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Staphylococcus aureus Virulence Expression Is Impaired by *Lactococcus lactis* in Mixed Cultures▽†

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***Staphylococcus aureus* is responsible for numerous food poisonings due to the production of enterotoxins by strains contaminating foodstuffs, especially dairy products. Several parameters, including interaction with antagonistic flora such as *Lactococcus lactis*, a lactic acid bacterium widely used in the dairy industry, can modulate *S. aureus* proliferation and virulence expression. We developed a dedicated *S. aureus* microarray to investigate the effect of *L. lactis* on staphylococcal gene expression in mixed cultures. This microarray was used to establish the transcriptomic profile of *S. aureus* in mixed cultures with *L. lactis* in a chemically defined medium held at a constant pH (6.6). Under these conditions, *L. lactis* hardly affected *S. aureus* growth. The expression of most genes involved in the cellular machinery, carbohydrate and nitrogen metabolism, and stress responses was only slightly modulated: a short time lag in mixed compared to pure cultures was observed. Interestingly, the induction of several virulence factors and regulators, including the *agr* locus, *sarA*, and some enterotoxins, was strongly affected. This work clearly underlines the complexity of *L. lactis* antagonistic potential for *S. aureus* and yields promising leads for investigations into nonantibiotic biocontrol of this major pathogen.**

Staphylococcus aureus is a major human pathogen that causes a wide range of diseases, such as septicemia, meningitis, endocarditis, osteomyelitis, and toxic shock syndrome (77). *S. aureus* is also a major causative agent of food poisonings (41). Staphylococcal food poisonings (SFP) are due to the production of staphylococcal enterotoxins (SEs) by *S. aureus* strains contaminating foodstuffs. Milk and dairy products are often incriminated in SFP, especially in France (19, 20, 35). During the cheese-making process, contamination by *S. aureus* may come from several sources. Raw milk (notably milk from mastitic cows) may be a source of contamination; however, strains endemic in the processing plant environment (present on fomites and in biofilms), as well as strains present in healthy human carriers, may also contaminate dairy products. A complex and balanced ecosystem is required throughout the cheese-making process for the development of the organoleptic properties of the final product. The inhibition of *S. aureus* growth and of the production of SE in cheeses must thus be achieved by nonantibiotic means so that the biodiversity and correct microbial ecology of the environment may be maintained, yielding a satisfactory final product. To this end, the appropriate and rational use of lactic acid bacteria (LAB) appears to be a promising strategy in the fight against *S. aureus*.

Interactions between *S. aureus* and LAB in several ecosystems, including fermented foodstuffs but also nasal and vaginal environments, have been explored for years (11). However, apart from offering general observations, studies have not yet comprehensively described the mechanism of inhibition of *S. aureus* by LAB. LAB have frequently been considered to be a factor that influences *S. aureus* growth by physically and chemically changing the environment. Several possibilities have been proposed to explain the inhibition of *S. aureus* by LAB, including the production of bacteriocins (2) and hydrogen peroxide (2, 58), competition for nutrients (28), and needless to say, acidification (4, 20, 52), even if the impact of the latter mechanism has been questioned (12). Studies of the inhibition of *S. aureus* virulence expression by LAB, including the inhibition of SE production, are quite scarce. Few studies have described the inhibition of enterotoxin production in the presence of LAB, and none have unraveled the mechanisms involved in such antagonism (26, 29, 50, 51, 57, 68).

Virulence expression in *S. aureus* is controlled by complex regulatory networks that include two-component systems (e.g., AgrAC, SrrAB, SaeRS, and ArlRS) and transcription factors (e.g., SarA and its homologs and Rot) but also the Clp proteolytic complex and the alternative sigma factor σ^B (5, 13, 15, 21, 47, 48, 54, 60, 64, 72). Among these regulatory systems, the accessory gene regulator (*agr*) is a key modulater of virulence expression. It combines a two-component system and a quorum-sensing system (55, 64, 78). The *agr* system comprises two

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transcripts, RNAII and RNAIII, which are divergently transcribed. RNAII encodes AgrBDCA, the structural components of the quorum-sensing system. RNAIII is the effector molecule of the *agr* system but also encodes the delta-hemolysin (32, 55). The accessory gene regulator is responsible for the post-exponential-phase induction of several virulence factors, including exoproteins (21, 54, 62, 64, 72). The activity of *agr* itself can be modulated by other regulatory systems and in response to environmental variations.

Exploring the interactions between positive microbiota and *S. aureus* and understanding the mechanisms involved in the inhibition of *S. aureus* growth or virulence will enable the setting of rational screening criteria for positive microbiota to be used in food preservation and will also yield new strategies for fighting against this major human pathogen. Such an endeavor is, however, complex and requires the employment of new approaches. DNA microarrays are powerful tools for the investigation of these multifactorial interactions. The microarray technique has been widely used to investigate the global responses of *S. aureus* to several stimuli (5, 10, 17, 21, 38, 59, 65, 76). Nonetheless, a transcriptomic analysis of *S. aureus* in interaction with other bacterial species has, as of yet, not been reported. In this study, we have developed a dedicated *S. aureus*-specific microarray, usable for the study of mixed cultures of *S. aureus* and LAB. This microarray was used to investigate the transcriptomic response of *S. aureus* to the presence of *Lactococcus lactis* in a chemically defined medium (CDM) at a constant pH. Surprisingly, while *L. lactis* hardly affected *S. aureus* growth and the expression of genes involved in central metabolism processes under these conditions, it was able to dramatically impair the expression of several virulence genes.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. lactis* subsp. *lactis* biovar diacetylactis LD61 (kindly provided by R. Perrin, Soredab, La Boissière Ecole, France) was used throughout this work. The LD61 strain contains plasmids that enable optimal growth in milk (by facilitating lactose, protease, and citrate utilization) and is thus a highly acidifying strain which is industrially used in dairy fermentations. *L. lactis* LD61 does not produce any detectable bacteriocin active against *S. aureus* (data not shown). The previously sequenced strain *S. aureus* MW2 was chosen for the interaction experiments for the following reasons. The complete genome sequence of MW2 is publicly available, which ensures access to information on the gene content of the strain. Of note, MW2 possesses genes encoding several toxins, including six enterotoxins, of which the *sea* and *sec* toxins (3) are frequently involved in SFP (41). The expression of some enterotoxin genes, including *sec*, is known to be under *agr* control (62), which is functional in this strain (74) in contrast to other strains (e.g., N315 and RN4220). Furthermore, *S. aureus* MW2 was preferred over other sequenced strains because it is a typical community-acquired strain of *S. aureus*. It is actually more probable for community rather than clinical strains to contaminate foodstuffs. Moreover, contamination with *S. aureus* strains of human origin is reportedly prevalent in SFP outbreaks (37), and the MW2 strain belongs to one of the major clonal lineages found among human isolates of *S. aureus* (43).

Mixed cultures of *S. aureus* and *L. lactis* and pure cultures (used as controls) were grown on CDM at 30°C and a constant pH (6.6) in a 2-liter fermentor as described elsewhere (53). Both strains were inoculated at 10^6 CFU ml^{-1}. The kinetics of growth, consumption of glucose, and formation of fermentation products are discussed elsewhere (53).

Two additional *S. aureus* strains, clonally unrelated to the MW2 strain (which belongs to sequence type 1 [ST1] and clonal complex 1) (43), were used in complementary interaction experiments: SH1000 (30), a derivative of strain NCTC 8325 (ST8, clonal complex 8; *agrI*), and RN4850 (ST not available; *agrIV* [kindly provided by G. Lina, CNR des Staphylococcies, Lyon, France]) (33).

RNA extraction and purification. Volumes of culture each corresponding to 6 mg of cells (dry weight) were harvested and frozen immediately in liquid nitrogen. Samples were stored at −80°C until RNA extraction. Samples were thawed on ice and centrifuged (at 4°C for 5 min at 6,500 × *g*), and cell pellets were resuspended in 500 μl of Tris-EDTA buffer. Cells in tubes containing a mixture of 0.6 g of zirconium beads, 25 μl of sodium dodecyl sulfate (20%), 3.5 μl of β-mercaptoethanol, and 500 μl of phenol (pH 4.7) were disrupted for 4 min at 30 Hz by using a Retsch MM301 high-speed mixer mill (Grosseron, France). After the addition of 200 μl of chloroform and centrifugation (at 4°C for 25 min at 15,500 × *g*), the aqueous phase was extracted using a Nucleospin RNA L kit according to the instructions of the manufacturer (Macherey Nagel, Hoerdt, France). After precipitation with isopropanol, the RNA sample was incubated with RNase-free DNase I from a DNA-free kit (Applied Biosystems, Warrington, United Kingdom). RNA was quantified and its contamination by proteins was assessed using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Rockland, DE), and the quality of the preparation was evaluated using a model 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA). Noncontamination of the RNA sample by genomic DNA (gDNA) was confirmed by quantitative PCR.

gDNA extraction and purification. gDNA was prepared from overnight cultures grown in tryptic soy broth with agitation at 37°C (*S. aureus*) or grown under static conditions on M17 supplemented with 0.5% glucose at 30°C (*L. lactis*). Briefly, cells were centrifuged (at 4°C for 5 min at 6,500 × *g*), washed in 1 ml of Tris-HCl (50 mM; pH 8), and resuspended in 450 μl of Tris-EDTA buffer. Cells were disrupted with 0.6 g of zirconium beads for 4 min at 30 Hz by using an MM301 high-speed mixer mill. After centrifugation (at 4°C for 25 min at 15,500 × *g*), the lysate was incubated at 37°C with RNase (50 μg μl^{-1}) for 30 min and proteinase K (0.4 mg ml^{-1}) for one additional hour. Following extraction with phenol-chloroform-isoamyl alcohol (25/24/1) and chloroform, gDNA was precipitated with isopropanol and resuspended in water. DNA was quantified and contamination by proteins was assessed using a NanoDrop ND-1000 spectrophotometer.

Construction of the *S. aureus* microarray. A dedicated microarray containing 420 probes specific for *S. aureus* and 27 probes specific for *L. lactis* IL1403 was designed. The complete list of target genes is available in the supplemental material. The design of oligonucleotides (60-mers) was carried out using OligoArray 2.1 software (66, 67) and was based on the genome sequence of strain Mu50 (BA000017), which is one of the strains for which the complete genome sequence was publicly available at the beginning of this work and for which the annotation is regularly updated (http://www.bio.nite.go.jp/dogan/Top). The design took into account the use of the microarray in the context of mixed cultures with *L. lactis* (and possibly other LAB). Oligonucleotides generated by using OligoArray 2.1 were selected based on thermodynamic parameters and their specificities with regard to *S. aureus*, as determined by a BLAST analysis comparing the oligonucleotide sequences against genome sequences of *S. aureus* Mu50, LAB (*L. lactis* IL1403 [AE005176], *Streptococcus thermophilus* CNRZ1066 [CP000024], *Lactobacillus acidophilus* NCFM [CP000033], and *Lactobacillus plantarum* WCFS1 [AL935263]), and *Bifidobacterium longum* NCC2705 (AE014295). The specificity of the microarray with regard to *S. aureus* MW2 was further validated by a BLAST analysis comparing the oligonucleotide sequences against the MW2 genome sequence: 92.6% of oligonucleotides hybridized in silico with strain MW2. All remaining oligonucleotides which did not hybridize in silico corresponded to genes carried by a genomic island or mobile genetic elements in strain Mu50 and absent from strain MW2. The 5′-end-aminated oligonucleotides (Sigma Genosys, Suffolk, United Kingdom) were spotted onto Slide E epoxysilane slides (Schott Nexterion, Jena, Germany) in sextuplicate with a Biorobotics MicroGrid II as specified by the manufacturer (Genomic Solutions Ltd., Cambridgeshire, United Kingdom). This spotting step was carried out through the transcriptomic platform at Ouest Genopole (Rennes, France).

Fluorescent labeling. Five micrograms of total RNA was labeled with Cy3- or Cy5-dCTP (Perkin Elmer, Waltham, MA) by using a ChipShot kit according to the recommendations of the manufacturer (Promega, Madison, WI). Labeled cDNA was dried out and stored at −20°C until use. gDNA was labeled basically as described previously (23) with modifications as follows: gDNA was digested overnight with restriction endonuclease HinPII, precipitated with isopropanol, and resuspended in water. Samples of 400 ng of digested gDNA were denatured at 95°C for 5 min and employed as a template in a 50-μl reaction mixture containing reaction buffer (1×); dATP, dGTP, and dTTP (each at 20 μM); Cy3-dCTP (0.5 mM); bovine serum albumin (0.4 mg ml^{-1}); and a Klenow fragment (5 U) for the direct incorporation of Cy3 or Cy5-dCTP by using the Prime-a-Gene labeling system (Promega, Madison, WI). The reaction mixture was incubated for 6 h at 22°C, and the reaction was stopped by adding EDTA (20

TABLE 1. Oligonucleotides used in this study for quantitative real-time PCR

Gene identification no.	Gene name(s)	Forward primer	Reverse primer
MW0051	*seh*	TGGTCAATATAATCACCCATTCA	TCAAATCATTGCCACTATCACC
MW0084	*spa*	TTAAAGACGATCCTTCAGTGAGC	TGTTGTTGTCTTCCTCTTTTGGT
MW0124	*cap8A*	GCGCTATTGTTACATTTTTCGTC	TCTTGTGCCATAAACTGAGGATT
MW0759	*sec4*	AAACATGAAGGAAACCACTTTGA	TTTGCACTTCAAAAGAAATTGTG
MW0760	*sel2*	GGTTACCGCACAAGAAATAGATG	TGCCGTATTCTTTACCTTTACCA
MW0802		TCTAAGGGTCAACCTCAAGAACA	TCCAACCTACTCGTCTCAATTTC
MW1044	*hla*	ATGGATAGAAAAGCATCCAAACA	TTTCCAATTTGTTGAAGTCCAAT
MW1362	*hu*	AGAAGCTGGTTCAGCAGTAGATG	TACCTCAAAGTTACCGAAACCAA
MW1889	*sea*	TAATCGATTGACCGAAGAGAAAA	ATAACGTCTTGCTTGAAGATCCA
MW1937	*seg2*	TACGATTTGTTTTACACCGGAAC	TCCAAATGAAAATTCTCTGCATC
MW1938	*sek2*	CTACACAGGAGATGATGGGCTAC	CATCCAAATGGAATTTCTCAGAC
MW1959	RNAIII/*hld*	TAAGGAAGGAGTGATTTCAATGG	GTGAATTTGTTCACTGTGTCGAT
MW1963	*agrA*	CCTCGCAACTGATAATCCTTATG	ACGAATTTCACTGCCTAATTTGA
MW2108	*asp23*	AGACATGAAAGGTGGCTTAACTG	GCTTGTTTTTCACCAACTTCAAC
MW2469	*clpL*	AAGATGCACTGATTCGACTTGAT	TGTCATCATAACCGACATAACCA

mM; pH 8.0). Unincorporated nucleotides were removed using a Nucleospin Extract II kit (Macherey Nagel, Hoerdt, France). The reaction mixture was supplemented with 4 volumes of NTC buffer (Macherey Nagel) and loaded onto a column. Labeled gDNA was dried out and stored at −20°C until use, according to the specifications of the labeling system manufacturer.

Hybridization and scanning of microarrays. Hybridization and scanning of microarrays were performed using the transcriptomic platform of the IFR 140 Génétique Fonctionnelle, Agronomie et Santé (Université Rennes 1, France). The specificity of the *S. aureus* microarray was tested by hybridizing either labeled cDNA or gDNA from *S. aureus* MW2 and N315 and *L. lactis* IL1403, separately or in competition. The expression profile of *S. aureus* in pure cultures or mixed cultures with *L. lactis* was determined by hybridizing Cy5-labeled cDNA corresponding to each sample of RNA with Cy3-labeled gDNA of *S. aureus* MW2 and N315 (50:50). gDNA was used as a reference for normalization, as described previously (25, 70). Microarray analyses at all experimental time points were performed in triplicate (using three independent biological replicates).

Labeled cDNA and gDNA were resuspended in 200 μl of ChipHybe 80 (reference no. 760-127; Ventana). Hybridizations were carried out in a Discovery station at 42°C for 8 h according to the recommendations of the manufacturer (Ventana, Illkirch, France). Slides were then washed twice manually in Ribo-Wash solution (Ventana, Illkirch, F) and once in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), dried by centrifugation, and scanned with a Genepix 4000B scanner (Axon Instruments, Foster City, CA). Image analysis was performed with GenePix Pro4 software (Axon Instruments, Foster City, CA).

Data analysis. The median intensity of each spot was used as the signal value. To test species specificity, spots were considered to be detected when the signal value was higher than a background value, calculated as the mean intensity of spots containing buffer plus 2 standard deviations.

To determine the expression profiles for *S. aureus* in pure and mixed cultures, data were analyzed with R software (63). Oligonucleotides with very low signal values for gDNA (corresponding to a signal/noise ratio of less than 2 in more than 75% of microarrays) were flagged and removed from further analysis. Data were first normalized per spot (cDNA signal values were divided by corresponding gDNA signal values) and $\log_2$ transformed and then normalized per chip (to the 50th percentile). Statistical analyses were performed using the analysis of variance (ANOVA) test with the criteria that *P* values and false-discovery rates (FDRs) be lower than 0.05. Genes showing significant changes in expression in either pure or mixed cultures and a ≥3-fold change were considered to be differentially expressed.

qRT-PCR. To confirm microarray data, expression profiles of *agrA*, *hld*, *sigB*, *asp23*, *clpL*, *capA*, *katA*, *sel*, *sea*, MW2096, and MW802 were determined by quantitative reverse transcription-PCR (qRT-PCR) analyses. Expression profiles of *sarA*, *spa*, *hla*, and genes encoding enterotoxins not included in the *S. aureus* microarray, namely, *seh*, *sec4*, *sek*, and *seg*, were also determined by qRT-PCR. cDNA was synthesized using the high-capacity cDNA archive kit as recommended by the manufacturer (Applied Biosystems, Warrington, United Kingdom). Quantitative real-time PCR was performed using an Opticon 2 real-time PCR detector (Bio-Rad, Hercules, CA). The reaction mixture contained power Sybr green PCR master mix (1×; Applied Biosystems, Warrington, United Kingdom), each primer (0.5 μM; sequences are given in Table 1), and a cDNA template. Thermal cycling consisted of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. Each PCR product was further analyzed by generating melting curves to ensure the specificity of the assay. qRT-PCR analyses for all experimental time points were performed in triplicate (using three independent biological replicates). Standard curves were generated to calculate the copy number for each gene in each sample. The *hu* gene was used as an internal standard for normalization as described previously (25). Gene expression levels are thus reported relative to that for *hu* and were calculated by using following the relation: (number of copies of each gene in each cDNA preparation/number of copies of *hu* in each cDNA preparation) ×10. Statistical analysis was performed using the ANOVA test with the criterion of a *P* value lower than 0.05 to identify genes showing significant changes in expression in either pure or mixed cultures over time. Similarly, statistical analysis was performed using the *t* test with the criterion of a *P* value lower than 0.05 to identify, for each time point, genes showing significant changes in expression in pure versus mixed cultures.

Microarray data accession number. The microarray data were deposited in the public repository database Array Express under the accession number E-MEXP-1837.

RESULTS

Development and validation of an *S. aureus* microarray usable with mixed cultures. The goal of this study was to investigate *S. aureus* gene expression profiles during *S. aureus* interaction with *L. lactis* in mixed cultures. Bacterial populations of each species could not be separated or differentially lysed prior to retrotranscription and cDNA labeling. Probe specificity was thus a crucial parameter. The strategy for the design of *S. aureus*-specific probes focused on having only labeled cDNA generated from *S. aureus* RNA out of a bulk of total RNAs of both species that would hybridize on the microarray. Each probe had to be specific to only one gene within the *S. aureus* genome, and cross-hybridization with genes of the *L. lactis* genome had to be minimized as well. Oligonucleotides (60-mers) were preferred to PCR fragments due to the potential of the latter for cross-hybridization and the consideration of oligonucleotides, due to their short length, to be more specific. The 420 *S. aureus* genes selected for the design corresponded to genes involved in virulence and associated regulation pathways, stress adaptation, peptide transport, central metabolism (for carbohydrates and amino acids), cellular machinery (for replication, transcription, and translation), and cell wall biosynthesis and turnover (see the supplemental material). Twenty-

seven oligonucleotide probes specific to *L. lactis* IL1403 (as determined by genome subtraction) were included as controls. Oligonucleotides were designed and validated in silico as described in Materials and Methods to minimize the risk of cross-hybridization with LAB cDNA. The specificity of the microarray for *S. aureus* was experimentally validated by performing hybridizations with cDNAs or gDNA of *L. lactis* LD61 and *S. aureus* MW2 and/or N315, a strain closely related to strain Mu50 (39). A mixture of gDNA of strains MW2 and N315 was used in order to maximize the detection of oligonucleotides: 92.6 and 98.6% of oligonucleotides were indeed detected in silico (as determined by performing a BLAST analysis) with strains MW2 and N315, respectively. Major differences between strains corresponded to oligonucleotides targeting genes located on genomic islands or mobile genetic elements present in strain Mu50 but absent in other strains, as observed previously (3). Of the oligonucleotides designed based on *S. aureus* Mu50 genes, 76% were detected using a mixture of labeled gDNA extracts from *S. aureus* N315 and MW2. This yield slightly decreased to 70.4% with gDNA from *S. aureus* MW2 alone, as expected from in silico hybridization results. In vitro yields of hybridization were in good agreement with those generally observed with gDNA, which range from 65 to 80%, depending on the preparation of gDNA and labeling and hybridization conditions (70). In parallel, total RNA was extracted from MW2 or N315 cells harvested in post-exponential phase. Hybridization with cDNA obtained from these samples showed that 72 to 75% of *S. aureus* oligonucleotides were detected. In hybridization experiments using *L. lactis* LD61 cDNA and/or gDNA, only 16 of the 420 oligonucleotides (3.8%) targeting *S. aureus* genes gave a signal (see the supplemental material). These 16 oligonucleotides were removed from the analysis when the microarray was used further for mixed cultures. Among the oligonucleotides designed as positive controls for *L. lactis*, 78% were detected using *L. lactis* LD61 cDNA or gDNA. Altogether, these in vitro results validated the *S. aureus* specificity of the dedicated microarray developed in this study.

Time lag in the expression of *S. aureus* genes involved in the cellular machinery, carbohydrate metabolism, and stress responses in mixed cultures. As a first approach to study interactions between *S. aureus* and *L. lactis*, we investigated the transcriptomic responses of *S. aureus* MW2 in pure and mixed cultures with *L. lactis* LD61 at 30°C in CDM at a constant pH (6.6) with agitation to homogenize and allow moderate aeration of the medium. These conditions allowed us to monitor the kinetics of substrate and product formation and to control environmental parameters such as a drop in pH, which may have masked other phenomena. Furthermore, the effect of acidification on *S. aureus* expression has already been documented (7, 76). *S. aureus* growth rates at the beginning of the exponential phase in pure and mixed cultures were similar. In the pure culture, *S. aureus* MW2 growth slowed down and a transient plateau of growth appeared at the end of the exponential phase (Fig. 1). In the mixed culture, the growth rate decrease occurred earlier and the plateau lasted longer. Altogether, the microbiological analyses showed that, under the conditions used, *L. lactis* hardly affected *S. aureus* growth and the final staphylococcal population in the mixed culture was only threefold lower than that in the pure culture.

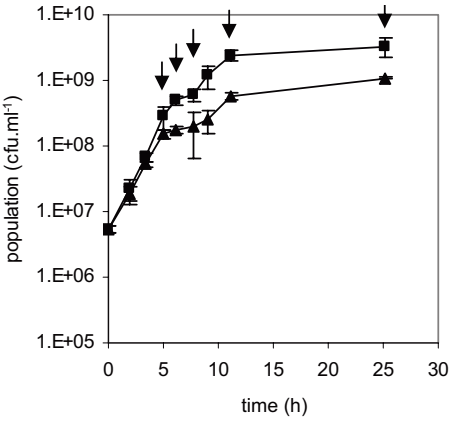

FIG. 1. Kinetics of growth of *S. aureus* MW2 in pure cultures (■) and mixed cultures with *L. lactis* LD61 (▲) on CDM medium at 30°C and a constant pH. Data are the averages of results from three independent experiments. Arrows indicate the time points at which samples were harvested for transcriptome analyses.

S. aureus MW2 gene expression profiles in the pure culture and in the mixed culture with *L. lactis* LD61 were investigated. Samples were harvested at different time points (5, 6.2, 7.8, 11.1, and 25 h after inoculation) in order to monitor the kinetics of gene expression under both growth conditions. Genes whose expression varied significantly (≥3-fold) in the pure and/or the mixed culture were identified by an ANOVA test as described in Materials and Methods (Tables 2, 3, and 4).

A general survey of the data indicated that the number of genes whose expression varied significantly in the pure culture was ~3-fold higher than that in the mixed culture. This pattern was emphasized when the analysis was restricted to virulence-related genes: the expression of 29 genes varied in the pure culture, versus that of only 4 genes in the mixed culture. Such a difference was related, at least partially, to the loss of a signal in the mixed culture. Hybridizations were indeed carried out with cDNAs resulting from a constant amount of total RNAs (i.e., a mixture of lactococcal and staphylococcal cDNAs in a mixed culture). The amount of *S. aureus* cDNA in mixed culture hybridizations was lower than that in pure-culture hybridizations, resulting in lower signals and a subsequent loss of sensitivity. Total RNA extracted from pure cultures gave a signal (signal/noise ratio of ≥2) in 90% of the spots retained for statistical analysis (see Materials and Methods), whereas total RNAs extracted from mixed cultures gave a signal in 64% of the spots. However, general trends in pure cultures, as well as in mixed cultures, were observed.

Several genes involved in the cellular machinery, cell division, cell envelope biosynthesis and turnover, carbohydrate and nitrogen metabolism, stress responses, and virulence displayed differential expression levels over the time course of the cultures (Tables 2, 3, and 4). The expression profiles of some genes belonging to various functional categories were clearly related to growth: genes involved in the translational machinery, cell division, the biosynthesis and turnover of the cell envelope, secretion, and the metabolism of nucleotides and nucleic acids maintained high levels of expression in the pure culture until h 11, when cells entered stationary phase. Likewise, the expression of genes associated with energetic metab-

TABLE 2. Expression profiles of genes involved in cellular machinery, cell envelope biosynthesis or turnover, or metabolism of carbohydrates, nitrogen, nucleotides, nucleic acids, or iron that exhibited significant variations in pure and/or mixed cultures[a]

ORF (Mu50)[b]	Corresponding ORF (MW2)[c]	Gene name[d]	Description of gene product or ORF[e]	cDNA/gDNA ratio[f] for pure culture at h:					cDNA/gDNA ratio[f] for mixed culture at h:				
				5	6.2	7.8	11.1	25	5	6.2	7.8	11.1	25
Transcription, translation, and cellular division genes													
SAV0535	MW0491	*nusG*	Transcription antitermination protein						4.0	1.8	1.1	1.3	1.1
SAV0547	MW0502	*fus*	Translational elongation factor G	25.8	48.3	30.0	20.3	1.5	7.7	14.0	5.2	4.3	2.3
SAV0548	MW0503	*tufA*	Translational elongation factor TU	20.2	27.5	17.0	15.6	1.3	8.6	10.0	4.3	4.2	2.1
SAV1678	MW1622	*rplT*	50S ribosomal protein L20	4.4	3.8	4.6	3.1	0.4	1.9	1.8	1.2	0.5	0.6
SAV1679	MW1623	*rpmI*	50S ribosomal protein L35	5.0	8.3	6.5	3.3	0.4	3.5	2.6	1.3	0.6	0.6
SAV1128	MW1010	*rpmF*	Ribosomal protein L32						5.2	4.4	2.0	1.7	1.3
IGS2			Intergenic sequence (16S to 23S)	14.4	25.9	13.6	10.2	1.0	20.7	9.7	3.1	1.1	0.9
SAV1180	MW1063	*ftsL*	Cell division protein	1.4	2.4	2.0	2.2	0.7					
SAV0511	MW0466	*ftsH*	Cell division protein	8.3	9.4	5.8	6.3	1.1					
Cell envelope biosynthesis and turnover genes													
SAV0307	MW0284		Similar to outer membrane protein precursor	2.7	2.8	2.0	3.0	0.4	1.9	1.4	0.8	0.9	0.6
SAV1433	MW1323		Similar to cell wall enzyme EbsB	0.3	0.4	0.3	0.3	1.0					
SAV1474	MW1363	*gpsA*	Glycerol-3-phosphate dehydrogenase	4.1	6.2	5.5	4.5	0.8					
SAV1261	MW1144	*cdsA*	Phosphatidate cytidylyltransferase	2.9	3.0	2.0	1.7	0.4					
SAV1228	MW1111		Transcription factor FapR (fatty acid and phospholipid biosynthesis regulator)	2.8	3.7	2.1	2.2	0.6					
SAV0465	MW0419		Probable autolysin (*N*-acetylmuramoyl-L-alanine amidase)	2.8	2.8	10.2	4.7	0.6	2.3	1.5	1.8	0.7	0.6
SAV2090	MW2013		Lipoprotein precursor	2.1	3.4	2.1	1.4	0.5					
SAV0464	MW0418		Lactococcal lipoprotein						2.9	2.6	1.8	1.0	0.8
SAV0642	MW0604	*pbp4*	Penicillin binding protein 4	2.9	3.0	2.1	1.7	0.5					
SAV1754	MW1697		Spore cortex protein homolog	1.5	1.5	1.7	1.8	0.5					
SAV0935	MW0817	*dltD*	Poly(glycerophosphate chain) D-alanine transfer protein						5.6	1.0	1.1	0.6	0.5
SAV2644	MW2565		Similar to autolysin precursor	0.7	0.4	0.8	1.0	2.1					
Carbohydrate metabolism genes													
SAV1698	MW1642	*pfk*	6-Phosphofructokinase	7.5	7.3	9.2	10.4	2.3	7.5	3.9	3.2	2.3	1.6
SAV1697	MW1641	*pykA*	Pyruvate kinase	4.9	4.0	6.5	6.1	1.3	3.2	2.3	1.8	1.2	1.0
SAV2609	MW2528		Acetate-CoA ligase	1.6	1.2	1.0	1.8	18.6	0.8	0.5	0.7	1.8	4.2
SAV0605	MW0568	*adh1*	Alcohol dehydrogenase I	8.3	4.8	10.0	17.0	45.9					
SAV1149	MW1032	*sdhB*	Iron sulfur subunit of succinate dehydrogenase	1.9	1.8	2.2	2.0	7.4					
SAV0269	MW0245	*rbsD*	Ribose permease	0.2	0.3	0.4	0.3	1.0					
SAV0214	MW0190		Similar to maltose/maltodextrin transport system	0.5	0.5	0.3	0.3	1.1					
SAV2158	MW2084	*mtlA*	PTS	0.9	0.9	0.5	0.6	7.3					
SAV0189	MW0163	*glcA*	PTS enzyme II	10.2	9.1	1.9	3.1	0.4	14.7	2.6	0.6	0.5	0.5
SAV2323	MW2244	*glvC*	PTS	0.3	0.4	0.3	0.3	4.2					
SAV0474	MW0428	*treP*	Phosphoenolpyruvate-dependent and trehalose-specific PTS enzyme II	0.3	0.4	0.2	0.3	1.1					
SAV0771	MW0733	*gapR*	Glycolytic operon regulator	17.8	24.7	5.9	13.9	9.1	10.5	10.4	4.1	1.3	1.6
Energetic metabolism genes													
SAV2607	MW2526	*mqo2*	Malate/quinone oxidoreductase	6.1	4.8	5.9	5.0	1.2	2.7	2.0	1.8	1.3	1.0
SAV1116	MW0999	*ctaB*	Cytochrome caa_3 oxidase homolog	6.5	6.3	3.9	4.7	2.3	2.2	3.0	1.5	1.3	0.9
SAV0623	MW0586		Na^+/H^+ antiporter	4.8	6.2	5.1	4.1	0.8	2.9	2.1	0.9	1.2	0.9
SAV0625	MW0588		MnhD homolog, similar to Na^+/H^+ antiporter subunit	5.7	5.9	3.6	3.2	1.3					
SAV1832	MW1772	*hemY*	Protoporphyrinogen oxidase	2.5	2.4	3.1	2.4	0.7					
SAV0627	MW0590		Similar to Na^+/H^+ antiporter	1.0	0.9	0.8	0.9	6.4					
Nitrogen metabolism genes													
SAV2056	MW1980	*ilvC*	Alpha-keto-beta-hydroxylacil reductoisomerase	0.1	0.2	0.2	0.1	0.7					
SAV1751	MW1694		Xaa-His dipeptidase homolog	2.0	3.0	3.3	3.0	0.9	1.5	1.1	1.1	0.9	0.5
SAV0986	MW0868	*oppB*	Oligopeptide transport system permease protein	0.8	1.2	8.6	6.9	2.1					
SAV1380	MW1268		Oligopeptide transport ATPase	0.5	0.8	0.9	0.7	4.3					
SAV1382	MW1270		Oligopeptide transporter membrane permease domain	2.1	2.3	1.6	1.4	0.4					
SAV0727	MW0689		Ditripeptide ABC transporter	3.9	3.0	4.7	4.3	0.5	7.2	1.6	1.8	0.6	0.4
SAV0989	MW0871	*oppF*	OppF homolog	0.6	0.8	6.9	4.1	0.5					

Continued on following page

TABLE 2—*Continued*

ORF (Mu50)[b]	Corresponding ORF (MW2)[c]	Gene name[d]	Description of gene product or ORF[e]	cDNA/gDNA ratio[f] for pure culture at h:					cDNA/gDNA ratio[f] for mixed culture at h:				
				5	6.2	7.8	11.1	25	5	6.2	7.8	11.1	25
SAV0205	MW0181	*oppF*	Oligopeptide transport ATP binding protein	0.2	0.2	0.2	0.2	0.6					
SAV0010	MW0010		Amino acid permease	0.3	0.3	0.6	0.4	1.5					
SAV2384	MW2304	*gltT*	Proton/sodium glutamate symport protein	9.3	8.6	11.0	7.0	1.0					
SAV1255	MW1138	*codY*	Pleiotropic transcription repressor	3.2	4.4	2.9	3.8	0.6					
Nucleotide and nucleic acid metabolism genes													
SAV1065	MW0948	*purK*	Phosphoribosylaminoimidazole carboxylase	0.3	0.4	0.8	2.3	0.6					
SAV0521	MW0476	*nupC*	Pyrimidine nucleoside transport protein	4.7	4.6	5.3	5.9	0.7	2.6	2.2	1.4	0.9	0.4
DNA replication, recombination, and repair genes													
SAV1146	MW1029	*uvrC*	Excinuclease ABC subunit C	0.7	0.8	0.7	0.7	3.2					
SAV1252	MW1135	*xerC*	Site-specific recombinase XerC homolog	1.9	2.8	1.7	1.8	0.5	3.1	0.7	0.5	0.8	0.9
SAV0432	MW0393	*hsdS*	Probable restriction modification system specificity subunit	0.1	0.1	0.1	0.1	0.3					
Secretion genes													
SAV0964	MW0846	*spsA*	Type I signal peptidase	1.9	1.5	1.4	0.9	0.6	3.7	4.6	5.6	0.9	0.7
SAV2230	MW2149	*secY*	Preprotein translocase SecY subunit	32.2	69.4	35.6	33.1	6.3	23.5	16.2	8.3	5.0	3.0
SAV0778	MW0740	*secG*	Probable protein export membrane protein	5.7	7.8	5.7	4.2	2.0					
Iron metabolism genes													
SAV1893	MW1834		Ferritin	6.5	7.2	7.6	9.6	48.3					
SAV0225	MW0200		Similar to periplasmic iron binding protein BitC	0.5	0.5	0.6	0.6	3.1					
SAV2550	MW2471	*feoB*	Ferrous iron transport protein B homolog	0.4	0.4	2.3	1.0	1.5					
SAV0113	MW0086	*sirC*	Lipoprotein	0.2	0.2	0.2	0.2	0.5					
Other genes													
SAV1837	MW1777		*ecsA* ABC transporter homolog	6.2	7.4	7.3	7.6	1.0					
SAV2698	MW2617		Similar to high-affinity nickel transport protein	0.2	0.2	0.2	0.3	1.0					
SAV0643	MW0605		ABC transporter A	0.7	0.7	0.8	1.2	2.1					
SAV1321	MW1208		Similar to two-component sensor histidine kinase	1.3	2.1	1.8	1.5	7.4					
SAV2146	MW2070	*czrB*	Cation efflux system membrane protein homolog	1.0	0.9	3.4	0.8	0.6					
SAV2631	MW2552		Similar to transcription regulator Crp/Fnr family protein	0.3	0.3	0.4	0.2	0.6	1.0	0.3	0.3	0.8	0.4
SAV1026	MW0906		Competence transcription factor	0.1	0.2	0.1	0.2	0.5					
SAV0070	MW2003	*kdpE*	Transcription regulator protein KdpE	0.1	0.1	0.1	0.1	0.4					
SAV2078	MW2002	*kdpD*	Sensor protein	0.2	0.2	0.2	0.2	0.6					
SAV0909			Cell wall hydrolase (encoded within bacteriophage ϕMu1)	0.6	0.7	0.8	0.9	33.6	1.1	1.3	1.6	2.4	8.1
SAV2462	MW2386		Antibiotic resistance protein	2.2	2.0	1.5	1.6	0.7					

[a] See Materials and Methods.
[b] Identification number of *S. aureus* Mu50 open reading frame (ORF) targeted on the microarray.
[c] Identification number of corresponding *S. aureus* MW2 ORF.
[d] Previously published gene name.
[e] Previously reported description of gene product or ORF. CoA, coenzyme A; PTS, phosphotransferase system.
[f] Expression levels in pure and mixed cultures at 5, 6.2, 7.8, 11.1, and 25 h, detected with the *S. aureus* microarray and presented as normalized cDNA/gDNA ratios. Only genes that showed significant variations in expression levels in pure and/or mixed cultures over time (as determined by the ANOVA test with the criterion of an FDR of <0.05) and a degree of change of $\geq$3-fold were defined as differentially expressed.

olism (e.g., *mnhD* and *ctaB*) decreased slightly during the first slowing down of growth and more drastically in the stationary phase. Similar variations in the expression of genes in these functional categories were observed in the mixed culture. Of note, the decrease of expression occurred earlier in the mixed culture than in the pure culture. This finding correlates well with the premature slowing down of *S. aureus* growth observed in the mixed culture. Hence, the expression of genes encoding transcriptional and translational machinery, as well as genes associated with energetic metabolism, the metabolism of nucleotides and nucleic acids, and secretion, started decreasing from the 7.8-h time point onward and even earlier for some genes (e.g., *nusG* and *xerC*). The premature slowing down of growth in the mixed culture may also account for the earlier

TABLE 3. Expression profiles of stress response genes showing significant variations in pure and/or mixed cultures[a]

ORF (Mu50)[b]	Corresponding ORF (MW2)[c]	Gene name[d]	Description of gene product[e]	cDNA/gDNA ratio[f] for pure culture at h:					cDNA/gDNA ratio[f] for mixed culture at h:					σ^B control[g]
				5	6.2	7.8	11.1	25	5	6.2	7.8	11.1	25	
SAV1253	MW1136	*clpQ*	Heat shock protein HslV	3.0	4.5	3.1	3.3	0.8						
SAV1254	MW1137	*clpY*	Heat shock protein HslU	2.6	4.3	2.6	2.9	0.8						
SAV0380	MW0356	*ahpF*	Alkyl hydroperoxide reductase subunit F	2.1	1.7	4.5	6.3	4.8						
SAV1553	MW1505	*sodA*	Superoxide dismutase	2.5	2.6	2.1	2.9	8.7						
SAV1334	MW1221	*katA*	Catalase	3.4	5.5	5.6	3.4	62.8						+
SAV0551	MW0506		Chaperone protein HchA (Hsp31)	3.3	3.2	2.1	2.4	21.2						
SAV2029	MW1953	*groEL*	GroEL protein	1.5	1.4	1.0	1.2	5.1						
SAV0975	MW0857	*clpB*	ClpB chaperone homolog	0.6	0.7	0.6	0.5	5.4						
SAV0525	MW0480	*clpC*	Endopeptidase	2.6	2.7	2.1	2.0	7.0						
SAV2182	MW2108	*asp23*	Alkaline shock protein 23	5.3	2.8	2.3	8.0	141.0	3.3	2.3	2.8	25.8	51.2	+
SAV1739	MW1682		General stress protein-like protein	5.3	4.9	6.3	7.6	59.6						+
SAV2548	MW2469	*clpL*	ATP-dependent Clp proteinase chain	4.1	2.4	1.5	4.1	58.8	3.2	0.9	1.0	5.5	19.2	+
SAV0828	MW0781		Similar to general stress protein	1.0	1.0	0.7	1.5	5.9						+
SAV0498	MW0453	*spoVG*	Stage V sporulation protein G homolog	1.3	1.1	1.4	8.6	21.3						+
SAV0704	MW0666		Similar to CsbB stress response protein	1.1	1.0	0.9	1.6	10.4	1.0	0.8	0.7	2.1	4.4	+
SAV1348	MW1236	*opuD*	Glycine betaine transporter	4.7	5.1	3.6	4.1	0.7	3.5	1.9	1.1	0.6	0.6	
SAV1339	MW1226	*lexA*	SOS regulatory LexA protein	1.6	2.5	2.9	6.3	15.7						
SAV0522	MW0477	*ctsR*	Transcription repressor of class III stress gene homologs	1.1	1.3	0.7	1.1	6.2						
SAV1491	MW1445	*srrB*	Staphylococcal respiratory response protein	1.5	1.8	2.6	1.9	0.8						
SAV1492	MW1446	*srrA*	Staphylococcal respiratory response protein	3.1	3.4	3.4	2.9	1.0						

[a] See Materials and Methods.
[b] Identification number of *S. aureus* Mu50 open reading frame (ORF) targeted on the microarray.
[c] Identification number of corresponding *S. aureus* MW2 ORF.
[d] Previously published gene name.
[e] Previously reported description of gene product.
[f] Expression levels in pure and mixed cultures at 5, 6.2, 7.8, 11.1, and 25 h, detected with the *S. aureus* microarray and presented as normalized cDNA/gDNA ratios. Only genes that showed significant variations in expression levels in pure and/or mixed cultures over time (as determined by the ANOVA test with the criterion of an FDR of <0.05) and a degree of change of $\geq$3-fold were defined as differentially expressed.
[g] + indicates that σ^B exerted positive control (5).

induction of genes belonging to the σ^B regulon under this condition, as observed for *asp23* and *clpL* (confirmed by qRT-PCR) (data not shown).

The expression of genes involved in carbohydrate metabolism and transport was linked to growth kinetics but also to glucose availability. The levels of expression of *pfk* and *pykA* seemed to be maintained until glucose exhaustion, which occurred in the pure culture after 11 h and in the mixed culture after ~8 h (53). In the pure culture, the exhaustion of glucose resulted in the upregulation of the expression of genes encoding several transporters for other carbon sources, like ribose (*rbsD*), maltose and maltodextrin (MW0190 [*mtlA*]), and trehalose (*treP*). Such regulation resulting from catabolic repression in several gram-positive bacteria, including *Staphylococcus* species, was reported previously (31, 71, 75).

Selective repression of enterotoxin expression in mixed cultures. In a food context, *S. aureus* is particularly undesirable because of the ability of some strains to produce SEs, which are the causative agents of SFP. Six enterotoxins are encoded within the *S. aureus* MW2 genome, including the two classical enterotoxins SE type A (SEA) and SEC and four SE-like (SE*l*) proteins: SE*l*G, SE*l*H, SE*l*K, and SE*l*L. SE*l*s have not been well characterized, and their involvement in food poisoning has not yet been established (42). Indeed, their emetic properties, except for those of SEH and SEG, still need to be evaluated (34, 49, 56, 69). Transcriptomic analyses revealed changes in the expression of enterotoxins A and L (Table 4). The expression of all enterotoxin genes harbored in strain MW2 was further investigated by qRT-PCR to confirm and complete the microarray data. *L. lactis* barely affected the expression of enterotoxins G, K, and H (Fig. 2A, B, and C; Table 5): the expression of *seg2* remained constant in both pure and mixed cultures. The expression of *sek* was maximal during the exponential phase and decreased in the stationary phase under both conditions. A moderate induction of *seh* in the stationary phase of the pure culture was observed, whereas *seh* expression was slightly impaired in the mixed culture. In contrast, the induction of *sec4* and *sel* expression in the exponential and/or post-exponential phase was dramatically affected or possibly delayed by the presence of *L. lactis*, as observed for genes of

TABLE 4. Expression profiles of virulence genes showing significant variations in pure and/or mixed cultures[a]

ORF (Mu50)[b]	Corresponding ORF (MW2)[c]	Gene name(s)[d]	Description of gene product[e]	cDNA/gDNA ratio[f] for pure culture at h:					cDNA/gDNA ratio[f] for mixed culture at h:					*agr* control[g]
				5	6.2	7.8	11.1	25	5	6.2	7.8	11.1	25	
Adhesin genes														
SAV0811	MW0764	*fnb*	Fibrinogen binding protein	0.8	0.9	1.0	2.9	37.7	1.1	0.6	1.4	2.3	10.1	
SAV1159	MW1041		Fibrinogen binding protein precursor	3.4	1.8	2.4	1.2	1.0						
SAV1208	MW1091		Fibrinogen binding protein	1.0	0.7	0.8	0.5	0.3						
SAV1481	MW1369	*ebpS*	Elastin binding protein	1.5	1.2	1.0	1.4	7.9						
SAV2371	MW2293		Similar to products for attachment to host cells and virulence	0.2	0.2	0.1	0.2	0.5						
Antigen genes														
SAV2569	MW2490	*isaA*[h]	Immunodominant antigen A	7.2	4.9	6.8	5.3	0.6	5.0	3.1	1.3	1.2	1.0	
SAV2638	MW2559	*isaB*	Immunodominant antigen B	3.5	3.1	10.0	6.5	10.3						
SAV0665	MW2217		Secretory antigen SsaA homolog	2.3	2.0	4.6	6.2	0.5						
Capsule genes														
SAV0149	MW0124	*capA*	Capsular polysaccharide synthesis enzyme Cap5A	0.2	0.2	0.2	0.4	15.5						
SAV0164	MW0139	*capP*	Capsular polysaccharide synthesis enzyme Cap5P	0.2	0.3	0.2	0.3	2.3						
Enzyme genes														
SAV0815	MW0769	*nuc*	Staphylococcal nuclease	0.9	2.2	5.0	4.8	0.5						
SAV1324	MW1211	*nuc*	Thermonuclease	6.0	6.0	7.3	4.8	0.6						
SAV1811	MW1753	*splC*	Serine protease	0.4	0.6	0.3	0.6	1.8						+
SAV1813	MW1755	*splA*	Serine protease	17.1	16.2	17.0	9.3	0.9	8.7	7.2	8.5	1.9	0.9	+
SAV1046	MW0930	*sspC*	Cysteine protease	0.2	0.3	0.3	0.3	2.2	1.7	2.3	1.7	0.6	0.9	+
SAV0320	MW0297	*geh*	Glycerol ester hydrolase	0.5	0.4	1.2	0.8	1.6						+
Hemolysin genes														
SAV2035	MW1959	*hld*/RNAIII	Delta-hemolysin	1.6	1.8	20.0	39.0	96.7						+
SAV0919	MW0802		Hemolysin	0.4	0.5	0.3	0.5	11.4						
SAV2170	MW2096		Hemolysin III	1.4	2.0	1.5	2.3	5.5						
Toxin genes														
SAV1948	MW1889 (*sea*)	*sep*	Enterotoxin P (encoded within bacteriophage ϕ Sa 3Mu)	2.2	1.7	1.6	1.6	0.6						
SAV2008	MW0760	*sel*	Extracellular enterotoxin L	1.5	1.5	1.8	2.1	5.4						
SAV1819	MW1767	*lukD*	Leukotoxin F subunit	0.1	0.1	0.1	0.2	0.4						+
SAV0422	MW0382 (*set16*)	*set6*	Exotoxin 6	0.3	0.3	0.2	0.4	1.4						
SAV0423	MW0383 (*set17*)	*set7*	Exotoxin 7	0.1	0.1	0.1	0.1	0.3						
SAV0424	MW0384 (*set18*)	*set8*	Exotoxin 8	0.1	0.1	0.1	0.1	0.3						
SAV0428	MW0390 (*set24*)	*set13*	Exotoxin 13	0.4	0.4	0.4	0.6	2.1						
Virulence regulation genes														
SAV2039	MW1963	*agrA*	Accessory gene regulator A	0.6	0.8	4.1	14.1	15.0						
SAV1884	MW1824	*vraR*	Two-component response regulator	3.4	3.1	1.1	1.9	0.6						
SAV0661	MW0623	*vraF*	ABC transporter ATP binding protein	1.1	0.8	1.3	1.5	2.7						

[a] See Materials and Methods.
[b] Identification number of *S. aureus* Mu50 open reading frame (ORF) targeted on the microarray.
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[d] Previously published gene name.
[e] Previously reported description of gene product.
[f] Expression levels in pure and mixed cultures at 5, 6.2, 7.8, 11.1, and 25 h, detected with the *S. aureus* microarray and presented as normalized cDNA/gDNA ratios. Only genes that showed significant variations in expression levels in pure and/or mixed cultures over time (as determined by the ANOVA test with the criterion of an FDR of <0.05) and a degree of change of $\geq$3-fold were defined as differentially expressed.
[g] + indicates that *agr* in strain MW2 exerted positive control (62).
[h] *isaA* was found to be positively regulated by σ^B, as its expression decreased in a σ^B mutant (5). However, its expression decreased in late log and stationary phases in both wild-type and mutant strains, as observed in this experiment.

other functional categories (Fig. 2D and E; Table 5). *sea* was expressed mainly during the exponential phase in the pure culture, and its expression decreased in the stationary phase (Fig. 2F; Table 5), as observed previously (8, 18). The level of expression of *sea* during the exponential phase in the mixed culture was slightly lower than that in the pure culture. However, in the mixed culture, *sea* expression remained constant during the stationary phase (to h 25) and was thus slightly (~2-fold) higher than that in the pure culture. Altogether, these results showed that the presence of *L. lactis* strongly reduced the expression of two of the six enterotoxin and enterotoxin-like proteins identified so far in *S. aureus* MW2.

Modified expression of other *S. aureus* virulence genes in mixed cultures. Virulence gene expression is tightly controlled

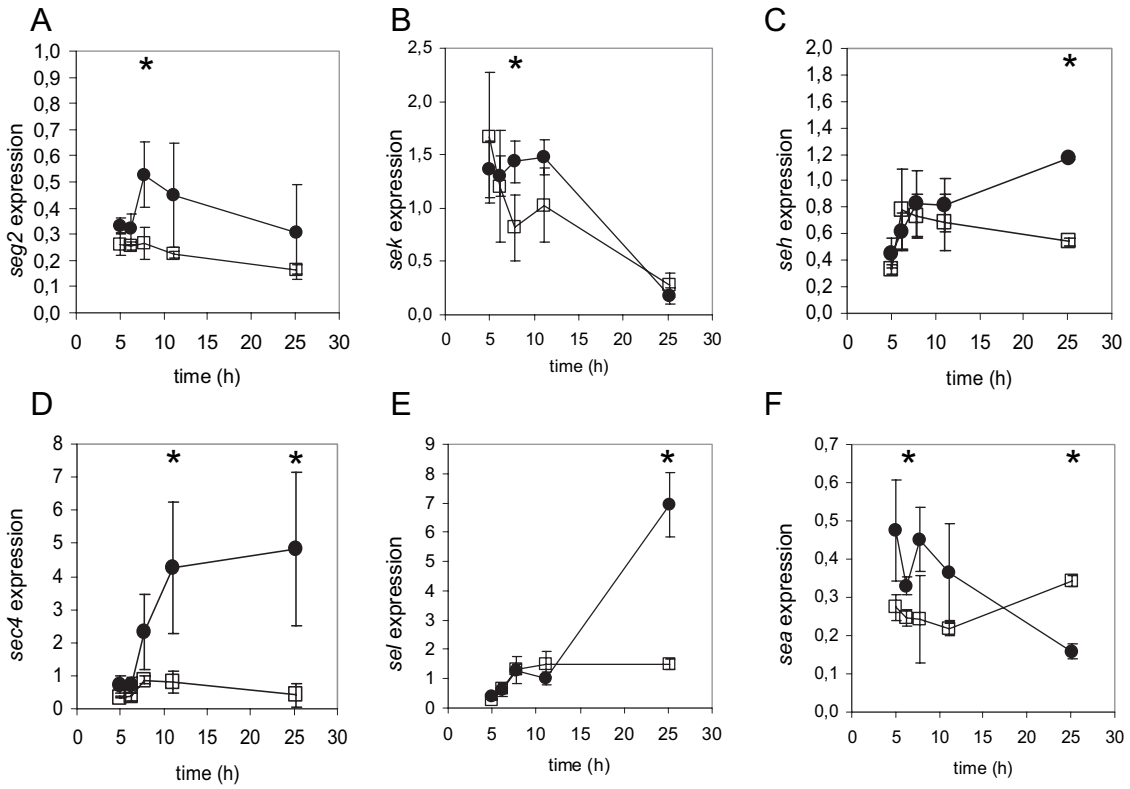

FIG. 2. Transcription profiles of genes encoding *S. aureus* MW2 enterotoxins as determined by qRT-PCR. Transcript levels in pure (●) and mixed (□) cultures of *S. aureus* between 5 and 25 h were measured. Data points were plotted as relative intensity values (see Materials and Methods). Profiles of transcript titers for enterotoxin type G2 (*seg2*), enterotoxin K (*sek*), enterotoxin H (*seh*), enterotoxin type C4 (*sec4*), enterotoxin L (*sel*), and enterotoxin A (*sea*) genes are presented. Statistical analyses (by an ANOVA test with the criterion of a P value of <0.05) indicated that these genes showed significant changes in expression in pure and mixed cultures over time, except for *sea* and *seh* in mixed cultures ($P > 0.05$). Time points at which genes showed significant changes in expression in pure versus mixed cultures, as determined by a t test ($P < 0.05$), are indicated by asterisks, and ratios of expression levels are presented in Table 5.

in *S. aureus* by complex and intricate systems, of which *agr* and *sar* are central elements (9, 54). As expected, the expression of several virulence genes in the pure culture of *S. aureus* MW2 was modified over time (Table 4). Genes encoding exoproteins (such as *geh*, *splC*, and *sspC*), hemolysins (MW0802 and MW2096), toxins (such as *sel* and *lukD*), and exotoxins (MW0382 to MW0384 and MW0390) and genes involved in capsule synthesis (*capA* and *capP*) were induced in the post-exponential and stationary phases of growth. This upregulation of exoprotein production was concomitant with the induction of the *agr* system. The expression of *agrA* and RNAIII was indeed 25- and 60-fold higher, respectively, in the stationary phase than in the exponential phase. Accordingly, some genes encoding cell surface proteins, such as MW1041 and MW1091 (encoding fibrinogen binding proteins), *isaA* (encoding immunodominant antigen A), and MW2217 (encoding a secretory antigen SsaA homolog) were repressed when cells entered the stationary phase (Table 4).

TABLE 5. Ratios of expression levels in pure versus mixed cultures[a]

Gene	Ratio of expression in pure culture to expression in mixed culture at:				
	5 h	6.2 h	7.8 h	11.1 h	25.2 h
seg	–	–	2.0	–	–
sek	–	–	1.8	–	–
seh	–	–	–	–	2.2
sec	–	–	–	5.2	11.6
sel	–	–	–	–	4.7
sea	–	1.3	–	–	0.5
agrA	–	–	–	–	4.7
RNAIII	–	–	–	9.2	73.9
MW802	1.8	2.3	–	–	5.6
capA	–	–	–	0.2	7.4
hla	2.8	1.7	–	–	–
spa	–	–	–	–	0.4
sarA	1.5	1.9	4.7	2.5	6.4

[a] Ratios were calculated by using qRT-PCR for genes showing significant changes in expression levels (as determined by a t test with the criterion of a P value of <0.05). – indicates the absence of an effect of *L. lactis* on gene expression.

Strikingly, in the mixed culture, the induction of exoprotein genes and the *agr* system was not observed. To confirm that these observations were not due to the lower sensitivity of the microarray hybridization analysis for mixed-culture samples, qRT-PCR experiments with several virulence-related genes were carried out (Fig. 3). In the pure culture, *agrA* and RNAIII expression was dramatically induced (37- and 1,555-fold, respectively) in post-exponential and stationary phases of growth, whereas it was strongly altered in the mixed culture, with maximum inductions of 12- and 25-fold, respectively, at

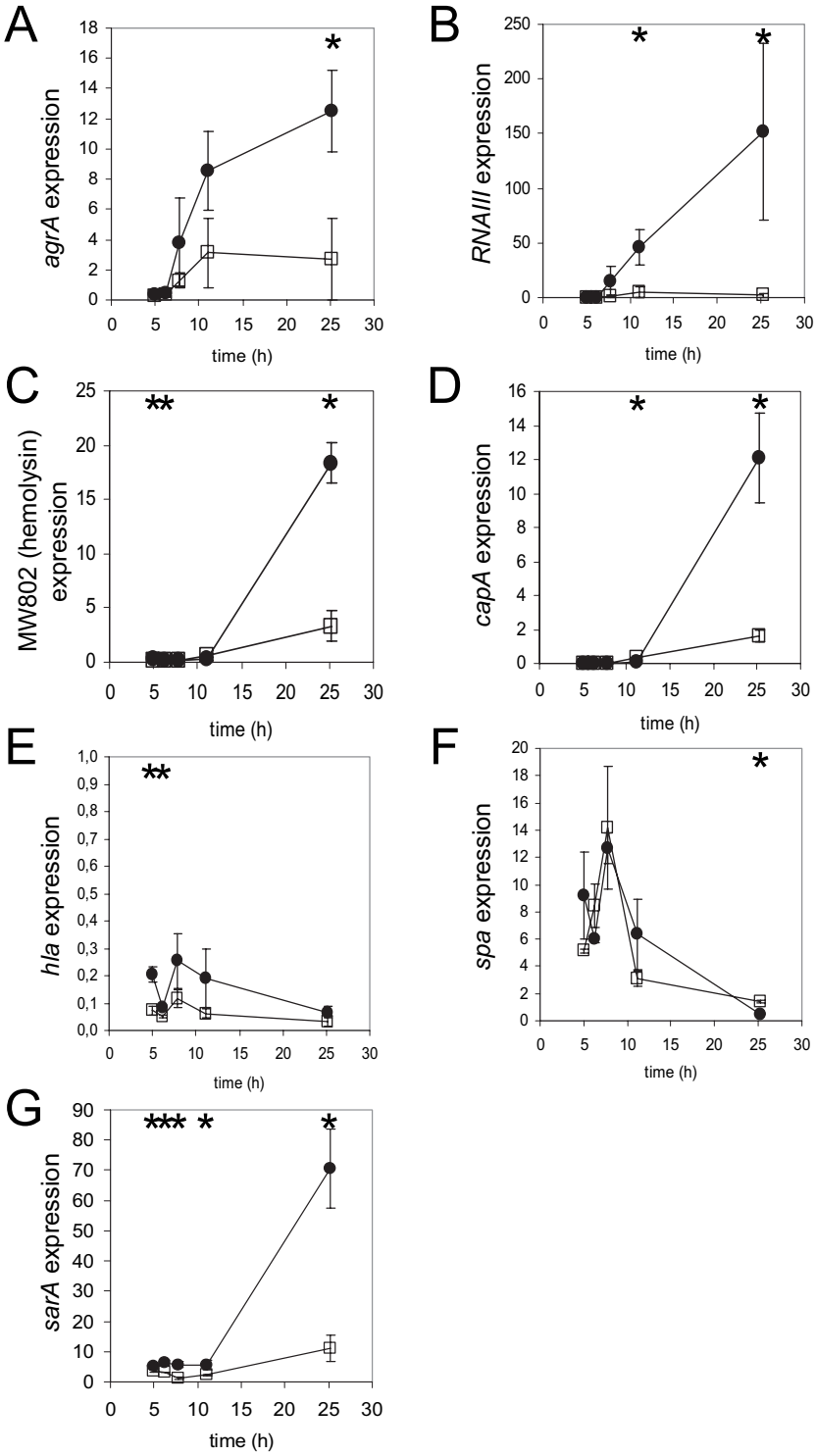

FIG. 3. Transcription profiles of virulence genes as determined by qRT-PCR. Transcript levels in pure (●) and mixed (□) cultures of *S. aureus* between 5 and 25 h were measured. Data points were plotted as relative intensity values (see Materials and Methods). Profiles of transcript titers for accessory gene regulator A (*agrA*), delta-hemolysin (RNAIII/*hld*), hemolysin (MW802), capsular polysaccharide synthesis enzyme Cap5A (*capA*), alpha-hemolysin (*hla*), protein A (*spa*), and staphylococcal accessory regulator (*sarA*) genes are presented. Statistical analyses (by an ANOVA test with the criterion of a P value of <0.05) indicated that these genes showed significant changes in expression in pure and mixed cultures over time, except for *agrA* and RNAIII in mixed cultures ($P > 0.05$). Time points at which genes showed significant changes in expression in pure versus mixed cultures, as determined by a t test ($P < 0.05$), are indicated by asterisks, and ratios of expression levels are presented in Table 5.

11 h (Fig. 3A and B; Table 5). Similarly, the expression of genes encoding MW0802 and Cap5A hemolysins was strongly induced in post-exponential and stationary phases in the pure culture, whereas only a moderate induction was observed in the mixed culture (Fig. 3C and D; Table 5). The expression of *hla* and *spa*, two well-characterized genes encoding alpha-hemolysin and protein A, respectively, was also assayed by qRT-PCR (Fig. 3E and F; Table 5). As observed previously (21, 24), the expression of *spa* was maximal during the exponential phase and decreased when cells entered post-exponential growth in the pure culture. A similar *spa* expression profile in the mixed culture was observed. Surprisingly, *hla* was not induced in the stationary phase, either in the pure culture, as observed previously in other strains (21), or in mixed cultures.

In order to complete the microarray data with regard to virulence regulation, the expression of *sarA*, one of the major and pleiotropic regulators of virulence in *S. aureus* (together with the *agr* system) (9, 21, 54), was assayed by qRT-PCR. The expression of *sarA* was constant throughout the exponential phase of the pure culture, as well as in the mixed culture, although at a slightly lower level (Fig. 3G; Table 5). However, strong (13-fold) induction of *sarA* expression in the stationary phase of the pure culture was observed, as reported previously (45), whereas the level of induction in the mixed culture (4-fold) was significantly lower. In conclusion, *L. lactis* was able to alter the induction of *agr* and *sarA*, the two main regulators of virulence in *S. aureus*.

Supernatant from an *L. lactis* culture does not affect RNAIII expression. RNAIII downregulation may be due to the release of an inhibitory molecule(s) into the culture supernatant by *L. lactis*. If so, supernatants from a pure culture of *L. lactis* LD61 or those from a mixed culture would have an impact on the expression of *hld*/RNAIII. To test this hypothesis, *S. aureus* MW2 was grown in pure cultures on CDM supplemented with supernatants harvested at 24 h from pure or mixed cultures of *L. lactis* (1 volume of supernatant was added to 1 volume of 2× CDM). The expression of RNAIII was investigated by qRT-PCR as described in Materials and Methods. The addition of a supernatant did not alter the kinetics of RNAIII expression, as similar profiles with and without supernatants were observed (data not shown). Moreover, the addition of supernatants obtained from pure or mixed cultures of *L. lactis* to a pure *S. aureus* culture in the post-exponential phase of growth, which corresponds to the apex of RNAIII induction, did not affect RNAIII expression (data not shown). Altogether, these results suggest that the downregulation of *agr* locus expression by *L. lactis* does not rely on the release of an inhibitory molecule or, alternatively, that the inhibitory molecule is labile.

***L. lactis* downregulates RNAIII expression in other *S. aureus* strains belonging to various *agr* groups.** The downregulation of a key virulence regulator like the *agr* system by *L. lactis* is of great interest. We therefore wondered whether this effect was restricted to strain MW2 or if it could be observed in other strains, notably strains belonging to different *agr* groups. We thus investigated the effect of *L. lactis* LD61 on two additional strains, namely, strain SH1000 (derived from the well-characterized strain NCTC 8325, of *agr* group I) and RN4850 (the prototype strain of *agr* group IV). The strains were grown in mixed cultures under conditions similar to those used for strain MW2, and the expression of RNAIII was monitored by qRT-PCR (data not shown). While RNAIII expression in both strains was strongly induced in post-exponential and stationary phases of growth in pure cultures, it was strongly altered in mixed cultures (>100-fold downregulation of expression in mixed cultures compared to that in pure cultures had occurred at 25 h). This result clearly indicates that the observed downregulation of RNAIII in strain MW2 by *L. lactis* is not unique, even though we cannot exclude the possibility that some *S. aureus* strains may be resistant to this inhibitory effect. Furthermore, it does not seem to be restricted to one *agr* group.

DISCUSSION

Successful exploration of the *S. aureus* transcriptome in mixed cultures with *L. lactis*. Interactions between *S. aureus* and other bacterial species, notably LAB, arouse more and more interest (1, 11, 40). A better understanding of bacterial interference phenomena will indeed lead to promising nonantibiotic means to control the growth of and/or pathogenesis by *S. aureus* (or other undesirable microorganisms) in ecosystems where keeping a complex and balanced microbial community is crucial (e.g., the vaginal ecosystem and food fermentation environments). Bacterial interactions involved in such contexts are quite complex and require global molecular approaches, such as transcriptome analyses. Only a few studies have investigated the behavior of *S. aureus* during interaction with a host or other microorganisms. One recent study reports the transcriptome analysis of *S. aureus* in interaction with (internalized by) human epithelial host cells (25). Another study reports a transcriptional analysis of the interaction between *S. aureus* and *Pseudomonas aeruginosa*, yet only the *P. aeruginosa* transcriptome was analyzed (46). We report here the first transcriptome analysis of *S. aureus* in interaction with another bacterial species, namely, *L. lactis*. A dedicated *S. aureus*-specific microarray was successfully developed and validated in order to minimize the cross-hybridizations that may result from hybridization with a pool of labeled cDNAs from two species. Genomes of other LAB species were taken into account in designing the microarray so that *S. aureus* gene expression profiles during interaction with other LAB ecosystems (e.g., that of a complex starter culture in the cheese-making process or vaginal lactobacilli) can be analyzed.

Culture with *L. lactis* induces a time lag in the kinetics of *S. aureus* gene expression. The inhibitory effect of *L. lactis* on *S. aureus* growth is widely documented (1, 12, 28, 36; for a review, see reference 11); however, its effect on the global physiology of *S. aureus* and on virulence expression had never been explored. Under the conditions tested in this work, i.e., a chemically defined culture medium at a constantly regulated pH, *L. lactis* hardly affected *S. aureus* growth and the final population of *S. aureus* in the presence of *L. lactis* was only threefold smaller than that in the pure culture. Despite the moderate effect on growth, the kinetics of *S. aureus* gene expression in the mixed culture with *L. lactis* was modified compared to that in the pure culture. For instance, most functions associated with growth (translation and cell envelope biosynthesis) showed reduced expression earlier in the mixed culture than in the pure culture, which may be related to the premature slowing down of growth in the mixed culture. Likewise, the kinetics of expression of genes involved in carbohydrate metabolism

was modified in the presence of *L. lactis*. Altogether, our results showed that coculturing with *L. lactis* LD61 inflicted a time lag on *S. aureus* transcriptomic profiles rather than wild variations in the whole transcriptomic profile. The altered profiles correlated with environmental changes provoked by the presence of *L. lactis*, such as the premature exhaustion of glucose (53).

Kinetic follow-up of *S. aureus* gene expression reveals an alteration in the expression of several virulence genes in mixed cultures with *L. lactis*. In the present study, we showed that *L. lactis* was able to impair the expression of several *S. aureus* virulence genes. Staphylococcal virulence is tightly controlled and temporally regulated. During the post-exponential and stationary phases of growth, many exoproteins, including some enterotoxins, exofoliative toxins, alpha-, beta-, gamma-, and delta-hemolytic toxins, toxic shock syndrome toxin 1, and several secreted enzymes, are upregulated whereas many cell surface-exposed factors, such as protein A, immunodominant antigen A, coagulase, and fibronectin binding protein, are downregulated (5, 21, 48, 54, 55, 64, 72). Accordingly, we found that several exoproteins, hemolysins, toxins, and proteins involved in capsule biosynthesis were induced in the post-exponential and stationary phases of growth in the pure culture of *S. aureus* MW2. Surprisingly, the expression of *hla* was rather constant throughout the time course of the culture, suggesting that the complex regulation of *hla* expression established in other strains may not apply to strain MW2 (21, 72). Furthermore, we observed that some cell surface protein genes, including *isaA*, *spa*, and genes encoding fibrinogen binding proteins, were repressed in the post-exponential phase, although no general downregulation of genes encoding cell surface proteins was observed. Thus, the expression profiles of virulence genes in strain MW2 fitted the temporal regulation patterns generally observed in other *S. aureus* strains. However, the gene expression profile was very much altered in the presence of *L. lactis* with, in particular, no general upregulation of many exoproteins.

***L. lactis* hinders *S. aureus* virulence expression through the alteration of *agr* expression.** In a pure culture of *S. aureus* MW2, we found that the induction of several exoprotein genes, including *geh*, *sspC*, *capA*, and *sec4*, was concomitant with the activation of the *agr* system. The *agr* regulon in strain MW2 was very recently characterized (62), revealing that virulence regulation by *agr* in strain MW2 partially fits the current model established for well-characterized laboratory strains (derived from strain NCTC 8325) (54): while several toxins and other exoproteins are upregulated by *agr*, the global downregulation of surface binding proteins by *agr* observed in other strains does not occur in strain MW2. Such discrepancies in the *agr* regulon profiles indeed explain why in our study, no general downregulation of surface binding protein expression occurred in the post-exponential phase of the MW2 pure culture. Strikingly, only transient and feeble induction of the *agr* locus was observed in the mixed culture, suggesting that the lack of induction of exoprotein expression in the mixed culture was, at least in part, due to the nonactivation of the *agr* system.

It is noteworthy that *agr* downregulation was not restricted to *S. aureus* strain MW2 and occurred in other *S. aureus* strains belonging to different *agr* groups. Hence, although the ability of *L. lactis* to downregulate the *agr* system cannot be generalized to all *S. aureus* strains without further investigations, the downregulation in MW2 is not an isolated case. This result strongly reinforces the interest in the observed inhibitory capacity of *L. lactis*, the mechanism of which has now to be identified. We did not observe any significant downregulation of RNAIII expression when *S. aureus* MW2 was grown in CDM supplemented with *L. lactis* pure- or mixed-culture supernatants. This finding suggests that *agr* downregulation was not due to the production of an inhibitory molecule or the release of an autoinducing-peptide-modifying or -degrading enzyme by *L. lactis*, although we cannot totally exclude the possibility that the inhibitory molecule(s)/enzyme is highly unstable. The production of an inhibitory molecule (metabolite or peptide) that interacts with the two-component system within the *agr* system and blocks signal transduction in different species of the *Staphylococcus* genus (27, 73) and in other gram-positive or negative bacteria (40, 61) has already been described. In particular, Laughton and coworkers showed that *Lactobacillus reuteri* has the potential to repress the expression of the superantigen-like protein 11 (SSL11) and RNAIII via the secretion of a soluble factor which still remains to be identified (40). Our study suggests that the signaling pathways involve the transcriptional regulator SarA. SarA acts as a global regulator of virulence expression in *S. aureus* (21, 44, 45) and is, in particular, required for the full expression of *agr* (6, 14, 16, 21). The lower level of *sarA* expression in the mixed culture with *L. lactis* than in the pure culture may thus account for the weaker induction of *agr* locus expression in the mixed culture. Further investigations will be necessary to unravel both the lactococcal factor and the signaling pathway responsible for *agr* downregulation.

Impact of *L. lactis* on *S. aureus* enterotoxigenesis. Among virulence factors, enterotoxins play a particular role and are the main threat in foodstuffs. Despite the importance of enterotoxins, the regulation of enterotoxins and enterotoxin-like protein expression is still poorly documented. We here report the expression profiles of the six SE and SE*l* proteins of *S. aureus* MW2. All enterotoxin genes were found to be expressed in strain MW2. *sea* was expressed mainly during the exponential phase, as established previously (8), as was *sek*, whereas the expression of *seg2* was constant throughout the culturing. In contrast, *sec*, *sel*, and *seh* were induced in the post-exponential and stationary phases of growth. The induction of *sec* expression in a pure culture of *S. aureus*, concomitant with the activation of the *agr* locus, corroborated the results of previous studies, as *sec* expression is controlled by RNAIII (62). Accordingly, *sec* induction was impaired in the presence of *L. lactis*, and the impairment correlated with *agr* downregulation. The impact of *L. lactis* on enterotoxin expression was enterotoxin type dependent. While *L. lactis* strongly affected the expression of *sec* and *sel*, it barely affected the expression of *sek*, *seg2*, and *seh* and even slightly favored the maintenance of *sea* expression in the stationary phase. This finding is of particular interest, as SEA is the main enterotoxin identified in SFP outbreaks (22, 37). The high prevalence of SEA involvement in SFP outbreaks associated with dairy products may be related to the lack of inhibition of *sea* expression by *L. lactis* while the expression of other enterotoxin genes, including *sec*, is affected.

Conclusions. This study reports a transcriptomic analysis of *S. aureus* in interaction with another bacterial species through the development and use of a dedicated species-specific microarray. Together with Nouaille et al. (53), we have provided the first reports of studies of bacterial interaction at the transcriptome level in which both partners are comprehensively analyzed. We showed that some staphylococcal virulence genes were selectively downregulated in a mixed culture. The inhibition of virulence expression correlated with the repression of the virulence-associated regulators *agr* and *sarA* in *S. aureus* MW2. To our knowledge, this is the first study that demonstrates the capability of *L. lactis*, the model LAB, of inhibiting *agr*, *sarA*, and some of the virulence genes in *S. aureus* under conditions in which *S. aureus* growth is not dramatically impaired. This study is a first step toward the identification of molecular mechanisms involved in the inhibition of *S. aureus* by LAB and opens avenues for the biocontrol of *S. aureus* contamination and/or virulence expression.

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