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RESEARCH ARTICLE

Toward a Semisynthetic Stress Response System To Engineer Microbial Solvent Tolerance

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ABSTRACT Strain tolerance to toxic metabolites is an important trait for many biotechnological applications, such as the production of solvents as biofuels or commodity chemicals. Engineering a complex cellular phenotype, such as solvent tolerance, requires the coordinated and tuned expression of several genes. Using combinations of heat shock proteins (HSPs), we engineered a semisynthetic stress response system in *Escherichia coli* capable of tolerating high levels of toxic solvents. Simultaneous overexpression of the HSPs GrpE and GroESL resulted in a 2-fold increase in viable cells (CFU) after exposure to 5% (vol/vol) ethanol for 24 h. Co-overexpression of GroESL and ClpB on coexisting plasmids resulted in 1,130%, 78%, and 25% increases in CFU after 24 h in 5% ethanol, 1% *n*-butanol, and 1% *i*-butanol, respectively. Co-overexpression of GrpE, GroESL, and ClpB on a single plasmid produced 200%, 390%, and 78% increases in CFU after 24 h in 7% ethanol, 1% *n*-butanol, or 25% 1,2,4-butanetriol, respectively. Overexpression of other autologous HSPs (DnaK, DnaJ, IbpA, and IbpB) alone or in combinations failed to improve tolerance. Expression levels of HSP genes, tuned through inducible promoters and the plasmid copy number, affected the effectiveness of the engineered stress response system. Taken together, these data demonstrate that tuned co-overexpression of GroES, GroEL, ClpB, and GrpE can be engaged to engineer a semisynthetic stress response system capable of greatly increasing the tolerance of *E. coli* to solvents and provides a starting platform for engineering customized tolerance to a wide variety of toxic chemicals.

IMPORTANCE Microbial production of useful chemicals is often limited by the toxicity of desired products, feedstock impurities, and undesired side products. Improving tolerance is an essential step in the development of practical platform organisms for production of a wide range of chemicals. By overexpressing autologous heat shock proteins in *Escherichia coli*, we have developed a modular semisynthetic stress response system capable of improving tolerance to ethanol, *n*-butanol, and potentially other toxic solvents. Using this system, we demonstrate that a practical stress response system requires both tuning of individual gene components and a reliable framework for gene expression. This system can be used to seek out new interacting partners to improve the tolerance phenotype and can be used in the development of more robust solvent production strains.

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Solvent tolerance is a complex, multigenic phenotype stemming from interactions of a variety of cellular programs and remains a limiting factor for the production of chemicals and biofuels from renewable resources (1–4). Improving tolerance in an organism requires a complex response utilizing simultaneous modifications to multiple genes and pathways. Due not only to the individual complexity of responses to stress but also to the interconnected nature of those responses, multigenic approaches utilizing a better understanding of those processes are essential to producing a more tolerant organism. Microorganisms engage several mechanisms for resolving solvent stress (1–3). The general stress response system is typically the first line of defense against a large number of stresses, including heat, cold, and solvent stress (5). The ubiquitous heat shock proteins (HSPs), the primary members of the general stress response system, play an essential role in the folding and transport of proteins, as well as remediation of damaged or misfolded proteins (Fig. 1). The *Escherichia coli* GroEL protein forms a set of two heptameric rings that are capped by the GroES protein, also in a heptameric conformation (Fig. 1). Upon binding of an unfolded or misfolded protein to the hydrophobic interior of the GroEL cylindrical structure, the GroES heptamer seals the compartment and causes the bound substrate protein to be released from the wall for a folding attempt. ATP is consumed upon release of the folded protein. The DnaK system, comprised of the chaperone DnaK and its cochaperones, DnaJ and GrpE, works on partially folded nascent proteins as well as denatured proteins (6). In this system, DnaJ binds to unfolded proteins facilitating interaction with DnaK, which hydrolyzes ATP to refold proteins (7). GrpE acts as a nucleotide exchange factor, removing the bound ADP, thus allowing a new ATP molecule to bind to DnaK and the folded protein to be released from the complex (8). The ClpB chaperone acts as a method to disaggregate proteins. The majority of the Clp proteins in *E. coli* function as part of a proteolytic system with ClpP (9). ClpB, however,

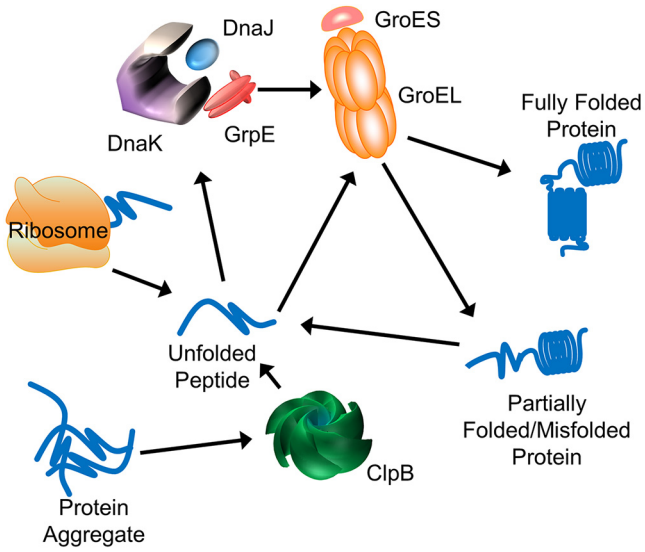

FIG 1 Schematic for folding pathways of both nascent and misfolded proteins in *E. coli*. The GroESL and DnaKJE systems function in folding of a wide variety of proteins, while ClpB functions to break apart protein aggregates, allowing for proper refolding.

acts specifically as part the DnaKJE multichaperone system to disaggregate proteins and allow their refolding (45).

HSPs have been identified as part of the solvent stress response in a variety of organisms, including *Clostridium acetobutylicum* and *Lactobacillus brevis* (10–13). It has been shown that overexpression of autologous GroESL in *C. acetobutylicum* results in both improved solvent tolerance and improved solvent production (14). Although upregulation of HSPs in response to solvent stress has been identified, studies using genomic libraries have often failed to identify them as overexpression targets (15, 16). More recently, transcriptional upregulation of HSP genes has been identified in tolerance enrichment studies for ethanol in *E. coli* (17). Additionally, transcriptional studies have identified upregulation of HSP genes in response to *n*-butanol (18) or *i*-butanol (18, 19) stress in *E. coli*. Finally, HSP genes were shown to be upregulated upon carboxylic acid (butyric and acetic) stress (e.g., see reference 10). Thus, HSP genes appear to be upregulated upon a broad variety of toxic chemical stresses. Heterologous HSPs have also been used to improve organic solvent tolerance in *E. coli* (20, 21).

Overexpression of combinations of autologous HSPs has been used to improve soluble protein yields of recombinantly expressed proteins in *E. coli* (22, 23). Chaperone systems, including GroESL, DnaKJE, ClpB, and the small heat shock proteins IbpA and IbpB, were overexpressed using a multiplasmid system to regulate their expression to stoichiometrically appropriate levels based on their presumed mechanisms of action. Expression levels were modulated by inducible promoters and the plasmid copy number. The resulting chaperone-based strategy, when combined with a modified protocol to allow for longer protein folding times, greatly increased soluble protein fractions (24). However, the system did not perform consistently, and different combinations of HSPs were needed for each of the recombinant proteins examined. Additionally, a substrate-optimized GroESL complex to further specialize the role of chaperones in folding recombinant proteins has been produced (25). This system successfully improved folding for a single protein, green fluorescent protein (GFP), but caused decreases in the more general function of GroESL within the cell.

We have recently demonstrated that overexpression of the *E. coli* GroESL proteins improved tolerance to a variety of toxic solvents, apparently in a solvent-agnostic manner (26). Using a plasmid-based expression system, we showed significant increases in growth and survivability in the presence of several toxic alcohols: ethanol, butanetriol (BT), and *n*-, *i*-, and 2-butanol. These results suggest that HSPs can be engaged to improve solvent tolerance in *E. coli*. In this study, we hypothesized that overexpression of other HSPs in *E. coli* could produce an improved solvent tolerance phenotype and that combinations of those HSPs could produce an additive or possibly supra-additive tolerance effect. Based on the biology of the HSP response, we examined the expression of GrpE and ClpB alone and in combination with the GroESL system. The two genes of the GroESL system were expressed from their stress-responsive native promoter, which was previously shown to be best for achieving enhanced tolerance (26). Expression of GrpE and ClpB was tuned based on the inducible *lac* promoter (P_{lac}) and the plasmid copy number. We show that tuned coexpression of combinations of these four genes can be used as a foundation to build a customized engineered system to achieve tolerance to toxic chemicals.

RESULTS

Co-overexpression of GroESL with GrpE or GrpE with ClpB imparts enhanced ethanol tolerance. We desired to test the hypothesis that co-overexpression of multiple HSPs could cooperate to provide additive benefits above single gene overexpression. We have previously shown that GroESL overexpression (by 25- to 70-fold) can improve solvent tolerance when overexpressed from its native promoter (26). Here we explored beneficial interactions and cooperative function when HSP genes are overexpressed in tandem. We chose to examine the combination of *grpE*, *clpB*, and the *groESL* operon. All members of the protein folding cascade (Fig. 1) were examined as overexpression targets. Of the HSPs examined, ClpB and GrpE demonstrated the most significant improvements in tolerance when overexpressed, and the data shown here present those results. Other HSPs, specifically DnaK, DnaJ, IbpA, and IbpB, when overexpressed alone or in combination with members of the cascade failed to produce improvements and often decreased survival (see below). The first combination examined was the three-gene combination: the *groESL* operon, expressed by its native promoter on a medium-copy-number vector (pAC-ES^N; 15 copies/cell [27]) (Table 1), and the *grpE* gene expressed under P_{lac} on a high-copy-number plasmid (pZE-G^L, 50 to 70 copies/cell [28]) (Table 1 and Fig. 2). Enhanced expression of GrpE was accomplished by induction with 0.1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) (see below). When grown in 5% ethanol with 3% ethanol pretreatment, MG1655(pZE-G^L, pAC-Ctl) and MG1655(pZE-Ctl, pAC-ES^N) reached higher final optical densities, of 0.356 and 0.384, respectively, compared to a final optical density of 0.314 for the control ($P = 0.007$ and $P = 0.005$, respectively). We note that this growth assay is a stringent test to assess tolerance under active growth conditions, but this does not imply that these strains would reach these relatively low cell densities. The co-overexpression strain, MG1655(pZE-G^L, pAC-ES^N), demonstrated the highest final optical density (0.441) at 24 h ($P = 0.002$). All test strains were growing after 24 h of expo-

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TABLE 1 Plasmids used in this work

Plasmid	Antibiotic resistance[a]	Gene(s) expressed (promoter[b])	Origin of replication	Copy no. (37°C)	Previous name or source (reference)
pAC-Ctl	Cm^r Tc^r		p15A	15	pACYC184[c] (23)
pAC-ES^N	Cm^r	*groES groEL* (nat)	p15A		pAC-groESL (22)
pZE-Ctl	Km^r		ColE1	5–70	pZE23MCS[d] (24)
pZE-G^L	Km^r	*grpE* (lac)	ColE1		This study
pZE-C^L	Km^r	*clpB* (lac)	ColE1		This study
pZS-Ctl	Ap^r		SC101	10–12	pZS13Luc[d] (24)
pZS-G^L	Ap^r	*grpE* (lac)	SC101		This study
pZS-C^L	Ap^r	*clpB* (lac)	SC101		This study
pZS-ES^N	Ap^r	*groES groEL* (nat)	SC101		This study
pZS-C^LG^L	Ap^r	*clpB* (lac) *grpE* (lac)	SC101		This study
pZS-C^L G^LES^N	Ap^r	*clpB* (lac) *grpE* (lac) *groES groEL* (nat)	SC101		This study

[a] Cm^r, chloramphenicol resistance; Ap^r, ampicillin; Km^r, kanamycin resistance.
[b] nat, native promoter; lac, *lac* promoter.
[c] NEB (New England Biolabs), Beverly, MA.
[d] Expressys, Ruelzheim, Germany.

sure to 5% ethanol, while the control strains appeared to have plateaued. Combined overexpression of GrpE and GroESL demonstrates the ability of HSPs to cooperate in enhancing tolerance above their individual contributions.

Tolerance was also tested using the 24-h cell viability (CFU) assay (Fig. 2B), which is meant to capture the ability of cells to survive and presumably produce a product under severe solvent stress conditions. Co-overexpression of GrpE and GroESL produced a 2.1-fold increase in the number of CFU/ml over that for the double-plasmid control. Overexpression of GroESL alone produces a 1.9-fold increase in CFU/ml, representing a significant improvement from results for the control, but no statistically significant difference was observed between the overexpression of GroESL and the combination of GroESL and GrpE. Overexpression of GrpE alone did not produce a significant difference from results for the double control. This same trend was observed after 48 h of exposure to 5% ethanol: overexpression of GroESL and GrpE produced a 1.9-fold increase in viable cells over the number for the double-plasmid control, and GroESL alone produced a 1.7-fold increase (Fig. 2C). Although the combination of GroESL and GrpE overexpression resulted in increased cell density and growth above those of the control, their combination failed to produce a significant increase in the CFU counts compared to the overexpression of GroESL alone. This difference highlights the importance of utilizing multiple assays to test tolerance. Cell density measurements allow for observation of cell growth under a high stress load, while viable cell counts provide a picture of the actual survival of a strain when exposed to a toxic solvent.

Next, we examined co-overexpression of ClpB and GrpE, whereby each gene was expressed on its own plasmid (pZE-C^L and pZS-G^L) and under control of P_{lac} (Fig. 3A). In this part of the study, we aimed to modulate the level of expression of these genes by varying the IPTG concentration and the copy numbers of the plasmids employed and then assess the impact of these modulated expression levels on ethanol tolerance. In this system, the best induction level we examined was seen with 0.1 mM IPTG (Fig. 3). With 0.1 mM IPTG, the double-plasmid control, MG1655(pZE-Ctl, pZS-Ctl), produced approximately 3 doublings and reached a final A_{600} value of 0.4 in 5% ethanol. The co-overexpression strain, MG1655(pZE-C^L, pZS-G^L), demonstrated a higher growth rate, reaching an A_{600} of 0.5 in the first 7 h of growth, and reached a final optical density of 0.548 at 24 h. Each of the individual gene expression sets, MG1655(pZE-C^L, pZS-Ctl) and MG1655(pZE-Ctl, pZS-G^L), however, performed worse than the double-plasmid control. This represented a significant increase for the dual-expression system over all other combinations with greater than 99% confidence. The supraadditive effect indicates a cooperative effect achieved by the overexpression of both genes that is not observed with their independent expression. Effects such as this further serve to highlight the necessity for screening combinations of genes rather than individual members to identify optimal improvements for complex cellular properties. The need to integrate multiple genes is an important feature of solvent tolerance and other complex phenotypes. Single gene mutations or integrations have produced only small increases in tolerance (2). Only through examinations of multiple genes can large steps forward be made in the development of more-tolerant organisms.

In order to interrogate the cause for the various phenotypes observed with the induction levels and copy numbers of the expression vectors, using quantitative reverse transcription-PCR (Q-RT-PCR), we examined the mRNA expression levels of *clpB* and *grpE* in the ClpB and GrpE overexpression strains (see Text S1 in the supplemental material). These data indicate that higher levels of expression of both *clpB* and *grpE* are necessary to produce the best tolerance phenotypes observed.

Expression of other autologous HSPs failed to improve the tolerance phenotype. Other HSP members were also examined, including overexpression of the *E. coli* autologous genes *ibpA*, *ibpB*, *dnaK*, and *dnaJ*. Overexpression of these genes alone and in combination with other HSP genes failed to produce significant improvements in tolerance for any of the strains examined and in several cases significantly reduced the tolerance of the strain (data not shown). A summary of the genes and solvent combinations tested is provided in Table 2. Previous attempts to overexpress the DnaKJ operon have been unsuccessful in both *E. coli* (29) and *C. acetobutylicum* (14). These HSPs, although routinely identified as being upregulated during stress (10, 13, 19, 30), fail to produce improvements to tolerance or growth when overexpressed. This important finding further reflects the need for careful regulation of stress response genes aiming to achieve a desirable outcome and highlights the possibility of negative effects on cell growth resulting from misregulated expression of HSP genes. In other words,

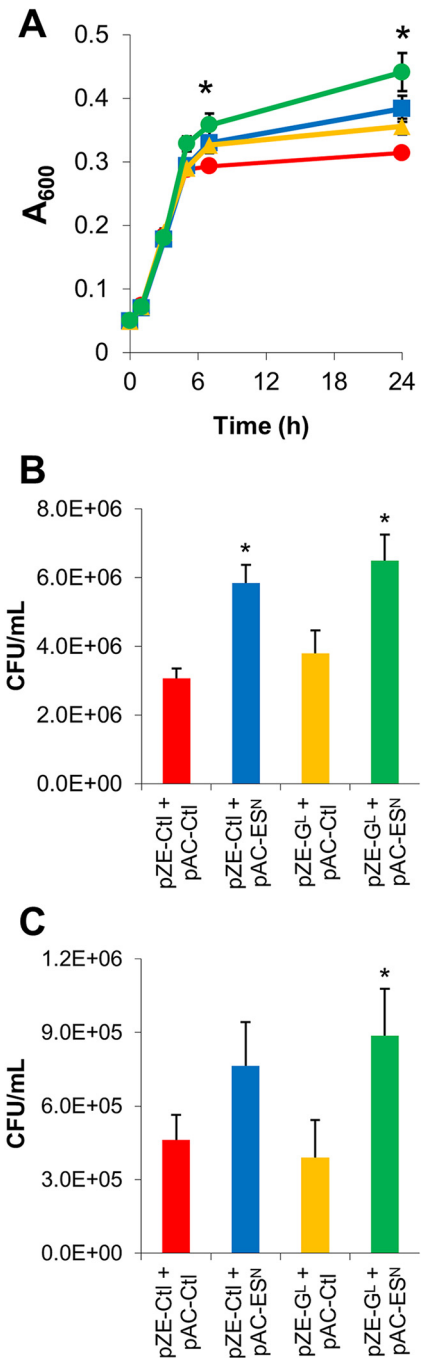

FIG 2 Tolerance assays for co-overexpression of autologous GroESL and GrpE with growth in 5% ethanol with 0.1 mM IPTG and a 3% ethanol pretreatment. (A) A_{600} growth-based measurements over 48 h for control (red), GroESL overexpression (blue), GrpE overexpression (yellow), and GroESL and GrpE overexpression (green) strains. "*" indicates a statistically significant increase of MG1655(pZE-G^L, pAC-ES^N), MG1655(pZE-G^L, pAC-Ctl), and MG1655(pZE-Ctl, pAC-ES^N) over MG1655(pZE-Ctl, pAC-Ctl) ($P < 0.05$). (B) Viable cell counts (CFU/ml) performed after 24 h of growth in the presence of 5% ethanol. "*" indicates a statistically significant increase from the control strain, MG1655(pZE-Ctl, pAC-Ctl) ($P < 0.05$). (C) Viable cell counts (CFU/ml) performed after 48 h growth in the presence of 5% ethanol. "*" indicates a statistically significant increase from MG1655(pZE-Ctl, pAC-Ctl) ($P < 0.05$). Data are from 3 biological replicates. Error bars indicate standard errors between replicates.

the fact that several HSP genes are routinely identified as overexpressed upon exposure to toxic chemicals does not imply that overexpression of these genes will lead to a tolerant phenotype. Several HSPs, including GroESL and DnaK, have been identified as regulatory elements both in the regulation of the general stress response through RpoH and in regulation of the cell cycle (31–33). This is a complicating factor, and thus development of a tunable HSP system for developing solvent tolerance requires careful screening of HSP genes and control of their expression level.

Coexisting plasmids enable rapid screening of combinations of genes but may impose additional stress on cells. We next examined combinations of all four genes: *grpE*, *groES*, *groEL*, and *clpB*. As before, *groESL* was overexpressed on pAC-ES^N (pA15 origin of replication; 15 copies/cell [27]) (Table 1) using its native promoter, *grpE* was overexpressed on pZE-G^L (ColE1 origin of replication; 50 to 70 copies/cell [28]) (Table 1) under P_{lac}, and *clpB* was overexpressed under P_{lac} using pZS-C^L (pSC101 origin of replication; 10 to 12 copies/cell [28]) (Table 1). The plasmids selected for these studies were chosen based upon the work examining each gene individually and previously examined combinations. Based on our work with GroESL (26), we chose to express the *groESL* genes from their native promoter using a plasmid with the pA15 origin of replication, while the expression of *grpE* and *clpB* on plasmids with the ColE1 and SC101 origins of replication was based on our work examining combinations of those genes alone. Induction with IPTG using the P_{lac} promoter enables tunable expression of HSP genes, as shown in other applications (24). These strains were examined for both growth (Fig. 4A) and CFU-based viability (Fig. 4B) under 5% ethanol stress with a 3% ethanol pretreatment and 0.1 mM IPTG induction. The best performers using growth as a measure of tolerance were the combinations pZE-G^L, pAC-ES^N, pZS-Ctl and pZE-Ctl, pAC-ES^N, pZS-C^L, both of which produced statistically significant increases from results for the plasmid control ($P < 0.001$ and $P < 0.001$, respectively). It was also observed that MG1655(pZE-Ctl, pAC-Ctl, pZS-C^L) and MG1655(pZE-Ctl, pAC-ES^N, pZS-Ctl) performed better than the triple-plasmid control strain MG1655(pZE-Ctl, pAC-Ctl, pZS-Ctl) ($P = 0.033$ and $P < 0.001$, respectively), but their average cell density was less than those for the combinations listed above. The remaining combinations were also examined; however, the resulting growth was comparable to or lower than the control data (see Fig. S1 in the supplemental material). For comparison purposes, the four strains presented in Fig. 4 were also grown without a stressor in order to assess the effects of overexpression of these HSP genes on cell growth rather than tolerance (see Fig. S2). These data show that overexpression of HSPs produces growth comparable to or below those of the plasmid control strains, thus showing that the benefit is related to solvent tolerance rather than cell growth.

Viability assays based on measurement of CFU after 24 and 48 h of growth under 5% ethanol resulted in statistically significant higher number of viable cells in MG1655(pZE-G^L, pAC-ES^N, pZS-Ctl), MG1655(pZE-Ctl, pAC-ES^N, pZS-Ctl), and MG1655(pZE-Ctl, pAC-ES^N, pZS-C^L) than in the triple plasmid control (Fig. 4B) ($P = 0.002$, $P < 0.001$, and $P < 0.001$, respectively). MG1655(pZE-Ctl, pAC-ES^N, pZS-C^L) produced 10.1-fold more CFU than the control and 3.1-fold more CFU than MG1655(pZE-G^L, pAC-ES^N, pZS-Ctl), indicating significant increases ($P < 0.001$ for both). The remaining plasmid combinations performed comparably to or worse than the triple-plasmid

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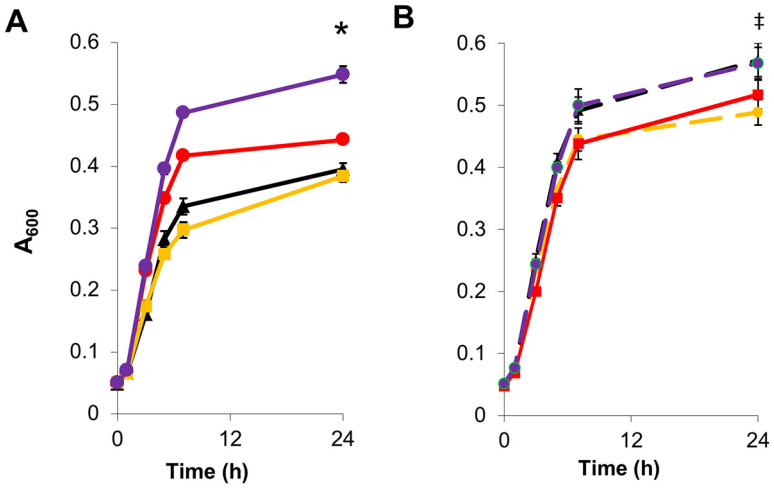

FIG 3 Impact of expression levels of *clpB* and *grpE* on cell growth under ethanol stress (5%) with IPTG induction. (A) ClpB overexpressed on the high-copy-number ColE1 ori-containing vector and GrpE overexpressed on the low-copy-number pSC101 ori-containing vector with 0.1 mM IPTG. MG1655(pZE-Ctl, pZS-Ctl) (red), MG1655,(pZE-C^L, pZS-Ctl) (black), MG1655(pZE-Ctl, pZS-G^L) (yellow), and MG1655(pZE-C^L, pZS-G^L) (purple) were used. "*" indicates a statistically significant increase of MG1655(pZE-C^L, pZS-G^L) compared to MG1655(pZE-Ctl, pZS-Ctl) ($P < 0.001$). (B) ClpB overexpressed on the low-copy-number pSC101 ori-containing vector and GrpE overexpressed on the high-copy-number ColE1 ori-containing vector with 0.1 mM IPTG. MG1655(pZE-Ctl, pZS-Ctl) (red), MG1655(pZE-Ctl, pZS-C^L) (black dashed), MG1655(pZE-G^L, pZS-Ctl) (yellow dashed), and MG1655(pZE-G^L, pZS-C^L) (purple dashed). "‡" indicates a statistically significant increase of MG1655(pZE-G^L, pZS-C^L) and MG1655(pZE-Ctl, pZS-C^L) compared to MG1655(pZE-Ctl, pZS-Ctl) ($P < 0.05$).

control. It should be noted that the increases observed could be the result of mutations; however, due to the short time frame in which the cells were exposed to the stress (48 h), it is unlikely that any significant mutations could accumulate and do so consistently for all biological replicates. We additionally examined the effect of *n*-butanol and *i*-butanol stress on the best-performing strain, namely, MG1655(pZE-Ctl, pAC-ES^N, pZS-C^L). This strain produced a 78% increase ($P < 0.001$) in viable cells over results for MG1655(pZE-Ctl, pAC-Ctl, pZS-Ctl) in 1% *n*-butanol and a 25% increase in 1% *i*-butanol (Fig. 4C). Thus, the tolerant phenotype is not restricted to ethanol.

Although combinations of HSPs, notably GrpE and GroESL or GroESL and ClpB, were shown to provide additive effects when co-overexpressed, combinations of all four genes expressed from three plasmids decreased growth and survival compared to results with the triple-plasmid control strain. This effect indicates that although these genes can cooperate, as evidenced by the combinations demonstrated to improve tolerance of all three combinations of GroESL, ClpB, and GrpE overexpression, their expression on three coexisting plasmids produced too much stress for the cell to properly tolerate ethanol exposure. To investigate the origin of this stress, we examined the impact of antibiotics on cell growth (see Text S2 in the supplemental material) and integrated the genes into a single expression vector. Cultures without antibiotics improved cell densities achieved by all strains but resulted in a decrease in CFU when plated on antibiotic-containing plates. Also, the three-plasmid, 4-gene expression strain performed only comparably to the control in growth and significantly worse in numbers of CFU, indicating that the presence of three antibiotics was not limiting the strain's performance.

TABLE 2 Effects of HSP overexpression on tolerance

Alcohol tested	Tolerance with overexpression of HSP[a]					
	GroESL[b]	DnaK	DnaJ	GrpE	IbpAB[b]	ClpB
Ethanol	+[c]	−	−	+[c]	−	+[c]
n-Butanol	+	N	N	+	N	+
i-Butanol	+[d]	N	N	−	N	+[d]
1,2,4-Butanetriol	+	N	N	+	N	+

[a] "+" indicates improved tolerance; "−" indicates that tolerance was not improved; N, not tested.
[b] Tested only as operons (individual genes not tested).
[c] Tested both individually and in combination for ethanol tolerance; both showed improvements.
[d] Beneficial for *i*-butanol tolerance when expressed using three-plasmid system [MG1655(pZE-Ctl, pAC-ES^N, pZS-C^L)] but not when tested on a single plasmid [MG1655(pZS-$C^LG^LES^N$)].

Tandem *groES*, *groEL*, *grpE*, and *clpB* overexpression from a single plasmid improves ethanol, 1,2,4-butanetriol, and *n*-butanol tolerance. To reduce the strain imposed on cells for maintaining three coexisting plasmids, we cloned the four genes expressing GrpE, GroESL, and ClpB into a single vector (pZS-$C^LG^LES^N$) which was cloned into MG1655. The tolerance of this strain was then compared to those of a plasmid control (pZS-Ctl), strains containing overexpression plasmids for each gene (pZS-C^L, pZS-G^L, and pZS-ES^N), and the previously successful (26) GroESL overexpression strain (carrying pAC-ES^N) (Fig. 5A). Tolerance assays were performed in a higher concentration of ethanol (7%), which we hypothesized constitutes a more stringent performance test. MG1655(pZS-$C^LG^LES^N$) improved the CFU counts by 2-fold over those of the control and 26% over those of MG1655(pZS-C^L), both in a statistically significant way (Fig. 5A). The overexpression strains (based on the vector pZS) for each individual gene, encoding ClpB, GrpE, and GroESL, improved CFU-based tolerance versus that of the control by 59% ($P < 0.001$), 15% ($P = 0.038$), and 27% ($P = 0.006$). CFU counts from MG1655(pAC-ES^N) demonstrated a 38% increase ($P = 0.001$) from those of the control strain.

Additionally, we examined the effect of overexpression of GrpE, GroESL, and ClpB on CFU-based tolerance to *n*-butanol, BT, and *i*-butanol (Fig. 5B). Previously, we have demonstrated that overexpression of GroESL using the plasmid pAC-ES^N produced increased tolerance to a variety of alcohols, including ethanol, *n*-butanol, BT, and *i*-butanol (26). MG1655(pZS-$C^LG^LES^N$) produced a 3.9-fold increase ($P < 0.001$) in numbers of CFU compared to results for the plasmid control when grown in 1% *n*-butanol and a 78% increase in 25% BT ($P = 0.007$). However, in 1% *i*-butanol the control strain proved to be much more tolerant. MG1655(pZS $C^LG^LES^N$) maintained only 20% of the surviving viable cells compared to the control. When ClpB and GroESL were

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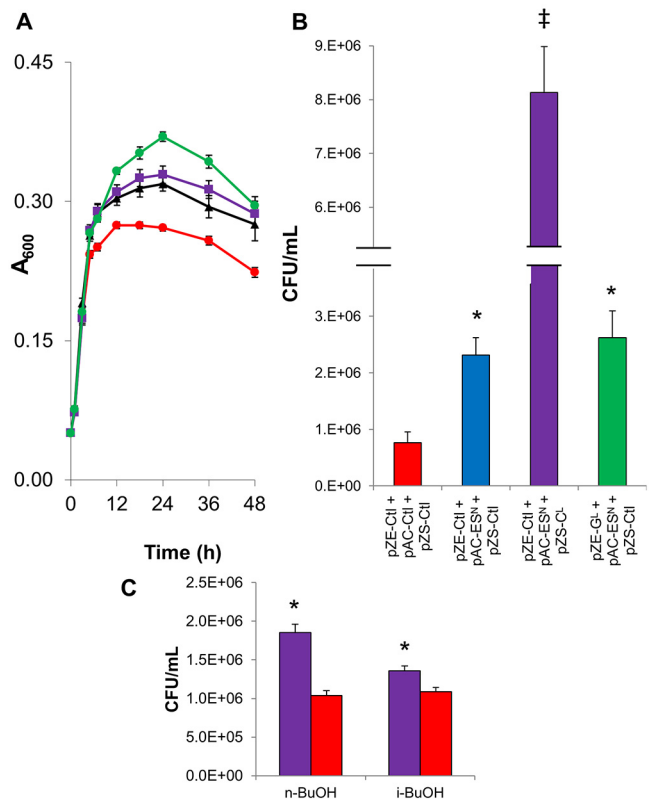

FIG 4 Ethanol tolerance assays for simultaneous overexpression of GrpE, GroESL, and ClpB using a three-plasmid system. GrpE was overexpressed on a ColE1 ori-containing vector, GroESL was expressed on a pA15 ori-containing vector, and ClpB was overexpressed on a pSC101 ori-containing vector. Samples were grown in 5% ethanol with a 3% ethanol pretreatment and induction with 0.1 mM IPTG. (A) Growth (A_{600}) data. MG1655(pZE-Ctl, pAC-Ctl, pZS-Ctl) (red), MG1655(pZE-Ctl, pAC-Ctl, pZS-C^L) (black), MG1655(pZE-G^L, pAC-ES^N, pZS-Ctl) (green), and MG1655(pZE-Ctl, pAC-ES^N, pZS-C^L) (purple). (B) Viable cell counts (CFU/ml). "*"indicates a statistically significant increase from results for the plasmid control, MG1655(pZE-Ctl, pAC-Ctl, pZS-Ctl) ($P < 0.005$). "‡" indicates a statistically significant increase from results for all other strains examined ($P < 0.05$). CFU were determined after 24 h of exposure to 5% ethanol. Remaining combinations of plasmids are presented in Fig. S1 in the supplemental material. (C) Viable cell counts (CFU/ml) in 1% *n*-butanol and 1% *i*-butanol for MG1655(pZE-Ctl, pAC-ES^N, pZS-C^L) (purple) and MG1655(pZE-Ctl, pAC-Ctl, pZS-Ctl) (red). "*" indicates a statistically significant increase from results for the plasmid control ($P < 0.005$). Data are from 3 biological replicates; error bars indicate standard errors.

overexpressed using the three-plasmid system, a 25% increase in survival under *i*-butanol stress was observed, as already discussed (Fig. 4C). This was lower than the improvements observed for those strains under ethanol and *n*-butanol stress (Fig. 4). This consistently less effective response to *i*-butanol stress suggests that this combinations of genes under these expression conditions may be suboptimal for handling *i*-butanol stress.

DISCUSSION

The current model for the protein refolding cascade describes DnaKJ and GrpE, along with GroESL, acting to fold both nascent and misfolded proteins, while disaggregation is performed in a parallel pathway by ClpB, with the resulting proteins fed into the refolding cascade (6, 22, 34). These systems then operate either in series or in parallel, indicating that upregulation of each step of the system might allow for a more active and in turn more beneficial stress response system. Our method of including the most beneficial genes, shown by upregulation of those genes individually, in a semisynthetic response system allows for the use of HSPs simultaneously producing a more efficient protein refolding system. Considering the role of GroESL as a multipurpose chaperone shown to interact with up to half of the soluble protein in *E. coli* (35) and ClpB being responsible for the breakdown of damaged and misfolded proteins, this combination fits well with the expectation that a more robust protein refolding system is beneficial for cell survival under solvent stress.

Since the DnaKJE, GroESL, and ClpB systems are known to operate independently while part of the protein disaggregation and refolding system, these proteins must act in a coordinated manner, requiring particular ratios of each protein to the other to function properly (36). To that end, variation of expression levels of each of the genes in our semisynthetic stress response system allowed us to examine the impact of different expression levels of these genes on the strain's effectiveness at tolerating solvent stress. This need for specific expression ratios dictates that an effective system be tuned to the appropriate level required for a particular solvent. The effects of tuning the expression levels and ratios of various HSP genes was also observed by de Marco (23) in the use of these proteins for improved heterologous, recombinant protein expression in *E. coli*, since each individual protein expressed required a different combination to generate improved protein expression.

Increases in tolerance to ethanol and *n*-butanol indicate that the co-overexpression of these four HSP genes examined here (Fig. 4 and 5) results in beneficial effects for surviving high levels of toxic solvent exposure. In view of the success of GroESL (26), lack of benefit for *i*-butanol tolerance with the four-HSP-gene overexpression is surprising. This further demonstrates the complexity of the tolerance phenotype and highlights the need for tailored resistance to each solvent. The additive effect resulting from HSP co-overexpression indicates that this system could be used in a practical setting for improving tolerance during production of ethanol, *n*-butanol, and potentially other useful but toxic solvents.

The use of a multiplasmid system allows us to regulate the expression level of each gene separately and to examine diverse expression levels; however, it also provides new difficulties in elucidating useful genes for increased alcohol tolerance. In particular, the presence of plasmids is known to cause an upregulation of the stress response system (37), which could potentially influence the expression levels of the genes that we have overexpressed, as well as other genes that may be responsible for regulation of the stress response cascade and other cellular responses to stress. However, maintaining the expression systems on a plasmid also provides some unique benefits, including the ability to test wide variation in expression levels, efficiently test the combinatorial effect of various genes, and investigate genetic libraries to screen for new functional partners. As indicated previously, the metabolic burden of three expression vectors likely plays a significant role in the effectiveness of our tolerance system. By reducing this burden, we were able to produce significant improvements in survival with high concentrations of ethanol (7%), BT (25%), and *n*-butanol exposure (1%). Although we could not identify improvements in tolerance in our three-plasmid-containing strain with overexpression of the four genes examined, we have consistently

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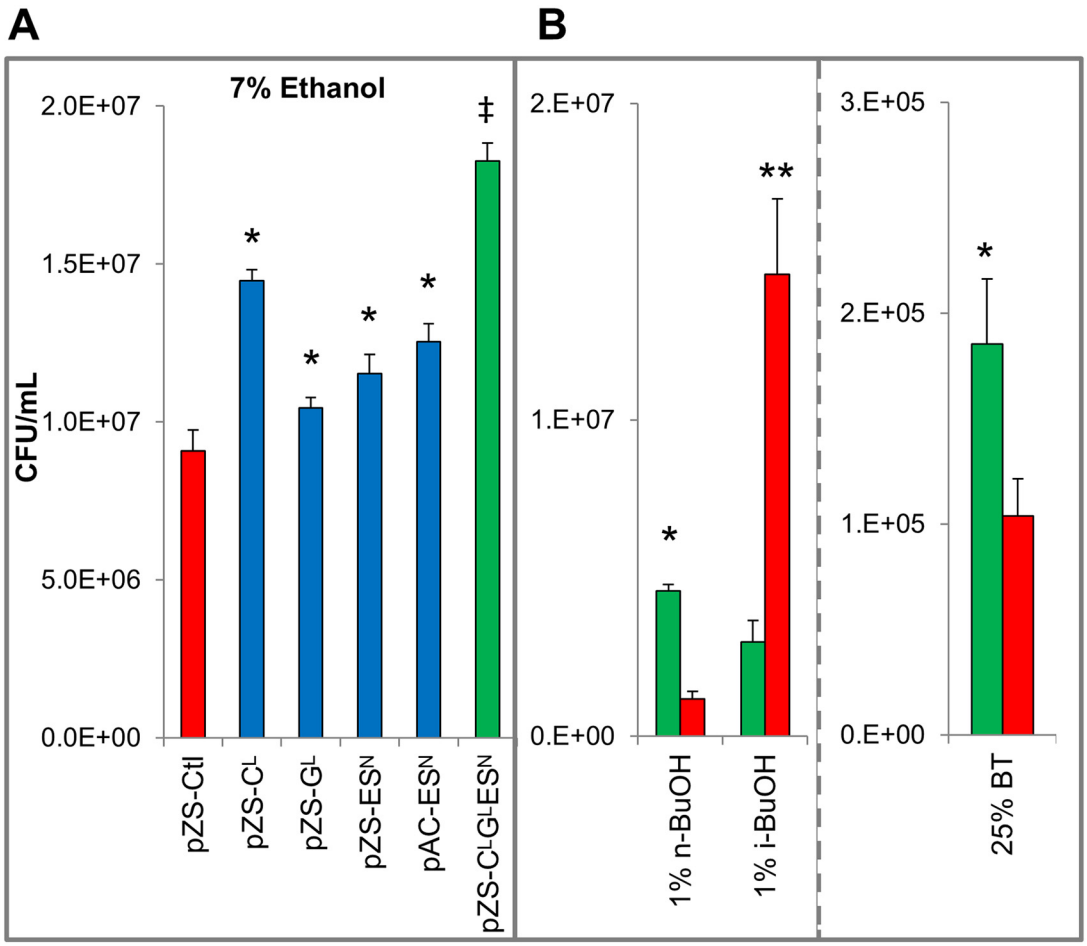

FIG 5 (A) Viable cell counts (CFU/ml) performed after 24 h of culture in 7% ethanol for the 4-gene, single-plasmid overexpression system and 0.1 mM IPTG induction. (B) Numbers of CFU/ml, measured after 24 h of culture in 1% *n*-butanol, 1% *i*-butanol, and 25% 1,2,4-butanetriol (BT). MG1655(pZS-C^LG^L ES^N) (green) and MG1655(pZS13Luc) (red) were used. Results are from 3 biological replicates; error bars indicate standard errors between replicates. "*" indicates a statistically significant improvement over the control strain, MG1655(pZS-Ctl) ($P < 0.05$). "**" indicates a statistically significant increase above results for the overexpression strain MG1655(pZS-$C^LG^LES^N$). "‡" indicates a statistically significant increase over results for all other strains examined ($P < 0.05$).

demonstrated that two-plasmid systems do not appear to significantly affect cell activities, and thus screening for combinations of genes using an established expression platform, such as the pZS-$C^LG^LES^N$ plasmid, still allows for examination of combinations of genes prior to their integration into any future tolerance system.

An important aspect of developing a tolerant organism is defining an appropriate assay for measuring tolerance. From an industrial perspective, solvent tolerance is an important aim because solvent titers for many production strains are limited by cell survival, let alone growth. Therefore, increasing cell productivity and viability in higher solvent concentrations is essential for making a successful industrial process. To that end, we have found that using cell growth as a sole measure is not sufficient for demonstrating solvent tolerance. Instead, we have chosen to examine both cell growth using a model assay and cell viability (by CFU), the latter likely being a more useful metric of potential industrial performance.

The improvement in both growth and survival generated by overexpressing combinations of HSPs represents significant improvements over other reported *E. coli* strains engineered for ethanol/alcohol tolerance. We have compared our GroESL overexpression strain 10-β(pAC-ES^N) to previously reported engineered strains (26). Our comparisons indicated improvements from previous efforts to generate solvent tolerance (17, 38–40). Here, our four-gene overexpression strain MG1655(pZS-$C^LG^LES^N$) has been found to show a significant improvement to overexpression of GroESL alone using MG1655(pAC-ES^N) in both ethanol and *n*-butanol tolerance. Improvements over our previous strain represent steps forward from the other engineered strains we have compared it against. Also, if HSP overexpression not only can impart solvent tolerance but also can improve solvent production titers, as we have shown previously (14), MG1655(pZS-$C^LG^LES^N$) may represent a strong platform strain for solvent production. These growth experiments, however, represent increased tolerance under laboratory conditions only, and further optimization of the system will be essential for the development of strains suitable for industrial applications.

For the development of practical industrial production strains, it will be necessary to further optimize and enhance the synthetic HSP systems we presented here. This will include optimization of the choice of HSP and partner genes that can be overexpressed, tuning of the expression levels for these genes, and integration of

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the system into the chromosome or the employment of a plasmid expression system that does not require complex maintenance under production conditions. To identify other partner genes that add to the effects of the currently developed system of four HSP genes, multiple coexisting genomic libraries can be employed in strain MG1655(pZS-$C^L G^L ES^N$) using the CoGeL technology we have recently developed (16). This technology will allow us to identify new cooperating or interacting partner genes. To optimize the expression levels, several powerful genomic tools have been described in the last few years. An important approach for tuning the expression of multiple genes is to employ combinatorial engineering based on libraries of tunable intergenic regions (41). Another approach is multiplex automated genome engineering (MAGE) technology (42), which enables the optimization of complex pathways or in our case an HSP-based synthetic program.

MATERIALS AND METHODS

Bacterial strain, plasmids, and primers. The wild-type (WT) *E. coli* strain MG1655 was used in this study. Plasmids are listed in Table 1. Primers used are listed in Table S1 in the supplemental material.

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.

Growth conditions. *E. coli* strains were grown aerobically in liquid Luria-Bertani (LB) medium and on agar-solidified LB at 37°C. The medium was supplemented with the appropriate antibiotics unless otherwise indicated: ampicillin at 50 μg/ml, chloramphenicol at 35 μg/ml, and kanamycin at 25 μg/ml. All solvent concentrations in media are reported as percentages (vol/vol). Frozen stocks were prepared from overnight cultures and were stored in 15% glycerol at −85°C.

DNA isolation, manipulation, and cell transformations. Isolation of plasmid DNA was performed using the Gerard Biotech (Oxford, OH) Hurricane miniprep kit (H240M). Transformations were carried out using electrocompetent *E. coli* MG1655 cells. All cloning enzymes were used according to the supplier's protocols (NEB). PCR products and digests were purified using the Qiagen (Valencia, CA) PCR purification kit (2810).

Plasmid constructs. The *E. coli clpB* gene was amplified from MG1655 genomic DNA via PCR in two separate reactions with the primers Eco-clpB KpnI F and Eco-clpB XmaI R and the primers Eco-clpB F KpnI and Eco-clpB SphI R. The PCR product flanked by the KpnI and XmaI sites was then cloned under P_{lac} of the plasmid pZE-Ctl, producing pZE-C^L. The nomenclature used here identifies the plasmid origin of replication by the first section (pZE, pZS, or pAC), the gene overexpressed by the letter in standard font (C [*clpB*], G [*grpE*], or ES [*groESL*]), and the regulatory elements as a superscript letter (superscript L [P_{lac}] or superscript N [native promoter]). The *clpB* PCR product flanked by KpnI and SphI sites was cloned under P_{lac} of pZS-Ctl, producing pZS-C^L.

The *E. coli grpE* gene was amplified from MG1655 genomic DNA via PCR in two separate reactions using the primers Eco-grpE KpnI F and Eco-grpE XmaI R and the primers Eco-grpE F KpnI and Eco-grpE SphI R. The PCR product flanked by KpnI and XmaI sites was then cloned under P_{lac} of the plasmid pZE-Ctl, producing pZE-G^L. The *grpE* PCR product flanked by KpnI and SphI sites was cloned under P_{lac} of pZS-Ctl, producing pZS-G^L.

The *E. coli* genes *groES* and *groEL*, including their native regulatory elements, were PCR amplified from MG1655 DNA using the primers Eco-groESL SalI F and Eco-groESL SalI R. Additionally, the pZS-Ctl plasmid was PCR amplified using the primers pZS13-No pLac F and pZS13-No pLac R to exclude P_{lac} and introduce desirable restriction sites. The resulting product was then self-ligated to produce pZS13-No pLac, to be used for construction of pZS-ES^N. Finally, the *groESL* genes were cloned into the SalI sites of pZS13-No pLac to produce pZS-ES^N.

To integrate multiple genes onto the single vector, plasmid pZS-C^L was PCR amplified with the primers pZS-C^L PstI F and pZS-C^L SmaI F and self-ligated to integrate the new restriction digest sites. Additionally, the *grpE* gene, under control of P_{lac} from pZE-G^L, was amplified using the primers pLac-grpE PstI F and pLac-grpE PstI R. This product was then cloned into the PstI site engineered into the pZS-C^L PCR product, producing pZS-C^L grpE. Finally, a linear blunt-ended PCR product containing the *groESL* genes was amplified using Eco-groESL F and Eco-groESL R from *E. coli* MG1655 chromosomal DNA. This product was then blunt end cloned into a blunt-ended SacI digest of pZS-$C^L G^L$, producing pZS-$C^L G^L ES^N$.

Alcohol tolerance assays. We employed 4 different alcohol tolerance assays as described previously (26). Two growth assays were used to measure growth under high ethanol stress starting from a low cell density (A_{600} = 0.05) with or without 3% ethanol pretreatment. The ethanol pretreatment was meant to simulate the continuous accumulation of toxic solvents in a fermentation broth and more pertinently the process of fed-batch operation, where the cells would be exposed to prior stress in later cycles. This would simulate the effects of increasing solvent stress experienced under solvent-producing conditions in extended batch or repeated fed-batch fermentations typically employed in the industrial setting. Although this assay may not directly represent a practical fermentation, it served as a measure of tolerance under high stress since the starting cell density is so low. Two viability assays were also used, with and without ethanol pretreatment as before, where CFU were measured after 24 and 48 h of solvent exposure to assess cellular production under stress. Cultures were serially diluted in 10-fold steps in LB medium 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 an agar solidified LB plate containing appropriate antibiotics. CFU were measured after 18 to 24 h of growth at 37°C. This assay serves as a better measure of potential industrial use because it is a direct measure of cells that are still biologically active during solvent exposure.

Q-RT-PCR. RNA was isolated from samples of cultures after 6 h of exposure to 5% ethanol and induced as described above. RNA isolation was performed using the Qiagen RNeasy minikit (74106; Qiagen) according to the manufacturer's protocols, including the optional DNase digestion. Reverse transcription and Q-RT-PCR were then carried out as described previously (43). The housekeeping gene *ihfB* was chosen for calculation of differential expression (44). Data were collected from five biological replicates. Primers used are listed in Table S1 in the supplemental material.

Statistical analysis. Data were statistically treated with an unpaired *t* test, and 95% confidence intervals for cell density, CFU, and mRNA fold differences were calculated to demonstrate a statistically significant difference in means of the samples.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00308-12/-/DCSupplemental.

Figure S1, DOCX file, 0.1 MB.
Figure S2, DOCX file, 0.1 MB.
Figure S3, DOCX file, 0.1 MB.
Figure S4, DOCX file, 0.1 MB.
Table S1, DOCX file, 0.1 MB.
Text S1, DOCX file, 0.1 MB.
Text S2, DOCX file, 0.1 MB.

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