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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.

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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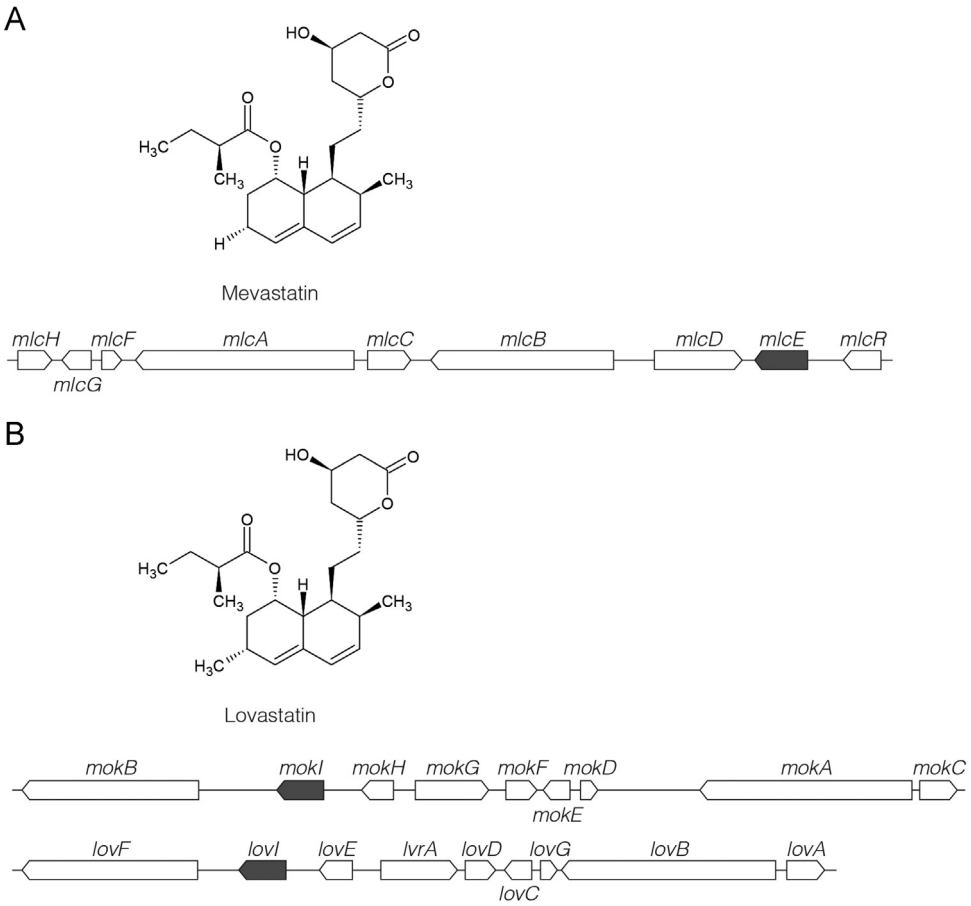

Fig. 1. Natural statins and their biosynthetic gene cluster: (A) Mevastatin and its gene cluster from *P. citrinum*. (B) Lovastatin and its gene clusters from *M. ruber* (*mok* genes) and *A. terreus* (*lov* genes). The putative efflux pump genes are shown in gray.

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* synthetized 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/