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Analysis of *Lactobacillus sakei* Mutants Selected after Adaptation to the Gastrointestinal Tracts of Axenic Mice▽†

Fabrizio Chiaramonte,[1,2]§ Patricia Anglade,[1] Fabienne Baraige,[1] Jean-Jacques Gratadoux,[2] Philippe Langella,[2] Marie-Christine Champomier-Vergès,[1] and Monique Zagorec[1]*

Unité Flore Lactique et Environnement Carné, UR309,[1] and Unité Ecologie et Physiologie du Système Digestif, UR902,[2] INRA, Domaine de Vilvert, F78350 Jouy en Josas, France

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We recently showed that *Lactobacillus sakei*, a natural meat-borne lactic acid bacterium, can colonize the gastrointestinal tracts (GIT) of axenic mice but that this colonization in the intestinal environment selects *L. sakei* mutants showing modified colony morphology (small and rough) and cell shape, most probably resulting from the accumulation of various mutations that confer a selective advantage for persistence in the GIT. In the present study, we analyzed such clones, issued from three different *L. sakei* strains, in order to determine which functions were modified in the mutants. In the elongated filamentous cells of the rough clones, transmission electron microscopy (TEM) analysis showed a septation defect and dotted and slanted black bands, suggesting the presence of a helical structure around the cells. Comparison of the cytoplasmic and cell wall/membrane proteomes of the meat isolate *L. sakei* 23K and of one of its rough derivatives revealed a modified expression for 38 spots. The expression of six oxidoreductases, several stress proteins, and four ABC transporters was strongly reduced in the GIT-adapted strain, while the actin-like MreB protein responsible for cell shaping was upregulated. In addition, the expression of several enzymes involved in carbohydrate metabolism was modified, which may correlate with the observation of modified growth of mutants on various carbon sources. These results suggest that the modifications leading to a better adaptation to the GIT are pleiotropic and are characterized in a rough mutant by a different stress status, a cell wall modification, and modified use of energy sources, leading to an improved fitness for the colonization of the GIT.

Lactobacilli are Gram-positive bacteria known mostly for their importance in the food industry or their potential beneficial effects on consumers. Their presence in the human gastrointestinal tract (GIT) has frequently been reported, and, although in small amounts (31), 16 different *Lactobacillus* species have been isolated at least once from human feces (35). Most of those species are probably not autochthonous but rather persisting species introduced in the GIT after food ingestion.

Lactobacillus sakei is known as a food-borne bacterium, frequently isolated from fresh or fermented meat but also found in fish or vegetal fermented products (6, 30). The importance of *L. sakei* in the meat industry significantly increased in recent years because of its technological properties that are optimal for sausage fermentation (5, 9) and its potential use as a bioprotective culture for the biopreservation of various meat products (3, 5, 36). *L. sakei* has been detected in human feces (20, 37), and some human strains have been isolated (7, 10). The estimated amount of this species in the fecal samples (10^6 CFU/g of feces) does not permit a conclusion about whether its presence is strictly food related or not. In addition, although the physiological functions involved in the adaptation to and growth of *L. sakei* in its preferred environment (meat essentially) are well documented (5, 21), those required for *L. sakei* transit through and/or colonization of the GIT are yet unknown.

We recently reported that three *L. sakei* strains from different origins (*L. sakei* 23K, a meat-borne strain, and *L. sakei* LTH5590 and FLEC01, both isolated from human feces) can survive the transit through conventional mice and durably colonize the GIT of axenic mice (7). However, we observed that the transit through the GIT of axenic mice led to the appearance of *L. sakei* subpopulations, characterized by atypical colony morphologies and cell shapes and probably resulting from the accumulation of mutations allowing a better adaptation to the GIT environment. The clones forming small colonies on MRS agar medium (12) were named S and the clones forming rough colonies R. In the present study, we aimed to determine which functions could be involved in this adaptation by comparing S and R clones to their parent strains.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. sakei* 23K is a plasmid-free strain issued from a natural isolate originating from fermented sausage (2). *L. sakei* LTH5590 (10) and *L. sakei* FLEC01 (7) were isolated from feces of healthy humans. *L. sakei* 23K-J10SN2, *L. sakei* 23K-J30RO2, *L. sakei* FLEC01-J6SN2, *L. sakei* FLEC01-J15RN2, *L. sakei* LTH5590-J21SO2, and *L. sakei* LTH5590-J40RN2 are clones which have acquired a different colony morphotype on MRS medium after passage through the GIT of axenic mice. They were named from a nomenclature indicating the parent strain they were derived from, the time after transit they were collected (J10, J21, for instance), their colony morphotype (R or S), and the incubation conditions used for their isolation (O2 or N2 for aerobic or anaerobic conditions, respectively), as previously described (7).

* Corresponding author. Mailing address: Unité Flore Lactique et Environnement Carné, UR309, INRA, Domaine de Vilvert, F78350 Jouy en Josas, France. Phone: 33 1 34 65 22 89. Fax: 33 1 34 65 21 05. E-mail: monique.zagorec@jouy.inra.fr.

§ Present address: Institut de Génétique et Microbiologie, Equipe Physiologie et Métabolisme des Corynebactéries, Université Paris 11 Sud, 15 Rue Georges Clémenceau, F91400 Orsay, France.

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Bacteria were routinely grown at 30°C with stirring at 70 rpm either in MRS medium (12) or, for physiological studies, in the chemically defined medium MCD (26) supplemented with 0.5% (wt/vol) various carbon sources. To measure growth rate for various carbon sources, bacteria were first grown overnight in MCD medium containing 0.5% (wt/vol) glucose. Aliquots were then inoculated at an initial optical density at 600 nm (OD_{600}) of 0.05 in fresh MCD medium supplemented with 0.5% (wt/vol) D-glucose (Merck), *N*-acetyl-D-glucosamine (Sigma), D-mannose (Sigma), D-fructose (Merck), or D-galactose (Sigma) as the sole carbon source. The OD_{600} was determined every 90 min until stationary phase. Doubling times reached during the exponential phase of growth were calculated. Experiments were repeated at least twice.

Stress response measurement. To evaluate response to acidic pH, hydrogen peroxide (H_2O_2), and bile salt stresses, bacteria were collected from 10-ml overnight cultures in MRS medium and resuspended into fresh MRS medium, to which various components were added. For H_2O_2 stress, bacteria were inoculated at 10% (vol/vol) in fresh MRS medium, to which H_2O_2 was added at a concentration of 4 or 10 mM. Bacteria were then incubated for 1 h at 30°C, H_2O_2 stress was stopped by catalase addition, and survival was finally measured by plating bacterial dilutions on MRS plates and CFU counting. For bile salt stress, a sodium deoxycholate solution at 1% was used. Sodium deoxycholate was added at a final concentration of 0.1 or 0.3%, and bacteria were incubated for 3 h at 30°C and treated as described above. For acid stress, drops of concentrated HCl were added in order to obtain pH values ranging from 2.5 to 6.0 every half pH unit (initial MRS pH was around 6.5). Bacteria were inoculated at 1%, incubated for 1 h, and treated as described above. Stress response was measured as the log CFU difference between control (no stress component added) and stress conditions. Experiments were performed at least twice.

Transmission electron microscopy (TEM). Bacterial pellets recovered from MRS cultures (OD_{600} of 0.5) were fixed with 2% glutaraldehyde in 0.1 M Na cacodylate buffer, pH 7.2, for 3 h at room temperature. Samples were then postfixed with 1% osmium tetroxide containing 1.5% potassium cyanoferrate, gradually dehydrated in ethanol (30% to 100%), and embedded in Epon. Thin sections (70 nm) were collected onto 200 mesh copper grids and counterstained with lead citrate before examination with a Zeiss EM 902 transmission electron microscope at 80 kV (MIMA2; Plateau de Microscopie Electronique, Unité GPL, Jouy-en-Josas, France). Microphotographs were acquired using a Mega-View III charge-coupled-device (CCD) camera and analyzed with ITEM software (Eloïse Sarl, Roissy CDG, France).

Preparation of protein samples and labeling of cell wall-associated proteins. *L. sakei* 23K and *L. sakei* 23K-J30RO2 were grown in 250 ml MCD medium supplemented with 0.5% (wt/vol) glucose until an OD_{600} of 0.8 and centrifuged 30 min at 6,000 × *g*. Supernatant was discarded, and the bacterial pellet was washed three times with Tris-HCl, 0.1 M, pH 7.5, and stored at −80°C until use.

In order to stain surface-exposed proteins, cells were resuspended in 1 ml Tris-HCl, pH 8.8, and mixed with 400 pmol of CyDye DIGE Fluor Cy5 (Amersham GE Healthcare) diluted in dimethylformamide (DFM) (Sigma) according to the manufacturer's instructions. Samples were incubated at 4°C for 20 min in the dark, then mixed with 1 μl of a 10 mM L-lysine (Sigma) solution, incubated 10 min at 4°C, and washed three times in Tris-HCl, 0.1 M, pH 7.5. Cells were broken by a single pass through a cell disrupter (Basic Z; Constant Systems Ltd., Daventry, United Kingdom) at 2.5×10^5 Pa. Unbroken cells and cell debris were removed by centrifugation at 4,500 × *g* for 15 min. To ensure that bacteria were not permeable to Cy5, bacteria were collected prior to cell disruption and were observed on an Axio Observer Z1 microscope used in combination with the Apotome system and the Colibri illumination system (all from Carl Zeiss, Goettingen, Germany). Apotome generates deblurred optical sections of fluorescent samples, and the Colibri module is constituted of several light-emitting diodes (LED). For Cy5 excitation, an LED module at 625 nm was used. Images were acquired, stored, and processed using Axio Vision software facilities (Carl Zeiss). No intracellular signal was observed.

Cytoplasmic proteins were obtained by recovering the supernatant after ultracentrifugation at 50,000 × *g* for 30 min at 4°C and treating the suspension as previously described (32). Membrane vesicles from the pellet obtained after ultracentrifugation were resuspended in 1 ml 10% (wt/vol) sodium dodecyl sulfate (SDS), incubated at 100°C for 15 min, and centrifuged for 10 min at 3,500 × *g*; supernatant was filtered using 0.22-μm sterile filters. The protein suspension was then treated for 30 min at 37°C with 1 μl benzonase (Merck) in the presence of 10 mM $MgSO_4$ to remove contaminating nucleic acids. Proteins were precipitated by adding trichloroacetic acid to the final concentrations of 1.2 M (Merck) and 0.2 mg ml^{-1} sodium deoxycholate (Merck). After incubation for 1 h at 4°C in the dark, samples were centrifuged at 16,000 × *g* for 30 min, and the pellet was washed three times in cold acetone and air dried. Proteins were solubilized as described previously (32) and used for two-dimensional (2-DE) gel electrophoresis.

2-DE. The protocol for 2-DE gel electrophoresis was performed as previously described (32). Briefly, 350 μg of proteins was focalized in 17-cm IPG strips (pH 4 to 7) for the analysis of the cytoplasmic proteome and in 17-cm strips (pH 3 to 10) for surface proteins (Bio-Rad, Hercules, CA). Strips were rehydrated for 12 h at 50 V using Protean IsoElectric Focusing Cell II (Bio-Rad) and then focused at 300 V for 1 h (exponential), 1,500 V for 1 h (exponential), 10,000 V for 6 h (linear), and 10,000 V for 2.5 h (exponential). The second dimension was performed in 12.5% (wt/vol) polyacrylamide gels (20 by 20 by 0.1 cm) run overnight at 26 mA per plate at 4°C. Gels were stained with BioSafe colloidal Coomassie blue (Bio-Rad) and scanned with an ImageScanner (Amersham Biosciences, Piscataway, NJ) for the analysis of cytoplasmic proteins. To discriminate surface-exposed proteins stained with Cy5 dye in the cell wall/membrane fraction, gels were first scanned with a Typhoon 9410 (Amersham Pharmacia Biotech) at 670 nm to detect Cy5 and then stained with Coomassie blue and scanned with an ImageScanner as described above. Comparative image analysis was performed with Progenesis SameSpots (Nonlinear Dynamics) software by analyzing gels from at least three independent experiments. The relative volume of each spot (percent volume) was obtained from its spot intensity normalized to the sum of the intensities of all spots of the gel. Statistical analysis of the spot expression variation was performed by analysis of variance (ANOVA) provided by the software. Only proteins displaying significantly up- or downregulation in $n - 1$ gels and with a mean of at least 1.5-fold volume variations were considered and selected for identification through peptide mass fingerprinting.

Peptide mass fingerprinting and protein identification. Spots were analyzed as described in reference 19. Briefly, target spots were excised and in-gel digested overnight with 250 ng of trypsin (Promega, Madison, WI). The mass of the peptides was determined by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) on a Voyager DE STR instrument (Applied Biosystems) at the PAPSSO platform of the INRA Center in Jouy-en-Josas. Internal calibration was automatically performed with autolytical fragments of trypsin. *L. sakei* peptides were searched against the *L. sakei* database of the annotated genome (5) using Mascot and MS-Fit software. Sequence coverage (above 40%) scores of the search and agreement between theoretical and experimental molecular weights and isoelectric points were taken into account for considering identifications positive.

DNA manipulations, PCR, and sequencing. DNA was extracted from *L. sakei* cultures using the High Pure PCR template preparation kit (Roche) according to the manufacturer's instructions. DNA fragments encompassing the LSA1317 and LSA0104 genes and the LSA0162 to LSA0170, LSA0462-LSA0463, and LSA0551-LSA0552 operons as well as their promoter regions, were amplified by PCR with primers listed in Table S1 in the supplemental material. PCR amplification was performed in a PCT-200 thermocycler (MJ Research). The 100-μl PCR mixtures contained PCR buffer, 1.5 nmol $MgCl_2$ $liter^{-1}$, 0.2 nmol $liter^{-1}$ of each dNTP, 0.5 μmol $liter^{-1}$ of each primer, 1 μg ml^{-1} of chromosomal DNA, and 2 U of *Taq* polymerase (Fermentas). The program used was as follows: 95°C for 4 min; 25 cycles of 95°C for 30 s, 55°C for 10 s, and 72°C for 1 min; and a final elongation step at 72°C for 5 min. For sequencing, PCR fragments were treated with 0.1 U shrimp alkaline phosphatase (USB) and 1 U exonuclease I (Biolabs) in 20 mM Tris-HCl, pH 8.0, and 10 mM $MgCl_2$ buffer for 1 h at 37°C and then sent for sequencing to Eurofins MWG Operon (Germany). Primers used for sequencing are listed in Table S2 in the supplemental material.

RESULTS

Morphological changes acquired during transit of *L. sakei* in the GIT are associated with septation defect or cell wall modification. TEM observations showed that long filaments issued from R clone mutants FLEC01-J15RN2 and 23K-J30RO2 were formed by cells lacking internal septation (Fig. 1B, C, and E) compared to their parent strains (Fig. 1A and D). Interestingly, we also noticed the presence of dark transversal bands (absent from wild-type [WT] strains) on the surface of R clones FLEC01-J15RN2 and 23K-J30RO2 (Fig. 1B, C, and E), suggesting a helical structure. In addition, images of the *L. sakei* LTH5590-J21SO2 S clone (Fig. 1G) revealed an abnormally deformed cell membrane and an apparently thicker cell wall than that of the wild-type parent, LTH5590

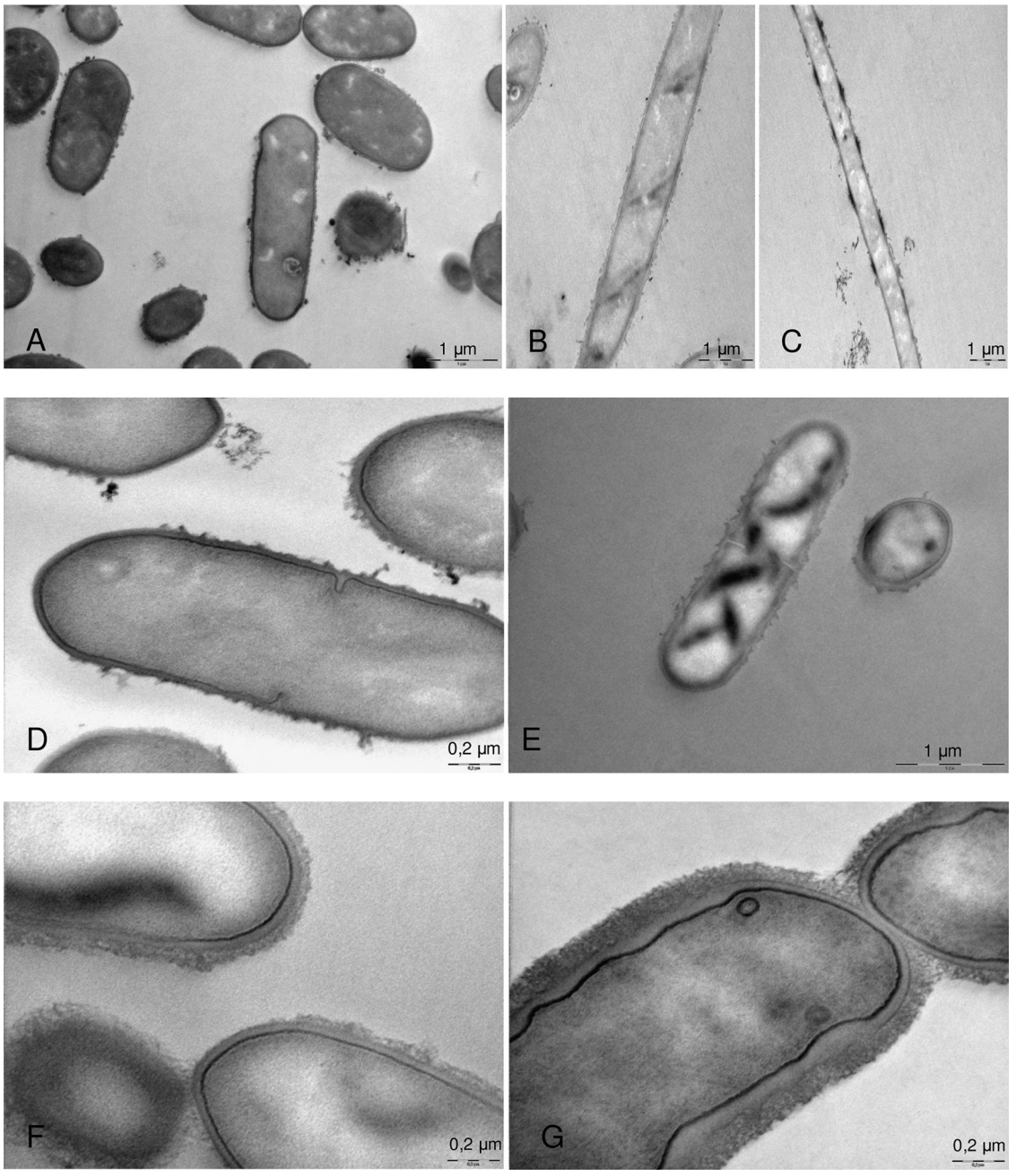

FIG. 1. Morphology of cells issued from S and R clones of the *L. sakei* 23K, FLEC01, or LTH5590 strain observed by transmission electron microscopy. *L. sakei* FLEC01 wild type (A) and its rough clone derivative FLEC01-J15RN2 (B and C); *L. sakei* 23K wild type (D) and its rough clone derivative *L. sakei* 23K-J30RO2 (E); *L. sakei* LTH5590 wild type (F) and it small clone derivative *L. sakei* LTH5590-J21SO2 (G) grown in MRS liquid medium.

(Fig. 1F), which were not observed with the R strains. This confirmed previous scanning electron microscopy observations that showed atypical cell shape and cell surface structures of the bacteria issued from S and R clones (7).

Modification of stress responses in *L. sakei* R and S clones. Responses to bile salt, H_2O_2, or acidic pH stress were evaluated as the difference of survival rates after the stress for the S and R clones and the three parent strains 23K, FLEC01, and LTH5590 from which they were derived. The S clones had, in general, a phenotype close to that of their WT or intermediary between those of their WT and the R clones (Table 1). We noticed that the behaviors of three WT strains regarding bile salt response were slightly different: at 0.3% bile salt, 23K was less sensitive than LTH5590 and FLEC01 (Table 1). However, the R clone 23K-J30RO2 became more sensitive than its WT parent, whereas the two R mutants FLEC01-J15RN2 and LTH5590-J40RN2 were more resistant than their parent strain (Table 1). After 10 mM H_2O_2 stress, the R clone LTH5590-

TABLE 1. Stress responses of WT and R clones[a]

Strain	Stress response to:			
	Bile salt (0.3%)	H_2O_2 (4 mM)	H_2O_2 (10 mM)	pH[b]
23K	0.90 ± 0.10	3.20 ± 0.30	>5	<0.4
23K-J10SN2	2.10 ± 0.90	3.80 ± 0.70	>5	ND
23K-J30RO2	3.90 ± 0.70	5.00 ± 0.20	>5	2.4 ± 0.20[c]
FLEC01	5.20 ± 0.00	4.05 ± 0.15	>5	<0.4
FLEC01-J6SN2	4.10 ± 0.10	3.40 ± 0.20	>5	ND
FLEC01-J15RN2	3.95 ± 0.15	3.40 ± 0.10	>5	<0.4
LTH5590	5.35 ± 0.05	>5	>5	<0.4
LTH5590-J21SO2	3.50 ± 0.10	3.80 ± 0.50	4.10 ± 0.20	ND
LTH5590-J40RN2	1.75 ± 0.15	0.60 ± 0.10	2.27 ± 1.0	<0.4

[a] Stress response is expressed as the loss of cell viability (Δlog CFU/ml) after the various stress conditions tested, as described in Materials and Methods.
[b] Values were similar for pH values ranging from 3 to 6.0. ND, not determined.
[c] Value is given for pH 3.

J40RN2 was the most resistant strain, whereas all other strains showed a high sensitivity (a loss of viability of >5 log CFU). At 4 mM H_2O_2, all strains showed a loss of viability of ~3 to 5 log CFU (Table 1). However, LTH5590-J40RN2 was clearly more resistant than its parent, and 23K-J30RO2 was slightly more sensitive (5.0 ± 0.2 versus 3.8 ± 0.1 log CFU) than its parent, *L. sakei* 23K. Concerning acidic stress, all strains were highly resistant to low pH (loss of viability did not exceed 0.4 log CFU, even at pH 3.0). The mutant 23K-J30RO2 showed a viability decrease but only at pH 3.0 (Table 1).

***L. sakei* mutants 23K-J30RO2 and 23K-J10SN2 have improved growth on some carbohydrates.** Ability to grow on various carbon sources of the S and R clones 23K-J10SN2 and 23K-J30RO2 was tested and compared to that of their parent strain, *L. sakei* 23K (Table 2). No significant differences were observed between the S and R mutants and their parent strain on glucose or mannose. In contrast, *L. sakei* 23K-J30RO2 exhibited slightly faster growth on *N*-acetylglucosamine ($P < 0.1$) and significantly faster growth on fructose, ribose, and galactose. 23K-J10SN2 had a similar but less pronounced phenotype.

Proteomic analysis of a clone forming rough colonies reveals multiple changes. In order to detect which proteins may be involved in the phenotype of the mutants, we chose to compare the proteomes of a WT strain and of one of its derived mutant. For that purpose, we chose *L. sakei* 23K as the WT parent, since its genome sequence is known (5), and the R clone *L. sakei* 23K-J30RO2. Indeed, since this mutant did not show any growth defect in MCD medium supplemented with glucose, no differences due to growth rate were expected from the comparison with the WT. In addition, its R phenotype was shown to be stable in MCD medium (7), avoiding the apparition of revertants and thus avoiding a mixed population that may have led to erroneous proteomic data. As TEM analysis suggested, cell wall modifications in the mutant, both cytosolic and cell wall/membrane compartments, were analyzed.

Twenty-four proteins were downregulated in *L. sakei* 23K-J30RO2 (Table 3): eight were cytoplasmic, 12 were cell wall or membrane associated, and one (LSA0494), the 30S ribosomal interface protein S30EA, was observed in both compartments. Five out of the 13 proteins of the cell wall/membrane fraction were surface exposed, as revealed by Cy5 labeling. In addition, for three proteins (LSA1366, LSA1188, and LSA1032), products of genes encoding, respectively, the ATP binding subunit of an ABC transporter, the pyruvate oxidase Pox1, and the pyruvate kinase, the differences between the parent and R-derived clone were observed only with the Cy5-labeled fraction and not with the membrane fraction stained with Coomassie blue. This suggests that the relative amounts of these three proteins did not vary, but rather their expositions to the surface of the cell. Among the less expressed spots in the R clone, those identified as proteins LSA0169, LSA0170, LSA1317, LSA0552, and LSA0104 were abundant in the proteome of the *L. sakei* 23K WT strain but were almost undetectable in the R clone. LSA0169 and LSA0170 are putative general stress proteins of the Asp family and are encoded by the same operon as LSA0165, also downregulated and identified as a putative oxidoreductase. LSA1317 is a putative chromate reductase. LSA0552 (OhrA, a hydroperoxide resistance protein) and LSA0104 (a thiol peroxidase) are both involved in the response to oxidative stresses. Five proteins (LSA0705, LSA0706, LSA0938, LSA0616, and LSA1366) are components of ABC

TABLE 2. Growth characteristics of *L. sakei* 23K, *L. sakei* 23K-J10SN2, and *L. sakei* 23K-J30RO2 in MCD medium supplemented with various carbon sources[a]

L. sakei strain	Doubling time[b]					
	Glucose	Mannose	Ribose	NAG	Fructose	Galactose
23K	86 ± 4	98 ± 9	117 ± 10	102 ± 4	113 ± 2	117 ± 3
23K-J10SN2	85 ± 7	91 ± 8	96 ± 6*	88 ± 8**	93 ± 8*	95 ± 5*
23K-J30RO2	89 ± 6	93 ± 9	93 ± 8*	90 ± 6**	91 ± 6*	98 ± 3*

[a] Carbon sources were added in the medium at the concentration of 0.5% (wt/vol).
[b] Doubling times are expressed in minutes. Results are the mean of three independent repeats. NAG, *N*-acetylglucosamine; *, values were significantly different from the WT ($P < 0.05$); **, values were significantly different from the WT ($P < 0.1$).

TABLE 3. Cytosolic and cell wall/membrane-associated proteins downregulated in R clone *L. sakei* 23K-J30RO2[a]

Protein type	Functional category	Function	Locus tag	ANOVA[a]	Fold vol change
Cytoplasm	Adaptation to atypical conditions	Putative general stress protein	LSA0169	0.003	34
		Putative general stress protein	LSA0170	0.017	6.2
		Universal stress protein (Usp5)	LSA0038	0.030	4.3
	Oxidoreduction	Putative chromate reductase (flavoprotein)	LSA1317	0.002	8.5
		Organic hydroperoxide resistance protein (OhrA)	LSA0552	0.030	6.5
		Putative oxidoreductase, short-chain dehydrogenase/reductase family	LSA0165	0.005	2.7
	Carbohydrate metabolism	Pyruvate dehydrogenase complex, E1 component, beta subunit (PdhB)	LSA1084	0.041	3.2
	Amino acid metabolism	Putative 2-hydroxyacid dehydrogenase	LSA0463	0.003	3.2
	Transcription-translation	30S ribosomal interface protein S30EA	LSA0494	0.007	4.0
Cell wall/membrane	Adaptation to atypical conditions	Universal stress protein (Usp6)	LSA0836	0.004	2.1
	Transport and binding	Oligopeptide ABC transporter, ATP-binding subunit (OppD)	LSA0705	0.022	3.5
		Oligopeptide ABC transporter, ATP-binding subunit (OppF)	LSA0706	0.007	3.4
		Putative drug ABC exporter, ATP-binding subunit	LSA0938	0.026	3.3
		Putative glycine/betaine/carnitine/choline ABC transporter, ATP-binding subunit	LSA0616	0.023	3.1
	Oxidoreduction	Thiol peroxidase (Tpx)	LSA0104	0.003	5.3
		Putative aldo/keto reductase (oxidoreductase)	LSA0030	0.001	2.6
		Putative nitroreductase (oxidoreductase)	LSA1712	0.001	2.5
	Protein modification	ATPase/chaperone (ClpC)	LSA1779	0.023	2.6
	Amino acid metabolism	Glycine/serine hydroxymethyltransferase (GlyA)	LSA1134	0.037	2.7
		Putative aminopeptidase	LSA1426	0.002	3.1
	Transcription-translation	30S ribosomal interface protein S30EA	LSA0494	0.004	3.7
	RNA restriction modification	Putative RNA methyltransferase	LSA1544	0.012	1.9
Surface exposed	Adaptation to atypical conditions	Universal stress protein (Usp6)	LSA0836	0.004	2.1
	Transport and binding	Putative glycine/betaine/carnitine/choline ABC transporter, ATP-binding subunit	LSA0616	0.0006	3.9
		Oligopeptide ABC transporter, ATP-binding subunit (OppD)	LSA0705	0.030	2.4
		Oligopeptide ABC transporter, ATP-binding subunit (OppF)	LSA0706	0.004	2.3
		Putative ABC exporter, ATP-binding subunit	LSA1366	0.046	2.1
	Oxidoreduction	Thiol peroxidase (Tpx)	LSA0104	0.037	2.4
	Carbohydrate metabolism	Pyruvate oxidase (Pox1)	LSA1188	0.012	2.3
		Pyruvate kinase (Pyk)	LSA1032	0.033	1.7

[a] Statistical validation was performed by the use of an ANOVA test provided by SameSpots (see Materials and Methods).

transporters, two proteins (LSA0038 and LSA0836) are involved in general stress response, and three, LSA1188 and LSA1032, mentioned above, and the beta subunit of the pyruvate dehydrogenase complex (LSA1084), are involved in the metabolism of pyruvate. The remaining proteins are involved in amino acid metabolism and protein modification (LSA0463, LSA1134, LSA1426, and LSA1779) or oxidoreduction (LSA0030 and LSA1712). It should be noted that the downregulation of spots LSA0170, LSA0522, and LSA0616 *per se* is not sufficient to explain the phenotype observed with the R mutant since mutants of the genes encoding these proteins, available in the laboratory (29; M. Zagorec, unpublished results), do not show the same phenotype as that of *L. sakei* 23K-J30RO2 (M. Champomier-Vergès and M. Zagorec, personal communication).

Fourteen spots were upregulated in the mutant *L. sakei* 23K-J30RO2: nine were cytoplasmic, two were found in the cell wall/membrane fraction, and one (LSA1629) was detected at both locations (Table 4). Three spots were detected as surface exposed (i.e., in the Cy5-labeled fraction); among the three spots, LSA0853 was already found in the cell wall/membrane fraction. Four upregulated proteins, GroEL (LSA0359), DnaK (LSA1236), the copper homeostasis protein LSA1440, and the surface-exposed GTP-binding protein LSA1079, are involved in stress response, two are ribosomal proteins with regulatory functions (LSA0007 and LSA1667), one is an ATP-dependent H^+ transporter (LSA1127), and one is a cysteinyl tRNA synthase (LSA1681). Interestingly, among the three proteins that were cell wall/membrane-associated proteins and surface exposed, the amount of which was increased in the R clone, we noticed the presence of the actin-like cell shape determining protein MreB (LSA0853). Three spots were identified as enzymes involved in carbohydrate metabolism: 6-phosphofructokinase (LSA1033), L-lactate dehydrogenase (LSA1606), and fructose-biphosphate aldolase (LSA1527). Finally, two proteins, CTP synthase (LSA1629) and xanthine phosphoribosyltransferase (LSA1557), are involved in nucleotide metabolism. Surprisingly, some proteins usually described

TABLE 4. Cytosolic and cell wall/membrane-associated proteins upregulated in the R clone *L. sakei* 23K-J30RO2[a]

Protein type	Functional category	Function	Locus tag	ANOVA[a]	Fold vol change
Cytoplasm	Adaptation to atypical conditions	Chaperonin GroEL	LSA0359	0.017	2.7
		Chaperone protein DnaK	LSA1236	0.032	2.6
		Copper homeostasis protein (CutC)	LSA1440	0.018	1.5
	Transport and binding	H^+-transporting two-sector ATPase (ATP synthase), gamma subunit	LSA1127	0.038	3.7
	Carbohydrate metabolism	6-Phosphofructokinase (Pfk)	LSA1033	0.044	2.5
	Transcription-translation	30S ribosomal protein S6 (RpsF)	LSA0007	0.025	2.1
		50S ribosomal protein L10 (RplJ)	LSA1667	0.046	2.0
	Translation	Cysteinyl tRNA synthase (CysS)	LSA1681	0.034	2.2
	Nucleotide metabolism	CTP synthase (PyrG)	LSA1629	0.041	3.2
		Xanthine phosphoribosyltransferase (Xpt)	LSA1557	0.021	1.6
Cell wall/membrane	Carbohydrate metabolization	L-Lactate dehydrogenase (L-Ldh)	LSA1606	0.007	1.5
	Nucleotide metabolism	CTP synthase (PyrG)	LSA1629	0.003	2.5
	Cell division	Cell shape-determining protein (MreB)	LSA0853	0.022	1.7
Surface exposed	Adaptation to atypical conditions	GTP-binding protein TypA	LSA1079	0.016	1.6
	Carbohydrate metabolism	Fructose-bisphosphate aldolase (Fba)	LSA1527	0.020	1.6
	Cell division	Cell shape-determining protein (MreB)	LSA0853	0.039	2.2

[a] See Table 3, footnote *a*.

as cytoplasmic were found in the cell wall/membrane-associated fraction. Such results have already been observed for so-called "moonlight" proteins and particularly with *L. sakei* (P. Anglade, unpublished results).

Spot disappearance in the R mutant does not result from *cis*-acting mutations. Since several spots, some being encoded by the same operon, strongly diminished or even became undetectable in the R clone, one could interpret this as the result of mutations in or upstream from the corresponding genes. Therefore, we amplified and sequenced those genes, with their promoter regions, from both *L. sakei* 23K and *L. sakei* 23K-J30RO2 and compared the DNA sequences. We chose operons and genes coding for proteins with the highest volume change according to Progenesys SameSpots image analysis: the operons LSA0162 to LSA0170 (from which the genes encoding spots corresponding to LSA0165, LSA0169, and LSA0170 disappeared in the mutant, with the other genes encoding proteins not being detectable in any strain), LSA0551-LSA0552, and LSA0462-LSA0463 and the two monocistronic genes LSA0104 and LSA1317. However, DNA sequences showed 100% identity between the parent and derivative strains, thus indicating that mutations suppressing the expression of those spots were located neither in their structural genes nor in their promoter regions.

DISCUSSION

We previously showed that colonization of *L. sakei* in the GIT of axenic mice induced the emergence of subpopulations, having the advantage to colonize the GIT and to partially displace the preexisting bacterial population (7). This confirmed that food is the preferred environment for *L. sakei* but suggested that mutations can be acquired for a better adaptation to the GIT in axenic mice. Here we show that mutants acquired a modified response to various stresses that can be encountered in the GIT and an increased growth ability on some carbon sources that are present in the GIT.

The appearance of phenotypic heterogeneity within an isogenic microbial population during transit through the GIT of mice has already been described for the Gram-negative *E. coli* MG1655 strain: within 10 days, small granular colonies reached more than 90% of the total population but could not completely displace the original population (17). This phenotype results from mutations in the *E. coli* EnvZ/OmpR system, a master regulator controlling more than 100 target genes. The authors proposed that mutations in regulatory genes promote a better adaptation and evolution than some improving a single function. Proteomic analysis performed with an R mutant issued from *L. sakei* 23K showed some variations, suggesting a modification in sugar catabolism and/or a pyruvate rerouting. Indeed, whereas L-lactate dehydrogenase (LSA1606), 6-phosphofructokinase (LSA1033), and 1,6-fructose-bisphosphate aldolase (LSA1527) are overexpressed, pyruvate oxidase (LSA1188), pyruvate kinase (LSA1032), and a subunit of the pyruvate dehydrogenase complex are underexpressed. Although no growth difference was observed between the parent strain and the R mutant on glucose and mannose, the mutant grew faster on ribose, galactose, fructose, and *N*-acetylglucosamine. This may lead to a better fitness due to more efficient use of alternative carbon sources that are present in the GIT, an environment poor in energy sources, particularly in glucose. These characteristics are similar to those reported for *E. coli* MG1655 mutants isolated from feces of streptomycin-treated mice, which showed improved growth on several sugars (27), some shown indeed to be utilized by *E. coli* in the GIT (1).

Proteomic analysis performed on an R clone suggested a modification of the stress status of the cells, which may correlate with modification of the survival rate after various stresses were applied. Four proteins involved in stress response (LSA0038, LSA0836, LSA0169, and LSA0170) were remarkably underrepresented in the proteome of the R clone; in contrast, the chaperones GroEL (LSA0359) and DnaK (LSA1236), the copper homeostasis protein CutC (LSA1440), and one GTP binding protein, TypA (LSA1079), all involved in stress response, were upregulated. The two paralogs LSA0169

and LSA0170 showed some similarity with the protein Gls24 of *Enterococcus faecalis*, and a *gls24* mutant exhibited phenotypes and modification of protein expression partially similar to those of the *L. sakei* 23K-J30RO2 R mutant (16). Some proteins involved in transcription and regulation were also differently expressed in the mutant. The wild-type *L. sakei* 23K expresses the LSA0494 ribosomal protein four times more than the R clone. In contrast, *L. sakei* 23K-J30RO2 overexpresses RpsF (LSA0007) and RplJ (LSA1667). Those two ribosomal proteins are overproduced in *L. sakei*, *E. faecalis*, and *Listeria monocytogenes* after a high-pressure treatment (23). RpsF is also reported to be upregulated in *B. subtilis* after cold shock (25), while both RpsF and RplJ are downregulated in stationary phase in *L. monocytogenes* (15). We also noticed that the *L. sakei* R clone overproduces an ATP-dependent H^+ transporter involved in the maintenance of the internal pH but was, however, less resistant than its parent after an external shock at pH 3.0. Cysteinyl tRNA synthase (LSA1681) is also upregulated in *L. sakei* 23K-J30RO2. The importance of sulfur amino acids in the mouse GIT colonization and in particular in the resistance to acid stress has already been reported. The *luxS* gene (absent from the *L. sakei* 23K genome), coding for the autoinducer-2 and involved in the metabolism of cysteine and methionine, has an essential role in the persistence of *Lactobacillus reuteri* 100-23 and *Lactobacillus rhamnosus* GG in the GIT (28, 34). In addition, *Bifidobacterium longum* 8809dpH, a mutant resistant to low pH, overexpresses proteins involved in the metabolism of sulfur amino acids compared to its parental strain (33). The increased amount of LSA1681 in the R mutant of *L. sakei* might thus contribute to its improved adaptation to the GIT. Several oxidoreductases (LSA1317, LSA0552, LSA0165, LSA0104, LSA0030, and LSA1712) involved in detoxification or oxidative stress protection were strongly downregulated in the R clone. This may explain the slightly higher sensitivity to H_2O_2 in the mutant, although observed in laboratory conditions. The modified expression of all these proteins suggests that the R mutant has acquired a different stress status than that of the parent strain.

Recently, the appearance of a heterogeneous population composed of microcolonies characterized by elongated cells and lack of septation after exposure to acid stress was described for *Lactobacillus plantarum* WCFS1 (22). As for *L. plantarum* WCFS1, the long cells or filaments of our R and S clones were lacking septa. In addition, we observed a modification of the membrane shape but only in the R mutant derived from LTH5590, suggesting that the factors affected in the various S and R clones are different. This is consistent with our previous observation of many different growth patterns in the collection of S and R clones (7). Interestingly, we noticed in our TEM observations the presence of transversal slanted black bands crossing filamentous cells located apparently at the surface of cells issued from R clones. Our proteomic analysis showed that the actin-like protein MreB, located at the surface of the cells, is upregulated in the R mutant. MreB is a protein involved in cell wall formation, cell elongation, and cell shape determination. MreB polymers can form dots and helical filamentous structures associated with the membranes of the cell (18, 24). Although further experiments would be required to demonstrate it, it is tempting to hypothesize that the helical structures observed with R mutants may be composed of MreB, which is overexpressed, and may consequently "modify" the cell shape but does not affect cell division. That could also explain the atypical morphology of colonies (rough) of the mutant and might possibly modify their adhesion properties, which are important for colonization of the GIT.

In eukaryotic cells, oxidative stress can disrupt the actin cytoskeleton by oxidative modification of the sulfhydryl group of its conserved Cys374 (11). Residues Cys113 and Cys324 of the MreB proteins are highly conserved in prokaryotes, and a role for the MreB protein in the response to oxidative stress was proposed for *Vibrio parahaemolyticus* by demonstrating how the configuration of the MreB cytoskeleton can change under stressing conditions (8). In particular, it was proposed that the oxidation of this protein could act as a neutralizer for reactive species generated when the cell is stressed. The overexpression of MreB detected in the *L. sakei* R strain might thus have a secondary effect as an alternative response toward oxidative stresses, compensating for the downregulation of several oxidoreductases.

It is also possible that transport of some molecules could be modified in the R clone, since five spots (LSA0705, LSA0706, LSA0938, LSA0616, and LSA1366) identified as components of ATP-dependent transporters for oligopeptides or osmoprotectants or for drug export were downregulated in *L. sakei* 23K-J30RO2. These results suggest the improved fitness, involving cell permeability, of the R mutant for transit through the GIT. Similarly, reduced permeability of the membranes has been reported for the *E. coli* MG1655 *ompB* mutants, naturally adapted to the GIT environment (17). As well, a decreased expression of genes encoding transporters has been observed during the transit of *Lactobacillus johnsonii* NCC533 in the jejunum and cecum intestine (13).

Sequencing of five DNA regions coding for proteins, the expression of which was strongly diminished in the R clone, did not reveal any mutation (7). We think that, as described for *E. coli* GIT-adapted mutants, only one or a few mutations having pleiotropic effects occurred. In addition, the modification of carbohydrate metabolism and the stress status of the cells may have secondary multiple effects, explaining the proteome differences we observed between the R strain and its parent.

Several methods were used to investigate the bacterial functions involved in GIT colonization. A proteomics-based analysis of *E. coli* cells collected directly from mice feces has been reported (1). Several transcriptomics analyses, aiming at the identification of bacterial genes expressed in the GIT of mice and using microarrays, *in vivo* expression technology (IVET), or resolvase IVET (R-IVET), have also been reported for various lactobacilli (4, 13, 14, 38, 39). Alternatively, our approach combining proteomic and phenotypic analyses of *L. sakei* mutants adapted to GIT colonization allowed us to define functions that are important for improving the fitness of this meat-borne bacterium in the GIT.

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