

Improvement of Multiple-Stress Tolerance and Lactic Acid Production in *Lactococcus lactis* NZ9000 under Conditions of Thermal Stress by Heterologous Expression of *Escherichia coli* *dnaK*[▽]

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The effects of nisin-induced *dnaK* expression in *Lactococcus lactis* were examined, and this expression was shown to improve stress tolerance and lactic acid fermentation efficiency. Using a nisin-inducible expression system, DnaK proteins from *L. lactis* (DnaK_{Lla}) and *Escherichia coli* (DnaK_{Eco}) were produced in *L. lactis* NZ9000. In comparison to a strain harboring the empty vector pNZ8048 (designated NZ-Vector) and one expressing *dnaK*_{Lla} (designated NZ-LDnaK), the *dnaK*_{Eco}-expressing strain, named NZ-EDnaK, exhibited more tolerance to heat stress at 40°C in GM17 liquid medium. The cell viability of NZ-Vector was reduced 4.6-fold after 6 h of heat treatment. However, NZ-EDnaK showed 13.5-fold increased viability under these conditions, with a very low concentration of DnaK_{Eco} production. Although the heterologous expression of *dnaK*_{Eco} did not effect DnaK_{Lla} production, heat treatment increased the DnaK_{Lla} level 3.5- and 3.6-fold in NZ-Vector and NZ-EDnaK, respectively. Moreover, NZ-EDnaK showed tolerance to multiple stresses, including 3% NaCl, 5% ethanol, and 0.5% lactic acid (pH 5.47). In CMG medium, the lactate yield and the maximum lactate productivity of NZ-EDnaK were higher than the corresponding values for NZ-Vector at 30°C. Interestingly, at 40°C, these values of NZ-EDnaK were not significantly different from the corresponding values for the control strain at 30°C. Lactate dehydrogenase (LDH) activity was also found to be stable at 40°C in the presence of DnaK_{Eco}. These findings suggest that the heterologous expression of *dnaK*_{Eco} enhances the quality control of proteins and enzymes, resulting in improved growth and lactic acid fermentation at high temperature.

Lactococcus lactis is a lactic acid bacterium used in the dairy industry to produce lactic acid and to synthesize various minor fermentation compounds that contribute to flavor development. The species has already acquired the status of generally recognized as safe (GRAS), and when incorporated into food products, it is frequently exposed to different stress conditions, such as those induced by temperature, salt, acid, and oxidative species. Much effort has been made to overcome these stress problems in lactic acid bacteria, and this has involved the application of different strategies, such as the use of oxygen-impermeable containers, microencapsulation (15), addition of nutrients, use of stress-resistant strains (46), and preincubation under sublethal stress conditions (14, 15, 43). Molecular genetic and proteomic studies have indicated the involvement of some genes in the stress responses of lactic acid bacteria. These include the genes for the molecular chaperones GroESL and DnaK (18, 43, 54), methionine sulfoxide reductase (55), and

F₁F₀-ATPase (33), which are expressed in response to heat, oxygen, and acid stress, respectively.

Heat shock proteins (HSPs), which are also known as molecular chaperones, are induced when cells are exposed to environmental stresses (35, 40). These proteins are involved in the protection of other proteins from denaturation and the reactivation of denatured or aggregated proteins. DnaK, an HSP70 homolog in bacteria, is believed to serve as a “cellular thermometer” that transduces signals to other cellular factors in response to an increase in temperature (11). Although *hsp70* genes are widely conserved in all kingdoms, with the exception of some archaea, extensive functional studies have been carried out mainly with *Saccharomyces cerevisiae*, *Escherichia coli*, and, more recently, *Bacillus subtilis* (25, 42, 45). Research on *E. coli* molecular chaperones has revealed that DnaK functions in cooperation with DnaJ and GrpE and that this complex plays a significant role in the refolding of thermally damaged proteins, besides its role in assisting in the folding of nascent protein chains under normal growth conditions (7–10, 20, 30, 47, 57). *In vitro* analysis revealed that DnaK requires ATP for its chaperone activity and that this activity is regulated by DnaJ and GrpE (34). In addition, this complex also participates in ATP-dependent proteolysis in the cell (37). It has also been reported that *L. lactis*, similar to other bacteria, exhibits a heat shock response in which molecular chaperones play central

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roles (2, 29, 56). Previously, GroESL-overproducing *L. lactis* was reported to show improved tolerance to stresses induced by heat (54°C for 30 min), salt (5 M NaCl for 1 h), and solvent (0.5% butanol) (16). Fiocco et al. (18) also reported improved adaptation to heat and cold and tolerance to solvent in *Lactobacillus plantarum* by overproduction of small HSPs. Sugimoto et al. (48) earlier reported that the aggregation of proteins induced by 5% (0.86 M) NaCl could be suppressed remarkably by the overproduction of *Tetragenococcus halophilus* DnaK in *E. coli*. However, they did not further their studies by using their transformants in fermentation setups. Tomas et al. (52) reported that the overexpression of *groESL* in *Clostridium acetobutylicum* resulted in 38% and 30% increases in acetone and butanol production, respectively, relative to that in the plasmid control strain during pH-controlled glucose fed-batch acetone-butanol fermentation. In comparison to the plasmid-controlled strain, the *groESL*-overexpressing strain also showed increased tolerance to butanol. However, the effects of DnaK overproduction on cell growth and fermentation in lactic acid bacteria are still unclear.

In this study, we constructed an *E. coli dnaK* (*dnaK_{Eco}*)-expressing *L. lactis* NZ9000 strain (NZ-EDnaK) that exhibited improved tolerance to stresses induced by salt (3% NaCl), acid (0.5% lactic acid), solvent (5% ethanol), and heat (40°C). Most importantly, we found that the lactic acid fermentation efficiency of NZ-EDnaK was greatly improved at high temperature (40°C). To the best of our knowledge, this is the first report that describes the multiple positive effects of heterologous DnaK production on stress tolerance and lactic acid fermentation in lactic acid bacteria.

MATERIALS AND METHODS

Bacterial strains and media. *L. lactis* subsp. *cremoris* NZ9000 and *E. coli* JM109 were used throughout the study. *L. lactis* NZ9000 was grown in GM17 medium (M17 broth supplemented with 0.5% glucose) or CMG medium (0.5% polypeptone, 0.5% yeast extract, 0.5% NaCl, and 0.5% glucose) at 30°C, unless stated otherwise. *E. coli* JM109 was grown aerobically in Luria-Bertani (LB) broth at 37°C, unless stated otherwise. To facilitate clonal selection, 5 µg/ml chloramphenicol was added to the media.

Construction of *dnaK*-expressing *L. lactis*. Chromosomal DNAs were isolated from *E. coli* JM109 and *L. lactis* NZ9000 by a combination of two previously reported methods (4, 36). The *dnaK_{Eco}* gene was amplified from *E. coli* JM109 chromosomal DNA by use of primers 5'-CCCTATTAGGATCCACAACCA CATGATGACCGAATATAT-3' and 5'-GTCAGTATAATTACCGTTTATAGACTCTTATT-3'. The BamHI and SacI sites were simultaneously inserted into the amplified *dnaK_{Eco}* gene (underlined in the primer sequences). A BamHI site was inserted into plasmid pNZ8048 (13) by inverse PCR, using the primers 5'-CTAGAGAGCTCAAGCTTCTTTGAACCAAA-3' and 5'-TTTTGTGGA TCCTTTTGAACGAAATC-3'. The *dnaK_{Lla}* gene was amplified from *L. lactis* NZ9000 by using primers 5'-ATATTGACCGCATGGCTTTAACTATTC-3' and 5'-ACTGACGAAACGATGAGCTCTTTTAAAA-3'. The NcoI and SacI sites were simultaneously inserted into the amplified *dnaK_{Lla}* gene (underlined in the primer sequences). The amplified PCR products were purified with a QIAquick PCR purification kit (Qiagen, West Sussex, United Kingdom). All of the amplified *dnaK* genes and the plasmid were digested with their respective restriction enzymes. To insert *dnaK_{Lla}*, plasmid pNZ8048 was digested with NcoI and SacI. The enzymatically digested products were then ligated using a Ligation High v. 2 kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The resulting plasmids, which contained *dnaK_{Eco}* and *dnaK_{Lla}*, were named pNZ-EDnaK and pNZ-LDnaK, respectively. Both plasmids were then transformed into *L. lactis* NZ9000 (24), and the transformants were designated NZ-EDnaK and NZ-LDnaK, respectively. The empty plasmid pNZ8048 was also transformed into *L. lactis* NZ9000, and the transformant was named NZ-Vector.

Expression of *dnaK* in *L. lactis* and investigation of stress tolerance. The *L. lactis* strains NZ-Vector, NZ-EDnaK, and NZ-LDnaK (if required) were grown

in GM17 medium (10 ml) containing 5 µg/ml chloramphenicol at 30°C. When the optical density at 600 nm (OD_{600}) reached a value of 0.5 to 0.6, the expression of *dnaK* was induced by adding 10 ng/ml nisin (from *Streptococcus lactis*; Sigma-Aldrich, St. Louis, MO) and incubating the culture overnight. Aliquots of the overnight cultures ($OD = 1.4$ to 1.6) were then transferred to fresh GM17 medium (30 ml) containing 5 µg/ml chloramphenicol and 10 ng/ml nisin, such that the OD_{600} was 0.04 to 0.05. The new cultures were then treated with 3% NaCl, 5% ethanol, or 0.5% lactic acid at 30°C and incubated with shaking. Bacterial cell growth was monitored by measuring the OD_{600} . Growth under heat stress conditions (40°C) was also examined using the same method. To compare growth under stress with the growth under nonstress conditions, the strains were grown at 30°C in the presence of nisin; such conditions are referred to as control conditions throughout this work. To determine the effect of nisin on growth, the strains were also grown in the absence of nisin at 30°C and 40°C without prior nisin induction. All experiments were repeated at least three times.

Relative viability under heat stress. Viable cell counts at 30°C and 40°C were estimated by plating cells on GM17 agar plates for cultivation times of 0, 6, and 12 h for each condition. All experiments were repeated at least three times.

SDS-PAGE and Western blotting. To check the production levels of the DnaK proteins after induction using different nisin concentrations, overnight cultures (10 ml) were harvested by centrifugation at $6,000 \times g$ for 5 min at 4°C. Bacterial cells were resuspended in chilled 50 mM potassium phosphate buffer (pH 7.4) and disrupted using a Multi-Beads Shocker instrument (Yasui Kikai, Osaka, Japan) at 2,500 rpm for 1 min at 4°C; this process was repeated five times with 1-min intervals. Cell extracts were obtained by centrifuging the disrupted sample ($2,000 \times g$ for 15 min) to remove the cell debris. The protein concentration was determined using a Bradford assay kit (Nacalai Tesque, Kyoto, Japan). The soluble proteins (20 µg) were then subjected to 9% (wt/vol) SDS-PAGE, and the protein bands were either stained with Coomassie brilliant blue (CBB) R-250 or transferred to a polyvinylidene difluoride membrane. Immunoblotting and detection of DnaK proteins with anti-*T. halophilus* DnaK polyclonal antibody were performed using 10 µg of sample proteins, as described earlier (49). The two DnaK bands, which had a very small difference in molecular mass (4.2 kDa), were separated in a 12% (wt/vol) SDS-PAGE gel. The same amount of *E. coli* extract was also used as a positive control for DnaK_{Eco}.

Lactic acid fermentation analysis. To investigate the fermentation efficiencies of the strains, nisin-induced bacterial cultures were inoculated into CMG medium (30 ml) containing 0.5% glucose as the sole carbon source, 5 µg/ml chloramphenicol, and 10 ng/ml nisin. Fermentation was carried out at 30°C and 40°C without pH control, and each experiment was repeated at least three times. Bacterial growth was monitored by measuring the OD_{600} , and the values were then converted to dry cell weight by using a standard curve (1 OD_{600} unit = 0.294 g/liter of dry cell weight). The concentrations of lactic acid and glucose were assessed by high-performance liquid chromatography (HPLC) (US-HPLC-1210 chromatograph; Jasco, Tokyo, Japan).

Enzyme activity assays. After 6 and 12 h of fermentation in CMG medium, cell extracts were prepared using the above-mentioned method. Lactate dehydrogenase (LDH) activity in the extracts was assayed by the NADH-dependent reduction of pyruvate to lactate at 25°C. The assay mixture contained 100 mM Tris-HCl buffer (pH 7.4), 10 mM MgCl₂, 0.2 mM β-NADH, 1 mM fructose-1,6-diphosphoric acid trisodium salt octahydrate (FDP), and 5 mM sodium pyruvate. The reaction was initiated by adding 20 µl of the extract, and the decrease in OD_{340} was monitored. To determine the dependence of the LDH activity on FDP, a separate experiment was conducted using a reaction mixture that did not contain FDP. To determine the NADH oxidase activity, we used an assay mixture that contained only Tris-HCl buffer (pH 7.4) and the above-mentioned concentration of β-NADH. The NADH oxidase activity was also estimated by measuring the decrease in OD_{340} at 25°C, as in the case of LDH activity determinations. One unit of LDH activity was defined as the amount of enzyme required to convert 1 µmol of pyruvate to lactate per minute at 25°C, and 1 unit of NADH oxidase activity was defined as the amount required to catalyze the oxidation of 1 µmol of NADH to NAD⁺ per minute at 25°C.

RESULTS

Homologous and heterologous expression of *dnaK* in *L. lactis*. In this study, we examined the effects of nisin-induced DnaK production on stress tolerance and fermentation of *L. lactis*. Since the pNZ8048 vector contains the nisin-inducible promoter and the chloramphenicol resistance gene, *L. lactis* NZ9000 carrying only the pNZ8048 plasmid (NZ-Vector) was

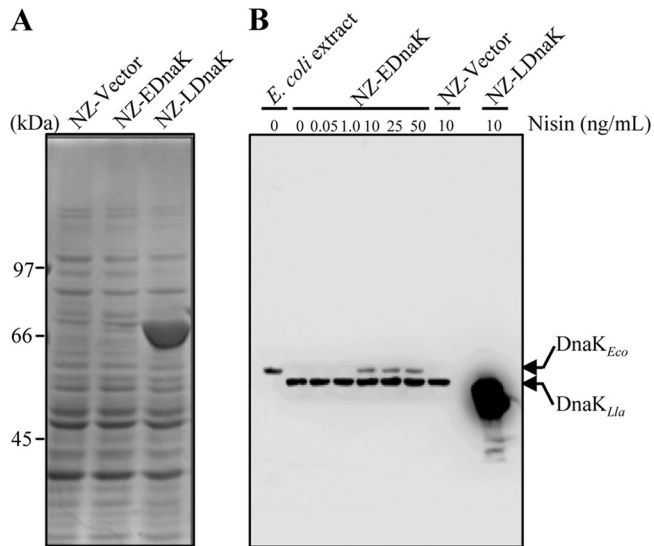


FIG. 1. DnaK production with nisin induction in *L. lactis*. (A) Cell extracts of NZ-Vector, NZ-EDnaK, and NZ-LDnaK were prepared from cultures that were induced overnight with 10 ng/ml nisin in M17 liquid medium supplemented with 0.5% glucose at 30°C. Protein samples were subjected to SDS-PAGE followed by CBB staining, as described in Materials and Methods. The positions of the molecular mass markers are shown on the left side of the panel. (B) Production of DnaK_{Eco} and DnaK_{Lla} was induced by the indicated concentrations of nisin. Proteins were separated by SDS-PAGE and subjected to Western blotting using the anti-DnaK antibody, as described in Materials and Methods. The same amount of *E. coli* extract was used as the amount of positive control for DnaK_{Eco}. Equal amounts of protein were used in all lanes. Arrows indicate the respective proteins.

used as the control strain instead of the plasmid-free strain. First, we checked the DnaK production level by CBB staining (Fig. 1A). In the presence of 10 ng/ml nisin, a concentration previously used to overproduce GroESL in *L. lactis* (16), a strong band corresponding to DnaK_{Lla} was visible upon CBB staining; however, no band corresponding to DnaK_{Eco} was detected (Fig. 1A). Therefore, DnaK_{Eco} production was followed at a different nisin concentration by Western blotting, a more sensitive method (Fig. 1B), using the anti-DnaK antibody (49). Since there is a difference in the molecular masses of DnaK_{Eco} (ca. 69.1 kDa) and DnaK_{Lla} (ca. 64.9 kDa), these proteins can be discriminated on the basis of the difference in their mobilities in an SDS-PAGE gel. In fact, the mobility of the signal from the *E. coli* extract was slightly greater than that for the signal from NZ-Vector (Fig. 1B). Notably, the nisin concentration did not affect the overnight level of DnaK_{Lla} in NZ-EDnaK. A weak signal corresponding to DnaK_{Eco} was detected beyond the signal of DnaK_{Lla} in the presence of 10 ng/ml nisin. However, in the presence of lower concentrations of nisin (0.05 to 1.0 ng/ml), no DnaK_{Eco} signal was observed (Fig. 1B), although the concentrations were sufficient to induce DnaK_{Lla} production in NZ-LDnaK (data not shown). In addition, use of much higher concentrations of nisin did not enhance the DnaK_{Eco} production level (Fig. 1B). Therefore, 10 ng/ml nisin was employed in subsequent experiments.

Improved heat stress tolerance of *dnaK_{Eco}*-expressing *L. lactis*. First, the effect of nisin on the growth of the three *L. lactis* strains was determined at a normal growth temperature (30°C) because nisin addition (10 ng/ml) was necessary for the production of plasmid-borne DnaK_{Eco} but had the potential to inhibit the growth of these strains. As summarized in Table 1,

TABLE 1. Generation times and maximum growth yields of *L. lactis* strains under nonstress and stress conditions

Condition(s)	Strain	Generation time (min) ^a	Generation time relative to that of NZ-Vector	Maximum OD ₆₀₀	Maximum OD ₆₀₀ relative to that of NZ-Vector
30°C, without nisin	NZ-Vector	25.4	1	1.67	1
	NZ-EDnaK	23.3	0.92	1.67	1
	NZ-LDnaK	21.5	0.85	1.65	0.99
30°C, with nisin	NZ-Vector	60.3	1	1.49	1
	NZ-EDnaK	24.5	0.45	1.67	1.12
	NZ-LDnaK	46.0	0.76	1.65	0.99
40°C, without nisin	NZ-Vector	39.33	1	1.2	1
	NZ-EDnaK	39.59	1.01	1.3	1.08
	NZ-LDnaK	37.00	0.94	1.3	1.08
40°C, with nisin	NZ-Vector	ND ^b	— ^c	0.16	1
	NZ-EDnaK	47.1	—	0.98	6.24
	NZ-LDnaK	218	—	0.76	4.85
3% NaCl, with nisin	NZ-Vector	137	1	1.08	1
	NZ-EDnaK	65.0	0.47	1.37	1.28
5% Ethanol, with nisin	NZ-Vector	150	1	1.07	1
	NZ-EDnaK	116	0.78	1.20	1.13
0.5% Lactic acid, with nisin	NZ-Vector	204	1	0.60	1
	NZ-EDnaK	78.5	0.39	0.86	1.44

^a Generation time was determined in the exponential growth phase.

^b ND, not detected (since the cells were not in exponential phase).

^c —, uncalculated.

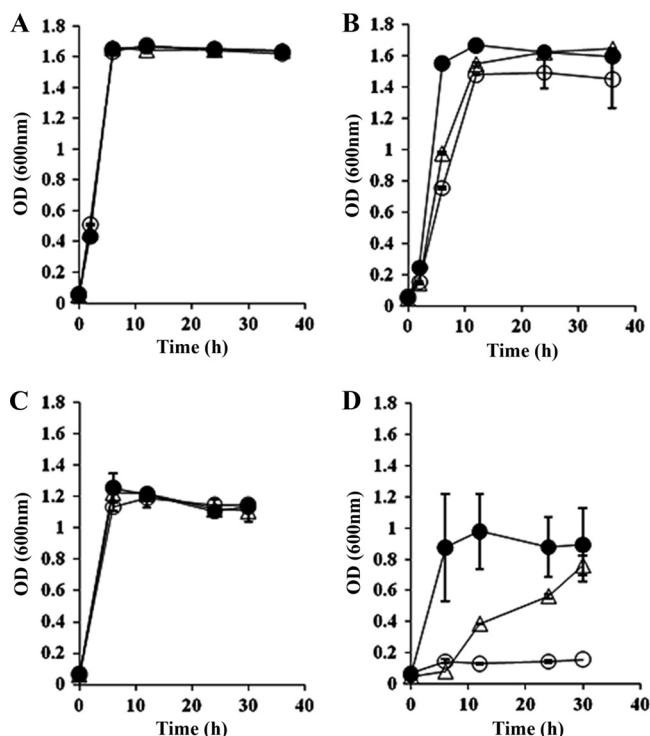


FIG. 2. Effects of heat and nisin on the growth of *L. lactis* strains. NZ-Vector (open circles), NZ-EDnaK (closed circles), and NZ-LDnaK (open triangles) were grown at 30°C in the absence of nisin (A), at 30°C in the presence of nisin (B), at 40°C in the absence of nisin (C), and at 40°C in the presence of nisin (D). Growth of the cultures was monitored by periodic measurement of the OD₆₀₀. The values shown are the means $\pm$ standard errors (error bars) for three independent experiments.

in the absence of nisin at 30°C, generation times and maximum OD₆₀₀ values for these strains were not significantly different. In contrast, in the presence of nisin, these values for NZ-Vector were 2.37-fold longer and 1.12-fold lower, respectively, than the corresponding values for the same strain in the absence of nisin. However, these values were not significantly different in the case of NZ-EDnaK. Although a 2.14-fold longer generation time was detected for NZ-LDnaK in the presence of nisin than that in the absence of nisin, the maximum OD₆₀₀ values were not different for the strain (Table 1). This could be attributed to the better maintenance of cellular proteins by DnaK_{Eco} and, to a lesser extent, by DnaK_{Lla}, which helped these strains to overcome the weak but significant stress arising from the presence of nisin.

Second, we wondered whether the production of DnaK_{Eco} and DnaK_{Lla} affects the heat stress tolerance of *L. lactis*. In the absence of nisin, all three strains gave reduced OD₆₀₀ values at 40°C (Fig. 2C) compared to those under control conditions. However, as expected, their growth patterns were similar (Fig. 2C) and showed similar generation times and maximum OD values (Table 1), since without nisin induction they acted to the heat stress similarly due to no expression of plasmid-borne *dnaK*. In the presence of nisin, although NZ-Vector was found to be very susceptible to a high temperature (40°C), NZ-EDnaK continued to grow and reached the stationary phase at 12 h (Fig. 2D). The maximum OD₆₀₀ of NZ-EDnaK was 6.24-fold

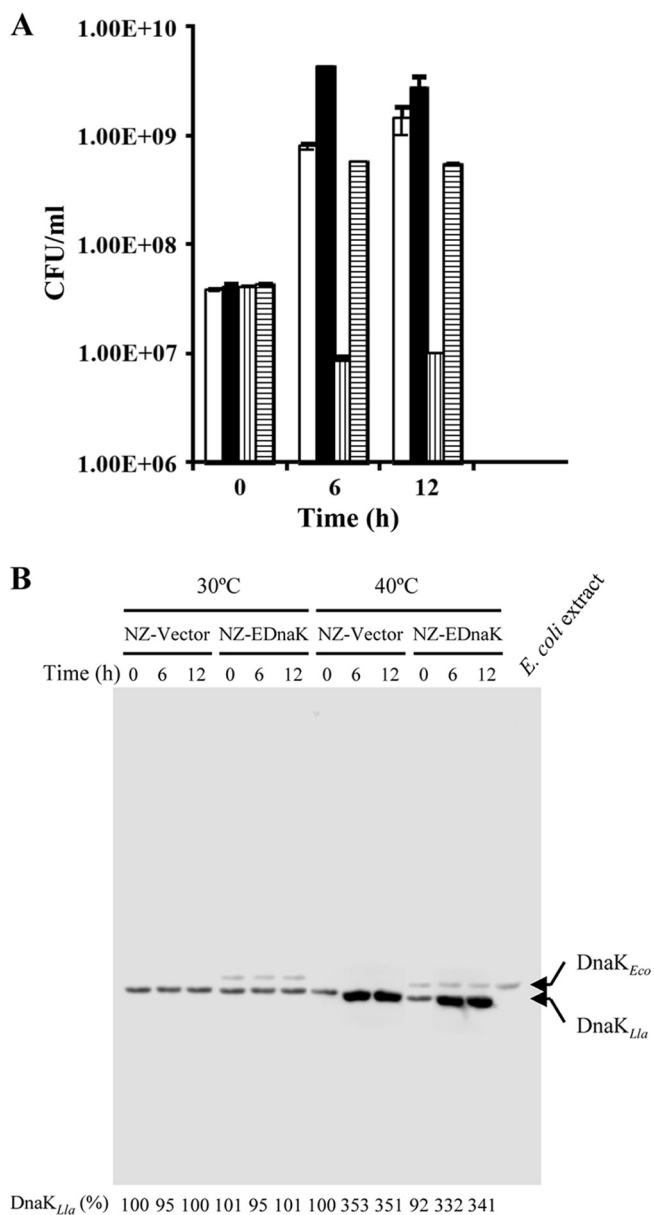
higher than the corresponding value of NZ-Vector (Table 1). In contrast, NZ-LDnaK exhibited less heat stress tolerance (Fig. 2D; Table 1), despite the fact that the DnaK_{Lla} level in NZ-LDnaK was much higher than those of DnaK_{Eco} and DnaK_{Lla} in NZ-EDnaK (Fig. 1). Since NZ-EDnaK showed more heat stress tolerance than NZ-LDnaK, we selected this strain for further study.

Third, we measured the viable cell counts of NZ-Vector and NZ-EDnaK at 30°C and 40°C (Fig. 3A). NZ-EDnaK showed slightly better viability than NZ-Vector at 30°C, which was consistent with the results from OD measurements (Fig. 2B). Under heat stress conditions at 40°C, the viable cell counts of NZ-Vector decreased 4.6-fold at 6 h and 4.1-fold at 12 h in comparison with the value at 0 h. However, the OD₆₀₀ values for NZ-Vector at 40°C showed increases of $\sim$ 2-fold after 6 and 12 h of incubation (Table 2), which can be explained by a very transient exponential phase of this strain followed by a viability reduction that started before 6 h of incubation. On the other hand, the viable cell counts of NZ-EDnaK increased (13.5-fold) during the first 6 h, and the viability was maintained in the subsequent 6 h, which supported the OD₆₀₀ values (Table 2). Western blot analysis revealed that individual DnaK molecules were produced at apparently similar levels at 30°C (Fig. 3B). In contrast, when the strains were exposed to heat stress at 40°C, the DnaK_{Lla} levels at 6 h were 3.5- and 3.6-fold higher in the case of NZ-Vector and NZ-EDnaK, respectively, while that of DnaK_{Eco} was unchanged in NZ-EDnaK. No significant increase in DnaK_{Lla} was found after DnaK_{Eco} production at both temperatures.

Multiple-stress tolerance of *dnaK_{Eco}*-expressing *L. lactis*. Lactic acid bacteria are exposed to several stresses, including lactic acid and alcohol, during lactic acid production and are exposed to salt stress during the preservation of cheese. In this study, we further investigated the tolerance of NZ-EDnaK to lactic acid, ethanol, and NaCl stresses.

In the presence of 3% NaCl, both NZ-Vector and NZ-EDnaK showed lower growth yields (OD₆₀₀ values) than those at 30°C in the presence of nisin (Fig. 2B), but the growth yield of NZ-EDnaK was higher than that of NZ-Vector (Fig. 4A). The generation time and maximum OD₆₀₀ for NZ-EDnaK were 2.11-fold shorter and 1.28-fold higher, respectively, than the corresponding values for NZ-Vector (Table 1). In comparison to growth under control conditions (Fig. 2B), ethanol stress also reduced the growth yields of both strains (Fig. 4B). In the presence of 5% ethanol, NZ-EDnaK showed a 1.29-fold shorter generation time and a 1.13-fold higher maximum OD₆₀₀ than those of NZ-Vector (Table 1). Lactic acid showed a more toxic effect on cell growth than that of ethanol (Fig. 4C). Similar to the other stress conditions mentioned above, under lactic acid stress conditions, NZ-EDnaK exhibited a 2.60-fold shorter generation time and a 1.44-fold higher maximum OD₆₀₀ than those of NZ-Vector. Based on these results, it can be concluded that the heterologous production of DnaK_{Eco} confers multiple-stress tolerance on *L. lactis*.

Lactic acid fermentation under normal and thermal stress conditions. Since the growth yield of NZ-EDnaK at 40°C was higher than that of NZ-Vector, the fermentation efficiencies of these two strains were compared in CMG medium enriched with 0.5% glucose at both 30°C and 40°C. CMG medium was used instead of GM17 medium because of its simple compo-



DnaK_{Lla} (%) 100 95 100 101 95 101 100 353 351 92 332 341

FIG. 3. Improvement of cell viability upon DnaK_{Eco} production at 40°C. (A) The viable cell counts of NZ-Vector and NZ-EDnaK at 30°C and 40°C were calculated at the indicated times. White bars, NZ-Vector at 30°C; black bars, NZ-EDnaK at 30°C; bars with vertical stripes, NZ-Vector at 40°C; bars with horizontal stripes, NZ-EDnaK at 40°C. The results are the averages for at least three independent experiments, and the error bars indicate the standard deviations. (B) The DnaK levels of NZ-Vector and NZ-EDnaK at 30°C and 40°C were determined by Western blotting. *E. coli* extract was used as the control for DnaK_{Eco}. Arrows indicate the respective proteins. The band intensities of DnaK_{Lla} were quantified by ImageQuant software (GE Healthcare). The intensity of the DnaK_{Lla} band of NZ-Vector at 30°C and 0 h was defined as 100%. Equal amounts of protein were used in all lanes.

sition, which allowed easy analysis of lactic acid fermentation. However, it should be noted that growth yields in CMG medium were found to be somewhat lower than those in GM17 medium. The study revealed that at both temperatures, the

growth patterns correlated with glucose utilization and lactic acid production (Fig. 5). At 30°C, NZ-EDnaK exhibited faster growth, glucose utilization, and lactic acid production than those of NZ-Vector (Fig. 5A). Expectedly, these differences were very significant at 40°C. The almost negligible growth of NZ-Vector resulted in little glucose utilization and lactic acid production (Fig. 5B). In addition, negligible lactic acid production was also confirmed by a slight change in pH at 40°C (Fig. 5B, inset).

We next analyzed the data to have a clear picture of the differences in the fermentation efficiencies of NZ-EDnaK and NZ-Vector at nonstress and stress temperatures. Comparison of the results shown in Fig. 5A and B revealed that growth, glucose utilization, and lactate production decreased when fermentation was carried out with NZ-EDnaK at 40°C; however, this decrease was found to be very minor in comparison to the efficiency of NZ-Vector. The important functionality of the DnaK protein was exhibited when the bacterial culture was subjected to a high temperature. In this case, maintenance of the stability of proteins required for growth and fermentation was reflected by the comparatively higher maximum lactate production level, lactate yield, maximum lactate productivity, and specific lactate productivity (Table 3). Although there were no significant differences in these fermentation parameters in the case of NZ-Vector and NZ-EDnaK at 30°C, NZ-EDnaK gave 12.0-, 4.76-, 11.8-, and 1.84-fold increased maximum lactate production, lactate yield, maximum lactate productivity, and specific lactate productivity, respectively, at 40°C (Table 3). These differences were expected, since the growth of NZ-Vector was ceased. More interestingly, lactate yield, maximum lactate productivity, and specific lactate productivity of NZ-EDnaK at 40°C were not significantly different from the corresponding values of NZ-Vector at 30°C. The very low lactate yield obtained with NZ-Vector at 40°C can be explained by the metabolic changes in the lactic acid fermentation pathway. Although no peaks were detected for acetate or formate in the HPLC analysis, these changes probably led to the production of other fermentation end products, such as acetoin, ethanol, diacetyl, butanediol, etc. Therefore, we concluded that in the case of NZ-EDnaK, heterologous production of DnaK_{Eco} confers increased heat stress tolerance and leads to enhanced fermentation performance.

LDH and NADH oxidase activity. To further analyze the improved fermentation resulting from the expression of *dnaK_{Eco}*, we investigated the activity of LDH (Table 4). The LDH activity was found to be higher in NZ-EDnaK than in

TABLE 2. OD₆₀₀ values and viable cell counts for *L. lactis* strains at 40°C

Strain	Incubation time (h)	OD ₆₀₀	OD ₆₀₀ relative to that at 0 h	Viable count (CFU/ml)	Viable count relative to that at 0 h
NZ-Vector	0	0.067	1	4.02E+07	1
	6	0.145	2.16	8.71E+06	0.22
	12	0.131	1.96	9.81E+06	0.24
NZ-EDnaK	0	0.053	1	4.16E+07	1
	6	0.877	16.55	5.61E+08	13.49
	12	0.979	18.47	5.31E+08	12.76

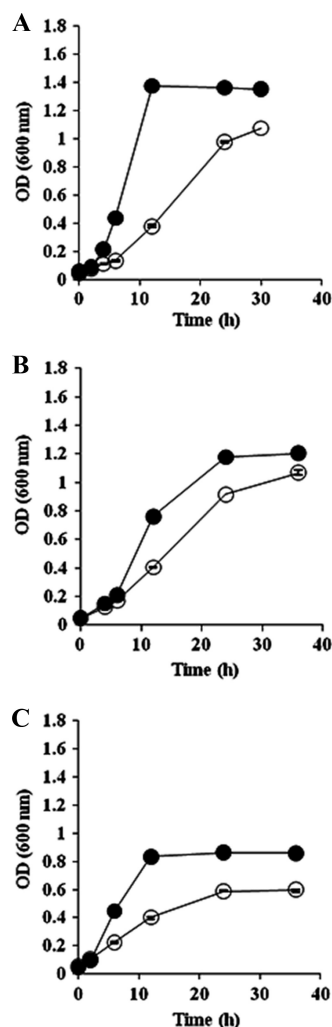


FIG. 4. Production of DnaK_{Eco} confers multiple-stress tolerance on *L. lactis*. NZ-Vector (open circles) and NZ-EDnaK (closed circles) were grown in the presence of 3% NaCl (A), 5% ethanol (B), and 0.5% lactic acid (C). In all cases, the cultures were grown in GM17 liquid medium supplemented with 10 ng/ml nisin at 30°C without pH control. Cell growth was monitored by periodic measurement of the OD₆₀₀. The values shown are the means $\pm$ standard errors (error bars) for three independent experiments.

NZ-Vector under both stress and nonstress conditions, indicating better protein quality control in the presence of DnaK_{Eco}. Cell viability reduction of NZ-Vector under heat stress conditions (Fig. 3A) correlated with its low LDH activity. The LDH activity of NZ-Vector at 40°C was found to be approximately 2.5-fold lower than that at 30°C. However, the LDH activity of NZ-EDnaK at 40°C was only slightly lower than that at 30°C. Moreover, the LDH activity of NZ-EDnaK at 40°C was not significantly different from that of the control strain at 30°C, suggesting a stabilizing effect of DnaK_{Eco} on the enzyme at high temperature. We also detected the presence of NADH oxidase activity in the cell extracts. This enzyme might have produced a false result by oxidizing NADH, which would lead to a decrease in the OD₃₄₀. Very low NADH oxidase activities were detected in both strains at 30°C and 40°C, which nullified the effect of NADH activities on LDH activities. Sim-

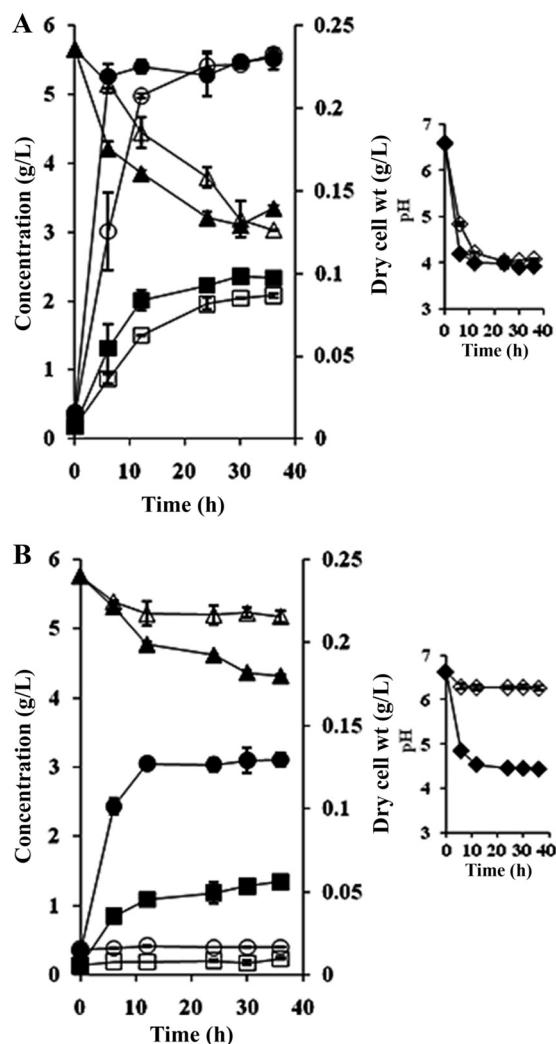


FIG. 5. Production of DnaK_{Eco} improves lactic acid fermentation by *L. lactis*. Lactic acid fermentation was carried out at 30°C (A) and 40°C (B). NZ-Vector (open symbols) and NZ-EDnaK (closed symbols) were grown in CMG medium containing 0.5% glucose as the sole carbon source, without pH control. Growth (circles) was monitored by measuring the OD₆₀₀ and is shown in the graph as dry cell weight after conversion. Glucose utilization (triangles) and lactic acid production (squares) were assessed by HPLC. Changes in the pH (diamonds) were also estimated and are shown in the inset. All values shown are the means $\pm$ standard errors (error bars) for three independent experiments.

ilar to other reports on the LDHs of *L. lactis* and some other bacteria (1, 12, 41), we also found that the LDH activity was FDP dependent in the cell extracts (Table 4).

DISCUSSION

In this study, both *dnaK_{Eco}* and *dnaK_{Lla}* were successfully expressed in *L. lactis* NZ9000, despite the difference in required nisin concentration for their expression. Differences in codon usage between *L. lactis* and *E. coli* might be the reason for the differences in the production levels, since research on the relationship of codon usage and protein expression revealed that codon biases might have significant impacts on the expression of heterologous proteins (22, 28). Based on the observation that NZ-LDnaK showed higher growth yields than

TABLE 3. Fermentation parameters for lactic acid fermentation by NZ-Vector and NZ-EDnaK in CMG medium without pH control^a

Temp (°C)	Strain	Maximum amt of lactate produced (g/liter)	Lactate yield (g/g consumed glucose)	Maximum lactate productivity (g/liter/h)	Specific lactate productivity (g/g/h)
30	NZ-Vector	1.91 ± 0.021	0.726 ± 0.010	0.116 ± 0.011	0.931 ± 0.091
	NZ-EDnaK	2.19 ± 0.035	0.935 ± 0.042	0.190 ± 0.057	0.863 ± 0.229
40	NZ-Vector	0.100 ± 0.001	0.174 ± 0.025	0.0100 ± 0.002	0.628 ± 0.132
	NZ-EDnaK	1.20 ± 0.113	0.829 ± 0.066	0.108 ± 0.015	1.16 ± 0.097

^a Values are averages ± standard errors for at least three independent experiments.

NZ-Vector under heat stress conditions (Fig. 2D), we concluded that although the DnaK_{Lla} level was high, it had no inhibitory effect on *L. lactis* growth. This result was in contrast to that reported for DnaK_{Eco} overproduction in *E. coli*, where *E. coli* growth was hampered (6).

We compared the heat stress tolerances of the NZ-Vector, NZ-LDnaK, and NZ-EDnaK strains. Although overnight nisin-induced cultures were used throughout the study, a 2-h nisin-induced culture of NZ-EDnaK showed a similar level of stress tolerance (data not shown). Furthermore, once the cultures of *L. lactis* strains, including the control, were grown under nonstress conditions up to mid-log phase (OD₆₀₀ of ~0.6), they showed similar growth under heat stress conditions at 40°C (data not shown). These phenomena can be explained by a growth-phase-dependent sensitivity to stresses. Duwat et al. (17) observed that *L. lactis* MG1363 showed more thermosensitivity during lag or early exponential phase (OD₆₀₀ of ~0.3) than during mid- or late exponential phase (OD₆₀₀ of 0.5 to 1.0). Growth-phase-dependent switching of gene expression would be related to the growth-phase-dependent stress resistance. Interestingly, in *Lactobacillus plantarum*, production of stress-related proteins, including GroEL, ClpC, ClpE, ClpL, and small HSPs, is significantly stimulated in the lag phase, which represents the transition from late stationary phase to new active growth (32). Thus, lactic acid bacteria seem to have an activated stress response when introduced into fresh media. In the case of our study, *L. lactis* strains likely required a greater activated stress response for their survival when they were introduced into fresh medium in the presence of heat and nisin stresses. In this sense, nisin-induced DnaK might have some effects on global regulatory factors required for expression of stress-related genes in lag phase, leading to improved stress tolerance. In *E. coli* and *B. subtilis*, the general stress response has been reported to be controlled by σ^{32} and σ^B , respectively, under different stress conditions (3, 23). However, in lactic acid bacteria, no homologue of σ^B has been identified

(50), and the regulation of the general stress response of these bacteria has yet to be clarified. DnaK was found to be involved in increasing the expression of heat shock proteins in a *dnaK* mutant of *L. lactis* (31). However, in this study, we found that DnaK_{Eco} did not induce the production of DnaK_{Lla} (Fig. 3B). This result further indicated that heterologous expression of *dnaK_{Eco}* at least did not affect the regulatory systems that regulate *dnaK_{Lla}* expression, leading to improve heat tolerance. Therefore, we concluded that the chaperone activity of DnaK_{Eco} was responsible for the increased heat stress tolerance.

The differences in the responses of heterologous and homologous DnaK proteins to enhanced stress tolerance (Fig. 2) might be due to differences in their chaperone activities. Zmijewski et al. (58) showed different substrate specificities of the DnaKs of *E. coli* and an archaeal organism, *Methanosarcina mazei*. Sugimoto et al. (49) also reported functional differences in *E. coli* and *T. halophilus* DnaKs, both *in vitro* and *in vivo*. Our study further suggests that there are differences in chaperone activities of different DnaKs, since heterologous production of only a low concentration of DnaK_{Eco} (Fig. 3B) conferred a dramatic effect on multiple-stress tolerance in *L. lactis*.

We demonstrated that DnaK_{Eco} conferred tolerance to nisin, which was used in this study for induction of DnaK. This antimicrobial peptide is known to affect the cell membranes of Gram-positive bacteria having the target site of lipid II (21, 44). We found that the combined effect of nisin and heat was much higher than the single effects of either of these stresses (Fig. 2). Increased sensitivities to nisin were reported earlier for heat-stressed *Bacillus cereus* and *L. lactis* (5, 26, 27). It was suggested that the presence of nisin prevented the repair of heat-damaged membranes. In this study, the synergistic action of heat and a very low concentration of nisin (10 ng/ml) probably showed effects on the cell viability of NZ-Vector because of the presence of some additional stressors, i.e., low pH due to lactic acid production, antibiotic, and high-copy-number plas-

TABLE 4. Effects of DnaK_{Eco} production on enzyme activity^a

Temp (°C)	Strain	LDH activity (U/mg protein)				NADH oxidase activity (U/mg) after 6 h of fermentation
		After 6 h of fermentation		After 12 h of fermentation		
		With FDP	Without FDP	With FDP	Without FDP	
30	NZ-Vector	6.92 ± 0.823	0.582 ± 0.028	6.40 ± 0.539	0.585 ± 0.081	0.0164 ± 0.001
	NZ-EDnaK	8.85 ± 1.79	0.782 ± 0.085	7.16 ± 0.369	0.635 ± 0.066	0.0275 ± 0.002
40	NZ-Vector	2.73 ± 0.394	0.192 ± 0.044	2.89 ± 0.227	0.121 ± 0.001	0.0119 ± 0.01
	NZ-EDnaK	6.18 ± 0.001	0.413 ± 0.018	6.23 ± 0.804	0.555 ± 0.009	0.0113 ± 0.004

^a Values are averages ± standard errors for at least three independent experiments.

mids. However, DnaK_{Eco} had the potential to rescue the growth of NZ-EDnaK.

L. lactis possesses a mechanism of accumulation of compatible solutes to avoid osmotic pressure (39). Meury and Kohiyama (38) reported that a *dnaK* mutant of *E. coli* was impaired in K⁺ accumulation and deplasmolysis followed by NaCl addition, suggesting the involvement of DnaK in the process. Overexpression of *groESL* was reported to enhance salt tolerance in *L. lactis* (16). In the present study, the salt tolerance conferred by DnaK also suggests the involvement of the chaperone protein in a similar type of protection.

The increases in maximum lactic acid production, lactate yield, and maximum lactate productivity at 30°C (Table 3) can be explained on the basis of the better maintenance of enzymes related to growth and fermentation due to the production of DnaK_{Eco}. The high specific productivity of lactic acid in NZ-EDnaK at 40°C (Table 3) indicated that the increase in lactic acid production was not only due to the high growth yield of bacterial cells but also a result of the improved lactic acid production efficiency of individual cells in the fermentation medium at this temperature. Improved growth and lactic acid production of NZ-EDnaK can also be explained by the enhanced tolerance of the strain to lactic acid (Fig. 4C). Another reason is that the enzymes involved in lactic acid fermentation are well maintained by DnaK_{Eco}. LDH denaturation under stress conditions has already been reported in a study by Gosslau et al. (19), where LDH was studied as a representative denatured protein under oxidative and heat stress conditions. In this study, the high stability of LDH in NZ-EDnaK at 40°C further demonstrated that DnaK_{Eco} production led to better maintenance of proteins and enzymes with respect to growth and lactic acid fermentation.

Finally, the approach used in this study, i.e., heterologous expression of molecular chaperone genes, is considered one of the most promising approaches for constructing stress-tolerant microorganisms. The approach can have important implications in fermentation technology, as it can lead to reductions in the cost of temperature maintenance in bioreactors. Increasing the tolerance to ethanol and lactic acid is also a possible approach for development of lactic acid bacteria that can improve the low fermentation yields resulting from growth inhibition when these products are released into the fermentation medium. Not considering restrictions on the use of genetically modified strains, the application of DnaK_{Eco}-expressing lactic acid bacteria can solve several problems in the food and cheese industry due to the improved salt tolerance of the strain. The knowledge can also be applied to developing robust lactic acid bacteria involved in lactic acid production for other industrial applications, such as production of poly-L-lactic acid (bioplastic), where the application of genetically modified organisms would not be restricted. The knowledge of improved tolerance conferred by GroESL and small HSPs can be combined with the present study for development of multichaperone overexpression systems to generate stronger lactic acid bacteria.

However, this study had certain drawbacks, such as the presence of two DnaKs in the transformant and of combinations of various stresses in the system, such as those induced by pH, plasmid, nisin, and antibiotic. A pH-controlled fermentation system using a strain with chromosomally inserted *dnaK*_{Eco}

would provide a better example of the increase in fermentation efficiency.

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