# Supporting Information

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## SI Experimental Methods

**Strains and Growth Conditions.** The *Saccharomyces cerevisiae* strain D273-10B was grown at 30 °C on rich [10 g/L yeast extract, 20 g/L bacto-peptone, and 20 g/L dextrose (YPD)] or minimal [yeast nitrogen base (YNB) without amino acids] media (Difco) containing 20 g/L dextrose unless indicated otherwise. YNB media were enriched by a "dropout" mixture containing all amino acids with the exception of arginine or proline where indicated. *Aspergillus niger* N402 was grown on complete medium (CM) containing 6.0 g/L $NaNO_3$, 1.5 g/L $KH_2PO_4$, 0.5 g/L KCl, 0.5 g/L $MgSO_4$, 25 mM glucose, 0.5% casamino acids, 1% yeast extract, and 200 μL of trace elements at 25 °C. Conidia of 10- to 12-d-old colonies were harvested and washed in cold N-(2-acetamido)-2-aminoethanesulfonic acid (ACES) buffer [10 mM ACES, 0.02% Tween-80 (pH 6.8)], as described by van Leeuwen et al. (1). Conidia were resuspended in cold CM and kept on ice until further processing.

**RNA Extraction.** For isolation of RNA, $3 \times 10^9$ conidia were inoculated in triplicate in 300 mL of CM. Cultures were incubated in the absence or presence of 3 or 10 μM natamycin (Royal DSM). After 2- and 8-h inoculations, $4.5 \times 10^8$ germinating conidia were centrifuged at 1,100 × *g* at 5 °C for 5 min and the pellet was stored at −80 °C. The samples were homogenized with a Qiagen Tissuelyser (2 times for 2 min at 30 strokes per $s^{-1}$) with RNeasy lysis (RLT) buffer (Qiagen), and RNA was extracted using the RNeasy Maxi kit (Qiagen). The elution volume was reduced to ∼100–400 μL with a SpeedVac (DNA 110; Savant). Subsequently, 600 μL of 2× T & C lysis buffer (Epicentre) was added, and the mixture was incubated on ice. After 5 min, 350 μL of MPC Protein Precipitation Reagent (Epicentre) was added, mixed vigorously, and centrifuged for 10 min at 12,000 × *g* at 4 °C. After isopropanol precipitation, the RNA was resuspended in 100 μL of RNase-free water and 700 μL of RLT buffer, and 500 μL of ethanol [96–100% (vol/vol)] was added. RNA concentrations were determined using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies), and the quality was assayed with an Agilent 2100 Bioanalyzer, using an RNA Nano LabChip (Agilent Technology).

**cDNA Labeling, Microarray Hybridization, and Data Analysis.** cDNA labeling, microarray hybridization, and scanning were performed at ServiceXS (Leiden, The Netherlands) according to Affymetrix protocols. For this, 2 μg of high-quality total RNA was used to generate biotin-labeled antisense cRNA with the Affymetrix Eukaryotic One-Cycle Target Labeling and Control reagents. Labeled cRNA was used for the hybridization to Affymetrix *A. niger* Genome Genechips. After an automated process of washing and staining, absolute values of expression were calculated from the scanned array using Affymetrix Command Console v1 software. For data analysis, the GeneSpring v1.0 software package (Agilent) was used. The Functional Catalogue (Munich Information Center for Protein Sequences) (2) was used for systematic classification of genes according to their cellular and molecular functions (3). Before comparison, all arrays were globally scaled to a target value of 100 utilizing the average signal from all gene features using Affymetrix Microarray Suite version 5.0 (MAS5.0) in Refiner Array Affymetrix IVT Arrays 5.2 of Genedata Expressionist. Arrays were normalized on the median with a reference value of 100 in Genedata Expressionist 5.3 Analyst. Filtering was performed by setting the value of all samples flagged A or M to a fixed value of 12 (4). Statistical assessment of differential expression of the subset of 29 transporter genes among samples was performed with a *t* test in Genedata Expressionist. A significance level of 0.01 was used, and a Benjamini–Hochberg false discovery rate of $P = 0.05$ (5) was applied.

**Uptake and Release of Arginine in Yeast.** Arginine can be taken up in yeast by Can1p (the specific transport protein for arginine) or the general amino acid permease (Gap1p). Because of transcriptional repression of Gap1p, this protein has low expression in cells grown in the presence of ammonium (6, 7). This makes it possible to study the uptake of arginine predominantly by Can1p in cells grown in YNB (8). In these experiments, a yeast culture was grown overnight in YPD. Cells were washed, resuspended in YNB without arginine, and grown until they reached an $OD_{600}$ between 0.3 and 0.8. At the start of the assay, cells were incubated with natamycin at indicated concentrations for 5 min; at each time point, the number of cfus was determined. To a portion of the cell suspension (between 200 and 600 μL), a mixture of radioactive and nonradioactive arginine (ratio of 1:5) was added at a final concentration of 30 μM. Samples of 100 μL were withdrawn at different time intervals, diluted in 1 mL of ice-cold water, and placed on ice. To determine background radioactivity, a similar amount of radioactive arginine was diluted in 1 mL of ice-cold water and treated similar to the other samples. The samples were filtered on membrane filters [cellulose acetate (CA), 0.45-μm pore size, 47-mm diameter] from Nalgene (Nunc, International), after which the filter was washed with 4 mL of ice-cold water. The filters were dissolved in 4 mL of Insta-Gel Plus Scintillation mixture (Packard Bioscience), and the radioactivity was counted using a Tri-Carb 2300TR liquid scintillation analyzer (Packard Bioscience). Signals were corrected for background radioactivity and differences in cell density. To determine the release of arginine, the same protocol was followed as for the uptake; however, instead of adding natamycin (20 μM) at the start of the assay, it was added 8 min after the addition of arginine to the cell suspension. The effect of natamycin on cell growth ($OD_{600}$) was determined after 3 h.

**Reversibility of the Inhibition of Arginine Uptake by Natamycin.** To determine if the yeast cells are able to recover from incubation with natamycin, an overnight grown yeast culture was diluted in YNB (−arginine) medium and grown until it reached an $OD_{600}$ between 0.3 and 0.5. At the start of the assay, cells were incubated without or with 20 μM natamycin for 5 min. Cells were washed and resuspended in YNB (−arginine) medium. All cultures were placed in an incubator at 30 °C. At each subsequent step (i.e., incubation with natamycin, removal of natamycin, 1 and 2 h of growth), the uptake of arginine (followed for 10 min) and the $OD_{600}$ and number of cfus were determined.

**Transport Assay of Glucose or Proline in Yeast.** The transport assays for glucose and proline were performed essentially as described for the transport assay of arginine, with the differences described below that were based on the method of Robl et al. (6). At the start of the glucose uptake assay, cells were resuspended to an $OD_{600}$ of ∼5 in YNB but without glucose. To monitor uptake of proline, the cells were adjusted to 50 mM potassium phosphate buffer (pH 6.6), 2 mM $MgSO_4$, and 1% glucose by incubating them for 1 h at an $OD_{600}$ of 5, after which the assay was started. The final concentrations and ratios of radioactive and nonradioactive compounds added to the cell suspensions were 1 mM glucose (ratio of 1:80) and 20 μM proline (ratio of 1:2). Cells untreated with natamycin reached an uptake of 9 or 1.2 nmol per OD unit after 10 min for

glucose and proline, respectively. These values were normalized to 100% to allow comparison of the effect of natamycin on both compounds.

**Uptake of Arginine, Proline, and Glucose in Conidia of *A. niger*.** To allow a comparison of the results with baker's yeast and fungal conidia, the substrate uptake assays were performed in a similar fashion. Harvested *A. niger* conidia were resuspended to $10^7$ conidia per milliliter in CM and germinated at 25 °C. To allow comparison with the transcriptome analysis, a portion of the conidia was kept in the presence of 10 μM natamycin. After ~5 h, the conidia were washed in minimal medium (MM) containing 1.5 g/L $(NH_4)_2SO_4$, 1.5 g/L $KH_2PO_4$, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 0.5 g/L KCl, and 200 μL of trace elements. The conidia were resuspended in MM, but glucose was omitted in MM to assay glucose uptake. Aliquots containing 100 μL of $5 \times 10^7$ conidia were divided in Eppendorf tubes and kept on ice. Before starting the assay, conidia were incubated with 0 or 10 μM natamycin for 5 min at 30 °C. A 100-μL mixture of radioactive and nonradioactive substrates was added at a final concentration of 30 μM, 20 μM, and 1 mM for arginine, proline, and glucose, respectively (similar to the uptake assay in yeast). The conidia were incubated for 0 or 10 min at 30 °C, diluted in 1 mL of ice-cold CM, and placed on ice. The samples were filtered and analyzed as described above with the difference that ice-cold CM was used instead of water for washing.

## SI Text

Twenty genes related to sugar transport in *A. niger* were analyzed during germination of conidia and in the presence of natamycin. Sixteen genes show very high expression after treatment in comparison to controls. This effect occurred after 2 h and 8 h of incubation with the polyene antibiotic and showed an increase on the following order: 2 h, 3 μM < 2 h, 10 μM < 8 h, 3 μM < 8 h, 10 μM). These most frequently expressed transcripts show high similarity to hexose, sugar, arabinose, and myoinositol transporters, as well as a glucose sensor. Interesting is the clear down-regulation of an HXT3-like protein with natamycin that is highly expressed in the control. Thirty-one proteins were related to amino acid transport, and 17 of them show clear up-regulation and similar trends as in case of the sugar transporters. The most strongly expressed genes are proteins with a high similarity to neutral amino acid permeases, choline transporters, and a proline permease.

The regrowth of natamycin-treated cells on the plates reported above indicates that the inhibition of natamycin on arginine uptake may be reversible. To test this directly, a culture was washed after incubation with natamycin and subsequently resuspended in fresh medium, and the arginine uptake was monitored in time. The control culture, which had not been incubated with natamycin, showed an increase in arginine uptake, which stabilized after 1 h (Fig. S2*A*). A control culture that had been incubated with natamycin, without washing afterward, is blocked in the uptake of arginine from the start to the end of the assay. The culture that had been washed after natamycin incubation was blocked in arginine uptake at the start of the assay and showed a steady increase of uptake over time, reaching the same level of uptake after 2 h as the control without natamycin (Fig. S2*A*). These results show that natamycin-induced blockade of arginine uptake in yeast cells can be reversed.

To determine if reversed blockade in arginine uptake also corresponds to reversed inhibition of growth, cell viability was monitored as the number of cfus in parallel to the arginine uptake as described above (Fig. S2*B*). As expected, the control culture without natamycin shows an increase in cell viability, whereas the natamycin control culture shows a decrease in cell viability over time. The culture that had been washed after natamycin treatment showed an initial decrease of 50% in cell viability, which did not change, meaning that the cells did not significantly regain their ability to grow in this time period (Fig. S2*B*). These results show that a reversed block in arginine uptake does not correspond to reversed inhibition of growth, which indicates that the inhibition of arginine uptake by natamycin alone cannot be responsible for the growth inhibition of yeast and suggests that there might be additional targets for natamycin.


1. Van Leeuwen MR, Smant W, de Boer W, Dijksterhuis J (2008) Filipin is a reliable in situ marker of ergosterol in the plasma membrane of germinating conidia (spores) of Penicillium discolor and stains intensively at the site of germ tube formation. *J Microbiol Methods* 74:64–73.
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4. Pepper SD, Saunders EK, Edwards LE, Wilson CL, Miller CJ (2007) The utility of MAS5 expression summary and detection call algorithms. *BMC Bioinformatics* 8:273.
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6. Robl I, Grassl R, Tanner W, Opekarová M (2001) Construction of phosphatidylethanolamine-less strain of Saccharomyces cerevisiae. Effect on amino acid transport. *Yeast* 18:251–260.
7. Andréasson C, Neve EP, Ljungdahl PO (2004) Four permeases import proline and the toxic proline analogue azetidine-2-carboxylate into yeast. *Yeast* 21:193–199.
8. Malínská K, Malínský J, Opekarová M, Tanner W (2003) Visualization of protein compartmentation within the plasma membrane of living yeast cells. *Mol Biol Cell* 14: 4427–4436.

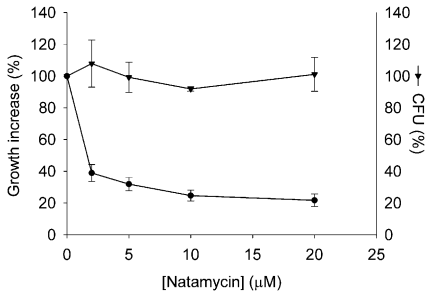


**Fig. S1.** Effect of natamycin on yeast cells while examining the uptake of arginine. Growth over 3 h (●) and the number of CFU (▼) of the yeast cultures were determined after the addition of different concentrations of natamycin and are given as a percentage of the value observed in the absence of natamycin (100%). The results shown are the averages of three separately performed experiments with SD. CFU, colony-forming units.

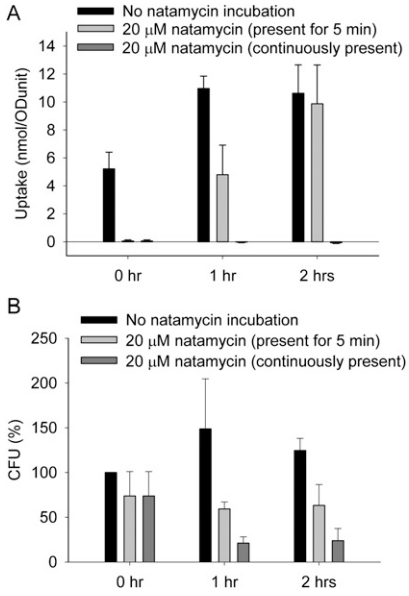


**Fig. S2.** Recovery of yeast cells after natamycin treatment. The uptake of arginine (*A*) and the number of CFU (*B*) were determined after washing the cells that were incubated with natamycin. CFU, colony-forming units. Cultures were incubated with or without 20 μM natamycin for 5 min. A control sample was used in which 20 μM natamycin was continuously present. A sample was taken immediately after the 5-min incubation with natamycin (0 h). Cultures were washed and resuspended in fresh medium, and samples were taken after 1 h or 2 h. The uptake of arginine was determined at 10 min and corrected for the amount of cells. The number of cfus is expressed as a percentage of the culture without natamycin at the start of the assay (100%). The results shown are the averages of two separately performed experiments with the spread of the data.

**Table S1. Effect of natamycin on the differential expression of sugar transporters and amino acid transporters in germinating *A. niger* conidia**

| No. | Gene no. | Description | 2 h | 2 h, 3 μM | 2 h, 10 μM | 8 h | 8 h, 3 μM | 8 h, 10 μM |
|---|---|---|---|---|---|---|---|---|
| Sugar transporters | | | | | | | | |
| 1 | An01g08780 | s.s. to high-affinity hexose transporter Hxt1, *S. cerevisiae* | 45 | 58 | 112 | 87 | 1,776 | 3,026 |
| 2 | An02g03540 | s.s. to hexose transport protein HXT3, *S. cerevisiae* | 2,028 | 1,650 | 1,143 | 1,581 | 123 | 21 |
| 3 | An02g07610 | s.s. to mannitol transporter Mat1, *Apium graveolens* | 12 | 18 | 29 | 12 | 278 | 498 |
| 4 | An03g02190 | s.s. to the sugar transporter Sut1, *Pichia stipitis* | 28 | 416 | 529 | 12 | 854 | 1,634 |
| 5 | An04g00340 | s.s. to myoinositol transport protein ITR2, *S. cerevisiae* | 43 | 41 | 54 | 65 | 133 | 152 |
| 6 | An05g00730 | s.s. to hexose transport protein HXT3, *S. cerevisiae* | 12 | 13 | 12 | 12 | 116 | 274 |
| 7 | An05g01290 | s.s. to hexose transport protein HXT3, *S. cerevisiae* | 112 | 424 | 796 | 41 | 437 | 133 |
| 8 | An06g02270 | Similarity to arabinose transport protein araE, *Escherichia coli* | 199 | 660 | 1,513 | 22 | 3,009 | 4,081 |
| 9 | An07g10370 | s.s. to high-affinity glucose transporter HGT1, *Kluyveromyces lactis* | 43 | 79 | 97 | 105 | 206 | 223 |
| 10 | An09g02930 | s.s. to high-affinity glucose transporter HGT1, *K. lactis* | 304 | 416 | 567 | 51 | 577 | 1,039 |
| 11 | An09g04810 | s.s. to high affinity glucose transporter HGT1, *K. lactis* | 13 | 12 | 12 | 285 | 16 | 16 |
| 12 | An11g01100 | s.s. to high-affinity glucose transporter HGT1, *K. lactis* | 25 | 31 | 41 | 26 | 358 | 128 |
| 13 | An11g05280 | s.s. to sugar transporter 1 SUT1, *P. stipitis* | 25 | 19 | 18 | 22 | 112 | 241 |
| 14 | An11g09600 | s.s. to glucose transporter rco-3, *Neurospora crassa* | 13 | 12 | 13 | 17 | 419 | 1,075 |
| 15 | An12g07450 | s.s. to glucose sensor RGT2, *S. cerevisiae* | 244 | 507 | 682 | 184 | 2,798 | 4,136 |
| 16 | An14g03990 | s.s. to hexose transport protein hxt5, *S. cerevisiae* | 30 | 46 | 55 | 215 | 24 | 23 |
| 17 | An15g00310 | s.s. to monosaccharide transporter Mst-1, *Amanita muscaria* | 35 | 52 | 50 | 15 | 284 | 174 |
| 18 | An15g03940 | s.s. to monosaccharide transporter Mst-1, *A. muscaria* | 573 | 1,146 | 2,028 | 486 | 3,100 | 3,280 |
| 19 | An16g07660 | s. to glucose and galactose transporter gluP, *Brucella abortus* | 20 | 16 | 20 | 28 | 63 | 98 |
| 20 | An17g01560 | s.s. to sucrose transport protein SUT1, *Oryza sativa* | 37 | 42 | 72 | 156 | 301 | 427 |
| Amino acid transporters | | | | | | | | |
| 21 | An01g09730 | s.s. to GABA permease gabA, *Aspergillus nidulans* | 16 | 15 | 24 | 794 | 45 | 40 |
| 22 | An01g13290 | s.s. to choline permease HNM1, *S. cerevisiae* | 17 | 37 | 107 | 230 | 441 | 767 |
| 23 | An02g14410 | s.s. to ammonium transport protein Mep3p, *S. cerevisiae* | 78 | 253 | 473 | 57 | 77 | 81 |
| 24 | An03g05360 | s.s. to neutral amino acid permease mtr, *N. crassa* | 254 | 522 | 930 | 121 | 471 | 367 |
| 25 | An04g00530 | s.s. to proline permease prnB, *A. nidulans* | 23 | 27 | 29 | 30 | 99 | 124 |
| 26 | An04g01390 | s.s. to the amino acid transporter ATA1, *Homo sapiens* | 56 | 53 | 71 | 147 | 165 | 123 |
| 27 | An04g02150 | s. to amino acid system *N* transporter SN1, *Rattus norvegicus* | 99 | 163 | 203 | 166 | 278 | 349 |
| 28 | An04g03940 | s.s. to methionine transport protein MUP1, *S. cerevisiae* | 296 | 626 | 748 | 104 | 41 | 12 |
| 29 | An04g09420 | s.s. to neutral amino acid permease mtr, *N. crassa* | 378 | 229 | 154 | 138 | 65 | 31 |
| 30 | An07g03690 | s.s. to amino acid transporter ata2, *H. sapiens* | 122 | 315 | 376 | 93 | 223 | 186 |
| 31 | An08g02900 | s.s. to neutral amino acid permease mtr, *N. crassa* | 175 | 322 | 353 | 65 | 170 | 220 |
| 32 | An08g07780 | s.s. to lysine-specific permease LysP, *E. coli* | 201 | 313 | 332 | 60 | 51 | 43 |
| 33 | An09g03250 | s. to the amino acid transport system SN2, *H. sapiens* | 27 | 89 | 153 | 76 | 128 | 173 |
| 34 | An09g05010 | s.s. to choline transporter HNM1, *S. cerevisiae* | 278 | 194 | 226 | 210 | 655 | 664 |
| 35 | An09g06730 | s.s. to lysine-specific high-affinity permease LYP1, *S. cerevisiae* | 711 | 1,158 | 1,232 | 190 | 323 | 321 |
| 36 | An11g06150 | s.s. to proline permease prnB, *A. nidulans* | 42 | 75 | 151 | 39 | 839 | 1,321 |
| 37 | An12g04180 | s.s. to dicarboxylic amino acid permease DIP5, *S. cerevisiae* | 15 | 17 | 29 | 34 | 86 | 147 |
| 38 | An12g09900 | s.s. to amino acid transporter BAT1, R. *norvegicus* | 34 | 35 | 38 | 103 | 34 | 35 |
| 39 | An13g00840 | s.s. to amino acid transport protein GAP1, *S. cerevisiae* | 13 | 28 | 62 | 12 | 673 | 1,583 |
| 40 | An13g03650 | s.s. to lysine permease LYP1, S. *cerevisiae* | 391 | 494 | 458 | 147 | 244 | 243 |
| 41 | An14g01850 | s.s. to GABA permease UGA4, *S. cerevisiae* | 93 | 132 | 253 | 227 | 171 | 209 |
| 42 | An14g02590 | s.s. to GABA permease gabA, *A. nidulans* | 229 | 277 | 262 | 39 | 137 | 274 |
| 43 | An14g02720 | s.s. to neutral amino acid permease mtr, *N. crassa* | 690 | 715 | 692 | 365 | 500 | 547 |
| 44 | An14g07130 | s.s. to neutral amino acid permease mtr, *N. crassa* | 1,459 | 1,327 | 1,200 | 337 | 2,590 | 1,857 |
| 45 | An15g00330 | s.s. to glycoprotein-associated amino acid transporter, *Mus musculus* | 58 | 99 | 119 | 61 | 75 | 65 |
| 46 | An15g07120 | s.s. to proline permease prnB, *A. nidulans* | 132 | 484 | 826 | 104 | 108 | 63 |
| 47 | An15g07550 | s.s. to neutral amino acid permease Mtr, *N. crassa* | 401 | 728 | 1,071 | 386 | 1,725 | 2,258 |
| 48 | An16g00350 | s.s. to neutral amino acid permease Mtr, *N. crassa* | 137 | 826 | 1,029 | 125 | 39 | 42 |
| 49 | An16g02000 | s.s. to γ-amino-n-butyrate permease gabA, *A. nidulans* | 21 | 26 | 157 | 14 | 258 | 533 |
| 50 | An16g05880 | s.s. to neutral amino acid transporter Mtr1, *N. crassa* | 596 | 780 | 771 | 251 | 214 | 124 |
| 51 | An18g00960 | s.s. to choline permease CTR1, *S. cerevisiae* | 12 | 12 | 12 | 101 | 12 | 12 |

Data are given from three separate hybridization experiments as average fluorescence data generated by hybridization on Affymetrix arrays. The selection of the genes is based on the Functional Catalogue (Munich Information Center for Protein Sequences). s.s., strong similarity; s., similarity.

**Table S2. Effect of natamycin on the differential expression of other plasma membrane proteins in germinating *A. niger* conidia**

| No. | Gene no. | Description | 2 h | 2 h, 3 μM | 2 h, 10 μM | 8 h | 8 h, 3 μM | 8 h, 10 μM |
|---|---|---|---|---|---|---|---|---|
| 1 | An02g11700 | s.s. to copper transport protein CTR2, *S. cerevisiae* | 14 | 28 | 56 | 133 | 141 | 192 |
| 2 | An02g12510 | s.s. to plasma membrane $H^+$-ATPase pmaA, *Aspergillus nidulans* | 1,113 | 1,215 | 1,225 | 1,123 | 820 | 291 |
| 3 | An07g09740 | s.s. to $Na^+/H^+$ antiporter ZrSOD2-22, *Zygosaccharomyces rouxii* | 43 | 44 | 48 | 16 | 50 | 61 |
| 4 | An09g05950 | s.s. to plasma membrane ATPase PMA1, *Kluyveromyces lactis* | 29 | 37 | 26 | 20 | 127 | 243 |
| 5 | An14g01710 | s.s. to $Na^+/H^+$-exchanging protein NapA, *Enterococcus hirae* | 22 | 23 | 24 | 16 | 36 | 48 |

Data are given from three separate hybridization experiments as average fluorescence data generated by hybridization on Affymetrix arrays. The selection of the genes is based on the Functional Catalogue (Munich Information Center for Protein Sequences). s.s., strong similarity.