNA samples from three different extracts of the same strain were run at least three times, or several dilutions of the DNA samples were electrophoresed. The plasmid copy number (N) was calculated according to Projan et al. (1983) from the equation N = Dp1 + 1.36 Dp2 × McDx Mp were Dp1 and Dp2 are the value obtained from densitometric quantification of open circular and covalently closed circle forms of the plasmid, Dc is the value obtained from densitometric quantification of the chromosomal DNA, Mc is the genome size for E. coli (4.64 × 109 bp according to Blattner et al., 1997) and Mcp is the plasmid size expressed in bp.

Determination of Chloramphenicol Acetyltransferase Activity

E. coli MG1683(pJD100) and SK7622(pJD100) strains were grown to an absorbance of 0.4 at 600 nm. Then, total extracts were prepared and CAT activity encoded by pJD100 determined as previously described (Ballester et al., 1990). One Unit of enzymatic activity is defined as the amount of enzyme catalyzing the acetylation of 1 nmol of chloramphenicol/min at 37°C.

Detection of RNase III in Polyacrylamide Gels by Western Blot Hybridization

To prepare bacterial extracts, L. diacetylactis CRL264[pCIT264] was grown at 30°C to an absorbance of 0.2 at 660 nm. Cultures were sedimented by centrifugation washed by suspension in buffer B (50 mM Tris-HCl pH 7.8, 1 mM dithiotreitol), centrifuged again and concentrated ten-fold by suspension in buffer B. Bacterial extracts were prepared by passing cell through a French pressure cell at 12,000 lbf/in2 and removing the cell debris by centrifugation at 20,000 X g for 15 min. Bacterial extracts of E. coli MG1683 and SK7622 strains, grown at 37°C to an absorbance of 0.5 at 620 nm, were prepared as previously described (Zillio et al., 1996).

For Western blot analysis, bacterial extracts containing 30 μg of total protein were fractionated by 10% SDS-PAGE and transferred to nitrocellulose (Schleicher and Schuell) by electroblotting. To check that the transfer has occurred and to detect molecular weight standards, membranes were stained with a Ponceau-S solution (Sigma). Western blot and hybridization was performed as previously described (Zillio et al., 1996). Polyclonal antibodies against E. coli RNase III kindly provided by Prof. Robert W. Simons and by Prof. Gabriele Klug were respectively diluted 1:10000 and 1:2000 and used as primary antibodies. Anti-rabbit IgG conjugated with alkaline phosphatase from Sigma was diluted 1:1000 and used as secondary antibody. Alkaline phosphatase was used for detection with enhanced chemiluminescence (ECL detection kit, Amersham). The blots were exposed to X-ray films.

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