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Starvation-Induced Stress Resistance in *Lactococcus lactis* subsp. *lactis* IL1403

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Carbohydrate-starved cultures of *Lactococcus lactis* subsp. *lactis* IL1403 showed enhanced resistance to heat, ethanol, acid, osmotic, and oxidative stresses. This cross-protection seems to be established progressively during the transitional growth phase, with maximum resistance occurring when cells enter the stationary phase. Chloramphenicol or rifamycin treatment does not abolish the development of a tolerant cell state but, on the contrary, seems to provoke this response in *L. lactis* subsp. *lactis*.

Stationary phase due to nutrient limitation corresponds to usual conditions that microorganisms encounter in their natural environments. Some bacteria respond to starvation by forming endospores which are able to survive under these deprivation conditions. On the other hand, the nondifferentiating bacteria do not show such drastic morphological changes upon starvation. Nevertheless, these organisms show some characteristics in common with the true differentiating bacteria. They are capable of developing a resistant cell state which allows survival for long periods under starvation conditions and withstanding of multiple environmental stresses (3, 7, 8, 16). Adaptation to starvation conditions is accompanied by changes in cell size (6, 15; unpublished observation) and fatty acid composition (19, 23), decrease in the overall rate of protein synthesis, and induction of distinct sets of proteins (4, 14, 17, 21, 22). These proteins are essential for effective long-term survival (18) and for cell tolerance against environmental challenges (8, 16). Furthermore, some of the starvation proteins are proteins synthesized in response to heat shock or oxidative or osmotic stress (7, 8). The major regulator of the general starvation response has been identified for *Escherichia coli* and is the product of the *katF* gene, which encodes a specific sigma factor named RpoS or σ^S (5, 11).

Lactococcus lactis subsp. *lactis* (20), formerly *Streptococcus lactis*, a species widely used in the dairy industry and other food fermentations (13), is of interest because of its dual potential for producing lactic acid and aroma molecules. This species is well known to be subjected, in the industrial process, to temperature shifts (e.g., pasteurization), so we have previously characterized the general properties of the heat shock response of *L. lactis* (2). Because the food ecosystem is also impacted by nongrowing bacteria, we have initiated studies aimed at increasing our fundamental knowledge about the physiological and biochemical responses in starved *L. lactis* subsp. *lactis*. If greater genetic analysis of this bacterium is to be achieved, a more complete understanding of its physiology must be obtained.

We report here that *L. lactis* subsp. *lactis* IL1403 developed strong cross-protection against heat, ethanol, acid, osmotic, and oxidative challenges when the growth rate became slower because of exhaustion of the carbohydrate source and that chloramphenicol or rifamycin treatment does not abolish but, on the contrary, seems to provoke this response in this strain.

Bacterial strain, culture conditions, and starvation protocol. The present study was performed with *L. lactis* subsp. *lactis* IL1403 (lactose-, protease-, restriction-, and modification-negative) obtained from the parental strain IL594 (1, 12). Cultures were grown at 30°C without shaking in 20-ml glass tubes containing 10 ml of M17 medium, pH 7.4 (24), supplemented with 0.1% glucose. Preliminary growth yield studies using different concentrations of glucose have led to the choice of 0.1% glucose to ensure that exhaustion of glucose triggered transition to the stationary phase. Cultures entered stationary phase under these conditions at an optical density at 600 nm (OD_{600}) of 1.2 (about 5×10^8 CFU/ml). The pH of the medium at the onset of starvation was 6.8. The starving culture

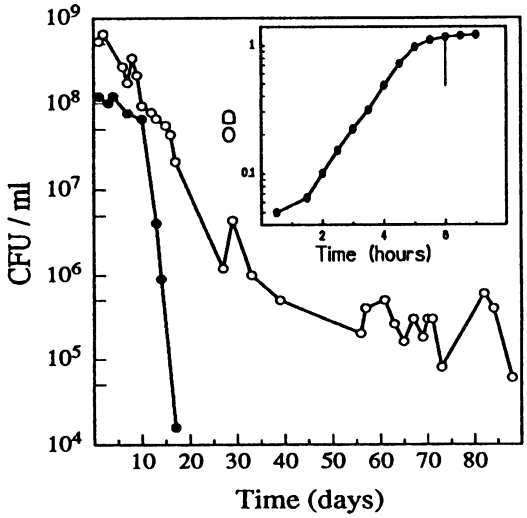

FIG. 1. Changes in survival of *L. lactis* subsp. *lactis* IL1403 in the stationary phase (○). ●, culture treated at the onset of starvation with chloramphenicol (100 μg/ml). A typical growth curve (OD_{600}) of *L. lactis* subsp. *lactis* IL1403 in M17 medium supplemented with 0.1% glucose is shown in the inset. The time point in the growth curve which we have defined as the onset of stationary phase is indicated (vertical line) in the inset.

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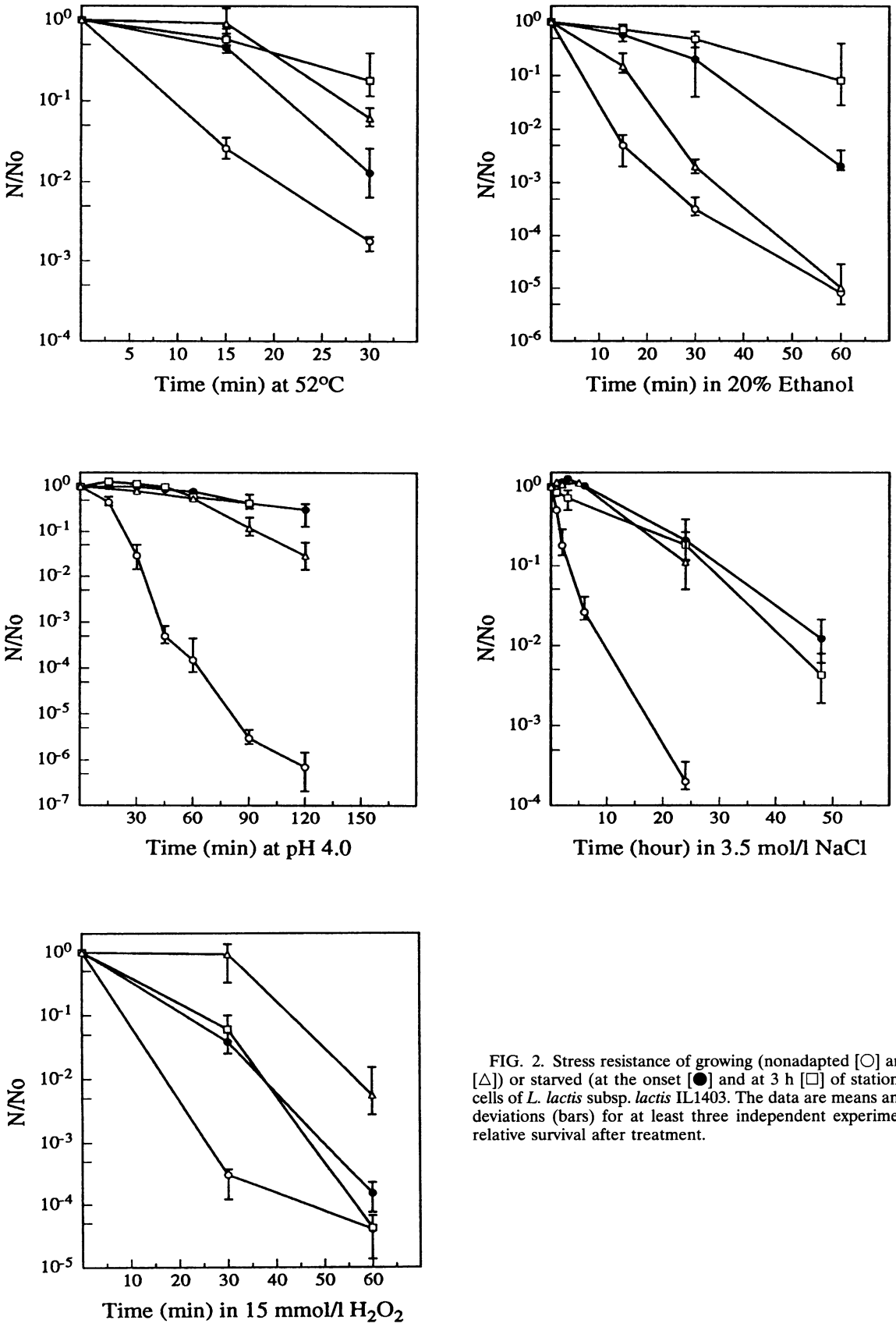

FIG. 2. Stress resistance of growing (nonadapted [○] and adapted [△]) or starved (at the onset [●] and at 3 h [□] of stationary phase) cells of *L. lactis* subsp. *lactis* IL1403. The data are means and standard deviations (bars) for at least three independent experiments. N/No, relative survival after treatment.

continued to be incubated under these conditions and was sampled at appropriate intervals.

Adaptation conditions. Cultures were grown as described above, and cells in the exponential growth phase were harvested at an OD_{600} of 0.4 and resuspended in fresh medium supplemented with 0.1% glucose. Adaptation was performed by exposure of cultures to a nonlethal level of a given stress agent before exposure to a challenge dose of the same stress. Adaptation treatments were conducted as follows: (i) 42°C, (ii) 4% ethanol at 30°C, (iii) pH 5.5 at 30°C, (iv) 0.35 M NaCl at 30°C, and (v) 1.5 M H_2O_2 at 30°C. In all cases the time of adaptation was 30 min. Preliminary experiments have shown that these conditions are optimal for conferring protection. Cells from the exponential growth phase (OD_{600} of 0.5, corresponding to about 2×10^8 CFU/ml) were used as a control.

Challenge conditions. Control (exponential-growth-phase), adapted, and starved cells were harvested by centrifugation, washed in 0.9% NaCl, and adjusted in fresh M17 medium without glucose to an OD_{600} of 1.0. Ten milliliters of each culture received one of the following treatments: (i) 52°C, (ii) 20% (vol/vol) ethanol, (iii) pH 4.0 (adjusted with concentrated lactic acid), (iv) 3.5 M NaCl (added in solid form), and (v) 15 mM H_2O_2. The temperature for treatments ii to v was 30°C. For experiments with chloramphenicol or rifamycin (rifampin; Sigma Chemical Co.), cells were incubated for 30 min with 100 or 75 μg of the antibiotic per ml, respectively, prior to challenge. Samples were immediately diluted in 0.9% NaCl after various periods of treatment and poured in M17 agar (Difco) (1.5%, wt/vol) supplemented with 0.5% glucose. CFU were determined after 48 h of incubation at 30°C.

Survival of *L. lactis* subsp. *lactis* in the stationary phase. Figure 1 shows the changes in survival of a culture of *L. lactis* subsp. *lactis* IL1403 in the stationary phase established by energy starvation (glucose deprivation) in M17 medium. After an initial decrease in survival during the first 40 days of approximately 3 orders of magnitude, the culture stabilizes at a density of 10^5 to 10^6 CFU/ml for up to 3 months. This shows that this strain is able to survive long periods of glucose deprivation. Treatment of the culture with 50 μg of chloramphenicol per ml at the onset of the stationary phase results in a rapid decrease in viable counts (Fig. 1). The same treatment after 3 h of carbohydrate deprivation has a lesser influence and results in survival comparable to that observed without chloramphenicol (data not shown). This suggests that protein synthesis is necessary for longevity of the culture and synthesis takes place in the first 3 h after the onset of starvation.

Starvation-induced cross-protection in *L. lactis* subsp. *lactis*. Compared with exponential-phase cells, stationary-phase cells of *L. lactis* subsp. *lactis* IL1403 were significantly more resistant to heat (52°C), ethanol (20%), acid (pH 4.0), and osmotic (NaCl, 3.5 M) stresses, and to a lesser degree, against an oxidative (H_2O_2, 15 mM) challenge (Fig. 2). Cells harvested after 3 h of starvation are more resistant to heat and ethanol for long exposure times than those harvested at the onset of starvation. Cultures starved for 24 h do not show a further augmentation of resistance (data not shown). For the other treatments (acid, osmotic, and oxidative challenge), cells are already completely resistant at the onset of starvation.

Adaptation-induced stress resistance in *L. lactis* subsp. *lactis*. We have compared starvation-induced protection with that induced in adapted exponential cells (Fig. 2). In the case of heat, acid, and osmotic stresses, adapted cells acquired resistance comparable to the resistance of cells in the stationary growth phase. In contrast, adapted cells are comparatively more sensitive to ethanol treatment but show enhanced resistance against the H_2O_2 treatment relative to cells in the stationary growth phase (Fig. 2). The survival rates of starved, adapted, and unstressed cells subjected to UV irradiation were similar (data not shown).

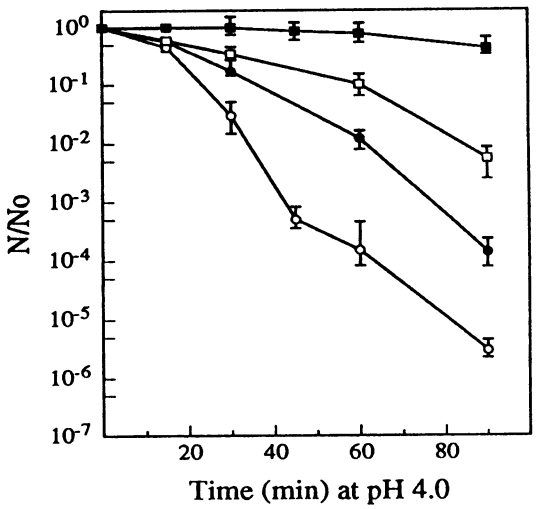

FIG. 3. Progressive induction of acid resistance during the transitional growth phase in *L. lactis* subsp. *lactis* IL1403. Cells were challenged to pH 4.0 after reaching OD_{600}s of 0.5 (exponential phase) (○), 0.7 (transitional phase) (●), and 0.9 (transitional phase) (□) and at the onset of the stationary growth phase (■). The data are means and standard deviations (bars) for at least three independent experiments. N/No, relative survival after treatment.

Development of resistance during the transitional growth phase. Since starvation-induced cross-protection is immediately present at the beginning of starvation, we investigated during the transitional growth phase the time course of this response in a representative case, the acid challenge (Fig. 3). Results show that cells develop resistance progressively against the acid treatment during the transitional growth phase, with maximum protection in cells entering the stationary phase. Similar results have been obtained for the heat and osmotic challenges (data not shown).

Starvation-induced cross-protection against heat and $CdCl_2$ stresses as against UV irradiation has been found for a marine *Vibrio* sp. (16). Carbon- or nitrogen-starved *E. coli* is more resistant to heat and H_2O_2 challenge (8) and osmotic stress (7) than exponential-phase cells. However, maximal resistance against these treatments has been observed after 4 h of starvation. This particular feature may be due to differences in the starvation protocol. In the case of *E. coli* and a *Vibrio* sp., exponential-growth-phase cells were subjected to carbon starvation by being transferred to a glucose-free medium. In our experiments cells entered the stationary phase after depletion of the energy source (glucose) by passing through the transitional phase.

Effect of protein synthesis inhibitors on starvation-induced cross-protection. The induction of specific starvation proteins has been reported for *L. lactis* ML3 (10). Using two-dimensional polyacrylamide gel electrophoresis, we have found the induction of at least 21 proteins in starved cells of *L. lactis* subsp. *lactis* IL1403 (data not shown). In *E. coli* and a *Vibrio* sp. the addition of chloramphenicol (100 and 200 μg/ml, respectively) reduced significantly the development of starvation-induced multiresistance (7, 8, 16). Obviously, induction of starvation proteins is necessary for the observed cross-protec-

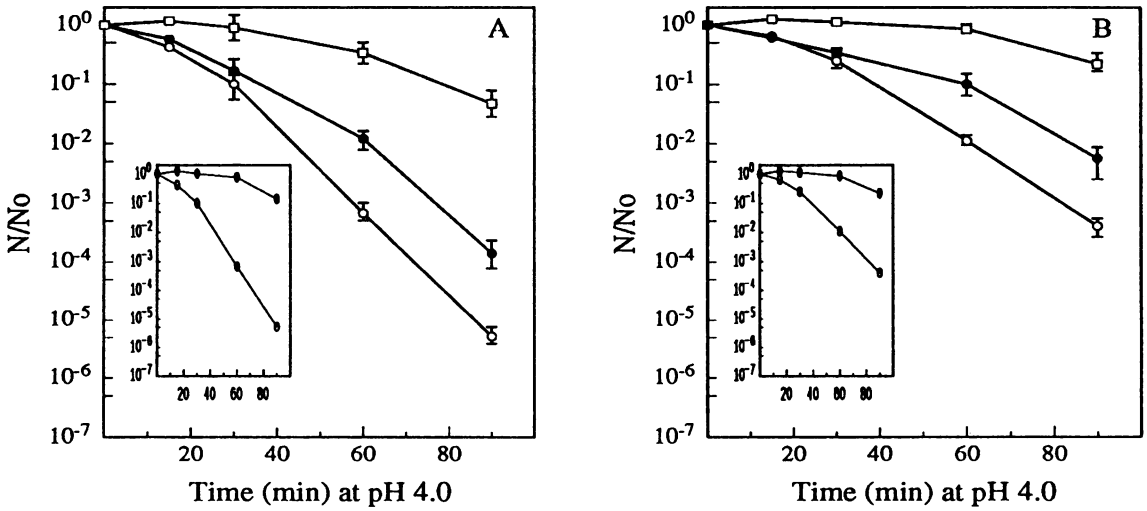

FIG. 4. Effect of chloramphenicol or rifamycin treatment on the development of acid resistance in growing cells of *L. lactis* subsp. *lactis* IL1403. Cells were challenged to pH 4.0 immediately after reaching an OD_{600} of 0.5 (A) or 0.7 (B) (○) and after a 30-min incubation at 30°C in the absence (●) or presence (□) of chloramphenicol (100 μg/ml). During the 30-min incubation the OD_{600} rose from 0.5 to 0.7 (A) and from 0.7 to 0.9 (B) independently of the presence or absence of chloramphenicol. Results obtained under the same experimental conditions but in the absence (○) or in the presence (●) of rifamycin (75 μg/ml) are shown in the insets. In each case the final concentration of the antibiotic was adjusted by adding an aliquot of a concentrated solution directly to the corresponding culture. An equal amount of solvent was added to the antibiotic-negative cultures as a control. The data are averages for at least three independent experiments. N/No, relative survival after treatment.

tion effects. However, Jouper-Jaan et al. (9), using another *Vibrio* sp. strain, have found that the development of increased thermotolerance was not affected by the inhibition of protein synthesis by chloramphenicol prior to heat stress at any time of starvation while an *E. coli* strain showed a great dependence on protein synthesis in the first hours of starvation but was independent of de novo protein synthesis after a relatively long-term starvation. To determine if induced proteins may mediate the cross-protection effects in *L. lactis*, we have conducted experiments in the presence of 100 μg of chloramphenicol per ml (the MIC of chloramphenicol for this strain of *L. lactis* subsp. *lactis* is in the range of 5 μg/ml). Under these conditions growth of the culture ceases completely 30 min (corresponding to an augmentation in the OD_{600} of 0.2) after the onset of treatment (data not shown). Surprisingly, cells from the exponential (OD_{600}, 0.5 [Fig. 4A]) and transitional (OD_{600}, 0.7 [Fig. 4B]) growth phases incubated for 30 min in the presence of chloramphenicol nevertheless developed an unexpected strong protection to the acid stress comparable to the results obtained with cells in the stationary phase. Even with a fivefold higher concentration of chloramphenicol we observed comparable resistance (data not shown). A similar effect was observed by using rifamycin (inset in Fig. 4) or a combination of the two antibiotics (data not shown). These data have been confirmed for the heat and NaCl challenges (data not shown). Pulse-labeling experiments in the presence of chloramphenicol or rifamycin showed that chloramphenicol (100 μg/ml) is active in this strain of *L. lactis* subsp. *lactis* and blocks completely protein synthesis (data not shown). On the contrary, cells treated with rifamycin (75 μg/ml) showed some residual protein synthesis (data not shown). This residual protein synthesis is probably due to the presence of preexisting RNA messengers prior to antibiotic treatment.

Our conflicting results show that in *L. lactis* subsp. *lactis* chloramphenicol or rifamycin treatment does not abolish the development of a tolerant cell state. On the contrary, these antibiotics seem to either induce this response or mimic, in slowing down the growth rate, the entrance to an artificial stationary phase. It seems that an additional, de novo protein-independent response triggers the development of a multiresistant phenotype in naturally starved and in chloramphenicol-and/or rifamycin-treated cells of *L. lactis* subsp. *lactis*. Preliminary experiments with two other antibiotics (erythromycin at 0.1 μg/ml and streptomycin at 100 μg/ml [concentrations equal to the respective MICs]) which alter the translational capacity of the bacterial cells seem to support this hypothesis. However, streptomycin seems to be less active than the other antibiotics used. The mechanism(s) by which these agents provoke the development of a tolerant cell state remains unknown.

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