Gene Expression and Biochemical Analysis of Cheese-Ripening Yeasts: Focus on Catabolism of L-Methionine, Lactate, and Lactose[∇][†]

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DNA microarrays of 86 genes from the yeasts *Debaryomyces hansenii*, *Kluyveromyces marxianus*, and *Yarrowia lipolytica* were developed to determine which genes were expressed in a medium mimicking a cheese-ripening environment. These genes were selected for potential involvement in lactose/lactate catabolism and the biosynthesis of sulfur-flavored compounds. Hybridization conditions to follow specifically the expression of homologous genes belonging to different species were set up. The microarray was first validated on pure cultures of each yeast; no interspecies cross-hybridization was observed. Expression patterns of targeted genes were studied in pure cultures of each yeast, as well as in coculture, and compared to biochemical data. As expected, a high expression of the *LAC* genes of *K. marxianus* was observed. This is a yeast that efficiently degrades lactose. Several lactate dehydrogenase-encoding genes were also expressed essentially in *D. hansenii* and *K. marxianus*, which are two efficient deacidifying yeasts in cheese ripening. A set of genes possibly involved in L-methionine catabolism was also used on the array. *Y. lipolytica*, which efficiently assimilates L-methionine, also exhibited a high expression of the *Saccharomyces cerevisiae* orthologs *BAT2* and *ARO8*, which are involved in the L-methionine degradation pathway. Our data provide the first evidence that the use of a multispecies microarray could be a powerful tool to investigate targeted metabolism and possible metabolic interactions between species within microbial cocultures.

Cheese ripening is a complex phenomenon in which the curd undergoes a set of biochemical reactions during the ripening process. Such reactions are catalyzed by the microbial ecosystem living within the cheese matrix—among others, yeasts such as *Debaryomyces hansenii*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, and *Yarrowia lipolytica*, which play key roles in deacidification and the development of flavor compounds (3, 11).

The mechanisms by which yeast growth influences the maturation process are fermentation of lactose, utilization of lactic acid (with consequent pH increase), proteolytic and lipolytic activities, and release of autolysis products (13, 17). However, the contributions of yeasts to cheese flavor development during ripening are generally underestimated, and their roles are generally not well established. They develop at the early stages of ripening (16, 21, 22), when they participate in the deacidification of the curd through lactate/lactose consumption (11), and they could also be involved in flavor compound biosynthesis. Yeasts like *K. lactis*, *K. marxianus*, *Y. lipolytica*, and *D. hansenii* are found in a wide range of cheeses (11, 12, 16, 21, 31) and are expected to play an important role in the ripening. For instance, *K. lactis*, *K. marxianus*, and *D. hansenii* assimilate lactose, whereas a majority of yeast species isolated from cheese efficiently degrade lactate (11, 12). In smear soft cheese, the rise in pH at the cheese surface was found to be related to lactate degradation by *D. hansenii* (4, 21). The same situation

prevails with *K. lactis* or *K. marxianus*, for which lactate degradation coincides with a dramatic increase in pH (4). The presence of yeasts during ripening is essential, since this rise in pH enables the acid-sensitive bacteria that are necessary for the cheese typicity to develop at the cheese surface. Due to the wide catabolic spectrum of *Y. lipolytica*, this yeast could be of interest in cheese making. A comparison of the technological characteristics of *D. hansenii* and *Y. lipolytica* has shown that *Y. lipolytica* was much more proteolytic and lipolytic than *D. hansenii* (30).

It has also been shown that cheese-ripening yeasts such as *Y. lipolytica*, *D. hansenii*, and *K. lactis* could produce volatile sulfur compounds (VSC) through L-methionine catabolism (3, 8, 19). The importance of such compounds in ripened cheese derives mainly from their reactivity and their high volatility at very low concentrations. Also, the involvement of a transamination step in L-methionine catabolism, as well as VSC production, has been demonstrated in *Y. lipolytica* (8) and *K. lactis* (19).

The recent publications of the genomes of some yeasts involved in cheese ripening (15, 28) opened new opportunities to investigate the metabolic capacities of such microorganisms during the making of dairy or other fermented food products. With the availability of whole-genome sequences, DNA microarray analysis offers the potential to monitor and compare the expression patterns of a wide range of mRNA species simultaneously (27). However, whole-genome DNA microarray analysis is generally used to examine differential expression patterns of genes of a single microorganism resulting, for example, from changes in the microbial environment, e.g., a stress effect, or in the microbial phenotype, e.g., biofilm formation (1, 23, 32). Analysis of gene expression is important, since changes in the physiology and metabolism of an organism

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are the consequences of changes in the pattern of gene expression. With the availability of an increasing number of genome sequences from microorganisms of food ecosystems (e.g., dairy products, fermented beverages, and meat), strategies using mixed genome microarrays can be now considered.

Until now, changes occurring during ripening were based essentially on global biochemical data, such as enzymatic activities, consumption of substrates, and biosynthesis of products, but could not fully describe the specific involvement of a given microorganism in the whole process. More precisely, we were not able to know which gene products, related to one protein and/or one function, were expressed in a given microorganism of the cheese ecosystem. The cheese-ripening yeasts *D. hansenii*, *K. marxianus*, and *Y. lipolytica* were used as model organisms of a cheese ecosystem.

The first part of this work was devoted to the design of species-specific oligonucleotide probes and to the validation of microarray data. In the second part, we identified which genes related to L-methionine and lactose/lactate catabolism were expressed in yeast pure cultures and which were expressed in yeast cocultures. The expression profiles of candidate genes were simultaneously followed for the three yeasts cultivated in coculture at three culturing times.

MATERIALS AND METHODS

Yeast strains and storage conditions. Yeast strains used in this work were *Debaryomyces hansenii* 304, *Kluyveromyces marxianus* 44$_{(8)}$ (initially named *K. lactis* 44$_{(8)}$) and *Yarrowia lipolytica* 370. These microorganisms were from our laboratory collection, having originally been isolated from French cheeses. They were selected on the basis of their efficiency in producing volatile sulfur compounds that contribute to the quality of cheeses (3). The microorganisms were stored in 5% glycerol/nonfat dried milk at −80°C.

Culture conditions and media. The yeasts were cultivated in pure cultures or in coculture in 500-ml flasks containing 100 ml of medium. Precultures were cultivated for 2 days in potato dextrose broth (PDB) (Difco Laboratories, Detroit, MI) at 25°C with agitation (150 rpm). The PDB medium was inoculated with 1 ml of thawed stock suspension. These precultures served as inocula (1% vol/vol) for the subsequent cultures. The synthetic cheese medium (SCM) used for all strains was composed (per liter of distilled water) of 1 g yeast extract (Gibco, Heidelberg, Germany), 15 g Bacto Casamino Acids (Difco Laboratories), 38 ml of a 60% sodium lactate stock solution (Prolabo, Fontenay-sous-Bois, France), 0.1 g CaCl$_2$ (Prolabo), 0.5 g MgSO$_4$ 7H$_2$O (Prolabo), 6.8 g KH$_2$PO$_4$ (Sigma-Aldrich, St-Quentin Fallavier, France), 10 g NaCl (Prolabo). After adjusting the pH to 5.5 ± 0.1, the medium was autoclaved (120°C, 20 min) and then supplemented with 20 g · liter^{-1} lactose (Prolabo) and 6 g · liter^{-1} L-methionine (Sigma-Aldrich) prior to inoculation. Cultures were incubated at 25°C (150 rpm) for either 84 h or 120 h. The composition of the SCM was chosen since it corresponds to the amount of substrates (e.g., lactate and lactose) present in the curd of a soft cheese, like Camembert. The L-methionine concentration was chosen since it corresponds to the maximum amount of free L-methionine present in the curd of a soft cheese, like Camembert. This medium was shown to reproduce growth conditions found during ripening (21, 10).

Microbial analyses. Viable cell counts were determined as CFU ml^{-1} following a standard aerobic plate count procedure with yeast extract glucose chloramphenicol agar (Biokar Diagnostics, Paris, France). Surface inoculation was carried out by using a spiral plater (Interconsciences, St-Nom La Bretèche, France) on 120-mm-diameter petri dishes to ensure immediate differentiation of the colonies based on their size, appearance, and pigmentation and to detect any microbial contamination. The dishes were incubated at 25°C, and colonies were counted after 48 to 72 h.

Oligonucleotide probes design. The appropriate design of oligonucleotide probes is crucial to ensure the success of transcriptional analyses. Therefore, one To-mer oligonucleotide probe was designed for each gene of interest, using ROSO software (26). *D. hansenii*, *K. lactis*, and *Y. lipolytica* sequence data were obtained from the Génolevures public database (http://cbi.labri.fr/Genolevures/). Oligonucleotide sequences were searched in the last 300 bases of each gene, with no stable secondary structure and several optimal thermodynamic properties as defined by ROSO (http://pbil.univ-lyon1.fr/roso/Home.php). As a final check, a low-homology search using BLASTN was performed against all genomes to ensure that each probe would not display any cross-hybridization. It should be noted that the *K. lactis* genome sequence was used to design the *K. marxianus* probes, because the two yeasts are closely related. The oligonucleotides used are listed in the supplemental material. They were synthesized by QIAGEN Operon (QIAGEN, Alameda, CA). In addition, six *Arabidopsis thaliana* probes were used as external positive controls and were provided by Stratagene (SpotReport Oligo array validation system; Stratagene, La Jolla, CA).

Oligonucleotide printing. Printing onto Corning UltraGAPS coated slides (gamma amino propyl silane surface; Corning Life Sciences, Corning, NY) was performed at the Transcriptome Biochips Platform in Toulouse using a spotter (VersArray ChipWriter Pro; Bio-Rad) with 12 pins (SMP3; Telechem International, Sunnyvale, CA) in a 3-by-4 format. Each microarray comprises 86 oligonucleotides (30 for *D. hansenii*, 29 for *K. lactis* [*K. marxianus*], and 27 for *Y. lipolytica*) deposited in duplicate and 6 *A. thaliana* species-positive controls replicated four times to generate sufficient data points, giving a total of 196 elements per microarray. The diameter of each spot was approximately 100 μm. After printing, DNA elements were cross-linked to the slides by UV irradiation (Stratalinker UV cross-linker; Stratagene) and stored in a vacuum chamber until use.

Extraction and purification of total RNA. (i) Sample preparation. Cells were centrifuged for 5 min at 8,200 × g and 4°C. The pellets were washed with Tris-EDTA (TE) buffer (1× TE; 10 mM Tris-HCl, 1 mM EDTA [pH 8.0]; Sigma-Aldrich) and then resuspended in 150 μl of 10% *N*-lauroylsarcosine (Sigma-Aldrich) and 1 ml of RNeasy lysis buffer (RLT; QIAGEN, Hilden, Germany)–β-mercaptoethanol (Sigma-Aldrich) (1:0.01). The suspension was mixed by vortex for approximately 3 min, poured into sterile 2-ml Eppendorf tubes (each aliquot containing 800 μl of suspension), and then stored at −80°C or used for the extraction.

(ii) Total RNA isolation. For the RNA extraction, 200 mg of zirconium beads (diameter, 0.1 mm; BioSpec Products, Bartlesville, OK) and 800 μl of RLT-β-mercaptoethanol were added to an aliquot. The mixture was shaken in a Fast-Prep FP120 bead beating system (Bio101, Vista, CA) for 30 s at a machine speed setting of 6.0 m · s^{-1}. Samples were cooled down on ice for 1 min, and the shaking procedure was repeated a second time. Phase separation was carried out after a centrifugation for 5 min at 1,700 × g and 4°C. The aqueous phase was transferred to a fresh tube, and an equal volume of 70% ethanol was added, after which the extraction was performed with an RNeasy Midi kit (QIAGEN), according to the manufacturer's instructions. Total RNA was eluted directly from the RNeasy silica-gel membrane into 500 μl of diethylpyrocarbonate-treated water and immediately precipitated by the addition of 50 μl of 3 M sodium acetate and 400 μl of absolute isopropanol (at 4°C). Tubes were mixed by inversion and placed at −20°C for at least 2 h. The RNA was collected by centrifugation (30 min, 20,800 × g, 4°C), and the pellets were washed twice with 250 μl of cold 70% ethanol, dried for 30 min at room temperature, and resuspended in 50 μl of 1× TE. Samples were then hydrated overnight at 4°C after the addition of 0.5 μl (20 U) of RNase inhibitor (RNasin; Promega, Madison, WI). The RNA integrity was visualized by electrophoresis at 6 V · cm^{-1} with a 1% agarose gel, which was stained with 0.3 μg · ml^{-1} ethidium bromide (Sigma-Aldrich) and photographed under UV light. Quantity and purity were assessed by measurement of the ratios A_{260}:A_{230} and A_{260}:A_{280} by using a spectrophotometer (Beckman DU640B; Beckman Instruments, Fullerton, CA).

Labeling of cDNA targets. External RNA controls (*A. thaliana* mRNA spikes; SpotReport oligo array validation system, Stratagen) were added into total RNA samples (i) prior to cDNA synthesis, to control all of the downstream steps, and (ii) at different concentrations, to cover the entire range of expression levels of mRNAs of interest. mRNA of *A. thaliana* and mRNA of biological samples were then reverse transcribed and simultaneously cyanine 3 labeled with a CyScribe first-strand cDNA labeling kit (Amersham Biosciences, Piscataway, NJ), without any amplification. Reverse transcription-labeling reactions were performed at 42°C for 90 min in a thermocycler (GeneAmp PCR system 9700; Perkin-Elmer Applied Biosystems, Foster City, CA) by direct incorporation of dCTP-Cy3 (Amersham Biosciences) according to the manufacturer's instructions. RNA template and unincorporated fluorescent nucleotides were then eliminated by a chemical treatment (15 min at 37°C with 2 M NaOH). After neutralization with 2 M HEPES (pH 6.8) (Sigma-Aldrich), labeled cDNA was purified on GFX columns (CyScribe GFX purification kit; Amersham Biosciences) and then concentrated using a Microcon YM-30 filter (Millipore, Bedford, MA).

Microarray hybridization and washing. To reduce the nonspecific adsorption of fluorescent probes to the surface, microarray slides were prehybridized by injecting 5 μl of 10 mg · ml^{-1} herring sperm DNA (Promega), previously heated at 95°C for 2 min, and 30 μl of DIGeasy hybridization buffer (Roche Diagnostics

GmbH, Mannheim, Germany) to the slide covered with a 22-by-40-mm coverslip (LifterSlip premium printed cover glass; Erie Scientific Company, Portsmouth, NH). The slide was then put into an individual hybridization chamber (Corning, Avon, France) and immersed in a water bath for 1 h at 60°C. It was then washed in 0.1× SSC (0.15 M NaCl plus 0.015 M sodium citrate) and dried by centrifugation at 150 × g for 3 min at room temperature before hybridization. Labeled targets and 5 μl of herring sperm DNA were heated at 95°C for 5 min for denaturation and then snap-cooled on ice. Twenty microliters of the hybridization buffer was added to the mixture and then injected under a new coverslip. The hybridization chamber was incubated in a 60°C water bath overnight. The slide was then immersed in a solution of 2× SSC, 0.1% sodium dodecyl sulfate (SDS) to remove the coverslip and washed in 2× SSC, 0.1% SDS at 55°C for 5 min and then in 1× SSC at room temperature, followed by a rinse in 0.2× SSC before being dried by centrifugation.

Scanning, and quantification of microarray data. Subsequently, slides were scanned at 532 nm (the wavelength for Cy3 fluorescence) using a robot Scan-Array 4000 (Packard Biosciences, Boston, MA) with 5-μm-pixel resolution. Pictures were generated by using appropriate gains on the photomultiplier tube to obtain the highest signal intensity without saturation. Hybridization signals were analyzed with QuantArray software (Packard BioChip Technologies, Billerica, MA). The mean fluorescence intensity for each spot was quantified, and the expression level of each gene was calculated as the average of two individual hybridizations (duplicate probes for each gene of interest).

Statistical analysis of cDNA microarray experiments. The glass plates contained few genes. The mean fluorescence intensity is, thus, biased by those genes that were most highly expressed and is therefore not a reliable value for the normalization of data. It should be noted that as a consequence, the variance cannot be relied upon either, as it is calculated with the mean value. We decided therefore to work with the median and minimum values under each experimental condition (24). The minimum value was taken as the zero (value). For normalization, the median was used instead of the mean value, and the standard deviation was calculated using the difference between the median and the minimum values. The new (normalized) value of a given gene, j, under a given experimental condition, c, was as follows: $x'_{jc} = (x_{jc} - min_c)/(med_c - min_c)$.

For statistical analyses, we applied the above procedure to the logged values. An analysis of variance (ANOVA) was performed using the GeneANOVA software (14). For each gene, the equation used was as follows: $Yikj = \mu + Ci + Bj + Rk + \epsilon ijk$, where $Yikj$ is the gene intensity; μ is the mean of the intensities of expression measured for the gene; Ci, Bj, and Rk are, respectively, the effects of analyzed "culture time," i (at early [36-h], mid- [72-h], or late [120-h] stationary phase); the biological repetition, j; and the spot replicate on microarray, k; and ϵijk is the residual error. The residual error, ϵijk, includes all the interactions. The threshold of 10% for the *false discovery rate* (FDR) was chosen to select genes whose expression changes in the "time course" are significant (the FDR is the expected proportion of erroneously rejected null hypotheses among the rejected ones) (6, 25).

High-performance liquid chromatography analyses. Culture samples stored at −20°C were thawed at 4°C, centrifuged (2,060 × g; 15 min), and filtered using a polyethersulfone membrane filter (pore size, 0.22 μm; diameter, 33 mm; Dutscher, Brumath, France) before analysis. α-Keto-γ-methylthiobutyric acid (KMTBA) and α-hydroxy-γ-methylthiobutyric acid (HMTBA) contents of the filtrates were determined by high-performance liquid chromatography (HPLC Waters TCM; Waters, Saint Quentin en Yvelines, France) with a cation exchange column (diameter, 7.8 mm; length, 300 mm; Aminex HPX-87H; Bio-Rad, Ivry-Sur-Seine, France) thermostatted at 65°C. The mobile phase was sulfuric acid (0.01 N) dispensed at a 0.6-ml · min⁻¹ flow rate. Detection of compounds of interest was performed with a Waters 486 tunable UV/visible detector regulated at 210 nm. Methionine was analyzed with a reversed-phase column (Symmetry C₁₈; pore size, 100 Å; diameter, 4.6 mm; length, 100 mm; Waters). A gradient of H₂O plus acetonitrile at a flow rate of 0.6 ml · min⁻¹ was applied as follows: 100% H₂O for 2.5 min, 100 to 90% for 0.5 min, 90 to 60% for 7 min, and 60 to 100% for 4 min. UV detection at 210 nm was used. All compounds were quantified from calibration curves established with pure chemicals.

Solid-phase microextraction gas chromatography-mass spectrometry analyses. The VSC production was analyzed by an automatic solid-phase microextraction (SPME) method coupled to a gas chromatograph (Varian CP-3800; Varian, Inc., Walnut Creek, CA) and a single-quadrupole mass spectrophotometer equipped with an impact electronic source (model 1200, Varian, Inc.). Automation of the extraction and injection was achieved with a CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland). Defrosted samples (5 ml) kept at 4°C were preincubated for 2 min at 40°C with agitation at 250 rpm. The extraction was carried out with 100-μm polydimethylsiloxane fiber (Supelco, Bellefonte, PA) for 40 min at 40°C and equal agitation conditions. The sample was injected

by desorption at 250°C for 60 s in splitless mode using the standard Varian split/splitless injector (model 1177, Varian, Inc.). The volatiles were carried onto a nonpolar capillary column (HP-5 mass spectrometry; 30 m by 0.25 mm; 0.25-μm film thickness) swept by helium at a constant flow rate (1.2 ml/min). The compounds were then separated using the following temperature program. First, the temperature was maintained at 15°C for 8 min. Subsequently, the temperature reached 220°C with an increment of 5°C/min. Separated compounds were detected with a mass spectrometry detector. Data were collected in the range of 30 to 400 atomic mass units at a rate of 2 scans/s. Volatile compounds were identified by comparison of their ion chromatograms with those in the NIST/02 Mass Spectral Library (National Institute of Standards and Technology, Gaithersburg, MD). Data were analyzed using Statgraphics Plus software (Statistical Graphics Corp., Englewood Cliffs, NJ). Values are presented as the means ± standard deviations of three replicates.

RESULTS

Choice of candidate genes for the multispecies microarray construction. Our first objective was to select genes possibly involved in the catabolism of lactose, lactate, and L-methionine. Taking *Saccharomyces cerevisiae* as the yeast reference, we therefore collected all the potential genes encoding such specific enzymes from the *Saccharomyces* Genome Database (http://www.yeastgenome.org/). Such genes were then searched for in the full genome of *D. hansenii* CBS767, *K. lactis* CLIB210, and *Y. lipolytica* CLIB122, using the Génolevures public database (http://cbi.labri.fr/Genolevures/). BLAST (2) was used to screen sequence databases for homology. Even distantly related homologs to *S. cerevisiae* genes were selected in order to ensure that potential genes of interest in *D. hansenii*, *K. lactis*, and *Y. lipolytica* were present on the microarray (Table 1). Possible related metabolic pathways were examined using the online service KEGG Pathway database (http://www.genome.ad.jp/kegg/pathway.html).

Growth, substrate assimilation, and metabolite production by yeasts *K. marxianus*, *D. hansenii*, and *Y. lipolytica* cultivated as pure cultures or in cocultures. (i) Growth of microorganisms and pH over time. Growth of the three yeasts cultivated separately or in coculture are shown in Fig. 1. The development of *K. marxianus* started (Fig. 1a) after a 24-h lag phase during which the initial population remained unchanged (approximately 2.10⁴ CFU ml⁻¹). The *K. marxianus* population steadily increased between 30 h and 62 h of culture, reaching around 3.10⁸ CFU ml⁻¹ until the end of the experiment. In contrast, *D. hansenii* and *Y. lipolytica* displayed a continuous and rapid development in the first hours of the cultivation (Fig. 1b and c), increasing from an initial population of almost 2.10⁴ CFU ml⁻¹ to between 4.10⁸ CFU ml⁻¹ and 8.10⁷ CFU ml⁻¹ at 38 h, respectively. Thereafter, *D. hansenii* showed a stationary phase until 80 h, whereas the *Y. lipolytica* population stabilized at 10⁸ CFU ml⁻¹ after 58 h of cultivation. When the strains were cultivated in coculture (Fig. 1d), whereas the development of *Y. lipolytica* did not differ significantly, the maximal populations were significantly decreased (<1 to 1.5 log units) for *D. hansenii* and *K. marxianus* compared to those of pure cultures. However, the initial growth of all yeasts was accelerated when cultivated in coculture. This was particularly clear with *K. marxianus*, for which no lag phase was observed when it was cultivated in coculture with *D. hansenii* and *Y. lipolytica* (Fig. 1d). The initial growth acceleration observed in coculture, compared to that in pure cultures, suggests that there is a competition for growth among the yeasts.

TABLE 1. List of selected genes from *S. cerevisiae* and their respective homologues in *D. hansenii*, *K. lactis*, and *Y. lipolytica*

Gene name	*S. cerevisiae* ORFs	SGD annotation[a]	Yeast type strain and accession no.[b]		
			D. hansenii CBS767	*K. lactis* CLIB210	*Y. lipolytica* CLIB122
LAC4	No orthologue	β-Galactosidase	*DEHA0G26345g*	*KLLA0B14883g*	NMF
LAC12	No orthologue	Lactose permease	*DEHA0G26367g*	*KLLA0B14861g*	NMF
JEN1	*YKL217w*	Lactate transporter	*DEHA0F18843g*	*KLLA0E16313g*	*YALI0D24607g*
			*DEHA0D20427g**	*KLLA0F10043g*	*YALI0D20108g*
			*DEHA0F28809g**		*YALI0C15488g*
					YALI0E32901g
					YALI0C21406g
					YALI0B19470g
CYB2	*YML054c*	Lactate dehydrogenase cytochrome b_2	*DEHA0E01166g*	*KLLA0D02640g*	*YALI0E21307g*
				KLLA0B14795g	
DLD1	*YDL174c*	Lactate ferrocytochrome *c* oxidoreductase	*DEHA0D09658g**	*KLLA0E19789g*	*YALI0E03212g*
				KLLA0D03344g	
DLD2	*YDL178w*	Lactate ferrocytochrome *c* oxidoreductase	*DEHA0F07612g**	*KLLA0B01397g**	NMF
ILV2	*YMR108w*	Acetolactate synthase (catalytic subunit)	*DEHA0G02574g**	*KLLA0B12584g*	*YALI0C00253g*
ILV6	*YCL009c*	Acetolactate synthase (regulatory subunit)	*DEHA0E22869g**	*KLLA0F12364g*	*YALI0C09636g*
PDA1	*YER178w*	E1α subunit of the PDH complex	*DEHA0G13728g**	*KLLA0F12001g*	*YALI0F20702g*
PDB1	*YBR221c*	E1β subunit of the pyruvate dehydrogenase (PDH) complex	*DEHA0C10065g*	*KLLA0F09603g*	*YALI0E27005g*
					*YALI0F05038g**
PDC1	*YLR044c*	Major of three pyruvate decarboxylase isozymes	*DEHA0G19525g**	*KLLA0E16357g*	*YALI0D10131g*
			DEHA0B03784g		
			*DEHA0D18381g**		
PDC6	*YGR087c*	Minor isoform of pyruvate decarboxylase	NMF	NMF	*YALI0D06930g*
THI3	*YDL080c*	Probable decarboxylase	NMF	*KLLA0F16962g*	NMF
ARO10	*YDR380w*	Phenylpyruvate decarboxylase	*DEHA0D11066g**	*KLLA0E02662g*	NMF
BAT1	*YHR208w*	Mitochondrial branched-chain amino acid aminotransferase	NMF	*KLLA0A10307g*	*YALI0D01265g*
BAT2	*YJR148w*	Cytosolic branched-chain amino acid aminotransferase	*DEHA0D07689g**	NMF	*YALI0F19910g*
ARO8	*YGL202w*	Aromatic aminotransferase	*DEHA0A06974g*	*KLLA0F10021g*	*YALI0E20977g*
			*DEHA0A00253g**	*KLLA0A04906g*	
			*DEHA0A00231g**		
ARO9	*YHR137w*	Aromatic aminotransferase	NMF	*KLLA0D11110g*	*YALI0C05258g*
MAP1	*YLR244c*	Methionine aminopeptidase	*DEHA0G13937g**	*KLLA0D18436g*	*YALI0D17138g*
MAP2	*YBL091c*	Methionine aminopeptidase	*DEHA0C04444g*	*KLLA0E06875g*	*YALI0D05159g*
SAM2	*YDR502c*	*S*-Adenosylmethionine synthase	*DEHA0E14751g*	*KLLA0C01782g*	*YALI0B14509g**
SAM4	*YPL273w*	*S*-Adenosylmethionine-homocysteine methyltransferase	*DEHA0A00869g**	*KLLA0D01551g*	*YALI0F25641g**
CYS3	*YAL012w*	Cystathionine γ-lyase	*DEHA0C16863g**	*KLLA0F07909g*	*YALI0F05874g*
CYS4	*YGR155w*	Cystathionine β-synthase	*DEHA0C15708g*	*KLLA0F09317g*	*YALI0E09108g*
STR3	*YGL184c*	Cystathionine β-lyase	*DEHA0A06897g**	*KLLA0C17028g*	*YALI0D00605g**
STR2	*YJR130c*	Cystathionine γ-synthase	*DEHA0D10285g**	*KLLA0B04378g*	NMF
	YLL058w	Hypothetical cystathionine γ-synthase	NMF	NMF	*YALI0D17402g**
	YHR112c	Cystathionine γ-synthase activity	*DEHA0A01144g*	*KLLA0E21406g*	NMF
			DEHA0A01529g		

[a] Annotations from the *Saccharomyces* Genome Database (SGD) (http://www.yeastgenome.org/).
[b] Accession numbers of sequences were obtained from Génolevures (http://cbi.labri.fr/Genolevures/). NMF, no matches found; *, distantly related homolog of *S. cerevisiae* (see putative designations in the supplemental material).

The pH measurements showed that the three yeasts were able to neutralize the medium when cultivated as pure cultures but not when cultivated in coculture. We can reasonably suspect that most metabolites responsible for the neutralization of the medium in yeast pure cultures are catabolized by the yeast coculture. The nature of such metabolites remains to be identified. For *K. marxianus* cultures (Fig. 1a), a rise in pH was observed after 38 h of incubation and reached a plateau (pH 7.0 ± 0.2) after 72 h. *D. hansenii* and *Y. lipolytica* cultures followed similar variation patterns (Fig. 1b and c), although *Y. lipolytica* was the most efficient at increasing the pH (Fig. 1c). For both yeasts, the pH started to increase after 16 h and kept increasing thereafter until the end of the experiment. The rise in pH by *D. hansenii* cultures (Fig. 1b) was slightly slower and

the final pH was lower than in *Y. lipolytica* cultures. In yeast cocultures, the pH started to increase after 16 h and reached a maximum (pH 6.3 ± 0.2) after 50 h (Fig. 1d), then steadily decreased thereafter, to increase again after 95 h of cultivation.

(ii) Lactose and lactate consumption. The initial concentration of lactate was 22.9 ± 0.1 g · liter^{-1} and that of lactose was 18.3 ± 0.3 g · liter^{-1} (Table 2). Most lactose (over 92%) was consumed between 30 h and 62 h in *K. marxianus* cultures, while lactate was hardly consumed during the remaining time. During the stationary phase, 16% of the lactate was consumed, and lactose was completely exhausted at 72 h. In *D. hansenii* cultures, lactose consumption was progressive and continuous: 10% of the lactose was catabolized at 30 h, 34% at 55 h, and over 67% at 72 h. The degradation of the lactate paralleled
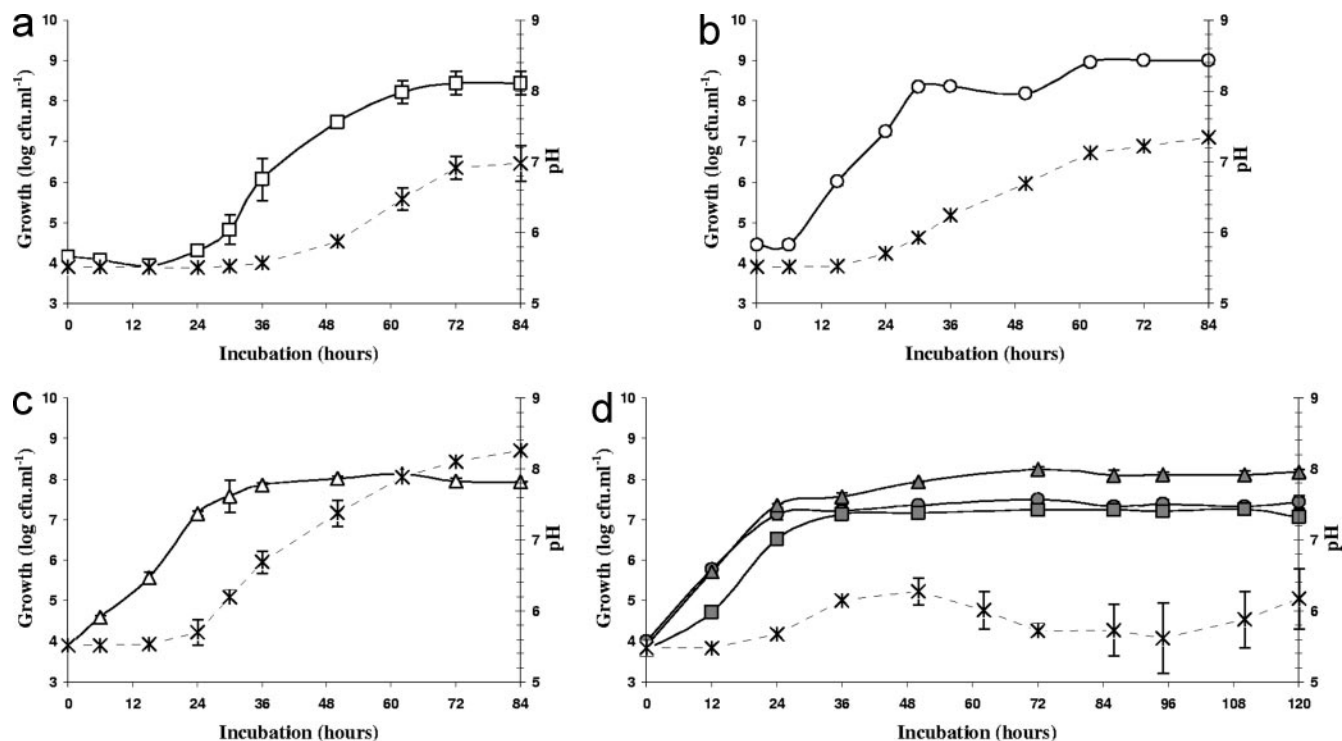
FIG. 1. pH and growth of three yeasts cultivated separately (open symbols) and in coculture (closed symbols) over time. (a) □, *K. marxianus* alone; (b) ○, *D. hansenii* alone; (c) △, *Y. lipolytica* alone; (d) ■, *K. marxianus*; ●, *D. hansenii*; and ▲, *Y. lipolytica* in coculture. ✳, pH levels. Error bars represent standard deviations calculated on the average values of six determinations.

lactose consumption after 30 h. In contrast to the other yeasts, *Y. lipolytica* consumed neither lactose nor lactate in the SCM, and initial concentrations remained unchanged during the 84 h of culture. In cocultures, lactose consumption was progressive, whereas lactate was hardly consumed. Also, the pH did not rise significantly in cocultures (Fig. 1d) compared to that of pure cultures (Fig. 1a, b, and c). Since all yeast species could develop in cocultures (Fig. 1d), this suggests a coculture effect among yeasts. It is possible that metabolites possibly involved in the neutralization process observed in yeast pure cultures are catabolized by the yeast coculture, resulting in no significant change in pH (Fig. 1d).

(iii) L-Methionine consumption and KMTBA and HMTBA biosynthesis. The results obtained showed that the consumption of L-methionine was much more important in *Y. lipolytica* cultures than in others (Table 2). Indeed, while *Y. lipolytica* consumed over 77% of L-methionine within 72 h, only 16% and almost 4% of the initial amount of this amino acid was utilized at this time by *K. marxianus* and *D. hansenii*, respectively (Table 2). In yeast cocultures, 22% of the L-methionine was consumed after 72 h and 32% after 120 h. In parallel to L-methionine degradation, a transient accumulation of the transamination product KMTBA was observed in the yeast coculture and in *Y. lipolytica* culture, although it was much more important in *Y. lipolytica* culture (Table 2). Moreover, HMTBA, which is the reduction product of KMTBA, was detected in *K. marxianus* and *D. hansenii* cultures, as well as in the yeast cocultures. The absence of HMTBA from *Y. lipolytica* cultures indicated that this strain is strongly oxidative.

(iv) Volatile sulfur compound biosynthesis. The production of VSC was measured in the pure cultures of the three yeasts, as well as in yeast cocultures (Table 3). *Y. lipolytica* was by far the most efficient of the yeasts at producing VSC, with dimethyl disulfide being the major sulfur compound produced. This is in agreement with the fact that *Y. lipolytica* can degrade L-methionine most efficiently among the three yeasts (Table 2). The thioester methylthioacetate was produced only by *D. hansenii* and *K. marxianus*. In yeast cocultures, VSC production was lower than in *Y. lipolytica* cultures and surpassed the VSC biosynthesis of the two other yeasts, *D. hansenii* and *K. marxianus*. This suggests that the presence of *Y. lipolytica* promotes VSC production within the yeast coculture.

Gene expression analyses. (i) Multispecies microarray validation with pure cultures. *D. hansenii*, *K. marxianus*, and *Y. lipolytica* were first cultivated separately in the SCM containing lactose, lactate, and L-methionine. Cells were grown to late exponential phase on the basis of kinetic studies performed as described above (Fig. 1), 36 h for *D. hansenii* and *Y. lipolytica* and 55 h for *K. marxianus*, and total RNA isolation was performed.

The presence of homologs on the same microarray increases the risk of cross-hybridizations. Effects of hybridization temperature on the hybridization efficiency and stringency were thus determined. First, assays were made at 42 and 52°C, and cross-hybridizations were observed between sequences of the three yeasts (data not shown). By increasing the hybridization temperature to 60°C, cross-hybridizations were avoided while keeping a high fluorescence signal (Fig. 2). For example, very

TABLE 2. Lactose, lactate, and L-methionine consumption and KMTBA and HMTBA production by *K. marxianus*, *D. hansenii*, and *Y. lipolytica* cultivated in pure cultures and in cocultures[a]

Yeast	Time (h)	% of consumption (g · liter^{-1}) of:			% of production (g · liter^{-1}) of:	
		Lactose	Lactate	L-Methionine	KMTBA	HMTBA
K. marxianus	0	18.4 ± 0.1	22.9 ± 0.1	5.73 ± 0.05	ND	ND
	30	18.3 ± 0.1	22.9 ± 0.1	5.77 ± 0.19	ND	ND
	36	18.2 ± 0.1	22.8 ± 0.2	5.82 ± 0.04	0.01 ± 0.00	0.03 ± 0.00
	55	8.9 ± 0.8	22.6 ± 0.7	5.39 ± 0.15	0.03 ± 0.00	0.09 ± 0.00
	62	1.4 ± 0.1	22.2 ± 0.3	5.05 ± 0.10	0.01 ± 0.00	0.12 ± 0.01
	72	ND	19.6 ± 0.6	4.79 ± 0.11	0.02 ± 0.00	0.16 ± 0.01
	84	ND	18.6 ± 0.7	4.69 ± 0.06	0.02 ± 0.00	0.22 ± 0.02
D. hansenii	0	18.3 ± 0.1	22.9 ± 0.1	5.81 ± 0.02	ND	ND
	30	16.5 ± 0.7	22.7 ± 0.1	5.93 ± 0.38	ND	ND
	36	14.1 ± 0.2	22.4 ± 0.2	5.95 ± 0.60	0.02 ± 0.00	0.04 ± 0.01
	55	12.0 ± 0.0	20.1 ± 0.7	5.86 ± 0.29	0.01 ± 0.00	0.05 ± 0.01
	62	9.6 ± 0.9	19.4 ± 0.4	5.75 ± 0.02	0.02 ± 0.00	0.14 ± 0.06
	72	6.1 ± 0.6	19.4 ± 0.9	5.59 ± 0.12	0.03 ± 0.01	0.32 ± 0.06
	84	4.7 ± 0.5	19.9 ± 0.4	5.26 ± 0.10	0.03 ± 0.01	0.55 ± 0.10
Y. lipolytica	0	18.3 ± 0.1	22.9 ± 0.1	5.87 ± 0.03	ND	ND
	30	18.5 ± 0.1	23.0 ± 0.1	5.75 ± 0.10	0.16 ± 0.01	ND
	36	18.6 ± 0.1	23.1 ± 0.1	5.29 ± 0.07	0.32 ± 0.01	ND
	55	18.6 ± 0.0	23.0 ± 0.1	2.95 ± 0.05	0.51 ± 0.02	ND
	62	18.5 ± 0.1	22.9 ± 0.1	2.10 ± 0.04	0.55 ± 0.01	ND
	72	18.7 ± 0.2	22.9 ± 0.1	1.37 ± 0.07	0.52 ± 0.02	ND
	84	18.6 ± 0.0	22.8 ± 0.1	0.72 ± 0.05	0.46 ± 0.02	ND
K. marxianus, *D. hansenii*, and *Y. lipolytica* in coculture	0	18.3 ± 0.1	22.9 ± 0.1	5.72 ± 0.09	ND	ND
	36	13.8 ± 0.3	23.0 ± 0.3	5.16 ± 0.00	0.13 ± 0.00	ND
	55	10.5 ± 0.3	23.0 ± 0.3	4.86 ± 0.06	0.20 ± 0.01	0.04 ± 0.01
	62	7.2 ± 0.4	22.9 ± 0.2	4.58 ± 0.03	0.27 ± 0.01	0.05 ± 0.01
	72	5.4 ± 0.2	22.9 ± 0.2	4.44 ± 0.06	0.32 ± 0.01	0.08 ± 0.01
	84	2.6 ± 0.1	23.0 ± 0.1	4.34 ± 0.03	0.34 ± 0.02	0.10 ± 0.02
	110	0.3 ± 0.1	22.8 ± 0.2	3.98 ± 0.07	0.44 ± 0.02	0.18 ± 0.03
	120	0.1 ± 0.0	21.8 ± 0.6	3.88 ± 0.03	0.45 ± 0.03	0.20 ± 0.02

[a] Results are the means ± standard deviations of six different experiments. ND, not detected.

low signal intensities were detected with *K. marxianus* strain- and *Y. lipolytica* strain-specific oligonucleotide probes when Cy3-labeled cDNA was prepared from *D. hansenii* mRNA (Fig. 2a).

Parallel analyses were performed with the three pure cultures to test the reproducibility of the methodology (i.e., cDNA

TABLE 3. Production of VSC by *K. marxianus*, *D. hansenii*, and *Y. lipolytica* cultivated as pure cultures or cocultures[a]

Yeast(s)	Time (h)	VSC production (peak area × 10^4 · CFU^{-1})			
		MTL	DMDS	DMTS	MTA
Cultivated as pure cultures					
K. marxianus	72	ND	7 ± 2	2 ± 1	59 ± 8
D. hansenii	72	ND	37 ± 7	4 ± 0	11 ± 0
Y. lipolytica	72	1,905 ± 136	12,575 ± 726	11 ± 2	ND
K. marxianus, *D. hansenii*, and *Y. lipolytica* cultivated in coculture	72	ND	98 ± 17	8 ± 2	ND
	120	ND	523 ± 57	24 ± 9	ND

[a] Results are shown as means ± standard deviations. MTL, methanethiol; DMDS, dimethyl disulfide; DMTS, dimethyl trisulfide; MTA, methylthioacetate; ND, not detected.

synthesis, purification, hybridization, and scanning parameters) when the transcripts of a given species were evaluated from a yeast coculture. As a representative example, signal intensities for *D. hansenii*-specific oligonucleotide probes were compared when Cy3-labeled cDNA was prepared from *D. hansenii* mRNA in pure culture versus signals from an artificial mixed culture with *K. marxianus* and *Y. lipolytica* mRNA. Prior to RNA extraction, the three pure cultures of *D. hansenii*, *K. marxianus*, and *Y. lipolytica* were pooled (1:1:1, vol/vol/vol). All RNAs were extracted, transcribed, and labeled by direct incorporation of dCTP-Cy3. The specific hybridization signals for each species were then compared to those obtained from pure cultures. For *D. hansenii* (Fig. 3), a good reproducibility between arrays was observed, with a correlation coefficient of 0.95. The same experiment was performed for *K. marxianus* and *Y. lipolytica*; in these two cases, a correlation coefficient of at least 0.96 was generated (data not shown).

(ii) Identification of expressed genes in yeast pure cultures. In order to identify genes expressed for each yeast, expression profiling of those genes possibly involved in lactose/lactate and L-methionine catabolism was carried out. The reproducibility of the measurements was confirmed by duplicate biological experiments. Hybridization signals were quantified using Quantarray (Packard BioChip Technologies) software and normalized by the median (see Materials and Methods) value.
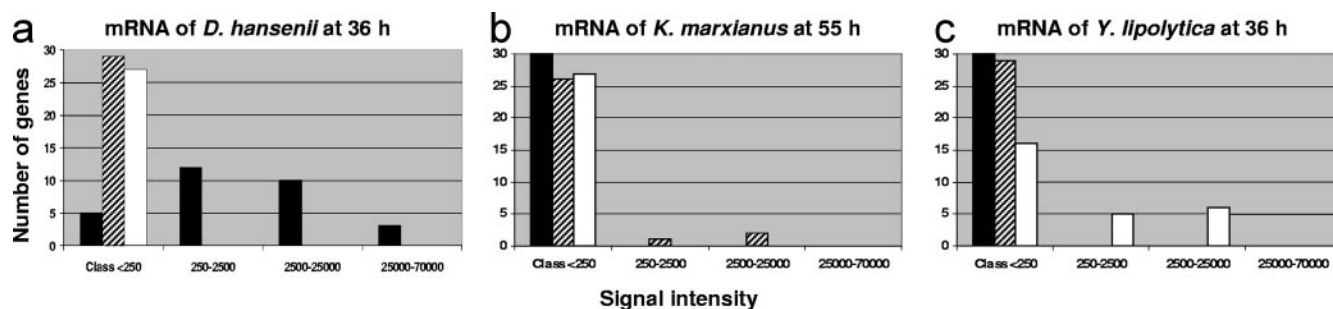
FIG. 2. Distribution of spot intensities (arbitrary units) under different experimental conditions: (a) Cy3-cDNA prepared from *D. hansenii* mRNA; (b) Cy3-cDNA prepared from *K. marxianus* mRNA; (c) Cy3-cDNA prepared from *Y. lipolytica* mRNA. The genes were ranked according to the intensities of the corresponding hybridization signals. The values were then arranged into four sets: Class <250; 250 to 2,500; 2,500 to 25,000; and 25,000 to 70,000, with increasing hybridization values. Black histograms, *D. hansenii* genes. Hatched histograms, *K. marxianus* genes. White histograms, *Y. lipolytica* genes.

Table 4 compares the expression level of each gene to the mean expression of all genes present on the array for a given species and, consequently, gives us biological information about the predominant biological processes in the yeast considered. Expression patterns varied considerably depending on the yeast, which suggests a complementary role for each given yeast during ripening. As expected, lactose catabolism was highly induced in *K. marxianus* as indicated by expression levels of both *LAC4* (β-galactosidase) and *LAC12* (lactose permease), while major genes involved in lactate and pyruvate catabolism were highly expressed in *D. hansenii*. In *Y. lipolytica*, the highest expression levels were reached by *BAT2* and *ARO8*, two genes involved in amino acid catabolism, and *PDC6*, which encodes a pyruvate decarboxylase.

(iii) Gene expression analysis of the yeast coculture. In order to give an insight into the relative importance of each yeast when cultivated in coculture, with respect to lactose/lactate and L-methionine catabolism, gene expression measurements were carried out at three different times, at early (36 h), mid- (72 h), and late (120 h) stationary phases of yeast coculture with the SCM (Table 5). The reproducibility of the measurements was confirmed by triplicate, independent experiments for each time point. Our experimental design included three experimental parameters ("culture time" analyzed, rep-

etition of experiments, and replication of spots on microarray). Statistical analysis was performed with GeneANOVA software (see Materials and Methods). ANOVA showed that the biological factors "gene identity" and "culture time" were responsible for most of the variance. Only genes whose expression levels changed significantly (false discovery rate of <10%) over time are indicated in Table 5. Lactose metabolism was highly induced within 72 h as indicated by high expression levels of the *LAC4* (β-galactosidase) and *LAC12* (lactose permease) genes. This induction was followed by a dramatic decline thereafter (Table 5), which correlates with complete lactose consumption (Table 2). In *Y. lipolytica*, L-methionine catabolism was highly induced during the whole duration of the cultivation, as indicated by the expression levels of *BAT2* and *ARO8*; this corresponds to a gradual accumulation of KMTBA observed in the yeast coculture (Table 2). In cocultures, lactate is less efficiently degraded compared to that in *D. hansenii* pure cultures (Table 2), although major genes involved in lactate/pyruvate catabolism were highly expressed (Table 5). Low expression levels of a *D. hansenii* lactate transporter at 36 to 72 h might explain this phenomenon.

DISCUSSION

In this work, we were able to identify distinct and/or complementary roles of three cheese-ripening yeasts cultivated in pure cultures or in cocultures, with respect to lactose/lactate and L-methionine catabolism.

We have shown that lactose and lactate consumption profiles varied depending on the yeast species considered. Both lactose and lactate were consumed in *K. marxianus* cultures, which is in agreement with the results obtained from analyses of ewe's cheeses (12). Indeed, high mRNA signals of genes encoding β-galactosidase and lactose permease corroborate the high lactose consumption in *K. marxianus* cultures. In yeast cocultures, our results show that both genes were most highly expressed between 36 h and 72 h, which corresponds to a continuous lactose consumption. This is well in agreement with results obtained with *K. lactis* in which *LAC4* and *LAC12* genes were up-regulated in response to lactose addition (29 and references therein). Our data also revealed that the lactate consumption began concomitantly with the pH rise in *K. marxianus* cultures. In *K. marxianus* culture, microarray results
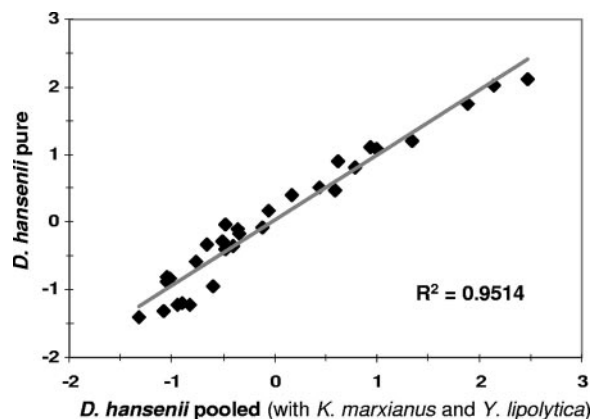

FIG. 3. Scatter plots of the signal intensities obtained from mRNA of *D. hansenii* in pure culture (pure) versus mRNA of *D. hansenii* in artificial mixed culture (pooled) with *K. marxianus* and *Y. lipolytica*.

TABLE 4. Transcript levels of *D. hansenii*, *K. marxianus*, or *Y. lipolytica* genes when yeasts were cultivated separately in SCM containing lactose, lactate, and L-methionine

Gene	Putative function[a]	D. hansenii 304		K. marxianus 44(8)		Y. lipolytica 370	
		Accession no.	Transcript level	Accession no.	Transcript level	Accession no.	Transcript level
LAC4	β-Galactosidase	*DEHA0G26345g*	−0.0	*KLLA0B14883g*	+10.7		
LAC12	Lactose permease	*DEHA0G26367g*	−1.1	*KLLA0B14861g*	+10.3		
JEN1	Lactate transporter	*DEHA0F18843g*	+0.5	*KLLA0E16313g*	−0.0	*YALI0D24607g*	−0.7
		DEHA0D20427g*	−1.2	*KLLA0F10043g*	−0.2	*YALI0D20108g*	−0.7
		DEHA0F28809g*	−1.3			*YALI0C15488g*	−0.2
						YALI0E32901g	−0.6
						YALI0C21406g	−0.5
						YALI0B19470g	−1.0
CYB2	Lactate dehydrogenase cytochrome b_2	*DEHA0E01166g*	+3.2	*KLLA0D02640g*	−1.0	*YALI0E21307g*	+1.1
				KLLA0B14795g	+0.5		
DLD1	Lactate ferricytochrome *c* oxidoreductase	*DEHA0D09658g**	+4.0	*KLLA0E19789g*	−0.2	*YALI0E03212g*	+5.0
				KLLA0D03344g	−0.4		
DLD2	Lactate ferricytochrome *c* oxidoreductase	*DEHA0F07612g**	+0.4	*KLLA0B01397g**	−0.2		
ILV2	Acetolactate synthase (catalytic subunit)	*DEHA0G02574g**	+1.0	*KLLA0B12584g*	−0.7	*YALI0C00253g*	+4.6
ILV6	Acetolactate synthase (regulatory subunit)	*DEHA0E22869g**	+4.0	*KLLA0F12364g*	−0.3	*YALI0C09636g*	+5.3
PDA1	E1α subunit of the PDH complex	*DEHA0G13728g**	+5.9	*KLLA0F12001g*	+0.3	*YALI0F20702g*	+4.9
PDB1	E1β subunit of the PDH complex	*DEHA0C10065g*	+6.1	*KLLA0F09603g*	−0.0	*YALI0E27005g*	+3.6
						YALI0F05038g*	+5.4
PDC1	Major pyruvate decarboxylase isozyme	*DEHA0G19525g**	+7.3	*KLLA0E16357g*	+7.9	*YALI0D10131g*	+5.5
		DEHA0B03784g	+0.5				
		DEHA0D18381g*	+4.3				
PDC6	Minor isoform of pyruvate decarboxylase					*YALI0D06930g*	+6.8
THI3	Probable decarboxylase			*KLLA0F16962g*	−0.4		
ARO10	Phenylpyruvate decarboxylase	*DEHA0D11066g**	+4.4	*KLLA0E02662g*	−0.1		
BAT1	Branched-chain amino acid aminotransferase			*KLLA0A10307g*	−0.6	*YALI0D01265g*	−0.9
BAT2	Branched-chain amino acid aminotransferase	*DEHA0D07689g**	+4.7			*YALI0F19910g*	+6.1
ARO8	Aromatic aminotransferase	*DEHA0A06974g*	+0.6	*KLLA0F10021g*	−0.3	*YALI0E20977g*	+7.3
		DEHA0A00253g*	+1.5	*KLLA0A04906g*	−0.3		
		DEHA0A00231g*	−0.9				
ARO9	Aromatic aminotransferase			*KLLA0D11110g*	−0.2	*YALI0C05258g*	−0.2
MAP1	Methionine aminopeptidase	*DEHA0G13937g**	+2.6	*KLLA0D18436g*	−0.6	*YALI0D17138g*	−0.5
MAP2	Methionine aminopeptidase	*DEHA0C04444g*	+2.9	*KLLA0E06875g*	−0.9	*YALI0D05159g*	−0.9
SAM2	S-Adenosylmethionine synthase	*DEHA0E14751g*	+4.4	*KLLA0C01782g*	−0.6	*YALI0B14509g**	+0.3
SAM4	S-Adenosylmethionine-homocysteine methyltransferase	*DEHA0A00869g**	−1.4	*KLLA0D01551g*	−0.7	*YALI0F25641g**	−0.2
CYS3	Cystathionine γ-lyase	*DEHA0C16863g**	+1.3	*KLLA0F07909g*	−0.6	*YALI0F05874g*	+0.3
CYS4	Cystathionine β-synthase	*DEHA0C15708g*	+1.8	*KLLA0F09317g*	−0.2	*YALI0E09108g*	+0.3
STR3	Cystathionine β-lyase	*DEHA0A06897g**	+0.7	*KLLA0C17028g*	−0.3	*YALI0D00605g**	−0.8
STR2	Cystathionine γ-synthase	*DEHA0D10285g**	+1.0	*KLLA0B04378g*	−0.3		
	Hypothetical cystathionine γ-synthase					*YALI0D17402g**	−0.4
	Cystathionine γ-synthase activity	*DEHA0A01144g*	+0.6	*KLLA0E21406g*	−0.2		
		DEHA0A01529g	−0.9				

[a] Annotations are from Génolevures (http://natchaug.labri.u-bordeaux.fr/Genolevures/index.php).

[b] Yeast cells were grown to late exponential phase (36 h for *D. hansenii* and *Y. lipolytica* and 55 h for *K. marxianus*). It should be noted that the transcript levels of two genes belonging to the same species can be compared but cannot be compared between homologous genes from two different species. *, distantly related homolog of *S. cerevisiae* (see putative designation in the supplemental material).

showed low transcript levels of the open reading frames (ORFs) corresponding to lactate dehydrogenases and lactate transporters; this is in accordance with limited lactate catabolism, since around 16% of lactate was consumed after 72 h. When cultivated in coculture, in which lactate is not extensively degraded, none of the genes involved in lactate catabolism was significantly expressed from *K. marxianus*. In *K. lactis*, only one gene was found to express a pyruvate decarboxylase (Pdc) activity (7). Our microarray results showed that the *K. marxianus PDC1* ortholog mRNA level was highly expressed when this strain was cultivated in pure culture. Also, the relative

transcript levels of the *K. marxianus PDA1* and *PDB1* genes encoding pyruvate dehydrogenases (PDH) were very low.

In *D. hansenii*, although this yeast was able to grow efficiently on lactose (Table 2), the *LAC4* and *LAC12* genes were poorly expressed (Table 4). In contrast, lactate is poorly degraded by *D. hansenii*, but our results also showed that genes putatively involved in lactate and/or pyruvate (e.g., Ldh, Pdh, Pdc, and acetolactate synthase) catabolic pathways were up-regulated in *D. hansenii* under all culture conditions (pure culture or coculture). When yeasts were cultivated in coculture, in which lactate is poorly degraded, major genes involved in lactate

TABLE 5. Significant up- and down-regulated genes with an FDR of <10% over time[a]

Gene	Accession no.[b]	FDR rate at:			Putative function[c]
		36 h	72 h	120 h	
LAC4	KLLA0B14883g	+7.2	+7.1	+0.6	β-Galactosidase
LAC12	KLLA0B14861g	+3.6	+2.4	−1.6	Lactose permease
JEN1	DEHA0F18843g	+2.2	−0.7	+5.0	Lactate transporter
ILV6	DEHA0E22869g*	+5.2	+4.7	+3.4	Acetolactate synthase (regulatory subunit)
PDA1	DEHA0G13728g*	+8.2	+7.2	+5.8	E1α subunit of the PDH complex
PDB1	DEHA0C10065g	+10.1	+8.6	+7.9	E1β subunit of the PDH complex
	YALI0F05038g*	+2.6	+1.9	+6.0	
PDC1	DEHA0D18381g*	+5.9	+6.8	+5.2	Major of three pyruvate decarboxylase isozymes
	DEHA0G19525g*	+8.8	+10.0	+7.0	
	YALI0D10131g	−0.4	+2.5	+1.0	
ARO10	DEHA0D11066g*	+6.0	+5.8	+8.6	Phenylpyruvate decarboxylase
BAT2	YALI0F19910g	+3.8	+6.3	+4.4	Branched-chain amino acid aminotransferase
ARO8	YALI0E20977g	+5.2	+7.5	+6.6	Aromatic aminotransferase

[a] Samples were taken at early (36 h), mid- (72 h), and late (120 h) stationary phase of yeasts cocultured in synthetic cheese medium. The FDR was determined using GeneANOVA software.

[b] *, distantly related homolog of *S. cerevisiae* (see putative designations in the supplemental material).

[c] Annotations are from Génolevures (http://cbi.labri.fr/Genolevures/).

and/or pyruvate catabolism were highly expressed, with the exception of the lactate permease gene *JEN1*. Since *D. hansenii* grows properly in coculture where lactose is efficiently degraded, we can suspect this yeast to degrade lactose by an as-yet-unknown pathway. Alternatively, we can suspect the yeast coculture (i) to produce an intermediate that induces the lactose degradation pathway in *D. hansenii* or (ii) to degrade lactose to an intermediate used by *D. hansenii*. Further work is required to clarify how lactose is degraded by this yeast.

In *Y. lipolytica* pure cultures, lactose and lactate concentrations remained unchanged. Neither *LAC4* nor *LAC12* genes were identified in the *Y. lipolytica* genome. This is in accordance with the natural inability of *Y. lipolytica* to use lactose as a carbon source (5). Moreover, none of the six *JEN1* homolog genes was induced by lactate at the transcriptional level in *Y. lipolytica* cultures. However, the deacidification rate was highest with *Y. lipolytica* cultures. It is clear, therefore, that the pH increase does not depend on the assimilation of lactate in *Y. lipolytica*. From our study, we can propose that this pH increase is due to the release of ammonia during L-methionine catabolism, since this amino acid is efficiently degraded by this yeast. The involvement of aminotransferase(s) in L-methionine catabolism in the cheese-ripening yeasts *Geotrichum candidum*, *D. hansenii*, *K. lactis*, and *Y. lipolytica* has been suggested (3, 9). Two ORFs encoding aromatic aminotransferases were identified in the *Y. lipolytica* genome on the basis of sequence homology with *ARO8* and *ARO9* of *S. cerevisiae*. Moreover, *Y. lipolytica* has two branched-chain aminotransferase genes, one with a mitochondrial targeting signal and one which is cytoplasmic, like *S. cerevisiae* (8). The mitochondrial gene *BAT1* is highly expressed during logarithmic phase and is repressed during stationary phase in *S. cerevisiae*, whereas the cytosolic isoenzyme *BAT2* has the opposite pattern of expression (20). In other species, there is only one branched-chain aminotransferase (Bat), and it has either cytoplasmic features (as in *D. hansenii*) or mitochondrial features (as in *K. lactis*) (8). Our microarray experiments showed high transcriptional expressions of *Y. lipolytica* *ARO8* (Yl*ARO8*) and *BAT2* (Yl*BAT2*), in pure cultures or in coculture with other yeasts, which corre-

lates with the rapid production of KMTBA in *Y. lipolytica* concomitant with the degradation of L-methionine. Interestingly, L-methionine is specifically transported by one high-affinity and two low-affinity permeases in *S. cerevisiae* (18). A homology search against the three genomes of *K. lactis*, *D. hansenii*, and *Y. lipolytica* revealed that they contain several ORFs whose products show extensive sequence similarities to these L-methionine permeases. These ORFs show similarities to the high-affinity L-methionine permeases encoded by *MUP1* (YGR055w). Two other genes carried by *K. lactis* and *D. hansenii* present high similarities with the low-affinity L-methionine permease gene *MUP3* (YHL036w). As a high redundancy of high-affinity L-methionine permeases is observed in the *Y. lipolytica* genome, we can suspect that such L-methionine transporters may give a competitive advantage to *Y. lipolytica*, allowing it to grow better on L-methionine.

In conclusion, multispecies microarrays were successfully employed to identify major genes involved in lactose/lactate and L-methionine catabolism by three cheese-ripening yeasts cultivated in cocultures. We observed good agreement between expressed genes in the array experiments and biochemical data. It provides evidence of the reliability of our metabolic array data. Furthermore, we also found no interspecies cross-hybridization. Our data open up new prospects in the use of tailor-made microarrays to study the simultaneous expression of targeted metabolism processes in several species within a microbial association (e.g., the cheese ecosystem). Such an approach could therefore be developed to investigate functional redundancy and possible interspecies metabolic interactions within complex microbial associations.

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