Evidence for Distinct l-Methionine Catabolic Pathways in the Yeast *Geotrichum candidum* and the Bacterium *Brevibacterium linens*

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Tracing experiments were carried out to identify volatile and nonvolatile l-methionine degradation intermediates and end products in the yeast *Geotrichum candidum* and in the bacterium *Brevibacterium linens*, both of which are present in the surface flora of certain soft cheeses and contribute to the ripening reactions. Since the acid-sensitive bacterium *B. linens* is known to produce larger amounts and a greater variety of volatile sulfur compounds (VSCs) than the yeast *G. candidum* produces, we examined whether the l-methionine degradation routes of these microorganisms differ. In both microorganisms, methanethiol and α-ketobutyrate are generated; the former compound is the precursor of other VSCs, and the latter is subsequently degraded to 2,3-pentanedione, which has not been described previously as an end product of l-methionine catabolism. However, the l-methionine degradation pathways differ in the first steps of l-methionine degradation. l-Methionine degradation is initiated by a one-step degradation process in the bacterium *B. linens*, whereas a two-step degradation pathway with 4-methylthio-2-oxobutyric acid (MOBA) and 4-methylthio-2-hydroxybutyric acid (MHBA) as intermediates is used in the yeast *G. candidum*. Since *G. candidum* develops earlier than *B. linens* during the ripening process, MOBA and MHBA generated by *G. candidum* could also be used as precursors for VSC production by *B. linens*.

Traditional fermented products result from the action of a set of microorganisms that give these products their diversity, their uniqueness, and their quality. In this context, sulfur compounds play a key role (18). Owing to their low detection thresholds and their strong reactivity, volatile sulfur compounds (VSCs) significantly influence the quality and unique flavor characteristics of many foodstuffs, such as cheese, wine, and beer (15, 16, 20). VSCs essentially arise from degradation of the sulfur-carbon bond of the amino acid precursor l-methionine, giving rise to methanethiol (MTL), a common precursor of VSCs (5).

A large number of microorganisms, including yeasts and bacteria, are employed in traditional food processing, such as cheese ripening and wine and beer making, and are often used in association with each other. In soft cheeses, yeasts (e.g., *Geotrichum candidum*) develop in the early stages of cheese ripening and participate in the decacidification of the curd and, to a lesser extent, in aromatization (2). In contrast, acid-sensitive surface bacteria (e.g., *Brevibacterium linens*) develop much later than yeasts during ripening, once the pH is sufficiently raised by the decacidifying yeast (3). *B. linens* is known to produce consistent amounts of VSCs (9). l-Methionine γ-lyase (MGL) catalyzes the one-step degradation of l-methionine to MTL, α-ketobutyrate (α-KB), and ammonium, and the gene encoding this enzyme in *B. linens* has been identified recently (1). In contrast, lactic acid bacteria (LAB) poorly convert l-methionine to VSCs (9) because they possess Met aminotransferase but no MGL activity. It therefore seems likely that the efficiency of conversion of l-methionine to VSCs depends on how the initial breakdown of this amino acid proceeds; a one-step pathway catalyzed by a MGL seems to be more favorable for efficient VSC production. In LAB, l-methionine degradation could be initiated by a transamination step that leads to 4-methylthio-2-oxobutyric acid (MOBA), which is subsequently converted to MTL through a chemical process (6, 10). In *Lactococcus lactis*, two aminotransferases (17), one aromatic aminotransferase and one branched-chain aminotransferase, were shown to be involved in the synthesis of volatile aroma compounds but can catalyze the transamination of l-methionine to MOBA only to a limited extent. It has been suggested that in the oenological bacterium *Oenococcus oeni*, the transamination of l-methionine is the first step in the pathway for degradation of l-methionine to VSCs (16). In *B. linens*, one aromatic amino acid aminotransferase and one aspartate aminotransferase were partially purified and characterized (11), but neither of these enzymes was tested with l-methionine as the transamination substrate. A transamination pathway was also suggested for several cheese-ripening yeasts, including *G. candidum* (7), as well as for the brewing yeast *Saccharomyces cerevisiae* (15), and this pathway could be involved in VSC production in such microorganisms.

Cheese-ripening bacteria like *B. linens* are known to produce larger amounts and a greater variety of VSCs than the yeast *G. candidum* produces (5). We therefore suspect that the l-methionine degradation routes of these microorganisms differ. Furthermore, the synthesis of VSCs depends not only on l-methionine catabolism but also on carbon metabolism (e.g., methylthioesters), which implies that complementary metabolic pathways that are relevant for both bacterial and yeast metabolism could be involved.

Due to the high reactivity of many sulfur compounds, l-methionine degradation products are not easy to identify. Furthermore, potential l-methionine degradation products, such
Aldrich.

fluoroacetamide (BSTFA), MOBA, and MHBA were purchased from Sigma-

O (gas chromatography grade) was purchased from Carlo Erba (Nanterre, France).

rinsed twice with distilled water and dried at 105°C until the weight was constant.

A.

B. linens

for 48 to 72 h at 25°C under aerobic conditions. Potato dextrose broth (Difco, Détroit, MI) was used for the precultures of G. candidum, while Trypticase soy yeast extract (TSYE) medium prepared as described previously (8) was used for B. linens. The precultures were used to inoculate (1%, vol/vol) the culture media.

The G. candidum cultures were grown in a medium containing 0.5 g liter−1 tryptone peptone (Difco), 1 g liter−1 yeast extract (Labosi, Oulchy-le-Château, France), 1 g liter−1 malt extract (Difco), 2 g liter−1 Casamino Acids (Difco), 2 g liter−1 KH2PO4, (Prolabo, Fontenay-Sous-Bois, France), 0.13 g liter−1 CaCl2 (Prolabo), 0.01 g liter−1 FeSO4 · 7H2O (Sigma-Aldrich, St. Quentin Fallavier, France), and 3 g liter−1 MgSO4 · 7H2O (Prolabo) whose pH was adjusted to 5.0 ± 0.1. The B. linens cultures were grown in 100 ml of TSYE medium (pH 7.0 ± 0.1). Five hundred-milliliter flasks containing 100 ml of medium were sterilized at 120°C for 20 min. Labeled or unlabeled L-methionine or glucose was added as a precursor to the media depending on the experiment, as described below. Cultures were agitated (150 rpm) under aerobic conditions at 25°C, and the culture broth (supernatant and pellets) was harvested after 24 h of incubation for G. candidum and after 48 h for B. linens. Volatile compound analyses were performed with the culture broth by purge and trap gas chromatography as described below. L-Methionine degradation intermediates (e.g., MOBA and MHBA) were analyzed with the supernatant obtained after centrifugation (12,000 × g for 10 min at 4°C) of the culture broth.

Dry weight.

Ten milliliters of a yeast or bacterial culture was filtered through a glass microfiber filter (diameter, 4.7 cm; Whatman, England). The filters were rinsed twice with distilled water and dried at 105°C until the weight was constant.

Metabolic tracing experiments: volatile compound analyses. Butyl acetate (gas chromatography grade) was purchased from Carlo Erba (Nanterre, France), O-(2,3,4,5,6-Pentafluorobenzyl)hydroxyamine (PFBHA), N-O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), MOBA, and MHBA were purchased from Sigma-Aldrich.

MOBA and MHBA that possibly formed from L-methionine catabolism were analyzed using a two-step derivatization technique. The procedure used in this study was adapted from a previously described method for identification of —O—CH2—COOH, and —C—O-containing compounds (19). In this method, —C—O groups react primarily with PFBHA to form oxime derivatives. Subsequently, —OH and —COOH groups react with BSTFA to form trimethylsilyl derivatives. The first derivatization step prevents conversion of carboxyls to enols, which could react with BSTFA and complicate interpretation of the data. Then the derivatives were separated by gas chromatography and identified by electronic ionization mass spectrometry. In our study, the first derivatization step was performed in aqueous medium. We therefore developed a solvent extraction methodology to efficiently recover hydroxyl acids and oxime derivatives to allow an effective silylation step, which requires a water-free medium. Standard solutions of MOBA and MHBA were prepared to deionized water at a concentration of 1 g liter−1. Five milliliters of each solution was derivatized with 500 μl of a 20 mM PFBHA acetonitrile–water solution (9:1, vol/vol). The reaction mixture was heated at 45°C for 2 h and, after cooling, acidified with 20 μl of concentrated sulfuric acid (96%). This step prevented excessive extraction of PFBHA and improved the extraction of acids. Oxime derivatives were then extracted with 2 ml of butyl acetate. Extraction was performed at 25°C by manually rolling the tubes along their length for 2 min. Then the solvent layer was recovered in a flask containing 1.5 g of anhydrous sodium sulfate. Fifty microliters of the solvent phase was transferred to a GC vial and silylated with 10 μl of BSTFA for 40 min at 60°C. Culture supernatants, which were kept at −20°C after sampling, were thawed at room temperature. Five milliliters of each sample was treated as described above, except that after oxime derivatization, the samples were centrifuged (6,400 × g, 4°C, 5 min) to remove the emulsion formed in our complex microbiological medium. The solvent layer was then recovered in a flask containing 1.5 g of anhydrous sodium sulfate, transferred to a GC vial, and dried under a gentle N2 stream. The dry material was resuspended in 50 μl of butyl acetate, subsequently silylated, and then analyzed. One milliliter of this mixture was analyzed by GC-MS.

GC-MS analyses were performed using an Agilent 6890 M gas chromatograph (Agilent Technologies, Palo Alto, CA) interfaced with a mass spectrometer detector (HP 5972 M quadrupole mass spectrometer; Agilent Technologies). The GC separation was carried out with an Agilent HP5 MS methyl siloxane capillary column (30 m by 0.25 mm; film thickness, 0.25 μm) using helium as the carrier gas (flow rate, 1.2 ml min−1). The following GC temperature program was used: injector temperature, 250°C; initial oven temperature, 60°C; initial holding time, 1 min; rate of temperature increase, 10°C min−1 up to 280°C; holding time, 5 min. Samples were injected in a splitless injection mode. The injector was switched to split mode 1 min after an injection was made.

Metabolic tracing experiments: volatile compound analyses. Unlabeled L-methionine (Sigma), L-methionine CD3 (Cambridge Isotope Laboratories, Andover, MA), l-[U-13C]methionine (Cambridge Isotope Laboratories), l-[U-13C]glucose (Martek Biosciences Corporation, Columbia, MD), l-[U-13C]leucine (Cambridge Isotope Laboratories), and unlabeled glucose (Prolabo) were used in this study. Cultures of B. linens and G. candidum were supplemented with 6.7 mM L-methionine CD3 or l-[U-13C]methionine, l-[U-13C]leucine was added to a culture of B. linens at a concentration of 6.7 mM. Control cultures were supplemented with 6.7 mM unlabeled L-methionine or l-leucine. A concentrated solution of l-methionine was sterilized by filtration (por size, 0.2 μm; diameter, 64 mm; Sartorius AG, Göttingen, Germany) at room temperature and added to the culture media prior to inoculation. Tracing experiments with glucose were also performed in this study; 10 g liter−1 and 3.3 g liter−1 of [U-13C]glucose were added to G. candidum and B. linens culture media, respectively. Control cultures were grown with an unlabeled carbon source.

Five milliliters of culture supernatant was analyzed using a headspace analyzer (HP 7695A purge and trap concentrator; Agilent Technologies, Palo Alto, CA) coupled with a gas chromatograph (HP 6890A; Agilent Technologies) and a mass spectrometer detector (HP 6890A quadrupole mass spectrometer; Agilent Technologies) as previously described (12). Sulfur compounds in control cultures (without any added labeled compound) were quantified using a dimethyl disulfide (DMDS) (Acros, Noisy-Le-Grand, France) standard calibration curve.

Labeling levels of the molecules in the cultures were expressed as percentages of the areas of the labeled molecules (sum of the areas of the different forms of labeling for the same molecule) over the total area of the molecule (sum of the areas of the different forms of labeling and the area of the unlabeled form for the same molecule). Interpretation of the spectra consisted of analyzing the various fragments of the labeled molecule compared to the unlabeled molecule.

RESULTS

Analysis of labeled nonvolatile sulfur-containing intermediates from L-methionine catabolism. In order to identify nonvolatile sulfur-containing intermediates, a two-step derivatization technique was employed with culture supernatants supplemented with l-[U-13C]methionine. MOBA and MHBA were properly derivatized and well separated on an HPS MS capillary column. Derivatized MOBA produced double peaks (16.4 and 16.75 min) for a nonsymmetric parent carbonyl because PFBHA forms two geometric isomers due to the nitrogen-carbon double bond. The two isomers have the same mass spectra, even if one isomer is always predominant (relative abundance ranging from 60% to 100%). Derivatized MOBA produced a single peak (retention time, 12.12 min). Derivatized MHBA and MOBA had a common ion fragment at m/z 73, [Si(CH3)2]1+ , resulting from the silylation of hydroxyl groups from —OH or —COOH function (Fig. 1). For MHBA, the m/z 147 ion had a very high relative abundance (90%). This fragment, postulated to be [(CH3)2Si=O]3+ by Yu et al. (19), should have
FIG. 1. Mass spectra of MOBA derivatives (A and B) and MHBA derivatives (C and D) following addition of L-[U-13C]methionine to cultures of G. candidum. (A and C) Unlabeled compounds; (B and D) labeled compounds.
TABLE 1. Growth and production of volatile sulfur compounds by *G. candidum* and *B. linens* over 72 h of incubation

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Time (h)</th>
<th>Growth (g liter⁻¹)</th>
<th>Concn (ppm) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>MTL</td>
</tr>
<tr>
<td><em>G. candidum</em></td>
<td>12</td>
<td>2.23 ± 0.18</td>
<td>0.49 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>6.03 ± 0.99</td>
<td>0.33 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>5.84 ± 0.09</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>5.49 ± 0.39</td>
<td>0.02 ± 0.00</td>
</tr>
<tr>
<td><em>B. linens</em></td>
<td>12</td>
<td>3.04 ± 0.08</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>4.83 ± 0.80</td>
<td>0.43 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>8.31 ± 0.35</td>
<td>0.65 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>10.72 ± 1.83</td>
<td>0.15 ± 0.00</td>
</tr>
</tbody>
</table>

* ND, not detected.

resulted from the two active H atoms of a hydroxyl-carboxylic acid. For MOBA, the oxime derivative contained a specific fragment ion at m/z 181 (CH₃C₆F₅) (Fig. 1) resulting from conversion of the carbonyl group to C=NOCH₂C₆F₅. Thus, selection of the m/z 73 ion can serve as an indicator that —OH or —COOH function is syphylated, while the m/z 147 ion is a good indicator of a hydroxyl-carboxylic acid (e.g., MHBA). These results explain the identification of MHBA and MOBA based on their functional groups, which was confirmed by comparison to pure commercial products (retention times and mass spectra).

The derivatives of MHBA and MOBA had molecular weights of 294 and 415, respectively. Mass spectra of these compounds (Fig. 1) were characterized by a low relative abun-

TABLE 2. Relative intensities of the major ions of D- and ¹³C-labeled compounds produced by *B. linens* or *G. candidum*

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Microorganism</th>
<th>Compound</th>
<th>Labeling efficiency (%)</th>
<th>Relative intensity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unlabeled precursor</td>
<td>BL, GC</td>
<td>MTL</td>
<td>39 ± 0.8 (BL), 42 ± 0.4 (GC)</td>
<td>49 (52), 45 (41), 47 (100), 48 (84)</td>
</tr>
<tr>
<td>GC</td>
<td>45 (84), 46 (90), 47 (93), 61 (34), 62 (100)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL, GC</td>
<td>DMDS</td>
<td>90 ± 0.3 (BL), 97 ± 1 (GC)</td>
<td>45 (56), 46 (29), 47 (22), 61 (94), 79 (56), 94 (100)</td>
<td></td>
</tr>
<tr>
<td>GC</td>
<td>45 (66), 46 (28), 47 (18), 61 (24), 79 (19), 94 (42), 111 (15), 126 (100)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL, GC</td>
<td>DMTS</td>
<td>39 ± 0.3 (BL), 98 ± 0.6 (GC)</td>
<td>45 (52), 46 (17), 47 (13), 48 (15), 71 (47), 75 (12), 118 (12)</td>
<td></td>
</tr>
<tr>
<td>GC</td>
<td>45 (54), 46 (17), 47 (13), 48 (15), 71 (47), 75 (12), 118 (12)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL</td>
<td>45 (19), 47 (19), 61 (7), 75 (100), 75 (15), 104 (37)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL</td>
<td>41 (53), 47 (12), 50 (100), 74 (13), 85 (11), 117 (4), 132 (2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL</td>
<td>29 (45), 32 (15), 43 (100), 57 (48), 85 (84), 100 (15)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>BL</td>
<td>43 (100), 44 (41), 47 (11), 90 (35)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL</td>
<td>41 (40), 43 (100), 45 (11), 47 (13), 48 (15), 71 (47), 75 (12), 118 (12)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL</td>
<td>45 (19), 47 (19), 61 (7), 75 (100), 75 (15), 104 (37)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL</td>
<td>41 (37), 43 (100), 45 (84), 47 (19), 71 (56), 76 (11), 118 (12), 119 (11)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL</td>
<td>29 (34), 32 (15), 43 (100), 57 (26), 60 (26), 100 (7), 103 (15)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>¹[U-¹³C]methionine</td>
<td>BL, GC</td>
<td>MTL</td>
<td>65 ± 0.8 (BL), 81 ± 4 (GC)</td>
<td>46 (53), 47 (62), 48 (56), 49 (81), 50 (40), 51 (100)</td>
</tr>
<tr>
<td>GC</td>
<td>45 (100), 46 (25), 47 (25), 48 (12), 50 (27), 64 (10), 65 (45)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL, GC</td>
<td>DMDS (one methyl)</td>
<td>88 ± 0.5 (BL), 97 ± 2 (GC)</td>
<td>46 (42), 47 (10), 48 (16), 50 (9), 64 (22), 79 (2), 82 (35), 94 (9), 97 (100)</td>
<td></td>
</tr>
<tr>
<td>GC</td>
<td>45 (58), 46 (49), 47 (35), 50 (100), 52 (5), 66 (26), 88 (27)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL, GC</td>
<td>DMDS (two methyls)</td>
<td>68 ± 0.7 (BL), 92 ± 0.1 (GC)</td>
<td>43 (100), 46 (12), 50 (10), 93 (36)</td>
<td></td>
</tr>
<tr>
<td>GC</td>
<td>41 (41), 43 (100), 46 (10), 50 (12), 51 (17), 71 (53), 78 (16), 121 (14)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL</td>
<td>46 (16), 48 (6), 50 (17), 51 (12), 70 (100), 84 (5), 78 (28), 107 (37)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL</td>
<td>41 (51), 50 (11), 70 (100), 78 (16), 85 (54), 117 (4), 135 (2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[U-¹³C]glucose</td>
<td>BL, GC</td>
<td>MTL</td>
<td>7 ± 0.2 (BL), 16 ± 5</td>
<td>43 (100), 45 (29), 47 (13), 90 (40), 91 (34), 92 (7)</td>
</tr>
<tr>
<td>GC</td>
<td>29 (73), 32 (27), 43 (24), 45 (90), 57 (100), 86 (12), 100 (2), 102 (13)</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

* BL, B. linens; GC, G. candidum.

b The molecular ion is indicated by boldface type.

c Two forms of the molecule (form bearing one labeled methyl and a form bearing two labeled methyls) are undissociated under one chromatographic peak.
dance or an absence of their molecular ions. This was probably due to electronic ionization, where the impact energy was high. Nevertheless, the pseudomolecular ion at \( m/z \) M-15, resulting from the loss of a methyl group from the neutral molecule, was identified with a relative abundance of 5 to 12%. Furthermore, ions at \( m/z \) M-117 and \( m/z \) M-197 resulted from the loss of \( \text{COOSi(CH}_3\text{)}_3 \) and \( \text{C}_6\text{F}_5\text{CH}_2\text{O} \), respectively, and had moderate to high abundance in MHBA and MOBA derivatives.

The double derivatization of culture supernatants of \( B. \) linens and \( G. \) candidum allowed identification of both MOBA and MHBA in \( G. \) candidum cultures, whereas these compounds were not produced in \( B. \) linens cultures. In \( G. \) candidum cultures, MOBA ion fragments (Fig. 1A), such as \( m/z \) 218 (corresponding to \( m-197 \)), \( m/z \) 298 (corresponding to \( m-117 \)), and \( m/z \) 400 (corresponding to \( m-15 \)), had a labeled equivalent in the mass spectrum of the labeled MOBA derivative (Fig. 1B). These labeled fragments, \( m/z \) 223, \( m/z \) 302, and \( m/z \) 405, respectively, exhibited a 4- or 5-mass unit increase due to \( ^{13}\text{C} \) labeling originating from \( \text{L-[U-}^{13}\text{C}]\text{methionine} \). For MHBA, ion fragments (Fig. 1C) such as \( m/z \) 177 (corresponding to \( m-117 \)), \( m/z \) 279 (corresponding to \( m-15 \)), and \( m/z \) 294 (corresponding molecular ion) had labeled equivalents (\( m/z \) 181, \( m/z \) 284, and \( m/z \) 299, respectively) in the mass spectrum of the labeled derivative (Fig. 1D).

These results clearly demonstrate that MOBA and MHBA are \( \text{L-methionine} \) degradation intermediates in \( G. \) candidum which are subsequently degraded to VSCs.

The analysis of labeled volatile compounds from \( \text{L-methionine catabolism} \). Several volatile compounds were detected in \( B. \) linens and \( G. \) candidum cultures (Tables 1 and 2). This con-

FIG. 2. Mass spectra of 2,3-pentanedione detected in \( G. \) candidum and \( B. \) linens cultures. (A) Cultures with unlabeled \( \text{L-methionine} \) and glucose. (B) Cultures with \( \text{L-[U-}^{13}\text{C}]\text{methionine} \) and unlabeled glucose. (C) Cultures with unlabeled \( \text{L-methionine} \) and [\( \text{U-}^{13}\text{C}]\text{glucose} \).
firms that B. linens produces larger amounts and a greater variety of VSCs than G. candidum produces. In B. linens cultures, MTL, DMDS, dimethyl trisulfide (DMTS), S-methyl thioacetate (MTA), S-methyl thioisovalerate (MTIV), and 2,3-pentanedione (PTD) were detected, while in G. candidum cultures, MTL, dimethyl sulfide (DMS), DMDS, DMTS, MTA, and PTD were produced.

When cultures were supplemented with L-methionine CD₃, all VSCs produced bore a CD₃ fragment that can be explained by cleavage of the C-S terminal bond of L-methionine (Table 2). High levels of labeling (>80%) were observed for sulfides produced by B. linens and G. candidum. For compounds containing two methyl fragments (DMS, DMDS, and DMTS), two types of labeled molecules were identified; one form had only one labeled methyl, and one form had two labeled methyls in addition to the unlabeled form accounting for approximately 20% of the total peak area. For instance, DMDS, which had an unlabeled mass ion at 

$$m/z \quad 94$$

(i.e., 

$$m+6$$

, corresponding to CD₃S=C=S=CD₃) and at 

$$m/z \quad 97$$

(i.e., 

$$m+3$$

, corresponding to CD₃S=C=S=CH₃). Analysis of the mass spectra of S-methyl thioesters produced by B. linens (Table 2) showed that more than 65% of these compounds were derived from labeled L-methionine, while more than 91% of the S-methyl thioacetate (the only S-methyl thioester detected in G. candidum cultures) was labeled. The difference is attributed to the presence of unlabeled L-methionine in the TSYE medium, the culture medium used for B. linens (8).

When cultures were supplemented with L-[U-¹³C]methionine, analysis of G. candidum and B. linens revealed that several labeled molecules had one or more ¹³C atoms in the carbon skeleton. These molecules were sulfides (MTL, DMS, DMDS, and DMTS) and S-methyl thioesters (MTA, MTB, MTP, and MTIV). More than 90% of the sulfides detected in both cultures were labeled with ¹³C. In these compounds, at least one methyl was labeled, corresponding to mass 

$$m+1$$

. However, compounds having two labeled methyls corresponding to mass 

$$m+2$$

 were prevalent. Labeling of methanethiol (40%) was also observed, and more than 70% labeling was obtained for S-methyl thioesters. Apart from VSCs, PTD was synthesized by both microorganisms and had three labeled carbons corresponding to mass 

$$m+3$$

 (Table 2 and Fig. 2). The analysis of the mass spectrum indicated that a \(^{13}p\text{CH}_{2}^-\text{CH}_{2}^-\text{CO}\) fragment was derived from the labeled L-[U-¹³C]methionine and, more precisely, from ɑ-ketobutyric acid, the keto acid resulting from the initial action of a transaminase or an L-methionine γ-lyase on L-methionine. In order to investigate possible connections between L-methionine and glucose catabolism, B. linens and G. candidum cultures containing added unlabeled L-methionine were sup-
plemented with \([U^{13}C]\text{glucose}\). A portion (7 to 16\%) of MTA had two labeled carbons in the acetyl group, indicating that this acyl moiety of MTA originated from glycolysis. Apart from MTA, more than 85\% of the PTD produced was labeled with \([^{13}C]\text{glucose}\). PTD had two labeled carbons in the \(\text{CH}_3--\text{CO}\) fragment (Table 2 and Fig. 2C), which most probably originated from activated acetaldehyde, as suggested by Ott et al. (14). Apart from MTA, glucose did not contribute to the production of other sulfur-bearing aroma compounds.

**DISCUSSION**

Our results showed that L-methionine catabolism was initiated by an aminotransferase in *G. candidum*. The transamination product MOBA was subsequently degraded to MTL and \(\alpha\)-KB. It has recently been suggested that in the brewing yeast *S. cerevisiae*, L-methionine can be transaminated to MOBA and then decarboxylated to methional and subsequently reduced to methionol (15). Knowledge of L-methionine catabolism is of technological interest since different degradation schemes could lead to different end products which may influence the product quality in a distinct manner. For instance, methional and/or methionol can be responsible for part of the overall aroma and/or off-flavors of beer or wine (13). In *G. candidum* MOBA is not decarboxylated to methional, since the latter compound was not identified among L-methionine degradation products.

In *B. linens*, L-methionine is predominantly degraded to MTL and \(\alpha\)-KB in one step by an MGL enzyme, in contrast to the oenological bacterium *O. oeni* or the LAB *L. lactis*, in which a transamination step is involved (16, 17). Although the *mgl* gene was recently identified in *B. linens* (1), whether an L-methionine transamination step could be an alternative pathway in this microorganism is still not known. This question was reinforced by the presence of several genes encoding aminotransferases in *B. linens* that we identified by analyzing the sequenced genome of *B. linens* ATCC 9174 (U.S. Department of Energy Joint Genome Institute [http://www.jgi.doe.gov]). However, our results show that these genes are unlikely to be involved in L-methionine catabolism in this microorganism since neither MOBA nor MHBA was detected in *B. linens*.

Although yeasts and bacteria do not develop simultaneously during ripening, it has been shown that L-methionine degradation intermediates (e.g., MOBA and MHBA) produced by the yeast *G. candidum* early during the ripening process (7) can serve as precursors for the efficient VSC producer *B. linens* (2). In *G. candidum* cultures, Met aminotransferase activity was found to be increased, together with the MOBA concentration, by increasing the L-methionine concentration (4). This occurs at low pH (pH \(<4\)), which shows that Met transamination is active in *G. candidum* under the acidic conditions which prevail during the early stages of ripening and which are detrimental to *B. linens*. Although *B. linens* is unable to convert L-methionine to MOBA, its ability to produce VSCs from this keto acid has been demonstrated (2). This result and the results of the present study emphasize the metabolic complementarities between *B. linens* and *G. candidum* with respect to L-methionine catabolism.

The results of our study also show that the \(\alpha\)-KB resulting from L-methionine conversion to MTL is subsequently degraded to PTD. The origin of the PTD in lactic acid bacteria has been studied previously by Ott et al. (14). These authors suggested that this diketone was formed from \(\alpha\)-ketobutyric acid and active acetaldehyde originating from pyruvate. These two precursors undergo condensation, which is catalyzed by an acetate synthase (EC 2.2.1.6), and produce the intermediate \(\alpha\)-acetoc-\(\alpha\)-hydroxybutyrate, which is decarboxylated under aerobic conditions to PTD (Fig. 3). The gene coding for an acetolactate synthase was found in the recently sequenced genome of *B. linens* (U.S. Department of Energy Joint Genome Institute [http://www.jgi.doe.gov]). We also suspect that this enzyme is present in *G. candidum*, but we were unable to confirm this hypothesis because of the absence of a sequenced genome for this microorganism. Using labeled glucose or L-methionine resulted in PTD labeling that provided evidence of a highly probable pathway at the crossroads of glycolysis and L-methionine catabolism in *B. linens* and *G. candidum*. Our results revealed that there is a close relationship between L-methionine and carbohydrate metabolism, illustrated by S-methyl thiosters and PTD biosynthesis (Fig. 3).

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