1 **Robustness of *Pseudomonas putida* KT2440 as a host for ethanol**
2 **biosynthesis**

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15 **Running Title:** Ethanol stress endurance in engineered *P. putida*

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Abstract

Expansion of the burgeoning biofuels agenda involves not only the design of suitable genetic and metabolic devices but also their deployment into suitable hosts that can endure the stress brought about by the products themselves. The microorganisms easiest to genetically manipulate for these endeavours (e.g., *Escherichia coli*) are often afflicted by an undesirable sensitivity to the very product that they are engineered to synthesize. In this context, we have examined the resistance to the stress arising from ethanol synthesis and/or its addition to cultures of recombinant *Pseudomonas putida*, using as a benchmark the same trait in an *E. coli* strain. To this end, ethanologenic strains of these two species were constructed by functionally expressing *pdc* (pyruvate decarboxylase) and *adhB* (alcohol dehydrogenase) from *Zymomonas mobilis*. Recombinants were compared under anoxic conditions as ethanol producers, and cell survival, stress resistance, and phenotypic stability were quantified in each case. *P. putida* consistently outperformed *E. coli* in every ethanol tolerance test conducted – whether the alcohol was produced endogenously or added exogenously. These results highlight the value of such bacterium as a microbial cell factory for the production of biofuels owing to its naturally pre-evolved ability to withstand different kinds of chemical stresses.

Introduction

Ongoing efforts to manipulate microorganisms for the production of valuable biochemicals mostly revolve around plugging-in and -out genes needed for their synthesis in a few bacterial chassis [1]. An interesting example that profits from Synthetic Biology tools and strategies is biofuel synthesis [2-5]. The production of fuel substitutes such as bioalcohols and biodiesel from renewable resources has gained significant attention over the last decades because of the continuously rising energy and oil prices and environmental concerns [6, 7]. So far, biofuel production has mainly focused on ethanol (EtOH) [8-11], which currently comprises > 90% of the total bioalcohols consumed in the United States [12]. The seminal work of Ingram and collaborators, who originally cloned the genes needed for EtOH biosynthesis from *Zymomonas mobilis* and expressed them in *Escherichia coli* [13-17], paved the way towards the construction

of efficient microbial cell factories for bioalcohol synthesis with a suite of desirable industrial traits. Other attempts within the increasingly explored biofuels production field also include butanol [18-20], and, most recently, gasoline (*i.e.*, triacylglycerides esters) [21]. In the recent years, the combination of model-based predictions and high-throughput technologies has further improved the design of efficient biocatalysts for biofuels production by investigating physiological traits at the systems level – thus extending metabolic engineering strategies far beyond the mere cloning and expression of a limited number of structural genes. Yet, several hurdles have to be considered and solved before bioalcohol production *en masse* becomes technically feasible and economically viable – some of these problems being caused by the very nature of the product at stake.

One of the major challenges in the bulk production of EtOH (mainly from biomass) is the effect of inhibitory compounds generated during the thermo-chemical pre-treatment of the substrate [22]. Inhibitory chemical species of this sort, such as weak acids, furans, and phenolic compounds, are very toxic to the fermenting microorganisms and their presence seriously compromises the final EtOH yield and overall productivity during the fermentation of lignocellulosic hydrolysates [23-25]. Along the line, tolerance to EtOH itself is another desirable trait in an efficient biocatalyst [16, 26, 27]. EtOH causes several changes at the level of the cell membrane [28, 29], ultimately altering its physical structure and giving rise to highly-damaging reactive oxygen species (ROS) [30, 31]. Thus, two sources of alcohol-related stresses can be identified in ethanologenic microorganisms, namely, [i] the endogenous stress caused by the *intracellular synthesis* of EtOH, and [ii] the exogenous stress produced by alcohol *accumulation* in the culture medium. Such insults can ultimately translate in DNA damage (which elicits the SOS response), heat shock, acid stress response, starvation response, and the envelope stress response. We have argued that processes that involve contact or production of chemical stressors may benefit from adopting whole-cell catalysts based on environmental bacteria that thrive in niches with a history of exposure to xenobiotic and recalcitrant compounds [32]. Among them, *Pseudomonas putida* comes into view as an appealing host for EtOH synthesis considering its [i] intrinsically high resistance to different stressors (including several solvents), [ii] amenability to genetic manipulation, [iii] ability to grow very fast on simple substrates, and [iv] GRAS (*i.e.*, generally recognised as safe) credentials of some strains of this species (*e.g.*, KT2440) [33-35].

In this work, we have examined the value of metabolically-engineered derivatives of *P. putida* strain KT2440 as active agents for bioalcohol production, that can be potentially used in bioprocesses conducted in the presence of high EtOH concentrations. To this end, the genes needed to activate an EtOH synthesis pathway in *P. putida* were recruited from *Z. mobilis*, and the performance of the resulting strain as a microbial cell factory for biofuel synthesis was thoroughly tested. Quantification of ROS production, overall fitness, extracellular metabolome patterns, cell growth and survival in the presence of ethanol, as well as the phenotypic stability among the ethanologenic strains, accredited the worth of *P. putida* as a promising host for engineering bioalcohol production.

Material and Methods

Bacterial strains, culture conditions, and general techniques

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* and *P. putida* strains were routinely grown at 37°C and 30°C, respectively, in complex LB medium under oxic conditions (*i.e.*, in 125-ml Erlenmeyer flasks containing medium up to one-fifth of their nominal volume with agitation at 170 rpm). *E. coli* DH5 α and CC118 λ *pir* were used for routine cloning procedures and plasmid maintenance and mating into *P. putida*. Recombinant *E. coli* and *P. putida* strains were grown in M9E medium, a modified culture medium based on M9. M9E medium contained 6 g l⁻¹ Na₂HPO₄, 3 g l⁻¹ KH₂PO₄, 1.4 g l⁻¹ (NH₄)₂SO₄, 0.5 g l⁻¹ NaCl, 0.2 g l⁻¹ MgSO₄·7H₂O, 5 mg l⁻¹ thiamine·HCl, 5 g l⁻¹ yeast extract, and 2.5 ml l⁻¹ of a trace elements solution [36, 37]. Glucose was added as the sole carbon source to all experiments at 20 g l⁻¹. Anoxic incubations were carried out in glass vials with teflon-stoppered metallic screw caps (Sigma-Aldrich Co., St. Louis, MO, USA) essentially as previously described by Nikel and de Lorenzo [37]. Briefly, the biomass from cultures previously grown under oxic conditions to mid-exponential phase was concentrated by centrifugation (15 min at 4,000 × *g* and 4°C), washed once with cold saline (9 g l⁻¹ NaCl), and finally re-suspended in the adequate volume of M9E medium. Fresh culture medium was inoculated at an optical density measured at 600 nm (OD₆₀₀) of *ca.* 2. Growth was estimated by measuring the OD₆₀₀ of appropriately diluted broth samples using an Ultrospec 3000 *pro* UV/Visible Spectrophotometer (GE Healthcare Bio-Sciences Corp.,

Piscataway, NJ, USA). All solid media contained 15 g l⁻¹ agar and kanamycin (Km, 50 µg ml⁻¹), or chloramphenicol (30 µg ml⁻¹), were added to the culture media, when appropriate, as filter-sterilised solutions. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added at 2.5 mM to induce the expression of genes under the control of LacI^Q/P_{trc}. Plasmids were transferred into *P. putida* KT2440 and its derivatives using the filter mating procedure described in detail by de Lorenzo and Timmis [38]. EtOH concentration in culture supernatants, as well as the presence of other fermentation metabolites (acetate, lactate, formate, and succinate), was determined in selected samples using enzymatic kits (R-Biopharm AG, Darmstadt, Germany) as per the manufacturer's instructions.

Alcohol tolerance assays and survival and plasmid stability

Colony forming units (CFUs) were counted after 6 h of solvent exposure at the concentrations indicated in the text to assess cellular fitness under EtOH stress. Cultures were serially diluted in 10-fold steps in LB medium containing no antibiotics. The dilution level was estimated based on OD₆₀₀ measurements of the samples, and 50 µl of the final dilution was then plated on LB agar with and without Km. After plating, CFUs were measured after 18 to 24 h of growth at either 37°C or 30°C. This assay served as a measure of potential industrial use because it is a direct evaluation of recombinant cells that are still biologically active during solvent exposure [27]. At the same time, plasmid segregational stability was estimated by comparing CFUs in plates with and without Km from samples withdrawn from the anoxic incubation experiments.

Quantification of ROS in bulk cultures

Strains were incubated in M9E medium containing 0%, 0.5%, 1%, 1.5%, and 5% (v/v) EtOH for 6 h. Aliquots of 100 µl of pre-cultured strains grown as described in the preceding section were each re-suspended in 5 ml M9E medium, and 140 µl of each diluted sample was transferred to a 96-well microtiter plate (clear-bottom, black polypropylene 96-well plates; Nunc A/S, Roskilde, Denmark) followed by incubation at 30°C or 37°C for 30 min. All samples were then added with 10 µl of 25 mM 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA; Invitrogen Co., Carlsbad, CA, USA), carefully mixed, and statically incubated for 15 min in the

1 dark [39, 40]. OD₆₀₀ values and fluorescence excitation/emission signals at 488/535 nm in each
2 sample were measured in quadruplicate in a SpectraMax Plus³⁸⁴ microplate reader (Molecular
3 Devices, Sunnyvale, CA, USA). *Tert*-butyl hydroperoxide (Sigma-Aldrich Co.), a known stressor
4 that produces intracellular H₂O₂, was added to some experiments as a positive control for the
5 ROS assay. These control experiments were prepared with the strains cultured without EtOH and
6 treated as above except for an initial 30 min incubation in the presence of 10 µl of 5.5 M *tert*-butyl
7 hydroperoxide.

8 9 *Phenotypic stability of recombinant strains*

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11 Mixtures of *p*-rosaniline and NaHSO₃ are often referred to as the Schiff's reagent and have been
12 widely used to detect aldehydes. Conway *et al.* [15] have incorporated these components into a
13 relatively non-toxic solid medium which can be used to easily identify clones expressing enzymes
14 that produce aldehydes. Aldehyde indicator plates were set by promptly adding 8 ml of a freshly-
15 prepared *p*-rosaniline solution [2.5 mg ml⁻¹ in 95% (v/v) ethanol] and 100 mg of NaHSO₃ to 400-
16 ml batches of pre-cooled (ca. 45°C) LB agar, previously amended with 0.5% (v/v) ethanol, 10 g l⁻¹
17 glucose, and 2.5 mM IPTG. Most of the dye is immediately converted to the leuco form by
18 reaction with NaHSO₃ to produce a rose-colored medium that was dispensed into Petri dishes.
19 Cells, anoxically incubated for 24 h, 48 h, and 96 h, were spread onto these plates and incubated
20 at the appropriate temperature for 4-6 h. Magenta-coloured colonies were considered positive
21 (AdhB⁺), and alcohol dehydrogenase activity was further confirmed in selected clones through a
22 direct spectrophotometric assay by measuring the acetaldehyde-dependent oxidation of NADH
23 (see details below). Fresh indicator plates were prepared no more than 2 days before use and
24 stored at room temperature thereafter.

25 26 *Preparation of cell-free extracts and in vitro determination of enzyme activities*

27
28 Cell-free extracts were obtained from cells incubated anoxically and harvested by centrifugation
29 from an appropriate culture volume at 4,000 × *g* at 4°C for 10 min. Pellets were suspended in 1
30 volume of 10 mM sodium phosphate buffer (pH = 7.5, previously refrigerated) containing 0.1 M 2-
31 mercaptoEtOH and centrifuged again as described above. Cells were finally re-suspended in 0.3-

0.5 volume of the same buffer and sonicated intermittently for 5 min in an ice bath. Sonicated cells were centrifuged at $7,500 \times g$ at 4°C for 30 min to remove cell debris and to reduce the NADH/NAD⁺ background in the lysate. Total protein concentration in cell extracts was measured by the Bradford method [41] using a commercially available kit from BioRad Laboratories, Inc. (Hercules, CA, USA), with crystalline bovine serum albumin as the standard. Pdc catalyses the decarboxylation of pyruvate to acetaldehyde. The assay is an indirect method in which the conversion is linked to the activity of alcohol dehydrogenase, that is supplied in excess and effectively converts acetaldehyde into NAD⁺ and ethanol [42, 43]. The assay mixture (1 ml final volume) contained 50 mM 2-(*N*-morpholino)ethanesulfonic acid·KOH (pH = 6.5), 5 mM MgCl₂, 0.1 mM thiamine pyrophosphate, 0.15 mM NADH, 10 units ml⁻¹ alcohol dehydrogenase (from *Saccharomyces cerevisiae*, Sigma-Aldrich Co.), and 5 mM pyruvate. AdhB activity was assayed by measuring the ethanol-dependent reduction of NAD⁺ [15, 44]. The assay mixture (1 ml) contained 50 mM sodium phosphate buffer (pH = 6.5), 350 mM ethanol, and 10 mM NAD⁺. In both Pdc and AdhB assays, the cell-free extract was added to the reaction mixture, and the rate of change of absorption at 340 nm was recorded at 30°C . An extinction coefficient (ϵ_{NADH}) of 6.22 mM⁻¹ cm⁻¹, representing the difference between the extinction coefficients of NADH and NAD⁺, was used in both assays. One unit of activity was defined as the quantity of enzyme that catalysed the formation of 1 μmol product in 1 min at 30°C .

Statistical analysis

Data were statistically treated with an unpaired *t* test, and 95% or 99% confidence intervals for each parameter were calculated to demonstrate a statistically significant difference in means among the experimental samples.

Results and Discussion

*Rationale for the construction of ethanologenic *P. putida* strains*

The starting point of this work was the examination of the native pathways through which *E. coli* naturally ferments sugars to a mixture of EtOH and organic acids under anoxic conditions (Fig. 1).

1 EtOH is produced from pyruvate through the activity of pyruvate-formate lyase (Pfl) [45], which
2 replaces (at least partially) the NAD⁺-dependent pyruvate dehydrogenase complex in the
3 absence of oxygen [46]. This fermentation pathway is, however, unbalanced, because one NADH
4 is generated for each pyruvate molecule arising from sugars, and two NADH molecules are
5 required to convert pyruvate into ethanol. *E. coli* balances this biochemical process by also
6 producing acetate, lactate, and succinate as co-products of the fermentative metabolism [45, 47].
7 Unlike Enterobacteria, *P. putida* exclusively uses the Entner-Doudoroff pathway (Fig. 1) for oxic
8 hexose processing [48, 49]. A side effect of this situation is the generation of one NADPH and
9 one NADH per glucose consumed, instead of the two NADH molecules per glucose consumed
10 through the Embden-Meyerhof-Parnas pathway [50]. On the other hand, yeast and *Z. mobilis* are
11 deemed homoethanogenic fermentative microorganisms because they convert pyruvate to
12 EtOH almost stoichiometrically by means of pyruvate decarboxylase (Pdc), which only consumes
13 one NADH for each EtOH produced (Fig. 2A) [51, 52]. Since the native alcohol dehydrogenase
14 activity of *E. coli* is not sufficient to achieve high EtOH yields, Ingram *et al.* [13, 15] originally
15 isolated *adhB* (encoding alcohol dehydrogenase II) from a genomic library of *Z. mobilis* to
16 complement the Pdc activity in a two-step biochemical conversion of pyruvate into ethanol. As a
17 consequence, *E. coli* transformed with a plasmid expressing *pdc* and *adhB* mostly produced
18 EtOH as the main fermentation metabolite [13]. Building on this background, we asked what
19 could be the physiological impact of entering a homoethanogenic fermentation pathway in a
20 metabolic network that operate different catabolic pathways as *E. coli* and *P. putida* do. To tackle
21 this issue, we constructed a synthetic operon in which *pdc* and *adhB* from *Z. mobilis* (Fig. 2A)
22 were co-expressed as a single transcriptional unit from the IPTG-inducible P_{trc} promoter (Fig. 2B).
23 The vector carrying these genes, termed pS234ET (Table 1), was introduced in parallel into the
24 wild-type hosts *E. coli* BW25113 and *P. putida* KT2440. This operation resulted in EtOH formation
25 (< 2 mM) by *E. coli* recombinants even under oxic growth conditions and in non-induced cultures.
26 Under the same conditions, no detectable EtOH was detected in *P. putida* recombinants.

27
28 When cells of the recombinants were shifted to anoxic conditions in tightly sealed glass vials,
29 both bacteria were able to produce EtOH from glucose as detected in culture supernatants after
30 24 h (Fig. 2C). Under these non-optimised conditions, *E. coli* and *P. putida* performed almost
31 equally as engineered ethanogenic bacteria, as the final EtOH titre reached 29.1 ± 3.5 mM and

23.8 ± 6.3 mM, respectively. Some EtOH was also detected in the absence of IPTG in the strains bearing pS234ET, its concentration attaining 32% and 54% of that observed in induced cultures of *E. coli* and *P. putida*, respectively. These figures point to a higher leakiness of the LacI^Q/P_{trc} expression system in pseudomonads as compared to its behaviour in enterobacteria [53, 54]. *E. coli* BW25113 carrying the empty pSEVA234 vector still produced a small but significant amount of alcohol, likely as result of the action of the native AdhE ($p < 0.05$, as compared with *P. putida* cultures). As previously noted by Ingram *et al.* [13] and Clark [45], EtOH represented < 5% of the total fermentation metabolites produced by *E. coli* when grown on glucose.

Other fermentation by-products (besides ethanol) were quantified in culture supernatants of the strains under study. While the extracellular fermentation metabolome of *P. putida* was entirely restricted to ethanol, ethanogenic *E. coli* strains also produced acetate, lactate, succinate, and formate as co-products of anoxic glucose utilization (Table 2). Among these metabolites, acetate prevailed as the main product of glucose fermentation by *E. coli* BW25113/pS234ET. As expected, over-expression of *pdc-adhBZ. mobilis* determined a decrease in the formation of all the remaining fermentative products, considering both [i] the draining of pyruvate away from the native fermentation pathways to be re-routed into EtOH synthesis, and [ii] the likely dearth of NADH (used in the AdhBZ. *mobilis*-dependent reduction of acetaldehyde), a co-factor used by LdhA (catalyzing the formation of lactate), the reductive branch of the tricarboxylic acid cycle (which generates succinate), and AdhE (the extant alcohol dehydrogenase of *E. coli*, which might contribute to EtOH formation in the recombinants to some extent) [47, 55]. Taken together, these results accredited the performance of a *P. putida*-based process for EtOH synthesis. In addition, the metabolome of either ethanogenic strain revealed an improved conversion of pyruvate to EtOH by *P. putida* (through the Entner-Doudoroff route, Fig. 1) as compared to the spill of by-products typically observed in the *E. coli* counterpart used as a reference.

EtOH synthesis vs. cell survival

The next question was whether the natural tolerance to organic solvents of *P. putida* could be also beneficial for its performance as an ethanol-producing microbial cell factory. Specifically: does EtOH produced by the recombinant strains cause stress to the cells? To answer this

question, we performed a simple survival test of the strains by plating dilutions of the cultures throughout the anoxic incubation period onto LB plates and scoring CFUs after 24 h (Fig. 3). EtOH synthesis was also assayed in these cultures. As the anoxic incubation proceeded, the EtOH concentration in the supernatant increased for both bacterial hosts. The pattern of cell survival for each strain was however very different. In *E. coli* BW25113/pS234ET, EtOH accumulation increased linearly with time, but survival of bacteria in the same culture followed a pronounced decline (Fig. 3A). In contrast, survival of *P. putida* was not affected at all by the presence of endogenously produced EtOH (Fig. 3B). At the highest alcohol concentration, the survival was in fact 1.2-fold higher than the maximal observed for the reference ethanologenic *E. coli* strain ($p < 0.05$). Moreover, the *P. putida* recombinant expressing *pdhB^{Z. mobilis}* had higher anoxic survival values (*i.e.*, $> 100\%$ for all data points) than the control strain carrying the empty plasmid. As described previously, NAD⁺ recycling through the activity of Adh^{*Z. mobilis*} represents a way to re-oxidize excess reducing power, thereby allowing for enhanced fitness of *P. putida* KT2440 in the absence of oxygen [37]. Taken together, these results reveal a superior tolerance of *P. putida* to the endogenous stress produced by EtOH production (especially at 24 h, at which point the level of alcohol in the medium was comparable to the reference *E. coli* strain).

P. putida is highly resistant to the oxidative stress caused by endogenous EtOH production

One of the primary effects of alcohols on bacteria is perturbation of cell membrane function, which is typically translated into generation of ROS [30, 40, 56, 57]. Production of these agents is not entirely circumscribed to oxic conditions [58], as cells thriving in micro-oxic environments can produce ROS as the respiratory chain is still partially active [59]. We hypothesized that the enhanced tolerance of *P. putida* to endogenously-produced EtOH could be traced to a decrease in the ensuing production of such ROS. To ascertain this question, the level of stress imposed by endogenous EtOH was directly quantified in the recombinants by using the fluorescent probe carboxy-H₂DCFDA (Fig. 3C). Carboxy-H₂DCFDA is a non-fluorescent molecule that can be readily converted into a green-fluorescent form when the acetate groups are removed by intracellular esterases, followed by oxidation (*via* the activity of ROS) occurring within the cell. As shown in Fig. 3C, the amount of ROS detected in EtOH-producing *P. putida* recombinants was *ca.* half that observed for *E. coli*, which inversely correlated with survival values. This is consistent

1 with the notion that ability to form CFUs when transferred into fresh medium is plausibly related to
2 endurance to the stress caused by the alcohol. Note, however, that levels of EtOH produced by
3 the recombinants under these culture conditions consistently remained below 2.5 g l⁻¹ [*i.e.*, *ca.*
4 0.32% (v/v)] which is still a very low concentration if one thinks on a large-scale production
5 process. How far up can *P. putida* go into an authentic industrial EtOH production setup, where
6 the target levels of this metabolite cannot go below 1% (v/v)?

7 8 *Resistance to endogenous vs. exogenous EtOH*

9
10 The results above prompted the question of whether resistance to exogenously added EtOH
11 might be also different among the strains. In a typical fed-batch process, products accumulate
12 over time. In such an industrial scenario applied to bioalcohol formation, alcohol concentrations
13 can reach as high as 3% (v/v) [8]. To investigate these possibilities, we examined survival and
14 stress related to the addition of EtOH at different concentrations to the growth culture. As shown
15 in Fig. 4, *E. coli* BW25113/pS234ET was much more sensitive to EtOH addition than *P. putida*
16 KT2440/pS234ET for all the concentrations tested within the range 0.5% to 5% (v/v). As the
17 percentage of added alcohol increased, the survival of all the strains trended downwards – more
18 pronouncedly so in the case of the reference *E. coli* strain: at the highest concentration assayed,
19 the survival of *P. putida* recombinants was ~2.2-fold higher ($p < 0.01$). Following the same
20 rationale above for the endogenous production of ethanol, we entertained that the stress
21 endurance of the *P. putida* biocatalyst should be higher than *E. coli*, a trait which would be finally
22 reflected in larger CFUs counts. To evaluate this possibility, we measured the oxidative status of
23 the recombinants as explained above (Fig. 5). As expected, addition of EtOH elicited an increase
24 of the ROS levels that paralleled the final alcohol concentration. The stressful effects of this
25 metabolite were more pronounced in the reference *E. coli* strain (as reflected in an 1.7-fold higher
26 carboxy-H₂DCFDA signal; Fig. 5A) as compared to *P. putida* (Fig. 5C) when challenged with 5%
27 (v/v) EtOH ($p < 0.05$). This behaviour was not limited to ethanol: addition of *tert*-butyl
28 hydroperoxide, which directly results in formation of H₂O₂, produced a more dramatic effect in *E.*
29 *coli* in terms of ROS generation (Fig. 5B and 5D). In this case, the ROS level was 1.5-fold higher
30 in the enterobacterial ethanologen, mirroring the differences observed when assessing the

ethanol-dependent formation of ROS. Again, there was a direct link between ROS generation and cell survival scores, that correlated well throughout the concentrations assayed.

P. putida shows enhanced activity of the heterologous enzymes needed for EtOH production

As the stress tolerance of *P. putida* ethanologens was higher than that of *E. coli* recombinants under all the conditions tested, we wondered whether the biocatalytic vigour of the cells shows any difference in the presence of the final product of the biotransformation. In a control experiment, the specific enzyme activities of Pdc and AdhB were quantified in cell-free extracts of the strains at stake (Table 3) after an anoxic 24-h incubation period. Surprisingly, no significant differences were observed between the two strains bearing pS234ET. A very small, but significant background level of alcohol dehydrogenase activity was detected in both *E. coli* and *P. putida* carrying the empty pSEVA234 vector (data not shown), revealing that either the native AdhE (in *E. coli*) [60] or one of the several putative alcohol dehydrogenases (in *P. putida*) [61, 62] contributed to the overall activity detected. In contrast, no pyruvate decarboxylase was present in the control strains. When EtOH was added at 1% and 5% (v/v), the specific Pdc^{*Z. mobilis*} activity was diminished in *E. coli* BW25113/pS234ET by 33% and 62%, respectively ($p < 0.05$). Likewise, the specific AdhB^{*Z. mobilis*} activity dropped to 31% and 80%, respectively, of that observed in the absence of any added alcohol ($p < 0.05$). When the same enzymes were assayed in *P. putida* KT2440/pS234ET, their levels were roughly similar irrespective of the presence of exogenous EtOH in the culture medium. Addition of 1% (v/v) EtOH did not translate into any significant difference of neither Pdc^{*Z. mobilis*} nor AdhB^{*Z. mobilis*} activities. At the highest alcohol concentration, on the contrary, both enzymatic activities were diminished *ex aequo* by ca. 20%. These results endorsed the vigour of *P. putida* under all the conditions tested, especially in the presence of an external stressor – an important advantage in bioprocesses in which the final product is toxic to the producing cells.

Phenotypic stability of ethanologens after extended anoxic incubation

As the enzymatic activities from which EtOH stem in the recombinants were subjected to significant fluctuations as the external EtOH concentration increased, it was also relevant to

1 assess whether the recombinants could maintain their catalytic properties after prolonged anoxic
 2 incubation – a condition pertinent to industrial setups, in which EtOH is to be accumulated over
 3 time. In this context, we assayed the gross alcohol dehydrogenase activity of the recombinants
 4 after 1, 2, and 4 days of incubation by means of a colorimetric test that allowed the visualization
 5 of positive clones as bright pink colonies onto aldehyde indicator plates. A representative
 6 example of the results obtained when plating the ethanologens onto these plates after 2 and 4
 7 days is shown in Fig. 6A and 6B. Visual inspection of the assay revealed that, while the reference
 8 *E. coli* strain seemed to quickly lose the AdhB⁺ phenotype (Fig. 6A), *P. putida* recombinants
 9 displayed a high level of aldehyde formation when transferred onto indicator medium plates (Fig.
 10 6B). By directly counting AdhB⁻ and AdhB⁺ colonies, the fraction of phenotypically stable clones
 11 was quantified (Fig. 6C). After 24 h of anoxic incubation, both strains almost completely retained
 12 the alcohol-producing phenotype bestowed by pS234ET. Significant differences, however, were
 13 observed at 48 h and 96 h, as the fraction of *E. coli* AdhB⁺ cells steadily decreased to reach
 14 $68.1\% \pm 1.5\%$. After the same time period, > 90% of the *P. putida* cells retained the AdhB⁺
 15 phenotype ($p < 0.01$). Moreover, the specific AdhB activity was assayed *in vitro* for some of the
 16 clones that gave a positive signal in aldehyde-indicator plates, and the enzymatic activity levels
 17 were comparable to those reported in Table 3 in the absence of any added alcohol. The ethanol-
 18 producing phenotype was not completely related to plasmid loss, as observed in other
 19 ethanologens [63]. We assessed the fraction of Km^R colonies in these cultures to estimate the
 20 segregational stability of pS234ET, and found that this parameter decreased slightly for *E. coli*,
 21 while it was maintained at > 87% in *P. putida* recombinants. However, *E. coli* retained > 80% of
 22 the ethanologenic plasmid after 96 h, which does not suffice to explain the loss of catalytic
 23 activity. It is thus plausible that the differences observed arise from metabolic changes in the cells
 24 bearing pS234ET rather than the mere plasmid loss – again pointing to the superior metabolic
 25 robustness of *P. putida*.

26 27 **Conclusion**

28
 29 The data presented above highlights the value of environmental bacteria that naturally thrive in
 30 sites polluted with xenobiotic contaminants as hosts for the expression of heterologous
 31 biochemical routes involving toxic compounds [32, 34, 48, 64]. This situations stems from the fact

that the evolutionary roadmap of such bacteria to degrade otherwise recalcitrant molecules can occur only if the biological vessel of the process can endure high levels of endogenous metabolic stress and physico-chemical insults. We argue that of hosts of this sort – like the *P. putida* strain employed in this work, are pre-evolved biological recipients that Nature offers as optimal hosts for biotechnological applications [34, 65]. The results above demonstrate the importance of stress resistance and catalytic robustness in processes that produce biochemicals (e.g., biofuels) that might act as stressors themselves, highlighting, at the same time, the choice of *P. putida* as a potential *host of reference* for biocatalysis at an industrial scale.

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References

- [1] Danchin A. Scaling up synthetic biology: do not forget the *chassis*. FEBS Lett 2012;586:2129-37.
- [2] Colin VL, Rodríguez A, Cristóbal HA. The role of synthetic biology in the design of microbial cell factories for biofuel production. J Biomed Biotechnol 2011;2011.
- [3] Jang YS, Park JM, Choi S, Choi YJ, Seung DY, Cho JH, *et al.* Engineering of microorganisms for the production of biofuels and perspectives based on systems metabolic engineering approaches. Biotechnol Adv 2012;30:989-1000.
- [4] Jarboe LR, Zhang X, Wang X, Moore JC, Shanmugam KT, Ingram LO. Metabolic engineering for production of biorenewable fuels and chemicals: contributions of synthetic biology. J Biomed Biotechnol 2010;2010:761042.
- [5] Wen M, Bond-Watts BB, Chang MC. Production of advanced biofuels in engineered *E. coli*. Curr Opin Chem Biol 2013;17:472-9.

- 1 [6] Li H, Cann AF, Liao JC. Biofuels: biomolecular engineering fundamentals and advances.
2 Annu Rev Chem Biomol Eng 2010;1:19-36.
- 3 [7] Demain AL. Biosolutions to the energy problem. J Ind Microbiol Biotechnol 2009;36:319-
4 32.
- 5 [8] Geddes CC, Nieves IU, Ingram LO. Advances in ethanol production. Curr Opin Biotechnol
6 2011;22:312-9.
- 7 [9] Vertès AA, Inui M, Yukawa H. Technological options for biological fuel ethanol. J Mol
8 Microbiol Biotechnol 2008;15:16-30.
- 9 [10] Wackett LP. Biomass to fuels *via* microbial transformations. Curr Opin Chem Biol
10 2008;12:187-93.
- 11 [11] Clomburg JM, Gonzalez R. Biofuel production in *Escherichia coli*: the role of metabolic
12 engineering and synthetic biology. Appl Microbiol Biotechnol 2010;86:419-34.
- 13 [12] Farrell AE, Plevin RJ, Turner BT, Jones AD, O'Hare M, Kammen DM. Ethanol can
14 contribute to energy and environmental goals. Science 2006;311:506-8.
- 15 [13] Ingram LO, Conway T, Clark DP, Sewell GW, Preston JF. Genetic engineering of ethanol
16 production in *Escherichia coli*. Appl Environ Microbiol 1987;53:2420-5.
- 17 [14] Ohta K, Beall DS, Mejia JP, Shanmugam KT, Ingram LO. Genetic improvement of
18 *Escherichia coli* for ethanol production: chromosomal integration of *Zymomonas mobilis*
19 genes encoding pyruvate decarboxylase and alcohol dehydrogenase II. Appl Environ
20 Microbiol 1991;57:893-900.
- 21 [15] Conway T, Sewell GW, Osman YA, Ingram LO. Cloning and sequencing of the alcohol
22 dehydrogenase II gene from *Zymomonas mobilis*. J Bacteriol 1987;169:2591-7.
- 23 [16] Jarboe LR, Grabar TB, Yomano LP, Shanmugam KT, Ingram LO. Development of
24 ethanologenic bacteria. Adv Biochem Eng Biotechnol 2007;108:237-61.
- 25 [17] Arfman N, Worrell V, Ingram LO. Use of the *tac* promoter and *lacI^q* for the controlled
26 expression of *Zymomonas mobilis* fermentative genes in *Escherichia coli* and *Zymomonas*
27 *mobilis*. J Bacteriol 1992;174:7370-8.
- 28 [18] Schiel-Bengelsdorf B, Montoya J, Linder S, Dürre P. Butanol fermentation. Environ
29 Technol 2013;34:1691-710.
- 30 [19] Zhang F, Rodriguez S, Keasling JD. Metabolic engineering of microbial pathways for
31 advanced biofuels production. Curr Opin Biotechnol 2011;22:775-83.

- 1 [20] Xue C, Zhao XQ, Liu CG, Chen LJ, Bai FW. Prospective and development of butanol as an
2 advanced biofuel. *Biotechnol Adv* 2013;31:1575-84.
- 3 [21] Choi YJ, Lee SY. Microbial production of short-chain alkanes. *Nature* 2013;502:571-4.
- 4 [22] Dunlop MJ. Engineering microbes for tolerance to next-generation biofuels. *Biotechnol*
5 *Biofuels* 2011;4:32.
- 6 [23] Parawira W, Tekere M. Biotechnological strategies to overcome inhibitors in lignocellulose
7 hydrolysates for ethanol production: review. *Crit Rev Biotechnol* 2011;31:20-31.
- 8 [24] Klinke HB, Thomsen AB, Ahring BK. Inhibition of ethanol-producing yeast and bacteria by
9 degradation products produced during pre-treatment of biomass. *Appl Microbiol Biotechnol*
10 2004;66:10-26.
- 11 [25] Taylor MP, Mulako I, Tuffin M, Cowan D. Understanding physiological responses to pre-
12 treatment inhibitors in ethanologenic fermentations. *Biotechnol J* 2012;7:1169-81.
- 13 [26] Nicolaou SA, Gaida SM, Papoutsakis ET. A comparative view of metabolite and substrate
14 stress and tolerance in microbial bioprocessing: from biofuels and chemicals, to
15 biocatalysis and bioremediation. *Metab Eng* 2010;12:307-31.
- 16 [27] Zingaro KA, Papoutsakis ET. Toward a semisynthetic stress response system to engineer
17 microbial solvent tolerance. *mBio* 2012;3:E00308-12.
- 18 [28] Liu S, Qureshi N. How microbes tolerate ethanol and butanol. *New Biotechnol*
19 2009;26:117-21.
- 20 [29] Ramos JL, Duque E, Gallegos MT, Godoy P, Ramos-González MI, Rojas A, *et al.*
21 Mechanisms of solvent tolerance in Gram-negative bacteria. *Annu Rev Microbiol*
22 2002;56:743-68.
- 23 [30] Ingram LO, Buttke TM. Effects of alcohols on micro-organisms. *Adv Microb Physiol*
24 1984;25:253-300.
- 25 [31] Ingram LO. Ethanol tolerance in bacteria. *Crit Rev Biotechnol* 1990;9:305-19.
- 26 [32] Pérez-Pantoja D, Nikel PI, Chavarría M, de Lorenzo V. Endogenous stress caused by
27 faulty oxidation reactions fosters evolution of 2,4-dinitrotoluene-degrading bacteria. *PLoS*
28 *Genet* 2013;9:e1003764.
- 29 [33] Martins dos Santos VAP, Heim S, Moore ER, Strätz M, Timmis KN. Insights into the
30 genomic basis of niche specificity of *Pseudomonas putida* KT2440. *Environ Microbiol*
31 2004;6:1264-86.

- [34] Nikel PI. A brief guide to *Pseudomonas putida* as a microbial cell factory. BioEssays 2012; Available on line at <http://goo.gl/DXF1y>.
- [35] Poblete-Castro I, Becker J, Dohnt K, Martins dos Santos VAP, Wittmann C. Industrial biotechnology of *Pseudomonas putida* and related species. Appl Microbiol Biotechnol 2012;93:2279-90.
- [36] Abril MA, Michan C, Timmis KN, Ramos JL. Regulator and enzyme specificities of the TOL plasmid-encoded upper pathway for degradation of aromatic hydrocarbons and expansion of the substrate range of the pathway. J Bacteriol 1989;171:6782-90.
- [37] Nikel PI, de Lorenzo V. Engineering an anaerobic metabolic regime in *Pseudomonas putida* KT2440 for the anoxic biodegradation of 1,3-dichloroprop-1-ene. Metab Eng 2013;15:98-112.
- [38] de Lorenzo V, Timmis KN. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5- and Tn10-derived minitransposons. Methods Enzymol 1994;235:386-405.
- [39] Chin WC, Lin KH, Chang JJ, Huang CC. Improvement of *n*-butanol tolerance in *Escherichia coli* by membrane-targeted tilapia metallothionein. Biotechnol Biofuels 2013;6:130.
- [40] Rutherford BJ, Dahl RH, Price RE, Szmidt HL, Benke PI, Mukhopadhyay A, *et al.* Functional genomic study of exogenous *n*-butanol stress in *Escherichia coli*. Appl Environ Microbiol 2010;76:1935-45.
- [41] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54.
- [42] Hoppner TC, Doelle HW. Purification and kinetic characteristics of pyruvate decarboxylase and ethanol dehydrogenase from *Zymomonas mobilis* in relation to ethanol production. Appl Microbiol Biotechnol 1983;17:152-7.
- [43] Diefenbach RJ, Duggleby RG. Pyruvate decarboxylase from *Zymomonas mobilis*. Structure and re-activation of apoenzyme by the cofactors thiamin diphosphate and magnesium ion. Biochem J 1991;276:439-45.
- [44] Neale AD, Scopes RK, Kelly JM, Wettenhall REH. The two alcohol dehydrogenases of *Zymomonas mobilis*. Purification by differential dye ligand chromatography, molecular characterisation and physiological roles. Eur J Biochem 1986;154:119-24.

- 1 [45] Clark DP. The fermentation pathways of *Escherichia coli*. FEMS Microbiol Rev 1989;5:223-
2 34.
- 3 [46] Murarka A, Clomburg JM, Moran S, Shanks JV, Gonzalez R. Metabolic analysis of wild-
4 type *Escherichia coli* and a pyruvate dehydrogenase complex (PDHc)-deficient derivative
5 reveals the role of PDHc in the fermentative metabolism of glucose. J Biol Chem
6 2010;285:31548-58.
- 7 [47] Nikel PI, Ramirez MC, Pettinari MJ, Méndez BS, Galvagno MA. Ethanol synthesis from
8 glycerol by *Escherichia coli* redox mutants expressing *adhE* from *Leuconostoc*
9 *mesenteroides*. J Appl Microbiol 2010;109:492-504.
- 10 [48] Chavarría M, Nikel PI, Pérez-Pantoja D, de Lorenzo V. The Entner-Doudoroff pathway
11 empowers *Pseudomonas putida* KT2440 with a high tolerance to oxidative stress. Environ
12 Microbiol 2013;15:1772-85.
- 13 [49] del Castillo T, Ramos JL, Rodríguez-Herva JJ, Fuhrer T, Sauer U, Duque E. Convergent
14 peripheral pathways catalyze initial glucose catabolism in *Pseudomonas putida*: genomic
15 and flux analysis. J Bacteriol 2007;189:5142-52.
- 16 [50] Fuhrer T, Fischer E, Sauer U. Experimental identification and quantification of glucose
17 metabolism in seven bacterial species. J Bacteriol 2005;187:1581-90.
- 18 [51] Rogers PL, Jeon YJ, Lee KJ, Lawford HG. *Zymomonas mobilis* for fuel ethanol and higher
19 value products. Adv Biochem Eng Biotechnol 2007;108:263-88.
- 20 [52] Nielsen J, Larsson C, van Maris A, Pronk J. Metabolic engineering of yeast for production
21 of fuels and chemicals. Curr Opin Biotechnol 2013;24:398-404.
- 22 [53] de Lorenzo V, Eltis L, Kessler B, Timmis KN. Analysis of *Pseudomonas* gene products
23 using *lacI^q/Ptrp-lac* plasmids and transposons that confer conditional phenotypes. Gene
24 1993;123:17-24.
- 25 [54] Nikel PI, de Lorenzo V. Implantation of unmarked regulatory and metabolic modules in
26 Gram-negative bacteria with specialised mini-transposon delivery vectors. J Biotechnol
27 2013;163:143-54.
- 28 [55] Díaz-Ricci JC, Tsu M, Bailey JE. Influence of expression of the *pet* operon on intracellular
29 metabolic fluxes of *Escherichia coli*. Biotechnol Bioeng 1992;39:59-65.

- [56] Atsumi S, Wu TY, Machado IM, Huang WC, Chen PY, Pellegrini M, *et al.* Evolution, genomic analysis, and reconstruction of isobutanol tolerance in *Escherichia coli*. *Mol Syst Biol* 2010;6:449.
- [57] Woodruff LB, Pandhal J, Ow SY, Karimpour-Fard A, Weiss SJ, Wright PC, *et al.* Genome-scale identification and characterization of ethanol tolerance genes in *Escherichia coli*. *Metab Eng* 2013;15:124-33.
- [58] Kang A, Tan MH, Ling H, Chang MW. Systems-level characterization and engineering of oxidative stress tolerance in *Escherichia coli* under anaerobic conditions. *Mol Biosyst* 2013;9:285-95.
- [59] Uden G, Bongaerts J. Alternative respiratory pathways of *Escherichia coli*: energetics and transcriptional regulation in response to electron acceptors. *Biochim Biophys Acta* 1997;1320:217-34.
- [60] Leonardo MR, Dailly Y, Clark DP. Role of NAD in regulating the *adhE* gene of *Escherichia coli*. *J Bacteriol* 1996;178:6013-8.
- [61] Nelson KE, Weinl C, Paulsen IT, Dodson RJ, Hilbert H, Martins dos Santos VAP, *et al.* Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ Microbiol* 2002;4:799-808.
- [62] Winsor GL, Lam DKW, Fleming L, Lo R, Whiteside MD, Yu NY, *et al.* *Pseudomonas* Genome Database: improved comparative analysis and population genomics capability for *Pseudomonas* genomes. *Nucleic Acids Res* 2011;39:D596-D600.
- [63] Nikel PI, Pettinari MJ, Galvagno MA, Méndez BS. Metabolic selective pressure stabilizes plasmids carrying biosynthetic genes for reduced biochemicals in *Escherichia coli* redox mutants. *Appl Microbiol Biotechnol* 2010;88:563-73.
- [64] Nikel PI, Pérez-Pantoja D, de Lorenzo V. Why are chlorinated pollutants so difficult to degrade aerobically? Redox stress limits 1,3-dichloroprop-1-ene metabolism by *Pseudomonas pavonaceae*. *Philos Trans R Soc Lond B Biol Sci* 2013;368:20120377.
- [65] Domínguez-Cuevas P, González-Pastor JE, Marqués S, Ramos JL, de Lorenzo V. Transcriptional tradeoff between metabolic and stress-response programs in *Pseudomonas putida* KT2440 cells exposed to toluene. *J Biol Chem* 2006;281:11981-91.
- [66] Hanahan D, Meselson M. Plasmid screening at high colony density. *Methods Enzymol* 1983;100:333-42.

- 1 [67] Herrero M, de Lorenzo V, Timmis KN. Transposon vectors containing non-antibiotic
2 resistance selection markers for cloning and stable chromosomal insertion of foreign genes
3 in Gram-negative bacteria. *J Bacteriol* 1990;172:6557-67.
- 4 [68] Boyer HW, Roulland-Dussoix D. A complementation analysis of the restriction and
5 modification of DNA in *Escherichia coli*. *J Mol Biol* 1969;41:459-72.
- 6 [69] Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, *et al.* Construction of
7 *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst*
8 *Biol* 2006;2:2006.0008.
- 9 [70] Bagdasarian M, Lurz R, Rückert B, Franklin FCH, Bagdasarian MM, Frey J, *et al.* Specific
10 purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF1010-
11 derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. *Gene*
12 1981;16:237-47.
- 13 [71] Kessler B, Herrero M, Timmis KN, de Lorenzo V. Genetic evidence that the XylS regulator
14 of the *Pseudomonas* TOL *meta* operon controls the *Pm* promoter through weak DNA-
15 protein interactions. *J Bacteriol* 1994;176:3171-6.
- 16 [72] Silva-Rocha R, Martínez-García E, Calles B, Chavarría M, Arce-Rodríguez A, de las Heras
17 A, *et al.* The Standard European Vector Architecture (SEVA): a coherent platform for the
18 analysis and deployment of complex prokaryotic phenotypes. *Nucleic Acids Res*
19 2013;41:D666-75.

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TABLES

TABLE 1. Bacterial strains and plasmids used in this study.

Bacterial strain or plasmid	Relevant characteristics ^a	Reference
<i>E. coli</i>		
DH5α	Cloning host; Φ 80/ <i>lacZ</i> ΔM15 <i>recA1 endA1 gyrA96 thi-1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 relA1 deoR</i> Δ(<i>lacZYA-argF</i>)U169	[66]
CC118λ <i>pir</i>	Cloning host; <i>araD139</i> Δ(<i>ara-leu</i>)7697 Δ <i>lacX74 galE galK phoA20 thi-1 rpsE rpoB</i> (Rif ^R) <i>argE</i> (Am) <i>recA1</i> , λ <i>pir</i> lysogen	[67]
HB101	Helper strain; F ⁻ λ ⁻ <i>mcrB mrr hsdS20</i> (r _B ⁻ m _B ⁻) <i>recA13 leuB6 ara-14</i> Δ(<i>proBA</i>)2 <i>lacY1 galK2 xyl-5 mtl-1 rpsL20</i> (Sm ^R) <i>glnV44</i>	[68]
BW25113 ^b	Considered wild type in this study; F ⁻ λ ⁻ Δ(<i>araD-araB</i>)567 Δ <i>lacZ4787</i> (::rrnB-3) <i>rph-1</i> Δ(<i>rhaD-rhaB</i>)568 <i>hsdR514</i>	[69]
<i>Pseudomonas putida</i>		
KT2440	Considered wild type in this study; spontaneous restriction-deficient derivative of strain mt-2 cured of the TOL plasmid pWW0	[70]
Plasmid		
pRK600	Helper plasmid used in tri-parental matings; <i>oriV</i> (ColE1) RK2 <i>tra</i> ⁺ <i>mob</i> ⁺ , Cm ^R	[71]
pSEVA234 ^c	Expression vector; <i>oriV</i> (pBBR1) <i>lacI</i> ^Q <i>P_{trc}</i> <i>aphA</i> , Km ^R	[72]
pS234ET	Same as pSEVA234, carrying <i>pdC</i> and <i>adhB</i> from <i>Zymomonas mobilis</i> as single transcriptional unit; Km ^R	[37]

^a Antibiotic resistance markers: Cm, chloramphenicol; Km, kanamycin; Rif, rifampicin; Sm, streptomycin.

^b Strain obtained from the *E. coli* Genetic Stock Center (Yale University, New Haven, CT, USA).

^c Plasmid belonging to the SEVA (Standard European Vector Architecture) collection.

TABLE 2. Pattern of fermentation metabolites after 24 h of anoxic incubation^a.

Bacterial strain	Plasmid	Metabolite concn. ^b (mM)			
		Acetate	Lactate	Formate	Succinate
<i>E. coli</i> BW25113	pSEVA234	68.1 ± 6.3	6.5 ± 0.9	27.8 ± 9.3	12.3 ± 1.8
	pS234ET	46.9 ± 3.7	4.2 ± 0.8	24.9 ± 5.4	8.4 ± 0.9
<i>P. putida</i> KT2440	pSEVA234	< 1.5	–	–	–
	pS234ET	< 0.5	–	–	–

^a Culture conditions were as described for Fig. 2, and the samples used to quantify EtOH were also processed to detect fermentation acids.

^b Results correspond to mean values ± standard deviations of triplicate measurements from at least two independent experiments; concn., concentration; –, not detected.

TABLE 3. Enzymatic activities of the heterologous EtOH synthesis pathway in the presence of exogenously added alcohol^a.

Bacterial host	EtOH concn. ^c (% v/v)	Sp. enzymatic activity ^b ($\mu\text{mol min}^{-1} \text{mg protein}^{-1}$) for:	
		Pyruvate decarboxylase	Alcohol dehydrogenase
<i>E. coli</i> BW25113	0	5.4 $\pm$ 0.9	2.1 $\pm$ 0.7
	1	3.7 $\pm$ 0.5	1.4 $\pm$ 0.2
	5	1.1 $\pm$ 0.2	0.8 $\pm$ 0.1
<i>P. putida</i> KT2440	0	4.9 $\pm$ 0.8	2.9 $\pm$ 0.3
	1	5.2 $\pm$ 0.5	2.7 $\pm$ 0.5
	5	3.8 $\pm$ 0.4	2.3 $\pm$ 0.6

^a Culture conditions were as described for Fig. 2. Both bacterial hosts were transformed with pS234ET (carrying *pdhB*^{Z. mobilis}).

^b Note that low concentrations of alcohol were still detected in experiments with no externally added ethanol, as detailed in Fig. 2C. Concn., concentration.

^c Pyruvate decarboxylase was not detected in cells transformed with the empty pSEVA234 vector; alcohol dehydrogenase activity was corrected by the background activity determined in such control experiments. Results correspond to mean values $\pm$ standard deviations of triplicate measurements from at least two independent experiments; Sp., specific.

FIGURES

FIG. 1. Simplified scheme of the pathways involved in glucose catabolism.

After an initial phosphorylation step, catalyzed either by an hexokinase or the phosphotransferase (PTS) system, the resulting glucose-6-*P* (G6P) can either (i) follow the Embden-Meyerhof-Parnas (EMP) route [via the sequential conversion of G6P into fructose-6-*P* (F6P) and fructose-1,6-*P*₂ (FBP)], or (ii) be channeled through the Entner-Doudoroff (ED) route [via the transformation of G6P into 6-*P*-gluconate (6PG) and 2-keto-3-deoxy-6-*P*-gluconate (KDPG)]. Note that both pathways finally yield glyceraldehyde-3-*P* (G3P) and pyruvate. The oxidative pentose phosphate (PP) pathway is not shown in the outline. While *Escherichia coli* mostly uses the EMP pathway for

1 glucose breakdown, *Pseudomonas putida* exclusively processes the hexose via the ED pathway.
2 Under oxic conditions, pyruvate is transformed into acetyl-coenzyme A (CoA) in both
3 microorganisms through the activity of the pyruvate dehydrogenase complex in a NAD⁺-
4 dependent reaction. Although acetyl-CoA can meet different fates depending on further catabolic
5 steps, it is mainly oxidized in the tricarboxylic acid cycle. Under anoxic conditions, *E. coli*
6 produces a number of fermentation metabolites from pyruvate or acetyl-CoA to meet the carbon
7 and redox balances. While some genes encoding putative fermentation enzymes are present in
8 *P. putida*, this microorganism does not ferment glucose in the absence of oxygen. The main
9 metabolic blocks are depicted in different colors, and the relevant enzymatic steps are identified.
10 The arrows connecting G3P and pyruvate represent several metabolic steps. Redundant
11 enzymes, branched pathways conducive to the same intermediates, co-factor dependence, and
12 multi-step transformations are not shown in this outline. Enzyme abbreviations are termed as
13 follows: Pgi, phosphoglucosomerase; Zwf, G6P dehydrogenase; Pfk, phosphofructokinase; Fda,
14 FBP aldolase; Edd, 6PG dehydratase; Eda, KDGP aldolase; Pfl, pyruvate-formate lyase; LdhA,
15 D-lactate dehydrogenase; Pta, phosphotransacetylase; AckA, acetate kinase; and AdhE, alcohol
16 dehydrogenase.

FIG. 2. Design of an ethanol (EtOH) synthesis pathway composed of enzymes recruited from *Zymomonas mobilis*.

(A) Pyruvate, generated from glucose either *via* the Embden-Meyerhof-Parnas pathway (in *Escherichia coli*) or *via* the Entner-Doudoroff pathway (in *Pseudomonas putida*), is decarboxylated to acetaldehyde by pyruvate decarboxylase ($Pdc^Z. mobilis$). Acetaldehyde is then converted into EtOH by means of alcohol dehydrogenase II ($AdhB^Z. mobilis$), together with the concomitant oxidation of one NADH molecule. (B) Schematic representation of the synthetic operon in which $pdc^Z. mobilis$ and $adhB^Z. mobilis$ are placed under the control of an inducible $LacI^Q/P_{trc}$ element as a single transcriptional unit, borne by plasmid pS234ET. Each coding sequence was preceded with a Shine-Dalgarno (SD) motif, and the stop codons were edited to TAA. The transcriptional terminator of the bacteriophage λ is depicted as T_0 . The elements in this outline are not drawn to scale. (C) Quantification of the EtOH concentration (concn.) in culture medium supernatants. Cells from oxic mid-exponential cultures were spun, washed, concentrated, and transferred to fresh culture medium (OD_{600} ca. 2) containing 20 g l⁻¹ glucose and with or without 2.5 mM IPTG. These cultures were incubated under anoxic conditions for 24 h before samples were taken to analyze EtOH formation. Control experiments (-) were carried out using either *E. coli* BW25113 or *P. putida* KT2440 bearing the empty vector (pSEVA234). Bars represent the mean value of the EtOH concn. $\pm$ standard deviation of triplicate measurements from at least two independent experiments.

FIG. 3. Evaluation of time-dependent cell survival, ethanol (EtOH) formation, and ROS levels.

These parameters were determined in cell suspensions of (A) *E. coli* BW25113/pS234ET and (B) *P. putida* KT2440/pS234ET incubated anoxically. Survival was assessed by plating appropriately diluted culture aliquots into fresh LB plates and incubating them for 18 to 24 h (survival percentages are referred to CFUs counts of the corresponding strain transformed with the empty pSEVA234 vector, and evaluated at the same anoxic incubation time as the ethanologenic recombinants). EtOH concentration (concn.) was determined in the supernatant of the same culture samples. Culture conditions were as indicated for Fig. 1. The levels of ROS produced by endogenous EtOH were estimated *in vivo* (C) by exposing the cells transformed with pS234ET to carboxy-H₂DCFDA, and measuring the fluorescence resulting from the ROS-dependent oxidation of the probe (AFU, arbitrary florescence units) followed by normalization by the biomass used in each assay [estimated as the optical density measured at 600 nm (OD₆₀₀)]. Bars and data points represent the mean value of the corresponding parameter ± standard deviation of triplicate or quadruplicate measurements from at least two independent experiments.

FIG. 4. Survival of ethanologens in the presence of exogenous ethanol (EtOH).

Survival was assessed by plating appropriately diluted culture aliquots into fresh LB plates and incubating them for 18 to 24 h (survival percentages are referred to CFUs counts of the corresponding strain transformed with the empty pSEVA234 vector, and evaluated at the same anoxic incubation time without any added alcohol as the ethanologenic recombinants). EtOH concentration (concn.) is indicated in each case. Bars represent the mean value of the survival score $\pm$ standard deviation of triplicate measurements from at least two independent experiments.

FIG. 5. Quantification of the ROS levels produced by exogenous ethanol (EtOH).

ROS formation was estimated *in vivo* by exposing the cells transformed with pS234ET to different concentrations (concn.) of ethanol, followed by staining with carboxy-H₂DCFDA. The fluorescence resulting from the ROS-dependent oxidation of the probe (AFU, arbitrary fluorescence units) was quantified and normalized by the biomass used in each assay [estimated as the optical density measured at 600 nm (OD₆₀₀)]. Note that low alcohol concn. were detected in experiments with no externally added ethanol, as detailed in Fig. 2C. Positive control experiments were added with *tert*-butyl hydroperoxide (TBHP). Bars represent the mean value of the ROS level $\pm$ standard deviation of quadruplicate measurements from at least two independent experiments.

FIG. 6. Phenotypic stability of the recombinants.

The ability of either *E. coli* BW25113/pS234ET or *P. putida* KT2440/pS234ET to retain the AdhB⁺ phenotype was evaluated after 24 h, 48 h, and 96 h of anoxic incubation *via* an *in vivo* test using aldehyde indicator plates. Cells were spread onto these plates and incubated for 4-6 h, after which colonies were scored and those exhibiting a magenta coloration were considered positive (AdhB⁺). Qualitative results of the test are shown for selected (A) *E. coli* and (B) *P. putida* recombinants after 48 h (left) and 96 h (right) anoxic incubations. (C) Quantification of the phenotypic stability in the ethanologens. Bars represent the mean value of the percentage of AdhB⁺ clones $\pm$ standard deviation of duplicate measurements from at least three independent experiments.