A fatty acyl-CoA reductase promotes wax ester accumulation in *Rhodococcus jostii* RHA1

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Running title: A rhodococcal fatty acyl-CoA reductase

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Abstract

Many rhodococci are oleaginous and, as such, have considerable potential for the sustainable production of lipid-based commodity chemicals. Herein, we demonstrated that *Rhodococcus jostii* RHA1, a soil bacterium that catabolizes a wide range of organic compounds, produced wax esters (WEs) up to 0.0002% of its cellular dry weight during exponential growth on glucose. These WEs were fully saturated, and contained primarily 31 to 34 carbon atoms. Moreover, they were present at higher levels during exponential growth than under lipid-accumulating conditions. Bioinformatics analyses revealed that RHA1 contains a gene encoding a putative fatty acyl-CoA reductase (FcrA). The purified enzyme catalyzed the NADPH-dependent transformation of stearoyl-CoA to stearyl alcohol with a specific activity of 45±3 nmol/mg·min and dodecanal to dodecanol with a specific activity of 5300±300 nmol/mg·min. Deletion of *fcrA* did not affect WE accumulation when grown in either carbon- or nitrogen-limited media. However, the Δ*fcrA* mutant accumulated less than 20% the amount of WEs as the wild type strain under conditions of nitric oxide stress. A strain of RHA1 overproducing FcrA accumulated WEs to ~13% cellular dry weight under lipid-accumulating conditions and their acyl moieties

had a longer average chain length than those in wild type cells (C17 vs. C16). The results provide insight into the biosynthesis of WEs in rhodococci and facilitate the development of this genus for the production of high-value neutral lipids.

Importance

Among the best-studied oleaginous bacteria, rhodococci have considerable potential for the sustainable production of lipid-based commodity chemicals such as wax esters. However, many aspects of lipid synthesis in these bacteria are poorly understood. The current study identifies a key enzyme in wax ester synthesis in rhodococci, and exploits it to significantly improve the yield of wax esters in bacteria. In so doing, this work contributes to the development of novel bioprocesses for an important class of oleochemicals that may ultimately allow us to phase out their unsustainable production from sources such as petroleum and palm oil.

41 **Introduction**

42 Many bacterial species, particularly the mycolic acid-producing Actinobacteria, synthesize
43 neutral lipids for energy storage. For example, the soil bacterium *Rhodococcus jostii* RHA1
44 (RHA1 hereafter) accumulates neutral lipids up to 70% of cellular dry weight (CDW) in response
45 to environmental stresses such as nitrogen limitation (1-3). These neutral lipids are primarily
46 triacylglycerides (TAGs), and are stored in lipid bodies within the cytoplasm (4). Proteins
47 associated with these carbon storage organelles (5) facilitate the dynamic shuffling of lipids
48 between utilization and storage, allowing the bacterium to sequester intracellular energy
49 reserves as needed. Due in part to their remarkable biosynthetic capacity, there is considerable
50 interest in using oleaginous microorganisms to sustainably produce neutral lipids to replace
51 oleochemicals currently derived from palm oils and petroleum (6-8). In rhodococci, research
52 has focused on the production of TAGs to manufacture biodiesel (7). However, the poor
53 economics of biodiesel has prevented the development and adoption of industrial-scale
54 processes (9, 10). An alternate approach would be to harness the biosynthetic potential of
55 rhodococci to produce higher-value lipids.

56 Wax esters (WEs), comprising a fatty alcohol and a fatty acid, are high-value neutral lipids used
57 extensively in cosmetics (11). Found throughout the domains of life, WEs have both functional
58 and structural roles (12-15). In some oleaginous bacteria, WEs are primarily used to store
59 energy (16, 17). In these bacteria, WE synthesis involves the esterification of a fatty alcohol and
60 an acyl-CoA in a reaction catalyzed by a bifunctional WE synthase/acyl-CoA:diacylglycerol
61 acyltransferase (WS/DGAT) (Figure 1) (18, 19). Lipid accumulation is induced by environmental
62 insult or stress, such as nitrogen-limitation, in the presence of excess carbon. Under such

63 conditions, fatty acyl-CoA substrates are predominantly provided through lipid biosynthesis.
64 When not available exogenously or generated as intermediates in the degradation of alkanes,
65 fatty alcohols are synthesized *de novo* from acyl-CoAs produced by fatty acid synthases (17, 20).
66 Some oleaginous Actinobacteria have been reported to produce WEs. For example,
67 *Mycobacterium tuberculosis* accumulates WEs to ~4% of total neutral lipids alongside TAGs
68 during periods of stress (21), contributing to dormancy and antibiotic resistance (22).
69 Rhodococci also produce WEs under lipid-accumulating conditions when supplied with
70 exogenous fatty alcohols or hydrocarbons (1). Moreover, transient *de novo* production of WEs
71 was reported in RHA1 by Barney *et al* (23). However, because this phenomenon has not been
72 further investigated, the capacity of rhodococci to produce WEs via a *de novo* route is unclear.
73 Interestingly, oleaginous rhodococci such as RHA1 and *Rhodococcus opacus* PD630 harbor over
74 a dozen WS/DGAT homologues, encoded by *atf* genes (1, 24). Among the characterized
75 rhodococcal WS/DGATs, $Atf1_{PD630}$ has higher WS than DGAT activity (25).

76 Fatty alcohols are produced through the reduction of an activated fatty acid. In bacteria, the
77 latter is usually a fatty acyl-CoA, and its reduction is accomplished by either a single enzyme or
78 consecutive reactions catalysed by two enzymes (Figure 1). The two-enzyme pathway was first
79 described in the WE-accumulating bacterium *Acinetobacter calcoaceticus* BD413. In this
80 bacterium, an NADPH-dependant fatty acyl-CoA reductase (FAR), encoded by *acr1*, performs
81 the two-electron reduction of the acyl-CoA to a fatty aldehyde. The second enzyme, responsible
82 for reducing the fatty aldehyde to a fatty alcohol, has yet to be identified (26). *Marinobacter*
83 *aquaeolei* VT8 accomplishes the same process using a FAR that catalyzes two successive two-
84 electron reductions of the acyl-CoA to the corresponding fatty alcohol. *M. aquaeolei* VT8

85 contain two FAR-encoding genes with this activity, *maqu_2220* and *maqu_2507* (27, 28). *M.*
86 *tuberculosis* contains both enzyme types; *fcr1* (*Rv3391*) is a homolog of *maqu_2507*, while *fcr2*
87 (*Rv1543*) is a homolog of *acr1* (22). Maqu_2507 and Fcr1 contain two NADPH-binding domains.
88 Interestingly, the C-terminal domain of these enzymes is homologous to the NADPH-binding
89 domain of Acr1 and Fcr2 (Figure 1D).

90 Herein, we studied the production of WEs in RHA1. WE levels were investigated in different
91 growth media. A fatty acyl-CoA reductase (FcrA) encoded by the RHA1 genome was purified
92 and characterized with respect to its ability to transform various substrates. A deletion mutant
93 and an FcrA overproduction strain were generated to examine the role of this fatty acyl-CoA
94 reductase in WE synthesis. The results are discussed in terms of the potential of rhodococcal
95 species for the production of high-value oleochemicals.

96

97 **Results**

98 *Production of WEs in RHA1* – Based on the ability of mycobacteria to produce WEs (21, 22) and
99 the similar physiologies of mycobacteria and rhodococci, we hypothesized that the latter also
100 produce WEs when supplied with a growth substrate other than a fatty alcohol or hydrocarbon
101 (1). To test this hypothesis, we grew RHA1 on M9 Goodies minimal medium containing 4 g/l
102 glucose (defined here as carbon-limiting, or C^- media as glucose is completely consumed before
103 nitrogen is depleted), extracted the neutral lipids from exponentially growing cells, and
104 fractionated them using flash chromatography. Initial GC/MS analyses revealed that cells
105 contained saturated WEs ranging from 31-34 carbons in length, with C32 species being the

most abundant (Figure 2A). Using electron ionization mass spectrometry, we analyzed the WEs using the $RCOOH_2^+$ and $RCO\text{-}H^{+\bullet}$ ions of their saturated and unsaturated fatty acid components, respectively (29). For example, the length of the fatty acyl component of the C32 WEs varied from 14 to 18 carbons, as seen from the $RCOOH_2^+$ ions with *m/z* values of 229, 243, 257, 271, 285, respectively (Figure 2B). These ions indicate that ~70% of the C32 WEs were C16:C16 species, while the remainder were a mixture of species varying from C14:C18 to C18:C14. Finally, using the highly abundant C16 ion, which is more abundant than the corresponding WE molecular ion, we were able to identify additional WEs with 29, 30, and 35 carbon atoms that were not apparent in the total ion trace (Figure 2C). In summary, the most abundant WEs in exponentially growing cells contained 32 carbons (Figure 3A). Moreover, C16 was the most abundant WE fatty acyl moiety in the detected WEs (Figure 3B). No unsaturated fatty acids were detected under these conditions. Quantification by GC/MS revealed that the cells contained 2.2 ± 0.2 µg of WEs/g cell dry weight (CDW). Interestingly, the cells contained lower amounts of WEs (0.6 ± 0.2 µg/g CDW) when grown in nitrogen-limiting (N^-) media (*i.e.*, media in which nitrogen is depleted before glucose is consumed) and examined in stationary phase (Table 1), conditions under which they accumulate TAGs to over 50% of their CDW (1). WEs produced in N^- media were similar in length and composition to those in C^- media (Figure 3).

Bioinformatic identification of FcrA – Having identified WEs in RHA1, we searched the strain's genome for homologs of *fcr1* or *fcr2* of *M. tuberculosis*, which encode fatty acyl-CoA reductases (FARs) (22). Using BLAST, we identified RHA1_RS30405 and RHA1_RS09420 as reciprocal best hits of Fcr1 and Fcr2, respectively. *RS30405* encodes a protein of 664 amino acid residues, identified here as FcrA, that shares 56% and 43% amino acid sequence identities with Fcr1 and

128 Maqu_2507 from *M. aquaeolei* VT8, respectively. The three enzymes share a similar two-
129 domain structure in which each domain contains a predicted nucleotide-binding site (Figure
130 1D). *RS30405* is conserved in all rhodococci whose genomes have been sequenced. *RS09420*
131 encodes a protein of 280 amino acids that shares 48% and 46% amino acid sequence identities
132 with Fcr2 and Acr1 from *A. calcoaceticus*, respectively, as well as 46% identity with the second
133 nucleotide-binding domain of FcrA.

134 To gain further insight into the physiological role of the identified FARs in RHA1, we considered
135 their transcript levels in previous RNA-seq studies aimed at understanding TAG biosynthesis (3,
136 GEO accession number GSE77158). In N^- media, the transcript levels of both *fcrA* and *RS09420*
137 were low, with reads per kilobase per million mapped reads (RPKM) values between 6 and 11 in
138 exponential and stationary phase. Interestingly, *fcrA* appears to be more highly expressed in C^-
139 media, with RPKMs of 19 ± 9 and 87 ± 6 in exponential and stationary phase, respectively. By
140 contrast, *RS09420* transcript levels were also low in C^- media: the RPKM value was 9 ± 5 in
141 exponential phase and no transcripts were detected in stationary phase.

142 The RNA-seq data also provides insight into the operon structures of *fcrA*. Thus, *RS30410*,
143 located immediately upstream of *fcrA* and encoding a putative membrane protein, was not co-
144 transcribed with *fcrA*. Interestingly, *RS30410* is conserved upstream of *fcrA* across the diverse
145 phylogenetic clades of rhodococci (30), while the surrounding genomic context is not. By
146 contrast, a homolog of *RS30410* is not present upstream of *fcr1* in mycobacterial species,
147 suggesting that *RS30410* is not essential for the activity of *fcrA*. The low transcription levels for
148 *RS09420* did not allow us to make meaningful observations about the structure of its transcript.

149 *Production, purification, and characterization of recombinant FcrA* – We focused further efforts
150 on exploring the function of FcrA as related two-domain enzymes completely reduce acyl-CoAs
151 to fatty alcohols (28) and have been used in biotechnology applications (31, 32). To investigate
152 the activity of FcrA, we produced it as a C-terminally His-tagged protein in RHA1 and purified it
153 to greater than 95% apparent homogeneity (Figure 4A). To check for post-translational
154 modification, purified FcrA was subjected to mass spectrometry. The molecular mass of FcrA-
155 His_6 was 73627 Da, which corresponds to the mass of the protein (73758 Da) less the N-terminal
156 methionine (131 Da). This result indicates that the heterologoulsy produced enzyme was not
157 subjected to other post-translational modifications.

158 To evaluate the ability of FcrA to catalyze the formation of fatty alcohols, 1.4 µM FcrA was
159 incubated with 400 µM NADPH and 100 µM oleoyl-CoA in 1 mL 20 mM Tris-HCl, pH 7.0, 50 mM
160 NaCl for 20 h at room temperature. The reaction was quenched with 1 mL saturated NaCl and
161 100 µM of tetradecanol was added as a standard. Analysis of the extracted and derivatized
162 reaction products by GC/MS revealed that FcrA converted oleoyl-CoA to oleyl alcohol (Figure
163 4B). No alcohol was detected in control reactions in which FcrA was omitted.

164 Using a spectrophotometric assay following either the production of 2-nitro-5-thiobenzoate
165 (NTB^{2-}) or the consumption of NADPH, FcrA catalyzed the reduction of various acyl-CoAs. As
166 with previously characterized FARs (22, 28), activity was maximal at pH 7.0. Moreover, no
167 activity was detected when NADPH was substituted with NADH. Similar to Maqu_2507 (28), the
168 rate of NADPH consumption was twice that of NTB^{2-} production, indicating that FcrA oxidizes
169 two equivalents of NADPH for every molecule of CoA released. NTB^{2-} production was used to

query a range of acyl-CoA substrates. Among the tested substrates, FcrA had highest specific activity for stearoyl-CoA (C18-CoA), reducing it at a rate of 45 ± 3 nmol/mg·min. The specific activity of FcrA dropped off with decreasing chain length of the acyl-CoA substrate (Table 2). By contrast, oleoyl-CoA (C18:1-CoA), a monounsaturated acyl-CoA, was reduced at a similar rate to stearoyl-CoA.

As previously characterized FARs also reduce fatty aldehydes, we used a spectrophotometric assay to investigate whether FcrA also catalyses this transformation. Like Maqu_2507 (28), FcrA reduced fatty aldehyde at a rate ~100-fold greater than fatty acyl-CoAs. Among the three tested aldehydes, FcrA had highest specific activity for dodecanal, reducing it at a rate of 5300 ± 300 nmol/mg·min (Table 2).

Wax ester production in a Δfcr A mutant – Having established the identity of RS30405 as a fatty acyl-CoA reductase, we further investigated the physiological role of *fcrA* by deleting the gene in RHA1. Interestingly, the Δ*fcrA* mutant contained similar amounts of WEs as wild type (WT) RHA1 when grown in both C^- and N^- media, in exponential and stationary phase, respectively (Table 1).

In *M. tuberculosis*, a Δ*fcr1* mutant did not show a decrease in WEs when starved for both carbon and nitrogen, but did so under conditions of nitric oxide (NO) stress (22). Therefore, we sought to investigate whether WE production by *fcrA* is linked to the presence of reactive nitrogen species in RHA1 by using sodium nitroprusside (SNP), to generate NO. When stressed with NO, WT cells growing exponentially on glucose minimal medium produced similar levels of WEs as non-stressed cells. The majority of the WE species observed in WT RHA1 are similar in

chain length and composition to those seen under other growth conditions (Figure 3). However, trace amounts of unsaturated C32-34 WEs were detected in the SNP-treated cells. The production of WEs in response to NO stress was strikingly different in the Δ*fcrA* mutant (Figure S1), with a 6-fold reduction in accumulated WEs (Table 1).

Overexpression of fcrA – To investigate the potential of FcrA to promote WE accumulation, we overproduced a tag-less form of the enzyme in RHA1 using a pTip vector. We performed this experiment in N^- media, which promotes neutral lipid accumulation (1). Indeed, TLC analyses of cells overproducing FcrA indicated that they contained significant amounts of WEs under these conditions (Figure 5A). Thus, overproduction of FcrA resulted in the appearance of a large band that ran similarly to stearyl stearate. Gravimetric analysis indicated that the FcrA-overproducing strain accumulated WEs to 13±5 % of CDW in N^- media. No significant accumulation of WEs was observed in cells growing exponentially or at stationary phase in C^- media. These WEs ranged from 30 to 38 C atoms in length (Figure 5B). The WEs were on average a couple of carbon atoms longer than those in WT Cells (Figure 3A). Moreover, a significant portion (~20%) of the WEs were unsaturated. Analysis of the fragmentation ions further revealed that the most abundant saturated and unsaturated fatty acyl chain lengths were C17 (Figure 3B). Finally, only trace amounts (<1% of total) of unsaturated fatty alcohols were detected, signifying that the unsaturation was primarily located within the acyl moiety.

209 **Discussion**

210 This study presents evidence for the *de novo* synthesis of WEs in rhodococci from non-alkane
211 substrates and establishes that FcrA is an alcohol-forming fatty acyl-CoA reductase that
212 contributes to the biosynthesis of WEs in RHA1. More specifically, purified FcrA catalyzed the
213 reduction of various fatty acyl-CoAs to the corresponding fatty alcohol. Although the enzyme
214 did not appear to contribute significantly to the synthesis of WEs under normal growth
215 conditions, deletion of *fcrA* resulted in significantly lower production of WEs under NO stress.
216 Finally, overproduction of FcrA in RHA1 resulted in the bacterium accumulating WEs to greater
217 than 10% of CDW under conditions that promote TAG production.

218 FcrA of RHA1 had comparable activity to the other two-domain FARs that have been
219 characterized to date, but had a slightly different substrate preference. For example, FcrA
220 showed a strong preference for C18-CoAs, in contrast to Maqu_2507, which had highest activity
221 for C16-CoA (28). Both enzymes showed decreased activity with shorter-chain acyl-CoAs.
222 Monounsaturation of the acyl-CoA substrate appears to have no significant effect on the
223 activity of either enzyme. In contrast, Fcr1 of *M. tuberculosis* had a strong preference for C18:1-
224 CoA over saturated acyl-CoAs (22). Maqu_2507 and FcrA reduced fatty aldehydes at
225 comparable rates. However, while Maqu_2507 had maximal activity with decanal (C10), FcrA
226 had maximal activity with dodecanal (C12). Nevertheless, additional studies are required to
227 properly compare the substrate preferences of the two enzymes.

228 Our data indicate that, like *M. tuberculosis* and *M. aquaeolei* VT8, RHA1 has more than one WE
229 biosynthesis pathway. Moreover, FcrA only contributes to WE biosynthesis under specific

stresses. Thus, deletion of *fcrA* had no effect on WE synthesis during nutrient-limited growth, similar to the phenotypes of the *fcr1* mutant in *M. tuberculosis* (22) and the *maqu_2507* mutant in *M. aquaeolei* VT8 (16), a Gram-negative WE-accumulating bacterium isolated from an offshore oil-producing well. In further similarity to the phenotype of the *fcr1* mutant in *M. tuberculosis* (22), deletion of *fcrA* impacted WE synthesis in the presence of reactive nitrogen species. Consistent with the apparently minor role of FcrA in WE biosynthesis during exponential growth, the WE composition of RHA1 under these conditions did not reflect the substrate preference of FcrA. More specifically, FcrA had a preference for longer acyl-CoAs than the average chain length of the WE alcohols under these conditions. By contrast, the WE content of RHA1 cells was more reflective of FcrA's substrate preference in cells overproducing the enzyme in N^- media. That is, the WEs contained alcohols with longer chain lengths. Overall, this suggests that the two-domain FARs may be responsible for producing WEs only in response to specific stresses (22).

Aldehyde-forming fatty acyl-CoA reductases may play a larger role in WE synthesis in mycolic acid-producing Actinobacteria. For example, the *fcr2* mutant of *M. tuberculosis* contained lower amounts of WEs under all conditions examined. It seems reasonable that in RHA1, RS09420 plays a similar role. Both Fcr2 and RS09420 are homologs of Acr1 (26), the aldehyde-forming fatty acyl-CoA reductase of *A. calcoaceticus* BD413, and could therefore function as such. Sirakova *et al*. reported that Fcr2 generated fatty alcohols from acyl-CoA substrates (22). However, Fcr2 was characterized in *E. coli* lysate, which contains an unidentified enzyme that reduces fatty aldehydes to fatty alcohols (26). Indeed, the presence of this enzyme has been

exploited to produce fatty alcohols in *E. coli* expressing *acr1* (8). Genetic and biochemical characterization of *RS09420* are required to definitively assign this gene's function.

The amount of WEs in RHA1 is significantly less than what has been reported in *M. tuberculosis*. More specifically, WEs comprised a larger portion of the neutral lipids in *M. tuberculosis* than in WT RHA1 under all the conditions we tested and, as quantified by [1-^{14}C]-oleate incorporation into neutral lipid pools after 6h, were approximately 10,000-fold higher than in RHA1 under stress conditions (21, 22). However, it is unclear whether the WE content of *M. tuberculosis* is representative of mycolic acid-containing bacteria. For example, WEs were not detected in *Mycobacterium smegmatis* under nitrogen-limited conditions by TLC (18), an observation that does not preclude their presence in the trace amounts reported here for RHA1. WEs have been suggested to contribute to dormancy and membrane permeability in *M. tuberculosis* (22). Interestingly, *M. tuberculosis* is one of the few mycobacterial species that does not produce mycolate-derived WEs (33), a class of lipids synthesized from keto-mycolic acids by a Baeyer-Villiger monooxygenase (34). These mycolate-derived WEs are integral components of the mycolic acid layer of mycobacteria (35) and appear to be replaced by methoxy-mycolates in *M. tuberculosis*. More studies are required to elucidate the role of WEs in mycolic acid-containing bacteria.

The current data highlight the potential of rhodococci for the sustainable production of WEs. More specifically, simply overproducing FcrA in RHA1 yielded a system that accumulated ~13% of its CDW in WEs. This exceeds the levels of any natural or engineered strains reported to date, including *A. calcoaceticus* and *M. aquaeolei* VT8, which accumulated *de novo* WEs to ~6% and

~10% CDW, respectively (16, 36). Similarly, engineered strains of *E. coli* and *Acinetobacter* accumulated WEs to 1% and 3% CDW, respectively (37, 38), or resulted in large reductions to growth yields (39). Further metabolic engineering should significantly increase WE yields in RHA1 strains. This could be achieved by increasing carbon flow into WEs, modulating redox co-factors, analogously to what was accomplished in the oleaginous yeast *Yarrowia lipolytica* (40), and/or simultaneous overproduction of a WE synthase. Interestingly, the WEs accumulated by our engineered strain are remarkably similar to spermaceti WEs, which were historically valued as lubricants and cosmetics due to their excellent physicochemical properties (41). The rhodococcal and spermaceti WEs are comparable with respect to several characteristics, including range of chain lengths (with C34 WEs being the majority species), lengths of acyl- and alcohol-components, and degree of unsaturation (14). The similarity of rhodococcal and spermaceti WEs enhances the former's industrial relevance and justifies further research into the production of rhodococcal WEs.

286 **Materials and Methods**

287 *Strains and culture conditions* – *Escherichia coli* DH5α was used to propagate DNA. *E. coli* S17.1
288 was used to conjugate pK18-derived plasmids into RHA1. *E. coli* strains were grown in LB broth
289 at 37 °C, 200 rpm. RHA1 was grown at 30 °C while shaking at 200 rpm. For protein production,
290 RHA1 was grown in LB. For lipid production, RHA1 was grown in M9 minimal medium
291 supplemented with trace elements plus thiamin (Goodies mix), and 4 g/L glucose as growth
292 substrate (42, 43). In carbon-limited (C^-) media (*i.e.*, medium in which the carbon concentration
293 limits maximum cell growth), the M9 medium contained 1 g/L ammonium chloride. In nitrogen-
294 limiting (N^-) media (*i.e.*, medium in which the nitrogen concentration limits maximum cell
295 growth), this concentration was 0.05 g/L, as previously described (3). For solid medium, LB
296 broth was supplemented with Bacto agar (1.5% [w/v]; Difco). Media were further
297 supplemented with 100 µg/mL ampicillin (*E. coli* carrying pTip-derived plasmids), 50 µg/mL
298 kanamycin (*E. coli* carrying pK18-derived plasmids), 34 µg/mL chloramphenicol (RHA1 carrying
299 pTip-derived plasmids) or 10 µg/mL neomycin (RHA1 carrying pK18-derived plasmids) as
300 appropriate.

301 *Reagents* – Enzymes for cloning were purchased from New England Biolabs unless otherwise
302 noted. Primers were ordered from Integrated DNA Technologies. Chemicals were of at least
303 reagent grade unless otherwise noted. Buffers were prepared using water purified on a
304 Barnstead NANOpure UV apparatus to a resistivity of greater than 17 MΩcm.

305 *DNA manipulation, plasmid construction, and gene deletion* – DNA was isolated, manipulated,
306 and analyzed using standard protocols (43). *E. coli* and RHA1 were transformed with DNA by

electroporation using a MicroPulser with GenePulser cuvettes (Bio-Rad). To produce a C-terminal His_6-tag FcrA, RHA1_RS30405 was amplified from RHA1 genomic DNA using Phusion Polymerase™ with the primers 5'-TTTAAGAAGGAGATATACATATGGCCACCTACCTCGTCACCG-3' and 5'-ATGGTGATGGTGATGCTCGAGGCTGCTGCCCTGGAAATACAAGTTTTCTCTGCCGCTGCTCCA-GTGGGTGCCGGGCAC-3'. The resulting amplicon was inserted into pTip-QC2 linearized with Nde1 and Xho1 using Gibson Assembly. Tagless FcrA was produced as above using the primers 5'-TTTAAGAAGGAGATATACATATGGCCACCTACCTCGTCACC-3' and 5'-GTGCCGGTGGGTCGACTA-GTCACCAGTGGGTGCCGGG-3'. The nucleotide sequence of the cloned genes was verified. The Δ*fcrA* mutant was constructed using a *sacB* counter selection system (44). Two 500 bp flanking regions of RHA1_RS30405 were amplified from RHA1 genomic DNA using the upstream primers 5'-TGAGGTGCGAGGACGTGGATCTCGGCTG-3' and 5'-CGGGTACCGAGCTCGAATTCGTCTCCGCGCC-ATCACGC-3' and the downstream primers 5'-GCTATGACATGATTACGAATTCGGCCGGGGTGCGG-GTCA-3' and 5'-ACGTCCTCGCACCTCAGCACACACCGGAACCC-3'. The resulting amplicons were inserted into pK18mobsacB linearized with EcoR1 using Gibson Assembly. The nucleotide sequence of the resulting construct was verified. Kanamycin-sensitive/sucrose-resistant colonies were screened using PCR and the gene deletion was confirmed by sequencing.

FcrA production and purification – RHA1 freshly transformed with pTip-*fcrA* were grown overnight in LB. These cultures were used to inoculate 1 L fresh LB medium to an optical density at 600 nm (OD_{600}) of 0.05. Cultures were grown to an OD_{600} ~0.8, the expression of *fcrA* was induced with 10 µg/mL of thiostrepton, and cultures were incubated for a further 24 h. Cells were harvested by centrifugation and stored at -80 °C.

Cells from 2 L of culture were suspended in 40 mL of lysis buffer (50 mM Na-phosphate, pH 8.5, 500 mM NaCl, 2.5 mM imidazole) containing cOmplete Mini EDTA-Free protease inhibitors (Roche Diagnostics). The cell suspension was split between four 15 mL conical tubes each containing ~1 mL 0.1 mm zirconium/silica beads and ~1 mL 0.5 mm glass beads (BioSpec Products) and subjected to three rounds of bead-beating at 5-6 m/s using a FastPrep®-24 (MP Biomedicals) with 5 min on ice between rounds. The lysate was centrifuged (4000 × g for 5 min) to remove unbroken cells. The supernatant lysate was removed and stored on ice. The pellets were suspended in 5 mL lysis buffer and subjected to another 3 rounds of bead-beating. The supernatants were combined and further clarified by ultracentrifugation (40,000 × g for 60 min) and passage through a 0.22 µm filter.

The clarified lysate was incubated with 5 mL Ni Sepharose 6 fast flow resin (GE Healthcare Bio-Sciences) for 3 h. After incubation, the resin was poured into a column, then washed with 50 mL lysis buffer, 50 mL lysis buffer containing 50 mM imidazole, and 15 mL lysis buffer containing 75 mM imidazole. FcrA was eluted with 15 mL lysis buffer containing 250 mM imidazole. FcrA-containing fractions, as judged by SDS-PAGE, were pooled, exchanged into 50 mM Na-phosphate, pH 8.5, 500 mM NaCl, and concentrated to ~10 mg/mL using an Amicon Ultra-15 centrifugal filtration unit (Merck KGaA) equipped with a 30 kDa cut-off membrane. Protein was flash frozen as beads in liquid nitrogen and stored at -80 °C. Protein concentration was determined by Micro BCA (Thermo Fisher Scientific, Rockford, IL) and by absorbance at 280 nm.

348 *Mass spectrometry* – Purified FcrA (~4 µg/mL in 5% acetonitrile, 0.1% formic Acid (v/v)) was
349 injected onto a 5mm C4 column connected to a Waters Xevo GS-2 QTof mass spectromter via a
350 NanoAquity UPLC system operated at 20 µL/min. Samples were eluted in a 40 µL gradient of 5-
351 100% acetonitrile at 20 µL/min. MS spectra were summed and deconvoluted using Waters'
352 MaxEnt algorithm.

353 *Activity assays* – FcrA activity was evaluated using two spectrophotometric assays (28). Assays
354 were performed at 25 °C in 1 mL 20 mM MOPS, 80 mM NaCl, pH 7.0 (I = 0.1 M) containing 5 µM
355 acyl-CoA or 60 µM aldehyde, and 200 µM NADPH. Reactions were initiated by the addition of
356 FcrA to a final concentration of 0.1 to 2 µM. In one assay, the oxidation of NADPH was followed
357 at 340 nm (ε = 6.3 mM mM^{-1} cm^{-1} (45)). In the second assay, the reaction mixture also
358 contained 0.1 mg/mL 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), and the formation of NTB^{2-}
359 was followed at 412 nm (ε = 14.15 mM^{-1} cm^{-1} for NTB^{2-} (46)). Reaction rates were calculated
360 from progress curves using Cary WinUV Kinetics Application (Agilent). The pH-dependence of
361 the reaction was evaluated using the DTNB assay and a series of 20 mM Good's buffers (MES,
362 MOPS, HEPPS, or TAPS), 80 mM NaCl (I = 0.1 M), pH 6 to 9.

363 *Production of neutral lipids and WEs* – Fresh media were inoculated to an OD_{600} of 0.1 using
364 washed RHA1 cells harvested from cultures grown overnight in C^- media. For analyses of C^-
365 cultures, cells were harvested during exponential growth, approximately 24 h after inoculation.
366 For analyses of N^- cultures, cells were harvested after approximately 72 h (3). To induce nitric
367 oxide (NO) stress, cells were grown in C^- media to an OD_{600} of 0.5-0.6 at which point sodium
368 nitroprusside (SNP) was added to the cultures to a concentration 1 mM at two-hour intervals

for six hours. NO-stressed cells were harvested two hours after the final addition of SNP. For RHA1 transformed with pTip-*fcrA* or empty pTipQC2, cultures were grown as above, except that when they reached an OD_{600} between 0.6-1.0, thiostrepton was added to 10 µg/mL. Cells were pelleted at 4,000 × *g* for 30 min at 4 °C, washed with distilled water, and stored at -80 °C.

Lipid extraction and thin layer chromatography (TLC) – Frozen cell pellets were lyophilized for 24 to 48 h using a FreeZone 2.5 (LABCONCO). Dried cells were suspended in water and sonicated to lyse cells. Myristyl myristate (Nu-Chek Prep) and/or ethyl myristate (Sigma) was added as a standard. Total lipids were extracted from lysate using 2:1 chloroform:methanol with 1% acetic acid (47). The organic phase was collected, and dried using a rotary evaporator (Buchi) and/or nitrogen gas. Dried extracts were suspended in chloroform and stored at -20 °C. Lipid extracts were analyzed using silica-TLC and a mobile phase of 90:6:1 v/v of hexane/diethyl ether/acetic acid. Lipids were visualized by staining with 10% cupric sulphate in 8% aqueous phosphoric acid (v/v) and charring for 5 min at 200°C.

Isolation, fractionation, and gravimetric quantification of neutral lipids – Neutral cellular lipids were purified using flash chromatography. Total lipid extracts were applied to a column of silica resin (10 mg lipids per 300 mg resin (48)) equilibrated with 5 column volumes (CV) of chloroform. Neutral lipids eluted with 5 CV of chloroform, were dried as described above, and quantified by weight using an AT200 analytical balance (METTLER). The neutral lipids were suspended in hexanes and fractionated using silica equilibrated with 5 CV of hexanes. Neutral lipid extracts were applied to the column and the hydrocarbon fraction was eluted with 10 CV of hexanes, the WE fraction with 10 CV of 98:2 v/v of hexanes/diethyl ether, and the remaining

390 TAGs and neutral lipids with 10 CV of chloroform. The fractions were dried, weighed, and
391 suspended in chloroform for storage at -20 °C.

392 To analyze fatty alcohols produced *in vitro*, quenched reactions of 2 mL were extracted with 5
393 mL of 2:1 chloroform:methanol (v/v). The organic phase was collected, washed once with 1:1
394 H_2O:methanol, and three times with H_2O. The organic phase was removed and dried under
395 nitrogen stream. The extract was suspended in pyridine and derivatized with tetramethylsilane
396 for 1 h at 60 °C. Derivatized extracts were analyzed by gas chromatography-mass spectrometry
397 (GC/MS) as described below.

398 *GC/MS analyses* – Analyses were performed using an Agilent 6890n gas chromatograph system
399 fitted with an HP-5 MS 30 m × 0.25 mm capillary column (Hewlett-Packard) and an Agilent
400 5973n mass-selective detector. The GC was operated at an injector temperature of 300 °C, a
401 transfer line temperature of 320 °C, a quad temperature of 150 °C, a source temperature of
402 230 °C, and a helium flow rate of 1 mL/min. Samples of 1 µL were injected in splitless mode. For
403 fatty alcohol analyses, the temperature program of the oven was 40 °C for 2 min, increased to
404 160 °C at a rate of 40 °C per min, then increased to 240 °C at a rate of 5 °C per min, and finally
405 increased to 300 °C at a rate of 60 °C per min and held for 5 min. The mass spectrometer was
406 operated in electron emission scanning mode at 40 to 800 m/z and 1.97 scans per second. For
407 WE analysis, the temperature program of the oven was 40 °C for 2 min, increased to 180 °C at a
408 rate of 40 °C per min, and then increased to 320 °C at a rate of 2.5 °C per min and held for
409 20 min. Derivatized fatty alcohols and WEs were identified using Chemstation E.02.02.1431
410 (Agilent) and the NIST08 Library. The identity of fatty alcohols was further verified using

411 similarly derivatized authentic fatty alcohols. WEs were further identified by comparison to an
412 authentic stearyl stearate standard and were quantified using a standard curve generated using
413 palmityl myristate (Nu-Chek Prep).

414

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544 **Figures**

545

546 **Figure 1. Bacterial WE biosynthesis pathways.** Fatty alcohols can be generated through either a
547 single enzyme (A) or consecutive reactions catalysed by two enzymes (B). Fatty alcohols and an
548 acyl-CoA are esterified to form WEs (C). The enzymes are: Fcr, alcohol-forming fatty acyl-CoA
549 reductase; Acr, aldehyde-forming fatty acyl-CoA reductase; FaldR, fatty aldehyde reductase;
550 WS/DGAT, WE synthase/acyl-CoA:diacylglycerol acyltransferase. RS30405 and RS09420 are the
551 homologs identified in RHA1. (D) Domain structure of bacterial FARs. White boxes represent
552 NADPH-binding domains. Black triangles indicate regions containing conserved nucleotide-
553 binding residues. Amino acids lengths and percent identity (%ID) to $FcrA_{RHA1}$ are reported.

554

555 **Figure 2. Analyses of WEs in RHA1.** (A) GC/MS chromatogram of WE-containing lipid fraction.
556 Identified WEs are labeled according to total number of carbon atoms. (B) Mass spectrum of
557 the detected C32 WEs. Molecular ion and major fatty acyl fragments labeled. (C) WEs detected
558 using the C16 $RCOOH_2^+$ ion (m/z = 257), the most abundant ion fragment associated with the
559 WEs. Cells were grown on glucose and neutral lipids were extracted and fractionated using flash
560 chromatography.

561

562 **Figure 3. WE content of RHA1 under different conditions.** (A) Distribution of WE chain lengths
563 detected. (B) Distribution of acyl-chain lengths of detected WEs. Weighted mean chain lengths
564 were calculated, and are displayed above each column. Values were obtained from biological
565 duplicates. Errors ranged from 0.01 to 0.17. Conditions were: C-, carbon-limited (exponential);
566 N-, nitrogen limited (stationary); SNP, SNP-treated RHA1; pTip-FcrA, RHA1 overproducing FcrA
567 in N^- media. Sat. and Unsat. indicated saturated and unsaturated WEs/acyl-chains, respectively.

568

569 **Figure 4. Purification and activity of recombinant FcrA.** (A) SDS-PAGE of 8 µg His_6-tagged FcrA
570 purified from RHA1. (B) GC/MS trace of a reaction mixture containing 1.4 µM FcrA-His_6, 100 µM
571 oleoyl-CoA and 400 µM NADPH (20 mM Tris-HCl, pH 7.0, 50 mM NaCl) incubated for 20 hours.

572

573 **Figure 5. WE content of RHA1 overproducing FcrA.** (A) Total lipid extracts as visualized by TLC.
574 Lanes were loaded with: 1, extract of RHA1 carrying an empty vector (pTipQC2); 2, extract of
575 RHA1 overproducing FcrA (pTip-*fcrA*); and 3, stearyl stearate and glyceryl tripalmitate. (B)
576 GC/MS analysis of WE fraction isolated from RHA1 overproducing FcrA from pTip-*fcrA*.

Tables

Table 1. WE levels[a] in RHA1 strains grown on different media and at different growth stages.

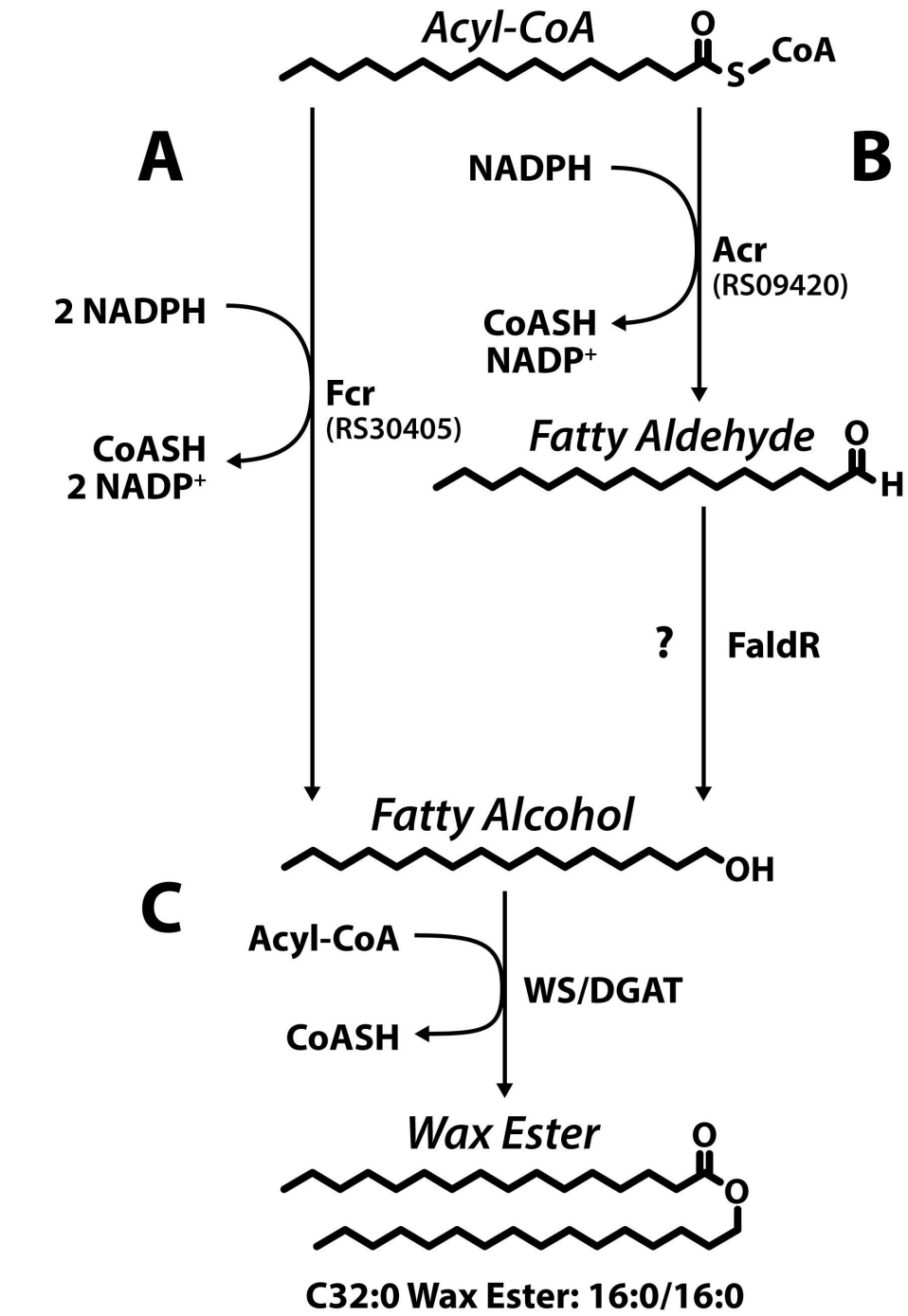
RHA1 strain	Growth condition		
	N^- (stationary)	**C^- (exponential)**	**C^- & SNP-treated (exponential)**
WT	0.6 ± 0.2	2.2 ± 0.3	3.0 ± 0.3
Δ*fcrA*	0.7 ± 0.1	3.2 ± 0.5	0.5 ± 0.1

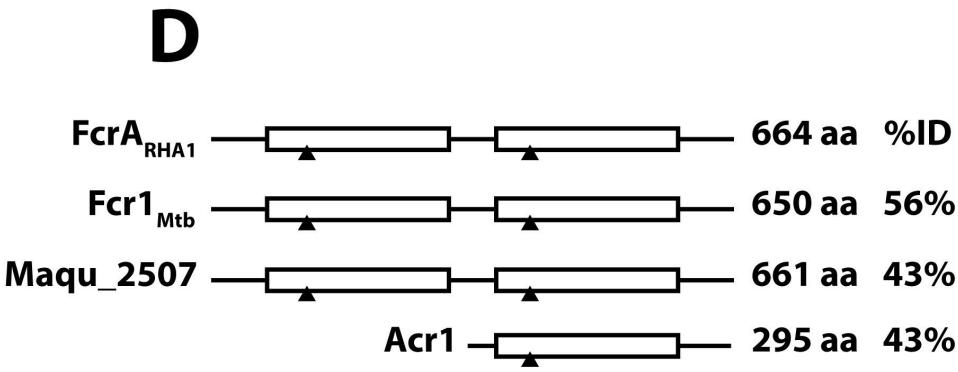
[a]Experimental values represent μg WE / g CDW. Values represent the mean of biological duplicates. Errors indicate the standard deviation.

Table 2. Specific activities of FcrA with various acyl-CoA and fatty aldehyde substrates.

Substrate	**Chain Length**	**Specific Activity**[a,b] (nmol/min/(mg protein))
Stearoyl-CoA	C18	45 ± 3
Oleoyl-CoA	C18:1	41 ± 1
Palmitoyl-CoA	C16	7.0 ± 0.1
Lauroyl-CoA	C12	1.5 ± 0.3
Decanal	C10	3300 ± 200
Dodecanal	C12	5300 ± 300
cis-11-Hexadecanal	C16:1	2800 ± 300

[a]Specific activity for acyl-CoA substrates was determined from $NTB2^-$ ion formation as detected at 412 nm. [b]Specific activity for aldehyde substrates was determined from $NADP^+$ formation as detected at 340 nm. Values represent means determined from triplicate experiments. Errors represent the standard deviation.





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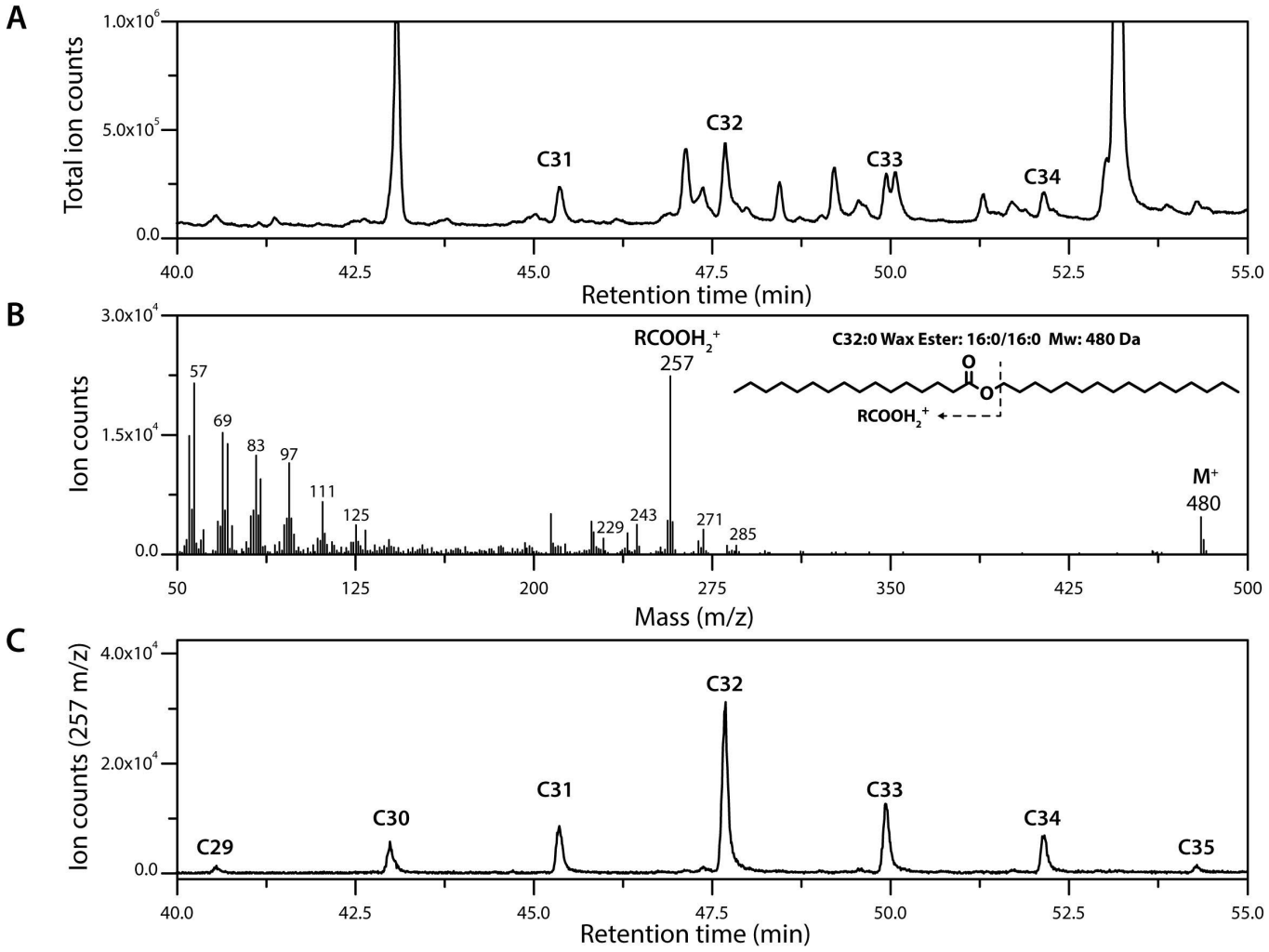

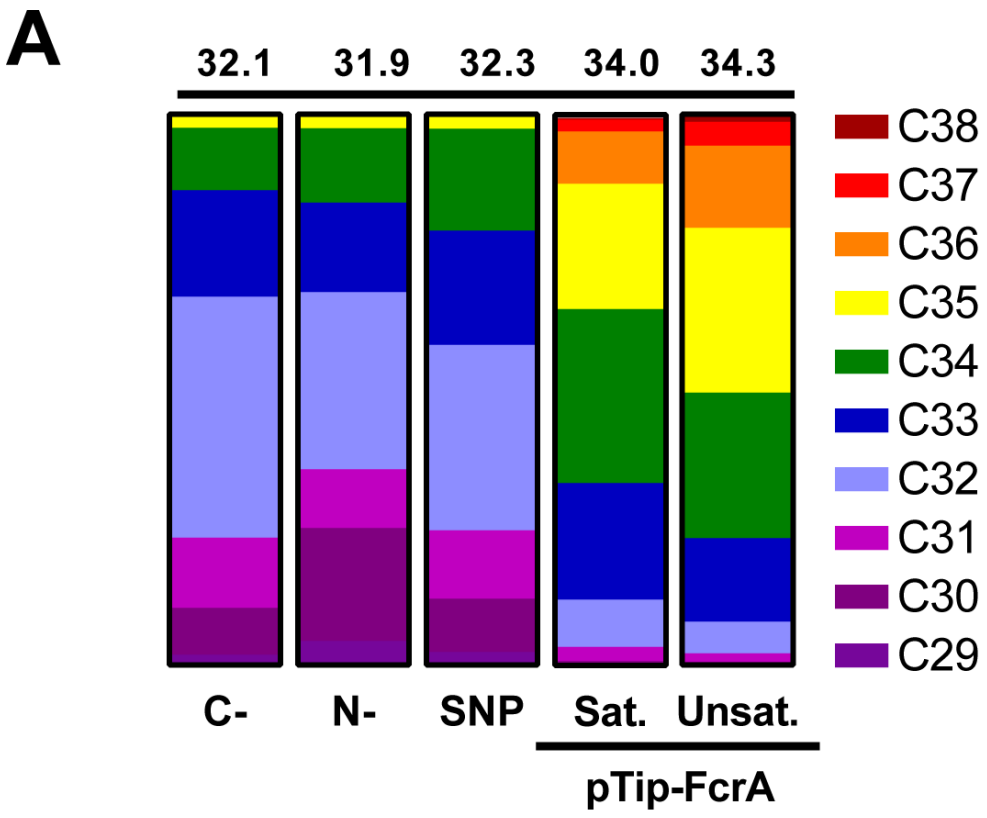

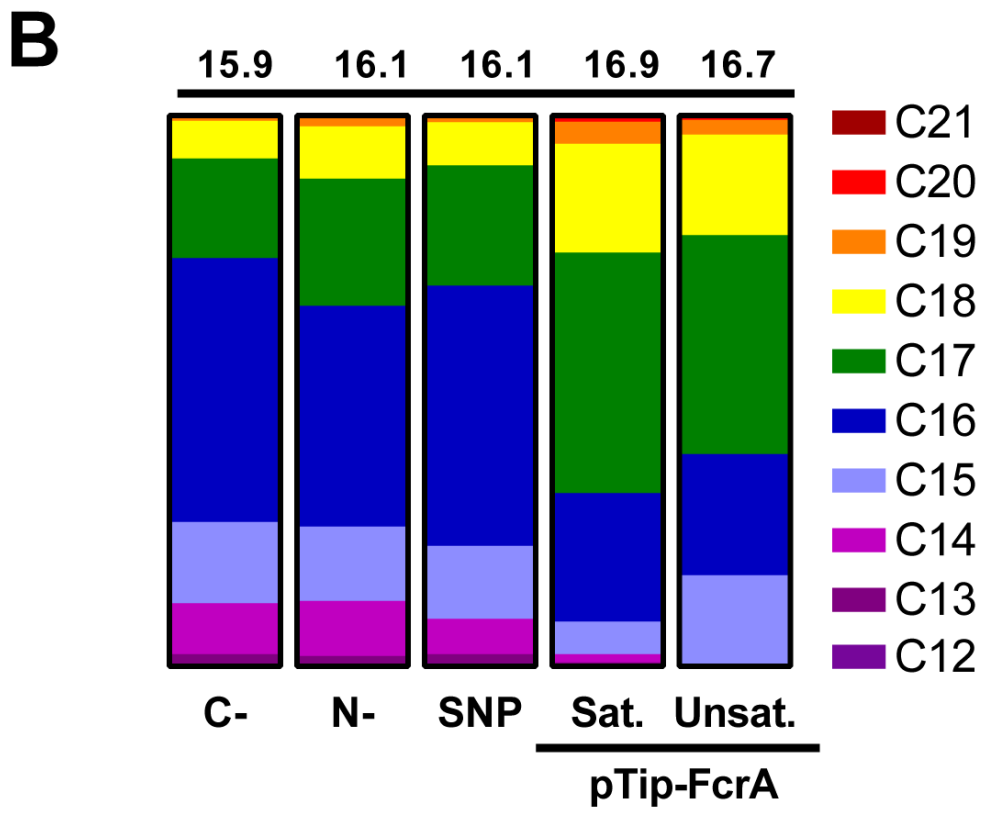

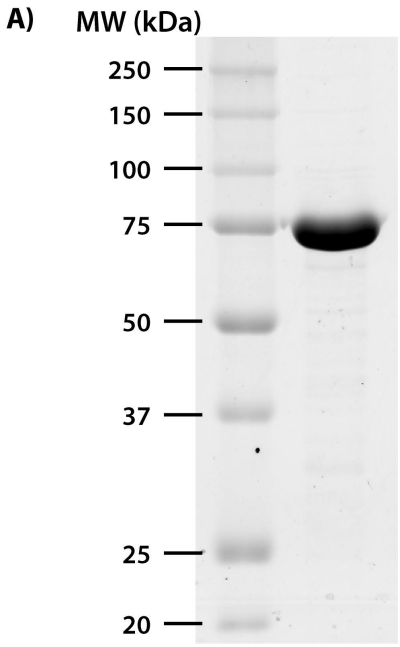

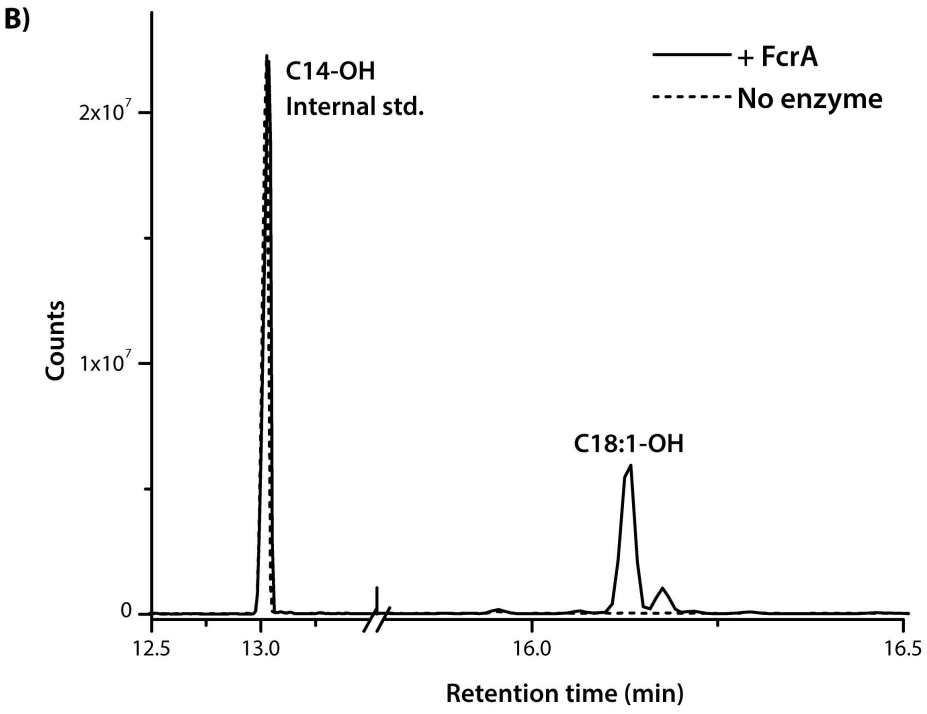

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