

# Exploring the Transportome of the Biosurfactant Producing Yeast *Starmerella Bombicola*

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## Research Article

**Keywords:** *Starmerella bombicola*, transportome, sophorolipid, yeast, ABC transporters

**Posted Date:** August 30th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-778149/v1>

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**Version of Record:** A version of this preprint was published at BMC Genomics on January 9th, 2022. See the published version at <https://doi.org/10.1186/s12864-021-08177-x>.

# Abstract

*Starmerella bombicola* is a non-conventional yeast mainly known for its capacity to produce high amounts of the glycolipids 'sophorolipids'. Although its product has been used as biological detergent for a couple of decades, the genetics of *S. bombicola* are still largely unknown. Computational analysis of the yeast's genome enabled us to identify 254 putative transporter genes that make up the entire transportome. For each of them, a potential substrate was predicted using homology analysis, subcellular localization prediction and RNA sequencing in different stages of growth. One transporter family is of exceptional importance to this yeast: the ATP Binding Cassette (ABC) transporter Superfamily, because it harbors the main driver behind the highly efficient sophorolipid export. Furthermore, members of this superfamily translocate a variety of compounds ranging from antibiotics to hydrophobic molecules. We conducted an analysis of this family by creating deletion mutants to understand their role in the export of hydrophobic compounds, antibiotics and sophorolipids. Doing this, we could experimentally confirm the transporters participating in the efflux of medium chain fatty alcohols, particularly decanol and undecanol, and identify a second sophorolipid transporter that is located outside the sophorolipid biosynthetic gene cluster.

## Background

Phospholipid-based cell membranes facilitate the possibility of cellular life, but at the same time create the need for the cell to access to the exterior environment; this need is provided for by membrane transporter proteins. Their crucial roles range from nutrient uptake to export of undesired product, from cell volume control to extracellular nutrient sensing and much more [1]. The set of genes encoding proteins contributing to this purpose is called the 'transportome'. The metabolic cost for keeping the transportome operational cannot be underestimated; up to 60% of the total ATP requirement of the cell is required for activity of the transportome [2]. Transport reactions account for roughly one third of the reactions in the consensus yeast metabolic network reconstruction [3]. Yet, despite their apparent importance, membrane transporters remain widely neglected [4, 5]. Analogous to the Enzyme Commission (EC) system, the Transport Classification Database (<http://www.tcdb.org>) aims to structure membrane transport proteins based on both functional and phylogenetic information [6]. Different types of transport proteins are separated into classes, subclasses, superfamilies, families and subfamilies. Three main classes of transporters can be distinguished. Channels or pores (TC.1) create an aqueous pathway for polar compounds of a specific size and charge to pass through the membrane. The transport rates are high, up to  $10^8$  molecules per second, and are driven by the electrochemical gradient of the molecule, in an energy-independent process. Carriers (TC.2) bind their substrate and catalyze its crossing after conformational change without the need for utilization of a primary source of energy. This can be for a single substrate, termed 'uniporter', or two or more substrate species in the opposite direction, termed 'antiporter' or two or more substrate species in the same direction, termed 'symporter'. Antiporters and symporters are 'secondary active' systems; the energy stored in the electrochemical gradient of one of the two substrate species drives the other substrate against its electrochemical gradient. The largest and

best-known superfamily within this class is the MFS superfamily of transporters [7]. They are single-polypeptide secondary carriers involved in symport, antiport or uniport of a vast array of substrates, across all kingdoms of life. The pumps (TC.3) are 'primary active' transporters and use the hydrolysis of ATP to drive active transport of the substrate against its gradient. The largest and best-known superfamily of this class are the ATP-Binding Cassette (ABC) transporters. Together with the MFS superfamily, this ABC superfamily accounts for nearly half of the known solute transporters. The TC.9 designation is used for transporters that have an incompletely characterized transport system. Mechanisms of bulk transport via endocytosis/exocytosis and intracellular trafficking are however not included in the TCDB and are therefore not discussed in this manuscript.

The non-conventional yeast *Starmerella bombicola* is mostly known for its high capacity (>300g/L) for producing sophorolipids (SL; Fig. 1) [8]. Despite the fact that it was discovered five decades ago, and is used in an industrial setup for the production of its sophorolipid biosurfactants, relative little is known about its genetics. Four genome assemblies are publicly available in the NCBI database (GCA\_001599315.1, GCA\_004124885.1, GCA\_000950655.1, GCA\_003033785.1) yet annotation is lacking. Few genes are characterized resulting in 343 nucleotide sequence records (NCBI database consulted at 12-03-2021). Roughly 10% thereof is characterized in depth because of their role in sophorolipid biosynthesis and degradation or in the lipid metabolism [9]. Most importantly, the 13-kb long SL biosynthetic cluster with its central SL transporter 'Mdr' was found at the end of chromosome 2, and is well described [10]. Its expression pattern in the stationary phase was attributed to the subtelomeric position of the cluster and associated regulatory elements [11]. Recently, our group identified two mitochondrial citrate carriers and the ATP citrate lyase, and demonstrated their importance in sophorolipid production [12]. Furthermore, two fatty acid importers located in the peroxisome membrane have been described [13]. Besides being a notoriously good biosurfactant producer, *S. bombicola* has other exceptional features that involve compounds crossing the lipid bilayers; it can easily take up hydrophobic compounds such as fatty acids, alkanes, fatty alcohols and hydroxylated fatty acids [14, 15]. Furthermore, it is exceptionally resistant to a wide range of antibiotics, even though it is not pathogenic [16]. This manuscript presents a complete inventory of *S. bombicola*'s transporters gathered through sequence-based analysis, complemented by selected functional studies in search for key transport processes.

## Results

### Identification of the *S. bombicola* transportome

The 4626 predicted protein sequences in the *S. bombicola* genome were scanned with the transporter identification pipeline. 254 genes were identified as putative transporter genes and subsequently subjected to sequence-based functional grouping: 180 genes were found to be of the type 'channels', 24 of the type 'carrier' and 44 of type 'pump'. 7 genes belong to the group of incompletely characterized transport systems. For a complete list of the putative transporter gene designation to Saier's TC system and their predicted function, the reader is referred to the additional file. All transporters, their class, their

substrate group, potential substrate and subcellular localization are summarized in Fig. 2. In Fig. 3, the distribution of predicted transportome members among different (super)families is given. By means of comparison, the number of family members in the three yeasts present in TransportDB, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Cryptococcus neoformans*, is given as well. According to this analysis, the *S. bombicola* transportome accounts for 5.49% of its genome (*S. cerevisiae* 5.68%) with a transporter gene density of 26.75 transporter genes per Mb (*S. cerevisiae*: 28.05). These proportions accord with what is found for the fungal branch of transportome analyses throughout all living cells [2]. The largest superfamily within the *S. bombicola* transportome is the MFS superfamily (TC 2.A.1) with 65 members, clustered into 15 families, most notably the Sugar Porter Family (TC 2.A.1.1) with 15 members and the Drug:H<sup>+</sup> Antiporter-1 (DHA1) Family (TC 2.A.1.2) with 11 members.

## Mapping the expression profile of the *S. bombicola* transportome

It is unlikely that the entire transportome is present in the yeast cell's membrane at any given time. Rather, subsets of the transportome are expressed according to their function in the yeast's growth phase or environmental conditions. Whole-cell RNA sequencing was performed in three different growth stages in order to evaluate the expression time frame of the transportome: (1) in the exponential growth phase, (2) in early stationary phase when SLs are produced as a kind of secondary metabolite and (3) very late in the fermentation process, when all carbon sources are depleted except for the extracellularly produced SLs, which are being metabolized at that stage. From Fig. 4, it is clear that in all three growth stages, transporters are on average higher expressed. The expression of SbStl1.1 and SbVht1.1 was not detected.

The datasets were analyzed to find differentially expressed genes in the three growth stages (Fig 5). The large majority of transporters has a Log<sub>2</sub> Fold Change (FC) of around 0 when comparing the exponential growth phase with the stationary growth phase, indicating that expression levels do not change over time (Fig5a). Yet, 23 transporters display a significant change. Transporters associated with growth are indeed more represented in the exponential phase. Most obviously the sugar uptake carriers SbHgt1.3, SbStl.4, SbHgt1.2, the amino acid uptake carriers SbMup1.1, SbST.29 and sulfate uptake carrier SbSul2. Towards the stationary phase, central cellular processes are slowed down, and other transporters play a bigger role. The most striking differentially expressed one is the SbSLMdr.1. Together with the other SL biosynthetic genes, this transporter is highly upregulated in the stationary phase. Furthermore, transporters implicated in nitrogen metabolism are upregulated under SL producing conditions. SbMep2.1 channeling ammonium, SbDur3 carrying urea and polyamines, SbFur4 for uracil, SbYl31 and SbFcy21 purines. Also, the amino acid transporter orthologues SbGap1.2 and SbGap1.3 are upregulated. SbFtra is highly expressed, suggesting an active iron uptake in this later growth phase. It is also in this stationary phase that most multidrug transporters (SbYor1.1, SbYor1.2, SbFnx1, SbYcf1.3) are being expressed. In Fig 5b, gene expression very late in the fermentation stage (i.e. 'metabolizing' conditions) is compared with the gene expression in the early stationary phase. In this metabolizing stage, all carbon sources are depleted, and the produced SLs are hydrolyzed and metabolized for the cell's survival. There

is a large shift in the expression profile: 80 of the 254 transporter genes (31.50%) are significantly differentially expressed, 1029 of the 4626 genes (22.24%) on genome level. The most remarkable differences are found in the Major Facilitator Superfamily, where the presumed sugar transporters SbHgt1.1 and Sbltr1 stand out. Many other members of this family with unknown substrate preference are also upregulated (SbStl1.4, SbCpaT and SbMFS.1-14, except SbMFS.3, SbMFS.7, SbMFS.12). Important members of the ABC transporter family on the other hand (most prominently SbSLMdr.1, but also SbYcf1.2-3), are downregulated. Also the nitrogen transporters highly upregulated in the stationary phase are now significantly downregulated.

## Highlighting the ABC transporter family

*S. bombicola* is not known to cause any disease, and consequently is not exposed to antibiotics other than the ones in its natural environment. Yet, it shows a remarkably high resistance to a variety of antibiotics and antifungals. The multidrug resistance phenotype is driven by a complex interactive network of transcriptional regulators and ABC efflux pumps. In the model yeast *S. cerevisiae*, the main detoxification transporters are Pdr5, Snq2 and Yor1, all members of the ABC Superfamily [17]. The physiological functions performed by yeast ABC transporters are diverse, and include mating factor secretion, regulation of mitochondrial function, vacuolar detoxification and pleiotropic drug resistance [18]. Furthermore, they are involved in the transport of hydrophobic molecules [19]. Which means that these transporters are also likely candidates to explain why *S. bombicola* is exceptionally good at export of fatty acids and derivatives thereof [14, 20].

In total, 18 members of the ABC Superfamily are retrieved in the *S. bombicola* transportome, including the described SL transporter SbSLMdr.1 and two peroxisomal fatty acid importers SbPxa1 and SbPxa2 [10, 13]. 12 out of the 18 are annotated as 'full size' transporters (2 Nucleotide Binding Domains (NBD) and at least 2 Transmembrane Domains (TMD)) and 5 as 'half size' transporters (1 NBD and 1 TMD). One transporter, SbABC.1, is predicted to be a close homolog of *S. cerevisiae* YDR061W, a protein which remarkably lacks membrane-spanning regions, yet is an uncharacterized family member of ABC transporters. All putative ABC transporters are listed in Table 1.

Table 1  
Predicted *S. bombicola* ABC transporters

ID	Size (AA)	Topology			TCDB no.	Subcellular location	Closest homolog
		NBD	TMD	TMH			
SbSLMdr.1	1299	2	2	12	3.A.1.201	Plasma membrane	H6TB12
SbSLMdr.2	1331	2	2	12	3.A.1.201	Plasma membrane	H6TB12
SbHst6	1261	2	2	12	3.A.1.206	Membrane	P53706
SbYbt1	1626	2	3	17	3.A.1.208.12	Vacuole	P32386
SbYcf1.1	1497	2	3	17	3.A.1.208.11	Vacuole	P39109
SbYcf1.2	1480	2	3	17	3.A.1.208.11	Vacuole	P39109
SbYor1.1	1445	2	2	12	3.A.1.208.3	Plasma membrane	P53049
SbYor1.2	1358	2	2	12	3.A.1.208.3	Plasma membrane	P53049
SbAbcc1	1231	2	2	10	3.A.1.208	Plasma membrane	O35379
SbYcf1.3	1450	2	3	16	3.A.1.208.11	Vacuole	P39109
SbYor1.3	1361	2	2	12	3.A.1.208.3	Cell membrane	P53049
SbCdr4	1543	2	2	13	3.A.1.205	Plasma membrane	O74676
SbMdr2	718	1	1	6	3.A.1.201	Membrane	Q4WPP6
SbAtm1	561	1	1	4	3.A.1.210	Mitochondrial membrane	Q6CX96
SbABC.1	469	0	0	0	3.A.1	Mitochondrial membrane	Q12298
SbABCG1	1322	1	1	7	3.A.1.204	Endoplasmic reticulum	D4AYW0
SbPxa2	698	1	1	5	3.A.1.203.6	Peroxisome membrane	P34230
SbPxa1	746	1	1	5	3.A.1.203.6	Peroxisome membrane	P41909

The 18 ABC transporters segregate into seven subfamilies, according to the phylogenetic tree pictured in Fig. 6a and sequence comparison with their closest homologs. An alternative classification according to the Human Genome Organization (HUGO), where all eukaryotic ABC proteins are subdivided in eight subfamilies ABC-A to H, is also given. Figure 6b shows the predicted topology for every subfamily member. The largest subfamily within the ABC transporters, with 8 members, is the Drug Conjugate Transporter family (DCT), unlike other yeasts as *S. cerevisiae* or *C. albicans*, where the PDR subfamily is largest [21]. The entire fungal ABC-C subfamily of ABC transporters is very poorly characterized except for

some of *S. cerevisiae* transporters, most notably *Yor1* and *Ycf1*, both responsible for drug extrusion. In the ABC-B group, one can find an orthologue to the first ABC transporter ever to be characterized, SbHst6, orthologous to the *S. cerevisiae* *STE6* and responsible for the export of the farnesylated peptide pheromone a-factor [22]. This subfamily contains both full-size and half-size transporters, and have a wide variety of functions [23]. ABC-D transporters are half-size transporters that dimerize at the peroxisomal membrane, where they allow fatty acids to enter the peroxisome in the form of acyl-CoA [24]. *S. bombicola* has, like *S. cerevisiae*, two members of this family [13]. The ABC-G subfamily can be distinguished from the others on regard of their 'reverse orientation': the signature NBD precedes the TMD domains instead of the more common 'forward orientation' where a TMD is most N-terminal. In the *S. cerevisiae* genome, 10 ABC-G members can be found. All of them are intensively studied because of their involvement in the pleiotropic drug resistance (PDR) phenotype. Curiously, this subfamily is rather underrepresented in *S. bombicola*, with only SbCdr4 and SbABCG1 predicted to export a range of drugs.

Four transporters were selected to proceed with functional studies: SbYcf1.2, SbYor1.1, SbYor1.2 and SbCdr4. All four are full size transporters and predicted to be localized at the plasma membrane, which flags them as candidates for participation in drug tolerance and hydrophobic compound export [25, 26]. Besides homology to *Candida albicans* *CDR4*, SbCdr4 shows high similarity to the *Y. lipolytica* ABC1 alkane transporter [27]. SbYcf1.2 is also predicted to be situated at the vacuole membrane, indicating an alternative manner of detoxification, by sequestration in the vacuole. Founded on their similarity to *S. cerevisiae* *YOR1*, SbYor1.1 and SbYor1.2 are predicted to export a range of drugs as well as phospholipids [28, 29]. The differential expression analysis in Fig. 4 showed that both are significantly upregulated in the stationary phase. SbYcf1.2 and SbCdr4 are highly expressed throughout the yeast's entire life cycle. Creating ABC transporter knockout mutants proved to be a valuable method to understand their function in *S. cerevisiae* [30]. Only three ABC proteins are essential for survival of *S. cerevisiae*: ScYef3p, ScArb1p and ScRli1p, of which none are involved in transport [31]. Hence, single gene deletions were made. Firstly, the viability of the knockout mutants was checked and appeared to be affected compared to wild-type *S. bombicola*, but not significantly different from each other (see Table S5).

Secondly, the knockout mutants were subjected to a disc diffusion assay, applying a list of structurally unrelated compounds in various concentrations to screen for translocation abilities (Table S6). From these results, no clear difference between wild-type and the deletion mutants could be observed in the transport of several flavonoids, aniline, rhodamine B and 2-nitrophenol. Nor did the detergents Triton X-100 and Triton X-144 result in inhibitory effect. Lauroylsarcosine was equally toxic to all strains. Interestingly, the mild non-ionic detergent polyoxyethylene-9-lauryl ether (P9Le) had a strong inhibitory effect on *S. bombicola*  $\Delta$ *SbCdr4* and *S. bombicola*  $\Delta$ *SbYor1.2*, associated with increasing concentration, whilst only a minor toxic affect was observed in *S. bombicola*  $\Delta$ *SbYor1.1* and *S. bombicola*  $\Delta$ *SbYcf1.2*. Wild-type growth was not affected at all (Fig. 7a). P9Le consists of a long carboxyl group linked to a long ethylene oxide chain (Fig. 7b).

These results indicate a hydrophobic substrate preference for both *SbCdr4* and *SbYor1.2*. Therefore, the toxicity of medium chain alkanes and fatty alcohols were tested in a next screening round in a disc diffusion assay (Fig S2). A strong inhibitory effect of medium chain fatty alcohols was observed for *S. bombicola*  $\Delta$ *SbCdr4* and *S. bombicola*  $\Delta$ *SbYor1.2*. The disc diffusion assay was followed by toxicity tests in liquid culture based on the outcome of the fast screening technique (Fig. 8). The results are expressed as relative growth, calculated as OD<sub>600</sub> measured after 48h corrected by OD<sub>600</sub> without the addition of the tested compound. It can be seen that all strains show decreased growth with the addition of 200 µg/mL decanol, most significantly in *S. bombicola*  $\Delta$ *SbYor1.2*, where growth was completely abolished. *S. bombicola*  $\Delta$ *SbCdr4* and *S. bombicola*  $\Delta$ *SbYcf1.1* showed similar growth inhibition. Also for undecanol, *S. bombicola*  $\Delta$ *SbYor1.2* seems the most sensitive among tested ABC transporter knockouts, followed by  $\Delta$ *SbYcf1.1*. To conclude, these results show that *SbCdr4*, *SbYcf1.1* and *SbYor1.2* all contribute to the efflux of medium chain fatty alcohols decanol and undecanol with high substrate specificity.

## SLMdr.2: the second SL transporter

Previous research demonstrated that the *SbSLMdr.1*, present in the SL biosynthetic gene cluster, is indeed largely responsible for export of SLs [10]. Being a member of the MDR subfamily of ABC transporters, it shows high similarity to known multidrug pumps, yet it does not appear to contribute to the high resistance phenotype with phleomycin, G418 and zeocin tested [10]. Quantification of SL production after deleting this transporter showed a large reduction in SL yield, never reaching more than 10% of wild-type strains. The transportome analysis uncovered a similar transporter in the *S. bombicola* genome, named *SbSLMdr.2*. This gene translates to 1331 amino acids (AA) which corresponds to a molecular weight of 146 kDa without considering post-translational modifications. In comparison; *SbSLMdr.1* is 1299 AA in length, and 142 kDa. Alignment of both AA sequences results in an alignment length of 1345, with 59.18% AA identity and 72.57% AA similarity. In order to confirm the potential complementary role that *SbSLMdr.2* might have to SL export, *S. bombicola* knockout mutants were created: *S. bombicola*  $\Delta$ *SbSLMdr.1*, *S. bombicola*  $\Delta$ *SbSLMdr.2* and *S. bombicola*  $\Delta$ *SbSLMdr.1* $\Delta$ *SbSLMdr.2*. SL production was evaluated by performing a growth experiment with and without the addition of 37.5 g/L rapeseed oil to the culture broth. Rapeseed oil is commonly added to SL production medium because its triglycerides contain mostly oleic acid, which in turn is the most prevalent hydrophobic backbone of wildtype SL mixtures. *S. bombicola* readily takes up these fatty acids and immediately incorporates them in the SL resulting in higher SL titers. The viability and relative growth among the different SL transporter deletion mutants was monitored by means of OD<sub>600</sub> and pH measurements every 24h and in both experiments, no differences could be observed between the strains (Fig S3). The SL titers of the resulting end extracts are depicted in Fig. 9. When rapeseed oil was added to the culture medium, a highly significant drop in SL export could be observed between wild type and both single SL transporter knockouts. Furthermore, the double deletion mutant produced even less SLs, indicating functional redundancy of the two transporters. Different results are obtained without adding rapeseed oil. As expected, the overall yield is drastically lower. The extracellular medium of *S. bombicola*  $\Delta$ *SbSLMdr.1* shake flasks contained the same amount

of SLs compared to wild-type. *S. bombycolia*  $\Delta$ *SbSLMdr.2* on the other hand showed significantly less SL export, the same as the double deletion mutant.

## Discussion

### Identification of the *S. bombycolia* transportome

The importance of transport processes i.e. the uptake of nutrients, flux of signaling molecules or the extrusion of toxic compounds is evident. In industrial biotechnology, tinkering with the main driver can lead to increased production rates [32], the usage of waste feedstocks [33] or alternate product export [34]. Fundamental knowledge about these transporters is essential, yet so far limited. The bioinformatic analysis presented in this article is the first step on the way to functionally characterize the *S. bombycolia* transportome. The transporter percentage and its density is in line with what is expected for small eukaryotic transportomes, with 100–500 members [2]. Furthermore, the distribution among transporter families follows roughly the same subdivision as the three yeasts present in the TransportDB database. The largest (super)family by far is the MFS superfamily of secondary active channels. The primary active pumps, ABC transporters, are the second largest family, making up less than a third in size. This finding follows the evolutionary trend that was observed from prokaryotes to higher eukaryotes; low-energy-demanding transport classes occur more frequently in higher eukaryotes compared to prokaryotes because of an evolutionary selection pressure for lower energy [2].

Arguably the most challenging aspect of fully understanding the transportome in any given organism, is the designation of the substrates and their specificity fitting each transport system. Generally, this knowledge cannot be gathered in a high-throughput setup but has a high technical and resource-intensive demand. Preliminary computational analysis is therefore often valuable to start from. This *in silico* analysis identified 254 putative transporters, and linked them to their respective orthologous genes in better known yeasts. Curated database TCDB as well as RefSeq were searched for substrate information providing the first clue regarding their function (Table S2). To improve accuracy of substrate prediction, evolutionary information and the AAIndex as implemented in the web server 'TrSSP: The Transporter Substrate Specificity Prediction Server' helped to confirm the TransAAP division of the transporters in the substrate groups depicted in Fig. 2 [35]. Knowledge of a protein's subcellular localization is an additional help in understanding its proper function.

### Mapping the expression profile of the *S. bombycolia* transportome

RNA sequencing is a tool that is conceptually unbiased; mapping the reads to the genome sequence helped to identify ORFs and therefore correctly annotate genes, finetuning the results that were obtained from the automated annotation pipeline to 254 transporters. The expression of two transporters, *SbStl1.1* and *SbVht1.1* could not be detected, which could imply that specific growth conditions are needed for

their expression or that they might be candidate pseudogenes. We believe the latter to be the most likely, because both have at least one gene duplicate. The expression of all other transporter genes not only confirmed their presence, but also indicate their potential biological relevance.

The transporters with highest expression in the exponential phase are mostly glucose transporters, transporters involved in adenine and methionine synthesis, sulfur metabolism and cell wall. Also in *S. cerevisiae*, these functionalities are clustered with a similar expression pattern [36]. Martinez *et al.* chose ScMup1 as reference transporter, which shows a similar expression pattern as SbMup1.1 in this analysis: it is highly expressed in the exponential phase only to go down in the stationary phase. Later in the growth curve, reaching the stationary phase and therefore SL production, transporters involved in nitrogen metabolism are more highly expressed. A similar observation was made in *Pseudomonas aphidis*, during the production of mannosylerythritol lipids, just as sophorolipids a type of glycolipid [37]. This can be explained by the high carbon to nitrogen ratio at that timeframe; transcriptional changes occur with the release of nitrogen catabolite repression [38]. Nitrogen depletion switches on the transcription of *S. bombicola's* nitrogen transporters (SbGap1.2, SbGap1.3, SbDur3, SbUga4.2, SbFur4, SbHnm1, SbMep2.1) similarly to what is seen in *Y. lipolytica* and *S. cerevisiae* [39]. The biggest effect of this high C/N ratio however, is observed on the SL biosynthetic genes, including the transporter SbSLMdr.1. This is supported by previous results and *S. bombicola's* natural niche, in the midst of flowers, bees, and therefore high sugar content [40]. SLs are produced extracellular as excess carbon storage, unavailable for consumption by competitive microorganisms. Because of this, SL production is only observed in the stationary phase. Indeed, all genes present in the SL biosynthetic gene cluster are induced in the stationary phase [41]. The expression of the entire SL biosynthetic cluster is regulated simultaneously, at least partially by a subtelomeric effect [11]. How exactly the C/N ratio is steering the cluster regulation is still to be determined in detail.

After analysis of the differential expression between the metabolizing sample and the stationary phase sample in Fig. 4b, it is clear that the cells are in survival mode. Because all nutrients enter the cell via transport proteins, the cell heavily invests in the presence of transporters with a large variety in capacity and affinity to increase the chances of gating the scarce nutrients that are left in the media into the cell. The best example of this phenomenon was found in *S. cerevisiae* and its hexose transport family encoded by *HXT* genes [42]. This family of glucose transporters contains 20 members that are differentially expressed depending on the glucose concentration in the medium. In excess of glucose, low affinity transporters are prevalent, while they are downregulated to be replaced by high affinity transporters when glucose becomes limited. How this regulation occurs in cooperation with glucose sensors ScSnf3 and ScRgt2, on transcriptional level, but also on post-translational level, was reviewed by Bisson *et al.* [43]. Following this reasoning, it can be speculated that SbHgt1.1, SbItr1, SbCpaT and SbMFS.9 are high affinity transporters for *S. bombicola's* preferred carbon source, glucose. SbHgt1.3 on the other hand, that is very highly expressed in the exponential phase, will more likely be a high capacity and low affinity glucose transporter. Besides glucose, *S. bombicola* is in search for other forms of carbon and/or energy, which is reflected by the upregulation of amino acid and peptide transporters SbPtr2, SbMtr.1, SbOpt2.1 and SbMup1.1.

The same nitrogen transporters, i.e. SbGap1.3, SbDur31.1, SbMep3, under influence of the nitrogen regulation are again repressed when the C/N ratio drops to growth-limiting conditions in the metabolizing phase. Yet, some orthologues of the same amino acid transporters are actually upregulated (SbGap1.2, SbGap3.3). Most likely, the cell turns to a second strategy besides the higher glucose affinity; it upregulates transporters that can scavenge amino acids as alternative carbon source [42]. More information on how amino acid transporters are degraded by endocytosis in response to nitrogen excess or starvation in *S. cerevisiae* and *Aspergillus* spp. can be found in the recent review of Barata-Antunes *et al.* [44]. The SLs that are stored extracellularly are now actively consumed for the cell's survival, so the SL biosynthetic genes and its central transporter are largely silenced in response to this lower C/N ratio. Other members of the ABC transporter family are also significantly downregulated. This can be explained by the functional mechanism of ABC transporters in eukaryotes that are mainly – but not all, as was believed until recently [45] - exporters. MFS transporters belonging to the Drug:H<sup>+</sup> Antiporter-1 Family and therefore mainly responsible for export, such as SbQdr2, are downregulated. The same holds true for SbFfz1; this transporter previously described by Goncalves *et al.* as a specific, low affinity and high capacity fructose transporter that allows a fructophilic metabolism [46]. SbCtp1, the mitochondrial citrate carrier that was linked to SL production is also downregulated in SL metabolizing conditions [12].

## Highlighting the ABC transporter family

Yeast ABC transporters are shown to translocate structurally and functionally unrelated molecules. These include fungicides, antibiotics, flavonoids, detergents etc. [17]. For this reason, this group of transporters is investigated first in the search for the multidrug resistance phenotype culprits. Even though this yeast is not pathogenic, overcoming the multi-resistance phenotype is still beneficial from a biotechnological point of view. In recent years, some progress has been made toward engineering this rather unconventional yeast, yet genetic modification of it is still in its infancy compared to the myriad of engineering tools that are available in model yeasts nowadays [9]. Only two dominant drug selective markers can be used and this in rather high concentrations: 500 µg/mL hygromycin or 600 µg/mL nourseothricin which can be counteracted by the introduction of the *hygR* gene or *nat* gene respectively [47]. Understanding and restricting this multidrug resistance can help the development of new selective tools that can be used for genetic modification. Members of the MDR family within the ABC Superfamily are also known to transport lipids, lipopolysaccharides and/or lipoproteins [48]. It is therefore not a surprise that both the previously identified SbSLMdr.1 and the newly discovered SbSLMdr.2 can be categorized in this family.

Four transporters were chosen to study more in detail: SbCdr4, SbYcf1.2, SbYor1.1 and SbYor1.2 From Table S5, it can be seen that the selected transporters are not essential for viability, but they do have an influence on the yeast's fitness. The closest homolog to SbCdr4, *CDR4* from *C. albicans* is one of the seven full-sized members of the Pdr protein subfamily that is largely responsible for the notorious multidrug resistance of that yeast [49]. However, *CDR4* does not have a role in antifungal resistance, yet it does function as a phospholipid translocator [50]. The second highest homology is shared with *Yarrowia lipolytica* ABC1, the long chain (C14/C16) alkane importer. Three other highly similar *Y. lipolytica* ABC

transporters - ABC2-ABC4 - were also suggested to be involved in alkane transport, both in- and outward [19]. SbYor1.2 in its turn shows the closest homology to *YOR1*-like transporters from *S. cerevisiae* that are known to export a broad range of substrates, including hydrophobic molecules [17]. Indeed, Cui et al. (1996) showed that *S. cerevisiae*  $\Delta$ *YOR1* is especially sensitive to carboxyl group containing compounds such as propionic acid or reveromycin A [28].

*S. bombicola* wild type is still able to grow in the presence of 200  $\mu$ g/mL decanol, and only moderately affected by 100  $\mu$ g/mL undecanol, whereas the model yeast *S. cerevisiae* already stops growing at 50  $\mu$ g/mL and 25  $\mu$ g/mL respectively [51]. The liquid toxicity assay suggests that this four times higher tolerance to medium chain fatty alcohols is attributed to the activity of ABC transporters. This knowledge can be of use in an industrial biotechnology set-up: medium chain fatty alcohols are broadly used in various industries, yet are currently extracted from vegetable oils and animal fat. The transition to a more ecological, microbial production of fatty alcohols would be beneficial. To that end, genetically engineered *E. coli* was already developed in 2009 [52]. Better results were obtained more recently using the oleaginous yeast *Y. lipolytica*, that reached maximum undecanol production titers of 550 mg/L [53]. In these works, the aspect of export was not taken into account, leading to growth impairing accumulation of the fatty alcohols inside the cell [39]. Furthermore, costly extraction procedures prohibit upscaling to industrial levels. The chief reason for this neglect is the lack of functional knowledge of transporters. Hu et al. (2018) even turned to domain shuffling of human fatty alcohol transporters, to improve fatty alcohol secretion in *S. cerevisiae* [32]. The *S. bombicola* transporters described in this manuscript are the first experimentally confirmed transporters that participate in the efflux of medium chain fatty alcohols and could therefore be used when aiming at higher production and efflux of these compounds.

## SLMdr.2: the second SL transporter

Secondary metabolites are non-essential for growth and reproduction, but are commonly produced to function as communication signals. Transport systems are often found within gene clusters encoding their biosynthetic genes, being responsible for the secretion of the secondary metabolite where they can interact with other (micro)organisms [54]. Furthermore, several ABC-transporters associated with secondary metabolism are highly conserved among fungi [23]. All fungal glycolipid biosurfactants produced by yeasts that are characterized so far are also organized in such clustered architecture with a transporter in its center, as was recently reviewed by our group [55]. Taken together, it is not surprising that SLMdr.1 is under heavy transcriptional regulations that coincide with the rest of the cluster genes. From the RNAseq results depicted in Fig. 5, it can be clearly seen that the SLMdr.1 transporter gene expression is highly upregulated in the stationary growth phase, governed by a high C/N ratio (1757.52 cpm exponential vs 10943.07 cpm stationary). In metabolizing conditions, when the C/N ratio drops and SLs are being consumed rather than produced, the SLMdr.1 centrally located in the SL biosynthetic gene cluster, is again largely downregulated, yet its expression remains rather high (781.68 cpm). Nevertheless, secondary metabolite clusters are among the most mobile genetic elements, driven by processes such as gene loss, duplication and horizontal gene transfer [56]. This might explain the existence of a paralogous gene, SbSLMdr.2, that has the same function but is subject to different regulatory rules. SbSLMdr.2 is

more steadily expressed in all three studied stages of growth, albeit slightly - but not significantly- higher in the metabolizing stage (852.00 cpm exponential, 941.01 cpm stationary and 1479.31 cpm metabolizing). These results clearly indicate a different regulatory system between these two highly similar transporters. Transcription of SLMdr.1 most likely is regulated as part of the SL biosynthetic gene cluster, a mechanism that is up to now only partially resolved [11]. A high C/N ratio is needed for induction of the cluster enzymes, and at least partially, the subteleomeric positioning of the cluster has an effect. SLMdr.2, located on another chromosome not surrounded by any other SL biosynthetic enzymes is no subject to this cluster regulation.

Analysis of SL formation with either *S. bombicola*  $\Delta$ SLMdr.1 or *S. bombicola*  $\Delta$ SLMdr.2 demonstrated a drop in product titer to approximately one third of what is achieved with wild type *S. bombicola*. The double knockout strain yielded even less (17%) compared to both single SL transporter knockouts, which means there is redundancy in their functionality. This can also be derived from the fact that the drop in SL export is equally large for both knockouts. Based on the UPLC chromatograms, no shift in composition of the SL mixture could be observed compared to the SL mixture produced by wild-type *S. bombicola*, which indicates that there is no marked substrate preference within the SL spectrum for either of the transporters. Even in the double deletion mutant, SL molecules could still be detected in the extracellular environment. Alternative secretion routes are probably still in play. Most likely they are less specific and consequently less efficient. A different outcome can be observed when no rapeseed oil is added to boost SL production. As expected, the overall yield is much lower (5–10%). Moreover, the SL production titers of both wild-type *S. bombicola* and *S. bombicola*  $\Delta$ SbSLMdr.1 is approximately the same. Only in *S. bombicola*  $\Delta$ SbSLMdr.2 and *S. bombicola*  $\Delta$ SbSLMdr.1 $\Delta$ SbSLMdr.2, a reduction in SL titer could be observed. This suggests that when no rapeseed oil is added to the fermentation broth, the SL export is predominantly governed by SbSLMdr.2, as opposed to the SbSLMdr.1, the transporter present in the biosynthetic gene cluster. Altogether, these results confirm the hypothesized role of SLMdr.2 as a SL exporter.

## Conclusions

The present study brings forward a complete inventory of transporter genes present in the genome of *S. bombicola*, based on *in silico* prediction and sequence similarities with characterized transporters from closely related yeasts or other organisms. With the RNA sequencing results in three different life cycle stages, some insight is given on the global expression profile, and compared to the expression profile of the transportome. The ABC transporter superfamily was highlighted by the creation of five deletion mutants. Four were tested for their capacity to export a large variety of compounds, including hydrophobic molecules. *S. bombicola*  $\Delta$ SbCdr4 and *S. bombicola*  $\Delta$ SbYor1.2 showed a strong inhibitory effect by the detergent P9LE as well as the medium chain fatty alcohols decanol and undecanol. *S. bombicola*  $\Delta$ SbYcf1.1 showed decreased relative growth compared to wildtype in the presence of decanol. So far, no microbial transporter with an active role in medium chain fatty alcohol export was described. The findings from this manuscript can thus be used for industrial biotechnology purposes,

where a transition to microbial fatty alcohol production would be beneficial, compared to the extraction from vegetable oils and animal fat that is used nowadays.

Furthermore, aside from the already established SL exporter present in the biosynthetic gene cluster, a second SL exporter was found by *in silico* analysis: SbSLMdr.2. SL production assays with the corresponding deletion mutant confirmed that SbSLMdr.2 is indeed the main exporter of SLs when no rapeseed oil is added to the production medium, and has a secondary role in SL export when oil is added.

The unravelling of the *S. bombycolia* transportome also provides a route for further exploitation of *S. bombycolia* as non-conventional microbial cell factory, by its contribution to the primary insight into its cellular capabilities for utilizing nutrients or extruding non-natural compounds.

## Methods

### Identification of transporter proteins

Genome sequencing, assembly and ORF prediction was performed as described before [41]. All the protein sequences were then analyzed with the membrane transporter analysis tool TransAAP, to informatically identify putative transporters [57, 58]. A local version of this was run which allows for a more permissive inclusion of transporters than the publicly available version, to maximize the chances of characterizing novel transporters, and additionally utilizes eukaryotic reference sequences and HMMs which are not employed in the online version due to computational restraints. The 254 putative transporter sequences were implemented in OmicsBox version 1.2.4 for functional annotation by performing BLASTp analysis against the UniProtKB/Swiss-Prot database (version 2019 11). Classification according to the Transporter Classification Database (<http://www.tcdb.org/>) was performed using provided BLASTp results. The TOPCONS web server (<http://topcons.net/>) was used for prediction of membrane spanning regions. Subcellular localization of the putative transporter genes was predicted by DeepLoc-1.0 and compared with the input from the BLASTp results and the associated experimental data [59]. Sequences were deposited in GenBank under bioproject MZ671253-MZ671487. The fungal transportomes other than *S. bombycolia* were retrieved from the TransportDB database [57]. ABC transporters were classified as such with HMMSearch 3.1b2 (February 2015), part of HMMER (<http://hmmer.org/>), in search for the HMM model 'ABC\_tran' (Pfam accession PF00005). Top hits with a domain score higher than 50 and e-value below  $1e^{-15}$  were withheld as true members of the ABC Superfamily. The amino acid sequences were used for phylogenetic analysis after alignment using Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). A tree was inferred using the maximum-likelihood method bootstrapped with 1000 replicates implemented in MEGA X version 10.0.5. Amino acid identity and similarity is calculated after alignment with Clustal Omega with the online 'Ident and Sim' tool ([https://www.bioinformatics.org/sms2/ident\\_sim.html](https://www.bioinformatics.org/sms2/ident_sim.html))

### Strains and culture conditions

The *S. bombicola* strains used in this research are listed in Table S3. *Escherichia coli* Top10 cells (Invitrogen, Carlsbad, U.S.A.) were used for the purpose of cloning and propagation of plasmids. All *S. bombicola* strains were maintained on YPD (2% glucose, 1% yeast extract, 2% bactopectone and 2% agar) plates at 30°C. Selection for *S. bombicola* transformants was done with YPD plates supplemented with 500 µg/mL hygromycin B in the case of *S. bombicola*  $\Delta$ *SbSLMdr.2*, selection on SD-ura (2% glucose, 0.67% yeast nitrogen base without amino acids (DIFCO), complemented with complete supplement mixture without uracil (MP Biomedicals Europe)) plates was performed for all other *S. bombicola* mutants. *E. coli* transformants were cultivated on Luria-Bertani (LB) (1% tryptone, 0.5% yeast extract, 0.5% NaCl) medium supplemented with 100 mg/L ampicillin. Sophorolipid production on shake flask scale was performed in medium optimized by Lang et al. [60] (132 g/L glucose.H<sub>2</sub>O, 4 g/L yeast extract, 5 g/L Na.citrate.2H<sub>2</sub>O, 1.5 g/L NH<sub>4</sub>Cl, 1g/L KH<sub>2</sub>PO<sub>4</sub>, 0.16 g/L K<sub>2</sub>HPO<sub>4</sub>, 0.7 g/L MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.5 g/L NaCl, 0.27 g/L CaCl<sub>2</sub>.2H<sub>2</sub>O).

## RNA sequencing

Wild-type *S. bombicola* ATCC 22214 was grown in 100 mL Lang medium without the addition of rapeseed oil at 200 rpm and 30°C in 500 mL shake flask. 20 mL of culture broth was centrifuged at 4000g after 24 h, 80 h and 240h, for isolation of total RNA, representing the exponential, stationary and metabolizing growth phase. Cell pellets were resuspended in 15 mL RNase-Free water and lyophilized. After crushing the lyophilized residue in liquid nitrogen, the RNA was extracted with the RNeasy Midi kit (Qiagen). Single end, stranded shotgun RNA sequencing was subsequently performed by FASTERIS SA (Switzerland), according to the Illumina library preparation protocol and kit. RNAseq reads were trimmed and quality controlled with FastQC (<https://github.com/s-andrews/FastQC>). The clean reads were then aligned to the reference genome with Hisat2 [61] and gene expression estimates were reported as raw counts by HTSeq [62]. To identify differentially expressed genes between different experimental conditions, the edgeR package (version 3.6.2) implemented in R was used, only taking into account the genes that have count-per-million (CPM) above 10 in all samples [63]. Multiplicity correction of the results was performed by applying the Benjamini-Hochberg method to control the false discovery rate (FDR). Genes were considered differentially expressed if they possessed an absolute value of log<sub>2</sub>-fold change  $\geq 1$  and an adjusted P-value  $\leq 0.05$ .

## Cloning and strain engineering

Single gene deletion *S. bombicola* strains were obtained by homologous recombination between the target locus and linear transformation cassette containing the *URA3* selection marker when the *S. bombicola* uracil auxotrophic mutant PT36 was used as background strain. Hygromycin resistance was used to perform genetic modifications in the SLMDR knockout mutant and to knockout *SbSLMdr.2*, with the introduction of an *E. coli* hygromycin resistance gene (*hyg*) codon-harmonized for *S. bombicola* using the Eugene v1.4.0 software based on the *S. bombicola* codon usage table found

at <https://www.kazusa.or.jp/>, and under control of the *S. bombycol*a GPD promoter [64]. The linear transformation cassettes were created by circular polymerase extension cloning (CPEC) assembly, encompassing the following 4 fragments: pJet vector backbone (Thermo Fischer),  $\pm 1000$ bp up- and downstream homology regions and the selection marker under control of its endogenous promoter and terminator sequence, amplified from *S. bombycol*a genomic DNA. 1  $\mu$ g of the linear transformation cassette was introduced in *S. bombycol*a through electroporation as described previously [65]. The genotype of the transformants was checked by yeast colony PCR. All primers used for cloning in this study are listed in Table S4.

## Sophorolipid production, extraction and analysis

5 mL of Lang medium was inoculated with the strains under consideration and incubated at 30°C and 200 rpm for 48h. Cells were then transferred to 50 mL fresh Lang medium with or without the addition of 37.5 g/L rapeseed oil in 250 mL sterile shake flasks, where they were incubated for 8 days at 30°C and 200 rpm. 500  $\mu$ L samples were taken every 24h to be analyzed for glucose consumption, OD<sub>600</sub>, pH and product formation. Sample supernatant was analyzed for the presence of glucose by HPLC as described before [12]. After 8 days of incubation, the production experiment was stopped and sophorolipids extracted by adding 50 mL ethyl acetate. After shaking for 5 minutes, shake flasks were left to sediment at room temperature for 30 min. The upper fraction was subjected to analysis by UPLC as described in Jezierska et al. [14]. Purified wild-type SL mixes were used as external standards for quantification.

## Specific growth rate determination

Specific growth rates for *S. bombycol*a strains were determined by incubating 200  $\mu$ L of cell culture in Lang medium in a flat bottom, 96-well microtiter plate (Greiner Bio-One) at 30°C in a Tecan Infinite® m200 Pro plate reader (Tecan, Mannedorf, Switzerland) with an initial OD<sub>600</sub> of 0.05, sealed off with a Breath-Easy® sealing membrane (Sigma-Aldrich). The OD<sub>600</sub> was measured every 15 minutes for 40h, while shaking in between measurements at 200 rpm (orbital mode, amplitude 2 mm). The maximum specific growth rate ( $\mu$ ) was calculated by fitting the Richards model in with the 'curve\_fit' function in the 'SciPy' module in Python 3.7 [66].

## Statistical analysis

All experiments were performed in triplicates. The results are expressed as the mean values  $\pm$  standard error of the mean (SE). Statistical differences among groups were found with two-way analysis of variance (ANOVA) with Tukey's multiple comparisons test using the 'statsmodels' module in Python 3.7, unless specified otherwise [67].

# Susceptibility assays

A list of compounds (Table S6) in various concentrations was used to screen for the translocation capacity of putative multidrug exporters in a disc diffusion assay. Strains were grown in 5 mL SD medium for 48h at 30°C and 200 rpm. 300 µL of the cell culture (OD<sub>600</sub> 0.3) was streaked on SD plates (20 mL medium for each plate). A 10 mm paper disk filter impregnated with 60 µL of filter-sterilized tested compound diluted in DMSO was subsequently placed on the plate. After three days of incubation at 30°C, growth inhibition zones were compared. Susceptibility tests in liquid culture were performed as described for the specific maximum growth rate, with the addition of the filter-sterilized compound to be tested in the concentration as listed in Table S6.

## Declarations

## Ethics approval and consent to participate

The article does not contain any studies with human participants or animals performed by any of the authors.

## Consent for publication

Not applicable

## Availability of data and material

The *Starmerella bombicola* genome can be accessed at the publicly available NCBI Genome database with the accession number PRJDB2962. Transcriptome data were described before in reference [41], co-authored by the corresponding author, who consequently has personal and administrative access to this. Transporter sequences were deposited in GenBank for public access under bioproject BankIt2485497: MZ671253-MZ671487.

## Competing interests

The authors declare that they have no competing interests.

## Funding

This work was funded by the Aspirant Research grant from the Research Foundation Flanders (FWO) (PhD fellowship of Silke Claus, Grant Number 1117519N), the Strategic Basic Research grant from the

FWO, (PhD fellowship of Sylwia Jezierska, Grant Number 151610), and the Flemish Agency for Innovation by Science and Technology (Grant IWT80050). The authors have no conflict of interest to declare.

## Author's contributions

S.C. wrote the manuscript, did bioinformatics including RNA sequencing analysis and prepared the figures. S.J. performed wet lab experiments on the ABC transporter part. LE ran the local TransAAP tool, identifying the membrane transporters computationally and guided bioinformatics. INA VB was involved in the conception, guiding and reviewing the text. All authors read and approved the manuscript.

## Acknowledgements

Not applicable

## References

1. Van Belle D, André B. A genomic view of yeast membrane transporters. *Figure 1*:389–98.
2. Darbani B, Kell DB, Borodina I. Energetic evolution of cellular Transportomes. 2018;;1–11. doi:10.1186/s12864-018-4816-5.
3. Herrgård MJ, Swainston N, Dobson P, Dunn WB, Yalçın K, Arvas M, et al. A consensus yeast metabolic network reconstruction obtained from a community approach to systems biology. *Nat Biotechnol.* 2008;26:1155–60.
4. César-Razquin A, Snijder B, Frappier-brinton T, Isserlin R, Gyimesi G, Bai X, et al. Perspective A Call for Systematic Research on Solute Carriers. *Cell.* 2015;162:478–87.
5. Kell DB, Swainston N, Pir P, Oliver SG. Membrane transporter engineering in industrial biotechnology and whole cell biocatalysis. *Trends Biotechnol.* 2015;33:237–46. doi:10.1016/j.tibtech.2015.02.001.
6. Saier MH, Reddy VS, Tsu B V, Ahmed MS, Li C, Moreno-Hagelsieb G. The Transporter Classification Database (TCDB): Recent advances. *Nucleic Acids Res.* 2016;44:D372–9.
7. Pao SS, Paulsen IT, Saier MH. Major Facilitator Superfamily. *Microbiol Mol Biol Rev.* 1998;62:1–34.
8. Gao R, Falkeborg M, Xu X, Guo Z. Production of sophorolipids with enhanced volumetric productivity by means of high cell density fermentation. *Appl Microbiol Biotechnol.* 2013;97:1103–11.
9. De Graeve M, De Maeseneire SL, Roelants SLKW, Soetaert W. *Starmerella bombicola*, an industrially relevant, yet fundamentally underexplored yeast. *FEMS Yeast Res.* 2018;18:1–13.
10. Van Bogaert INA, Holvoet K, Roelants SLKW, Li B, Lin YC, Van de Peer Y, et al. The biosynthetic gene cluster for sophorolipids: A biotechnological interesting biosurfactant produced by *Starmerella bombicola*. *Mol Microbiol.* 2013;88:501–9.
11. Lodens S, Roelants SLKW, Luyten G, Geys R, Coussement P, De Maeseneire SL, et al. Unraveling the regulation of sophorolipid biosynthesis in *Starmerella bombicola*. *FEMS Yeast Res.* 2020;20:1–11.

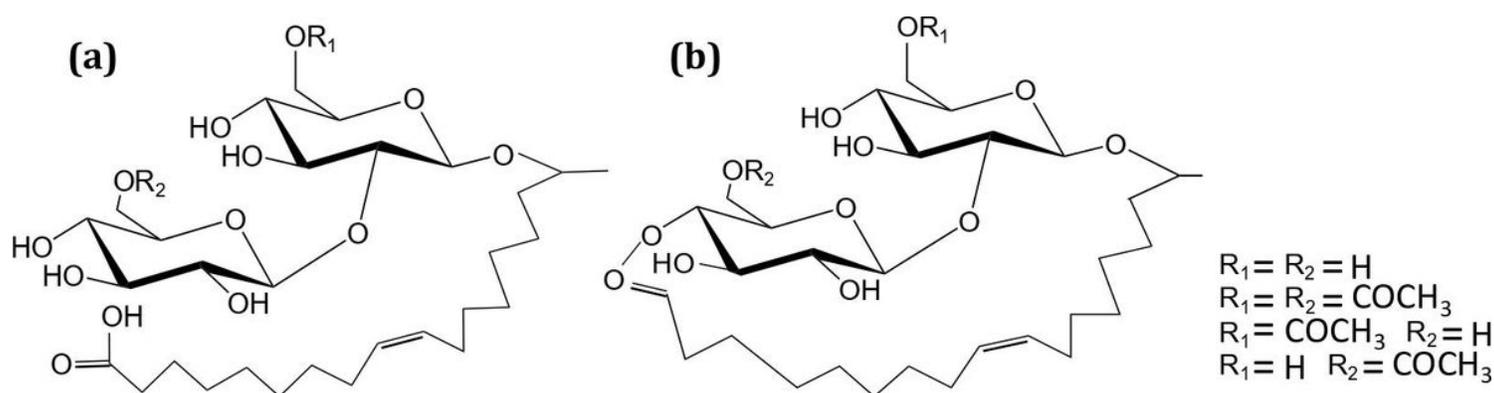
12. Jezierska S, Claus S, Van Bogaert I. Identification and importance of mitochondrial citrate carriers and ATP citrate lyase for glycolipid production in *Starmerella bombicola*. *Appl Microbiol Biotechnol*. 2020;104:6235–48.
13. Ichihara T, Tohata M, Hayase A, Fumikazu T. Sophorolipid Highly-Productive Mutant Strain. 2019; 2019/0062723 A1. <http://www.freepatentsonline.com/y2019/0062723.html>.
14. Jezierska S, Claus S, Ledesma-Amaro R, Van Bogaert I. Redirecting the lipid metabolism of the yeast *Starmerella bombicola* from glycolipid to fatty acid production. *J Ind Microbiol Biotechnol*. 2019;46:1697–706. doi:10.1007/s10295-019-02234-x.
15. Van Bogaert I, Fleurackers S, Van Kerrebroeck S, Develter D, Soetaert W. Production of New-to-Nature Sophorolipids by Cultivating the Yeast *Candida bombicola* on Unconventional Hydrophobic Substrates. *Biotechnol Bioeng*. 2011;108:734–41.
16. Van Bogaert INA, De Maeseneire SL, Develter D, Soetaert W, Vandamme EJ. Development of a transformation and selection system for the glycolipid-producing yeast *Candida bombicola*. *Yeast*. 2008;25:273–8.
17. Rogers B, Decottignies A, Kolaczowski M, Carvajal E, Balzi E, Goffeau A, et al. The pleiotropic drug ABC transporters from *Saccharomyces cerevisiae*. *J Mol Microbiol Biotechnol*. 2001;3:207–14.
18. Jungwirth H, Kuchler K. Yeast ABC transporters - A tale of sex, stress, drugs and aging. *FEBS Lett*. 2006;580:1131–8.
19. Claus S, Jezierska S, Van Bogaert INA. Protein-facilitated transport of hydrophobic molecules across the yeast plasma membrane. *FEBS Lett*. 2019;:1873–3468.13469. doi:10.1002/1873-3468.13469.
20. De Graeve M, Van de Velde I, Saey L, Chys M, Oorts H, Kahrman H, et al. Production of long-chain hydroxy fatty acids by *Starmerella bombicola*. *FEMS Yeast Res*. 2019;19:1–9.
21. Gaur M, Choudhury D, Prasad R. Complete inventory of ABC proteins in human pathogenic yeast, *Candida albicans*. *J Mol Microbiol Biotechnol*. 2005;9:3–15.
22. Kuchler K, Sterne RE, Thorner J. *Saccharomyces cerevisiae* STE6 gene product: a novel pathway for protein export in eukaryotic cells. *EMBO J*. 1989;813:3973–84.
23. Kovalchuk A, Driessen AJM. Phylogenetic analysis of fungal ABC transporters. *BMC Genomics*. 2010;11.
24. Van Roermund CWT, Ijlst L, Majczak W, Waterham HR, Folkerts H, Wanders RJA, et al. Peroxisomal fatty acid uptake mechanism in *Saccharomyces cerevisiae*. *J Biol Chem*. 2012;287:20144–53.
25. Karamanou DA, Aliferis KA. The yeast (*Saccharomyces cerevisiae*) YCF1 vacuole transporter: Evidence on its implication into the yeast resistance to flusilazole as revealed by GC/EI/MS metabolomics. *Pestic Biochem Physiol*. 2020;165:104475.
26. Prasad R, Goffeau A. Yeast ATP-binding cassette transporters conferring multidrug resistance. *Annu Rev Microbiol*. 2012;66:39–63.
27. Chen B, Ling H, Chang MW. Transporter engineering for improved tolerance against alkane biofuels in *Saccharomyces cerevisiae*. *Biotechnol Biofuels*. 2013;6:21. doi:10.1186/1754-6834-6-21.

28. Cui Z, Hirata D, Tsuchiya E, Osada H, Miyakawa T. The multidrug resistance-associated protein (MRP) subfamily (Yrs1/Yor1) of *Saccharomyces cerevisiae* is important for the tolerance to a broad range of organic anions. *J Biol Chem.* 1996;271:14712–6.
29. Decottignies A, Grant AM, Nichols W, Wet H De, David B, Decottignies A, et al. ATPase and Multidrug Transport Activities of the Overexpressed Yeast ABC Protein Yor1p. 1998;273:12612–22.
30. Paumi CM, Chuk M, Snider J, Stagljar I, Michaelis S. ABC Transporters in *Saccharomyces cerevisiae* and Their Interactors: New Technology Advances the Biology of the ABCC (MRP) Subfamily. *Microbiol Mol Biol Rev.* 2009;73:577–93. doi:10.1128/membr.00020-09.
31. Schüller C, Bauer BE, Kuchler K. Inventory and Evolution of Fungal ABC Protein Genes. In: *ABC Proteins.* Elsevier; 2003. p. 279–93.
32. Hu Y, Zhu Z, Nielsen J, Siewers V. Heterologous transporter expression for improved fatty alcohol secretion in yeast. *Metab Eng.* 2018;45 November 2017:51–8. doi:10.1016/j.ymben.2017.11.008.
33. Farwick A, Bruder S, Schadeweg V, Oreb M, Boles E. Engineering of yeast hexose transporters to transport D-xylose without inhibition by D-glucose. *Proc Natl Acad Sci U S A.* 2014;111:5159–64.
34. Darbani B, Stovicek V, van der Hoek SA, Borodina I. Engineering energetically efficient transport of dicarboxylic acids in yeast *Saccharomyces cerevisiae*. *Proc Natl Acad Sci.* 2019;116:201900287.
35. Mishra NK, Chang J, Zhao PX. Prediction of membrane transport proteins and their substrate specificities using primary sequence information. *PLoS One.* 2014;9:3–6.
36. Martinez MJ, Roy S, Archuletta AB, Wentzell PD, Anna-Arriola SS, Rodriguez AL, et al. Genomic Analysis of Stationary-Phase and Exit in *Saccharomyces cerevisiae*: Gene expression and Identification of Novel Essential Genes. *Mol Biol Cell.* 2004;15 December:5295–305.
37. Günther M, Grumaz C, Lorenz S, Stevens P, Lindemann E, Hirth T, et al. The transcriptomic profile of *Pseudozyma aphidis* during production of mannosylerythritol lipids. *Appl Microbiol Biotechnol.* 2015;99:1375–88.
38. ter Schure EG, van Riel NAW, Verrips CT. The role of ammonia metabolism in nitrogen catabolite repression in *Saccharomyces cerevisiae*. *FEMS Microbiol Rev.* 2000;24:67–83. doi:10.1111/j.1574-6976.2000.tb00533.x.
39. Dahlin J, Holkenbrink C, Marella ER, Wang G, Liebal U, Lieven C, et al. Multi-Omics Analysis of Fatty Alcohol Production in Engineered Yeasts *Saccharomyces cerevisiae* and *Yarrowia lipolytica*. *Front Genet.* 2019;10 JUL:747. doi:10.3389/fgene.2019.00747.
40. Van Bogaert INA, Holvoet K, Roelants SLKW, Li B, Lin Y-C, Van de Peer Y, et al. The biosynthetic gene cluster for sophorolipids: a biotechnological interesting biosurfactant produced by *Starmerella bombicola*. *Mol Microbiol.* 2013;88:501–9.
41. Ciesielska K, Li B, Groeneboer S, Van Bogaert INA, Lin YC, Soetaert W, et al. SILAC-based proteome analysis of *Starmerella bombicola* sophorolipid production. *J Proteome Res.* 2013;12:4376–92.
42. Boer VM, De Winde JH, Pronk JT, Piper MDW. The genome-wide transcriptional responses of *Saccharomyces cerevisiae* grown on glucose in aerobic chemostat cultures limited for carbon, nitrogen, phosphorus, or sulfur. *J Biol Chem.* 2003;278:3265–74. doi:10.1074/jbc.M209759200.

43. Bisson LF, Fan Q, Walker GA. Sugar and glycerol transport in *Saccharomyces cerevisiae*. In: Advances in Experimental Medicine and Biology. Springer New York LLC; 2016. p. 125–68. doi:10.1007/978-3-319-25304-6\_6.
44. Barata-Antunes C, Alves R, Talaia G, Casal M, Gerós H, Mans R, et al. Endocytosis of nutrient transporters in fungi: The ART of connecting signaling and trafficking. Comput Struct Biotechnol J. 2021;19:1713–37.
45. Choi CC, Ford RC. ATP binding cassette importers in eukaryotic organisms. Biol Rev. 2021.
46. Gonçalves C, Wisecaver JH, Kominek J, Salema Oom M, Leandro MJ, Shen XX, et al. Evidence for loss and reacquisition of alcoholic fermentation in a fructophilic yeast lineage. Elife. 2018;7:1–28.
47. Lodens S, De Graeve M, Roelants S, De Maeseneire S, Soetaert W. Transformation of an exotic yeast species into a platform organism: a case study for engineering glycolipid production in the yeast *Starmerella bombicola*. In: Braman JC, editor. Synthetic biology: methods and protocols. Springer Humana Press; 2018. p. 95–123. [http://dx.doi.org/10.1007/978-1-4939-7795-6\\_5](http://dx.doi.org/10.1007/978-1-4939-7795-6_5).
48. Nagao K, Kimura Y, Mastuo M, Ueda K. Lipid outward translocation by ABC proteins. FEBS Lett. 2010;584:2717–23. doi:10.1016/j.febslet.2010.04.036.
49. Prasad R, Goffeau A. Yeast ATP-Binding Cassette Transporters Conferring Multidrug Resistance. Annu Rev Microbiol. 2012;66:39–63. doi:10.1146/annurev-micro-092611-150111.
50. Sanglard D, Ischer F, Monod M, Dogra S, Prasad R, Bille J. Analysis of the ATP-binding cassette (ABC)-transporter gene CDR4 from *Candida albicans*. In: ASM Conference on Candida and Candidiasis, Charleston, SC, USA, March. 1999. p. 1–4.
51. Kubo I, Cespedes CL. Antifungal activity of alkanols: Inhibition of growth of spoilage yeasts. Phytochem Rev. 2013;12:961–77.
52. Doan TTP, Carlsson AS, Hamberg M, Bülow L, Stymne S, Olsson P. Functional expression of five Arabidopsis fatty acyl-CoA reductase genes in *Escherichia coli*. J Plant Physiol. 2009;166:787–96.
53. Rutter CD, Rao C V. Production of 1-decanol by metabolically engineered *Yarrowia lipolytica*. Metab Eng. 2016;38:139–47.
54. Martín JF, Casqueiro J, Liras P. Secretion systems for secondary metabolites: How producer cells send out messages of intercellular communication. Curr Opin Microbiol. 2005;8:282–93.
55. Claus S, Jenkins Sánchez L, Van Bogaert INA. The role of transport proteins in the production of microbial glycolipid biosurfactants. Appl Microbiol Biotechnol. 2021;:1779–93.
56. Fischbach MA, Walsh CT, Clardy J. The evolution of gene collectives: How natural selection drives chemical innovation. Proc Natl Acad Sci. 2008;105:4601–4608.
57. Elbourne LDH, Tetu SG, Hassan KA, Paulsen IT. TransportDB 2.0: a database for exploring membrane transporters in sequenced genomes from all domains of life. Nucleic Acids Res. 2017;45 November 2016:320–4.
58. Elbourne L, Hassan K, Tetu S, Paulsen I. TransportDB 2.0. <http://www.membranetransport.org/transportDB2/index.html>. Accessed 9 Jun 2021.

59. Almagro Armenteros JJ, Sønderby CK, Sønderby SK, Nielsen H, Winther O. DeepLoc: prediction of protein subcellular localization using deep learning. *Bioinformatics*. 2017;33:3387–95.
60. Lang S, Brakemeier A, Heckmann R, Spöckner S, Rau U. Production of native and modified sophorose lipids. *Chim oggi-Chem Today*. 2000;18:76–9. <http://cat.inist.fr/?aModele=afficheN&cpsidt=816695>. Accessed 15 Mar 2016.
61. Kim D, Paggi JM, Park C, Bennett C, Salzberg SL. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nat Biotechnol*. 2019;37:907–15. doi:10.1038/s41587-019-0201-4.
62. Anders S, Pyl PT, Huber W. HTSeq - A Python framework to work with high-throughput sequencing data. *bioRxiv*. 2014;:1–4. doi:10.1101/002824.
63. Robinson MD, McCarthy DJ, Smyth GK. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*. 2009;26:139–40.
64. VanBogaert I, De Maeseneire S, Develter D, Soetaert W, Vandamme EJ. Cloning and characterisation of the glyceraldehyde 3-phosphate dehydrogenase gene of *Candida bombicola* and use of its promoter. *J Ind Microbiol Biotechnol*. 2008;35:1085–92.
65. Saerens KMJ, Saey L, Soetaert W. One-step production of unacetylated sophorolipids by an acetyltransferase negative *Candida bombicola*. *Biotechnol Bioeng*. 2011;108:2923–31. doi:10.1002/bit.23248.
66. Kahm M, Hasenbrink G, Lichtenberg-Fraté H, Ludwig J, Kschischo M. Grofit: Fitting biological growth curves with R. *J Stat Softw*. 2010;33:1–21.
67. Seabold S, Perktold J. statsmodels: Econometric and statistical modeling with python. In: 9th Python in Science Conference. 2010.

## Figures



**Figure 1**

Generic chemical structure of (a) acidic sophorolipids and (b) lactonic sophorolipids.

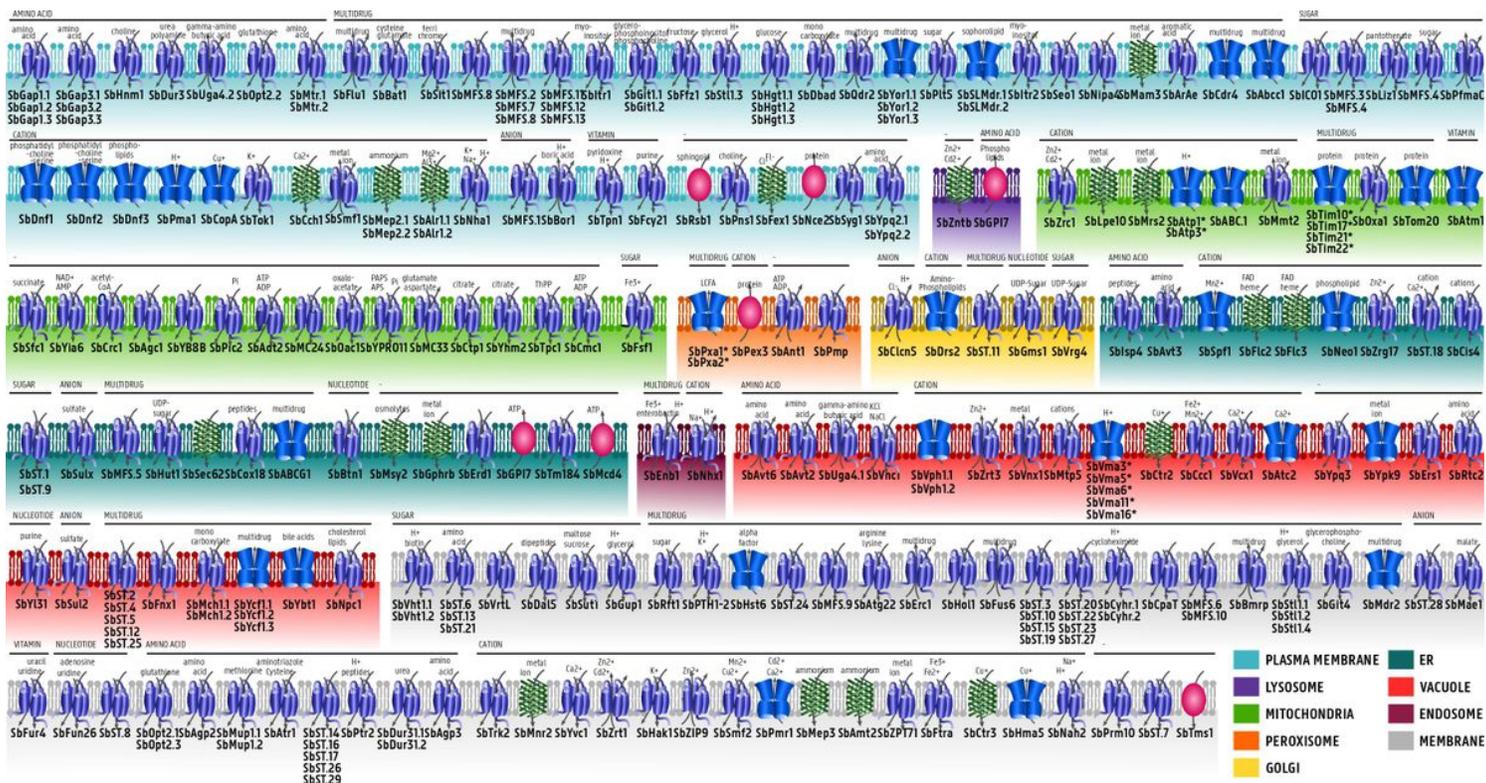


Figure 2

Overview of the predicted transporters in *S. bombycolia* with their substrates predicted according to homology. Pumps are depicted in blue, channels in green and carriers in purple. Transporters of unknown mechanism are depicted as green oval. The color of the membrane structure represents the subcellular localization of the transporter as predicted by the DeepLoc online tool and curated by the experimental evidence of the closest homologs. Transporters are grouped according to the substrate specificity found by the TransAAP algorithm: amino acids, multidrug, anions, vitamins, nucleotides and sugar. More detailed information on the substrate groups can be found in the Supplementary Table S2.

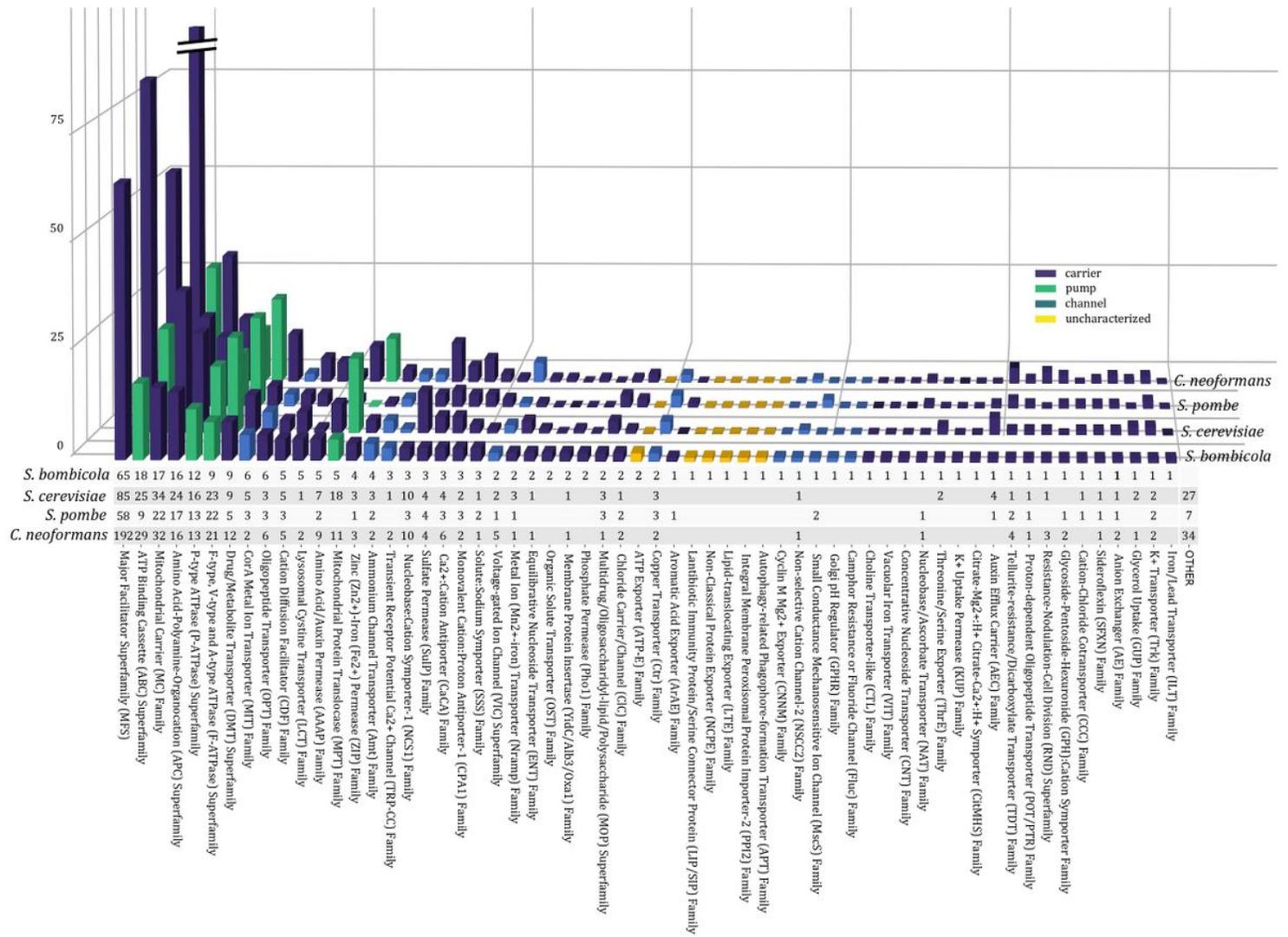
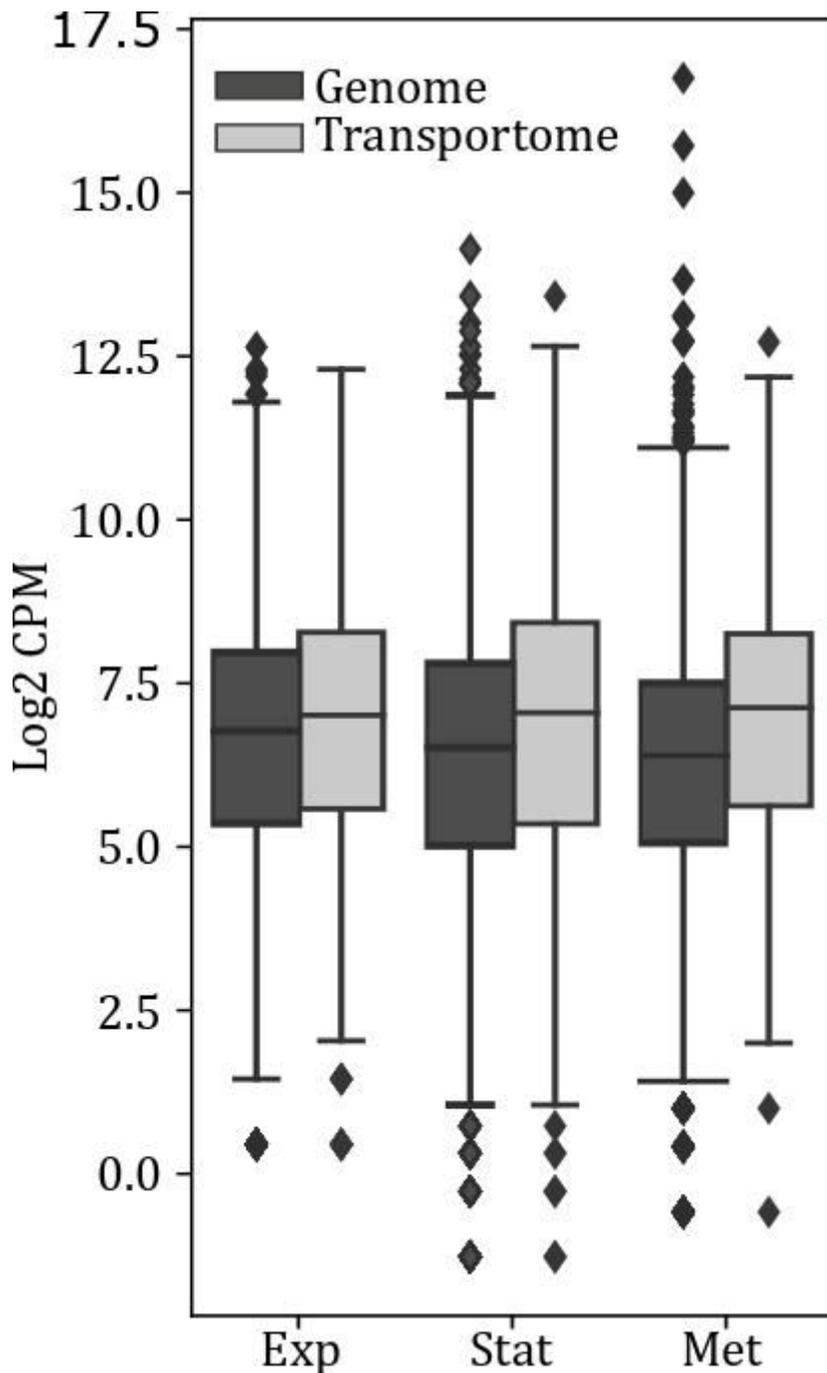


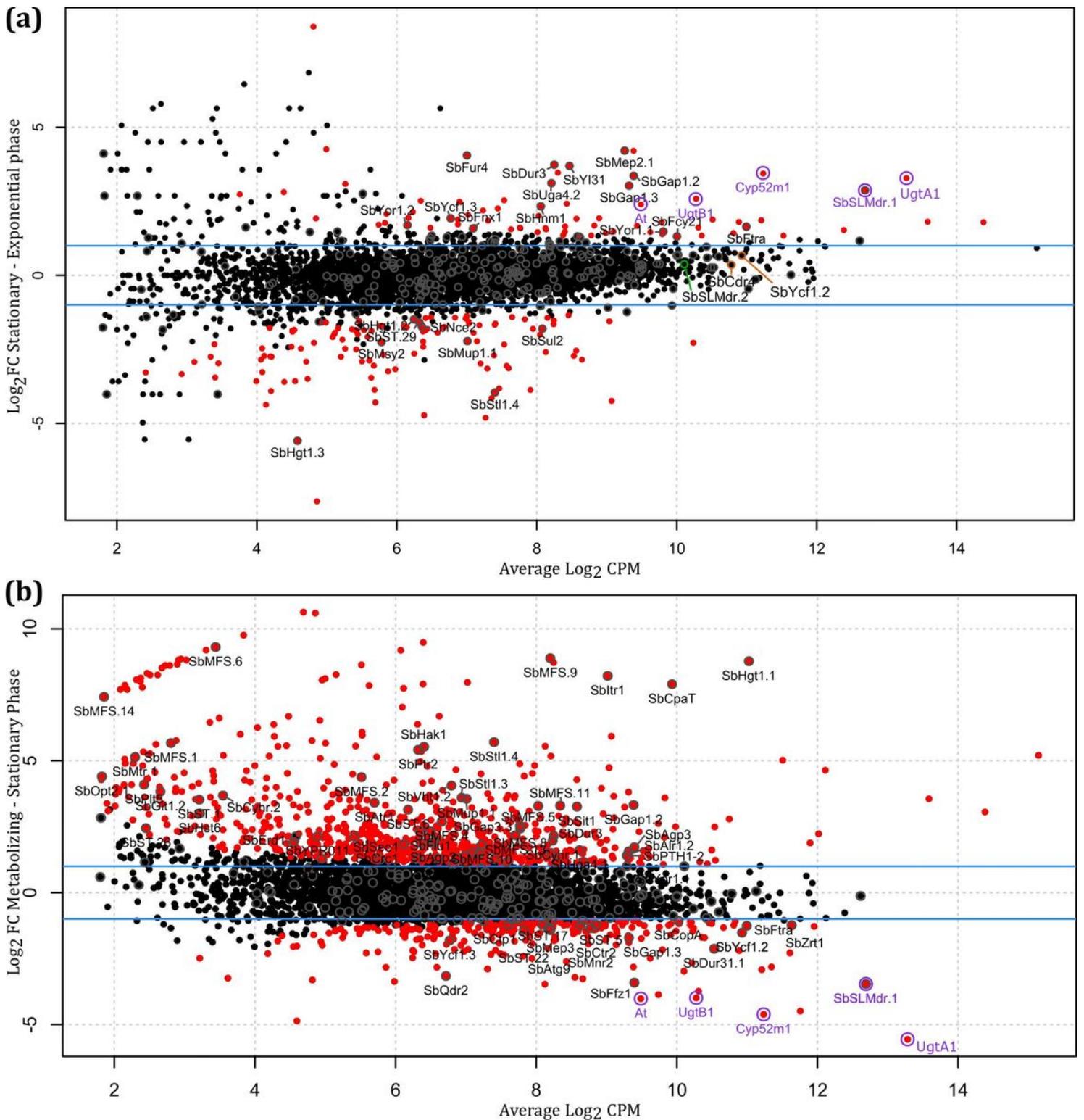
Figure 3

Transporter (super)family distribution of predicted transporters of *S. bombicola* in comparison with *S. cerevisiae*, *S. pombe* and *C. neoformans* according to the TCDB classification system.



**Figure 4**

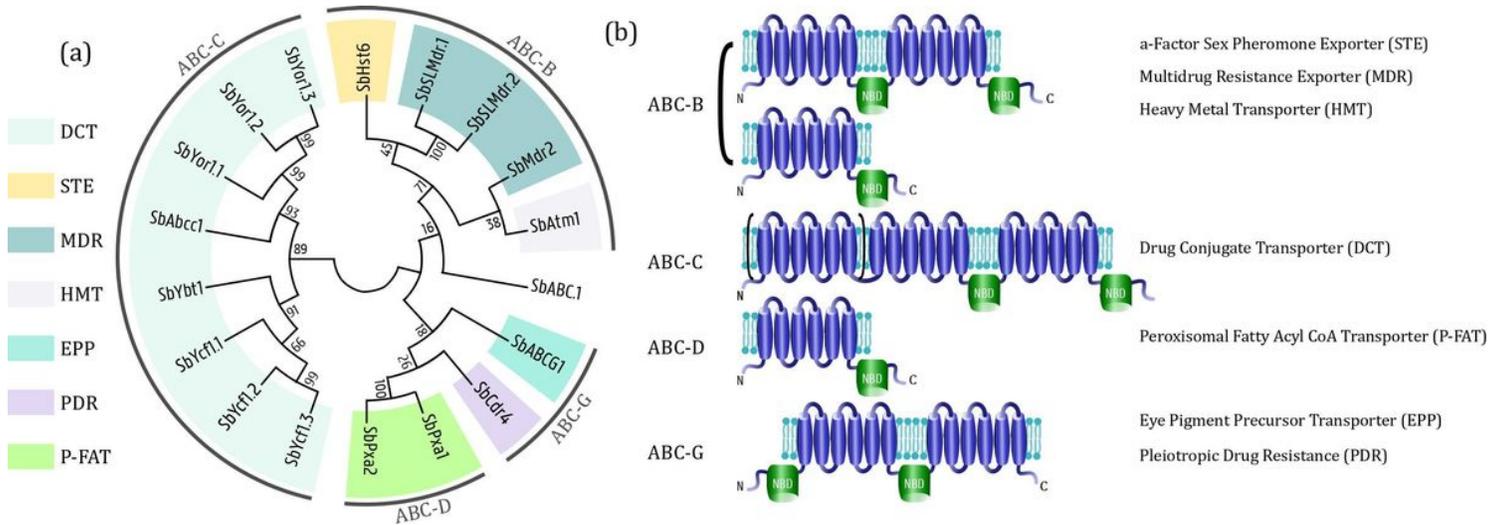
RNA Seq Count Data distribution after TMM normalization of the entire genome compared to the data distribution for the transportome. 'Exp': data sampled in the exponential phase, 'Stat': data samples in the stationary phase and 'Met': data sampled very late in the growth stage where the assembled SLs are the sole carbon source left. The horizontal line within the box represents the median, the lower and upper boundary of the box represent the first and third quantile respectively. Whiskers above and below the box extend to the most extreme data point which is no more than 1.5 times the interquartile range. Outliers beyond this range are represented by diamonds.



**Figure 5**

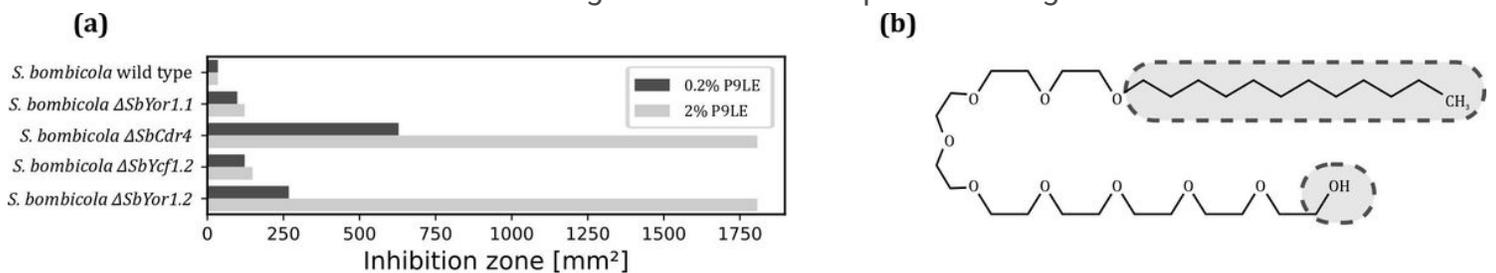
Smear plot showing the log<sub>2</sub> fold change (FC) versus the average log<sub>2</sub> count per million (CPM) of the change in gene expression. (a) Genes with positive Log<sub>2</sub> FC are upregulated in the stationary phase compared to the exponential phase, while genes with negative FC are downregulated. (b) Genes with positive FC are upregulated under metabolizing conditions compared to the stationary phase, while genes with negative FC are downregulated. The horizontal blue lines delineate log<sub>2</sub> FC ≥ 1, indicating genes with

two fold differences in expression. Each dot represents one gene, transporters present in the predicted transportome are circled in grey. Genes from the SL biosynthetic gene cluster are indicated in purple. Differentially expressed genes (FDR<0.05) are indicated in red.



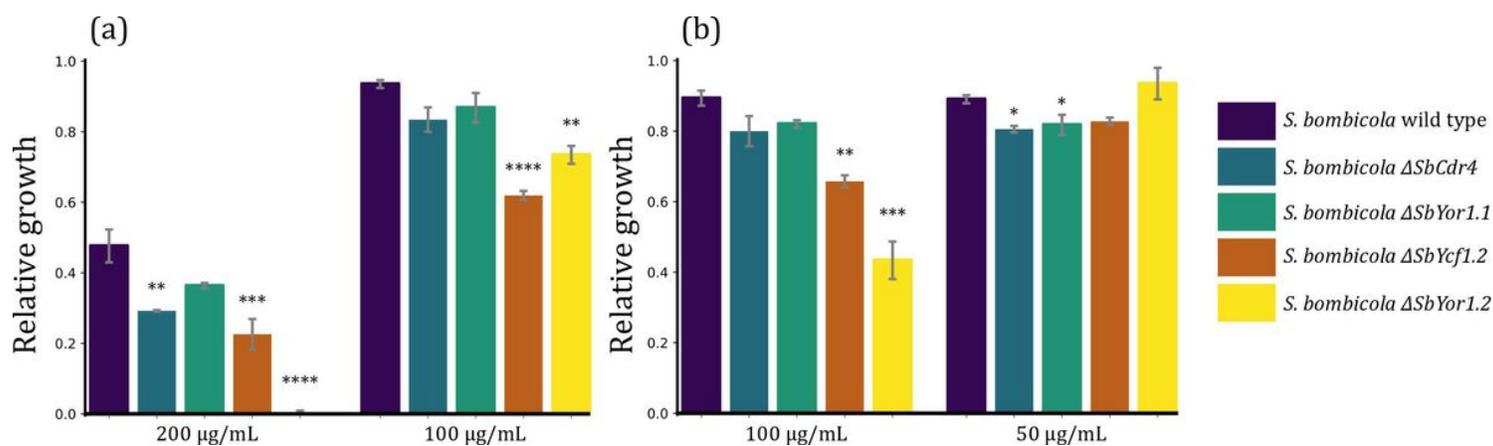
**Figure 6**

(a) The maximum-likelihood phylogenetic tree using 1000 replicates of the ABC Superfamily members in *S. bombicola* transportome, after alignment using Clustal Omega. Gene names are given at the terminal nodes. Subfamily Classification according to the Human Genome Organization (ABC-A to ABC-H) as well as the TCDB family designation is indicated (see legend) (b) Predicted topology according to their HUGO subfamily grouping and confirmed by sequence analysis by the Scan Prosite online tool (<http://prosite.expasy.org>). TMDs consists of six transmembrane helices, represented in blue. NBDs with the Walker A and B motifs and the ABC signature motif are represented in green.



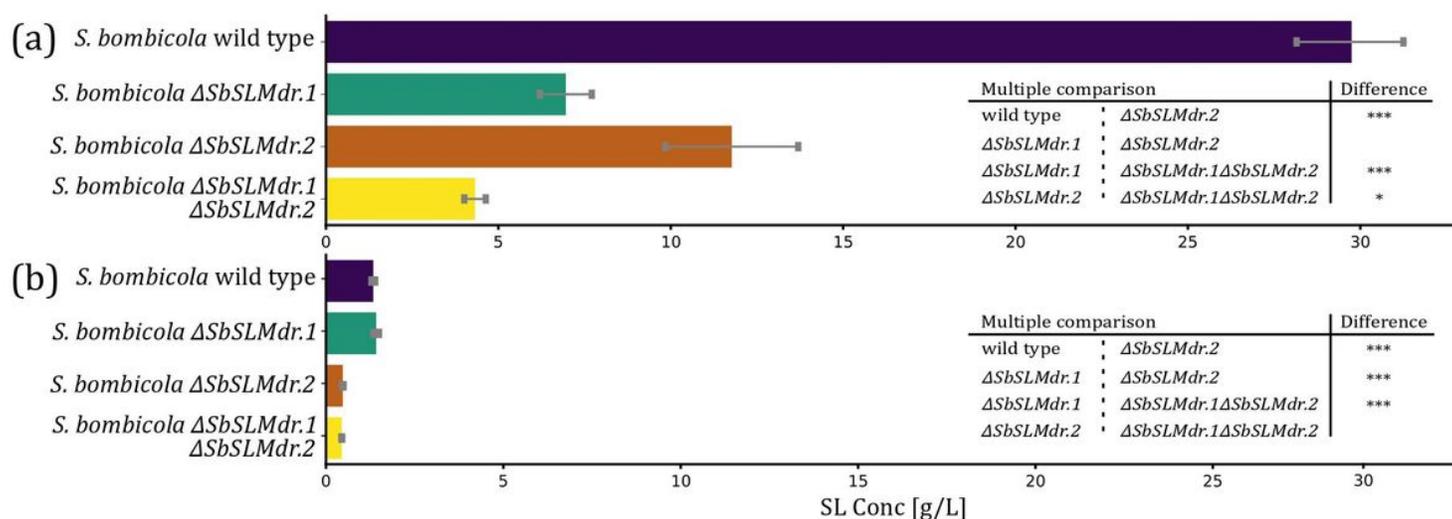
**Figure 7**

(a) The inhibitory effect of polyoxyethylene 9 lauryl ether (P9Le) as represented by the area of clearing zone [mm<sup>2</sup>] (b) Graphical representation of the chemical structure of P9Le. Dashed lines indicate the potential transport recognition sites.



**Figure 8**

Relative growth of *S. bombicola* ABC transporter knockout mutants in SD medium supplemented with (a) decanol and (b) undecanol. The results are shown as mean  $\pm$  standard error of the mean. Statistically significant differences compared to wild-type relative growth are indicated with asterisks. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ .



**Figure 9**

Multiple comparison of mean SL production on shake flask scale (a) with the addition of 37.5 g/L rapeseed oil and (b) without the addition of rapeseed oil. Statistically significant differences between wild type and SL transporter deletion mutants are indicated with asterisks, as calculated using one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ .

## Supplementary Files

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