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Cloning and Expression in *Escherichia coli* of the *dnaK* Gene of *Zymomonas mobilis*

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The DnaK protein of *Zymomonas mobilis* (DnaKz) was identified and found to be 80% identical to the DnaK protein of *Escherichia coli* on the basis of the sequence of the N-terminal 21 amino acids. The *dnaKz* gene was cloned and found to be expressed in a thermosensitive *dnaK* mutant of *Escherichia coli*. Expression of the foreign gene restored a thermoresistant phenotype but failed to modulate the heat shock response in *E. coli*.

The *Escherichia coli* heat shock protein (HSP) DnaK belongs to the most highly conserved HSP family in evolution, the HSP 70 (16). DnaK acts as a molecular chaperone (25), catalyzes ATPase activity (9), and is involved in thermotolerance (3, 8, 10, 13, 22). It also modulates the heat shock response of *E. coli* (23) by binding to the sigma^{32} transcription factor (15).

Synthesis of HSPs has also been shown to be stimulated in the ethanol-producing gram-negative bacterium *Zymomonas mobilis* upon heat or ethanol stress (18) or in the stationary growth phase (19).

As a molecular chaperone, DnaK would have to carry out specific interactions with diverse cellular proteins. However, at least some of these interactions might not be conserved among the HSP 70 family, since it has been reported that the *dnaK* gene of *Mycobacterium tuberculosis* cannot complement the *dnaK52* mutation of *E. coli* (3). This observation stimulated questions about the conservation of the DnaK functions in various bacterial species and led us to clone the *dnaK* gene of *Z. mobilis* in order to study its expression in a thermosensitive *dnaK* mutant of *E. coli*. In this report, we present some results which indicate that a DnaK-like protein from *Z. mobilis* can complement only some of the functions of the corresponding protein of *E. coli*.

As previously reported (18, 19), *Z. mobilis* cells increased the rate of synthesis of a set of proteins (stress proteins) upon heat or ethanol shock. The major stress proteins detected in our experimental conditions were designated C55, C68, D38, E18.5, and C14 (19). Among these proteins, C55 and C68 were thought to correspond to *E. coli* GroEL and DnaK, respectively, on the basis of their location on two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) maps. Proteins from heat-shocked *E. coli* (HB101) and *Z. mobilis* (ZM1) cells (bacterial strains used in this study are listed in Table 1) were separated by sodium dodecyl sulfate (SDS)-PAGE and blotted onto a nitrocellulose filter. Immunodetection was carried out with *Z. mobilis* antisera directed against stress proteins C55, C68, D38, and E18.5. Our results showed that C55 antiserum cross-reacted with a protein doublet in *E. coli* extracts (Fig. 1A). Since protein C55 (60 kDa) exhibited an electrophoretic mobility on SDS-PAGE similar to that of the immunodetected protein doublet (58 to 62 kDa) of *E. coli*, it was thought to correspond to GroEL (HSP 60). Moreover, appearance of GroEL as a protein doublet on immunoblots has been previously observed by other investigators (1). Immunodetection of proteins reacting with C55 antiserum was also performed after Western blotting (immunoblotting) of 2D gels loaded with a mixture of *Z. mobilis* and *E. coli* protein extracts. Two major immunoreactive proteins corresponding to C55 and to a more acidic protein were detected, showing that although these proteins exhibited a similar molecular mass, they were characterized by different isoelectric points (Fig. 1B). Therefore, we concluded that protein C55 was most probably the GroEL equivalent of *Z. mobilis* but that it differed from the protein of *E. coli* by its charge. No cross-reaction was observed in *E. coli* protein extracts with antisera against D.38 or E.18.5. The absence of immunoreactive response of DnaK with C68 antiserum was particularly surprising since this protein is known to be highly conserved throughout evolution (2, 12, 16). Thus, we assumed that although characterized by a similar molecular mass, C68 and DnaK proteins could differ by their amino acid composition or by their structural organization.

Regions of identity extend over large portions of DnaK proteins in species from bacteria to man (16), while the amino-terminal portion of the protein is much more highly conserved than the carboxy-terminal one (7). Therefore, in order to compare DnaK and C68 proteins, sequencing of the N-terminal end of C68 was carried out. C68 was purified according to a three-step electrophoretic procedure. Proteins from heat-shocked *Z. mobilis* ZM1 cells were separated by preparative SDS-PAGE, gel strips containing protein C68 were loaded onto isoelectric focusing gels, and proteins were separated by 2D-PAGE according to the method of O'Farrell (21). Then, the purified protein C68 was blotted onto a Glassy bond membrane (Biometra). N-terminal end sequence analysis of the blotted protein was carried out directly on the Glassy bond membrane by stepwise Edman degradation with a gas-phase sequencer. As shown in Fig. 2, a high degree of identity was observed for the DnaK protein of *E. coli* (80%) and the HSP 70 of *Drosophila melanogaster* (65%), strongly suggesting that the C68 protein of *Z. mobilis* is a DnaK-like protein (DnaKz) belonging to the HSP 70 family.

Further information was obtained by cloning the gene encoding protein DnaKz. *E. coli* RR1 cells were transformed with plasmids obtained from the genomic library of *Z. mobilis* ZM1 constructed in pUC19 as previously described (11). Transformants were screened for DnaKz production by colony blotting since the DnaKz antiserum was shown not to cross-react with *E. coli* proteins. Immunodetection revealed one clone which produced DnaKz (strain RR1 [pZK1]).

Plasmid pZK1 was extracted from the strain RR1 (pZK1)

TABLE 1. Bacterial strains, phage, and plasmids

Organism and strain, phage, or plasmid	Description or genotype
Strains	
Z. mobilis ZM1	ATCC 10988
E. coli HB101	*recA13 ara-14 proA lacY1 galK2 xyl-5 mtl-1, supE44 hsd20* ($r^- m^-$ *rpsL20*) (Sm^r)
E. coli RR1	*leu pro thi rpsL hsdR hsdM lacZ*Δ *M15* (F' $lacI^q$ *lacZ*Δ*M15 pro*$^+$)
E. coli CG333	*dnaK103 thr*::Tn*10* (obtained from C. Georgopoulos)
E. coli MC4100 (PR13)	*araD139* Δ(*lac*IPOZYA-*argF*) *rpsL thi* Mu c(Ts) PR13
E. coli MC1061	*araD139* (*ara, leu*) *lacX174 galU galK hsr rpsL*
Phage	
Mini-Mu PR13	MudII PR13 (Cm^r, *lac* transcription defective)
Plasmids	
pUC19	ColE1, Ap^r, ϕ80Δ*lacZ*
pBR322	Tc^r Ap^r, replicon ColE1
pZK1	Obtained by cloning a 10-kb fragment from ZM1 DNA into the *Bam*HI site of the multiple cloning sites of pUC19
pZK2	Obtained by subcloning a 3.5-kb *Sma*I-*Sal*I fragment of pZK1 into *Nru*I-*Sal*I sites of pBR322
pZK31, pZK32, pZK33, and pZK34	pZK2 with different mini-Mu insertions in the *dnaKz* gene

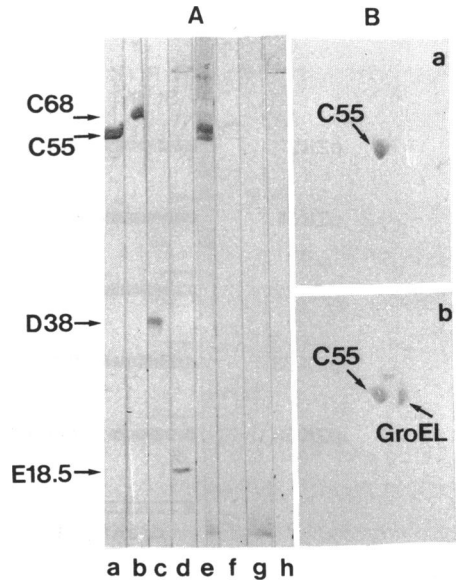

FIG. 1. Immunoblots of heat shock proteins from *Z. mobilis* and *E. coli*. (A) Immunoblot of cellular proteins separated by SDS-PAGE (50 μg of protein was loaded on the gels) from *Z. mobilis* (lanes a to d) or *E. coli* (lanes e to h). Lanes: a and e, C55 antiserum; b and f, C68 antiserum; c and g, D38 antiserum; d and h, E18.5 antiserum. (B) Immunoblot of cellular proteins separated by 2D-PAGE (20 μg of protein was loaded on isoelectric focusing gels). The immunoreaction was carried out with C55 antiserum and from *Z. mobilis* protein extract (a) or a mixture of *Z. mobilis* and *E. coli* protein extracts (b). Antisera were obtained as described by Diano et al. (5) from proteins purified by 2D-PAGE.

and used to transform a thermosensitive *E. coli* mutant (*dnaK103*) that was unable to form a colony at 43°C. Introduction of plasmid pZK1 into the strain carrying *dnaK103* restored colony formation on solid medium and growth in liquid medium at 43°C, showing that DnaKz can complement the *dnaK* mutation in *E. coli* (data not shown).

Regulation of the expression of the heat shock gene *dnaKz* cannot be studied by using a high-copy-number plasmid such as pUC19. However, it has been reported that cloning a suppressor gene involved in thermoresistance on a low-copy-number plasmid is not sufficient to fully rescue the thermosensitive phenotype of an *htrB* mutant of *E. coli* (14). Therefore, a 3.5-kb *Sal*I-*Sma*I fragment from plasmid pZK1 carrying the *dnaKz* gene was subcloned into the *Sal*I-*Nru*I sites of the multicopy plasmid pBR322 (plasmid pZK2; Table 1). In order to localize the structural gene and direction of transcription, the *dnaKz* gene was inactivated by insertion mutagenesis with mini-Mu PR13 (4) as previously described for the *phoA* gene of *Z. mobilis* (17). Plasmids were extracted from thermosensitive *dnaK103* transductants and transferred to strain MC1061 Δ*lac*. Mini-Mu insertions into the plasmid pZK2 (plasmids pZK31 to pZK34); (Table 1 and Fig. 3) and deletion experiments (data not shown) delimited a 1.8-kb region corresponding to the *dnaKz* gene. This result was in good agreement with the molecular mass of the protein (68 kDa). The gene was localized between the left *Hin*dIII site and the *Pst*I site of the insert and its promoter next to the left *Hin*dIII site (Fig. 3, plasmid pZK34).

In other respects, the Mini-Mu insertion did not greatly modify the plasmid copy number (estimated according to the method of O'Callaghan et al. [20]) since 50 to 55 copies were found in the *dnaK* (pZK2) strain and 35 to 40 copies were found in the *dnaK* (pZK31) transductant.

Cegielska and Georgopoulos (6) suggested that thermoresistance could be related either to the N-terminal end of DnaK or to a conformational change affecting the ATPase active site of the protein. Mini-Mu insertion between the central portion and the C-terminal end of the DnaKz protein from *Z. mobilis* (plasmid pZK32) restored thermosensitivity of the *dnaK103* mutant of *E. coli*. However, since no *dnaKz* gene product was identified with polyclonal antibodies by immunoblotting analysis (Fig. 3), a clear conclusion cannot be drawn from these results. Indeed, truncated products from the *Z. mobilis* gene could be rapidly degraded in *E. coli* or could have lost the antigenic moiety.

lac gene fusion to promoter from gene *dnaKz* allowed expression of *lac* genes under the control of that promoter with the formation of an active β-galactosidase. We thought that the pZK31 construction could be useful in the study of the regulation of *dnaKz* gene expression and could also help us to quantitate the enhancement of DnaKz protein synthesis under stress conditions. β-Galactosidase was assayed as

1																				21	
Met	Gly	Lys	Val	Ile	Gly	Ile	Asp	Leu	Gly	Thr	Thr	Asn	Ser	Val	Val	Ala	Val	Met	Gly	Gly	
*	*	*	Ile	*	*	*	*	*	*	*	*	*	*	Cys	*	*	Ile	*	Asp	*	(1)
*	Pro	*	Ala	*	*	*	*	*	*	*	*	Tyr	*	Cys	*	Gly	Val	Tyr	Gly	His	(2)

FIG. 2. N-terminal end sequencing of protein C68. The amino acids identical in sequences are shown by an asterisk (*). The *E. coli* (sequence labelled 1) and *Drosophila* (sequence labelled 2) HSP 70 sequences were obtained from Bardwell and Craig (2).

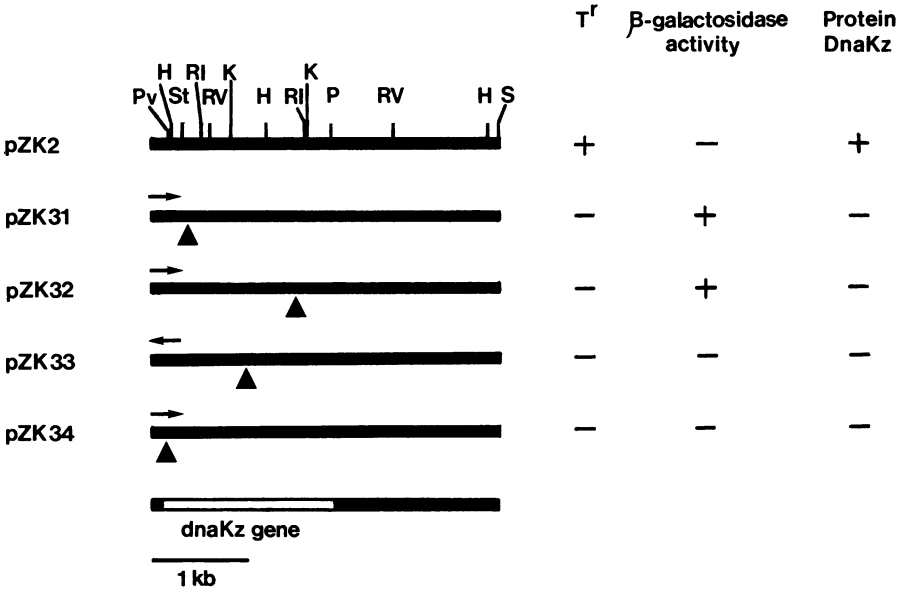

FIG. 3. Localization of the *dnaKz* structural gene and direction of transcription. Mini-Mu PR13 containing the *lac* operon structural genes was used for gene insertions and *lac* gene fusions. Only the ZM1 DNA inserted in pBR322 is shown. Restriction sites are indicated as follows: RI, *Eco*RI; RV, *Eco*RV; St, *Sst*I; S, *Sal*I; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II. ▲ indicates mini-Mu PR13 insertions. Thermoresistance (T^r), activity of hybrid β-galactosidase protein, and the presence of DnaKz (detected by immunoblot) are indicated. Arrows indicate the direction of transcription.

described by Wallenfels (24) in sonicated cell extracts. Our results with strain MC1061 (pZK31) showed that 5% ethanol (vol/vol) and heat (upshift from 30 to 45°C) enhanced β-galactosidase specific activity by a factor 4 (670 ± 40 U/mg and 613 ± 63 U/mg, respectively) compared with the control (170 ± 37 U/mg). No striking differences were observed between alcohol and heat stresses, suggesting that these stress agents caused the same stimulus that acted on the heat shock response. During growth, the expression of the enzyme remained relatively low and constant in unstressed cells, while it increased in ethanol-shocked cells up to 30 min after the alcoholic stress. Then, β-galactosidase activity tended to progressively abate (Fig. 4). Thus, the *dnaKz* gene is likely expressed in *E. coli* under the *rpoH* control.

dnaK mutants of *E. coli* have been previously shown to fail to turn off the heat shock response, even at the permissive temperature (23). Expression of the *dnaKz* gene in the mutant *dnaK103* suppressed thermosensitivity, showing that DnaKz can serve as a substitute for DnaK. We expected that transfer of the *dnaKz* gene into the *dnaK* mutant strain would also turn off the heat shock response at the permissive temperature. In this study, our attention was focused on the expression of two of the most important HSPs of *E. coli*: GroEL and DnaK. In $dnaK^+$ cells, temperature upshift increased synthesis of GroEL and DnaK (Fig. 5a and d). Overproduction of these proteins ceased when the temperature of the cells returned to 30°C (data not shown). As expected, GroEL was overproduced in the *dnaK* cells grown at the permissive temperature, showing that heat shock response was not turned off, even in unstressed conditions (Fig. 5b and e). However, GroEL overproduction was not lowered in the dnaK (pZK2) strain, demonstrating that DnaKz failed to efficiently turn off the heat shock response at the permissive temperature (Fig. 5c and f).

In conclusion, our results showed that some of the functions of DnaK, especially thermoresistance, can be carried out by DnaKz. In agreement with the results of Liberek et al. (15), our results suggest that the interaction between the *rpoH* product and DnaK depends on specific protein domains, since DnaKz cannot turn off the heat shock response in a *dnaK* mutant of *E. coli*. As described by Cegielska and Georgopoulos (6), DnaK is organized in several specific

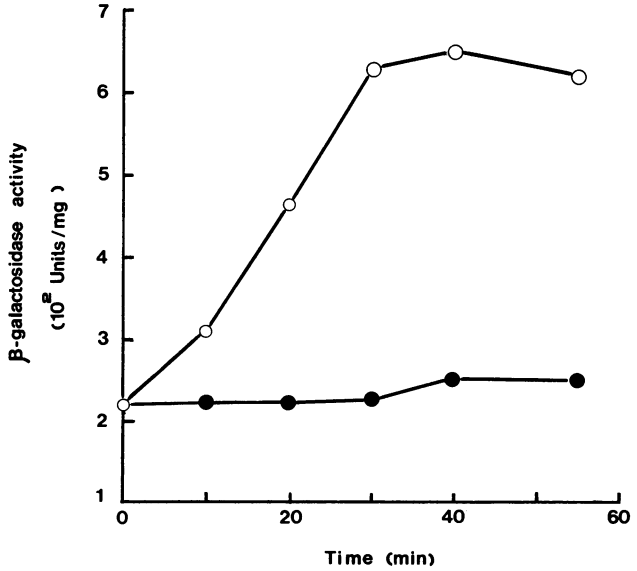

FIG. 4. β-Galactosidase expression in ethanol-stressed MC1061 (pZK31) cells during growth. Cells were grown at 30°C up to the mid-exponential growth phase, and 5% (vol/vol) ethanol (○) and 5% (vol/vol) water (control, ●) were added in separate cultures. Growth was carried on at 30°C, samples were withdrawn at time intervals, and β-galactosidase was assayed in sonicated cell extracts.

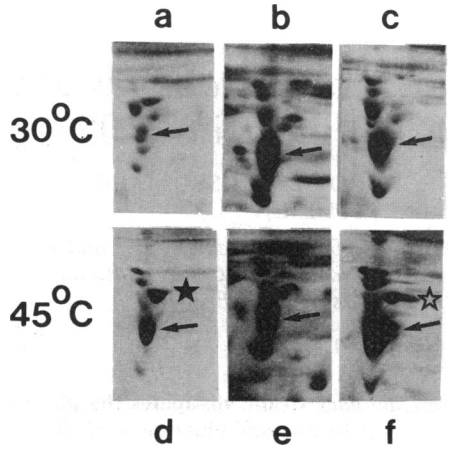

FIG. 5. Influence of protein DnaKz on the modulation of the heat shock response in the *dnaK103* mutant. *E. coli* cells were grown in M9 medium supplemented with 0.4% glucose (wt/vol), 1 μg of thiamine per ml, 20 μg of threonine per ml, and 10 mg of Difco methionine assay medium per ml (final concentrations). Proteins were radiolabelled with 25 μCi of [^{35}S]-Trans Label (specific activity, 1,120 Ci/mmol; ICN Biochemicals) per ml for 10 min. Labelling was stopped in ice by the addition of unlabelled methionine and cysteine solutions (1 mM, final concentration). (a, b, and c) Control cells grown at 30°C; (d, e, and f) cells radiolabelled 10 min after temperature upshift from 30 to 45°C; (a and d) *dnaK*$^+$ strain; (b and e) *dnaK* strain; (c and f) *dnaK* (pZK2) strain. ↙, GroEL; ★, DnaK; ☆, DnaKz. Isoelectric focusing gels were loaded with 20 μg of protein.

domains. It cannot be excluded, as shown by the absence of an immunological relationship between DnaKz and DnaK, that the two proteins differ by some domains and, thus, adopt different conformations. The quite unusual alcoholic environment of *Z. mobilis* might require a specific structural organization of the protein, but this structure could be inadequate for interaction with the *rpoH* product of *E. coli*. Furthermore, the failure of DnaKz to shut off the heat shock response in *E. coli* could also be due to a defective interaction with either GrpE or DnaJ, since DnaK functioning has been shown to depend on interaction with these HSPs (15). On the other hand, the thermoresistance function of DnaK would depend on less-specific protein domains since protein DnaKz can suppress thermosensitivity caused by the *dnaK* mutation.

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