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Lactococcus lactis SpOx Spontaneous Mutants: a Family of Oxidative-Stress-Resistant Dairy Strains§

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Numerous industrial bacteria generate hydrogen peroxide (H_2O_2), which may inhibit the growth of other bacteria in mixed ecosystems. We isolated *sp*ontaneous *ox*idative-stress-resistant (SpOx) *Lactococcus lactis* mutants by using a natural selection method with milk-adapted strains on dairy culture medium containing H_2O_2. Three SpOx mutants displayed greater H_2O_2 resistance. One of them, SpOx3, demonstrated better behavior in different oxidative-stress situations: (i) higher long-term survival upon aeration in LM17 and milk and (ii) the ability to grow with H_2O_2-producing *Lactobacillus delbrueckii* subsp. *delbrueckii* strains. Furthermore, the transit kinetics of the SpOx3 mutant in the digestive tract of a human flora-associated mouse model was not affected.

The dairy bacterium *Lactococcus lactis* is sensitive to the oxidative stress to which it is exposed during its use in an industrial environment or during its transit in the digestive tract (DT). More-robust strains could provide improvements in traditional uses of lactococci in industrial fermentations (5, 27).

During industrial dairy processes, milk can contain dissolved oxygen and hydrogen peroxide (H_2O_2). Some species of lactobacilli grown in the presence of oxygen also produce H_2O_2; this can lead to a premature shift from exponential into stationary phase and, consequently, a reduced biomass (16). As dairy fermentations often comprise oxygen-sensitive lactic acid bacteria (LAB) (26), the presence of H_2O_2-producing lactobacilli could have negative effects on bacterial yields and the quality of the products. Growth and survival of LAB may be spared from oxygen disturbances by the addition of either purified catalase to remove H_2O_2 from milk (2) or cysteine as a reducing agent. H_2O_2 production may also be exploited as a disinfectant in sterilization processes. In 1999, the feasibility of using H_2O_2-producing LAB strains in new biotherapeutic applications was tested to inhibit vaginal infections by *Staphylococcus aureus* (19).

Furthermore, robust strains could also be attractive as live oral vehicles to deliver proteins of medical interest to the DT (17). Oxidative stress is generated in vivo via the inflammatory response: macrophage activation induced by biological stimuli (such as microbes) engages oxidative metabolism, liberating reactive oxygen species inside the DT (23). Such stress could hamper LAB survival and limit any potential health benefits associated with their consumption.

The use of resistant strains is a promising strategy to avoid the growth inhibitory effects of H_2O_2-producing lactobacilli or other sources of oxidative stress. Such oxidative-stress-resistant strains could be isolated using recombinant DNA techniques, including either random mutagenesis as already performed to obtain and characterize *L. lactis* acid-resistant mutants (22) and *Streptococcus thermophilus* H_2O_2-sensitive or -resistant mutants (25) or the introduction of heterologous genes encoding antioxidant functions (e.g., the *Bacillus subtilis* hemin-dependent catalase KatE [T. Rochat et al., manuscript in preparation]).

Here, we developed a strategy to isolate oxidative-stress-resistant *L. lactis* MG1363 derivatives, with the following two original aspects: (i) the use of milk-adapted strains (rendered protease and β-galactosidase proficient) with verification of phenotypes in a milk-based medium and (ii) the isolation of nongenetically manipulated (non-GM) H_2O_2-resistant mutants, hereafter referred to as *sp*ontaneous *ox*idative-stress-resistant (SpOx) mutants. The resistance of SpOx mutants was evaluated after exposure to H_2O_2 and under aerated conditions. No putative cross-resistance phenotype to two other oxidative reagents (menadione and paraquat), to acidic conditions, or to bile salts was found in three of these SpOx mutants confirmed as H_2O_2-resistant mutants. One of these mutants, SpOx3, was further examined for its resistance in milk and its capacity to grow and to survive in coculture with H_2O_2-producing lactobacilli. We also evaluated how H_2O_2 resistance of *L. lactis* could influence its survival after passage in the DTs of human flora-associated mice. Our results show that H_2O_2-resistant lactococci gain a survival advantage in the context of a mixed bacterial ecosystem, such as that present in dairy fermentations (20).

Strains, media, and growth conditions. *L. lactis* subsp. *cremoris* MG1363 (7) was grown in M17 medium (Difco) containing 0.5% glucose (GM17). Dairy strains of *L. lactis* were cul-

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tured at 30°C in M17 medium containing 0.5% lactose (LM17) or 5% lactose (L_{10}M17) and in reconstituted dried skim milk (Compagnie Laitière Food Service, Le Pecq, France). When necessary, erythromycin (Em) was added at 5 μg/ml. *Lactobacillus delbrueckii* subsp. *delbrueckii* strains (19) were grown at 37°C in LAPT (1.5% peptone, 1% tryptone, 1% glucose, 1% yeast extract, 0.1% Tween 80, pH 6.5), which had been autoclaved for 15 min at 121°C, with 0.5% lactose. For aeration, cultures were stirred in circular agitators (Infors AG) at 240 rpm. The appropriate sample dilutions (prepared in cold peptone water; 1 g/liter) were plated on GM17 or LM17 and incubated at 30°C for 48 h before counting was done. Aerated cultures (45 ml) were performed with 250-ml Erlenmeyer flasks, and nonaerated cultures (10 ml) were performed with closed tubes of 25 ml.

Construction of a dairy derivative *L. lactis* MG1363 strain. *L. lactis* MG1363 is not able to grow on milk due to the curing of the pLP712 conjugative plasmid, which carries two genes essential for growth in milk: *lacZ* and *prtP* (encoding β-galactosidase and proteinase, respectively). The dairy *L. lactis* MG1363 strain (hereafter called J60011) was obtained by conjugation as described previously (12). This mating was performed between the pLP712 donor strain *L. lactis* NCDO712 (7) and the recipient strain MG1363 containing the Em^r thermosensitive replicative plasmid pGhost9 used to select the desired Em^r clone; pGhost9 was then eliminated by a temperature shift (15). Transconjugants were first selected on LM17 with Em. The presence of pLP712 was confirmed by analysis of the plasmid content and detection of both β-galactosidase and proteinase activities on Fast Slow differential agar medium (10). Plasmid pGhost9 was eliminated from transconjugants after ~100 generations without Em at 30°C. *L. lactis* J60011 displays the same clotting time of milk as industrial dairy strains.

Selection of robust SpOx mutants after exposure to H_2O_2. The parental J60011 dairy strain was submitted to a range of H_2O_2 concentrations to determine the threshold of lethality. Cells are more sensitive to H_2O_2 in exponential phase (lethality of 10^4 CFU/ml after exposure to 2 mM H_2O_2) than in stationary phase (lethality of 10^3 CFU/ml after exposure to 10 mM H_2O_2). These results are in accordance with previous observations showing that bacteria are generally more resistant to numerous stress conditions in stationary phase. A key role of metabolism in the stress response of lactococci was previously suggested by the identification of mutants involved in both phenomena (6, 22). Mutagenesis was performed with the J60011 *L. lactis* strain on plates of Fast Slow differential agar medium (agar, 6 g/liter) in the presence of 2 mM or 5 mM H_2O_2 and incubated at 37°C. Eleven SpOx mutants were initially selected as H_2O_2 resistant at frequencies ranging from 10^{-9} to 10^{-6} at 5 and 2 mM of H_2O_2, respectively. Among these 11 SpOx mutants, 7 mutants having good acidification capacities in milk (with 6 h of clotting time or less following a 1% inoculation with saturated bacterial cultures) were selected for further examination. The survival of SpOx mutants was monitored on both exponential- and stationary-phase cultures after H_2O_2 shock in either laboratory medium or milk. The H_2O_2 shock was performed by incubating these *L. lactis* cultures in the presence of H_2O_2 (0, 2, and 4 mM for exponential-phase cultures and 0 and 10 mM for stationary-phase cultures) at 30°C. After 1 h, H_2O_2 was eliminated by the addition of bovine catalase (10 U/ml; Sigma), and viable bacterial counts were determined by plating. The results given correspond to the average for three different assays. Error bars correspond to the standard errors of the means.

(i) Survival of SpOx mutants in laboratory medium. To test the levels of H_2O_2 resistance of SpOx mutants in LM17 in exponential phase, saturated overnight cultures were diluted at least 100-fold and grown until the optical density at 600 nm (OD_{600}) was 0.1. Stationary-phase cultures were prepared by 10-fold dilution of an overnight culture. Among the seven SpOx mutants, three were confirmed as H_2O_2 resistant (Fig. 1A): SpOx1 exhibited improved H_2O_2 resistance compared to the parental J60011 strain in exponential phase, whereas SpOx2 and SpOx3 were both confirmed to show significantly higher resistance than J60011 in both exponential phase and stationary phase. When challenged during exponential phase with 2 mM or 4 mM H_2O_2, SpOx2 and SpOx3 both showed ~6,000-fold (2 mM H_2O_2) and ~7,000-fold (4 mM H_2O_2) better survival than that of J60011. Stationary-phase cultures of these mutants showed 200-fold better survival than did J60011 after incubation with 10 mM H_2O_2. These results are in accordance with observations that stress resistance mechanisms vary depending on the growth phase (5). The SpOx3 mutant, resistant in both culture phases, was chosen for further studies under conditions simulating those used in dairy technologies.

(ii) Survival of the SpOx3 mutant after H_2O_2 shock in milk. One *L. lactis* strain encounters different environments when grown in milk or in LM17. These differences are reflected in variations in the *L. lactis* proteome profile as a function of growth medium (8). We therefore examined SpOx3 survival upon H_2O_2 stress in milk in both exponential- and stationary-phase cultures. To execute shocks in milk, growth was performed in LM17 until an OD_{600} of ~0.1 was reached; cells were then washed (in cold peptone water) and resuspended at a 10% concentration in milk for H_2O_2 incubation. In exponential phase, the SpOx3 mutant displayed ~100- and ~10,000-fold better survival than the J60011 strain with 2 and 4 mM of H_2O_2, respectively (Fig. 1B). In stationary phase, SpOx3 survival was ~60- and ~80-fold better than that of J60011 (Fig. 1B) after incubation with 10 and 20 mM of H_2O_2, respectively. The SpOx3 mutant isolated by natural selection thus possesses significant H_2O_2 resistance in both laboratory and dairy media.

Does the H_2O_2 resistance of SpOx mutants also provide cross-resistance to other stresses? To determine the potential cross-resistance to other stresses, wild-type (wt) and mutant strains were exposed to various stress conditions: other reactive oxygen species, acidity, and bile salts. We first evaluated the growth inhibition of the wt strain J60011 and of the three mutants SpOx1, SpOx2, and SpOx3 in the presence of superoxide ions generated in aerated cultures by addition of either paraquat or menadione. Paraquat was added at the beginning of the culture, whereas menadione was added once an OD_{600} of ~0.3 was reached, at final concentrations of 5 mM and 10 mM, respectively. No difference in OD_{600} measurement was observed between the wt strain and the three SpOx mutants for which the growth levels were affected to the same extent (data not shown). The same results were obtained when resistance to acidic stress and bile salts was tested. For the test for acidic stress, an overnight culture was mixed (1:1, vol/vol) with LM17 acidified at pH 2 (by the addition of HCl) and the survival

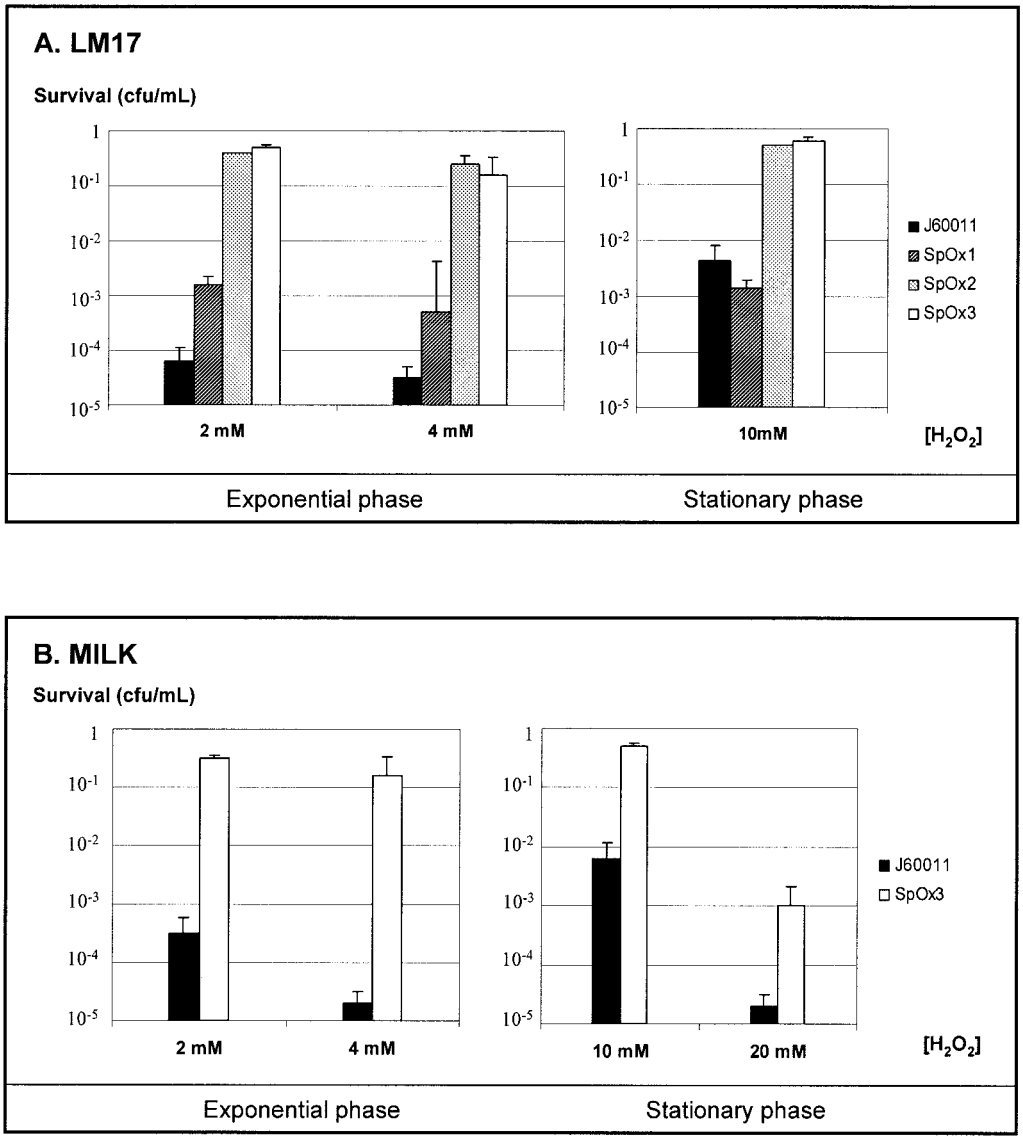

FIG. 1. Survival of *Lactococcus lactis* SpOx mutants after H_2O_2 shock. Exponential- or stationary-phase cultures of J60011 or SpOx mutants were incubated for 1 h at 30°C with different concentrations of H_2O_2 and then plated in LM17 to determine the number of CFU. Survival is formulated as the ratio of CFU per ml in the presence of H_2O_2 to that in the absence of H_2O_2. The behavior of the *L. lactis* J60011 strain was compared to that of seven SpOx mutants in LM17 (A), three of which were H_2O_2 resistant, and to that of the SpOx3 mutant in milk (B).

levels of the *L. lactis* strains were compared after 3 h of incubation at 37°C by plating. For the test of resistance to bile salts, an overnight culture was diluted at 1/100 in fresh LM17 and incubated for 3 h at 37°C with 0 or 0.05% of bile salts (composed of sodium cholate and sodium deoxycholate [1:1, vol/vol]). Viable bacterial counts were determined by plating at the time of inoculation of the culture and after 3 h of incubation. An increase in lethality of 2 orders of magnitude after 3 h of incubation was measured for all the tested strains (data not shown). Altogether, these results suggest that the three SpOx mutants selected on H_2O_2 are not resistant to other tested stress conditions, suggesting an absence of cross-resistance.

The H_2O_2-resistant SpOx3 mutant survives better under aerated conditions. In the presence of dissolved oxygen, reactive oxygen species other than H_2O_2 can be generated and can affect growth and viability of LAB. We tested the capacity of the H_2O_2-resistant SpOx3 mutant to grow and survive under aeration conditions similar to the type of oxidative stress encountered in industry. The lag phase of J60011 and SpOx3 in cultures aerated by stirring at 240 rpm was measured during the first 5 h by plating, and growth of the two strains was evaluated by the pH decrease of the culture. The results for the two strains were comparable: changes in pH (measured 6 h after inoculation) of ~1 in aerated milk and of ~1.65 under static conditions. An overnight culture was diluted 1,000-fold in adequate medium. Long-term survival was determined by bacterial enumerations at days 1, 2, and 3. The results given correspond to the average for three different assays. After 2 days in stationary phase, the viability of SpOx3 was higher than that of J60011: (i) in LM17, a 200-fold improvement was ob-

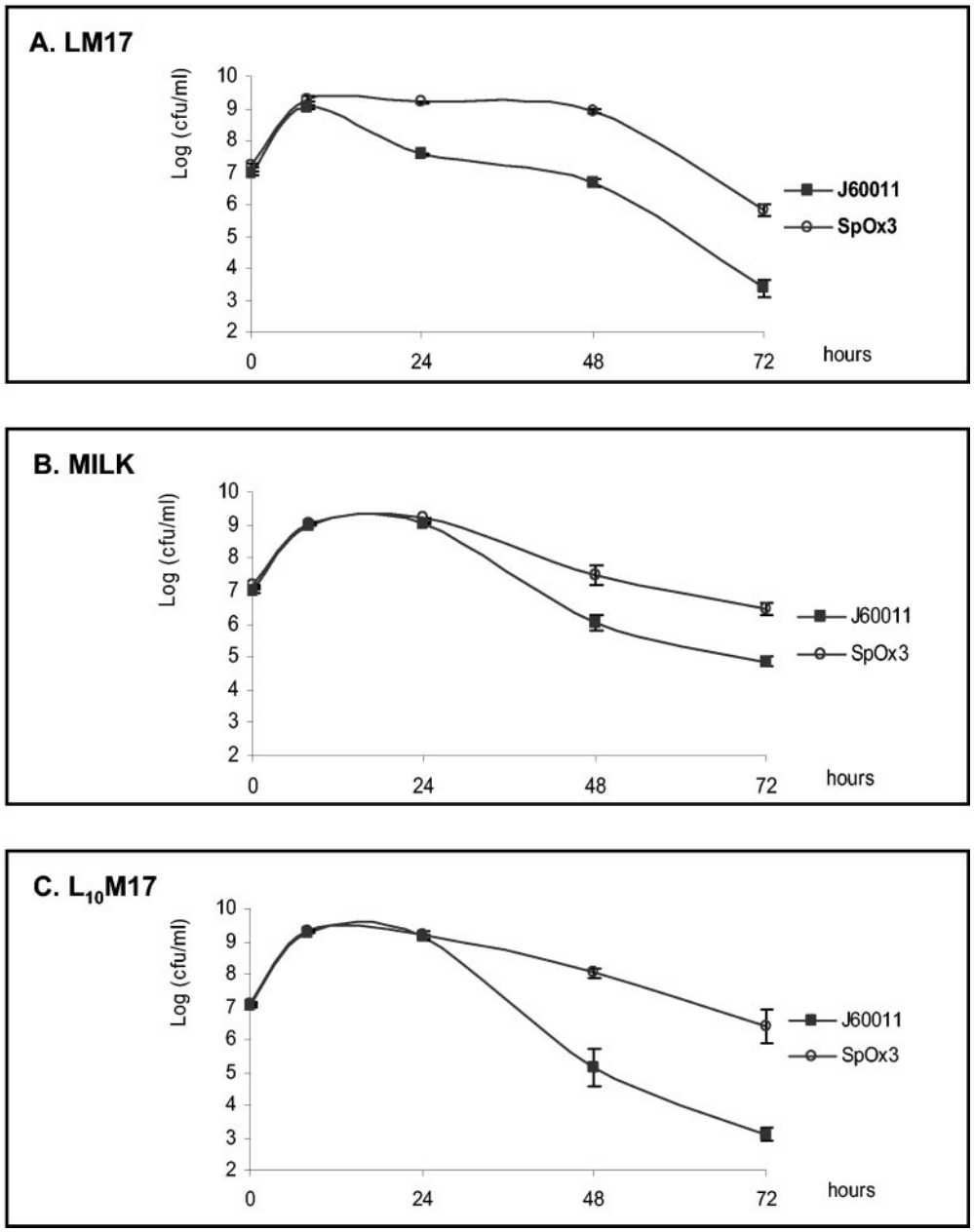

FIG. 2. Long-term survival of *L. lactis* SpOx3 mutant under aerated conditions. An overnight culture was diluted 1,000-fold in LM17 (A), milk (B), and L_{10}M17 (C). Aeration was performed by stirring the culture at 240 rpm at 30°C. The survival levels of J60011 and SpOx3 *L. lactis* strains were compared by performing plate counts on LM17 every 24 h for 4 days.

served (6×10^6 CFU/ml for J60011 compared to 1×10^9 CFU/ml for SpOx3) (Fig. 2A); and (ii) in milk, SpOx3 viability is 40-fold higher than that of J60011 (6×10^4 CFU/ml for J60011 compared to 3×10^6 CFU/ml for SpOx3). The improved survival of SpOx3 versus the survival of J60011 lasted for the duration of the experiment (Fig. 2B).

We noted that the loss of viability starts later in milk than in LM17 for J60011 (1×10^9 CFU/ml in milk compared to 4×10^7 CFU/ml in LM17 after 24 h of aeration). As the lactose concentration is 10-fold higher in milk than in LM17, the impact of this higher concentration was tested on bacterial survival by using LM17 containing the same lactose level as that in milk (L_{10}M17). The loss of J60011 viability in L_{10}M17 was delayed, compared to that in LM17, and occurred only after 48 h of growth (Fig. 2C). Nevertheless, even after adjustment of the lactose concentration, a better survival was observed in milk (1×10^3 CFU/ml in L_{10}M17 versus 6×10^4 CFU/ml in milk for J60011 after 72 h of aeration). The larger bacterial population after 24 h of aeration in milk could be explained by the higher lactose concentration and, consequently, by a residual growth. We propose that although a high lactose concentration favors a better survival under aeration, other protections against oxygen are also provided in milk. The above-described results establish that the SpOx mutant se-

lected for H_2O_2 resistance also displays improved survival under aeration in different growth media.

The SpOx3 mutant can grow and survive in coculture with H_2O_2-producing *L. delbrueckii*. The development of new fermented products could involve new combinations such as cocultures of lactococci and lactobacilli (Claude Buchet, personal communication). H_2O_2 is generated by dairy bacteria during growth and may accumulate in stationary phase, leading to detrimental effects on survival and final biomass (16). In the presence of oxygen, some species of lactobacilli can produce H_2O_2 that could inhibit the growth of facultative anaerobe lactococci in aerated coculture. The growth and survival of the J60011 strain and the H_2O_2-resistant SpOx3 mutant in the presence of two H_2O_2-producing *Lactobacillus delbrueckii* subsp. *delbrueckii* strains, F29 and F86 (19), were monitored under aerated conditions. Pure cultures and cocultures of *L. lactis* and *L. delbrueckii* were performed in LAPT with 0.5% lactose with or without stirring at 37°C. Lactococci and lactobacilli were inoculated, respectively, at 10^6 and 10^7 CFU/ml. Viable cell counts of the two species were determined by plating appropriate dilutions of the coculture (i) on LM17 at 30°C (for *L. lactis*) and (ii) on LAPT supplemented with glucose (0.5%) at 42°C (for *L. delbrueckii*). H_2O_2 produced during bacterial growth was determined for each culture condition by the modified *o*-dianisidine–horseradish peroxidase method (18). The results given are representative of three independent assays. Compared to nonaerated conditions under which H_2O_2 was not produced, aerated cultures of both lactobacilli led to reduced growth and a decrease in viability as soon as they produced H_2O_2, confirming the oxidative-stress sensitivity of these LAB species (data not shown). F29 grows and produces H_2O_2 faster than F86 (Fig. 3A and B); thus, the decrease in survival occurs earlier for F29 than for F86 (data not shown). For cocultures with either F29 or F86, lactococcal development had no effect on the kinetics of H_2O_2 production compared to production of the pure lactobacillus cultures (Fig. 3A and B, respectively). In aerated cocultures with both H_2O_2-producing lactobacilli, the J60011 strain, inoculated at 10^6 CFU/ml, did not grow, even in the first hours of the culture, when the H_2O_2 concentration was still low (especially with F86). The survival of J60011 after 12 h was also dramatically reduced (10^2 and 10^3 CFU/ml in the presence of F29 and F86, respectively [Fig. 3C and D]). In marked contrast, the SpOx3 mutant grew well and reached 10^7 and 10^8 CFU/ml in coculture with F29 and F86, respectively (Fig. 3C and D). Furthermore, its survival was also significantly better than that of J60011 (1,000-fold better after 10 h of aeration) (Fig. 3C and D). Interestingly, growth of both of the H_2O_2-producing lactobacillus strains was better in the presence of SpOx3 than in the presence of J60011 (Fig. 3E and F). The H_2O_2 concentrations detected in the supernatants of cocultures containing either J60011 or SpOx3 are very close (Fig. 3A and B), suggesting that the beneficial effects observed in the presence of SpOx3 were not due to degradation of H_2O_2.

These experiments showed that the SpOx3 mutant could be cocultivated with H_2O_2-producing lactobacilli which will keep their bactericidal properties. They also demonstrate the great impact of the bactericidal effect of H_2O_2 on the growth of two partners of a coculture.

Behavior of the SpOx3 mutant in the DTs of human flora-associated mice. About 0.1 to 2% of *L. lactis* cells survive after passage through the human DT (11), while stable populations are obtained when lactococci are implanted in germfree mice (3). Numerous factors, including nutritional competition and survival in response to endogenous stresses, notably induced by intestinal flora, may explain the poor survival of lactococci when they are placed in a competitive situation. To determine whether improved oxidative-stress resistance could affect lactococcal survival in the DT, survival levels of both the parental J60011 and the mutant SpOx3 strains were compared in a human flora-associated mouse model (9). To analyze their behavior in the DT, these strains were genetically marked by the introduction of plasmid pGK12ΔCm (13), which harbors an Em^r gene. The Em^r marker did not affect the survival of *L. lactis* strains in the mouse DT (data not shown). Mice were reared in sterile Texler-type isolators (La Calhène, Vélizy, France), and a defined human microflora was conferred in an environmentally controlled room (21°C) with a 12-h light-dark cycle as described previously (21). Mice received irradiated food (UAR, Villemoisson, France) and sterilized water. Each mouse received by intragastric administration 0.5 ml of a bacterial cell suspension containing ~10^9 CFU/ml of an overnight culture of *L. lactis* and ~10^8 CFU/ml *Bacillus subtilis* spores, which served as a microbial marker for transit. Bacterial survival of *L. lactis* J60011 and the SpOx3 mutant was examined in the feces of mice 8 h after ingestion and then every 24 h for 7 days. Two groups of five mice were used for the three experiments. Spores in the fecal samples were enumerated after 24 h at 56°C. To count surviving lactococci, samples were plated on LM17 with Em (20 μg/ml) supplemented with nalidixic acid (40 μg/ml) as an inhibitor of gram-negative bacteria and then incubated for 48 h at 30°C. Survival of *L. lactis* after transit in the DT was calculated with respect to the number of spores (4). We observed that the J60011 and SpOx3 *L. lactis* strains showed similar elimination kinetics during the course of the experiment. Both lactococcal populations decreased rapidly: population levels dropped to ~10^3 CFU/g fecal sample at 48 h postinoculation of 10^8 CFU/ml. These results suggest that H_2O_2 resistance cannot be considered a major factor that influences lactococcal survival in the DT of the human flora-associated mouse model. In addition, they also show that the persistence of the SpOx3 mutant is not affected and that this mutant can be used for traditional purposes.

In summary, our strategy was to isolate SpOx mutants of *L. lactis* by use of nonrecombinant methods, thus ensuring the applicability of the approach. As these SpOx mutants underwent strictly "natural" selection pressures, they can be introduced directly in industrial processes. This presents a clear advantage over recombinant strains, whose use in the food industry is strictly regulated and forbidden in many countries. Adjunct bacteria present in dairy productions can generate significant amounts of H_2O_2 during growth, which can disturb the growth of *L. lactis* and hamper the reproducibility of industrial processes where *L. lactis* plays a major role (27). We reasoned that a reproducible bacterial balance could be maintained if the oxidative-stress resistance of *L. lactis* was improved. These non-GM mutants display growth properties similar to the parent's. However, non-GM mutants show superior long-term survival when grown singly and better growth when associated with other LAB. This phenotype is quite stable even

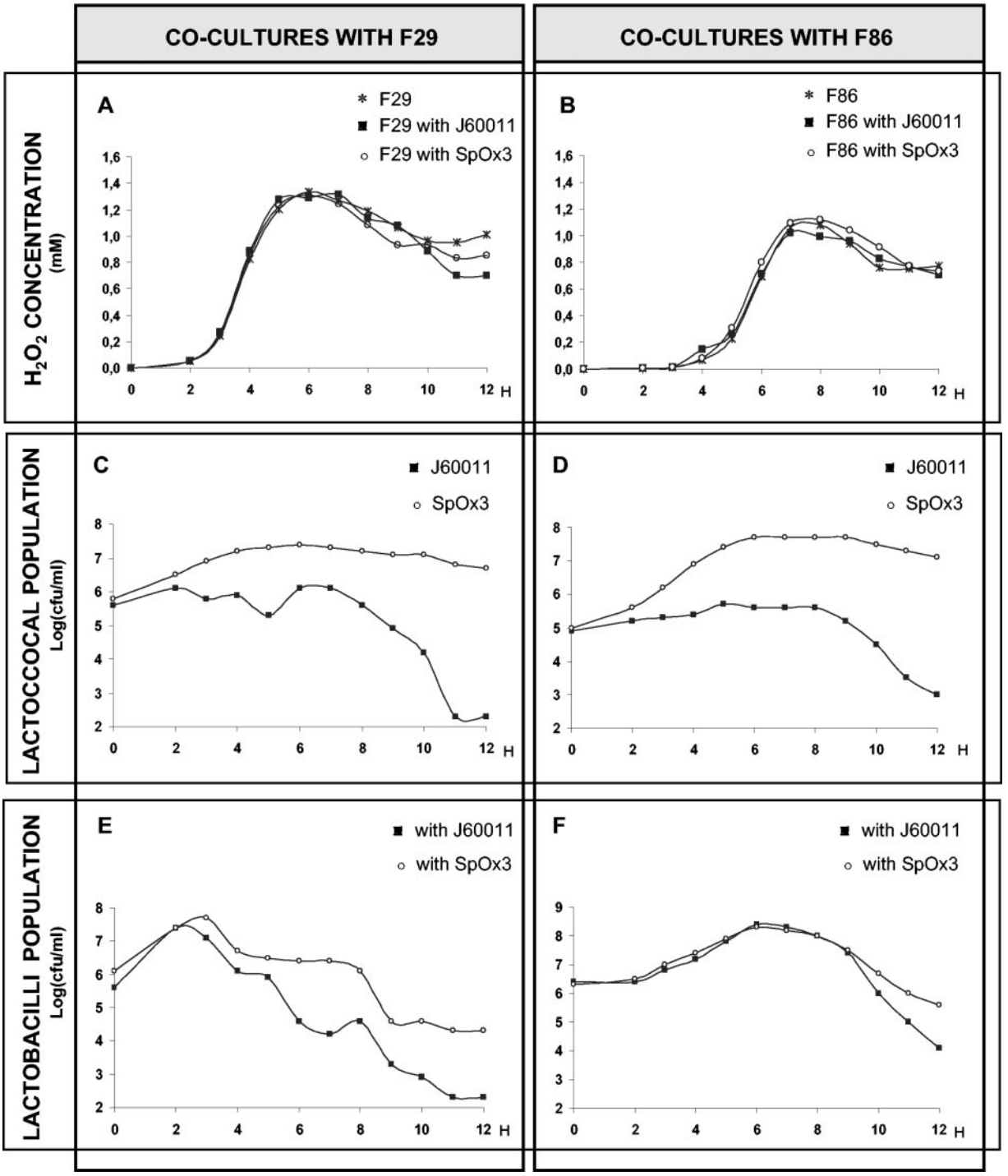

FIG. 3. Coculture of *L. lactis* SpOx3 mutant with H_2O_2-producing *L. delbrueckii* F29 or F86. Single and mixed cultures were grown in LAPT at 37°C and aerated by stirring at 240 rpm. A and B, H_2O_2 production was determined for the supernatants of pure lactobacillus cultures and of each coculture. Growth and survival levels of J60011 and SpOx3 *L. lactis* strains were compared by determining their population levels in aerated cocultures with either *L. delbrueckii* F29 or F86. C and D, *L. lactis* counts were determined on LM17 agar after incubation at 30°C. E and F, *L. delbrueckii* counts were determined on LAPT agar after incubation at 42°C. The results given are representative of three independent assays.

after numerous freezing and spray-drying procedures and several days after oral administration to mice. The non-GM mutants render possible new combinations of LAB starters (such as the one tested here with H_2O_2-producing lactobacilli and H_2O_2-resistant lactococci) and, consequently, new potential fermented products.

Compared to a conventional recombinant strategy such as random insertional mutagenesis using pGh9:IS*S1* (15), the

nonrecombinant strategy used does not allow direct identification of the genetic event(s) responsible for a given phenotype. The genetic modifications responsible for the observed oxidative-stress-resistance phenotype of the SpOx mutants thus remain to be identified. Genetic studies and analysis of the *L. lactis* IL1403 genome sequence revealed the presence of several genes known to influence tolerance to oxidative stress, such as the *nox*, *ahpC* and *ahpF*, *gpo* and *gshR*, and *sodA* genes encoding, respectively, NADH dehydrogenases, alkyl hyperoxide reductases, glutathione peroxidase/reductase, and superoxide dismutase (1, 14, 24). A modification of these functions could enhance the oxidative-stress resistance of *L. lactis*. Recently, Li et al. (14) showed that the addition of glutathione in culture medium with lactococci could activate a glutathione peroxidase/reductase system which catalyzes the reduction of H_2O_2 and protects *L. lactis* against oxidative stress. A comparative analysis of the parental and SpOx strains using transcriptomic and proteomic approaches is in progress and should be valuable in characterizing the physiological modifications responsible for the improved oxidative-stress tolerance in SpOx mutants. In parallel to the nonrecombinant strategy, experiments with random insertional mutagenesis to isolate clones from *L. lactis* modified in their capacities to tolerate oxidative stress are currently in progress.

Tests in a mouse model indicate that the H_2O_2 resistance phenotype did not affect the *L. lactis* survival rate in vivo, suggesting that other drastic stresses due to, e.g., bile salts, acidic conditions, or killing by other bacteria could be the major determinants of survival of *L. lactis* in the DT. This result would facilitate the direct utilization of the SpOx mutant in food industry fermentations. Concerning the new potential medical uses of LAB (for a review, see reference 17), these SpOx mutants are not better candidates for in situ delivery of therapeutic molecules than the parental strain because of their similar lifetimes in vivo. A more customized approach comprising passages of *L. lactis* in the DT could be envisioned as a pertinent strategy to isolate useful DT stress-resistant *L. lactis* mutants.

The nonrecombinant strategy, used here in a milk-adapted *L. lactis* strain, could potentially be applied to virtually any bacteria (such as other LAB species) and any stress (e.g., acidity or high temperature) to isolate spontaneously stress-tolerant strains. This work illustrates the impact of a "straightforward preselection" of one stress-resistant strain on the balance between two species in a bacterial ecosystem. This approach could be useful to better understand the interactions between bacterial partners of more-complex ecosystems in both traditional domains and novel probiotic uses of LAB. It could also be used to optimize the behavior of *L. lactis* in the presence of other LAB species in dairy, animal, or human ecosystems.

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REFERENCES

1. **Bolotin, A., P. Wincker, S. Mauger, O. Jaillon, K. Malarme, J. Weissenbach, S. D. Ehrlich, and A. Sorokin.** 2001. The complete genome sequence of the lactic acid bacterium Lactococcus lactis ssp. lactis IL1403. Genome Res. **11:**731–753.
2. **Chu, H. D., J. G. Leeder, and S. G. Gilbert.** 1975. Immobilized catalase reactor for use in peroxide sterilization of dairy products. J. Food Sci. **40:**641–643.
3. **Corthier, G., C. Delorme, S. D. Ehrlich, and P. Renault.** 1998. Use of luciferase genes as biosensors to study bacterial physiology in the digestive tract. Appl. Environ. Microbiol. **64:**2721–2722.
4. **Drouault, S., G. Corthier, S. D. Ehrlich, and P. Renault.** 1999. Survival, physiology, and lysis of *Lactococcus lactis* in the digestive tract. Appl. Environ. Microbiol. **65:**4881–4886.
5. **Duwat, P., B. Cesselin, S. Sourice, and A. Gruss.** 2000. Lactococcus lactis, a bacterial model for stress responses and survival. Int. J. Food Microbiol. **55:**83–86.
6. **Duwat, P., S. D. Ehrlich, and A. Gruss.** 1999. Effects of metabolic flux on stress response pathways in Lactococcus lactis. Mol. Microbiol. **31:**845–858.
7. **Gasson, M. J.** 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. **154:**1–9.
8. **Guillot, A., C. Gitton, P. Anglade, and M. Y. Mistou.** 2003. Proteomic analysis of Lactococcus lactis, a lactic acid bacterium. Proteomics **3:**337–354.
9. **Hirayama, K.** 1999. Ex-germfree mice harboring intestinal microbiota derived from other animal species as an experimental model for ecology and metabolism of intestinal bacteria. Exp. Anim. **48:**219–227.
10. **Huggins, A. R., and Sandine, W. E.** 1984. Differentiation of fast and slow acid producing strains of lactic streptococci. J. Dairy Sci. **67:**1674–1679.
11. **Klijn, N., A. H. Weerkamp, and W. M. de Vos.** 1995. Genetic marking of *Lactococcus lactis* shows its survival in the human gastrointestinal tract. Appl. Environ. Microbiol. **61:**2771–2774.
12. **Langella, P., Y. Le Loir, S. D. Ehrlich, and A. Gruss.** 1993. Efficient plasmid mobilization by pIP501 in *Lactococcus lactis* subsp. *lactis*. J. Bacteriol. **175:** 5806–5813.
13. **Le Loir, Y., A. Gruss, S. D. Ehrlich, and P. Langella.** 1994. Direct screening of recombinants in gram-positive bacteria using the secreted staphylococcal nuclease as a reporter. J. Bacteriol. **176:**5135–5139.
14. **Li, Y., J. Hugenholtz, T. Abee, and D. Molenaar.** 2003. Glutathione protects *Lactococcus lactis* against oxidative stress. Appl. Environ. Microbiol. **69:** 5739–5745.
15. **Maguin, E., H. Prévost, S. D. Ehrlich, and A. Gruss.** 1996. Efficient insertional mutagenesis in lactococci and other gram-positive bacteria. J. Bacteriol. **178:**931–935.
16. **Marty-Teysset, C., F. de la Torre, and J. R. Garel.** 2000. Increased production of hydrogen peroxide by *Lactobacillus delbrueckii* subsp. *bulgaricus* upon aeration: involvement of an NADH oxidase in oxidative stress. Appl. Environ. Microbiol. **66:**262–267.
17. **Nouaille, S., L. A. Ribeiro, A. Miyoshi, D. Pontes, Y. Le Loir, S. C. Oliveira, P. Langella, and V. Azevedo.** 2003. Heterologous protein production and delivery systems for Lactococcus lactis. Genet. Mol. Res. **2:**102–111.
18. **Nunez de Kairuz, M. S., M. E. Olazabal, G. Oliver, A. A. Pesce de Ruiz Holgado, E. Massa, and R. N. Farias.** 1988. Fatty acid dependent hydrogen peroxide production in Lactobacillus. Biochem. Biophys. Res. Commun. **152:**113–121.
19. **Ocana, V. S., A. A. Pesce de Ruiz Holgado, and M. E. Nader-Macias.** 1999. Selection of vaginal H2O2-generating Lactobacillus species for probiotic use. Curr. Microbiol. **38:**279–284.
20. **Ogier, J.-C., O. Son, A. Gruss, P. Tailliez, and A. Delacroix-Buchet.** 2002. Identification of the bacterial microflora in dairy products by temporal temperature gradient gel electrophoresis. Appl. Environ. Microbiol. **68:**3691–3701.
21. **Oozeer, R., N. Goupil-Feuillerat, C. A. Alpert, M. van de Guchte, J. Anba, J. Mengaud, and G. Corthier.** 2002. *Lactobacillus casei* is able to survive and initiate protein synthesis during its transit in the digestive tract of human flora-associated mice. Appl. Environ. Microbiol. **68:**3570–3574.
22. **Rallu, F., A. Gruss, S. D. Ehrlich, and E. Maguin.** 2000. Acid- and multistress-resistant mutants of Lactococcus lactis: identification of intracellular stress signals. Mol. Microbiol. **35:**517–528.
23. **Roos, D.** 1991. The involvement of oxygen radicals in microbicidal mechanisms of leukocytes and macrophages. Klin. Wochenschr. **69:**975–980.
24. **Sanders, J. W., K. J. Leenhouts, A. J. Haandrikman, G. Venema, and J. Kok.** 1995. Stress response in *Lactococcus lactis*: cloning, expression analysis, and mutation of the lactococcal superoxide dismutase gene. J. Bacteriol. **177:** 5254–5260.
25. **Thibessard, A., A. Fernandez, B. Gintz, N. Leblond-Bourget, and B. Decaris.** 2002. Effects of *rodA* and *pbp2b* disruption on cell morphology and oxidative stress response of *Streptococcus thermophilus* CNRZ368. J. Bacteriol. **184:** 2821–2826.
26. **van de Guchte, M., S. D. Ehrlich, and E. Maguin.** 2001. Production of growth-inhibiting factors by Lactobacillus delbrueckii. J. Appl. Microbiol. **91:**147–153.
27. **van de Guchte, M., P. Serror, C. Chervaux, T. Smokvina, S. D. Ehrlich, and E. Maguin.** 2002. Stress responses in lactic acid bacteria. Antonie Leeuwenhoek **82:**187–216.