

Heterologous expression of MlcE in *Saccharomyces cerevisiae* provides resistance to natural and semi-synthetic statins



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ABSTRACT

Statins are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the key enzyme in cholesterol biosynthesis. Their extensive use in treatment and prevention of cardiovascular diseases placed statins among the best selling drugs. Construction of *Saccharomyces cerevisiae* cell factory for the production of high concentrations of natural statins will require establishment of a non-destructive self-resistance mechanism to overcome the undesirable growth inhibition effects of statins. To establish active export of statins from yeast, and thereby detoxification, we integrated a putative efflux pump-encoding gene *mlcE* from the mevastatin-producing *Penicillium citrinum* into the *S. cerevisiae* genome. The resulting strain showed increased resistance to both natural statins (mevastatin and lovastatin) and semi-synthetic statin (simvastatin) when compared to the wild type strain. Expression of RFP-tagged *mlcE* showed that MlcE is localized to the yeast plasma and vacuolar membranes. We provide a possible engineering strategy for improvement of future yeast based production of natural and semi-synthetic statins.

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1. Introduction

Statins are used as cholesterol-lowering drugs in treatment and prevention of coronary heart diseases, and their extensive worldwide usage placed them among the best selling pharmaceuticals in the past decade (GBI Research, 2013). The application of statins in medicine is based on their ability to inhibit the catalytic action of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR). HMGCR constitutes the rate-limiting enzyme in the mevalonate pathway, which is responsible for the production of sterols, such as cholesterol in animal cells, and ergosterol in fungi (Maury et al., 2005). Natural statins are synthesized as secondary metabolites by filamentous fungi; mevastatin (Fig. 1A) by *Penicillium citrinum* (Endo et al., 1976), and lovastatin (Fig. 1B) by *Aspergillus terreus* (Alberts et al., 1980) and *Monascus ruber* (Endo, 1979). Industrial scale production of natural statins and their semi-synthetic derivatives (e.g. simvastatin and pravastatin) is based on fermentation of statin-producing filamentous fungi (Manzoni and Rollini, 2002; Singh and Pandey, 2013). Production limitations associated with the unique physiology and morphology of these natural producers can be overcome by heterologous expression of the biosynthetic pathway in a fast-growing host, such as *Saccharomyces cerevisiae*.

It will, however be crucial to establish a nondestructive resistance mechanism in yeast to overcome the undesirable growth inhibition effects of statins. One such mechanism could be active export of statins. Export systems have previously proved to be efficient in increasing the tolerance of microorganisms to the produced compounds, either relying on native efflux pumps, as it has been shown for the production of several antibiotics (Malla et al., 2010; Ullán et al., 2002; Xu et al., 2012), or via heterologous pumps as shown in *Escherichia coli* in connection with biofuel synthesis (Dunlop et al., 2011).

Secondary metabolite gene clusters, in addition to the catalytic enzymes, often encode proteins for secretion of the produced bioactive compounds and thereby also a self-resistance mechanism (reviewed in Martín et al., 2005). This is also likely the case for the known statin clusters, where putative efflux pump encoding genes are present; *mlcE* in the mevastatin cluster (Fig. 1A) (Abe et al., 2002), and *lovI* or *mokI* in the lovastatin cluster of *A. terreus* (Kennedy et al., 1999) or *M. ruber* (Chen et al., 2008), respectively (Fig. 1B). Given the industrial importance of the microbial statin-producing cell factories it is surprising that only limited evidence concerning the function of the putative efflux pumps in the statin gene clusters has been provided so far. Hutchinson et al. found that *A. terreus lovI* mutants did not produce lovastatin or any of its known precursors, and that heterologous expression of *lovI* in *Aspergillus nidulans*, a lovastatin sensitive species, did not result in increased lovastatin resistance (unpublished result in Hutchinson et al., 2000). These findings did not clarify the function of the

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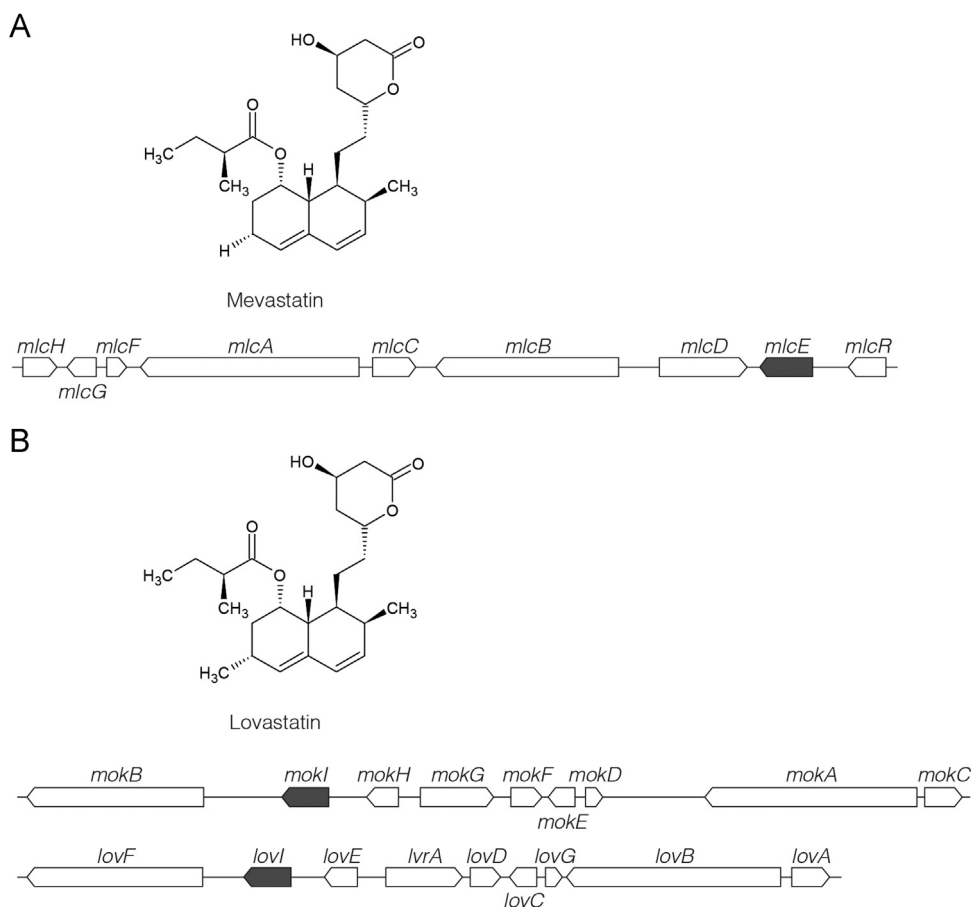


Fig. 1. Natural statins and their biosynthetic gene cluster: (A) Mevastatin and its gene cluster from *P. citrinum*. (B) Lovastatin and its gene clusters from *M. ruber* (*mok* genes) and *A. terreus* (*lov* genes). The putative efflux pump genes are shown in gray.

putative efflux pumps in the statin-producing fungi. Nevertheless, understanding the statin transport mechanism could open up an alternative avenue to classical metabolic engineering strategies aimed at increased productivity of the natural statin-producing strains (Barrios-González and Miranda, 2010). Moreover, genes encoding for the statin transporters can represent a pool of candidates for co-expression in a heterologous host, such as *S. cerevisiae*, thus open up a possibility to establish the necessary self-resistance mechanism for the production of statins in yeast.

In this study, we investigate the function of the putative efflux pump MlcE from the *P. citrinum* mevastatin gene cluster and explore its potential to confer statin resistance in *S. cerevisiae*.

2. Materials and methods

2.1. Bioinformatics

Protein sequences were obtained from UniProtKB (Consortium, 2013). Protein topology prediction was carried out using TOPCONS web server (Bernsel et al., 2009). Prediction of subcellular localization was performed with CELLO v.2.5 (Yu et al., 2006). For phylogenetic tree construction the protein sequences were aligned with the multiple sequence alignment tool Multiple sequence Alignment using Fast Fourier Transform (MAFFT) (Katoh et al., 2009) available at the European Bioinformatics Institute (EMBL-EBI) (McWilliam et al., 2013). See Supplementary Table S1 for the list of protein sequences used for the tree construction. The phylogenetic tree was generated with the ClustalW2 alignment extension (Larkin et al., 2007) at EMBL-EBI using the Neighbor

joining clustering method, with the following setting: distance correction on, exclude gaps on. FigTree software, version 1.4 was used for displaying the tree.

2.2. Construction of plasmids and strains

A yeast codon-optimized version of the *mlcE* gene, *de novo* synthesized by Genscript, was PCR amplified from the plasmid pEN669 with primers *mlcE*-F and *mlcE*-R. The *S. cerevisiae* TEF1 promoter was amplified from the plasmid pSP-G2 (Partow et al., 2010) using primers TEF1-d and PGK1-s. The amplified fragments were cloned into the pX-3 targeting vector (Mikkelsen et al., 2012) via the USER cloning technique (Nour-Eldin et al., 2006) resulting in plasmid pX3-TEF1-*mlcE*-CYC1. The subcellular localization of MlcE was determined by tagging it C-terminally with monomeric red fluorescent protein (RFP). For that plasmid pX3-TEF1-*mlcE*-RFP-CYC1 and a control plasmid pX3-TEF1-RFP-CYC1 were constructed as follows: the coding sequence of *mlcE* lacking the stop codon was amplified using the primer pair *mlcE*-F and *mlcE*-RFP-R, and a yeast codon-optimized RFP was amplified from plasmid pWJ1350 (Lisby et al., 2003) using the primers RFP_R+ and either RFP-F (for tagging *mlcE*) or RFP_F+ (for the control plasmid). All fragments were amplified by PCR using a USER cloning compatible PfuX7 polymerase (Nørholm, 2010). *Escherichia coli* DH5α (Woodcock et al., 1989) was used as host for USER cloning experiments and for the propagation of the constructed plasmids. The inserts of the resulting plasmids were verified by sequencing (StarSEQ). The constructed plasmids were digested with the NotI enzyme (New England Biolabs), and the obtained linear fragments were used for yeast transformation using the lithium acetate/

Table 1
Oligonucleotides, plasmids and strains used in this study. U=2-deoxyuridine.

Primer name	Primer sequence (5'–3')	Use
mlcE-F	AGCGATACGUAAAAATGAGTGAACCATACC	Amplification of <i>mlcE</i> from plasmid pEN669
mlcE-R	CACCGGAUATTATGCATCAGTCTCAG	
TEF1-d	ACGTATCCUGTGTGAGTCGTATTACGGATCCCTTG	Amplification of promoter sequence from plasmid pSP-G2
PGK1-s	CGTGC GAUGCCGCTGTTTATATTGTTG	
RFP_F+	ATGGCCTCCUCCGAGGACGTCATCAAGGAG	Amplification of <i>RFP</i> from plasmid pWJ1350
RFP_R+	CACCGGAUCTAGGCGCCGTTGGAGTGGCCG	
mlcE-RFP-R	AGGAGGCCAUTGCATCAGTCTCAGGGAC	Amplification of <i>mlcE</i> from plasmid pX3-TEF1-mlcE-CYC1
RFP-F	AGCGATACGUAAAAATGGCCTCTCCGAG	Amplification of <i>RFP</i> from plasmid pX3-TEF1-mlcE-RFP-CYC1
X-3-up-out-sq	TGACGAATCGTTAGGCACAG	Strain confirmation via colony PCR
C1_TADH1_F	CTTGAGTAACTTTCTCTGTA	
Plasmid name	Description	Reference or source
pEN669	Template for amplifying <i>mlcE</i> (<i>S. cerevisiae</i> codon optimized)	From Evolva Holding SA
pWJ1350	Template for amplifying <i>RFP</i>	Lisby et al. (2003)
pSP-G2	Template for amplifying <i>TEF1</i>	Partow et al. (2010)
pX3	USER cloning vector equipped with the <i>CYC1</i> terminator designed to target site 3 on chromosome X.	Mikkelsen et al. (2012)
pX3-TEF1-mlcE-CYC1	Plasmid carrying a gene-targeting cassette for expressing <i>mlcE</i> in yeast.	This study
pX3-TEF1-RFP-CYC1	Plasmid carrying a gene-targeting cassette for expressing <i>RFP</i> -tagged <i>mlcE</i> in yeast.	This study
pX3-TEF1-mlcE-RFP-CYC1	Plasmid carrying a gene-targeting cassette for expressing <i>RFP</i> in yeast.	This study
Strain name	Genotype	Reference or source
<i>Escherichia coli</i>		
DH5 α	F- ϕ 80lacZ Δ M15 Δ (<i>lacZYA-argF</i>) U169 <i>recA1 endA1 hsdR17</i> (rK-, mK+) <i>phoA supE44</i> λ - <i>thi-1 gyrA96 relA1</i>	Woodcock et al. (1989)
<i>Saccharomyces cerevisiae</i>		
CEN.PK113-11C (Wild type strain)	<i>MATα MAL2-8C SUC2 his3Δ ura3-52</i>	Dr. Petter Kötter, Institut für Mikrobiologie, der Johan Wolfgang Goethe-Universität, Frankfurt am Main, Germany
ARX1	<i>MATα MAL2-8C SUC2 his3Δ ura3-52X3::PTEF1mlcE-RFP-Tcyc1</i>	This study
ARX2	<i>MATα MAL2-8C SUC2 his3Δ ura3-52X3::PTEF1-RFP-Tcyc1</i>	This study
ARX3	<i>MATα MAL2-8C SUC2 his3Δ ura3-52X3::PTEF1-mlcE-Tcyc1</i>	This study

single-stranded carrier DNA/polyethylene glycol transformation method (Gietz and Schiestl, 2007). The linear gene targeting cassettes were integrated into the X-3 locus of the reference yeast strain, *S. cerevisiae* CEN.PK 113-11C as described by Mikkelsen et al. (2012). The *URA3* markers in the constructed strains were removed by direct repeat recombination using 5-FOA (Melford) counter selection. Correct integration of substrates was verified by diagnostic colony PCR with one primer annealing outside of the integration site in the yeast genome (X-3-up-out-sq), and one substrate specific primer (C1_TADH1_F). Oligonucleotides, plasmids and strains used in this study are listed in Table 1.

2.3. Media

The *E. coli* transformants were selected on lysogeny broth (LB) medium containing 100 μ g/mL of ampicillin. Yeast strains were cultivated in standard liquid or solid yeast peptone dextrose medium (YPD), synthetic complete medium (SC), or synthetic medium (SM). SC medium was prepared according to Sherman et al. (1986), with the minor modification that the L-leucine concentration was doubled to 60 mg/L. Yeast transformants were selected on SC medium lacking uracil. Removal of the *URA3* marker, via direct repeat recombination, was achieved by growing the strain on SC medium containing 5-fluororotic acid (5-FOA; 740 mg/L, Sigma-Aldrich) and uracil (30 mg/L).

For susceptibility experiments strains were grown aerobically either on YPD plates or in SM, supplemented with compounds as described below. SM was prepared according to Verduyn et al. (1992), but concentrations of $(\text{NH}_4)_2\text{SO}_4$ and KH_2PO_4 were modified to 7.5 g/L and 14.4 g/L, respectively. SM was supplemented with uracil (150 mg/L; Sigma-Aldrich) and L-Histidine (125 mg/L;

Sigma-Aldrich) (Pronk, 2002). The pH was adjusted to 6.5 with a 2 M NaOH solution. Glucose was added as carbon source to a final concentration of 20 g/L. The compounds used in the susceptibility experiments were prepared as follows: stock solutions of vanillin (320 mM), mycophenolic acid (MPA, 50 mM) and atorvastatin (10 mM) were prepared by dissolving the compounds in 99% ethanol. Mevastatin, lovastatin, and simvastatin stock solutions (50 mM) were prepared as described previously (Morimoto et al., 2013). Briefly, the solid compounds were dissolved in 1 mL of 99% ethanol, preheated to 50 °C, alkalized with 0.5 mL of 0.6 M NaOH and incubated at 50 °C for 2 h. The pH of the solutions was then adjusted to 7.2 by adding 0.4 M HCl. The final volume of the solutions was adjusted to 2 mL with water, resulting in stock solutions of 50 mM. All stock solutions were filter-sterilized and stored at -20 °C. Mevastatin and atorvastatin were purchased from Toronto Research Chemicals, lovastatin from Tokyo Chemical Industry, MPA and vanillin from Sigma-Aldrich, and simvastatin from Ark Pharm.

2.4. Fluorescent microscopy

For fluorescent microscopy the *mlcE-RFP*- and *RFP*-expressing strains (ARX1 and ARX2, respectively) were cultured in liquid SC medium at 30 °C with 150 rpm agitation overnight and analyzed by fluorescence and visible light microscopy using a Nikon Eclipse E1000 microscope equipped with an oil-immersed objective at 100 \times magnification. The images were captured with QImaging Retiga Exi digital camera using Image Pro Plus 5.1 software. The brightness of images to be compared was adjusted pairwise using Adobe Photoshop CS6.

2.5. Susceptibility experiments

For susceptibility assays on solid media tenfold dilution series of *S. cerevisiae* WT and ARX3 strains (Table 1), starting with an OD₆₀₀ of 0.02 were prepared from overnight cultures in SC medium (30 °C/150 rpm). 4.5 µL of each dilution were plated on a set of YPD agar plates containing different cytotoxic compounds. The plates were incubated at 30 °C for 3 days, after which the growth of the yeast strains was recorded by photography.

For susceptibility assay in liquid medium, strains were grown aerobically in SM, containing different concentrations of lovastatin. Yeast optical density measurements were performed in 48 wells plates in a plate reader (BioTek's Synergy™ Mx Microplate Reader) at 30 °C with fast shaking intensity setting (19 Hz speed, linear shake, which translates into 1140 rpm according to the BioTek's instructions) in 400 µL of SM. Cells were harvested from overnight shake flask cultures (30 °C/150 rpm) in late exponential phase and diluted to an OD₆₀₀ of 0.1 in SM medium with 0.7, 1.2 or 2.0 mM of activated lovastatin or an equal volume of control solution (99% ethanol treated as described above—preparation of the compounds for the susceptibility experiments). Triplicate OD₆₀₀ measurements were taken every 5 min for 24 h.

3. Results and discussion

3.1. Topology prediction and phylogenetic clustering of MlcE

MlcE, a putative efflux pump from the *P. citrinum* mevastatin biosynthetic gene cluster shows significant sequence similarity to drug resistance proteins of the major facilitator superfamily (MFS) (Marger and Saier, 1993). MFS transporters are characterized by using the proton gradient across the plasma membrane as an energy source for the translocation they mediate (Pao et al., 1998). The drug resistance protein subfamily of MFS transporters is further divided into two families, depending on the number of transmembrane spanning regions (TMS) the proteins consist of:

12-TMS family and 14-TMS family (Paulsen and Skurray, 1993; Paulsen et al., 1996), also termed as Drug:H⁺ antiporter 12 TMS (DHA12) family, and 14 TMS (DHA14) family, respectively (Pao et al., 1998). The performed phylogenetic analysis showed that MlcE, as well as Lov1 and Mok1 (Fig. 2), clustered with known members of 14-TMS family of drug resistance proteins, such as the cercosporin facilitator protein (CFP) from *Cercospora kikuchii* (Callahan et al., 1999) and HC-toxin efflux pump (ToxA) from *Cochliobolus carbonum* (Pitkin et al., 1996) (further proteins are listed in Supplementary Table S1). This classification is supported by the performed topology prediction, which showed that MlcE comprises of 14 TMS (data not shown), indicating that it is indeed a member of 14-TMS family. We next performed an *in silico* prediction of MlcE's subcellular localization, using CELLO v.2.5 to see where in eukaryotic cells the protein would be localized. The prediction suggests that it is most likely localized at the plasma membrane (score=4.942 and a combined reliability score of 0.997 for the five used prediction methods). Collectively, this proposes that MlcE is likely localized in the plasma membrane and functions as a statin efflux pump driven by the proton gradient found across the plasma membrane.

3.2. Subcellular localization of MlcE

To experimentally determine the subcellular localization of MlcE in *S. cerevisiae* we tagged MlcE with the red fluorescent protein (RFP) at its carboxylic terminus and expressed it as a single copy gene from the yeast genome (Fig. 3A). Fluorescent microscopy of the fluorescent strain, ARX1, revealed a ring-like distribution of the fluorescent protein at the periphery of the cells and inside the vacuole (Fig. 3B), indicating that the RFP-tagged MlcE was localized to the plasma and vacuolar membranes. In contrast, when RFP was expressed alone it was found to have a uniform cytoplasmic distribution in the control cells ARX2. This subcellular localization of MlcE in *S. cerevisiae* supports the hypothesis that MlcE is a transmembrane protein, which is localized to the plasma membrane.

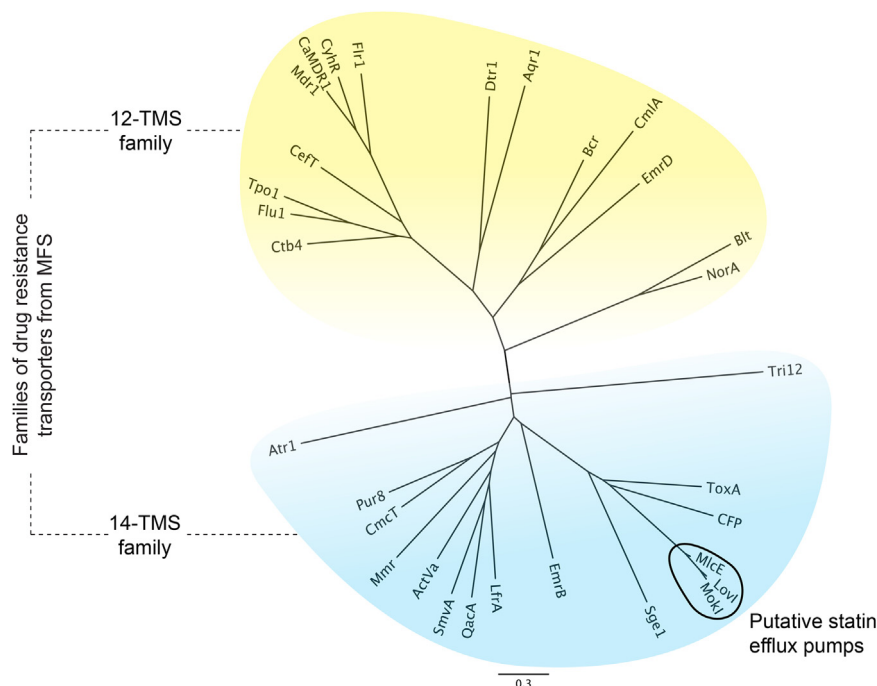


Fig. 2. Phylogenetic clustering of putative statin efflux pumps (MlcE, Mok1 and Lov1) with major facilitator superfamily (MFS) transporters involved in the efflux of toxic compounds, belonging to the subfamily of proteins with 14 transmembrane domains (14-TMS family). Proteins used to construct the phylogenetic tree are listed in Supplementary Table S1, where information about the source organism and the substrate of each protein is provided.

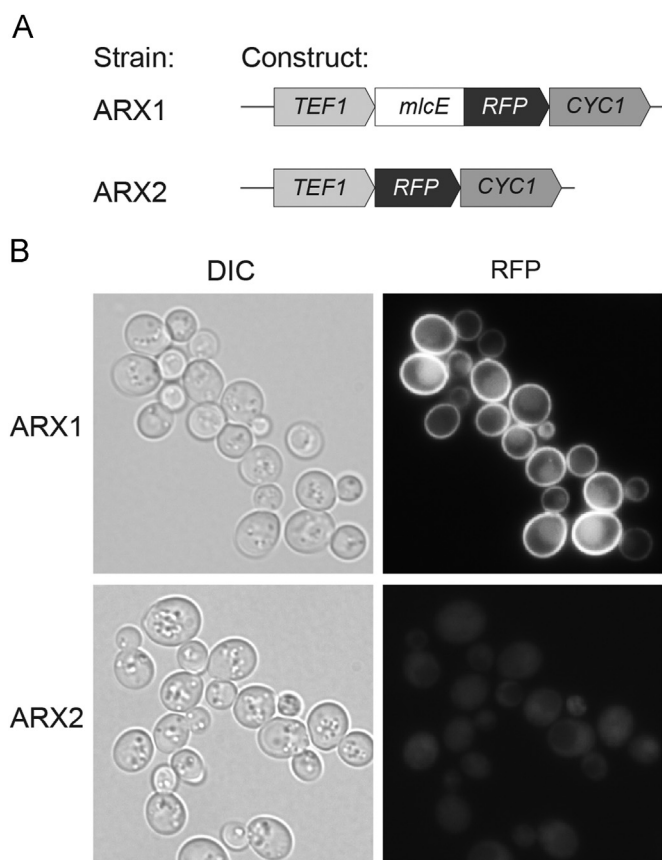


Fig. 3. Subcellular localization of MlcE in *S. cerevisiae*: (A) strain construction summary and (B) fluorescent microscopy of the constructed strains (see Section 2 for experimental details).

3.3. Investigation of the potential of MlcE to confer the resistance to statins in *S. cerevisiae*

We next tested if the localization of MlcE to the yeast plasma membrane would enable it to export statins from yeast, and thereby increase the yeast's resistance to statins. For that, *mlcE* was expressed from a defined genomic locus in *S. cerevisiae* as a single copy gene under the control of the strong constitutive promoter TEF1 (Fig. 4A). The resulting strain ARX3 was tested for its susceptibility to mevastatin, MlcE's predicted natural substrate, by serial dilution plating on YPD agar plates supplemented with the active form of mevastatin. The *mlcE*-expressing strain showed an increased resistance to mevastatin compared to the reference strain (Fig. 4B).

To determine if MlcE would be able to excrete other structurally related compounds, we tested the effects of lovastatin and simvastatin. Again, the *mlcE*-expressing strain displays an increased resistance compared to the reference strain, and the putative pump was able to protect the cells against both the natural statin lovastatin and its semi-synthetic derivative simvastatin (Fig. 4B). This shows that MlcE is able to accept not only its native substrate but also structurally related natural compounds, and even compounds it has not encountered during evolution, when expressed in yeast. To determine whether MlcE should be considered as a general pleiotropic efflux pump, or a dedicated statin pump, we tested the susceptibility of the ARX3 strain to other toxic compounds. This analysis showed that MlcE was not able to protect yeast against the lethal effects of the synthetic statin, atorvastatin or the effects of the natural compounds vanillin and mycophenolic acid (MPA) (Fig. 4B). These results suggest that MlcE is not a multi-drug resistance efflux pump. The specificity of MlcE and its

presence in the mevastatin biosynthetic gene cluster suggest that it has likely evolved as a statin efflux pump; however, testing in the endogenous species is still required to confirm this.

3.4. MlcE and a future *S. cerevisiae* based statin cell factory

Statins are currently commercially produced by fermentation of natural statin-producing species of filamentous fungi. The highest titers reported for these systems have been achieved by submerged cultivation, reaching levels up to 950 mg/L (2.35 mM) of lovastatin (Jia et al., 2010) and 1200 mg/L (3.07 mM) of mevastatin (Choi et al., 2004), respectively. For future heterologous production of statins to be competitive, these titers will likely have to be matched and preferably exceeded. For the last decade several groups have been working on establishing *S. cerevisiae* based statin cell factories, and in 2013, Xu et al. succeed in producing dihydromonacolin L acid (0.11 mM), the first stable intermediate in the lovastatin pathway (Xu et al., 2013). However, no one has yet reported whether *S. cerevisiae* will be able to cope with the required product levels. To test this, we cultured the reference wild type strain (WT) in liquid synthetic medium supplemented with increasing concentrations of activated lovastatin in a micro-fermentation setup. The analysis revealed that the IC₅₀ value for extracellularly added lovastatin is approx. 1 mM (less than half of the required concentration) in the wild type and that even low concentrations of lovastatin greatly reduced the aerobic maximum specific growth rate and final optical density (Table 2).

These results show the necessity of establishing a non-destructive self-resistance mechanism in a future yeast statin cell factory to allow for titers similar to those reported for statin-producing filamentous fungi. For this, MlcE constitutes a potential tool for tackling the described self-intoxication problem. To investigate if it would also provide protection from statins in liquid cultures, the MlcE expressing strain (ARX3) was tested as described above for the reference wild type strain (Table 2). The analysis showed that while the growth of *S. cerevisiae* wild type strain was almost completely inhibited at lovastatin concentrations similar to those achieved by fermentation of *A. terreus*, growth of the ARX3 strain was only slightly affected by the same high concentration of lovastatin. The liquid culture experiment also allowed for determination of the strains growth efficiencies (Table 2), which revealed that expression of *mlcE* did have a cost (9% reduction), but that this cost did not change as function of the statin concentration, within the tested concentration range.

Direct proof of the effects of implementing the MlcE based resistance in a *S. cerevisiae* statin cell factory is currently not possible as only part of the biosynthetic pathway at this time has been established in yeast (Xu et al., 2013). However, implementation could likely have additional benefits such as increasing titers by reducing feedback inhibition of the pathway enzymes caused by high intracellular concentrations of statins and furthermore reduce product purification costs.

4. Conclusions

We provide evidence that *mlcE* from the *P. citrinum* mevastatin biosynthetic gene cluster encodes a transmembrane protein that localizes to the plasma and vacuolar membranes in *S. cerevisiae*. Moreover, MlcE significantly increases yeast resistance to both, natural and semi-synthetic statins, likely by exporting the compounds from the cells. This resistance mechanism has a potential to improve future yeast based production of natural and semi-synthetic statins.

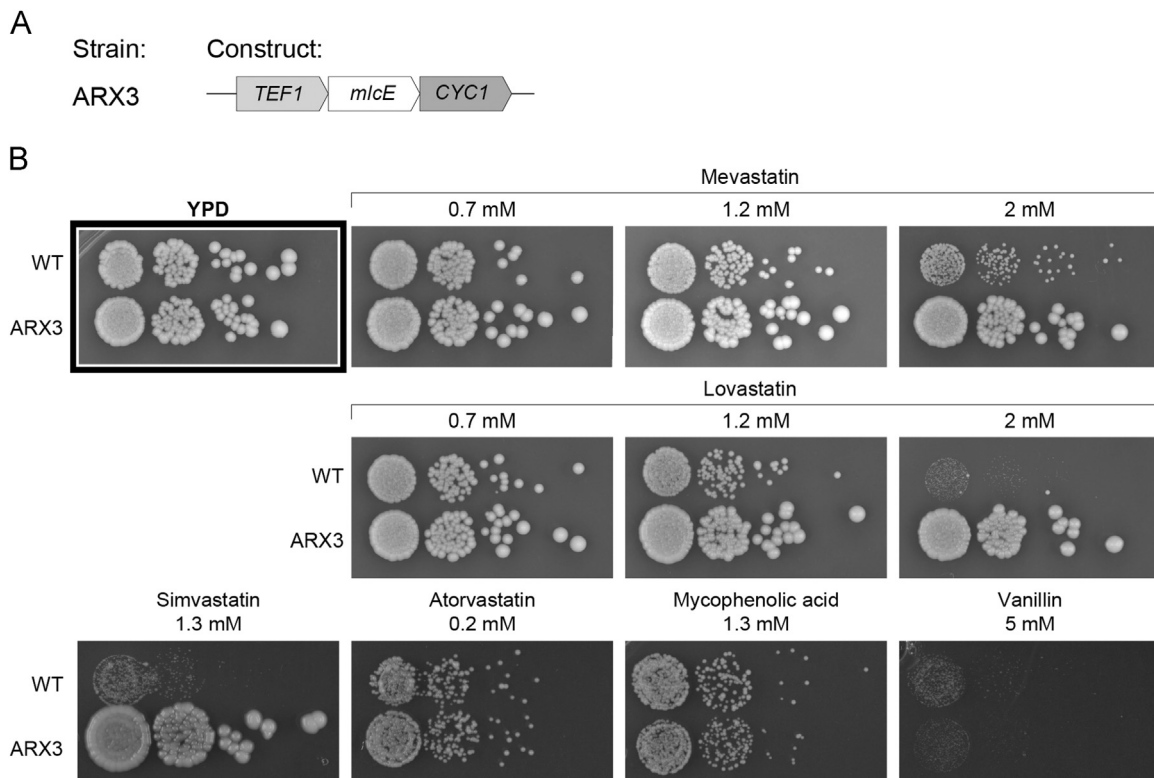


Fig. 4. Investigation of the potential of MlcE to confer the resistance to statins in *S. cerevisiae*: (A) strain construction summary and (B) susceptibility assay. Ten-fold dilution series of WT (CEN.PK 113-11C) and ARX3 strains (harboring MlcE efflux pump), starting with an OD₆₀₀ of 0.02 were prepared from overnight cultures and plated on a set of YPD agar plates containing different cytotoxic compounds. The plates were incubated at 30 °C for 3 days, after which the growth of the strains was recorded by photography. The plate in the black square represents the reference plate (no compounds added to YPD) (for experimental details see Section 2).

Table 2

Aerobic maximum specific growth rates and growth efficiencies calculated as $\Delta(OD_{600,max} - OD_{600,t=0})$ of *S. cerevisiae* strains WT (CEN.PK 113-11C) and ARX3 (harboring MlcE efflux pump) on glucose and different concentrations of activated lovastatin. In the samples with 0 mM lovastatin, an equal volume of solvent was added. Averages and standard deviations were obtained from triplicate experiments.

Lovastatin concentration (mM)	Growth rate (h ⁻¹)		Growth efficiency	
	WT	ARX3	WT	ARX3
0	0.28 ± 0.004	0.31 ± 0.01	0.90 ± 0.008	0.82 ± 0.04
0.7	0.18 ± 0.01	0.34 ± 0.008	0.37 ± 0.04	0.87 ± 0.02
1.2	0.10 ± 0.008	0.27 ± 0.003	0.28 ± 0.01	0.84 ± 0.02
2.0	0.04 ± 0.005	0.27 ± 0.005	0.10 ± 0.01	0.86 ± 0.02

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.meten.2015.09.003>.

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