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Diversity in Robustness of *Lactococcus lactis* Strains during Heat Stress, Oxidative Stress, and Spray Drying Stress

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In this study we tested 39 *Lactococcus lactis* strains isolated from diverse habitats for their robustness under heat and oxidative stress, demonstrating high diversity in survival (up to 4 log units). Strains with an *L. lactis* subsp. *lactis* phenotype generally displayed more-robust phenotypes than strains with an *L. lactis* subsp. *cremoris* phenotype, whereas the habitat from which the strains had been isolated did not appear to influence stress survival. Comparison of the stress survival phenotypes with already available comparative genomic data sets revealed that the absence or presence of specific genes, including genes encoding a GntR family transcriptional regulator, a manganese ABC transporter permease, a cellobiose phosphotransferase system (PTS) component, the FtsY protein, and hypothetical proteins, was associated with heat or oxidative stress survival. Finally, 14 selected strains also displayed diversity in survival after spray drying, ranging from 20% survival for the most robust strains, which appears acceptable for industrial application, to 0.1% survival for the least-tolerant strains. The high and low levels of survival upon spray drying correlated clearly with the combined robustness under heat and oxidative stress. These results demonstrate the relevance of screening culture collections for robustness under heat and oxidative stress on top of the typical screening for acidifying and flavor-forming properties.

Based on their spoilage-preventing and flavor-enhancing characteristics, lactic acid bacteria (LAB) have been employed since ancient times in the fermentation of foods, e.g., fruits, vegetables, cereal grains, meat, and milk (1, 2). Nowadays, many of these processes have been industrialized, and fermentation is typically initiated by the addition of starter cultures, which contain high concentrations of one or multiple LAB strains (1, 2). As starter cultures require metabolic activity to contribute to the taste and texture of the fermentation end products, there has been an increasing industrial interest in studying robustness phenotypes during industrial production and processing (1), which involves preservation by either freezing or drying techniques (3–5). The major disadvantages of frozen starter cultures are the inconvenience and costs of transport and storage at low temperature, and, therefore, drying techniques are preferred (3–5). During spray drying, cultures are exposed to severe heat and oxidative stress (6, 7), typically resulting in lower survival rates of starter cultures than freeze-drying (3, 4). Therefore, freeze-drying is currently the most often applied industrial drying method (3–5). However, spray drying appears a more cost-effective and energy-efficient drying alternative for the preservation of starter cultures (3–5), providing strains that display high robustness under the stresses encountered in this process can be identified. This appears feasible, as studies on stress phenotypes typically result in highly strain-specific robustness phenotypes, e.g., for the gastrointestinal survival of *Lactobacillus plantarum* strains (8) and the robustness of several *Lactobacillus* strains during acid, alkaline, heat, oxidative, osmotic, detergent, and starvation stresses (9).

Lactococcus lactis is one of the most widely used LAB for industrial food fermentations, including the production of cheese and butter(milk) (2). *L. lactis* strains used in industry are mainly of dairy origin, and within this group of strains a high diversity has been observed in functional characteristics such as bacteriocin production (10) and proteolytic activity (11). Interest in strains from other habitats has increased over the past decade, as diversity studies including nondairy strains demonstrated even more distinct phenotypes than studies including solely dairy strains, e.g., in flavor formation (12, 13). Furthermore, the potential for the application of nondairy strains in dairy starter cultures was demonstrated by adaptation of the plant-derived *L. lactis* strain KF147 to a dairy environment by long-term propagation (14). Comparative genomics approaches have pinpointed differences between *L. lactis* strains with respect to genes predicted to be involved in stress responses (15, 16), suggesting differences in stress survival characteristics between *L. lactis* strains. Nevertheless, diversity in stress survival phenotypes has been minimally studied for this LAB.

In this study we tested 39 *L. lactis* strains isolated from diverse habitats for their robustness under heat and oxidative stress and compared these data with robustness during lab scale spray drying. The heat and oxidative stress survival data were also correlated to the habitat and subspecies of the strains and to genomic content data (15) to identify genes associated with robustness, an approach that previously has been successfully employed for *L. plantarum* (17–19).

MATERIALS AND METHODS

Bacterial isolates and media. The 39 *L. lactis* strains used in this study were selected from a large collection of phenotypically and genotypically

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TABLE 1 Strains employed for heat and oxidative stress survival screening

Strain code[a]	NIZO code	Isolation source	Other information
L. lactis subsp. *lactis* genotype, *L. lactis* subsp. *lactis* phenotype			
ML8	20	Dairy starter	
LMG8526	26	Chinese radish seeds	
ATCC 19435^T	29	Milk (dairy starter)	
UC317	644	Dairy starter	
M20	844	Soil	*L. lactis* subsp. *lactis* biovar diacetylactis
Li-1	1156	Grass	
E34	1173	Silage	
DRA4	1592	Dairy starter A	*L. lactis* subsp. *lactis* biovar diacetylactis
LMG9446	2123	Frozen peas	
LMG9447	2124	Frozen peas	
K231	2199	White kimchi	
K337	2202	White kimchi	
P7266	2206	Litter on pastures	
P7304	2207	Litter on pastures	
NCDO895	2211	Dairy starter	
KF7	2219	Alfalfa sprouts	
KF24	2220	Alfalfa sprouts	
KF67	2223	Grapefruit juice	
KF134	2226	Alfalfa and radish sprouts	
KF146	2229	Alfalfa and radish sprouts	
KF147	2230	Mung bean sprouts	
KF196	2236	Japanese kaiware shoots	
KF201	2238	Sliced mixed vegetables	
NIZO2244B	3919	Mustard and cress	
KF282	3920	Mustard and cress	
N42	1230	Soil and grass	
LMG14418	2424	Bovine milk	
IL1403	2441	Dairy starter	
L. lactis subsp. *cremoris* genotype, *L. lactis* subsp. *lactis* phenotype			
NCDO763	643	Dairy starter	Derivative of NCDO712
V4	1157	Raw sheep milk	
N41	1175	Soil and grass	
MG1363	1492	Cheese starter	Plasmid-free derivative of NCDO712
KW10	2249	Kaanga wai	
L. lactis subsp. *cremoris* genotype and phenotype			
SK11	32	Dairy starter	
AM2	33	Dairy starter	
HP	42	Dairy starter	Same as LMG6897^T but from different collection
FG2	2252	Dairy starter	
LMG6897^T	2418	Cheese starter	Same as HP but from different collection
L. lactis subsp. *hordniae* LMG8520^T	24	Leaf hopper (insect)	

[a] Underlined strains were included in the spray drying analysis.

characterized strains (15, 20) and are listed in Table 1. This set contains *L. lactis* strains of three different subspecies: *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, and *L. lactis* subsp. *hordniae*, which were isolated from dairy as well as nondairy environments. All strains were grown in M17 broth (Oxoid, Basingstoke, United Kingdom) supplemented with 0.5% (wt/vol) glucose (Merck, Darmstadt, Germany) (GM17) at 30°C.

Heat and oxidative stress survival assays. From a preculture, a 1% (vol/vol) inoculum was added to 2 ml of fresh GM17 in duplicate in a 96-well Masterblock (Greiner BioOne GmbH, Frickenhausen, Germany) and incubated for 16 h at 30°C. In stationary phase, cells were harvested from 0.5 ml of culture by centrifugation at 1,865 × *g* for 15 min and resuspended in 1 ml sterile 50 mM sodium phosphate buffer (Merck, Darmstadt, Germany), pH 7.2. To measure heat stress survival, 100 μl of the cell suspensions was incubated at 50°C for 30 min in a 0.1-ml 96-well PCR plate (MicroAmp; Applied BioSystems, Foster City, CA) in a GeneAmp PCR system 9600 (Applied BioSystems). Controls were left at room temperature for 30 min. For assessment of oxidative stress survival, 1 ml of culture was centrifuged at 1,865 × *g* for 15 min and resuspended in the same volume of phosphate buffer. Hydrogen peroxide (Merck) in phosphate buffer was added to 0.25 ml of the cell suspensions to a final concentration of 5 mM and an end volume of 0.5 ml, followed by incubation for 3 h at 30°C in a water bath. To the controls, buffer without hydrogen

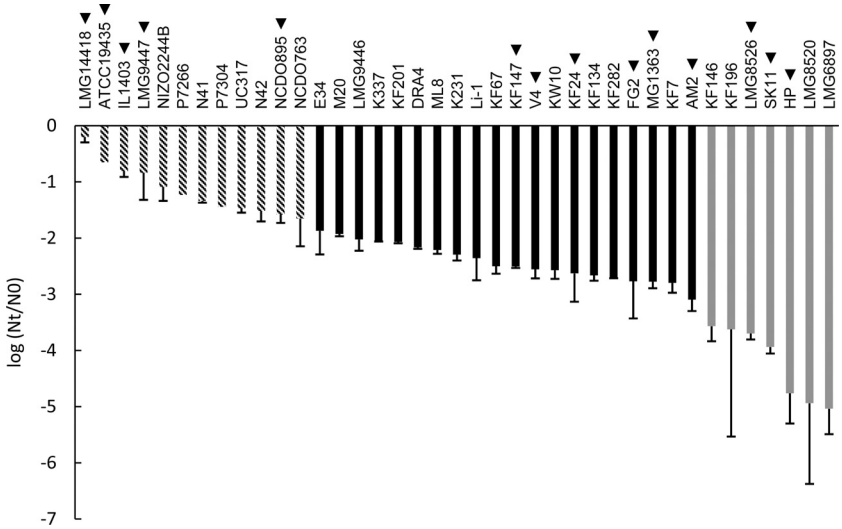

FIG 1 Robustness of *L. lactis* strains upon exposure to heat stress expressed as the difference between log CFU/ml after heat stress (Nt) and control (N0). For genotype-phenotype matching, the strains were divided into groups of robust (striped bars), intermediate (black bars), and sensitive (gray bars) strains. Strain names indicated with a triangle represent strains that were selected for the spray drying analysis (see Fig. 4). The data represent averages of two biological replicates. Error bars indicate standard deviations.

peroxide was added, and these cell suspensions were also incubated for 3 h at 30°C. After incubation, samples were centrifuged at 1,865 × *g* for 15 min and cells were resuspended in 0.5 ml of phosphate buffer. Survival was measured by spotting serial dilutions in triplicate on GM17 agar plates (21). CFU were assessed after incubation of the plates for 72 h at 30°C.

Genotype-phenotype comparison. Heat stress and oxidative stress survival data were associated with the gene presence/absence matrix derived from the pangenome-based comparative genome hybridization analysis performed by Siezen et al. (15). The Web tool PhenoLink (22), which applies the Random Forest classification algorithm (23), was used to identify genes associated with stress survival. Default parameters were used, except for the bagging parameter, which was set to 100, and the "ratio of largest phenotype size to smallest phenotype size" parameter (explained below), which was set to 1 for the heat stress data. These parameters were modified from the default setting to deal with imbalance in sample group sizes for some survival parameters measured. Class imbalance can severely skew importance estimation of genes due to overtraining of the classification model to the larger sample group. Briefly, from the larger class (sample group), 100 (bagging parameter) times the number of samples (ratio of 1) from the smallest class were drawn to ensure that, even for very unbalanced sample groups, all samples of the larger group were taken. Three groups of strains were defined (see Fig. 1 and 2), based on the heat and oxidative stress survival phenotypes (robust, intermediate, and sensitive groups). The presence of a gene in 75% of the strains in the robust or sensitive group and the absence of this gene in 75% of the strains in the reciprocal group were used as a minimum cutoff to pinpoint genes possibly associated with robustness under the applied stress.

Spray drying. From the set of 39 *L. lactis* strains, 14 strains (LMG8526, ATCC 19435, LMG9447, NCDO895, KF24, KF147, LMG14418, IL1403, V4, MG1363, SK11, AM2, HP, and FG2) were selected to determine survival during spray drying. Strains were incubated in duplicate at 30°C in 200 ml GM17, and stationary-phase cells were harvested by centrifugation at 3,315 × *g* for 7 min and dissolved in 200 ml 20% (wt/vol) skim milk powder. Cell suspensions were dried in a mini-lab-scale spray dryer (model B-290; Büchi Labortechnik AG, Flawil, Switzerland) by using an inlet temperature of 200°C and an outlet temperature of 100°C. Ice water was continuously used to cool the nozzle. The moisture content of the resulting powder was determined in duplicate by measuring weight loss during incubation at 102°C for 3 h. To determine survival rates, the generated powders were rehydrated in water (1% [wt/vol]) for 1 h at room temperature. The rehydrated cell suspension and the feed cell suspension were serially diluted in duplicate and spotted in triplicate on GM17 agar plates. After 3 days of incubation at 30°C, CFU were assessed. Dry weight of the cell suspensions was determined in triplicate by measuring the weight of 5 ml of sample after incubation at 55°C for 5 days.

Statistical analysis. Significance of the differences in robustness of groups with different origins, genotypes, or phenotypes was assessed with a *t* test. Significance of the correlations of survival during heat stress, oxidative stress, and spray drying was assessed by a linear model. All statistic calculations were done in R (version 2.15; R Foundation for Statistical Computing, Vienna, Austria [http://www.R-project.org]).

RESULTS

Heat and oxidative stress survival phenotypes are highly diverse. As LAB robustness can be highly strain specific (8, 9), we assessed the heat and oxidative stress survival of a collection of 39 *L. lactis* strains of diverse origins (Table 1). The collection of *L. lactis* strains displayed highly variable heat survival characteristics at 50°C, with the decrease in viability ranging from 0.2 log unit for the most robust strain (LMG14418) to 5.0 log units for the least robust strain (LMG6897) (Fig. 1). Moreover, the phenotype did not appear binary, as a continuum of intermediate survival levels was observed.

The same collection of strains was assessed for their oxidative stress tolerance (Fig. 2). Similar to results for heat stress, high diversity was observed (more than 4 log units). Strain P7266 displayed the highest robustness under oxidative stress, with a viability loss of 0.1 log unit. By contrast, strain AM2 was by far the most sensitive to oxidative stress, with a viability loss of 4.7 log units, approximately 1 log unit more than the second-most-sensitive strain (SK11). The stress analyses revealed that strains HP and LMG6897, which represent the same strain derived from different culture collections, displayed similar sensitivities to both heat stress (5.0- and 4.8-log-unit decreases, respectively) and oxidative stress (3.1- and 2.7-log-unit decreases, respectively), confirming the reproducibility of both assays and demonstrating that the strain-specific survival differences observed are far greater than

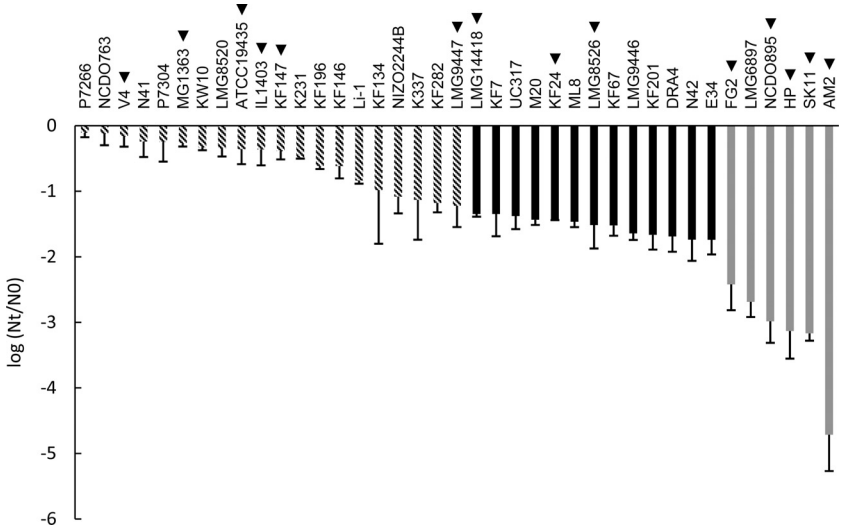

FIG 2 Robustness of *L. lactis* strains upon oxidative stress exposure expressed as the difference between log CFU/ml after oxidative stress (Nt) and control (N0). For genotype-phenotype matching, groups were made up of robust (striped bars), intermediate (black bars), and sensitive (gray bars) strains. Strain names indicated with a triangle represent strains that were selected for the spray drying analysis (see Fig. 4). The data represent averages of two biological replicates. Error bars indicate standard deviations.

the technical variability in our assays. Furthermore, statistical analysis revealed that the robustness of each of the three groups (as represented in Fig. 1 and 2) was significantly different from the robustness of the other two groups of strains ($P < 0.001$).

Robustness under heat stress and robustness under oxidative stress are correlated. Combining the data generated in both stress analyses, two strains, ATCC 19435 and IL1403, appeared relatively robust under both heat and oxidative stress (less than 1 log unit loss of viability). LMG6897, HP, and SK11 were among the most sensitive strains upon exposure to both heat and oxidative stress. However, some strains showed larger differences in their robustness under the two stresses. For example, LMG8520 was robust under oxidative stress (0.3 log unit), whereas this strain displayed a larger decrease in viability (4.9 log units) when exposed to heat stress. Other strains, like NCDO895, were more robust under heat stress (1.6 log units) than under oxidative stress (3.0 log units). Nevertheless, a correlation between responses to heat stress and oxidative stress was observed ($P < 0.05$).

Robustness is related to *L. lactis* subsp. *lactis* or *L. lactis* subsp. *cremoris* phenotype but not to the habitat from which strains originated. Differences in robustness under both heat and oxidative stress between the different phenotypic groups were assessed. Strains with an *L. lactis* subsp. *lactis* phenotype displayed more robustness under heat as well as oxidative stress than strains with an *L. lactis* subsp. *cremoris* phenotype ($P < 0.01$) (Fig. 3A and B). Besides the phenotype-based division, currently the species is divided genotypically into *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* (24, 25). These genotypic groups are not clearly re-

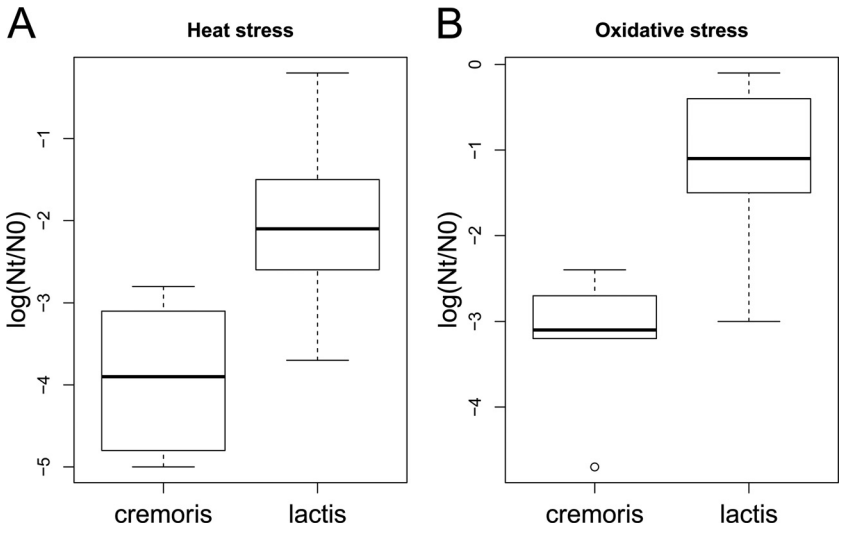

FIG 3 Box plots of heat stress (A) and oxidative stress (B) survival phenotypes of strains with an *L. lactis* subsp. *cremoris* or *L. lactis* subsp. *lactis* phenotype. Strains with an *L. lactis* subsp. *lactis* phenotype display more robustness than strains with an *L. lactis* subsp. *cremoris* phenotype ($P < 0.01$).

flected in the phenotype-based grouping (Table 1). The groups based on genotype differ less in stress survival phenotype; strains with an *L. lactis* subsp. *cremoris* genotype are more sensitive to heat stress than strains with an *L. lactis* subsp. *lactis* genotype ($P < 0.05$) but respond similarly to oxidative stress ($P = 0.3$).

Similarly, we assessed differences in stress tolerance phenotypes of isolates from different origins. Dairy and nondairy isolates did not significantly differ in robustness under heat stress ($P = 0.9$) or oxidative stress ($P = 0.054$).

Identification of genes associated with robustness under heat stress. A genotype-phenotype comparison was made by matching the heat stress survival data with the gene presence/absence matrix available from a pangenome-based comparative genome hybridization analysis (15). Based on their stress survival phenotypes, the set of strains was manually divided into three groups (robust, intermediate, and sensitive) (Fig. 1). If a gene resulting from the PhenoLink analysis (22) was present in 75% of the strains in the robust or sensitive group and absent in 75% of the strains in the opposite group, this gene was considered to be correlated or anticorrelated, respectively, with robustness under heat stress.

Matching the genotype with the heat stress survival phenotype revealed that the presence of a gene (*LLKF_1440* [ortholog, *LACR_1472*]) encoding a GntR family transcriptional regulator negatively correlated with robustness under heat stress (Table 2). This gene is genetically linked (i.e., part of a group of genes that are adjacent and colinear and that have intergenic spacing smaller than 100 bp) to genes encoding a sugar transporter (*LLKF_1444* [*LACR_1477*], *LLKF_1445* [*LACR_1478*]), a beta-glucosidase (*LLKF_1441* [*LACR_1473*]), and a hypothetical protein (*LLKF_1442* [*LACR_1475*]) that are also negatively correlated with robustness under heat stress. The presence of a gene encoding part of a manganese ABC transporter (*mtsC*) positively correlated with robustness under heat stress. Although this gene is present in the heat-sensitive strain SK11, it appears to be absent in all the other heat-sensitive strains (15). Moreover, the presence of a gene encoding an ABC transporter ATP-binding protein (*yabE*) and two genes encoding hypothetical proteins (*yliD* and *ymgH*) positively correlated with robustness under heat stress. Furthermore, some genes that were associated with robustness under heat stress encode phage-related functions, which are highly variable among strains (26) and which were not regarded as possible robustness markers and therefore were excluded from Table 2.

Identification of genes associated with robustness under oxidative stress. Gene presence and absence patterns were also compared with oxidative stress survival phenotypes using the same criteria as described above for heat stress. Genes that positively correlated with robustness under oxidative stress included a gene (*L31294* [*LLKF_0836*]) encoding part of a cellobiose-specific phosphotransferase system (PTS) (Table 2). Oxidative stress survival was also associated with the presence of the gene *ftsY*, encoding a signal recognition particle docking protein. Furthermore, the neighboring genes *ymgH* and *ymgI*, encoding hypothetical proteins, were associated with oxidative stress survival. Gene *ymgH* of strain IL1403 (*L66209*), which was associated with heat stress, is orthologous (defined by the orthology prediction program InParanoid [27]) to the MG1363 gene *llmg_1259* and the KF147 genes *LLKF_0279* and *LLKF_2282*, which appeared in the genotype-phenotype matching results for the oxidative stress phenotypes. These genes were not assigned to the same orthologous group (15) because strict criteria were required for adequate orthology grouping due to the high similarity of the strains. All genes in a group were required to be orthologs of one another, and any gene in that group should not have other orthologs. In contrast to the other genes, *L66209* was not an ortholog of *LACR_2147* and, therefore, was excluded from the group.

Most genes associated with oxidative stress anticorrelated with robustness, and these genes were often found to encode hypothetical proteins (Table 2). However, genes encoding four transcriptional regulators and four glycosyltransferases were also found to negatively correlate with robustness under oxidative stress.

Heat and oxidative stress survival predicts spray drying robustness. During spray drying, cultures are exposed to a combination of heat stress and oxidative stress, which both lead to loss of viability (6, 7). To assess whether robustness under heat and oxidative stress can therefore predict robustness under spray drying, stationary-phase cells of 14 *L. lactis* strains with diverse responses to heat and oxidative stress (Fig. 1 and 2) were subjected to lab scale spray drying. Inlet and outlet temperatures were optimized to obtain powders with a moisture content that did not exceed 4%, which is required for powder stability and spoilage prevention (28, 29). Survival was determined by comparing the viability of the rehydrated powder with the viability of the feed and was expressed in CFU per gram of dry weight. Relative survival compared to strain IL1403 was calculated to compensate for technical variability. The most robust strains (MG1363, LMG14418, and NCDO895) displayed a more-than-200-fold-better survival during spray drying than the most sensitive strains (SK11, AM2, and LMG8526) (Fig. 4). Most strains with a sensitive or intermediate response to both heat and oxidative stress displayed a larger decrease of viability during spray drying than strains with a robust phenotype under at least one of the stresses, which is in line with the fact that during spray drying cells are exposed to heat as well as oxidative stress (6, 7). Of the individual stresses, robustness under heat stress appeared to have the highest correlation with survival during spray drying (Fig. 5). To further analyze whether the robustness under spray drying could be predicted by measuring robustness under heat and oxidative stress, we employed linear modeling using spray drying as the response variable and measurement series, robustness under heat stress, and robustness under oxidative stress as explanatory variables. Survival upon spray drying appeared to be related to robustness under heat stress (R^2 is 0.59, $P < 0.001$) and to robustness under oxidative stress (R^2 is 0.41, $P < 0.01$). Combining heat and oxidative stress robustness in a single model improved the predictive power of the model (R^2 is 0.61, $P < 0.001$), demonstrating the feasibility of our relatively simple, high-throughput heat and oxidative stress assays to predict spray drying robustness, which can be assessed only in more-tedious experiments.

DISCUSSION

High, strain-specific diversity in stress responses and functional characteristics makes it worthwhile to identify novel (more-robust) strains for application in starter cultures. The diversity of the 39 *L. lactis* strains employed in this study, which were selected from a larger collection and are anticipated to be a good representation of the *L. lactis* species as a whole (20), appeared high (up to 4 log units) in both heat and oxidative stress survival assays. The heat stress survival level of strain IL1403 corresponds with results in a study of Hartke et al., in which heat resistance of IL1403 at

TABLE 2 Genes associated with robustness against heat and oxidative stress resulting from the genotype-phenotype comparison

Locus(i)[a]	Gene	Product	Presence of gene in robust phenotype
Heat stress			
LACR_1479 (*LLKF_1446*)		Hypothetical protein	Absent
LLKF_1440 (*LACR_1472*)		GntR family transcriptional regulator	Absent
L183932	*yliD*	Hypothetical protein	Present
LLKF_1406 (*llmg_1137*, *LACR_1439*, *L149891*)	*mtsC*	Manganese ABC transporter permease	Present
LLKF_1445 (*LACR_1478*)		Sugar ABC transporter	Absent
LACR_1475 (*LLKF_1442*)		Hypothetical protein	Absent
LLKF_1441 (*LACR_1473*)		Beta-glucosidase	Absent
L66209	*ymgH*	Hypothetical protein	Present
LLKF_0011 (*L15262*)	*yabE*	ABC transporter ATP-binding protein	Present
LACR_1477 (*LLKF_1444*)		Sugar ABC transporter permease	Absent
Oxidative stress			
L31294 (*LLKF_0836*)	*yidB*	Cellobiose-specific PTS IIC component	Present
LACR_0155		Hypothetical protein	Absent
LACR_1321		Hypothetical protein	Absent
llmg_1634 (*LACR_0981*)		ABC transporter permease	Absent
LACR_0651		Surface antigen	Absent
LACR_1297		Saccharopine dehydrogenase-related protein	Absent
LACR_1259		Hypothetical protein	Absent
LACR_0861		Hypothetical protein	Absent
LACR_1215		Type I restriction-modification system methyltransferase subunit	Absent
LACR_1347		Transcriptional regulator	Absent
LACR_1992		Hypothetical protein	Absent
LACR_2213		Hypothetical protein	Absent
LACR_2214		Hypothetical protein	Absent
LACR_0503		Hypothetical protein	Absent
LACR_0994		Lipopolysaccharide biosynthesis glycosyltransferase	Absent
LACR_0995		Lipopolysaccharide biosynthesis glycosyltransferase	Absent
LACR_1262		Hypothetical protein	Absent
LACR_1322		Hypothetical protein	Absent
LACR_1393		Hypothetical protein	Absent
LACR_0354		Molybdopterin/thiamine biosynthesis dinucleotide-utilizing protein	Absent
LACR_1164		Hypothetical protein	Absent
LACR_1257		Glycosyltransferase	Absent
LACR_1261		Hypothetical protein	Absent
LACR_1963		Hypothetical protein	Absent
LACR_2216		Hypothetical protein	Absent
LACR_2218		Hypothetical protein	Absent
LACR_2480		Hypothetical protein	Absent
LACR_1318		Hypothetical protein	Absent
LACR_1319		ADP-ribose pyrophosphatase	Absent
LACR_1348		Arabinose efflux permease	Absent
LACR_1930		Hypothetical protein	Absent
LACR_0803		Hypothetical protein	Absent
LACR_1260		Hypothetical protein	Absent
LACR_2551		XRE family transcriptional regulator	Absent
LACR_2447		LacI family transcription regulator	Absent
LACR_0292		Hypothetical protein	Absent
LACR_1300		Hypothetical protein	Absent
LACR_2549		Serine/threonine protein kinase	Absent
LLKF_0831 (*L0206*, *llmg_1744*)	*ftsY*	Signal recognition particle docking protein FtsY	Present
LACR_0846		Hypothetical protein	Absent
LACR_1296		Putative intracellular protease/amidase	Absent
LACR_1931		Hypothetical protein	Absent
LACR_2086		Glycosyltransferase	Absent
LACR_0154		Cell surface protein	Absent
LACR_1038		Hypothetical protein	Absent
LACR_1264		Hypothetical protein	Absent
LACR_1388		Hypothetical protein	Absent
LACR_2121 (*llmg_0814*)	*ps323*	Hypothetical protein	Absent
LACR_0157		XRE family transcriptional regulator	Absent
LACR_1834 (*LLKF_1826*)	*yrbH*	Hypothetical protein	Absent
LLKF_2283 (*llmg_1258*, *L66407*)	*ymgI*	Hypothetical protein	Present
llmg_1259 (*LLKF_0279*, *LLKF_2282*)	*ymgH*	Hypothetical protein	Present

[a] Loci with prefixes of *LACR*, *LLKF*, *llmg*, and *L* represent strains SK11, KF147, MG1363, and IL1403, respectively.

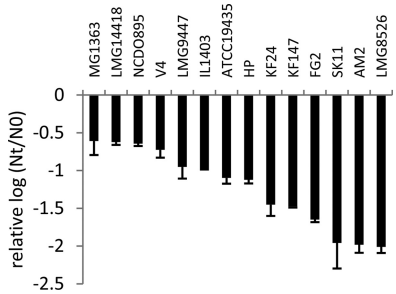

FIG 4 Viability of *L. lactis* strains after spray drying expressed as the relative difference between log CFU/g dry weight after spray drying (Nt) and before spray drying (N0), compared to that of strain IL1403. The data represent averages of two biological replicates. Error bars indicate standard deviations.

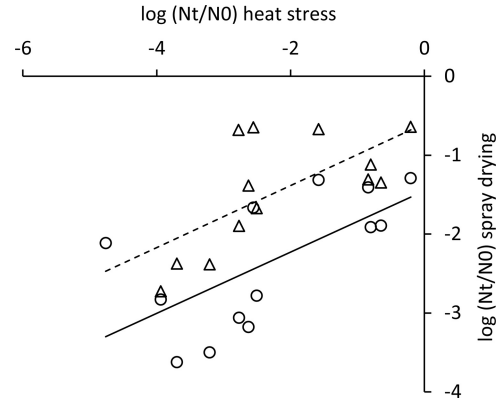

FIG 5 Correlation of spray drying survival and heat stress survival. Different markers indicate the two series of spray drying analyses.

52°C was assessed (30). Although specific strains displayed different responses to the two stresses, overall correlation between the response to heat stress and oxidative stress was observed, suggesting that heat and oxidative stress responses are partly driven by general stress mechanisms, as has been shown in strain MG1363 (31, 32). Moreover, these observations also corroborate earlier studies demonstrating that preexposure of *L. lactis* strains to a specific stress provides cross-protection to another stress condition, e.g., increased robustness under both heat and oxidative stress during carbohydrate starvation (30) and mildly acid conditions (33).

Furthermore, robustness under both heat and oxidative stress appeared to be related to phenotype and only partly to genotype. Therefore, the phenotype-based nomenclature of *L. lactis* strains appears more helpful for selection of robust strains than the nowadays often applied genotype-based nomenclature. Robustness under both heat and oxidative stress appeared unrelated to the habitat from which strains were originally isolated, despite the fact that genomic analyses have demonstrated the presence of additional genes involved in stress response in nondairy strains (15, 16).

Correlation of robustness under heat and oxidative stress and the gene absence/presence pattern of the collection of *L. lactis* strains revealed several genes associated with robustness phenotypes. Robustness under heat stress was associated with the presence of a gene encoding a manganese transporter (*mtsC*). Interestingly, manganese is often associated with stress survival, specifically oxidative stress (34, 35). The presence of genes encoding a cellobiose transporter (*yidB*), a signal recognition particle docking protein (*ftsY*), and two hypothetical proteins (*ymgH* and *ymgI*) was associated with oxidative stress survival. Involvement in the stress response of these genes was also suggested by the facts that in *L. plantarum* a transporter of cellobiose was upregulated in an oxidative-stress-sensitive *trxB1* deletion mutant compared to the wild-type strain (36), a *Streptococcus mutans ftsY* deletion mutant was sensitive to both acid and salt stress (37), and the neighboring genes *ymgH* and *ymgI*, encoding hypothetical proteins, are located near the general stress-inducible gene *gls24* (38). Mutants of strain MG1363 with deletions of the gene encoding part of a manganese ABC transporter (*mtsC*) and the genes *ymgH* and *ymgI*, encoding hypothetical proteins, however, did not display an altered robustness phenotype (data not shown), indicating that not all genes associated with the robustness phenotypes are indeed required for improved robustness. Neither in strain MG1363 nor in IL1403 could the gene *ftsY* be deleted (data not shown), suggesting that this gene is essential in both strains, as it is in *Escherichia coli* (39). Taking these results together, we have not been able to confirm the involvement of these genes in robustness phenotypes by a gene deletion approach. Possibly, complementary genes that take over the function originally performed by the gene targeted by deletion are present in strain MG1363. Furthermore, besides the presence or absence of genes, inactivation or differential regulation of conserved genes could be the basis for the high diversity in robustness. These differences in gene activity intrinsically cannot be revealed by genotype-phenotype matching but rather require a transcriptome-phenotype matching approach, as was recently demonstrated in *L. plantarum* (40). Nevertheless, although the selected genes associated with robustness could not be established as genetic robustness biomarkers, the gene presence/absence profile of the entire group of robustness-associated genes, resulting from the genotype-phenotype matching, might be indicative of robustness under heat and oxidative stress.

As expected from the observed high diversity of *L. lactis* strains in robustness under heat and oxidative stress, a high diversity in robustness under spray drying was displayed. The strains that were most robust under spray drying displayed survival levels (10 to 20% survival) which are similar to those of commercial *L. lactis* starter bacteria after freeze-drying (41) and thus are acceptable for practical applications. This suggests that by selection of *L. lactis* strains for robustness under heat and oxidative stress, spray drying could become a feasible method for preservation of selected strains for dairy starter cultures. Our results not only demonstrate the importance of selection of starter culture strains for robustness characteristics along with acidifying and flavor-forming properties but also provide relatively simple methods for assessment of strains for spray drying-related robustness, which ultimately should aid industry in the identification of novel strains with optimal combinations of industrially relevant traits.

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