

Global Regulation of the Response to Sulfur Availability in the Cheese-Related Bacterium *Brevibacterium aurantiacum*^{∇†‡}

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In this study, we combined metabolic reconstruction, growth assays, and metabolome and transcriptome analyses to obtain a global view of the sulfur metabolic network and of the response to sulfur availability in *Brevibacterium aurantiacum*. In agreement with the growth of *B. aurantiacum* in the presence of sulfate and cystine, the metabolic reconstruction showed the presence of a sulfate assimilation pathway, thiolation pathways that produce cysteine (*cysE* and *cysK*) or homocysteine (*metX* and *metY*) from sulfide, at least one gene of the transsulfuration pathway (*accD*), and genes encoding three MetE-type methionine synthases. We also compared the expression profiles of *B. aurantiacum* ATCC 9175 during sulfur starvation or in the presence of sulfate. Under sulfur starvation, 690 genes, including 21 genes involved in sulfur metabolism and 29 genes encoding amino acids and peptide transporters, were differentially expressed. We also investigated changes in pools of sulfur-containing metabolites and in expression profiles after growth in the presence of sulfate, cystine, or methionine plus cystine. The expression of genes involved in sulfate assimilation and cysteine synthesis was repressed in the presence of cystine, whereas the expression of *metX*, *metY*, *metE1*, *metE2*, and *BL613*, encoding a probable cystathionine- γ -synthase, decreased in the presence of methionine. We identified three ABC transporters: two operons encoding transporters were transcribed more strongly during cysteine limitation, and one was transcribed more strongly during methionine depletion. Finally, the expression of genes encoding a methionine γ -lyase (*BL929*) and a methionine transporter (*metPS*) was induced in the presence of methionine in conjunction with a significant increase in volatile sulfur compound production.

Brevibacteriaceae play a major role in the cheese microbial community (36, 40) and may constitute over 5% of the total bacterial counts of cheese surfaces (5). *Brevibacterium linens* and *Brevibacterium aurantiacum* strains have been used for a long time as ripening starters by the cheese industry. These bacteria are used for their ability to produce orange pigmentation and to release a variety of sulfur aromas during cheese ripening and for their inhibitory potential against food pathogens. *B. linens* and *B. aurantiacum* produce volatile sulfur compounds (VSCs), which are key aromas of cheese flavor. The metabolic pathways leading to VSC production have been investigated (1, 2, 14, 15). During the ripening process, amino acids such as methionine are gradually released due to the hydrolysis of casein by lactic acid bacteria (LAB) and yeasts (7,

12). In *Brevibacteriaceae*, VSCs arise from the degradation of methionine to methanethiol (MTL) by a methionine γ -lyase. MTL is then used as a common precursor for a wide variety of VSCs found in cheese, including dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), and *S*-methylthioesters (6). However, little is known about the central sulfur metabolism in *Brevibacteriaceae* (27).

Microorganisms frequently use sulfate for the synthesis of organic sulfur metabolites, mostly cysteine, methionine, and *S*-adenosylmethionine (SAM). The sulfate assimilation pathway involves uptake and activation of inorganic sulfate, followed by a stepwise reduction to sulfite. Sulfite could also be obtained from organic sulfonates via their oxidation by monooxygenases (48). Sulfite is then converted into sulfide by a sulfite reductase (48). An *O*-acetylserine-thiol-lyase, CysK, further catalyzes the reaction of sulfide and *O*-acetylserine (OAS) to generate cysteine (48). Homocysteine could be obtained from *O*-acylhomoserine and cysteine by the transsulfuration pathway via the intermediary formation of cystathionine or directly from *O*-acylhomoserine and sulfide by the thiolation pathway (24). Cystathionine γ -synthase and cystathionine β -lyase are required for transsulfuration, whereas *O*-acylhomoserine thiol-lyase is involved in the thiolation pathway. Homo-

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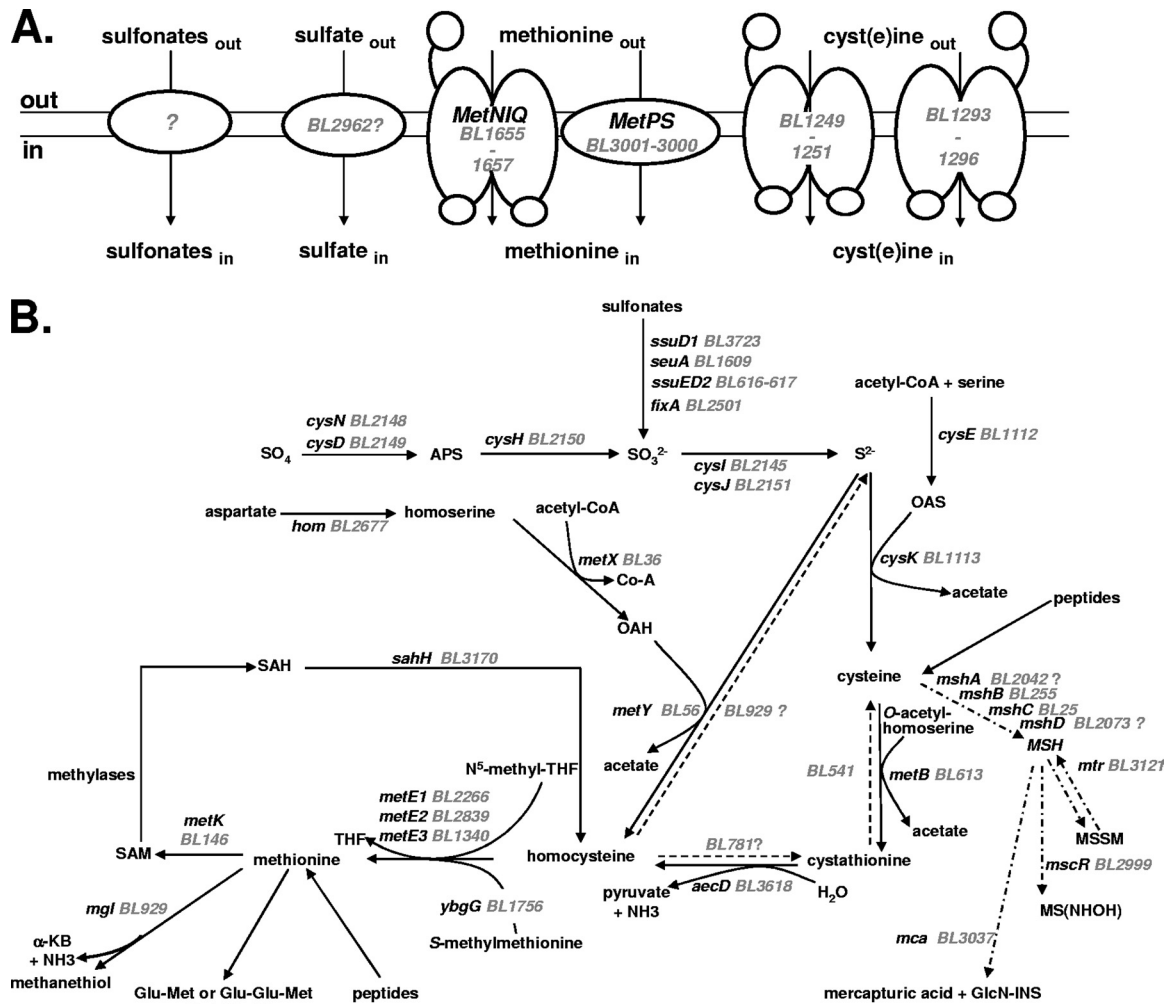


FIG. 1. Sulfur metabolism in *B. aurantiacum*. The BL numbers for *B. aurantiacum* genes correspond to those of Agmlia (<http://genome.jouy.inra.fr/~vloux/publications/forquin08/>) (8). A systematic search for *E. coli*, *B. subtilis*, *C. glutamicum*, and *M. tuberculosis* genes involved in sulfur metabolism was performed. The high-GC-content Gram-positive bacteria *C. glutamicum* and *M. tuberculosis* are phylogenetically related to *Brevibacterium* strains (33, 49). The genes were renamed according to *B. subtilis* and *C. glutamicum* orthologues. "?" indicates a step or a pathway for which the gene proposed remains to be more clearly identified. (A) Transport of sulfur compounds. Methionine transporters (MetNIQ, MetPS), cyst(e)ine transporters (BL1249 to BL1251, BL1293 to BL1296), sulfate transporters and sulfonate transporters are indicated. (B) Central sulfur metabolism. ATP sulfurylase, *cysDN*; APS reductase, *cysH*; sulfite reductase, *cysJI*; serine *O*-acetyltransferase, *cysE*; *O*AS thiol-lyase, *cysK*; homoserine dehydrogenase, *hom*; homoserine acetyl-transferase, *metX*; OAH thiol-lyase, *metY*; cystathionine β -lyase, *aecD*; cystathionine γ -synthase, *metB*; methionine synthase, *metE1-3*; methionine γ -lyase, *mgl*; SAM synthetase, *metK*; SAH hydrolase, *sahH*; alkanesulfonate monooxygenase, *ssuD1-2*; flavin mononucleotide (FMN) reductase, *ssuI*; FMN₂-dependent alkanesulfonate monooxygenase, *seuA*; electron transfer flavoprotein, *fixA*; glycosyltransferase, *mshA*; MSH deacetylase, *mshB*; MSH ligase, *mshC*; MSH synthase, *mshD*; MSH disulfide reductase, *mtr*; NAD/mycothiol-dependent formaldehyde dehydrogenase/nitrosothiol reductase, *mcr*; MSH-S-conjugate amidase, *mca*; Ac-CoA, acetyl coenzyme A; APS, adenylyl sulfate; PP_i, diphosphate; OAS, *O*-acetylserine; OAH, *O*-acetylhomoserine; MSH, mycothiol; MSSM, mycothiol disulfide; GlcN-INS, glucoaminonylinositol; THF, tetrahydrofolate; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; α -KB, α -ketobutyrate.

cysteine is then methylated by methionine synthases to form methionine (28). Methionine is also a sulfur source for several bacteria, indicating an efficient conversion of methionine into cysteine, usually through the SAM recycling pathway and then reverse transsulfuration (Fig. 1). This pathway, present in mammals, yeasts, *Pseudomonas aeruginosa*, and *Bacillus subtilis*, requires the sequential action of a cystathionine β -synthase and a cystathionine γ -lyase (48, 55). Sulfur-containing compounds are also efficiently taken up by specific transporters. Three main families of inorganic sulfate transporters are described (23, 29, 57). Moreover, ABC transporters and a sym-

porter participate in the uptake of cystine in *B. subtilis* and *Escherichia coli* (9). Methionine can be taken up by high-affinity ABC transporters of the MUT family (48) or by a recently characterized MetPS symporter in *Corynebacterium glutamicum* (53). Finally, two different types of ABC transporters, the TauABCD and the SsuABC systems, are involved in sulfonate uptake (26, 48, 54).

The unfinished genome sequence of the *B. aurantiacum* ATCC 9174 strain (20) has been recently released by the Joint Genome Institute (<http://genome.jgi-psf.org/>). In the present study, we performed a reconstruction of sulfur metabolism in

B. aurantiacum. We further analyzed the metabolic and expression profiles of a strain of *B. aurantiacum* after growth under conditions of sulfur starvation or in the presence of various sulfur sources.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *B. aurantiacum* strains ATCC 9174 and ATCC 9175 were used in the present study. These strains were grown in a synthetic medium (SM), adapted from the medium described by Mansour et al. (30), in 100-ml flasks containing 20 ml of medium at 25°C (180 rpm). In this medium, a residual growth in the absence of any added sulfur source was observed. To avoid this problem, a sulfur-depleted medium (SDM) was obtained by growing *B. aurantiacum* ATCC 9175 in a sulfur-free SM, followed by centrifugation (5,000 × g, 10 min, 4°C) and filtration of the supernatant. There was a complete absence of growth of *B. aurantiacum* strain ATCC 9175 in SDM without an added sulfur source. SDM was supplemented with a sulfur source as stated: 500 μM sulfate, 500 μM homocysteine, 500 μM sulfite, 500 μM S-methyl-L-cysteine, 500 μM thiosulfate, 500 μM cystine, or 10 mM methionine. In cow casein, methionine is ~10-fold more abundant than cysteine (21). We then used SDM containing 500 μM L-cystine and 10 mM methionine to mimic the ratio present in casein.

Volatile sulfur compound extraction and analysis. The volatile compounds were analyzed by a dynamic headspace analyzer (Purge and Trap HP 3547A; Agilent Technologies, Garches, France) fitted with a sorbent trap (Tenax) and a cryofocusing module. The concentrator was coupled to a gas chromatograph (GC G1530A; Agilent Technologies) connected to a mass spectrometer detector (MSD 5973; Agilent Technologies). Portions (5 ml) of nonfiltered culture were heated to 60°C and purged for 15 min with high-purity helium at 15 ml min⁻¹. Volatile compounds were concentrated by adsorption to the Tenax trap maintained at 40°C. The trap was heated to 225°C for 2 min to desorb the volatile compounds, which were directly transferred at 150°C to the head of a capillary column and condensed by cryofocusing at -150°C. The condensed volatile compounds were then injected by heating (180°C) and analyzed as previously described (2). The concentrations of each volatile compound were quantified by using a standard curve of pure DMDS (Sigma-Aldrich, Saint Quentin Fallavier, France). Measurements were made in triplicate for each concentration in the noninoculated media, and the concentrations of volatile compounds were expressed as equivalents of DMDS in μg kg⁻¹.

RNA extraction, quantitative real-time reverse transcription-PCR (qRT-PCR), and 5'RACE (rapid amplification of cDNA ends) analysis. Exponentially growing cells (optical density at 600 nm of 5) were collected and broken by shaking in a FastPrep apparatus (Bio101; Qiogene, Illkirch, France). Total RNA was extracted from cells of three independent cultures for each growth condition. The total RNA was then extracted by TRIzol treatment (Invitrogen, Carlsbad, CA), as described previously (22). RNA quality was assessed with an Agilent 2100 Bioanalyzer. After RNA extraction, the cDNAs were synthesized by using the SuperScript III first-strand synthesis system (Invitrogen) according to the manufacturer's recommendations. The primers used are listed in Table S1 in the supplemental material. In each sample, the quantity of cDNA for each gene was normalized to the quantity of the *rpoB* cDNA (35, 52), which is stably expressed in the transcriptome during growth in the presence of sulfate, cystine, and methionine plus cystine. The relative change in gene expression was recorded as the ratio of normalized target concentrations ($\Delta\Delta C_T$). The transcriptional start sites of the *BL613*, *metY*, *BL2267*, *BL929*, *BL3001*, *cysK*, and *cysI* genes were determined by using a 5'/3'RACE kit (Roche, Neuilly-sur-Seine, France) according to the manufacturer's recommendations. RNA preparations extracted from strain ATCC 9175 grown in the presence of various sulfur sources were used as a template. The primers used for 5'RACE analysis are listed in Table S1 in the supplemental material.

Transcriptome analysis. The expression profiles of *B. aurantiacum* ATCC 9175 grown in the presence of various sulfur sources were analyzed by using DNA microarrays (Agilent Technologies). The DNA microarray was described in a previous study (20). RNAs were labeled with either Cy3 or Cy5 fluorescent dye (GE Healthcare, Little Chalfont, United Kingdom) by using a SuperScript Direct cDNA labeling system kit (Invitrogen) according to the manufacturer's recommendations. The levels of Cy3-dCTP and Cy5-dCTP incorporation were quantified by absorbance measurements. Hybridizations were then performed for 17 h at 65°C in dedicated microchambers with 150-pmol portions of the different samples. Array scanning was carried out with a GenePix 4000A dual-channel (635- and 532-nm) laser scanner (GenePix, Sunnyvale, CA) with a resolution of 10 nm per pixel. The laser power was set to 100, and the photo-

multiplier tension was adjusted to between 680 and 800 V according to the average intensity of the hybridization of each slide in order to optimize the dynamic range of measurements. The complete experimental data set was deposited in the GEO database under accession number GSE25422. The analyses were performed twice by swapping the fluorescent dyes, Cy3 and Cy5, to reduce the dye bias and the technical variance. The statistical analysis was based on two dye swaps. For each array, the raw data comprised the logarithm of median feature pixel intensity at wavelengths of 635 nm (red) and 532 nm (green). No background was subtracted. In the following description, log ratio refers to the differential expression between two conditions. It is either log₂ (red/green) or log₂ (green/red) according to the experiment design. An array-by-array normalization was performed to remove systematic biases. First, we excluded spots that were considered to be badly formed features. We then performed a global intensity-dependent normalization with the Loess procedure to correct the dye bias (58). Finally, for each block, the log-ratio median calculated over the values for the entire block was subtracted from each individual log-ratio value to correct print tip effects. To determine differentially expressed genes, we performed a paired *t* test on the log ratios implemented in the Anapuce R package (Tools for Microarray Data Analysis 2010, R package [http://www.R-project.org]; J. Aubert [INRA], Anapuce). The variance was divided between subgroups of genes with homogeneous variance (13). Statistical raw *P* values were adjusted for multiple comparisons by using the Benjamini-Hochberg procedure (4), which controls the false discovery rate after estimation of the proportion of differentially expressed genes by using the smoother method (50). The level of statistical significance was set at 0.05 or 0.1 for all of the comparisons. Each experiment was performed in three biological replicates and two technical replicates.

Extraction of intracellular metabolites and analysis by liquid chromatography coupled to mass spectrometry. Cells of *B. aurantiacum* ATCC 9175 were grown in SDM with various sulfur sources. Exponentially growing cells (10¹⁰ bacteria) were harvested by centrifugation (5,000 × g, 2 min, 4°C). Cells were washed with 20 ml of Milli-Q water to prevent contamination with SDM, centrifuged again (5,000 × g, 2 min, 4°C), and then stored at -80°C. The cells were resuspended in 1 ml of formic acid 1%, incubated for 10 min at 95°C, and centrifuged (5,000 × g, 30 min, 4°C). The supernatant was evaporated to dryness, stored at -80°C, and then resuspended in water containing 0.1% formic acid prior to injection. Chromatographic separation was performed on a Discovery HS-F5 (2.1 by 250 mm, 5 μm) from Supelco Analytical (Interchim, Montluçon, France) by using a Surveyor LC system (Thermo Fisher Scientific, Courtaboeuf, France). Before sample injection (20 μl), the samples were resuspended with 400 μl of formic acid 1% and stored at 4°C in the tray of the autosampler. Separations were carried out using the following gradient at 200 μl min⁻¹: 0 to 3 min, 0% solvent B; 3 to 20 min, from 0 to 100% solvent B; 20 to 25 min, 100% solvent B; and 25 to 45 min, 0% solvent B. Solvent A was water, and solvent B was acetonitrile; both contained 0.1% formic acid. The column temperature was set to 30°C. Mass spectrometric detection was performed with a LTQ/Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific) fitted to an electrospray source operated in the positive and negative ionization modes. The detection was achieved from 75 to 1,000 U at the maximum resolving power of 30,000 (expressed as full width at half-maximum for an ion at 400 U). The mass spectrometer was operated with the capillary voltage at 4 kV and the capillary temperature at 275°C. The sheath gas pressure and the auxiliary gas pressure were set at 45 and 10 arbitrary units, respectively, with nitrogen gas.

Data processing and statistical analyses. Data were processed by using the Xcalibur Qualbrowser module, version 2.0.7 (Thermo Fisher Scientific), and its chemical formula generator was used to provide elemental formulas. Peak detection from liquid chromatography-mass spectrometry chromatograms was performed using XCMS software (47), version 1.14.1, run under R version 2.8.1. The matched filter algorithm was used, and the default values were set for all parameters except for the fwhm, step, steps, mzdiff, mzwid, bw, and snthresh parameters, which were set at 25, 0.01, 3, 0.1, 0.1, 5, and 3, respectively. The areas of the peaks detected were obtained for each sample, using a combination of the retention time and the *m/z* ratio as an identifier. An arbitrary number was assigned to each of these retention time-*m/z* pairs. The data were then combined into a single matrix by aligning peaks with the same mass-retention time pair from each data file in the data set. The XCMS datasets were annotated by using tools developed in-house: the spectral database of the laboratory that contains MS and MS/MS spectra of 300 metabolites and an informatics tool for automatic query of public metabolic and metabolomic databases (i.e., HMDB, KEGG, and METLIN) with the measured accurate masses.

The resulting data matrix was processed by the SIMCA P software (version 12.0; Umetrics, Malmö, Sweden) for multivariate analyses (MVAs). The data were first mean centered and scaled either to unit variance or to Pareto variance and then subjected to principal component analysis (PCA) and projec-

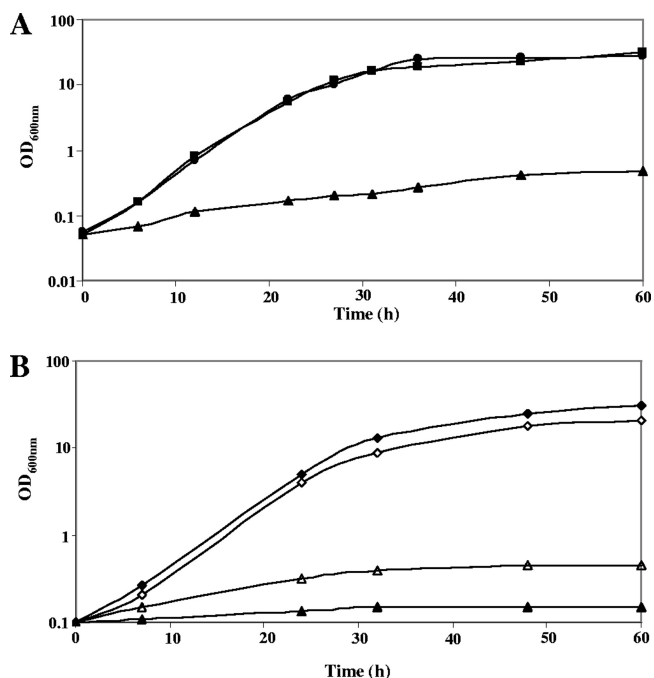


FIG. 2. Growth of *B. aurantiacum* strains in the presence of various sulfur sources. (A) Growth of strain ATCC 9175 in SDM in the presence of various sulfur sources: 10 mM methionine–500 μ M cystine (■), 10 mM methionine (▲), or 500 μ M cystine (●). The growth of strain ATCC 9175 in SDM in the presence of 500 μ M sulfite, 500 μ M homocysteine, 500 μ M cystathionine, and 500 μ M thiosulfate was similar to the growth in the presence of 500 μ M cystine (data not shown). (B) Growth of strains ATCC 9174 (open symbols) and ATCC 9175 (closed symbols) in SDM in the presence of sulfate or sulfonates: 500 μ M sulfate (□, ■) or 500 μ M taurine (△, ▲). The growth of strains ATCC 9175 and ATCC 9174 in SDM in the presence of 500 μ M methanesulfonate or ethanesulfonate was similar to the growth in the presence of 500 μ M taurine (data not shown).

tion on latent structure-discriminant analysis (PLS-DA). PLS-DA models were validated by using the cross-validation function of SIMCA P and were tested with permutation tests ($k = 20$). The relevance of the discriminant variables highlighted by MVAs was confirmed by one-way analysis of variance (ANOVA) performed on the areas of chromatographic peaks. The mean values of each group were then compared to a control group using Dunnett's test (16). Normality was checked prior to ANOVA. P values of <0.05 were considered significant. Calculations were done using GraphPad Prism version 5 software (GraphPad Software, Inc., La Jolla, CA).

RESULTS

Reconstruction of sulfur metabolism in *B. aurantiacum*. We performed a systematic search (Fig. 1) in the *B. aurantiacum* genome of strain ATCC 9174 (formerly *B. linens*) for genes of *E. coli*, *B. subtilis*, *C. glutamicum*, and *Mycobacterium tuberculosis* involved in the assimilation pathways of sulfur-containing compounds. Growth of *B. aurantiacum* ATCC 9174 and ATCC 9175 in SDM with various sulfur sources supported the proposed reconstruction (Fig. 2). Most of the genes of ATCC 9174 involved in sulfur metabolism, with two exceptions, are present in ATCC 9175 (20). Both strains were able to grow on sulfate as the sole sulfur source, an observation in agreement with the presence of a sulfate assimilation pathway (CysIHDN-BL2146-BL2147-CysJ) in the *B. aurantiacum* genome. Interestingly, a

gene encoding an APS kinase, CysC, is absent, indicating that PAPS is probably not produced and that CysH is an APS reductase rather than a PAPS reductase. This three-step pathway for sulfide synthesis has been proposed for *C. glutamicum* and *M. tuberculosis* (41, 45). Strain ATCC 9174 also grew in the presence of several organic sulfonates (taurine, methanesulfonate, and ethanesulfonate), in agreement with the presence of several operons possibly involved in sulfonate assimilation (Fig. 2B). However, sulfonates were poor sulfur sources, since the growth rate and the growth yield of ATCC 9174 in the presence of sulfonates remained low. Interestingly, *B. aurantiacum* ATCC 9175 cannot use taurine, methanesulfonate, or ethanesulfonate, whereas this strain grew similarly to ATCC 9174 with sulfate or cystine (Fig. 2B and data not shown). This result may be correlated with the deletion of the BL615-to-BL621 region containing genes similar to *ssuD* and *ssuE* in the genome of ATCC 9175, as observed by CGH analysis (20). *B. aurantiacum* further utilized sulfide for cysteine synthesis using the OAS thiol-lyase, CysK, and the serine acetyltransferase, CysE (Fig. 1B). Both strains grew in the presence of cysteine or cystine, indicating efficient methionine biosynthesis.

Two main pathways for homocysteine production could be involved: the thiolation pathway, involving direct synthesis of homocysteine from sulfide by an *O*-acetylhomoserine (OAH) thiol-lyase, and the transsulfuration pathway, requiring a cystathionine γ -synthase and a cystathionine β -lyase. Both pathways first required the synthesis of OAH by a homoserine acetyl-transferase encoded by *metX* (Fig. 1B). Cystathionine β -lyase, cystathionine γ -synthase, OAH thiol-lyase, methionine γ -lyase, and cystathionine γ -lyase belong to the same family of pyridoxal-P-dependent enzymes (34). Transcriptomic analyses will therefore be helpful in assigning functions to the four members of this family: BL56, BL541, BL613, and BL929. However, the BL56 protein (MetY) has similarities to the OAH thiol-lyases of *C. glutamicum* (54% identity) and *M. tuberculosis* (61% identity), and contains a 30-amino-acid insertion usually present in OAH thiol-lyases (10). A gene (*aecD*) encoding a cystathionine β -lyase of the PatC family is also present in the genome. Homocysteine is further methylated by MetE-type methionine synthases: BL2266, BL2839, and/or BL1340. Methionine can be converted into homocysteine by the SAM recycling pathway, which requires MetK, the SAM synthase, SAM-dependent methylases, and SahH, the *S*-adenosylhomocysteine (SAH) hydrolase (Fig. 1B). The ATCC 9174 and ATCC 9175 strains grew poorly in the presence of methionine as the sole sulfur source. The doubling time is 24 h in the presence of methionine compared to 3.5 h in the presence of sulfate or cystine (Fig. 2B). In contrast, the growth rate with 500 μ M homocysteine was similar to that observed in the presence of cystine or sulfate (data not shown), indicating the existence of an efficient conversion of homocysteine to cysteine in *B. aurantiacum*.

Regulation of sulfur metabolism in strain ATCC 9175 in response to sulfur availability. To analyze the global response of *B. aurantiacum* to sulfur starvation, we first compared the expression profiles of strain ATCC 9175 after growth in the presence of sulfate or after resuspension of the cells 2 h in the absence of any sulfur source (Table 1). We used strain ATCC 9175, a technological strain with a better cheese matrix colonization capability than the sequenced strain ATCC 9174. A

TABLE 1. Genes related to sulfur metabolism differentially expressed in *B. aurantiacum* ATCC 9175 grown in the presence of cystine, sulfate, or methionine plus cystine or in the absence of any sulfur source

Gene	Function and/or similarity	Ratio and <i>P</i> value ^a							
		S/no sulfur		S/MC		C/MC		S/C	
		Ratio	<i>P</i>	Ratio	<i>P</i>	Ratio	<i>P</i>	Ratio	<i>P</i>
<i>BL146</i>	MetK, SAM synthase	0.34	4.5E-6	—*	2.8E-2				
<i>BL613</i>	MetB, putative cystathionine γ -synthase	0.26	2.8E-7	7.55	1.8E-4				
<i>BL1112</i>	CysE, serine acetyltransferase	0.16	1.1E-12	2.82	2.1E-3			2.37	1.7E-2
<i>BL1113</i>	CysK, OAS thiol-lyase	0.32	2.5E-5	8.65	1.2E-6			3.39	1.8E-5
<i>BL2145</i>	CysJ, sulfite reductase, flavoprotein	0.38	6.1E-6	3.50	4.5E-2			2.86	2.8E-2
<i>BL2147</i>	Uroporphyrin III C-methyltransferase, C terminal	0.47	2.9E-3	4.51	1.7E-3			2.47	8.5E-5
<i>BL2148</i>	CysN, sulfate adenylyltransferase	0.33	5.1E-6	14.21	4.3E-5			3.53	2.41E-5
<i>BL2149</i>	CysD, sulfate adenylyltransferase	0.44	2.5E-3	16.95	1.4E-7			3.38	3.1E-3
<i>BL2150</i>	CysH, phosphoadenosine 5'-phosphosulfate sulfotransferase	0.26	4.5E-7	7.04	1.5E-3			2.79	6.1E-3
<i>BL2151</i>	CysI, sulfite reductase, hemoprotein	0.25	3.0E-6	10.39	3.1E-3			3.58	7.4E-6
<i>BL2839</i>	MetE2, cobalamin-independent methionine synthase	0.30	4.9E-7	4.02	2.2E-2				
<i>BL2146</i>	Unknown function	0.46	7.1E-4	4.51	1.7E-3				
<i>BL1340</i>	MetE3, cobalamin-independent methionine synthase	0.31	1.1E-6						
<i>BL2496</i>	Protein sharing similarity with part of MetH	0.27	9.0E-9						
<i>BL1114</i>	Formylmethionine deformylase	0.39	1.2E-5						
<i>BL3618</i>	AecD, cystathionine β -lyase	0.21	3.4E-10						
<i>BL2478</i>	Glutamate-cysteine ligase	0.18	8.0E-8						
<i>BL3579</i>	IscS, cysteine desulfurase	3.85	1.1E-7						
<i>BL36</i>	MetX, homoserine acetyltransferase	0.50	9.6E-3	5.26	7.4E-3	—†		—‡	
<i>BL56</i>	MetY, OAH thiol-lyase	—**	1.7E-3	18.77	1.9E-4	16.13	8.0E-2	2.2	5.1E-2
<i>BL2266</i>	MetE1, cobalamin-independent methionine synthase	16.10	0.0E+0	11.84	5.5E-10	32.40	5.5E-4	0.45	4.0E-2
<i>BL2267</i>	Unknown function	19.3	0.0E+0	13.8	1.9E-10	32.4	3.5E-2		
<i>BL891</i>	MerR-like regulator			0.10	5.9E-06	0.07	3.6E-2		
<i>BL929</i>	Mgl, methionine γ -lyase			0.06	5.5E-10	0.07	8.2E-2		

^a Abbreviations: S, sulfate; MC, methionine plus cystine; C, cystine. A *P* value of ≤ 0.05 was obtained using a Benjamini-Hochberg (BH) test under the S/no sulfur condition, and a *P* value of ≤ 0.1 was obtained using the BH test under S/MC, S/C, and C/MC conditions, where the difference is > 2 or < 0.5 . *, *BL146* = 1.8 under this condition; **, *BL56* = 0.52 under this condition; †, based on qRT-PCR, the C/MC ratio is 7.0; ‡, based on qRT-PCR, the S/C ratio is 1.6.

total of 690 genes were differentially expressed at least 2-fold in the presence of sulfate compared to sulfur starvation. About 300 and 390 genes were more and less expressed during sulfur starvation, respectively. A total of 113 genes differentially expressed in the presence of sulfate or during sulfur starvation participated in central cellular processes such as transcription, translation, replication, and cell wall and macromolecule degradation or respiration. Fourteen genes related to stress response were differentially expressed. There were 19, 31, 1, 21, 56, and 28 genes involved in nucleotide, carbohydrate, secondary metabolite, lipid/energy, amino acid, and cofactor/vitamin metabolisms, respectively, that were also differentially expressed. In addition, 36 genes encoding regulators were identified: 9 were induced in the presence of sulfate, and 27 were induced during sulfur starvation. Such regulators may help *B. aurantiacum* to adapt to environmental changes.

Finally, 21 genes involved in sulfur metabolism were identified in this analysis (Table 1). During sulfur starvation, we observed an increased expression of genes involved in sulfate assimilation (CysND, CysH, and CysJI), in cysteine biosynthesis from sulfide (CysE and CysK), in methionine synthesis via transsulfuration or thiolation (MetX, BL613, AecD, MetY, and two MetE-like enzymes [BL1340 and BL2839]), and in SAM synthesis (MetK) (Fig. 1B). The expression of *BL2478*, encoding a glutamate-cysteine ligase, also increased during

sulfur depletion. In contrast, only three genes involved in sulfur metabolism were more transcribed in the presence of sulfate: *BL3579*, encoding a cysteine desulfurase that incorporates sulfur in iron-sulfur clusters using cysteine as a donor, as well as *BL2266* and *BL2267*, encoding a MetE-type protein and a protein of unknown function, respectively.

Changes in the metabolome of strain ATCC 9175 grown in the presence of various sulfur sources. To obtain new insights into regulation in response to sulfur availability, we compared the metabolome of strain ATCC 9175 after growth in the presence of sulfate (S), cystine (C), or methionine plus cystine (MC). We first focused our metabolomic analyses on the intermediary compounds of sulfur metabolism. The cysteine remained undetectable under the three growth conditions (Table 2). OAH and cystathionine were only detectable during growth with cystine or sulfate (Table 2). The methionine concentration increased 8- and 11-fold during growth in the presence of methionine plus cystine compared to cystine or sulfate, respectively. The homocysteine concentration similarly increased 5- and 1,000-fold during growth in the presence of methionine plus cystine.

Furthermore, a global approach was performed using automatic peak detection and integration with XCMS software, leading to the detection of 4,335 and 1,225 signals in positive (ESI+) and negative (ESI-) ion detection mode, respectively.

TABLE 2. Sulfur-containing compounds and amino acids detected with the metabolomic analysis

Identification ^a	<i>m/z</i>	Retention time (min)	Detection mode ^b	MS/MS	Ratio ^c		
					MC/S	C/S	MC/C
Target analysis							
Cysteine*	ND ^d	ND	ND	ND	ND	ND	ND
Cystine*	ND	ND	ND	ND	ND	ND	ND
Cystathionine*	222.06797	3.6	+	No	ND in MC	2.2*	ND in MC
Homocysteine*	135.03556	5.1	+	No	1,000*	230*	4.6*
Methionine*	149.05126	9.1	+/-	No	11*	1.4*	8.0*
OAS	ND	ND	ND	ND	ND	ND	ND
OAH	161.06901	6.3	+/-	Yes	0.003*	1.0*	0.003*
Global analysis							
Glutamate*	147.05341	3.7	+/-	No	0.6*	0.8*	0.8*
Isoleucine*	131.09453	11.8	+/-	No	4.2*	1.2*	3.6*
Leucine*	131.09453	12.5	+/-	No	1.2*	1.5*	0.8*
Proline*	115.06344	4.8	+/-	No	1.7*	1.8*	0.9*
Aspartic acid*	133.03754	3.6	+/-	No	1.3*	1.8*	0.7*
Phenylalanine*	165.07909	13.6	+/-	No	1.0	1.2*	0.8*
Tryptophan*	204.08993	17.0	+/-	No	1.0	1.6*	0.6*
Valine*	117.07901	7.6	+/-	No	0.2	1.0*	0.2
Tyrosine*	181.07383	11.8	+/-	No	0.8*	1.9*	0.4*
Ophthalmic acid†	289.12827	5.3	+/-	Yes	0.03*	0.2	0.2
Glu-Met	278.09199	12.3	+/-	Yes	8.3*	2.2*	3.7*
Glu-Met sulfoxide	294.08895	5.2	+/-	Yes	58*	4.7*	13.0*
5-Methylthioadenosine†	297.08831	14.7	+/-	Yes	2.8	1.2*	2.3
Putative mycothiol†	486.15231	5.2	+/-	No	0.3*	1.2*	0.2*
Putative Glu-Glu-Met or Met-Glu-Glu	407.13559	14.6	+/-	No	ND in S	ND in S	7.3*
Putative <i>N</i> -acetylcystathionine	264.07855	11.8	+/-	No	12*	62*	0.2*
Putative sulfur compound 1	378.10787	12.0	+/-	No	ND in S*	ND in S*	3.0*
Putative sulfur compound 2	343.09281	15.3	-	No	5.7*	1.8*	3.3*
Putative sulfur compound 3	266.15407	19.8	-	No	1.0*	0.6*	1.5*
Putative sulfur compound 4	256.05309	7.0	-	No	0.4*	0.6*	0.7*
Putative sulfur compound 5	424.13834	10.9	+	No	83*	1.3*	63*
Putative sulfur compound 6	345.11127	14.0	+	No	0.1*	8.6*	0.01*

^a *, compounds identified on the basis of the similarity of the retention time, accurate mass, and mass spectrum compared to those of an authentic standard; †, compounds characterized on the basis of the elemental formula provided by accurate mass measurements, by the presence of sulfur in the molecule assessed by the isotopic pattern observed in the mass spectra, and by the interpretation of the fragmentation spectra (for MTA and mycothiol).

^b +, signal in positive (ESI+) ion detection mode; -, signal in negative (ESI-) ion detection mode.

^c MC, methionine plus cystine; C, cystine; S, sulfate. *P* values of <0.05 are indicated by an asterisk.

^d ND, not detected.

The score plot obtained from PCA exhibited a clear separation between our three conditions, and more particularly in the presence of methionine, suggesting that the sulfur source was the main source of variance. The metabolites of biological relevance are listed in Table 2. Some of them were identified on the basis of the similarity of their retention time, accurate mass, and multistage tandem mass spectrometry (MSn) spectra, compared to those of standards. The other metabolites were annotated according to the definition established by the Metabolomics Standard Initiative (51): the matching was based on the elemental formula obtained from accurate mass measurements or on the basis of the interpretation of fragmentation spectra. This analysis highlighted the presence of 23 putative dipeptides and tripeptides in *B. aurantiacum* cells (data not shown). Among them, we identified a Glu-Met dipeptide, its oxidized form, Glu-methionine sulfoxide, and a tripeptide (Glu-Glu-Met or Met-Glu-Glu). The global analysis also strongly suggested the presence of mycothiol, 5-methylthioadenosine (MTA) and ophthalmic acid, a tripeptide analogue of glutathione in which the cysteine group is replaced by L-2-aminobutyrate (Table 2 and see Fig. S1 in the supplemental material). The levels of

mycothiol and ophthalmic acid decreased under MC conditions, whereas those of MTA increased. A pathway for the biosynthesis of mycothiol (*mshABCD*) and a gene encoding a mycothiol disulfide reductase (*mtr*) are found in the *B. aurantiacum* genome (Fig. 1B). The mycothiol present in *Actinomyetales* protects cells against oxidative stress. MTA might be a by-product of spermidine synthesis. However, a SpeE protein is present in the genome of *B. aurantiacum* ATCC 9174, but we were unable to identify a SpeD protein, a less-conserved protein (46). Finally, seven putative sulfur-containing compounds were detected on the basis of their isotopic patterns with the presence of the sulfur-34 ion (Table 2). Using metabolomic databases, we identified one of these compounds as *N*-acetylcystathionine. Its concentration increased 62- and 5-fold in the presence of cystine compared to sulfate or methionine plus cystine. We also observed an impact of the sulfur source on the concentrations of isoleucine, valine, or glutamate. The decreased levels of the latter under MC conditions could be explained by its use for the synthesis of glutamyl-containing peptides, which accumulated under these conditions (see Fig. S1 in the supplemental material).

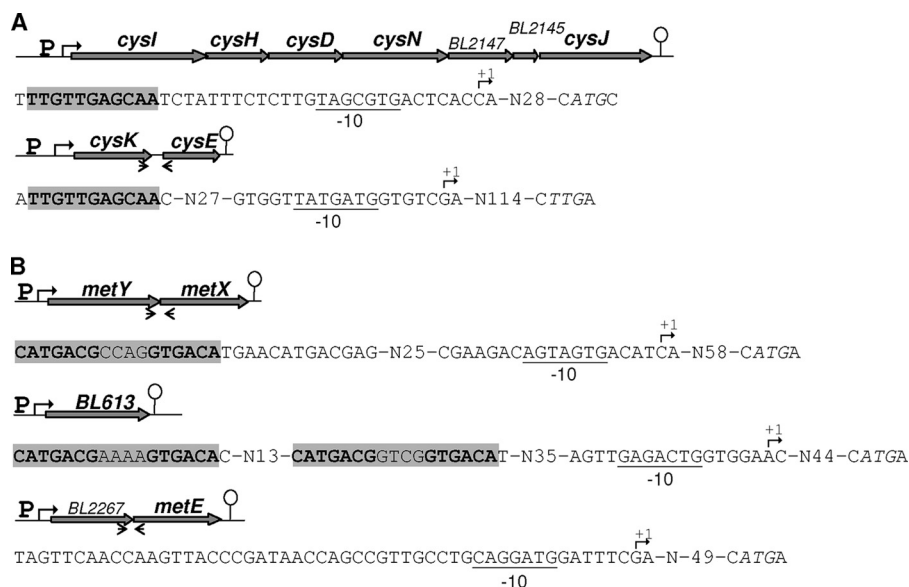


FIG. 3. Genetic organization of operons involved in sulfate assimilation or cysteine biosynthesis (A) and in methionine biosynthesis (B). A bent arrow indicates the experimental transcriptional start site. The potential -10 boxes for the promoters are underlined. The translational start codon is indicated in italics. The gray boxes correspond to conserved motifs. The terminators are represented by a loop. The positions of the primers used for the determination of the operon by RT-PCR are indicated by arrows.

Comparison of the expression profiles after the growth of strain ATCC 9175 in the presence of various sulfur sources.

After the growth of *B. aurantiacum* ATCC 9175 in the presence of sulfate, cystine, or cystine plus methionine, we observed major changes in the pools of sulfur-containing compounds that may influence the transcription of genes involved in sulfur metabolism. We then compared the expression profiles of strain ATCC 9175 grown under these conditions (Table 1 and data not shown). Totals of 15 and 30 genes were differentially expressed after the growth of strain ATCC 9175 in the presence of sulfate compared to growth in the presence of cystine or methionine plus cystine, respectively. The expression profiles of 15 genes also differed when we compared growth with cystine and growth with cystine plus methionine. Most of the genes differentially expressed participate in sulfur metabolism. However, the expression of genes encoding a peptidase (BL978), a putative carboxymuconolactone decarboxylase (BL1185), and a putative monooxygenase (BL1738), as well as proteins of unknown function, changed in S or MC conditions.

Sulfate assimilation and cysteine biosynthesis from sulfide are coordinately regulated. A first set of genes was derepressed during sulfur starvation and was more expressed in strain ATCC 9175 grown in the presence of sulfate than in the presence of cystine or methionine-cystine (Table 1). The *cysN*, *cysD*, *cysH*, *cysI*, *cysJ*, *cysE*, and *cysK* genes were clearly transcribed more weakly in the presence of cystine. The *metY* gene encoding the OAH thiol-lyase that also uses sulfide as a substrate was similarly regulated. However, *metY* expression also responded to methionine availability, indicating a pattern of expression different from that of the *cys* genes. The genes involved in sulfate assimilation (*cysIHDN-BL2146-BL2147-cysJ*) and in cysteine biosynthesis (*cysKE*) are organized into two operons (Fig. 3A). We then determined the transcriptional start site of these operons by 5' RACE. The transcription of the

sulfate assimilation operon is initiated 32 bp upstream of the *cysI* start codon, whereas that of the *cysKE* operon is initiated 117 bp upstream of the *cysK* start codon (Fig. 3A). Upstream of these transcriptional start sites, we did not identify the TATAAT and TTGACA sequences characteristic of promoters in *E. coli* and *B. subtilis*. A TAN₃TG motif that shares similarities with part of the consensus of the -10 region of *C. glutamicum* [tgngnTA(c/t)aaTgg] (uppercase letters indicate highly conserved; lowercase indicates poorly conserved) (37) is present upstream of the transcriptional start site of *cysK* and *cysJ* (Fig. 3A).

Regulation of methionine biosynthesis and catabolism in response to methionine availability. Comparison of the expression profiles of strain ATCC 9175 in the presence of cystine or cystine-methionine revealed a few genes specifically regulated in response to methionine availability (Table 1). These genes probably participated in either methionine biosynthesis or degradation. Only two adjacent genes, *BL929* (encoding a putative methionine γ -lyase) and *BL891* (encoding a regulator), were upregulated in the presence of methionine (Table 1). We demonstrated by RT-PCR the existence of a common transcript for these genes (data not shown). Surprisingly, the transcription of the *BL929* operon is initiated at the ATG start codon of *BL929* (Fig. 4A), as observed for several genes in actinomycetes (37). Accordingly, no RBS is present upstream of the ATG of *BL929*. Methionine is usually regarded as the VSC precursor. We thus measured the total production of VSCs after the growth of strain ATCC 9175 in the presence of various sulfur sources (Table 3). This strain did not produce VSCs in the presence of cystine or sulfate. Large amounts of VSCs were detected when methionine was added to the SDM containing cystine. DMDS and DMTS were the major VSCs produced under these conditions, with 643.5 ppb and 201.6 ppb, respectively.

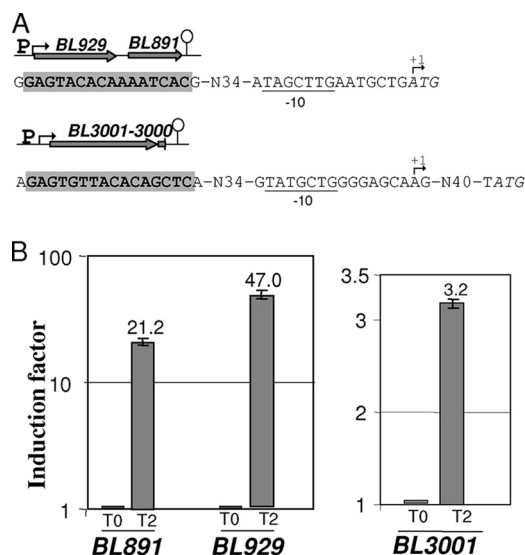


FIG. 4. Operons induced in the presence of methionine. (A) Genetic organization of the *BL929-to-BL891* and *BL3000-BL3001* operons. A bent arrow indicates the transcriptional start site. The potential -10 boxes of the promoters are underlined. The start codons of *BL929* and *BL3001* are indicated in italics. The gray box corresponds to a conserved motif. The terminator is represented by a loop. (B) qRT-PCR analysis of *BL929*, *BL891*, and *BL3001* expression after the addition of methionine. The $2^{-\Delta\Delta CT}$ method was applied to calculate the relative amount of cDNAs of interest. Methionine (10 mM) was added to a culture of strain ATCC 9175 in SDM containing 500 μ M cystine, and the cells were then harvested before (T0) and 2 h (T2) after methionine addition (induction factor = 1 at T0). This experiment was performed in three biological replicates.

The expression of *metY*, encoding the OAH thiol-lyase, and of *BL2266* (*metE1*), encoding a methionine synthase, was 16- and 32-fold repressed, respectively, when methionine was added to the medium (Table 1). This was also the case for *BL2267*, encoding a protein of unknown function, which is adjacent to *metE1*. Using RT-PCR, we demonstrated that *BL2267* and *BL2266*, as well as *metY* and *metX*, form operons (Fig. 3B). The methionine-dependent repression of these operons suggested that MetY, MetX, and MetE1 play an important role in methionine biosynthesis in *B. aurantiacum*. The function of *BL613* and *BL541*, which share similarities with MetY and *BL929*, remains unclear. The expression of *BL613* was higher in the presence of sulfate than in the presence of methionine plus cystine. A 6-fold repression was also observed in qRT-PCR, 2 h after methionine addition to a medium containing cystine (see Table S2 in the supplemental material). Finally, *BL613* expression decreased 30-fold in the presence of homocysteine compared to sulfate or cystine, suggesting that *BL613* could participate in methionine synthesis via transsulfuration. In contrast, *BL541* expression was not modulated under any of the conditions tested.

Regulation of transporter synthesis in response to sulfur, methionine, or cystine starvation. Genes encoding several transporters were differentially expressed in the presence of sulfate compared to sulfur limitation (Table 4). Eleven peptide transporters are present in *B. aurantiacum*. Among them, the expression of an operon encoding an oligopeptide ABC transporter (*BL1734* to *BL1737*) increased 5-fold during sulfur depletion. In contrast, other genes encoding ABC transporters

for oligopeptides (*BL250* to *BL253*, *BL634* to *BL637*, *BL2377*, and *BL2733*) or a dipeptide/tripeptide transporter (*BL1264*) were expressed more during growth with sulfate. Interestingly, the expression of five genes encoding proteases (*AprE/BL4033*, *BL2348*, and *BL494*) or peptidases (*BL1078* and *BL1007*) also increased 2- to 4-fold in the presence of sulfate. These enzymes may contribute to the production or degradation of peptides.

Genes encoding two amino acid ABC transporters (*BL1657* and *BL1293-BL1296*) were transcribed stronger during growth with sulfate (Table 4). *BL1655* to *BL1657* and *BL1293* to *BL1296* share similarities with MetNPQ, a high-affinity ABC transporter of *B. subtilis* (53, 35, and 36% identities) (48) and a cystine ABC transporter of *E. coli* (48, 31, 33, and 26% identities), respectively. Other putative cystine or methionine transporters are present in the *B. aurantiacum* genome. The *BL1249-BL1250-BL1251* proteins correspond to an ABC transporter that shares similarities with *BL1293* to *BL1296*. *BL3001* and *BL3000* are 48 and 30% identical to MetPS, a low-affinity methionine transporter of *C. glutamicum* (53). We tested the effect of cysteine or methionine limitation on their expression. Strain ATCC 9175 was first grown in the presence of methionine plus cystine and then resuspended either in the presence of cysteine alone (methionine depletion) or in the presence of methionine alone (cysteine depletion). The expression of *BL1250* and *BL1296* increased 2.1- and 3.2-fold, respectively, during cysteine limitation (see Table S2 in the supplemental material). Both ABC transporters (*BL1249* to *BL1251* and *BL1293* to *BL1296*) probably constitute uptake systems for cystine and/or cysteine (Fig. 1A).

During methionine limitation, *BL1657* gene expression increased 25-fold in agreement with a key role of the *BL1655-1657* ABC transporter for methionine supply. In contrast, the *BL3001* expression did not change during methionine starvation but increased 3.2-fold when methionine was added. Interestingly, the expression of *BL929* encoding a probable methionine γ -lyase also increased in MC medium. An additional qRT-PCR analysis was performed to analyze the induction of the *BL929-to-BL891* and *BL3001-BL3000* operons. The transcription of *BL891*, *BL929*, and *BL3001* increased 21-, 47-, and 3-fold, respectively, 2 h after the addition of 10 mM methionine in SDM containing cystine (Fig. 4B), indicating a methionine-specific induction.

TABLE 3. Specific production of volatile sulfur compounds by *B. aurantiacum* ATCC 9175 in SDM with different sulfur sources^a

Volatile sulfur compound	Mean sulfur compound concn (μ g kg ⁻¹) \pm SD		
	MC	C	S
Methanethiol	1.04 \pm 0.40	ND	ND
DMDS	643.51 \pm 136.35	0.70 \pm 0.06	0.23 \pm 0.05
2,4-Dithiapentane	0.08 \pm 0.05	ND	ND
DMTS	201.63 \pm 50.45	0.05 \pm 0.02	ND
DMQS	0.59 \pm 0.31	ND	ND

^a ND, not detected. Abbreviations: MC, methionine plus cystine (10 mM L-methionine plus 500 μ M L-cystine); C, cystine (500 μ M L-cystine); S, sulfate (500 μ M sulfate); DMDS, dimethyl disulfide; DMTS, dimethyl trisulfide; DMQS, dimethyl tetrasulfide. All experiments were performed in triplicate.

TABLE 4. Genes encoding oligopeptide or amino acid transporters differentially expressed in *B. aurantiacum* ATCC 9175 grown in the presence of sulfate, grown in the presence of methionine plus cystine, or grown during sulfur limitation

Gene	Function and/or similarity	Ratio and <i>P</i> value ^a			
		S/no sulfur		S/MC	
		Ratio	<i>P</i>	Ratio	<i>P</i>
<i>BL1734</i>	Oligopeptide ABC transporter, solute-binding protein	0.14	5.4E-12		
<i>BL1736</i>	Oligopeptide ABC transporter, permease	0.22	7.9E-10		
<i>BL1735</i>	Oligopeptide ABC transporter, permease	0.46	6.3E-10		
<i>BL1737^b</i>	Oligopeptide ABC transporter, ATP-binding protein	0.19	5.3E-10		
<i>BL380</i>	Probable glutamine ABC transporter, ATP-binding protein	0.47	6.6E-3		
<i>BL250</i>	Oligopeptide ABC transporter, solute-binding component	2.15	2.7E-3		
<i>BL251</i>	Oligopeptide ABC transporter, permease	9.00	2.1E-14		
<i>BL252</i>	Oligopeptide ABC transporter solute-binding protein	14.23	2.1E-14		
<i>BL253</i>	Oligopeptide ABC transporter ATP-binding protein	12.30	2.1E-14		
<i>BL634</i>	Oligopeptide ABC transporter ATP-binding protein	2.95	5.6E-6		
<i>BL635</i>	Oligopeptide ABC transporter, permease	3.25	1.5E-6		
<i>BL636</i>	Oligopeptide ABC transporter solute-binding protein	3.40	4.8E-7		
<i>BL637</i>	Oligopeptide ABC transporter solute-binding protein	5.90	4.4E-5		
<i>BL1103</i>	Oligopeptide ABC transporter, solute-binding protein	9.95	0.0E+0		
<i>BL1104</i>	Oligopeptide ABC transporter, ATP-binding protein	2.25	8.1E-3		
<i>BL2377</i>	Oligopeptide ABC transporter, permease	2.96	5.2E-5		
<i>BL2733</i>	Oligopeptide ABC transporter, permease	2.50	6.7E-4		
<i>BL1197</i>	Amino acid transporter	2.05	2.0E-4		
<i>BL1264</i>	Dipeptide/tripeptide permease	5.16	2.7E-11		
<i>BL1657</i>	Methionine ABC transporter, MetQ	6.90	5.1E-4		
<i>BL2207</i>	Lysine transporter	2.14	1.4E-3		
<i>BL3604</i>	LivF leucine/isoleucine/valine transporter, ATP-binding protein	3.51	6.2E-7		
<i>BL3605</i>	LivJ leucine/isoleucine/valine transporter, solute-binding protein	2.93	4.2E-6		
<i>BL3607</i>	LivM leucine/isoleucine/valine transporter, permease	3.32	3.5E-7		
<i>BL3608</i>	LivH leucine/isoleucine/valine transporter, permease	2.24	7.3E-4		
<i>BL1293</i>	Probable cystine ABC transporter ATP-binding protein	8.27	0.0E+0	1.82	7.9E-2
<i>BL1294</i>	Probable cystine ABC transporter, permease	10.74	2.1E-14	2.01	3.9E-2
<i>BL1295</i>	Probable cystine ABC transporter, permease	6.01	6.2E-13	1.92	2.6E-2
<i>BL1296</i>	Probable cystine ABC transporter, solute-binding component	2.14	2.1E-3		

^a S, sulfate; MC, methionine plus cystine.

^b The expression of this gene increased 2-fold in the presence of sulfate compared to cystine.

DISCUSSION

We combined metabolic reconstruction and growth assays with expression and metabolic profiling to obtain a global view of the sulfur metabolic network and of the response to sulfur availability in *B. aurantiacum*, a major VSC producer in cheese-ripening bacteria. The sulfate assimilation pathway encoded by the *cysI* operon and pathways required for direct formation of cysteine or homocysteine from sulfide are present in the *B. aurantiacum* genome. The expression of the *cysI* and *cysK* operons is high in the presence of sulfate and reduced with cystine, as expected for genes involved in cysteine production from sulfate. Using IMOMI software (38), we identified a conserved TTGTTGAGCAA motif upstream of *cysK* and *cysI* (Fig. 3A), suggesting a cysteine-dependent repression by a common regulator. Proteins sharing similarities with regulators controlling sulfate assimilation and/or cysteine biosynthesis in other Gram-positive bacteria, such as McbR and CysR of *C. glutamicum* (41, 43) or CymR of *B. subtilis* (17), are absent in *B. aurantiacum*, suggesting the involvement of a still-uncharacterized regulator. In addition, effectors of regulators of these pathways, such as cysteine, OAS, and OAH, are either undetectable or not differentially accumulated during growth with sulfate or cystine.

Two methionine biosynthesis pathways, direct thiolation and transsulfuration, are present in the genome of *B. aurantiacum*.

C. glutamicum utilizes both pathways with equal efficiency (42). While the OAH-thiol-lyase, MetY, is clearly identified, the annotation of the enzymes for the transsulfuration pathway, the cystathionine γ -synthase (MetB) and the cystathionine β -lyases (MetC- or AecD-type), is less evident. BL613, which is more similar to MetB of *C. glutamicum* and *M. tuberculosis* than is BL541, is the probable cystathionine γ -synthase. Furthermore, homocysteine and, to a lesser extent, methionine repressed *BL613* expression. The synthesis of AecD, a probable cystathionine β -lyase, is constitutive, as observed for AecD of *C. glutamicum* and PatB of *B. subtilis* (3). BL541, which shares similarities with Cg3086, could be either a second cystathionine β -lyase or a cystathionine γ -lyase (44). Three cobalamin-independent methionine synthases required for homocysteine methylation are found in the *B. aurantiacum* genome. MetE2 and MetE3 share 56 and 43% identity with MetE of *C. glutamicum*. Interestingly, MetE1 (BL2266) is highly similar to MetE from *Arthrobacter*, *Pseudomonas*, *Xanthomonas*, *Psychrobacter*, and *Acinetobacter* (78 to 82% identity), suggesting the existence of a horizontal gene transfer between gamma-proteobacteria and *Micrococcineae* in common biotopes such as soils or foods. The *metE1* and *metE2* genes are repressed in the presence of methionine, as observed for *BL613*, *metX*, *metY*, and *BL1657*. A conserved motif, CATGACG-N₄-GT-GACA, is present ~50 bp upstream of the *metY* and *BL613*

transcriptional start sites (Fig. 3B). A coordinated regulation of these genes by a still-uncharacterized regulator could be proposed. However, this motif is absent in the promoter region of the *BL2267-metE1* and *BL1655-to-BL1657* operons and *metE2*, suggesting the possible existence of several regulators for methionine biosynthesis, as observed in *E. coli* (56). In the presence of methionine, the pools of methionine and homocysteine increase whereas cystathionine and OAH are depleted. All of these compounds might be effectors of these regulators.

Surprisingly, the doubling time of ATCC 9175 on methionine (24 h) is very high compared to that observed with cystine or homocysteine (3.5 h). Its ability to grow on homocysteine indicates an efficient conversion of this compound into cysteine. Several pathways could be involved: the reverse transsulfuration pathway (48), the presence of an homocysteine γ -lyase, or the degradation of methionine to MTL and its further conversion into methanesulfonate (55). This latter possibility seems unlikely since strain ATCC 9175 grows poorly in the presence of methionine. Genes encoding proteins sharing only weak similarities with cystathionine β -synthases (BL781) or cystathionine γ -lyases (BL541) are present in the *B. aurantiacum* genome. Interestingly, the methionine γ -lyase of *B. aurantiacum* BL2 (formerly *B. linens*) also has a homocysteine γ -lyase activity (15). The expression of *BL929* is induced in the presence of homocysteine (see Table S2 in the supplemental material). The BL929 enzyme could participate in cysteine production from homocysteine via the intermediary formation of sulfide (Fig. 1B). Two hypotheses for methionine being a poor sulfur source for *B. aurantiacum* can be proposed: (i) methionine is inefficiently taken up, and (ii) the SAM recycling pathway is limiting. Two methionine transporters are present in the *B. aurantiacum* genome: one, similar to the high-affinity transporter MetNPQ of *B. subtilis* (48), is induced during methionine limitation, and the second, which shares similarities with the low-affinity transporter MetPS of *C. glutamicum* (53), is induced in the presence of methionine. In addition, the intracellular concentration of methionine strongly increased when methionine was added to the medium, indicating an efficient uptake of this amino acid (Table 2).

The catabolism of methionine in *Brevibacteriaceae* has been the subject of several studies because this amino acid is the precursor of MTL, which is required for the production of VSCs (11, 14, 15). The methionine γ -lyase is the key enzyme for methionine degradation in *Brevibacteriaceae* and other bacteria (25, 32). The expression of genes encoding methionine γ -lyases is induced by methionine in *Pseudomonas putida* (25) and *Citrobacter freundii* (31). Methionine γ -lyase activity in *B. antiquum* CNRZ918 (formerly *B. linens*) also increases in the presence of methionine (18). BL929 is the only enzyme similar to methionine γ -lyase, whose synthesis is induced by methionine. This indicated that BL929 is the major methionine γ -lyase of *B. aurantiacum* ATCC 9175. In addition to MTL, methionine degradation leads to the production of α -ketobutyrate, a precursor of isoleucine (39). Interestingly, we observed an increase in isoleucine concentration in the presence of methionine (Table 2; see also Fig. S1 in the supplemental material). The BL3000-BL3001 transporter, similar to the MetPS system of *C. glutamicum*, probably corresponds to the Na⁺-stimulated L-methionine transporter identified in *B. anti-*

quum CNRZ918 (19). The expression of the *BL3000-BL3001* operon, in contrast to that of *metPS* in *C. glutamicum* (53), is induced in the presence of 10 mM methionine. *BL929* forms an operon with *BL891* encoding a MerR-type regulator. This regulator is a good candidate to participate to the control of methionine γ -lyase and MetPS synthesis. Interestingly, a common motif, GAGTRYWMMAMAKCWC, is located 51 bp upstream of the transcriptional start site of the *BL929* and *BL3001* operons. The existence of a coordinated regulation of methionine transport and degradation could be an adaptation to the cheese matrix in which amino acids constitute a major substrate for growth and/or aroma production (7, 12). This work represents the first picture of sulfur metabolism in *B. aurantiacum*, a bacterium of major importance in cheese ripening. It also gives an overview of the global regulation of sulfur metabolism through transcriptomic and metabolomic approaches. This bacterium develops at the cheese surface and is then exposed to oxygen. The production and/or accumulation of reducing thiol molecules either extracellularly (sulfide, MTL) or intracellularly (mycothiol, homocysteine) could be an interesting adaptive feature to its biotope.

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