Supplemental Material

Applied and Environmental Microbiology
11 pages
7 Figures

Synthesis of short-chain diols and unsaturated alcohols from secondary alcoholic substrates by the Rieske non-heme mononuclear iron oxygenase MdpJ

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1. Purity and supply sources of secondary alcohols and other chemicals

MTBE (≥ 99% pure), TBA (≥ 99% pure) and 3-methyl-2-butanol (> 98% pure, racemate) were purchased from Merck Schuchardt (Hohenbrunn, Germany). Isobutene (≥ 99% pure), 1,2-propanediol (highest purity available), (*R*)-(-)-2-butanol (99% pure), (*S*)-(+)-2-butanol (99% pure), 2-butanone (99% pure), 3-methyl-2-butanone (≥ 98.5% pure), and 3-pentanol (98% pure) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). 2-Propanol (>99.95% pure), diethylether (>99.8% pure) and acetone (>99.57% pure) were purchased from Carl Roth GmbH & Co KG (Karlsruhe, Germany). 2-Butanol (>99.5% pure, racemate), sodium sulfate (Na_2SO_4, >99% pure) and potassium carbonate (K_2CO_3, >99% pure) were purchased from Merck KGAa (Darmstadt, Germany). (*S*)-(+)-1,2-Propandiol (99% pure) and 3-buten-2-one (90% pure) were purchased from ABCR GmbH & Co KG (Karlsruhe, Germany). 3-Buten-2-ol (97% pure) was purchased from Alfa Aesar GmbH & Co KG (Karlsruhe, Germany). Pyridine (>99.5% pure) was purchased from Riedel-de Haën AG (Selze, Germany), and MPD at the highest purity available was from Taros Chemicals (Dortmund, Germany).

2. Detailed description of MSM culture medium

The mineral salt medium (MSM) contained in mg L^{-1}: NH_4Cl, 760; KH_2PO_4, 680; K_2HPO_4, 970; $CaCl_2 \times 6\ H_2O$, 27; $MgSO_4 \times 7\ H_2O$, 71.2; initial pH was 7.5. MSM also contained trace elements (in mg L^{-1}): $FeSO_4 \times 7\ H_2O$, 14.94; $CuSO_4 \times 5\ H_2O$, 0.785; $MnSO_4 \times 4\ H_2O$, 0.81; $ZnSO_4 \times 7\ H_2O$, 0.44; $Na_2MoO_4 \times 2\ H_2O$, 0.25; $CoCl_2$ x 6 H_2O, 0.040. A vitamin solution was added (in μg L^{-1}): biotin, 20; folic acid, 20; pyridoxine-HCl, 100; thiamine-HCl, 50; riboflavin, 50; nicotinic acid, 50; DL-Ca-pantothenate, 50; p-amino-benzoic acid, 50; lipoic acid, 50, and cobalamin, 50.

3. Heterologous expression, purification of MdpJK proteins and monooxygenase enzyme assay

MdpJ and MdpK were heterologously expressed in *Escherichia coli* Arctic Express (DE3) (Merck KGaA, Darmstadt, Germany) using pASGIBA43::mdpJ (N-terminal Strep and C-terminal His tag, IBA, Goettingen, Germany) and pET3b::mdpK (N-terminal Strep tag was added, Merck KGaA, Darmstadt, Germany) as expression vectors. Purification of the recombinant protein was performed using a Strep-Tactin Superflow high capacity column according to the manufacturer´s instruction (IBA, Goettingen, Germany).

The activity of MdpJK was measured at 30°C spectrophotometrically (spectrophotometer U-2000, Hitachi, Tokyo, Japan) in quartz cuvettes at 340 nm as NADH consumption. The reaction conditions were varied for pH (6 to 9), buffer system (Tris buffer, potassium phosphate buffer and TEG buffer containing Tris, ethanol, glycerol and dithiothreitol according to Ensley et al. 1982) and TBA (3 to 11 mM) as well as for the concentration of NADH, Fe^{2+} as $(NH_4)_2Fe(SO_4)_2$, and polyethylene glycol as stabilizing agent. The assay was performed using equimolar ratios of purified recombinant subunits MdpJ and MdpK at concentrations of 3 to 6 µg mL^{-1}.

Although expression of both subunits was successful and sufficient amounts of soluble target proteins were obtained, the MdpJK assay using the purified recombinant protein components failed with all tested buffer combinations and additives. The reason for this inactivity is not apparent at present. Likewise, it has also been found that other non-heme mononuclear iron enzymes were completely inactive in vitro. The CobG of *Pseudomonas denitrificans*, for example, a monooxygenase involved in cobalamin biosynthesis, was shown to be completely inactive under in vitro conditions after purification due to the non-reducibility of the mononuclear non-heme iron center (Schröder et al. 2009).

4. Induction of MdpJK in *A. tertiaricarbonis* L108 wild-type strain by incubation on TBA

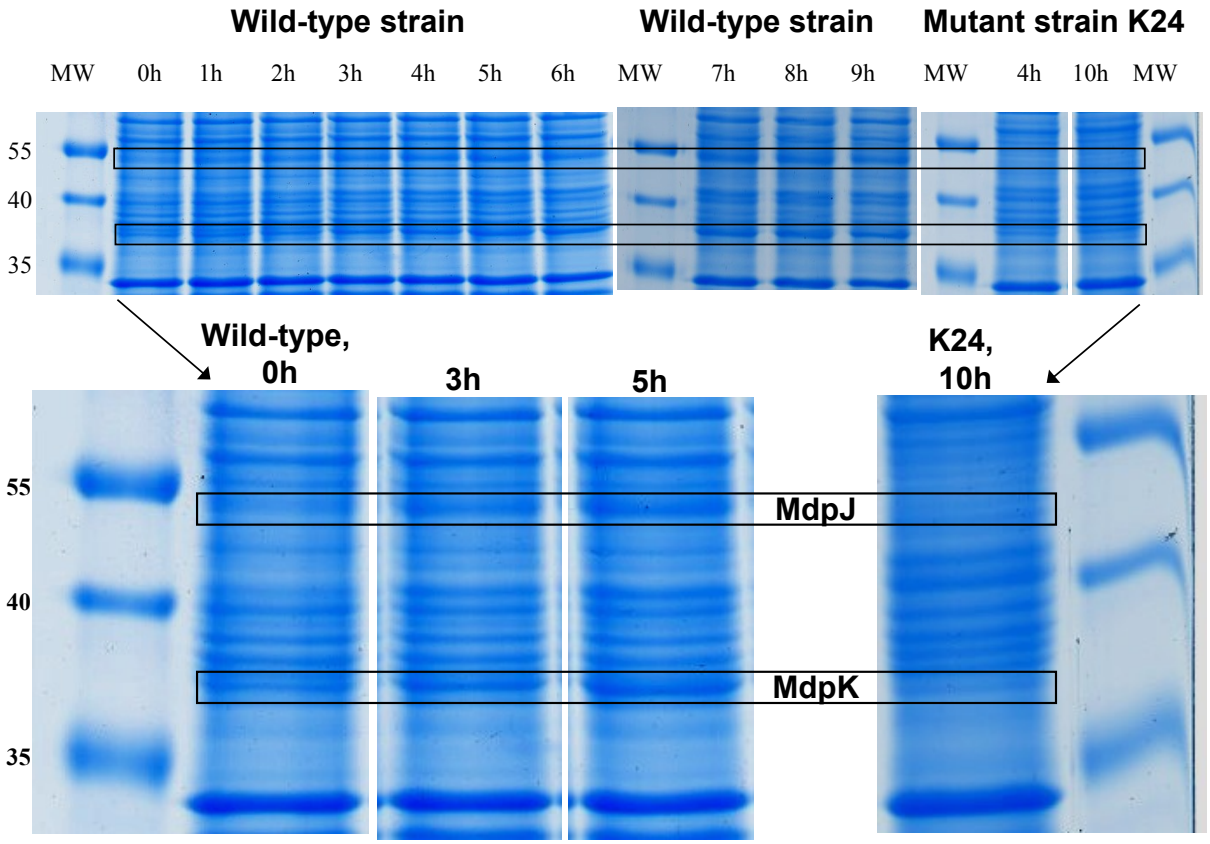


Figure S1. SDS-PAGE of samples from resting-cell experiments incubating *A. tertiaricarbonis* L108 wild-type strain and knockout mutant strain (Δ*mdpJ*) K24 in the presence of TBA. Protein patterns of cells pregrown on MPD and then shifted to TBA are shown for samples taken after 0 to 9 hours of incubation for cells of the wild-type strain and after 4 and 10 hours for cells of the mutant strain as indicated. In the presence of TBA, cells of the wild-type strain induced MdpJ and also MdpK within a few hours, whereas these proteins were not detectable in cells of the mutant strain even after 10 hours of incubation. SDS-PAGE was performed according to protocols of Schägger et al. (1987) and Laemmli (1970). MdpJ (52.9 kDa) and MdpK (37.0 kDa) proteins were assigned according to Schäfer et al. (2007). Each sample contained 20 µg of protein. (MW: kDa molecular weight marker, PageRuler Prestained Protein Ladder; Fermentas GmbH, St. Leon-Rot, Germany).

5. Degradation of TBA by *A. tertiaricarbonis* L108 wild-type strain and *mdpJ* knockout mutant K24 in resting-cell experiments

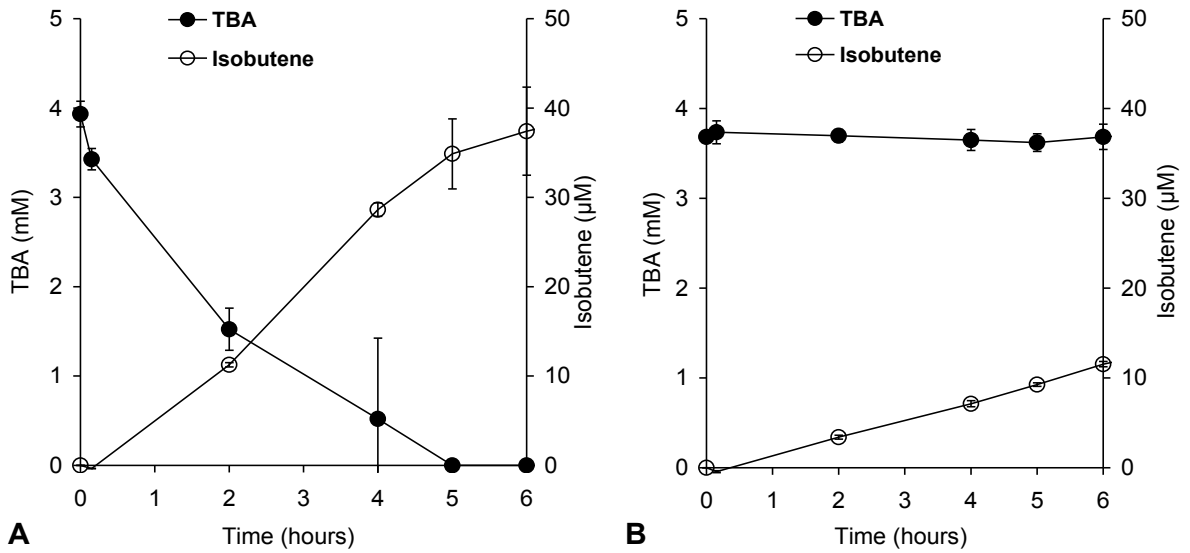


Figure S2. Degradation of TBA in resting-cell experiments (1.4 g biomass dry weight per liter). (**A**) Complete degradation of TBA and isobutene formation by wild-type strain L108. (**B**) No significant degradation of TBA but isobutene formation by *mdpJ* knockout strain L108 (*ΔmdpJ*) K24. For a direct comparison with TBA conversion, isobutene values refer to concentrations in the liquid phase, although it was exclusively found in the gas phase of the close incubation bottles.

6. Identification of the 2-butanol metabolites 2-butanone and 3-buten-2-one

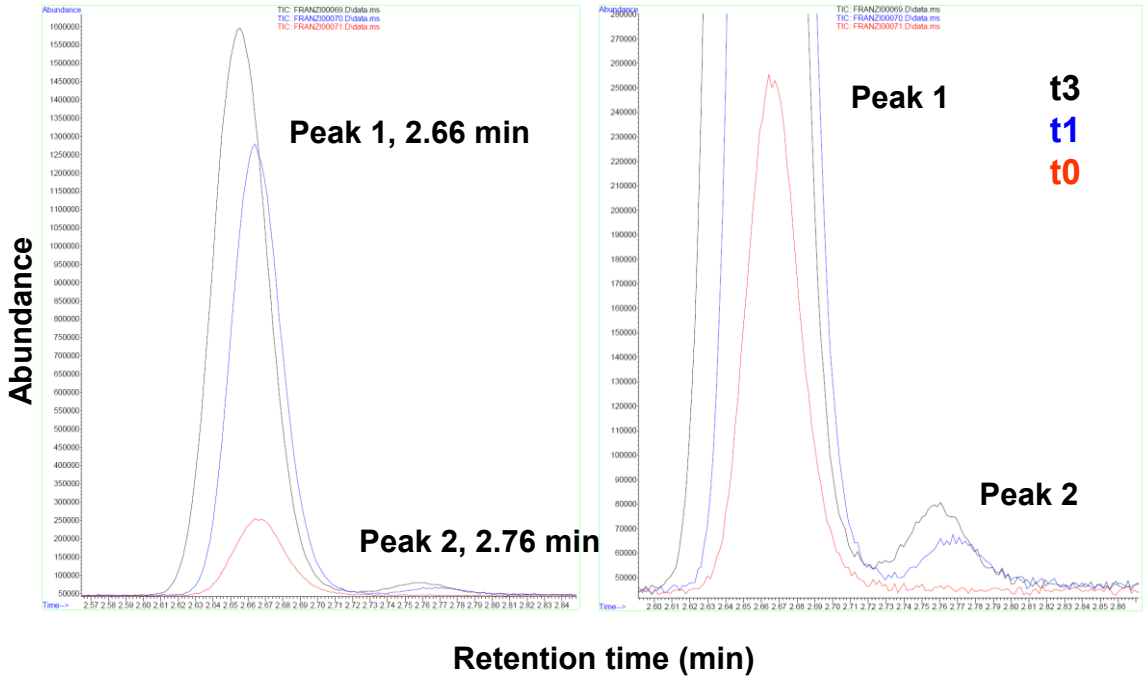
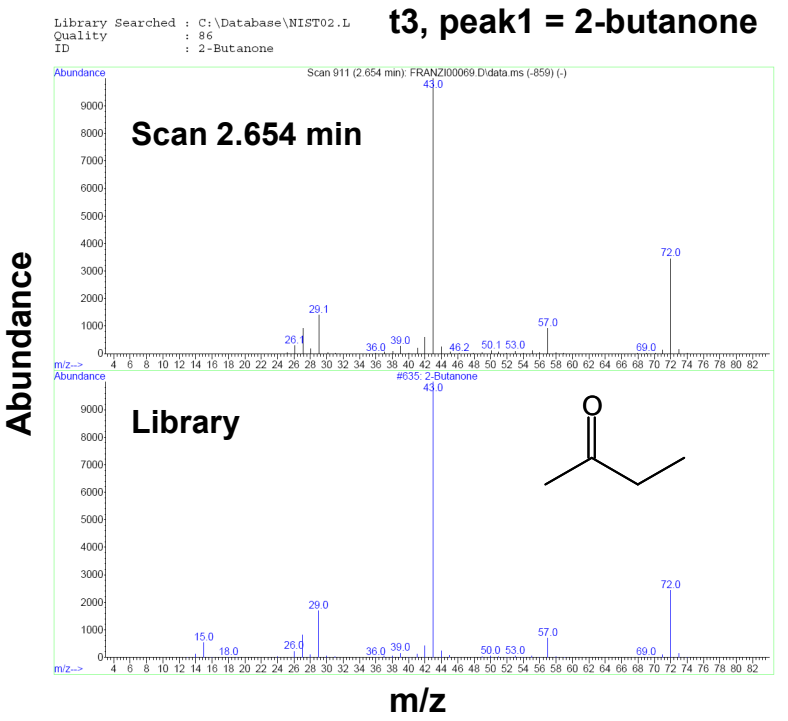


Figure S3. GC-MS analysis of 2-butanol metabolites. Overlay of total ion chromatograms (TIC) of samples from resting-cell experiments incubating *A. tertiaricarbonis* L108 wild-type cells with 2-butanol. Incubation times are indicated as t0 = 0, t1 = 1, and t3 = 3 hours. An increase of peaks 1 and 2 was observed which were identified as 2-butanone and 3-buten-2-one (see Figure S4), respectively. **Left:** overview showing complete areas of peaks 1 and 2. **Right:** blow-up of peak 2. GC method: Ten-milliliter headspace vials were filled with 1 mL of a 3 M NaCl solution and 1 mL of liquid sample. After incubation of the sample vials at 70°C for 20 min, 0.1 mL of gas phase was removed and injected manually into the GC system. Analyses were carried out using a HP 6890 GC instrument with mass detector HP 5973 MSD (Agilent) and a DB-23 column (30 m, 0.25 mm, 0.25 µm, Agilent). Helium was used as carrier gas at 0.8 mL min^{-1}. Temperature program: initial oven temperature 50°C for 2.8 min, then increase to 120°C at 30°C min^{-1}.



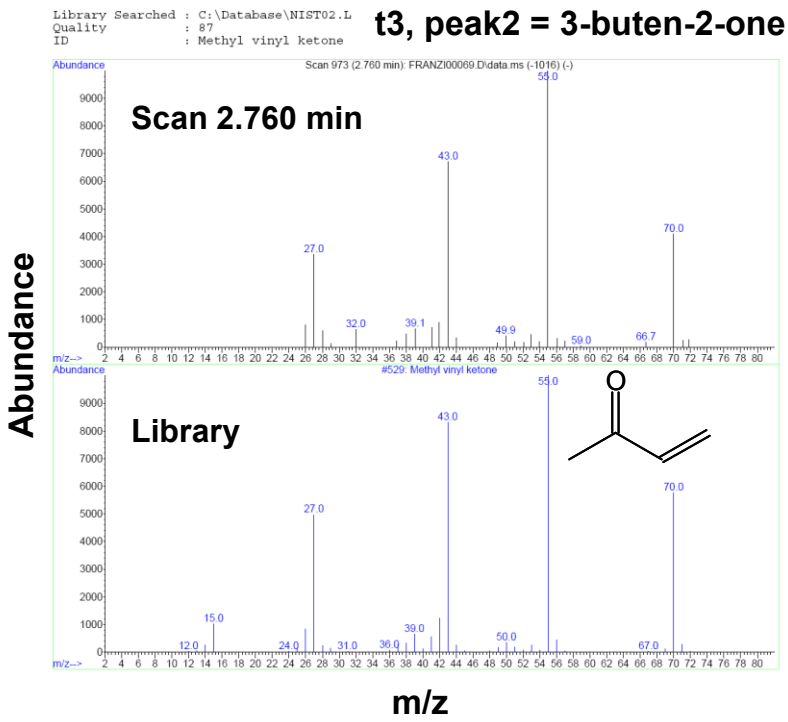


Figure S4. GC-MS analysis of 2-butanol metabolites. Mass spectra of peaks 1 and 2 of sample t3 (see Figure S3) and most probable matches by GC-MS spectral NIST data base (NIST 02).

7. Identification of 3-methyl-2-butanol metabolites

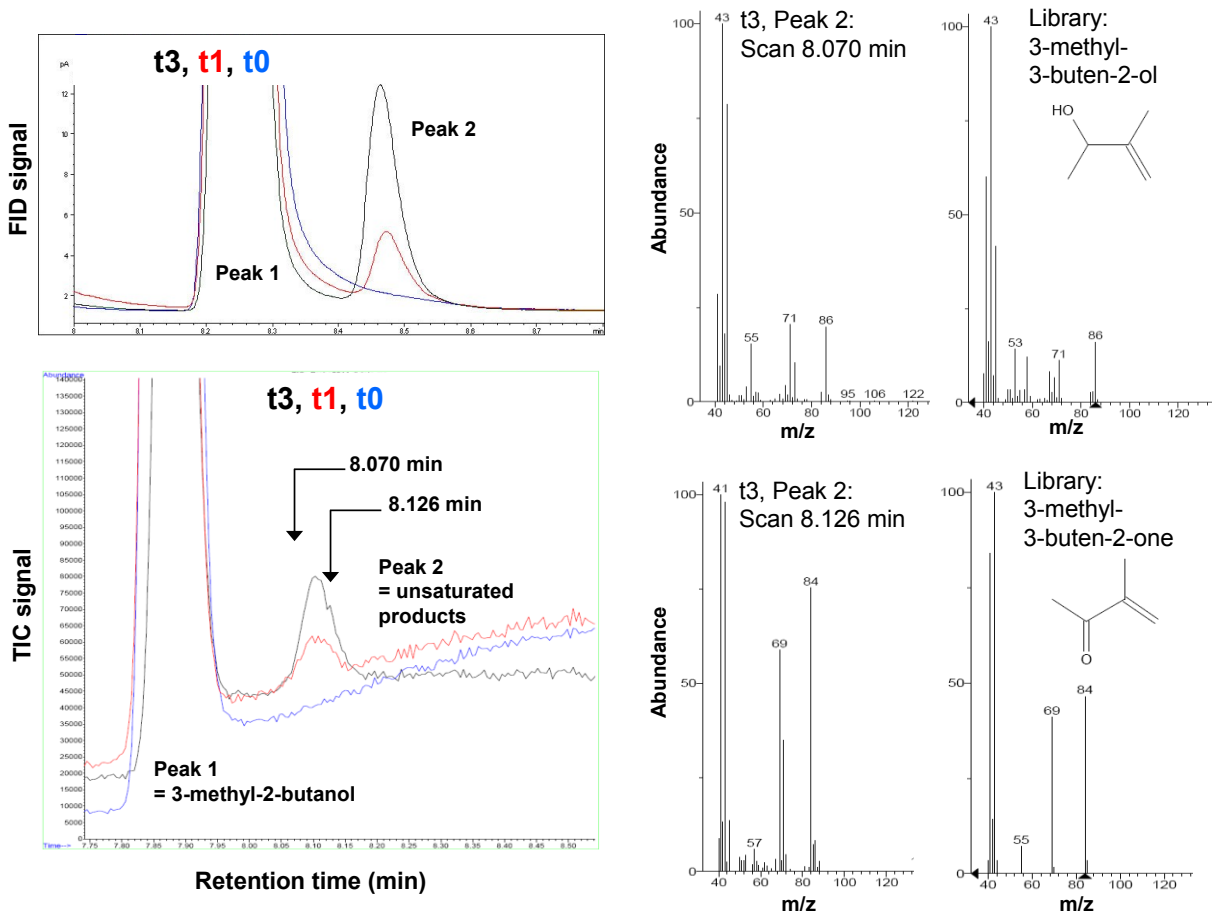


Figure S5. GC-FID and GC-MS analysis of 3-methyl-2-butanol metabolites. **Left:** Overlay of FID and TIC signals of samples from resting-cell experiments incubating *A. tertiaricarbonis* L108 with 3-methyl-2-butanol. Incubation times are indicated as t0 = 0, t1 = 2, and t3 = 8 hours. The substrate (peak 1) was mainly oxidized to 3-methyl-2-butanone by cells of the wild-type strain and knockout strain K24 (not shown). In addition, exclusively in samples from the wild-type culture, an increase of peak 2 was observed which was identified as a mixture of 3-methyl-3-buten-2-ol and 3-methyl-3-buten-2-one, respectively. Analyses were carried out using two HP 6890 GC instruments (Agilent) with FID and mass selective detector (HP 5973 MSD), respectively, and an Optima Delta 3 column (60 m, 0.32 mm, 0.35 µm, Macherey-Nagel). Ten-milliliter headspace vials were filled with 1 mL of a 3 M NaCl solution and 1 mL of liquid sample. GC-FID method: After incubation of the sample vials at 70°C for 20 min, 1 mL of gas phase was removed and injected into the GC system by using a G1888 autosampler. Nitrogen was used as carrier gas at 2.0 mL min^{-1}, oven temperature was held at 50°C, and detector temperature was 220°C. GC-MS method: After incubation of the sample vials at 95°C for 20 min, 0.1 mL of gas phase was removed and injected manually into the GC system. Helium was used as carrier gas at 1.3 mL min^{-1} and oven temperature was held at 50°C. **Right:** Mass spectra of peak 2 of sample t3 for two retention times as indicated and most probable matches by GC-MS spectral NIST data base (NIST 02).

8. Identification of 3-pentanol metabolites

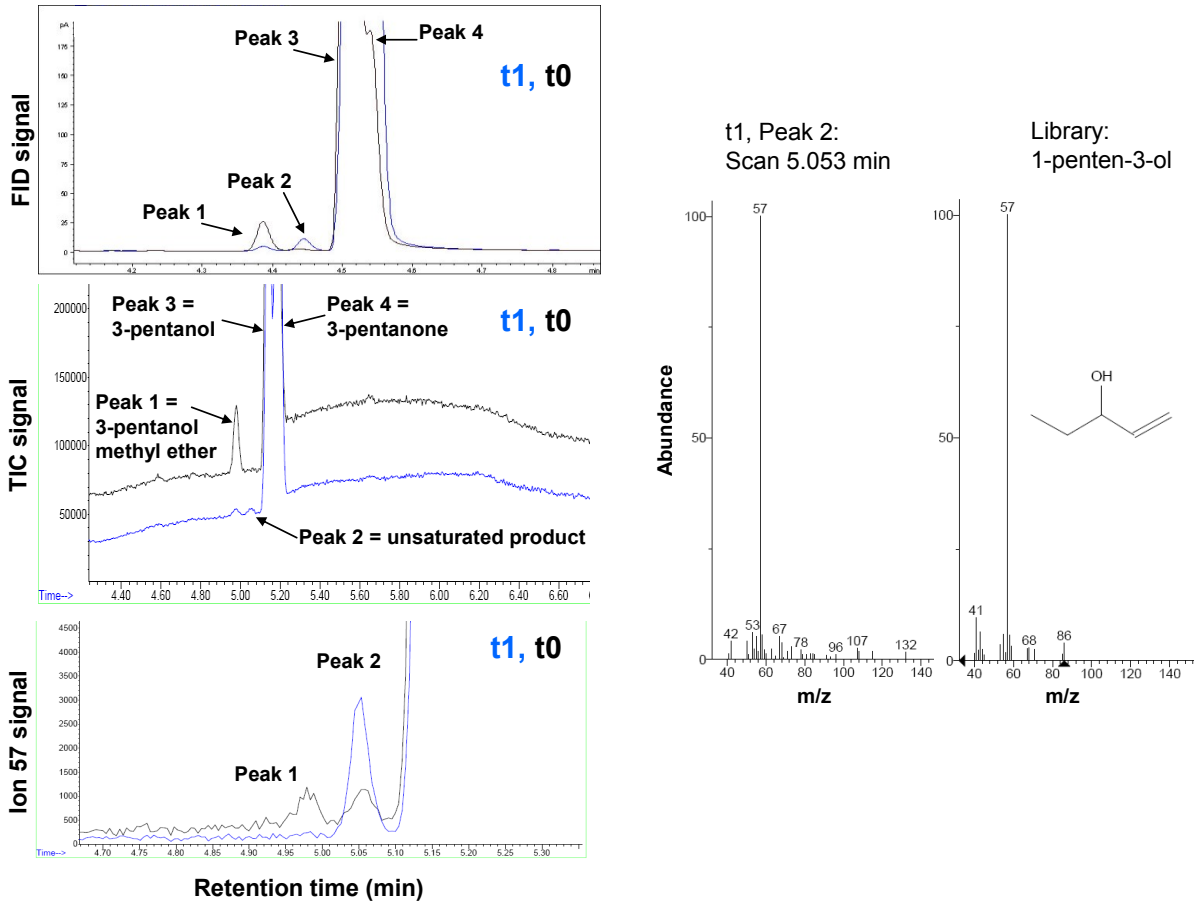


Figure S6. GC-FID and GC-MS analysis of 3-pentanol metabolites. **Left:** Overlay of FID, TIC and Ion 57 signals of samples from resting-cell experiments incubating *A. tertiaricarbonis* L108 with 3-pentanol. Incubation times are indicated as t0 = 0 and t1 = 2 hours. The substrate 3-pentanol (peak 3) contained an impurity of 3-pentanol methyl ether (peak 1) which was rapidly degraded by the wild-type cells. In addition, formation of 3-pentanone (peak 4) and 1-penten-3-ol (peak 2) was observed. In contrast, cells of the mutant strain K24 oxidized 3-pentanol exclusively to 3-pentanone (not shown). The same GC systems already employed for analyzing the 3-methyl-2-butanol metabolites were used (see caption of Fig. S5). GC-FID method: After incubation of the sample vials at 90°C for 30 min, 1 mL of gas phase was removed and injected into the GC system by using a G1888 autosampler. Nitrogen was used as carrier gas at 2.0 ml min^{-1}. Oven temperature was held at 100°C for 2 min, increased from 100 to 180°C at a rate of 15°C min^{-1}, and finally kept at 180°C. Detector temperature was 250°C. GC-MS method: After incubation of the sample vials at 95°C for 30 min, 0.1 mL of gas phase was removed and injected manually into the GC system. Helium was used as carrier gas at 1.3 mL min^{-1}. Oven temperature was held at 100°C for 2 min, increased from 100 to 180°C at a rate of 15°C min^{-1}, and finally kept at 180°C. **Right:** Mass spectrum of peak 2 of sample t1 and most probable match by GC-MS spectral NIST data base (NIST 02).

9. Substrates and products of MdpJ-catalyzed reactions

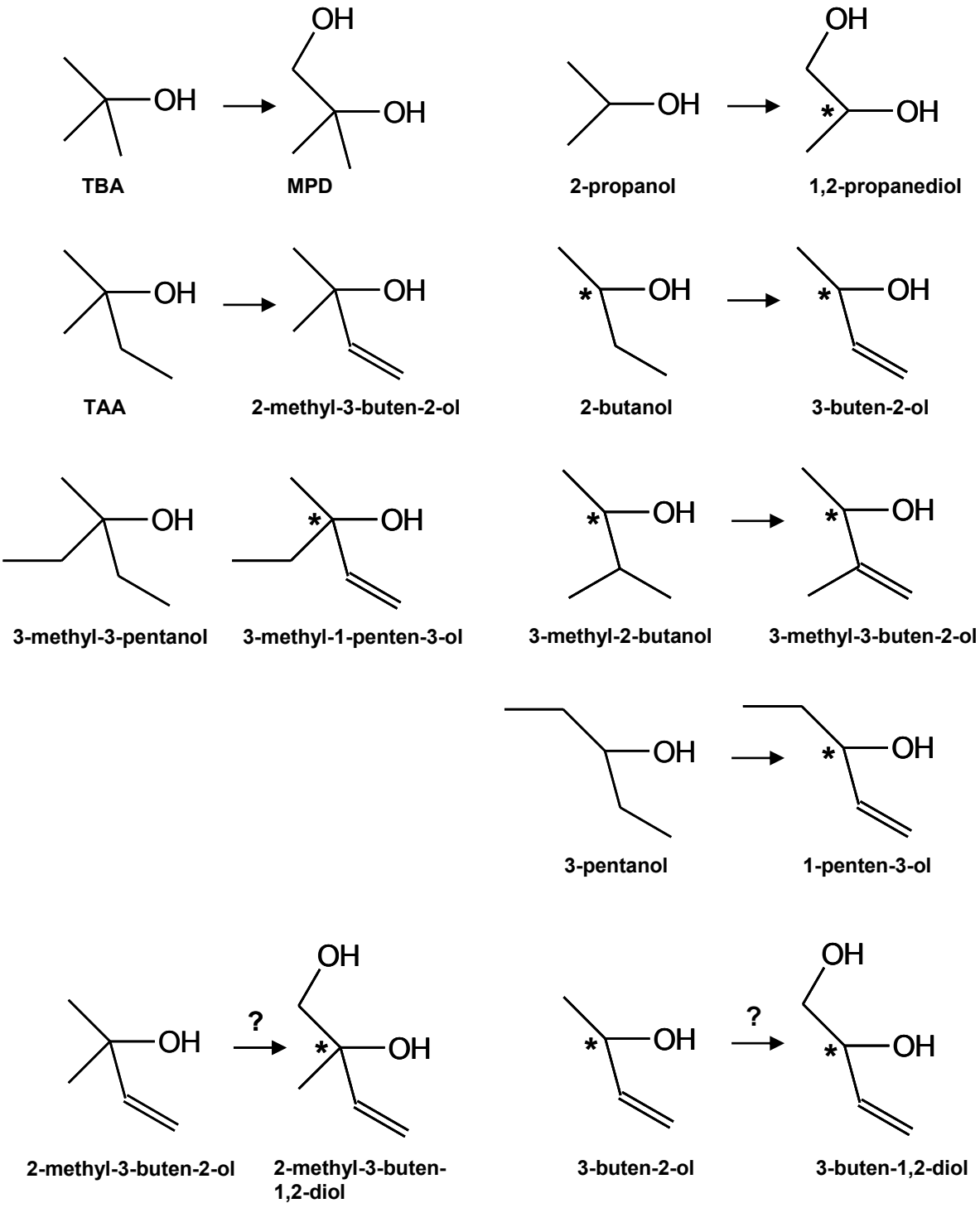


Figure S7. Substrates and corresponding products of MdpJ-catalyzed hydroxylation and desaturation of tertiary (left) and secondary (right) alcohols. Hydroxylation of unsaturated alcohols by MdpJ has not yet been established by GC-MS analysis (indicated by "?"). Chiral centers of substrates and products are indicated by an asterisk.

References

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