A comparative analysis of the citrate permease P mRNA stability in *Lactococcus lactis* biovar *diacetylactis* and *Escherichia coli*

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Received 18 December 1998; received in revised form 13 January 1999; accepted 14 January 1999

Abstract

The role of ribonucleases in the control of gene expression remains unknown in lactic acid bacteria. In the present work, we analysed the expression of the *citP* gene, which encodes the lactococcal citrate permease P, through the stability of the *citQRP* messenger in both *Lactococcus lactis* biovar *diacetylactis* (*L. diacetylactis*) and *Escherichia coli*. The chemical half-life for *citQRP* mRNA observed in *L. diacetylactis* wild-type strain was abnormally long for bacteria. It was even longer than that detected in *E. coli* RNase E or RNase III mutant strains. A model of processing and fate of RNA species containing *citP* gene is presented. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: *Lactococcus lactis* biovar *diacetylactis*; *Escherichia coli*; Citrate permease P; Gene expression; Ribonuclease; mRNA degradation

1. Introduction

Citrate, present in milk, is co-metabolised together with sugars by several strains of lactic acid bacteria, including *Lactococcus lactis* biovar *diacetylactis* (*L. diacetylactis*) [1]. The metabolism of citrate leads to the synthesis of several products, such as diacetyl, which is responsible for the flavour of some dairy products, like butter, buttermilk and cottage cheese. Thus, in the dairy industry, the control of diacetyl production is an important parameter, which could be achieved by understanding the mechanisms involved in the citrate utilisation by lactic acid bacte-
plex secondary structure comprising the central region of \textit{citQ} and the 5'-end of \textit{citR} [4,8].

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

The bacteria used in this work were: \textit{Lactococcus lactis} biovar \textit{diacetylactis} CRL264[pCIT264] [5] and the \textit{Escherichia coli} strains: MG1693 (\textit{thyA} 715) designated wild-type [9], the isogenic SK5665 (\textit{thyA} 715 \textit{ams-1\textit{rme1}}(ts)) RNase E thermosensitive mutant [10] and SK7622 (\textit{thyA} 715 \textit{\Delta}rnc\textit{38} \textit{Km}) RNase III deletion mutant [11]. \textit{L. diacetylactis} was grown at 30°C without shaking in M17 medium supplemented with glucose at 0.5%. The \textit{E. coli} strains were grown at 30°C with shaking, in Luria–Bertani medium supplemented with thymine at 50 \textmu g ml\(^{-1}\) and with kanamycin at 50 \textmu g ml\(^{-1}\) in the case of SK7622 strain. The plasmids used in this work were: pCIT264, the natural lactococcal \textit{Cit} plasmid and pFS21, a hybrid plasmid composed of pUC18 and pCIT264 [6].

2.2. RNA isolation

\textit{L. diacetylactis} was grown in M17 medium to an absorbance of 0.4 at 660 nm. Then, rifampicin was added at 500 \textmu g ml\(^{-1}\) to stop synthesis of new RNAs, and total RNA was extracted from samples withdrawn at the times indicated in Section 3 as described previously [4]. \textit{E. coli} strains harbouring plasmid pFS21 were grown at 30°C to an absorbance of 0.4 at 600 nm. Initiation of transcription was stopped by the concomitant addition of rifampicin at 500 \textmu g ml\(^{-1}\) and nalidixic acid at 20 \textmu g ml\(^{-1}\), 20 s prior to shift to the non-permissive temperature of 44°C to inactivate the RNase E thermosensitive enzyme in the SK7622 strain. The RNAs used in this work were: pCIT264, the natural lactococcal \textit{Cit} plasmid and pFS21, a hybrid plasmid composed of pUC18 and pCIT264 [6].

2.3. Northern blot hybridisation

For the Northern analysis, total RNA and molecular RNA markers (0.28–6.5 kb) (Promega) were denatured and fractionated on 5% denaturing polyacrylamide gels containing 7 M urea as previously described [8]. Staining of the gels with ethidium bromide allowed us to check for 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 and hybridised at 42°C to a \textit{32P}-labelled probe (which encompasses the \textit{citP} gene) as previously described [8]. For autoradiography, filters were exposed to Kodak X-Omat S films. The radioactivity present in the filter bands was directly quantified with a PhosphorImager ImageQuant equipment and software from Molecular Dynamics.

2.4. Primer extension analysis and DNA sequencing

Primer extension analysis was performed as previously described [4]. The primer used was the 19-mer oligonucleotide 5'-AGGGTTTTGTTTTTGGTTT-3', which is complementary to \textit{cit} mRNA from nucleotides 1233–1251 in GenBank (sequence S77101). One pmol of the primer was annealed to 15 \mu g of total RNA obtained from \textit{E. coli} 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. DNA sequencing was performed by the dideoxy chain terminator method [12] using T7 polymerase sequencing kit from Pharmacia and [\alpha-\textit{32P}]dCTP. Plasmid pFS21 was used as template.

3. Results and discussion

3.1. Fate of \textit{citQRP} mRNA in \textit{E. coli}

We have previously shown, by Northern blot analysis, that in \textit{L. diacetylactis} [4] and in \textit{E. coli} RNase
III deficient strain [8] citQRP mRNA is processed at a complex secondary structure (structure I in Fig. 1), which contains the central region of citQ and the 5'-end of citR. Additionally, it was also established that this processing occurs at the same location in L. diacetylactis and E. coli wild-type strain [8]. However, it should be noted that the plasmid pDJD2, previously used for the analysis of citQRP mRNA decay in E. coli [8], although containing structure I, harbours a deletion of 0.8 kb within the citQRP operon. In the present work, plasmid pFS21 was used to characterise the fate of citP mRNA. pFS21 is a hybrid plasmid composed of pUC18 and the Cit+ lactococcal plasmid pCIT264, which contains the intact citQRP operon (Fig. 1). The processing at structure I of cit mRNA encoded by pFS21 was analysed in E. coli RNase III deletion mutant, RNase E thermosensitive mutant and wild-type strains by primer extension. This analysis revealed two major processing sites at nucleotides 172 and 173 in all three strains tested (Fig. 2). The localisation of these cleavages at nucleotides 172 and 173 is in good agreement with that previously obtained from plasmids pDJD2 in E. coli and pCIT264 in L. lactis [5]. A citQRP transcript of 2.9 kb is encoded from promoter P1 ([4] and Fig. 1), whereas messengers of 1.2 and 2.0 kb are encoded by pDJD2 and pFS21 from P2 promoter ([7] and Fig. 1). Thus, it could be argued that the processing of citQRP mRNA at structure I takes place irrespective of the length of the regions located upstream or downstream of this structure. Therefore, it seems that the structure is conserved and the sole determining factor for cleavage. Moreover, in E. coli, the detection of the extended products in the RNase III null strain (Fig. 2, lane 3) ruled out involvement of this enzyme in this processing, whereas the low levels observed in the RNase E thermosensitive mutant at the permissive temperature (Fig. 2, lane 2), which decreased after transfer to 44°C (data not shown) suggested a role of this endonuclease in this processing. On these bases, the fate of citQRP en-
coded by pFS21 was analysed in E. coli strains by Northern blot hybridisation (Fig. 3) utilising a probe which encompasses the central and 3’-region of citP gene (see Fig. 1). The analysis of cit mRNA decay performed in E. coli wild-type strain indicated that the half-life of this messenger is less than 2 min without accumulation of processed species (data not shown). The pattern of degradation of citP mRNA was clearly different in RNase III (Fig. 3, lanes 1–6) and RNase E (Fig. 4, lanes 7–12) mutants. A major band (designated A) was detected in the RNase E mutant. This band could correspond to the 2.0 kb transcript encoded by pFS21, and decreased 80% after 2 min of transcription inhibition (Fig. 3, lane 7 versus lane 8). Moreover, no accumulation of processed species was observed in this strain. These results indicate that in the absence of RNase E, the cit transcript is mainly subjected to exonucleolytic degradation. In the RNase III mutant several bands were detected. Two of them (B and C) could contain the 1.3 kb citP gene, and they showed a differential fate. Band B, of 1.9 kb, that could correspond to processing at structure I was not detectable 4 min after addition of rifampicin (Fig. 3, lane 3), whereas band C first accumulates to low levels (Fig. 3, lane 2 versus lane 1) and then slowly decreased during the sampling period (Fig. 3, lanes 3–6). The other major bands detected (D–J), which ranged in size from 1.0 to 0.18 kb remain stable even after 30 min of transcription inhibition. These species could arise from endonucleolytic cleavages within the proper citP mRNA, and they were stabilised by the absence of endoribonuclease III (Fig. 3, lanes 1–6), but not by inactivation of RNase E (Fig. 3, lanes 7–12). This observation strongly implicates RNase III in the decay of small degradation products.

3.2. Analysis of citP mRNA decay in L. diacetylactis

The aforementioned results raised the question whether the processed species detected in the E. coli RNase III mutant were also generated in L. diacetylactis. We have previously analysed the fate of citQRP mRNA encoded by the natural lactococcal plasmid pCIT264 in L. diacetylactis by Northern blot hybridisation [4]. However, this analysis in agarose gels only permitted identification of processing at structure I. Here, to correlate the result obtained in

![Fig. 2. Localisation of the endonucleolytic cleavages within the secondary structure I of citQRP mRNA in E. coli. Cultures of MG1693 wild-type (wt), SK5665 RNase E mutant (rne1) and SK7622 RNase III mutant (Δrne) strains harbouring pFS21 were grown at 30°C to the middle of the exponential phase, total RNA extracted and primer extensions carried out as described in Section 2. DNA sequence ladders (labelled A, C, G, T) used as size standards were generated with the same oligonucleotide used for primer extension.](image-url)
*E. coli*, the decay of this transcript was investigated by Northern blot analysis of RNAs extracted after stopping transcription. The results obtained from this analysis (Fig. 4, lanes 1–6) showed the detection of the expected lactococcal full-transcript of 2.9 kb (designated mRNA), and various processed RNA species which were numbered from I to VIII (Fig. 3). Quantification of the radioactivity present at the position of the transcript revealed that its half-life is more than 20 min. This data is unusual given the short half-lives of mRNAs in prokaryotes, averaging about 3 min [13]. Bands I–VIII were detected at high levels after 30 min of rifampicin treatment (Fig. 4, lane 6). The deduced size of these bands indicated that some of these processed species correspond to that detected in the *E. coli* RNase III mutant (Fig. 3., lanes 1–6). Band I should correspond to band B and represent processing at structure I. Bands III–V should be the counterparts of bands C–E, and bands VII and VIII should correspond to bands F and G.

![Fig. 3. Analysis of citQP mRNA decay in *E. coli* mutant strains.](image)

Cultures of SK7622 RNase III mutant (*Δrnc*) and SK5665 RNase E mutant (*rne1*) strains harbouring pFS21 were grown at 30°C to the middle of the exponential phase, treated with rifampicin and nalidixic acid (time 0) and transferred to 44°C. At the times indicated, samples were withdrawn and total RNA was extracted and fractionated on 5% polyacrylamide gel as described in Section 2. Detection of RNA species containing *citP* was performed by Northern blot hybridisation with the probe depicted in Fig. 1 as described in Section 2. The major species detected are labelled A–I on the left. The sizes of the bands were deduced from their relative migration rates in relation to RNA size markers of 0.28–6.5 kb (MW) from Promega run in parallel and stained with ethidium bromide.
These results indicate a similar fate of citQRP mRNA in both hosts regarding to the nature of the RNA species generated although the decay was clearly different in the two hosts. Some of the detected bands could be generated from endonucleolytic cleavages at stem-loop structures. Therefore, in order to assess a location to the processed species detected we have analysed the secondary structure of the cit mRNA with the aid of the Fold Program [14,15]. Unfortunately, the long length of the transcript (2.9 kb) encoded by pCIT264 did not allow the prediction of its total folding. Thus, the folding of the entire citQRP mRNA encoded by pFS21 was predicted (Fig. 5). In addition to the structure I, two other structures (II and III) were observed that could be substrates for processing. Structures II and III are respectively located within the citR and the citP genes (see Fig. 1). Cleavage at one of these locations could generate either band II or band III detected in L. diacetylmaltis. Moreover, processing at structure III seems to occur in E. coli (band C in Fig. 3), but not very efficiently since low levels of band C were detected. Interestingly, no significant secondary structures were observed at locations expected for generation of the other RNA species observed. However, when the product of cleavage at structure I was folded a dramatic change of the total structure was observed (Fig. 5). In addition to structures II and III, structures IV–VIII were detected (see location in Fig. 1). These structures could well be the substrate for generation of bands IV–VIII in L. diacetylactis (Fig. 4). Moreover, the expected products of processing at structures IV, V, VII and VIII were also detected in E. coli (bands D–G in Fig. 3). On these basis and the results obtained by primer extension analysis (Fig. 2 and data not shown), we propose that at least in E. coli the transcript folds and is mainly cleaved at structure I. This processing is not performed by RNase III and it is tempting to assume that RNase E catalyses this cleavage. Then, upon folding of the 3′-processed species generated, endonucleolytic cleavages at structures IV, V, VII and VIII take place, and the resulting 3′-processed species are stabilised in absence of RNase III.

3.3. Concluding remarks

The work presented in this report constitutes a good tool for studying expression of industrial enzymes like citrate permease P. The study carried out in E. coli reveals that endoribonucleases, at least in this host, play an important role in the control of citP mRNA stability and therefore could modulate synthesis and expression of this enzyme. In L. diacetylactis endonucleolytic cleavages seem to control expression of citP. However, it remains unknown why the citP mRNA is so stable in lactococci. Moreover, our results even arise the question of whether an RNase III-type enzyme exists in this micro-organism. More experiments have to be performed in order to gain more insight on the role of ribonucleases in L. diacetylactis.
Acknowledgments

We thank Prof. Dr. Manuel Espinosa for the critical reading of the manuscript and to Ma. Angeles Corrales for technical assistance. Part of this work was supported by a Spanish and Portuguese joint program (Acciones Integradas) to P.L. and C.M.A. Research at the Centro de Investigaciones Biológicas was under the auspices of the CSIC and was supported by the Comisión Interministerial de Ciencia y Tecnología grants BIO95-0795 and BIO97-0347, and Comunidad Autónoma de Madrid (CAM) Grant

Fig. 5. Model of folding and fate of citQRP mRNA encoded by pFS21. Secondary structure of cit transcript encoded by pFS21 and of 3'-RNA species generated by processing at structure I are depicted. Putative cleavage targets for endoribonucleases (structures) are labelled I–VIII. See details in the text.
06G/002/96. The work at Instituto de Tecnologia Química e Biológica was partially supported by sectoral grants from PRAXIS XXI. D.D. was supported by a postdoctoral fellowship from C.A.M. N.G. and J.M.D. were recipients of predoctoral fellowships from Spanish Ministry of Science and Education and PRAXIS XXI, respectively.

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