Improved thermotolerance of genome-reduced *Pseudomonas putida* EM42 enables effective functioning of the P_L/cI857 system

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Abbreviations: PCN, Plasmid Copy Number; TIR, translation initiation region; RBS, ribosome-binding site; MCS, multiple cloning site; *msf*GFP, monomeric super-folder GFP; PI, propidium iodide.

Abstract

The higher intracellular ATP levels of genome-edited strains of *P. putida* that result from deleting various energy-consuming functions has been exploited for expanding the window of thermal tolerance of this bacterium. Unlike instant growth halt and eventual death of the naturally-occurring strain *P. putida* KT2440 at 42 °C, the EM42 variant maintained growth and viability of most of the population at the higher temperature for at least 6 h. We took advantage of this quality for implementing a robust thermo-inducible heterologous expression device in this species. To this end, the cI857/P_L pair of the lambda phage of *Escherichia coli* was reshaped as a functional cargo that followed the SEVA (Standard European Vector Architecture) format. Quantitation of the transcriptional output of the resulting expression device with GFP reporter technology in various gene dosages identified conditions of unprecedented induced/uninduced ratios (>300 fold) and very high total transcriptional capacity in this bacterial host. The broad-host range nature of the cognate replication origins makes expression vectors pSEVA2214 (low plasmid copy number), pSEVA2314 (medium) and pSEVA2514 (high) to cover a wide range of heterologous expression needs in *P. putida* and possibly other Gram-negative species.

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1 Introduction

Owing to their environmental endurance and their distinct redox metabolism *Pseudomonas putida* strains are growingly consolidating as platforms of choice for hosting biotransformations of industrial interest [1-3]. Alas, the typical window of thermal tolerance of *Pseudomonas* species in their natural niches is narrow, many of them being psychrophiles but most having their growth optima in the range 25-30 °C [4]. This not only limits their catalytic efficacy at higher temperatures but it also prevents the use of thermo-inducible transcriptional devices when pulses of heterologous expression—rather than continuous

production of the gene(s) of interest—are required. The most conspicuous response of most bacteria towards heat stress is the transient generation of chaperones (typically the chaperonin GroEL and its co-chaperone GroES, DnaK and similar) mediated by σ^{32} [5,6]. These molecular machines aid the proper folding of polypeptides through cycles of ATP hydrolysis that help proteins to keep a functional conformation upon thermal perturbations [7,8]. The drain of ATP may contribute to deplete the energy currency needed for feeding such mechanism. The prediction that stems from this scenario is that artificially increasing intracellular ATP levels should help prolonging endurance to temperatures higher than their nominal optimum. But how can such an increase in ATP be brought about?

P. putida EM42 is a genome-edited variant of *P. putida* KT2440, itself a plasmid-less derivative of isolate *P. putida* mt-2 [9,10]. In order to increase its predictability and make it more suited for industrial applications, *P. putida* EM42 was deleted of all prophages and other instability determinants that account for 4.3% of the genome and approximately 300 genes. This included elimination of the entire flagellar machinery. The resulting non-motile strain turned out to be way superior to the wild-type counterpart in enduring different types of physicochemical stresses. And—most important for the sake of the present work—genome-reduced strains increased their intracellular levels of ATP and NAD(P)H by >20% [9]. Additional benefits of the genome-reduced strain include a better genomic stability upon deletion of prophages and other mobile elements [9,10].

In this work, we have investigated whether the ATP surplus and superior stress endurance of *P. putida* EM42 could be exploited for adapting to this bacterium the well-known heat-inducible system based on regulatory parts recruited the *E. coli* lambda phage. Typically, the expression device includes either (or both) of the strong P_L/P_R promoters and the *c*I857 gene, that encodes a thermo-labile variant of the cognate repressor *c*I [11]. This basic set has been

shaped in many configurations either as a chromosomal insert [12,13] or, more often in plasmid vectors [14-16]. While bacteria are grown at 28-32 °C transcription from P_L/P_R is altogether repressed, but shifting the temperature above 37 °C (e.g. 42 °C) turns on expression of any downstream gene of interest [17]. The duo P_L-*c*I857 has been extensively used not only in *E. coli* and related Gram-negative bacteria like *Serratia* and *Erwinia* [18] but also in *B. subtilis* [19]. However, its application to genera such as *Pseudomonas* has been limited by the circumstances explained above.

The data below documents the adequacy of *P. putida* EM42 as a host of a thermo-inducible heterologous gene expression system based on P_L-*c*I857. The tangible outcome of this work is the setting and validation of a set of broad host range plasmids assembled with the SEVA (Standard European Vector Architecture) format and covering a wide scope of needs of heterologous expression in *Pseudomonas.*

2 Materials and methods

2.1 Strains, media and general procedures

DNA manipulations were done using standard protocols [20] and recommendations from manufacturers. *E. coli* CC118 strain [21] was used for cloning purposes. *P. putida* strain KT2440 and its genome-reduced derivative EM42 have been described before [9]. Rich LB liquid and solid media were routinely used for bacterial growth and supplemented when necessary with 50 μg/mL kanamycin (Km) or ampicillin (Ap). Liquid cultures were always incubated aerobically with shaking (170 rpm) at 30 °C, 37 °C, 40 °C and 42 °C as needed and indicated in each case. In all instances, ligations with SEVA plasmids were transformed in competent cells of strain *E. coli* CC118, plated on LB-Km plates and incubated 24 h at 30 °C. *oriT*-containing plasmids were mobilized towards *P. putida* EM42 through triparental

conjugal transfer from *E. coli* CC118 (harboring the plasmid to be delivered) and helper *E. coli* HB101 (pRK600) strain [22] as described [23].

2.2 Growth curves and Survival Assays

For assessing the ability of *P. putida* KT2440 and EM42 strains to grow at different temperatures, an overnight culture (30 °C) was diluted to OD_{600} ~ 0.05 in 40 ml of LB and incubated in 250 ml Erlenmeyer flasks at 30 °C, 37 °C, 40 °C and 42 °C. Growth was monitored at 1 h intervals for 24 hours by taking 1 ml aliquots (diluted if necessary) and OD_{600} measured in a Ultrospec 2100-pro spectrophotometer (Biochrom, Harvard Bioscience, Inc.). To determine the survival rate of *P. putida* strains at 42 °C, 20 ml of LB were inoculated to OD_{600} = 0.1 from overnight cultures and grown at 30 °C till mid-exponential phase. OD_{600} was adjusted to 0.5 in 20 ml LB and transferred to a water bath at 42 °C, incubated 5 minutes (to make temperature rise quickly), placed in an air shaker at 42 °C and further incubated with shaking for the next 3 hours. Samples were taken at different times (0, 30´, 1h, 2h and 3h) and appropriate dilutions plated in LB solid media. Duplicate Petri dishes were incubated at 30 °C for 18 hours and colonies counted. Survival rates were calculated as the percentage of CFUs at a given time (CFU_t) in relation to the initial number of CFUs (CFU_i) applying the formula: (CFUt/CFUi)*100. Means and standard deviations were calculated from two biological replicas of the corresponding experiment.

2.3 Plasmid construction

The cI857/P_L heat-inducible system of lambda phage [11,24,25] were constructed in the frame of vectors pSEVA2214 (RK2 origin of replication, low PCN), pSEVA2314 (pBBR1 origin, medium PCN) and pSEVA2514 (RSF1010 origin, high PCN), the sequences of which can be found in GenBank under accession numbers MH650998, MH650997 and MH638301 respectively. Their PCNs for *E. coli* were accurately determined by Jahn *et al* with a droplet

digital PCR method [26]. Note however that RSF1010 replicon borne by the SEVA plasmid pSEVA2514 utilized in this work is not identical to the wild-type replication, but has a stronger promoter in front of the replication proteins that increases copy number by 3-fold [27]. Regardless of specific copy numbers, the hierarchy of relative plasmid abundance in *P. putida* of the vectors used is RS1010 (high) > pBBR1 (medium) > RK2 (low). On this basis, the [cI857/P_L →] expression cargo and a downstream fluorescent reporter was prepared as a synthetic 2226 bp DNA segment flanked by PacI/SpeI restriction sites and cloned in vector pUC57 (GeneCust Europe, Luxembourg). The parts included in such a synthetic segment and their arrangement are shown in Fig. 1A. The *c*I857 gene (714 bp) was endowed with a heterologous TIR motif [28] upstream the start codon and the whole placed under the control of the strong, constitutive P_{Kan} promoter. A neutral spacer sequence of 150 bp was then placed upstream of P_{Kan} for insulating expression of divergent and extended P_L promoter (384 bp) recruited from plasmid pORTMAGE [16]. This was followed by the complete SEVA multiple cloning site (MCS) and a downstream reporter unit composed of a canonical RBS (5´-AGGAGG-3´) and the gene for the monomeric super-folder green fluorescent protein *msf*GFP [29,30] as a HindIII/SpeI fragment. The detail of the intergenic region between cI857 and *msf*GFP is shown in Fig. 1A. Vector pUC57 inserted with such a segment was cleaved with PacI/SpeI for excising the 2.2 Kb DNA fragment of interest and ligated with pSEVA221, pSEVA231 and pSEVA251 vectors digested with the same enzymes. Since the [cI857/P_L →] expression system was given a SEVA cargo code #14, the resulting plasmids were designated pSEVA2214-*msfGFP* (low PCN), pSEVA2314-*msfGFP* (medium PCN) and pSEVA2514-*msfGFP* (high PCN). The control, *msf*GFP-less, empty expression vector named pSEVA2514 (Fig. 1B) was made by digesting the pSEVA2514-*msfGFP* with PacI/AvrII an ligating the excised 1.4 Kb fragment in pSEVA251 cut with the same enzymes. Plasmid constructs were sequenced (Macrogen; Spain) with PS1 (5´-AGGGCGGCGGATTTGTCC-3´ [31]), PS2 (5´-GCGGCAACCGAGCGTTC-3´ [31]), R24 (5´-AGCGGATAACAATTTCACACAGGA-3´) and 860 (5´-

ACCCATCTCTCCGCATCACC-3´) primers to verify the sequence of the insert (see location in Fig. 1A). All plasmids are available upon request through the SEVA repository (http://seva.cnb.csic.es).

2.4 Flow cytometry

P. putida EM42 transconjugants carrying plasmids pSEVA2214-*msf*GFP, pSEVA2314-*msf*GFP, pSEVA2514-*msf*GFP and pSEVA2514 (Fig. 1B) were used to test the responsiveness of the cognate expression cargoes driving induction of the reporter *msf*GFP gene at different temperatures. To this end, each strain was grown overnight in LB-Km at 30 °C and used to inoculate 70 ml of filtered LB-Km to OD_{600} = 0.1. Cultures were incubated at 30 °C to mid-exponential phase OD_{600} = 0.5. For each strain three culture aliquots of 15 ml were separately added to Erlenmeyer flasks (150 ml) and each one incubated at the desired temperatures (30°C, 37°C and 42°C). Samples were taken at the times indicated and the OD_{600} adjusted to OD_{600} ~ 0.1 by spinning down the cultures and resuspending the pellet in filtered 1× phosphate-buffered saline (PBS; 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 3 mM KCl, 137 mM NaCl, pH = 7.0). Fluorescence analysis and quantification was performed in a MACSQuant^{TM} VYB cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany) as indicated in [32]. *msf*GFP was excited at 488 nm and the fluorescence signal recovered with a 525 ± 40 nm band-pass filter. At least 100000 cells were analyzed per sample and the results processed using the FlowJo v. 9.6.2 software (FlowJo LLC, Ashland, OR, USA). Fold-inductions changes were calculated by normalizing the average fluorescence of the induced samples at 37 °C y 42 °C with the levels of the same samples at 30 °C. The threshold fluorescence for induced populations set as 10^0 arbitrary units (1.0 a. u.). Samples incubated at 42 °C were also analyzed for assesing *msf*GFP expression *vs.* cell mortality. To this end, 500 μl of the PBS-suspended cells were added with 5 μl of 0.5 mg/ml propidium iodide (PI) for 20 minutes and analyzed in the cytometer. *msf*GFP signal was recovered as explained before while PI was excited at 561 nm and the fluorescence

signal recovered with a 586 ± 15 nm band-pass filter. Two biological replicas were performed and at least 100,000 cells analyzed per sample. Results were processed using the FlowJo v. 9.6.2 software and percentages of the four different sub-populations (±PI/±GFP) were plotted as shown.

3 Results and Discussion

3.1 Thermal endurance of *P. putida* KT2440 and its genome edited variant EM42

The starting point of this work was the investigation of the thermal sensitivity of *Pseudomonas putida* KT2440 and how deletion of genomic segments encoding major ATP-consuming functions (e.g. the flagellar machinery) could influence it. To this end, we first tested the ability of these strains to grow at temperatures 30 °C, 37 °C, 40 °C and 42 °C. Fig. 2A shows the growth kinetics of *P. putida* KT2440 and EM42 in LB medium at the temperatures indicated. Note that growth profiles of both strains at 30 °C and 37 °C are virtually indistinguishable, reaching identical OD_{600} above 7.0 after 24 h of growth. Similarly, the growth of the same strains is arrested at the upper temperature (42 °C) and beyond. The situation is however different at the intermediate heating (40 °C): both strains do grow during the first 3h of thermal induction (albeit at different rates). But after t = 4h, the behavior of either strain conspicuously diverges. The wild-type genome strain *P. putida* KT2440 stops growth altogether and initiates a declining trend that goes on until the end of the experiment (24 h) reaching a final OD_{600} of 0.12 ± 0.08. In contrast, the genome reduced *P. putida* EM42 strain keeps on growing for at least 6 hours after thermal induction. The ensuing OD_{600} of the culture then levels off but maintains a slow but consistent increase to reach a final value of 0.22 ± 0.06 i.e. approximately twice as much as the wild-type strain.

Taken together, these data indicated that genome-reduced EM42 variant did better than the

wild-type KT2440 in tolerating higher temperatures. Yet, 40 °C is still insufficient for inducing the P_L-*c*I857 as intended (see above), as the thermosensitive lambda repressor is fully inactivated only above 42 °C. Although the growth of both strains was halted at such higher temperature (Fig. 2A), we wondered whether the cells were either killed also by the stress or just resting but still active and viable. To clarify this, we inspected the survival of mid-logarithmic cells of either strain of *P. putida* after exposing cells to 42 °C for the critical first 3 h. Fig. 2B shows a clear divergence in the viability of either strain when subject to such high temperature. While *P. putida* EM42 cells remain virtually unscathed during the first hour of thermal stress, viability of the wild-type bacterium decreases by >40% under the same conditions. This different exacerbates with time of heat exposure: after 3 h, the CFU count of the wild-type comes down to ~10% of the total population, while the genome-reduced variant still keeps a good 43% of viability.

How do the data above compare with the known thermal sensitivity of other *P. putida* strains? [33] reported 40 °C as the upper growth limit for *P. putida* SUBP03—total arrest was observed at 41 °C. Similarly, screening of a collection of 2500 *P. putid*a samples for tolerance to high temperatures [34] identified just one strain which survived to 40 °C. These reports suggest that there is a growth temperature threshold around 40 °C which might be a common feature for other members of the species. Beyond this threshold, 42 °C seems to be a non-permissive temperature for cell division and survival due to excessive heat stress. But as shown above, the genome-reduced EM42 strain performed noticeably better than KT2440 in terms of growth at 40 °C and survival at 42 °C. It is plausible that such an advantage stems from the higher levels of intracellular ATP found in the genome-reduced strains that is drained for fueling the chaperonins that counteract the thermal perturbation caused by excessive heat. In any case, the broadening of the survival and physiological activity of *P. putida* EM42 during at least 3 h of exposure to 42 °C exposed a window of opportunity for

optimally deploying a heterologous expression system based on the thermo-inducible platform derived from lambda phage P_L-*c*I857.

3.2 Refactoring the P_L-*c*I857 device for optimal performance in *P. putida* EM42

Although most heterologous expression devices maintain their gross induction pattern when transferred among different bacterial species, parameters may change very significantly depending on the specific host and gene dosage. In order to benchmark the P_L-*c*I857 pair for operating in *P. putida* we adopted the SEVA standard for arraying regulatory elements in a genetic node shaped as a heterologous expression cargo. This standard, which was first adopted in the platform for the cyclohexanone-responsive expression system of pSEVA2311 [35] was implemented on the P_L and *c*I857 parts as shown in Fig 1A and 1B. First, the *c*I857 mutant allele of the *c*I repressor of lambda phage was placed under the control of a constitutive promoter and a TIR motif upstream of the start codon. Second, an extended P_L promoter sequence was located in an opposite orientation in respect to the *c*I857 gene but separated of it by a neutral spacer sequence that insulates each of the promoters in respect to the other. Third, the standard multiple cloning site (MCS) of pSEVA plasmids [31] was placed downstream of P_L. This cargo [cI857/P_L →] was then followed by the sequence of the *msf*GFP gene preceded by a canonical RBS and the whole (i.e. cargo plus reporter segment) inserted in Km^R broad host range vectors pSEVA221 (*oriV* RK2, low copy number), pSEVA231 (*oriV* pBBR1, medium copy number) and pSEVA251 (*oriV* RSF1010, high copy number). The resulting plasmids pSEVA2214-*msfGFP*, pSEVA2314-*msfGFP*, pSEVA2514-*msfGFP* were used in the ensuing experiments along with the null-control (i.e. *msf*GFP-less) plasmid pSEVA2514 (see Materials and Methods for details). These constructs thus cover a range of gene dosages that allowed us to define optimal conditions for utilization of the cognate thermo-inducible expression system.

3.3 Performance of *c*I857/P_L expression system in *P. putida* EM42

In order to inspect the performance of the [cI857/P_L →] cargo in the genome-reduced host, *P. putida* EM42 was added with plasmids pSEVA2214-*msfGFP*, pSEVA2314-*msfGFP*, pSEVA2514-*msfGFP* as well as with pSEVA2514 (a non-fluorescent control) as explained in Materials and Methods. Once cultures reached and OD_{600} ~ 0.5, samples were split and further grown at 30 °C, 37 °C and 42 °C for the next 20 h. *msf*GFP expression of each of the resulting strains was monitored by fluorescent flow cytometry with the Results shown in Fig. 3. One important detail of the cytometry plots is that the signal output of cells bearing *msf*GFP constructs at the permissive temperature (30 °C) was indistinguishable of those carrying the *msf*GFP-less control plasmid pSEVA2514. That the background signal is below detectable levels at the lower temperature certifies the tight repression of the corresponding promoter in such conditions regardless the PCN of the vector backbone. Also shown in Fig. 3, the situation changes at 37 °C and low induction levels are detected for the three plasmids between 2 and 4 hours of incubation, with fold changes at 3 hours ranging from 1.5 to 5 compared to the basal level at 30 °C. Maximum induction at 37 °C is reached after 20 hours with average fold changes that increase with PCN: ~ 5.0 for RK2, ~ 9 for pBBR1 and ~ 20 for RSF1010 origin of replication (Fig. 4A). It was also noticeable that *msf*GFP expression at 37 °C was mono-modal, the whole bacterial population moving gradually towards higher fluorescence levels with the time of exposure.

The mild-induction scenario at 37 °C changed when cultures were taken to 42 °C. As shown in Fig. 3A, the 3 tested strains displayed high inductions of the *msf*GFP signal as early as 30 minutes after the heat-shift, with increasing fluorescent emission along time until a maximum at 3-4 h. But even at this point differences between the constructs became evident in an unexpected fashion. While levels of *msf*GFP expression were predicted to correlate with the PCN of the constructs, it turned out that the pBBR1 derivative (pSEVA2314-*msfGFP*, medium

PCN) delivered ~ 10-fold more signal than the RSF1010-based vector (pSEVA2514-*msfGFP*, high PCN). Furthermore, the low PCN plasmid (pSEVA2214-*msfGFP*, RK2 origin of replication) showed (expectedly) lower output than the medium-copy counterpart but (unexpectedly) way more than the high PCN constructs (see Fig. 4A for a summary). This pattern is kept not just in terms of induction-fold but also as absolute promoter capacity (Fig. 4B). It was also noticeable that, unlike at 37 °C, the behavior of the [cI857/P_L →] device was apparently bimodal during the first 3-4 of exposure to 42 °C in all cases, with abrupt changes between non-induced to induced states along with the persistence of a population that seems to never express the reporter gene. The proportion of cells that fail to produce a fluorescent signal grew also with time in all cases and by t = 20 h little, if any, active GFP could be detected in the 42 °C cultures. While these data pinpointed the conditions for optimal performance of the [cI857/P_L →] expression cargo in *P. putida* EM42 (medium PCN vector pSEVA2314, induction at 42 °C for 3 h), they also raised some questions on plasmid stability and strain physiology at higher temperatures, which were addressed next.

3.4 Long-term exposure of *P. putida* EM42 at 42 °C

Fig. 2B shows that despite the advantage of the genome reduced strain to do well under higher temperatures as compared to the precursor *P. putida* KT2440, culture viability decreased by half after 3 h of thermal stress. Note, however, that during the first hour of heating, cells seem to be perfectly viable. There seems to be a difference between short-term and long-term exposure to high temperature. The cytometry data at 42 °C shown in Fig. 3A may thus reflect two separate phenomena i.e. the inherent viability of the bacteria at the restrictive temperature and the stability and impact of the plasmids on the cells under the heat stress conditions. In order to inspect the relative share of the two effects on the observed behavior of the strains at stake we subjected cultures to a treatment with propidium iodide (PI) under various induction conditions. This non-permeable dye endows a bright red

fluorescence to dead cells upon DNA-binding but it is excluded from active, viable cells. After staining, bacteria were passed through the cell cytometer adjusted to detect double fluorescence (PI and GFP) in single bacteria. A boiled sample of strain *P. putida* EM42/ pSEVA2314-*msf*GFP was used as a control of no-GFP emission but full-IP binding. The second control was the sample of PI-stained cells under non-induced conditions with a nominal 100% of viability. Inspection of the double fluorescence plots at 3 hours (Fig. 5; when the 3 systems seem to be induced to their respective maxima) at that temperature indicated that the activity of the [cI857/P_L→] cargo was accompanied also by an increase of cell mortality (less in the case of bacteria with pSEVA2314-*msf*GFP). However, under these conditions, the activity of the expression module was much higher in the case of cell bearing medium PCN construct than the others. After 20 h of exposure to high temperatures, the GFP signal disappeared and the PI readout took over the whole population, suggesting that most cells were dead by then.

It is surprising that bacteria with the highest token plasmid copy number (RSF1010 replicon) gives the least transcription signal even at the optimal induction time of 3-4 h. In fact, it has been observed before that the related pSEVA254 vector (also bearing an RSF1010 origin of replication) is lost by a good share of the population when used for expression of heterologous proteins [34]. It is plausible that the poor performance of pSEVA2514-*msf*GFP can be traced to the sum of the metabolic burden of its complex replication (as compared to the other plasmids) and the thermal sensitivity of some of the proteins encoded in the replicon, which may result in a factually lower plasmid copy number. In fact, it is not infrequent that plasmids are lost at high temperatures [36-38]. Regardless of the specific mechanism, the results of Fig. 3 and 4 favor the use of the medium PCN version of the [cI857/P_L→] expression cargo for heterologous gene expression.

The second conspicuous feature of the GFP expression pattern at 42 °C in all strains under examination was the bimodal profile of fluorescent emission (Fig. 3A) even at short induction times. By 3h, the subpopulation of non-induced cells amount to roughly 10% of the whole and regardless of the specific construct. Since this figure is roughly coincident with the share of dead cells under the same conditions (Fig. 5), it is likely that the two are related and that the distribution reflects the virtually instant split of the population upon heating between cells that succumb to the stress and survivors that turn on the expression device to high levels. Moreover, the tailing of the fluorescence peaks along time up to 3 h observed in all cases could be the result of progressive cell death with incubation at the high temperature, resulting in a more heterogeneous expression of *msf*GFP at the single cell level. At the longest incubation times (20 h), most cells are non-fluorescent, surely due to massive cell death.

4 Concluding remarks

The nominal thermal sensitivity of *Pseudomonas putida* strains has traditionally discouraged the use of any of the variants of the popular cI857/P_L-R_L -based devices for engineering genetic circuits or heterologous gene expression. The main advantage of this platform is the ease of removing the induction signal after a defined period of thermal shock, as is required in a growing number of applications e.g. [16]. Although the reference strain *P. putida* KT2440 can survive short periods of exposure to 42 °C (i.e., 15-30 min; Fig. 2), we show above that its genome-reduced variant *P. putida* EM42 expands significantly its window of thermal tolerance and thus now becomes a suitable host for a standardized [cI857/P_L →] expression cargo (#14 in the SEVA nomenclature; [39]). Furthermore, the data derived from the 3 versions of the same device (low, medium and high PCN) pinpoints the conditions under which the system can be optimally used. Despite some shortcomings (e.g. long-term sensitivity of cells to 42 °C) the hereby described platform has one of the most favorable non-induced/induced ratios available for heterologous expression in *P. putida* [40]. Furthermore,

its availability as SEVA plasmids eases the reuse of the same plasmids in a variety of Gram-negative bacteria. Finally, the poor performance of the high PCN construct at 42 °C is a warning about the pursuit of the highest expression levels against the background of the host's retroactivity [41]. In any case, the work above accredits the value of combining improved hosts with matching genetic devices for the sake of building more robust Cell Factories. Both *P. putida* EM42 and vectors pSEVA2214, pSEVA2314 and pSEVA2514 are freely available through the SEVA webpage http://seva.cnb.csic.es.

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Conflict of interest

The authors declare no financial or commercial conflict of interest

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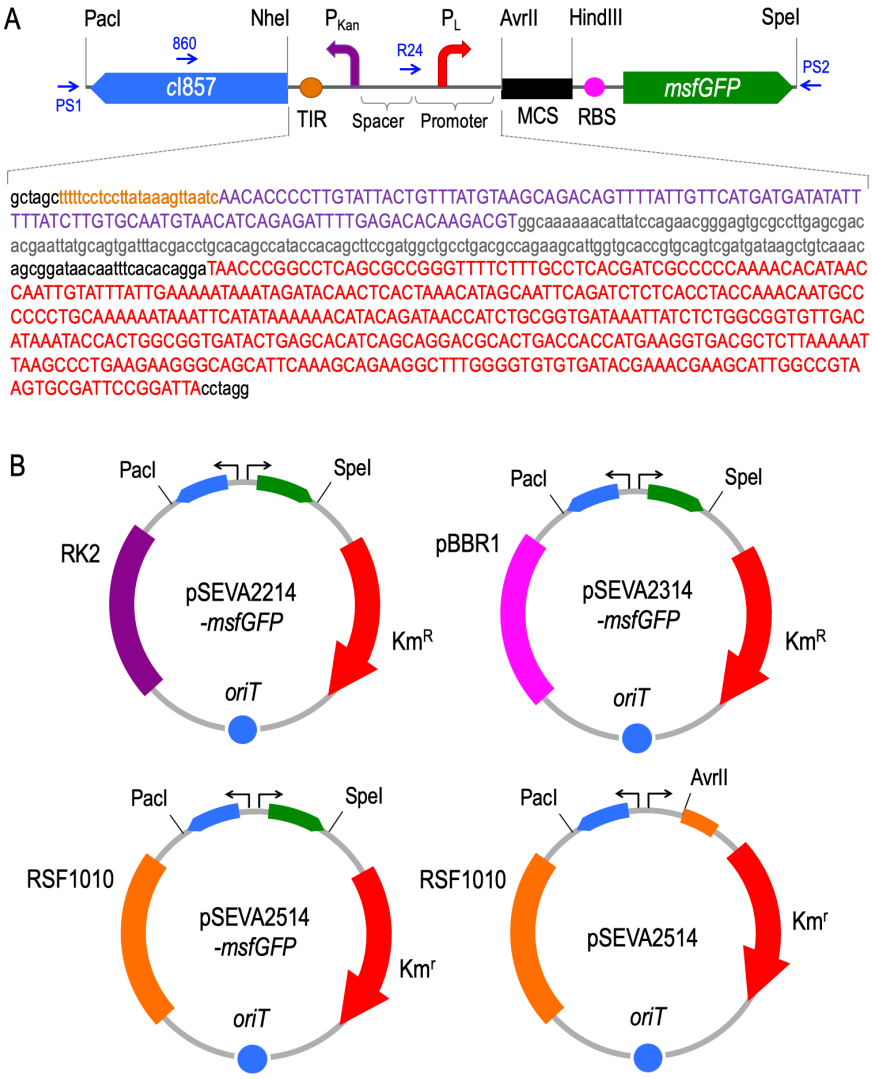
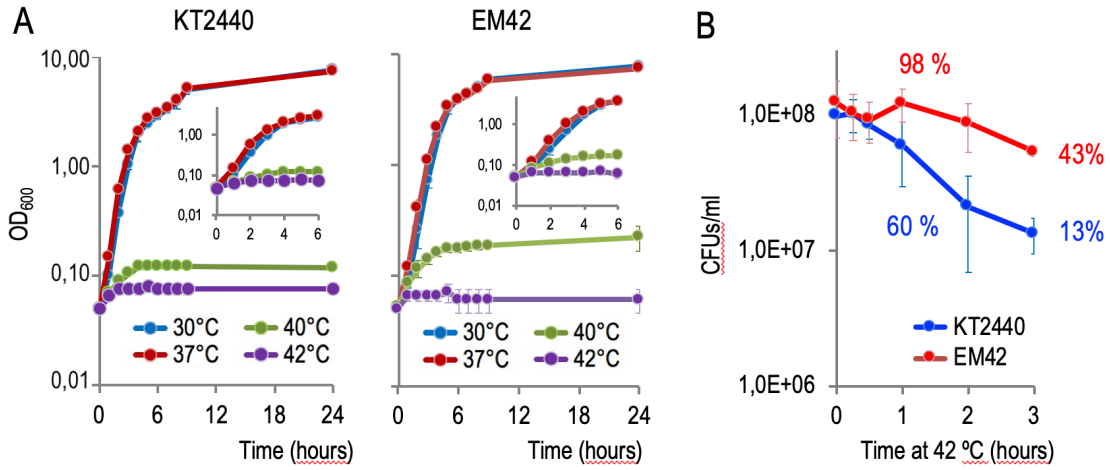
Figures

Figure 1. Arrangement of a heat-inducible system and organization of expression plasmids.



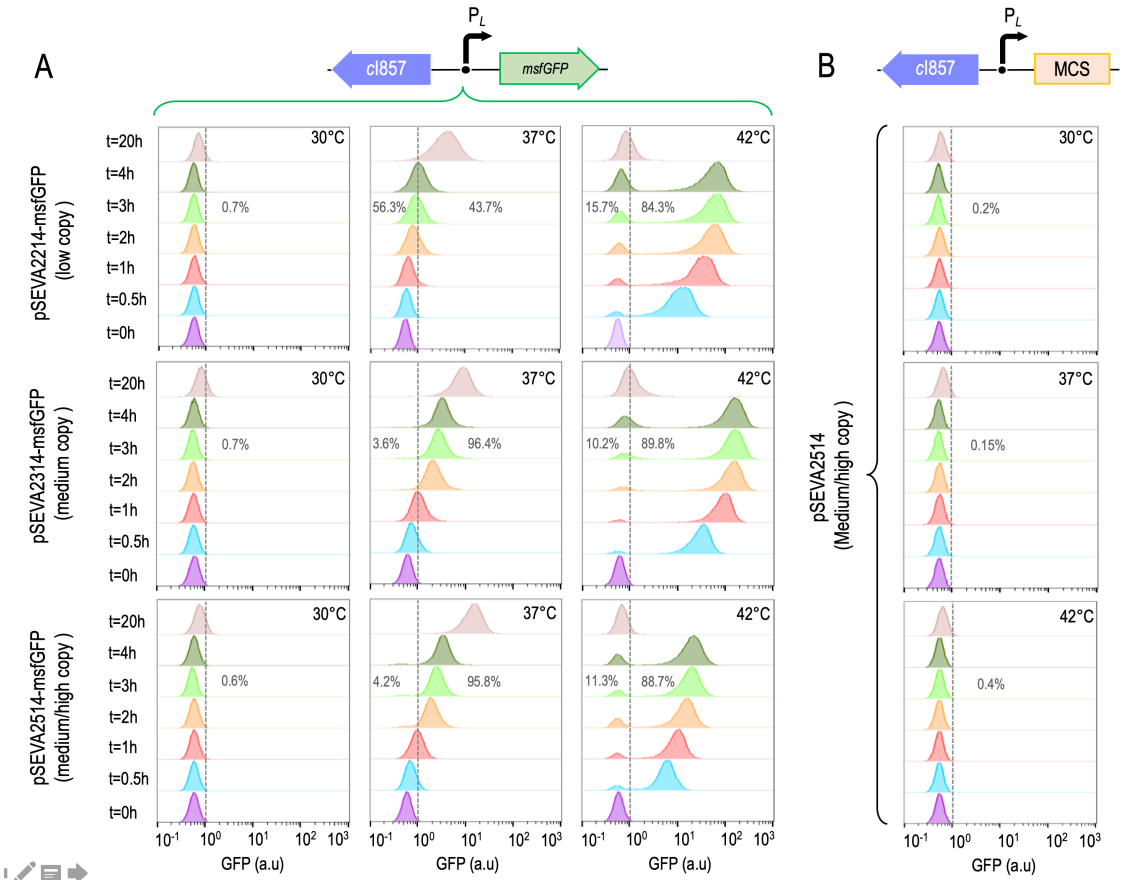
A. Components of the cI857/P_L cargo. The main parts of the genetic device for transcription of *msf*GFP are shown. Only relevant restriction sites are shown. TIR, translation initiation region (orange); P_{kan} promoter (purple); P_L promoter (red); MCS, Multiple Cloning Site (black); RBS, ribosome binding site (pink). Sites that hybridize diagnostic primers PS1, 860, R24 and PS2 are indicated. **B.** Configuration of cI857/P_L-based expression plasmids. The three *msf*GFP-harboring constructs used to characterize the expression system in *P. putida* are sketched, each one having a broad-host range replicon but different copy number. The control vector *msf*GFP-less pSEVA2514 is shown also (not to scale).

Figure 2. Effect of temperature on growth and survival of *P. putida* strains.



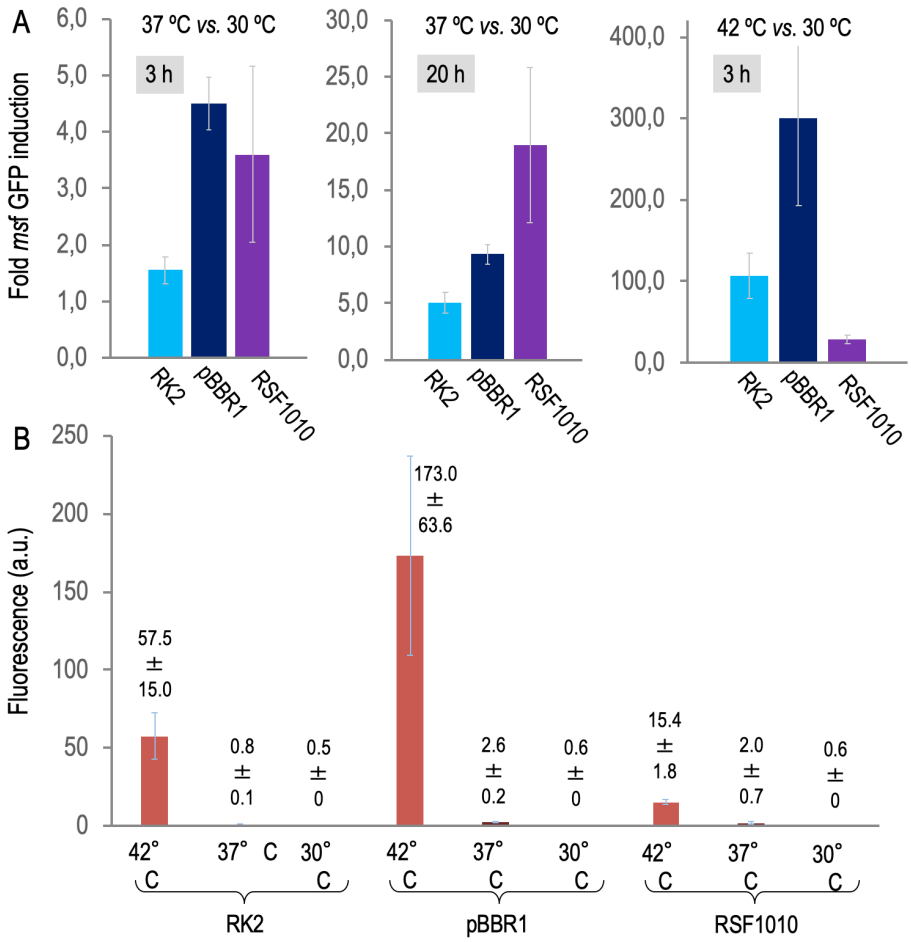
A. Growth of *P. putida* strains at different temperatures. *P. putida* KT2440 and EM42 strains were grown in liquid LB at 30°C, 37°C, 40°C and 42°C and OD_{600} were monitored for 24 hours (blowups of the first 6 h are shown as inserts). **B.** Viability of *P. putida* at high temperature. Survival at 42 °C for mid-logarithmic cultures was followed for 3 hours after the thermal shift and represented as number of viable cells per ml. Percentage survival is marked at 1 and 3 hours. The experiments shown represent the average data from two biological replicates with the standard deviation indicated.

Figure 3. Induction dynamics of the cI857/P_L device in *P. putida*.



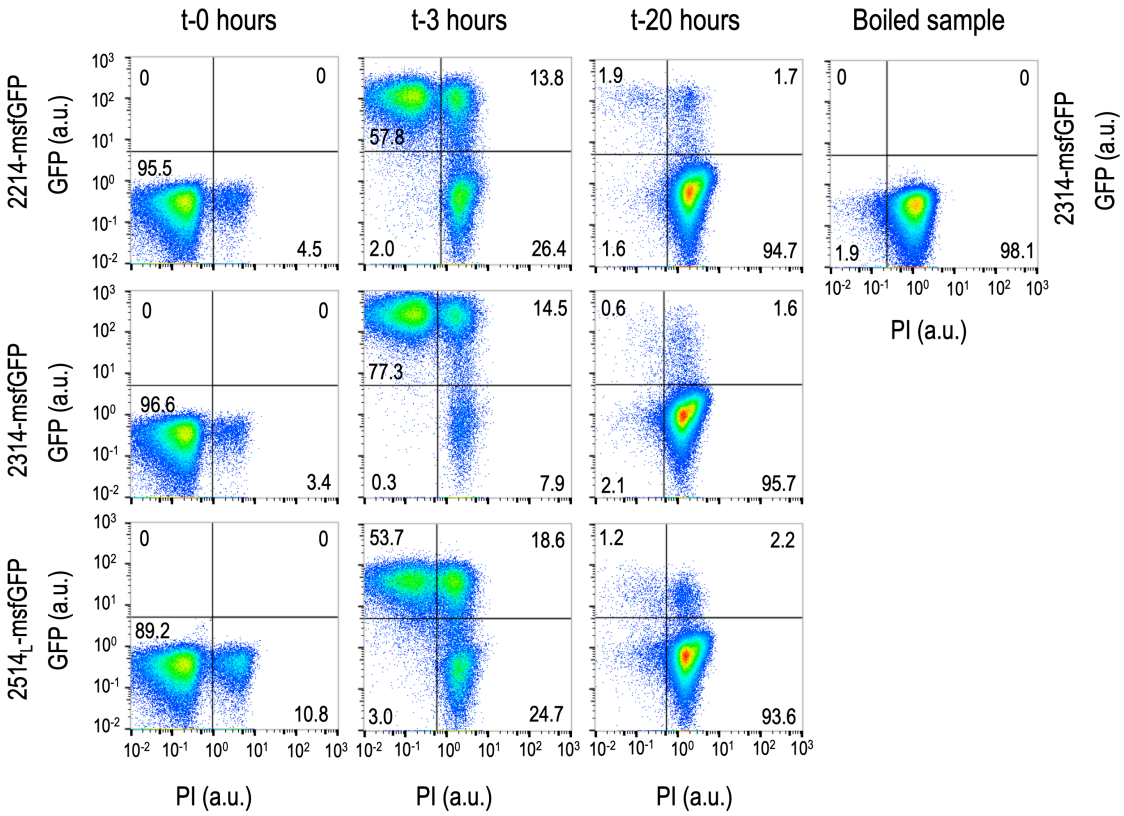
A total of 100,000 events were analyzed in each experiment. The dotted lines within the charts represent an arbitrary threshold value set to count fluorescence-positive cells. Percentages of fluorescent *vs.* non-fluorescent populations after 3 h of induction are indicated. One representative dataset of the two (virtually identical) biological replicas is shown. **A.** Flow cytometry of *P. putida* EM42 strains harboring expression plasmids under study. *msf*GFP emissions were recorded at 0, 0.5, 1, 2, 3, 4 and 20 hours of incubation at three temperatures (30 °C, 37 °C and 42 °C) for each strain. **B.** Control cytometry of *msf*GFP-less vector pSEVA2514.

Figure 4. Inducibility and capacity of the cI857/P_L device.



A. Fold induction values. Fluorescent emission levels were calculated from the cytometry data as explained in Materials and Methods. Data for pSEVA2214-*msfGFP* (RK2, low PCN), pSEVA2314-*msfGFP* (pBBR1, medium PCN) and pSEVA2514-*msfGFP* (RSF1010, high PCN) are represented for 37 °C inductions (3 h and 20 h) and for 42 °C induction (3 h). Bars indicate the mean values of the corresponding samples +/- standard deviations of two biological replicas (note that fold-change scales are different in each case). **B.** Full capacity. Raw fluorescences (in arbitrary units, a.u.) of cells bearing plasmids with the cI857/P_L expression system are shown after 3 h of induction at 30°C, 37°C and 42 °C. Some numerical values are indicated for reference.

Figure 5. Influence of high temperature in cell mortality and reporter output.



P. putida EM42 strains harboring the three plasmids under examination were incubated at 0, 3 and 20 hours at 42 °C, stained with PI (a proxy of cell mortality, see Material and Methods for more details) and analyzed by flow cytometry, recording fluorescence and PI emissions. The straight lines define four distinct sub-populations: living cells with no GFP signal (PI^-/GFP^-, left-bottom quadrant), living cells with GFP signal (PI^-/GFP^+, left-top quadrant), dead cells with GFP signal (PI^+/GFP^+, right-top quadrant) and dead cells with no GFP signal (PI^+/GFP^-, right-bottom quadrant). Percentages for each population are shown. One representative dataset of the two (virtually identical) biological replicas is shown.