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Microbial sensors for small molecules: Development of a mevalonate biosensor

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Abstract

We describe a novel biosensor strain for detection and quantification of a small molecule, mevalonate. The biosensor strain is an *Escherichia coli* mevalonate auxotroph that expresses the green fluorescent protein and reports on the mevalonate concentration in the growth medium through a change in growth rate. A model describing the growth rate dependence on mevalonate was developed in order to use the biosensor strain for high-throughput screening (HTS) and quantitative measurement of mevalonate in the extracellular environment. In general, this method should be applicable to the quantification of any small molecule for which an auxotroph can be developed and will be useful for HTS of evolved metabolic pathways for which there is no readily available screen or selection.
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1. Introduction

In recent years, an increasing number of mutagenic/combinatorial strategies capable of constructing large libraries of DNA sequences, proteins, and novel chemicals have been developed. However, these strategies are only as good as the screens used to distinguish individual library members (Kuchner and Arnold, 1997). In the case of non-chromophoric small molecule production, the screen is typically performed using chromatography, a very low-throughput method that limits the number of clones that can be screened from any given library. Therefore, new methods are needed for screening small molecules that are not readily detectable using existing high-throughput assays.

We describe here a method using a biosensor strain for detection and quantification of a small molecule, mevalonate. Mevalonate is an intermediate in the biosynthesis of isoprenoids (Khosla and Keasling, 2003; Maury et al., 2005; Mijts and Schmidt-Dannert, 2003; Martin et al., 2003), a large class of industrially important secondary metabolites that includes flavors, fragrances, anti-oxidants, steroids, and pharmaceuticals including the anti-malarial, artemisinin (Ro et al., 2006). The production of any isoprenoid in a heterologous host will require high flux through the involved biosynthetic pathways. As the precursors to isoprenoids are present in very low amounts in *Escherichia coli* and other potential heterologous hosts, these organisms must be engineered to produce large quantities of precursors. When the host is utilizing the mevalonate pathway, either natively or through heterologous expression (Martin et al., 2003), mevalonate is a key precursor whose production needs to be increased. In order to apply the available mutagenic strategies to develop this

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host, a screen for mevalonate is needed. In this report we show that a mevalonate biosensor can be used as that screen.

The biosensor strain is an engineered *E. coli* mevalonate auxotroph that reports on the mevalonate concentration in the growth medium through a change in growth rate. The auxotroph harbors the gene encoding the green fluorescent protein (Zimmer, 2002), translating growth rate into a fluorescent readout. The growth rate dependence has been modeled, and a method using the biosensor strain has been successfully evaluated for high-throughput screening (HTS) and quantitative measurement of engineered mevalonate pathway clones (Pfleger et al., 2006). In general, this method should be applicable to the quantification of other metabolites for which viable auxotrophs can be developed.

2. Materials and methods

2.1. General

The strains, plasmids and oligonucleotides used in this study are listed in Tables 1 and 2. Enzymes used in the study were purchased from New England Biolabs (Beverly, MA) and Stratagene (La Jolla, CA).

2.2. Construction of the auxotroph/biosensor

To construct the mevalonate auxotroph, a chromosomal insertion of the MBIS operon was combined with a deletion of the native *E. coli ispC* (*dxr*) gene, which encodes the 1-deoxy-D-xylulose 5-phosphate (DXP) reductoisomerase of the 1-deoxy-D-xylulose 5-phosphate (DXP) isoprenoid biosynthetic pathway. First, the MBIS operon, which contains the genes encoding the enzymes that convert mevalonate to farnesyl pyrophosphate (FPP), was inserted into the *E. coli* chromosome in *ispA* by homologous recombination (Martinez-Morales et al., 1999). The MBIS operon, including the *E. coli lac* promoter, was amplified by PCR from plasmid pMBIS using primers FMBISAscI and RMBISAscI (Operon Biotechnologies, Alameda, CA) and inserted into the *AscI* site of plasmid pLOI2224. Plasmid pLOI2224 (Martinez-Morales et al., 1999) contains the R6K conditional replicon, which requires the trans-acting π protein to replicate, and a kanamycin resistance marker flanked by FRT sites (FLP recombinase recognition sequence). The ligation product, pLOIMBIS, was screened and propagated in *E. coli* strain S17-1 (*pir*+) (Martinez-Morales et al., 1999).

Integration of MBIS into *ispA* required the activity of RecA, which is absent in *E. coli* DH10B. In order to produce this protein for the purpose of gene integration, a *recA*-containing helper plasmid was constructed from plasmid pKD46 (Datsenko and Wanner, 2000). pKD46 has a temperature-sensitive origin of replication and contains the λ Red recombinase system under control of the arabinose-inducible *araBAD* promoter (P_{BAD}). *recA* was amplified from *E. coli* MG1655 genomic DNA by PCR using primers RecA-For and RecA-Rev. The PCR product was digested and ligated into the *SacI* and *XmaI* sites of pKD46 after excising the three λ Red genes (γ, β, exo). The resulting plasmid, pDPRec, was propagated at 30 °C.

To perform the integration, *E. coli* DH10B containing pDPRec was grown at 30 °C in LB medium containing 0.2% arabinose and 100 μg/ml ampicillin to an optical density at a wavelength of 600 nm (OD_{600}) of 0.6 and made electrocompetent according to the methods of Sambrook et al. (1989). Competent *E. coli* DH10B-expressing *recA* was then transformed with pLOIMBIS, incubated at 37 °C for 2 h, and then spread onto LB agar plates containing 25 μg/ml kanamycin to select for integrants. After 48 h of incubation, kanamycin-resistant colonies were selected and

Table 1
Strains and plasmids

Strain/plasmid	Genotype/description	Source/reference
DH10B	F, mcrA, Δ(mrr-hsdRMS-mcrBC), F80lacZΔM15, ΔlacX74, recA1, endA1, araD139, Δ (ara, leu)7697, galU, galK, λ-, rpsL (StrR), nupG	Invitrogen
DP3	DH10B, ispA::P_{LAC}-(MK, PMK, MPD, idi, ispA)::ispA	This work
DP5	DH10B, ispA::P_{LAC}-(MK, PMK, MPD, idi, ispA)::ispA, Δ ispC	This work
S17-1	recA, pro hsdR RP4-2-Tc::Mu-Km::Tn7, pir+	Martinez-Morales et al. (1999)
pCP20	Temperature sensitive vector carrying flippase (FLP) recombinase	Datsenko and Wanner
pDPRec	*E. coli* recA cloned behind arabinose promoter in pKD46	This work
pBBR1MCS-3	Tc^R, Mob^+, Tra^-	Kovach et al. (1995)
pMBIS	pBBR1MCS-3 containing MK, PMK, MPD, idi, ispA under P_{LAC}; Tc^R	Martin et al. (2003)
pLOI2224	Integration vector containing FRT—KmR—FRT fragment, R6K origin	Martinez-Morales et al. (1999)
pLOIMBIS	pLOI2224 containing the MBIS operon	This work
pKD46	Temperature sensitive vector carrying the λ Red recombinase	Datsenko and Wanner (2000)
pBAD33	Cloning vector containing Cm^R, pACYC origin, *araC*, and P_{BAD} promoter	Guzman et al. (1995)
pKD13	Gene deletion vector containing FRT—KmR—FRT fragment, R6K origin	Datsenko and Wanner (2000)
pBAD33MevT	pBAD33 with *atoB*, *HMGS*, *tHMGR* from pMevT	Martin et al. (2003)
pKLN59	GFP biosensor plasmid containing, Amp^R Kan^R, *gfp*.	Newman et al. (2003)
pMevT	pBAD33 derivative containing the *atoB*, HMGS and tHMGR genes under control of P_{LAC}; Cm^R	Martin et al. (2003)

Table 2
Oligonucleotides PCR primer used in this study

Primer name	Sequence (5′–3′)
FMBISAscI	ATTGGCGCGCCCGGAGAACTGTGAATGCGCAA
RMBISAscI	TAAGGCGCGCCAGCGCGCGTAATACGACTCAC
RecA-For	GTTCGAGCTCTAAGGAGGTTATAAAAATGGCTATCGACGAAAACAAACAG
RecA-Rev	ACTCCCGGGTTAAAAATCTTCGTTAGTTTCTGC
dxr13-for	TGTCTCAACTCTGGATGTTTCATGAAGCAACTCACCATTCTGGGTGTAGGCTGGAGCTGCTTC
dxr13-rev	TCTCTGTAGCCGGATTATCCTCAGCTTGCGAGACGCATCACCTATTCCGGGGATCCGTCGACC

screened for loss of the pDPRec helper plasmid (ampicillin sensitivity). To excise the kanamycin marker and a portion of pLOIMBIS exterior to the MBIS operon, selected colonies were grown in LB medium with 10 μg/ml kanamycin, made electrocompetent and transformed with pCP20 (Datsenko and Wanner, 2000), a temperature-sensitive plasmid containing the FLP recombinase under thermal control. Cells were plated on LB agar containing 16 μg/ml of fosmidomycin (known inhibitor of IspC) and 2 mM mevalonate to screen for functional MBIS insertions. Colonies that grew on the selective agar were screened for loss of the kanamycin resistance marker and pCP20 by antibiotic sensitivity, and chromosomal integrants were confirmed by PCR amplification of the gene encoding mevalonate kinase from the MBIS operon. The exact location of the integrated MBIS operon in the resulting strain DP3 was confirmed by a series of PCR reactions that amplified integration junctions.

To disrupt the native isoprenoid pathway in DP3, the *ispC* gene was deleted using the λ Red recombinase system described by Datsenko and Wanner (2000). The PCR product for gene inactivation was amplified from pKD13 (Datsenko and Wanner, 2000) using primers dxr13-for and dxr13-rev, and the resulting mutants were selected for and propagated on medium containing 2 mM mevalonate. The *ispC* knockout was confirmed by PCR and by the inability of the strain to grow without mevalonate. The resulting auxotrophic strain (DP5) was transformed with pKLN59 (Newman et al., 2003) for constitutive expression of *gfp* to make the biosensor strain.

2.3. Plate spraying/testing

To prepare a solution of the biosensor for spraying on plates, the biosensor strain was grown overnight in LB medium and 2 mM mevalonate. The culture was centrifuged at 1000*g* for 10 min at 4 °C, the supernatant was removed, and the cells were re-suspended in fresh LB medium (no antibiotics or mevalonate) to an OD_{600} of 0.3. The dilute biosensor culture was kept on ice until spraying.

Overnight cultures of the mevalonate producer strains (*E. coli* DH10B containing pBAD33MevT) were also centrifuged at 1000*g* for 10 min and re-suspended in fresh LB medium. The cell suspensions were either spotted directly onto LB agar plates with antibiotics in 1 μL aliquots or diluted to approximately 500 CFU/ml and then spread onto LB agar to isolate individual colonies. The dilute culture of the mevalonate biosensor was applied to plates containing the mevalonate producer cells by airbrush (paasche Airbrush V-SET) in a sterile laminar flow hood. Prior to application, the airbrush was flushed with 70% ethanol, followed by sterile LB medium to remove the remaining ethanol. The biosensor cells were carefully sprayed onto individual agar plates using helium at 20 psig as the carrier gas. Multiple passes were made with the airbrush to ensure consistent coverage over each plate. After application of the biosensor cells, the sprayed plates were incubated at 37 °C.

2.4. Growth studies using the biosensor

To determine the growth dependence of the auxotrophic biosensor for mevalonate, a solution of D,L-mevalolactone (Sigma) converted to mevalonate (Martin et al., 2003) was added to biosensor cells and cell growth was assayed. Increasing concentrations of D,L-mevalonate from 50 μM to 10 mM were added to 200 μL of biosensor cells diluted to an OD_{600} of 0.02 in C medium (Helmstetter and Cooper, 1968; Pfleger et al., 2006) supplemented with 3.4% glycerol, 1% Casamino acids (Difco), micronutrients and 25 μg/ml kanamycin. The OD_{600} was monitored for 48 h using a Tecan Safire 96-well plate reader (Maennedorf, Switzerland).

2.5. Mevalonate production assays

Cultures for the mevalonate production assays were started from individual colonies of fresh transformants grown on LB containing 0.1% glucose to repress P_{BAD}. The various producer strains were grown overnight in C medium containing 0.06% glucose, diluted to an OD_{600} of 0.02 in fresh medium without glucose and grown for an additional 24 h in the presence of inducers. The cultures were then centrifuged to remove the cells, and the amount of mevalonate in the spent medium was measured in triplicate with biosensor cells inoculated to an OD_{600} of 0.02 in cultures containing 5% and 0.5% (vol/vol) spent medium. The OD_{600} and GFP fluorescence were monitored over the next 48 h in a 96-well plate reader. The growth rates were determined for each well by determining the slope of the growth curves plotted logarithmically. The growth rates from the two dilutions of spent medium were

used to determine the mevalonate concentration via an inhibited growth model and a standard curve for D,L-mevalonate. The resulting average mevalonate concentrations were normalized for the difference in mevalonate standards and divided by two to account for the fact that the biosensor strain (mevalonate kinase) only metabolizes R-mevalonate.

2.6. GC–MS measurement of mevalonate

The mevalonate concentration of producing strains was also determined by GC–MS analysis. Briefly, 560 μL of *E. coli* culture was mixed with 140 μL of 500 mM HCl in a glass GC vial to convert mevalonate to mevalonic acid lactone. Ethyl acetate (700 μL) was added, and the samples were vortexed for 3 min. Mevalonic acid lactone from ethyl acetate extracts were analyzed by GC–MS (Agilent Technologies Inc., Palo Alto, CA) using a DB-5 column and helium carrier gas. The temperature cycle was 70 °C for 2 min, followed by a gradient of 20 °C/min to 150 °C, a second gradient of 15 °C/min to 250 °C, a final gradient of 50 °C/min to 300 °C and a hold at 300 °C for 2 min. The resolved samples were analyzed by selected ion monitoring with an Agilent 5973 mass selective detector that monitored ions 71 and 58 m/z. The retention time and mass spectrum of the extracted mevalonic acid lactone were confirmed using commercial D,L-mevalonic acid lactone (Sigma).

2.7. Z-scores

Z-scores (Zhang et al., 1999) were calculated to determine the usefulness of the biosensor as a screen. Cultures in 96-well plates were grown in parallel in the presence of 0, 200, or 700 μM mevalonate. The average (μ) and standard deviation (σ) of the OD_{600} and GFP fluorescence were calculated at each time point and the Z and Z' scores were calculated using the following formula:

$$Z' = 1 - \frac{(3\sigma_{c+} + 3\sigma_{c-})}{|\mu_{s+} - \mu_{c-}|}, \quad Z = 1 - \frac{(3\sigma_{s} + 3\sigma_{c})}{|\mu_{s} - \mu_{c}|},$$

where σ is the standard deviation and μ the mean of the OD_{600} or fluorescence value. C+ is the positive control, C− the negative control, C the control, and S the sample. The Z′ score used measurements of the positive (700 μM mevalonate) and negative (0 μM mevalonate) controls to evaluate the range of the measurement. The *Z*-score used the limits of the linear range of the screen (200 and 700 μM mevalonate) to determine if samples could be reliably distinguished.

2.8. Screening production cultures

The biosensor strain was tested on a library of mevalonate-producing clones containing combinatorial libraries of tunable intergenic regions (Pfleger et al., 2006). The initial library was enriched for functional clones by transforming DP5 cells with the plasmid library. The surviving clones were pooled, and the isolated plasmids from this collection of cells were transformed into DH10B for screening. Individual colonies from the enriched library were grown in 96-well plates in the presence of 0.2% arabinose and 34 μg/ml chloramphenicol. After 24 h of growth, the cells were removed by centrifugation and the supernatant was probed for mevalonate using the biosensor. The biosensor cultures were set up by adding 10 μL of the supernatant media and 10 μL of a 1:10 dilution of the supernatant to 190 μL of C-medium containing 50 μg/ml kanamycin. The dilutions were set up in order to place the mevalonate concentration generated by the original vector at the bottom of the linear range so that improved versions would fall in the linear region of the model for at least one dilution. The two dilutions were required to discern samples that fell into the inhibited regime of the model. The fluorescence of each well was measured periodically using a Typhoon laser scanner (Amersham Biosciences) so that multiple plates could be scanned simultaneously. The fluorescence of each well was compared to the wells representing the original vector. Internal controls and replicate biosensor cultures from the same producing culture were used to determine the significance of the improved wells.

3. Results and discussion

3.1. Construction of a mevalonate biosensor

An *E. coli* mevalonate auxotroph was constructed for use as a biosensor to measure mevalonate concentrations and identify improved producers from libraries of mutants. *E. coli* was first engineered to convert exogenous mevalonate to isopentenyl pyrophosphate (IPP), dimethylallyl pyrophosphate (DMAPP) and FPP by employing a hybrid pathway containing both heterologous genes from *Saccharomyces cerevisiae* and genes native to *E. coli*. An operon containing the last five genes in the mevalonate pathway (*ERG12* encoding mevalonate kinase (*S. cerevisiae*), *ERG8* encoding phosphomevalonate kinase (*S. cerevisiae*), *MVD1* encoding mevalonate pyrophosphate decarboxylase (*S. cerevisiae*), *idi* encoding IPP isomerase (*E. coli*), and *ispA* encoding FPP synthase (*E. coli*)), called MBIS (Martin et al., 2003), all under the control of the *lac* promoter (P_{lac}), was integrated into the chromosome by recombination (Martinez-Morales et al., 1999). Instead of employing an extra gene as a "leader" sequence for recombination, recombination was allowed to occur between one of the native *E. coli* genes contained in the operon and the chromosomal copy. Strains with MBIS integrated into either chromosomal *idi* or *ispA* were isolated. However, recombination of the operon with chromosomal *idi* left the last gene of the operon under *idi*'s native promoter and not P_{lac}. Recombination of the operon at the chromosomal *ispA* (strain DP3) retained the integrity of the MBIS operon under P_{lac} control (Fig. 1).

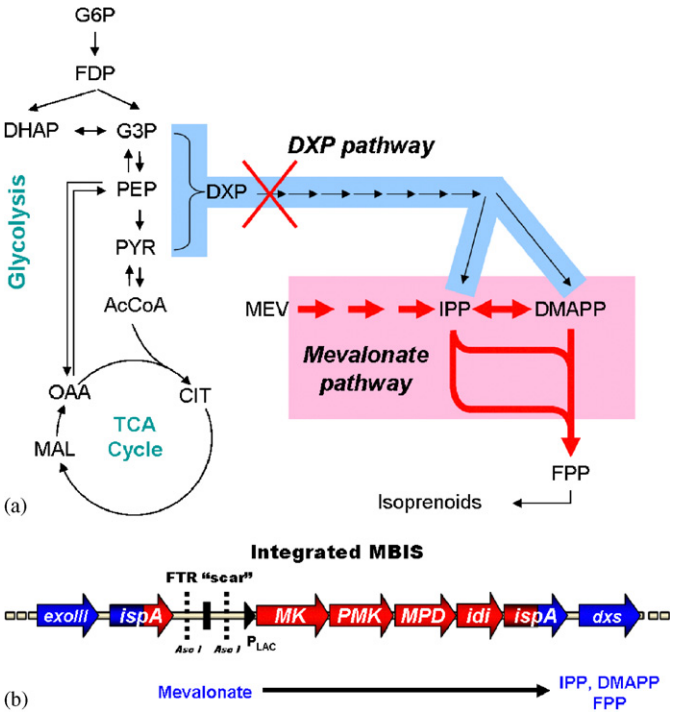

Fig. 1. Construction of the mevalonate biosensor. (a) Biosynthesis of isoprenoids in the mevalonate biosensor. The native DXP pathway (blue box) was eliminated by deleting the second gene in the DXP pathway, *ispC*. The last three enzymes from the mevalonate pathway (MK, mevalonate kinase; PMK, phosphomevalonate kinase; and MPD, mevalonate pyrophosphate decarboxylase, pink box) as well as the IPP isomerase (*idi*) and FPP synthase (*ispA*) were integrated into the chromosome allowing for exogenous mevalonate (MEV) to be converted to isoprenoids. (b) Organization of the MBIS operon integrated into the chromosome at the native *ispA*. The "red" genes originated from the operon genes that have been integrated into the "blue" chromosomal genes. The cross-over occurred in *ispA*, so that a portion of each copy is from the integrated operon with the remainder coming from the chromosomal copy.

The mevalonate auxotroph was constructed by disrupting *E. coli*'s native DXP isoprenoid biosynthetic pathway (Hoeffler et al., 2002; Proteau, 2004), so that the cell cannot produce IPP and DMAPP, both of which are necessary for growth (Fig. 1a) (Hoeffler et al., 2002; Kuzuyama, 2002; Proteau, 2004). Specifically, the *ispC* (*dxr*) gene was deleted from DP3 using λ Red recombination (Datsenko and Wanner, 2000). The *ispC* gene encodes the subunits of the DXP reductoisomerase, which converts DXP to 2-C-methyl-D-erythritol-4-phosphate in the first committed step of the DXP pathway (Kuzuyama et al., 2000). Flux through the DXP pathway and cell growth can be restored upon addition of 2-methyl-erythritol (Takahashi et al., 1998). Alternatively, IPP and DMAPP can be produced and cell growth restored by the integrated mevalonate pathway upon the addition of mevalonate, as in strain DP5.

To construct the mevalonate biosensor strain, DP5 cells were transformed with pKLN59 (Newman et al., 2003), an ampicillin- and kanamycin-resistant plasmid carrying a constitutively expressed *gfp*. The addition of this plasmid permits the monitoring of growth via fluorescence and eliminates the contribution of any contaminating non-sensing strains. The mevalonate biosensor strain was first tested for its utility in plate assays. In these assays, cultures containing the recombinant mevalonate operon (described below) under control of the arabinose-inducible promoter (on plasmid pBAD33MevT) were grown in the absence of inducer to mid-log phase and spotted onto plates that had been aseptically sprayed with the biosensor culture (Newman et al., 2003). When these strains were induced upon plating, mevalonate was produced and exported from the cell. The extracellular mevalonate was consumed by the lawn of surrounding auxotrophic cells, which grew and produced GFP. Mevalonate was spotted onto one sector of the plate as a positive control for growth of the auxotrophic biosensor strain (Fig. 2b) and this spot produced a visible green circle of biosensor cells. These fluorescent spots (Fig. 2b and 2c) were readily distinguished from spots where the producing strains were not induced (Fig. 2a), i.e., where there was no mevalonate.

To increase the number of individual clones that could be tested on a single plate, mevalonate-producing cultures were plated at a dilution that would ensure discrete single colonies. Then, the plate was sprayed with the biosensor auxotroph (Fig. 2d and 2e). The plates were grown for 2 days before the colonies were imaged with a Leica DM400B microscope. The results demonstrate that colonies producing mevalonate (induced with arabinose, Fig. 2e) are distinguishable from colonies that produce no mevalonate (uninduced, Fig. 2d). Thus, by spraying replica plates with the biosensor, this technique is applicable for qualitatively determining mevalonate production from a library of producing strains.

3.2. Mevalonate measurement

Quantitative discrimination of mevalonate production was simplified by working in liquid culture. Because the biosensor cells could not produce IPP and DMAPP using the DXP pathway, the growth of the biosensor cells depended on the concentration of mevalonate in the medium. The growth rates of cells growing in media containing different concentrations of mevalonate were determined by fitting an exponential curve to the OD_{600} measurements between 0.2 and 0.8. The Monod model (Blanch and Clark, 1996) fit the growth rate data up to approximately 700 μM mevalonate (Fig. 3 top). However, above this range, the growth rates saturated and eventually decreased with increasing mevalonate concentrations. Instead, the data was fit to a substrate-inhibited Monod model (Shuler and Kargi, 1992):

$$\mu = \frac{\mu_{\mathrm{Max}} S}{K_S + S + K_I S^2}.$$

Parameters were derived from a quadratic fit of the data plotted as the specific growth rate/[mevalonate] versus [mevalonate] (Fig. 3 bottom). We suspect that substrate

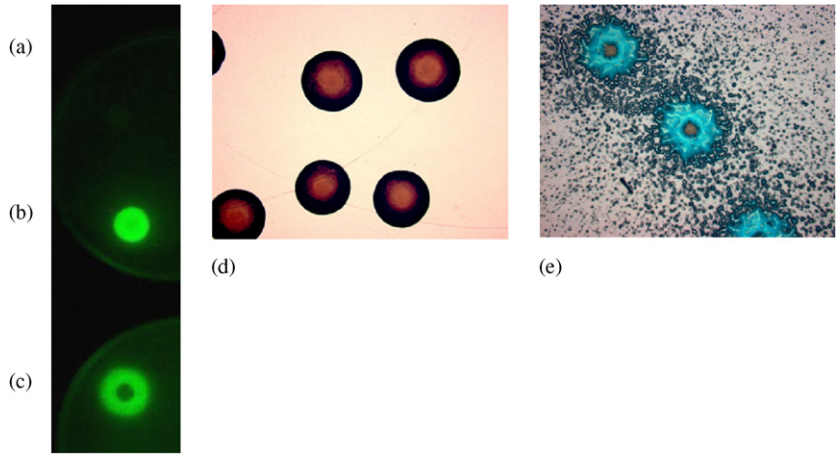

Fig. 2. Biosensor detection of mevalonate on solid media. (a–c) Cultures containing mevalonate biosynthetic genes (pBAD33MevT) were spotted (1 μL) onto plates without (a) or with (c) arabinose and aseptically sprayed with the biosensor (mevalonate auxotroph). Mevalonate was also spotted (b) as a positive control. Only those spots containing mevalonate (b and c) fluoresced. (d–e) Agar plates without (d) or with (e) arabinose spread with *E. coli* containing pBAD33MevT were aseptically sprayed with biosensor and imaged with both visible and epi-fluorescent light sources after 2 days of growth. The small, red dots (mevalonate-producing cells) in the center of colonies in (e) are the same cells that formed the red colonies in (d). These micrographs show that individual colonies of mevalonate-producing cells can be distinguished from those that do no produce mevalonate.

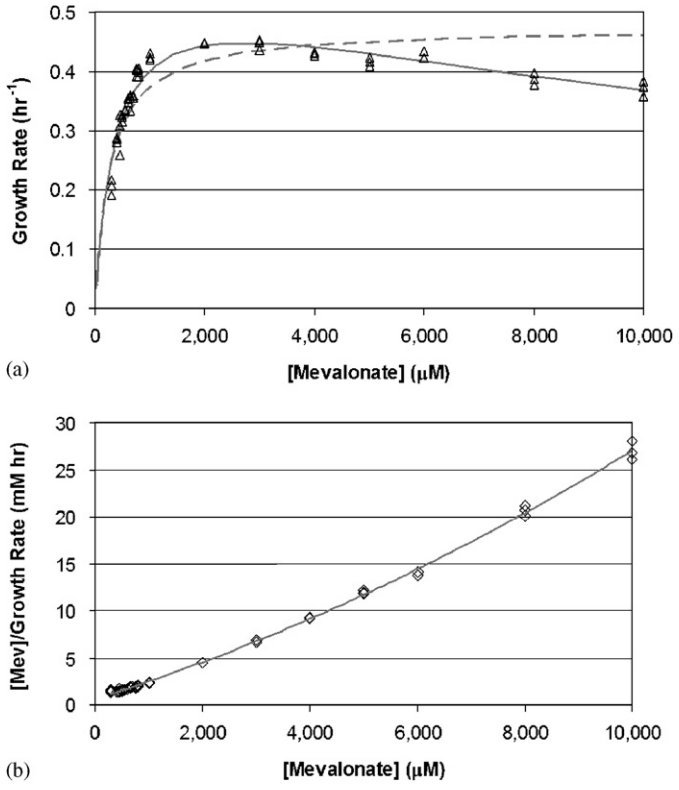

Fig. 3. Effect of mevalonate concentration on biosensor growth rate. Growth curves were used to determine the exponential growth rates of the biosensor between OD_{600} 0.2 and 0.8. (top) Growth rate dependence on mevalonate. The dashed line is a Monod model fit to the data. The solid line is the inhibited model fit to the same data. (bottom) Quadratic fit of the growth rates to mevalonate concentration.

inhibition was caused by elevated levels of IPP and FPP due to the increased flux through the integrated MBIS pathway. Prior work with the mevalonate pathway genes demonstrated a similar growth inhibition caused by elevated levels of the prenyl pyrophosphate intermediates (Martin et al., 2003).

Recently, a strain of *E. coli* was engineered with the complete mevalonate pathway for increased production of isoprenoids, specifically amorphadiene, the sesquiterpene precursor to the anti-malarial drug artemisinin (Ro et al., 2006; Martin et al., 2003). This strain harbors two plasmids expressing the genes of the mevalonate pathway. The first plasmid, pMBIS, harbors the genes that encode enzymes to transform mevalonate to FPP, which were necessary to construct the mevalonate auxotroph described above. The second plasmid, pMevT, harbors the first three genes of the mevalonate pathway (*E. coli*'s *atoB* encoding acetoacetyl-CoA thiolase, *S. cerevisiae*'s *HMGS* encoding HMG-CoA synthase and *tHMGR* encoding a truncated HMG-CoA reductase). As a test, the biosensor strain was used to measure the mevalonate production from a series of strains harboring variations of the MevT operon (Pfleger et al., 2006). Extracellular mevalonate concentrations were determined using both the biosensor and GC–MS. For the biosensor strain, the growth rates of three biosensor cultures were used in conjunction with the inhibited growth model whose parameters were derived from a standard curve run in parallel with the assays. For the GC–MS analysis, the peak area was interpolated into a standard curve made from the same mevalonate standard. The measurements obtained by the two methods were nearly identical (Fig. 4) except for a small systemic overestimation of the mevalonate concentration by the biosensor. This may be due to artifacts in the preparation of the standards used by the biosensor strain. For instance, if the mevalolactone were not completely converted to mevalonate prior to analysis, the biosensor output would be lower than if all of the mevalolactone had been converted to mevalonate. This error would lead to the overestimation of mevalonate concentrations in the samples. Despite the

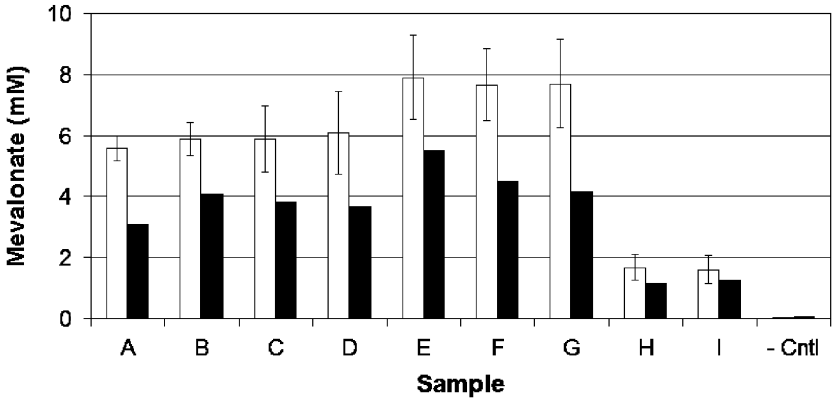

Fig. 4. Comparison of mevalonate measurements by the biosensor and by GC–MS. Unfilled bars represent the biosensor measurements, with error bars determined from measurements of the three wells of the same sample. Filled bars represent mevalonate measurement from ethyl acetate extractions of each sample quantified by GC–MS. Each sample (A–H) was generated from a different mevalonate-producing sample isolated from the libraries described by Pfleger et al., 2006. Sample represents the original producer, pBAD33MevT. Cntl refers to the empty vector pBad33.

error, the relative concentrations of the ten samples were in agreement between the two measurement techniques, indicating that the biosensor assay can be used to discriminate mevalonate concentrations between samples.

3.3. Biosensor screening of mevalonate-producing libraries

To increase the throughput beyond a single 96-well plate, the green fluorescence of the biosensor strain was assayed using a laser scanner, which permitted quick comparison of many 96-well plates at once. This measurement technique makes the biosensor very amenable to HTS. The biosensor strain was tested as a candidate for HTS by determining its Z-score (Zhang et al., 1999). Z-scores combine the effects of precision and signal-to-noise into one parameter and have been used to evaluate the usefulness of high-throughput screens. Plots of Z'-scores (comparing the positive and negative controls) for the biosensor assay continually increased over the first few hours and eventually settled on a value of approximately 0.9 after 15–20 h of growth (Supplementary Fig. S1, left). The Z-score (comparing the limits of differential growth, 200–700 μM mevalonate) approached a maximum value of 0.9 for the OD_{600} measurements (0.7 for fluorescence) when the 200 μM sample began to show significant growth around 26 h (Supplementary Fig. S1, right). These results fell into the "excellent assay" category used by the developers of the parameter (Zhang et al., 1999). The Z-scores also demonstrated that libraries should be screened before the lowest control well begins to grow in order to maximize the ability of the screen to discern improved samples.

A library of mevalonate producers generated by varying the sequence of the intergenic regions of the mevalonate pathway operon MevT was used as a test of the biosensor method (Pfleger et al., 2006). Prior to screening, the library was enriched for mevalonate-producing strains by transforming the library into DP5, which could not grow without a functional MevT pathway. Plasmids from the surviving strains were prepared and transformed into DH10B for mevalonate production (Section 2). The spent media of the MevT library clones were analyzed using the biosensor (Fig. 5). Mevalonate standards (spiked into wells) and the original producer, pBAD33MevT, were used to normalize data across the different plates. Two sets of replicates were used on each plate to estimate the standard deviation of the producing cells. A threshold was then set at three times the average standard deviation (0.04 RFU) of all replicates. Of the more than 600 colonies screened in this manner, 18% were significantly more fluorescent than the original vector used as a control (more than three standard deviations greater, calculated from two sets of triplicate controls on each plate). This large percentage can be partially attributed to the prescreen selection where clones that did not produce sufficient mevalonate for auxotroph survival were not enriched. At the other end of the spectrum, nearly 50% of the samples did not fluorescence significantly above background after 24 h of biosensor growth. The screen identified four strains that produced seven-fold more mevalonate than the original strain (Martin et al., 2003). Future libraries can be screened by altering the dilutions of spent media used to inoculate the biosensor cultures such that the best identified producer falls at the bottom of the range. With an average standard deviation of 0.04 from this library, the screening methodology should be able to discern a 10% improvement with 95% confidence, making the screen ideal for most directed evolution experiments.

4. Conclusions

Laboratory evolution is an extremely important technique for optimizing the function of metabolic pathways. However, in the absence of a high-throughput screen, it may be difficult or impossible to screen through the many mutants that would be generated using highly mutagenic strategies. We have developed a microbial biosensor strain for small molecules that allows relatively high throughput

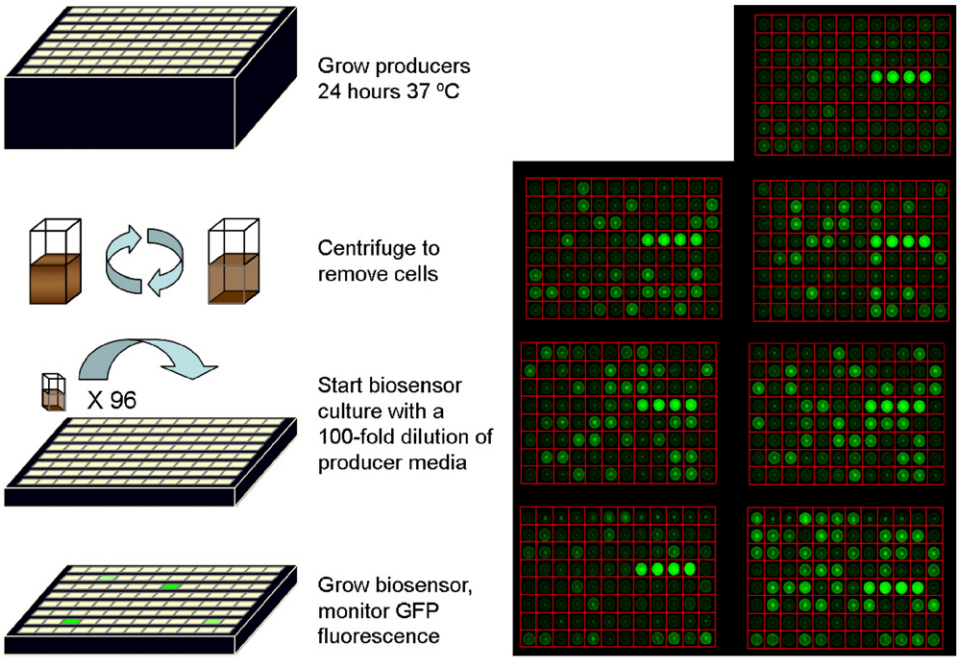

Fig. 5. Mevalonate biosensor screening strategy. (a) Screening methodology. Mevalonate producers were grown in 96-well format under standard conditions. Producer cells were removed by centrifugation, and the spent medium was passed to new cultures inoculated with the biosensor. Wells containing the most mevalonate were the most fluorescent. (b) Mevalonate library. Seven 96-well plates are shown 15 h post biosensor inoculation. The four intense wells on each plate are mevalonate controls.

and accurate screening of many mutants. Not only is this screen simple, it is relatively inexpensive to implement, not requiring the expensive equipment often used with most HTS procedures. Using auxotrophic strains as both biosensors and selection filters should enable the screening of larger libraries in order to identify strains that produce even larger quantities of mevalonate. In the future, we envision similar biosensors being developed for other molecules that can be imported into the cell and metabolized.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymben.2006.08.002.

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