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Candida glabrata tryptophan-based pigment production via the Ehrlich pathway

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Summary

Pigments contribute to the pathogenicity of many fungi, mainly by protecting fungal cells from host defence activities. Here, we have dissected the biosynthetic pathway of a tryptophan-derived pigment of the human pathogen *Candida glabrata*, identified key genes involved in pigment production and have begun to elucidate the possible biological function of the pigment. Using transcriptional analyses and a transposon insertion library, we have identified genes associated with pigment production. Targeted deletion mutants revealed that the pigment is a by-product of the Ehrlich pathway of tryptophan degradation: a mutant lacking a tryptophan-upregulated aromatic aminotransferase (Aro8) displayed significantly reduced pigmentation and a recombinantly expressed version of this protein was sufficient for pigment production *in vitro*. Pigment production is tightly regulated as the synthesis is affected by the presence of alternative nitrogen sources, carbon sources, cyclic AMP and oxygen. Growth of *C. glabrata* on pigment inducing medium leads to an increased resistance to hydrogen peroxide, an effect which was not observed with a mutant defective in pigmentation. Furthermore, pigmented yeast cells had a higher survival rate when exposed to human neutrophils and caused increased damage in a monolayer model of human epithelia, indicating a possible role of pigmentation during interactions with host cells.

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Introduction

Although bacteria are still the leading cause of nosocomial bloodstream infections, the importance of fungi in these hospital acquired life-threatening diseases has been rising over the last decades (Perlroth *et al.*, 2007). Among fungemia, *Candida* species are the most common isolates. In many groups of patients susceptible to opportunistic infections, *Candida glabrata* has become the second most common cause for disseminated candidemia, surpassed only by *Candida albicans* (Pfaller and Diekema, 2007).

While the pathogenicity of *C. albicans* has been the subject of many studies and a broad array of pathogenicity attributes, such as the ability to form true hyphae, adhesion factors, thigmotropism, invasins, immune evasion factors, secreted hydrolases and other factors, have been identified (Calderone, 2002), the pathogenicity mechanisms of *C. glabrata* are less well studied and few virulence or fitness-related attributes have been recognized (Kaur *et al.*, 2005). Among these are the Epa family of adhesins (Cormack *et al.*, 1999; De Las Penas *et al.*, 2003) and the phenotypic switching phenomenon (Lachke *et al.*, 2000; Lachke *et al.*, 2002), which has been implicated in virulence (Brockert *et al.*, 2003; Srikantha *et al.*, 2008). Furthermore, *C. glabrata* has an unusually high innate resistance towards a broad array of antimycotics (Tortorano *et al.*, 2006).

Surprisingly, the production of an indole-derived pigment was recently described for *C. glabrata*, which is usually described as a non-pigmented yeast (Mayser *et al.*, 2007). Pigments have been implicated in a wide range of biological effects (Jacobson, 2000), and can be produced by many different fungal pathogens, ranging from plant pests such as *Magnaporthe grisea* (Howard and Valent, 1996) or *Colletotrichum lagenarium* (Takano *et al.*, 1997) to the occasionally human pathogenic 'black yeasts' (De Hoog *et al.*, 2000) and important human pathogens such as *Cryptococcus neoformans* (Kwon-Chung *et al.*, 1983),

Histoplasma capsulatum (Nosanchuk *et al.*, 2002) and *Aspergillus fumigatus* (Jahn *et al.*, 1997; Langfelder *et al.*, 1998; Youngchim *et al.*, 2004).

In many of these fungi, pigment production is based on the synthesis of melanin; *C. neoformans*, for example, synthesizes melanin when a precursor like L-DOPA is provided (Kwon-Chung *et al.*, 1983). The production of mutant strains lacking the laccase gene, *CnLAC1*, essential for pigment production in *C. neoformans*, was among the pioneering work which provided the first genetic evidence for a virulence factor of a human pathogenic fungus (Salas *et al.*, 1996). Similarly, the identification and characterization of genes responsible for melanin production in *A. fumigatus* led to the first targeted mutants of this fungus which had attenuated virulence potential in animal models (Jahn *et al.*, 1997; Langfelder *et al.*, 1998; Tsai *et al.*, 1998; 1999). Since its discovery, numerous functions for this pigment have been identified (Howard and Valent, 1996; Langfelder *et al.*, 2003; Nosanchuk and Casadevall, 2003). For example, melanin shields fungi against the detrimental effects of UV light (Wang and Casadevall, 1994a) and oxidative stress (Jacobson and Tinnell, 1993), which may contribute to the fungal response against host defence systems (Wang *et al.*, 1995). Furthermore, in *M. grisea*, *C. neoformans* and *A. fumigatus*, melanin has an important structural role – for example during the production of appressoria, or by stabilizing the cell wall against mechanical and chemical stresses. This was most strikingly demonstrated by the presence of 'melanin ghosts' of *C. neoformans* after the destruction of the cell wall by very harsh conditions, including treatment with concentrated acids (Wang *et al.*, 1996). In addition, melanin plays a role in the protection against antimycotic drugs (Wang and Casadevall, 1994b). These examples illustrate why melanin is often called a 'fungal shield', which provides protection against adverse environments.

The pigment recently described for *C. glabrata* (Mayser *et al.*, 2007) differs from melanin and is much more similar to the pigment produced by the skin pathogen *Malassezia furfur* (Mayser *et al.*, 1998). In both fungi, pigmentation occurs when tryptophan is provided as the sole source of nitrogen (Mayser *et al.*, 2007), and the pigment is composed of a wide range of indolic compounds. In *M. furfur*, this pigment has been demonstrated to protect cells against UV light (Mayser *et al.*, 2002a; Machowinski *et al.*, 2006). Furthermore, isolated pigment compounds induced apoptosis in human melanocytes (Kramer *et al.*, 2005a) and suppressed the oxidative burst by phagocytes (Kramer *et al.*, 2005b). These pigment properties have been implicated in the pathogenesis of *M. furfur* infections (Kramer *et al.*, 2005b; Thoma *et al.*, 2005), and may also play a role in the pathogenesis of *C. glabrata*.

Very little is known about the genetic basis of *M. furfur* pigment synthesis, mainly due to the lack of molecular tools for this fungus (Hort *et al.*, 2008). However, the discovery of a tryptophan-induced pigment in *C. glabrata* with many chemical similarities to the *M. furfur* pigment has opened up the possibility of using this yeast as a model for studying the molecular basis of the pigmentation process and its putative role in human fungal diseases. In this study, we have employed molecular tools available for *C. glabrata* to shed light on the synthesis and biological role of this novel pigment in human pathogenic fungi.

Results

Characterization of pigment production in C. glabrata

When grown on either solid or in liquid minimal medium with tryptophan as the sole nitrogen source, *C. glabrata* produced a reddish to dark brown pigment. In liquid media, the pigment is visible in both the supernatant and the cell pellet (Fig. 1A and data not shown). Furthermore, unlike in melanized fungi (Wang *et al.*, 1996), treatment with guanidinium isothiocyanate and hydrochloric acid did not result in any residual particles or pigment 'ghosts' (not shown) suggesting that the tryptophan-induced pigment of *C. glabrata* has different characteristics compared with melanin. Similar to a recently described tryptophan-induced pigment in *Cryptococcus gattii*, this pigment therefore seems unrelated to melanins (Chaskes *et al.*, 2008). A feature shared by melanins and this pigment seems to be the protection against UV light, which was demonstrated for *M. furfur* (Mayser *et al.*, 2002b) and was, to a moderate extent, also observed in *C. glabrata* (see *Supporting information*). We therefore investigated the biosynthesis and possible further biological functions of the *C. glabrata* pigment.

Dynamics of pigment production

Visible pigmentation in liquid media was observed several hours after entry of the culture into stationary phase (1–2 days' incubation) and maximal pigment production occurred following 3–4 days of incubation (Fig. 1A). To investigate whether the relationship between growth phase and pigmentation reflected an increase in pigment production by *C. glabrata* or another cell-independent mechanism, we isolated supernatants from induced liquid cultures and quantified the concentration of pigment (Fig. 2A); in parallel, cell-free samples from each time point were incubated in the dark for an additional 3 days before pigment quantification. For all time points, this additional 3 day incubation resulted in increased pigmentation (Fig. 1B). Furthermore, the pigmentation dynamics

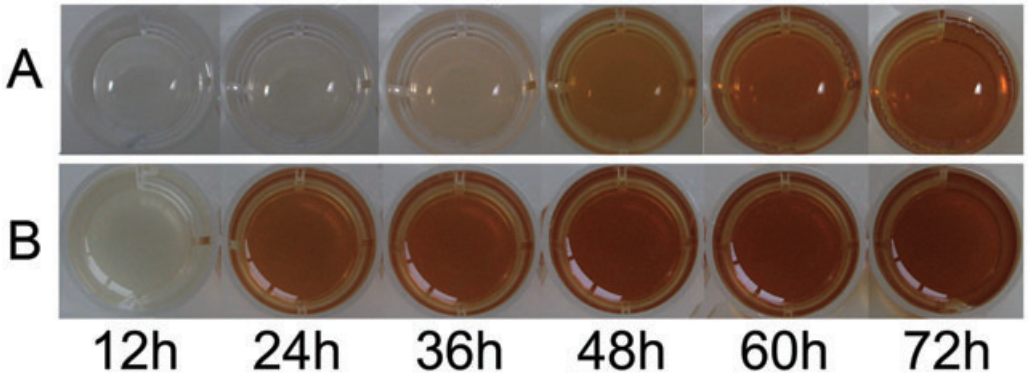

Fig. 1. Pigment formation of *C. glabrata*. In inducing, tryptophan-based minimal medium, *C. glabrata* forms pigment over the course of several days. The red-brown pigment is both secreted into the supernatant (shown here, A) and found associated with fungal cells (not shown). The sterile filtered supernatant darkened following an additional 3 day incubation in darkness (B, cf. Fig. 2A).

of these treated samples correlated directly with increasing cell density (Fig. 2A). This result demonstrates that the lag between growth phase and pigment production is due to an extracellular reaction in the media independent from direct fungal activity.

Pigment production requires oxygen

To investigate the role of oxygen in pigmentation, *C. glabrata* cells were grown in an oxygen-free atmosphere. Although growth was slow under this condition (not shown), no visible pigment was observed, even after extended (5 days) incubation, suggesting that oxygen is necessary for pigment production. When cell-free supernatants of the oxygen-free cultures were exposed to normal atmosphere, pigmentation became visible over the course of several hours (Fig. 2C). However, the total amount of pigment produced under these anaerobic conditions never reached the level of the control culture grown in the presence of oxygen. It can therefore be concluded that *C. glabrata* secretes a precursor into the media, which then reacts, in the presence of oxygen, to produce visible pigment.

The influence of additional nitrogen sources on tryptophan-induced pigment production

For *M. furfur* and *C. glabrata*, pigment production has been reported only when tryptophan was present as the sole source of nitrogen (Mayser *et al*., 1998; 2007). To investigate the role and influence of other nitrogen sources on pigment production, we incubated *C. glabrata* with all 20 standard amino acids, ammonium sulphate, or mixtures of these with tryptophan, and monitored any possible inhibitory or enhancing effects of additional nitrogen sources on pigment production.

In agreement with previous data (Mayser *et al*., 2007), the presence of tryptophan was essential for pigment production to occur (data not shown). When additional nitrogen sources were added to tryptophan-containing medium, pigmentation was inhibited to various degrees, depending on the nitrogen source added (Fig. 2B). There was no clear correlation between the type of amino acid (aromatic, polar, non-polar) and the reduction in pigmentation. For example, while tyrosine led only to a minor decrease in pigment production (down 27% as compared with tryptophan only), addition of phenylalanine strongly inhibited pigment production (reduction of 85%) – although both are aromatic amino acids like tryptophan. Asparagine and arginine were most effective in suppressing pigmentation, reducing by 98% the OD_{400} of tryptophan alone. We concluded that the presence of other amino acids reduce, but do not necessarily abolish, pigment production by *C. glabrata* and that the type of alternative nitrogen sources clearly influences pigment production to different extents.

However, when the colourless supernatant from yeast cells grown anaerobically with tryptophan was then mixed with different amino acids, pigmentation developed normally (Fig. 2C and D). There was no evident inhibition of pigment formation in the oxygen-dependent secondary step, with the notable exception of cysteine. In the presence of cysteine, visible pigmentation was severely inhibited, reaching only 40% of the OD_{400} of the control supernatant (Fig. 2D). We therefore concluded that the reduced pigment formation in media due to the presence of additional nitrogen sources is not due to an inhibitory effect on the oxygen-dependent secondary reaction step, with the possible exception of cysteine.

Influence of the carbon source on pigment production

All experiments described above were performed with glucose containing media. To test whether the type of carbon source influenced pigment synthesis, we used different non-fermentable substrates together with tryptophan. Although the growth rate and final OD_{600} of

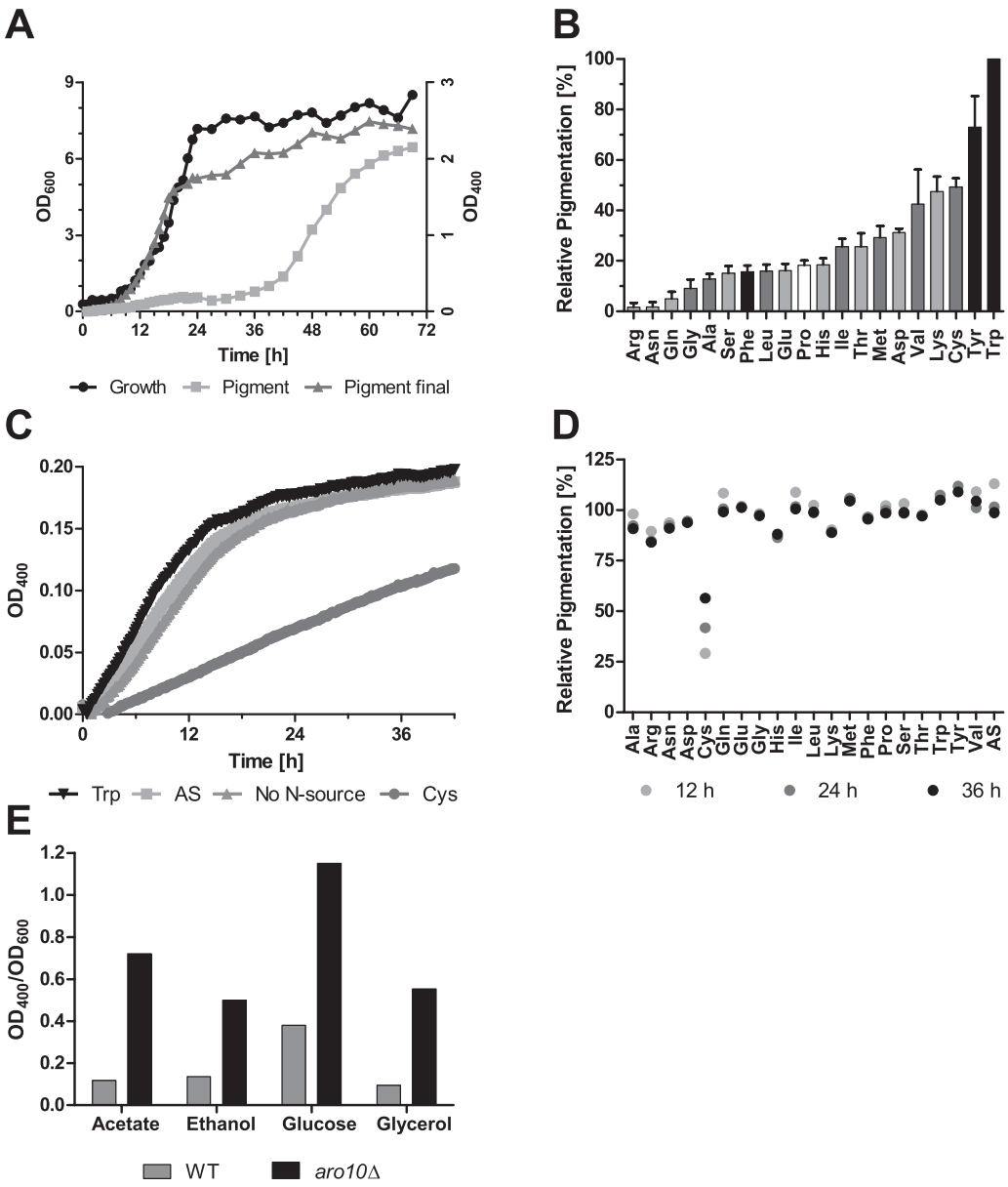

Fig. 2. A. Pigment is produced in a cell-dependent and a cell-independent step. Supernatants were taken from a culture growing in medium with tryptophan as the sole nitrogen source and pigment levels were measured either directly (Pigment) or following incubation for 3 days in the dark (Pigment final). While pigment in the untreated samples was visible at about 24 h when cells had entered the stationary phase, pigment of treated supernatant samples appeared earlier and correlated directly with cell growth (Growth).
B. Different nitrogen sources inhibit pigment formation to different extents. Tryptophan was mixed with other amino acids in minimal medium and pigment formation was measured as OD_{400} of the supernatants after 3 days. Relative pigment abundance is given compared with cultures with tryptophan only. Black bars, aromatic amino acids; dark grey, non-polar; light grey, polar; white bar, proline.
C. Pigment formation in cell-free supernatants is not influenced by most alternative N-sources. *C. glabrata* was grown anaerobically in medium with tryptophan as the sole nitrogen source. Supernatants were filtered and mixed with minimal medium without (SD) or with different nitrogen sources. Addition of ammonium sulphate (AS) or tryptophan (Trp) or several other amino acids (not shown) to the cell-free supernatants did not effect the kinetics of pigment formation as compared with SD. In contrast, cysteine (Cys) reduced the rate of pigment formation in the supernatant.
D. The same experiment as shown in C for the addition of 21 different additional alternative nitrogen sources. OD_{400} of mixtures with different N-sources compared with OD_{400} of supernatants from tryptophan cultures after 12, 24 or 36 h. Only cysteine reduced pigment formation.
E. Alternative carbon sources reduce pigment formation. Pigmentation measured (OD_{600}) after 3 days' growth with 2% of different carbon sources and normalized to the cell density (OD_{600}). The wild type showed a strong reduction in pigment formation with all non-glucose carbon sources tested. In contrast, the *aro10*Δ mutant produced more pigment than the wild type under all conditions.

Table 1. *C. glabrata* mutants with decreased pigmentation found in the transposon insertion library.

Name of disrupted gene	Function of most similar *S. cerevisiae* gene	Number of mutants found
FIG1	Integral membrane protein, involved in mating, probably involved in Ca^{2+} signalling (Factor-Induced Gene 1)	1
GID8	Function unknown. Involved in proteasome-dependent catabolite inactivation of fructose-1,6-bisphosphatase (Glucose Induced Degradation deficient 8)	18
YTA12	Component of the mitochondrial inner membrane m-AAA protease required for correct assembly of mitochondrial enzyme complexes (Yeast Tat binding Analog 12)	3
MSP1	Mitochondrial protein involved in sorting of proteins (Mitochondrial Sorting of Proteins 1)	1
FYV10	Function unknown. Involved in proteasome-dependent catabolite inactivation of fructose-1,6-bisphosphatase. Required for survival upon K1 killer toxin treatment (Function required for Yeast Viability 10)	1
VID24	Peripheral membrane protein located at Vid vesicles. Involved in proteasome-dependent catabolite inactivation of fructose-1,6-bisphosphatase (Vacuolar Import and Degradation 24)	1
VID30	Involved in proteasome-dependent catabolite inactivation of fructose-1,6-bisphosphatase. Shifts the balance of nitrogen metabolism toward glutamate production (Vacuolar Import and Degradation 30)	1
Total number of mutants with decreased pigmentation		26

The gene name and function is given according to the most similar *S. cerevisiae* gene, and the number of independent mutants of the same gene found in the library is given.

C. glabrata in glycerol, acetate and ethanol containing media was moderately to strongly reduced (approximate doubling times 16 h, 15 h and 30 h; final OD_{600} 9.1, 4.6 and 4.6 respectively), the relative pigment production efficiency in all three media was significantly lower than with glucose (doubling time 5 h, final OD_{600} 9.1; Fig. 2E). To compare pigmentation in these different media, an OD_{400}/OD_{600} ratio was used to determine the production of pigment relative to cell density. Glucose grown cells reached an OD_{400}/OD_{600} of 0.4 after 60 h. In contrast, cells grown in acetate, ethanol, or glycerol media only reached an OD_{400}/OD_{600} of 0.05–0.1 (Fig. 2E). Therefore, the carbon source significantly influences the levels of pigment formed.

Influence of cyclic AMP signalling on pigment production

As we observed differences in pigmentation depending on the nitrogen or carbon sources present, we next investigated whether signalling pathways involved in nutrient sensing may be involved in the regulation of pigment synthesis. We therefore supplemented *C. glabrata* cultures growing on a tryptophan-containing, non pigment-inducing medium with the cAMP analogue, dibutyryl cAMP. Following 3–4 days of incubation, a halo of pigmented cells could be observed (see *Supporting information*) in the presence of dibutyryl cAMP, suggesting that the cAMP signalling pathway plays a role in the regulation of pigment synthesis.

Identification of genes associated with pigment production

Our experiments demonstrated that pigment production in *C. glabrata* is tryptophan-dependent, influenced by different nitrogen and carbon sources, cAMP levels and oxygen. Based on these data, we postulated that a distinct biochemical pathway is responsible for the production of an essential and secreted precursor of the final pigment compound(s). In order to identify genes associated with pigment synthesis, we employed two independent approaches. First, we screened a library of random transposon insertion *C. glabrata* mutants, kindly provided by B. Cormack (Castano *et al*., 2003), to identify clones with defects or modifications in pigmentation. Second, we performed genome-wide transcriptional analyses of *C. glabrata* under pigment inducing versus non-inducing conditions to identify genes expressed during pigment production.

A mutant library screening identified mutants with abnormal pigment production

Altogether, over 17 000 clones were tested for their ability to form pigment on minimal medium with tryptophan as the sole nitrogen source. As expected, the overwhelming majority (98.96%) showed no discernible difference to the wild type. Of the remainder, most (0.81%) were unable to grow on the inducing medium, but 26 mutants (0.15%) grew and showed reduced pigment production. Sequencing identified seven genes which were disrupted in these mutants. Several different clones with transposons integrated in the same gene, but with different transposon insertion sites, were identified. Of these genes, four seem to encode proteins associated with glucose-dependent catabolite repression, as determined by the closest *S. cerevisiae* homologues (sequence similarity to *GID8*, *FYV10*, *VID24* and *VID30*) (Table 1). Another two genes are possibly involved in the folding and sorting of mito-

chondrial proteins (similar to *S. cerevisiae MSP1* and *YTA12*).

Interestingly, 10 mutants (0.06%) with a significant increase in pigmentation were identified. Sequencing revealed that in all 10 cases, independent transposon insertions have occurred in the putative promoter region of one single gene, CAGL0A03102g. This gene displays high sequence similarity to the *S. cerevisiae* gene encoding a phenylpyruvate decarboxylase, *ARO10*, which is involved in the degradation of aromatic amino acids via the Ehrlich pathway (Vuralhan *et al.*, 2003).

Identification of pigment-associated genes by transcriptional profiling

In a second approach, *C. glabrata* was grown with tryptophan (pigment-inducing) or arginine (non-inducing) as the sole source of nitrogen and the RNA then isolated for transcriptional analysis at different time points. We then compared the transcriptional response to both growth conditions using microarrays designed for *C. glabrata*, which represented 5063 open reading frames (ORFs).

A pre-culture was diluted to an OD_{600} of 0.2 in glucose-containing, defined medium with arginine or tryptophan. RNA was isolated from the stationary phase pre-culture and following 8, 24 and 48 h incubation from both main cultures, to represent: no visible pigment, medium and full pigmentation respectively. The OD_{600} of the cultures at these time points reached, respectively, around 1, 6 and 9 with tryptophan and 2, 8 and 9 for arginine. Hierarchical clustering showed that for arginine cultures, the transcription profiles at late time points (48 h) were highly similar to the stationary phase of the pre-culture, and a GO term enrichment analysis indicated starvation and cessation of growth (data not shown). The 48 h time points were therefore not investigated further. For the remaining conditions, the scheme depicted in Fig. 3 was used.

First, those genes which were upregulated at least threefold in tryptophan medium at both 8 and 24 h (as compared with the pre-culture) were selected. Second, from this list of 172 genes, those which were also at least threefold upregulated in arginine medium (125 genes) were removed; these 125 commonly upregulated genes were removed because they were considered to be associated with growth in minimal media, and not specifically associated with pigment induction.

The remaining 47 genes are therefore specifically upregulated in tryptophan medium during pigment synthesis (Table 2; Fig. 4). Many of the highest upregulated genes are directly related to arginine biosynthesis (e.g. homologues to the *S. cerevisiae* genes *ARG3*, *CPA1*, *ARG4* and *ARG1*) and were therefore excluded from

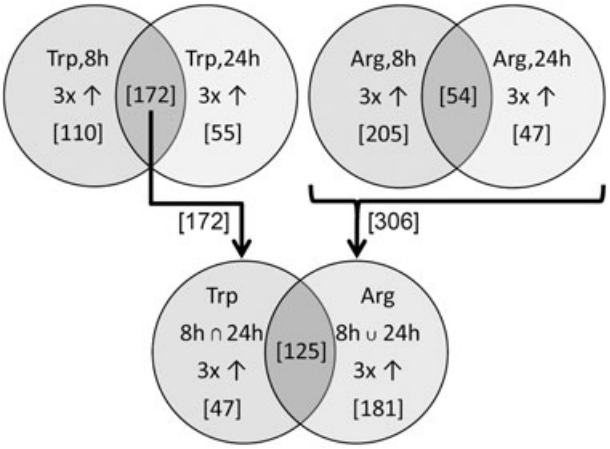

Fig. 3. Strategy for the identification of pigmentation-associated genes based on genome wide gene expression. Transcriptional profiles from cells grown for 8 or 24 h in minimal medium with either arginine (Arg) or tryptophan (Trp) as nitrogen sources were compared. In total, 172 genes were at least 3× upregulated at both time points in tryptophan medium, and 306 genes upregulated at either time point in arginine medium. Genes upregulated in both lists (125) were considered as induced by growth in minimal medium independent of the nitrogen source and subtracted from the tryptophan-upregulated set. This left 47 genes which were upregulated at both time points with tryptophan, but at no time point with arginine as the nitrogen source. These genes were defined as pigmentation-associated.

further analysis. Among the other upregulated genes, several were highly similar to *S. cerevisiae* genes involved in the Ehrlich pathway of amino acid degradation. These included *ARO8*, coding for an aromatic aminotransferase (up 7.2-fold after 8 h in tryptophan), and *ARO10*, which had also been identified in the transposon insertion library screening (up 12.8-fold after 8 h in tryptophan).

Other genes of interest included homologues of the multidrug-transporter gene *FLR1* (up 7.6-fold) and of the ABC transporter gene *PDR12* (up 25-fold after 24 h), which has been implicated in resistance to organic acids (Piper *et al.*, 1998). A gene similar to *VID24*, which is involved in the glucose-induced vacuolar degradation of fructose-1,6-bisphosphatase (Chiang and Chiang, 1998), was upregulated in tryptophan (5.6-fold after 8 h) and had also been identified in the transposon insertion library.

In silico *analysis of* C. glabrata *genes putatively involved in pigment production*

We have identified *C. glabrata* genes associated with pigment synthesis; however, the annotation of these genes was based on their 'best hit' in the *S. cerevisiae* genome. Using the BLASTP algorithm (Altschul *et al.*, 1997), we further investigated the similarities of the predicted protein sequences of our genes of interest with their *S. cerevisiae* counterparts.

Table 2. Expression levels of genes upregulated during pigment synthesis.

C. glabrata name	Fold regulation[a]				*S. cerevisiae*[b] homologue	Similarity[c]	Category[d]
	[Arg, 8]	[Arg, 24]	[Trp, 8]	[Trp, 24]			
CAGL0A03102g	0.85	0.80	12.84	10.38	ARO10	3	Ehrlich pathway
CAGL0I10791g	0.61	0.73	8.82	6.27	ARG3	4	Arginine synthesis
CAGL0H06017g	1.87	2.21	7.60	5.10	FLR1	3	Transporter/permease
CAGL0J11242g	1.91	1.44	7.53	6.62	RHO5	3	Regulation/signal transduction
CAGL0G01254g	2.63	2.09	7.22	3.90	ARO8	4	Ehrlich pathway
CAGL0K02145g	2.94	1.22	6.89	5.94	YER130C	1	Hypothetical protein
CAGL0C04917g	0.65	0.88	6.61	4.33	CPA2	4	Arginine synthesis
CAGL0G04389g	1.32	1.07	5.97	4.27	GZF3	1	Regulation/signal transduction
CAGL0M11660g	2.51	0.90	5.92	4.58	RHR2	4	Stress response
CAGL0K10780g	2.31	2.35	5.87	3.54	IMD4	4	Nucleotide metabolism
CAGL0M07293g	1.10	0.99	5.73	24.95	PDR12	3	Transporter/permease
CAGL0M05775g	2.27	2.22	5.70	3.16	NIP7	4	Ribosome
CAGL0I06248g	2.74	1.46	5.69	3.79	HAL5	3	Regulation/signal transduction
CAGL0K12254g	1.73	1.41	5.63	6.36	VID24	3	Glucose-induced degradation
CAGL0I08987g	1.31	0.59	5.34	3.77	ARG4	4	Arginine synthesis
CAGL0C05115g	1.27	1.10	5.21	4.10	ARG1	4	Arginine synthesis
CAGL0L00671g	1.98	1.84	4.96	3.12	FCY2	4	Transporter/permease
CAGL0L03157g	2.77	0.89	4.69	4.21	GZF3	1	Regulation/signal transduction
CAGL0M05841g	2.16	0.84	4.54	3.30	KTR2	3	Glycosylation
CAGL0J04554g	2.96	1.34	4.51	3.86	AAT2	3	Amino acid metabolism
CAGL0B01507g	0.86	0.57	4.48	3.76	ARG8	4	Arginine synthesis
CAGL0B04895g	2.26	2.71	4.46	4.54	RFX1	2	Regulation/signal transduction
CAGL0G03531g	1.21	2.43	4.33	4.80	SPR6	1	Unknown function
CAGL0I10747g	2.54	1.79	4.30	4.54	MEP3	4	Transporter/permease
CAGL0F02563g	1.40	0.61	4.28	3.27	HPT1	4	Nucleotide metabolism
CAGL0C05555g	2.87	1.51	4.19	3.84	GUD1	3	Nucleotide metabolism
CAGL0G04763g	2.84	2.45	4.17	5.11	RGS2	1	Regulation/signal transduction
CAGL0J09790g	1.92	1.12	3.90	3.09	GGC1	4	Transporter/permease
CAGL0M08580g	2.03	2.39	3.66	3.86	DON1	1	Meiosis
CAGL0M11198g	2.23	2.94	3.57	3.76	UBP8	3	Regulation/signal transduction
CAGL0I10626g	1.18	1.26	3.48	3.25	YGR125W	3	Unknown function
CAGL0H08393g	1.50	1.39	3.45	4.90	BAP3	4	Transporter/permease
CAGL0E01793g	1.63	0.53	3.44	3.76	YPS1	3	Protease
CAGL0D01826g	2.55	2.92	3.40	3.43	GCN1	3	Regulation/signal transduction
CAGL0G04213g	2.99	1.60	3.39	3.03	RNR4	3	Nucleotide metabolism
CAGL0J07502g	2.21	2.06	3.38	3.99	YNL234W	3	Regulation/signal transduction
CAGL0L03388g	0.91	0.44	3.36	3.35	–		No similarity
CAGL0I04862g	1.68	2.00	3.31	3.49	SNQ2	4	Transporter/permease
CAGL0K05753g	2.63	1.38	3.31	4.66	GNP1	4	Transporter/permease
CAGL0H02519g	2.52	2.87	3.28	3.78	YMR253C	3	Unknown function
CAGL0K12540g	2.04	2.31	3.27	3.77	HAC1	1	Regulation/signal transduction
CAGL0E05522g	2.26	2.25	3.21	3.85	YOR342C	3	Unknown function
CAGL0B02057g	2.65	2.74	3.15	3.02	FIN1	1	Mitosis
CAGL0I10901g	2.49	1.13	3.11	3.10	SLK19	3	Meiosis
CAGL0F02387g	2.13	1.69	3.06	3.26	PHO87	3	Transporter/permease
CAGL0L05038g	2.47	1.48	3.06	3.00	MUD2	2	Splicing
CAGL0B01683g	2.83	1.86	3.04	3.07	TMN2	4	Unknown function

a. Compared with pre-culture.
b. Most similar *S. cerevisiae* gene according to Génolevures.
c. Level of similarity according to Génolevures (highly similar – 4, similar – 3, weakly similar – 2, some similarities – 1).
d. Generic category of *S. cerevisiae* homologue function.
Fold regulation compared with pre-culture in YPD medium. Sorted by expression level after 8 h in pigment inducing medium (Trp).

This approach indicated that the product of gene CAGL0G01254g shares 69% identical and 82% similar amino acids with ScAro8, but also 29% identical and 51% similar amino acids with ScAro9. The *C. glabrata* gene designated CAGL0G06028g codes for a protein with 57% identity and 75% similarity to ScAro9, and 28% identity and 48% similarity to ScAro8. Interestingly, though, expression of the former gene is induced by the presence of tryptophan, whereas expression of the latter is not. This is in contrast to the behaviour of their respective *S. cerevisiae* counterparts, where *ScARO9* can be induced by aromatic amino acids (Iraqui *et al.*, 1999), while *ScARO8* is described as non-regulated under these circumstances (Iraqui *et al.*, 1998; Godard *et al.*, 2007).

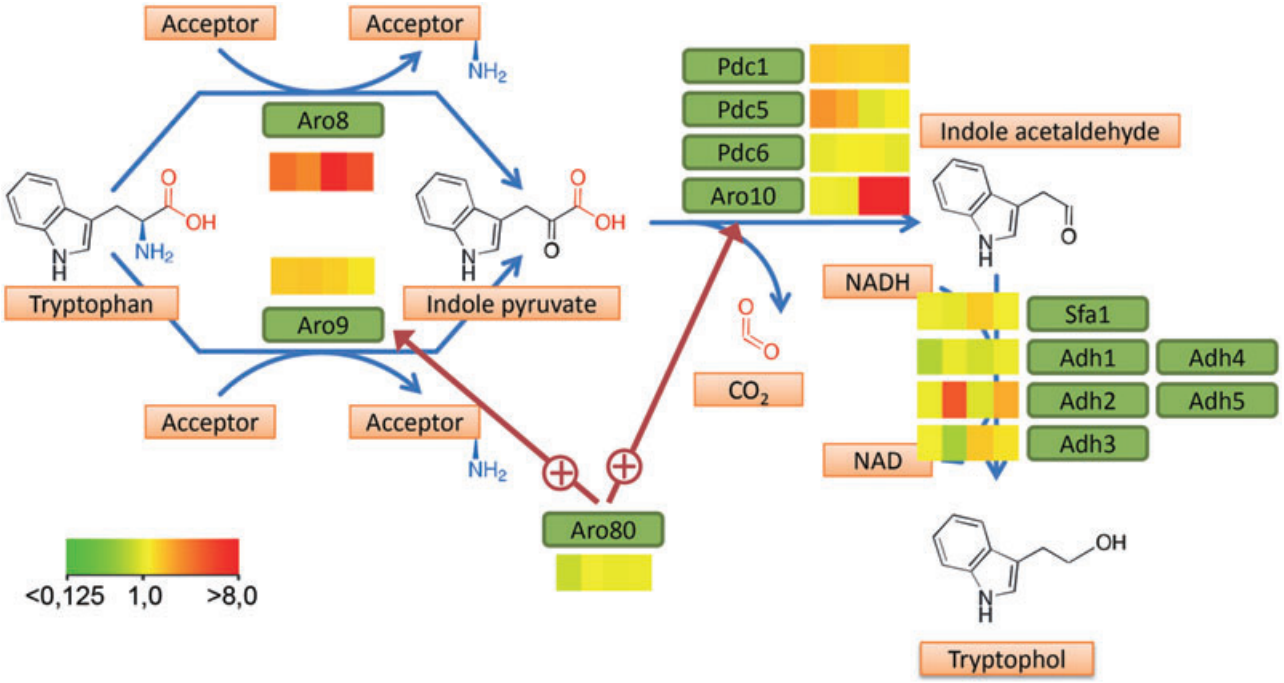

Fig. 4. Expression data of different genes projected onto a model of the Ehrlich pathway from *S. cerevisiae*. Expression of different genes is plotted as compared with the pre-culture. Green, downregulated; yellow, unchanged; red, upregulated. Expression patterns for each gene, from left to right: arginine 8 h, arginine 24 h, tryptophan 8 h, tryptophan 24 h. *ARO8* and *ARO10* were strongly induced in tryptophan-containing minimal medium, while there was no induction of *ARO9*, or other phenyldecarboxylase genes. No clear differences were observed in expression of the alcohol dehydrogenase genes. All *C. glabrata* homologous genes are assigned to *S. cerevisiae* genes according to tblastx results. The *PDC* and *ADH* genes are unclear in assignment and are shown in arbitrary order.

Targeted deletion of genes of the Ehrlich pathway

Microbes can biochemically modify aromatic amino acids by replacing the amino group with hydroxyl and releasing carbon dioxide. In the case of tryptophan, this process gives rise to tryptophol or 3-indole ethyl alcohol, first described by Ehrlich in 1907 (Ehrlich, 1907; Neubauer and Fromherz, 1911) and now recognized as one of the end-products of the so-called Ehrlich pathway (Hazelwood *et al*., 2008).

Many genes found to be upregulated during pigmentation were involved in the Ehrlich pathway of aromatic amino acid degradation. Therefore, we decided to produce targeted deletion mutants of different genes in this pathway, and tested their ability to produce pigment.

To this end, histidine or leucine auxotrophic mutants of the standard wild-type strain ATCC2001 were transformed with specific knock-out cassettes containing 500 base pairs of flanking sequence and the appropriate prototrophic marker gene. The mutants were then tested for their ability to grow and form pigment on tryptophan containing medium.

Functional analysis of genes of the Ehrlich pathway

ARO10. In *S. cerevisiae*, ScAro10 catalyses the decarboxylation of branched chain and aromatic 2-oxo acids, like the tryptophan-derived 3-indole pyruvate, to their corresponding aldehydes (Dickinson *et al*., 2003; Vuralhan *et al*., 2005). Deletion of the *ARO10* homologue in *C. glabrata* (58% identity, 71% similarity) had no effect on growth in standard medium, but resulted in significantly increased pigmentation under inducing conditions, confirming the results from the transposon insertion library screen (Fig. 5). This was not only the case when glucose was provided as a carbon source (OD_{400}/OD_{600} of 1.2, compared with wild-type level of 0.4), but also during growth on the alternative carbon sources acetate, ethanol and glycerol. Using these alternative carbon sources, growth rates were slower as compared with the wild type; however, pigment production was much higher (Fig. 2E). Growth of *aro10*Δ was also slightly delayed on media with tryptophan as the sole nitrogen source (Fig. 6) with a minimal generation time t_g of 254 min versus 200 min for the wild type. Similar results were obtained with other aromatic amino acids such as tyrosine (t_g 148 versus 107 min) and phenylalanine (t_g 143 versus 73 min). In contrast, the growth rate of *aro10*Δ was comparable to the wild type with all other tested amino acids or ammonium sulphate as nitrogen sources, and in complex medium (YPD, t_g 37 and 39 min). Interestingly, the *aro10*Δ mutant formed small aggregates when grown in pigment inducing conditions, while the wild type did not (not shown).

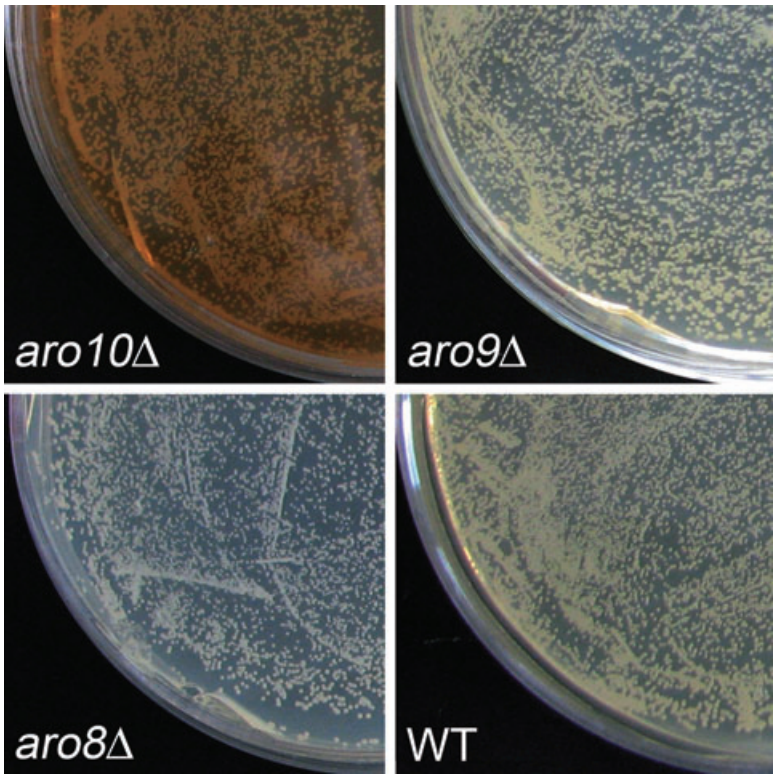

Fig. 5. Pigmentation phenotype of *C. glabrata* mutants lacking *ARO* genes. Wild type and mutants were grown on minimal media with tryptophan and threonine as nitrogen sources to allow growth of all strains. The mutant *aro9*Δ did not show any visible difference to the wild type, whereas *aro10*Δ produced more and *aro8*Δ considerably less pigment than the wild type.

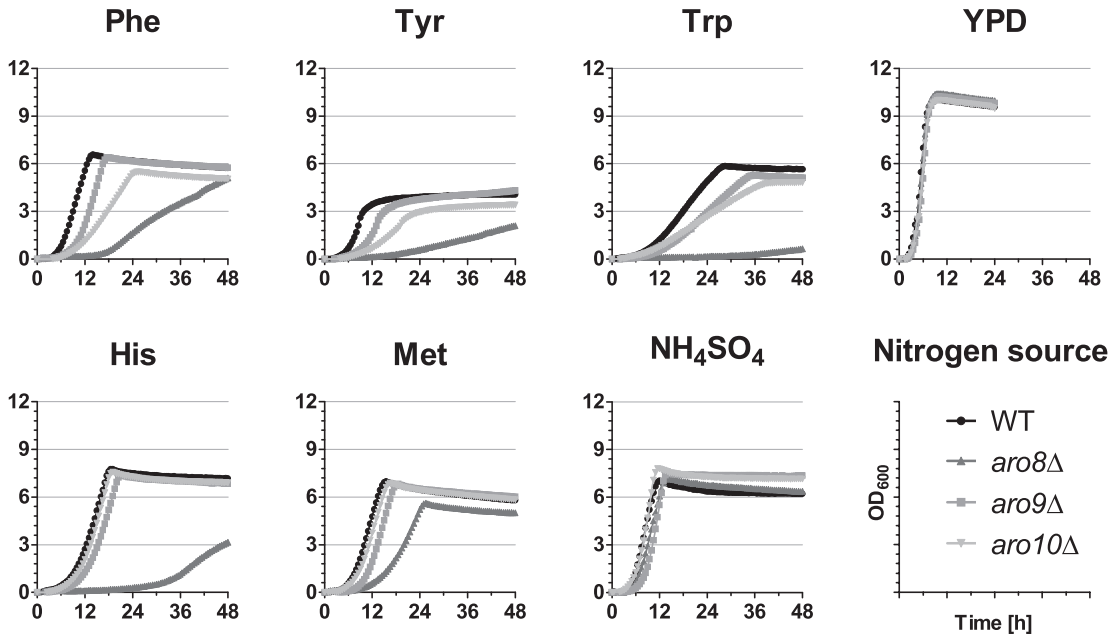

Fig. 6. Growth of mutants in media with different nitrogen sources. The *aro8*Δ, *aro9*Δ and *aro10*Δ mutants were grown in minimal media with 10 mM of each of the 20 standard amino acids, 0.5% ammonium sulphate or in YPD complex medium. A reduced growth rate was observed mainly for *aro8*Δ with the aromatic amino acids tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe), and with histidine (His). Both *aro9*Δ and *aro10*Δ had a slight growth defect with aromatic amino acids, and *aro8*Δ also with methionine as nitrogen sources. The growth rates were comparable for growth in complex and minimal media with ammonium sulphate, as well as with other amino acids (not shown here).

In order to investigate whether *ARO10* is involved in the synthesis of tryptophol, the end-product of the Ehrlich pathway, we analysed the tryptophol production of *aro10*Δ during growth with 15 mM tryptophan by semi-quantitative high-performance liquid chromatography (HPLC). Supernatants of *aro10*Δ cultures had significantly reduced amounts of tryptophol as compared with the wild type (data not shown), suggesting that the gene product of *ARO10* in *C. glabrata*, similar to its *S. cerevisiae* homologue, is most likely involved in the degradation of 3-indole pyruvate.

ARO9. In *S. cerevisiae*, ScAro9 is an inducible aromatic aminotransferase II (Iraqui *et al.*, 1999), which catalyses the first step in degradation of phenylalanine, tyrosine and tryptophan to their respective 2-oxo acids and which is induced by the presence of aromatic amino acids via the transcription factor ScAro80 (Iraqui *et al.*, 1998; 1999). In contrast, *ARO9* (CAGL0g06028g) is not upregulated by the presence of tryptophan (Fig. 4). Deletion of this gene had no discernable effect on pigment production. Similar to the *aro10*Δ mutant, deletion of *ARO9* resulted in a slight growth defect in media with aromatic amino acids as nitrogen sources.

ARO8. The *S. cerevisiae* gene *ScARO8* codes for the aromatic aminotransferase I, and is not induced by aromatic amino acids (Iraqui *et al.*, 1998). The expression pattern of *ARO8* (CAGL0G01254g), however, like *ScARO9*, showed a significant transcriptional upregulation in pigment-inducing medium (Table 2; Fig. 4). The *aro8*Δ mutant has a severe growth defect in tryptophan medium (Fig. 6; t_g 999 min) and showed significantly reduced growth in medium with phenylalanine (t_g 226 min), tyrosine (t_g 409 min), or methionine (t_g 173 min, 105 min for wild type) as sole nitrogen sources. The growth defects of the *aro8*Δ mutant were more severe than those of the *aro9*Δ and *aro10*Δ mutants. Furthermore, growth was significantly reduced with histidine as the sole source of nitrogen (t_g 351 min, 159 min for wild type). In contrast, no significant differences in growth rate were observed in complex (YPD) or in minimal medium with ammonium sulphate or other proteinogenic amino acids as nitrogen sources (t_g in YPD 38 min).

Pigmentation of *aro8*Δ was significantly reduced, both in tryptophan medium (which does not support growth of this mutant) and in a mixture of tryptophan and threonine – which does support growth, and caused only a moderate reduction in pigment production by the wild type (Fig. 2B).

Recombinant expression of Aro8

The mutant phenotypes strongly suggested that Aro8 is responsible for pigment production in *C. glabrata*. We therefore expressed the *ARO8* gene with an N-terminal histidine tag in *Escherichia coli*. After purification, the ability of this recombinant Aro8* protein to form pigment from tryptophan was analysed *in vitro*. A *lacZ* protein, expressed and purified using the same system, served as a negative control.

First, the ability of Aro8* to perform a transamination reaction with tryptophan as the substrate was tested. The deaminated product, indole pyruvate, has an absorption maximum at 338 nm. A rapid increase in OD_{338} could be observed in a mixture of 10 mM L-tryptophan, Aro8* enzyme and the cofactor pyridoxale phosphate, after the amino group acceptor α-ketoglutarate was added. The aminotransferase activity under these conditions was determined to be 120 mU mg^{-1}. This demonstrated that Aro8* is in fact an aminotransferase, and that it accepts tryptophan as a substrate. Interestingly, over the course of several hours, a brown pigment appeared in the reaction mixture, similar to the supernatant of pigmentation induced *C. glabrata* (Fig. 7A, cf. Fig. 1). As in *C. glabrata* culture supernatants, the secondary pigment forming reaction required several hours (Fig. 7B, cf. Fig. 2A). We speculated that the fast reaction is identical to the oxygen-independent reaction observed in *C. glabrata* cultures, and that the slow reaction is the oxygen-dependent pigment formation step.

Therefore, we performed the enzymatic reaction in an oxygen-free atmosphere. Even after 72 h, the solution remains colourless, and there was no measurable increase in OD_{400}. Indole pyruvate was formed, as can be seen by the high absorption at 338 nm (Fig. 7C). This shows that the first, enzymatically catalysed reaction can take place in the absence of oxygen. As soon as the mixture was exposed to a normal atmosphere, visible pigment started to form, and a slow rise in OD_{400} took place (Fig. 7C). We concluded that this is the oxygen-dependent step.

When the enzyme was heat-inactivated after the formation of indole pyruvate, pigment was still formed (Fig. 7D). Heat inactivation before the transaminase reaction, on the other hand, abolished both indole pyruvate and pigment formation.

Taken together, this shows that the pigment formation is based on a two-step mechanism, as suggested by the *in vivo* experiments with *C. glabrata* yeast cells. The first step in this reaction seems to be the oxygen-independent, enzymatic formation of indole pyruvate, and the second step the spontaneous, oxygen-dependent generation of the pigment from this precursor.

In *C. glabrata* supernatant, addition of cysteine, which possesses a reducing power due to its SH-group and which is able to scavenge oxygen radicals, strongly delayed pigment formation even under aerobic conditions (see above). To investigate the cysteine-mediated

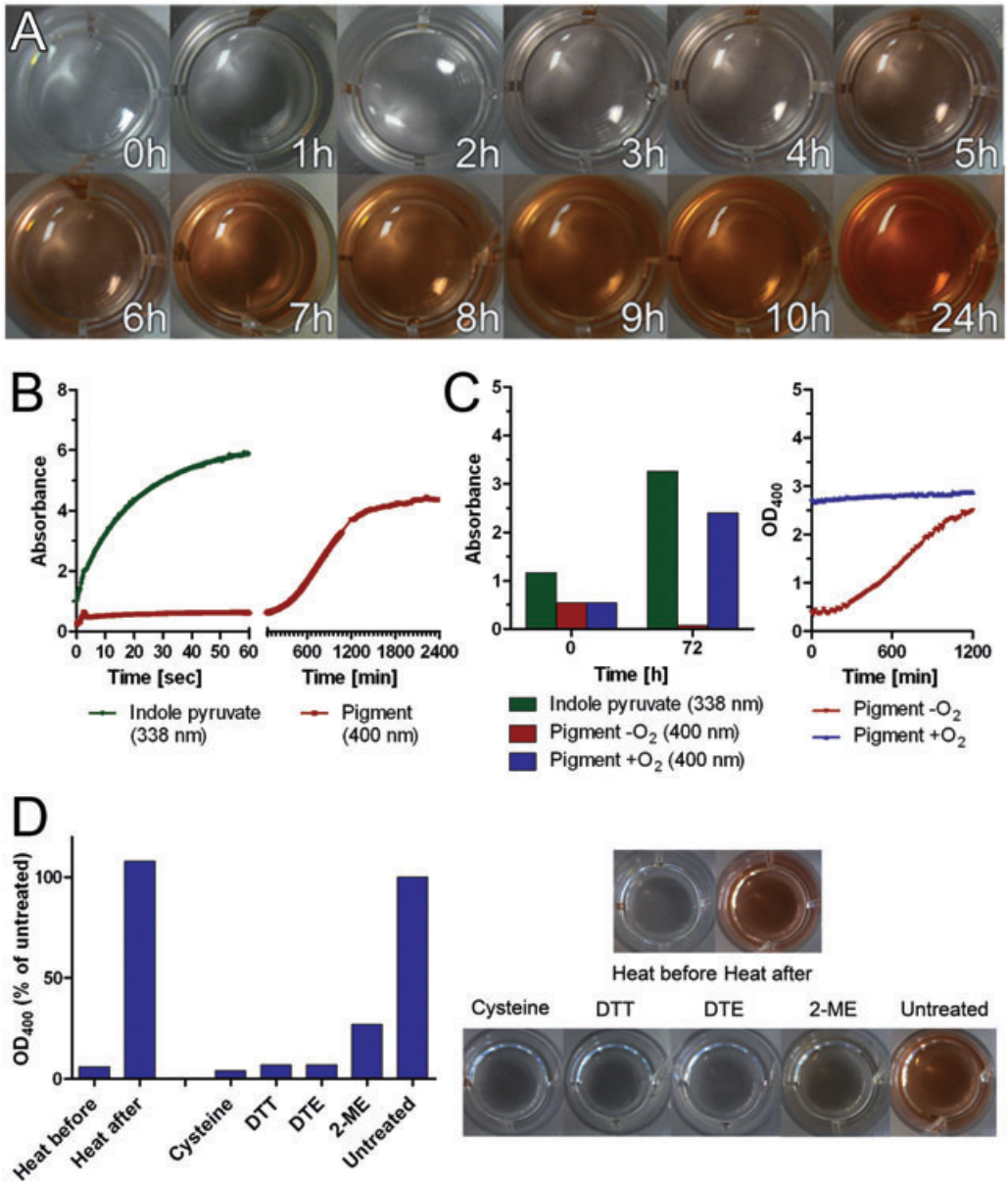

Fig. 7. *In vitro* pigment production by purified Aro8* enzyme.
A. Visual appearance of pigment in a solution of 10 mM tryptophan, 10 mM of the acceptor α-ketoglutarate and 10 mU ml^{-1} Aro8*. The coloration is highly similar to the supernatant of pigmentation induced *C. glabrata* cells (Fig. 1).
B. Time-course of the formation of indole pyruvate (green) and pigment (red) in an *in vitro* assay with 10 mU Aro8*. Indole pyruvate was formed within the first few minutes, while the pigment appeared over the course of several hours. Note that the early levelling of the indole pyruvate absorption may be due to measurement limits.
C. Indole pyruvate, but not pigment is formed in an oxygen-free atmosphere. Even after 72 h, an *in vitro* reaction with tryptophan and Aro8* did not result in pigment formation (OD_{400}, red bar), although indole pyruvate was formed (OD_{338}, green bar). In a parallel reaction in normal atmosphere, pigment was formed (OD_{400}, blue bar). Only after the sample was removed from the oxygen-free atmosphere, the brown colour started to appear (OD_{400}, red line) and reached an OD_{400} similar to the control sample (blue line) after several hours.
D. Inhibition of the pigment formation reaction. (Left) Appearance of the pigment was inhibited when the pigment was heat-inactivated before the indole pyruvate forming reaction, but not after. Different sulphydryl-group containing reducing agents inhibited the pigment formation from indole pyruvate, according to their reductive power. (Right) Visual appearance of the solutions after inhibition of the pigment forming reaction *in vitro*.

inhibition in more detail we performed *in vitro* experiments with purified recombinant Aro8*. Interestingly, cysteine at concentrations of 5 and 2.5 mM, inhibited enzymatic transamination of tryptophan by 85% and 65% respectively. This inhibition seemed competitive in respect to tryptophan, because increasing tryptophan concentrations restored activity (data not shown). Therefore, inhibition of pigment formation *in vivo* in the presence of cysteine and tryptophan as nitrogen sources could have been due to a competitive inhibition of indole pyruvate formation. However, as colour formation was also strongly delayed when cysteine was applied to the cell-free medium supernatant (see Fig. 1C and D), a competitive inhibition of cysteine can only partially explain this effect. Therefore, different sulphydryl-containing reducing agents were tested for their ability to inhibit Aro8* activity and, subsequently, pigment formation. Dithiothreitol (DTT), dithioerythrol (DTE) and 2-mercaptoethanol (2-ME) were applied at a concentration of 5 mM to the Aro8* assay system. DTT, DTE or 2-ME did not inhibit the formation of indole pyruvate from tryptophan, but when the colour formation was tested after 48 h, all tests with DTT, DTE and cysteine remained mostly colourless, whereas some coloration appeared in the presence of 2-ME (Fig. 7D). For those pure hydroxy radical scavengers (DTT, DTE and 2-ME), which do not affect the catalytic activity of Aro8, this result is in agreement with their respective reducing power, which follows the order of DTT $\geq$ DTE > 2-ME (Surdhar and Armstrong, 1987). Therefore, sulphydryl-containing agents are able to prevent the oxygen-dependent colour formation from indole pyruvate and tryptophan, independent of inhibition of the Aro8 transaminase reaction.

The pigment protects C. glabrata *from hydrogen peroxide-mediated killing*

In comparison with *C. albicans* and *S. cerevisiae*, *C. glabrata* is relatively resistant to hydrogen peroxide-mediated killing, mainly due to the action of a single extracellular catalase (Cuellar-cruz *et al*., 2008). As pigmentation is known to protect against reactive oxygen species in other fungi (Jacobson and Tinnell, 1993; Romero-Martinez *et al*., 2000; Brakhage and Liebmann, 2005), we hypothesized that the pigment of *C. glabrata* may contribute to the relatively high oxidative stress resistance of this yeast. We therefore tested the survival of pigmented and non-pigmented *C. glabrata* cells after incubation for 60 min in buffer containing 100 or 200 mM hydrogen peroxide.

Pigmented *C. glabrata* cells exhibited significantly higher survival following treatment with hydrogen peroxide at both concentrations as compared with non-pigmented cells (Fig. 8A). Upon exposure to 100 mM H_2O_2, about 60% of non-pigmented yeasts (grown with arginine) survived, while 90% of pigmented cells grown in tryptophan survived ($P < 0.0001$). The effect was similar with 200 mM hydrogen peroxide, where 50% of pigmented and only 35% of non-pigmented cells survived ($P < 0.05$), indicating that pigmentation provides resistance to oxidative stress.

To investigate whether this protective effect was dependent on the growth medium which induced pigmentation, or whether the protection was mediated predominantly by the pigmentation itself, we also tested selected mutants of the Ehrlich pathway for their resistance to hydrogen peroxide. As the hyper-pigmented deletion mutant *aro10*Δ grew in clumps under all pigment-inducing conditions tested, determination of surviving colony forming units was impossible. Therefore, this mutant was not included in these experiments. For testing the other mutants (*aro8*Δ and *aro9*Δ), a modified pigment-inducing medium containing a mixture of tryptophan and threonine was used prior to incubation with hydrogen peroxide. This mixture allowed growth for tryptophan catabolism mutants and wild type, while the pigment synthesis was only moderately reduced.

As shown in Fig. 8B, pigmented *aro9*Δ mutant cells were killed at about the same range as wild-type cells (87% versus 81%, $P = 0.6$ at 100 mM, and 45% versus 37%, $P = 0.6$ at 200 mM). In contrast, the hypopigmented *aro8*Δ mutant was more efficiently killed, with 52% survival at 100 mM H_2O_2 ($P < 0.05$) and only 11% at 200 mM ($P < 0.001$). In addition, survival of all strains was reduced when cells were cultured in arginine medium (which prevented pigmentation) and, in this non-inducing medium, no differences between the wild type and the two mutants were observed. Thus, the pigmented wild type and *aro9*Δ mutant survived exposure to hydrogen peroxide better than the hypo-pigmented *aro8*Δ mutant after growth on tryptophan.

Pigmentation reduces damage of C. glabrata *by neutrophils*

Neutrophilic granulocytes play a key role during *C. albicans* bloodstream infections (Fradin *et al*., 2005). Reactive oxygen species are potential antimicrobial agents produced by neutrophils to kill microbes. H_2O_2 is the key oxidizing agent produced by neutrophils and other immunoactive cells, which in turn generates other extremely toxic radicals that are active against microorganisms (Kowanko *et al*., 1991). As non-pigmented *C. glabrata* cells had significantly reduced resistance against treatment with H_2O_2, we investigated whether pigmentation might also protect against neutrophils.

Neutrophils were isolated from human whole blood and incubated at a ratio of 1:1 together with either pigmented or non-pigmented *C. glabrata* yeast cells. Control experi-

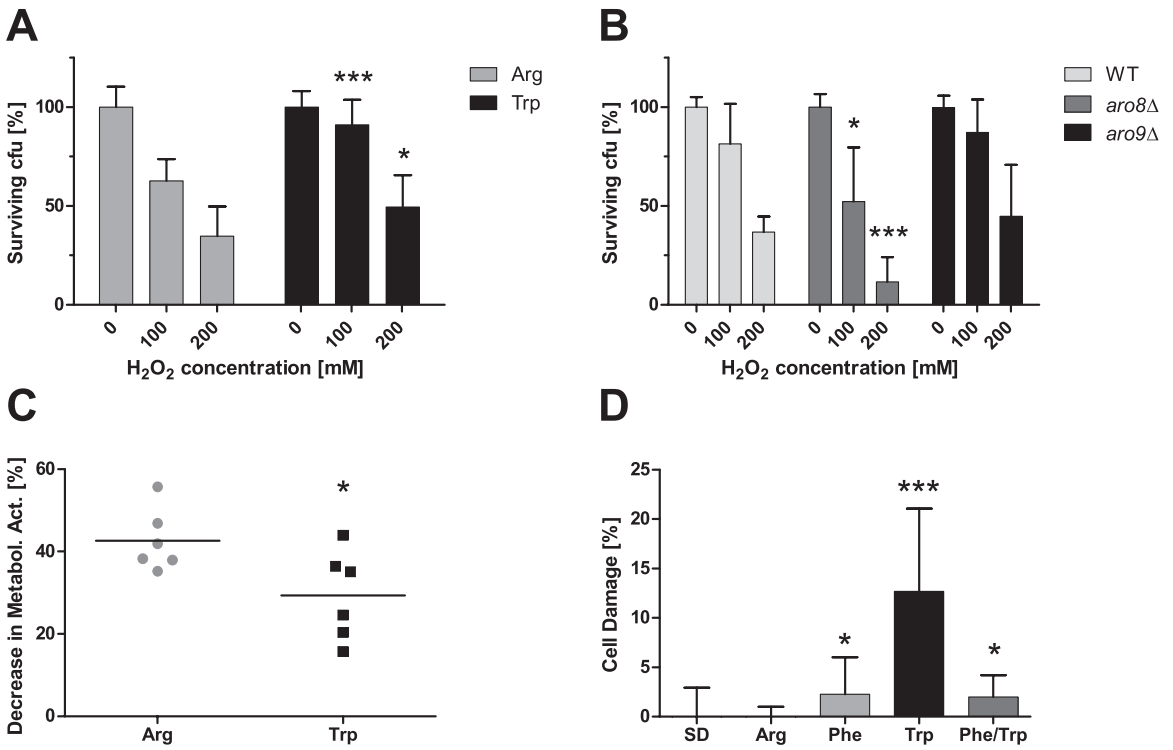

Fig. 8. A. Pigmented cells are protected against hydrogen peroxide. Yeasts exposed to 100 or 200 mM hydrogen peroxide for 1 h displayed significantly increased survival when pre-grown on pigment-inducing tryptophan containing medium (Trp) than pre-grown on arginine medium (Arg). Bars show surviving CFUs compared with control with 0 mM H_2O_2. * $p < 0.5$, *** $p < 0.01$ compared to arginine cultures.
B. A mutant with reduced pigmentation is more susceptible to H_2O_2. Yeasts were grown on medium with tryptophan and threonine to allow growth of all mutants. After treatment with H_2O_2, the *aro8*Δ mutant, which is severely reduced in pigmentation, was significantly more susceptible. * $p < 0.5$, *** $p < 0.01$ compared to wild type.
C. Pigmented yeasts are better protected against neutrophils. After co-incubation with human neutrophils, the reduction of metabolic activity was significantly less pronounced in yeasts pre-cultured under pigment-inducing conditions (Trp) than after pre-culture under non-inducing conditions (Arg). Circles and squares represent single experiment with different charges of neutrophils, bars the mean values of the conditions. * $p < 0.5$ compared to arginine cultures.
D. Pigmented yeasts damage human epithelial cells. Host cell damage was measured as percentage of maximum LDH release. Yeasts pre-cultured on pigment-inducing tryptophan medium showed a significant cell damage as compared with the uninfected control. Yeasts pre-cultured on non-inducing media with ammonium sulphate (SD), arginine (Arg), phenylalanine (Phe) or a mixture of phenylalanine and tryptophan (Phe/Trp) only exhibited a negligible to small host cell damage. * $p < 0.5$, *** $p < 0.01$ compared to SD and arginine cultures.

ments with pigmented or non-pigmented *C. glabrata* cells, but without neutrophils were also performed. After 1 h incubation with neutrophils, damage of yeast cells was determined by an XTT-based metabolic activity assay. The comparison of yeast cells pre-cultured in arginine medium and then incubated with or without neutrophils showed that 43 ± 8% of non-pigmented fungal cells were damaged (Fig. 8C). In contrast, only 29 ± 11% of the pigmented, tryptophan-grown cells were damaged. This corresponds to a significant ($P < 0.05$; Wilcoxon signed rank test) decrease in neutrophil-mediated damage due to the presence of pigment.

Pigmentation increases epithelial damage by C. glabrata

The potential of *C. glabrata* to damage epithelial cells is very low when compared with *C. albicans* (Li and Dongari-Bagtzoglou, 2007). In order to determine whether the production of pigment may have an impact on the pathogenic potential of *C. glabrata*, pigmented and non-pigmented yeast cells were used to infect monolayers of the human oral epithelial cell line TR146. Cell damage was monitored by measuring the activity of released lactate dehydrogenase (LDH) (Albrecht *et al.*, 2006; Schaller *et al.*, 2006).

As a control, *C. glabrata* was grown under three non-pigment inducing conditions, with medium containing arginine, phenylalanine or ammonium sulphate as sole source of nitrogen. Yeasts from these non-inducing pre-cultures caused very low-to-negligible damage to the epithelial monolayer after 24 h (Fig. 8D): LDH release induced by yeasts grown on ammonium sulphate or arginine medium was indistinguishable from the uninfected control, similar to the results obtained by Li and Dongari-Bagtzoglou (2007). Similarly, incubation with *C. glabrata*

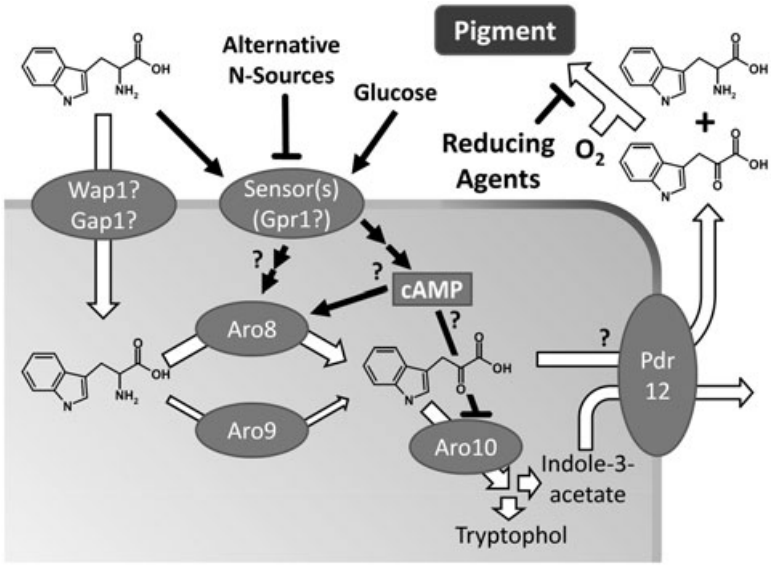

Fig. 9. Model of pigment synthesis in *C. glabrata*. This model is based on findings from this work and previous data from *S. cerevisiae*. Tryptophan is taken up (probably by homologues of the amino acid permeases) and then deaminated by the *ARO8* product and to a lesser extent by the *ARO9* product. Indole pyruvate is either further decarboxylated by Aro10 and follows the Ehrlich pathway to tryptophol, or the intermediate is secreted into the supernatant, possibly via Pdr12 or a similar transporter. Here it spontaneously reacts in the presence of oxygen to form the final pigment. The presence of glucose, tryptophan and alternative nitrogen sources modulates the production of pigment, presumably in part via cAMP signalling pathways, e.g. via a homologue of the ScGpr1 protein. Cysteine in the supernatant can slow down the pigment formation process.

cells grown on phenylalanine medium resulted only in very slight damage (2.3 ± 3.7%). In contrast, pigmented yeasts grown on tryptophan medium caused 12.7 ± 8.4% cell destruction of the monolayer, significantly more than cells pre-cultured on SD medium ($P = 6 \times 10^{-8}$).

On a medium where tryptophan was mixed 1:1 with phenylalanine, no pigment production was observed. Under this condition, LDH values were nearly identical to values generated by yeasts from pure phenylalanine medium (2.0 ± 2.2%; $P = 0.8$), demonstrating that the presence of tryptophan alone in the pre-culture media does not influence epithelial damage. Again, damage caused by these non-pigmented cells was significantly lower than the damage caused by pigmented cells ($P = 2 \times 10^{-8}$). Evidently, pigmented *C. glabrata* cells possess an increased ability to cause epithelial damage in this monolayer model.

Discussion

Traditionally, the human pathogenic ascomycete *C. glabrata* has been described as a white or colourless yeast (Calderone, 2002). Therefore, the recent observation that *C. glabrata* has the potential to produce a pigment on media with tryptophan as nitrogen source was unexpected (Mayser *et al*., 2007). While it seems clear that the pigment is similar in nature to the well-characterized indole-derived compounds of the lipophilic yeast *M. furfur* (causing diseases such as pityriasis versicolor) (Mayser *et al*., 1998; Mayser *et al*., 2007), the biochemical pathway of tryptophan-induced pigment production, the genes involved in the biosynthesis of this pigment by human pathogenic fungi in general and the biological roles of pigmentation for *C. glabrata* in particular have remained elusive (Hort *et al*., 2008). In this study, we have identified the key genes involved in pigment production in *C. glabrata* and created knock-out mutants of relevant pigmentation pathway genes, which were either strongly reduced in pigment production or which showed hyper-pigmentation. Additionally, we have investigated the function of one of the gene products, Aro8, with a purified enzyme after recombinant expression in *E. coli*. Based on these data we suggest a model of tryptophan-based pigment production (Fig. 9). Furthermore, our data provide the first insights into the regulation of pigment production and suggest possible roles for this pigment in the lifestyle of *C. glabrata*.

The pigment of C. glabrata *is a by-product of tryptophan catabolism*

Our results show that the *C. glabrata* pigment is not produced by a dedicated secondary metabolism pathway, as is the case for fungal melanins (Gomez and Nosanchuk, 2003; Langfelder *et al*., 2003; Nosanchuk and Casadevall, 2003), but instead is formed as a by-product of tryptophan catabolism via the Ehrlich pathway. This is supported by data obtained from the screening of a transposon insertion library and analysis of selected mutants, a comparative microarray transcription analysis, targeted gene disruptions and heterologous expression of one gene of this pathway in *E. coli*. These data strongly suggest that the putative aromatic aminotransferase Aro8 (encoded by ORF CAGL0G01254g) plays a major role in pigment production. In *S. cerevisiae*, the Ehrlich pathway includes two aromatic aminotransferases (28% identity), named aromatic aminotransferase I (ScAro8) and II (ScAro9), encoded by *ScARO8* and *ScARO9* respectively

(Iraqui *et al*., 1998). Expression of *ScARO8* is regulated by the general control of amino acid biosynthesis and ScAro8 has only minor tryptophan catabolic activity (Kradolfer *et al*., 1982; Urrestarazu *et al*., 1998). In contrast, *ScARO9* is induced by aromatic amino acids (Iraqui *et al*., 1998; Iraqui *et al*., 1999) and ScAro9 is the main catabolic aromatic aminotransferase acting on tryptophan (Kradolfer *et al*., 1982; Urrestarazu *et al*., 1998).

In our study, we identified two genes homologous to *ScARO8* and *ScARO9* (ORFs CAGL0G01254g and CAGL0G06028g). As CAGL0G06028g has a higher similarity to *ScARO9* (75% similarity to ScAro9 and 48% to ScAro8) and CAGL0G01254g to *ScARO8* (82% similarity to ScAro8 and 52% to ScAro9) both genes have been annotated accordingly at the Génolevures database (http://www.genolevures.org/cagl.html). However, our transcription data show that CAGL0G01254g is induced early in the presence of tryptophan, with the expression level declining over time – probably in correlation with the decreasing amount of available tryptophan. Furthermore, disruption of CAGL0G01254g had a major impact on pigment production, and, more importantly in this context, on growth with tryptophan as the sole nitrogen source. In contrast, CAGL0G06028g was not specifically induced by tryptophan, and its deletion had no effect on pigment production and only a minor effect on the ability of *C. glabrata* to grow with tryptophan as a nitrogen source. This suggests that in *C. glabrata*, Aro8 plays a role more similar to ScAro9, and Aro9 may function more similarly to ScAro8.

The expression of the phenylpyruvate decarboxylase gene homologue, *ARO10* (CAGL0A03102g), also followed an expression pattern similar to its corresponding gene in *S. cerevisiae* (*ScARO10*) when cells were grown in the presence of aromatic amino acids (Vuralhan *et al*., 2005; Godard *et al*., 2007): *ARO10* was highly induced by growth in tryptophan medium, but not with arginine as the sole nitrogen source. Three other phenylpyruvate decarboxylase genes, putatively involved in aromatic amino acid catabolism (Vuralhan *et al*., 2003), with similarities to *ScPDC1*, *ScPDC5* and *ScPDC6*, were not upregulated in either arginine- or tryptophan-based media, again similar to the regulation of their homologues in *S. cerevisiae* (Vuralhan *et al*., 2005; Godard *et al*., 2007).

Further enzymatic steps in the branch of the Ehrlich pathway, which lead to fusel alcohols, require alcohol dehydrogenases. Among the alcohol dehydrogenase genes identified in the *C. glabrata* genome, assignment to *S. cerevisiae* homologues is difficult due to a high degree of similarity among these genes. No gene homologous to *S. cerevisiae ScADH4*, the deduced protein sequence of which is more similar to bacterial alcohol dehydrogenases (Williamson and Paquin, 1987), was found in the *C. glabrata* genome. Of the remaining putative alcohol dehydrogenase genes, no clear difference in expression could be detected when *C. glabrata* was grown in media containing either tryptophan or arginine as single nitrogen sources. Under both conditions, CAGL0M14047g (*ADH6*) expression increased over time, while expression of CAGL0J01441g (*ADH3*) decreased.

Taken together, the expression pattern of putative Ehrlich pathway genes in *C. glabrata* is highly similar to their *S. cerevisiae* homologues when grown in media with tryptophan as the sole source of nitrogen. Furthermore, we show that heterologously expressed Aro8 from *C. glabrata* has aminotransferase activity with L-tryptophan as the amino donor, and α-ketoglutarate as the acceptor. The function and transcriptional regulation of this catabolic pathway may therefore be very similar between these two fungi, with the exception of *ScADH4*.

Based on these data, it can be concluded that the Ehrlich pathway is active under pigment-inducing conditions in *C. glabrata*. The gene *ARO8* is transcriptionally induced in medium with tryptophan as the sole nitrogen source, and its product Aro8 can generate indole pyruvate from tryptophan. Presumably, this intermediate is then either catabolized further to indole acetaldehyde by *ARO10*, or is secreted into the supernatant (Fig. 9). How it is secreted is unknown, but among the genes upregulated during pigmentation, were two genes coding for putative transporters: Flr1, a member of the major facilitator protein superfamily, and Pdr12, which has been implicated in the transmembrane transport of fusel acids from the Ehrlich pathway in *S. cerevisiae* (Hazelwood *et al*., 2006) and in resistance to organic acids (Piper *et al*., 1998). In fact, *PDR12* was as highly as 25-fold upregulated after 24 h of growth under pigment-inducing conditions. It is feasible that the same transporter may export the pigment intermediate.

Our data suggest that, once outside the yeast cell, the intermediate spontaneously reacts to form the pigment only if oxygen is present. This was demonstrated by *in vitro* reactions with a recombinant Aro8* protein: the production of indole pyruvate by a transaminase reaction was sufficient to start the formation of the brown pigment, even when the enzyme was heat-inactivated after the first step. In both *C. glabrata* supernatants and recombinant enzyme reaction mixtures, this second step required the presence of oxygen. Furthermore, the addition of sulphydryl-containing reducing agents reduced pigment formation to different degrees, corresponding to their relative reducing power. With the exception of cysteine, this inhibition was not based on a reduced enzymatic production of indole pyruvate by Aro8. The most likely explanation is that these agents directly inhibit oxidative reaction steps by means of their sulphydryl groups.

Therefore, together with the fact that *C. glabrata aro8*Δ mutants produced significantly less pigment, it can be

concluded that in the presence of tryptophan and oxygen, Aro8 is sufficient and (to the most part) necessary for the pigment production of *C. glabrata*. This is in agreement with a recent publication on a very similar pigment from *Ustilago maydis*, where an aminotransferase has also been identified as the sole step necessary for initiating this reaction (Zuther *et al.*, 2008). As *U. maydis* is a basidiomycete relatively closely related to *M. furfur*, it was concluded that the pigment synthesis in *M. furfur*, where this indolic pigment was first described (Mayser *et al.*, 1998), most probably follows a similar route. This postulation is supported by our data and provides further evidence that this pathway of pigment production is conserved between basidiomycetes and ascomycetes. It is likely that similar pathways exist, and are active, in other fungi; for example in *Cryptococcus* species or *C. albicans*, where similar pigments were recently demonstrated (Chaskes *et al.*, 2008), and where the genes of the Ehrlich pathway have been shown to exist (Ghosh *et al.*, 2008).

The growth of aro*-mutants in media with different amino acids as nitrogen sources*

The main functions of the Ehrlich pathway are assimilation of amino acids and the catabolic degradation of amino acids as carbon and nitrogen sources (Hazelwood *et al.*, 2006). Therefore, genes of the Ehrlich pathway may be essential for growth under conditions where amino acids are provided as sole nitrogen sources.

The growth patterns of *C. glabrata* mutants lacking *ARO* genes resemble the described phenotypes of *S. cerevisiae* mutants of the homologous genes (Iraqui *et al.*, 1998; Iraqui *et al.*, 1999). In all three media with aromatic amino acids as nitrogen sources, the mutants lacking the aromatic aminotransferases Aro9 or Aro8 showed a growth defect. The *aro8*Δ mutant exhibited the slowest growth under all conditions, while the *aro9*Δ mutant was only slightly reduced in growth. Evidently, deletion of the gene encoding the constitutively expressed aminotransferase Aro9 can be compensated by another aminotransferase, presumably Aro8.

The *aro8*Δ mutant also shows a severe growth defect with histidine as the sole nitrogen source. Interestingly, this is the only amino acid which *C. glabrata* can use for growth (this study), but *S. cerevisiae* cannot (Large, 1986). Like most fungi, both organisms lack any protein similar to the histidine-ammonium-lyases, of bacteria and animals, necessary for the utilization of histidine as a nitrogen source (Taylor *et al.*, 1990; Suchi *et al.*, 1995; Fernandez and Zuniga, 2006). However, while this leaves *S. cerevisiae* unable to extract nitrogen from histidine, our results indicate that *C. glabrata* may instead use its aromatic aminotransferase, Aro8, for this purpose. This additional catabolic pathway may add to the metabolic flexibility of *C. glabrata* as compared with baker's yeast, an attribute which is recognized as an important factor in the lifestyle of pathogenic fungi (Brock, 2009).

Regulation of tryptophan-based pigment production

Our data suggest that *C. glabrata* relies on the Ehrlich catabolic pathway to obtain nitrogen from tryptophan. This is supported by the fact that the alternative route of tryptophan degradation via kynurenine has been lost in *C. glabrata* (Li and Bao, 2007). As degradation of tryptophan occurs via the Ehrlich pathway, and the pigment is a by-product of this degradation, how can pigment synthesis be regulated? The amount of pigment formed is dependent on the opposing action of at least two proteins. The aromatic aminotransferases Aro8, and – to a lesser extent – Aro9 convert tryptophan to the pigment intermediate indole pyruvate, while the decarboxylase, Aro10 reduces pigmentation by processing indole pyruvate further (Fig. 9). Therefore even enzymatically, the regulation of pigment production may occur on at least two levels: the induction and activation of the aromatic aminotransferases or the decarboxylase.

Interestingly, it has been shown that the induction of ScAro10 activity in *S. cerevisiae* is not only regulated on the transcriptional level (Vuralhan *et al.*, 2005). When *ScARO10* was placed under the control of a constitutive promoter, decarboxylase activity could be detected in ethanol-grown cells, but not in cells grown with glucose as a carbon source. Therefore, activity of the decarboxylase depends on the carbon source available, and is regulated post-transcriptionally. This finding correlates well with our observation that the pigment is synthesized from tryptophan more efficiently in the presence of glucose (where Aro10 activity should be repressed) than with other carbon sources. The activity of Aro10 may therefore play an important role in the regulation of pigment production.

In this context, it is interesting to note that within the group of transposon-disrupted genes resulting in hypopigmented mutants, many are involved in glucose-induced catabolite repression. The corresponding genes are highly similar to *S. cerevisiae ScGID8*, *ScFYV10*, *ScVID24* and *ScVID30*. However, a direct connection between these genes and pigmentation is not evident.

As in *M. furfur* (Mayser *et al.*, 1998), the presence of alternative nitrogen sources in tryptophan-containing medium inhibits the pigment production to different degrees. However, this reduction is not due to a reaction of the precursor product with other amino acids – except, perhaps, in the case of cysteine. When *C. glabrata* was grown in an oxygen-free atmosphere with tryptophan, no pigment became visible in the supernatant. The pigmentation process only started when the supernatant was

exposed to the normal atmosphere, and was independent of the presence of amino acids other than tryptophan. The only notable exception was cysteine, which severely reduced the development of pigmentation. This shows that the inhibitory effect of alternative amino acids most likely occurs intracellularly, on the regulatory level in *C. glabrata*, and not during the spontaneous reaction in the supernatant.

Taken together, these results show that the presence and type of carbon and nitrogen sources strongly influence pigment production by *C. glabrata*. As exogenous cAMP can stimulate pigmentation under otherwise non-inducing conditions, the cAMP signalling pathway may play a major role in this regulation. In *S. cerevisiae*, the G protein-coupled receptor ScGpr1 is involved in sensing both glucose and nitrogen starvation (Lorenz *et al.*, 2000), and a structurally similar protein in *C. neoformans* signals the presence of methionine in the medium (Xue *et al.*, 2006). It is therefore feasible that the presence of glucose and the type of amino acid may be detected by a *C. glabrata* Gpr1 homologue (and possibly other sensors), which in turn regulates the pigment synthesis via cAMP.

Effect of pigmentation on resistance to oxidative stress and neutrophils

Neutrophils are important immune effector cells which kill pathogenic fungi and neutropenic patients have an increased risk of suffering from life-threatening *Candida* infections (Krcmery and Barnes, 2002; Perlroth *et al.*, 2007). The oxidative burst inside the neutrophil phagosome is essential for the killing of many microorganisms (Hampton *et al.*, 1998), including *C. glabrata* (Kowanko *et al.*, 1991). Our experiments demonstrated that pigmentation of *C. glabrata* elicited a low, but measurable protective effect against killing by neutrophils.

At least two general mechanisms are conceivable to explain this effect: either the fungicidal activity of the neutrophils is repressed by the pigment, or the pigmented yeasts can better withstand the killing by the immune cells. *In vitro* experiments have shown that substances in the secreted pigment of *M. furfur* are able to suppress the oxidative burst of chemically activated granulocytes via inhibition of kinases (Kramer *et al.*, 2005b). It is feasible that cell-associated pigment reaches sufficiently high local concentrations during contact with neutrophils and thus inhibiting the production of reactive oxidative species in a similar way. It has been demonstrated that another compound in the pigment of *M. furfur*, Malassezin, elicits apoptosis in human melanocytes (Kramer *et al.*, 2005a). This action is mediated by the binding of Malassezin to the melanocytes' aryl hydrocarbon receptor, thereby leading to apoptosis. Neutrophils also express this receptor, and its activation by dioxins and other compounds is known to induce immunosuppression (Lawrence and Kerkvliet, 2006). In addition, a similar effect has been observed when leucocytes were exposed to low millimolar doses of tryptophol (Kosalec *et al.*, 2008). Thus, different components of the *C. glabrata* pigment may explain some of the observed effect. Clearly, future experiments are needed to shed light on the precise mechanisms involved and the extent to which this effect is relevant to the *in vivo* infection situation.

The alternative explanation, that fungal cells are directly protected by the pigment, is supported by our observation that pigmented *C. glabrata* exhibit decreased killing by hydrogen peroxide. A correlation between H_2O_2 sensitivity and neutrophil killing has been observed for following gene deletion in *C. glabrata* (*ERG11*) (Kan *et al.*, 1996) and *C. albicans* (*SOD5*) (Martchenko *et al.*, 2004; Fradin *et al.*, 2005). Pigmentation may therefore directly protect the yeast from the oxidative burst, similar to the effect of melanin on fungi such as *C. neoformans* (Wang *et al.*, 1995) or *Aspergillus fumigatus* (Brakhage and Liebmann, 2005), or bacterial pigments like the *Staphylococcus aureus* carotenoid pigment (Liu *et al.*, 2005). The common notion of melanin as a 'fungal shield' may thus also apply to this pigment.

Pigmented C. glabrata *cells cause increased damage of epithelial cells*

Pigmented *C. glabrata* cells also caused increased damage of human epithelial cells in a monolayer infection model. A possible explanation for this phenomenon again roots in the composition of the cell wall-bound pigment. As described above, part of the *M. furfur* pigment, the Malassezin, has been shown to induce apoptosis in human melanocytes (Kramer *et al.*, 2005a), with micromolar concentration leading to cellular damage. With the close contact between epithelial cells and the pigment-containing *C. glabrata* cell wall, a Malassezin-like compound, or other components of the pigment, may reach concentrations sufficiently high to cause damage to the host cells. It is also feasible that this damage to epithelial cells may aid in the invasion process of pigmented cells during an infection.

Can pigmentation occur in vivo*?*

The possible benefits of pigment production *in vivo* are evident from the data presented here. Pigmented *C. glabrata* are able to withstand killing by neutrophils better than non-pigmented yeasts. Furthermore, expression of the pigment *in vivo* may explain in part the pathogenic potential of *C. glabrata*, because pigmented yeasts caused an increased damage of epithelial cells in a monolayer model.

The question remains whether pigment production can take place *in vivo*. Although we do not have direct evidence, *in vivo* production of pigment is conceivable.

Until now, it appeared that pigment production by *C. glabrata* is limited to very specific conditions, as pigmentation predominantly occurs on tryptophan as the sole nitrogen source as has been shown for *M. furfur* (Mayser *et al.*, 1998). We believe that such a specific environmental situation is a rather unlikely event in any relevant *in vivo* scenario of *C. glabrata* infections, which would in fact make *in vivo* production of pigment unlikely. Indeed, it is more likely that several nitrogen sources including non-tryptophan amino acids are simultaneously available within the host. In this study, we were able to show that *C. glabrata* can produce pigment even in the presence of other amino acids. Furthermore, pigment production can be increased by the transcriptional downregulation of *ARO10* and possibly post-transcriptional downregulation of its product, Aro10. Therefore, pigment synthesis could take place *in vivo* providing tryptophan is present. This may be viewed as analogous to the situation in *C. neoformans*, which is dependent on phenolic precursor molecules such as L-DOPA for melanization. Although it is less probable that L-DOPA molecules are freely available in sufficient amounts *in vivo*, melanized *C. neoformans* have in fact been identified in infected animals, obviously using the host's own molecules as building blocks (Rosas *et al.*, 2000).

In addition, *in vivo* pigment production has previously been shown indirectly for *M. furfur*, which presumably uses a similar pathway to *C. glabrata*. Pityriasis versicolour, a typical disease caused by *M. furfur*, is characterized by dark and light patches on the skin (Gupta *et al.*, 2003; Thoma *et al.*, 2005), the former possibly consisting of pigment (Mayser *et al.*, 1998). Evidently, *M. furfur* is able to synthesize large amounts of the pigment in the course of an infection. While *M. furfur* is mainly limited to the upper epidermis, *C. glabrata* may be able to use a similar mechanism to produce the pigment on mucosal surfaces or in deeper tissues during systemic infections.

As discussed below, pigment production is difficult to analyse *in vivo*. But if pigmentation does occur *in vivo*, where may *C. glabrata* acquire the tryptophan necessary to synthesize the pigment? The gastrointestinal tract is a known reservoir for *Candida* species (Cole *et al.*, 1996; Wells *et al.*, 2007) and systemic *C. glabrata* infections may originate from oral (Redding *et al.*, 2004) or intestinal colonization. In these niches, tryptophan is readily available from food. For example, tryptophan is found up to a weight proportion of 0.5% in some cheese, and 0.25% in pork (http://www.ars.usda.gov/nutrientdata). Also, although in the current study, relatively high amounts of tryptophan were used, pigmentation in both *M. furfur* and *C. glabrata* occurs at much lower concentrations in the upper micromolar range (Mayser *et al.*, 1998 and own observations). Thus, dietary tryptophan supplementation may be sufficient for pigment production *in vivo*.

Further investigations into this are complicated by the fact that pigment production is intertwined with important metabolic routes. The mutants lacking genes of the Ehrlich pathway show severe growth defects under several tested *in vitro* conditions, which would bias any *in vivo* experiments. Furthermore, a direct demonstration of pigment production by wild-type cells *in vivo* may prove difficult because large samples would have to be isolated to chemically identify the pigment *in vivo*, while indirect detection would require a specific antibody against purified compounds of the pigment. However, new tools and adequate models may be available in the future to better understand the effect of pigmentation on the life style of this pathogenic fungus.

In summary, we have elucidated the biochemical pathway by which pigment is produced in *C. glabrata,* have demonstrated a number of different conditions and factors which influence or regulate pigment production, and have identified putative functions of the pigment. While the biochemical pathway demonstrates the metabolic flexibility of *C. glabrata*, the ability to produce pigment may add to the immune evasion strategies and thus to the ability to cause disease in the host.

Experimental procedures

Strains and growth conditions

The *C. glabrata* strain ATCC2001 was used for all experiments. For the targeted deletion mutants, histidine or leucine auxotrophic strains were used with the *HIS3* or *LEU2* gene deleted (provided by Karl Kuchler, Vienna). Pigment was induced in medium with 15 mM tryptophan (Roth, Germany) in 1× YNB (BD Biosciences, CA, USA) with 2% glucose (Roth) and 100 mM phosphate buffer (pH 5.8). For growth experiments, tryptophan was replaced with 10 mM of the respective amino acid (Roth), or 0.5% NH_4SO_4 (Roth). Growth was measured in a 24-well plate (TPP, Switzerland) in a Tecan Infinite M200 microplate reader at 37°C with intermittent shaking.

Measurement of pigment production

The amount of pigment in the supernatant was measured following sterile filtration by reading the optical density at 400 nm in an Amersham Ultrospec 3100pro spectrophotometer. Because of the intrinsic darkening of tryptophan solutions over time, a medium control was treated identically in all experiments and its OD_{400} subtracted from the measurements.

Microarray experiments

RNA from liquid cultures was isolated using the RNeasy kit (Qiagen, Germany) and quality checked with an Agilent Bio-

Analyzer. For the labelling reaction, Cy5-CTPs (GE Healthcare, UK) were incorporated using the Low Input Linear Amplification kit (Agilent, CA, USA). As a common reference for all samples, RNA from mid-log phase *C. glabrata* was labelled with Cy3-CTP (GE Healthcare) following the same protocol. Of the Cy-labelled RNAs, 1 μg of each was mixed in DIG Easy-Hyb (Roche, Switzerland) and hybridized to a custom-made *C. glabrata* array. Microarrays were produced (S. Rupp lab) by spotting 5908 69- or 70-mer oligonucleotides (kindly provided by Ken Haynes) synthesized at the Pasteur Institute to Nexterion E slides in duplicates, using a MicroGrid II spotter (Biorobotics). Slides were post-processed by incubation in a wet chamber followed by baking at 80°C overnight. After washing in 2× SSC/1% SDS (15 min), 1× SSC/0.2% SDS (8 min) and 0.1× SSC/0.2% SDS (5 min), the arrays were dried and scanned with a GenePix 4000B scanner (Molecular Devices, CA, USA) at 10 μm resolution. Data extraction was performed with the GenePix 4.1 software (Molecular Devices) and the files imported into GeneSpring GX 10.0 (Agilent) for LOWESS normalization and data analysis. For the GO-Term enrichment analysis and annotation of *C. glabrata* genes, data from the Génolevures database (Sherman *et al*., 2009) and the Saccharomyces Genome Database (SGD project; Hong *et al*., 2008) were combined: for missing *C. glabrata* gene functions or GO-terms, *S. cerevisiae* data was used if the annotation showed the genes to be 'similar' or 'highly similar'. The microarray data will be stored in the ArrayExpress database of transcription profiles.

Targeted gene deletions

For the *aro8*Δ mutant, two flanking regions of about 500 bp each were amplified with primers ARO8-1 (GGATCCTGTAG GCTTGCTTTGTATTTG) and ARO8-2 (GGATCCGATTTAT GCTAGTGCTTGATG) for the upstream region and ARO8-3 (CTCGAGTCGATGTATTGGTAAGAAGTT) and ARO8-4 (CTCGAGATCAACATTTTACGCCAAA) for the downstream region. A leucine cassette was amplified from wild-type genomic DNA with primers LEU-1 (GGATCCTACCAATAGAT AGCGGTTTAA) and LEU-2 (CTCGAGGTTCGTTTCCGATA CATGC) and cloned into a pCR2.1 vector using the TOPO TA cloning kit (Invitrogen, CA, USA). The flanking regions were integrated next to the leucine cassette using the newly created BamHI (upstream) and XhoI (downstream) sites using T4 ligase (NEB).

The flanking regions for the *aro9*Δ mutant were generated with the primers ARO9-1 (TCTTCTTGCGGCGTATCAT) and ARO9-2 (ACTGGGATCCAGATGACACAATTTCCAGC) for the BamHI cloning and ARO9-3 (ACTCTCGAGTGTCTTG CATTTTACATATGG) and ARO9-4 (TTATTGGTGTGACGT TGCTGG) for XhoI cloning. Here, PCR products were directly cut with the respective enzymes, ligated to the leucine cassette and the ligation product was then cloned into pCR2.1 using the TOPO TA kit.

The primer pairings for ARO10 were ARO10-1 (ACG GCTGGATCCTGTGTGCCTGGGATAATA) and ARO10-2 (ACGGCTGGATCCTTCAGCTCGTTACAGTTG) for BamHI cloning, or ARO10-3 (ACGGCTCTCGAGAGATGTTCGA AGCATTCG) and ARO10-4 (CGGCTCTCGAGCCTG GTTTCTTTCAATAAG) for XhoI cloning purposes. A histidine prototrophy cassette was amplified with the primers HIS3-1 (AGCTGGATCCAATTATGGATCCAGTTGAA) and HIS3-2 (ATGCTCTCGAGGTAGCTCTCGAGAAATACTC) and the fragments ligated as before.

For transformation, the construct was PCR-amplified from the pCR2.1 vector. *C. glabrata* leucine or histidine auxotrophic strains were transformed using a modified heat shock method (Sanglard *et al*., 1996), with 45°C heat shock for 15 min. Yeasts were then plated on YNB medium with ammonium sulphate and the transformants checked for the absence of the wild-type allele and the presence of the prototrophy cassette by PCR and Southern blotting. At least two independent transformants were tested for their ability to form pigment for each gene.

Recombinant expression of Aro8

Using primers Aro8Ex-1 (ATGACTCTGCCAGAATCTAAA) and Aro8Ex-2 (TTATGCCAATTGAAATTCTTC), *ARO8* was amplified from genomic DNA of *C. glabrata* wild-type strain ATCC2001. The proof-reading polymerase Phusion (Finnzymes) was used in the PCR reaction according to manufacturer's instructions. The product was subcloned into the pCR2.1 vector using the TOPO-TA system (Invitrogen). An EcoRI fragment containing the *ARO8* gene was then isolated and cloned into the pRSET-B expression vector (Invitrogen). This construct was used to transform Rosetta *E. coli* cells (Novagen) for expression.

Expression of the protein was induced by adding 1 mM IPTG to 200 ml cells in logarithmic growth phase (OD_{600} 0.5–0.8), and the cells were harvested after 5 h of incubation at 30°C with rigorous shaking (300 rpm). Cells were resuspended in 50 mM HEPES buffer, pH 7.5, and disrupted by ultrasound treatment. The soluble fraction was isolated by centrifugation and the his-tagged protein purified in a ÄKTA Explorer (Amersham). A 14 ml Ni-Sepharose column was equilibrated with 50 mM HEPES, pH 7.5, and loaded with the soluble fraction, followed by 3 column volumes (CV) of 50 mM HEPES. After washing with 5 CV of 50 mM HEPES with 40 mM imidazole, the bound protein was eluted with 5 CV 50 mM HEPES with 200 mM imidazole, and concentrated using a 30 kDa Amicon Centrifugal Filter Unit (Millipore). SDS-PAGE analysis was used to verify the correct size of the expressed protein (61 kDa), and a Bradford assay (Biorad) employed to determine the protein concentration.

Transaminase activity was determined in an assay solution containing 3 mM L-tryptophan, 2 mM α-ketoglutarate, 100 mM HEPES (pH 7.5), 10 μM pyridoxale phosphate and the purified enzyme. The reaction was started by addition of the acceptor α-ketoglutarate, and formation of indole pyruvate from tryptophan was monitored by measuring the absorbance at 338 nm ($\varepsilon = 9300\ M^{-1}\ cm^{-1}$). One unit of enzyme was defined by the conversion of 1 μmol L-tryptophan to indole pyruvate per minute, as measured by the increase in OD_{338}. Pigment formation by the isolated Aro8* enzyme was assayed in a solution containing 10 mM L-tryptophan, 10 mM α-ketoglutarate, 100 mM HEPES (pH 7.5), 10 μM pyridoxale phosphate, and the purified enzyme.

Hydrogen peroxide survival assay

Candida glabrata yeasts were grown on inducing or non-inducing medium plates for 3 days until pigmentation was clearly visible. After washing, the cells were adjusted to 5×10^6 cells in YNB medium without nitrogen source, but with hydrogen peroxide (AppliChem, Germany) at the desired concentration. After 1 h of shaking incubation at 37°C, the yeast cell solutions were diluted in YNB medium and plated on YPD. Colony-forming units were determined the next day and compared with the control without hydrogen peroxide (0 mM). All experiments were performed in triplicate at least three times.

Neutrophil survival assay

Neutrophils were isolated from human donor blood by a density gradient centrifugation in Histopaque 1077 and 1119 (Sigma, MO, USA) according to the manufacturer's instructions. After centrifugation at 700 *g* for 30 min at room temperature, the polymorphonuclear cell fraction (PMN) was transferred to PBS. Residual erythrocytes were then removed in a lysis buffer (0.83% NH_4Cl, 10 mM HEPES, pH 7.0), the PMNs washed in PBS and resuspended in 100 µl Gibco HBSS buffer (Invitrogen, CA, USA). Yeasts were grown on inducing (tryptophan) or non-inducing (arginine) plates for 3 days until pigmentation was clearly visible. After washing in PBS, the cells were diluted in 100 µl HBSS buffer and mixed with the neutrophils at a ration of 1:1. After 1 h of co-incubation at 37°C, the metabolic activity was determined with an XTT assay following a modified method from (Meshulam *et al*., 1995): two volumes of XTT solution (0.5 mg ml^{-1} XTT, 50 µg ml^{-1} Coenzyme Q) were added, and the samples incubated for 1 h at 37°C. After centrifugation at 14 000 *g* for 1 min, 100 µl of the supernatant was used for measuring absorption at 451 nm. Relative decrease in metabolic activity was determined by subtracting the absorbance of a control sample with neutrophils alone, and then dividing by a sample of *C. glabrata* without neutrophils:

$$\text{Relative decrease} = 1 - (OD_{451}(C.g. + \text{neutrophils}) - OD_{451}(\text{neutrophils}))/OD_{451}(C.g.).$$

Epithelial cell damage assay

Human epithelial cells from the cell line TR146 (Rupniak *et al*., 1985) were grown to confluence in RPMI 1640 medium supplemented with FBS (PAA, Austria) at 37°C with 5% CO_2 in a humidified incubator. For infection, the monolayers were washed twice with PBS and the media replaced with serum-free RPMI 1640 medium. *C. glabrata* were grown as described before, washed, and 5×10^6 yeast cells were used to infect the monolayer.

Epithelial cell damage was estimated after 24 h via LDH release into the medium using the Roche Cytotoxicity Detection Kit. LDH levels of non-infected epithelial cells and yeasts alone were both subtracted as the base value from the measurements. For calculation of relative cytotoxicity, 100% lysis was determined by treating uninfected epithelial cells with 0.2% Triton-X 100 (Serva, Germany). All experiments were performed in triplicate at least three times and the controls were determined for each experiment individually.

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