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 *groEL* 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...
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, were diluted 50-fold in 50 ml liquid medium 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 and the RNA was precipitated overnight at −40 °C, with 0.1 vol. of 3 M sodium acetate pH 5.5 and 2.5 vol. ice-cold ethanol. Samples were centrifuged at 4 °C, 12,000 r.p.m., for 30 min, then the pellets were washed in 70% (v/v) ethanol and dried in a Hetoac (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 2 h, to remove potential RNase activity. Using this protocol, undegraded RNA of high purity (A260/A280 > 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 x MOPS buffer, 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 x 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 Sau3AI 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 BamHI-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 HindIII fragment of the cloned L. lactis subsp. lactis strain 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 fitH 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 & Kibichen (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 (Qiagen). 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. of 3 M sodium acetate pH 5.5 and 2.5 vol. ice-cold ethanol. Samples were centrifuged at 4 °C, 12,000 r.p.m., for 30 min, then the pellets were washed in 70% (v/v) ethanol and dried in a Hetoac (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 2 h, to remove potential RNase activity. Using this protocol, undegraded RNA of high purity (A260/A280 > 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 x MOPS buffer, 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 x 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).

Amplication of the groEL gene of strain MG1363 by PCR. The sequence of the groEL 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
Heat shock gene expression in *L. lactis*

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 OD$_{600}$ 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
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.
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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-groEL-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 dnaK, groEL and dnaJ, 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 \textit{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 \textit{E. coli} (Akiyama et al., 1994; Herman et al., 1993; Ogura et al., 1991). As in \textit{B. subtilis}, the \textit{L. lactis} \textit{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 \textit{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 \textit{orf4} in defined medium (Fig. 2e). A transcription terminator sequence located downstream from the adjacent \textit{dnaK} gene and a vegetative promoter were found upstream of the \textit{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 \textit{dnaK} operon is efficiently processed in \textit{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 \textit{orf1} and a terminator structure separated \textit{dnaK} from \textit{orf4} (Eaton et al., 1993). The major processing products include a monocistronic \textit{dnaK} mRNA, and two bicistronic mRNA species (\textit{orf1-dnaK} and \textit{grpE-dnaK}). However, further experiments on the regulation of the \textit{dnaK} operon are required to confirm the suggested processing.

The \textit{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 \textit{E. coli} and \textit{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 \textit{ftsH} (data not shown).

The procedure described here for the study of heat shock gene expression in \textit{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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