DAMAGE TO THE MICROBIAL CELL MEMBRANE DURING PYROLYTIC SUGAR UTILIZATION AND STRATEGIES FOR INCREASING RESISTANCE

Tao Jin[a], Marjorie R. Rover[b], Elspeth M Petersen[a], Zhanyou Chi[b], Ryan G. Smith[b], Robert C. Brown[b,c], Zhiyou Wen[c], Laura R. Jarboe[a*]

[a]*Department of Chemical and Biological Engineering, Iowa State University, Ames, IA. tjin@iastate.edu, ljarboe@iastate.edu*

[b]*Bioeconomy Institute, Iowa State University, mrrover@iastate.edu, rgsmith@iastate.edu*

[c]*Department of Mechanical Engineering, Iowa State University, rcbrown3@iastate.edu*

[d]*Department of Food Science and Human Nutrition, Iowa State University, wenz@iastate.edu*

Abstract

Lignocellulosic biomass is an appealing feedstock for the production of biorenewable fuels and chemicals, and thermochemical processing is a promising method for depolymerizing it into sugars. However, trace compounds in this pyrolytic sugar syrup are inhibitory to microbial biocatalysts. This study demonstrates that hydrophobic inhibitors damage the cell membrane of ethanologenic *Escherichia coli* KO11+*lgk*. Adaptive evolution was employed to identify design strategies for improving pyrolytic sugar tolerance and utilization. Characterization of the resulting evolved strain indicates that increased resistance to the membrane-damaging effects of the pyrolytic sugars can be attributed to a glutamine to leucine mutation at position 29 of carbon storage regulator CsrA. This single amino acid change is sufficient for decreasing EPS protein production and increasing membrane integrity when exposed to pyrolytic sugars.

Keywords

Pyrolytic sugars; Membrane damage; evolution; *csrA*; Extracellular polymeric substances

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Introduction

Lignocellulosic biomass, consisting mainly of cellulose, hemicellulose and lignin [19], is an attractive feedstock for the production of biofuels and chemicals [34,30,21,54]. Sugars from cellulose and hemicellulose are an attractive carbon and energy source for the microbial production of fuels and chemicals. There are a variety of methods to release these sugars, including acid hydrolysis [18,68,81], alkali hydrolysis [75], ozonolysis [79], ionic liquid [43] and steam explosion [2], as reviewed elsewhere [51,33,25]. Progress has also been made in the recovery and upgrading of the lignin component of biomass [59,10].

Fast pyrolysis of lignocellulosic biomass results in "pyrolytic syrup" rich in sugars. This fast pyrolysis is the rapid thermal decomposition of biomass in the absence of oxygen to produce mostly liquid products [7,36,80]. As conventionally produced, the liquid is an emulsion of lignin-derived phenolic compounds in an aqueous phase of oxygenated compounds derived from the decomposition of polysaccharides. These oxygenated components include aldehydes, ketones, furans, and carboxylic acids, with sugars normally being only a small fraction of the carbohydrate-derived compounds [8]. However, it has been shown that the recovery of a variety of hexose and pentose sugars and anhydrosugars (collectively referred to as pyrolytic sugars) can be dramatically increased by pretreating the biomass prior to pyrolysis with a small amount of sulfuric or phosphoric acid to passivate the alkali and alkaline earth metals (AAEM) and impede their catalytic action on the polysaccharides [35,55].

A second advance in the recovery of pyrolytic sugars is the development of a two-step fractionation process [65]. The first step condenses high boiling point compounds (heavy ends) from vapors exiting the pyrolysis reactor. The heavy ends contain lignin-derived water-insoluble phenolic oligomers and water-soluble pyrolytic sugars, the latter of which are washed out of the heavy ends with water to produce pyrolytic syrup. Although this syrup is an attractive fermentation substrate, it unfortunately contains compounds that are inhibitory to microbial biocatalysts, including phenolic compounds, alcohols, aldehydes and acids [64,6]. The inhibitory effect of these compounds limits the amount of sugar that can be provided to the fermentation and thus limits the amount of fuels and chemicals that the microbes can produce.

Biocatalyst inhibition is a common problem in utilization of biomass-derived sugars and production of biorenewable fuels and chemicals [29]. As mentioned above, a variety of biomass processing techniques have been described, for which many of them, the resulting sugar stream contains compounds that are inhibitory to the microbial biocatalyst [50,64,32,56], including but not limited to, phenolics, alcohols, aldehydes, and organic acids. This inhibition has been characterized for a variety of the most prominent compounds present in the sugar stream [11,24,84,83,82,53]. This inhibition is synergistic for many of these compounds, making it difficult to identify which inhibitor is the most problematic [84,83].

One approach for dealing with this problem is to remove the inhibitory compounds via a pretreatment step. For example, treating pyrolytic sugars with alkali decreases the phenolic content and the toxicity for microbial biocatalysts, leading to higher ethanol titers [64,6], though toxicity is still problematic. Adding a pretreatment step will increase the overall process cost, that may or may not be justified by the subsequent improvement in sugar utilization. An alternative approach is to identify the mechanisms of inhibition and engineering the microorganisms for improved robustness [29,16,5,77]. However, when inhibition mechanisms are unknown or a rational mitigation strategy is not apparent, adaptive evolution can be a useful tool [78]. Subsequent reverse engineering of such evolved strains can in turn, lead to the identification of new design strategies to combat microbial inhibition.

Previous studies have identified a variety of non-sugar compounds in pyrolytic sugars, many of which are hydrophobic [64,6]. These include, but are not limited to: hydroquinone, vanillin, catechol, cyclotene, syringol, syringaldehyde and pyrogallol. Overton's Rule states that membrane permeability is a function of a molecule's hydrophobicity [31], leading us to hypothesize that pyrolytic sugar toxicity may be due, at least in part, to membrane disruption by these hydrophobic compounds. One symptom of membrane damage is a loss of membrane integrity, leading to leakage of valuable cellular content [72,66,23]. Another important metric of membrane health is membrane fluidity, which is a function of the chain length, saturation, isomerization, branching, and cyclization of the lipid bilayers and the membrane-embedded proteins [52]. Membrane fluidity can change in response to growth conditions [47,12,49,3] or the presence of membrane-damaging compounds [66,73]. Previous studies have demonstrated that exposure to these inhibitory compounds can also lead to changes in membrane lipid composition, which can lead to further perturbation of membrane fluidity [44,62].

We hypothesize that pyrolytic sugar toxicity arises from damage of the cell membrane by hydrophobic compounds in the syrup, particularly phenolic compounds. The goals of this study were to both test this hypothesis and to identify design strategies that mitigate or prevent this damage.

Materials and methods

Strains and medium

All strains used in this work are listed in Table 1. *E. coli* strain KO11 (ATCC strain 55124) was previously engineered for levoglucosan utilization by genomic integration of the levoglucosan kinase gene (*lgk*) from *Lipomyces starkeyi* [40], here referred to as KO11+*lgk*. Seed cultures were grown overnight in 250 mL flasks at 37 °C with horizontal shaking at 200 rpm in LB medium containing 40 μg/ml chloramphenicol.

Production of raw pyrolytic sugars

Red oak (*Quercus rubra*; Wood Residual Solutions, LLC of Montecello, WI), with moisture content of approximately 10% (w/v), was used for production of the pyrolytic sugar syrup [64]. It was milled using an Artsway 60 hp hammer mill equipped with a 3 mm screen. The feedstock was then pyrolyzed at approximately 500 °C in a fluidized bed reactor to produce two fraction of bio-oil: a sugar-rich heavy ends and an aqueous phase, as described elsewhere [65].

Immediately after production, the heavy ends were mixed with an equal weight of deionized water to separate water-soluble pyrolytic sugars from water-insoluble phenolic oligomers. The mixture of heavy ends and water was thoroughly mixed using a batch lab scale mixer, placed on a shaker table (MaxQ 2506, Thermo Scientific, Hanover Park, IL) for 30 min at 250 motions min^{-1} and then centrifuged (accuSpin 1R, Thermo Scientific, Hanover Park, IL) at 2,561*ig* and room temperature for 30 min. The aqueous phase, containing pyrolytic sugars, was decanted and rotary evaporated at 40 °C until no condensation remained on the sides of the evaporation flask or condenser [64].

Overliming treatment of pyrolytic sugar syrup

Raw pyrolytic sugar syrup was detoxified according to the previously described overliming procedure [6]. Briefly, chemical grade $Ca(OH)_2$ was added to an aqueous solution containing 10 wt% of the raw pyrolytic sugars syrup, so that the final $Ca(OH)_2$ concentration was 18.5 g/L. The mixture was then

held at 60 °C for 4 h with stirring, centrifuged at 8,817*g*, and the supernatant was collected and adjusted to pH 7.0 by addition of 50% sulfuric acid. After incubation at room temperature for 24 h with shaking (150 rpm), the sample was centrifuged again and the supernatant was collected.

Determination of Cell Membrane Integrity

1. Magnesium leakage

Leakage of magnesium out of damaged cells was quantified according to previously described methods [66]. Cells were grown to mid-log in LB medium, centrifuged at 4000 × g and 4 °C for 15 min, washed twice in phosphate-buffer saline (PBS) buffer (pH 7.0), and resuspended in PBS buffer to a final OD_{550} of 15 and treated with either pyrolytic sugars, glucose or chloroform. Chloroform was added at a volume ratio of 1 part chloroform to 10 parts cells suspension. The resulting mixtures were vortexed and incubated at 37 °C, 250 rpm for 1.5 h, and then centrifuged at 21,000 × g and 4 °C for 5 min. Two µl of the supernatant were added to 3 ml of the magnesium reagent (SEKISUI Chemical Co) and incubated at 25 °C for 5 min. The amount of magnesium present in the supernatant was measured via absorbance at 660 nm.

2. SYTOX Green permeability

Cells were harvested in the mid-log phase and processed in the same way as for the magnesium leakage analysis. The cell pellets were then resuspended in PBS at a final OD_{550} of 1.0 and treated with glucose or pyrolytic sugars. After 1.5 h incubation, 100 µl of the cell suspensions were added to 900 µl PBS with 1 µl SYTOX Green (Life Technologies) nucleic acid stain, as described previously [41,63]. BD Biosciences FACSCanto II flow cytometer (-20 mW, 488 nm Argon laser with optical fixed-alignment as the excitation source) at the ISU Flow Cytometry Facility was employed for the analysis. Simultaneous measurements of forward and side laser scatter and SYTOX Green fluorescence were made. Background and non-cellular events were eliminated from data acquisition using a minimum side scatter threshold.

Membrane fluidity analysis

Cells were harvested in the mid-log phase and processed in the same way as with the magnesium leakage analysis. The collected cells were resuspended in PBS buffer at a final OD_{550} of approximately 0.6. After 1.5 h treatment with pyrolytic sugars, 6-diphenyl-1,3,5-hexatriene (DPH; Life Technologies) in tetrahydrofuran was added to a final concentration of 0.2 µM, followed by 30 min of incubation in the dark at 37 °C with shaking (250 rpm). Fluorescence polarization values were quantified as previously described

[66]. A Synergy 2 Multi-Mode microplate reader from BioTek was employed for analysis at 360/40 nm excitation and 460/40 nm emission. To eliminate the background of glucose and pyrolytic sugars, the corresponding samples without cells were analyzed as a control.

Membrane lipid composition analysis

1. Measurement of phospholipid fatty acids distribution

Cells were harvested in the mid-log phase and processed in the same way as for magnesium leakage analysis. The collected cells were resuspended in PBS and treated with raw pyrolytic sugars at 37 °C for 1.5 h. Extraction of fatty acids was performed as described elsewhere [66]. Briefly, the cell pellets were washed in ice-cold water and then resuspended in methanol. The cell suspensions were sonicated and incubated at 70 °C for 15 min with tridecanoic (C13) and nonadecanoic (C19) acids internal standards. After centrifugation at 2,200× g, 4 °C for 5 min, the supernatant was separated and mixed with water, and the cell pellets were resuspended in chloroform. Subsequently, the two solutions were combined and mixed by vortexing. After centrifugation, fatty acids were extracted from the chloroform layer of the mixture and concentrated by an N-Evap nitrogen tree evaporator (Organomation Associates). The fatty acids were methylated into esters and analyzed by gas chromatograph-flame ionization detector/mass spectrometer (GC-FID/MS) (Agilent Technologies Model 6890 Gas Chromatograph coupled to a Model 5973 Mass Selective Detector).

2. Measurement of phospholipid head groups distribution

Cells were harvested in the mid-log phase and then processed and treated in the same manner as described above. The distribution of phospholipid head groups was measured by HPLC-ELSD [1].

Fermentation and adaptive evolution

Seed cultures were inoculated at an initial OD550 of 0.05 in 500 ml fleakers containing 350 ml LB medium containing with 40 μg/ml chloramphenicol and 10% (w/v) of a mixture of glucose and raw pyrolytic sugars. Batch fermentations were performed in a 37 °C water bath with stirring at 200 rpm. The pH was maintained at 7.0 by automated addition of 2M KOH. Two replicates were performed for each concentration of raw pyrolytic sugars. Pyrolytic sugar concentration at the beginning of the evolutionary procedure was 0.25% (w/v). Cultures were sequentially diluted into fresh medium at 12 hr intervals or when the optical density (OD_{550}) reached 2.0. Three serial transfers were conducted for each sugar

concentration, followed by an increase of 0.05% (w/v) in the pyrolytic sugar content. One colony was picked at the end of the adaptive evolution and designated TJE1.

Cell encapsulation

Seed cultures of *E. coli* KO11+*lgk* were prepared in 10 ml LB medium and shaken at 250 rpm, 37 °C overnight. Seed cultures were inoculated into flasks containing 50 ml LB at an initial OD_{550} of 0.1, and grown to OD_{550} ~1.0 at 37 °C. Preparation of encapsulated cells is described in [37]. Briefly, the cell suspensions were washed with water and mixed with 1.3% (w/v) sodium alginate solution at a 1:4 ratio (v/v). Then the polymer/cell suspension was extruded into 20 g/L calcium chloride at a rate of 1.0 ml/min. The distance between the droplets of suspension and the calcium chloride solution was approximately 2.5 cm. The resulting beads were further hardened in fresh calcium chloride solution (20 g/L) for 1 h at room temperature, washed with 0.9% (w/v) saline solution and water, and resuspended in LB medium. Finally, the beads were added to an equal volume of fresh medium containing pyrolytic sugars.

Whole-Genome Sequencing

Genomic DNA was extracted and purified using DNeasy Blood & Tissue Kit (QIAGEN) and submitted to the Iowa State University DNA facility for Illumina sequencing. The short reads produced from MiSEQ 600-Cycle (2×300) were assembled by Lasergene software by DNASTAR Inc (Madison, WI). Possible mutations identified from the assembly results were investigated by Sanger sequencing at the Iowa State University DNA facility, with primers given in the supplementary information.

Genetic manipulation

Gene fragments including 20-40 bp upstream and downstream were amplified from purified genomic DNA by using Bio-Rad thermal cyclers and purified by using QIAquick PCR Purification Kit (QIAGEN). Kanamycin resistance gene (Km^R) was amplified from plasmid pKD4 flanked by 30 or 50 bp of homology to the 5' and 3' termini of each gene fragment for gene deletion or gene overlapping. Primer sequences are given in the supplementary information. Overlapping of gene fragments and the marker was achieved by PCR with Q5 high fidelity polymerase (NEB). Constructed fragments were chromosomally inserted into *E. coli* strains expressing the pKD46 plasmid, as previously described [9].

Extracellular polymeric substance (EPS) extraction and quantification

Procedures followed previously described methods [45]. *E. coli* were grown on LB plates overnight at 37 °C. Approximately 0.9~1.5×10^{11} cells were collected and suspended in 30 ml 0.85% (w/v) NaCl solution. Cell concentration was measured by a Cellometer M10 (Nexcelom Bioscience), and cell suspensions were centrifuged at 16,300×g at 4 °C for 30 min. The supernatant was passed through a polypropylene filter (0.45μm pore size) and three volumes of ice-cold 100% ethanol were added. The mixture was incubated at -20 °C for 24 hr, and centrifuged at 16,300×g at 4 °C for 30 min. The supernatant was decanted and the pellets were dried at room temperature and then resuspended with 5.5 ml DI water. This resuspension is referred to here as EPS extract.

For protein analysis, 3 ml alkaline copper reagent (1 wt% $CuSO_4$: 2 wt% Na Tartrate: 2 wt% $NaCO_3$ in 0.1M NaOH=1:1:98) was added to 0.6 ml of the EPS extract; the mixture incubated for 10 min at room temperature without agitation. Then, 0.15 ml Folin-Ciocalteu reagent (2N Folin-Ciocalteu Phenol:water=1:1) was added and the mixture incubated for 30 min at room temperature without agitation. The absorbance at 500 nm was measured, and protein content estimated using bovine serum albumin (BSA) as standard. For polysaccharide analysis, 25 μl of 80% phenol (w/v) was mixed with 1 ml of EPS extract, and 2.5 ml of 98% sulfuric acid was then added. The mixture was incubated for 10 min at room temperature and then at 30 °C for another 20 min.The samples were held for 4 hr at room temperature. Absorbance was measured at 488 nm and polysaccharide content estimated with xanthan gum as standard.

Results and Discussion

Pyrolytic sugars inhibit the growth and damage the membrane of *E. coli* KO11+*lgk*

1. Pyrolytic sugars inhibit microbial growth

Pyrolytic sugars contain a variety of organic compounds, such as phenolics, aldehydes and acids [65], which have been characterized as toxic to microbes [83,82]. To establish the concentration of pyrolytic sugars that *E. coli* KO11+*lgk* could tolerate, batch fermentations were performed (Figure 1). Note that *E. coli* KO11 was previously evolved for the production of ethanol at high yield and titer from up to 10% (w/v) sugars [28]. Our long-term goal is to replace these pure sugars with pyrolytic sugars; therefore, a high total sugar concentration was maintained in these experiments. As shown in Figure 1, provision of pyrolytic sugars at concentrations as low as 0.5% (w/v) was sufficient for complete inhibition; no growth

was observed over the course of 72 h. Cultures containing 0.25% (w/v) pyrolytic sugar syrup did grow, although with a longer lag phase than the pure glucose cultures.

This result demonstrates the magnitude of pyrolytic sugar toxicity and the need to either decrease toxicity of the sugar syrup and/or increase tolerance of the microbial biocatalyst. Understanding the mechanisms of microbial inhibition can guide such efforts.

2. Pyrolytic sugars induce membrane leakage

We have hypothesized that hydrophobic compounds in pyrolytic sugars damage the cell membrane, impacting overall microbial growth and fermentation. Consistent with this proposition, we observed that raw pyrolytic sugars cause dose-dependent decreases in membrane integrity (Figure 2A). These leakage experiments used an assay that measures the amount of magnesium released from damaged cells into the extracellular medium. Chloroform was used as a positive control and leakage values are presented relative to leakage measured with chloroform. Consistent with expectations, cells challenged with increasing concentrations of raw pyrolytic sugars showed substantial increases in membrane leakage. Specifically, cells treated with 0.5% (w/v) raw pyrolytic sugars released 40% as much magnesium as the cells treated with chloroform. However, beyond concentrations of 0.3 wt%, the amount of leakage induced remained relatively stable. In contrast, the membrane damage resulting from pure glucose never exceeded 10%, indicating that damage observed with raw pyrolytic sugars is due to non-sugar components.

The cell membrane plays a critical role in separation of the interior of the cell from the external environment, and in transporting molecules and ions into and out of cells. This is attributed to its selective permeability, which helps to exclude inhibitors and import nutrients. Our data suggests that hydrophobic inhibitors in pyrolytic sugar syrup disrupt this barrier due to their membrane permeability, resulting in a decrease in membrane integrity and leakage of essential metabolites. This phenomenon of membrane damage has been reviewed elsewhere [74]. Increasing cell membrane integrity could potentially improve pyrolytic sugar tolerance and utilization. Other researchers have demonstrated that the membrane can be strengthened through genetic modification [4,46,77].

Our group previously observed that overliming treatment reduces pyrolytic sugar toxicity without decreasing sugar content [6]. If membrane damage observed during pyrolytic sugar challenge does contribute to toxicity of pyrolytic sugars, sugars detoxified by overliming should cause less membrane

damage. Consistent with this supposition, overlimed pyrolytic sugars caused less magnesium leakage than raw sugars, even at concentrations as high as 1.0% (w/v) (Figure 2A). Specifically, while leakage values of 40% were observed even in the presence of 0.3% (w/v) raw pyrolytic sugars, leakage values of only 26% were observed in the presence of 1.0% (w/v) overlimed sugars. Thus, in addition to our previous observations that overlimed pyrolytic sugars have increased fermentability and decreased concentrations of certain inhibitory compounds [6], here we have shown that overlimed sugars cause less membrane leakage than the corresponding raw sugars.

3. Membrane fluidity increases during pyrolytic sugar challenge

The physical state of the membrane can change in response to changes in growth conditions, such as environmental stress [52]. One measurable aspect of the state of membrane is membrane fluidity, which must be maintained within a critical range in order to support normal membrane function [27]. Membrane fluidity can be measured by membrane fluorescence polarization, the decreased membrane polarization corresponds to increased membrane fluidity. Similar to our observation of decreased membrane integrity, we observed a sharp decrease in the membrane polarization of *E. coli* KO11+*lgk* when challenged with 0.1% (w/v) pyrolytic sugars (Figure 2B). Specifically, fluorescence polarization sharply decreased to 0.02, an order of magnitude lower than the value measured for the non-sugar control (0.3). Note that the concentration of 0.1% (w/v) sugars is within the tolerance limit of KO11+*lgk*. Pyrolytic sugar concentrations as low as 0.1% (w/v) perturbed polarization to a great extent relative to both the non-sugar control and cells treated with pure glucose. Since the fluorescence polarization value already approximated zero in the presence of 0.1% (w/v) of pyrolytic sugars, measurements were not performed for higher concentrations.

Previous studies have shown that phenolic compounds, which are one class of major non-sugar components in the pyrolytic sugars [6], can alter the membrane fluidity and permeability [14]. We have shown that pyrolytic sugars have a similar damaging effect. As previously reviewed [13], the affinity of lipophilic compounds for the phospholipid membrane leads to their infiltration of the membrane lipid bilayer and accumulation within it. Their interaction with lipid acyl chains causes disordered lipid packing and abnormal membrane configuration [15]. Therefore, changing the propensity of these compounds to

partition into the membrane by changing membrane composition could be a useful strategy for excluding these compounds.

Encapsulation helps to alleviate the toxicity of pyrolytic sugars

The cell membrane normally serves as a barrier to inhibitory compounds that could disturb metabolism. However, the native *E. coli* membrane apparently does not sufficiently block inhibitory compounds in pyrolytic sugar syrup. Therefore, creation of a secondary artificial "membrane" outside of the cell could possibly provide protection from inhibitors in pyrolytic sugar syrup. Encapsulation has been previously demonstrated to reduce toxicity of hydrolysates to microbes, since the calcium alginate matrix can selectively enable passage of some compounds while blocking others [58]. Here, we investigated this method as a means of providing protection from inhibitors in pyrolytic sugar syrup. Ethanol production was used to evaluate sugar tolerance and utilization.

In the absence of pyrolytic sugars or when provided with low concentrations of pyrolytic sugars at 0.4% (w/v), the encapsulated cells produced similar titers of ethanol compared to non-encapsulated, cells. However, when pyrolytic sugar concentration was increased to 0.8% (w/v) only encapsulated cells were able to produce ethanol (Figure 3). Note that this concentration is beyond the tolerance limit of KO11+*lgk*. Therefore, encapsulation was demonstrated to improve performance of *E. coli* cells in the presence of a concentration of pyrolytic sugars which was beyond the tolerance limit of non-encapsulated cells. When pyrolytic sugars were added at concentrations of 0.9 wt% and higher, no ethanol production was detected by the encapsulated cells (*data not shown*). The identification and implementation of genetic changes that decrease the permeability of these cells to the toxins in the pyrolytic sugar syrup could improve pyrolytic sugar utilization, without the increased cost associated with cell encapsulation.

Adaptive evolution improved the physiology of the cell membrane

We employed adaptive evolution to select for mutations that improve the resistance of *E. coli* to pyrolytic sugars, following Orgel's Second Rule that "evolution is cleverer than you are" [78]. By gradually increasing the concentrations of raw pyrolytic sugars in the medium, an evolved strain was obtained. A single colony was isolated and named TJE1.

This final evolved strain TJE1 was characterized in using either pure glucose as a control or the final concentration of pyrolytic sugars used during the adaptive evolution process (Figure 4A). Even though

the parental KO11+*lgk* strain and evolved strain TJE1 exhibited similar growth trends in LB medium containing pure glucose, the evolved strain showed higher OD values at each time point when pyrolytic sugars were used. Moreover, only the evolved strain TJE1 was able to grow in the presence of 0.65% (w/v) pyrolytic sugars (Figure 4A). No growth of parent KO11+*lgk* was observed at this condition. These results demonstrate enhanced pyrolytic sugar tolerance acquired by TJE1 during adaptive evolution.

1. Evolved strain TJE1 has increased resistance to membrane damage during pyrolytic sugar utilization

Since our hypothesis is that the primary mechanism of pyrolytic sugar toxicity is damage to the cell membrane, we compared membrane integrity of the parent and evolved strains during pyrolytic sugar challenge. Here we used a different assay to assess the integrity of cell membranes. Specifically, we assessed permeability to nucleic acid-binding SYTOX dye rather than Mg^{2+} leakage [41,38]. Values are presented as the percent of the cell population that was measured as SYTOX-positive. As with the magnesium leakage assay, when the concentration of raw pyrolytic sugars increased, the percent of cells permeable to SYTOX Green increased for both parental KO11+*lgk* strain and evolved TJE1 strain (Figure 4B). However, the degree of damage to the TJE1 membrane by pyrolytic sugars is lower than that inflicted on parent KO11+*lgk*. This difference was especially pronounced at higher pyrolytic sugars concentrations: when the concentration was higher than 0.5% (w/v), the percent of TJE1 population permeated by SYTOX Green was approximately half of the value for the corresponding parent strain. For example, at 1.0% (w/v) pyrolytic sugars, which is double the concentration of sugars that are completely inhibitory to our parent strain, more than 80% of the KO11+*lgk* population was SYTOX-permeable, compared to less than 50% of the population of TJE1. It should be noted that for both KO11+*lgk* and TJE1, membrane leakage induced by glucose never exceeded 20%. This confirms that components of the pyrolytic sugar syrup can damage the cell membrane of *E. coli*, which is consistent with the magnesium-based assay. More importantly, this also demonstrates that strain TJE1 has acquired mutations that result in a membrane with increased resistance to the damage inflicted by the pyrolytic sugar syrup.

Note that our metabolic product, ethanol, has previously been characterized as also causing membrane damage [84]. We observed that even 40 g/L ethanol, the maximum theoretical titer achieved by this strain from fermentation of 10% sugars, caused less than 40% of cells to be permeable to SYTOX

Green, which represents similar membrane leakage to that caused by raw pyrolytic sugars at a concentration of 1 g/L (*data not shown*).

We also measured membrane fluidity of both strains in the presence of either glucose or raw pyrolytic sugars (Figure 4C). When challenged with glucose, both KO11+*lgk* and TJE1 exhibited similar fluorescence polarization values, which were relatively constant from 0.1 – 1% (w/v) glucose. This means that glucose is not causing the perturbation of the membrane fluidity. However, when challenged by 0.1% (w/v) pyrolytic sugars, the fluorescence polarization of TJE1 was approximately 0.2, which is much higher than the value of 0.02 observed for KO11+*lgk*. This indicates that the membrane of TJE1 was less fluid than the KO11+*lgk* membrane in the presence of a low concentration of pyrolytic sugars. Evolved strain TJE1 did still show a decrease in fluorescence polarization, indicating increased fluidity, as pyrolytic sugar concentration increased to 0.5 wt%. Thus, TJE1 is still sensitive to the fluidizing effect of the pyrolytic sugars, but the magnitude of the effect is dampened relative to the parent strain.

This increased resistance to membrane damage imposed by pyrolytic sugars, both in terms of leakage and fluidity, is consistent with our hypothesis that membrane damage is the major mechanism of pyrolytic sugar toxicity. The fact that evolved strain TJE1 has increased resistance to these effects demonstrates that it is possible to implement genetic changes that decrease the vulnerability of the cell membrane to this damage.

2. The evolved strain has altered fatty acid tail distribution and extracellular protein production

It has been previously demonstrated that both short-term adaption and long-term evolution can result in alterations in membrane lipid composition of *E. coli* in response to stressful environments [66,26]. According to this observation, rational engineering of the membrane lipids, such as changing the length, degree of saturation, or geometry of the lipid tails within the membrane, has improved *E. coli*'s tolerance to some inhibitors [48,42,14,22,70]. With the goal of identifying "clever" strategies used by the evolved strain to strengthen resistance to the membrane-damaging effects of pyrolytic sugars, we characterized the membrane composition at the level of phospholipid tails, phospholipid heads, and extracellular proteins and sugars (Figure 5).

The distribution of phospholipid tails was measured for both strains, with and without pyrolytic sugars (Figure 5A). In both the 0% and 0.3% pyrolytic sugar conditions, relative abundance of each lipid

significantly ($P<0.05$) differed between parent strain and evolved strain. Specifically, the relative abundance of C14:0, C16:1, and C18:0 was lower in the evolved strain, and the relative abundance of C16:0, C17cyc, C18:1 was higher. The relative C19cyc content was higher in the evolved strain in the control condition, but lower in the 0.3 wt% pyrolytic sugar condition.

These relative abundance values were used to calculate the saturated:unsaturated (S:U) ratio and the average lipid length (Figure 5B). The S:U ratio was significantly higher ($P=0.003$) in the evolved strain relative to the parent during challenge with 0.3 wt% pyrolytic sugars. Similarly, the average lipid length was significantly higher ($P=0.004$) in the evolved strain relative to the parent at 0 wt% sugar.

In addition to comparing the lipid composition between the two strains at the same condition, it is also interesting to consider the trends for each strain as pyrolytic sugar concentration increased. In the parent strain, the relative abundance of C14:0, C16:1, C16:0, C17cyc and C18:0 each decreased, while C18:1 and C19cyc increased (Figure 5A). These changes led to a significant increase in the average lipid length, from 16.43 to 16.48 ($P=4x10^{-5}$) and a significant decrease in the S:U ratio, from 0.88 to 0.83 ($P=1x10^{-4}$). In the evolved strain, increasing pyrolytic sugar concentration was associated with increased C16:1, decreased C17cyc and C19cyc, and no significant trends for C14:0, C16:0, C18:1, and C18:0 across the three tested conditions. Consistent with the relatively stable membrane composition, there was no significant change in the evolved strain's S:U ratio as pyrolytic sugar concentration increased, and average lipid length differed significantly only when comparing the 0.6 wt% and 0 wt% conditions, with an increase from 16.49 to 16.52 ($P=0.044$). In summary, for the KO11+*lgk* parent strain, exposure to pyrolytic sugars resulted in a significant decrease in the S:U ratio and a significant increase in the average lipid length. In contrast, evolved strain TJE1 seems to have acquired longer-length lipids with slightly lower S:U ratio throughout the evolutionary improvement. This result suggests that the sustained presence of pyrolytic sugars promotes the production of unsaturated and longer membrane lipids. While these changes in membrane lipid content are significant, they are of relatively small magnitude compared to other changes in the lipid distribution for other evolved *E. coli* strains [67]. Thus, other changes in the membrane may be responsible for the observed changes in integrity and fluidity.

Each lipid within the membrane is attached to a corresponding phospholipid head. *E. coli* normally produces three head groups: phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and

cardiolipin (CL) [57]. We measured the relative abundance of these three components in the absence and presence of pyrolytic sugars (Figure 5C). CL was not detected in any of the samples and no significant differences were observed in the PE/PG ratio when comparing strains or growth condition. Thus, the change in membrane integrity in the evolved strain cannot be attributed to a change in phospholipid head distribution.

Production of extracellular polymeric protein is decreased in evolved strain TJE1

Since providing cells with a protective barrier by encapsulation increased utilization of pyrolytic sugars, we next sought to characterize the cell's native protective barriers. EPS are located outside the surface of the cells and thus are the first part of *E. coli* to contact the chemical compounds in pyrolytic sugar syrup. Therefore, we measured the two major components of EPS, proteins and polysaccharides, to determine if their production was altered in the evolved strain relative to the parent (Figure 6).

The results indicate that proteins are the major constituents of EPS of the parent strain, which is consistent with previous reports [39]. While the amount of EPS sugars produced by the two strains is similar, a substantial difference in EPS protein abundance was observed. Specifically, the EPS protein content of evolved strain TJE1 is approximately two-fold lower than parental strain KO11+*lgk*. Thus, adaptive evolution for pyrolytic sugar tolerance and utilization resulted in decreased EPS protein production.

EPS proteins are known to play an important role in cell aggregation [17] and other interactions with the environment, including sorption of xenobiotics and binding to organic materials [20,39]. The fact that acquisition of increased pyrolytic sugar tolerance was associated with decreased production of EPS proteins suggests that this change serves to decrease the cells' vulnerability to inhibitors in pyrolytic sugars.

Mutation of *csrA* is associated with decreased EPS protein production

In our study, the evolved strain TJE1 acquired both increased membrane resistance to membrane damage imposed by pyrolytic sugars and decreased EPS protein production. We conducted whole-genome sequencing (Illumina MiSEQ) to identify mutations acquired during adaptive evolution. Sequenced short reads were mapped to the reference backbone, KO11, to generate consensus sequences of both parental KO11+*lgk* and evolved TJE1. After further verification by Sanger sequencing, the genes *csrA* and *wcaF* were confirmed to contain mutations within the coding region.

csrA encodes a global carbon storage regulator that controls the expression of many genes by affecting their translation. Its product, a 61-amino-acid RNA-binding protein, CsrA, regulates a variety of metabolic pathways, including glycogen accumulation and catabolism, glycolysis, biofilm formation and motility [61,60]. In evolved strain TJE1, the 86th nucleotide of *csrA* gene changed from A to T, changing the associated 29th amino acid from glutamine to leucine. Henceforth, this is referred to as *csrA**.

To assess the contribution of this mutation to the evolved strain phenotype, we replaced the wild-type *csrA* in the parent strain KO11+*lgk* with mutant *csrA** from evolved TJE1. This engineered strain was then characterized in terms of EPS production (Figure 6) and membrane integrity (Figure 7). Replacement of wild-type *csrA* with *csrA** confers both decreased production of EPS proteins and decreased vulnerability to pyrolytic sugar-induced membrane leakage.

The other mutation verified in our evolved strain is within *wcaF*, which encodes an acetyltransferase involved in biosynthesis of the extracellular polysaccharide colonic acid [76]. Sanger sequencing revealed multiple mutations and insertion of two fragments of the IS4 transposase within *wcaF* in the evolved strain. Since these mutations presumably eliminated WcaF activity, we characterized their effect by deleting *wcaF* in parent KO11+*lgk* and measured production of EPS proteins and polysaccharides (*data not shown*). However, neither EPS protein nor polysaccharide content were changed significantly. Thus, we conclude that the mutation in *wcaF* does not contribute to decreased EPS protein production by the evolved strain.

<u>*csrA** enables increased membrane integrity</u>

The evolved strain TJE1 has altered membrane integrity and fluidity relative to the parent strain during pyrolytic sugar exposure (Figure 4B, 4C). We have proposed that these altered membrane properties contribute to increased pyrolytic sugar tolerance. The evolved strain also encodes a mutant version of *csrA**, which seems to be responsible for decreased production of EPS proteins (Figure 6). Here, we investigate the contribution of *csrA** to membrane integrity.

We observed that, as with the original parent strain, membrane integrity of KO11+*lgk* (*csrA**) decreased in a dose-dependent manner during pyrolytic sugar challenge (Figure 7). However, at pyrolytic sugar concentrations below 1.0% (w/v), the loss of integrity observed for parental strain KO11+*lgk* expressing *csrA** was less than the strain expressing the wild-type *csrA*. For example, during challenge

with 0.5% (w/v) pyrolytic sugars, 69.77±0.12% of the cells expressing wild-type *csrA* were SYTOX-permeable, compared to 53.96±0.75% of the cells expressing *csrA**, $P=1.77\times10^{-6}$. However, the increased membrane resistance associated with expression of *csrA** was not observed at 1.0% (w/v) pyrolytic sugars.

In addition to characterizing membrane integrity, we also measured the membrane fluidity of parent strain KO11+lgk with *csrA**. It was observed that this engineered strain exhibited similar fluidity as evolved TJE1 in the presence of 0.5% (w/v) of pyrolytic sugars (*data not shown*). This is consistent with our finding that the *csrA** mutation contributes to resistance of the membrane to damage caused by low concentrations of pyrolytic sugars.

These results demonstrate that reduction of extracellular proteins is associated with improved membrane integrity to a certain extent. EPS provides a three-dimensional, enclosed matrix for microbes. The composition of EPS, which can change according to culturing conditions, is of great importance to cell surface properties. It has been reported that an increase in the ratio of carbohydrates to proteins resulted in a decrease of cell hydrophobicity [71]. Since most of the non-sugar components of the pyrolytic sugar syrup are hydrophobic [64], it is possible that these compounds interact with the hydrophobic residues of the EPS proteins. Such an interaction could form channels, or leaks, in the EPS matrix, leading to the exposure of cells to the inhibitors. It is also possible that some of the extracellular proteins may serve as transporters for the inhibitors. However, the composition of extracellular proteins needs further study.

Conclusions

Thermochemical processing is a promising method for releasing fermentable sugars from biomass. However, as with many other methods of biomass depolymerization, toxicity of the sugar product is problematic. Here we have demonstrated that one of the problems associated with microbial utilization of pyrolytic sugars is damage of the cell membrane, both in terms of increased membrane fluidity and decreased membrane integrity. This damage is similar to the damage observed during production of a variety of biorenewable fuels and chemicals.

Encapsulation of the cells, in essence providing another layer of protection, enabled production of ethanol at sugar concentrations in which no ethanol production was observed by free cells (Fig. 3). This provides supporting evidence that efforts to prevent cell entry by the inhibitory compounds can improve utilization of these sugars. An alternative to the cell encapsulation is strengthening of the cell membrane by

altering the biological pathways associated with membrane construction, maintenance and repair. This idea of engineering the microbial membrane for increased resistance to such damage is showing increasing promise. For example, engineering strategies focused on the membrane lipid tails, such as altering the saturated/unsaturated ratio [42,48], average lipid length [69], or lipid conformation [77], have been shown to increase tolerance of some membrane-damaging compounds.

Our development and characterization of a strain evolved for pyrolytic sugar tolerance demonstrates that it is possible for genetic changes to increase resistance of the membrane to damage incurred during pyrolytic sugar utilization. While our evolved strain does show significant changes in the lipid tail distribution, including a significantly lower S:U ratio than the parent strain, it also shows decreased production of EPS proteins. These EPS proteins possibly serve as an entry point for membrane-damaging compounds, and decreasing their abundance reduces the ability of these compounds to enter, and 1therefore damage, the membrane. This decrease in EPS protein production and increased membrane resistance to leakage induced by low concentrations of pyrolytic sugars appears to be due to a single amino acid change within the well-characterized regulator CsrA. Thus, we have identified a single genetic engineering strategy for increasing pyrolytic sugar tolerance and demonstrated that decreasing EPS protein production may be a strategy for increasing resistance to membrane-damaging compounds present in the pyrolytic sugar syrup. The fact that a decrease in EPS abundance is associated with improved membrane integrity is a surprising contrast to the cell encapsulation strategy, in which the addition of extracellular material increased resistance.

Our observation that KO11 does not produce the cardiolipin phospholipid head group was surprising. It is not clear if this trait was derived from KO11's parent strain, *E. coli* W, or if cardiolipin production was eliminated during the evolutionary-based development of this strain [28].

Figure 1. Pyrolytic sugars inhibit growth of *E. coli* KO11+*lgk*. Batch fermentations were performed at pH 7.0, 37 °C, 200 rpm in Luria Broth containing 10% (w/v) of a mixture of pyrolytic sugar syrup and pure glucose, as indicated. Data is the average of two biological replicates with error bars indicating the standard deviation.

Figure 2. Pyrolytic sugars damage the membrane of *E. coli* KO11+*lgk*.

(A) Membrane leakage: cells were grown to mid-log phase in Luria Broth and then incubated with the indicated sugar at 37 °C for 1.5 h, and assessed for membrane damage via Mg^{2+} leakage, which is presented as a percentage of the leakage induced by chloroform. The data is the average of three replicates with error bars indicating the standard deviation.

(B) Membrane fluidity: cells were grown to mid-log phase in Luria Broth and then incubated with the indicated sugar at 37 °C for 1.5 h. The membrane polarization was measured via DPH. The data is the average of eight replicates with error bars indicating the standard deviation.

Figure 3. Cell encapsulation partially mitigates pyrolytic sugar toxicity. The titers of ethanol produced by encapsulated cells and free cells of parent KO11+*lgk* which were cultured in Luria Broth supplemented with 1.5 % (w/v) glucose plus the indicated amount of pyrolytic sugars at 37 ºC, 200rpm for 24 hours. The data is the average of two replicates with error bars indicating the standard deviation. No ethanol production was observed for the free cells in the presence of 0.8 wt% pyrolytic sugars.

Figure 4. Evolved strain TJE1 shows increased tolerance to pyrolytic sugars.

(A) Growth: batch fermentations were performed at pH 7.0, 37 ºC, 200 rpm in Luria Broth containing 10% (w/v) of a mixture of pyrolytic sugar syrup and pure glucose, as indicated.

(B) Membrane integrity: cells were grown to mid-log phase in Luria Broth, and incubated with the indicated wt% sugar at 37 °C for 1.5 h, and then assessed for membrane damage via nucleic acid stain SYTOX Green. Data is the average of three replicates with error bars indicating the standard deviation. The control condition is simply LB media, with no added sugars.

(C) Membrane fluidity: cells were grown to mid-log phase in Luria Broth and then incubated with the indicated sugar at 37 ℃ for 1.5 h. The membrane polarization was measured via DPH. The data is the average of eight replicates.

Figure 5. Membrane composition of parent KO11+*lgk* and evolved TJE1 during pyrolytic sugar challenge. (A) Membrane lipid composition: cells were grown to mid-log phase in Luria Broth and then incubated with the indicated sugar at 37 °C for 1.5 h. The membrane lipid distribution was measured by GC-MS.
(B) Characteristics of membrane lipids.
(C) Membrane phospholipid head content.
(*) indicates values that are significantly ($P<0.05$) different from the same strain in the 0% pyrolytic sugar condition
(+) indicates values that are significantly ($P<0.05$) different in the evolved strain relative to the parent strain at the same sugar concentration.
For all measurements, data is the average of at least two biological replicates, with error bars indicating the standard deviation.

Figure 6. Replacement of the wild-type *csrA* gene with mutant *csrA** in parental strain KO11+*lgk* resulted in a decrease in EPS protein abundance, comparable to the amount of EPS protein produced by evolved strain TJE1. Cells were collected for EPS analysis from LB agar plates. Data is the average of three replicates with error bars indicating the standard deviation.

Figure 7. Replacement of the wild-type *csrA* with mutant *csrA** in the parent strain increases resistance to the membrane leakage induced by pyrolytic sugars (ps). Cells were grown to mid-log phase in Luria Broth and then incubated with the indicated sugar at 37 °C for 1.5 h. Membrane damage was assessed via the nucleic acid stain SYTOX Green. The data is the average of three replicates with error bars indicating the standard deviations.

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