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Growth of Aerobic Ripening Bacteria at the Cheese Surface Is Limited by the Availability of Iron

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The microflora on the surface of smear-ripened cheeses is composed of various species of bacteria and yeasts that contribute to the production of the desired organoleptic properties. The objective of the present study was to show that iron availability is a limiting factor in the growth of typical aerobic ripening bacteria in cheese. For that purpose, we investigated the effect of iron or siderophore addition in model cheeses that were coinoculated with a yeast and a ripening bacterium. Both iron and the siderophore desferrioxamine B stimulated the growth of ripening bacteria belonging to the genera *Arthrobacter*, *Corynebacterium*, and *Brevibacterium*. The extent of stimulation was strain dependent, and generally, the effect of desferrioxamine B was greater than that of iron. Measurements of the expression of genes related to the metabolism of iron by *Arthrobacter arilaitensis* Re117 by real-time reverse transcription-PCR showed that these genes were transcribed during growth in cheese. The addition of desferrioxamine B increased the expression of two genes encoding iron-siderophore ABC transport binding proteins. The addition of iron decreased the expression of siderophore biosynthesis genes and of part of the genes encoding iron-siderophore ABC transport components. It was concluded that iron availability is a limiting factor in the growth of typical cheese surface bacteria. The selection of strains with efficient iron acquisition systems may be useful for the development of defined-strain surface cultures. Furthermore, the importance of iron metabolism in the microbial ecology of cheeses should be investigated since it may result in positive or negative microbial interactions.

Smear-ripened cheeses such as livarot, maroilles, munster, limburger, and tilsit are characterized by a complex surface microflora composed of various species of yeasts and bacteria (4, 7, 9, 10). During the first hours of the manufacturing procedure, lactic acid bacteria grow in the milk, resulting in a decrease in the pH. This acidification, in combination with the effect of rennet, results in the formation of a coagulum. Fresh curd blocks are then shaped from the curd grains and brined or salted with dry salt, and colonization by acid-tolerant yeasts occurs within a few days. The yeasts increase the pH by assimilating lactate and producing ammonia, thereby favoring the growth of less acid-tolerant ripening bacteria, whose concentration may exceed 10^{10} CFU/g (16). The sources of ripening bacteria are cheese milk, brine baths, ripening room air, wooden shelves on which the cheese rests during ripening, human skin, and deliberately added cultures (18, 25). These bacteria contribute to a large extent to the development of the typical color, flavor, and texture of smear-ripened cheeses. Their rapid growth also reduces the risk of contamination with spoilage microorganisms or pathogens such as *Listeria monocytogenes* (14). A better understanding of the microbial ecology of the surface of smear-ripened cheeses is needed to improve the control of microorganism growth on the surface of smear-ripened cheeses, which could, for example, facilitate the development of appropriate defined-strain surface cultures (6, 8, 18). Noordman et al. (26) reported that *Brevibacterium* strains of dairy origin produce and/or utilize siderophores, small, high-affinity iron-chelating compounds (36) that have been detected in several types of cheese (27). In cocultivation experiments, the production of hydroxamate siderophores by a *Brevibacterium linens* strain strongly stimulated the growth of another *Brevibacterium linens* strain, which was siderophore auxotrophic (27). In addition, the study of the genomes of two strains that belong to typical species found on the surface of cheese, *Arthrobacter arilaitensis* and *Corynebacterium variabile*, showed the presence of numerous genes involved in iron acquisition (23, 30). Iron acquisition may thus be instrumental in enabling bacteria to grow at the cheese surface. The objective of the present study was to show that iron availability is a limiting factor in the growth of typical surface bacteria in cheese. For that purpose, we investigated the effect of iron and siderophore addition on model cheeses. The present study is also an example of gene expression measurements in cheese by real-time reverse transcription-PCR. Such analyses are increasingly used for a better understanding of the physiology of microorganisms and their expression of beneficial or undesirable properties during cheese production (1, 12, 13, 15, 20, 24, 29, 31, 33, 34).

MATERIALS AND METHODS

Strains and growth conditions. The strains used in the present study are listed in Table S1 in the supplemental material. All of these strains were originally isolated from cheese. The lactic acid bacteria *Lactococcus lactis* subsp. *lactis* S3+ and its protease-negative variant S3− were routinely grown under static conditions at 30°C in M17 lactose (0.5%) broth (32). The ripening bacteria were grown in 50-ml conical flasks containing 10 ml of brain heart infusion broth (Biokar Diagnostics, Beauvais, France). The yeast *Debaryomyces hansenii* 304 was grown in 50-ml conical flasks containing 10 ml of potato dextrose broth (Biokar Diagnostics). The ripening bacteria and the yeast strains were incubated for 48 h at 25°C on a rotary

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shaker at 150 rpm. Two successive cultures inoculated at 1:20 were performed before inoculation of the cheese curd.

Model cheese experiments. Curd production (coagulation, cutting, molding, and draining of the curd) was done as previously described (22), except that the commercial starter culture was replaced with a mixture of *Lactococcus lactis* subsp. *lactis* S3+ and S3− strains, which were inoculated at concentrations of 2×10^6 and 4×10^6 CFU/ml, respectively. These strains were previously grown for 15 h at 30°C in reconstituted skim milk (100 g/liter; Difco Laboratories, Detroit, MI) that had been heated for 10 min at 110°C. Fifty-seven milliliters of a saline solution (92 g/liter NaCl) was added to 246 g of curd (pH ~4.64) under sterile conditions and mixed four times for 10 s at maximum speed using a Waring blender (Fisher Scientific, Elancourt, France). The cultures of the ripening bacteria in brain heart infusion broth were centrifuged for 10 min at 4°C and 3,000 × *g*, and the cell pellets were resuspended in physiological saline and subsequently added to the curd at a final concentration of 10^6 CFU/g. The cheese curd was inoculated with the yeast *Debaryomyces hansenii* 304, grown in potato dextrose broth, but at a final concentration of 10^4 CFU/g. In some cases, iron (added as a filter-sterilized solution of $FeCl_3 \cdot 6H_2O$) or the siderophore desferrioxamine B (desferrioxamine mesylate salt; Sigma, St-Quentin-Fallavier, France) was added to the cheese curd at a final concentration of 1 mg of iron or 50 μmol of desferrioxamine B per kg of cheese curd. Seventeen grams of inoculated curd was then transferred onto a plastic grid (diameter, 5.3 cm) in sterile crystallizing basins (diameter, 5.6 cm). These model cheeses were incubated for 24 h at 21°C and then at 14°C and 93% relative humidity. Sampling was done at 12 and 16 days, and three separate cheeses were used for each condition. The model cheese system used in the present study is representative mainly of the surface of soft cheeses due to the low thickness of the loaf (approximately 1 cm).

Microbiological analyses. One gram of cheese was mixed with 9 ml of physiological saline (9 g/liter NaCl). After dispersion with a mechanical blender (Ultra-Turrax model T25; Ika Labortechnik, Staufen, Germany) for 1 min at 11,500 rpm, 10-fold serial dilutions in physiological saline were prepared and plated in triplicate on agar plates. The ripening bacteria were counted on brain heart infusion agar supplemented with 50 mg/liter amphotericin (Biokar Diagnostics), which inhibits the growth of fungi, after 3 days of incubation at 25°C. The lactic acid bacteria from the starter culture (*Lactococcus lactis* subsp. *lactis* S3+ and S3−) were enumerated on deMan-Rogosa-Sharpe agar (pH 6.5; Biokar Diagnostics) supplemented with 50 mg/liter amphotericin after 3 days of incubation at 30°C under anaerobic conditions. *Debaryomyces hansenii* 304 yeast cells were counted on yeast extract-glucose-chloramphenicol agar (Biokar Diagnostics) after 3 days of incubation at 25°C.

Extraction of RNA from cheese samples. RNA was extracted from cheese as previously described (24), except that the amount of cheese processed in each tube was increased to 500 mg, using 7-ml bead-beating tubes that contained 1.6 g of 0.1 mm-diameter zirconium beads (Biospec Products, Bartlesville, OK) and 1.6 g of 0.5 mm-diameter beads. After the addition of 5 ml of TRIzol reagent (Invitrogen, Cergy Pontoise, France), the tubes were vigorously shaken in a BeadBeater (Precellys 24 Dual, Saint-Quentin-en-Yvelines, France) using two 20-s mixing sequences at a speed of 6,500 rpm. The tubes were cooled on ice for 5 min after each mixing. The tubes were then stored at −80°C, and the extraction procedure was carried out later. After thawing, the content of the tubes was transferred to 15-ml RNase-free centrifugation tubes, and centrifugation was performed for 10 min at 4,000 × *g* and 4°C. The supernatant (approximately 4,600 μl) was transferred to 15-ml tubes containing a gel that improves separation between the aqueous and organic phases (Phase Lock Gel Heavy; Eppendorf, Hamburg, Germany). In some cases, a red layer of approximately 5 mm formed just above the beads. This layer was also transferred. However, the fat layer, which was present at the top of the liquid phase, was not transferred. The tubes were incubated for 5 min at room temperature before the addition of 900 μl (20% of the volume of the TRIzol-cheese mixture) of chloroform. They were then shaken for 15 s, incubated for 3 min at room temperature and for 2 min on ice, and centrifuged for 15 min at 3,500 × *g* and 4°C. The aqueous phase (approximately 2,600 μl) was recovered in 15-ml centrifugation tubes, and 2,600 μl (100% of the aqueous phase volume) of phenol-chloroform-isoamyl alcohol (125/24/1, pH 4.7) was added. Tubes were then shaken for 15 s and centrifuged for 10 min at 3,500 × *g* and 4°C. The aqueous phase (approximately 2,000 μl) was recovered in 15-ml RNase-free centrifugation tubes, taking care not to recover any part of the organic phase, and 1,100 μl of absolute ethanol (corresponding to 55% of the volume of the aqueous phase) was added. Seven hundred microliters of the sample was loaded onto RNeasy spin columns (Qiagen, Courtaboeuf, France), which were then centrifuged for a few seconds at 12,000 × *g* at room temperature. After elimination of the flowthrough, the remainder of the sample was loaded onto the columns and treated in the same way until the entire sample was used. A total of 350 μl of RW1 buffer (Qiagen) was loaded onto the columns. After 5 min of incubation at room temperature, the tubes were centrifuged for a few seconds at 12,000 × *g*. The flowthrough was discarded, and a second washing step with 350 μl of RW1 buffer was performed. Two washing steps were then performed with 500 μl of RPE buffer (Qiagen), and the tubes were subsequently centrifuged for 1 min in order to eliminate all traces of RPE buffer. The RNA was recovered after the addition of 30 μl of RNase-free water, incubation for 2 min at room temperature, and centrifugation for 1 min. Purified RNA was quantified at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The quality of the RNA was analyzed with a 2100 Bioanalyzer (Agilent, Palo Alto, CA) using RNA 6000 NANO chips according to the manufacturer's instructions.

DNase treatment and reverse transcription. The concentration of extracted RNA was adjusted to 125 ng/μl by the addition of RNase-free water, and DNase treatment was performed using the TURBO DNA-free kit (Invitrogen) according to the manufacturer's instructions. Absence of DNA contamination in RNA samples was confirmed with non-reverse-transcribed control samples. Reverse transcription was performed using the SuperScript VILO cDNA synthesis kit (Invitrogen). The reaction mixture contained 500 ng of DNase-treated RNA in a final volume of 20 μl. The reverse transcription procedure was performed according to the manufacturer's recommendations, except that incubation was done for 30 min at 50°C.

Real-time PCR. Oligonucleotide primers were designed using LightCycler probe design software (v1.0; Roche Applied Science, Mannheim, Germany) and synthesized by Eurogentec (Seraing, Belgium). The thermocycling program consisted of initial denaturation at 95°C for 8 min, followed by 45 cycles of denaturation (95°C/10 s), annealing (60°C/7 s), and extension (72°C/6 s). Fluorescence measurement (530 nm) was done at the end of each extension. After real-time PCR, a melting curve analysis was performed by measuring fluorescence during heating from 65 to 95°C at a transition rate of 0.1°C/s. Quantification cycle (C_q) values were determined with LightCycler software (version 3.3) using the second-derivative method. Standard curves were generated by plotting C_q values as a function of the log cDNA concentrations. PCR efficiency (E) was calculated for each primer pair from the slopes of the standard curves using the following formula: $E = 10^{-1/\text{slope}}$ (28). The genes investigated in the present study and the corresponding primer pairs are presented in Table S2 in the supplemental material. Real-time PCR measurements of each sample were performed using 1/10 and 1/20 dilutions of the reverse-transcribed RNA.

Real-time PCR data analysis. The quantities of RNA targets were normalized to the quantities of internal reference genes. The stability of potential reference genes was evaluated by using the geNorm VBA applet for Microsoft Excel (35). This program calculates the gene expression stability measure (M) for a potential reference gene as the average pairwise variation in that gene with respect to all of the other reference genes tested. After the selection of reference genes, the C_q values for each gene of interest were transformed into relative quantities (Q) with a calibrator (cal) sample that, using the gene-specific PCR efficiency (E) value, were calcu-

lated as follows: $Q = E^{(\text{calC}q - \text{sampleC}q)}$. Normalization was then applied by dividing the relative quantities of the genes of interest by the geometric mean of the relative quantities of selected reference genes (normalization factor).

RESULTS

Impact of iron and desferrioxamine B on microorganism growth in a model cheese. Cheese curds were produced using a lactic starter culture and inoculated with *Debaryomyces hansenii* 304, whose main function is to raise the pH, thereby favoring the growth of acido-sensitive ripening bacteria. The aerobic bacteria that were inoculated belong to the genera *Arthrobacter* (seven strains), *Corynebacterium* (five strains), and *Brevibacterium* (seven strains) and correspond to species frequently found in smear-ripened cheeses. Some cheeses were supplemented with ferric chloride or with the siderophore desferrioxamine B, which is a bacterial siderophore produced by the actinobacterium *Streptomyces pilosus* (5).

The counts of *Lactococcus lactis*, of *Debaryomyces hansenii*, and of the ripening bacteria were measured at 12 and 16 days. Addition of iron or desferrioxamine B had no significant effect on the counts of *Debaryomyces hansenii* (see Table S3 in the supplemental material) and only a limited effect on the counts of *Lactococcus lactis* bacteria (see Table S4 in the supplemental material). In addition, in control cheeses that were not inoculated with ripening bacteria, the addition of iron or desferrioxamine B had no effect on the rate of pH increase (results not shown), which confirmed that these compounds did not stimulate *Debaryomyces hansenii*. The concentration of ripening bacteria ranged from 8.0×10^5 to 1.6×10^{10} CFU/g at 12 days and from 2.1×10^7 to 1.9×10^{10} CFU/g at 16 days (Table 1). Except for *Corynebacterium flavescens* Mu128, stimulation resulting from the addition of iron or desferrioxamine B was observed in counts at 12 or 16 days. The stimulation was 50-fold for iron at 12 days (*Arthrobacter arilaitensis* Ep104) and 300-fold for desferrioxamine B at 16 days (*Brevibacterium linens* ATCC 9172) (see Table S5 in the supplemental material). Growth was not monitored after 16 days of ripening because some cheeses became too liquid and passed through the plastic grids.

Expression of genes related to the metabolism of iron during the growth of *Arthrobacter arilaitensis* Re117 in cheese. We measured the expression of genes related to the metabolism of iron for *Arthrobacter arilaitensis* Re117, because its genome sequence is known and stimulation was observed with both iron and desferrioxamine B (10- to 15-fold) after 16 days of ripening. Three separate extractions (technical replicates) of RNA, from one control cheese, from one cheese supplemented with iron, and from one cheese supplemented with desferrioxamine B, were performed. Capillary electrophoresis analyses showed that most of the RNA was of fungal origin because 16S and 23S RNA peaks were low in comparison to the 18S and 26S peaks (see Fig. S1 in the supplemental material).

Eight genes involved in different cellular functions (16S rRNA, *dnaG*, *recA*, *gyrB*, *ftsZ*, *rpoA*, *rpoB*, and *tuf*) were evaluated for their potential as good internal standard controls for gene expression experiments. For the nine samples (three repetitions of three conditions), the stability of expression (M) of the candidate reference genes was measured using the geNorm VBA applet. This method is based on the assumption that two ideal reference genes are expected to have the same expression ratio in all samples, regardless of the experimental conditions. The genes were ranked according to their M values, with the lowest value indicating the greatest stability. All of the genes had M values below 1.5 (between 0.30 and 0.61), which is the default limit defined by Vandesompele et al. (35). A combination of three genes, *ftsZ*, *tuf*, and *rpoB*, was selected since it satisfied the criteria for reliable data normalization, with an average M value of 0.17 (see Fig. S2 in the supplemental material).

Remarkable stability of expression of most of the housekeeping genes, even those that were not used as reference genes, was observed (Table 2). However, the normalized 16S rRNA expression level was higher in the cheeses supplemented with iron (2.57 ± 0.26) or desferrioxamine B (4.00 ± 0.18) than in the control cheeses. This means that compared to the amount of ribosomes, the basic gene transcription activity of protein-encoding sequences was lower in the samples supplemented with iron or desferrioxamine B. One explanation could be that growth had stopped in these samples, in contrast to that in the control sample.

Two clusters of genes involved in the biosynthesis of two different siderophores have been identified in *Arthrobacter arilaitensis* (23). We chose to measure the expression of the three genes belonging to the first cluster (AARI_09550 to AARI_09570), which corresponds to a hydroxamate siderophore, and of four of the genes from the second cluster (AARI_32890 to AARI_32920), which corresponds to a catecholate or mixed catecholate-hydroxamate siderophore. The addition of iron repressed the expression of the three genes involved in the biosynthesis of the hydroxamate siderophore (AARI_09550 to AARI_09570) (Table 2). The normalized expression level was 0.22 for AARI_09570, which means that the abundance of transcripts of this gene was about five times lower in the presence of iron than in the control cheeses. It is likely that this repression occurs via the IdeR (iron-dependent regulator) binding site that is present upstream of AARI_09570. Iron also repressed the four genes (AARI_32890 to AARI_32920) involved in the biosynthesis of the catecholate siderophore, and the values of the normalized expression level for these genes were similar (from 0.29 to 0.37). This repression probably occurs via the IdeR binding site that is present upstream of AARI_32920. The expression of all of the nine substrate binding proteins from iron-siderophore ABC transporters identified in the *Arthrobacter arilaitensis* genome was measured. Iron decreased the expression of four of these genes, namely, AARI_15020, AARI_26370, AARI_32450, and AARI_32790. Interestingly, an IdeR binding site is present upstream of these genes. The expression of the five other genes possibly involved in the transport of iron that were identified during the genome annotation (AARI_16210, AARI_18090, AARI_26120, AARI_29890, and AARI_30460) was constant. Likewise, iron had no effect on the expression of the IdeR regulator and the ferritin-like protein AARI_26600. There was thus a good correlation between repression by iron and the presence of IdeR binding sites upstream of the corresponding genes. The mean of the normalized measured expression level of the 11 genes related to iron metabolism and for which an IdeR binding site is present was 0.33. The mean value obtained for the 11 other genes related to iron metabolism was 1.17. In summary, iron repressed the expression of the genes involved in siderophore biosynthesis and of some of the iron-siderophore ABC transporters.

The addition of desferrioxamine B, a hydroxamate siderophore, had no effect on the expression of the four genes

TABLE 1 Effect of addition of iron or desferrioxamine B on counts of ripening bacteria in model cheeses[a]

Aerobic ripening bacterium	Mean ripening bacterium count (CFU/g) ± SD at:					
	12 days			16 days		
	Control	With iron	With desferrioxamine B	Control	With iron	With desferrioxamine B
A. arilaitensis 3M03	1.7E+09 ± 7.8E+08	**5.9E+09 ± 1.9E+09**	**5.9E+09 ± 1.9E+09**	8.5E+09 ± 3.6E+09	1.3E+10 ± 3.0E+09	1.5E+10 ± 7.7E+09
A. arilaitensis Mu107	1.0E+08 ± 3.9E+07	1.2E+08 ± 6.8E+07	**2.6E+09 ± 9.2E+08**	1.1E+09 ± 3.4E+08	**7.7E+09 ± 3.0E+09**	**3.6E+09 ± 1.0E+09**
A. arilaitensis Ma107	1.9E+08 ± 8.4E+07	**3.8E+09 ± 1.1E+09**	**3.9E+09 ± 1.7E+09**	1.3E+09 ± 4.3E+08	**6.4E+09 ± 2.0E+09**	**5.2E+09 ± 1.6E+09**
A. arilaitensis GMPA29	7.1E+07 ± 2.4E+07	**5.4E+08 ± 2.1E+08**	**1.6E+10 ± 6.9E+09**	2.2E+09 ± 6.5E+08	**8.1E+09 ± 2.3E+09**	**6.0E+09 ± 1.6E+09**
A. arilaitensis 2L34	1.3E+08 ± 4.9E+07	**1.2E+09 ± 4.9E+08**	**2.8E+09 ± 1.2E+09**	7.9E+08 ± 2.6E+08	**6.3E+09 ± 2.7E+09**	**9.5E+09 ± 3.1E+09**
A. arilaitensis Ep104	2.0E+07 ± 7.2E+06	**9.2E+08 ± 1.1E+08**	**1.6E+09 ± 3.4E+08**	4.0E+08 ± 2.4E+08	**5.2E+09 ± 1.7E+09**	**4.0E+09 ± 1.2E+09**
A. arilaitensis Re117	7.3E+07 ± 1.0E+07	**2.3E+08 ± 6.0E+07**	**8.8E+08 ± 1.6E+08**	3.8E+08 ± 1.0E+08	**4.1E+09 ± 1.6E+09**	**5.6E+09 ± 1.1E+09**
C. casei DPC 5298	8.0E+05 ± 3.3E+05	**1.1E+07 ± 4.2E+06**	**4.6E+07 ± 4.1E+06**	1.8E+08 ± 1.0E+08	**8.8E+08 ± 3.2E+08**	**5.9E+08 ± 1.5E+08**
C. casei UCMA 3821	7.8E+07 ± 5.8E+06	**3.5E+08 ± 1.0E+08**	8.5E+07 ± 2.5E+07	8.9E+08 ± 3.2E+08	1.3E+09 ± 5.0E+08	**2.7E+09 ± 6.2E+08**
C. variabile DPC 5310	1.4E+08 ± 2.7E+07	2.8E+08 ± 9.9E+07	**1.6E+09 ± 7.4E+08**	3.2E+08 ± 6.5E+07	8.5E+08 ± 3.0E+08	**4.8E+09 ± 2.2E+09**
C. variabile Mu133	2.4E+08 ± 7.8E+07	5.7E+08 ± 3.4E+08	**5.9E+09 ± 2.0E+09**	4.1E+08 ± 2.4E+08	**2.4E+09 ± 9.2E+08**	**6.1E+09 ± 2.6E+09**
C. flavescens Mu128	8.2E+08 ± 4.6E+08	1.3E+09 ± 6.7E+08	1.4E+09 ± 5.9E+08	2.7E+09 ± 7.4E+08	3.2E+09 ± 9.0E+08	2.3E+09 ± 1.3E+09
B. aurantiacum BLE3	4.1E+07 ± 2.3E+07	**1.6E+08 ± 5.1E+07**	**1.2E+09 ± 4.8E+08**	3.9E+09 ± 3.3E+09	3.7E+09 ± 2.1E+09	4.7E+09 ± 1.9E+09
B. aurantiacum ATCC 9174	5.7E+07 ± 2.4E+07	2.5E+07 ± 1.3E+07	**4.4E+08 ± 1.4E+08**	1.8E+09 ± 1.0E+09	**7.1E+09 ± 2.1E+09**	**1.9E+10 ± 5.6E+09**
B. aurantiacum ATCC 9175	3.5E+08 ± 1.8E+08	7.9E+08 ± 3.9E+08	**2.6E+09 ± 9.9E+08**	3.5E+09 ± 2.0E+09	8.2E+09 ± 2.7E+09	7.2E+09 ± 3.2E+09
B. aurantiacum BA171	3.4E+08 ± 1.8E+08	**1.2E+09 ± 2.1E+08**	**1.1E+09 ± 3.4E+08**	1.1E+10 ± 5.3E+09	1.1E+10 ± 4.7E+09	1.4E+10 ± 4.7E+09
B. aurantiacum 2M23	5.0E+06 ± 3.0E+06	**3.5E+07 ± 4.6E+06**	**3.7E+08 ± 1.4E+08**	5.7E+07 ± 1.9E+07	**2.2E+09 ± 5.8E+08**	**1.2E+10 ± 3.4E+09**
B. antiquum 918	1.1E+08 ± 3.2E+07	**4.5E+08 ± 2.0E+08**	**3.4E+09 ± 1.1E+09**	2.3E+09 ± 6.8E+08	3.6E+09 ± 1.4E+09	**9.3E+09 ± 3.3E+09**
B. linens ATCC 9172	7.7E+06 ± 9.9E+05	1.2E+07 ± 5.0E+06	**1.5E+09 ± 2.7E+08**	2.1E+07 ± 1.2E+07	**2.5E+08 ± 7.4E+07**	**6.1E+09 ± 2.6E+09**

[a] Cheese curds were inoculated with a yeast (*Debaryomyces hansenii*) and different strains of aerobic ripening bacteria. The values are the means of three biological replicates (separate cheeses). Values in bold indicate significant differences (Student's *t* test, $P < 0.05$) between the cheeses supplemented with iron or desferrioxamine B and the corresponding control cheeses.

TABLE 2 Effect of iron or desferrioxamine B (siderophore) addition on the expression of *Arthrobacter arilaitensis* Re117 genes in model cheeses after 16 days of ripening[c]

Gene(s)	Product	IdeR binding site[a]	Mean NEL[b] ± SD	
			Iron	Desferrioxamine B
16S rRNA (AARI_36310, AARI_36340, AARI_36380, AARI_36520, AARI_36580, and AARI_36690)	16S rRNA	No	2.57 ± 0.26	4.00 ± 0.18
Housekeeping genes				
dnaG (AARI_09340)	DNA primase	No	1.51 ± 0.19	2.16 ± 0.13
recA (ARI_11010)	RecA bacterial DNA recombination protein	No	1.24 ± 0.08	1.35 ± 0.04
gyrB (AARI_20200)	Putative DNA gyrase subunit B	No	1.19 ± 0.20	1.52 ± 0.23
ftsZ (AARI_20710)	Cell division protein FtsZ	No	1.01 ± 0.11	1.12 ± 0.06
rpoA (AARI_23210)	DNA-directed RNA polymerase alpha chain	No	0.95 ± 0.27	1.33 ± 0.44
rpoB (AARI_23600)	DNA-directed RNA polymerase beta chain	No	0.94 ± 0.04	0.94 ± 0.01
tuf (AARI_23550)	Elongation factor Tu	No	1.06 ± 0.07	0.95 ± 0.05
Siderophore biosynthesis genes				
Hydroxamate				
AARI_09550	Siderophore biosynthesis protein	Upstream of AARI_09570	0.46 ± 0.10	0.85 ± 0.07
AARI_09560	L-Lysine 6-monooxygenase (NADPH), EC 1.14.13.59	Upstream of AARI_09570	0.29 ± 0.04	0.86 ± 0.19
AARI_09570	Pyridoxal-dependent amino acid decarboxylase	Upstream of AARI_09570	0.22 ± 0.04	0.31 ± 0.08
Catecholate				
AARI_32890	Nonribosomal siderophore peptide synthetase component, EC 2.7.7.58	Upstream of AARI_32920	0.32 ± 0.03	1.44 ± 0.19
AARI_32900	Isochorismate synthase, EC 5.4.4.2	Upstream of AARI_32920	0.31 ± 0.01	1.32 ± 0.07
AARI_32910	2,3-Dihydro-2.3-dihydroxybenzoate dehydrogenase, EC 1.3.1.28	Upstream of AARI_32920	0.29 ± 0.04	1.26 ± 0.09
AARI_32920	Isochorismatase, EC 3.3.2.1	Upstream of AARI_32920	0.37 ± 0.17	1.32 ± 0.15
Iron-siderophore binding protein genes				
AARI_02870	Iron-siderophore ABC transporter, substrate binding protein	No	0.84 ± 0.15	2.10 ± 0.30
AARI_03970	Iron-siderophore ABC transporter, substrate binding protein	No	1.49 ± 0.31	3.99 ± 0.46
AARI_03980	Iron-siderophore ABC transporter, substrate binding protein	No	1.24 ± 0.21	4.45 ± 0.43
AARI_10870	Iron-siderophore ABC transporter, substrate binding protein	No	0.79 ± 0.40	1.22 ± 0.40
AARI_15020	Iron-siderophore ABC transporter, substrate binding protein	Upstream of AARI_15020	0.35 ± 0.03	0.67 ± 0.04
AARI_26370	Iron-siderophore ABC transporter, substrate binding protein	Upstream of AARI_26370	0.28 ± 0.03	0.96 ± 0.02
AARI_30210	Iron-siderophore ABC transporter, substrate binding protein	No	1.85 ± 0.04	1.66 ± 0.17
AARI_32450	Iron-siderophore ABC transporter, substrate binding protein	Upstream of AARI_32450	0.34 ± 0.02	0.74 ± 0.08
AARI_32790	Iron-siderophore ABC transporter, substrate binding protein	Upstream of AARI_32790	0.40 ± 0.03	0.90 ± 0.16
Other possible iron transporters				
AARI_16210	Putative divalent metal ion transporter	No	1.16 ± 0.20	1.57 ± 0.09
AARI_18090	Metal ion transporter	No	1.13 ± 0.14	1.08 ± 0.03
AARI_26120	Iron-manganese-zinc ABC transporter, substrate binding protein	No	0.85 ± 0.123	1.00 ± 0.15
AARI_29890 and AARI_30460 (duplication)	Iron-manganese-zinc ABC transporter, substrate binding protein	No	1.00 ± 0.05	0.85 ± 0.05
Other genes related to iron metabolism				
ideR (AARI_05610)	Iron-dependent repressor IdeR	No	1.14 ± 0.07	1.60 ± 0.03
AARI_26600	Ferritin-like protein	No	1.37 ± 0.48	1.66 ± 0.59

[a] Consensus sequence, TTAGGTTAGGCTAACCTAA; up to five mismatches allowed.

[b] NEL, normalized expression level (expression of the gene under consideration in cheese supplemented with iron or desferrioxamine B divided by its expression in control cheese [calibrator] using *ftsZ*, *tuf*, and *rpoB* as reference genes).

[c] Values are the means obtained from three separate RNA extractions (technical replicates) of a control cheese and a cheese supplemented with 1 mg of iron or 50 μmol of desferrioxamine B per kg.

(AARI_32890 to AARI_32920) involved in the biosynthesis of the catecholate siderophore (Table 2) when expression was measured after 16 days of ripening. However, a lower expression level was observed for AARI_09570, which is the first of the three genes involved in the biosynthesis of the hydroxamate siderophore. No major change was observed in the other genes involved in the metabolism of iron, except for those that encode the iron-siderophore ABC transporter substrate binding proteins, AARI_03970 and AARI_03980, whose abundance increased about four times. It can therefore be hypothesized that these genes are involved in the transport of the iron-desferrioxamine B complexes.

The expression of several genes in the two other biological replicates of cheese samples was also measured. These results confirmed that the addition of iron decreased the level of expression of the genes AARI_32900, AARI_26370, and AARI_32790, which were preceded by IdeR binding sites (see Table S6 in the supplemental material). Higher expression of AARI_03970 in response to desferrioxamine B addition was also confirmed (see Table S7 in the supplemental material).

DISCUSSION

In model cheese experiments, the addition of iron or the siderophore desferrioxamine B stimulated the growth of aerobic ripening bacteria belonging to the genera *Arthrobacter*, *Corynebacterium*, and *Brevibacterium*. The extent of stimulation was strain dependent, and the effect of desferrioxamine B was generally greater than that of iron. We may thus conclude that iron availability is a limiting factor in the growth of typical surface bacteria in cheese. Cheese is a highly iron-restricted medium because bovine milk is low in iron (0.2 to 0.4 mg/liter) (17) and contains lactoferrin, a glycoprotein that has an antibacterial effect due to its ability to chelate iron (19). In addition, because of the presence of oxygen, iron at the surface of cheese is essentially in the oxidized ferric form, Fe^{3+}, which is extremely insoluble, especially at neutral and basic pHs. Diffusional limitations of iron and iron-containing compounds may also exist due to the solid cheese matrix. Moreover, available iron may be sequestered by yeast cells (40), the growth of which precedes that of surface bacteria. During the manufacturing of smear-ripened cheeses, several bacterial strains are always present. They originate from cultures that are deliberately added or from other sources. It is likely that strains that have efficient iron acquisition systems are more competitive than other strains and may thus become dominant. It is also possible that iron acquisition is at the origin of major interactions between the microorganisms that grow at the surface of cheese. In fact, siderophores excreted by one strain may be utilized by another strain, whose growth would then be stimulated (26). In contrast, siderophores may also chelate most of the available iron and, as a result, inhibit the growth of strains that are devoid of the corresponding iron-siderophore transport system (39). The genomes of four aerobic cheese-ripening bacteria (including strain Re117), which all belong to the *Actinomycetales* order, are available in public databases at this time. Interestingly, the number of iron-siderophore transport system components from cheese *Actinomycetales* bacteria (from 20 to 56 genes) is greater than the mean number obtained for other *Actinomycetales* bacteria (Table 3). We suggest that this is due to a strong selective pressure at the cheese surface for strains with efficient iron acquisition systems. Furthermore, cheese *Actinomycetales* bacteria have fewer proteins with iron-sulfur cluster domains (4 to 16 versus 25). One possible explanation is that the low iron availability in cheese promotes the elimination of proteins requiring iron. In *Corynebacterium glutamicum*, it has been shown that several genes encoding iron-containing proteins are repressed under iron limitation (38). It is interesting that lactic acid bacteria of dairy origin have a smaller number of iron-siderophore transport system components than cheese *Actinomycetales* bacteria do (Table 3). In addition, they seem to be devoid of proteins with iron-sulfur cluster domains. It is well-known that lactic acid bacteria have either no or only limited iron requirements (3, 11), which may explain their ability to grow in iron-restricted environments such as milk and the natural gut flora of breast-fed infants (37). However, important energy sources at the surface of cheese such as lipids, amino acids, or lactic acid, which are rarely used by the fermentative lactic acid bacteria, are more effectively catabolized by aerobic microorganisms with iron-requiring respiratory activity. Lactic acid bacteria are thus well adapted to the inner cheese environment, whereas there is probably a selective advantage for aerobic strains with efficient iron acquisition systems at the surface.

The expression of several genes involved in the metabolism of iron by *Arthrobacter arilaitensis* Re117 was measured by real-time reverse transcription-PCR in model cheeses after 16 days of ripening. In the RNA samples recovered from these cheeses, the amount of RNA from Re117 was much lower than that from the yeast *Debaryomyces hansenii* 311. In addition, the Re117 counts in the cheeses that were compared (i.e., control cheese and cheeses supplemented with iron or desferrioxamine B) varied up to 15-fold. Despite this, we were able to find a combination of reference genes that showed remarkable expression stability. This reveals that normalization of real-time reverse transcription-PCR data by a combination of housekeeping genes can be appropriate, even for studies with mixed cultures and with complex media such as cheese. Transcripts of all of the genes that were investigated were detected, showing that the strain was active at the time of sampling. The addition of iron to the model cheeses decreased the expression of the genes involved in siderophore biosynthesis and of some of the iron-siderophore transport components. IdeR binding sites were present upstream of all of the genes that were repressed. This shows that the genes involved in iron acquisition are active during the growth of *Arthrobacter arilaitensis* Re117 in cheese and that when more iron is available, a regulator, probably IdeR (AARI_05610), decreases the transcription of some of these genes. Repression of siderophore biosynthesis genes when iron is present in excess is well documented in the literature (2). It aims to avoid intracellular accumulation of iron to toxic levels and to limit the consumption of energy needed for the synthesis and transport of siderophores. Except for one gene (AARI_09570), the addition of desferrioxamine B did not decrease the expression of the siderophore biosynthesis genes. However, it increased the expression of the genes encoding two iron-siderophore ABC transporter substrate binding proteins (AARI_03970 and AARI_03980). It would be interesting to determine if these proteins are involved in the transport of the iron-desferrioxamine B complexes.

In conclusion, the present study shows that iron availability is a limiting factor in the growth of typical surface bacteria in cheese. The selection of strains with efficient iron acquisition systems may be useful for the development of defined-strain surface cultures. Furthermore, the importance of iron metabolism in the microbial

TABLE 3 Comparison of abundances of ABC-type iron-siderophore transport components and iron-sulfur cluster domains in selected bacterial genomes[d]

Function	Pfam domains or COG groups	No. of genes[a]					
		Aerobic cheese-ripening bacteria				Lactic acid bacteria from dairy products[b]	*Actinomycetales*[c]
		Arthrobacter arilaitensis Re117	*Brevibacterium aurantiacum* ATCC 9174	*Corynebacterium casei* UCMA 3821	*Corynebacterium variabile* DPC 5310		
ABC-type iron-siderophore transport system, ATPase component	COG1120, COG4604	5	4	6	9	0.7	3.2
ABC-type iron-siderophore transport system, permease component	COG0609, COG4605, COG4606, COG4779	14	8	14	14	1.2	5.0
ABC-type iron-siderophore transport system, substrate binding component	COG0614, COG4592, COG4607	9	8	13	33	0.8	4.9
Total no. of putative iron-siderophore transport system components		28	20	33	56	2.7	13.0
Iron-sulfur cluster domains	Pfam00037, Pfam00111, Pfam00142, Pfam00355, Pfam01077, Pfam01799, Pfam04060, Pfam04324, Pfam05484, Pfam10588, Pfam10589, Pfam11575	13	16	4	15	0	25.0

[a] EMBL genome accession numbers: FQ311875 (*Arthrobacter arilaitensis* Re117), CAFW01000001 to CAFW01000106 (*Corynebacterium casei* UCMA 3821), and CP002917 (*Corynebacterium variabile* DPC 5310). The sequence data from *Brevibacterium aurantiacum* ATCC 9174 (formerly *Brevibacterium linens*) were produced by the U.S. Department of Energy Joint Genome Institute (http://genome.jgi-psf.org/breli/breli.home.html) in collaboration with the user community.
[b] Mean values obtained for the 21 genomes of lactic acid bacteria isolated from dairy products present in the Integrated Microbial Genomes (IMG) database (October 2011): *Lactobacillus casei* ATCC 334, Zhang, and BL34; *Lactobacillus delbrueckii* subsp. *bulgaricus* 2038, ATCC 11842, ATCC BAA-365, and ND02; *Lactobacillus helveticus* DPC 4571, DSM 20075, and H10; *Lactobacillus paracasei* ATCC 25302; *Lactococcus lactis* subsp. *cremoris* MG1363, NZ9000, and SK11; *Lactococcus lactis* subsp. *lactis* IL1403 and KF147; *Leuconostoc mesenteroides* subsp. *cremoris* ATCC 19254; and *Streptococcus thermophilus* CNRZ 1066, LMD-9, LMG 18311, and ND03.
[c] Mean values obtained for the 240 genomes belonging to the order *Actinomycetales* present in the IMG database (October 2011).
[d] Assignments were done according to COG (clusters of orthologous groups) or Pfam domains using the IMG system (21).

ecology of cheese should be investigated since it may result in positive or negative microbial interactions.

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