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A new player in the biorefineries field: phasin PhaP enhances tolerance to solvents and boosts ethanol and 1,3-propanediol synthesis in *Escherichia coli*

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**Running title:** PhaP boosts tolerance and production of added-value chemicals.
Abstract. The microbial production of biofuels and other added-value chemicals is often limited by the intrinsic toxicity of these compounds. Phasin PhaP from the soil bacterium *Azotobacter* sp. strain FA8 is a polyhydroxyalkanoate granule-associated protein that protects recombinant *Escherichia coli* against several kinds of stress. PhaP enhances growth and poly(3-hydroxybutyrate) synthesis in polymer-producing recombinant strains and reduces the formation of inclusion bodies during overproduction of heterologous proteins. In this work, the heterologous expression of this phasin in *E. coli* was used as a strategy to increase tolerance to several biotechnologically relevant chemicals. PhaP was observed to enhance bacterial fitness in the presence of biofuels, such as ethanol and butanol, and to other chemicals, such as 1,3-propanediol. The effect of PhaP was also studied in a *groELS* mutant strain, in which both GroELS and PhaP were observed to exert a beneficial effect that varied depending on the chemical tested. Lastly, the potential of PhaP and GroEL to enhance the accumulation of ethanol or 1,3-propanediol was analyzed in recombinant *E. coli*. Strains that overexpressed either *groEL* or *phaP* had increased growth, reflected in a higher final biomass and product titer compared to the control strain. Taken together, these results add a novel application to the already multifaceted phasin protein group, suggesting that expression of these proteins or other chaperones can be used to improve biofuels and chemicals production.

Importance. This work has both basic and applied aspects. Our results demonstrate that a phasin with chaperone-like properties can increase bacterial tolerance to several biochemicals, providing further evidence of the diverse properties of these proteins. Additionally, both the PhaP phasin and the well-known chaperone GroEL were used to increase the biosynthesis of the biotechnologically-relevant compounds ethanol and 1,3-propanediol in recombinant *E. coli*. These findings open the road for the use of these proteins for the manipulation of bacterial strains to optimize the synthesis of diverse bioproducts from renewable carbon sources.
Introduction

Fossil oil, or petroleum, has been utilized by humankind for many centuries, but since the mid-XVIII century the number and variety of applications for this product have sharply increased, so that nowadays derivatives of this non-renewable substrate are present in almost every aspect of modern life (1). We use petroleum derivatives as our main source of energy, but also as a starting point for the synthesis of many different chemical precursors that are in turn converted to a large variety of materials (2). In recent times, dwindling petroleum availability and an increasing concern about the environmental impact of petroleum consumption has prompted the development of environmentally friendly and sustainable alternatives (3), such as the use of microorganisms to obtain biofuels and other chemicals from renewable carbon sources (4–7). Analogous to the term oil refinery, that refers to the obtainment of a variety of products from oil, the term biorefinery comprises the use of biomass to obtain different products, normally by means of a biological process (8, 9).

Among the different products that can be obtained in this way are biofuels such as ethanol and butanol, and chemicals such as 1,3-propanediol (1,3-PDO). Many bacteria and yeast can synthesize ethanol, the best known and most widely used biofuel (10–14). Butanol, that has several advantages over ethanol as a biofuel due to its lower vapor pressure and hydrophilicity, can be produced by several bacterial groups from a variety of feedstocks (15–18). 1,3-PDO is used in industry for the synthesis of polymers. This diol is currently synthesized from petroleum-derived chemicals such as ethylene oxide or acrolein, but it can also be obtained by the fermentation of glycerol or glucose using microorganisms (19–23).

One of the main problems limiting the application of microorganisms in the production of biofuels and other compounds is their toxicity, which prevents the accumulation of high concentrations of these products (24–26). Apart from the toxicity of the final product, microbial metabolism may also be affected by the presence of inhibitors from complex feedstocks and toxic by-products produced as a part of the fermentation process (27, 28). The presence and accumulation of these toxic compounds can adversely affect growth rates and reduce cell viability, limiting production capacity (29–31). For this reason, the development of strains that tolerate high solvent concentrations is critical to attain high productivity and thus economically-feasible industrial production of
biochemicals. Many approaches can be used to improve tolerance in producing strains, among them, the expression of genes that reduce the stress produced by high concentrations of this kind of chemicals (31–33).

Solvents, such as butanol, isobutanol, and ethanol have been shown to induce a stress response in *Escherichia coli* analogous to the heat shock response, as the expression of genes that encode heat shock proteins (HSPs) and chaperones are upregulated in the presence of these compounds (33–38). Additionally, studies performed in *Clostridium acetobutylicum* and solvent-tolerant *Clostridium beijerinckii* have shown a correlation between the abundance of the chaperone GroEL and an increase in butanol tolerance and yield (39, 40). Furthermore, previous studies have demonstrated that overproduction of chaperones can alleviate the inhibitory effects of various biofuels and other biochemicals on several bacterial strains (29, 33), most likely by stabilizing or refolding proteins that are crucial for cell metabolism and survival, and which are sensitive to solvent stress (33). In this sense, overexpression of the genes encoding the GroESL chaperone system with its natural promoter in *E. coli* increased tolerance to several toxic alcohols such as ethanol, n-butanol, isobutanol, and 1,2,4-butanetriol (41). Building on these observations, several combinations of the HSPs GroE, GroESL, and ClpB were used to engineer an *E. coli* strain capable of tolerating high levels of these toxic solvents (29). Overexpression of chaperones from other organisms, such as *groELS* from *C. acetobutylicum*, also resulted in increased tolerance of *E. coli* to several stressors, such as butanol, isobutanol, and ethanol (42). Thus, co-expression of chaperone systems together with homologous or heterologous pathways leading to the production of biofuels and chemicals seems to be a promising strategy to improve strain tolerance towards these added-value bioproducts (30). This approach has been tested some years ago in the well-known butanol producer *C. acetobutylicum*, in which overproduction of GroELS resulted in enhanced tolerance to butanol and in a 40% increase in butanol titers (43).

Phasin PhaP from *Azotobacter* sp. strain FA8 is a polyhydroxyalkanoate granule-associated protein that has chaperone activity and exerts a stress-alleviating effect in recombinant *E. coli* cells (44, 45). Moreover, PhaP has a general protective effect in *E. coli* under both normal and stress conditions, evidenced by i) a reduction in the expression of heat shock-related genes, ii) lower levels of RpoH, the main heat shock regulator, iii) increased growth, and iv) higher resistance to both heat shock and superoxide stress exerted by paraquat (44). Overexpression of PhaP in poly(3-hydroxybutyrate)–producing *E. coli* strains was observed to enhance growth and polymer accumulation, and resulted in a dramatic reduction in the expression of stress-related genes such as *ibpA*.
(a small HSP) and dnaK (a chaperone) compared to a strain that does not synthesize the phasin. This protein also has a protective effect against stress in cells that do not accumulate poly(3-hydroxybutyrate) (44), and was observed to exert a beneficial effect in E. coli cells producing heterologous proteins, playing an active role in protein folding and/or unfolding prevention, that reduced the number and size of inclusion bodies (45).

The increased resistance to toxic chemicals reported in strains that overexpress different chaperone systems, together with the stress-alleviating effect observed in strains that overproduce PhaP, suggested that this protein could increase solvent tolerance in E. coli. To elucidate this possibility, ethanol, butanol, and 1,3-PDO tolerance was assessed in E. coli strains expressing phaP. Additionally, the effect of PhaP and of the known chaperone GroEL on growth, tolerance, and product titers was studied in ethanol- and 1,3-PDO-producing E. coli. These studies pave the road for new strategies to obtain improved strains for the production of biofuels and added-value chemicals, and expand the landscape of possible applications for the multifaceted phasins.

Results

Overexpression of phaP in E. coli results in high tolerance to ethanol, butanol, and 1,3-PDO. Since chaperone expression has been reported to enhance solvent tolerance, and considering that PhaP has chaperone-like properties, we analyzed the effect of this phasin on solvent tolerance in E. coli strains. For this purpose, strain ADA100 (Table 1) was transformed with plasmid pADP (expressing phaP from Azotobacter sp. strain FA8 under control of the lac promoter), or with plasmid pBBR1MCS-1 (control strain). Both strains were challenged with the stressors ethanol, butanol, or 1,3-PDO in sub-inhibitory concentrations, that were added to the culture medium after the first hour of incubation as indicated in Materials and Methods. Growth in the presence of these solvents was determined by monitoring the optical density measured at 600 nm (OD600) during 24 h. When grown without solvents, no significant differences in the maximum specific growth rate (\( \mu_{\text{max}} \)) (1.04 ± 0.06 h\(^{-1}\) vs 0.97 ± 0.01 h\(^{-1}\)) and OD600 at 24 h were observed between the phaP expressing strain and the control strain (Fig. 1A).

When grown in the presence of 5% (vol/vol) ethanol, the PhaP producing strain showed increased growth compared to the control strain (Fig. 1B), as it displayed a 1.3-times higher \( \mu_{\text{max}} \) than the control strain and a 30% increase in the biomass attained after 24 h (Table 2). The percentage of tolerance (Eq. 1) relative to the...
unchallenged culture was estimated at different sampling times to further quantify the solvent resistance of the strains under study. The percentage of ethanol tolerance in the PhaP producing strain was higher (1.4 times) compared to the control, both after 6 h and 24 h of exposure. Also, when the percentage of growth inhibition (Eq. 2) was calculated for both strains, the strain expressing phaP was less inhibited in the presence of the alcohol than the control strain, indicating that PhaP exerts a protective effect against ethanol (Table 2).

Butanol tolerance of both strains was also determined. Cultures were grown in the presence of 0.5% (vol/vol) butanol and growth was monitored by measuring OD$_{600}$ during 24 h. A significant difference ($P$-value = 0.0072) between $\mu_{\text{max}}$ of both strains was observed, as the PhaP-producing strain displayed faster growth in the presence of this solvent, which resulted in a higher fitness compared to the control strain (Fig. 1C). Also, the phaP expressing strain presented a higher tolerance to the solvent at late exponential phase (6-h cultures) and a 40% increase in biomass concentration. However, no differences in tolerance were observed at 24 h, indicating that the protective effect of PhaP against this solvent occurs during the first stages of growth and until late exponential phase (Table 2).

Since PhaP increased tolerance to ethanol and butanol in a similar manner as previously observed with the known chaperone GroEL; the effect of PhaP in the tolerance of *E. coli* to another added-value chemical, 1,3-PDO, was assayed (Fig. 1D). Addition of 1,3-PDO at 8% (vol/vol) was observed to reduce growth in the control strain, while a lower percentage of inhibition was observed for the PhaP-producing strain, that exhibited a higher $\mu_{\text{max}}$ and a 40% increase in biomass after 6 h when compared to the control strain in the presence of the chemical (Table 2). During stationary phase, and after reaching a certain cellular density, cells of both strains growing in the presence of this chemical started to form aggregates and to sediment, thereby interfering with absorbance measurements.

For all the solvents tested, the ratio between the $\mu_{\text{max}}$ of the strain that overexpresses phaP relative to the control strain under each stress condition was assessed through the relative fitness coefficient $s$ (Eq. 3). Positive values of $s$ reflect a higher fitness in the presence of the solvent of the strain that expresses phaP in comparison to the control strain. Under all the conditions tested, this parameter showed positive values, indicating that expression of phaP results in an increment in fitness, allowing *E. coli* cells to increase their tolerance when growing in the presence of external stressors such as ethanol, butanol, or 1,3-PDO (Table 2). Taken together, the results...
presented above suggest that the heterologous expression of PhaP in *E. coli* improves tolerance to all chemicals tested.

A *groELS* mutant of *E. coli* that overexpresses *phaP* shows increased tolerance to solvents. To further characterize the effect of PhaP on solvent tolerance in *E. coli*, strain T850 (46), which contains a chromosomal *groEL* locus with a mutation resulting in a R268C change (47), was used to evaluate if *phaP* expression could complement the phenotype of this mutant. For this purpose, growth and solvent tolerance were assessed in the mutant strain transformed with plasmid pGroELS1 (expressing *groELS*), plasmid pADP (expressing *phaP*), or the empty vector pBBR1MCS-1. As expected, when grown in the absence of solvents, overexpression of *groELS* in the mutant resulted in an increment in the final cell density as compared to the control strain. The strain that overexpressed *phaP* also showed a 1.1-times increase in the $\mu_{\text{max}}$ compared to the control strain ($0.82 \pm 0.01$ h$^{-1}$ and $0.72 \pm 0.02$ h$^{-1}$, respectively). Surprisingly, the *phaP* expressing strain also displayed faster growth than the strain overexpressing *groELS* ($0.82 \pm 0.01$ h$^{-1}$ and $0.76 \pm 0.01$ h$^{-1}$, respectively). These results indicate that PhaP can functionally complement the growth defects brought about by the mutation in *groELS* in the absence of any stressor (Fig. 2A).

The next step was to test if GroELS and PhaP could also increase solvent tolerance in the *groEL* mutant strain and, for this purpose, the different strains were challenged with the solvents and chemicals tested in the previous section. In the presence of 5% (vol/vol) ethanol, both PhaP and GroELS were able to increase tolerance, resulting in higher OD$_{600}$ values at 24 h in the presence of this alcohol (Fig. 2B). Furthermore, the increase in ethanol tolerance observed in the presence of PhaP was slightly higher than in the *groELS* expressing strain at 6 h (1.3- vs 1.1-times when compared to the control strain), but at 24 h no significant differences were observed in tolerance to this alcohol between both strains (Table 3).

On the other hand, when 0.5% (vol/vol) butanol was added to the cultures, no significant differences were observed in the $\mu_{\text{max}}$ of the strains (Table 3), but cells expressing *phaP* or *groELS* showed a 70% and a 50% increase in biomass at 24 h, respectively, indicating that these strains presented a higher tolerance to this chemical as compared to the control strain (Fig. 2C). Lastly, when 1,3-PDO was added to the cultures, both the *phaP* and *groELS* expressing strains showed higher tolerance to the chemical than the control strain after 24 h, even when no
significant differences in $\mu_{\text{max}}$ were observed in these experiments (Table 3). The positive effect on 1,3-PDO tolerance was slightly more pronounced in the GroELS producing strain (Fig. 2D).
PhaP enhances ethanol accumulation in *E. coli*. Chaperones have been observed to increase tolerance to ethanol, presumably by reducing the stress caused by the solvent, so these proteins could help increase the fitness of solvent-producing *E. coli* strains. Additionally, since overproduction of GroELS has been reported to increase butanol yield in *C. acetobutylicum* (43), it was hypothesized that chaperone co-expression could also result in an enhanced ethanol yield in ethanologenic *E. coli*. As PhaP has been shown to reduce the levels of expression of stress related genes (44), and it was observed to increase exogenous ethanol tolerance in *E. coli* as indicated in the previous section, the effects of the expression of this phasin were analyzed in an ethanol-producing *E. coli* strain.

Strain ADA100 was transformed with plasmid pET<sub>Lm</sub> (10) that allows for the overexpression of *adhE* from *Leuconostoc mesenteroides*, encoding a bifunctional alcohol-acetaldehyde dehydrogenase (AdhE<sub>Lm</sub>) that has been shown to enhance the synthesis of ethanol in *E. coli* (10, 48). This ethanol-producing strain was then transformed with plasmid pADP, expressing phaP, or the empty vector pBBR1MCS-1. These strains were grown in microaerobic conditions LB medium supplemented with 10 g/L glucose. Expression of phaP resulted in an increase in growth, reflected in a 1.1-times higher $\mu_{\text{max}}$ compared to the control strain (0.94 ± 0.02 h<sup>-1</sup> and 0.84 ± 0.02 h<sup>-1</sup>, respectively) and a 50% increase in the final OD<sub>600</sub> in 24-h cultures. Furthermore, a 70% increase in final ethanol concentration was obtained in the PhaP-producing strain compared to the control strain (Fig. 3). These results indicate that PhaP helps to alleviate ethanol stress, exerting a positive effect on cell growth and thus enhancing ethanol yield in ethanologenic *E. coli*.

Role of PhaP in 1,3-PDO production in *E. coli*. Taking into account that PhaP was shown to increase tolerance to exogenous 1,3-PDO and that it enhances ethanol tolerance and titer, the effect of this protein was also investigated in 1,3-PDO–producing *E. coli* strains. 1,3-PDO can be synthesized from glycerol in a pathway that involves two steps: the first one is the dehydration of glycerol to 3-hydroxypropionaldehyde catalyzed by a glycerol dehydratase, such as the one encoded by *dhaB* in *Klebsiella pneumoniae*, a coenzyme B<sub>12</sub>-dependent enzyme. The second step is a NADH-dependent reduction of the aldehyde to 1,3-PDO catalyzed by a 1,3-PDO oxidoreductase, such as the one encoded by *dhaT* in *K. pneumoniae* (19). An *E. coli* strain capable of synthesizing 1,3-PDO was obtained by transforming strain ADA100 with plasmid pS221-PDO, that contains *dhaR*, *dhaG*, *dhaT*, *dhaB*, *dhaC*, *dhaE*, and *dhaF* from *K. pneumoniae* GLC29 under control of their native regulatory elements. Cultures of the 1,3-PDO–producing strain carrying additional plasmids pADP (expressing phaP) or the empty vector pBBR1MCS-1 were performed in LB medium with 10 g/L glycerol as a carbon source. The strain with PhaP grew more and produced...
more 1,3-PDO than the control strain (final OD_{600} 21.1 vs 7.87, and 1.24 vs 0.34 g/L 1,3-PDO, respectively). When
the amount of remaining substrate was determined after 24 h in the culture supernatants, it was observed that the
strain with phasin consumed up all the glycerol in the medium, while approximately half the initial amount of glycerol
(6.04 g/L) remained in the control strain cultures.

When the experiment was repeated using 40 g/L glycerol in the culture medium in an attempt to further boost 1,3-
PDO synthesis, the strain with phasin grew up approximately to the same OD_{600} reached in the cultures containing
10 g/L glycerol (Fig. 4), but synthesized much more 1,3-PDO than the control. In cultures supplemented with 40 g/L
glycerol, the strain bearing PhaP grew 2.2-times more than the control strain with a significantly higher μ_{max} (0.96 ±
0.01 h^{-1} vs 0.91 ± 0.01 h^{-1}, P-value = 0.0004), and produced 12.8-times more 1,3-PDO after 24 h. After 8 h, growth
of the control strain reached a plateau, while the strain with PhaP continued growing for several hours. The
synthesis of the diol increased sharply in this strain during this last phase of growth, and continued to increase even
after cell growth ceased, reaching a 1,3-PDO concentration more than 10-times higher than that produced by the
control strain after 24 h.

**PhaP and GroEL have similar effects on ethanol and 1,3-PDO accumulation.** Since PhaP was observed to
increase both growth and product accumulation in the _E. coli_ recombinants tested, and GroEL was reported to
increase butanol yields in butanologenic _Clostridium_ strains, the effect of this well-known chaperone was tested in
the ethanol– and 1,3-PDO–producing _E. coli_ strains. Cultures of the ethanologenic recombinants carrying additional
plasmids pADP (expressing _phaP_), pGroELS1 (expressing _groELS_), or the empty vector pBBR1MCS-1 were
performed in LB medium with 10 g/L glucose in microaerobiosis. Both PhaP and GroELS elicited similar phenotypic
effects, increasing growth, glucose consumption, and ethanol concentration as compared to the control strain
(Table 4). Derivatives of the 1,3-PDO–producing strain carrying the three plasmids were grown in LB medium with
40 g/L glycerol in aerobic conditions. Growth was approximately doubled in strains expressing PhaP or GroELS
compared to the control, with an even higher increase in glycerol consumption and 1,3-PDO accumulation, clearly
showing that both proteins have a very dramatic effect on the synthesis of this bioproduct (Table 4).
This study had two main objectives: i) to analyze if PhaP from Azotobacter sp. strain FA8, that was previously demonstrated to reduce the impact of different kinds of stress in recombinant E. coli, could also enhance tolerance to solvents and chemicals, and ii) to evaluate if PhaP and the known chaperone GroEL could be used to improve the synthesis of these bioproducts by engineered E. coli strains.

Although PhaP is a polyhydroxyalkanoate granule-associated protein, previous work performed in our laboratory showed that it has a general protective effect in E. coli that was not only associated to polymer metabolism, as expression of phaP was observed to protect cells that do not synthesize any polymer against heat and oxidative stress agents (44). Further work showed that PhaP has both in vivo and in vitro chaperone-like activity (45). Since expression of known chaperones and other HSPs was observed to increase tolerance of different bacteria to solvents and other chemical agents (29, 41–43), the effect of PhaP production in tolerance to different chemicals was tested. The results obtained in this work demonstrate that heterologous expression of phaP in E. coli increases tolerance to biofuels such as ethanol and butanol, and to bulk chemicals, such as 1,3-PDO, protecting cells against solvent stress, further supporting the general protective role observed for PhaP in E. coli. The effects of phaP expression in chemical tolerance were shown to be solvent dependent, as different growth patterns and diverse responses were observed in the cells when exposed to different stressors. These variations are similar to those reported when cells overexpressing well known chaperones and heat shock proteins were challenged with several solvents and chemicals (29, 41).

The stress produced in bacteria by the type of compounds explored in our study has been analyzed by other research groups. Stress produced by solvents such as ethanol and butanol has been extensively studied, and shown to affect different components in the cells, including proteins, nucleic acids, and cell membranes (33). Fewer studies have analyzed stress produced by 1,3-PDO. One of such studies, performed on the natural producer C. butyricum growing on glycerol, proposed that the inhibitory effect of this compound was similar to that produced by other diols, including membrane destabilization (49). Proteomic studies performed in E. coli strains accumulating ethanol and butanol have reported an increase in the content of HSPs (35, 50), while the accumulation of 1,3-PDO in C. butyricum was also observed to trigger the synthesis of HSPs in a similar fashion as that observed in solvent.
producing bacteria (51). Analysis of the genetic basis for tolerance to biofuels and other chemicals in several bacteria has revealed that it is a complex phenomenon, in which many different aspects and genes are involved, obviously including HSPs (52, 53). The mechanism through which HSPs have been proposed to protect cells from stress caused by solvents and other chemicals, thus increasing tolerance, is by stabilizing or refolding proteins important for cell metabolism and survival that are affected by these compounds (33). Since PhaP was demonstrated to have in vitro and in vivo chaperone-like activity (45), it is possible that the increase in tolerance to the chemicals tested is related to its capability to aid in protein stabilization and refolding.

In an effort to characterize the effect produced by PhaP expression on chemical tolerance, this phenomenon was analyzed in strain T850, that contains a mutation in groEL (46). The phenotype of this mutant can be complemented by HSPs such as IbpA and IbpB (47). As expected, complementation of this strain with a plasmid expressing groELS increased growth in the absence of stressors, and also resulted in increased tolerance of this strain to solvents and 1,3-PDO. The same result was obtained when a plasmid expressing phaP was introduced in the cells, indicating that PhaP was able to complement the phenotypic effects caused by the groEL mutation in a similar manner as the GroELS protein. Although PhaP has no sequence or structure similarity to GroELS or to other chaperones (54), its capability to increase the tolerance of the groEL mutant to the chemicals tested further supports the possibility that this could be due to its chaperone-like properties.

One of the main technical problems that limit the yield of solvents and other products in microbial processes is their toxicity, so that different strategies are used to increase the tolerance of producer microorganisms to these compounds (41). For example, ethanologenic microorganisms have been shown to suffer both exogenous stress produced by alcohol accumulation in the culture medium and also endogenous stress caused by the intracellular synthesis of ethanol, which induces different kinds of stress responses in E. coli, among them the heat shock response (55). For this reason, modifications that are able to increase solvent tolerance, such as the chaperones analyzed in previous studies (29) or other proteins with chaperone like properties such as PhaP would be expected to enhance yields of these products. To explore this hypothesis, the effect of the known chaperone GroELS and of PhaP on growth and ethanol production was analyzed in recombinant E.coli. Ethanol-producing strains that overexpressed groELS or phaP were observed to have increased growth, reflected in an increment in optical density and also in a higher end-product final titer compared to the control strain. These results demonstrate that
both proteins are not only able to increase tolerance, but they are also capable of enhancing the yields of ethanol. In *C. butyricum*, GroELS overproduction was shown to result in an increase in growth and butanol accumulation (43). This same phenomenon was observed in ethanologenic *E. coli* strains with GroELS in this work, but also in strains with PhaP, indicating that both proteins were able to help cells coping with solvent-induced damage. Additionally, the increase in ethanol accumulation obtained with both strains (70%) was even higher than the increase observed in *Clostridium*, in which *groELS* overexpression resulted in a 40% increase in butanol titers (43).

The results obtained in the ethanol-producing strain raised the possibility that PhaP and GroELS could also enhance growth and 1,3-PDO synthesis in a recombinant *E. coli* strain that synthesizes this compound from glycerol. The effects of 1,3-PDO have been studied in the natural producer *C. butyricum*, in which it was observed that the presence of 1,3-PDO (both endogenous and exogenous) induced the synthesis of numerous proteins, including HSPs such as Hsp60 (GroEL), and the small HSP Hsp20 (56). Expression of *phaP* was observed to increase growth in a 1,3-PDO–synthesizing recombinant, reflected in an increment in OD600 and also in a higher end-product final titer compared to the control strain. Unlike what was observed in the ethanol-producing recombinants, in the case of the strains accumulating 1,3-PDO the presence of PhaP not only enhanced growth, but also affected substrate conversion. While control cells ceased both growth and 1,3-PDO accumulation after around 8 h of growth, cells with PhaP had a longer growth phase, and continued to accumulate 1,3-PDO even after the cells reached the stationary phase. This effect can be compared to what was observed in butanol-producing *Clostridium*, in which GroELS overproduction was shown to result in an increase in metabolic rates (substrate uptake and product formation) and an extension of the active phase of growth (43). When GroELS was overexpressed in the 1,3-PDO–synthesizing recombinant, a similar effect was observed, clearly showing that chaperones can increase yields of this bioproduct, and further supporting the hypothesis that the effects brought about by PhaP can be associated to its chaperone-like properties.

This work demonstrates that both known chaperones (such as GroELS) and chaperone-like proteins (such as PhaP) enhance tolerance to solvents such as ethanol, butanol, and 1,3-PDO, and increase the synthesis of ethanol and 1,3-PDO by recombinant *E. coli*. The results obtained with the strains expressing PhaP further expand the variety of possible applications for the multifaceted phasin family, that already include recombinant protein purification and other biotechnologically relevant processes (57). These findings open the possibility of developing...
new strategies to optimize strains for the synthesis of a diversity of added-value chemicals, such as biofuels and chemical precursors. It has been reported that in high density cultures, such as those used in industrial fermentations, there is a significant increase in the levels of several chaperones, such as DnaK and GroEL (58). While the results presented in this study constitute a proof of concept, demonstrating that expression of GroEL and PhaP can enhance solvent tolerance and bioproduct yield in small-scale cultivations, further research will be necessary to analyze the beneficial effects of these proteins in bioreactor cultures, in which cells are exposed to multiple stresses. Finally, the use of proteins that increase the fitness of producing strains is expected to enable significant advances in the development of environmentally-friendly processes to obtain biotechnologically relevant chemicals from different renewable substrates.
Materials and methods

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids are listed in Table 1. E. coli strains were grown in LB medium at 37°C. For plasmid maintenance, 50 μg/mL kanamycin, 20 μg/mL chloramphenicol, or 100 μg/mL ampicillin were added when needed.

Construction of a suitable plasmid for the expression of genes from Klebsiella pneumoniae needed for 1,3-PDO synthesis. Plasmid pBBR1MCS-2::dha (59) carries a 9.8-kb DNA fragment spanning dhaR, dhaG, dhaT, dhaB, dhaC, dhaE, and dhaF containing their native regulatory signals. These genes were amplified from the genome of the 1,3-PDO producer Klebsiella pneumoniae strain GLC29 (60), and can be excised from plasmid pBBR1MCS-2::dha by double digestion with HindIII and SpeI. This DNA segment was cloned into vector pSEVA221 restricted with the same enzymes. After cloning, insertion of the dha gene cluster in pSEVA221 vector was checked by colony PCR of selected candidates using the following combination of primer pairs: i) PS1_SEVA_F (5'-AGG GCG GAT TTG TCC-3', Tm = 64.8°C) and dhaR_SEVA-R (5'-GGC GAT GGC CAG CGT CA-3', Tm = 65.2°C), expected amplicon = 949 bp; ii) dhaF_SEVA-F (5'-TGG AGG CCA ACA TGG CCA TCG-3', Tm = 65.9°C) and PS2_SEVA-R (5'-GCG GCA ACC GAG CGT TC-3', Tm = 65.9°C), expected amplicon = 755 bp; and iii) dhaB_check-F (5'-GTG GAG GCC GCC ACC TAC-3', Tm = 65.1°C) and dhaC_check-R (5'-ACG TCG GAC GTG CGC AGA ATG-3', Tm = 65.7°C), expected amplicon = 611 bp.

Construction of a suitable plasmid for the expression of genes encoding the GroELS chaperone from E. coli. Plasmid pT-groE (61) carries a 2.1-kb DNA fragment containing the GroESL-coding region. These genes were amplified by PCR using the primer pair GroESL-F (5'-ATT AAG CTT ACA CAG GAA ACA ATG AAT ATT CGT CCA TTG-3', Tm = 63.5°C) and GroESL-R (5'-AAA GGA TCC TTA CAT CAT GAT GCC TGG CCC GCT-3', Tm = 64.4°C). The resulting amplicon was restricted with HindIII and BamHI and cloned into vector pBBR1MCS-1, under the control of a lac promoter, cut with the same enzymes. After cloning, insertion of the groELS gene cluster in this vector was verified by colony PCR of selected candidates using the same primers pair used for amplification.

Solvent tolerance assay. Pre-cultures were started from a single bacterial colony dispersed in 5 mL of LB medium supplemented with the appropriate antibiotics and incubated at 37°C in a rotatory shaker at 200 rpm for 18 h. Test
cultures of *E. coli* ADA100 and T850 were prepared in 50-mL Falcon tubes containing 20 mL of medium, added with the appropriate antibiotics and inoculated at an initial OD_{600} of 0.1. For T850 cultures, IPTG was added at 1 mM after 30 min of incubation, and the corresponding solvent to be tested was then added after an extra 30 min. For ADA100 cultures, cells were incubated for 1 h and at this point the corresponding solvent was added to the cultures. For both strains, 5 mL of the solvent (ethanol, butanol, or 1,3-PDO), previously diluted in LB medium, was added to the culture in order to obtain a final volume of 25 mL at the final concentration used in each assay. The OD_{600} of these test cultures was then monitored over a period of 24 h with shaking at 200 rpm. Control cultures without the stressor solvent were carried out in an identical manner. All cultivations were repeated in independent biological triplicates.

**Calculation of growth kinetic parameters.** Several growth kinetic parameters were calculated in order to compare performance of the different strains in the presence of solvents. The *percentage of tolerance* (T%) was calculated using Eq. (1) (62). This parameter was calculated using the measured growth parameters (OD_{600}) after 6 and 24 h. In addition, the *percentage of inhibition* (I) and the *relative fitness coefficient* (s) were calculated using Eqs. (2) and (3) respectively (63). These parameters were calculated using the measured maximum specific growth rate (μ_max) of each strain, which was determined during early exponential growth according to Eq. (4).

\[
Tolerance \ (T) \ (%) = \frac{OD_{600} \ with \ solvent - OD_{600} \ without \ solvent}{OD_{600} \ with \ solvent - OD_{600} \ without \ solvent} \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ Eq. \ (1)
\]

\[
Inhibition \ (I) \ (%) = \left[1 - \left( \frac{\mu_{solvent}}{\mu_{absence \ of \ solvent}} \right) \right] \times 100 \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ Eq. \ (2)
\]

\[
Relative \ Fitness \ Coefficient \ (s) \ (%) = \left[\left( \frac{\mu_{PhA \ solvent}}{\mu_{control \ solvent}} \right) - 1 \right] \times 100 \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ Eq. \ (3)
\]

\[
\mu_{max} = \ln\left(\frac{OD_{600}}{OD_{600}}\right) \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ Eq. \ (4)
\]

**Ethanol production.** Ethanol production experiments were performed using strain ADA100 transformed with plasmid pET_Lm and pADP. The same strain, transformed with pET_Lm and vector pBBR1MCS-1, was used as a control. Pre-cultures were started from a single colony dispersed in 5 mL of LB medium supplemented with the appropriate antibiotics and incubated at 37°C in a rotatory shaker at 150 rpm for 18 h (microaerobic conditions).
Cultures were prepared in 15-mL Falcon tubes containing 10 mL of LB medium supplemented with 10 g/L glucose, 20 μg/mL chloramphenicol, and 100 μg/mL ampicillin, and inoculated at an initial OD<sub>600</sub> = 0.1. Cultures were incubated at 37°C and shaken at 150 rpm. The OD<sub>600</sub> and ethanol production of these test cultures was then monitored over a period of 24 h. Ethanol concentration was determined by gas chromatography (GC) as detailed below.

**1,3-PDO production.** 1,3-PDO production experiments were performed using strain ADA100 transformed with plasmid pS221-PDO and pADP. The same strain, transformed with pS221-PDO and the empty vector pBBR1MCS-1, was used as a control. Pre-cultures were started from a single colony dispersed in 5 mL of LB medium supplemented with appropriate antibiotics and incubated at 37°C in a rotatory shaker at 200 rpm for 18 h. Cultures were prepared in 50-mL cylindrical glass flasks containing 5 mL of LB medium supplemented with 40 g/L glycerol, 20 μg/mL chloramphenicol, 50 μg/mL kanamycin, and 0.15 mM vitamin B12, and inoculated at an initial OD<sub>600</sub> = 0.1. Cultures were incubated at 37°C with shaking at 200 rpm. The OD<sub>600</sub> and 1,3-PDO concentration in these test cultures was then monitored over a period of 24 h. 1,3-PDO concentration was determined by GC as detailed below.

**Ethanol and 1,3-PDO determination by GC.** Standards and stock solutions of both ethanol and 1,3-PDO were prepared in LB medium. For GC determinations, 250 μL of the corresponding standard was diluted in 750 μL of either ethanol or acetone for 1,3-PDO and ethanol determinations, respectively. These solutions were vortexed during 1 min and centrifuged at 13,000 × g for 3 min at 4°C to precipitate salts. Biological samples were filtered through 0.22-μm nylon membranes (MSI, USA), diluted in the corresponding solvent, and centrifuged as described above.

Compounds were measured using an Agilent 7820 chromatographic system equipped with a flame ionization detector and automatic liquid sampler ALS 7693. The separation was conducted on a HP-INNOWAX capillary column (30 m, 0.25 μm film thickness, and 0.25 mm internal diameter). For 1,3-PDO determinations, a method that allows for the simultaneous determination of this compound and glycerol was used (64). Briefly, the GC oven was initially heated at 185°C for 3 min, then to 220°C with a heat ramp of 40°C/min, and held for 1 min. The injector and flame ionization detector temperatures were set at 290°C and 300°C, respectively. Nitrogen was used as carrier.
gas at a column flow of 2.5 mL/min. The injection volume was 2 μL with a 30:1 split ratio. For ethanol
determinations, the GC oven temperature was set at 35°C for 8 min. Injector and detector temperatures were
150°C and 300°C, respectively. Nitrogen flow was 1 mL/min and the injection volume was 1 μL with 90:1 split ratio.

**Statistical analysis.** The reported experiments were independently repeated at least three times and the mean
value of the corresponding parameter ± standard deviation is presented. When comparing two strains, differences
in results were evaluated via a two-tailed Student’s t-test defining a P-value < 0.05 as significant. For solvent
tolerance assays of strain T850, differences between the strains were evaluated via a one way ANOVA, with post-
hoc Tukey honestly significant difference test, defining a P-value < 0.05 as significant.

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The funders had no role in study design, data collection and interpretation, or the decision to submit the work for
publication.


41. Zingaro KA, Papoutsakis ET. 2013. GroESL overexpression imparts Escherichia coli tolerance to i-, n-, and 2-butanol, 1,2,4-butanol and ethanol with complex and unpredictable patterns. Metab Eng 15:196–205.


**Figure legends**

**Figure 1: Solvent tolerance assays.** Growth curves of *E. coli* ADA100/pADP (PhaP) and ADA100/pBBR1MCS-1 (control) without solvent (A), with the addition of 5% (vol/vol) ethanol (B), 0.5% (vol/vol) butanol (C), or 8% (vol/vol) 1,3-propanediol (1,3-PDO) (D). Error bars indicate ± standard deviation between triplicates. OD$_{600}$, optical density measured at 600 nm.

**Figure 2: Solvent tolerance assays in a chaperone defective mutant of *E. coli*.** Growth curves of *E. coli* T850 (groEL) transformed with plasmid pADP (PhaP), pBBR1MCS-1 (control), or pGroELS1 (GroELS). Cells were incubated without solvent (A), with the addition of 5% (vol/vol) ethanol (B), 0.5% (vol/vol) butanol (C), or 8% (vol/vol) 1,3-propanediol (D). Error bars indicate ± standard deviation between triplicates.

**Figure 3: Effect of PhaP in an *E. coli* ethanol producer.** Growth (continuous lines) and ethanol synthesis (dashed lines) were evaluated in *E. coli* ADA100 carrying plasmids pET$_{Lm}$ (Adh$_{E_{Lm}}$) and pADP (PhaP), or pET$_{Lm}$ (Adh$_{E_{Lm}}$) and pBBR1MCS-1 (control). Error bars indicate ± standard deviation between triplicates.

**Figure 4: Effect of PhaP in an *E. coli* 1,3-propanediol producer.** Growth curve and 1,3-propanediol (1,3-PDO) synthesis *E. coli* ADA100 carrying plasmids pS221-PDO (DhaRGTBCDEF$_{Kp}$) and pADP (PhaP), or pS221-PDO and pBBR1MCS-1 (control). Error bars indicate ± standard deviation between triplicates.
Table 1: *E. coli* strains and plasmids used in this study.

<table>
<thead>
<tr>
<th><em>E. coli</em> strains or plasmids</th>
<th>Relevant characteristics&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADA100</td>
<td>araD139 Δ(argF-lac)U19 rpsL150 relA1 fliB5301 deoC1 ptsF25 rbsR λφ(lbp::lacZ)</td>
<td>(65)</td>
</tr>
<tr>
<td>T850</td>
<td>F- fhuA2::IS2? proA44 lacY1 gltX44(AS)? gal- λ hisG1 malT1(ΔR) xyl7 mtlA2 ΔargH1 rpl9(L?) thiE1 groE1</td>
<td>(46)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBBR1MCS-1</td>
<td>Broad host range vector; lacPOZ&lt;sup&gt;′&lt;/sup&gt; mob(RP4); Cm&lt;sup&gt;i&lt;/sup&gt;</td>
<td>(66)</td>
</tr>
<tr>
<td>pBBR1MCS-2</td>
<td>Broad host range vector; lacPOZ&lt;sup&gt;′&lt;/sup&gt; mob(RP4); Km&lt;sup&gt;i&lt;/sup&gt;</td>
<td>(66)</td>
</tr>
<tr>
<td>pBluescript II KS(-)</td>
<td>Cloning vector; T3 and T7 promoters, lacPOZ&lt;sup&gt;′&lt;/sup&gt;; Amp&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Fermentas Inc., Glen Burnie, MD, USA</td>
</tr>
<tr>
<td>pSEVA221</td>
<td>Broad host range vector; oriRK2, oriT; Km&lt;sup&gt;i&lt;/sup&gt;</td>
<td>(67)</td>
</tr>
<tr>
<td>pADP</td>
<td>Derivative of vector pBBR1MCS-1 carrying phaP from <em>Azotobacter</em> sp. strain FA8; Cm&lt;sup&gt;i&lt;/sup&gt;</td>
<td>(68)</td>
</tr>
<tr>
<td>pET&lt;sub&gt;LM&lt;/sub&gt;</td>
<td>Derivative of vector pBluescript II KS(-) carrying adhE from <em>Leuconostoc mesenteroides</em>; Amp&lt;sup&gt;i&lt;/sup&gt;</td>
<td>(10)</td>
</tr>
<tr>
<td>pBBR1MCS-2::dha</td>
<td>Derivative of vector pBBR1MCS-2 carrying dhaR, dhaG, dhaT, dhaB, dhaC, dhaE, and dhaF from <em>Klebsiella pneumoniae</em> GLC29</td>
<td>(59, 60)</td>
</tr>
<tr>
<td>pS221-PDO</td>
<td>Derivative of vector pSEVA221 carrying dhaR, dhaG, dhaT, dhaB, dhaC, dhaE, and dhaF from <em>K. pneumoniae</em> GLC29; Km&lt;sup&gt;i&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pT-groE</td>
<td>Derivative of vector pACYC-Duet1 carrying groELS from <em>E. coli</em>; Km&lt;sup&gt;i&lt;/sup&gt;</td>
<td>(61)</td>
</tr>
<tr>
<td>pGroELS1</td>
<td>Derivative of vector pBBR1MCS-1 carrying groELS from <em>E. coli</em>; Cm&lt;sup&gt;i&lt;/sup&gt;</td>
<td>This work</td>
</tr>
</tbody>
</table>

<sup>a</sup> The antibiotic resistance markers are identified as follows: Amp, ampicillin; Cm, chloramphenicol; and Km, kanamycin.
Table 2: Growth kinetic parameters of solvent tolerance assays.

<table>
<thead>
<tr>
<th>Stress agent and concn.</th>
<th>Additional plasmids (protein)</th>
<th>$\mu_{\text{max}}$ (h$^{-1}$)</th>
<th>Inhibition (%$\text{I}$)</th>
<th>Tolerance ($T%$)</th>
<th>Relative fitness coefficient (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol 5% (vol/vol)</td>
<td>pADP (PhaP)</td>
<td>0.67 ± 0.04*</td>
<td>36 ± 7*</td>
<td>30 ± 2*</td>
<td>22 ± 1*</td>
</tr>
<tr>
<td></td>
<td>pBBR1MCS-1 (none)</td>
<td>0.51 ± 0.01</td>
<td>48 ± 2</td>
<td>22 ± 2</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>Butanol 0.5% (vol/vol)</td>
<td>pADP (PhaP)</td>
<td>0.54 ± 0.03*</td>
<td>48 ± 4</td>
<td>65 ± 7*</td>
<td>61 ± 5</td>
</tr>
<tr>
<td></td>
<td>pBBR1MCS-1 (none)</td>
<td>0.44 ± 0.02</td>
<td>54 ± 2</td>
<td>48 ± 1</td>
<td>62 ± 7</td>
</tr>
<tr>
<td>1,3-PDO 8% (vol/vol)</td>
<td>pADP (PhaP)</td>
<td>0.85 ± 0.02*</td>
<td>19 ± 3*</td>
<td>46 ± 2*</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>pBBR1MCS-1 (none)</td>
<td>0.68 ± 0.05</td>
<td>30 ± 5</td>
<td>38 ± 2</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

All strains are derivatives of *E. coli* ADA100. All cultivations were carried out in independent biological triplicates and the mean value of the corresponding parameter ± standard deviation is presented. Differences in results when comparing strains producing PhaP and control strains were evaluated via a two-tailed Student's t-test defining a $P$-value < 0.05 as significant. The asterisk (*) indicates significant differences between strains. 1,3-PDO, 1,3-propanediol; N.D., not detected.
Table 3: Solvent tolerance assays in a groEL mutant.

<table>
<thead>
<tr>
<th>Stress agent and concn.</th>
<th>Complementing plasmid (protein)</th>
<th>μ_{max} (h^{-1})</th>
<th>Tolerance (T%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>6 h</td>
<td>24 h</td>
</tr>
<tr>
<td>Ethanol 5% (vol/vol)</td>
<td>pADP (PhaP)</td>
<td>0.18 ± 0.01*</td>
<td>15 ± 1*</td>
</tr>
<tr>
<td></td>
<td>pBBR1MCS-1 (none)</td>
<td>0.12 ± 0.01</td>
<td>11 ± 1</td>
</tr>
<tr>
<td></td>
<td>pGroELS1 (GroELS)</td>
<td>0.12 ± 0.01</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>Butanol 0.5% (vol/vol)</td>
<td>pADP (PhaP)</td>
<td>0.43 ± 0.01</td>
<td>65 ± 5</td>
</tr>
<tr>
<td></td>
<td>pBBR1MCS-1 (none)</td>
<td>0.41 ± 0.01</td>
<td>60 ± 4</td>
</tr>
<tr>
<td></td>
<td>pGroELS1 (GroELS)</td>
<td>0.41 ± 0.02</td>
<td>60 ± 4</td>
</tr>
<tr>
<td>1,3-PDO 8% (vol/vol)</td>
<td>pADP (PhaP)</td>
<td>0.33 ± 0.01</td>
<td>35 ± 1</td>
</tr>
<tr>
<td></td>
<td>pBBR1MCS-1 (none)</td>
<td>0.33 ± 0.01</td>
<td>35 ± 2</td>
</tr>
<tr>
<td></td>
<td>pGroELS1 (GroELS)</td>
<td>0.35 ± 0.01</td>
<td>40 ± 2*</td>
</tr>
</tbody>
</table>

All strains are derivatives of *E. coli* T850. All cultivations were carried out in independent biological triplicates and the mean value of the corresponding parameter ± standard deviation is presented. Differences in results when comparing strains producing PhaP, GroELS or control strains were evaluated via one way ANOVA, with a post-hoc Tukey honestly significant difference test defining a *P*-value < 0.05 as significant. The asterisk (*) indicates that values for a given strain differ significantly from those of the other two. 1,3-PDO, 1,3-propanediol.
Table 4: Effects of PhaP and GroEL on ethanol and 1,3-PDO accumulation.

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>Additional plasmids (protein)</th>
<th>OD&lt;sub&gt;600&lt;/sub&gt; at 24 h</th>
<th>Residual glucose (g/L)</th>
<th>Residual glycerol (g/L)</th>
<th>Ethanol (g/L)</th>
<th>1,3-PDO (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADA100/pET&lt;sub&gt;Lm&lt;/sub&gt; (ethanol producer)</td>
<td>pADP (PhaP)</td>
<td>1.94 ± 0.05*</td>
<td>6.0 ± 0.2*</td>
<td>-</td>
<td>0.65 ± 0.02*</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>pBBR1MCS-1 (none)</td>
<td>1.32 ± 0.05*</td>
<td>7.9 ± 0.2*</td>
<td>-</td>
<td>0.41 ± 0.04*</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>pGroELS1 (GroELS)</td>
<td>1.75 ± 0.03*</td>
<td>6.6 ± 0.2*</td>
<td>-</td>
<td>0.58 ± 0.01*</td>
<td>-</td>
</tr>
<tr>
<td>ADA100/pS221-PDO (1,3-PDO producer)</td>
<td>pADP (PhaP)</td>
<td>19 ± 1</td>
<td>-</td>
<td>8 ± 1</td>
<td>-</td>
<td>7.8 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>pBBR1MCS-1 (none)</td>
<td>9 ± 1*</td>
<td>-</td>
<td>32 ± 1*</td>
<td>-</td>
<td>0.4 ± 0.1*</td>
</tr>
<tr>
<td></td>
<td>pGroELS1 (GroELS)</td>
<td>19 ± 1</td>
<td>-</td>
<td>9 ± 1</td>
<td>-</td>
<td>6.7 ± 0.5</td>
</tr>
</tbody>
</table>

Ethanol production experiments were performed in microaerobic conditions in LB medium with 10 g/L glucose as the substrate. 1,3-PDO production experiments were performed in aerobic conditions in LB medium with 40 g/L glycerol as the substrate (see Materials and Methods). All cultivations were carried out in independent biological triplicates and the mean value of the corresponding parameter ± standard deviation is presented. Differences in results were evaluated via a one way ANOVA, with post-hoc Tukey honestly significant difference test, defining a P-value < 0.05 as significant. The asterisk (*) indicates that values for a given strain differ significantly from those of the other two. OD<sub>600</sub>, optical density measured at 600 nm.