SUT1-promoted sterol uptake involves the ABC transporter Aus1 and the mannoprotein Dan1 whose synergistic action is sufficient for this process

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Efficient sterol influx in the yeast *Saccharomyces cerevisiae* is restricted to anaerobiosis or to haem deficiency resulting from mutations. Constitutive expression of *SUT1*, an hypoxic gene encoding a transcriptional regulator, induces sterol uptake in aerobiosis. A genome-wide approach using DNA microarray was used to identify the mediators of *SUT1* effects on aerobic sterol uptake. A total of 121 ORFs (open reading frames) were significantly and differentially expressed after *SUT1* overexpression, 61 down-regulated and 60 up-regulated. Among these genes, the role of the putative ABC transporter (ATP-binding-cassette transporter) Aus1, and of the cell-wall mannoprotein Dan1, was characterized better. These two genes play an essential role in aerobic sterol uptake, since their deletion compromised the *SUT1* effects, but individual overexpression of either of these genes in a wild-type background was not sufficient for this process. However, constitutive co-expression of *AUS1* and *DAN1* in a wild-type background resulted in sterol influx in aerobiosis. These results suggest that the corresponding proteins may act synergistically *in vivo* to promote sterol uptake.

Key words: ATP-binding-cassette transporter (ABC transporter), *AUS1*/*YOR011W*, *DAN1*, sterol uptake, *SUT1*, *UPC2*.

INTRODUCTION

Budding yeast *Saccharomyces cerevisiae* can alternatively utilize respiration or fermentation for its metabolic requirements. Its adaptation to aerobiosis and anaerobiosis is achieved by the differential expression of a large number of genes whose regulation depends on oxygen availability [1–3].

Biosynthesis of sterols and unsaturated fatty acids are strict aerobic processes in this organism. Although sterol synthesis is an energy-consuming process, under aerobiosis, yeast does not take up a significant amount of exogenous sterols. In contrast, under anaerobiosis, when sterol biosynthesis is compromised, cells become capable of importing these essential molecules, provided that they are present in the medium. This physiological phenomenon is generally referred to as aerobic sterol exclusion and its regulation is at least partly mediated by haem availability [4].

In contrast with lipoprotein-mediated sterol uptake, free sterol influx by eukaryotic cells is still poorly understood. This saturable process depends on many factors that are not yet formally identified. However, de-regulation of two transcriptional regulators has been described as favouring exogenous sterol accumulation in aerobiosis.

Indeed, Lewis et al. [5] isolated a yeast mutant, *upc2-1* (uptake control), which leads to aerobic sterol uptake in haem- and ergosterol-competent cells. It was suggested that the *upc2-1* allele corresponds to a gain-of-function mutation, since deletion of *UPC2* does not result in sterol uptake. Upc2 contains a Zn(II)2Cys6 dinuclear cluster DNA-binding domain, a motif unique to fungal proteins shared by transcriptional regulators. Upc2, and its paralogue Ecm22, activate transcription through binding to sterol-regulatory elements of ergosterol biosynthesis genes [6,7]. Therefore Upc2-mediated sterol uptake in yeast results from altered transcriptional regulation of one or more target genes of Upc2. Recently, Wilcox et al. [8] used a genome-wide approach to identify all the putative Upc2 target genes. Starting from 82 genes induced in the *upc2-1* mutant, only nine genes were identified as putative actors of sterol uptake. Among these candidates, deletion of *DAN1*, a gene of the *DAN/TIR* family, or inactivation of *YOR011W* (subsequently named as *AUS1*), and its close paralogue *PDR11*, which encode putative ABC transporters (ATP-binding-cassette transporters), significantly decreased aerobic sterol influx in the *upc2-1* background, although to variable extents. Most interestingly, the combination of the two mutations *aus1*Δ*pdr11*Δ severely compromised growth in anaerobiosis, thereby suggesting that they play a key role in exogenous sterol uptake [8].

A second transcription factor involved in sterol uptake has been identified. Assuming that the genes involved in sterol uptake are down-regulated in aerobiosis, a high-copy-number library was screened in our laboratory to identify genes whose overexpression may overcome aerobic sterol exclusion. The only gene identified using this approach was *SUT1* [9]. Deletion of this hypoxic gene does not compromise growth, irrespective of oxygen availability. Its overexpression under the control of a strong constitutive promoter results in a high increase (up to 18-fold) in sterol influx in aerobiosis [10]. *SUT1* encodes a nuclear protein which, similar to Upc2, belongs to the Zn(II)2Cys6 family. However, in contrast with Upc2, Sut1 does not appear to bind to DNA directly. Rather, this protein acts at the transcriptional level by relieving hypoxic genes from Cyc8–Tup1 repression through its physical interaction with Cyc8 [11]. Cyc8–Tup1 acts as a co-repressor complex that is recruited to many promoters via specific interactions with DNA-binding regulatory proteins [12], such as Rox1, involved in the repression of many hypoxic genes [13]. *SUT1* transcription is induced in anaerobiosis or in haem-deficient strains; although putative Rox1-binding sites reside in its promoter, *SUT1* appears to be only moderately down-regulated by Rox1 [9].

It has been reported that in some cases, the Cyc8–Tup1 protein complex can shift from a transcriptional co-repressor to a transcriptional co-activator [14–16]. In a previous work, we have reported that in the presence of Sut1, the Cyc8–Tup1 complex is also

Abbreviations used: ABC transporter, ATP-binding-cassette transporter; GPI, glycosylphosphatidylinositol; NP40, Nonidet P40; ORF, open reading frame.

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Table 1 Yeast strains

Strains	Genotype/characteristics	Source
FY1679α	*MAT*α, *leu2 his3 trp1 ura3*	Progeny of diploid FY1679 [28]
YPA1-2	*MAT*α, *his3-*Δ*1 leu2-*Δ*0 ura3-*Δ*0 met15-*Δ*0 upc2::kanMX4*	This study, progeny of Y23572 (EUROSCARF)
YPA3	*MAT*α, *his3-*Δ*1 leu2-*Δ*0 ura3-*Δ*0 lys2-*Δ*0 met15-*Δ*0*	This study, progeny of BY4743 (EUROSCARF)
Y11787	*MAT*α, *his3-*Δ*1 leu2-*Δ*0 ura3-*Δ*0 lys2-*Δ*0 yor011w::kanMX4*	EUROSCARF
YPA4	*MAT*α, *his3-*Δ*1 leu2-*Δ*0 ura3-*Δ*0 dan1::kanMX4*	This study, progeny of Y26940 (EUROSCARF)
YPA4-1	*MAT*α, *his3-*Δ*1 leu2-*Δ*0 ura3-*Δ*0 lys2-*Δ*0 met15-*Δ*0 dan1::kanMX4*	This study, progeny of Y26940 (EUROSCARF)
RZ53-6Δrox1	*MAT*α, *trp1-289 leu2-3 leu2-112 ura3-52 ade1-100 rox1::LEU2*	[29]
YPA5	*MAT*α, *leu2 his3 trp1 ura3 rox1::LEU2*	This study, progeny of two backcrosses between RZ53-6Δrox1 $\times$ FY1679a
Y04529	*MAT*α, *his3-*Δ*1 leu2-*Δ*0 ura3-*Δ*0 met15-*Δ*0 sut1::kanMX4*	EUROSCARF
YPA6	*MAT*α, *leu2 his3 ura3 rox1::LEU2 dan1::kanMX4*	This study, progeny of YPA5 $\times$ YPA4-1

Table 2 List of plasmids

Plasmids	Characteristics/marker	Source
pNEV-N	*PMA1* expression cassette in 2μ-derivative, *URA3*	[30]
pNF1	$PMA1_{prom}$*::SUT1* in pNEV-N	[10]
YEp13	2μ-derivative, *LEU2*	[31]
YEp13-SUT1	$PMA1_{prom}$*::SUT1* in YEp13	This study
pNEV-AUS1	$PMA1_{prom}$*::AUS1* in pNEV-N	This study
pNEV-DAN1	$PMA1_{prom}$*::DAN1* in pNEV-N	This study
YEp13-AUS1	$PMA1_{prom}$*::AUS1* in YEp13	This study

converted into a transcriptional co-activator, resulting in the induction of several hypoxic genes [11]. The present study allowed us to characterize better the transcriptional effects of Sut1, but we failed to identify components of free sterol influx, with the exception of *CSR1*, a *SEC14* homologue, whose overexpression promotes increased sterol influx in aerobiosis [17].

In the present work, we performed a genome-wide transcriptional analysis to identify all the genes differentially transcribed after *SUT1* constitutive expression by comparison with the wild-type in aerobiosis. We show that co-expression of *DAN1* and *AUS1*, already identified as being involved in sterol uptake [8], is sufficient to promote the uptake of sterol in aerobiosis, indicating that they may act synergistically *in vivo* to promote sterol entry. However, the massive sterol influx observed in anaerobiosis, in haem-depleted cells or as a result of *upc2-1* mutation or *SUT1* overexpression, may still require other as yet uncharacterized components.

EXPERIMENTAL

Strains and plasmids

The yeast strains and plasmids used throughout the present study are listed in Tables 1 and 2 respectively. Strains were grown in standard minimal media [YNB: 1.7 g/l Difco™ yeast nitrogen base without amino acids and ammonium sulphate (Difco Laboratories), 5 g/l ammonium sulphate and 1 % D-glucose], supplemented with the appropriate amino acids and/or bases for plasmid selection. Fenpropimorph resistance was analysed on YPD solid medium, containing 1 μg/ml (31.5 μM) fenpropimorph and 80 μg/ml ergosterol solubilized in Tergitol NP40 (Nonidet P40)/ethanol (1:1, v/v). When ergosterol was omitted, Tergitol NP40/ethanol (1:1, v/v) was added to a final concentration of 2 %. The media used for anaerobic growth were supplemented with 1 % (v/v) Tween 80 (polyethylene sorbitan mono-oleate) and 80 μg/ml ergosterol in Tergitol NP40/ethanol (1:1, v/v). Anaerobic conditions were obtained with the BBL GasPak Anaerobic System (Becton Dickinson) in an anaerobic chamber.

RNA extraction and mRNA purification

Cells were grown in selective medium, harvested in mid-exponential phase, washed with RNase-free water and immediately frozen in liquid nitrogen. Cells were broken using TRIzol® reagent (Invitrogen). After extraction, RNAs were precipitated with isopropyl alcohol, washed with 75 % (v/v) ethanol and resuspended in RNase-free water. Poly(A)$^+$ (polyadenylated) RNAs were purified using PolyATract mRNA Isolation System IV (Promega). For Northern-blot analysis, we performed electrophoresis, blotting and hybridization with total RNA as described previously [18].

Generation of Cy3- and Cy5-labelled cDNA probes

Double-strand labelled cDNA was obtained by *in vitro* transcription using CyScribe first-strand cDNA-labelling kit (Amersham Biosciences) with 1.5 μg of poly(A)$^+$ RNA. Reverse transcription was performed in the presence of CyScript reverse transcriptase. RNAs were hydrolysed with 2.5 M NaOH at 37 °C for 15 min. Probes were purified using the QIAquick nucleotide removal kit (Qiagen). For each experimental condition, an independent set of probes was generated.

Microarray hybridization and scanning

S. cerevisiae microarray slides (Eurogentec, Angers, France) were used. For hybridization, 10 μl of probe, 30 μl of DIG Easy Hyb buffer (Boehringer Mannheim, Mannheim, Germany) and 2 μl (10 mg/ml) of salmon sperm DNA were mixed. Microarray experiments with FY1679α + [pNEV-N] and FY1679α + [pNF1] were performed twice with each pair of probes (Cy3 or Cy5 label), which were reversed for each experiment (generating four sets of data). The mixture was heated for 5 min at 95 °C and centrifuged at 12000 ***g*** for 2 min. Hybridization was performed at 37 °C overnight. After hybridization, the arrays were washed, dried and subsequently scanned with a GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA, U.S.A.).

Data analysis

The GenePix 4000B software package (Axon Instruments) was used to locate spots in the microarrays. Taking into account the numerous intrinsic variations of transcriptional profiling on arrays, a statistical analysis was performed to ensure the significance of the conclusions drawn from the data. Signal intensities of each hybridization were first normalized by the method of Yang et al.

[19] to remove the variation due to differences in the labelling between the two fluorescent dyes. For the statistical analysis, the normalized data for four independent hybridizations were compared using the tpWY software, allowing us to calculate an adjusted *P* value [20].

Respiration measurements

Exogenous respiration was determined with cells grown on selective medium (YNB) for 24 h. Cells were suspended in sterile distilled water to a density of 1.6×10^8 cells/ml. Enumeration of cells was performed by hematocytometer counts. For each measurement, 1.6×10^7 cells were suspended in 2 ml of oxygen-saturated water. Oxygen consumption was measured at 30 °C using an oxygraph (Hansatech Instruments, Norfolk, U.K.). Respiration rates are expressed as nmol of oxygen consumed $ml^{-1} \cdot min^{-1}$. Statistical significance of differences in oxygen consumption was assessed by the Mann–Whitney test.

Analysis of sterol uptake

Sterol uptake was measured essentially as described in [21], from independent cultures ($n = 7$) grown for 24 h in 5 ml of selective medium containing 26 μM [4-^{14}C]cholesterol (2600 d.p.m./μg) prepared from a stock supplied by NEN (Boston, MA, U.S.A.; at 46 mCi/mmol). Samples were collected by removing 4 ml of culture. After centrifugation, the cells were washed four times with 0.5 % (v/v) Tergitol NP40 and once with water. The net accumulation of [4-^{14}C]cholesterol in the cells was measured by scintillation counting and is expressed as μg of cholesterol/mg dry weight.

RESULTS

UPC2 inactivation does not compromise the *SUT1* effect on sterol uptake

Since strains bearing either the gain-of-function *upc2-1* mutation, or constitutively expressing *SUT1*, both promote free sterol influx in aerobiosis, we wondered whether *SUT1* may indirectly promote sterol uptake through activation of the *UPC2* gene. To rule out this possibility, plasmid pNF1 (*SUT1*) was introduced both in a *upc2*Δ strain and in the isogenic wild-type. As a control, the same strains were transformed with the empty plasmid (pNEV-N). Sterol import was assayed by spotting serial dilutions of transformants on a fenpropimorph-containing medium supplemented with ergosterol, under standard aerobic conditions. This assay has been widely used in previous studies to evaluate the ability of the cells to import exogenous sterol in aerobiosis, thereby enabling them to resist the inhibitor [9,10,17]. As a control, cell suspensions were also plated on to a medium containing the inhibitor, but no ergosterol, to assess that growth was not due to an ergosterol-independent resistance to the inhibitor. In addition, this control allowed us to evaluate spontaneous fenpropimorph-resistant strains that arise commonly within the most concentrated suspensions: however, since the phenotype of these mutants is ergosterol-independent, they can be easily discriminated from cells that do import ergosterol (Figure 1). From these experiments, we observed that *UPC2* inactivation does not impair *SUT1*-induced sterol import under these conditions significantly (Figure 1).

This result indicates that the effect on sterol uptake induced by constitutive expression of *SUT1* is not mediated by *UPC2*. Hence, we decided that it will be more appropriate to characterize the *SUT1* effects on transcription on a genome-wide level.

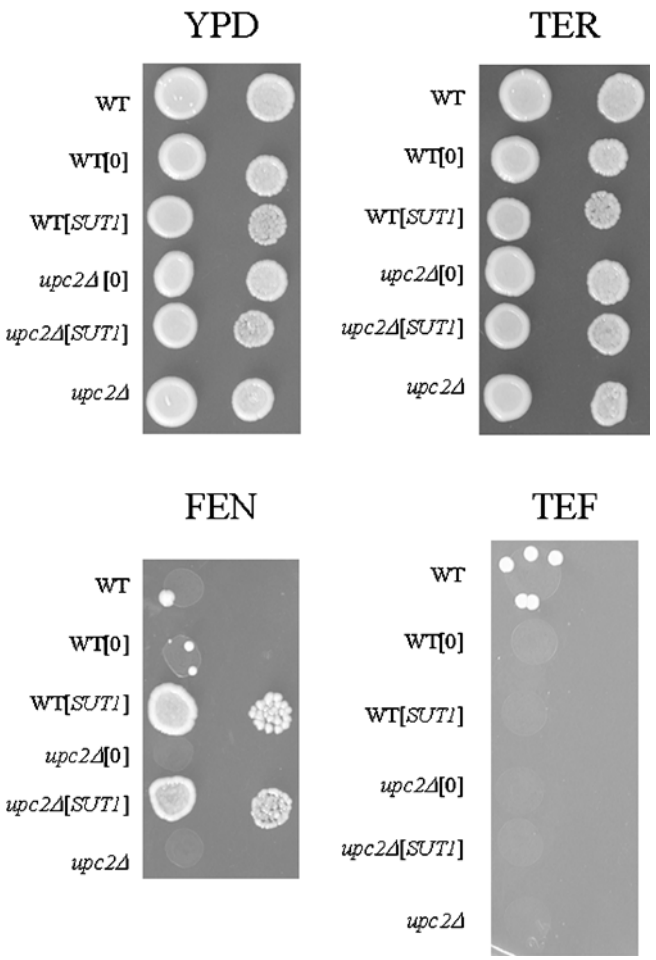


Figure 1 *UPC2* inactivation does not compromise the *SUT1* effects on sterol uptake

Serial 10-fold dilutions of cell suspensions of YPA1-1 (*upc2*Δ) and YPA3 (WT, wild-type), transformed with plasmid pNF1 harbouring the *SUT1* gene (resp. *upc2*Δ[*SUT1*] and WT[*SUT1*]) or with the empty vector pNEV-N (resp. *upc2*Δ[0] and WT[0]), were propagated on selective medium and subsequently spotted on to complete medium (YPD), YPD containing 80 μg/ml ergosterol in Tergitol/ethanol (1:1) with 1 μg/ml fenpropimorph (FEN) or without the inhibitor (TER) and YPD containing 2 % (v/v) Tergitol/ethanol (1:1) and 1 μg/ml fenpropimorph (TEF and ergosterol omitted).

Genome-wide analysis of *SUT1* effects on transcription

We have reported that Sut1 is a nuclear protein whose constitutive expression alters the transcriptional regulation of several genes [10,11,17]. In particular, we observed that hypoxic genes were up-regulated in aerobiosis as a result of *SUT1* overexpression. To achieve an exhaustive identification of all the genes whose transcription is altered after *SUT1* overexpression, we performed a DNA microarray-assisted screening. For this purpose, we compared the transcriptional profile of a wild-type strain (FY1679α) with the same strain transformed with pNF1, a high-copy-number plasmid in which *SUT1* expression is driven by the strong constitutive *PMA1* promoter [10]. Both strains were grown under standard aerobic conditions and RNAs were isolated from mid-exponential phase cultures. ORFs (open reading frames) with differential expressions were detected from four experiments with reverse labelling. Only ORFs with a significant transcriptional alteration (adjusted $P < 0.05$) were selected, irrespective of the signal intensity relative to the control condition (Tables 3 and 4, repressed and induced ORFs respectively). A total of 121 ORFs

Table 3 List of ORFs with decreased signal in an *SUT1*-overexpressing strain relative to the wild-type in aerobiosis

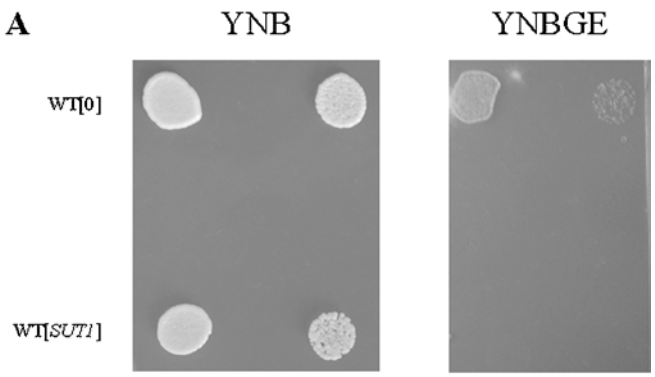
Results are expressed as the average of four experiments. Only genes with adjusted $P < 0.05$ were considered. The classification was based on the Comprehensive Yeast Gene Genome Database (http://mips.gsf.de/genre/proj/yeast/index.jsp); NC, not calculated.

ORF	Gene	Ratio	*P*
Mitochondrial function			
YLL018c-a	*COX19*	9.4	0.0081
YER053c		6.4	0.0036
YDL174c	*DLD1*	4.1	0.0188
YKL150w	*MCR1*	3.8	0.0006
YMR145c	*NDH1*	3.8	0.0336
YFR033c	*QCR6*	2.9	0.0081
YLR327c		2.3	0.0030
YEL024w	*RIP1*	2.2	0.0463
YJR077c	*MIR1*	2.2	0.0472
YNL055c	*POR1*	2.0	0.0030
Transport			
YML123c	*PHO84*	5.9	0.0005
YEL017c-a	*PMP2*	2.0	0.0030
YKR093w	*PTR2*	1.6	0.0451
Other putative transporters			
YPL221w	*BOP1*	4.4	0.0025
YGR142w	*BTN2*	4.1	0.0031
YPR149w	*NCE2*	4.0	0.0003
YKL051w		2.8	0.0039
Transcription			
YKL109w	*HAP4*	14.8	0.0019
YMR136w	*GAT2*	5.8	0.0174
YFR034c	*PHO4*	5.1	0.0019
YER088c	*DOT6*	3.0	0.0096
YMR070w	*MOT3*	3.0	0.0379
Nucleic acids processing			
YPR179c	*HDA3*	5.2	0.0011
YDL048c	*STP4*	5.1	0.0006
YDR243c	*PRP28*	4.2	0.0047
Stress response			
YFL014w	*HSP12*	10.8	0.0008
YOL151w	*GRE2*	7.1	0.0019
YER150w	*SPI1*	5.3	0.0005
YDR033w	*MRH1*	5.0	0.0003
YKL062w	*MSN4*	3.3	0.0325
YFL059w	*SNZ3*	3.1	0.0019
Metabolism			
YHR037w	*PUT2*	9.6	0.0306
YGL184c	*STR3*	5.6	0.0087
YGL157w		3.9	0.0033
YEL021w	*URA3*	3.1	0.0095
YBR111c	*YSA1*	2.7	0.0016
YML100w	*TSL1*	2.1	0.0137
YHR128w	*FUR1*	2.0	0.0315
Lipid metabolism			
YBR183w	*YPC1*	6.4	0.0012
YPR140w		2.3	0.0157
YGR157w	*CHO2*	2.1	0.0406
Cell wall			
YKL096w	*CWP1*	19.4	0.0006
YDL049c	*KNH1*	10.2	0.0019
Other			
YMR032w	*HOF1*	6.6	0.0267
YOR107w	*RGS2*	3.0	0.042
Unknown, hypothetical protein			
YER067w		9.2	0.0012
YPL014w		7.3	0.0036
YDL129w		7.2	0.0011
YGR001c		5.8	0.0039
YGR250c		5.1	0.0127
YOR062c		4.7	0.0006
YDR222w		4.0	0.0137
YOR138c		3.4	0.0230
YML101c		3.3	0.0028
YMR097c		3.2	0.0392
YBR230c		2.6	0.0395
YFR007w		2.0	0.0253
YBL006c		2.0	0.0458
YKL071w		1.8	0.0471
YBR157c	*ICS2*	1.4	0.0030
YHR087w		NC	0.0213

Table 4 List of ORFs with increased signal in an *SUT1*-overexpressing strain relative to the wild-type in aerobiosis

Only genes with adjusted $P < 0.05$ were considered. Hypoxic genes are underlined, whereas ORFs down-regulated by Rox1 in aerobiosis are indicated with an asterisk [3]; NC, not calculated.

ORF	Gene	Ratio	*P*
DAN, PAU and TIR gene family			
YOR009w	*TIR4*	79.9	0.0003
YJR150c	*DAN1*	54.5	0.0006
*YOR010c**	*TIR2*	22.2	0.0003
YAL068c		10.2	0.0003
YLL064c		7.3	0.0003
YHL046c		6.6	0.0005
YIL176c		6.6	0.0006
YEL049w	*PAU2*	5.5	0.0016
YGL261c		5.3	0.0006
YNR076w	*PAU6*	4.8	0.0005
*YBR067c**	*TIP1*	3.4	0.0012
YLR037c	*DAN2*	3.3	0.0031
YKL224c		3.1	0.0471
YLL025w		2.9	0.0123
YIL011w	*TIR3*	NC	0.0003
Cell wall			
YNR067c	*DSE4*	7.5	0.0006
YGL028c	*SCW11*	2.3	0.0019
YEL040w	*UTR2*	1.4	0.0019
Lipid metabolism			
YKL008c	*LAC1*	13.2	0.0006
*YLR413w**		8.1	0.0051
*YNL111c**	*CYB5*	2.1	0.0252
Transport			
YLL055w		13.7	0.0025
YOR011w	*AUS1*	9.6	0.0012
YLL061w	*MMP1*	5.4	0.0381
YPL274w	*SAM3*	3.2	0.0328
YBR294w	*SUL1*	3.1	0.0016
YHL016c	*DUR3*	1.5	0.0235
Intracellular trafficking			
YBR105c	*VID24*	2.7	0.0014
YHL002w	*HSE1*	1.5	0.0037
Sporulation			
YIL099w	*SGA1*	22.7	0.0084
DNA maintenance			
YGL107c		17.1	0.0005
YBR238c		2.7	0.0006
Transcription and translation			
*YOR032c**	*HMS1*	298	0.0096
YJR066w	*TOR1*	8.8	0.0003
YGL096w	*TOS8*	6.1	0.0148
YKL043w	*PHD1*	4.5	0.0019
*YJR047c**	*ANB1*	2.1	0.0182
Stress response			
YKL026c	*GPX1*	25.6	0.0003
YML058w-a	*HUG1*	NC	0.0014
Metabolism			
YHR033w		39.9	0.0017
YGR286c	*BIO2*	2.1	0.0051
YJL088w	*ARG3*	2.1	0.0364
*YGR180c**	*RNR4*	1.7	0.0087
YML082w		1.6	0.0235
Oxidoreduction, energy generation			
*YEL047c**	*FRDS1*	5.2	0.0117
*YHR039c**	*MSC7*	3.8	0.0030
*YIL111w**	*COX5B*	2.4	0.0039
Unknown, hypothetical proteins			
YDL241w		53.9	0.0308
YOL154w		24.1	0.0003
YLR346c		14.9	0.0236
YPR136c	*FYV15*	9.5	0.0006
YOR376w		8.7	0.0277
YPL068c		7.8	0.0042
YPL272c		7.6	0.0006
*YOR306c**		4.0	0.0449
YPL278c		3.7	0.0398
YOL164w		3.6	0.0228
*YGR079w**		3.3	0.0141
*YDR186c**		2.8	0.0317
YHR049w		2.2	0.0123



B

Strain	WT[0]	WT[*SUT1*]
Oxygen consumption (nmol ml^{-1} min^{-1})	0.282±0.021	0.129±0.008

Figure 2 *SUT1* overexpression impairs respiration

(**A**) Serial 10-fold dilutions of cell suspensions of YPA3, transformed with pNEV-N (WT[0]) or pNF1 (WT[*SUT1*]), were spotted on to selective medium containing glucose (20 g/l) as a carbon source (YNB) or a mixture of glycerol (20 g/l) and 1 % ethanol (YNBGE). The plates were incubated at 30 °C for 2 days (YNB) or 1 week (YNBGE). (**B**) Oxygen consumption of the same cells. Results are expressed as the means ± S.D. for three independent experiments. Statistical significance of differences was examined by the Mann–Whitney test ($P < 0.05$, $n = 3$).

were differentially regulated after *SUT1* overexpression; 61 of them were down-regulated and 60 were up-regulated. Changes in expression level varied from 1.4-fold up to 298-fold, but most genes exhibited a ratio of differential expression between 2 and 10. Many of the ORFs whose transcription is decreased are directly or indirectly related to mitochondrial functions (Table 3). Down-regulation of these genes may explain why the growth of *SUT1*-overexpressing strains is slower compared with the wild-type, as a consequence of a defect in the respiratory metabolism.

To address this question, cell suspensions of the wild-type strain (YPA3), transformed with pNF1 (*SUT1*) or with the control plasmid (pNEV-N), were spotted on to a medium containing a non-fermentable carbon source (glycerol/ethanol selective medium). As seen in Figure 2(A), non-fermentative growth of the *SUT1*-overexpressing strain was severely impaired. To confirm this result, oxygen consumption of the cells was measured. The respiration rate of the cells transformed with pNF1 was decreased by approx. 2.2-fold (Mann–Whitney test, $P < 0.05$, $n = 3$) when compared with the control strain (Figure 2B). Taken together, these results, which indicate that respiration is impaired after *SUT1* overexpression, are in good agreement with the microarray data.

Other essential metabolic function(s) may be affected by *SUT1*-induced de-regulation of the ORFs listed in Table 3. We have already reported that *SUT1* constitutive expression has a general negative effect on transcription [11]. Therefore we decided to focus on the genes that were activated (listed in Table 4).

Our results were compared with those obtained by Kwast et al. [3] in a study aimed at investigating the specific role of Rox1 in the adaptation to anaerobiosis [3], because our experimental conditions (batch cultures) were similar to those used by these authors. Among the genes induced by *SUT1* overexpression, 29 genes were identified as hypoxic genes, of which 13 were negatively regulated by Rox1 [3]. Altogether, these results are in full agreement with our previous report in which we have shown that *SUT1* constitutive expression relieved hypoxic genes from Cyc8–Tup1-mediated repression in aerobiosis [11]. It is noteworthy that, because of the stringency of our statistical analysis, some genes may have been discarded from our selection. Indeed, in a previous study, we identified *CSR1* as a clear *SUT1* target [17], besides *HES1* (M. Régnacq, unpublished work), but neither of these genes were selected in our microarray screening.

Among the up-regulated ORFs, the DAN (delayed anaerobic, cell wall mannoprotein induced during anerobic growth)/TIR (TIP1-related, cell wall mannoprotein) mannoproteins are widely represented (approx. 1/4). At least five other ORFs are also related to cell-wall modelling. Indeed, *YGL028c* (*SCW1*) and *YEL040w* (*UTR2*) encode glucanases, and *YNR067c* is similar to another bacterial glucanase, whereas *YKL008c* (*LAC1*) is involved in the transport of GPI (glycosylphosphatidylinositol)-anchored proteins. The function of *YLR413w* is still unknown, but it is highly similar to other members of a family of proteins involved in the elongation of fatty acids, which are themselves components of sphingolipids and GPI anchors. Several characterized or putative transporters are also up-regulated. *YLL061w* (*MMP1*), *YPL274w* (*SAM3*), *YBR294w* (*SUL1*), *YHL016c* (*DUR3*) and *YLL055w* belong to the major facilitator superfamily, whereas *YOR011w* (*AUS1*) belongs to the ABC transporter superfamily. So far, with the exception of Aus1 (see below), the function of these proteins has never been attributed to sterol uptake and/or metabolism.

A similar approach has been reported using *upc2-1* strains in which free sterol influx is stimulated in aerobiosis [8]. We reasoned that the genes that are most probably involved directly in sterol uptake should lie among those being regulated by *upc2-1* and *SUT1*. We thus compared their respective effects on transcription. A list of the ORFs regulated by both *SUT1* and *upc2-1* is presented in Table 5. All the 15 candidates have been shown to be up-regulated by the *upc2-1* gain-of-function mutation [8]. Most of them correspond to DAN/TIR mannoproteins. Wilcox et al. [8] identified Aus1 and Pdr11, members of the ABC transporter superfamily, and Dan1, a member of the DAN/TIR mannoprotein family, as being involved in sterol influx, since deletion of either of theses genes impairs sterol uptake. Interestingly, *AUS1* and *DAN1*, but not *PDR11*, are also significantly up-regulated after *SUT1* constitutive expression in our screening (adjusted $P < 0.001$

Table 5 ORFs differentially regulated both by Sut1 and by *upc2-1*

ORF	Gene	Ratio
DAN, TIR and PAU gene family		
YOR009w	*TIR4*	79.9
YJR150c	*DAN1*	54.5
YOR010c	*TIR2*	22.2
YAL068c		10.2
YLL064c		7.3
YHL046c		6.6
YIL176c		6.6
YEL049w	*PAU2*	5.5
YGL261c		5.3
YNR076w	*PAU6*	4.8
YBR067c	*TIP1*	3.4
YIL011w	*TIR3*	NC
Transport		
YOR011w	*AUS1*	9.6
Lipid metabolism		
YNL111c	*CYB5*	2.1
Unknown function, hypothetical protein		
YPL272c		7.6

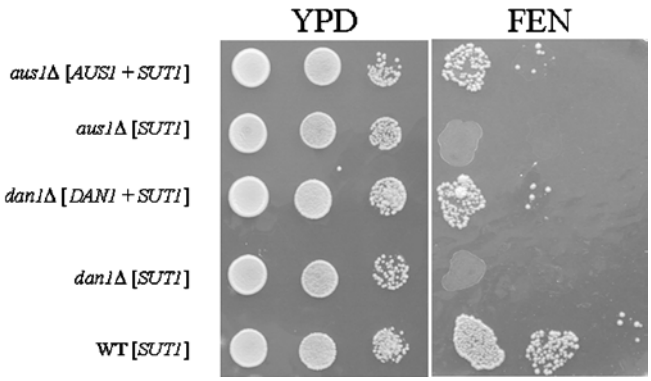


Figure 3 Effects of *AUS1* and *DAN1* on sterol uptake induced by *SUT1*

Serial 10-fold dilutions of a suspension of Y11787 (*aus1Δ*) and YPA4 (*dan1Δ*) transformed with Yep13-SUT1 were propagated on selective medium and subsequently spotted on to complete medium (YPD) and complete medium containing 1 μg/ml fenpropimorph, 80 μg/ml ergosterol in Tergitol/ethanol (1:1, v/v) (FEN). The same strains co-transformed with Yep13-SUT1 and pNEV-AUS1 or pNEV-DAN1 were used to ensure that the effects could be complemented by *AUS1* or *DAN1* respectively. The YPA3 strain (WT) transformed with Yep13-SUT1 was used as a positive control.

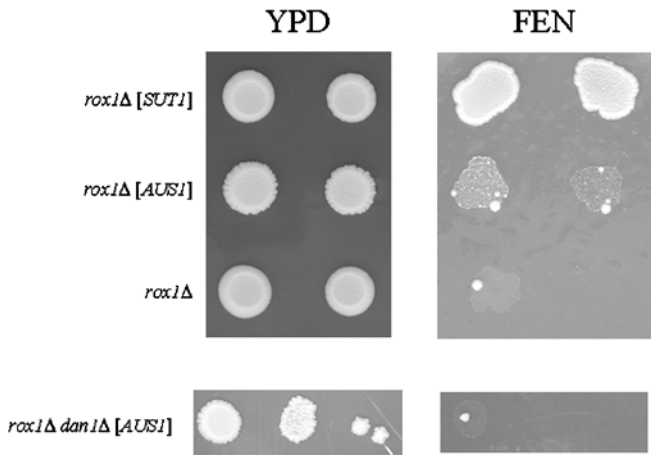


Figure 4 Effect of *AUS1* constitutive expression in a *rox1Δ* strain

Serial 10-fold dilutions of cell suspensions of YPA5 (*rox1Δ*) untransformed or transformed with either pNEV-AUS1 or pNF1 harbouring the *SUT1* gene, were propagated on selective medium and subsequently spotted on to either complete medium (YPD) or complete medium containing 1 μg/ml fenpropimorph, 80 μg/ml ergosterol in Tergitol/ethanol (1:1) (FEN). The strain YPA6 (*rox1Δdan1Δ*) transformed with pNF1 was also spotted on to the same media (lower panels). The largest isolated colonies growing in the presence of fenpropimorph (with the exception of the *rox1Δ*[SUT1] strain) correspond to spontaneous resistant mutants.

for both of them). This result was also confirmed by Northern-blot analysis (results not shown). Among the ORFs whose expression is commonly perturbed by *upc2-1* and *SUT1*, *CYB5*-encoding cytochrome b_5 and *YPL272c* (unknown function) are other potentially interesting candidates, since they were reported in a recent study to be also induced after azole exposure [22]. Their putative function in sterol uptake has not been examined so far.

AUS1 and *DAN1* are required for sterol uptake induced by *SUT1*

To characterize further the effects of *AUS1* and *DAN1* on sterol import, *SUT1* constitutive expression was assayed in an *AUS1*-deleted strain and in a *DAN1*-deleted strain. These strains were transformed with Yep13-SUT1 and tested for fenpropimorph resistance in the presence of ergosterol; the strains were then compared with the isogenic wild-type harbouring the same plasmid. As shown in Figure 3, *AUS1* or *DAN1* deletion abolished *SUT1* effects on sterol import in aerobiosis. As a control, the same strains were transformed with a plasmid carrying the wild-type *AUS1* or *DAN1* allele. The corresponding strains recovered their resistance to fenpropimorph. Therefore both *AUS1* and *DAN1* are required for *SUT1*-induced sterol import in aerobiosis.

AUS1 constitutive expression mediates sterol uptake in a *rox1Δ* strain

AUS1 encodes a putative ABC transporter. As shown by Wilcox et al. [8], this gene is necessary for sterol uptake and inhibition of ATP production compromises free sterol influx. However, so far, no direct positive role in sterol uptake has been reported for this gene. If Aus1 protein is directly involved in exogenous sterol uptake, its constitutive expression should result in enhanced sterol influx in aerobiosis. To test this hypothesis, we made a construct in which the *AUS1* ORF is under the control of the constitutive *PMA1* promoter, on a replicative plasmid (pNEV-AUS1). This construct was introduced into a wild-type strain (YPA3), which was subsequently challenged for growth in the presence of fenpropimorph and ergosterol. The sensitivity to the inhibitor did not differ from that of the control untransformed strain (results not shown). These results indicate that Aus1 is necessary but not sufficient to promote sterol uptake and Aus1 may require additional protein(s). Since *AUS1* does not seem to be down-regulated by Rox1 in contrast with several of the *SUT1*-regulated genes [3], we tested the possibility that the proteins co-operating with Aus1 for sterol uptake could be negatively regulated by Rox1. Therefore we introduced pNEV-AUS1 into an *ROX1*-deleted strain. Interestingly, in this genetic background, a moderate but significant fenpropimorph resistance related to sterol entry was observed, whereas the untransformed isogenic strain remains sensitive to the inhibitor (Figure 4). This is observed only if ergosterol is added to the medium. Notably, this effect is lost in a *rox1Δdan1Δ* background (Figure 4), indicating that *DAN1* is required for assisting *AUS1* to induce sterol uptake in aerobiosis.

AUS1 and *DAN1* co-expression results in enhanced sterol influx

We then questioned whether constitutive co-expression of *AUS1* and *DAN1* might be sufficient to mediate sterol uptake in aerobiosis. For this purpose, we co-transformed the wild-type YPA3 strain with two plasmids, each containing one of the two genes under the control of the *PMA1* constitutive promoter. In a first step, sterol entry was tested using the fenpropimorph assay. Colonies were obtained, whereas the control untransformed strain did not grow at all; however, the size of the colonies was much smaller than those obtained with pNF1 (*SUT1* overexpressed), which was used as a positive control (results not shown). Quantification of sterol uptake using [4-^{14}C]cholesterol confirmed that co-expression of *AUS1* and *DAN1* in aerobiosis increases sterol influx approx. 1.7-fold compared with the wild-type (Table 6). Although moderate, this sterol influx is highly reproducible and its significance was confirmed by a statistical analysis (Mann–Whitney test, $P < 0.05$, $n = 7$). However, exogenous sterol accumulation remained much lower than the *SUT1* effect (> 18-fold stimulation), suggesting that additional factors are required to promote full permeation.

DISCUSSION

Despite intensive investigations performed in several laboratories, the molecular mechanisms allowing sterol uptake in anaerobiosis,

Table 6 Cholesterol uptake

Results are expressed as the means ± S.D. for seven independent experiments. Statistical significance of differences was examined by the Mann–Whitney test ($P < 0.05$, $n = 7$).

Strain	Cholesterol uptake (μg/mg of dry weight)	Relative increase	Fenpropimorph resistance
YPA3+ [pNEV-N]	0.46 ± 0.04	1	−
[*SUT1*]	8.66 ± 1.02	18.8	+++
[*AUS1* + *DAN1*]	0.78 ± 0.10	1.70	+/−

and their exclusion in aerobiosis, are still poorly understood. So far, only a few genes involved in sterol influx have been characterized, two of them encoding transcriptional regulators, namely *UPC2* and *SUT1*. Their de-regulation, as a result of *upc2-1* gain-of-function mutation [5] or *SUT1* constitutive overexpression [9], is capable of promoting free sterol influx. Since both of them perturb the transcription of several genes, this suggests that exogenous sterol accumulation probably requires a cell competency ensured by several proteins whose genes are normally down-regulated in aerobiosis. Using a genome-wide transcriptional analysis, Wilcox et al. [8] have identified three Upc2 target genes whose deletion partly abolished *upc2-1* effects on free sterol influx, indicating that they are either directly or indirectly involved in this process [8]. One of these genes, *DAN1*, encodes a mannoprotein that belongs to a family of stress proteins, and its role in exogenous sterol accumulation is still puzzling. Dan1 is a putative cell-wall protein with a signature of a GPI anchor at its C-terminus. The putative role of *AUS1*, and of its close homologue *PDR11*, seems easier to understand. Indeed, their primary sequence indicates that the corresponding proteins belong to the family of ABC transporters. These proteins mediate the ATP-dependent efflux of a wide variety of molecules such as xenobiotic substrates (MDR, multi-drug resistance protein; [23]) or even lipids (ABC transporter A1, involved in cellular cholesterol efflux in mammals; [24]). Although it was reported that, consistent with the putative mode of action of Aus1 and Pdr11, the use of energy-depleting molecules abolished *upc2-1* effect on sterol influx [8], the direct implication of these two proteins in sterol uptake has not been formally proven so far. In two recent reports, it has been proposed that ABC transporters are involved in the influx of lipophilic molecules as well. Indeed CjMDR1, an MDR-type ABC transporter isolated from the plant *Coptis japonica*, was capable of transporting the alkaloid berberine in the inward direction when expressed in a *Xenopus* oocyte expression system, and this observation is in good agreement with its putative role *in planta* [25]. It has also been reported that Cdr3, an ABC transporter of *Candida albicans*, mediated out-to-in translocation of a phospholipid analogue between the two bilayers of the plasma membrane [26]. However, the *in vivo* function of these proteins has not been clearly established so far.

Since *UPC2* and *SUT1* have almost similar effects on exogenous sterol accumulation in aerobiosis, we first examined whether *SUT1* effect on sterol influx might be mediated indirectly by *UPC2*. This does not appear to be the case since deletion of *UPC2* did not abolish *SUT1* effects (Figure 1). Therefore we decided to identify the genes whose transcription is modified after *SUT1* overexpression, in a genome-wide screening using microarrays. For this purpose, we compared the transcriptional profiling of a strain overexpressing *SUT1* with that of the wild-type, both of which were cultivated in aerobiosis. This led us to identify 121 ORFs differentially regulated in the two strains. This relatively large number might be explained by the fact that the analysis was performed with RNA extracted at a single time point, when the culture had reached the mid-exponential phase. Thus it is not excluded that the differential regulation of at least some of these genes is the result of indirect cumulative effects of *SUT1* overexpression. However, we noticed that among these genes, 15 are also differentially regulated in the *upc2-1* strain (Table 5; [8]). Considering the similar effects of these two genes on free sterol influx, we reasoned that the most interesting candidates for controlling this process were genes that are commonly regulated by both the transcription factors. We thus focused more particularly on *DAN1* and *AUS1* to characterize further their role.

We confirmed that they do mediate *SUT1* effect on exogenous sterol accumulation, since the individual inactivation of either of these genes abolished *SUT1*-induced fenpropimorph resistance on a medium containing ergosterol, in aerobiosis (Figure 3). These results are in perfect agreement with the observations of Wilcox et al. [8]. No effect could be observed on exogenous sterol accumulation when these genes were overexpressed individually, indicating that this process requires more than a single protein. This is in good agreement with the results of genetic screenings performed so far.

Many but not all (e.g. *AUS1*) hypoxic genes are down-regulated by Rox1 in aerobiosis. Based on the fact that a *ROX1*-deleted strain is unable to import sterols in aerobiosis, we hypothesized that this phenomenon requires both Rox1-independent and -dependent proteins. Interestingly, *MOT3*, a gene encoding a transcriptional regulator which has been shown recently to act in synergy with Rox1 to repress anaerobic genes [27], was negatively regulated after *SUT1* overexpression (Table 3). We decided to overexpress *AUS1* in a *ROX1*-deleted background. All the clones tested were capable of growing on a fenpropimorph-containing medium, supplemented with ergosterol, demonstrating an efficient exogenous sterol accumulation in aerobiosis (Figure 4). This phenotype required the presence of a wild-type copy of *DAN1*.

This latter observation prompted us to overexpress both *AUS1* and *DAN1* in a single wild-type strain. Although growth on fenpropimorph was low, radiolabelled cholesterol accumulated significantly and reproducibly in this strain (Table 6). The level of sterol influx does not reach that obtained in a haem-incompetent strain or in a strain overexpressing *SUT1*, indicating that the cells are not fully permeable. However, it must be stressed that the uptake (1.7-fold increase) is close to that obtained when *SUT1* expression is directed by its own promoter [10], a level which allowed the cloning of this gene [9].

In conclusion, both deletion and overexpression of *AUS1* and *DAN1* support the notion that these proteins are key components of sterol import in yeast. However, their precise contribution is still not clear. Particularly, it is not yet proven that any of the actors identified correspond to a sterol transporter; so far, with the exception of transcriptional regulators such as *SUT1* or *UPC2*, no single gene capable of promoting full permeation in aerobiosis has been identified. This shows that this process requires additional components. Further investigation on the results of de-regulating the genes identified in the present study would be helpful to address their functional implications in sterol import.

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