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Exploring the Heterologous Genomic Space for Building, Stepwise, Complex, Multicomponent Tolerance to Toxic Chemicals

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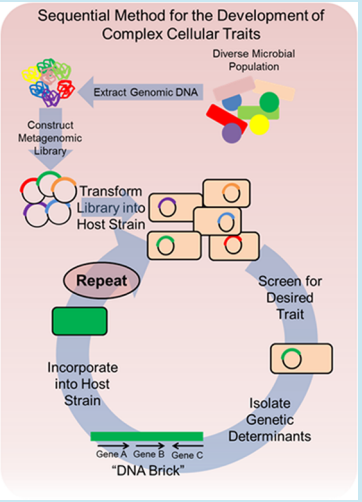
Supporting Information

ABSTRACT: Modern bioprocessing depends on superior cellular traits, many stemming from unknown genes and gene interactions. Tolerance to toxic chemicals is such an industrially important complex trait, which frequently limits the economic feasibility of producing commodity chemicals and biofuels. Chemical tolerance encompasses both improved cell viability and growth under chemical stress. Building upon the success of our recently reported semisynthetic stress response system expressed off plasmid pHSP (Heat Shock Protein), we probed the genomic space of the solvent tolerant *Lactobacillus plantarum* to identify genetic determinants that impart solvent tolerance in combination with pHSP. Using two targeted enrichments, one for superior viability and one for better growth under ethanol stress, we identified several beneficial heterologous DNA determinants that act synergistically with pHSP. In separate strains, a 209% improvement in survival and an 83% improvement in growth over previously engineered strains based on pHSP were thus generated. We then developed a composite phenotype of improved growth and survival by combining the identified *L. plantarum* genetic fragments. This demonstrates the concept for a sequential, iterative assembly strategy for building multigenic traits by exploring the synergistic effects of genetic determinants from a much broader genomic space. The best performing strain produced a 3.7-fold improved survival under 8% ethanol stress, as well as a 32% increase in growth under 4% ethanol. This strain also shows significantly improved tolerance to *n*-butanol. Improved solvent production is rarely examined in tolerance engineering studies. Here, we show that our system significantly improves ethanol productivity in a Melle-Boinot-like fermentation process.



KEYWORDS: *heterologous and metagenomic spaces, genomic libraries, biofuels, heat shock proteins, complex phenotypes*

Cellular traits often stem from multiple genes, gene interactions, regulatory pathways, and programs.[1,2] The genes and systems enabling such complex traits are often poorly understood making attempts to engineer them with *a priori* knowledge exceedingly difficult. Most cellular properties, including industrially relevant traits, such as ability to produce toxic metabolites in a robust, industrially relevant manner, are the result of the coordinated action of several cellular subsystems involving biophysical, metabolic, or regulatory ensembles. Tolerance of an organism to toxic chemicals is one such trait relevant to the production of small molecules, including biofuels and commodity chemicals, but also for bioremediation applications.[2−4]

Microbial tolerance to toxic chemicals (whether desirable toxic metabolites, toxic intermediates, or toxic substrates) is multifaceted, but for practical applications, two key aspects are crucial.[5] One is the ability of the cells to maintain viability thus continuing to produce the desirable toxic metabolite or to continue remediating. The second is the ability of the cells to grow under chemical stress, which practically means ability to grow under moderate concentrations of a toxic chemical. These two traits address virtually all practical bioprocessing situations.[5]

Attempts to improve solvent tolerance through rational design or directed evolution have met with some success. Approaches ranging from completely random, such as screening genomic and metagenomic libraries,[6−11] TRMR,[12] and protoplast fusion,[13,14] to more targeted screening approaches such as specific gene libraries (e.g., export pumps),[15] and to entirely rational approaches targeting specific genes have produced significant improvements in cellular tolerance to industrially important chemicals.[16−19]

Heat shock proteins (HSPs), members of the general stress response system, have been identified as part of the solvent and generally, toxic-chemical, stress response in a variety of organisms.[2,20−23] For example, transcriptional upregulation of HSP genes has been identified in tolerance enrichment studies for ethanol,[24] *n*-butanol,[11] or isobutanol[11,25] stress in *Escherichia coli*. Overexpression of the autologous GroESL in *Clostridium acetobutylicum* improved solvent tolerance and

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solvent production.[17] Heterologous HSPs have also been used to improve organic solvent tolerance in *E. coli*.[26,27] We have recently shown that overexpression of an autologous set of HSPs, namely GroES, GroEL, ClpB, and GrpE, increased *E. coli* tolerance to ethanol, *n*-, 2-, and i-butanol, as well as 1,2,4-butanetriol.[18,19]

Genomic libraries for screening to identify beneficial genetic determinants can be constructed from digested or sheared genomic DNA inserted into a replicating vector. The library is then propagated in a screening host (frequently *E. coli*) and individual clones are identified by sequencing or microarray analysis of an enriched pool.[7,9,10,28] Although most frequently the genomic DNA used in library construction for screening to identify determinants that impart a desirable traits are autologous, in principle, heterologous and metagenomic traits could be also screened, and in fact, that would vastly enlarge the genomic space that can be screened for desirable traits.[1] Although heterologous and metagenomic libraries offer a tremendous wealth of new genetic material, the ability of the host organism to express genes from large library inserts (which makes it possible to screen a larger set of genes in a single cell) is often severely limited due to the inability of the screening host to recognize most of the heterologous or metagenomic promoters.[29,30] A second problem in screening genomic libraries for complex traits is due the fact that complex traits such as tolerance to toxic chemicals depend on multigenic programs, the ability to screen for beneficial interactions of genetic determinants is of paramount importance.[10]

Here, we sought to explore the synergism and beneficial interactions between our previously developed semisynthetic HSP system and heterologous genetic determinants from the *Lactobacillus plantarum* genome through enrichment for beneficial interactions for solvent tolerance. To do so, we used the method we have recently developed to enable screening for beneficial genetic interactions.[10] We chose to probe the genomic space of *L. plantarum* (henceforth abbreviated as *Lpl*) for identifying beneficial determinants for solvent tolerance based on the fact *Lpl* is innately tolerant to a variety of toxic chemicals including ethanol and butanols.[31] To increase the ability of the screening host, *E. coli*, to recognize *Lpl* promoters, we used an *E. coli* strain that had integrated into its genome in the location of the *lacZ* gene, the gene (*rpoD*) for the main *Lpl* sigma factor.[29,30] We sought to identify genetic determinants that would separately promote better survival and growth under solvent stress (here, ethanol stress). To do so, we hypothesized, based on some previous work,[32] that the screening method determines the screening outcomes, thus leading us to utilize two separate enrichment methods for identifying genetic determinants for better survival and growth separately. Then, we hypothesized and aimed to show that these genetic determinants could be combined to develop simultaneous improvements to growth and survival in a single strain. This also served as a proof of concept for a sequential approach for developing complex trait, whereby genetic elements can be added after each screening step to build an increasingly stronger multigenic trait or program. Such a screening-based method has not been previously reported, let alone one that engages a heterologous genomic space.

RESULTS AND DISCUSSION

Targeted Enrichment to Identify *Lpl* Determinants That Work with the Synthetic HSP System to Enhance Survival of *E. coli* under Strong Ethanol Stress. We constructed plasmid pZS-$C^LG^LES^N$ (referred to as pHSP below for simplicity) to express four *E. coli* HSPs, GroES, GroEL, ClpB, and GrpE on a single expression vector. Expression of pHSP produced a significant improvement in *E. coli* tolerance to ethanol, *n*- and i-butanol, and 1,2,4-butanetriol compared to wild type and to expression of each gene separately.[18] This shows that the genes on pHSP act cooperatively against solvent stress. Here, we aimed to identify synergistic and beneficial interactions between pHSP and additional genetic determinants from the genome of the more tolerant *Lpl* prokaryote. Using an *E. coli* strain (MG1655 *lacZ::rpoD*)[29,30] with an integrated, inducible copy of the *Lpl* sigma factor gene *rpoD*, we screened a plasmid based *Lpl* genomic library with an average insert size of 4 kb in combination with pHSP. As mentioned above, we used two enrichment protocols, one to identify genetic *Lpl* determinants that enhance survival under high ethanol stress and the other that improves growth under moderate ethanol stress. To enrich clones for enhanced survival, we used a high, 8% (v/v), ethanol concentration, and a long time exposure of 24 h, with a 24 h recovery step in fresh media without stressant after every exposure. We used a 0.15 starting optical density (A_{600}) for each exposure and a 10% inoculum for each recovery step. During each exposure, survival was monitored using viable cell counts (colony-forming units; CFUs) at both the start and end of the solvent exposures. These enrichments were performed both on cells bearing the plasmid library and cells bearing a plasmid control (both types of cells coexpressed pHSP) in order to demonstrate that improvements in tolerance observed were derived from enrichment of the library population and not from random mutations acquired during the process. The enrichments were carried out in biological triplicates. CFU counting was used to determine percent survival during the enrichment process (Figure 1A).

The enrichment for survival demonstrated highly reproducible significant improvements for the *Lpl* library over the plasmid control during the fourth and all subsequent exposures (Figure 1A). From each of the three biological replicates of the library enrichment, 10 colonies (clones) were isolated from the sixth exposure in the survival screening, for a total of 30 tolerant clones. Library plasmids were then isolated and sequenced (Supporting Information (SI) Table 1). The inserts present with the highest frequency (SI Figure 1) were selected to test for improved survival in ethanol. The plasmids containing these *Lpl* fragments were renamed as pS plasmids (Table 1) and transformed into fresh MG1655 *lacZ::rpoD* (pHSP) to eliminate the impact of possible mutations to either genomic DNA or pHSP DNA that might have accumulated during the enrichment.

Survival of the strains containing the pS plasmids was assessed in a survival assay using 8% (v/v) ethanol for a 24 h exposure (Figure 1B). As had been seen previously, overexpression of the autologous pHSP genes results in a 2-fold increase in survival under high ethanol stress.[18] Of the four identified plasmids from the survival screen, only pS1 and pS2 produced significant improvements in survival when coexpressed with pHSP, 45% and 34% survival, respectively, compared to the plasmid-control strain with the pHSP, which had a 21% survival ($p < 0.001$ for both) (Figure 1B). These represent a 209% and 58% increase, respectively, from the beneficial expression of the pHSP genes alone.

Next we assessed the impact of *Lpl rpoD* expression on the effect the identified *Lpl* determinants had on tolerance, and also of the synergism, as affecting tolerance, between pHSP and the

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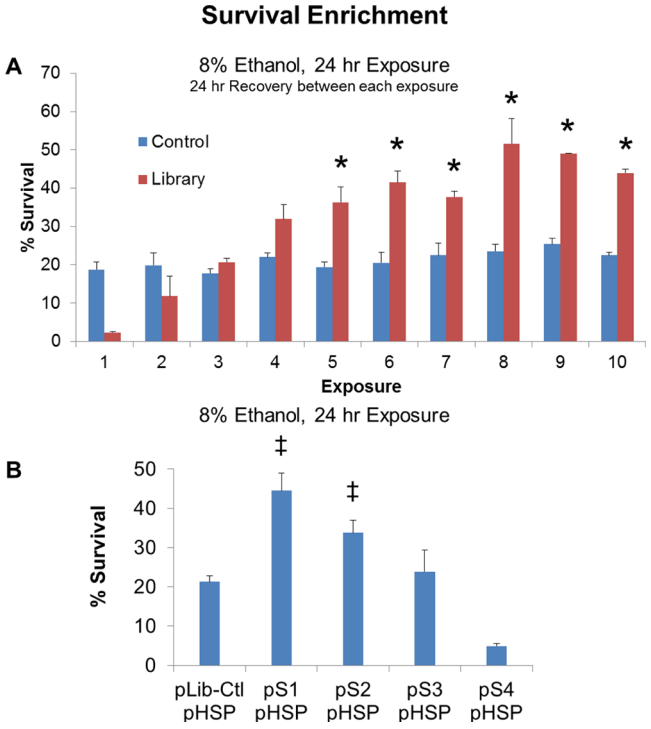

Figure 1. Enrichment for survival of *L. plantarum* plasmid library in *E. coli* MG1655 *lacZ::rpoD* cells bearing pHSP. (A) Percent survival of control [MG1655 *lacZ::rpoD* (pLib-Ctl, pHSP), blue] and library [MG1655 *lacZ::rpoD* (pLib-Ctl, pHSP), red] strains exposed to 8% ethanol for 24 h with a 24 h recovery in media without stressant after each exposure. Library and control were screened in biological triplicates each; error bars indicate standard error of percent survival; * indicates statistically significant improvement from library control, MG1655 *lacZ::rpoD* (pLib-Ctl, pHSP) ($p < 0.05$). (B) The genetic determinants with the highest frequency from the sequenced, enriched library were isolated and retransformed into fresh MG1655 *lacZ::rpoD* cells. Percent survival during 8% ethanol, 24 h exposure, was monitored to verify improved tolerance. Data reported are the averages of three biological replicates with error bars indicating standard error; ‡ indicates a statistically significant improvement from the HSP overexpression strain, MG1655 *lacZ::rpoD* (pLib-Ctl, pHSP) ($p < 0.05$).

Table 1. Plasmids Selected from *L. plantarum* Library Survival and Growth Enrichments

plasmid name	start	end	size	genes in fragment[a]
pS1	73816	77209	3394	(*fusA1*) hypothetical protein [lp_0077], permease/drug-metabolite transporter [lp_0078], (GNAT acetyltransferase)
pS2	1004096	1006110	2015	(*mae*) *citC citD* (*citE*)
pS3	2866824	2868276	1453	(*pts26BCA*)
pS4	3158907	3162902	3996	*tal2* [lp_3539], [lp_3540], *pts34B, pts34A*, (lp_3543)
pG1	3199864	3203643	3780	(*clpL*), (*lox*)
pG2	1429291	1430754	1464	Lp_1564 membrane protein, (LyTR family transcriptional regulator)
pG3	1086051	1088519	2469	(*rfbC*), *rfbB*, *rfbD*, (transposase, fragment)
pG4	1024357	1026809	2453	(*cydA*), *cydB*, (*cydC*)

[a]Incomplete genes indicated by parentheses.

identified *Lpl* genetic determinants (Figure 2). Expression of either pS1 or pS2 without the pHSP plasmid produced a lower

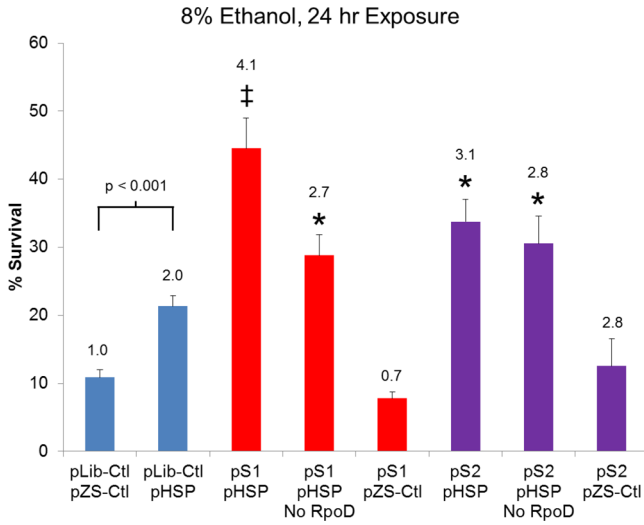

Figure 2. Outcomes from survival assays for pS1 and pS2 plasmids identified from ethanol survival enrichment performed in 8% ethanol with a 24 h exposure for *E. coli* MG1655 *lacZ::rpoD* or MG1655 cells bearing the plasmid combinations indicated above. No RpoD indicates that strain MG1655 was used. Percent survival is reported as the ratio of the number of viable cells after 24 h exposure to the initial viable cell count. The fold improvement of each strain compared with the double plasmid control strain [MG1655 *lacZ::rpoD*(pLib-Ctl, pZS-Ctl)] is shown above each survival bar. Data reported are the averages of three biological replicates with error bars indicating standard error. * indicates a statistically significant improvement from the HSP overexpression strain, MG1655 *lacZ::rpoD* (pLib-Ctl, pHSP) ($p < 0.05$). ‡ indicates a statistically significant improvement from both the HSP overexpression strain and from MG1655 (pS1, pHSP) ($p < 0.05$).

survival than coexpression with the pHSP plasmid, resulting in tolerance comparable to the double plasmid-control strain. This indicates that neither plasmid offers significant benefit without overexpression of the *E. coli* pHSP genes. Without expression of *Lpl rpoD*, pS1 expression with pHSP resulted in a 29% increased tolerance over the plasmid control strain, compared to a 45% increase when coexpressed with *rpoD*. Plasmid pS2 produced an equivalent improvement with or without the *Lpl rpoD* expression (34% versus 31%) indicating that the genes on plasmid pS2 can be expressed using a native *E. coli* sigma factor.

Screening for Growth: Targeted Enrichment to Identify *Lpl* Determinants That Work with pHSP to Enhance *E. coli* Growth under Moderate Ethanol Stress. After preliminary titration studies to assess what levels of ethanol would be suitable for screening for enhanced growth, we settled on a protocol using a moderate ethanol concentration (4% (v/v)), a short time exposure time (6 h), with three exposure cycles followed by a 6 h recovery step in media containing no stressor. For these screening experiments, we used two metrics to assess growth, using easy to implement assays that can be carried out effectively and reproducibly without undue burden. The first is the **Viable 6-h Growth Extent** ($\mathbf{GE_{V6}}$), defined as the ratio of viable cells after the 6-h exposure to the viable cell count prior to the exposure. The second is the **Biomass (X) 6-h Growth Extent** ($\mathbf{GE_{X6}}$), defined as the ratio of biomass concentration (X, measured as A_{600}) after the 6-h solvent exposure to the A_{600} prior to the exposure. Both metrics are useful, but GE_{V6} provides logically a better

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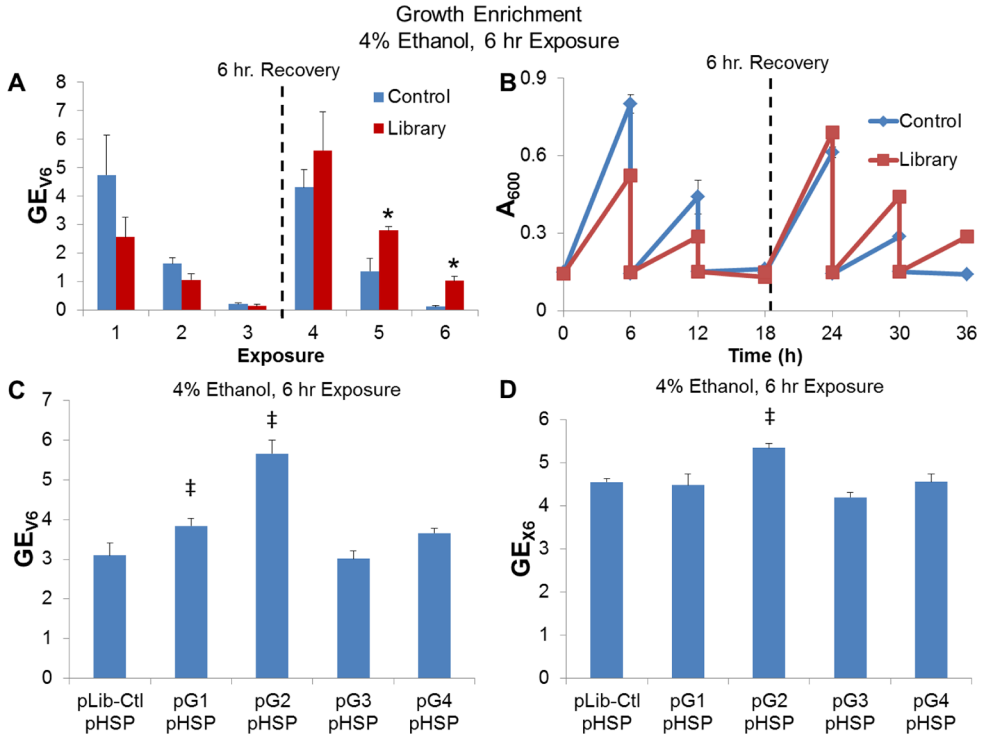

Figure 3. Outcomes from growth enrichment for *L. plantarum* plasmid library in *E. coli* MG1655 *lacZ::rpoD* (pHSP). (A) GE_{V6} of control [MG1655 *lacZ::rpoD* (pLib-Ctl, pHSP), blue] and library [MG1655 *lacZ::rpoD* (pLib-Ctl, pHSP), red] strains exposed to 4% ethanol for 6 h. GE_{V6} is defined as the ratio of the viable cell count at 6 h over the viable cell count at 0 h. (B) Cell density of control [MG1655 *lacZ::rpoD* (pLib-Ctl, pHSP), blue] and library [MG1655 *lacZ::rpoD* (pLib-Ctl, pHSP), red] strains exposed to 4% ethanol for 6 h. A single 6 h recovery step was included after the third exposure of the enrichment. * indicates a statistically significant improvement from the control strain, MG1655 *lacZ::rpoD* (pLib-Ctl, pHSP) ($p < 0.05$). Genetic determinants identified as the most enriched from the sequenced library were selected for analysis using growth tolerance assays. (C) GE_{V6} during 4% ethanol, 6-h exposure. (D) GE_{X6} for 4% ethanol, 6-h exposure. GE_{X6} is defined as the ratio of A_{600} at 6 h to A_{600} at 0 h. Data reported are the averages of three biological replicates with error bars indicating standard error. ‡ indicates a statistically significant improvement from HSP overexpression ($p < 0.05$).

assessment of tolerance. Upon screening, the library bearing strain displayed consistently improved GE_{V6} (Figure 3A) and cell density (Figure 3B) during the growth enrichment for the fourth and all subsequent exposures. As with the survival screening, 10 colonies from each biological replicate were isolated from the sixth exposure (30 colonies total). The *Lpl* genomic inserts identified most frequently and which were larger than 1000 base pairs in length were isolated and renamed as the pG plasmids (SI Figure 1 and Table 1). The 1000 bp cutoff point was intended to select for inserts which contained intact genes which could function with our previously identified HSPs as opposed to selection of partial genes, sRNAs or other small genomic pieces. These plasmids were then transformed into fresh MG1655 *lacZ::rpoD* (pHSP) to eliminate the impact of possible mutations to either genomic DNA or pHSP during the enrichment and screening process.

GE_{V6} (Figure 3C) and GE_{X6} (Figure 3D) were monitored for the growth plasmids (pG plasmids) using the aforementioned screening protocol with 6-h exposure to 4% (v/v) ethanol. Together with pHSP, both strains expressing pG1 and pG2 produced a statistically significant increases in GE_{V6} ($p = 0.031$ and $p < 0.001$, respectively), with the pG2 expressing strain having the highest GE_{V6}, an 83% improvement over the strain expressing pHSP alone. The strain expressing pG2 in combination with pHSP was the only strain tested to produce a statistically significant increase in GE_{X6}, compared to the strain expressing pHSP alone.

The effects of pG1 and pG2 were explored further (SI Figure 2 and Figure 4, respectively). Notably, in the presence of 4% (v/v) ethanol, the strain expressing pHSP alone resulted in a 27% decrease in GE_{V6} and a 7% decrease in GE_{X6} compared to the double plasmid control. pG2 in combination with pHSP resulted in the only observed increase in GE_{X6} compared to the double plasmid control (Figure 4). Without pHSP, MG1655 *lacZ::rpoD*(pZS-Ctl, pG2) resulted in an 8% decrease in GE_{V6} and an 8% decrease in GE_{X6} compared to the plasmid control. Additionally, coexpression of pHSP and pG2 without expression of the *Lpl* RpoD produced a 13% decrease in GE_{V6} and a 10% decrease in GE_{X6}. Taken together, these data show that the benefit to tolerance produced by expression of pG2 is dependent upon simultaneous overexpression of the *E. coli* HSPs and that the expression of the genes on pG2 requires expression of the *Lpl* RpoD.

Identified *Lpl* Genes Represent a Diverse Pool of Heterologous Genes for Engineering Tolerance. The beneficial genes identified by our growth and survival screens were the genes in plasmids pS1, pS2, pG1, and pG2 (Table 1), which include components of a variety of cellular systems. Plasmid pS1 contains an *Lpl* fragment encoding two intact hypothetical proteins. The second protein lp_0078 encodes a gene with a conserved drug or metabolite transporter domain, thus suggesting that these may function as a two-component transporter system. Plasmid pS2 expresses two complete *Lpl* genes, *citC* and *citD*, both components of a citrate lyase involved in fatty acid biosynthesis. They may contribute to

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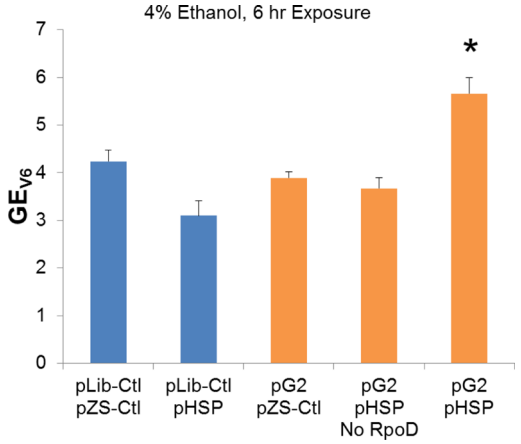

Figure 4. Outcomes (GE_{V6} data) from growth assays for pG2 plasmid performed in 4% ethanol with a 6 h exposure. Assay performed in *E. coli* MG1655 *lacZ::rpoD* or MG1655 cells bearing the plasmids listed above. No RpoD indicates that the strain MG1655 was used. GE_{V6} is defined as the ratio of viable cell counts at 6 h over the viable cell counts at 0 h for cells grown in 4% ethanol. Data reported are the averages of three biological replicates with error bars indicating standard error; * indicates a statistically significant improvement from double plasmid control, MG1655 *lacZ::rpoD* (pLib-Ctl, pZS-Ctl) ($p < 0.05$).

tolerance by promoting the biosynthesis of membrane components which impart tolerance. Plasmid pG1 contains an *Lpl* fragment encoding parts of the *clpL* and *lox* genes, both HSPs involved in the general stress response. Additionally, it contains a large untranslated region between the two partial genes that may encode a small RNA that may affect cellular processes in *E. coli*. Several small, noncoding RNAs play a role in imparting natural stress response and tolerance and can be used to develop superior synthetic tolerance.[33] Finally, the pG2 insert encodes a hypothetical membrane protein, lp_1564. This protein may be benefiting membrane structure, acting as a transporter, or be involved in some other membrane associated process. Further characterization of these proteins and better annotation of the *Lpl* genome may provide better understanding of the mechanisms by which these genes contribute to tolerance.

Combining Related Traits Leads to an Improved Complex Trait: Better Survival and Growth Together. Using pHSP together with genetic elements identified from the *Lpl* genomic library, we generated strains that separately improved growth and survival under ethanol stress (Table 2).

Table 2. Outcomes from Survival and Growth Assays for Best Performing Strains Identified from Enriching the *L .plantarum* Library for Growth and Survival[a]

strain	percent survival in 8% ethanol	GE_{V6}	GE_{X6}
wild type (plasmid control)	11% ± 3.3%	4.24 ± 0.73	4.91 ± 0.55
HSP overexpression (pHSP)	21% ± 4.6%	3.10 ± 0.93	4.55 ± 0.24
pS1 + pHSP	45% ± 13.3%	3.57 ± 0.55	4.10 ± 0.28
pS2 + pHSP	34% ± 9.8%	3.06 ± 0.09	4.00 ± 0.25
pG1 + pHSP	12% ± 5.3%	3.84 ± 0.18	4.49 ± 0.78
pG2 + pHSP	16% ± 9.2%	5.66 ± 0.34	5.34 ± 0.30

[a]Standard deviations of the measurements are indicated.

We next sought to examine if combining these beneficial effects might result in a further improved phenotype. The best performers from the growth and survival screenings were combined and assayed to identify strains with simultaneous improvements to growth and survival in ethanol. Thus, four combinations were examined combining (on a new set of plasmids) the genomic fragments identified on the pS1, pS2, pG1, and pG2 plasmids. The resulting plasmids (pS1G1, pS1G2, pS2G1, and pS2G2) were then transformed into MG1655 *lacZ::rpoD* (pHSP) and the strains were assayed for improved tolerance using growth and survival assays.

The combined expression of pS1G2 and pHSP resulted in a 3.7-fold increase in survival over the double plasmid control, comparable to the best performing survival strain MG1655 *lacZ::rpoD*(pHSP, pS1) (Figure 5A). MG1655 *lacZ::rpoD*(pHSP, pS1G2) also demonstrated a 32% increase in GE_{V6} over the double-plasmid control (Figure 5B) and a 21% increase in GE_{X6} compared to the double-plasmid control (SI Figure 6). Expression of pS1G2 without pHSP overexpression resulted in a 27% increase in GE_{X6} over the double plasmid control, producing the highest average GE_{X6} observed. However, it only produced a 51% increase in survival over the double plasmid control indicating that the combination of pHSP and pS1G2 results in a clearly superior strain. Plasmids pS1G1, pS2G1, and pS2G2 failed to produce improvements to growth and survival (SI Figures 4 and 5). These data show that the combinations of beneficial genetic determinants identified from the separate survival and growth enrichments cannot consistently be combined to produce improvements to both phenotypes. Instead, the beneficial combinatorial effects are not predictable; rather, only a limited subset of gene combinations will produce the desired increase to both growth and survival under ethanol stress.

Genes Imparting Tolerance Distinctly for Growth vs Viability. Solvent tolerance is an inherently complex cellular phenotype that can be further classified into the ability of a population to grow in the presence of a toxic chemical and the ability of the population to remain viable under chemical stress. Our tailored screening approaches sought to identify DNA determinants from the *Lpl* genome that impart separately improved viability and growth under stress. These determinants were examined in combination with our previously developed HSP system[18] in order to identify synergistic effects and beneficial interactions between the heterologous *Lpl* genes and the overexpressed autologous HSPs. Our data show that the genes we identified were indeed tailored to achieve the objective of the assay. For example, as shown in Figure 5, expression of pS1+ pHSP resulted in superior viability, but not growth. Conversely, expression of pG2+ pHSP resulted in superior growth, but not viability. It was only the combination of the genetic determinants identified on the plasmids pS1 and pG2, simultaneously expressed on pS1G2, in combination with pHSP that resulted in superior tolerance, both in growth and viability.

More than Ethanol: A Broader Alcohol Tolerance. We have previously shown that overexpression of HSP proteins originally developed for ethanol tolerance results in strains tolerant to a broader set of alcohol solvents.[18,19] We wanted to test if this also holds for the best strain developed on this study. Based on our previous experience,[5,18,19] *n*-BuOH is one of the most toxic and yet industrially relevant solvent to use for testing. Thus, we next tested the survival and growth MG1655 *lacZ::rpoD* (pHSP, pS1G2) in the presence of *n*-

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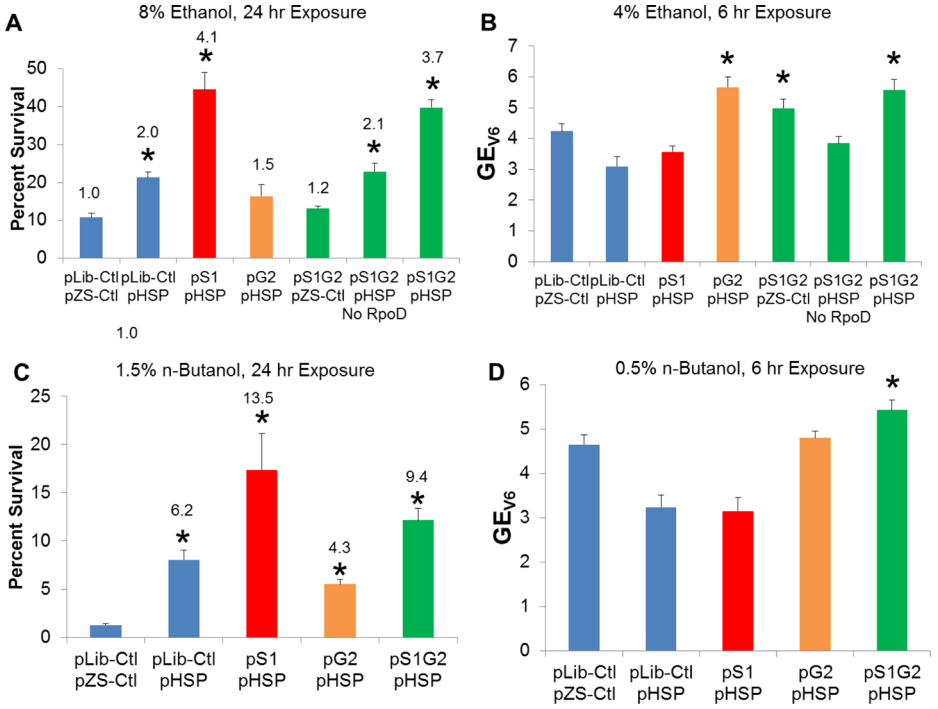

Figure 5. Outcomes from survival and growth assays under ethanol or *n*-butanol stress for *E. coli* cells bearing the pS1G2 plasmid. Assays performed in *E. coli* MG1655 *lacZ::rpoD* or MG1655 cells bearing the plasmid combinations listed. No RpoD indicates that the strain MG1655 was used. (A) Survival assays performed in 8% ethanol (v/v) over 24 h; percent survival reported as the ratio of the number of viable cells after 24 h exposure to initial viable cell count. The fold improvement of each strain compared with the double plasmid control strain [MG1655 *lacZ::rpoD*(pLib-Ctl, pZS-Ctl)] is shown above each survival bar. (B) GE_{V6} data. GE_{V6} is defined as the ratio of viable cell counts at 6 h over the viable cell counts at 0 h for cells grown in 4% ethanol (v/v). (C) Survival assays performed in 1.5% *n*-butanol (v/v) over 24 h; percent survival reported as the ratio of the number of viable cells after 24 h exposure to initial viable cell count. The fold improvement of each strain compared with the double plasmid control strain [MG1655 *lacZ::rpoD*(pLib-Ctl, pZS-Ctl)] is shown above each survival bar. (D) GE_{V6} data. GE_{V6} is defined as the ratio of viable cell counts at 6 h over the viable cell counts at 0 h for cells grown in 0.5% *n*-butanol (v/v). Data reported are the averages of three biological replicates with error bars indicating standard error; * indicates a statistically significant improvement from double plasmid control, MG1655 *lacZ::rpoD* (pLib-Ctl, pZS-Ctl) ($p < 0.05$).

butanol (Figure 5C and D). MG1655 *lacZ::rpoD* (pHSP, pS1G2) displayed in a 13-fold increase in survival in 1.5% (v/v) *n*-butanol compared to the double plasmid control, and a 2-fold increase compared to MG1655 *lacZ::rpoD* (pHSP, pLib-Ctl). Additionally, this strain displayed a 17% increase in GE_{V6} under 0.5% (v/v) *n*-butanol stress compared to the double plasmid-control strain. This same improvement in growth under n-butanol stress was not seen for MG1655 *lacZ::rpoD* (pHSP, pG2). Since expression of the pS1 plasmid caused a decrease in observed growth under *n*-butanol stress, this indicates that there may be a cooperative effect between the genes identified for improving survival and growth that benefitted the growth of the strain when both were expressed. These data show that the genetic determinants identified through the survival and growth enrichments in combination with autologous HSP over-expression are beneficial not only to ethanol tolerance but also improve growth and survival under *n*-butanol stress. This indicates that the solvent tolerance genes expressed in this system are not specific for ethanol and that the gene combination from MG1655 *lacZ::rpoD* (pHSP, pS1G2) could be beneficial for a broad spectrum of solvents.

Tolerance and Ability to Produce Higher Product Levels: Engineering for Improved Ethanol Tolerance Leads to Improved Ethanol Production. Although solvent tolerance is an important cellular trait, the much more industrially relevant characteristic[5] is the ability to produce the desired toxic chemical (here, ethanol) at higher titers and faster. This attribute has been rarely examined in studies attempting to improve microbial tolerance. Production titers have only been examined previously in *E. coli* with production of limonene using efflux pumps selected from an efflux pump library for improved tolerance to limonene[15] and in attempts to improve productivity of ethanologenic *E. coli* strains through selection-based enrichments for improved tolerance.[34] Additionally, multiscalar analysis of library enrichments was used to identify genes that enhanced both survival in ethanol and productivity of an ethanologenic *E. coli* strain.[35] *E. coli* strains engineered for improved isobutanol tolerance were found to not have improved isobutanol titers.[36] Attempts to improve tolerance of *E. coli* during free fatty acid production were also shown to not have an effect on production titers.[37] Due to both the increased growth and survival of our engineered strain, MG1655 *lacZ::rpoD* (pS1G2, pHSP), we hypothesized that engineering an ethanol-producing strain using this set of genes would result in increased ethanol titers in a Melle−Boinot-type of fermentation. The Melle−Boinot process, widely used in industry for ethanol production based on the yeast fermentation, employs a repeated batch fermentation to reduce fermentation times and increase ethanol productivity.[5] After the first batch cycle, all the cells are harvested and used to inoculate the second batch cycle. At the end of the second batch cycle, all the cells are harvested and used to inoculate the third batch cycle, and so on. In order to maintain expression of the *Lpl* genes, we first constructed the plasmid pS1G2RpoD, which

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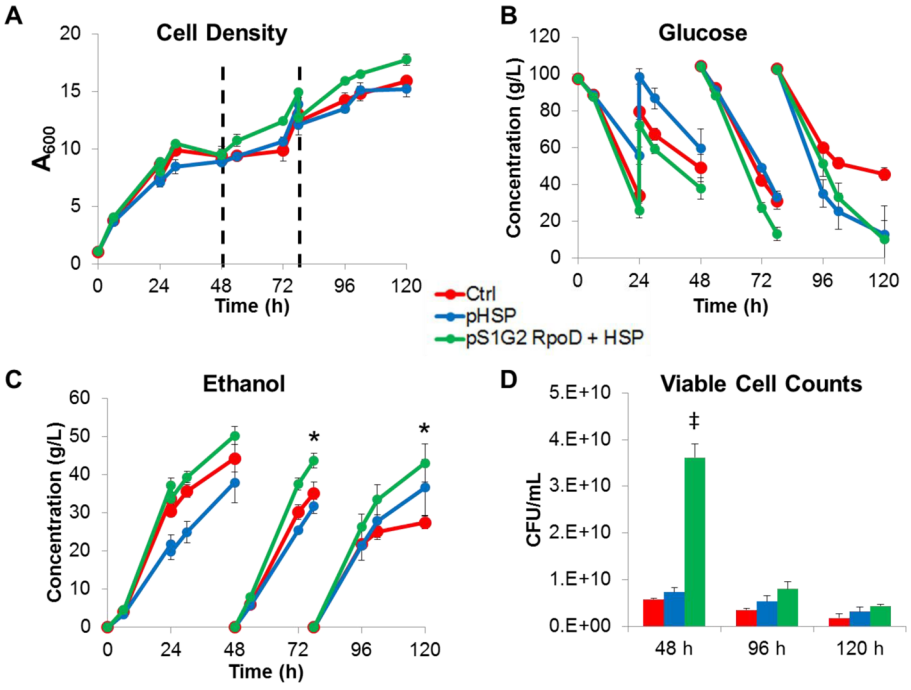

Figure 6. Time profiles for fermentations with cell recycle of *E. coli* KO11 strains bearing the plasmid combinations listed. Ctrl:KO11 (pLib-Ctl, pZS-Ctl); pHSP:KO11 (pLib-Ctl, pHSP); pS1G2RpoD + pHSP:KO11 (pS1G2RpoD, pHSP). The whole biomass was harvested by centrifugation and resuspended in fresh media at 48 and 78 h as indicated by the dashed lines on the cell density plot. Average values of cell density (A), glucose concentration (B), ethanol concentration (C), and viable cell counts (D) are presented. * indicates ethanol titer of KO11 (pS1G2RpoD, pHSP) was statistically greater than control strain ($p < 0.05$). ‡ indicates statistically higher viable cell count ($p < 0.05$). Reported values are averages from three biological replicates.

contains a copy of the *Lpl rpoD* under the Lac promoter in combination with our identified genetic determinants. We transformed pS1G2RpoD into the ethanologenic *E. coli* KO11 strain.[38] The KO11 strain has been previously shown to generate high titers of ethanol in previous fermentation studies and serves as a good model for *E. coli* ethanol fermentations.[38] Our fermentation experiments sought to emulate the Melle–Boinot process by using a repeated batch fermentation, which used concentrated cells from the end of one fermentation cycle to inoculate the next cycle. For the first cycle, we added 1.5 g of glucose after 24 h. After 48 h, the entire biomass from the fermentation was harvested via centrifugation and used to inoculate the second batch cycle in fresh media. This was repeated 30 h later (78 h into the fermentation experiments), whereby the entire biomass was again harvested and used to inoculate the third batch cycle (Figure 6). Cell density, glucose and ethanol concentrations, and viable cell counts were monitored throughout this three-cycle fermentation process (Figure 6). At the end of the first cycle (48 h), strain KO11 (pS1G2RpoD, pHSP) produced a higher ethanol titer than the control, although, due to the small number of replicates (three), this was not highly statistically significant ($p = 0.134$). However, at 48 h, the engineered strain had significantly higher number of viable cells than either the control or the HSP overexpression strain ($p = 0.005$). After the second cycle (72 h), KO11 (pS1G2RpoD, pHSP) produced 43.8 g/L ethanol compared to 35.1 g/L for the control strain ($p = 0.048$). After the third cycle (120 h), KO11 (pS1G2RpoD, pHSP) produced 43.1 g/L in 42 h, compared to 27.5 g/L for KO11 (pLib-Ctl, pZS-Ctl). KO11 (pS1G2RpoD, pHSP) also demonstrated increased glucose utilization (virtually exhausting glucose at the end of the second and third cycle, thus producing lower ethanol concentrations compared to hour 48) and biomass generation compared to the other strains examined during the second and third cycles of the fermentation. The strain overexpressing the HSPs only, namely KO11 (pLib-Ctl, pHSP), produced an equivalent final titer of ethanol to the control strain. Due to the slower growth rate of the KO11 (pLib-Ctl, pHSP), it benefitted less from the recycle process because of its lower cell density. As a final note, these fermentation experiments were performed in small-scale flasks, and experiments in such flasks typically produce substantially lower ethanol titers than experiments employing bioreactors (data not shown).

Building on the HSP Strength: Adding to It and/or Benefitting from It. The enrichment process to identify *Lpl* genetic determinants imparting tolerance was performed in combination with the previously developed HSP overexpression system[18] using pHSP. Our data show that the improvements to tolerance, either in growth or survival, were only observed for the identified *Lpl* DNA determinants in combination with the HSP overexpression system. This suggests the identified DNA determinants are either complementing the HSP overexpression system, possibly acting as alternative systems benefiting tolerance, or the HSP overexpression system is aiding in the function of the *Lpl* genomic material and/or their coded proteins. It has been shown that expression of functional, heterologous proteins in *E. coli* can be enhanced through overexpression of members of general stress response system, including components of the HSPs overexpressed in our system.[39] This indicates that the identified *Lpl* genomic fragments may impart tolerance by protein activities requiring the enhanced protein folding capabilities of our pHSP-based system.

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"Another" Synthetic Biology: Building Multigenic Complex Phenotypes by Functional Selection One "DNA Brick" At a Time. We have previously developed a semisynthetic HSP system (expressed on pHSP) by screening combinatorial expression of several core HSP genes thus identifying the most beneficial interactions and tuning their expression of maximum benefit to tolerance.[18,19] Here, we aimed to generate a stronger composite tolerance, for viability and growth, separately and together, by identifying and overexpressing heterologous *Lpl* DNA determinants that act synergistically with pHSP. By combining the fragments responsible for individual increases in survival and growth, we could generate a strain capable of both growing faster under moderate solvent stress and surviving longer in high concentrations of solvent. Among the resulting plasmid constructs only the plasmid pS1G2 produced improvements to both growth and survival when expressed in our HSP overexpression strain. The remaining combinations of determinants, expressed on the plasmids pS1G1, pS2G1, and pS2G2, failed to improve tolerance alone or in combination with the HSP system. This highlights the complexity of the solvent tolerance phenotype. These data suggest that due to interactions of the overexpressed genes, the remaining three combinations produced deleterious effects to growth, survival, or both. This loss of tolerance may stem from the increased demands of energy and resources on the cell created by expressing several proteins at the same time. In the case of the DNA determinants expressed on pS1G2, the benefit to tolerance is apparently greater than the increased metabolic burden of their expression.

Using this sequential method, we have identified a strain capable of higher growth rate, biomass accumulation, and survival under higher concentrations of toxic solvents. This indicates that with limited *a priori* knowledge of the mechanisms involved, we can engineer a complex phenotype stemming from a large number of cellular systems using a sequential approach. This strategy can thus be exploited for the development of other complex phenotypes, as long as there is a screening assay available. A generalizable approach for adding "DNA bricks" identified through enrichment of libraries offers an exciting prospect for the development of complex phenotypes (Figure 7). By developing a trait sequentially it becomes possible to develop multigenic effects over multiple rounds of enrichment. Such a strategy could seek to utilize specialized libraries engineered to include genes responsible for beneficial biophysical or metabolic/biocatalytic effects identified from a broad range of hosts.

This sequential and iterative method offers a means of discovering synergistic effects between multiple cellular systems from a combination of homologous, heterologous, and/or metagenomic sources, thus greatly expanding the searchable genomic space. Following the identification of beneficially interacting genes, their expression can be tuned using many of the powerful technologies developed in recent years. These include tunable intergenic regions and multiplex recombineering, which provide the capacity to adjust gene expression for multiple genes and screen for improved traits and phenotypes.[40−42]

MATERIAL AND METHODS

Bacterial Strains, Plasmids, and Primers. The WT *E. coli* strain MG1655 and a strain with an integrated copy of the *L. plantarum rpoD* gene under regulation of the P_{Lac} promoter

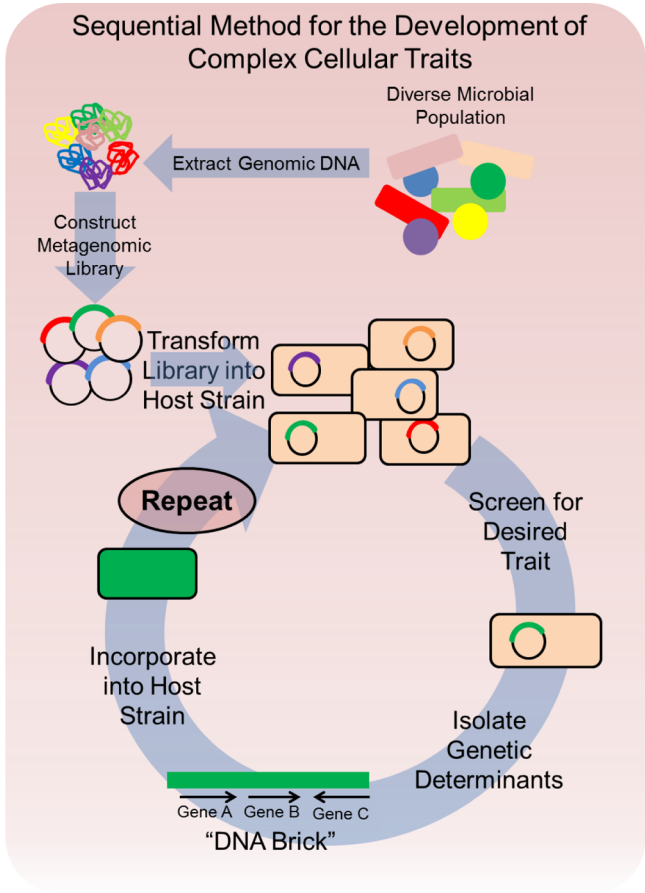

Figure 7. Sequential construction of complex, multigenic phenotypes. Using the diverse enzymatic and biophysical traits available in heterologous or metagenomic DNA, a sequential method of identification and incorporation of beneficial genetic determinants can be used to develop complex, multigenic cellular traits. Identification of novel "DNA Bricks" that can act synergistically with the host strain allows for development of composite phenotypes by screening broader specialized libraries utilizing targeted enrichment protocols to select for desirable traits.

(MG1655 *lacZ::rpoD*[29,30]) were used in the ethanol and *n*-butanol tolerance assays in this study. The ethanologenic *E. coli* strain KO11[38] (*Escherichia coli* (ATCC 55124)) was used for fermentation studies. Libraries, plasmids, and primers used in this study are listed in SI Table 3.

Analytical Methods. Cell growth was determined by measuring the absorbance at 600 nm (A_{600}) with a Beckman Coulter DU730 spectrophotometer. Samples were diluted in the appropriate medium to ensure an absorbance below 0.50. Glucose and ethanol concentrations were quantified using a high-pressure liquid chromatograph (Agilent Technologies, Santa Clara, CA, U.S.A.) using previously described methods.[17]

Growth Conditions. *E. coli* strains were grown aerobically in liquid Luria−Bertani (LB) medium and on agar-solidified LB at 37 °C. KO11 strains were regularly grown in LB + 1% glucose. The medium was supplemented with the appropriate antibiotics unless otherwise indicated: ampicillin at 50 μg/mL, chloramphenicol at 35 ug/mL, and spectinomycin at 100 μg/mL. All solvent concentrations in media are reported as percent (v/v) unless otherwise indicated. Frozen stocks were prepared from overnight cultures and were stored in 15% glycerol at −85 °C.

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DNA Isolation, Manipulation, and Cell Transformations. Isolation of plasmid DNA was performed using the Qiagen QIAprep Spin Miniprep Kit (27106; Valencia, CA). All cloning enzymes were used according to the supplier's protocols (NEB). PCR products and digests were purified with the Qiagen PCR Purification kit (28106).

L. plantarum genomic DNA was sheared using Covaris S220 Focused Ultrasonicator. Genomic DNA (15 μg) was suspended in 200 μL tris EDTA buffer (pH 8.0) and sheared using the Covaris DNA miniTUBE, red (520066; Woburn, MA) protocol with a target peak of 5 kb. Sheared DNA was resolved on a 0.7% agarose gel to confirm the distribution of fragment sizes, and the remaining DNA was concentrated via speed-vacuum centrifugation. The concentrated DNA was purified by gel purification using a QIAquick gel extraction kit (Qiagen).

Library construction. Sheared genomic DNA (gDNA) was blunt-ended, dephosphorylated, and adenylated. All enzymes used were obtained from New England Biolabs (NEB). A 50 μL PCR mixture with T4 polymerase (36.5 μL concentrated gDNA, 5 μL bovine serum albumin (100 μg/mL), 5 μL NEBuffer 2 ((100 mM Tris-HCl,100 mM MgCl2, 500 mM NaCl, 1 mM dithiothreitol [pH 7.9]), 2.5 μL of 2.5 mM deoxynucleoside triphosphates and 1 μL T4 polymerase) was kept at 12 °C for 2 h and heated to 75 °C for 20 min, and then, 0.5 μL of calf intestinal phosphatase (CIP) was added at 37 °C to dephosphorylate the blunt ended DNA for 1 h. DNA was purified using a PCR purification column (Qiagen). DNA fragments were adenylated in a Taq polymerase reaction (50 μL DNA, 5.7 μL 10× ThermoPol buffer, 1 μL 10 mM dNTPs, and 1 μL Taq Pol) at 72 °C for 30 min, and the end PCR product was column purified (Qiagen). The final gDNA was used in a TOPO reaction with the pCR8/GW/TOPO TA Cloning kit (Invitrogen).

Plasmids were transformed in 5-α cells (NEB) to obtain ~1.66×10^6 clones. Sequencing of 20 clones yielded an average insert size of 4 kb. This library was designated as 1Lp4Z. The library was then outgrown in 100 mL media to A_{600} ~ 1.0. Plasmid library DNA was extracted from 20 mL of the library outrgrowth and the remainder was stored in 2 mL aliquots at −85 °C. The 1Lp4Z library plasmids isolated from the 5-α cells was transformed into electrocompetent MG1655 *lacZ::rpoD* (pHSP) cells yielding ~53.1×10^6 clones. This library was then outgrown in 100 mL media to A_{600} ~ 1.0 and was stored in 2 mL aliquots at −85 °C.

Plasmid Construction. Fragments of *L. plantarum* genomic DNA identified during the growth screening in plasmids pG1 was amplified using the primers pG1 XhoI F and pG1 XhoI R; the fragment from plasmid pG2 was amplified via PCR using the primers pG2 XhoI F and pG2 XhoI R. Both were amplified from *L. plantarum* genomic DNA. The PCR products, flanked by *XhoI* sites, were then cloned into the plasmids pG1 and pG2 generating the four combination plasmids pS1G1, pS1G2, pS2G1, and pS2G2.

The *L. plantarum rpoD* gene under the regulation of the Lac promoter was PCR amplified from plasmid pACYC-RpoD[29] using primers pLac RpoD AhdI F and pLac RpoD AhdI R. The PCR product, flanked by AhdI sites was then cloned into plasmid pS1G2 generating the plasmid pS1G2RpoD.

Alcohol Tolerance Assays. Cultures were inoculated from a single colony in 5 mL of media supplemented with appropriate antibiotics in a 15 mL Falcon Tube. One milliliter of this overnight culture was used to inoculate a 20 mL preculture in a 50 mL Falcon tube. This preculture was grown to A_{600} ~ 1.0 and then used to inoculate the test cultures to an initial A_{600} of 0.15. Test cultures utilized a 10 mL total volume in a 50 mL Falcon Tube containing LB media, appropriate antibiotics, 1 mM IPTG, and ethanol (concentrations as specified). Cultures were grown shaking at 220 rpm. Two tolerance assays were performed on the test cultures, a survival assay and a growth assay. The survival assay was performed in 8% (v/v) ethanol and both cell density and viable cell counts were monitored at the start of the assay and after 24 h of exposure. The growth assay was performed with 4% (v/v) ethanol and measured both cell density and viable cell counts at the start of the assay and after 6 h of exposure. Cell density was then used to determine the biomass 6 h growth extent (GE_{X6}), defined as the ratio of the A_{600} at 6 h over the A_{600} at 0 h. Viable cell counts were used to determine the viable 6 h growth extent (GE_{V6}), defined as the ratio of the viable cell count at 6 h divided by the viable cell count at 0 h. Viable cell counts were determined by serially diluting the culture in 10-fold steps in LB media containing no antibiotics. The dilution level was determined based on A_{600} measurements of the samples. Fifty microliters of the final dilution was then plated on agar solidified LB plate containing appropriate antibiotics. CFUs were measured after 18−24 h of growth at 37 °C.

Survival and growth assays were also performed in *n*-butanol following the same protocols. The survival assay was done in 1.5% (v/v) *n*-butanol with a 24 h exposure, and the growth assay was performed in 0.5% (v/v) *n*-butanol with a 6 h exposure.

Ethanol Fermentations to Emulate the Melle−Boinot Process. Repeated batch fermentation experiments using *E. coli* KO11 strains were performed in 30 mL of LB + glucose with only ampicillin and spectinomycin in 125 mL screw cap flasks. The fermentation media was inoculated with overnight cultures grown in 30 mL of LB + 1% glucose media in 50 mL Falcon Tubes at 37 °C. Sufficient volume of the overnight culture was centrifuged and resuspended in 30 mL of fresh LB + 100 g/L glucose and transferred to the 125 mL fermentation flask to produce a starting A_{600} of 1.0. Fermentations were grown at 30 °C and 100 rpm. Cell density and ethanol and glucose concentrations were measured throughout the fermentation. Additional glucose, 3 mL of 500 g/L glucose, was added after the 24 h measurement. After 48 h, the entire biomass from the fermentation was harvested via centrifugation and used to inoculate the second batch cycle in fresh media. This was repeated 30 h later (78 h into the fermentation experiments), whereby the entire biomass was again harvested and used to inoculate the third batch cycle. Cultures were monitored for 120 h from the start of the fermentation experiments. The pH of the cultures was corrected to 7.0 using addition of 2N NaOH during each measurement.

Statistical Analysis. Data was statistically treated with an unpaired *t*-test and 95% confidence intervals for GE_{V6} and GE_{X6}, and percent survival were calculated to demonstrate a statistically significant difference in means of the samples.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental Tables 1−3 and Figures 1−7 as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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