Supplementary Material for

Aureobasidin A arrests growth of yeast cells through both, ceramide intoxication and deprivation of essential Inositolphosphorylceramides

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Supplementary results

Rationale for choosing YPK9 background for the study of lipid profiles generated by Ydc1p.

W303 *lag1*Δ *lac1*Δ and W303 *lag1*Δ *lac1*Δ *ypc1*Δ *ydc1*Δ cells still make several mild base resistant inositol-phosphorylsphingoids, indicating that they dispose of a yet uncharacterized pathway of making sphingolipids (Schorling et al., 2001; Guillas et al., 2001). Moreover, they proved to be highly unstable as many clones, when labeled with [^{3}Hinositol, displayed lipid profiles quite different from the one previously found in the original W303 *lag1*Δ *lac1*Δ strain and contained some new microsomal activity allowing to acylate [^{3}H]DHS through an uncharacterized

pathway (Guillas et al., 2001). Some clones started to produce a major mild base resistant lipid comigrating with $M(IP)_2C$ and/or ceased to make the residual amounts of IPC/C, IPC/D, MIPC or the abnormal lipids a and b that had been found in the original W303 *lag1*Δ *lac1*Δ cells (not shown). We concluded that these cells rapidly got outraced by spontaneous suppressors, which survived freezing and thawing better than unsuppressed cells, and that sphingolipid biosynthesis of W303 *lag1*Δ *lac1*Δ cells was genetically unstable so that the strain was not suitable to study the effects of overexpression of *YDC1*. We therefore decided to work in the YPK9 background, in which the *lag1*Δ *lac1*Δ double deletion is lethal (Jiang et al., 1998), but which can be maintained by pBM150-*LAG1*, a centromeric plasmid bearing *LAG1* under the control of the *GAL1* promoter (Guillas et al., 2001). The YPK9 *lag1*Δ *lac1*Δ pBM150-*LAG1* (2Δ.LAG1) strain grows at a normal rate on galactose and therefore is probably not accumulating suppressors; when put on glucose containing media, cells undergo numerous cell divisions but then switch to a slow growth rate.

Supplementary Discussion

Our data establishes, clarifies or confirms numerous aspects concerning the specificity of the enzymes involved in sphingolipid biosynthesis in yeast:
First, the rather similar prevalence of C18, C20, C22, C24 and C26 fatty acids on IPCs made by 2Δ.YDC1 (Fig. 4A) argues that the Elo2p- and Elo3p-dependent microsomal fatty acid elongation system somehow can furnish fatty acids of all lengths between C20 and C26. Wild type cells contain significant amounts of C24 and C26, but very low levels of C22 and C20 unless one deletes the acyl-CoA synthase Fat1p, which is proposed to activate intermediate length (C20, C22) fatty

acids in order to channel them into a degradation pathway (Choi and Martin, 1999). Thus, it may be that in the context of 2Δ.YDC1, Ydc1p draws on a physiological pool of fatty acids, but it may also be that the accumulation of C26-CoA or DHS in these *lag1*Δ *lac1*Δ cells causes a perturbation of the microsomal elongation system or inhibits Fat1p and thus causes the even representation of the various fatty acids in the sphingolipid fractions of 2Δ.YDC1 and 3Δ.YDC1.

Second, in 2Δ.YDC1 cells the Sur2p hydroxylase only hydroxylates free LCBs but not the more mature sphingolipids although Sur2p has been shown to have activity in vitro towards both, free DHS and DHS-containing ceramides (Fig. 1) (Grilley et al., 1998). Indeed, we find that IPCs, MIPCs or $M(IP)_2Cs$ of 2Δ.YDC1 and 3Δ.YDC1, have the same mobilities on TLC (Fig. 3C, Fig. S1), that their sphingolipids contain an abnormally high fraction of ceramides with only 2 hydroxyls (Fig. 4A - 4C) (consisting by necessity of a non hydroxylated fatty acid attached to DHS) and that these ceramides are hydroxylated to the same degree in both strains (Fig. 4A - 4C). This suggests that Sur2p does not hydroxylate DHS-containing ceramides or DHS-containing mature sphingolipids (IPCs, MIPCs and $M(IP)_2Cs$) and that the sphingolipid species containing 3 hydroxyls in their ceramide consist of a DHS plus a mono-hydroxylated fatty acid, not only in 3Δ.YDC1 but also in 2Δ.YDC1 (Fig.4A).

Third, the fact that Sur2p apparently does not hydroxylate ceramides or IPCs makes us believe that the minor IPC44-4 and IPC44-5 species seen in 2Δ.YDC1 but absent from 3Δ.YDC1 (Fig. 4A) does not originate from a DHS-containing but rather a PHS-containing ceramide, made by the endogenous Ypc1p. Indeed, Ypc1p uses PHS and DHS for reverse ceramidase activity with similar efficiency (Mao et al., 2000b).

Moreover, our data confirms that the fatty acid hydroxylase Scs7p (Fig. 1) prefers PHS-containing sphingolipids to DHS-containing ones (Haak et al., 1997). Most IPCs of wt cells contain hydroxylated C26 fatty acids (Smith and Lester, 1974). This

explains the prevalence of sphingolipids with only 2 hydroxyls in 2Δ.YDC1 and 3Δ.YDC1 cells. Also, the data shows that Scs7p can work on ceramides. Indeed it was not clear in the original report if Scs7p works on ceramides or on IPCs (Haak et al., 1997). The strong increase of C26:0-OH under AbA (Fig. 7C) argues strongly that it can act on the free ceramides, which accumulate under AbA.
Finally, the mannosyltransferase Csg1p/Csh1p/Csg2p (Fig. 1) seems to prefer IPCs with C24 or C26 fatty acids to those having shorter fatty acids (Fig. 4A vs. 4B). Yet, an earlier study had shown that IPC34-2 is efficiently mannosylated, if absolutely no IPCs with longer fatty acids are available (Cerantola et al., 2007).

Supplementary experimental procedures

Radiochemicals. [2-^{3}H]myo-inositol ([^{3}H]inositol) was obtained from ANAWA trading SA. [^{3}H]DHS was synthesized by NEN (3Ci/mmol) and purified on silica gel columns. For this, a 1 ml column was equilibrated in chloroform and [^{3}H]DHS in chloroform was loaded. The column was washed successively with 10 ml of chloroform, then 10 ml and 5 ml of chloroform:methanol (9:1,v/v) and (8:2, v/v), respectively. [^{3}H]DHS was recovered with 2 ml of methanol.

Electron microscopy. Sodium phosphate buffer (0.1M, pH 7.2) was used throughout. 10 A_{600} units of exponentially growing cells were washed twice with ice cold buffer, resuspended for fixation in 5 ml 2.5% glutaraldehyde in buffer and left at 4°C for 30 min. Cells were centrifuged at 1000 g, and washed three times in 5 ml of buffer at 4°C. Then the cells are treated with 1% OsO_4 in buffer for 20 min at room temperature. Thereafter cells were washed 3 times at room temperature and taken up in 2% agar in distilled water, melted at 30°C. Cells were dehydrated in ethanol and finally in propylene oxide before being embedded in epon.

Supplementary Tables

Table SI. Quantification of Fig. 3A and a second identical experiment by radioscanning.

	YPK9 (WT)	2Δ.YDC1	2Δ.LAG1*
PI'	0.1 +/- 0.1	4.35 +/- 1.8	4.0
PI	48.1 +/- 10	80.7 +/- 3.8	86.7
IPC-4	20.6 +/- 9	2.1 +/- 0.4	0.80
IPC-5 + MIPC	15.8 +/- 6.5	1.1 +/- 0.3	0.9

* The data on 2Δ.LAG1 grown on glucose were calculated from Fig. 2A.

Results indicate the incorporation of radioactivity into various lipids expressed as % of the total radioactivity in lipids of the strain and were calculated by taking into account both, lanes with deacylated as well as non-deacylated extracts.

Table SII. Masses corresponding to data in Figure 4.

IPC		**MIPC**		**M(IP)$_2$C**	
short name*	m/z	short name*	m/z	short name*	m/z
34-2	780.5696	34-2	942.5924	34-2	1183.6043
34-3	796.5345	34-3	958.5874	34-3	1199.5992
34-4	812.5295	34-4	974.5823	34-4	1215.5942
34-5	828.5244	34-5	990.5772	34-5	1231.5891
36-2	808.5709	36-2	970.6237	36-2	1211.6356
36-3	824.5658	36-3	986.6187	36-3	1227.6305
36-4	840.5608	36-4	1002.6136	36-4	1243.6255
36-5	856.5557	36-5	1018.6085	36-5	1259.6204
38-2	836.6022	38-2	998.655	38-2	1239.6669
38-3	852.5971	38-3	1014.65	38-3	1255.6618
38-4	868.5921	38-4	1030.6449	38-4	1271.6568
38-5	884.587	38-5	1046.6398	38-5	1287.6517
40-2	864.6335	40-2	1026.6863	40-2	1267.6982
40-3	880.6284	40-3	1042.6813	40-3	1283.6931
40-4	896.6234	40-4	1058.6762	40-4	1299.6881
40-5	912.6183	40-5	1074.6711	40-5	1315.683
42-2	892.6648	42-2	1054.7176	42-2	1295.7295
42-3	908.6597	42-3	1070.7126	42-3	1311.7244
42-4	924.6547	42-4	1086.7075	42-4	1327.7194
42-5	940.6496	42-5	1102.7024	42-5	1343.7143
44-2	920.6961	44-2	1082.7489	44-2	1323.7608
44-3	936.691	44-3	1098.7439	44-3	1339.7557
44-4	952.686	44-4	1114.7388	44-4	1355.7507
44-5	968.6809	44-5	1130.7337	44-5	1371.7456
46-2	948.7274	46-2	1110.7802	46-2	1351.7921
46-3	964.7223	46-3	1126.7752	46-3	1367.787
46-4	980.7173	46-4	1142.7701	46-4	1383.782
46-5	996.7122	46-5	1158.765	46-5	1399.7769

* First and second figure correspond to number of carbon atoms and hydroxyl groups in the ceramide moiety of the IPC, MIPC or M(IP)$_2$C.

Supplementary figures

Fig. S1. Incorporation of [^{3}H]inositol into lipids. The experiment shown in Fig. 3C was repeated as described in the legend to Fig. 3C.

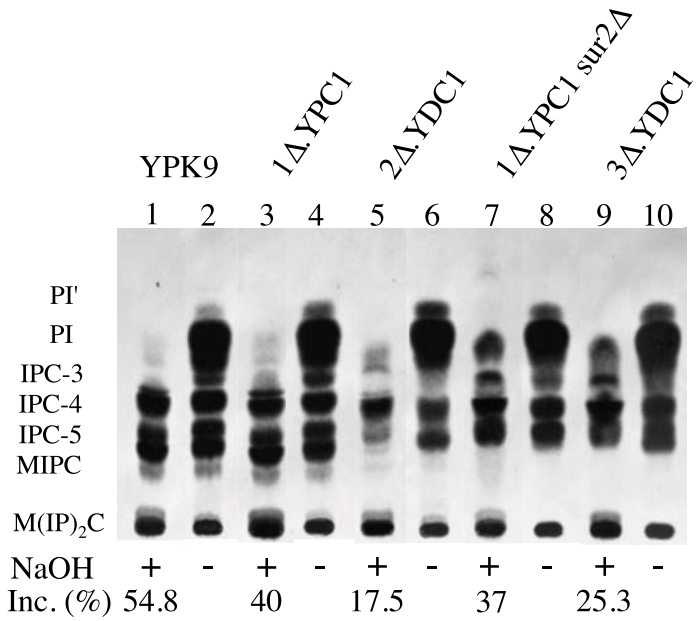

YPK9
1Δ YPC1
2Δ YDC1
1Δ YPC1 sur2Δ
3Δ YDC1
1 2 3 4 5 6 7 8 9 10
PI'
PI
IPC-3
IPC-4
IPC-5
MIPC
M(IP)2C
NaOH + - + - + - + - + -
Inc. (%) 54.8 40 17.5 37 25.3

Fig. S1

Fig. S2. 2Δ.YDC1 and 3Δ.YDC1 cells are relatively resistant to stress conditions that affect other sphingolipid-deficient cells. A, ten fold dilutions of the various strains were deposited onto plates containing various additions as indicated. Plates containing NaCl, $ZnCl_2$ or $CaCl_2$ were buffered to pH 5.5. Plates were incubated at 30°C for 3 days. B, as A, but plates were incubated for 4 days; YPK9 and YPK11 wild type strains contained the empty YEP13 2μ *LEU2* plasmid. Conditions were chosen based on previous reports: The *lcb1Δ SLC1-1* cells lack PHS and all complex sphingolipids (Fig. 1) and are known to be unable to grow in high salt, at low pH or at 37°C (Patton et al., 1992). *Elo3*Δ cells are deficient in fatty acid elongation, accumulate PHS and make less than normal amounts of complex sphingolipids. *Elo3*Δ cells were reported to be calcium and zinc hypersensitive and unable to grow at high pH or on glycerol, although calcium hypersensitivity was not detectable if *ELO3* was deleted in the YPK9 background (Oh et al., 1997; Chung et al., 2003; Cerantola et al., 2007). The differences between mutant and wt cells were: 2Δ.YDC1 cells grew slightly less well than 3Δ.YDC1 and YPK9 already in the absence of any stress and their growth was also weaker in most stress conditions. 2Δ.YDC1 and 3Δ.YDC1 cells were particularly sensitive to media containing glycerol as a carbon source. Curiously, 2Δ.YDC1 and 3Δ.YDC1 grew better than wt on $ZnCl_2$. Interestingly, 3Δ.YDC1 and even 2Δ.YDC1 were more heat resistant at 39°C than wt (data not shown). The same was also observed in 1Δ.YPC1. The reason may be the relative absence of ceramides, the higher amounts of LCBs or the trp prototrophy of those strains.

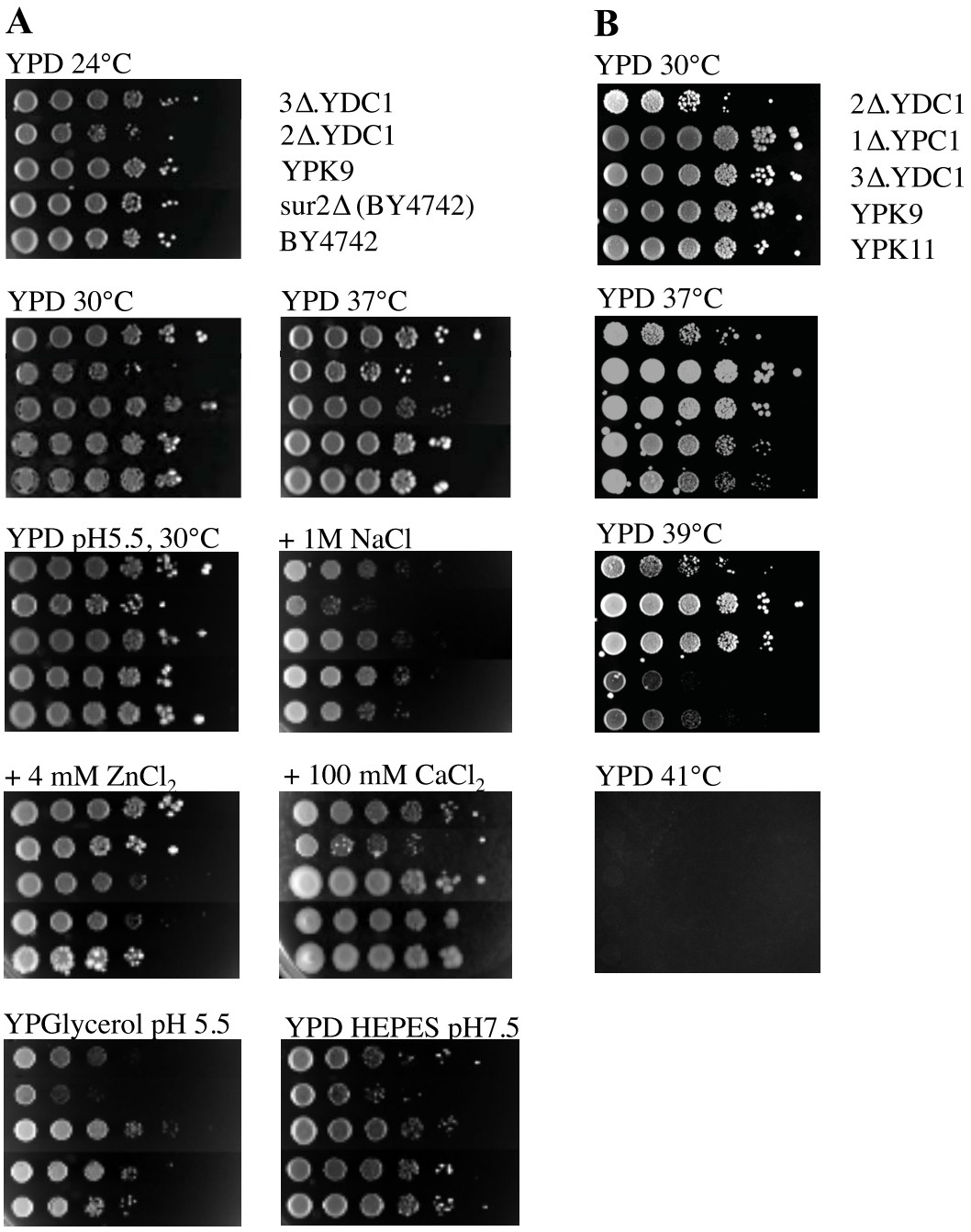
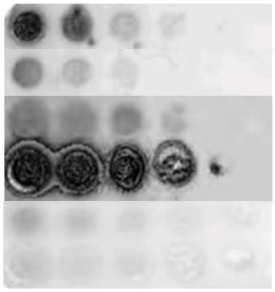

A
YPD 24°C
3Δ.YDC1
2Δ.YDC1
YPK9
sur2Δ (BY4742)
BY4742
YPD 30°C
YPD 37°C
YPD pH5.5, 30°C
+ 1M NaCl
+ 4 mM ZnCl2
+ 100 mM CaCl2
YPGlycerol pH 5.5
YPD HEPES pH7.5
B
YPD 30°C
2Δ.YDC1
1Δ.YPC1
3Δ.YDC1
YPK9
YPK11
YPD 37°C
YPD 39°C
YPD 41°C

Fig. S2

Fig. S3. CPY secretion and Calcofluor sensitivity of 2Δ.YDC1 cells. A, tenfold dilutions of the various strains were deposited onto YPD plates, incubated for 3 days at 30°C and overlaid with nitrocellulose. The nitrocellulose filter was processed for Western blotting using anti-CPY antibodies. Vps4Δ are deficient in vacuolar targeting and serve as positive control. B, tenfold dilutions of the various strains were placed onto plates containing Calcofluor white (CFW) and incubated for 3 days at 30°C. 3Δ.YDC1 secreted low amounts of carboxypeptidase Y (CPY), which may indicate a difficulty in vacuolar targeting. On the other hand, they were not hypersensitive to CFW.

A CPY secretion



B

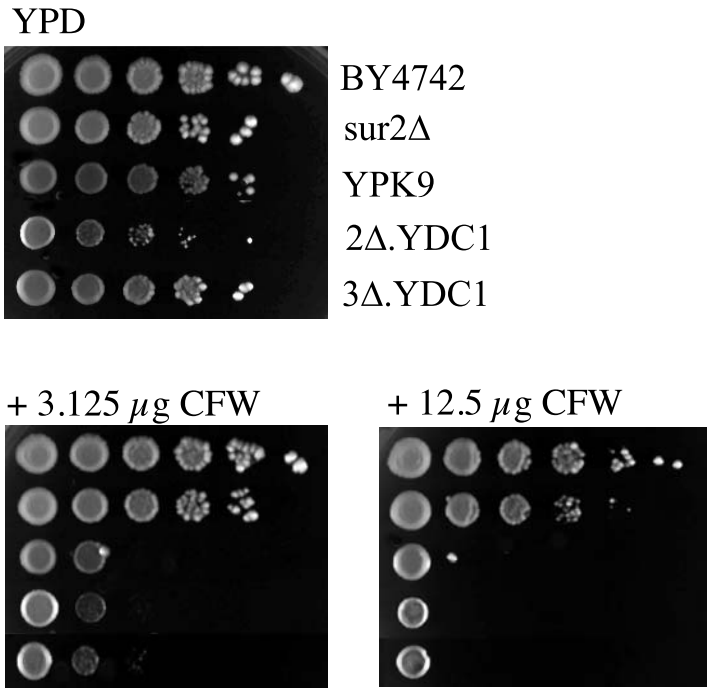


Fig. S3

Fig. S4. The abnormal cell wall morphology of *lag1*Δ *lac1*Δ cells is not corrected by overexpression of *YDC1*. The cell types indicated at the right were grown for 30 hours at 30°C in SDaaUA lacking inositol, causing partial depletion of Lag1p in 2Δ.LAG1. Cells were then processed for electron microscopy.

Barz and Walter (1999) have used electron microscopy to show that in W303 *lag1*Δ *lac1*Δ cells, the mother cells display thickened multilayered cell walls and noted that the cells are Calcofluor white (CFW) hypersensitive. In panels A and B, we can see the same picture in Lag1p-depleted 2Δ.LAG1 cells. While in wt cells we can observe occasionally a localized thickening of the cell wall next to a recent bud scar (panel D), a significant fraction of Lag1p-depleted 2Δ.LAG1 cells have multilayered cell walls of variable thickness. While the W303 *lag1*Δ *lac1*Δ cells observed by Barz and Walter (1999) deposited excessive cell wall material evenly over the entire body of the mother cell, we find that most mother cells showed uneven cell wall thickening limited to certain zones (panels A, B). Similar local cell wall thickening was recently described for las17Δ, vrp1Δ and kre5Δ mutants which make either increased or reduced amounts of β1,6 glucan, respectively (Li et al., 2002; Levinson et al., 2002). We also frequently observed strings of up to 5 cells that seem not to detach from each other properly as in panel B. The multilayered cell wall areas are still to be observed at a certain frequency in 2Δ.YDC1 (panels E, F). 2Δ.YDC1 also often show an increase of internal membranes of undefined nature (panels E, F). Ceramides are believed to form membrane domains in the ER, which are essential for the export of GPI proteins out of the ER (Bagnat et al., 2000). The very low levels of ceramides found in 2Δ.YDC1 cells (Fig. 7A and B) leads one to expect a cell wall fragility and CFW hypersensitivity due to compensatory chitin biosynthesis. Yet, 2Δ.YDC1 cells are not hypersensitive to Calcofluor white, but nevertheless have thickened cell walls.

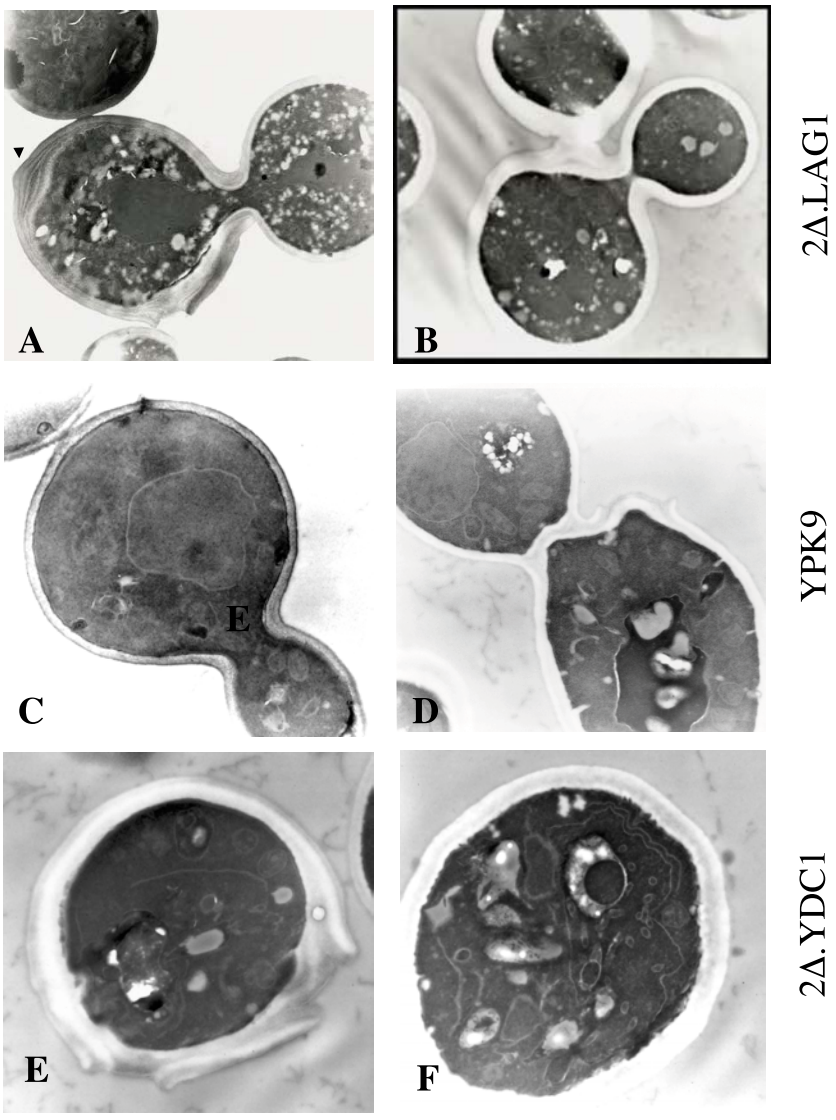


Fig. S4

Fig. S5. Myriocin hypersensitivity of 2Δ.YDC1 cells is cured only partially by exogenous DHS. Ten fold dilutions of the various strains were deposited on YPD plates containing myriocin (myr) and DHS as indicated. Cells were incubated for 4 days at 30°C.

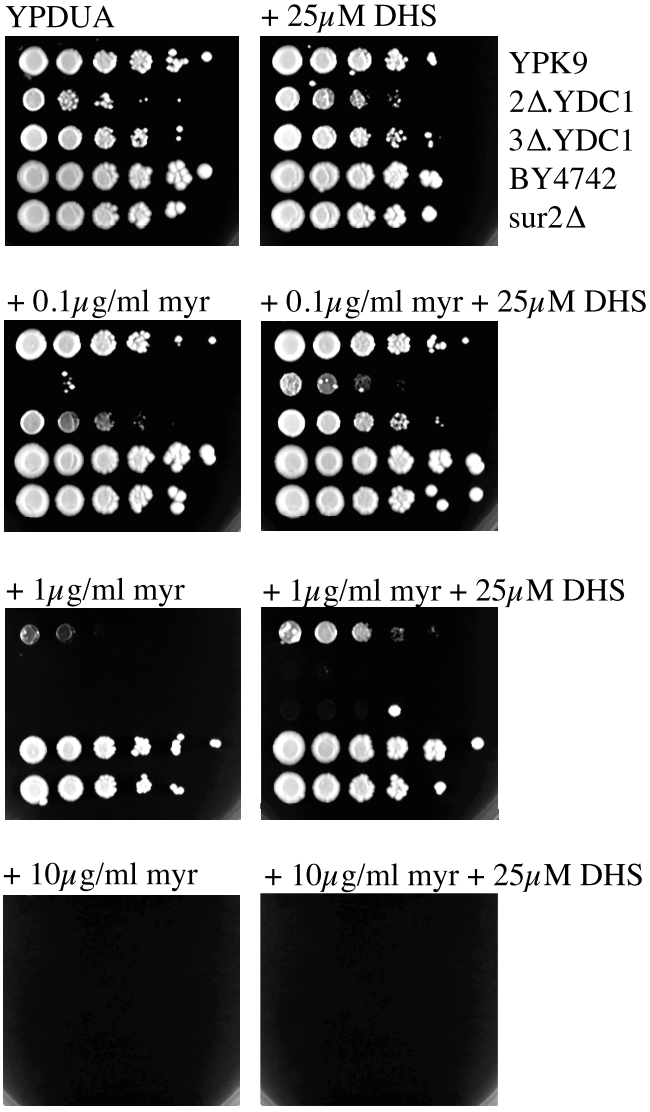

YPDUA
+ 25μM DHS
YPK9
2Δ.YDC1
3Δ.YDC1
BY4742
sur2Δ
+ 0.1μg/ml myr
+ 0.1μg/ml myr + 25μM DHS
+ 1μg/ml myr
+ 1μg/ml myr + 25μM DHS
+ 10μg/ml myr
+ 10μg/ml myr + 25μM DHS

Fig. S5