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Regulation of expression of the *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* citrate transport system

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Abstract — Citrate metabolism performed by a number of lactic acid bacteria yields volatile compounds, such as diacetyl and acetaldehyde, which are important for flavour development in fermented milk products. Citrate uptake in *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* and *Leuconostoc mesenteroides* subsp. *mesenteroides* is catalyzed by a secondary carrier, the citrate permease P (CitP). The presence of CitP is essential for citrate utilization, since in its absence no citrate metabolism is observed although all enzymes involved in conversion of citrate are present inside the cells. In this review the genetic organization of the plasmid encoding the citrate transport system of lactococci is described, the posttranscriptional regulation of the *citQRP* operon is presented and the influence of external pH on *citP* transcription, citrate uptake and cometabolism of citrate and glucose is discussed. These last studies reveal a novel molecular mechanism that improves the adaptation of *L. diacetylactis* to acidic pH. © Inra/Elsevier, Paris.

***Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* / citrate transport / regulation of gene expression / lactic acid bacteria / acidic stress**

Résumé — Régulation de l'expression du système de transport du citrate chez *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*. Le métabolisme du citrate chez certaines bactéries lactiques se traduit par la synthèse de composés d'arôme tels que le diacétyle et l'acétaldéhyde. Ces composés contribuent à l'élaboration de la saveur de certains aliments issus de la fermentation lactique. Le transport du citrate chez *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* et *Leuconostoc mesenteroides* subsp. *mesenteroides* est catalysé par un transporteur secondaire, la citrate perméase P. Cette perméase est essentielle pour l'utilisation du citrate. En son absence le citrate n'est pas métabolisé, bien que toutes les enzymes impliquées dans sa conversion soient présentes au niveau de la cellule. Dans cette revue, nous décrivons l'organisation génétique du plasmide codant pour le transport du citrate chez *L. diacetylactis*, la régulation post-transcriptionnelle de l'opéron *citQRP* et l'influence du pH extracellulaire sur la transcription du gène *citP*. Par ailleurs, l'étude du transport du citrate et du cométabolisme citrate-glucose a révélé un nouveau mécanisme moléculaire permettant une meilleure adaptation de *L. diacetylactis* à des pH acides. © Inra/Elsevier, Paris.

***Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* / transport du citrate/ génétique/ bactérie lactique/ stress acide**

* Correspondence and reprints

1. INTRODUCTION

Citrate is present in milk and is co-metabolized together with sugars by many strains of lactic acid bacteria (LAB) including *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* [2]. The breakdown of citrate results in the production of carbon dioxide responsible for the texture of some cheeses and, for the flavour compound diacetyl, essential in dairy products such as butter, buttermilk and cottage cheese. Therefore, there is a strong need to control the production of diacetyl in the dairy industry. This can only be achieved by a complete understanding of the mechanisms involved in citrate utilization by LAB. This process is determined by the presence of intracellular enzymes responsible for its metabolism (reviewed in [5]) and, of a membrane bound protein that catalyses the uptake of this compound by the cells. Thus, the transport of citrate limits the rate of citrate metabolization and may modify the bioconversion yield of citrate into aroma compounds. This uptake

is catalyzed by the citrate permease P (CitP) encoded by the plasmidic *citP* gene in *L. diacetylactis* [3] and *Leuconostoc* [13]. Here, we will review the actual knowledge concerning the citrate transport in LAB with special emphasis on the molecular basis of this process. Moreover, we will report our recent results concerning to induced expression of *L. diacetylactis citP* by acidification of the growth medium. This stress response results in an increase of citrate transport, which leads to a more efficient glucose utilization with a consequent growth advantage for lactococci at acidic pHs.

2. GENETIC ORGANIZATION OF THE PLASMID ENCODING THE CITRATE TRANSPORT SYSTEM OF *L. DIACETYLLACTIS*

The lactococcal *citP* gene is located in an 8 kb plasmid called pCIT264 [10] (figure 1), whereas its counterpart from *Leuconostoc* [15] is harboured by at least

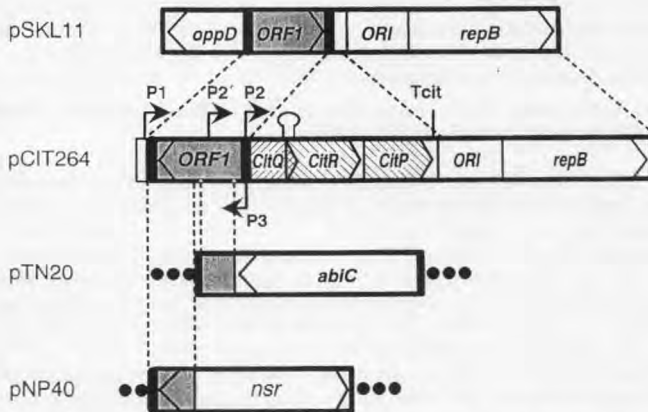


Figure 1. Comparison of lactococcal plasmids carrying regions of IS982. See text for details. P1, P2, P2', *citQRP* transcriptional promoters; *Tcit*, transcriptional terminator for *citQRP*; P3, promoter for ORF1; regions of IS982, shaded boxes; 17-mer inverted repeats flanking IS982, black boxes.

Figure 1. Comparaison des plasmides de lactocoques portant des régions de IS982. Voir les détails dans le texte. P1, P2, P2', promoteurs de transcription de *citQRP*; *Tcit*, terminateur de transcription pour *citQRP*; P3, promoteur pour l'ORF1; régions d'IS982, boîtes ombrées; régions inversées répétées (17-mer) encadrant IS982, boîtes noires.

two large plasmids [7, 13]. The two *citP* genes have 99% identity. However, the genetic structure and regulation of these two citrate transport systems seem to be different. The lactococcal *citP* is preceded by two partially overlapping genes (*citQ* and *citR*), and together these three genes constitute the *citQRP* operon [8]. In *Leuconostoc*, the existence of a *cit* operon and its transcription have not been investigated, but determination of the DNA sequence located upstream and adjacent to *citP* revealed that the integrity of *citR* is disrupted by several mutations [15]. Moreover, in this system the levels of CitP seem to increase when citrate is present in the growth medium [14], whereas in *L. diacetylactis* this compound does not influence either transcription or CitP synthesis [10]. In *L. diacetylactis*, transcription of the *citQRP* operon yields a 2900 nt *cit* transcript. The synthesis of this mRNA1 is driven from the P1 promoter located 1 kb upstream of *citQ*, and the transcript terminates at a ρ -independent terminator located next to the 3'-end of *citP* [8], (figure 1). The long leader untranslated region present in mRNA1 contains the insertion sequence-like element IS982, which is flanked by two 17 nt identical inverted repeats [11, 16]. These sequences bracket the ORF1 located in opposite orientation to *citP* (see figure 1). This ORF1 is homologous to two overlapping ORFs of a putative IS element located upstream of the *glgC* gene from *Bacillus stearothermophilus* [8].

Plasmid pCIT264 seems to have derived from a parental Cit⁺ plasmid by the occurrence of a transpositional event during evolution. The introduction of the IS982 generated two new transcriptional promoters P3 and P2. P3 supports expression of ORF1 of unknown function [11], and P2 increases expression of the *citQRP* operon in *L. diacetylactis* [9]. Moreover, IS982 contains P2', which is the only functional promoter for expression of *citP* in

Escherichia coli. Thus, the presence of IS982 in pCIT264 activates the operon in the heterologous host. The IS982 has a wide distribution among the lactococcal genome. Several copies of this element were detected in the chromosome of various lactococcal strains [11, 16]. Analysis of the NBRF data bank reveals that pTN20 and pNP40 plasmids contain regions of the IS982 (figure 1). A fragment of 236 nt of the element has 99% identity with a region of pTN20 located next to the 3'-end of the abortive infection phage gene (*abiC*), and upstream of the nisin resistance gene (*nsr*) of pNP40 are present 190 nt out of 192 of the IS982 left region. In addition, one copy of the entire IS982 is located between the origin of replication (*ori*) and the oligopeptide transport system (*opp*) of plasmid pSKL11 [16]. Interestingly, pCIT264 and pSKL11 replication regions are highly homologous, and belong to the pWV02 family which replicates via θ mode [6]. Thus, the conspicuous genetic fluidity, which is characteristic of LAB seems to be responsible for the actual genetic organization of the plasmid involved in citrate transport in *L. diacetylactis*.

3. POSTTRANSCRIPTIONAL REGULATION OF EXPRESSION OF THE *CITQRP* OPERON

The *citP* gene and its product CitP are the only requirements for the citrate transport system in *L. diacetylactis* [10]. The *citQ* and *citR* genes seem to play a role in regulation of levels of CitP in the cell. The central region of *citQ* and the 5'-end of *citR* are included in a complex secondary structure, and the *cit* mRNA1 is processed at this location in *L. diacetylactis* [8]. The identification of the 5'-end of the processed species showed that the endonucleolytic cleavage should interfere with translation of *citQ* and *citR* [4]. This processing also occurs in *E. coli*, and its ana-

lysis in RNase mutants of this host indicates that the endoribonuclease RNase III is involved in this process [4]. Utilization of an *E. coli* in vitro transcription-translation system has allowed to identify the products of translation of *citQ* and *citR*. The molecular mass of the products indicates that translation of both genes start at the first AUG of *citQ*, which is located upstream of the secondary structure. Provided that *citQ* and *citR* overlap but they are respectively in 0 and -1 frame, the synthesis of the CitQ-CitR polypeptide should be due to a frameshifting event catalyzed by the ribosomes. Thus, the interplay of translation of *citQ* and processing of the *cit* mRNA seems to control the expression of these genes. Moreover, these processes are connected with the expression of *citP*, because interruption of *citQ* at the major processing site results in an enhancement of CitP synthesis. At the present stage the nature of this complex regulation is not fully understood and further research should be developed on this subject.

4. ACTIVATION OF THE CITRATE TRANSPORT SYSTEM AT ACIDIC PH

The citrate uptake is influenced by the external pH in lactococci. CitP shows optimal activity within a narrow pH range of 4.5–5.5 [12], and the specific rates of citrate utilization by growing cells drastically increase upon decrease of pH from 6.5 to 5.5 [1, 5]. Moreover, both acidification of the medium and shift to acidic pH result in induction of transcription of the *citQRP* operon as well as enhancement of the citrate transport activity in *L. diacetylactis* (García-Quintáns, personal communication). In this report, influence of pH of the medium in growth and metabolism of CRL264/pCIT264 (Cit⁺) and CRL30 (isogenic strain cured of pCIT264) (Cit⁻) was investigated (figure 2). At neutral pH, both strains utilize efficiently glu-

cose as energy source, and release of lactate was accompanied by acidification of the medium. In contrast, at pH 4.5 glucose supports poor growth of both strains. The final cellular mass was 4-fold lower than that at pH 7.0. The substitution of glucose by citrate impaired the growth of both strains at neutral pH. These results were expected since citrate does not support growth at neutral pH [5]. CRL264, but not CRL30, consumed 40% of citrate at pH 4.5, and this consumption was accompanied by alkalinisation of the medium. This citrate metabolism allowed cellular growth at the same rate and final absorbance than that supported by glucose at acidic pH. However, it only provided to CRL264 slight growth advantage as compared with CRL30. In contrast, the utilization of citrate by CRL264 in the presence of glucose had a drastical influence. At neutral pH, this strain cometabolizes very efficiently glucose and citrate. At acidic pH, CRL264 reached a final absorbance as high as that obtained in M17G at pH 7.0, and 4-fold higher than that displayed by CRL30 at acidic pH. Moreover, in these conditions the growth of CRL264 results in alkalization of the medium. This effect should be ascribable to citrate transport inside the cells, since it was not detected with CRL30. Furthermore, the slope of the pH curve correlates with consumption of citrate up to 7.5 h of incubation. When the pH reached a value above 4.75, the rate of decay of citrate dramatically increased indicating that a maximum transport activity was achieved. This behaviour was accompanied by a diauxic growth curve like, and was not correlated with the production of lactate. When the pH was over 5.0 a very efficient consumption of glucose started to take place, not ascribable to the increase in cellular mass. This utilization of sugar correlated with lactate production, whose secretion presumably provoked the observed acidification of the medium at longer incubation times. It has been reported that

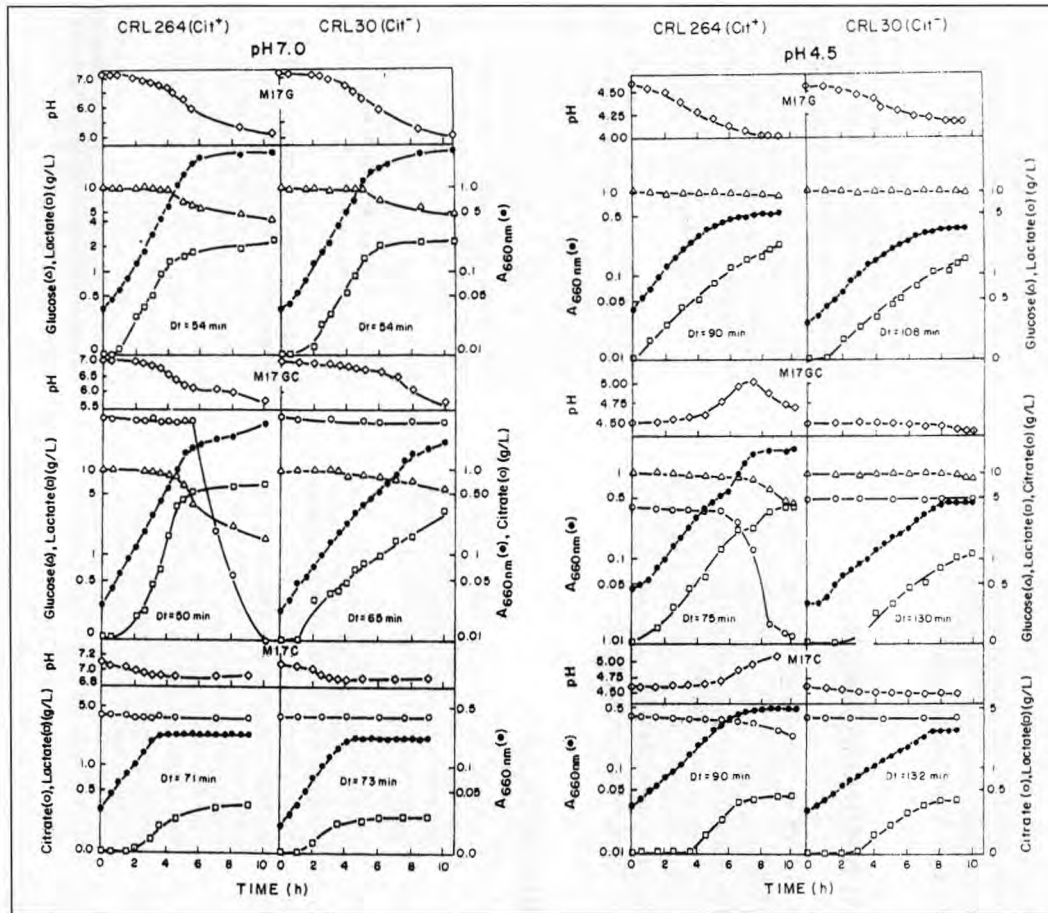


Figure 2. Characteristics of fermentation at non-controlled pH. Lactococcal strains were grown in M17 medium supplemented with glucose at 10 g/L (M17G), citrate at 4 g/L (M17C), or both compounds (M17GC). Samples of the cultures were taken at the times indicated, centrifuged and the supernatants analyzed. Lactic and citric acids were assayed enzymatically by using the appropriate kits from Boehringer Mannheim. Glucose was assayed enzymatically by using the test kit 510A from Sigma.

Figure 2. Caractéristiques de fermentation à pH non régulé. Les souches de lactocoques étaient cultivées sur milieu M17 supplémenté avec du glucose à 10 g/L (M17G), citrate à 4 g/L (M17C), ou glucose + citrate (M17GC). Des échantillons étaient prélevés de la culture aux temps indiqués, centrifugés puis les surnageants étaient analysés. Les acides citrique et lactique étaient dosés enzymatiquement en utilisant les kits fournis par Boehringer Mannheim. Le glucose était dosé enzymatiquement en utilisant le kit test 510A fourni par Sigma.

citrate-glucose cometabolism provides growth advantage to *L. mesenteroides* and that utilization of citrate alkalises the external medium [14]. Here, we have observed that this alkalisation results in an enhancement of glucose metabolism and strongly increases the rate of citrate utilization in lactococci. Thus, it seems that the interplay of citrate and glucose metabolisms achieved by *L. diacetylactis* counteracts the acidic stress during growth. Therefore, the induction of synthesis and activity of CitP at acidic pHs seems to be designed to support an efficient citrate-sugar cometabolism. This phenomenon could provide to *L. diacetylactis* a selective advantage, which should enlarge its survival in fermented products.

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