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Authors

Kirby, James

Nishimoto, Minobu

Chow, Ruthie WN

et al.

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Use of Nonionic Surfactants for Improvement of Terpene Production in *Saccharomyces cerevisiae*

James Kirby,[a,b] Minobu Nishimoto,[a,b] Ruthie W. N. Chow,[a] Venkata N. Pasumarthi,[a] Rossana Chan,[a] Leanne Jade G. Chan,[b] Christopher J. Petzold,[b] Jay D. Keasling[a,b,c,d]

California Institute of Quantitative Biosciences (QB3), University of California, Berkeley, Berkeley, California, USA[a]; Joint BioEnergy Institute, Emeryville, California, USA[b]; Department of Chemical Biomolecular Engineering, University of California, Berkeley, Berkeley, California, USA[c]; Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, Berkeley, California, USA[d]

To facilitate enzyme and pathway engineering, a selection was developed for improved sesquiterpene titers in *Saccharomyces cerevisiae*. α-Bisabolene, a candidate advanced biofuel, was found to protect yeast against the disruptive action of nonionic surfactants such as Tween 20 (T20). An experiment employing competition between two strains of yeast, one of which makes twice as much bisabolene as the other, demonstrated that growth in the presence of T20 provided sufficient selective pressure to enrich the high-titer strain to form 97% of the population. Following this, various methods were used to mutagenize the bisabolene synthase (BIS) coding sequence, coupled with selection by subculturing in the presence of T20. Mutagenesis targeting the BIS active site did not yield an improvement in bisabolene titers, although mutants were found which made a mixture of α-bisabolene and β-farnesene, another candidate biofuel. Based on evidence that the 3′ end of the BIS mRNA may be unstable in yeast, we randomly recoded the last 20 amino acids of the enzyme and, following selection in T20, found a variant which increased specific production of bisabolene by more than 30%. Since T20 could enrich a mixed population, efficiently removing strains that produced little or no bisabolene, we investigated whether it could also be applied to sustain high product titers in a monoculture for an extended period. Cultures grown in the presence of T20 for 14 days produced bisabolene at titers up to 4-fold higher than cultures grown with an overlay of dodecane, used to sequester the terpene product, and 20-fold higher than cultures grown without dodecane.

Terpenes, being a large family of natural products with interesting biological and chemical properties, have found a multitude of applications ranging from flavors and fragrances to medicines and advanced biofuels (1, 2). Microbial production presents an attractive route for industrial terpene biosynthesis, as evidenced by the successful engineering of yeast to provide a stable supply of the antimalarial drug artemisinin (2–4). We have recently investigated the sesquiterpene olefin α-bisabolene (Fig. 1) as a candidate biodiesel, achieving moderate titers through expression of a codon-optimized bisabolene synthase (BIS) gene from *Abies grandis* in an engineered strain of *Saccharomyces cerevisiae* (5). Our ability to further increase bisabolene titers through engineering of metabolic pathways or individual enzymes is most limited by the growth and product quantitation phase of this iterative process. To facilitate high-throughput engineering of metabolic networks, pathways, and enzymes for improvement of terpene titers, we undertook a search for a chemical agent that could act as a selection agent for higher sesquiterpene levels in yeast cells.

Considering that sesquiterpenes such as bisabolene are predominantly cell associated in yeast when cultured in the absence of an organic overlay such as dodecane (6), we reasoned that an altered membrane composition may result in an increased tolerance of membrane-disrupting agents. Modification of yeast sterol or fatty acid composition has led to differences in growth rates and has also altered susceptibility to drugs or ethanol as a result of changes in membrane fluidity (7–10). Of various groups of candidates evaluated as agents for selection of higher bisabolene levels in *S. cerevisiae*, nonionic surfactants were found to be the most promising.

Although surfactants may be used to improve extraction of olefins and pigments from microbial cultures, their use as a selective agent has not to our knowledge been investigated previously (11, 12). To initially evaluate candidates, we utilized two strains of *S. cerevisiae*, one of which makes twice as much bisabolene as the other, initially comparing growth levels of these strains in the presence of inhibitory concentrations of nonionic surfactants. Tween 20 (T20) was the most promising of the surfactants tested and is this focus of this work, but other candidates such as Brij 35 were also found to be effective. A proof-of-concept study was undertaken to investigate if T20 could be used to enrich for a strain that makes more bisabolene in a mixed population. Following this, we targeted the bisabolene synthase gene from *A. grandis*, taking various approaches to improve the kinetics or stability of the enzyme though mutagenesis with a goal of enhancing bisabolene production in yeast.

In addition to demonstrating that T20 could be used as a selection agent for higher terpene production in a mixed population, we also investigated whether it might be used to improve production titers as well as strain stability in a monoculture. We considered that an agent which selects for the metabolic product of an

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J.K. and M.N. contributed equally to this article.
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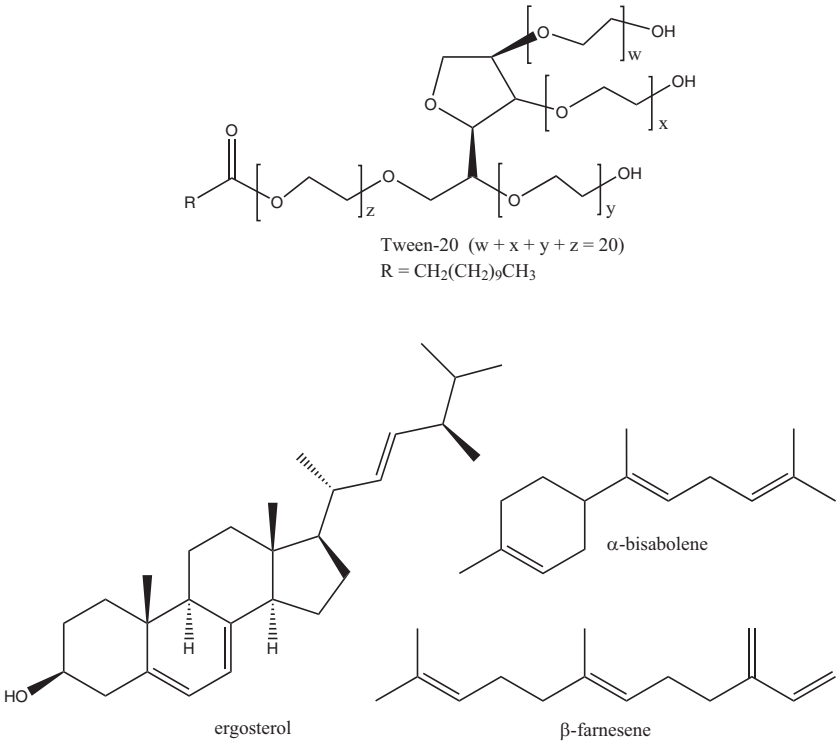

FIG 1 Structures of the nonionic surfactant, Tween 20, yeast membrane component, ergosterol, and sesquiterpenes α-bisabolene and β-farnesene.

engineered pathway, rather than just for the biosynthetic genes, may help to address strain stability issues associated with the larger-scale and longer-duration industrial production process. Overall, the use of nonionic surfactants such as T20 presents an attractive approach for both pathway and enzyme engineering and also for stabilizing and enhancing industrial production.

MATERIALS AND METHODS

Strains and growth conditions. Strains described here can be found in the Joint BioEnergy Institute Strain Registry (https://public-registry.jbei.org/) and are available upon request. All work was performed in EPY300 (JBEI-3343), a strain of *S. cerevisiae* BY4742 engineered for overproduction of the sesquiterpene precursor farnesyl pyrophosphate (FPP), described previously (13). For bisabolene production, EPY300 was transformed either with plasmid pRSLeu2d-BISopt (JBEI-2922), which harbors the codon-optimized *Abies grandis* bisabolene synthase gene under the control of the GAL1 promoter (5), or with pESCLeu2d-BISopt (JBEI-2951), which harbors the same gene under the control of the GAL10 promoter. pESCLeu2d-BISopt was constructed by cloning the *A. grandis* BISopt coding sequence (cds) between the SpeI and SacI sites of plasmid pESCLeu2d (JBEI-2951) (14).

Synthetic defined (SD) media were made following manufacturers' directions but containing twice the normal amount of Difco yeast nitrogen base (Becton, Dickinson & Co., Sparks, MD) and Complete Supplemental Mixture (CSM; MB Biomedicals, Solon, OH). Engineered yeast strains were maintained and grown on SD medium lacking the amino acids methionine, histidine, and leucine throughout. For growth under noninducing conditions, SD contained 2% (wt/vol) D-glucose as the carbon source (SD-glu). Bisabolene production was induced by inoculating an SD-glu preculture into SD plus 0.2% D-glucose plus 1.8% D-galactose (D-gal) at a starting optical density at 600 nm (OD_{600}) of 0.03 to 0.05.

Measurement of bisabolene and ergosterol. For strains grown in the presence of a dodecane overlay, bisabolene production was measured by sampling the dodecane followed by quantitation by gas chromatography-mass spectrometry (GC-MS) as described previously (6). Bisabolene was extracted from yeast cultures that were grown in the absence of dodecane by adding an equal volume of ethyl acetate (EtOAc) to a sample of culture, vortex mixing horizontally at top speed for 5 min, and centrifuging at top speed in a microcentrifuge for 5 min at room temperature. The presence of T20 made no difference to the efficiency of bisabolene extraction from whole culture. Bisabolene was extracted from yeast cells by briefly centrifuging a 0.7-ml aliquot of culture, resuspending cells in 0.7 ml fresh medium, and extracting with an equal quantity of EtOAc as described above. Ergosterol was extracted from cells and quantified by GC-MS as described previously (15).

Growth of mixed populations in the presence of T20. Experiments employing competition between strains pRS (EPY300 harboring pRSLeu2d-BISopt) and pESC (EPY300 harboring pESCLeu2d-BISopt) were performed as follows. The two strains were grown in SD plus 2% (wt/vol) D-galactose (SD-gal) to similar ODs and then mixed together in a ratio of equal amounts and inoculated at a 1:100 dilution into either SD-gal or SD-gal containing 12% (vol/vol) Tween 20 (SD-gal-T20). When cultures reached early to mid-log phase, they were subcultured at 1:100 dilutions into their respective media. Subculturing was repeated one more time, and a sample from each mixed culture was diluted at mid-log phase and plated onto SD-glu agar plates to yield around 400 colonies per plate. Colony PCR using a forward primer matching the GAL1 promoter and a reverse primer matching the CYC terminator was then used to differentiate the strains, yielding a 2.6-kb product for the pRS strain and a 0.4-kb product for the pESC strain. Three separate competition trials were performed.

Construction and screening of mutant BIS libraries. The initial approach for mutagenesis of the *Abies grandis* bisabolene synthase (BIS), based on an evaluation of various directed evolution strategies (16, 17), was to target individual amino acids within 5 Å of the active site (FPP and Mg^{2+} binding sites) using the recently elucidated BIS crystal structure (18). The list of candidate amino acids was narrowed from 26 to 19 resi-

dues following alignment of the BIS protein sequence with the 35 most similar proteins in GenBank (sesquiterpene and monoterpene synthases) and elimination of highly conserved residues (for example, aspartates from the DDxxD motif). Primers were designed and utilized to individually mutagenize the 19 amino acid targets following the QuikChange method (Agilent Technologies, Santa Clara, CA), replacing each amino acid codon with the sequence NNK (see Table S1 in the supplemental material). Mutant libraries were transformed into *Escherichia coli* XL10-Gold Ultracompetent cells (Agilent Technologies), and the quality was assessed by sequencing the BIS cds from a sample of clones. Plasmid was prepared from the remaining mutants by pooling colonies and was subsequently transformed into EPY300 using the high-efficiency lithium acetate method (19).

We undertook four additional approaches for mutagenesis of BIS: combinatorial active-site mutagenesis, substitution of hydrophobic surface residues, error-prone PCR, and chemical mutagenesis. These methods are described in Materials and Methods in the supplemental material.

Following transformation of EPY300 with plasmid libraries, yeast colonies were pooled and grown for 16 h in SD containing 0.2% D-glucose and 1.8% D-galactose to allow acclimation to galactose and induction of bisabolene production. Libraries were then subcultured in SD-gal-T20 (containing 12% [vol/vol] Tween 20) at early- to mid-log phase (generally between an OD of 0.2 and an OD of 1.0), starting each new culture at an OD of around 0.03. Average production over the course of T20 subcultures was monitored by extraction of a sample of culture with an equal volume of EtOAc and analysis by GC-MS as described above, and a diluted sample was plated onto SD-glu agar in order to measure bisabolene production in individual strains. Trials were also conducted at lower concentrations of T20, but 12% T20 provided the optimal level of selective pressure (judged by the average bisabolene production trend), while levels above 12% were too growth inhibitory.

Plasmid was isolated from strains that produced high titers of bisabolene by a modified version of the QIAprep Spin Miniprep kit protocol (Qiagen, Valencia, CA). Following centrifugation of 1.5 ml of culture and resuspension of cells in 250 μl of P1 buffer, 250 μl of P2 buffer was added along with enough 0.5-mm-diameter acid-washed glass beads to reach the surface of the liquid. Cells were disrupted by bead beating for 1 min, and, following completion of the plasmid Miniprep procedure according to the manufacturer's protocol, 1 μl was transformed into *E. coli* to propagate plasmids. Plasmids were sequenced to identify any mutations present and retransformed into EPY300 in order to measure bisabolene production independently from any genomic mutations that might have occurred during growth on T20.

Procedures for targeted proteomics and plasmid stability determination are described in Materials and Methods in the supplemental material.

RESULTS

Tween 20 can be used as a selective agent for bisabolene production in *S. cerevisiae*. In order to evaluate the utility of nonionic surfactants as a selective agent for higher bisabolene titers in yeast, we used two similar strains that differ only in their capacities for bisabolene production. Both strains are derived from EPY300, a strain of *S. cerevisiae* BY4742 in which the mevalonate pathway was engineered for enhanced farnesyl pyrophosphate (FPP) production (13). Strain pRS contains the *Abies grandis* bisabolene synthase (BIS) gene under the control of the galactose-inducible GAL1 promoter in plasmid pRSLeu2d-BISopt (JBEI-2922) (5), while strain pESC contains the same gene under the control of the GAL10 promoter in plasmid pESCLeu2d-BISopt (JBEI-2951).

We initially compared growth levels of the pESC strain in media containing various detergents at concentrations of 4%, 7%, and 10% (vol/vol). While Tween 80, Triton X-100, Brij 58, and Tergitol NP-40 had little effect on growth at concentrations of up to 10% (vol/vol), Triton X-114 had a moderate effect and Tween 20 and Brij 35 effectuated the strongest growth inhibition (data not shown). Following this, we selected T20 to investigate whether a growth differential would be observed between strains that produce different amounts of bisabolene.

Strain pRS made about twice as much bisabolene as pESC when grown in SD-gal, synthetic defined medium containing 2% (wt/vol) galactose, which served both as the carbon source and as the inducer of BIS expression (Fig. 2A). While these two strains normally grow at about the same rate in SD-glu and SD-gal, pRS exhibited an appreciably shorter lag phase than pESC when grown in SD-gal containing 12% (vol/vol) T20 (SD-gal-T20) (Fig. 2B). Since the T20 cultures shown in Fig. 2B were inoculated directly from a glucose-grown preculture into SD-gal-T20, we found that the length of the lag phase in T20 could be reduced by preinducing bisabolene production with galactose prior to exposure to T20. All subsequent experiments were performed by acclimating cells to galactose before inoculation into T20 media. When grown under noninducing conditions (in SD-glu), production of bisabolene was minimal and neither strain was found to grow in the presence of 12% T20 (data not shown).

Together, these results indicate that growth in the presence of high concentrations of T20 is facilitated by production of bisabolene. To investigate whether this difference in growth rates could be exploited as a selection method, we performed experiments employing competition between the two strains. Equal amounts of pRS and pESC cells, taken from SD-gal cultures that were grown to similar mid-log-phase ODs, were mixed together and then used to inoculate fresh media (either SD-gal or SD-gal-T20). Following three subcultures in their respective media, cells were plated on agar and colony PCR was performed to differentiate the pRS and pESC strains. In three trials conducted in this manner, the lower-titer pESC strain was marginally more abundant in the population after subculturing in the SD-gal medium, while the pRS strain was dominant (97% ± 2%) after growth in SD-gal-T20 (Fig. 2C).

Mechanism of Tween 20 resistance. It was clear that T20 could enrich for a strain that makes more bisabolene from a mixed population. We considered two likely mechanisms for this selection: either selection on an individual-cell level based on changes in membrane fluidity as a result of bisabolene accumulation or a more global effect based on the interaction of secreted bisabolene with T20 in the medium. A possible mechanism for the latter option could be via reduction of the cloud point of T20 by interaction with bisabolene released from the cells; a reduction in cloud point temperatures for nonionic surfactants has been demonstrated by the addition of hydrocarbons such as isopentanol and octanol (20). To investigate the cloud point theory, cultures of the pRS strain were set up in SD-gal and SD-gal-T20. As expected, the addition of T20 to the culture reduced the growth rate, but exogenous addition of bisabolene to the culture at concentrations around the range produced by the pRS strain did not alleviate the growth inhibition caused by T20 (Fig. 3A). On the other hand, measurement of the cell-associated bisabolene levels in both the pRS and pESC strains grown in the presence of T20 revealed that the pRS cells accumulated 3-fold more bisabolene, while ergosterol levels were not substantially different between the two strains (Fig. 3B). Together, these findings indicate that the mechanism for increased T20 tolerance in the pRS strain is due to cell-associated rather than exogenous bisabolene. Since the mechanism is local to the individual cell, we next explored the prospect of using T20 as a

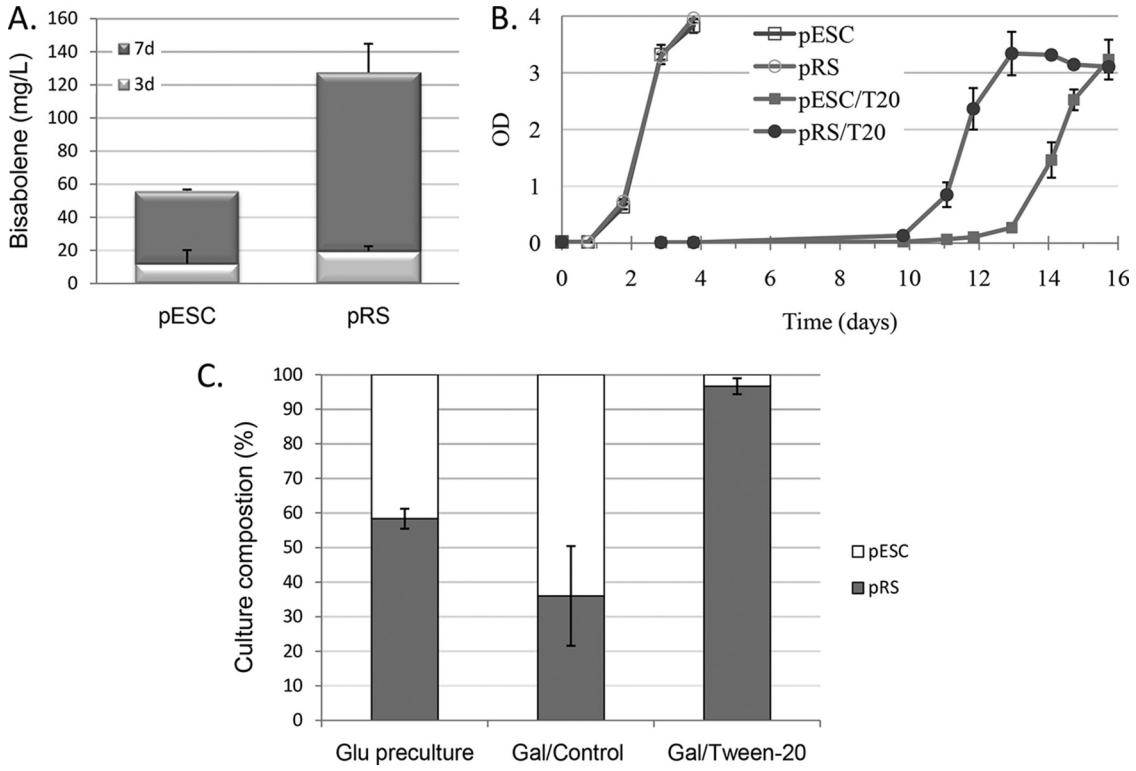

FIG 2 Proof-of-concept studies to evaluate T20 as a selective agent for improving bisabolene production in yeast. (A) Bisabolene production in pESC and pRS grown in SD-gal. (B) Growth of yeast strains pESC and pRS in the presence or absence of T20. (C) Composition of a mixed pRS/pESC culture prior to selection (Glu preculture) and following three subcultures in SD-gal (Gal/Control) and SD-gal-T20 (Gal/Tween 20).

selective agent for screening mutant libraries for improved bisabolene production.

Construction and screening of mutant BIS libraries. Since switching the BIS promoter from GAL10 to GAL1 increased bisabolene production 2-fold (comparing the pESC strain to the pRS strain), it seemed a reasonable assumption that BIS is a limiting factor in the yeast bisabolene biosynthetic pathway. This is in agreement with unpublished data on bisabolene production in yeast together with findings that, due to their relatively slow kinetics, terpene synthases are commonly found to be the limiting enzymes in terpene biosynthetic pathways (21–23).

We took several different approaches to mutagenizing the BIS cds in order to generate diverse libraries for selection on T20. A total of 19 amino acids close to the BIS active site (see Table S1 in the supplemental material) were individually targeted for saturation mutagenesis. In addition, we targeted four amino acids from the BIS active site for combinatorial mutagenesis (see Table S2 and Fig. S1 in the supplemental material). As an alternative to active-site mutagenesis, based on evidence that the majority of BIS protein in *S. cerevisiae* is insoluble (unpublished data), we targeted hydrophobic amino acids on the enzyme surface, hypothesizing that the protein may have been aggregating in the cell due to hydrophobic interactions (see Table S3 in the supplemental material). Solvent-exposed hydrophobic amino acids have been previously linked to protein insolubility (24, 25). We also took two random mutagenesis approaches, error-prone PCR targeting the BIS cds and chemical mutagenesis of the BIS plasmid DNA.

Each library was subcultured in SD-gal-T20 (usually containing 12% Tween, although we also ran trials at lower concentrations) for up to a month. Average production in the library was monitored by extraction of bisabolene from an aliquot of culture and quantitation by GC-MS. At each subculture, an aliquot was diluted and plated onto agar to generate colonies for bisabolene quantitation in a selection of individual strains from the population over the course of the experiment. Figure 4 shows the result of one representative trial. In this case, the combined site-directed mutagenesis libraries targeting individual active-site residues and hydrophobic surface residues were subcultured nine successive times in the presence of 12% T20 for a total of 24 days. Average bisabolene production in the mixed population was observed to peak in cultures 6 and 7 (Fig. 4A). A total of 40 individual colonies were selected from plates inoculated from the initial preculture which contained no T20, and another 40 colonies were picked from plates derived from cultures 6 and 7. Bisabolene production in isolates from the preculture was less than 60 mg/liter for all but three strains and was strongly biased toward negligible or zero titers, perhaps not surprisingly, since many active-site mutants abolish or significantly reduce catalytic activity (Fig. 4B). In contrast, the 40 isolates from the sixth and seventh cultures all produced more than 60 mg/liter bisabolene and titers in the majority of cases were close to the levels expected for the pRS strain containing the wild-type (wt) BIS gene (Fig. 4C).

Plasmids were isolated from strains producing the highest titers of bisabolene, and, following sequencing of the BIS expression cassette (and, in some cases, the origin of replication and selection marker), the plasmids were transformed into fresh EPY300 cells in order to eliminate any influence from chromosomal mutations that may have taken place during

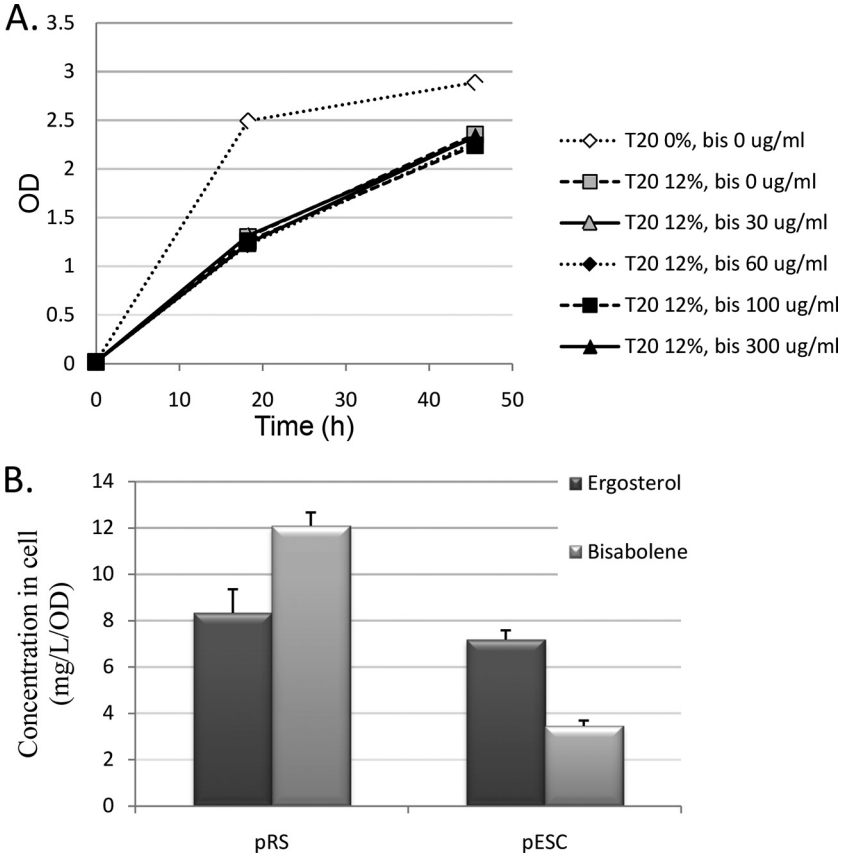

FIG 3 Mode of action of bisabolene-mediated T20 resistance in yeast cultures. (A) Growth of pRS strain in the absence or presence of T20; exogenous addition of bisabolene to the culture does not alleviate growth inhibition by T20. (B) Ergosterol and bisabolene concentrations in pRS and pESC cells grown in SD-gal-T20.

growth under conditions of selective pressure. In many repeated trials of this procedure using the various mutant libraries, we did not find any BIS mutants which enabled higher bisabolene titers in EPY300; in fact, all plasmids recovered at the end of the selection contained the wt BIS cds. Since it was clear that T20 acted as a selection agent for higher bisabolene production, eliminating or reducing low-titer strains, we considered the possibility that the level of bisabolene produced in the pRS strain might be optimal for survival in the presence of T20 or that BIS may no longer be a limitation in this strain.

Following this logic, we constructed similar libraries in the pESCLeu2d-BISopt plasmid since bisabolene production in the pESC strain was demonstrated to be suboptimal for growth in T20, having been quickly outgrown by the pRS strain in mixed cultures (Fig. 2C). A similar pattern was observed when mutant libraries in the pESC backbone were screened, in that subculturing in the presence of Tween 20 significantly reduced the proportion of zero- or low-bisabolene producers in the population (see Fig. S2 in the supplemental material). We also tested Brij 35 as a selective agent for these libraries and found that this surfactant worked at least as well as T20 in terms of population enrichment. However, the outcome was also the same in both cases—following recovery of plasmids isolated from the best bisabolene producers isolated postselection, and transformation into fresh EPY300 cells, all resulted in bisabolene titers matching the original pESC strain, and all contained the wt BIS sequence.

Screening BIS libraries with alternative 3′ coding sequences. Since the selection did not uncover BIS mutants in either the pRS or the pESC backbone, we considered it a strong possibility that amino acid substitutions that improve BIS kinetics or stability are rare and that there may be little room for improvement in titers using the approaches we had used thus far. In order to address this possibility, we undertook a different approach. A previous study has shown that total protein levels for a BIS-FPPS fusion expressed in *S. cerevisiae* were significantly higher than those for BIS alone; bisabolene production was also improved 30% to 60% by using the fusion (6). One of the hypotheses that we considered to explain this was that the 3′ end of the BIS-FPPS fusion mRNA may be more stable than that of BIS alone. RNA folding software (mfold and RNAfold) predicted a more stable (lower minimum free energy) structure for the fusion, substantiating this theory.

Based on this hypothesis, we constructed a library of BIS 3′ mutants in the pRS backbone by randomizing the DNA sequence encoding the last 20 amino acids of BIS (using a degenerate PCR primer; see Table S4 in the supplemental material), keeping the protein sequence unchanged. In this case, selection in the presence of Tween returned several promising mutants which appeared to produce more bisabolene than the wt BIS. Isolation of these plasmids and retransformation back into the EPY300 host strain followed by measurement of bisabolene production alongside the wt pRS plasmid revealed that the best of these, BIS-91 from the sev-

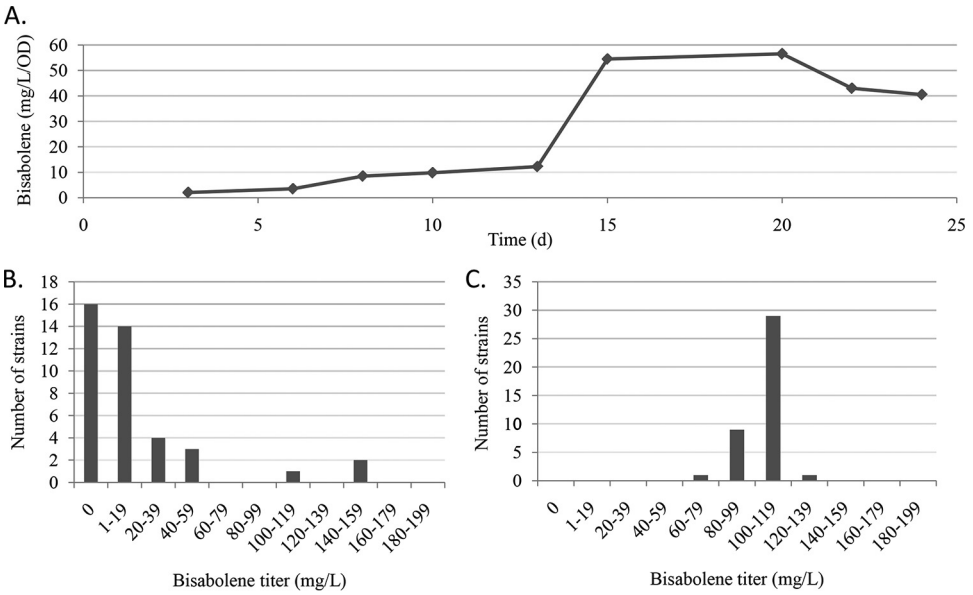

FIG 4 (A) Average bisabolene production in a library of site-directed BIS mutants (targeting active-site and hydrophobic-surface residues) over 24 days of subculturing in SD-gal-T20 (12% T20). The nine points correspond to extracts from the mixed culture, sampled before subculturing. (B and C) Distribution of bisabolene production titers across 40 individual strains isolated preselection (B) and from the sixth and seventh cultures postselection (C).

enth Tween 20 subculture, enabled a 30% improvement in bisabolene-specific production (Fig. 5A).

No additional mutations were found in the plasmid other than the recoded C-terminal 20 amino acids, indicating that the increased production was a result of altered mRNA stability (Fig. 5B). Minimum free energy predictions using RNAfold and mfold were around 50% lower for the 3′ sequence of BIS-91 (consisting of the last 65 nucleotides [nt] of the cds combined with the first 65 nt of the 3′ untranscribed region [UTR]) than for the corresponding region of BIS-wt. Proteomic analysis of EPY300

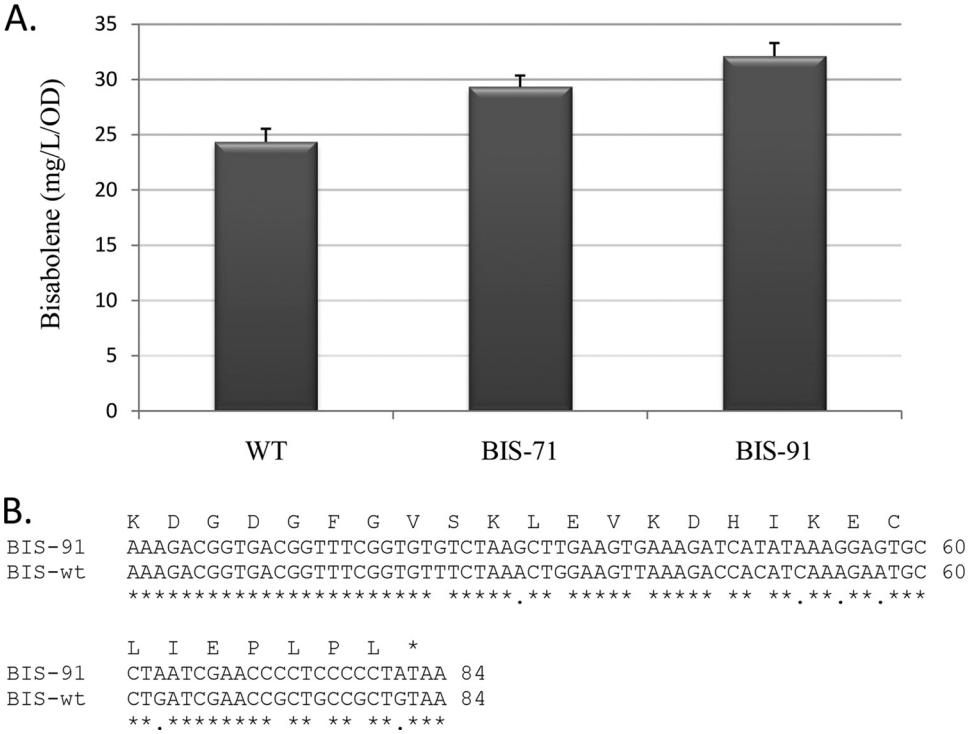

FIG 5 Analysis of recoded BIS mutants, isolated by selection in T20. (A) Bisabolene-specific production in EPY300 containing either the original pRS plasmid (WT) or plasmids isolated from a library of alternative BIS 3′ coding sequences following selection in T20 (BIS-71 and BIS-91). (B) Sequence alignment of the BIS-91 sequence with the original (wt) BIS sequence; the protein sequence is unchanged.

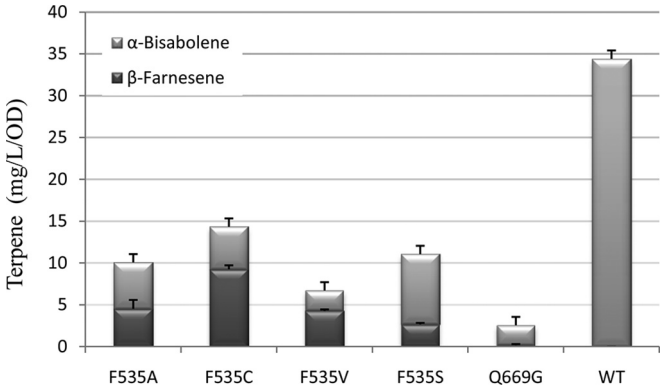

FIG 6 Terpene (α-bisabolene and β-farnesene) production in EPY300 strains harboring one of the five BIS active-site mutants or the original pRS plasmid (WT).

BIS-wt and BIS-91 SD-gal cultures harvested at 6 days revealed 2-fold-higher BIS protein levels in the BIS-91 cells, substantiating the notion that the alteration in mRNA led to higher mRNA stability and/or translation (see Fig. S3 in the supplemental material).

Isolation of mutants with altered reaction products. During comparison of the pre- versus postselection pRS active-site mutant libraries, we identified five mutants which produced a mixture of α-bisabolene and β-farnesene. All five of them were recovered from the preselection culture, and none were isolated postselection. Four of the mutations were at the F535 locus (changed to A, C, V, and S), and the fifth was Q669G. Transformation of the mutant plasmids alongside the wt plasmid into EPY300 demonstrated that total sesquiterpene production titers resulting from these mutations were lower than those obtained with the wt pRSLeu2d-BISopt plasmid (Fig. 6). This reduction in titers is not unexpected in terpene synthases with altered product profiles (26) and likely explains the fact that none of the mutants were enriched during selection. A terpene synthase that made a mixture of bisabolene and farnesene might be of interest from a biofuels perspective if production titers were improved. Farnesane, the fully reduced form of the linear sesquiterpene farnesene, is being pursued as an alternative biosynthetic diesel (27).

Tween 20 enhances bisabolene titers and production stability in yeast. Since T20 acts as a selective agent for higher bisabolene titers in a mixed population, we investigated whether it might also improve production titers in a monoculture. We reasoned that selective pressure for product formation may facilitate increased genetic and metabolic stability and enable longer production runs than those seen with selection for maintenance of heterologous DNA alone. Initial experiments performed by growing the pRS strain in culture tubes demonstrated that addition of T20 significantly enhanced bisabolene production titers. Bisabolene titers at the end of 7 days were up to 6-fold higher (296 mg/liter) in T20 cultures than in cultures with no additive and were 2-fold higher than in cultures with a 20% dodecane overlay used to sequester bisabolene, while specific production was enhanced around 12-fold and 3-fold, respectively (see Fig. S4 in the supplemental material).

To test these hypotheses on a larger scale for an extended period, we grew pRS cultures in 250-ml shake flasks containing 50 ml SD-gal with or without either a 10% (vol/vol) dodecane overlay or various concentrations of T20, and we used these cultures at the end of a 7-day production cycle to inoculate a fresh set of flask cultures which were grown for another 7 days. Bisbabolene titers and specific production were both enhanced by the addition of T20 at the end of the first 7 days, but the effect was even more marked at the end of 14 days (Fig. 7). Bisabolene titers dropped sharply by the end of 14 days in cultures grown without additives or with a dodecane overlay (11 or 55 mg/liter, respectively) whereas titers in cultures supplemented with 3% T20 were still relatively high (207 mg/liter). Specific production was also around 4-fold higher in cultures grown with T20 (40 mg/liter/OD) than in those grown with a dodecane overlay and around 20-fold higher than in cultures grown without additives.

Since all cultures were grown in media lacking appropriate amino acids to select for maintenance of the EPY300 chromosomal modifications and the pRSLeu2d-BISopt plasmid, these data suggest that T20 enhances titers, particularly for extended production cycles, by acting as a more direct selection agent to maintain production of the terpene product. We tested plasmid stability, one of several factors that contribute to the robustness of an engineered strain, in these cultures. The pRSLeu2d-BISopt plasmid was maintained at a higher rate in cultures containing 3% Tween (85% of cells contained the plasmid at the end of the 14 days) than in cultures containing dodecane (50%).

DISCUSSION

Production of terpenes in engineered microbes such as *S. cerevisiae* offers an attractive means for industrial production of a wide range of useful compounds from flavors and fragrances to biofuels and medicines (1). Significant improvements in yeast terpene titers have been achieved over the last decade through metabolic-engineering approaches, particularly as assembly of DNA constructs has become faster and easier (3). There is, however, a scarcity of tools available for high-throughput screening or selection of improved strains. An FPP analog which releases methanol upon enzyme-mediated cyclization has been used as a synthetic substrate to successfully increase the thermostability of a sesquiterpene synthase (22). This approach reduces processing time for screening a mutant library but still requires lysis and assay of each mutant on an individual basis.

A selection was developed in a strain of *E. coli* engineered to overproduce the 5-carbon terpene precursors isopentenyl pyrophosphate and dimethylallyl pyrophosphate, based on the premise that high levels of these prenyl-phosphate precursors inhibit growth (28). Screening of a *Bacillus subtilis* genomic DNA library in this strain resulted in the discovery of two isopentenol synthases through mitigation of the prenyl-phosphate-mediated growth inhibition. A similar approach should in theory be viable to screen for improved sesquiterpene production in yeast if the strain accumulates growth-inhibitory levels of the FPP precursor. That approach, however, may not be sufficiently sensitive to distinguish modest increments in sesquiterpene levels; indeed, we found that the two strains investigated here, pESC and pRS, although differing by around 2-fold in sesquiterpene titers as a result of differences in BIS expression levels, grew at the same rate in standard media. In fact, the pESC strain, which produces less bisabolene, was slightly dominant after the two strains were mixed and subcultured twice under inducing conditions (Fig. 2C). In contrast, the surfactant-based selection described here demonstrated that the 2-fold difference in levels of bisabolene content between the

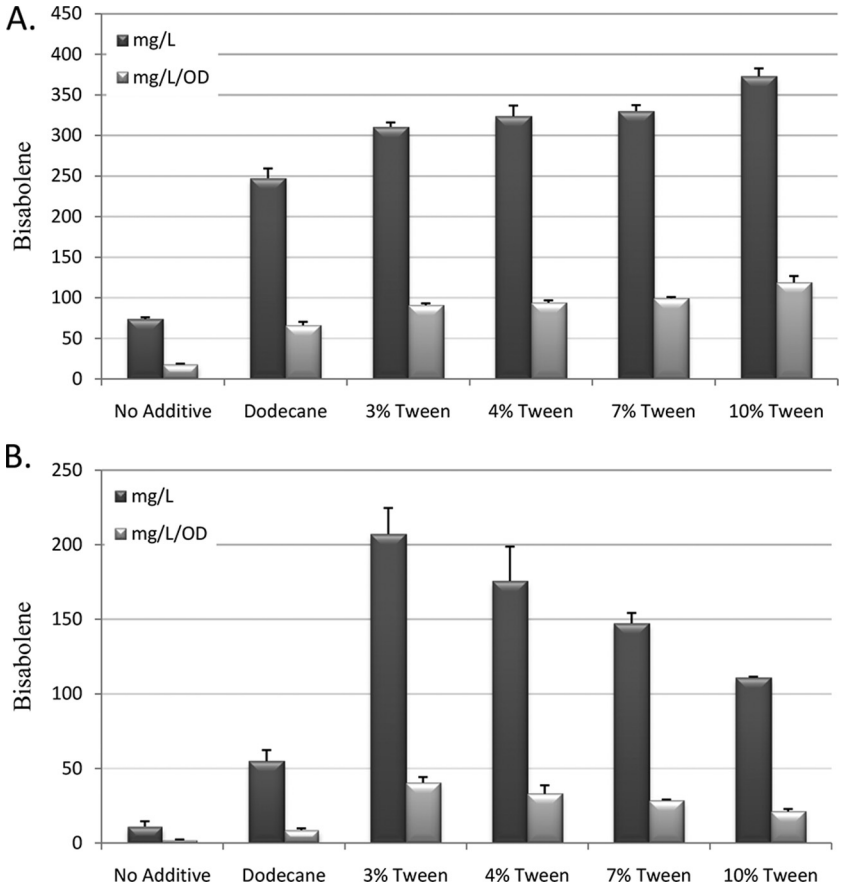

FIG 7 Bisabolene production in 50-ml SD-gal cultures of the pRS strain grown with no additional additive or a 10% (vol/vol) dodecane overlay or various concentrations of T20. Bisabolene was measured at the end of 7 days in the initial shake flasks (A) and also at the end of an additional 7 days in a second set of equivalent shake flasks inoculated from the first set (B).

otherwise equivalent pESC and pRS strains resulted in significantly increased resistance to T20.

One of the benefits of this selection, since it is driven by product levels rather than by alleviating prenyl-phosphate toxicity or some other indirect mechanism, is that it may be used to select for improvements at any level, from an individual enzyme to a metabolic network, that results in higher bisabolene titers. We chose bisabolene synthase as our first target for improvement with this selection, in part due to evidence that it is a limiting factor in our strain and in part to address the longstanding issue that terpene synthases are kinetically slow and that improvement of their kinetics through engineering is challenging (6, 26, 29, 30). Processing of mutant libraries by subculturing in the presence of T20 proved to be an effective method for removing strains in a mixed population that produce little or no bisabolene. Although the selection generated populations with much higher average bisabolene titers relative to preselection levels, none of the postselection isolates turned out to harbor mutations that changed the amino acid sequence.

Based on evidence that the BIS mRNA was unstable in yeast, we undertook mutagenesis to change the BIS cds without altering the encoded protein sequence. After applying selective pressure by subculturing a recoded BIS library in the presence of T20, mutants were isolated that yielded improved bisabolene titers in yeast. There is a bias in *S. cerevisiae* toward local mRNA secondary structures in coding sequences, and the more stable structure predicted for the improved BIS-91 mutant may effectuate changes in mRNA stability and translation that benefit gene expression (31). Consistent with this, BIS protein levels were found to be higher in the BIS-91 strain than in the corresponding BIS-wt strain.

In addition to its use as a selective agent in mixed populations, we found that the presence of T20 significantly increased bisabolene production in a monoculture, particularly over an extended growth period. Metabolic engineering approaches have led to significant improvements in terpene production titers in yeast, but maintenance of high production levels on a larger scale or for prolonged periods can be challenging (3). In addition to parameters such as productivity and yield, the ability of the strain to sustain production for longer fermentation runs is a key factor in determining success on a commercial scale (27).

Selective pressure for maintenance of plasmids and chromosomal modifications such as antibiotic resistance, complementation of auxotrophies, and addiction/lethality systems can be effective for basic maintenance of heterologous gene constructs. However, these approaches will not prevent mutations which abort or reduce production and can quickly take over a population, particularly when growth rates are compromised by the heterologous pathway. A selection for the desired metabolic product

itself would appear to be the most logical way to maintain long-term, high-level production of nonessential metabolites.

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