Analysis of heat shock gene expression in *Lactococcus lactis* MG1363

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The induction of the heat shock response in *Lactococcus lactis* subsp. *cremoris* strain MG1363 was analysed at the RNA level using a novel RNA isolation procedure to prevent degradation. Cloning of the *dnaJ* and *groEL* homologues was carried out. Northern blot analysis showed a similar induction pattern for *dnaK*, *dnaJ* and *groELS* after transfer from 30 °C to 43 °C when MG1363 was grown in defined medium. The *dnaK* gene showed a 100-fold induction level 15 min after temperature shifting. Induction of the first two genes in the *dnaK* operon, *orf1* and *grpE*, resembled the pattern observed for the above genes, although maximum induction was observed earlier for *orf1* and *grpE*. Novel transcript sizes were detected in heat-shocked cells. The induction kinetics observed for *ftsH* suggested a different regulation for this gene. Experimental evidence for a pronounced transcriptional regulation being involved in the heat shock response in *L. lactis* MG1363 is presented. A gene located downstream of the *dnaK* operon in strain MG1363, named *orf4*, was shown not to be regulated by heat shock.

Keywords: heat shock, *Lactococcus lactis*, stress

INTRODUCTION

Following an increase in temperature, the synthesis of a conserved set of proteins called heat shock proteins (HSPs) is induced in all living organisms, providing a means of protection against this physiological stress (Bukau, 1993; Georgopoulos & Welch, 1993). A detailed study of the heat shock response and its regulation has been carried out in bacteria, yeast and various higher organisms (Georgopoulos & Welch, 1993). Most HSPs are also synthesized at reduced rates in the absence of stress. In fact, HSPs have been implicated in central cellular processes such as protein renaturation, folding and degradation and DNA replication (Yura *et al.*, 1993). In *Escherichia coli* and *Bacillus subtilis*, the induction of HSPs is obtained through alternative sigma factors, which modify the promoter recognition specificity of the RNA polymerase to enable the expression of heat shock genes (Bukau, 1993; Riethdorf *et al.*, 1994; Schulz *et al.*, 1995). However, in *B. subtilis*, a number of heat shock genes

present a short inverted repeat element upstream from the coding region and a vegetative promoter (Zuber & Schumann, 1994). This inverted repeat, named CIRCE, plays a role in repression under normal growth (van Asseldonk *et al.*, 1993) and has also been found in other heat shock genes of Gram-positive bacteria including *Lactococcus lactis* (Kim & Batt, 1993).

L. lactis is a common mesophilic lactic starter used in the manufacture of fermented dairy products, where bacterial growth at different limiting temperatures is often required (Auffray *et al.*, 1992). In *L. lactis*, two subspecies have been defined that show different temperature ranges. Strains belonging to *L. lactis* subsp. *lactis* can grow at higher temperatures than strains of *L. lactis* subsp. *cremoris*. Recently, the model strain MG1363 has been reclassified as subsp. *cremoris*, based on DNA homology, despite a higher temperature optimum (Godon *et al.*, 1992; Swindell *et al.*, 1994). To investigate the effect of HSP induction in cheese manufacturing, we have initiated a characterization of the heat shock response in MG1363, to attempt the identification of gene(s) that allow this strain to grow at higher temperatures than other members of subsp. *cremoris*. Moreover, its small genome size (Le Bourgeois *et al.*, 1995) makes *L. lactis* a very suitable organism for the study of heat shock. A number of highly

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Abbreviations: DEPC, diethylpyrocarbonate; HSP, heat shock protein.

conserved heat shock genes have been cloned from different *L. lactis* strains belonging to either subspecies (Eaton *et al.*, 1993; Kim & Batt, 1993; Nilsson *et al.*, 1994; van Asseldonk *et al.*, 1993). To date, in spite of the fact that these genes have been cloned in *L. lactis*, only limited efforts have been made to study the heat shock response at a molecular level.

We have developed an RNA isolation procedure that ensures no detectable degradation of samples. We are interested in the cloning and analysis of novel heat shock genes in *L. lactis*, using RNA subtractive hybridization. As the first step towards this goal, a study of the induction of the heat shock genes in the model *L. lactis* subsp. *cremoris* strain MG1363 using the corresponding genes is presented here.

METHODS

Bacterial strains, plasmids, media and growth conditions. *E. coli* XL1 Blue MRF' (Stratagene), *L. lactis* subsp. *cremoris* MG1363 (Gasson, 1983) and *L. lactis* subsp. *lactis* NCDO 2118 were used. *E. coli* was grown in Luria-Bertani (LB) medium. *L. lactis* was grown on minimal SA medium (Jensen & Hammer, 1993), or on rich M17 medium (Terzaghi & Sandine, 1975), both supplemented with 1% glucose (GSA or GM17). For the studies on heat shock, overnight cultures, grown at 30 °C on either medium, were diluted 50-fold in 50 ml liquid medium and incubated at 30 °C in a 50 ml Erlenmeyer flask, with gentle continuous magnetic stirring, but without aeration (200 r.p.m.), to an OD₄₅₀ of 0.5. For RNA studies, heat shock was applied by shifting the cultures to 43 °C. The temperature was monitored using a digital thermometer (Ami, Germany), placing the electrode in an Erlenmeyer flask containing 50 ml medium. A 30 °C control sample was always included and allowed to grow for 10 min before collection. Samples were taken into centrifuge tubes containing liquid N₂ at different times, and kept on ice. This procedure prevented freezing of the samples and RNA degradation. After centrifugation at 4 °C, 7000 r.p.m. for 10 min, the pellets were kept at -40 °C, for RNA isolation (see below).

Plasmid pBR322 was used to construct a genomic library of strain MG1363 (see below). Plasmid pNZ2015, containing the *L. lactis* subsp. *lactis* R5 *dnaJ* gene (van Asseldonk *et al.*, 1993), was kindly provided by Dr Willem M. de Vos (NIZO, Ede, The Netherlands). Plasmid pFI573 was kindly provided by Dr Michael Gasson (IFR Norwich, UK); it contains the *dnaK* operon as well as the *orf4* gene cloned from *L. lactis* subsp. *cremoris* MG1363 (Eaton *et al.*, 1993). Plasmid pLN32 contains the entire *ftsH* gene from strain MG1363 (Nilsson *et al.*, 1994); it was a gift from Dr Dan Nilsson (CHL, Denmark).

The antibiotics used for selection were ampicillin (50 µg ml⁻¹) and tetracycline (12.5 µg ml⁻¹).

DNA manipulations. Chromosomal DNA from *L. lactis* was isolated as described by Johansen & Kibbenich (1992), with minor modifications. Overnight cultures (10–50 ml) were centrifuged (7000 r.p.m., 4 °C) for 10 min and the cell pellets were freeze-dried in a Heto FD-3.0 (Heto Lab Equipment, Denmark) for 12 h. After lysozyme treatment and the addition of SDS, samples were incubated at both 37 °C and 65 °C for 10 min. Plasmid DNA from *E. coli* was purified using Qiagen columns (Diagen). Restriction endonucleases, calf alkaline intestinal phosphatase and T4 DNA ligase were purchased from Boehringer Mannheim, New England Biolabs and Amersham,

and were used as recommended by the manufacturers. All DNA manipulations were performed as described by Sambrook *et al.* (1989).

RNA isolation and analysis. RNA extraction was carried out using the frozen cell pellets (see above), by adding 0.7 g glass beads (150 µm, Sigma) and 1 ml freshly prepared, pre-chilled RNA extraction buffer (4 M guanidine thiocyanate; 10 mM MES pH 7.0; 10 mM EDTA; 50 mM β-mercaptoethanol; 5%, v/v, Triton X-100). Samples were vortexed for 30 s and kept on ice for at least 30 s. This procedure was repeated a minimum of 15 times before the addition of 1 ml phenol/chloroform pH 4.7 (Sigma). After centrifugation at 4 °C, 5000 r.p.m., for 10 min, the aqueous phase was re-extracted with 1 ml phenol/chloroform and the RNA was precipitated overnight at -40 °C, with 0.1 vol. 3 M sodium acetate pH 5.5 and 2.5 vol. ice-cold ethanol. Samples were centrifuged at 4 °C, 12000 r.p.m., for 30 min, then the pellets were washed in 70% (v/v) ethanol and dried in a HetoVac (Heto Lab Equipment, Denmark). RNA was resuspended in DEPC-treated sterile water and kept at -40 °C. All solutions, plasticware and glassware were treated with 0.1% DEPC (Sigma) overnight and autoclaved or baked at 200 °C for 12 h, to remove potential RNase activity. Using this protocol, undegraded RNA of high purity ($A_{260}/A_{280} > 1.8$) was routinely obtained. For analysis of transcripts, approximately 10 µg RNA was denatured for 2 min at 100 °C in loading buffer [1 × MOPS buffer (200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA), 50% (v/v) formamide, 6% (v/v) formaldehyde, 0.6 mg ethidium bromide ml⁻¹, 5% (v/v) glycerol] containing a 50-fold dilution of a freshly made saturated solution of bromophenol blue in water, and separated on 1.2% (w/v) agarose gels containing 2% (v/v) formaldehyde in 1 × MOPS buffer, at 100 V for 2 h. The 0.36–9.49 kb RNA marker (Promega) was used for size determination. Gels were washed in 10 × SSC (1 × SSC is 1.5 M NaCl, 0.15 M sodium citrate) for 30 min before transferring the RNA to GeneScreen (Du Pont) membranes by capillary blotting, overnight in 10 × SSC (Sambrook *et al.*, 1989).

Construction of a genomic library for strain MG1363. Chromosomal DNA of strain MG1363 was partially digested with *Sau3A*I and aliquots taken at different times were run on a 0.7% agarose gel. Samples showing an average size of 3 kb were used to clone into *Bam*HI-digested, dephosphorylated pBR322. *E. coli* XL1-Blue MRF' (Stratagene) was transformed with the ligation mix, and filters were prepared for colony hybridization as described by Sambrook *et al.* (1989).

Cloning of the *dnaJ* gene of strain MG1363. A 726 bp internal *Hind*III fragment of the cloned *L. lactis* subsp. *lactis* strain R5 *dnaJ* gene (van Asseldonk *et al.*, 1993) was used in preliminary experiments to examine the expression of the corresponding gene in strain MG1363 (Fig. 1). No hybridization was observed to heat-shocked RNA preparations, suggesting a relatively low homology. When the same fragment was used as a probe in Southern hybridizations it detected a 4.5 kb *Eco*RI DNA fragment in MG1363 only when hybridizations were carried out at low stringency, with a final 20 min wash at 65 °C in 2 × SSC, 0.1% SDS (not shown). The probe was used to screen the MG1363 genomic library and a clone, pKS2, was identified.

Amplification of the *groEL* gene of strain MG1363 by PCR. The sequence of the *groESL* operon of the *L. lactis* subsp. *lactis* strain CC9 has been reported (Kim & Batt, 1993). Two primers, corresponding to the nucleotide positions 747–749 and 1733–1698 of the published sequence, within the *groEL* gene (Kim & Batt, 1993), were obtained (Hobolth DNA Syntese, Denmark) and used for amplification using DNA from strain MG1363 (Fig. 1). As a control, DNA of *L. lactis* subsp. *lactis* strain

Fig. 1. Overview of heat shock genes cloned in different *L. lactis* strains, detected mRNA sizes (thin lines) and probes (thick lines) used in this study are shown (see Results). The relevant restriction sites are also depicted: D, *DraI*; E, *EcoRV*; H, *HindIII*; S, *Sau3AI*. IR (black boxes), inverted repeat (CIRCE) present upstream of the *orf1*, *dnaJ* and *groES* genes. The positions of the primers used for amplification of the *groEL* fragment used as a probe are indicated as arrows. The relative positions of restriction sites and primer sequences are drawn to scale according to the published sequences for the *dnaK* region and *ftsH* gene from *L. lactis* subsp. *cremoris* MG1363 (Eaton *et al.*, 1993; Nilsson *et al.*, 1994), and the *dnaJ* gene and *groESL* operon from *L. lactis* subsp. *lactis* strains R5 and CC9, respectively (van Asseldonk *et al.*, 1993; Kim & Batt, 1993).

NCDO 2118 was used. The reactions were carried out in a 100 µl volume, using 2 units of *Taq* DNA polymerase (AH Diagnostics, Denmark) in a PREM thermal cycler (LEP Scientific), for 30 cycles of 92 °C, 1 min; 60 °C (or 55 °C, see Results), 1 min; 72 °C, 2 min. The expected 1 kb *groEL* DNA (Kim & Batt, 1993) fragment was isolated from the gel using GeneClean (BIO101).

Northern blot analysis. Only gene-specific probes were used for Northern hybridization, as follows: a 728 bp *DraI*–*HindIII* DNA fragment from pFI573 (*orf1*); 586 bp (*grpE*) and 1704 bp (*dnaK*) *DraI* DNA fragments from pFI573 (Eaton *et al.*, 1993); an 876 bp *Sau3AI* DNA fragment from pFI573 (*orf4*) (Eaton *et al.*, 1993); and a 1253 bp *EcoRV* fragment of pLN32 (*ftsH*) (Nilsson *et al.*, 1994). Additionally, a 1.5 kb *Sau3AI* fragment from pKS2 (*dnaJ*) and the amplified 1 kb *groEL* gene fragment from MG1363 were used (see above). Probes were labelled with [α -³²P]dATP, using the Multiprime DNA labelling kit (Amersham) for 2–3 h at 37 °C. Unincorporated nucleotides were removed using a NICK column (Pharmacia). Northern blot hybridization was carried out at 65 °C in 0.5 M sodium phosphate pH 7.2, 7% (w/v) SDS. Blots were washed at 65 °C in 20 mM sodium phosphate pH 7.2, 1% (w/v) SDS for three periods of 15 min.

Induction levels for *dnaK* and *dnaJ* were estimated using an Instant Imager 2024 (Packard) by measuring the radioactivity in each full-length lane directly from the nylon membrane, and normalizing to the values obtained in control samples in each case. All other induction levels were estimated by comparison.

RESULTS

An RNA isolation procedure for the study of heat shock gene expression in *L. lactis*

A few protocols for the study of heat shock gene expression by Northern blot analysis have been described in *L. lactis* (Kim & Batt, 1993; van Asseldonk *et al.*, 1993).

A critical step when studying the kinetics of heat shock gene expression is to ensure that samples are not exposed to additional stress during preparation, and that heat shock is homogeneously applied to a culture. We used magnetic stirring of the cultures to ensure a progressive, reproducible and fast increase in temperature without aeration. Heat shock was applied to exponentially growing cultures by shifting from 30 °C to 43 °C. Under these conditions, and using continuous gentle agitation, a rapid increase in temperature, up to 42 °C, occurred within the first 4 min, and the final temperature was reached after 8 min.

Samples were mixed with liquid nitrogen and placed on ice, preventing culture freezing and RNA degradation (Fig. 2a). Consistent high yields (approx. 100 µg total RNA per 50 ml culture at OD₄₅₀ 0.5) of undegraded RNA were obtained using a protocol that omits the frequently used macaloid clay (van Asseldonk *et al.*, 1993), which in our experience is a common source of RNase contamination. The new protocol incorporates the use of guanidine thiocyanate as a cell-disrupting agent, which is commonly used for eukaryotic RNA isolation (Sambrook *et al.*, 1989). Analysis of electrophoretically separated RNA prepared using this method did not show any detectable degradation of the major 23S and 16S rRNA species (Fig. 2a).

Cloning of the *dnaJ* and *groEL* homologues in strain MG1363

The use of an internal 726 bp *HindIII* DNA fragment of the *dnaJ* gene cloned from *L. lactis* subsp. *lactis* R5 (van Asseldonk *et al.*, 1993) as a probe in Northern analysis of heat-shocked MG1363 RNA did not succeed in the detection of the corresponding *dnaJ* mRNA (data not shown). A relatively low homology between the *dnaJ* genes of these two strains could account for the lack of hybridization. Thus, a genomic library of strain MG1363 was constructed in pBR322 and approximately 2500 clones were screened with the same probe, using low-stringency hybridization. A clone, pKS2, was isolated and analysis of the inserted DNA showed that a 1.5 kb *Sau3AI* DNA fragment that hybridized to the above probe was present in this clone. Using the 1.5 kb fragment as a probe against *EcoRI*-digested chromosomal DNA of strain MG1363, a 4.5 kb fragment was detected. A similar 4.5 kb *EcoRI* DNA band was identified in low-stringency hybridization, using the *HindIII* fragment of the previously characterized lactococcal *dnaJ* gene (van Asseldonk *et al.*, 1993), indicating that the 1.5 kb *Sau3AI* fragment of pKS2 contained the homologous gene from MG1363. Subsequent sequence analysis resulted in the identification of a DNA fragment showing 85% homology to the 3' end of the previously cloned *L. lactis* *dnaJ* gene (Fig. 1; data not shown). A 1 kb internal fragment of the *groEL* gene from strain MG1363 was obtained by PCR amplification using primers derived from conserved regions of the published sequence of the *L. lactis* subsp. *lactis* strain CC9 *groESL* operon (Kim & Batt, 1993). Another *L. lactis* subsp. *lactis* strain, NCDO 2118, was used as a positive control, since a higher degree of

Fig. 2. Northern blot analysis of heat shock gene expression in strain MG1363 grown on defined, GSA medium. RNA was isolated from cells growing at 30 °C (lanes 1) and at 5, 10, 15 and 20 min after shifting to 43 °C (lanes 2, 3, 4 and 5, respectively). Filters were hybridized with radioactively labelled gene specific probes (see Methods for details). (a) Example of RNA samples run on to a 1.2% (w/v) agarose/formaldehyde gel. Lane M, RNA size standards; sizes are indicated in kb to the left. (b–h) Northern blot hybridization, with *orf1* (b), *grpE* (c), *dnaK* [exposure time: 8 h (left) and 48 h (right)] (d), *orf4* (e), *dnaJ* (f), *groEL* (g) and *ftsH* (h). Autoradiograms were scanned using an HP Scanjet IIcx (Hewlett Packard) at 200 d.p.i. and assembled using Corel Draw version 5.0 software. Approximate transcript sizes are given in kb.

homology was expected between strains within this subspecies. The expected 1 kb DNA fragment was only amplified from strain MG1363 when an annealing temperature of 55 °C was used, whereas the *groEL* gene fragment of strain NCDO 2118 was also obtained using annealing at 60 °C (data not shown), suggesting a higher sequence homology for the *groEL* within subsp. *lactis* strains.

Induction of gene expression in strain MG1363 during heat shock

As mentioned above, no comprehensive study of the heat shock response has been reported in *L. lactis*, although a number of genes have been cloned in this organism (Eaton *et al.*, 1993; Kim & Batt, 1993; Nilsson *et al.*, 1994; van Asseldonk *et al.*, 1993).

Northern blot hybridizations were carried out on RNA isolated from strain MG1363 subjected to heat shock treatment for 5–20 min (Fig. 2). Recently, the *dnaK* region of strain MG1363 was cloned and sequenced. Four genes (in the order *orf1*–*grpE*–*dnaK*–*orf4*) were found (Eaton *et al.*, 1993). Sequence analysis from this region identified a consensus promoter and a CIRCE upstream of *orf1* and a

transcription terminator downstream of *dnaK*, suggesting that *orf1*, *grpE* and *dnaK* may constitute an operon. For *dnaK*, remarkably strong induction, 5-, 15- and 100-fold, respectively, 5, 10 and 15 min after heat shock was observed and a significantly higher level of expression (10-fold) was detected even after 20 min, as compared to control cells (Fig. 2d). A 1.8 kb transcript was observed in all samples which corresponds in size to the *dnaK* gene. Interestingly, no promoter sequence was reported upstream of this gene (Eaton *et al.*, 1993). A larger transcript (3.0 kb) which may include at least *grpE* (see below) was only observed in heat-shocked cells, together with a 1.2 kb mRNA (Fig. 2d).

In many bacteria, the *dnaJ* gene is found in the same operon as *grpE* and *dnaK* (Eaton *et al.*, 1993). However, in *L. lactis*, the *dnaJ* gene has been reported to be transcribed independently and to include a CIRCE upstream of the promoter sequence (van Asseldonk *et al.*, 1993). Using the MG1363 *dnaJ* gene, a low level of expression was observed in control cells (Fig. 2f, lane 1) and at least a 2- and 10-fold induction was found after 10 and 15 min at 43 °C, respectively (Fig. 2f, lanes 2–4). A 1.8 kb mRNA was observed in all samples, in agreement with the published Northern blot analysis of the *L. lactis* subsp. *lactis* strain

R5 *dnaJ* gene (van Asseldonk *et al.*, 1993), and a faint 3.2 kb band was only detected after 15 min (Fig. 2f, lane 4). No smaller transcripts were detected, in contrast to a 1.0 kb mRNA reported to hybridize to *dnaJ* and to be a specific breakdown product of the 1.8 kb RNA species (van Asseldonk *et al.*, 1993).

The *groESL* operon was cloned in *L. lactis* subsp. *lactis* CC9. A CIRCE was also found upstream of the *groES* gene (Kim & Batt, 1993). Using the *groEL* probe (Fig. 1), a major 2.2 kb transcript showed a 10-fold induction after 15 min (Fig. 2g). This may also include the *groES* mRNA, as reported for *E. coli* and *Agrobacterium tumefaciens* (Segal & Ron, 1995). The general induction pattern showed similar kinetics for *dnaK*, *dnaJ* and *groEL*, with an increasing level of mRNA up to 15 min after heat shock, and a decrease observed after 20 min (Fig. 2d, f and g). In contrast to the situation for all other genes studied, a lower level of *groEL* mRNA was observed after 20 min compared to control cells (Fig. 2g, lanes 1 and 5), suggesting a tight control of expression for this gene in heat-shocked cells. An additional 2.7 kb transcript was only detected after 15 min (Fig. 2g, lane 4).

The first gene in the *L. lactis* *dnaK* operon (Fig. 1), *orf1*, showed homology to the first gene in the *dnaK* operon of other Gram-positive bacteria and has been proposed to have a regulatory role (Eaton *et al.*, 1993; Naberhaus *et al.*, 1992; Schulz *et al.*, 1995; Wetzstein *et al.*, 1992). The order of the first three genes (*orf1-grpE-dnaK*) is conserved among this group of bacteria (Naberhaus *et al.*, 1992; Wetzstein *et al.*, 1992). At least a fivefold induction of *orf1* was observed after 10 min at 43 °C, and a low level of *orf1* mRNA was observed in control cells and after 20 min at 43 °C (Fig. 2b, lanes 3 and 5).

The *grpE* probe identified two mRNA species. A 1.7 kb mRNA, present at a low level in control cells, showed at least a fivefold induction after 10 min and decreased to normal levels after 20 min (Fig. 2c). An additional band, approximately 3.0 kb in size, was only observed after 10 min (Fig. 2c, lane 3). The *grpE* gene is located immediately downstream of the *orf1* gene in strain MG1363 (Fig. 1), and no transcription terminator sequence was found in the intergenic region (Eaton *et al.*, 1993). Since a similar-sized mRNA hybridized to the *orf1* and *grpE* probes, and the induction of both genes occurred at identical times, the two genes may be co-transcribed. The size of this RNA species, 1.7 kb (Fig. 1, Fig. 2b, c), correlated well with the DNA region including both genes (Eaton *et al.*, 1993). The size of the chromosomal region including *orf1*, *grpE* and *dnaK* in strain MG1363 is about 3.5 kb. No transcript of this size was detected with either gene probe.

A novel gene which codes for a protein of unknown function, *orf4*, was found downstream of the *dnaK* gene in strain MG1363 (Eaton *et al.*, 1993). Northern blot hybridization was carried out to study the expression of this gene during heat shock. Two transcripts (3.0 and 1.9 kb) were detected at similar levels in control and heat-shocked samples (Fig. 2e). Thus, expression of *orf4* was

not induced by heat shock in defined medium. No expression of *orf4* was detected when cells were grown in GM17 medium, regardless of the temperature, suggesting that this gene is not essential when growing in rich medium (data not shown). Interestingly, lower levels of *orf4* mRNA were found after 5 and 20 min, especially for the larger transcript. A similar situation was observed for the majority of genes studied (Fig. 2). Independent RNA samples and hybridization experiments were carried out to confirm that the difference in intensity of these bands was not due to different amounts of RNA being loaded, or to unspecific degradation (Fig. 2a, e).

The *B. subtilis* *ftsH* gene is induced after heat shock, despite the lack of CIRCE (Schulz *et al.*, 1995). The *L. lactis* *ftsH* gene was cloned from strain MG1363 and no CIRCE was found in the promoter region of this gene. A mutant carrying a truncated *ftsH* gene showed increased thermosensitivity, suggesting a role in heat shock (Nilsson *et al.*, 1994). Using the *ftsH* probe, a 2.5 kb mRNA, corresponding to the size of the *ftsH* gene (Fig. 1), was detected at very low levels only 10 min after temperature shift (Fig. 2h, lane 3). A smaller, 1.5 kb band was also observed after 10, 15 and 20 min; this may be a specific breakdown product of the 2.5 kb mRNA (Fig. 2h, lanes 3–5). A similar induction pattern was also observed when cells were grown in rich GM17 medium (data not shown).

Remarkably, lower levels of mRNA were observed after 5 min for all genes studied than in control cells, with the sole exception of *dnaK*. This included the constitutively expressed gene *orf4*, and was observed in duplicate experiments using independent RNA samples.

DISCUSSION

We have studied heat shock gene expression in *L. lactis* subsp. *cremoris* MG1363 growing in defined medium. In our conditions, a rapid increase in temperature occurs and cells are only subjected to heat stress. A new RNA isolation method allowed the study of the kinetics of induction during the first 20 min after temperature shift. The induction of gene expression after temperature shift from 30 °C to 43 °C was studied by Northern blot hybridization using gene-specific probes.

The known heat shock genes *dnaK*, *dnaJ* and *groEL*, as well as members of the suggested *dnaK* operon and *ftsH*, all showed induction of the corresponding mRNA(s), providing experimental evidence for a transcriptional regulation of the heat shock response in *L. lactis*, as shown for *E. coli* and *B. subtilis* (Bukau, 1993; Schulz *et al.*, 1995). For *dnaJ*, *groEL* and *dnaK*, a high level of induction was observed, ranging from 10- (*dnaJ* and *groEL*) to 100-fold (*dnaK*). The induction pattern for these three genes was similar, with an increasing level of mRNA up to 15 min after heat shock and a reduction observed after 20 min. This correlates with the presence of a CIRCE upstream of these genes.

A slightly different pattern was detected for *orf1* and *grpE*. Maximum induction was reached already after 10 min and

was still maintained after 15 min. A regulatory function has been proposed for these two genes in other bacteria (Bukau, 1993; Schulz *et al.*, 1995), which may account for this difference.

The *ftsH* gene codes for a membrane-bound ATP-dependent protease involved in many cellular processes, such as cell division, protein translocation and regulation of gene expression in *E. coli* (Akiyama *et al.*, 1994; Herman *et al.*, 1993; Ogura *et al.*, 1991). As in *B. subtilis*, the *L. lactis ftsH* gene was shown to affect temperature sensitivity, suggesting a role in heat shock, although no CIRCE was found in the promoter region of this gene (Schulz *et al.*, 1995; Nilsson *et al.*, 1994). To our knowledge, the expression of *ftsH* has been exclusively studied at the protein level, and no Northern blot hybridization has been included in any of the organisms where the gene has been cloned. The induction of this gene under heat shock followed a different kinetics, with a significant induction after 10 min that was still observed after 20 min (Fig. 2h). An alternative regulatory mechanism for heat shock genes lacking a CIRCE may be responsible for the differences observed.

No induction was observed for *orf4* in defined medium (Fig. 2e). A transcription terminator sequence located downstream from the adjacent *dnaK* gene and a vegetative promoter were found upstream of the *orf4* coding region. Interestingly, no expression was detected in GM17 in either control or heat-shocked cells (data not shown).

Assuming that a full-length transcript of the *dnaK* operon is efficiently processed in *L. lactis*, our results are consistent with the analysis of the reported DNA sequence, where a consensus vegetative promoter and a CIRCE were found upstream of *orf1* and a terminator structure separated *dnaK* from *orf4* (Eaton *et al.*, 1993). The major processing products include a monocistronic *dnaK* mRNA, and two bicistronic mRNA species (*orf1-grpE* and *grpE-dnaK*). However, further experiments on the regulation of the *dnaK* operon are required to confirm the suggested processing.

The *groEL* gene was found to be expressed in control samples grown at 30 °C in GSA (Fig. 2g), in contrast to the published results (Kim & Batt, 1993). Our results, which included a control experiment where heat shock was applied to cells growing in rich, GM17 medium (data not shown), are consistent with the observation that this gene is essential at all temperatures in both *E. coli* and *B. subtilis* (Burnett *et al.*, 1994; Kanemori *et al.*, 1994; Schmidt *et al.*, 1992).

The expression of heat shock genes was also studied in rich medium. Generally, lower and faster induction levels were observed for all genes studied except *ftsH* (data not shown).

The procedure described here for the study of heat shock gene expression in *L. lactis* allowed the detection of highly induced levels of mRNA for the genes known to be involved in the heat shock response. A significant difference in expression level between the induced and

non-induced state is advantageous for the cloning of novel heat shock genes. We are currently using RNA subtractive hybridization to attempt the cloning of such genes.

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