Acute limonene toxicity in *Escherichia coli* is caused by limonene-hydroperoxide and alleviated by a point mutation in alkyl hydroperoxidase (AhpC)

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Abstract

Limonene, a major component of citrus peel oil, has a number of applications related to microbiology. The anti-microbial properties of limonene make it a popular disinfectant and food preservative, while its potential as a biofuel component has made it the target of renewable production efforts through microbial metabolic engineering. For both applications, an understanding of microbial sensitivity or tolerance to limonene is crucial, but the mechanism of limonene toxicity remains enigmatic. In this study, we characterized a limonene-tolerant strain of *Escherichia coli* and found a mutation in *ahpC*, encoding for alkyl hydroperoxidase, which alleviated limonene toxicity. We show that the acute toxicity previously attributed to limonene is largely due to the common oxidation product limonene-hydroperoxide, which forms spontaneously in aerobic environments. The mutant protein AhpC^{L177Q} is able to alleviate this toxicity by reducing the hydroperoxide to a more benign compound. We show that the degree of limonene toxicity is a function of its oxidation level, and that non-oxidized limonene has relatively little toxicity to wild-type *E. coli*. Our results have implications for both the renewable production of limonene and the applications of limonene as an anti-microbial.

Introduction

Limonene, the major component of citrus peel oil, has a variety of industrial and microbiological applications. Its antimicrobial properties make it a popular component of disinfectants and food preservatives and an environmentally-friendly solvent used at industrial scale (1–4). More recently, limonene or its hydrogenated forms have been identified as potential jet fuel components (5–7). As such, the anticipation of a greater global demand for limonene has provided significant motivation for renewable

production of this compound from plant biomass through a microbial process (8–10), and recent efforts to optimize production have resulted in titers of over 400mg/L at bench scale (11). In this context, the toxicity of limonene to the microbial host presents a major challenge, as accumulation of toxic products limits growth and metabolic activity.

Curiously, the toxicity of limonene has been reported to be significantly higher than other monoterpenes or solvents with similar hydrophobicity (12, 13), suggesting that this acute toxicity is due to something other than its solvent-like properties. However, molecular-level studies of limonene toxicity in microbes are limited. While several studies in *E. coli* have noted an impact of limonene on lipid composition (14) and suggested a role for reactive oxygen species (15), no specific hypothesis for how limonene causes these cellular perturbations has been proposed.

In this work, we investigated the basis of limonene toxicity in the model gram-negative bacterium *E. coli*. We identified a mutant with significantly enhanced tolerance to limonene, and showed that the majority of the toxicity in wild-type cells is due not to limonene itself, but to a common oxidation product of limonene, limonene-hydroperoxide.

Materials and Methods

Strains

The original FM003 strain was isolated under the following conditions: LB agar plates were covered with a thin layer of 100% limonene and allowed to dry. After

streaking individual KEIO collection strains on these plates and incubating at room temperature, colonies of spontaneous mutants appeared within 2 days. One of these colonies was chosen for further analysis as FM003.

BW25113 (wild-type), *ΔahpC*, *ΔacrA*, and *ΔacrB* strains were obtained from the KEIO collection and the kanamycin-resistance cassette was removed by FLP-FRT recombination (16). $AhpC^{L177Q}$ and $AhpC^{WT}$ were amplified from genomic DNA of FM003 and BW25113, respectively, verified by Sanger sequencing, and cloned into the pBbS2K (Tet promoter) vector (17). J5 DNA assembly design software (18) was used for all primer and construct design, and plasmids were assembled using the Gibson method (19). A complete list of plasmids and strains used in this work can be obtained at http://public-registry.jbei.org, which also provides a mechanism for researchers to request strains (20).

Whole genome resequencing

DNA from both the parent stain and FM003 was randomly sheared into ~400 bp fragments and the resulting fragments were used to create Illumina libraries. These libraries were sequenced on Illumina sequencers generating 100 bp paired end reads. These reads were aligned to the reference genome NC_000913 using BWA (21), and down sampled to generate an average read depth of 250X. A total of 55 putative variants were identified using samtools and mpileup (22). Variants that failed our filtering criteria (minimum quality = 10, minimum P-value for strand bias = 0.0001, minimum P-value for end bias = 0.0001, SNP within 10 bp around a gap) were removed from the analysis, leaving 28 putative variants, 26 of which were found in both the parent strain

and FM003. The variants in the parent strain matched the expected genotype of BW25113 *ΔdmsD*. Of the two variants that were unique to FM003, the only non-synonymous one was in codon 177 of *ahpC (cTg/cAg)*, resulting in amino acid change L177Q. The other variant was a synonymous change in *frlA* at codon 123 *(gaT/gaC)*.

Media and growth conditions

All growth characterization experiments were performed at 30 °C in EZRich medium (Teknova), modified to contain 4 g/L glucose, 150 μM PO_4, and 1 μM SO_4. 40 nM anhydrotetracycline (aTc) was used for induction of strains containing Tet promoter constructs. Overnight cultures grown on LB + 50 mg/L kanamycin were diluted 1/250 into EZRich medium (with antibiotic and inducer if appropriate), grown to OD_{600} of 0.3-0.6, and rediluted 1/200 into 96-well plates containing appropriate amounts of limonene. Growth in 96-well plates was monitored using an automated reader, shaker, and incubator (F200 or F200pro, Tecan). To reduce differences in growth due to the use of antibiotic for plasmid-containing strains, a reduced concentration of kanamycin (5 mg/L) was used in the EZRich cultures. For experiments shown in Figure 5, conditions were identical except that 25 mL cultures in 250 mL non-baffled flasks were used.

Anaerobic growth experiments followed a similar protocol. Aerobic overnight LB cultures were diluted 1/250 into EZRich medium, sealed, sparged of air using a mixture of N_2 and CO_2, and grown to OD_{600} of 0.3. Main cultures in the same medium were prepared in an anaerobic chamber as 1/100 dilutions of the preculture. Growth was monitored in 100-well plates in a Bioscreen C MBR (Growth Curves Ab Ltd) reader, shaker, and incubator. All media used for anaerobic growth contained 20g/L glucose.

125

126 *Chemicals*

127

128 All chemicals were purchased from Sigma-Aldrich unless otherwise specified. S-
129 limonene was used for toxicity experiments shown in Figures 1, 3, 5, and 6, though
130 generally no difference in toxicity or hydroperoxide formation was observed between
131 the S- and R- enantiomers. The data shown in Figure 4 includes both enantiomers, from
132 stocks purchased from several different manufacturers (Sigma-Aldrich, Acros Organics,
133 Alfa Aesar) over several years. To anaerobically maintain non-oxidized limonene, new
134 bottles were purchased from Sigma-Aldrich and immediately sparged with N_2 gas and
135 sealed. LC-TOF-MS and GC-MS analysis showed minimal hydroperoxide content in these
136 samples.

137

138 *Metabolite analysis*

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140 For analysis of the oxidized forms of limonene, limonene samples were diluted
141 with ethyl acetate and analyzed directly via LC-TOF-MS or GC-MS. LC-TOF-MS was
142 performed on the Agilent 6210 TOF using atmospheric chemical pressure ionization
143 (APCI). Analyses were conducted at 55°C with a Kinetex XB-C18 column (100-mm
144 length, 3-mm internal diameter, 2.6-µm particle size; Phenomenex, Inc.) using a 1200
145 Series HPLC (high-performance liquid chromatography) system (Agilent Technologies).
146 The mobile phase was composed of water (solvent A) and methanol (solvent B) (HPLC
147 grade, Honeywell Burdick & Jackson, CA). Three microliter samples were injected and
148 separated with the following gradient: 60% to 98% B for 3.47 min, held at 98% B for
149 7.03 min, decreased to 60% B in 0.5 min, and held at 60% B for a further 1.5 min. A flow

rate of 0.42 mL/min was used from 0 to 8.67 min, increased to 0.65 mL/min in 0.33 min, and held at 0.65 mL/min for a further 3.5 min. The total analysis run time was 12.5 min. The selected mass range was 110-250 m/z. All other APCI-TOF MS conditions were as described previously (23). Data acquisition and processing were performed via Agilent MassHunter Workstation and Qualitative Analysis, respectively.

GC-MS was performed on the Agilent 6890 series GC equipped with an Agilent 5973 MS detector and an Agilent DB-5ms column (11). All integration and quantification was performed with software provided by the manufacturer. For compounds detected by both methods, results were quantitatively consistent. For final quantification, LC-TOF-MS data was used for limonene-hydroperoxide, while GC-MS data was used for limonene, limonene-oxide, carveol, and carvone.

For analysis of intracellular metabolites, 2 mL of culture was centrifuged at 14 000 × g for 1 minute, the supernatant was rapidly decanted, and the cell pellet frozen in liquid nitrogen. Pellets were extracted with 50/50 water/ethyl acetate. For extracellular metabolites, 800 µL of supernatant was extracted with 400 µL of ethyl acetate. In both cases, the organic phase was analyzed by LC-TOF-MS and GC-MS as above.

Results

Identification and genome sequencing of a limonene-resistant mutant

During routine screening of the KEIO gene deletion collection (16) for strains with differential sensitivity to limonene, we observed the spontaneous appearance of

mutants with high tolerance to limonene. One such strain, which arose from the *ΔdmsD* strain of the KEIO collection, was named FM003 and chosen for further analysis. The phenotype of growth on LB agar plates with limonene overlay was verified, and growth was also verified in liquid cultures with defined complex or minimal medium containing up to 5% v/v limonene. Given the robust phenotype, we sequenced the genome of FM003 to identify mutations responsible for limonene tolerance. The only non-silent mutation identified with high confidence in FM003, compared to the parent strain, was a point mutation in the coding sequence of *ahpC*, resulting in amino acid change L177Q.

To determine if the mutation in *ahpC* had resulted in loss of function, we assayed limonene tolerance in the *ΔahpC* strain taken from the KEIO collection. Deletion of *ahpC* failed to confer tolerance; in fact the *ΔahpC* strain was slightly more sensitive to limonene than wild-type, suggesting that the mutation had enhanced the role of AhpC in limonene tolerance. Therefore, we cloned the $ahpC^{L177Q}$ allele from FM003 into a low copy expression plasmid, and expressed $AhpC^{L177Q}$, $AhpC^{WT}$, or a red fluorescent protein (RFP) control in a *ΔahpC* background to avoid heterogeneity effects. Expression of $AhpC^{L177Q}$ was sufficient to essentially reconstitute the limonene-tolerant phenotype of FM003, whereas expression of $AhpC^{WT}$ resulted in approximately wild-type levels of limonene tolerance (Figure 1). These results strongly suggested that the phenotype of the evolved strain could be attributed to the $ahpC^{L177Q}$ mutation. No difference in phenotype was observed when $AhpC^{L177Q}$ was expressed in wild-type strains compared to the *ΔdmsD* background in which the mutation was originally isolated (data not shown), so the extremely slight fitness advantage that remained in the FM003 strain may be attributable to proper regulation of AhpC expression from its native locus.

The role of alkyl hydroperoxide reductase

AhpC forms a multimeric complex with AhpF to form the enzyme alkyl hydroperoxide reductase, which is responsible for relieving peroxide stress by reducing hydroperoxides to their corresponding alcohols, using NADH as an electron donor (24) (Figure 2A). AhpC is the peroxidase component of the enzyme, responsible for binding and reducing the peroxide substrate, while AhpF catalyzes the second step of reducing the AhpC disulfide bond and recycling AhpC. We hypothesized that $AhpC^{L177Q}$ may have an altered substrate specificity that allows reduction of a toxic hydroperoxide formed by oxidation of limonene (Figure 2B), which would be consistent with the increased sensitivity of the *ΔahpC* strain. Further support for this hypothesis came from a previous observation that oxidation products such as limonene-hydroperoxide are rapidly formed from limonene under aerobic conditions and are responsible for the allergenic properties of limonene to mammals (25). Additionally, previous work had uncovered a separate mutation in *ahpC* that conferred tolerance to tetralin via hydroperoxide reduction (26). In that study, the authors had sequenced a tetralin-resistant mutant and found an $ahpC^{G142V}$ mutation. The mutant protein was shown to reduce tetralin-hydroperoxide to 1,2,3,4-tetrahydro-1-naphthol *in vitro* and to alleviate toxicity of both tetralin and tetralin-hydroperoxide *in vivo*.

We hypothesized that limonene-hydroperoxide may form quickly *in vivo* due to the presence of reactive oxygen species (ROS) formed during aerobic respiration (27). In this case, we would expect limonene to be significantly less toxic during anaerobic growth. Surprisingly, we found no decrease in limonene toxicity under anaerobic conditions, and still observed a significant increase in tolerance provided by expression

of AhpC^{L177Q} (Figure 3). This result suggested that if the toxic species was indeed limonene-hydroperoxide, it was formed before addition of limonene to the *E. coli* culture.

Direct measurement of limonene oxidation products

To determine directly if limonene-hydroperoxide was present as a contaminant of the limonene used in our toxicity assays, we developed a method to quantify oxidized forms of limonene using liquid chromatography and time-of-flight mass spectrometry (LC-TOF-MS). Since pure standards for limonene-hydroperoxide were not commercially available, we oxidized limonene by leaving it exposed to air for three weeks with gentle stirring, a procedure previously shown to lead to the formation of significant quantities of limonene-hydroperoxide (25). The oxidized limonene was analyzed by LC-TOF-MS and showed a greater than 10-fold increase in the signal corresponding to the exact mass of limonene-hydroperoxide. While multiple chromatographic peaks were observed, we did not attempt to distinguish isomers such as limonene-1-hydroperoxide and limonene-2-hydroperoxide, or *cis* and *trans* forms. Several other likely oxidation products of limonene, such as limonene-1,2-oxide and carvone, were also observed, while approximately 50% of the sample remained in the form of pure limonene.

We also assayed toxicity of the oxidized limonene sample enriched in limonene-hydroperoxide to wild-type and AhpC^{L177Q}-expressing *E. coli.* The oxidized limonene sample was found to be significantly more toxic, completely inhibiting growth of wild-type *E. coli* even at 0.1% v/v. AhpC^{L177Q}-expressing strains were able to tolerate up to 0.5% v/v of the oxidized limonene with only minor defects in growth rate (Figure 4A).

Since these results further corroborated the hypothesis that limonene toxicity is caused by the presence of the limonene-hydroperoxide contaminant, we created a library of limonene samples oxidized to various degrees, and assayed their hydroperoxide content and toxicity. The library consisted of eleven different limonene bottles from several manufacturers, which had been subject to varying levels of routine laboratory use, as well as two bottles that were sparged with nitrogen gas immediately upon receipt from the vendor and stored anaerobically. We observed a remarkable correlation between toxicity and hydroperoxide content (Figure 4B), with the anaerobically stored limonene showing minimal toxicity to wild-type *E. coli*.

As mentioned above, mass spectrometry analysis showed several other oxidation products of limonene present in the oxidized samples. Using a combination of LC-TOF-MS and gas chromatography-mass spectrometry (GC-MS) analysis, we performed absolute quantification of carvone, carveol, and limonene-1,2-oxide, for which commercial standards were available. Carvone was determined to make up at most 5% of the sample, while carveol and limonene-1,2-oxide accounted for less than 1% (v/v). As mentioned above, about 50% of the sample was in the form of pure limonene, suggesting that another large fraction is accounted for by polymers of the oxidation products, which would be consistent with previous reports from a similar oxidation procedure (25).

To determine if carvone, carveol, or limonene-1,2,-oxide could be the toxic component of the oxidized limonene, we assayed their toxicity to both wild-type and $AhpC^{L177Q}$-expressing *E. coli.* The pure compounds were dissolved in non-oxidized (anaerobically stored) limonene at various concentrations, and the mixture was added

to the medium at 1% v/v. Limonene with up to 20% carvone or limonene-1,2-oxide, or with up to 5% carveol, had no effect on growth rate of either strain (Supplementary Figure S1). These results offer further evidence for the predominant toxic species being limonene-hydroperoxide.

To examine the role of AhpC^{L177Q} in alleviating toxicity, we quantified intracellular limonene-hydroperoxide levels in cells grown in the presence of limonene. Exponential phase cells expressing AhpC^{WT}, AhpC^{L177Q}, or RFP were treated with 1% v/v of partially oxidized limonene (i.e. imposing an intermediate level of toxicity), and cell samples were taken at several time points after limonene addition. LC-TOF-MS analysis of intracellular metabolites showed that significant quantities of intracellular limonene-hydroperoxide appeared directly after limonene addition in all three strains (Figure 5). In the strains expressing RFP or AhpC^{WT}, growth was dramatically impacted and the levels of intracellular limonene-hydroperoxide remained high. In contrast, the strain expressing AhpC^{L177Q} quickly resumed growth and limonene-hydroperoxide dropped below detection limit within 1 hour, strongly supporting the hypothesis that AhpC^{L177Q} reduces the toxic hydroperoxide to a less toxic compound.

Based on the known function of the AhpCF complex, the reduction product is likely to be carveol or the isomer p-mentha-2,8-dien-1-ol (Figure 2). We observed very slight accumulation of carveol and its isomers in the AhpC^{L177Q} strain (Supplementary Figure S2). However, the data were appreciably noisy due to the fact that these alcohols were already present in the oxidized limonene sample. Thus, our data are consistent with the proposed function of AhpC^{L177Q} but do not allow a definitive statement about the final detoxification product.

The role of the AcrAB efflux pump

Previous work had shown that even minimal levels of *E. coli* tolerance to limonene were completely dependent on the presence of a functional efflux pump such as the *E. coli* AcrAB-TolC complex (13). AcrAB-TolC is a broad-specificity efflux pump and has crucial roles in tolerance to a wide variety of other organic solvents as well as other toxins such as antibiotics (28). To determine if limonene efflux by AcrAB-TolC was still required in cells expressing AhpC^{L177Q}, we expressed AhpC^{L177Q} in strains with deletions in either *acrA* or *acrB*.

While the *ΔacrA* and *ΔacrB* strains showed almost no fitness defect in medium without limonene, growth was significantly impaired by limonene (as above, partially oxidized limonene with an intermediate level of toxicity was used). Expression of AhpC^{L177Q} conferred no additional tolerance to limonene in these strains (Figure 6). This suggests that, consistent with prior knowledge, intracellular limonene is efficiently secreted by the AcrAB-TolC efflux pump, relieving the toxicity of limonene itself. However, an entirely orthogonal stress is imposed by the oxidized form limonene-hydroperoxide, and this toxicity is relieved only by the mutant alkyl hydroperoxidase AhpC^{L177Q}.

Discussion

The toxicity of limonene to *E. coli* has been somewhat enigmatic. The working assumption for toxicity of organic solvents is that they work largely by disrupting the

cell membrane, which in turn interferes with a variety of processes such as respiration, transport, and maintenance of ion gradients (28). Under this model, the toxicity is largely a function of the compound's hydrophobicity, and several studies have shown the inverse correlation between toxicity and *log* P_{OW} (octanol-water partition coefficient, a measure of hydrophobicity) (29). However, limonene is significantly more toxic than other solvents in the same *log* P_{OW} range and very closely related compounds like pinene (12, 13). In this work we show that this is most likely due to the presence of the limonene oxidation product limonene-hydroperoxide as a contaminant of limonene.

A key finding in our report is that limonene-hydroperoxide is likely to be present in most laboratory limonene stocks, as we observed significant hydroperoxide formation and associated toxicity appear within several weeks of routine handling of limonene bottles. Special care, such as anaerobic storage, appears to be required for limonene if it is used for toxicity studies. Ideally, chemical manufacturers should also be encouraged to store and ship limonene under inert gas. With the recent interest in the mechanism of limonene toxicity in microbes (14, 15, 30, 31), it is critical to ensure that the toxicity attributed to limonene is not in fact due to limonene-hydroperoxide. Our findings suggest that non-oxidized limonene has effects similar to other solvents in the same *log* P_{OW} range. These solvents have relatively little effect on wild-type *E. coli*, although they are highly toxic to cells with impaired efflux capability such as *ΔacrA* or *ΔacrB* strains (13). The AcrAB-TolC efflux pump is particularly critical for compounds with a *log* P_{OW} in the range of 3.9-5.5 (29), thus it is not surprising that limonene, with a *log* P_{OW} of approximately 4.1, is efficiently effluxed by AcrAB-TolC.

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The AhpC^{L177Q} protein, discovered in a strain of *E. coli* that evolved on medium containing limonene and presumably limonene-hydroperoxide, appears to be highly efficient at reducing limonene-hydroperoxide to a less toxic product. Given the increased sensitivity of the *ΔahpC* mutant, wild-type AhpC likely has some minimal activity against limonene-hydroperoxide, but the activity is greatly enhanced by the L177Q mutation. Analysis of the protein structure could illuminate the effect of the mutation, and the structure of *E. coli* AhpC was recently solved by X-ray crystallography with 3.3Å resolution (32). Unfortunately, the C-terminal tail, which includes the L177 residue, could not be resolved in the structure. However, L177 is likely to be located close to the active site at C166, and the structure is consistent with the C-terminal tail being involved in substrate recognition. An alternative, though unlikely, scenario is that the L177Q mutation generally increases AhpC activity against a variety of substrates. Further *in vitro* studies with AhpC^{L177Q} and other mutant versions of AhpC would be necessary to resolve these questions.

We were mildly surprised to find that AhpC^{L177Q} was equally efficient in a wild-type and *ΔahpC* background (Figure 1B), since AhpC forms a decamer in its functional form (32, 33) and the mutation was originally isolated in a strain with only the single *ahpC^{L177Q}* allele. This strongly suggests that the heteromer is functional, which would be consistent with the fact that each decamer has ten independent substrate-accessible active sites. Alternatively, very small quantities of homomeric AhpC^{L177Q} may be sufficient for activity, which would also be consistent with our findings that even very low (leaky) expression of AhpC^{L177Q} was sufficient for tolerance and no increase in tolerance was associated with higher expression (Supplementary Figure S3).

Although the oxidation of limonene to limonene-hydroperoxide was observed half a century ago (34, 35), there is a limited body of literature on the biological properties of limonene-hydroperoxide. A large fraction of this literature focuses on the allergenic properties of limonene, which were shown to be due to limonene-hydroperoxide (25, 36–38). This direct parallel to bacterial toxicity suggests a general cellular damage mechanism for limonene-hydroperoxide. In general, hydroperoxides, like other reactive oxygen species, cause oxidative damage to a variety of cellular macromolecules, such as DNA, proteins, and lipids (39). Lipid peroxidation could be particularly relevant in the case of limonene-hydroperoxide, as the hydrophobic limonene moiety is likely to insert into the membrane.

Metabolic engineering of microbes for production of fuels and commodity chemicals has been challenging, and the toxicity of the final product is thought to be a major impediment to increasing production titers (40). In the specific case of microbial production of limonene, our findings are very encouraging, as they suggest that the limonene produced inside the cell will not be highly toxic as long as it is efficiently secreted. Consistent with this, expression of $AhpC^{L177Q}$ did not confer any significant improvement in limonene production in 24-72 hour batch cultures of engineered *E. coli* (Supplementary Figure S4). However, industrial fermentations can last several weeks or more, and we observed significant limonene oxidation to limonene-hydroperoxide on this time scale. While a scaled-up production process would be tailored towards preventing significant product oxidation, even small quantities of the hydroperoxide could be toxic, and strains not subject to this toxicity would be preferred. Since strains expressing $AhpC^{L177Q}$ showed no obvious defects in growth or metabolism, they are excellent candidates for microbial limonene production processes at the industrial scale.

399 Furthermore, the *ahpC*^{L177Q} mutation appeared after only a few days of growth in the
400 stress condition, and it is possible that even more tolerant strains could be obtained by
401 continued laboratory evolution.
402
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415 **Disclosure statement**
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418
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530

531 **Figure Legends**

532

533 **Figure 1**: Growth of *E. coli* expressing either AhpC^{WT} or AhpC^{L177Q}. **(A)** Six strains,
534 including the spontaneous mutant FM003 as well as the reconstituted strains expressing
535 AhpC^{L177Q}, growing in defined rich medium with 2% v/v limonene. Shaded areas
536 represent experimental variation (standard error of the mean, SEM) from three
537 biological replicates. **(B)** Specific growth rates at different limonene concentrations.
538 Error bars represent the SEM of three biological replicates.

539

540 **Figure 2: (A)** Reaction catalyzed by alkyl hydroperoxide reductase, formed by the
541 enzymes AhpC and AhpF, *in vivo*. **(B)** Hypothetical mechanism of limonene-
542 hydroperoxide formation and detoxification by AhpC^{L177Q}.

543

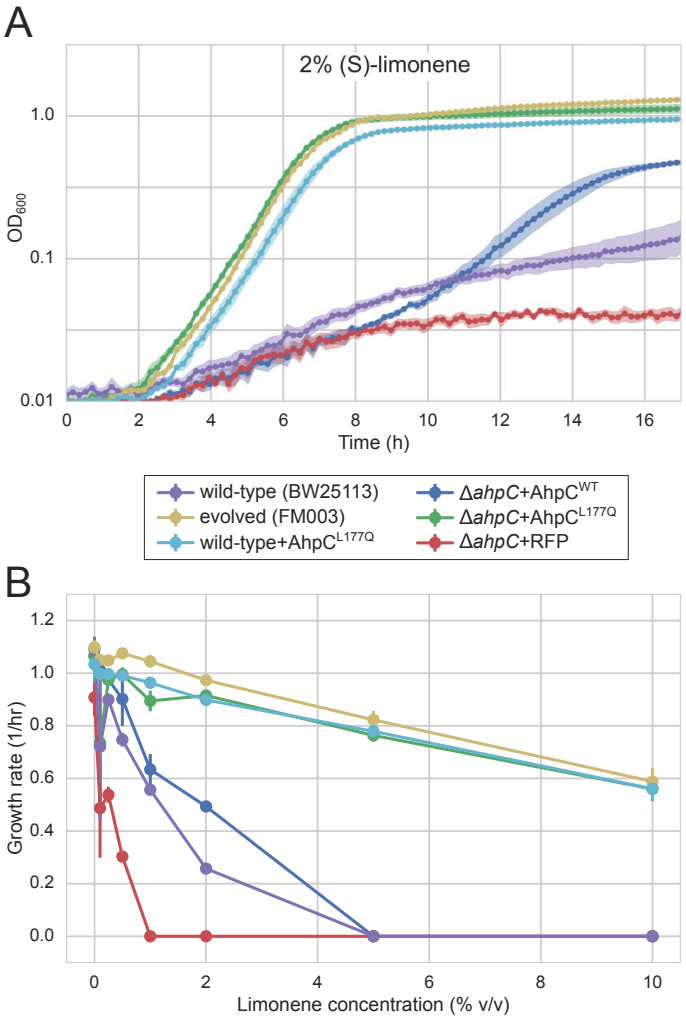
544 **Figure 3**: Anaerobic growth of *E. coli* expressing AhpC^{WT} or AhpC^{L177Q}. Specific growth
545 rate at different limonene concentrations is shown for *ΔahpC* expressing either of the

two AhpC variants or RFP, growing anaerobically in defined rich medium. Error bars represent SEM of three biological replicates.

Figure 4: Toxicity of limonene at different degrees of oxidation. **(A)** Specific growth rate plotted against limonene concentration for non-oxidized, anaerobically stored limonene (*Top*) and fully oxidized (3-week air and light exposed) limonene (*Bottom*). *Left*: wild-type BW25113 *Right*: *ΔahpC* expressing AhpC^{L177Q}. **(B)** Limonene toxicity, defined as the inverse of the shaded areas in panel A, plotted against the limonene-hydroperoxide content (LC-TOF-MS peak area, arbitrary units). Each point represents one of 13 limonene bottles subjected to varying degrees of oxidation. Blue points represent wild-type BW25113, while green points represent *ΔahpC* expressing AhpC^{L177Q}.

Figure 5: Kinetics of intracellular limonene-hydroperoxide upon limonene addition to growing cultures. 1% v/v of partially oxidized limonene was added at t=0. Four independent biological replicates (from two different days) are shown together. Lines are spline fits. n.d. = below detection limit.

Figure 6: Effect of *acrA* and *acrB* gene knockouts on limonene tolerance in strains expressing AhpC^{L177Q}. Error bars represent SEM of three biological replicates.



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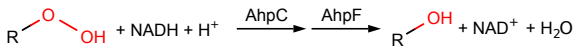
A



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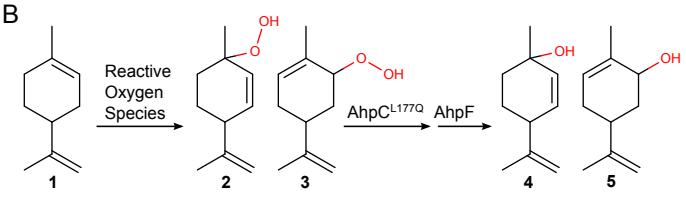

1: limonene

2: limonene-1-hydroperoxide

3: limonene-2-hydroperoxide

4: p-mentha-2,8-dien-1-ol

5: carveol