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Metabolism of Multiple Aromatic Compounds in Corn Stover Hydrolysate by *Rhodopseudomonas palustris*

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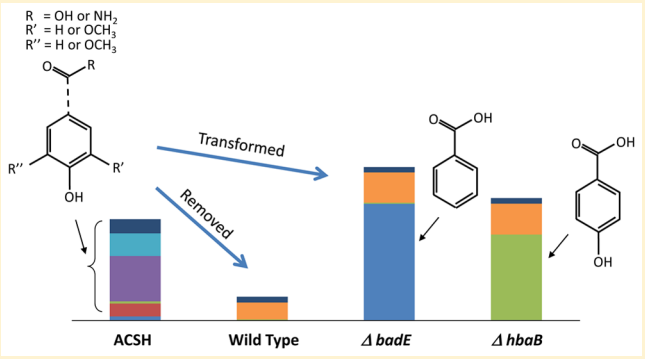
Supporting Information

ABSTRACT: Lignocellulosic biomass hydrolysates hold great potential as a feedstock for microbial biofuel production, due to their high concentration of fermentable sugars. Present at lower concentrations are a suite of aromatic compounds that can inhibit fermentation by biofuel-producing microbes. We have developed a microbial-mediated strategy for removing these aromatic compounds, using the purple nonsulfur bacterium *Rhodopseudomonas palustris*. When grown photoheterotrophically in an anaerobic environment, *R. palustris* removes most of the aromatics from ammonia fiber expansion (AFEX) treated corn stover hydrolysate (ACSH), while leaving the sugars mostly intact. We show that *R. palustris* can metabolize a host of aromatic substrates in ACSH that have either been previously described as unable to support growth, such as methoxylated aromatics, and those that have not yet been tested, such as aromatic amides. Removing the aromatics from ACSH with *R. palustris*, allowed growth of a second microbe that could not grow in the untreated ACSH. By using defined mutants, we show that most of these aromatic compounds are metabolized by the benzoyl-CoA pathway. We also show that loss of enzymes in the benzoyl-CoA pathway prevents total degradation of the aromatics in the hydrolysate, and instead allows for biological transformation of this suite of aromatics into selected aromatic compounds potentially recoverable as an additional bioproduct.



INTRODUCTION

The increasing worldwide demand for energy is accelerating fossil fuel consumption, depleting natural resources, and contributing to climate change.[1] With roughly 80% of the world's primary energy supply derived from fossil fuels, there is significant interest in increasing the contribution of renewable fuels to the overall energy production portfolio. Liquid fuels generated from lignocellulosic biomass are of particular interest as transportation fuels for long-term environmental and economic sustainability.

The Energy Independence and Security Act created a roadmap for increased industrial production of biofuels from lignocellulosic biomass in the United States.[2] According to the roadmap, the production of renewable fuels from lignocellulosic biomass was expected to reach 1.75 billion gallons by 2014.[2] The actual production was only 683 643 gallons,[3] and the first generation of commercial-scale biorefineries in the U.S., to be in full operation in 2015, will not exceed an annual capacity of 50 million gallons.[4]

Clearly, major bottlenecks still exist for the cost-effective production of biofuels from lignocellulosic biomass. Some of the challenges are economic and brought about by the large amounts of fossil fuels that can now be tapped with horizontal drilling and hydraulic fracturing, which contribute to instability in the price of fossil fuels. Other challenges are technical, requiring new scientific and engineering innovation to bring transformational changes and cost reductions to the lignocellulosic biofuels industry.

One of the persistent challenges to implement cost-effective fermentation processes is the presence of plant-derived aromatic compounds and other small bioactive molecules in hydrolysates derived from lignocellulosic biomass.[5,6] Some of these molecules have been shown to diminish biofuel

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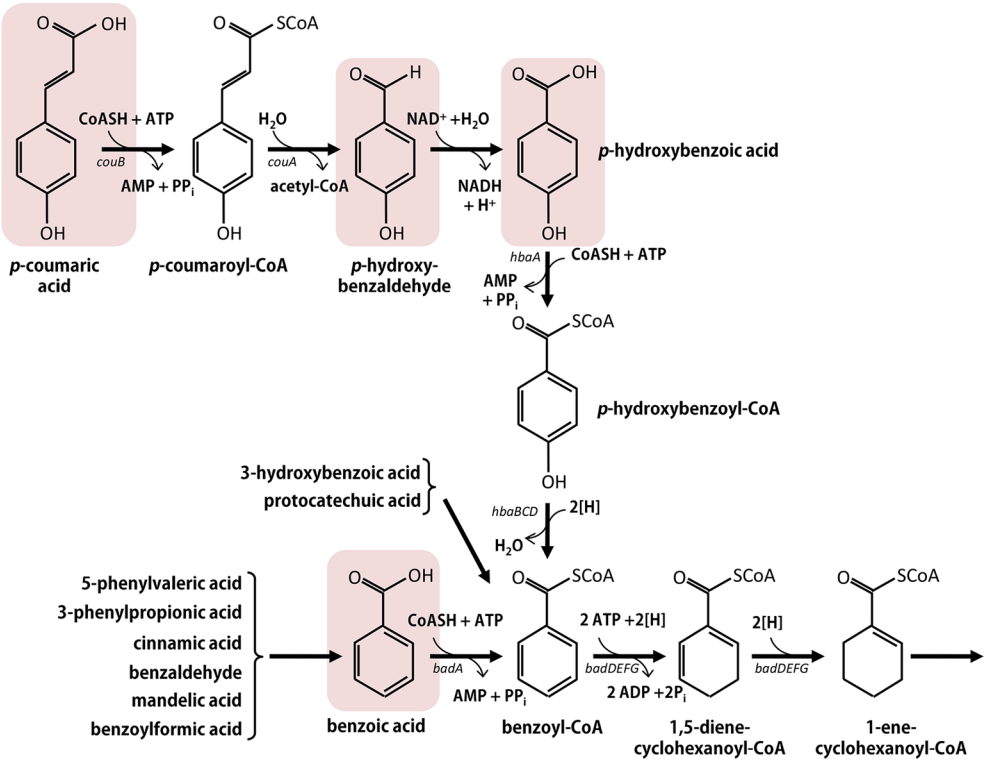

Figure 1. Aromatics known to be degraded by *R. palustris* CGA009 through the benzoyl-CoA pathway. The boxes indicate aromatics found in cellulosic biomass hydrolysates. References for the reactions shown in this figure are included in Table 1.

production by inhibiting growth and metabolism of sugars in fermenting organisms. For instance, acetic acid is known to affect cellular processes, reduce ethanol yields, and decrease sugar consumption in wild type and engineered strains of *Saccharomyces cerevisiae*,[7,8] whereas the negative effects of a variety of furans or aromatic compounds on ethanologens such as *S. cerevisiae*, *Zymomonas mobiliz*, and *Escherichia coli* are well documented.[9−14]

The suite of inhibitory molecules in lignocellulosic hydrolysates is diverse and highly dependent on the biomass pretreatment used to produce the hydrolysates. However, aromatic compounds are reported to be found in hydrolysates independent of the plant species or hydrolysis method.[6] Strategies to overcome the effect of these inhibitory bioactive molecules range from physical or chemical removal,[15] to microbial and enzymatic degradation.[16,17] Although removal can be achieved by different approaches, in most cases the removal of the inhibitory compounds is accompanied by an undesirable decrease in the amount of sugars (e.g., 5 to 35%).[17]

In this study, we showed that *Rhodopseudomonas palustris*, a bacterium known to anaerobically degrade aromatic compounds and utilize short chain organic acids,[18] can remove inhibitory aromatics from corn stover hydrolysate, without consuming the sugars needed for biofuel production. In addition, genetic removal of selected enzymes in the benzoyl-CoA pathway, used for anaerobic aromatic metabolism in *R. palustris*, resulted in the biotransformation of the large variety of plant-derived aromatics into selected phenolic compounds that could be potentially recovered and used for other applications. To our knowledge, this is the first demonstration that the diversity of aromatics in hydrolysates can be biotransformed to a single aromatic, a strategy that could add value to lignocellulosic biomass biorefineries, where production of multiple products can aid the cost-effective and sustainable production of biofuels and chemicals from lignocellulosic biomass.[4] Thus, the data in this paper (1) provides the first demonstration of using a microorganism for metabolism and removal of mixed aromatics present in hydrolysates produced by the ammonia fiber expansion (AFEX)[19] process, (2) demonstrates a process to remove these compounds that does not reduce the sugar content of the hydrolysates, and (3) demonstrates the possibility of not only removing inhibitory compounds from the hydrolysates, but also converting a chemically diverse suite of aromatics into single compounds (either benzoic acid or p-hydroxybenzoic acid), which can potentially be recovered as an additional valuable chemical coproduct in the biorefinery.

EXPERIMENTAL SECTION

Corn Stover Hydrolysate. AFEX-pretreated corn stover hydrolysate (ACSH) was prepared as described by Schwalbach et al.[20] and diluted ∼1:3 with sterile deionized water to reach ∼2% glucose content. The hydrolysates were filtered in series through 0.5 and 0.22 μm filters (Nalgene Disposable Bottle Top Filter, Thermo Fisher Scientific, Waltham, MA) prior to storage at 4 °C. Before inoculation, the pH of each hydrolysate batch, originally between 4.6 and 4.8, was adjusted to 7.0 using potassium hydroxide (KOH) pellets. After pH adjustment, the hydrolysates were filter-sterilized by passing through 0.22 μm filters.

Microbial Strains. *R. palustris* CGA009,[21] *R. palustris* CGA606 (CGA009-derived mutant lacking benzoyl-CoA reductase activity),[22] and *R. palustris* CGA506 (CGA009-derived mutant lacking 4-hydroxybenzoyl-CoA reductase activity)[23] were used in this study. In addition, *R. sphaeroides* 241EDD, an *R. sphaeroides* 2.4.1[24,25]-derived mutant with a

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Table 1. Anaerobic Transformation of Aromatic Compounds by *R. palustris* CGA009[a]

compound	degradation as sole carbon source		transformation in ACSH	
	transformed	ring fission	detected	transformed
acetosyringone			Y	N
acetovanillone			Y	N
benzaldehyde	Y[18]			
benzoic acid	Y[18]	Y[18]	Y	Y
benzoylformic acid	Y[18]	Y[18]		
caffeic acid	Y[18]	N[18]		
cinnamaldehyde	Y[18]			
cinnamic acid	Y[18]	Y[18]		
coumaroyl amide			Y	Y
p-coumaric acid	Y[18,34]	Y[18,34]	Y	Y
cyclohexanecarboxylic acid	Y[18]			
A-1-cyclohexenecarboxylic acid	Y[18]			
A-3-cyclohexenecarboxylic acid	Y[18]			
cyclohexanepropionic acid	Y[18]			
3,4-dihydroxybenzoic acid (protocatechuic acid)	N[18,36]		Y	Y[b]
ferulic acid	Y[18]	N[18]	Y	Y
feruloyl amide			Y	Y
hydrocaffeic acid	Y[18]	N[18]		
hydrocinnamaldehyde	Y[18]			
4-hydroxyacetophenone			Y	N
4-hydroxybenzamide			Y	N
4-hydroxybenzaldehyde	Y[18]		Y	Y
3-hydroxybenzoic acid	Y[18]	Y[18]		
4-hydroxybenzoic acid	Y[18]	Y[18]	Y	Y
4-hydroxybenzoylformic acid	Y[18]	Y[18]		
DL-mandelic acid	Y[18]	Y[18]		
4-phenylbutyric acid	Y[18]	N[18]		
3-phenylpropionic acid (hydrocinnamic acid)	Y[18]	Y[18]		
5-phenylvaleric acid	Y[18]	Y[18]		
syringaldehyde			Y	Y
syringamide			Y	N
syringic acid	N[18]		Y	N
vanillamide			Y	N
vanillic acid	N[18]		Y	N
vanillin	N[18]		Y	Y

[a]Harwood et al.[18] reports transformations in *R. palustris* CGA001, the parent strain of CGA009. Other compounds, not found in hydrolysates, and shown to not support anaerobic growth of *R. palustris* CGA001 are 4-aminobenzoic acid, anthranilate (2-aminobenzoic acid), catechol, 3-chlorobenzoic acid, coniferyl alcohol, 4-cresol, cyclohexanol, cyclohexanone, ethylvanillic acid, 2-fluorobenzoic acid, gallic acid (trihydroxybenzoic acid), gentisic acid, nicotinic acid, phenol, phenoxyacetic acid, 3-phenylbutyric acid, quinic acid, resorcinol, salicylic acid (2-hydroxybenzoic acid), shikimic acid, trimethoxybenzoic acid, trimethoxycinnamic acid, 3-toluic acid, 4-toluic acid, homogentisic acid, isovanillic acid, phenylacetic acid. Y = yes, N = no. [b]Protocatechuic acid has been shown to be degraded if benzoic acid or *p*-hydroxybenzoic acid are present in the medium.[36]

modification to restore function of the *edd* gene (see the Supporting Information) was also used.

Minimal Media. Photosynthetic medium (PM), prepared as described in Kim et al.[26] and containing succinate as the organic substrate, was used for *R. palustris* growth before inoculation in hydrolysate. Sistrom's minimal medium (SIS) containing succinate as the organic substrate, prepared as previously described,[27] was used for growing cultures of *R. sphaeroides* before inoculation into hydrolysate.

Experimental Conditions. Most experiments were conducted in an Applikon biofermenter (3L Autoclavable Microbial BioBundle, Applikon Biotechnology, Foster City, CA 94404) using 1000 mL of ACSH. In these experiments, the pH was controlled between 6.95 and 7.10 with 1 M H_2SO_4 and 10 M KOH, and the cultures were placed in front of continuous light generated by 10 W tungsten lamps. The temperature was kept at 28 °C, oxygen was removed by flushing with N_2 gas, and cell densities were measured using the Klett-Summerson colorimeter with a no. 66 filter (Klett MFG Co., NY). *R. sphaeroides* 241EDD and *R. palustris* CGA009 were pregrown in minimal media, and for each inoculation, 20 mL of culture was added to 1000 mL of ACSH.

Experiments with *R. palustris* CGA506 and CGA606 were conducted with ACSH (~1% glucose content) in the presence of 100 μg kanamycin mL^{-1}. The strain was initially grown in PM, and for each incubation, 150 μL of culture were added to 15 mL of hydrolysate. Glass reactor tubes were closed with rubber stoppers to ensure anaerobic conditions and placed in continuous illumination at 30 °C.

Analytical Procedures. Organic acids and sugars were analyzed by high performance liquid chromatography (HPLC) and quantified with a refractive index detector (RID-10A, Shimadzu) using a Bio-Rad Aminex HPX-87H column at 60 °C and mobile phase of 5 mM H_2SO_4 at 0.6 mL/min as described

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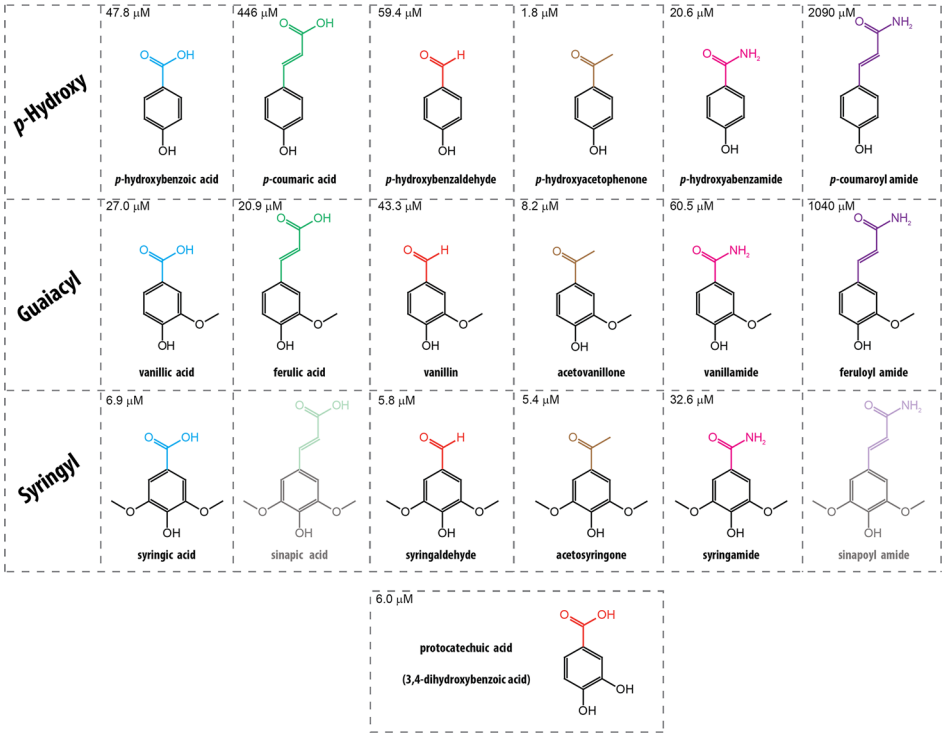

Figure 2. Chemical structures of aromatic compounds found in lignocellulosic hydrolysates. Concentrations in one batch of hydrolysate used in this study are indicated for each compound. The greyed out aromatics, sinapic acid and sinapoyl amide, were not detected.

by Schwalbach et al.[20] Samples were prepared by filtering (0.22 μm) aliquots of the culture and diluting the filtrate 10-fold before injection into the HPLC. The majority of phenolic compounds were quantified by reverse phase HPLC − high resolution/accurate mass mass spectrometry, as described in Keating et al.[28] Benzoic acid and 4-hydroxybenzoic acid were measured by high performance anion exchange chromatography−tandem mass spectrometry, using procedures also described in Keating et al.[28]

RESULTS AND DISCUSSION

Metabolism of Plant-Derived Aromatics in Corn Stover Hydrolysates by *R. palustris* CGA009. An effective microbial strategy to remove aromatic inhibitors from hydrolysates will require an organism that specifically degrades these compounds without compromising the conversion of glucose and xylose to biofuel. In addition to being deficient in sugar utilization,[21] *R. palustris* CGA009 efficiently grows using short chain organic acids[29] and is also known for its ability to utilize aromatic compounds as sole carbon sources under anaerobic conditions, using the benzoyl-CoA pathway.[18,30,31] Entrance into this pathway (Figure 1) occurs through activation of either benzoate or *p*-hydroxybenzoate by ligation to coenzyme A (CoA). A subsequent stepwise reduction of the aromatic ring yields 1-ene-cyclohexanoyl-CoA, leading to ring cleavage and further transformations to metabolites that enter central metabolism.[22,32]

Although *R. palustris* CGA009 has been shown to degrade several aromatic hydrocarbons (Table 1), little is known about its ability to utilize more complex plant-derived aromatics present in hydrolysates (Figure 2), which contain up to two methoxy functional groups and an alkyl side-chain with characteristics that depend on the methods used for biomass deconstruction.[6] Table 1 summarizes the aromatics that were known to be degraded by *R. palustris* and compares them to aromatics found in ACSH. In general, *R. palustris* CGA009 has been shown to degrade completely phenolic acids without ring substitutions or with only one hydroxyl group in the *meta* or *para* position (Table 1).[18] In addition, of the phenolic acids with a propanoid side-chain, *p*-coumaric acid can be completely degraded by *R. palustris* GGA009 (Figure 1),[33] whereas only partial degradation, without ring fission, was shown to occur with aromatic acids having more than one ring substitution, such as ferulic or caffeic acid (Table 1). *R. palustris* CGA009 has also been reported not to grow on vanillin, vanillic acid, or syringic acid when present as a sole organic carbon source.[18] Growth of *R. palustris* CGA009 on the aromatic amides that are both found in hydrolysates prepared with the AFEX pretreatment[19] and known to have inhibitory effects on ethanologenic microbes[6] has not been investigated so far.

Given the knowledge gap on the ability of *R. palustris* CGA009 to degrade aromatic amides and phenolics with more than one ring substitution, we evaluated the extent of aromatic transformation by this organism when grown in ACSH (Figure 3). Coumaroyl amide and feruloyl amide, the two aromatic amides present at the highest concentrations in ACSH, were removed from the medium (Figure 3A). Concomitant with the loss of these aromatics, 4-hydroxybenzoic acid, vanillic acid, and 3,4-dihydroxybenzoic acid accumulated (Figure 3C,D), suggesting that these aromatics are intermediates in the degradation of the propanoyl amides. Ferulic acid transiently accumulated, but was almost completely absent in the medium toward the end of the experiment (Figure 3D). Consistent with prior knowledge on its ability to serve as a carbon source,[34] *p*-coumaric acid was removed from the medium (Figure 3C). In addition, aromatic benzaldehydes (Figure 3E) were also transformed, regardless of the number of methoxy groups that they contained, while the extracellular level of aromatic phenones did not change, and a

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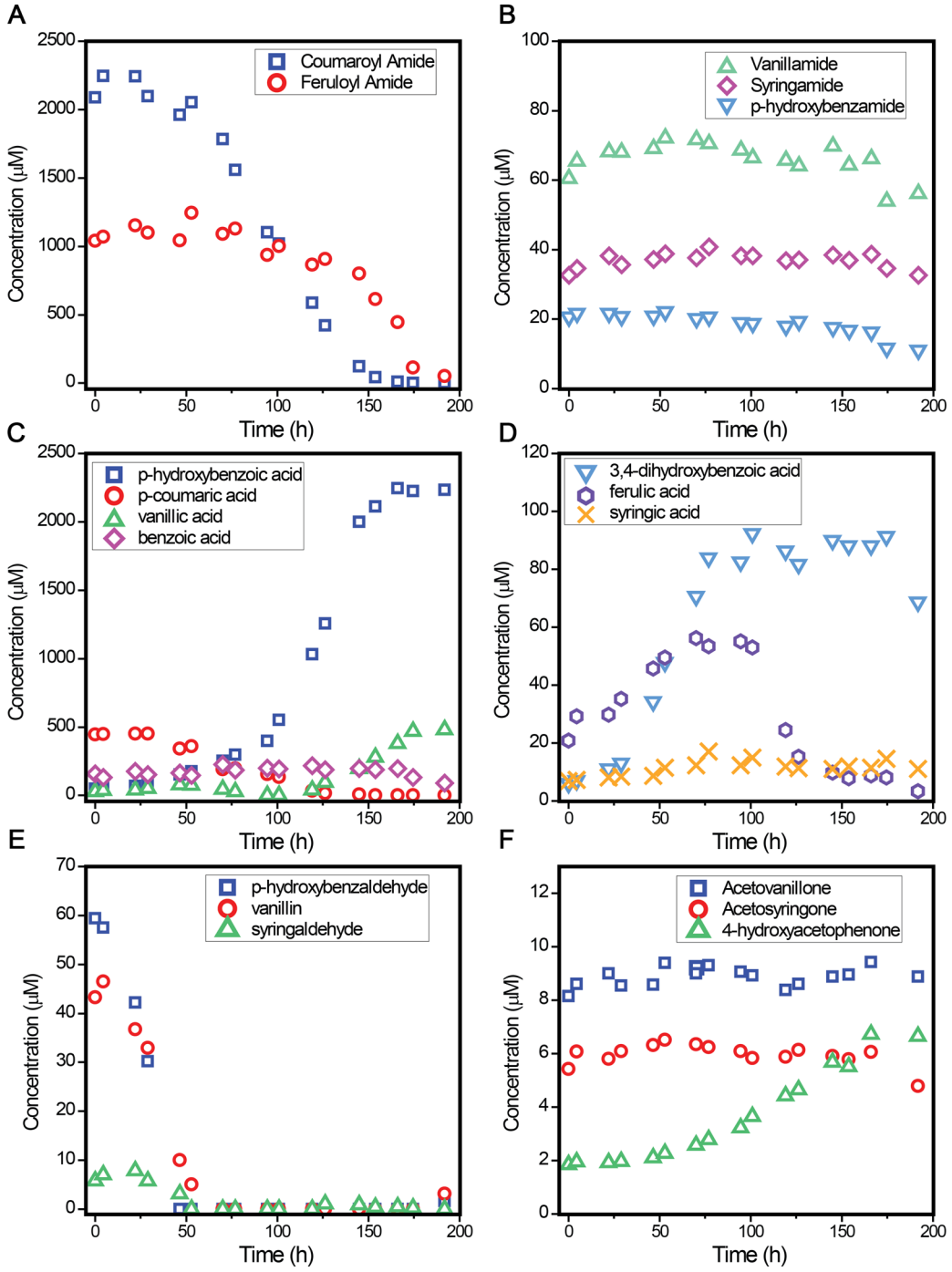

Figure 3. Transformation of aromatic compounds by *R. palustris* CGA009 in growth experiments using ACSH. Aromatic amides present at (A) high and (B) low concentrations; aromatic acids at (C) high and (D) low concentrations; (E) aromatic aldehydes; (F) phenones. The molecular structure of each compound is presented in Figure 2.

small accumulation of 4-hydroxyacetophenone was observed (Figure 3F).

This experiment demonstrated for the first time that *R. palustris* CGA009 has the ability to degrade coumaroyl amide and feruloyl amide, although three other aromatic amides present in ACSH, vanillamide, syringamide, and 4-hydroxybenzamide were not transformed (Figure 3B). On the basis of the predicted pathway for *p*-coumaric acid degradation (Figure 1),[34,35] a possible route for coumaroyl amide degradation may include an initial removal of the amine group and activation to coumaroyl-CoA, followed by removal of the alkyl chain leading to *p*-hydroxybenzaldehyde and oxidation to *p*-hydroxybenzoic acid, which then enters the benzoyl-CoA pathway after CoA-ligation. The accumulation of *p*-hydroxybenzoic acid in the medium (Figure 3C) suggests that CoA ligation of *p*-hydroxybenzoic acid is a limiting step in the use of coumaroyl amide and other aromatics in ACSH. Feruloyl amide may undergo similar transformations, with the removal of the alkyl chain after CoA ligation resulting in the formation of vanillin, and then accumulation of vanillic acid. Although it has been shown that vanillic acid is not degraded by *R. palustris* CGA001,[18] the parent strain of *R. palustris* CGA009 (CGA009

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is a chloramphenicol resistant derivative of CGA001), there is evidence that other *R. palustris* strains can use vanillic acid as a sole carbon source.[18] In the experiment with ACSH, the molar concentration of vanillic acid in the medium was about one-half of the initial concentration of feruloyl amide (Figure 3), suggesting that some degradation of vanillic acid occurred. In separate experiments with more dilute ACSH and longer incubation times (see the Supporting Information), we observed complete removal of vanillic acid, and therefore, we propose that the accumulation of vanillic acid in Figure 3C reflects a transient extracellular buildup of this putative pathway metabolite. In support of this hypothesis, we note that the extracellular accumulation of *p*-hydroxybenzoic acid is also transient (see the Supporting Information); this is not surprising because this compound is known to be metabolized via the benzoyl-CoA pathway (Figure 1).[23] Likewise, the seemingly stable accumulation of benzoic acid in the medium over the course of this experiment is likely due to it being actively produced and consumed during the experiment, as it is also a readily degradable aromatic by *R. palustris* (see the Supporting Information). The extracellular accumulation of 3,4-dihydroxybenzoic acid (protocatechuic acid) is also of note, because it is difficult to explain the source of this compound. One possibility is the removal of a methoxy group from the mono methoxylated aromatics during the degradation of vanillic acid and ferulic acid, although there is no prior knowledge that such a transformation is catalyzed by *R. palustris*. Another possibility is that protocatechuic acid is produced from the degradation of unidentified plant-derived aromatics present in ACSH. Regardless, we propose that protocatechuic acid is slowly degraded via the benzoyl-CoA pathway based on previous studies that showed that *R. palustris* CGA009 will degrade protocatechuic acid only when benzoic acid or *p*-hydroxybenzoic acid are also present, suggesting that these later compounds induce the benzoyl-CoA pathway, which does not get induced in the presence of protocatechuic acid alone.[36] The same synergy that allows *R. palustris* to degrade protocatechuic acid in the presence of other aromatics may be an explanation for the degradation of vanillic acid in ACSH.

While these experiments extend the knowledge on the range of plant-derived aromatics that *R. palustris* CGA009 can degrade, some compounds remained unutilized in ACSH. Specifically, there is no evidence that *R. palustris* CGA009 can degrade acetophenones, or dimethoxylated aromatics other than syringaldehyde present in ACSH (Figure 3).

***R. palustris* CGA009 Removes the Short Chain Organic Acids but Does Not Consume the Sugars Found in ACSH.** The removal of inhibitory compounds from hydrolysates is needed to alleviate metabolic stress in microorganisms used for fermentative production of ethanol or other compounds from lignocellulosic biomass hydrolysates.[12,28,37] However, an effective hydrolysate pretreatment needs to selectively remove the inhibitors, while leaving the sugars and other essential nutrients available for subsequent fermentation. As shown in Figure 4, *R. palustris* CGA009 does not consume glucose and xylose, the main sugars present in corn stover hydrolysate. In addition to metabolizing the aromatics in ACSH, acetate was the other main organic substrate that was removed from the medium in these cultures.

Biotransformation of Aromatics with Accumulation of Benzoic Acid Derivatives Using *R. palustris* Mutants. If the most abundant aromatics present in ACSH are biotransformed via the benzoyl-CoA pathway, we hypothesized

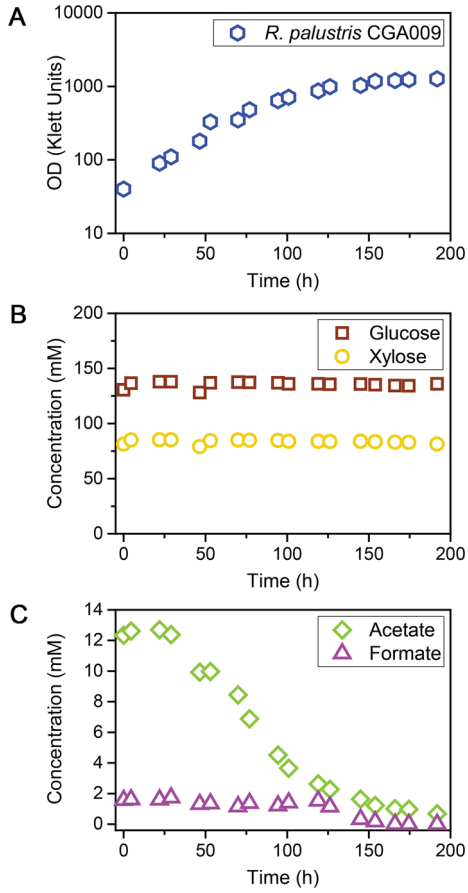

Figure 4. Growth (A), concentration of sugars (B), and concentration of short-chain organic acid (C) during growth of *R. palustris* CGA009 in ACSH.

that blocking this pathway could lead to partial transformations of some aromatics, but without ring cleavage. We tested this hypothesis with *R. palustris* CGA606, a mutant with an insertion in the *badE* gene that inactivates benzoyl-CoA reductase (Figure 1) and prevents dearomatization of benzoyl-CoA.[22] Experiments with the BadE mutant showed transformation of aromatic compounds, with a significant accumulation of benzoic acid in the medium (Figure 5). Because benzoic acid is not present at high levels in ACSH and remained at low concentrations during growth using *R. palustris* CGA009 (Figure 3), we conclude that its accumulation when using *R. palustris* CGA606 is a consequence of losing BadE activity (which normally uses benzoyl-CoA as a substrate).

We also tested growth of *R. palustris* CGA506, which lacks 4-hydroxybenzoyl-CoA reductase (HbaBCD) activity,[23] in ACSH. On the basis of what is known about the benzoyl-CoA pathway, the loss of HbaBCD should block degradation of *para*-hydroxylated aromatics but not benzoic acid. In experiments using CGA506, we found loss of aromatic compounds and accumulation of 4-hydroxybenzoic acid in the medium (Figure 5). HbaBCD uses 4-hydroxybenzoyl-CoA as a substrate (Figure 1), so the accumulation of 4-hydroxybenzoic acid in the medium predicts that metabolism of the aromatics in ACSH also uses this enzyme. More importantly, these experiment show that most of the aromatics are metabolized through the common benzoyl-CoA pathway where the aromatic ring is reduced (Figure 1). They also demonstrate the ability of engineered *R. palustris* strains to convert a diverse pool of

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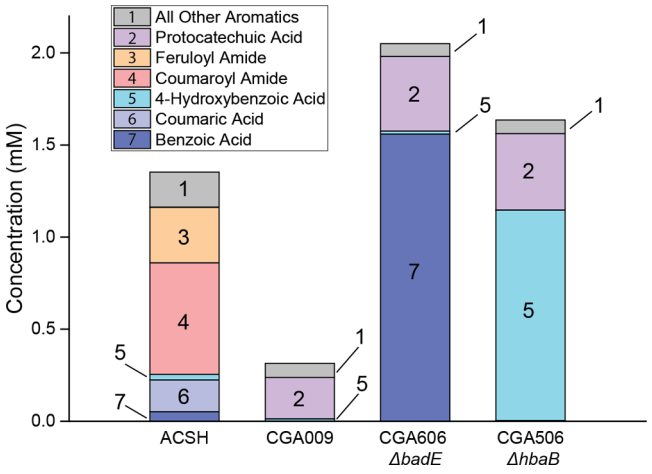

Figure 5. Concentrations of aromatic compounds in ACSH and extracellular concentrations of the same aromatics after growth of *R. palustris* CGA009 (wild type), CGA606 (Δ*badE*), and CGA506 (Δ*hbaB*).

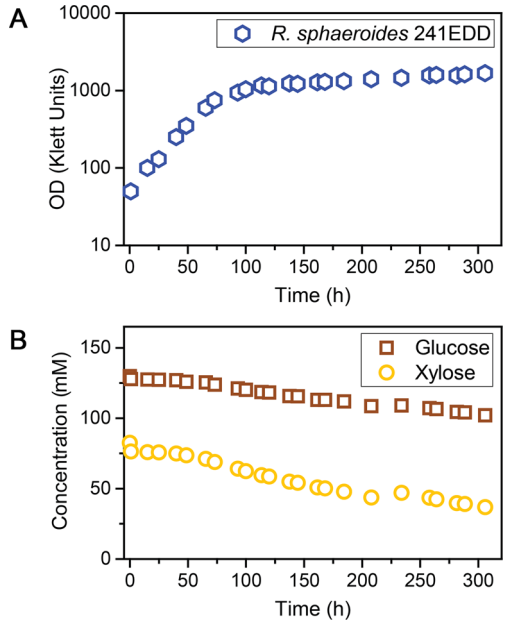

Figure 6. Growth of *R. sphaeroides* 241EDD (A) and sugar utilization (B) in ACSH that had been biologically conditioned with *R. palustris* CGA009. Note that *R. sphaeroides* 241EDD did not grow in ACSH that had not been conditioned with *R. palustris* CGA009 (see the Supporting Information).

aromatics into a single compound (either benzoic acid, 4-hydroxybenzoic acid, or possibly others based on the genetic block in the benzoyl-CoA pathway).

Biological Removal of Aromatics from ACSH Improves Growth of a Second Bacterium. The negative effect of aromatic compounds on ethanologenic fermentations has been well documented, with *p*-coumaric acid,[38] benzoic acid,[39] *p*-hydroxybenzaldehyde,[11,12] vanillin,[11,40] 4-hydroxyacetophenone,[11] acetovanillone,[11] and aromatic amides[28] reported to be inhibitory to bacterial or yeast ethanologens. Our experiments show that most of these compounds can be removed from ACSH using *R. palustris* CGA009 (Figure 3 and Table 1). Therefore, we considered the possibility that *R. palustris* can be used as a biobased method to reduce metabolic stress and improve ethanologenic or other biofuel fermentations. To test this hypothesis, we used *Rhodobacter sphaeroides*, an organism that we are investigating for production of advanced biofuels, such as long-chain fatty acids and furans.[41,42]

R. sphaeroides does not grow at the ACSH concentrations used in this study. However, when we inoculated *R. sphaeroides* 241EDD, a strain with an improved rate of glucose utilization (see the Supporting Information) in filter-sterilized ACSH that had been used to grow *R. palustris* CGA009, we found that *R. sphaeroides* could grow and metabolize the sugars. This demonstrates that biological conditioning of ACSH with *R. palustris* CGA009 allows growth and improved sugar metabolism by a second bacterium (Figure 6). From control experiments where *R. sphaeroides* is grown in the presence of the aromatic compounds found in ACSH, we know that *p*-coumaric acid, *p*-hydroxybenzaldehyde, and *p*-hydroxyacetophenone inhibited glucose utilization, whereas the other aromatics were not inhibitory.[43] ACSH conditioning with *R. palustris* effectively removed *p*-coumaric acid and *p*-hydroxybenzaldehyde, but not *p*-hydroxyacetophenone, which remained at low concentrations in the hydrolysate (Figure 3). Thus, the removal of the aromatics that were present at highest concentrations in ACSH by *R. palustris* was sufficient to allow *R. sphaeroides* growth and sugar utilization.

Implications of the Biological Removal of Aromatics from Lignocellulosic Biomass Hydrolysates. Our results demonstrate the possibility to exploit *R. palustris* metabolism for removal of aromatic compounds from lignocellulosic biomass hydrolysates. A key observation is that *R. palustris* CGA009 grown in ACSH leaves the sugars unaltered and available for biofuel production by a second microbe because it preferentially uses acetate and aromatics as organic electron donors. Although demonstrated in this study using ACSH, removal of inhibitory aromatics with *R. palustris* could be applied to other biomass pretreatments where aromatics in the hydrolysates are also a concern.[6] In addition, the removal of acetate from ACSH by *R. palustris* could also provide added benefit to the use yeast and other microbes in which fermentation performance is inhibited by the presence of this organic acid in the biomass hydrolysate.[5,44] To achieve the full potential of this process, it is necessary to engineer strains capable of growing in more concentrated hydrolysates, and extend the range of plant-derived aromatics that can be metabolized. Moreover, by engineering strains capable of channeling the aromatics into specific phenolic compounds, as demonstrated here with several *R. palustris* mutants, it should be possible to both remove inhibitors and convert them to valuable bioproducts recoverable from the hydrolysates. The accumulation of well-defined phenolic compounds by engineered strains of *R. palustris* adds to the diversity of biochemicals that could be produced in a biorefinery,[45] and contributes to increasing the fraction of the carbon present in the hydrolysates that is recovered as a valuable product instead of being released as organic waste.

■ ASSOCIATED CONTENT

Supporting Information

Text describing the construction of *R. sphaeroides* 241EDD strain, additional figures supporting the reproducibility of aromatic degradation in ACSH by *R. palustris* CGA009 (Figure S1) and the lack of growth of *R. sphaeroides* 241EDD in ACSH that has not been biologically conditioned by *R. palustris* CGA009 (Figure S2), and a table showing aromatic

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degradation in diluted ACSH (Table S1). The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b02062.

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Notes

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REFERENCES

(1) International Energy Outlook 2011; U.S. Energy Information Administration: Washington, D.C., 2011, www.eia.gov/forecasts/archive/ieo11/pdf/0484(2011).pdf.

(2) Energy Independence and Security Act of 2007. Public Law 110-140; 2007, www.gpo.gov/fdsys/pkg/BILLS-110hr6enr/pdf/BILLS-110hr6enr.pdf.

(3) U. S. Environmental Protection Agency. 2014 RFS2 Data, http://epa.gov/otaq/fuels/rfsdata/2014emts.htm.

(4) *Lignocellulosic biomass for advanced biofuels and bioproducts: Workshop Report, DOE/SC-0170*; U.S. Department of Energy Office of Science: Washington, D.C., 2015, http://genomicscience.energy.gov/biofuels/lignocellulose/.

(5) Palmqvist, E.; Hahn-Hägerdal, B. Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification. *Bioresour. Technol.* **2000**, *74* (1), 17−24.

(6) Piotrowski, J. S.; Zhang, Y. P.; Bates, D. M.; Keating, D. H.; Sato, T. K.; Ong, I. M.; Landick, R. Death by a thousand cuts: the challenges and diverse landscape of lignocellulosic hydrolysate inhibitors. *Front. Microbiol.* **2014**, *5*, 1−8.

(7) Bellissimi, E.; van Dijken, J. P.; Pronk, J. T.; van Maris, A. J. A. Effects of acetic acid on the kinetics of xylose fermentation by an engineered, xylose-isomerase-based *Saccharomyces cerevisiae* strain. *FEMS Yeast Res.* **2009**, *9* (3), 358−364.

(8) Swinnen, S.; Fernandez-Nino, M.; Gonzalez-Ramos, D.; van Maris, A. J. A.; Nevoigt, E. The fraction of cells that resume growth after acetic acid addition is a strain-dependent parameter of acetic acid tolerance in *Saccharomyces cerevisiae*. *FEMS Yeast Res.* **2014**, *14* (4), 642−653.

(9) Chambel, A.; Viegas, C. A.; Sa-Correia, I. Effect of cinnamic acid on the growth and on plasma membrane H+-ATPase activity of *Saccharomyces cerevisiae*. *Int. J. Food Microbiol.* **1999**, *50* (3), 173−179.

(10) Iwaki, A.; Ohnuki, S.; Suga, Y.; Izawa, S.; Ohya, Y. Vanillin Inhibits Translation and Induces Messenger Ribonucleoprotein (mRNP) Granule Formation in *Saccharomyces cerevisiae*: Application and Validation of High-Content, Image-Based Profiling. *PLoS One* **2013**, *8* (4), 1−10.

(11) Klinke, H. B.; Olsson, L.; Thomsen, A. B.; Ahring, B. K. Potential inhibitors from wet oxidation of wheat straw and their effect on ethanol production of *Saccharomyces cerevisiae*: Wet oxidation and fermentation by yeast. *Biotechnol. Bioeng.* **2003**, *81* (6), 738−747.

(12) Delgenes, J. P.; Moletta, R.; Navarro, J. M. Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Pichia stipitis*, and *Candida shehatae*. *Enzyme Microb. Technol.* **1996**, *19* (3), 220−225.

(13) Zaldivar, J.; Martinez, A.; Ingram, L. O. Effect of selected aldehydes on the growth and fermentation of ethanologenic *Escherichia coli*. *Biotechnol. Bioeng.* **1999**, *65* (1), 24−33.

(14) Sato, T.; Liu, T.; Parreiras, L.; Williams, D.; Wohlbach, D.; Bice, B.; Ong, I.; Breuer, R.; Qin, L.; Busalacchi, D.; Deshpande, S.; Gasch, A.; Hodge, D. Harnessing Genetic Diversity in *Saccharomyces cerevisiae* for Improved Fermentation of Xylose in Hydrolysates of Alkaline Hydrogen Peroxide Pretreated Biomass. *Appl. Environ. Microbiol.* **2014**, *80* (2), 540−554.

(15) Larsson, S.; Reimann, A.; Nilvebrant, N. O.; Jonsson, L. J. Comparison of different methods for the detoxification of lignocellulose hydrolyzates of spruce. *Appl. Biochem. Biotechnol.* **1999**, *77*−*9*, 91−103.

(16) Jonsson, L. J.; Palmqvist, E.; Nilvebrant, N. O.; Hahn-Hagerdal, B. Detoxification of wood hydrolysates with laccase and peroxidase from the white-rot fungus *Trametes versicolor*. *Appl. Microbiol. Biotechnol.* **1998**, *49* (6), 691−697.

(17) Parawira, W.; Tekere, M. Biotechnological strategies to overcome inhibitors in lignocellulose hydrolysates for ethanol production: review. *Crit. Rev. Biotechnol.* **2011**, *31* (1), 20−31.

(18) Harwood, C. S.; Gibson, J. Anaerobic and aerobic metabolism of diverse aromatic compounds by the photosynthetic bacterium *Rhodopseudomonas palustris*. *Appl. Environ. Microbiol.* **1988**, *54* (3), 712−717.

(19) Lau, M. W.; Dale, B. E. Cellulosic ethanol production from AFEX-treated corn stover using *Saccharomyces cerevisiae* 424A(LNH-ST). *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106* (5), 1368−1373.

(20) Schwalbach, M. S.; Keating, D. H.; Tremaine, M.; Marner, W. D.; Zhang, Y. P.; Bothfeld, W.; Higbee, A.; Grass, J. A.; Cotten, C.; Reed, J. L.; Sousa, L. D.; Jin, M. J.; Balan, V.; Ellinger, J.; Dale, B.; Kiley, P. J.; Landick, R. Complex Physiology and Compound Stress Responses during Fermentation of Alkali-Pretreated Corn Stover Hydrolysate by an *Escherichia coli* Ethanologen. *Appl. Environ. Microbiol.* **2012**, *78* (9), 3442−3457.

(21) Larimer, F. W.; Chain, P.; Hauser, L.; Lamerdin, J.; Malfatti, S.; Do, L.; Land, M. L.; Pelletier, D. A.; Beatty, J. T.; Lang, A. S.; Tabita, F. R.; Gibson, J. L.; Hanson, T. E.; Bobst, C.; Torres, J.; Peres, C.; Harrison, F. H.; Gibson, J.; Harwood, C. S. Complete genome sequence of the metabolically versatile photosynthetic bacterium *Rhodopseudomonas palustris*. *Nat. Biotechnol.* **2004**, *22* (1), 55−61.

(22) Egland, P. G.; Pelletier, D. A.; Dispensa, M.; Gibson, J.; Harwood, C. S. A cluster of bacterial genes for anaerobic benzene ring biodegradation. *Proc. Natl. Acad. Sci. U. S. A.* **1997**, *94* (12), 6484−6489.

(23) Gibson, J.; Dispensa, M.; Harwood, C. S. 4-hydroxybenzoyl coenzyme A reductase (dehydroxylating) is required for anaerobic degradation of 4-hydroxybenzoate by *Rhodopseudomonas palustris* and shares features with molybdenum-containing hydroxylases. *J. Bacteriol.* **1997**, *179* (3), 634−642.

(24) Mackenzie, C.; Choudhary, M.; Larimer, F. W.; Predki, P. F.; Stilwagen, S.; Armitage, J. P.; Barber, R. D.; Donohue, T. J.; Hosler, J. P.; Newman, J. E.; Shapleigh, J. P.; Sockett, R. E.; Zeilstra-Ryalls, J.; Kaplan, S. The home stretch, a first analysis of the nearly completed genome of *Rhodobacter sphaeroides* 2.4.1. *Photosynth. Res.* **2001**, *70* (1), 19−41.

(25) Kontur, W. S.; Shackwitz, W.; Ivanova, N.; Martin, J.; LaButti, K.; Deshpande, S.; Tice, H.; Pennachio, C.; Sodergren, E.; Weinstock, G.; Noguera, D. R.; Donohue, T. J. Revised sequence and annotation of the *Rhodobacter sphaeroides* 2.4.1 genome. *J. Bacteriol.* **2012**, *194*, 7016−7017.

(26) Kim, M. K.; Harwood, C. S. Regulation of benzoate-CoA ligase in *Rhodopseudomonas palustris*. *FEMS Microbiol. Lett.* **1991**, *83* (2), 199−203.

DOI: 10.1021/acs.est.5b02062
Environ. Sci. Technol. 2015, 49, 8914−8922

(27) Sistrom, W. R. The kinetics of the synthesis of photopigments in *Rhodopseudomonas spheroides*. *J. Gen. Microbiol.* **1962**, *28*, 607−16.
(28) Keating, D. H.; Zhang, Y.; Ong, I. M.; McIlwain, S.; Morales, E. H.; Grass, J. A.; Tremaine, M.; Bothfeld, W.; Higbee, A.; Ulbrich, A.; Balloon, A.; Westphall, M. S.; Aldrich, J.; Lipton, M. S.; Kim, J.; Moskvin, O.; Bukhman, Y. V.; Coon, J.; Kiley, P. J.; Bates, D. M.; Landick, R. Aromatic inhibitors derived from ammonia-pretreated lignocellulose hinder bacterial ethanologenesis by activating regulatory circuits controlling inhibitor efflux and detoxification. *Front. Microbiol.* **2014**, *5*, 1−17.
(29) Barbosa, M. J.; Rocha, J. M. S.; Tramper, J.; Wijffels, R. H. Acetate as a carbon source for hydrogen production by photosynthetic bacteria. *J. Biotechnol.* **2001**, *85* (1), 25−33.
(30) Dutton, P. L.; Evans, W. C. The Metabolism of Aromatic Compounds by *Rhodopseudomonas palustris*. *Biochem. J.* **1969**, *113*, 525.
(31) Harwood, C. S.; Burchhardt, G.; Herrmann, H.; Fuchs, G. Anaerobic metabolism of aromatic compounds via the benzoyl-CoA pathway. *FEMS Microbiol. Rev.* **1998**, *22* (5), 439−458.
(32) Harrison, F. H.; Harwood, C. S. The pimFABCDE operon from *Rhodopseudomonas palustris* mediates dicarboxylic acid degradation and participates in anaerobic benzoate degradation. *Microbiology* **2005**, *151*, 727−736.
(33) Hirakawa, H.; Schaefer, A. L.; Greenberg, E. P.; Harwood, C. S. Anaerobic p-Coumarate Degradation by *Rhodopseudomonas palustris* and Identification of CouR, a MarR Repressor Protein That Binds p-Coumaroyl Coenzyme A. *J. Bacteriol.* **2012**, *194* (8), 1960−1967.
(34) Pan, C.; Oda, Y.; Lankford, P. K.; Zhang, B.; Samatova, N. F.; Pelletier, D. A.; Harwood, C. S.; Hettich, R. L. Characterization of anaerobic catabolism of p-coumarate in *Rhodopseudomonas palustris* by integrating transcriptomics and quantitative proteomics. *Mol. Cell. Proteomics* **2008**, *7* (5), 938−948.
(35) Phattarasukol, S.; Radey, M. C.; Lappala, C. R.; Oda, Y.; Hirakawa, H.; Brittnacher, M. J.; Harwood, C. S. Identification of a p-Coumarate Degradation Regulon in *Rhodopseudomonas palustris* by Xpression, an Integrated Tool for Prokaryotic RNA-Seq Data Processing. *Appl. Environ. Microbiol.* **2012**, *78* (19), 6812−6818.
(36) Gall, D. L.; Ralph, J.; Donohue, T. J.; Noguera, D. R. Benzoyl coenzyme A pathway-mediated metabolism of meta-hydroxy-aromatic acids in *Rhodopseudomonas palustris*. *J. Bacteriol.* **2013**, *195* (18), 4112−20.
(37) Larsson, S.; Quintana-Sainz, A.; Reimann, A.; Nilvebrant, N. O.; Jonsson, L. J. Influence of lignocellulose-derived aromatic compounds on oxygen-limited growth and ethanolic fermentation by *Saccharomyces cerevisiae*. *Appl. Biochem. Biotechnol.* **2000**, *84*−*86*, 617−32.
(38) Gu, H. Q.; Zhang, J.; Bao, J. Inhibitor analysis and adaptive evolution of *Saccharomyces cerevisiae* for simultaneous saccharification and ethanol fermentation from industrial waste corncob residues. *Bioresour. Technol.* **2014**, *157*, 6−13.
(39) Verduyn, C.; Postma, E.; Scheffers, W. A.; Vandijken, J. P. Effect of benzoic acid on metabolic fluxes in yeasts - A continuous-culture study on the regulation of respiration and alcoholic fermentation. *Yeast* **1992**, *8* (7), 501−517.
(40) Fitzgerald, D. J.; Stratford, M.; Gasson, M. J.; Ueckert, J.; Bos, A.; Narbad, A. Mode of antimicrobial action of vanillin against *Escherichia coli*, *Lactobacillus plantarum* and *Listeria innocua*. *J. Appl. Microbiol.* **2004**, *97* (1), 104−113.
(41) Lemke, R. A. S.; Peterson, A. C.; Ziegelhoffer, E. C.; Westphall, M. S.; Tjellstrom, H.; Coon, J. J.; Donohue, T. J. Synthesis and scavenging role of furan fatty acids. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111* (33), E3450−E3457.
(42) Lemmer, K.; Dohnalkova, A.; Noguera, D. R.; Donohue, T. J. Oxygen-dependent regulation of bacterial lipid production. *J. Bacteriol.* **2015**, *197* (9), 1649−1658.
(43) Austin, S. Utilization of corn stover hydrolysates by *Rhodobacter sphaeroides* and *Rhodopseudomonas palustris* under photoheterotrophic conditions. MS Thesis, University of Wisconsin−Madison, Madison, WI, 2013.
(44) Roe, A. J.; O'Byrne, C.; McLaggan, D.; Booth, I. R. Inhibition of Escherichia coli growth by acetic acid: a problem with methionine biosynthesis and homocysteine toxicity. *Microbiology-Sgm* **2002**, *148*, 2215−2222.
(45) Ragauskas, A. J.; Williams, C. K.; Davison, B. H.; Britovsek, G.; Cairney, J.; Eckert, C. A.; Frederick, W. J.; Hallett, J. P.; Leak, D. J.; Liotta, C. L.; Mielenz, J. R.; Murphy, R.; Templer, R.; Tschaplinski, T. The path forward for biofuels and biomaterials. *Science* **2006**, *311* (5760), 484−489.

DOI: 10.1021/acs.est.5b02062
Environ. Sci. Technol. 2015, 49, 8914−8922