

Heat Shock Proteome Analysis of Wild-Type *Corynebacterium glutamicum* ATCC 13032 and a Spontaneous Mutant Lacking GroEL1, a Dispensable Chaperone

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Proteome analysis of *Corynebacterium glutamicum* ATCC 13032 showed that levels of several proteins increased drastically in response to heat shock. These proteins were identified as DnaK, GroEL1, GroEL2, ClpB, GrpE, and PoxB, and their heat response was in agreement with previous transcriptomic results. A major heat-induced protein was absent in the proteome of strain 13032B of *C. glutamicum*, used for genome sequencing in Germany, compared with the wild-type ATCC 13032 strain. The missing protein was identified as GroEL1 by matrix-assisted laser desorption ionization–time of flight peptide mass fingerprinting, and the mutation was found to be due to an insertion sequence, IsCg1, that was integrated at position 327 downstream of the translation start codon of the *groEL1* gene, resulting in a truncated transcript of this gene, as shown by Northern analysis. The GroEL1 chaperone is, therefore, dispensable in *C. glutamicum*. On the other hand, GroEL2 appears to be essential for growth. Based on these results, the role of the duplicate *groEL1* and *groEL2* genes is analyzed.

Corynebacterium glutamicum (initially named *Micrococcus glutamicus*) was isolated from a soil sample of the Ueno Zoo in Tokyo as a high producer of glutamic acid (22). The type strain Kyowa Hakko Kogyo strain 534 was reclassified as *C. glutamicum* (37) and deposited as ATCC 13032.

A large number of mutants derived from *C. glutamicum* ATCC 13032 have been obtained in several laboratories and used for the industrial production of L-glutamic acid, L-lysine, and L-threonine. Due to its industrial relevance, a large research effort for the last two decades has focused on the molecular genetics of this bacterium (4, 7, 11, 23, 25, 27). The genome of the Kyowa type strain ATCC 13032 was sequenced in Japan (19). In parallel, the genome of a different ATCC 13032 derivative (hereafter named *C. glutamicum* 13032B) was mapped (4) and sequenced in Germany (20).

Heat shock of *C. glutamicum* cultures increases the secretion of glutamic acid (9) and lysine (30), and it favors the entry of exogenous DNA during transformation by electroporation (38). As in other microorganisms, heat treatment triggers a heat shock response in *C. glutamicum* (3, 29).

There is considerable interest in the analysis of the heat shock-induced proteins in *C. glutamicum* and the heat shock effect on (i) wide-domain regulatory mechanisms and (ii) specific enzymatic steps involved in biosynthesis (24) and secretion (6) of amino acids. The availability of the genome sequence

and the improvement of the two-dimensional (2D) protein electrophoresis systems (17, 18, 35) have allowed good progress in the resolution and identification of many proteins of the *C. glutamicum* proteome. During a collaboration study on the proteome response to heat shock, we observed that a major heat-induced protein was absent in the *C. glutamicum* 13032B strain available in Bielefeld, Germany, which was present in the ATCC 13032 strain in our laboratory in León, Spain. It was therefore interesting to perform a detailed analysis of the heat shock response of the proteome of both *C. glutamicum* strains. In this article we report a comparative analysis of the proteome and the heat shock response of both strains and conclude that the GroEL1 protein is, therefore, dispensable in *C. glutamicum*. Moreover, we compare the proteome data with the transcriptome analysis of *C. glutamicum* under heat shock conditions.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Two different strains of *C. glutamicum* ATCC 13032 were used in this work: *C. glutamicum* 13032L was obtained from the American Type Culture Collection (ATCC) in 1990 and kept frozen in 20% glycerol in the University of León, and *C. glutamicum* 13032B also was obtained from ATCC and was cultured in the University of Bielefeld. *Escherichia coli* DH5 α (Bethesda Research Laboratory) was used as a host in DNA manipulation procedures. *E. coli* was grown in Luria-Bertani broth (34) at 37°C. Both *C. glutamicum* strains were grown in 2 \times Ty + 2% glucose (TYG) at 30°C. *E. coli* transformants were selected in the presence of ampicillin (100 μ g/ml).

Plasmid pGEM-T Easy (Promega) was used in this work for routine subcloning of PCR-amplified DNA fragments.

DNA isolation and manipulation. *E. coli* plasmid DNA was obtained by alkaline lysis as described by Birnboim and Doly (5). Total *C. glutamicum* DNA was prepared as described by Martín and Gil (26). DNA manipulations were per-

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formed as described by Sambrook and Russell (34). DNA fragments were isolated from agarose gels by using the GeneClean II kit (BIO 101). *E. coli* cells were transformed as described by Hanahan (16).

RNA extraction. Total RNA from corynebacteria was extracted by a method based on that of Eikmanns and colleagues (12) except that the cell pellet, obtained after centrifugation, was frozen with liquid nitrogen and kept at -70°C before RNA extraction (2). The RNA concentration was determined spectrophotometrically at 260 nm.

Northern hybridization. Denaturing RNA electrophoresis was performed in 0.9% agarose gels in MOPS buffer (20 mM morpholinepropanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA [pH 7.0]) with 17% (vol/vol) formaldehyde. RNA (30 μg) was dissolved in denaturing buffer (50% formamide, 20% formaldehyde, 20% morpholinepropanesulfonic acid [5 \times] with 10% DYE [34]) and 1% ethidium bromide. RNA probes were labeled and Northern hybridizations were performed according to the procedures described in the DIG Northern starter kit (Roche). The hybridization temperature was 68°C . The positive-hybridization bands were detected by using the CDP-star reagent (Roche) with exposition times between 30 s and 5 min.

Preparation of protein extracts. *C. glutamicum* protein extracts were prepared from cells grown to the mid-exponential growth phase in liquid medium TYG. Fifty milliliters of bacterial culture was centrifuged at $5,500 \times g$ for 10 min. Cell pellets were washed twice in 10 ml of cold water and once with washing buffer (50 mM Tris-HCl, pH 7.2) and finally centrifuged for 10 min at $5,500 \times g$. After removal of the supernatant, cells were suspended in 800 μl of washing buffer containing a protease inhibitor mix (COMPLETE; Roche) and added to a FastProtein BLUE tube (BIO 101) containing a silica-ceramic matrix. Cell disruption was carried out in a Fastprep (BIO 101) machine at a speed ratio of 6.5 for three time intervals of 30 s. Cell debris was removed by centrifugation at $14,000 \times g$ for 30 min. The supernatant was treated with Benzonase (Merck) for 30 min. Proteins were concentrated by acetone precipitation and finally centrifuged for 15 min at $14,000 \times g$ and 4°C . The protein was air dried and resuspended in 400 μl of rehydration buffer (8 M urea, 2% [wt/vol] 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.01% bromophenol blue). Protein concentrations of the crude extracts were determined by the Bradford method. Cell extracts were used immediately or frozen in aliquots at -80°C .

Proteome analysis. For 2D polyacrylamide gel electrophoresis of proteins, 350 μg of crude protein extract was resuspended in 350 μl of rehydration buffer plus 0.5% (vol/vol) IPG buffer (Amersham Biosciences), 15 mM dithiothreitol (DTT), and a few grains of bromophenol blue. For isoelectric focusing, precast IPG strips with linear pH gradients of 4.5 to 5.5, 4.0 to 7.0, and 3.0 to 10.0 were used in an IPGphor isoelectric focusing unit (Amersham Pharmacia Biotech). Proteins were focused according to the following programme: 1 h, 0 V; 12 h, 30 V; 2 h, 60 V; 1 h, 500 V; 1 h, 1,000 V; 7 h, 8,000 V. Focused IPG gels were equilibrated twice for 15 min in a buffer containing 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (vol/vol) glycerol, 2% (wt/vol) sodium dodecyl sulfate, and 1% (wt/vol) DTT. For the second equilibration step, DTT was replaced by 4.5% (wt/vol) iodoacetamide. The second dimension was run in sodium dodecyl sulfate-12.5% polyacrylamide gels in an Ettan Dalt apparatus (Amersham Biosciences) as recommended by the manufacturer, and gels were subsequently stained with Coomassie brilliant blue (34). Precision Plus protein Standards (Bio-Rad) were used as markers.

Protein spots were excised from gels and digested with modified trypsin (Promega) as described by Hermann et al. (17), and peptide mass fingerprints were determined with an Ultraflex III mass spectrometer (Bruker Daltoniks) and analyzed with Flex Analysis and Biotools (Bruker Daltoniks), using the MAS-COT software (31).

RESULTS

Proteome changes in response to heat shock. In a previous work, transcriptional analysis of expression of several *C. glutamicum* genes following heat shock was done (3). To confirm that the selected heat conditions based on transcriptional studies result in significant changes in the heat shock-induced proteins, anti-DnaK and anti-GroEL antibodies were used to probe total *C. glutamicum* proteins. Results showed that heating at 40°C for 60 min is adequate for observing the complete heat shock response in the proteome and correlates well with the changes observed at the transcriptional level (3).

Protein changes in 2D gels were studied after heat shock

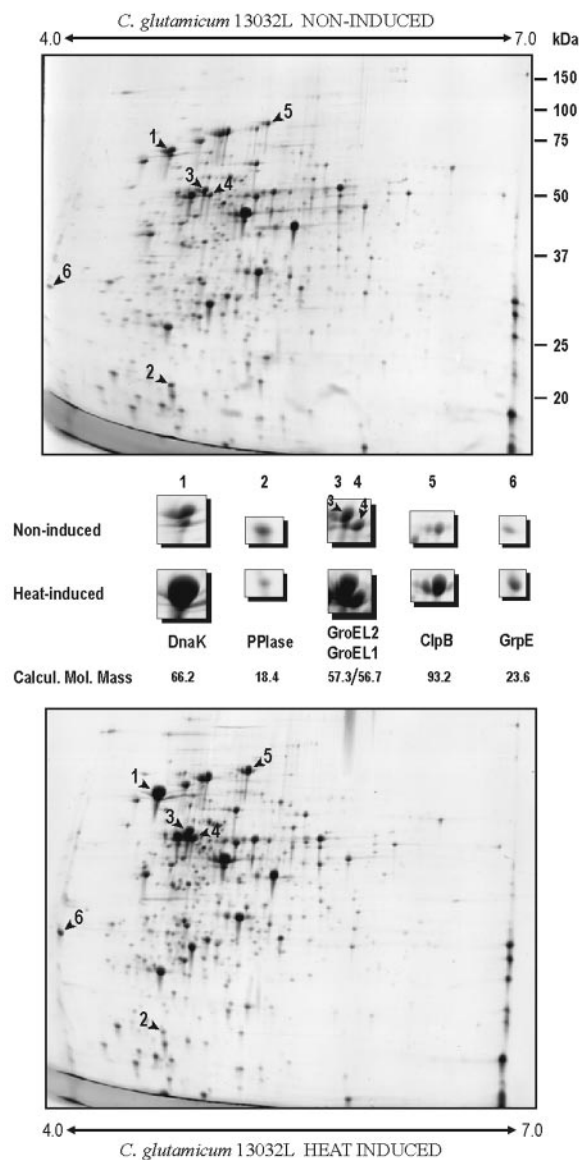


FIG. 1. Comparative 2D-gel analysis of heat shock-induced cytoplasmic proteins (lower panel) versus noninduced cells (upper panel) of *C. glutamicum* ATCC 13032L. pH range in first dimension is 4.0 to 7.0 (shown by arrows). The proteins identified by MALDI-TOF PMF are shown enlarged in the area between the upper and lower panels together with the calculated molecular masses in kilodaltons (Calcul. Mol. Mass). Molecular sizes (in kilodaltons) are shown on the right.

under the conditions defined above. Precast IPG strips with ampholites in the range of 3.0 to 10.0 and 4.0 to 7.0 were used initially. All proteins that showed significant changes in response to heat shock were located in the pH range of 4.0 to 7.0. Five proteins showed a drastic increase in response to heat shock and one protein clearly decreased in intensity when the *C. glutamicum* culture was heated at 40°C for 60 min (Fig. 1). A more detailed resolution of most of these proteins was achieved by using an ampholite pH range of 4.5 to 5.5. An additional, sixth protein that increased moderately in intensity

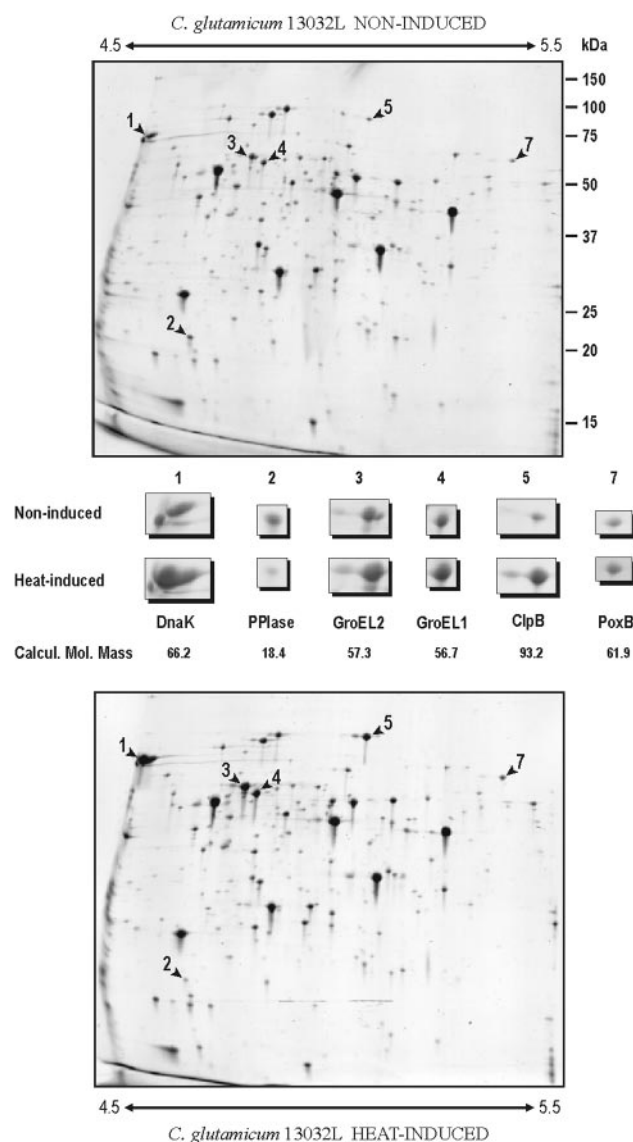


FIG. 2. Comparative “zoom-in” 2D-gel analysis of heat shock-induced cytoplasmic proteins of *C. glutamicum* 13032L. A pH range of 4.5 to 5.5 was used for better resolution (shown by arrows). The proteins identified by MALDI-TOF are shown enlarged in the area between the upper (noninduced) and lower (heat shock induced) panels together with the calculated molecular mass in kilodaltons (Calcul. Mol. Mass). Molecular sizes (in kilodaltons) are shown on the right. Note the presence of GroEL1 (spot 4) and its response to heat shock.

following heat shock was observed under these “zoom-in” 2D-gel conditions (Fig. 2).

All of these proteins were unequivocally identified by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) peptide mass fingerprinting (PMF). The analyses were repeated two or three times for each spot. As shown in Table 1, the five proteins whose levels increased clearly in response to heat shock were DnaK, GroEL1, GroEL2, ClpB, and GrpE, all of which are chaperones.

A sixth protein that increased moderately in response to heat shock was identified as PoxB, an enzyme catalyzing the

conversion of pyruvate to acetate that plays an important role in the production of amino acids in *C. glutamicum* (20). Finally, the protein that decreased in response to heat shock was identified as peptidyl-prolyl *cis-trans* isomerase, a foldase that catalyzes the isomerization of peptidyl prolyl bonds necessary for the functional folding of proteins.

At least five other proteins showed minor changes in response to heat shock. However, their changes were not considered as significant as those of the proteins described above and were not studied further.

A spontaneous mutant of *C. glutamicum* lacks GroEL1. There are two *groEL* genes in the genome of *C. glutamicum*, namely *groEL1*, which forms part of a bicistronic *groES-groEL1* operon, and a separate one, *groEL2*, expressed as a monocistronic transcript (3).

The presence of *groEL1* and *groEL2* was confirmed when the genome sequence of *C. glutamicum* ATCC 13032 was made available (accession number NC_003450 [19]).

During a study of the proteome of the ATCC 13032B strain, we observed that this strain lacked a major heat-induced protein that was present in the ATCC 13032L strain (used in the University of León). This protein was identified unequivocally by PMF as GroEL1 (Fig. 3). When the genome of the ATCC 13032B strain was sequenced (accession number BX927147 [20]), the *groEL1* gene was shown to contain an integrated insertion sequence, *ISCG1* (copy c). Thus, the lack of the GroEL1 protein in *C. glutamicum* 13032B appeared to be due to a copy of *ISCG1* that interrupts the gene (see below).

Heat shock induction shows a truncated *groES-groEL1* transcript in *C. glutamicum* 13032B. To confirm that the *ISCG1* insertion was indeed absent in the 13032L strain, PCR amplification of the *groEL1* open reading frame (ORF) was performed with primers P₁ (CGT CGA GAA GTA GGG GAT AAG) and P₂ (CCA CGG TGT TTT TCA CAG A). When the 13032L strain DNA and the ATCC original strain were used as a template, a 1,617-bp fragment (*groEL1* ORF) was amplified, whereas the amplified fragment from ATCC 13032B was of 3,078 bp (*groEL1* ORF plus *ISCG1*). Both PCR fragments were mapped with several restriction enzymes and sequenced, confirming the presence of the *ISCG1* element (data not shown). As shown in Fig. 4A, *ISCG1* is integrated at position 327 downstream of the translational start codon of the *groEL1* gene.

After confirmation of the presence of *ISCG1* in the 13032B strain, we studied how the expression of the *groES-groEL1* operon in both strains is affected, since *groES* and *groEL1* genes are expressed coordinately as a bicistronic transcript in *C. glutamicum* (3). Northern hybridization was performed with a 320-bp antisense *groES* probe, since internal probes to *groEL1* cannot be used because they hybridize with both *groEL1* and *groEL2*. Results (Fig. 4B) showed clearly that in *C. glutamicum* 13032L a transcript of 2,300 nucleotides was found, corresponding to the bicistronic operon (3), whereas strain 13032B showed an 850-bp transcript that corresponds to a truncated *groES-groEL1* mRNA due to the insertion of *ISCG1*. This truncated 850-bp transcript responded clearly to heat shock, as did the complete transcript.

Northern hybridizations using a *groEL2*-specific 15-nucleotide oligonucleotide as a probe (3) were carried out to detect the effect of *groEL1* deletion on the *groEL2* transcription pattern. As shown in Fig. 4C, the *groEL2* transcription was slightly

TABLE 1. Proteins whose levels increase or decrease significantly in response to heat shock^a

Spot	Name	Accession no.	Function	Molecular mass (kDa)	Calculated pI	Mowse factor ^c	Heat shock induction factor ^d
1	DnaK	cg3098	Heat shock protein HSP70	66.2	4.29	281	20.6
2	PPIase ^b	cg0048	Peptidyl-prolyl <i>cis-trans</i> isomerase B	18.4	4.70	99	
3	GroEL2	cg3011	Chaperonin cpn60	57.3	4.75	166	3.2–5.6
4	GroEL1	cg0691	Chaperonin cpn60	56.7	4.77	98	7.86
5	ClpB	cg3079	ATP-dependent protease	93.2	5.0	136	3.9–38.8
6	GrpE	cg3099	Molecular chaperone	23.6	4.12	88	4.2–14.0
7	PoxB	cg2891	Pyruvate quinone oxidoreductase (pyruvate oxidase)	61.9	5.20	177	

^a Only one protein was proposed by MASCOT software for each spot.

^b Peptidyl-prolyl *cis-trans* isomerase decreases after heat shock, whereas all other proteins increase in response to heat shock.

^c Mowse factor, significance level provided by MASCOT software (31).

^d Induction factor using microarrays (13, 29).

increased under heat shock conditions in the 13032B strain compared with results for the 13032L strain.

DISCUSSION

Heat shock results in changes in the transcriptional pattern of expression of a significant number of genes. Using the shotgun arrays approach and severe heat shock conditions (50°C, 7 min), Muffler and colleagues (29) observed several genes that showed a pattern of increased expression. The increased expression of the DnaK, GrpE, ClpB, and GroEL2 proteins observed in the proteome studies correlates perfectly with the changes in the transcriptional pattern of the *dnaK*, *grpE*, *clpB*, and *groEL2* genes detected by microarrays. In contrast, the overexpression of the *groEL1* gene, a well-known heat shock gene in *C. glutamicum* (3), was not detected using microarrays (29), suggesting that there is a problem with the expression of this gene in the strain used by these authors.

Two groups of genes have been identified as heat induced by microarray assays but not by proteome studies. The first one encodes the components of the Clp holoenzyme (*clpC*, *clpP1*, and *clpP2* genes), which responds to heat shock only under severe conditions (30 to 50°C), not under moderate stimuli (30 to 40°C), as described recently by Engels and colleagues (13). This differential response to heat shock typically has been described for gram-negative bacteria (41) and has been described for only one gram-positive bacterium, *Mycobacterium tuberculosis* (40). The results with *C. glutamicum* indicate that the differential response to severe heat shock also occurs in this gram-positive bacterium. These results suggest the existence of (i) a primary response of refolding the misfolded proteins by chaperones (e.g., DnaK and GroEL), since these genes do not show differential expression between 40 and 50°C (data not shown), and (ii) a drastic secondary response mediated by the ClpC and ClpP proteases.

A second group of heat-induced genes detected by microarray analysis that do not show large increases in the proteome analyses is involved in response to oxidative stress. This difference could be due to mechanisms of translational control that limit protein synthesis, even when the mRNA is overexpressed. In several studies the number of genes that respond to a given stress is always smaller in proteome analysis than in transcriptome studies (28). Alternatively, the difference may be due to the distinct induction conditions (7 min, 50°C) used in the

transcriptome analysis, since the high temperature and the short time could enhance the incomplete reduction of molecular oxygen by respiration and therefore the generation of a higher level of peroxide anions (29), switching on the oxidative response. In contrast, the proteome study was performed with longer induction periods and moderate temperature (40°C, 60 min), since these are the best conditions for long-term fermentations compatible with culture survival.

Our results showed that two proteins, DnaK and GrpE, which are translated from the same transcripts (*dnaK-grpE-dnaJ-hspR* and *dnaK-grpE* [3]), occur in very different amounts in the proteome (Fig. 1), indicating the existence of posttranscriptional controls affecting the synthesis or degradation of GrpE compared to that of DnaK. A similar observation has been described for the *groES-groEL* operon in *Agrobacterium tumefaciens* (33).

Interestingly, the proteome studies showed that the heat-induced GroEL1 protein is not present in the *C. glutamicum* 13032B strain. The absence of this protein was due to truncation of the *groES-groEL1* transcript as the result of the presence of an insertion sequence, IS*Cg1* (39), in the *groEL1* gene. This insertion sequence is present in four copies in the genome of *C. glutamicum* 13032B (20), but only three copies are present in the genome of the Kyowa ATCC 13032 strain. Since *C. glutamicum* 13032L and also the Kyowa strain (ATCC 13032, currently available from ATCC) show the presence of GroEL1, it is likely that the insertion of IS*Cg1* in *C. glutamicum* 13032B is a recent event, probably the result of laboratory replication of this strain. Differences in number and genomic position of a different insertion sequence, IS*Cg2*, have also been observed in *C. glutamicum*, since the IS*Cg2a* copy was present only in clones from a cosmid library but not in the sequenced genome (32). Transposition of IS elements was also observed when cultures of *Brevibacterium* (syn. *Corynebacterium*) *lactofermentum* were maintained in plates in our laboratory (8). The proteome analysis is, therefore, a simple and reliable tool for distinguishing null mutants that lack specific proteins.

The duplication of *groEL* (*hsp60*) genes occurs in many gram-positive bacteria (10, 15) as well as in other bacteria, in archaea, and in eukaryotic cell organelles (1). Duplication and transposition of the *groEL* genes is particularly interesting in the α -proteobacteria (21), where some species (e.g., *Bradyrhizobium japonicum*) contain some backup copies that are func-

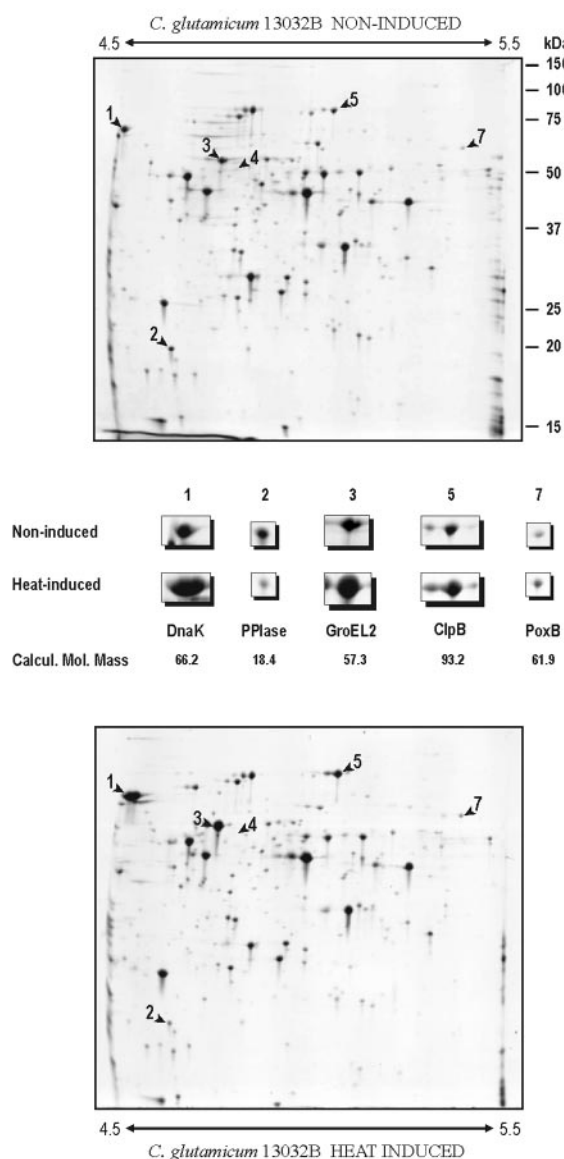


FIG. 3. Comparative 2D-gel analysis of heat shock-induced cytoplasmic proteins of *C. glutamicum* 13032B. A pH range of 4.5 to 5.5 was used for better resolution (shown by arrows). The proteins identified by MALDI-TOF PMF are shown enlarged in the area between the upper (control) and lower (heat-induced) panels together with the calculated molecular mass in kilodaltons (Calcul. Mol. Mass). Molecular sizes (in kilodaltons) are shown on the right. Note the absence of GroEL1 in *C. glutamicum* 13032B (arrowhead 4).

tionally exchangeable (14). In *C. glutamicum* the deletion of the *groEL1* gene causes only a small increase in the transcription of *groEL2*. This slight increment suggests that one copy is enough for survival or that each copy has different functions. In *Streptomyces albus* it has been impossible to delete the *groEL2* gene, whereas *groEL1* is dispensable (36). In *Mycobacterium smegmatis* the mycobacteriophage Bxb1, which integrates into the 3'-end of the *groEL1* gene, has never been found inserted into *groEL2*. In our work an analysis of the nucleotide sequence at the insertion site in *groEL1* in *C. glutamicum* revealed that *ISCg1* was integrated at a specific sequence, result-

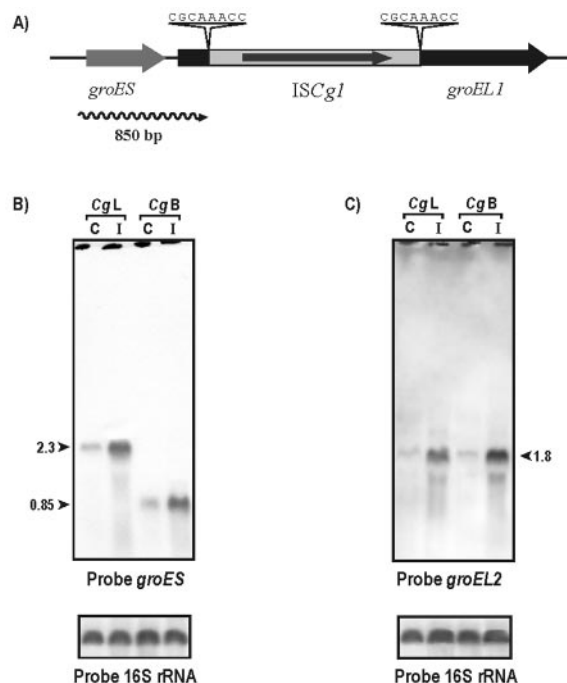


FIG. 4. (A) Organization scheme of the insertion element *ISCg1* in the *groEL1* region of *C. glutamicum*. The direct repetitions are shown, and the *groES* transcript in the mutant strain 13032B (850 bp) is indicated by a wavy line. (B) Northern analysis of the *groES-groEL1* operon in *C. glutamicum* 13032L (lanes Cg L) and 13032B (lanes Cg B) under normal conditions (C) (30°C) and heat-induced condition (I) (40°C, 40 min). Note that the 2.3-kb transcript is truncated to a size of 850 bp in *C. glutamicum* 13032B. (C) Northern analysis of the *groEL2* gene in *C. glutamicum* 13032L and 13032B under normal and heat-induced conditions. 16S rRNA was used as an amount control.

ing in duplication of the octanucleotide CGCAAACC at both ends of the insertion element. This octanucleotide does not occur in the *groEL2* gene, and therefore, the insertion is specific for *groEL1*.

Some microorganisms, such as *S. albus* and *M. tuberculosis*, present different functional motifs in the C-terminal ends of the HSP60 proteins (GGM for GroEL2 and multiple histidines for GroEL1) (21, 36). These motifs have been detected in the GroEL proteins from *C. glutamicum* (MGGMGGF for GroEL2 and HAGHHHH for GroEL1). These different motifs may explain distinct roles and interactions with proteins for GroEL1 and GroEL2. Moreover, there is no difference in the morphology or growth rate of the GroEL1-lacking *C. glutamicum* strain, although we cannot exclude that folding of some specific proteins may be affected by the lack of GroEL1.

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