Stable Isotopic Studies of *n*-Alkane Metabolism by a Sulfate-Reducing Bacterial Enrichment Culture

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Gas chromatography-mass spectrometry and nuclear magnetic resonance spectroscopy were used to study the metabolism of deuterated *n*-alkanes (C_6 to C_{12}) and 1-^{13}C-labeled *n*-hexane by a highly enriched sulfate-reducing bacterial culture. All substrates were activated via fumarate addition to form the corresponding alkylsuccinic acid derivatives as transient metabolites. Formation of d_{14}-hexylsuccinic acid in cell extracts from exogenously added, fully deuterated *n*-hexane confirmed that this reaction was the initial step in anaerobic alkane metabolism. Analysis of resting cell suspensions amended with 1-^{13}C-labeled *n*-hexane confirmed that addition of the fumarate occurred at the C-2 carbon of the parent substrate. Subsequent metabolism of hexylsuccinic acid resulted in the formation of 4-methyloctanoic acid, and 3-hydroxy-4-methyloctanoic acid was tentatively identified. We also found that ^{13}C nuclei from 1-^{13}C-labeled *n*-hexane became incorporated into the succinyl portion of the initial metabolite in a manner that indicated that ^{13}C-labeled fumarate was formed and recycled during alkane metabolism. Collectively, the findings obtained with a sulfate-reducing culture using isotopically labeled alkanes augment and support the previously proposed pathway (H. Wilkes, R. Rabus, T. Fischer, A. Armstroff, A. Behrends, and F. Widdel, Arch. Microbiol. 177:235–243, 2002) for metabolism of deuterated *n*-hexane by a denitrifying bacterium.

The anaerobic biodegradation of *n*-alkanes is now a well-documented process that has been demonstrated to occur under nitrate-reducing, sulfate-reducing, and methanogenic conditions. Several sulfate-reducing and denitrifying bacterial strains and enrichment cultures capable of complete *n*-alkane mineralization have been described (1, 7, 8, 13, 16, 17, 19, 20). Anaerobic metabolism of *n*-alkanes has been shown to occur by at least two mechanisms, both of which include addition of carbon to the parent substrate. An activation mechanism involves an initial carboxylation reaction. Using the sulfate-reducing isolate strain Hxd3, So et al. (21) showed that ^{13}C-bicarbonate was added to the C-3 position of the parent alkane, while subsequent reactions involved elimination of the two adjacent terminal carbon atoms, yielding a fatty acid with one less carbon atom than the original hydrocarbon. Alternately, the primary attack on an alkane can be initiated by addition to the double bond of fumarate with the formation of alkylsuccinic acid derivatives (13, 18). This mechanism seems to be more widespread as it has now been found in three separate anaerobic cultures (6, 7, 13, 18, 24). This reaction resembles the anaerobic biodegradation of toluene and xylene, in which the aryl methyl carbon of the substrate is added across the double bond of fumarate to form benzylsuccinate or methylated derivatives (2, 3). However, alkanes are not activated at the terminal methyl group. The succinyl moiety is attached to the parent alkane at the C-2 or C-3 position (7, 13, 18). The biochemistry of this conversion is incomplete, but electron paramagnetic resonance spectroscopy has suggested a possible

radical mechanism since an organic radical was detected in *n*-hexane-grown cells of the denitrifying bacterium strain HxN1 (18). Similarly, the benzylsuccinate synthase catalyzing toluene activation was shown to be a glycyl radical enzyme (12, 14).

Subsequent steps in anaerobic alkane metabolism are beginning to be elucidated. Studies of strain HxN1 grown with deuterated *n*-hexane or fumarate revealed the formation of a suite of fatty acid metabolites (23). The labeling patterns associated with identification of 4-methyloctanoic acid suggested that hexylsuccinate [(1-methylpentyl)succinate] underwent a carbon skeleton rearrangement and decarboxylation step prior to further oxidation. The identification of other transient metabolites, such as 4-methyloct-2-enoic acid and 3-hydroxy-4-methyloctanoic acid, allowed Wilkes et al. (23) to propose an anaerobic alkane pathway that included the regeneration of fumarate from propionate in a reaction sequence involving yet another carbon skeleton rearrangement similar to the known methylmalonyl-coenzyme A (methylmalonyl-CoA) pathway (11).

We examined the metabolism of isotopically labeled *n*-alkanes by a highly enriched sulfate-reducing bacterial culture. We focused on the metabolic fate of 1-^{13}C-labeled *n*-hexane rather than on deuterium-labeled parent alkanes. Our findings largely agree with the pathway for alkane degradation by a denitrifying bacterium initially proposed by Wilkes et al. (23). However, we could neither confirm nor rule out the possibility that a carbon skeleton rearrangement metabolite was produced during alkane metabolism.

MATERIALS AND METHODS

Sulfate-reducing enrichment culture. An alkane-degrading culture (ALDC) was enriched from oily sludge collected from a U.S. Navy wastewater storage facility. The enrichment was cultivated under strictly anaerobic conditions (sulfate reducing) in a brackish mineral medium (22) as previously described (13).

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After repeated transfers (20%) and preparation of dilution series in the mineral medium with hexane or decane as a growth substrate, the culture consisted of morphologically similar short oval rods and less than 1% other bacteria. The culture was routinely grown in 160-ml serum bottles containing 25 ml of the mineral medium (22) supplemented with 15 to 25 μl of sterile anoxic *n*-C$_6$, *n*-C$_8$, *n*-C$_{10}$, or *n*-C$_{12}$. Rates of alkane metabolism were determined in exponentially growing cultures before the optical density at 600 nm reached 0.2. All preparations were sealed with Teflon-lined stoppers and incubated inverted at 31°C.

When isotopically labeled substrates were employed, hexane-, octane-, or decane-grown ALDC cells were incubated with 20 μl of the corresponding protonated or fully deuterated (CDN Isotopes, Pointe-Claire, Quebec, Canada) alkane. Cultures used for ^{13}C nuclear magnetic resonance (NMR) analysis were grown under sulfate-limiting conditions with 15 μl of 1-^{13}C-labeled *n*-hexane (Isotec, Miamisburg, OH) and incubated for 6 weeks. The alkane degradation results were in good stoichiometric agreement with the theoretically expected amount of sulfate reduction (94% ± 5%), so the progress of various experiments was monitored by electron acceptor consumption by ion chromatography (5) or by protein accumulation (BCA protein assay reagent kit; Pierce, Rockford, IL). Alkane losses were monitored by gas chromatography (GC) as previously described (13).

Preparation of cell extracts and suspensions. The cells in the ALDC (1 liter) were anaerobically collected in the late exponential phase by centrifugation (16,000 × *g*, 15 min, 4°C) in sealed bottles and washed once in anoxic 20 mM morpholinepropanesulfonic acid (MOPS) buffer (pH 7.2) containing 5 mM MgCl$_2$ and 1 mM dithiothreitol. The washed cells were resuspended in 10 ml of the same buffer and divided into two aliquots. Each aliquot was centrifuged again and suspended in MOPS buffer (pH 7.2) containing TiCl$_3$ (1 mM) or Tris-HCl buffer (100 mM; pH 7.8) containing 5 mM MgCl$_2$ and 1 mM TiCl$_3$. Two milliliters of each cell suspension was disrupted with a French press (69 mPa), and the resulting crude extracts were used for in vitro assays. Unbroken cells were diluted 2.5-fold in the appropriate buffers and used for comparative purposes. All incubations were conducted inside sealed test tubes in a well-working anaerobic glove box containing an N$_2$-H$_2$ (95:5) atmosphere. The in vitro assay mixtures (total volume, 1.0 ml) contained 0.9 ml of crude extract, 1.5 μmol [1,2,3,4-^{13}C]fumaric acid (CIL, Andover, MA), and 10 μl fully deuterated d_{14}-*n*-hexane (CDN Isotopes, Pointe-Claire, PQ, Canada). The protein concentration in crude extracts was 2.8 mg/ml. The reaction was stopped after 16 or 26 h of incubation by addition of 50 μl of 6 N HCl, and the assay mixtures were extracted and analyzed by gas chromatography coupled with mass spectrometry (MS) (see below). Concentrated cell suspensions were incubated identically and used for comparative purposes. However, some preparations were also supplemented with 0.14 mmol sulfate. The final volume was adjusted to 1.0 ml, and the cell suspensions were incubated for 3 days prior to extraction and metabolite analysis.

Extraction and metabolite analysis. All preparations were acidified to pH <2 with H$_2$SO$_4$ prior to extraction with ethyl acetate. In some cases, cultures were initially treated with NaOH (pH >12) for 30 min to cleave presumed CoA thioester derivatives. Organic extracts were concentrated by rotary evaporation and then reacted with *N,O*-bis(trimethylsilyl)trifluoroacetamide (Pierce Chemical Co., Rockford, IL) to obtain trimethylsilyl (TMS) derivatives. Derivatized metabolites were separated using an HP 5890 GC equipped with a DB-5 capillary column (30 m by 0.25 mm [inside diameter]; J&W Scientific, Folsom, CA). The temperature was held at 40°C for 2 min, raised at a rate of 4°C per min to 240°C, and then held at 240°C for 8 min. The temperature of the injector and the transfer line from the GC to the MS was kept at 250°C. An HP 5970 MS detector was used in the scan mode from 50 to 600 mass units. Metabolites were identified based on matching the GC retention times and mass spectral profiles with those of authentic standards or by comparison with previously published mass spectra. 4-Methyloctanoic acid was acquired from Sigma-Aldrich (St. Louis, MO). *n*-Octylsuccinic acid was prepared by base hydrolysis from *n*-octylsuccinic anhydride (TCI America, Portland, OR) as previously described (9).

^{13}C nuclear magnetic resonance spectroscopic analysis. Underivatized ethyl acetate extracts of ^{13}C-amended cultures were concentrated by rotary evaporation and transferred into 5-mm NMR tubes. Extracts were dried under N$_2$ and redissolved in 1,4-dioxane-d_8 (CIL, Andover, MA). An additional culture was amended with unlabeled hexane to ensure that the observed NMR signals did not result from ^{13}C impurities present in the solvents used. The NMR spectra of the organic solvent-extracted samples were obtained with a Unity INOVA 400-MHz NMR (Varian) with a ^{13}C resonance frequency of 100.567 MHz. The proton-decoupled ^{13}C spectra were obtained at 30°C using a standard inverse-gated pulse sequence. The following experimental parameters were employed: sweep width, 25,000 Hz; acquisition time, 1.199 s; recycle time, 2 s; and number of acquisitions, 35,000. The data were processed with 1-Hz line broadening. Chemical shifts were referenced relative to the center peak of the 1,4-dioxane

pentet centered at 66.0 ppm. Metabolite chemical shift values were predicted using ChemNMR 5.0 (CambridgeSoft, Cambridge, MA). Predicted chemical shifts were allowed a tolerance of ±2 ppm. Pure (1-phenylethyl)succinic acid (ethylbenzylsuccinic acid) used for comparative NMR experiments was synthesized and purified as described by Bickford et al. (4). Additional NMR experiments included distortionless enhancement by polarization transfer (number of protons attached to each carbon), heteronuclear single quantum coherence (^{1}H-^{13}C coupling), and heteronuclear multiple-bond correlation (long-range ^{1}H-^{13}C coupling).

RESULTS

Growth and metabolite formation with H- and *d*-alkanes. The ALDC grew with *n*-alkanes ranging from C$_6$ to C$_{12}$. The biodegradation rate with individual alkanes decreased as the alkane chain length increased, presumably reflecting differences in the relative aqueous solubilities of the substrates. Hexane, decane, and dodecane were degraded at rates of 2.45, 1.76, and 0.95 μmol alkane/day (±5%), respectively. The fumarate addition metabolites associated with protonated or deuterated hexane, octane, and decane showed the characteristic mass spectral features associated with anaerobic alkane degradation (9, 13; data not shown). All six protonated succinyl metabolites showed abundant MS fragments at *m/z* 73 and *m/z* 147, attesting to their diderivatization with *N,O*-bis(trimethylsilyl)trifluoroacetamide (16). The McLafferty rearrangement ion associated with the GC-MS analysis of these metabolites was also observed (13, 15). This included major ions at *m/z* 262 and *m/z* 264 for the protonated and deuterated fumarate addition metabolites, respectively (9, 13). Fragmentation of the McLafferty rearrangement signal [HOSi(CH$_3$)$_3$ and DOSi(CH$_3$)$_3$] that resulted in ions at *m/z* 172 and 173, respectively, was also noted. Losses of CO$_2$H or CO$_2$D (or both) from the McLafferty rearrangement signal accounted for the *m/z* 217 ion in the spectra of the fumarate addition metabolites obtained from protonated alkanes or the *m/z* 218 and 219 ions in the spectra of the corresponding deuterated alkane metabolites. The ion with the largest mass in the spectra of these metabolites occurred at *m/z* 331, 359, 387, or 415 with protonated hexane, octane, decane, or dodecane as the parent substrate, while the corresponding fragments for the deuterated alkanes were at *m/z* 345, 377, 409, and 441. The latter ions were 14, 18, 22, and 26 atomic mass units larger than the corresponding protonated derivatives and accounted for all of the deuterium atoms present in the isotopically labeled parent alkane. However, these masses are not the masses for the molecular ions (M$^+$) of the metabolites but rather are the masses for the M$^+$-15 ions, as is typical of TMS esters (16). Thus, all of the alkanes studied were metabolized by the ALDC in an analogous fashion.

Multiple GC peaks that had the same MS features were typically observed for the succinyl metabolites produced during anaerobic alkane biodegradation. This finding presumably reflected the formation of diasteriomers, as previously described (13, 18). A synthesized authentic standard of *n*-octylsuccinic acid analyzed in the same fashion resolved as a single GC peak with a different retention time but the same MS fragmentation pattern as the fumarate addition metabolite of octane. Therefore, the alkane metabolites detected also likely bore the succinyl moiety at a subterminal position in a manner similar to that reported previously (13, 18).
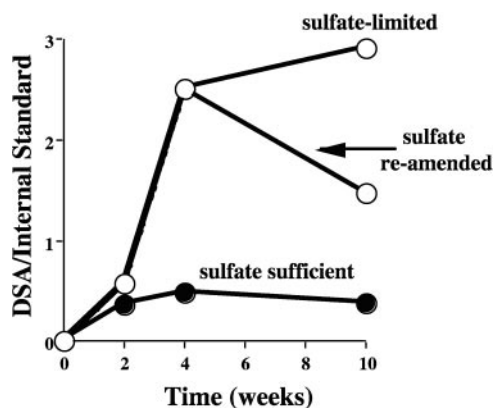
FIG. 1. Formation of *d*-decylsuccinic acid (DSA) from deuterated decane by the ALDC with different amounts of sulfate. The amount of decylsuccinic acid is expressed as the GC-MS response of the metabolite peak relative to a hexadecane internal standard (0.033 μl) added to each extract prior to analysis.

The pattern of alkylsuccinic acid accumulation was monitored as the ALDC was grown on fully deuterated decane ($C_{10}D_{22}$) under sulfate-sufficient and sulfate-limited conditions. Use of the deuterated substrate allowed us to track the formation of the addition product de novo and to distinguish it from the nonisotopically labeled metabolite pool. Under sulfate-sufficient conditions, complete mineralization of decane according to the theoretically expected stoichiometry was observed (25 mM Na_2SO_4 for 3 mM decane), while only about one-half of the initial decane added could be mineralized with 10 mM Na_2SO_4. Metabolites were typically found only at trace levels but accumulated when sulfate was limiting (Fig. 1). Subsequent reamendment with sulfate caused the level of decyl-

succinic acid to decrease, attesting to the transient nature of the metabolite (Fig. 1).

Formation of hexylsuccinic acid in vitro and in cell suspension experiments. In experiments with crude cell extracts and nongrowing cell suspensions, d_{14}-*n*-hexane and [1,2,3,4-^{13}C]fumarate were used to distinguish reaction products from preexisting metabolites. The reactions were performed at two different pH values (pH 7.2 and 7.8) to determine the optimal conditions. Hexylsuccinic acid was formed both in the crude extracts and in the whole-cell suspensions. Crude extracts catalyzed the formation of hexylsuccinic acid only at pH 7.2; no metabolite was formed at pH 7.8. However, in the cell suspensions, hexylsuccinate was formed at both pHs. The presence of sulfate in the cell suspensions did not influence the pattern of metabolite accumulation as the fumarate addition product was detected both in its presence and in its absence. The hexylsuccinic acid metabolite contained all of the deuterium atoms from d_{14}-*n*-hexane, but it did not incorporate any ^{13}C from [1,2,3,4-^{13}C]fumarate (Fig. 2).

GC-MS results with ^{13}C-labeled hexane. In cultures incubated with 1-^{13}C-labeled *n*-hexane, hexylsuccinic acid was also detected (as the di-TMS ester). The MS profile of this metabolite revealed M^+-15 ions at *m/z* 331, 332, 333, and 334, suggesting that the extracted molecule incorporated up to three ^{13}C atoms (Fig. 3). Such a molecule could have resulted if the ^{13}C label of the *n*-hexane substrate ultimately produced fumarate that was then repeatedly recycled back into the metabolic scheme. The relative abundance of the M^+-15 fragment at *m/z* 334 was consistently one-half that of the fragment at *m/z* 332 and 333; this value is much greater than would be expected based upon the natural abundance alone.

In addition, 4-methyloctanoic acid was positively identified in culture supernatants by GC-MS (Fig. 4A and B). In the organic
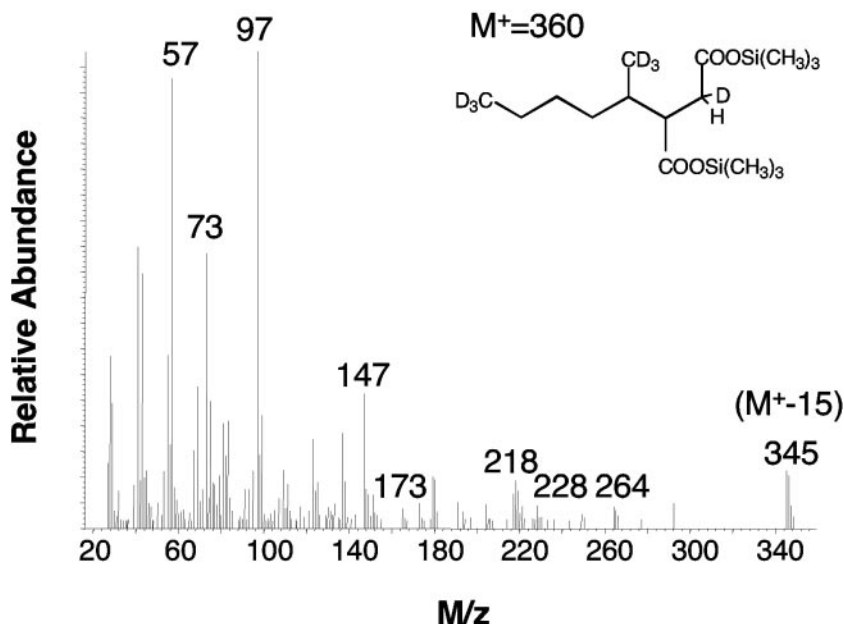

FIG. 2. Mass spectrum of TMS-derivatized d_{14}-hexylsuccinic acid formed during incubation of cell-free ALDC extracts incubated with d_{14}-*n*-hexane.
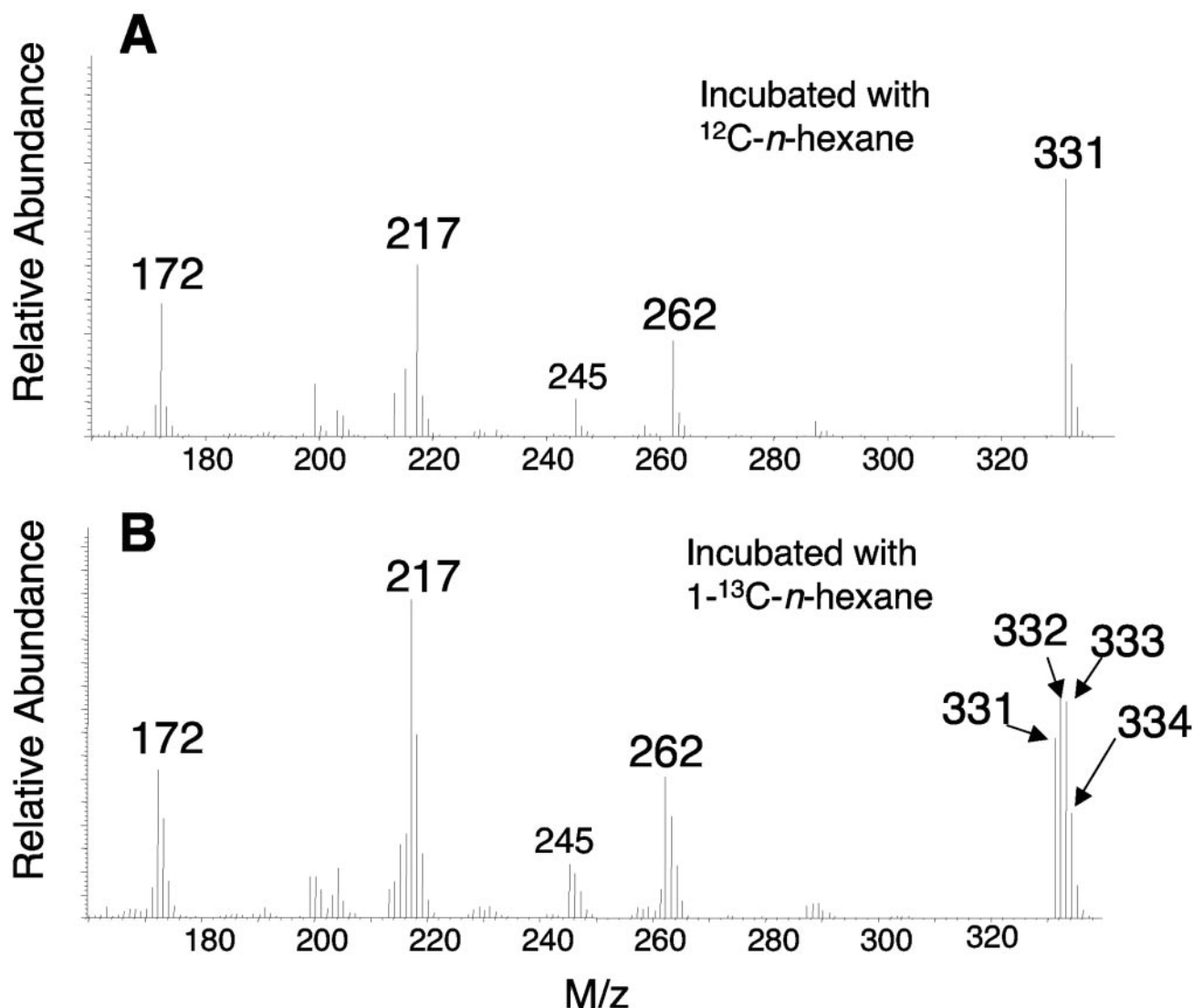
FIG. 3. Portion of the total mass spectrum of the di-TMS-derivatized hexylsuccinic acid formed by the ALDC during *n*-hexane (A) or 1-^{13}C-labeled *n*-hexane (B) metabolism.

extracts of ^{12}C-hexane-amended culture supernatants, the GC retention time and the MS profile of this metabolite were identical to those of an authentic standard (not shown). This metabolite was previously shown to be produced during hexane degradation by a nitrate-reducing isolate (23). For the ^{13}C-amended culture extracts, there was a peak whose GC retention time also matched the GC retention time of authentic 4-methyloctanoic acid, but the MS profile differed slightly in that fragment ions were shifted up to 2 mass units higher than those of the standard (Fig. 4B). A third metabolite (Fig. 4C and D) was tentatively identified as TMS-derivatized 3-hydroxy-4-methyloctanoic acid based on fragmentation comparisons with the same methyl-esterified metabolite proposed previously (23). In the MS profile reported by Wilkes et al. (23), this metabolite had a mass of 188 and a base peak at *m/z* 103, a difference of 85 mass units. Similarly, in the MS profile of the peak detected in our unlabeled culture extracts, a presumptive M[+]-15 of *m/z* 303 was seen, resulting in a molecular mass of 318 (Fig. 4C). With this metabolite, a base

peak was observed at *m/z* 233, also a difference of 85 mass units, like the difference observed by Wilkes et al. (23). Other key ions were at *m/z* 73, 147 (indicating that two functional groups were derivatized with TMS), and 187 [loss of —CH_2-COO-Si(CH_3)$_3$]. In the MS profile presented by Wilkes et al. (23), a comparable fragment was also seen at *m/z* 115 (loss of —CH_2-COO-CH_3). In the [13C]hexane-amended cultures, a metabolite with similar MS features was also detected and eluted at the same GC retention time, but it had fragments up to 2 mass units higher (Fig. 4D). Based on these comparisons we tentatively identified this metabolite, but confirmation awaits the availability of an authentic standard.

[13]C nuclear magnetic resonance spectroscopic analysis. The ^{13}C NMR spectrum of the unlabeled control sample did not display any signals other than that of the solvent 1,4 dioxane-d_8 (data not shown), demonstrating that all the signals present in the ^{13}C-labeled sample spectrum resulted from 1-^{13}C-labeled *n*-hexane and metabolites. Figure 5 shows the ^{13}C NMR spec-
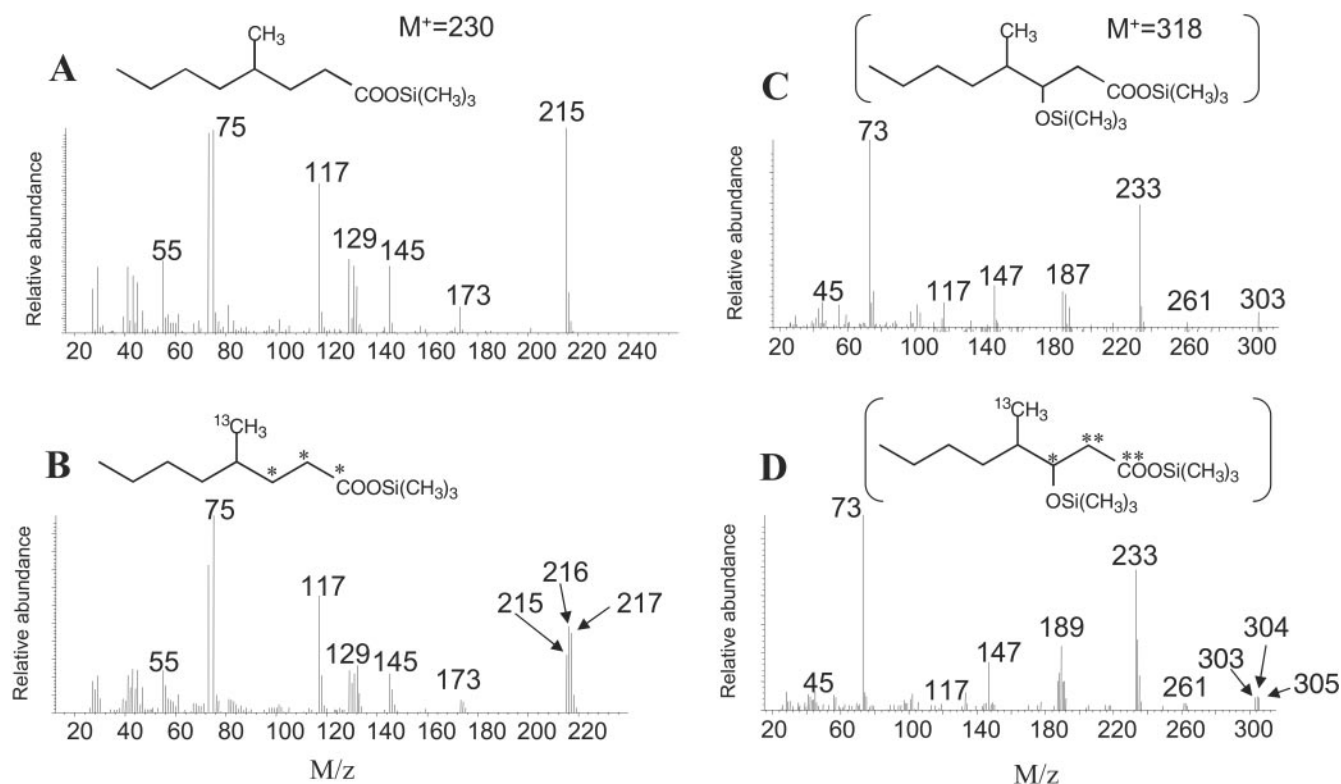
FIG. 4. (A and B) Mass spectra of 4-methyloctanoic acid (as the TMS derivative) produced during *n*-hexane (A) or 1-^{13}C-labeled *n*-hexane (B) metabolism by the ALDC. (C and D) Mass spectra of tentatively identified 3-hydroxy-4-methylocanoic acid (as the TMS derivative) produced during *n*-hexane (C) or ^{13}C-labeled *n*-hexane (D) degradation. The asterisks in panels B and D indicate possible sites of ^{13}C incorporation from labeled regenerated fumarate molecules.

trum of the ^{13}C-labeled sample after 6 weeks of incubation. ^{13}C NMR signals were detected only in the alkyl (0 to 50 ppm) and carboxylic acid (170 to 180 ppm) regions. Table 1 shows the chemical shift of each signal and indicates its functional group. The presence of a limited number of distinct ^{13}C NMR signals indicates that there was formation of discrete ^{13}C-labeled metabolites rather than uniform incorporation of the ^{13}C label into microbial lipid molecules, which, if it had occurred, would have given rise to a great array of fatty acid methylene signals. The comparison shown in Table 2 of ^{13}C NMR spectral chemical shifts with predicted ^{13}C chemical shifts for carbons expected to be ^{13}C labeled according to the pathway outlined by Wilkes et al. (23) and compounds observed by GC-MS supports the presence of hexylsuccinic acid and 4-methyloctanoic acid. Figure 6A shows the carbon atoms expected to be ^{13}C labeled based on the proposed pathway of Wilkes et al. (23) and demonstrates that hexylsuccinic acid is unique in that it contains two chiral centers, one at the C-2 carbon of hexane and the other at the methine carbon of the succinic acid moiety (carbon B in Fig. 6A). Depending on the isomeric configuration of these sites, it is possible that the ^{13}C-labeled carbons A, B, C, D, and E are in different chemical environments. If these chemical environments are unique, each ^{13}C-labeled carbon should produce two ^{13}C NMR signals, one for each of the enantiomeric configurations. As noted in Table 1, this effect was observed for all the ^{13}C-labeled carbons in hexylsuccinic acid. Likewise, 4-methyloctanoic acid has a chiral center at the

C-2 carbon of the hexane backbone, resulting in two ^{13}C NMR signals for the methylene carbon labeled G in Fig. 6A. This NMR chiral effect was observed and confirmed using an authentic (1-phenylethyl)succinic acid standard, which also has two chiral carbons (Fig. 6B). The ^{13}C NMR spectrum of the latter compound had "doublets" for the carbons present in the 1-phenylethyl and succinic acid moieties. Other NMR experiments (distortionless enhancement by polarization transfer, heteronuclear single quantum coherence, and heteronuclear multiple-bond correlation) confirmed that these "doublets" resulted from different enantiomeric configurations of (1-phenylethyl)succinic acid (data not shown). Finally, the ^{13}C NMR results also strongly indicate that the C-2 carbon of hexane is the prominent site for addition to fumarate, rather than the C-1 or C-3 carbon. Fumarate addition to the C-1 and C-3 carbons of hexane results in predicted chemical shifts of 26.7 and 11.8 ppm, respectively, neither of which was detected in the ^{13}C NMR spectrum. Moreover, the presence of carboxylic acid signals between 171 and 176 ppm illustrated that there was incorporation of ^{13}C label into the carboxylic acid functional groups of hexylsuccinic acid and 4-methyloctanoic acid.

DISCUSSION

Our study confirmed that fumarate addition reactions represent a common mechanism for anaerobic alkane oxidation independent of alkyl chain length. All alkanes tested, ranging
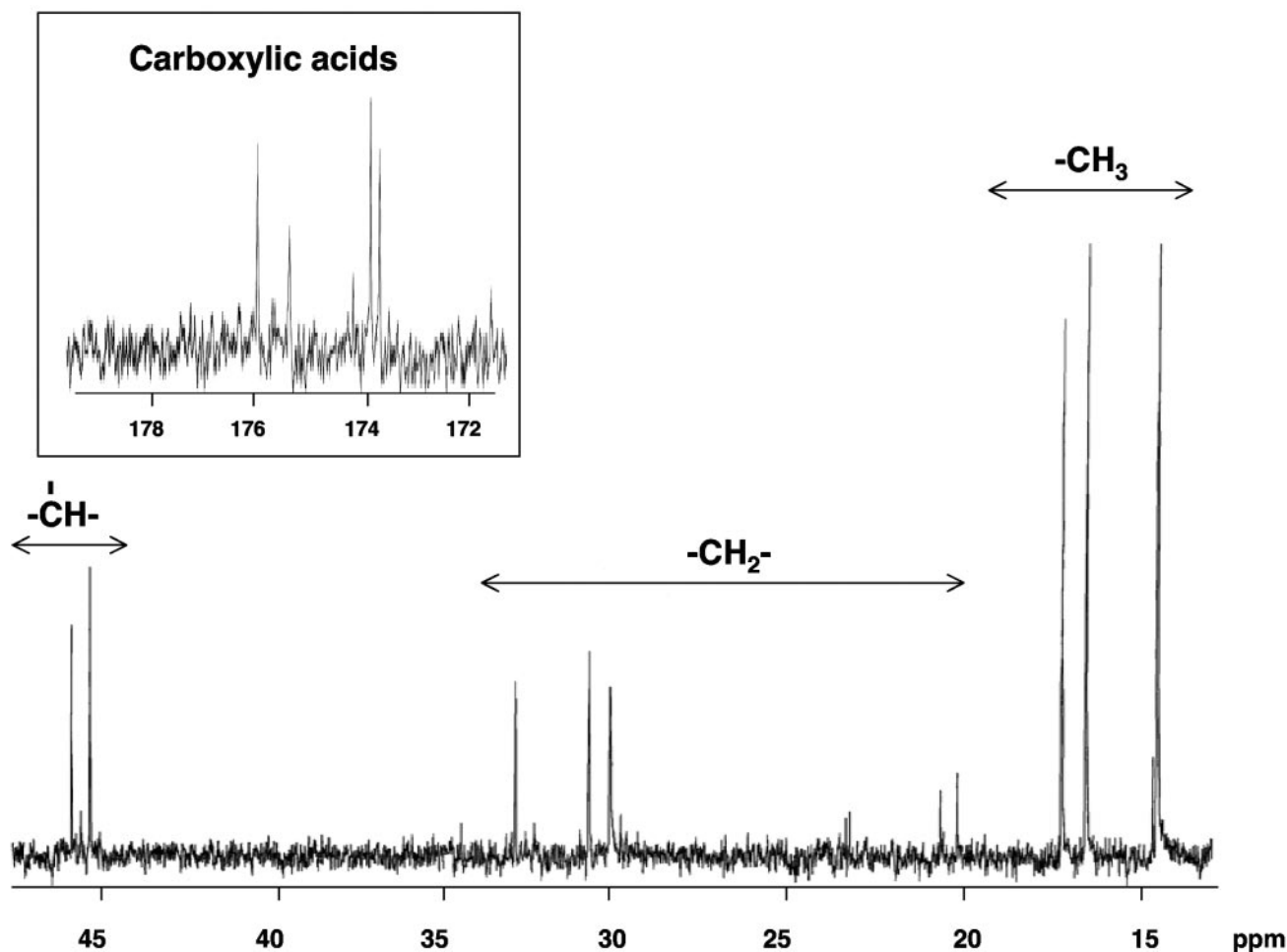
FIG. 5. ^{13}C NMR spectrum of the ^{13}C-labeled metabolites produced by the ALDC after 6 weeks of incubation.

from C_6 to C_{12}, formed corresponding alkylsuccinic acid derivatives. The transient nature of alkylsuccinate formation confirmed that the addition product is the first step leading to complete oxidation, rather than a dead-end product. The in

TABLE 1. NMR data for signals detected in cultures
grown on [1-^{13}C]-labeled *n*-hexane

^{13}C (ppm)	Functional group
14.34	Methyl (C-1 hexane)
14.80	Methyl
16.41	Methyl
17.12	Methyl
20.16	Methyl
20.62	Methyl
30.38	Methylene
30.98	Methylene
33.18	Methylene
45.70	Methine
46.25	Methine
173.88	Carboxylic acid
174.02	Carboxylic acid
175.32	Carboxylic acid
175.84	Carboxylic acid

vitro experiment showing the formation of hexylsuccinic acid from fully deuterated hexane with retention of all deuterium atoms in crude cell extracts confirmed that this is indeed an initial step in the anaerobic alkane degradation pathway. Subsequent reactions following alkylsuccinate formation are not well documented. The pathway proposed by Wilkes et al. (23) was based on metabolic studies of denitrifying alkane-degrading strain HxN1 cultures growing with unlabeled and deuterated hexane and amended with 2,3-d_2-fumarate. The detection of 4-methyloctanoic acid with deuterium in the C-3 position and also 4-methyloct-2-enoic acid, 3-hydroxy-4-methyloctanoic acid, and 2-methylhexanoic acid led to a hypothetical cycle in which alkane oxidation might be coupled to fumarate regeneration. The identification of 4-methyloctanoic acid with deuterium in the C-3 position was crucial evidence that supported the proposed model. A deuterium atom in the C-3 position suggested that the product of fumarate addition, hexylsuccinic acid, could possibly undergo carbon rearrangement of the carbon skeleton prior to decarboxylation. If such a rearrangement occurred, a deuterium atom from the C-2 position was moved toward the methyl group with opposite migration of a carboxyl group (or its thioester), yielding (2-methylhexyl)malonyl-CoA. After decarboxylation the product, 4-methyloctanoyl-CoA, would go through β-oxidation, yielding 2-methylhexanoyl-

TABLE 2. Actual and predicted ^{13}C NMR signals
for proposed metabolites[a]

Predicted results (ppm)	NMR results (ppm)	Functional group
Hexylsuccinic acid		
14.0	14.34	Methyl (if addition to fumarate occurred at C-5 rather than at C-2)
17.0	16.41	Methyl closest to succinic acid
32.7	30.98	Methylene in succinic acid
44.0	45.70, 46.25	Methine in succinic acid
4-Methyloctanoic acid		
14.0	14.34	Methyl (if addition to fumarate occurred at C-5 rather than at C-2)
19.7	17.12	Methyl closest to succinic acid
32.0	30.38	Methylene "alpha" to carboxylic
33.2	33.58	Methylene "beta" to carboxylic

[a] For hexane the 14.34 ppm signal is also from unreacted ^{13}C-labeled hexane. This signal also appears for metabolites in which the fumarate is added to the unlabeled end of hexane.
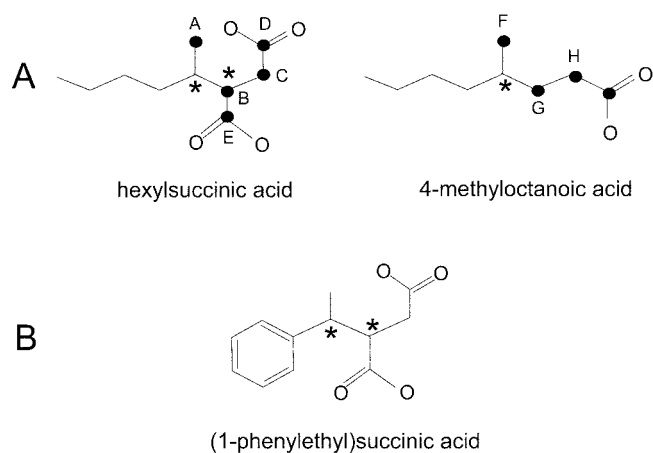

FIG. 6. (A) Molecular structure of hexylsuccinic acid and 4-methyloctanoic acid showing ^{13}C-labeled carbons (solid circles), as predicted by the cycling of fumarate in the proposed pathway (23), and chiral carbons (asterisks). (B) Molecular structure of (1-phenylethyl)succinic acid showing chiral carbons (asterisks) used for comparison in NMR experiments.

CoA, as well as the intermediate metabolites 4-methyloct-2-enoic acid and 3-hydroxy-4-methyloctanoic acid, all of which were identified as methyl esters in strain HxN1 culture extracts. Another cycle of β-oxidation would produce propionyl-CoA and butyryl-CoA from 2-methylhexanoyl-CoA. According to the model, butyryl-CoA would be oxidized further to acetyl-CoA and CO_2, and propionyl-CoA would be transformed to fumarate via transcarboxylation with (2-methylhexyl)malonyl-CoA as the carboxyl donor, thus regenerating a cosubstrate and closing the cycle. (2-Methylhexyl)malonyl-CoA was not detected in the study. However, while the detection of $3\text{-}d_1\text{-}4$-methyloctanoic acid as a product strongly argued in favor of simultaneous rearrangement and decarboxylation, proof was lacking.

Our studies on [^{13}C]hexane metabolism produced more data that support the proposed model. The GC-MS and ^{13}C NMR results indicate that fumarate addition indeed occurs at the C-2 carbon of n-hexane. Moreover, our results illustrate that multiple ^{13}C nuclei from ^{13}C-labeled hexane molecules are incorporated into the metabolites, demonstrating that ^{13}C-labeled fumarate is produced and recycled in this process. In extracts of cultures grown with ^{13}C-labeled n-hexane, GC-MS detection of M^+-15 ions at m/z 331, 332, 333, and 334 (Fig. 3B) illustrated that the extracted hexylsuccinic acid molecule contained up to three ^{13}C atoms, showing that the ^{13}C label at the C-1 carbon of hexane had been recycled multiple times through the degradation pathway. Likewise, the lack of ^{13}C incorporation from labeled [1,2,3,4-^{13}C]fumarate into hexylsuccinic acid in the in vitro and the cell suspension experiments suggested that preformed fumarate from internal cellular pools was used preferentially, thereby indirectly supporting the model for cosubstrate regeneration. Such selectivity in the formation of a fumarate addition product could also be explained by the fact that substitution of heavy isotopes into molecules produces a stronger bond (10), and therefore more energy may be required for formation of a new $^{12}\text{C-}^{13}\text{C}$ bond in the alkyl addi-

tion reaction, thereby possibly placing thermodynamic constraints on the involvement of exogenously added [1,2,3,4-^{13}C]fumarate in cell metabolism.

Figure 7 shows the pathway for anaerobic alkane metabolism that was initially proposed by Wilkes et al. (23) and that is supported by the results of the present study. If fumarate adds to the C-2 carbon adjacent to the ^{13}C-labeled C-1 carbon of a molecule of n-hexane, the ^{13}C-labeled C-1 carbon is incorporated as the C-2 carbon of fumarate upon completion of one metabolic cycle (carbon 1 of fumarate in Fig. 7). Addition of this ^{13}C-labeled fumarate to another molecule of 1-^{13}C-labeled n-hexane, either to the ^{13}C-labeled C-2 carbon or to the unlabeled C-3 carbon of fumarate, results in a covalent bond with the C-2 carbon of the hexane. If the former occurs, then a ^{13}C-labeled methine site (carbon 2a of hexylsuccinic acid in Fig. 7) is created with a predicted chemical shift of 44.0 ppm. If the latter occurs, a ^{13}C-labeled methylene site (carbon 2b of hexylsuccinic acid in Fig. 7) is produced with a predicted chemical shift of 32.7 ppm. Signals with very similar chemical shifts (i.e., 45.70 and 46.25, and 30.98 and 33.18 ppm) were detected in our ^{13}C NMR spectrum. The presence of two methine signals, as well as the two methylene signals, results from the fact that hexylsuccinic acid contains two chiral centers; therefore, methine and methylene carbons 2a and 2b reside in slightly different chemical environments formed by the enantiomers of hexylsuccinic acid, thereby giving rise to two signals for each carbon atom. The nearly equal peak areas (Fig. 5) illustrate that there is minimal stereochemical specificity upon addition of hexane to fumarate. This chiral "doublet" effect is also observed with the ^{13}C NMR signals of the ^{13}C-labeled methyl group of the hexane backbone and both of the ^{13}C-labeled carboxylic acids (Fig. 5, inset). The fact that the carboxylic acid functional groups in the succinic acid moiety of hexylsuccinic acid are ^{13}C labeled strongly indicates that multiple cycling of fumarate occurs. The fact that the experimentally observed ^{13}C NMR chemical shifts closely match those predicted based on the original proposed pathway (23) further supports the valid-
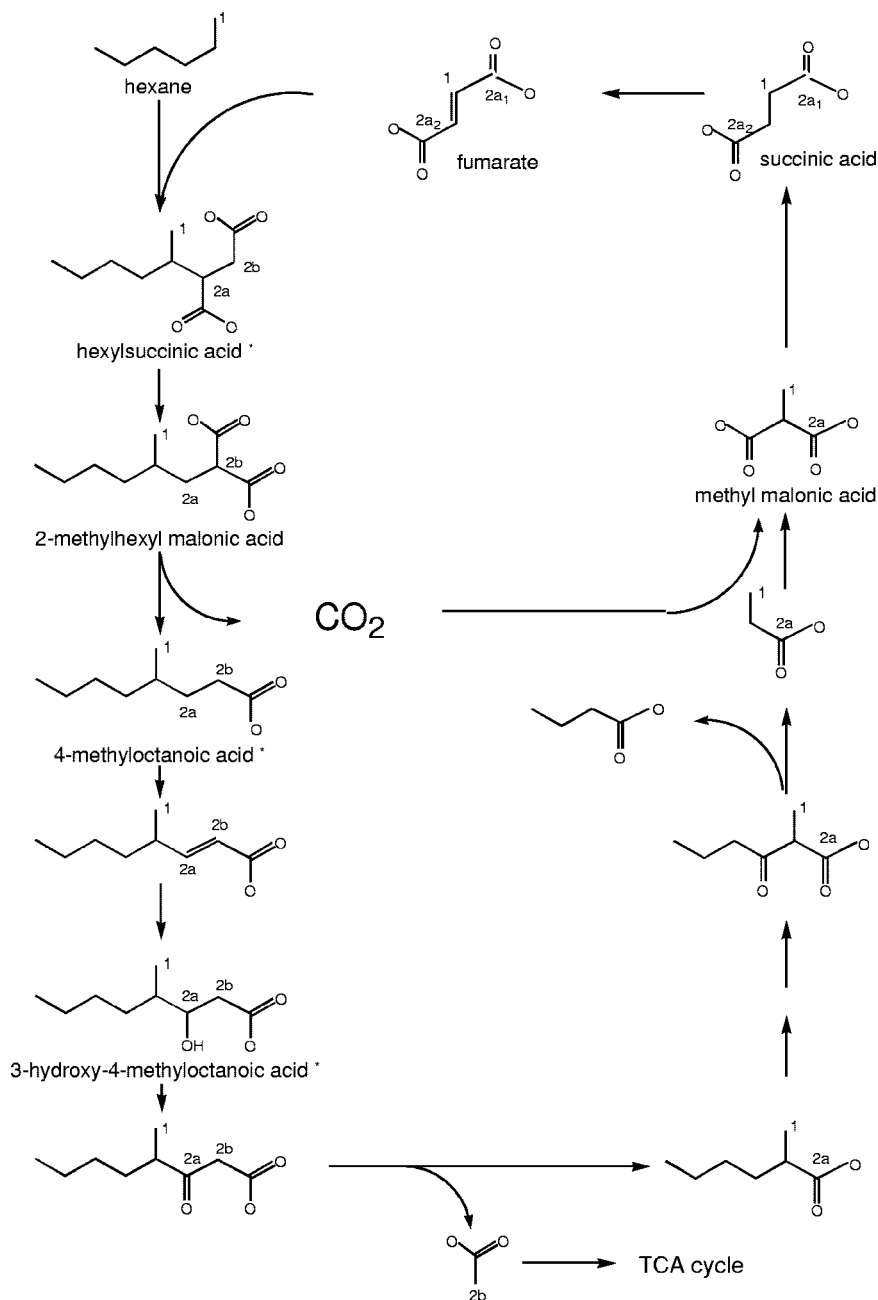
FIG. 7. Pathway of alkane metabolism under sulfate-reducing conditions based on the pathway proposed by Wilkes et al. (23). The numbered carbons indicate the positions of isotopically heavy carbon atoms (^{13}C). The number indicates the metabolic cycle number, and the lowercase letter and subscript indicate the sites of additional label incorporation as a result of pathway cycling. Metabolites that were experimentally detected using ^{13}C NMR and/or GC-MS are indicated by asterisks.

ity of the pathway. Moreover, the presence of multiple ^{13}C nuclei in metabolites supports the idea of a cyclical pathway, with ^{13}C becoming incorporated into fumarate.

The detection of 4-methyloctanoic acid with a deuterium in the C-3 position laid the foundation for the proposed alkane degradation pathway (23). Our studies with [^{13}C]hexane strongly suggest that this metabolite belongs in the pathway as it was identified by GC-MS during alkane metabolism, its presence was supported by ^{13}C NMR analysis, and cultivation experiments showed that the ALDC could use it as a growth

substrate (data not shown). The presence of this metabolite indicated that decarboxylation of hexylsuccinic acid had occurred and apparently requires carbon skeleton rearrangement in a manner similar to that proposed by Wilkes et al. (23). However, the presence of (2-methylhexyl)malonyl-CoA to prove this contention directly was not detected. Nevertheless, the existence of this molecule cannot be ruled out, as this compound could conceivably be destroyed under the acidic conditions used for metabolite extraction. Alternately, the relative rates of the reactions leading from hexylsuccinic acid to

4-methyloctanoic acid could preclude accumulation of the re-arrangement intermediate at a detectable level.

Common metabolites formed during the course of alkane biodegradation, as well as the presence of multiple ^{13}C atoms in the metabolites at the positions predicted by the pathway of Wilkes et al. (23), strongly suggest that the sulfate-reducing culture, ALDC, and a denitrifying strain, HxN1, not only both initiate alkane degradation by fumarate addition but most probably share the entire pathway.

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