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Microbial Transformation of Aliphatic Aldehydes by *Bacillus megaterium* to 2,3-Dialkylacroleins

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The biotransformation of a series of aliphatic aldehydes (C_8-C_{12}) by *Bacillus megaterium* isolated from strawberry leaf surfaces was investigated. Products were isolated by liquid/liquid extraction and analyzed by gas chromatography (GC) combined with mass spectrometry (MS). In addition to aliphatic alcohols and the remaining aldehydes, major transformation products included the corresponding acids as well as 2,3-dialkylacroleins, dehydrated aldol addition products, which were detected for the first time as biotransformation products. To verify the structures, 2,3-dialkylacroleins were chemically synthesized from the appropriate aldehydes by base-catalyzed aldol condensation reactions and characterized by ^{1}H and ^{13}C NMR spectroscopy. Time-course studies showed that the maximum yield of the acrolein derivatives was obtained after 6 days of incubation.

KEYWORDS: Biotransformation; *Bacillus megaterium*; aliphatic aldehydes; acroleins

INTRODUCTION

Aliphatic aldehydes are important volatiles that display a variety of biological activities. They are known to be emitted by plant leaves, flowers, fruits, stems, and roots; synthesized by microorganisms; contained in the scent emitted by the giraffe (*Giraffa camelopardalis reticulate*); and released by insects such as cockroaches, fruit-spotting bug (*Amblypelta nitida*), and ants (*1*−*5*). Numerous investigations have shown that they exhibit antimicrobial properties against pathogenic fungi and bacteria (*6*, *7*), but they can act also as allelochemicals and pheromones (*4*). When administered to mice and rats, nonanal mediates diarrhea (*8*).

The metabolism of unsubstituted aliphatic aldehydes has been studied in mice and rats (*9*). Aldehydes are readily oxidized in the body to the corresponding fatty acids, and subsequently, the resulting short and medium chain length fatty acids are oxidized primarily to CO_2 and water (*10*). Such oxidation takes place rapidly both in the liver and other tissues (*11*). It can be assumed that similar metabolic processes take place in plants and microorganisms. Indeed, the corresponding alcohols and derived esters have been identified as the major transformation products in plants (*12*). However, the microbial transformation of straight chain aldehydes has not been reported in detail until now.

Recently, we isolated a number of bacterial strains from strawberry leaves and tested their ability to metabolize volatiles emitted by the leaves, since volatiles represent one possible nutrition source for epiphytes. It was demonstrated that the bacteria were not capable of using any of the volatiles as a sole carbon source, but some bacteria, such as *Bacillus megaterium*, metabolized a variety of the plant products (unpublished data). *Bacillus* species are versatile chemoheterotrophs capable of respiration using a number of simple organic compounds, and species, such as *B. megaterium*, require no organic growth factors. Since cytochrome P_{450} enzymes from *B. megaterium* catalyze the regio- and enantioselective hydroxylation of a number of organic compounds, this strain has been widely used for biocatalysis purposes (*13*−*15*). We have investigated the biotransformation of C_8-C_{12} aliphatic aldehydes by *B. megaterium* because saturated straight-chain aldehydes consisting of 8−12 carbon atoms constitute important volatiles emitted by plants and are among the frequent volatiles in the atmosphere. All formed products were identified by gas chromatography combined with mass spectrometry (GC−MS). For the first time, 2,3-dialkylacroleins were found as biotransformation products and were confirmed by comparison to the ^{1}H and ^{13}C NMR spectra of the synthesized aldol condensation products.

MATERIALS AND METHODS

Materials and Bacterial Strain. Nonanal, decanal, undecanal, and dodecanal were obtained from Fluka (Deisenhofen, Germany), and octanal and hexanal were purchased from Aldrich (Deisenhofen, Germany). All reagents were of analytical grade or the highest commercial grade available. The strain AY131222 of *B. megaterium* was isolated from strawberry (*Fragaria* × *ananassa*) leaf surfaces and identified on the basis of its biochemical properties and 16 S rDNA sequence. *B. megaterium* (AY131222) can be obtained from Lukas Schreiber, Institute of Cellular and Molecular Botany (IZMB), University Bonn (Kirschallee 1, Bonn, Germany). The strain was maintained on plate count agar (bioMérieux, Marcy l'Etoile, France) and incubated at 30 °C.

Enrichment Culture. The *B. megaterium* strain was cultivated in a 300-mL flask filled with a mixture consisting of 75 mL of Dworkin solution (2 g of $(NH_4)_2SO_4$, 4 g of KH_2PO_4, 6 g of Na_2HPO_4, and 0.2

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g of $MgSO_4$ in 1 L of distilled water) and 375 μL of micronutrient solution (0.3 g each of $CuSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, $CoSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, and $MnSO_4 \cdot 4H_2O$ in 100 mL of distilled water). After sterilization (15 min at 121 °C) and subsequent cooling of the media, 1 mL of 50% filter-sterilized glucose solution was added. The flask was inoculated with *B. megaterium* (2×10^6 cfu/mL) and incubated on a rotary shaker (120 rpm, 30 °C) for 24 h.

Biotransformation of Aldehydes by *B. megaterium*. For the biotransformation experiments, the Dworkin broth described above was supplemented with 50 μL of each aldehyde (hexanal, octanal, nonanal, decanal, undecanal, or dodecanal) after 24 h of bacterial growth. After 3 days of incubation (after the addition of aldehyde), the cultures were extracted with diethyl ether (3×40 mL), and the extract was carefully concentrated to 0.1 mL using a Vigreux column (45 °C). After the addition of a standard solution consisting of 0.1% (v/v) geraniol, the samples were directly analyzed by GC−MS. Control experiments without *B. megaterium* were carried out in a similar manner.

Capillary Gas Chromatography−Mass Spectrometry (GC−MS) Analysis. GC analyses were performed with a Thermo Finnigan Trace DSQ mass spectrometer coupled to a Thermo Finnigan Trace GC with a split injector (1:20) equipped with Xcalibur software (version 1.4). A 30 m × 0.25 mm i.d., 0.25 μm BPX5 20 M fused silica capillary column was used, which was held isothermal at 40 °C for 3 min and then increased to 250 °C at 5 °C/min intervals, with helium at a flow rate of 3 mL/min. The EI-MS operating parameters were as follows: ionization voltage, 70 eV (electron impact ionization); ion source and interface temperature, 230 °C and 240 °C, respectively. Compounds were identified by comparing their mass spectra and retention indices to the National Institute of Standards and Technology (NIST) mass spectra library and reference compounds. The metabolites produced were quantified as equivalents of geraniol.

NMR. ^{13}C NMR experiments were performed on Bruker AV-360 spectrometer and ^1H NMR measurements on a Bruker AMX 400-III spectrometer (Bruker, Rheinstetten, Germany). Evaluation of the experiments was carried out using 1D- and 2D-WIN NMR as well as XWin-NMR software (version 3.5; Bruker, Rheinstetten, Germany). $CDCl_3$ containing TMS was used as solvent.

Synthesis of the Aldol Condensation Products. The aldehyde (10 mmol) was dissolved in a solution of 20 mL of dichloromethane and 20 mL of tetrahydrofuran. NaH (24 mg) was added and the solution was stirred at 0 °C. The reaction solutions showed a pH value of 10.9. After 5 h the mixture was acidified with 5% HCl and extracted with dichloromethane (3×20 mL). The extracts were washed with water, and the combined organic layer was dried over Na_2SO_4, filtered through a 15-cm-diameter folded paper filter, concentrated, and separated on a silica gel column by elution with pentane/diethyl ether (9:1) to give the desired product.

(E)-2-Hexyl-3-heptylacrolein: ^1H NMR ($CDCl_3$, 400 MHz) δ 0.86−0.89 (m, 6H), 1.25−1.51 (m, 18H), 2.23 (t, $J = 7.2$ Hz, 2H), 2.35 (q, $J = 7.3$ Hz, 2H), 6.42 (t, $J = 7.3$ Hz, 1H), 9.36 (s, 1H); ^{13}C NMR ($CDCl_3$, 360 MHz) δ 14.1, 22.6, 24.0, 28.7, 28.8, 28.9, 29.2, 29.3, 29.4, 29.4, 29.5, 29.7, 31.9, 143.8 (C═C), 155.4 (C═C), 195.4 (C═O); MS (70 eV) *m*/*z* (%) 238 (M^+, 80), 223 (4), 195 (4), 177 (4), 167 (48), 153 (40), 139 (100), 121 (14), 109 (16), 95 (92), 83 (74), 69 (62), 55 (68).

(E)-2-Heptyl-3-octylacrolein: ^1H NMR ($CDCl_3$, 400 MHz) δ 0.86−0.92 (m, 6H), 1.23−1.32 (m, 22H), 2.25 (t, $J = 7.4$ Hz, 2H), 2.38 (q, $J = 7.4$ Hz, 2H), 6.46 (t, $J = 7.4$ Hz, 1H), 9.39 (s, 1H); ^{13}C NMR ($CDCl_3$, 360 MHz) δ 14.1, 22.7, 24.1, 28.7, 28.8, 28.9, 29.1, 29.2, 29.2, 29.3, 29.4, 29.4, 29.7, 31.8, 31.8, 143.9 (C═C), 155.3 (C═C), 195.3 (C═O); MS (70 eV) *m*/*z* 266 (M^+, 55), 209 (10), 181 (40), 167 (40), 153 (87), 135 (25), 109 (65), 97 (70), 83 (87), 67 (66), 55 (100), 41 (87).

(E)-2-Octyl-3-nonylacrolein: ^1H NMR ($CDCl_3$, 400 MHz) δ 0.86−0.90 (m, 6H), 1.26−1.51 (m, 26H), 2.20 (t, $J = 7.3$ Hz, 2H), 2.31 (q, $J = 7.3$ Hz, 2H), 6.41 (t, $J = 7.4$ Hz, 1H), 9.30 (s, 1H); ^{13}C NMR ($CDCl_3$, 360 MHz) δ 14.1, 22.7, 24.0, 28.7, 28.8, 28.9, 29.3, 29.4, 29.4, 29.5, 29.5, 29.5, 29.6, 29.7, 30.3, 31.9, 32.1, 143.8 (C═C), 155.4 (C═C), 195.4 (C═O); MS (70 eV) *m*/*z* 294 (M^+, 34), 223 (9), 195 (28), 181 (31), 167 (56), 149 (16), 123 (31), 97 (69), 83 (81), 55 (100), 41 (84).

(E)-2-Nonyl-3-decylacrolein: ^1H NMR ($CDCl_3$, 400 MHz) δ 0.85−0.89 (m, 6H), 1.26−1.33 (m, 30H), 2.20 (t, $J = 8.1$ Hz, 2H), 2.31 (q, $J = 7.9$ Hz, 2H), 6.41 (t, $J = 8.3$ Hz, 1H), 9.35 (s, 1H); ^{13}C NMR ($CDCl_3$, 360 MHz) δ 14.1, 22.7, 24.0, 28.6, 28.7, 28.8, 28.9, 29.3, 29.3, 29.4, 29.4, 29.4, 29.5, 29.5, 29.6, 29.7, 30.3, 31.6, 31.7, 143.8 (C═C), 155.4 (C═C), 195.4 (C═O); MS (70 eV) *m*/*z* 322 (M^+, 76), 237 (12), 195 (45), 181 (82), 163 (12), 137 (27), 111 (36), 95 (72), 83 (79), 67 (61), 55 (94), 43 (100).

(E)-2-Decyl-3-undecylacrolein: ^1H NMR ($CDCl_3$, 400 MHz) δ 0.86−0.90 (m, 6H), 1.25−1.51 (m, 34H), 2.23 (t, $J = 7.3$ Hz, 2H), 2.35 (q, $J = 7.3$ Hz, 2H), 6.42 (t, $J = 7.4$ Hz, 1H), 9.35 (s, 1H); ^{13}C NMR ($CDCl_3$, 360 MHz) δ 14.1, 22.7, 24.0, 28.6, 28.7, 28.8, 28.8, 28.9, 29.3, 29.4, 29.4, 29.4, 29.5, 29.5, 29.6, 29.6, 29.6, 29.7, 31.6, 31.7, 31.9, 143.8 (C═C), 155.4 (C═C), 195.4 (C═O); MS (70 eV) *m*/*z* 350 (M^+, 61), 314 (7), 251 (9), 233 (15), 223 (27), 209 (36), 195 (62), 165 (16), 135 (18), 121 (30), 109 (36), 95 (70), 83 (69), 69 (60), 55 (88), 43 (100).

RESULTS

The biotransformation of a number of aliphatic aldehydes by *B. megaterium* was studied in Dworkin medium containing 1% glucose and the respective aldehydes. The pH value of the liquid cultures prior to the addition of the aldehydes was 6.8, dropping slightly at the end of the bioconversion period to pH 6.7. After 3 days of incubation, products were isolated by liquid/liquid extraction and analyzed by GC−MS.

The strawberry leaf epiphyte *B. megaterium* was able to convert octanal, nonanal, decanal, undecanal, and dodecanal to their corresponding alcohols, acids, and 2,3-dialkylacroleins (**Figure 1**). Hexanal was completely consumed under similar conditions and no products were detected (data not shown). Although the main products of the biotransformation of C_8−C_{12} aldehydes were acids (58−80% of recovered products), small yet notable amounts of alcohols (5−22%) and 2,3-dialkylacroleins (8−19%) were also formed. The amount of the recovered aldehyde transformation products was low, ranging from 0.5% of the original applied amount for nonanal to 2.7% of the original applied amount for decanal. Poor recovery of low molecular weight compounds is typically attributed to their volatility (*16*). In comparison to the bacterial experiments, control experiments resulted in larger amounts of acids and unreacted aldehydes. Alcohols were not detected in control samples, but traces of the corresponding 2,3-dialkylacroleins were found (**Figure 1**). The same product pattern was observed when the aldehydes were incubated under similar conditions in distilled water (data not shown). Aliphatic aldehydes represented less than 10% of the compounds recovered after 3 days of incubation in the control samples and in the samples inoculated with *B. megaterium*.

Time course experiments showed that the maximum concentration of the 2,3-dialkylacroleins was reached after 6 days of incubation and remained relatively stable at the maximum level of 0.4 μg/mL (**Figure 2**). In contrast, the concentration of the acid increased constantly until the aldehyde had been completely consumed. To determine whether aliphatic acids act as acrolein precursors, we performed experiments replacing the aldehydes with their corresponding acids and found that no 2,3-dialkylacroleins were produced (data not shown). **Figure 3** summarizes the transformation of straight-chain C_8−C_{12} aliphatic aldehydes by *B. megaterium*.

DISCUSSION

We present the first report on the microbial transformation of a series of straight-chain aliphatic aldehydes by *B. megaterium* to the corresponding 2,3-dialkylacroleins. Since these

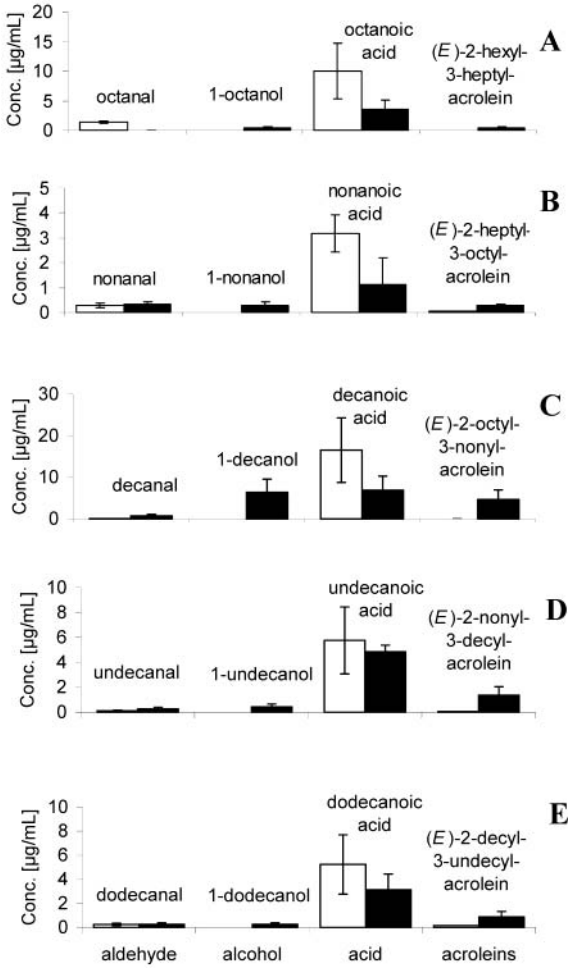

Figure 1. Main metabolites formed by the biotransformation of aliphatic aldehydes (C_8–C_{12}) by *B.* megaterium strain AY131222 after 3 days of incubation with the aldehydes (■); control experiments were carried out without *B. megaterium* (□): octanal (**A**); nonanal (**B**); decanal (**C**); undecanal (**D**); dodecanal (**E**). Vertical bars represent ± SD of the mean ($n = 3$).

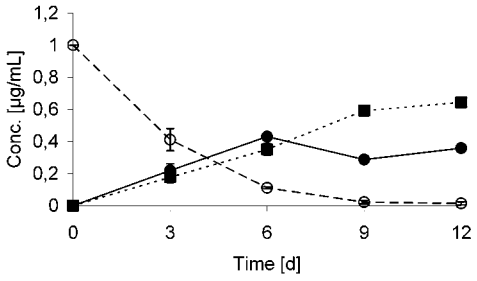

Figure 2. Conversion of nonanal (○) to (*E*)-2-heptyl-3-octylacrolein (●) and nonanoic acid (■) by *B. megaterium* strain AY131222 over the incubation time. Vertical bars represent ± SD of the mean ($n = 2$).

aldehydes are common exudates of leaves, it is reasonable that microbial epiphytes such as the strawberry leaf epiphyte *B. megaterium* used in this study should be able to metabolize these compounds. Despite a number of published reports concerning the bioconversion of aldehydes and other volatiles compounds by fungi and bacteria (*17*−*21*), the bioformation of 2,3-dialkylacroleins by a microorganism has not been described until now.

The aldol condensation is an important reaction for carbonyl compound coupling via C−C bond formation. Chemically,

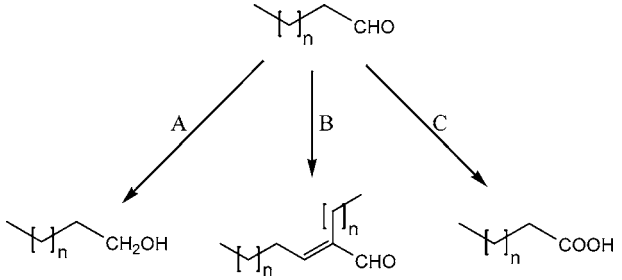

Figure 3. Biotransformation reactions of aldehydes ($n = 5$–9) catalyzed by *B. megaterium* strain AY131222: (**A**) reduction of aldehydes to alcohols; (**B**) condensation of aldehydes to acroleins; (**C**) oxidation of aldehydes to acids.

under the influence of a dilute acid or base, two molecules of an aldehyde or ketone can readily undergo aldol condensation, often referred to as a self-condensation (*22*). Since only minor amounts of the acroleins were detected in control samples, it is not likely that they are artifacts formed by self-condensation in the biotransformation experiments, as the pH value remained between 6.7 and 6.8. Furthermore, incubation experiments with aliphatic acids and time course studies showed that aldehydes rather than the acids are the primary precursor of the acroleins. Although we are not able to establish the mechanism for the formation of 2,3-dialkylacroleins by *B. megaterium*, it can be concluded from our results that the aldol condensation products are formed from aldehydes via an enzymatic reaction.

2,3-Dialkylacroleins have been described as natural components of Valencia orange peel oil, as volatile constituents of green and ripened pineapple (*Ananas comosus*), as constituents of the mandibular gland secretion of males of several species of *Myrmecocystus*, as alarm pheromone components of *Oecophylla longinoda*, and as volatile constituents of the scent gland reservoir of the fruit-spotting bug *Amblypelta nitida* (*1*, *4*, *23*). However, for none of these 2,3-dialkylacroleins has the biosynthetic pathway been described. Our study demonstrates that some of the formation of these compounds by plants or insects could possibly be attributed to a microbial partner. Symbiotic bacteria occur ubiquitous in nature and it is conceivable that they are involved in the formation. Recently, it has been shown that some ant species are using antibiotic-producing bacteria to control parasites (*24*). But more data are needed to demonstrate the involvement of bacteria in the case 2,3-acroleins. Enzymatic aldol reactions catalyzed by aldolases and transketolases are known for more than 60 years and in the closely related acyloin condensation, an acyl anion equivalent originating from the decarboxylation of pyruvate is transferred to an aldehyde to form an (*R*)-α-hydroxy ketone (acyloin) (*25*). None of these reactions can explain the formation of 2,3-dialkylacroleins formed by *B. megaterium*.

It is clear that the microbial transformation of straight-chain aliphatic aldehydes to the corresponding 2,3-dialkylacroleins by *B. megaterium* constitutes a first step to elucidating the enzymatic pathway of the aldol condensation reaction. Our findings not only provide the first clues to its biosynthetic origin but, due to the industrial importance of the synthetic reaction, could potentially help provide a means of enzymatically producing acroleins.

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