Anaerobic 1-alkene Metabolism by the Alkane- and Alkene-Degrading Sulfate Reducer *Desulfatibacillum aliphaticivorans* Strain CV2803<sup>TV</sup>

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The alkane- and alkene-degrading, marine sulfate-reducing bacterium *Desulfatibacillum aliphaticivorans* strain CV2803<sup>3</sup>, known to oxidize n-alkanes anaerobically by fumarate addition at C-2, was investigated for its 1-alkene metabolism. The total cellular fatty acids of this strain were predominantly C-(even number) (C-even) when it was grown on C-even 1-alkanes and predominantly C-(odd number) (C-odd) when it was grown on C-odd 1-alkenes. Detailed analyses of those fatty acids by gas chromatography-mass spectrometry after 6- to 10-week incubations allowed the identification of saturated 2- and 4-ethyl-, 2- and 4-methyl-, and monounsaturated 4-methyl-branched fatty acids with chain lengths that correlated with those of the 1-alkane. The growth of *D. aliphaticivorans* on (per)deuterated 1-alkenes provided direct evidence of the anaerobic transformation of these alkenes into the corresponding 1-alcohols and into linear as well as 10- and 4-methyl-branched fatty acids. Experiments performed with [13C]bicarbonate indicated that the initial activation of 1-alkene by the addition of inorganic carbon does not occur. These results demonstrate that *D. aliphaticivorans* metabolizes 1-alkene by the oxidation of the double bond at C-1 and by the subterminal addition of organic carbon at both ends of the molecule [C-2 and C-(ω-1)]. The detection of ethyl-branched fatty acids from unlabeled 1-alkenes further suggests that carbon addition also occurs at C-3. Alkylsuccinates were not observed as potential initial intermediates in alkene metabolism. Based on our observations, the first pathways for anaerobic 1-alkene metabolism in an anaerobic bacterium are proposed. Those pathways indicate that diverse initial reactions of 1-alkene activation can occur simultaneously in the same strain of sulfate-reducing bacterium.

Different studies during the past decades have demonstrated the utilization of hydrocarbons under anoxic conditions (32, 36). The initial reactions involved in the degradation of some aliphatic, alicyclic, and aromatic hydrocarbons by anaerobic bacteria have been subsequently elucidated elsewhere (4, 8, 24, 25, 29, 37, 38), but still, many gaps remain in the understanding of the anaerobic oxidation of some of these nonpolar molecules.

 Unsaturated linear hydrocarbons (n-alkenes) are ubiquitous naturally occurring compounds that can be biosynthesized by different (micro)organisms and plants (3, 34). Phytolankton appears, however, to be one of the major sources of these compounds in aquatic environments. Linear alkenes with 15 to 39 carbon atoms and with one to seven double bonds are known to be produced more or less specifically by microalgae (14). Short-chain n-C<sub>15</sub>, n-C<sub>17</sub>, and n-C<sub>19</sub> monoenes as well as the highly unsaturated n-C<sub>21</sub>:6 are among the most commonly encountered. Specific bacterial n-alkenes have also been identified in microbial mats (33, 34).

 Alkenes are more reactive than aromatic hydrocarbons and alkanes and, thus, are rarely identified as constituents of diagenetic organic matter. Different laboratory incubation studies as well as field studies have indeed revealed the low potential for preservation of n-alkenes during early diagenesis, although differences in the chemical structure and/or diagenetic conditions (e.g., anoxia) may be responsible for differences in reactivity between isomers (14, 18). Still, alkenes are found in some crude oils and condensates, but their origin seems to be related to an abiogenic alteration of n-alkanes in the trap rather than to a direct preservation of original molecules (12).

 Although the anaerobic degradation of n-alkenes by enrichment cultures (28) and pure denitrifying (17) and sulfate-reducing bacteria (1, 2, 9, 10, 11, 30, 31) has been reported, the initial reactions of these unsaturated hydrocarbons in anaerobic bacteria are still unknown (32). The degradation of 1-hexadecene by methanogenic enrichment cultures was supposed to occur by initial oxidation to 1-hexadecanol and subsequent β-oxidation of hexadecanoic acid (28), but no intermediate metabolite other than acetate could be detected. On the other hand, in some early studies of alkane-degrading microorganis-
isms, an oxygen-independent initial metabolism of alkanes via dehydrogenation to 1-alkenes and oxidation to primary alcohols was suggested (6, 23, 35). In recent investigations, however, alkane dehydrogenation to 1-alkenes was viewed critically (1, 2, 30), and two alternative pathways for the initial oxidation of alkanes in denitrifying or sulfate-reducing bacteria were subsequently described: the addition of fumarate at C-2 (5, 8, 24, 38) and carboxylation at C-3 (5, 29).

In the present report, we investigated the anaerobic degradation of 1-alkenes by the sulfate-reducing bacterium Desulfitobacillus aliphaticivorans strain CV2803T, recently isolated from a polluted marine sediment. This strain, which was proposed as the type strain of a novel species in a new genus of the family Desulfbacteraceae (class Deltaproteobacteria), is able to oxidize C13-C18 n-alkanes and C7-C23 n-alkenes into carbon dioxide during sulfate reduction (10). It was also recently demonstrated to transform anaerobically n-alkanes into fatty acids by the addition of fumarate at C-2 (8). By carefully examining the total cellular fatty acids of D. aliphaticivorans grown on different unlabeled 1-alkanes (C14, C15, C16, and C17) and on per)deuterated 1-pentadecene and 1-hexadecene, we could demonstrate that strain CV2803T oxidizes alkanes into fatty acids by the oxidation of the double bond at C-1 (via a primary alcohol) and by the addition of an undefined carbon unit(s) at C-2 and C-3 and at the subterminal carbon of the saturated end of the molecule (C-ω-1). Besides describing the first metabolic pathway of n-alkanes in an anaerobic bacterial isolate, our work demonstrates that diverse initial reactions of n-alkene activation can occur simultaneously in the same strain of sulfate-reducing bacteria.

MATERIALS AND METHODS

Chemicals. 1-Tetradecane, 1-pentadecane, 1-hexadecane, and 1-heptadecane (>99% pure) were purchased from Fluka Chemical Co. 1d-1-pentadecane and perdeuterated 1-hexadecane (C6-D6, COOH; Euriso-top), respectively: (i) reduction of the acid with LiAlD4 (Aldrich) in diethyl ether, (ii) reaction of the resulting alcohol with SOCl2 in pyridine (reflux for 1.5 h), and (iii) conversion of the produced 1-alkyl chloride into 1-alkene with potassium tert-butoxide in dry dimethyl sulfoxide (1). The double bond was located principally at C-11, but different positional isomers were detected.

Source of bacterial and cultivation. The sulfate-reducing bacterium D. aliphaticivorans strain CV2803T was isolated from sediment of the Gulf of Fos (Mediterranean Sea, France) (10). It belongs to the family Desulfobacteraceae in the Deltaproteobacteria and is phylogenetically closely related to a group of unnamed alkane-oxidizing strains. The most closely related species is Desulfocurvina variabilis (10). Strain CV2803 was grown in 500 ml of defined anoxic sulfate-reducing medium with pure unlabeled 1-alkane (1 L.1 to 1.4 mM) or ldl-1-1-pentadecane (1.1 mM) as the growth substrate, as described previously (9). Precultures were all grown on unlabeled n-alkanes. Cultures with perdeuterated 1-hexadecane were prepared by mixing equal volumes of unlabeled and labeled 1-hexadecane (1 mM final concentrations) in order to facilitate growth (8, 24, 38). Cultures in the presence of [13C]bicarbonate (Aldrich) were prepared under aerobic conditions with argon as the head space, as described previously (8). Bacterial cultures were grown on MOPS (3 g liter−1; pH 7.5) instead of bicarbonate buffer. [13C]bicarbonate and 1-hexadecane were added at concentrations of 5 mM and 1 mM, respectively. For each substrate, two experimental cultures and one sterile control (with autoclaved inoculum) were carried out. Cultures were analyzed after 6 to 10 weeks (depending on growth) of incubation at 30°C.

Extraction and analysis of cellular fatty acids. Cells were collected by filtration through glass microfiber filters (GF/B; Whatman) and treated with 1 N KOH in methanol-water (1:1, vol/vol). Unsaponifiable (neutral) and saponifiable (acid) lipids were extracted from the basic and the acidified solutions, respectively, as described previously (8, 9). Neutrals were silylated by reaction with N,O-bis(trimethylsilyl)trifluoroacetamide in pyridine (8), whereas free fatty acids were methylated with 1% sulfuric acid in methanol (7). A portion of the methylated fatty acids was hydrogenated (with PO2 and H2 at room temperature overnight) in ethyl acetate containing one drop of acetic acid. The assignment of the methyl (Me) branch and double-bond positions in branched and/or unsaturated fatty acids was based on the formation of pyrrolidide derivatives (7), and the positions of the double bonds were further confirmed by oxidation with O3 and subsequent trimethylsilylation (26).

RESULTS

Transformation of 1-alkenes into fatty acids by D. aliphaticivorans. Although D. aliphaticivorans strain CV2803T utilizes 1-alkanes from C7 to C23 (10), efficient growth is observed in the range from C14 to C17. This range was thus chosen for the present study (Table 1). In a preliminary report focused on the growth of D. aliphaticivorans on 1-alkenes (9), the major cellular fatty acids of strain CV2803T grown on 1-pentadecane and 1-hexadecene were described. This report already indicated that the chain length of the alkene influences the fatty acid composition of the strain. In the present study, new cultures with a broader spectrum of substrates further demonstrated that the chain lengths of the cellular fatty acids of D. aliphaticivorans are strongly dependent on those of the alkene

| Fatty acid | Relative abundance of fatty acid (% of total) of D. aliphaticivorans strain CV2803T grown on: |
|------------|-----------------------------------------------|
| 13:0       | 4.7                                           |
| 14:0       | 37.4                                          |
| 10-Me-14:0 | 3.6                                           |
| i-15:0     | 0.7                                           |
| a-15:0     | 0.9                                           |
| 15:0       | 2.5                                           |
| 10-Me-15:0 | 3.6                                           |
| 16:1Δ9     | 5.0                                           |
| 16:0       | 26.7                                          |
| 10-Me-16:0 | 4.9                                           |
| 4-Me-16:0  | 0.9                                           |
| 17:0       | 1.1                                           |
| 4-Me-17:1b | 1.9                                           |
| 4-Me-17:0  | 1.0                                           |
| 18:2Δ9Δ11  | 1.6                                           |
| 18:1Δ11    | 5.4                                           |
| 18:1Δ9     | 0.7                                           |
| 18:0       | 8.8                                           |
| 4-Me-18:1b | 1.9                                           |
| 4-Me-18:0  | 1.0                                           |
| 4-Me-19:1b | 1.1                                           |

*Data for acids constituting less than 0.5% of the total are not presented.

The double bond was located principally at C-11, but different positional isomers were detected.

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TABLE 1. Relative abundances of cellular fatty acids* of D. aliphaticivorans strain CV2803T grown on:

| 1-Tetradecene | 1-Pentadecene | 1-Hexadecene | 1-Heptadecane |
|---------------|---------------|--------------|--------------|
| 13:0          | 4.7           | 6.5          |
| 14:0          | 37.4          | 25.3         | 8.7          |
| 10-Me-14:0    | 3.6           | 1.0          | 1.0          |
| i-15:0        | 0.7           | 1.0          | 1.0          |
| a-15:0        | 0.9           | 1.4          | 29.3         |
| 15:0          | 2.5           | 16.2         | 16.1         |
| 16:1Δ9        | 5.0           | 2.0          | 1.5          |
| 16:0          | 26.7          | 28.6         | 11.1         |
| 10-Me-16:0    | 4.9           | 17.9         | 1.5          |
| 4-Me-16:0     | 0.9           |              |              |
| 17:0          | 1.1           | 0.5          | 13.2         |
| 4-Me-17:1b    | 1.9           |              |              |
| 4-Me-17:0     | 1.0           |              |              |
| 18:2Δ9Δ11     | 1.6           | 4.5          | 1.7          |
| 18:1Δ11       | 5.4           | 3.7          | 1.8          |
| 18:1Δ9        | 0.7           | 2.3          | 1.0          |
| 18:0          | 8.8           | 5.5          | 5.8          |
| 4-Me-18:1b    | 5.0           |              |              |
| 4-Me-18:0     | 1.0           |              |              |
| 4-Me-19:1b    | 1.1           |              |              |

*Data for acids constituting less than 0.5% of the total are not presented.
substrates (Table 1). Cells grown on C-even alkenes contain mainly fatty acids with C-even carbon chains, and similarly, fatty acids with C-odd chains predominate when cells are grown on C-odd alkenes.

The detailed fatty acid composition of the strain grown on 1-alkenes was further analyzed by gas chromatography-mass spectrometry (GC-MS). Linear saturated fatty acids are accompanied by branched (iso-, anteiso-, and 10-Me-) and unsaturated (e.g., 18:1Δ9 and 18:1Δ11) fatty acids commonly encountered in bacteria and by less common saturated and monounsaturated 4-Me-branched fatty acids whose chain lengths are specifically correlated with those of the alkene substrates (Table 1). 4-Me-16:0, 4-Me-17:0, 4-Me-18:0, and 4-Me-19:0 and their monounsaturated homologues were detected only after growth on 1-tetradecene, 1-pentadecene, 1-hexadecene, and 1-heptadecene, respectively (Table 1 and Fig. 1a). Those saturated and monounsaturated 4-Me-branched fatty acids were detected neither in sterile controls nor in cultures grown on fatty acids (data not shown). The identification of all the saturated Me-branched fatty acids was based on the interpretation of their mass spectra, which show specific ions due to cleavage to the branch carbon atoms, and on previously reported mass spectral data (9, 30, 38). The position of the methyl branch was further confirmed by the analysis of the pyrrolidide derivatives (data not shown) (9). Pyrrolidide derivatives were also used to determine the positions of both the methyl branch and the double bond in the monounsaturated homologues (Fig. 2a). Branching and unsaturation positions can be identified by fragments differing by 28

FIG. 1. Partial total-ion chromatograms of intact (a) and hydrogenated (b) fatty acid methyl esters of D. aliphaticivorans strain CV2803 grown on 1-hexadecene.

FIG. 2. Mass spectra of 4-methylheptadec-11-enoylpyrrolidide formed after the derivatization of 4-methylheptadec-11-enoic acid from a culture of D. aliphaticivorans strain CV2803 grown on 1-pentadecene (a), 4-ethylheptadecanoic fatty acid methyl ester from a culture grown on 1-hexadecene (b), 2-ethylhexadecanoic fatty acid methyl ester from a culture grown on 1-heptadecene (c), and d2-4-methylheptadecanoic fatty acid methyl esters from a culture grown on 1d2-1-pentadecene (d).
and 12 atomic mass units (amu), respectively, instead of the regular 14 amu (8, 26). For any alkene substrate, the mono-ununsaturated 4-Me-branched fatty acid formed has its double bond located preferentially at C-11 (Fig. 2a), but other positional isomers (with the double bond at C-12, for instance) were sometimes observed (Fig. 1). The position of the double bond was further confirmed by oxidation with OsO₄ (data not shown) (8, 9, 26), whereas that of the methyl branch was confirmed after catalytic hydrogenation. Indeed, monounsaturated methyl-branched fatty acids were all converted to the corresponding saturated 4-Me-branched fatty acids upon hydrogenation (Fig. 1).

GC-MS analysis of the minor cellular fatty acids (constituting <0.5% of the total) of D. aliphaticivorans strain CV2803 grown on 1-alkenes also allowed the identification of saturated 2-Me-branched fatty acids, as well as saturated monoethyl-branched fatty acids with an ethyl (Et) group located at C-2 or C-4 (Fig. 1, 2b, and 2c). Like the aforementioned 4-Me-branched fatty acids, these 2-Me-, 2-Et-, and 4-Et-branched fatty acids were detected neither in sterile controls nor in cultures grown on fatty acids (data not shown) and had a carbon chain length directly dependent on that of the alkene substrate (Fig. 1, 2b, and 2c). For instance, 2-Me-16:0, 2-Et-15:0, and 4-Et-17:0 were detected only after growth on 1-hexadecene (Fig. 1 and 2b), whereas 2-Me-17:0, 2-Et-16:0 (Fig. 2c), and 4-Et-18:0 were detected only after growth on 1-heptadecene. It should be noted that, due to their low abundance and their possible coelution with other major fatty acids, some of the Et-branched fatty acids may not have been detected without the catalytic hydrogenation of the fatty acid extract (Fig. 1). Mass spectra of 2-Me- and 2-Et-branched fatty acid methyl esters showed characteristic McLafferty base peaks at m/z of 88 and 102, respectively (see Fig. 2c for Et branching) (22, 30, 38). Although the mass spectra of 4-Et-branched fatty acids show similarities with those of 4-Me-branched fatty acids (Fig. 2b and d), the identification of the former compounds is based on their relative retention times (Fig. 1) and on the presence of an intense (M-71)⁺ ion in their mass spectra (Fig. 2b). This fragment, being analogous to the (M-57)⁺ ion observed for 4-Me-branched fatty acid methyl esters (Fig. 2b and d), corresponds to the elimination of the segment from C-2 to C-4 of the molecule, including the 4-ethyl group (and an additional hydrogen) (22).

The direct relationship observed between the chain lengths of the Me- and Et-branched fatty acids and those of the alkene substrates strongly suggests that 1-alkenes are (at least partly) transformed into fatty acids by the action of exogenous carbon onto the ethylenic C-2 and onto the allylic C-3, yielding a molecule, including the 4-ethyl group (and an additional hydrogen). Indeed, monounsaturated methyl-branched fatty acids were all converted to the corresponding saturated 4-Me-branched fatty acids upon hydrogenation (Fig. 1).

Identification of 1d₃₋₁-pentadecene-derived fatty acids. The growth of D. aliphaticivorans on pure 1d₃₋₁-pentadecene yielded essentially deuterated 14:0, 16:0, 18:0, and 10-Me-16:0 fatty acids (Fig. 3); deuterated 4-Me-18:0, 4-Me-18:1, 2-Me-16:0, 2-Et-15:0, and 4-Et-17:0 could not be detected. Although labeling experiments were performed using a mixture of unlabeled and perdeuterated 1-hexadecene, the labeled fatty acids have shorter chromatographic retention times than the unlabeled fatty acids and, thus, generally appeared completely separated during GC-MS analyses. However, labeled Me-branched fatty acids have retention times similar to those of the unlabeled linear fatty acids, which likely induced coelution problems and, consequently, the nondetection of some minor deuterated branched fatty acids, even after catalytic hydrogenation. It should be noticed that during these labeling experiments, unlabeled 4-Me-18:1, 4-Me-18:0, 2-Me-16:0, and 4-Et-17:0 formed from unlabeled 1-hexadecene were observed. Figure 3a shows the mass spectrum of the deuterated 16:0 fatty acid methyl ester. The molecular ion (m/z of 300) indicates the presence of 30 deuterium atoms in the molecule. The McLafferty ion (m/z of 76) is upshifted by 2 amu compared to the corresponding ion in the unlabeled analogue (m/z of 74), indicating the presence of two deuterium atoms in the labeled ion. Methanol used for methylation was not deuterated, and one deuterium atom would come from the McLafferty rearrangement, which indicates the presence of one deuterium and one hydrogen atom at C-2.

Similar analysis of the mass spectrum of the deuterated 14:0 fatty acid methyl ester indicates that it is fully deuterated (thus containing 27 deuterium atoms) (Fig. 3b), which suggests that it was formed by β-oxidation (loss of a C-2 unit) of the 1d₁₇₋₁-16:0 fatty acid. Similar analysis of the mass spectrum of the deuterated 18:0 fatty acid shows that it contains a total of 30 deuterium atoms, together with five hydrogen atoms located be-
between C-1 and C-5 (Fig. 3c). Although the exact positions of the hydrogen atoms cannot be deduced from the mass spectrum, the \(d_{30}^{-18:0}\) fatty acid is likely formed by chain elongation of the \(d_{30}^{-16:0}\) fatty acid with two nondeuterated carbon atoms. Traces of a \(d_{30}^{-20:0}\) fatty acid were also detected (data not shown).

Examination of the deuterated 10-Me-16:0 fatty acid indicates that it contains a total of 29 deuterium atoms together with one hydrogen atom at C-2 and three other ones at the methyl branch (Fig. 3d). This suggests that the 10-Me-16:0 fatty acid is formed by the exogenous addition of a methyl group at C-10 of the corresponding \(n\)-saturated fatty acid (\(d_{30}^{-16:0}\)). Traces of \(d_{26}^{-10\text{-Me}-14:0}\) fatty acid were also detected (data not shown).

These observations demonstrate that, along with the aforementioned addition of exogenous carbon at C-2, C-3, and C-(\(\omega\)-1), the initial reactions of anaerobic 1-alkene degradation by \(D.\ aliphaticivorans\) also involve the oxidation of the double bond at C-1 and the further metabolism of the resulting fatty acid (formed via the corresponding 1-alcohol).

Fatty acids formed in the presence of [\(^{13}\text{C}\)]bicarbonate. The fatty acids formed in cultures of \(D.\ aliphaticivorans\) grown on unlabeled 1-alkene in the presence of [\(^{13}\text{C}\)]bicarbonate were similar to those formed in the presence of unlabeled bicarbonate. Mass spectral analysis of the Me- and Et-branched fatty acids formed in the presence of [\(^{13}\text{C}\)]bicarbonate showed that they were not [\(^{13}\text{C}\)]-labeled (data not shown), indicating that the incorporation of carbon at C-2, C-3 or C-(\(\omega\)-1) in the original alkene does not proceed by incorporation of an inorganic carboxyl group.

Identification of (per)deuterated fatty alcohols. Owing to the major formation of \(d_{30}^{-16:0}\) fatty acid from \(d_{32}^{-1}\)-hexadecene (Fig. 3a) and to the dominance of linear fatty acids with chain lengths similar to those of the substrates during growth on unlabeled 1-alkenes (Table 1), we searched for the presence of labeled fatty alcohols among the unsaponifiable lipids of \(D.\ aliphaticivorans\) grown on (per)deuterated 1-alkenes. \(d_{2}^{-1}\)-pentadecanol and \(d_{32}^{-1}\)-hexadecanol were detected after growth on \(d_{2}^{-1}\)-pentadecene and \(d_{32}^{-1}\)-hexadecene, respectively (Fig. 4). For both alcohols, the ion with the largest mass in the spectrum corresponds to the \((M-15)^{-}\) ion resulting from the loss of one methyl from the trimethylsilyl group. This ion indicates the number of deuterium atoms present in each labeled alcohol. Moreover, for both alcohols, the ion fragment at \(m/z\) of 105 (corresponding to the ion at \(m/z\) of 103 for the unlabeled analogues) indicates the presence of two deuterium atoms at C-1 (Fig. 4).

The presence of two deuterium atoms on the carbon bearing the hydroxyl group (C-1) and that of one deuterium and one hydrogen atom at C-2 in one or the other 1-alcohol further support the initial oxidation of the double bond of the alkene substrates by \(D.\ aliphaticivorans\) and the subsequent transformation of the resulting 1-alcohols to the corresponding linear fatty acids.

DISCUSSION

Previous studies have reported the biodegradation of 1-alkenes by pure sulfate-reducing bacteria (namely, strains Hxd3 [1, 2], Pnd3 [2], and AK-01 [30]), and partial information on...
the cellular fatty acid compositions of these strains grown on 1-alkenes in the range from C\textsubscript{15} to C\textsubscript{17} was given. Like \textit{D. aliphaticivorans}, strains Hxd3, Pnd3, and AK-01 form essentially C-even fatty acids from C-even 1-alkenes and C-odd fatty acids from C-odd 1-alkenes. Aekersberg et al. (2) further showed that the major fatty acid of strain Hxd3 grown on 1-hexadecene is 16:0, whereas 15:0 and 17:0 fatty acids dominate after growth on 1-heptadecene. However, these studies were essentially designed to demonstrate that 1-alkenes are not intermediates of the anaerobic \textit{n}-alkane metabolism in strains Pnd3, Hxd3, and AK-01 (which were all originally isolated on \textit{n}-alkanes), and the initial reactions of 1-alkene degradation were not studied in detail.

In the present study, we demonstrated that \textit{D. aliphaticivorans} strain CV2803\textsuperscript{T} transforms a 1-alkene into the corresponding 1-alcohol and into linear and branched fatty acids on the basis of the recovery of (per)deuterated fatty alcohols and fatty acids from cultures grown on labeled 1-alkenes. \textit{D. aliphaticivorans} metabolizes 1-alkenes by both the oxidation of the double bond at C-1 and the addition of organic carbon at C-2, C-3, and C-(\textit{\omega}-1). The relationship between the carbon numbers of 1-alkene substrates and the predominant cellular \textit{n}-saturated fatty acids (Table 1) may indicate that the oxidation of the double bond is the major pathway. However, our experimental approach of taking a single snapshot of metabolites rather than conducting time series or in vitro (e.g., fumarate addition) studies of metabolites may have resulted in our overlooking key transient but low-concentration metabolites involved in alternative pathways. The exact mechanism involved in the transformation of the double bond into a primary alcohol remains unknown. The hydration of double bonds seems common in the metabolisms of many organisms (32). This reaction has been hypothesized to occur during the anaerobic biotransformation of some isoprenoid alkenes (19, 27) and during the mineralization of 1-hexadecene by a methanogenic enrichment culture that also continued to grow on primary long-chain alcohols (28). However, as far as we know, hydration of a double bond to yield a primary alcohol via an anti-Markovnikov-oriented reaction has never been proven to occur in an alkene-degrading anaerobic bacterium.

Additionally, the results from our \textsuperscript{[13]C}bicarbonate experiments indicate that the initial activation of 1-alkene by carboxylation does not occur in \textit{D. aliphaticivorans}. The positions of the two deuterium atoms (either at the Me branch or at the other side of the molecule \textit{[C-\omega]}) in the two isotopomers of \textit{d}_{2}-4-Me-17:0 formed from \textit{d}_{2}-1-pentadecene further demonstrate that an organic carbon unit is added to the initial 1-alkene subterminally either at the ethylenic C-2 or at the saturated terminus. \textit{D. aliphaticivorans} has been previously demonstrated to activate saturated \textit{n}-alkanes by the addition of a molecule of fumarate at C-2 to yield 4-Me-branched fatty acids via alkylsuccinic acids (8). In such catabolic reactions, the
alkylsuccinic acid undergoes a rearrangement of the carbon skeleton to form a (2-methyl-alkyl)malonate which is subsequently decarboxylated into a 4-Me-branched fatty acid possessing three carbon atoms more than the original n-alkane (38). The lack of detection of alkylsuccinates in the present study may be indicative of an alternative pathway for organic carbon addition to the alkene. However, alkylsuccinates are transient metabolites which may have been overlooked due to the analysis of cultures at a single time point and to the absence of in vitro assays (13); it is noteworthy that alkylsuccinates were not systematically observed among the fatty acids of D. aliphaticivorans during growth on n-alkanes (V. Grossi, unpublished results). We can thus still speculate that an addition of fumarate is also involved in the activation of 1-alkene by D. aliphaticivorans. Addition at C-2 and at C-(ω-1) of the 1-alkene followed by the rearrangement of the initial intermediate would yield a 4-Me fatty acid. A similar addition reaction at the carbon adjacent to the double bond (C-3) would explain the formation of 4-Et-branched fatty acids (Fig. 2b and c). Unfortunately, experiments with labeled fumarate which may have allowed confirmation of the above-stated hypothesis (24) have not given reliable results so far, probably because D. aliphaticivorans readily uses this molecule as a carbon source (10).

Thus, the possibility that D. aliphaticivorans transforms 1-alkenes into branched fatty acids by the subterminal addition of a carbon unit other than fumarate cannot be ruled out. Owing to the likely difference in reactivity between the saturated and the unsaturated ends of the alkene, the addition of different organic carbon units at each side may also be envisaged.

A pathway for anaerobic 1-alkene metabolism by D. aliphaticivorans strain CV2803T is proposed in Fig. 5. Although the acid metabolites were detected as free carboxylic acids, many of them presumably exist in vivo as coenzyme A thioesters (which are likely hydrolyzed during the analytical procedure) (38). The alcohol formed by the oxidation of the double bond is transformed into the corresponding linear fatty acid, which is mineralized into CO₂ or, alternatively, transformed by chain elongation and/or C-10 methylation. On the other hand, the 4-Et- and 4-Me-branched fatty acids formed by the addition of organic carbon at C-3 and C-2 [or C-(ω-1)] can be degraded by β-oxidation into the corresponding 2-Et- and 2-Me-branched homologues, respectively (Fig. 5). These can be further transformed into linear fatty acids, which are subsequently mineralized into CO₂ by other sequences of β-oxidation. Alternatively, the 4-Me fatty acid formed following the addition of carbon at C-2 [or C-(ω-1)] can be desaturated at C-11 (or, to a lesser extent, at other carbons), constituting anabolic reactions (Fig. 5).

Phylogenetic analyses showed that D. aliphaticivorans strain CV2803T is closely related to strains AK-01 and Pnd3, which may represent other species in the same genus (10). Moreover, as mentioned above, the carbon number of 1-alkene substrates has the same effect on the fatty acid compositions of the three strains. These similarities suggest that reactions of 1-alkene metabolism in strains AK-01 and Pnd3 are similar to those in D. aliphaticivorans. Strain Hxd3 is less phylogenetically related to D. aliphaticivorans than strains AK-01 and Pnd3 (10), but its fatty acid composition shows the same dependence on the carbon chain length of 1-alkenes when these compounds are used as growth substrates (2). It is thus possible that strain Hxd3 also possesses the same alkene-activating enzyme(s) as D. aliphaticivorans, catalyzing the oxidation of the double

FIG. 5. Proposed pathways for anaerobic 1-alkene metabolism by the sulfate-reducing bacterium D. aliphaticivorans strain CV2803T.
It is noteworthy that the growth of *D. aliphaticivorans* on *d*31-1-hexadecane led to the formation of deuterated 10-methylated fatty acids (*d*15-10-Me-16:0 and *d*16-10-Me-14:0) containing only one deuterium atom less (at C-10) than their *n*-saturated precursors (*d*16-16:0 and *d*18-14:0, respectively) (Fig. 3). Interestingly, a similar feature (the loss of only one deuterium atom at C-10 during C-10 methylation of *n*-saturated fatty acids) was observed during the anaerobic metabolism of perdeuterated pentadecane by strain Hx3 (29). Saturated midchain methyl-branched fatty acids are relatively common in bacteria (15) and are often assumed to be biosynthesized by the methylation of a monounsaturated linear fatty acid with *S*-adenosylmethionine as the methyl donor (2, 20). In the present case, this mechanism of methylation does not seem to be involved since desaturation and subsequent methylation at C-10 of the deuterated *n*-saturated fatty acids would have caused the loss of at least two deuterium atoms. Moreover, monounsaturated deuterated fatty acids that could have constituted potential intermediates in 10-Me-branched fatty acid biosynthesis were not detected. The present mechanism of methylation remains unknown but undoubtedly deserves further attention.

In summary, we provide direct evidence that 1-alkenes are anaerobically transformed into fatty acids by *D. aliphaticivorans* strain CV28033 and that different initial reactions of anaerobic 1-alkene degradation can occur simultaneously in this strain. The detailed characterization of labeled fatty alcohol and fatty acid metabolites demonstrates that *D. aliphaticivorans* metabolizes 1-alkene by the oxidation of the double bond at C-1 and by the subterminal addition of organic carbon(s) at both ends of the carbon chain. Although 1-alkenes are clearly not intermediates of the anaerobic *n*-alkene metabolism in *D. aliphaticivorans*, the present results suggest that the two classes of compounds may have some common pathways for their anaerobic oxidation.

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