Differentially regulated high-affinity iron assimilation systems support growth of the various cell types in the dimorphic pathogen *Talaromyces marneffe*.

Shivani Pasricha¹, Lukas Schaffner², Herbert Lindner², Kylie Joanne Boyce¹, Hubertus Haas², Alex Andrianopoulos^{1*}

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1. Department of Genetics, University of Melbourne, Victoria 3010, Australia

2. Division of Molecular Biology and Division of Clinical Biochemistry and the Protein Micro-Analysis Facility, Innsbruck Medical University, Innsbruck, Innrain 80-82, A-6020 Innsbruck, Austria

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*Corresponding author: Alex Andrianopoulos, Department of Genetics, University of Melbourne, Victoria 3010, Australia. Telephone + 61 3 8344 5164. Facsimile + 61 3 8344 5139. Email alex.a@unimelb.edu.au

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34 Abstract

Iron is a key trace element important for many biochemical processes and its
36 availability varies with the environment. For human pathogenic fungi iron acquisition
can be particularly problematical because host cells sequester free iron as part of the
38 acute-phase response to infection. Fungi rely on high-affinity iron uptake systems,
such as reductive iron assimilation (RIA) and siderophore-mediated iron uptake (non-
40 RIA). These have been extensively studied in pathogenic fungi that exist outside of
host cells, but much less is known for intracellular fungal pathogens. *Talaromyces*
42 *marneffei* is a dimorphic fungal pathogen endemic to Southeast Asia. In the host *T.*
marneffei resides within macrophages where it grows as a fission yeast. *T. marneffei*
44 has genes of both iron assimilation systems as well as a paralogue of the siderophore
biosynthetic gene *sidA*, designated *sidX*. Unlike other fungi, deletion of *sidA* or *sidX*
46 resulted in cell type-specific effects. Mutant analysis showed that *T. marneffei* yeast
cells also employ RIA for iron acquisition, providing an additional system in this cell
48 type that differs substantially from hyphal cells. These data illustrate the specialised
iron acquisition systems used by the different cell types of a dimorphic fungal
50 pathogen and highlight the complexity in siderophore-biosynthetic pathways amongst
fungi.

52

Author Summary

54 The opportunistic fungal pathogen *Talaromyces marneffei* (formerly known as
Penicillium marneffei) is endemic to Southeast Asia and represents an important and
56 escalating health threat to immunocompromised individuals. *T. marneffei* undergoes a
thermally dependent morphological switch from non-pathogenic hyphal cells at 25°C
58 to pathogenic yeast cells at 37°C. The ability of pathogenic fungi to proliferate within
the host is dictated by strategic acquisition of nutrients, such as iron. Two iron-uptake
60 mechanisms have been identified in fungi for the acquisition of iron under low iron
conditions, these include the reductive and non-reductive iron assimilation systems.
62 Restricting the ability of fungi to acquire iron through these systems has been shown
to reduce virulence and is a possible avenue for disease control. In this study we show
64 that *T. marneffei* yeast cells rely on both iron uptake systems, highlighting a key
difference between hyphal and yeast cell types, and this may be an explanation for
66 their success within host cells as they may scavenge iron more effectively. In addition

we identified the presence of a duplicated non-reductive iron assimilation gene,
68 relatively uncommon amongst other fungi. Deletion of this gene resulted in severe
growth defects under low iron conditions and presents itself as a good target for
70 antifungal drugs.

72 Introduction

Iron can gain or lose electrons to alternate between the oxidized ferric (Fe^{3+}) and
74 reduced ferrous (Fe^{2+}) state. Through this ability to transfer electrons, iron acts as a
redox mediator in an array of cellular processes and is essential for the survival of all
76 eukaryotes (Van Ho *et al.*, 2002, Philpott, 2006, Haas *et al.*, 2008). Ferric iron reacts
with atmospheric oxygen, resulting in insoluble ferric hydroxide and must be reduced
78 or bound to iron chelators before entering a cell (Cotton *et al.*, 1999). The ability of
pathogenic microbes to survive within mammalian hosts is governed by the interplay
80 between the microbe's capacity to grow and the host's response to inhibit this growth
through immune and nutritional restriction mechanisms (Brock, 2009, Fleck *et al.*,
82 2011, Rohmer *et al.*, 2011). For microbial infections, the host restricts free iron
through sequestration as part of the acute-phase response by reducing the release of
84 iron into the blood and extracellular fluids as well as increasing intracellular ferritin-
bound iron storage (reviewed in (Parrow *et al.*, 2013)). For a number of pathogenic
86 fungi it has been shown that disruption of iron uptake results in virulence defects
(Jung, 2006 *et al.*, Hwang *et al.*, 2008, Weinberg *et al.*, 2008). Under iron limited
88 conditions, such as during infection of a host, fungi employ high-affinity iron uptake
systems such as siderophore-mediated iron uptake (non-reductive iron assimilation,
90 non-RIA) and reductive iron assimilation (RIA) (Weinberg *et al.*, 2008). The level of
conservation of these systems varies between species, and candidate gene studies have
92 highlighted their roles in fungal growth and pathogenicity, often inconsistently
between fungal species, reflecting adaptive complexities to iron acquisition (Ramanan
94 *et al.*, 2000, Schrettl *et al.*, 2004, Hwang *et al.*, 2008, Jung *et al.*, 2008).

96 For ferric iron to be available for RIA it must first be reduced to ferrous. In the well-
characterized *Saccharomyces cerevisiae* system, this reduction is completed by the
98 metalloreductases, Fre1 and Fre2. Fre1 and Fre2 reduced ferrous iron is taken up by a
high-affinity ferrous-specific transport complex consisting of a plasma membrane

100 multicopper ferroxidase (Fet3) that oxidizes the iron in a copper- and oxygen-
dependent reaction, which is then transported to the cytosol by a permease (Ftr1)
102 (Eide *et al.*, 1992, Askwith *et al.*, 1994, De Silva *et al.*, 1995). In the absence of Fet3,
Ftr1 does not localize to the cell surface and vice versa (Johnson, 2008).

104
The second iron uptake system involves the secretion of siderophores, low molecular
106 mass ferric iron specific chelators, to mobilize and transport iron (Timmerman *et al.*,
1999, Schrettl *et al.*, 2004). Siderophore chelated ferric iron is recovered by cells
108 either by specific high-affinity siderophore transporters that take up the entire
siderophore-iron complex or through delivery to the RIA system (Ardon *et al.*, 2001,
110 Yun *et al.*, 2001). Most characterised fungal siderophores fall into the hydroxamate
class and operate as either extracellular or intracellular siderophores. Furthermore,
112 hydroxamates can be divided into four structural families; the rhodotorulic acid,
fusarinine (e.g. fusarinine C and triacetylusarinine C (TAFC)), coprogen (typically
114 secreted extracellular siderophores, important for scavenging iron) and ferrichrome
(e.g. ferrichrome A, ferricrocin and hydroxyferricrocin) (intracellular siderophores,
116 important for the appropriate storage of iron) families (Haas, 2003, Johnson, 2008).
The proposed biosynthetic pathway for hydroxamate siderophore production begins
118 with the enzyme L-ornithine N5-oxygenase, which catalyses the N-hydroxylation of
L-ornithine, and is followed by the addition of an acyl group. The completion of
120 siderophore biosynthesis involves the use of non-ribosomal peptide synthetases
(NRPS) that link the hydroxamate groups (Haas, 2003). The best characterised
122 siderophore biosynthetic pathway is in the pathogenic fungus *Aspergillus fumigatus*,
in which genes involved in both extracellular (TAFC) and intracellular (ferricrocin)
124 siderophore production have been identified (Haas, 2003, Hissen *et al.*, 2004).

126 In fungi, both the uptake and storage of iron is regulated by conserved transcriptional
mechanisms, mediated by iron availability (Haas *et al.*, 1999, Haas, 2003,
128 Hortschansky, 2007 *et al.*, Mercier *et al.*, 2009). This is achieved either through the
use of transcriptional activators or GATA-type transcriptional repressors (Philpott,
130 2006). Dysregulation and/or iron overload has varied consequences both on the
growth and the pathogenic potential of fungi. Most fungi (with the exception of the
132 *Saccharomycotina* family and *Kluyveromyces lactis*) use iron-responsive GATA-
factors (Haas, 2003, Haas *et al.*, 2008, Johnson, 2008). Under high iron conditions

these regulators have been shown to repress the transcription of their targets (with the exception of *C. neoformans* Cir1) (Jung *et al.*, 2006), particularly of RIA and non-RIA associated genes (Voisard *et al.*, 1993, Hoe *et al.*, 1996, An *et al.*, 1997a, An *et al.*, 1997b, Zhou *et al.*, 1998, Haas *et al.*, 1999, Yuan *et al.*, 2001, Harrison *et al.*, 2002, Pelletier *et al.*, 2002, Lan *et al.*, 2004, Jung *et al.*, 2006, Chao *et al.*, 2008). Recent studies in both *A. fumigatus* and *Aspergillus nidulans* have shown that the GATA factor SreA regulates iron assimilation in conjunction with the CCAAT binding bZIP transcriptional regulator HapX, working together in a negative feedback loop (Hortschansky *et al.*, 2007, Schrettl *et al.*, 2010, Haas, 2012).

Considered the third most common pathogen in HIV related diseases in Northern Thailand, *Talaromyces marneffe* (renamed from *Penicillium marneffe* (Samson *et al.*, 2011) targets those with compromised immune systems (Rongrungruang *et al.*, 1999, Walsh *et al.*, 1999). *T. marneffe* can undergo dimorphic switching such that at 25°C it grows in a multicellular hyphal form that can differentiate to produce asexual conidia, the infectious agent. Infection begins with the inhalation of conidia, which then switch to the pathogenic yeast cell form in the 37°C host environment. In the host *T. marneffe* resides within immune phagocytic cells (macrophages), predicted to be an iron limited environment, where it then grows as fission yeast (Wong *et al.*, 2011). The dimorphic nature of *T. marneffe* and its interaction with the host makes it an ideal organism for comparing non-pathogenic and pathogenic attributes within this organism. Expression profiling studies have highlighted the differential gene expression of a selection of high-affinity iron-assimilation genes between the hyphal and yeast cells of *T. marneffe* (Pasricha *et al.*, 2013). These include *fetC* that encodes the multi-copper oxidase in the RIA pathway, as well as two genes (*sidD* and *sidF*) homologous to those involved in the biosynthesis of extracellular siderophores. These genes were all upregulated early during yeast morphogenesis and late during yeast cell growth. Beyond this little is known about how the iron uptake systems work in each of the *T. marneffe* cell types and their role in pathogenicity.

To understand how iron assimilation is controlled during the saprophytic and pathogenic growth phases of fungi this study examined the cell-type specific nature of high-affinity iron assimilation in *T. marneffe*. Firstly, compared to hyphal cells, the pathogenic yeast cells rely more heavily on iron acquisition through the two high-

168 affinity iron assimilation systems. This is evident in the upregulation of both RIA and
extracellular siderophore biosynthetic genes in this cell type and appears to be driven
170 by the uncoupling of transcriptional regulation by SreA. Consistently, deletion of the
RIA gene *ftrA* results in reduced growth of yeast cells under iron limiting conditions
172 but not of hyphal cells. Secondly, the siderophore biosynthetic pathway of *T. marneffei*
is different from that of other fungi. Not only is a paralogue of the
174 siderophore biosynthetic gene *sidA*, denoted *sidX*, found in *T. marneffei* but SidA and
sidX display different short-chain dehydrogenases/reductases (SDR) motifs. In fungi
176 such as *A. fumigatus*, *sidA* is responsible for encoding the first step of both
extracellular and intracellular siderophore production. In *T. marneffei* the *sidA* and
178 *sidX* genes have non-redundant roles in siderophore biosynthesis where *sidA* is
important for intracellular but not extracellular siderophore production in hyphal cells.
180 In contrast, *sidX* is important for both hyphal and yeast intracellular siderophore
production and extracellular siderophore production in hyphal cells and possibly yeast
182 cells. Finally, deletion of *sidA* showed increased growth under low ferrous iron
conditions in both hyphal and yeast cells, while deletion of *sidX* led to an inability to
184 grow under the same conditions, suggesting these paralogues have important and
balancing roles in iron acquisition.

186

Results

188 *The RIA system is conserved in T. marneffei*

Microarray-based transcriptional profiling analyses showed that *fetC* was upregulated
190 within 6 hours of inducing the hyphal to yeast dimorphic switch (log2 fold-change
increase of 4.32, *p*-value of 0.0001) and late during the yeast growth phase at 37°C
192 (log2 fold-change increase of 3.94, *p*-value of 0.0001) (Pasricha *et al.*, 2013). To
investigate whether the *T. marneffei* genome encodes and expresses additional
194 components of the RIA system, in particular the permease-encoding *ftrA*, BLAST
searches using either the *S. cerevisiae* or *A. fumigatus* RIA genes were performed on
196 the genome sequence of *T. marneffei* and its close, non-pathogenic relative *T.*
stipitatus. Orthologues encoding both components of the RIA system are present and
198 clustered (similar to most other fungal species, but not *S. cerevisiae*) in the *T.*
marneffei (PMAA_057450; *fetC* and PMAA_057440; *ftrA*) and *T. stipitatus* genomes
200 (Fig. S1). The conservation of both *ftrA* and *fetC* in *T. marneffei* suggests that it has

the potential to utilise the RIA system for iron uptake.

202

Identified RIA system orthologues in *T. marneffei* and *T. stipitatus* were used for
204 BLAST searches against a diverse collection of other fungi that varied in phylogenetic
placement, mode of reproduction (sexual versus asexual), mating (homothallic versus
206 heterothallic), pathogenic potential and dimorphism (Materials and Methods).
Sequence homology was based on the top hit for both bit score and *e*-values. In the
208 rare situation where these approaches resulted in different best hits in reciprocal
BLAST searches, the gene with the lowest *e*-value was selected. Consistent with
210 previous reports, bioinformatic analysis confirmed that *A. nidulans* lacks both *fetC*
and *ftrA* homologues (Haas, 2003). Interestingly the analysis also suggests the
212 absence of RIA in *Coccidioides immitis*, *Paracoccidioides brasiliensis* and
Uncinocarpus ressei as they lacked *ftrA* and *fetC* orthologues (Table. S1). This
214 highlights the complex divergence of conservation of high-affinity iron assimilation
amongst closely related fungi.

216

RIA genes are upregulated in yeast cells compared to hyphal cells

218 Reverse transcriptase polymerase chain reaction (RT-PCR) was performed on *T.*
marneffei yeast and hyphal cells cultured with varying levels of available iron to
220 investigate whether cell type and/or iron levels influence the expression of RIA genes.
RT-PCR performed on RNA samples from cultures grown under standard laboratory,
222 iron rich conditions in liquid brain heart infusion (BHI) complex medium at 25°C (2
days) or 37°C (cultured for 4 days and then 10 mL transferred to fresh medium for an
224 additional 2 days) revealed that both *fetC* and *ftrA* are upregulated in yeast cells
compared to hyphal cells (Fig. 1A). These results are consistent with the previous
226 microarray expression profiling data (Pasricha, 2013). RT-PCR performed on RNA
samples from cultures grown under low iron conditions (SD plus (NH₄)₂SO₄, 0.2 mM
228 BPS, 6μM FeSO₄) showed higher expression levels in hyphal cells compared to
standard growth conditions and a similar marked differentiation between yeast and
230 hyphal cells as noted for standard growth conditions. These data suggest that RIA
gene expression is regulated in response to cell type as well as iron availability Fig.
232 1A.

234 To investigate the role the transcriptional regulator SreA (PMAA_041450) plays in
the regulation of *ftrA* in hyphal and yeast cells, and in response to available iron levels,
236 a Δ *sreA* strain was generated (Materials and Methods). Briefly, a construct consisting
of 3.8 kb of 5' and 3' flanking non-coding regions of *sreA* with the *pyrG* selectable
238 marker replacing the coding region of *sreA* was transformed into *T. marneffei* strain
 Δ *ligD*⁻ *pyrG*⁻ and *PyrG*⁺ transformants selected. These putative Δ *sreA* strains were
240 confirmed by Southern blot hybridisation. Quantitative reverse transcriptase PCR
(qRT-PCR) was performed on RNA extracted from the wild type (*sreA*⁺) and Δ *sreA*
242 strains grown under low and high iron conditions in BHI medium at 25°C and 37°C
(Materials and Methods). The results show that *ftrA* has ~100- and 50-fold higher
244 steady state mRNA levels under low and high iron conditions, respectively, in hyphal
cells of the Δ *sreA* strain relative to the wild type (*sreA*⁺)(Fig. 1B). This suggests that
246 in hyphal cells SreA strongly negatively regulates the RIA system. Expression levels
of *ftrA* were ~2-fold higher under low iron conditions in hyphal cells of the Δ *sreA*
248 strain. Similarly, the difference between low and high iron conditions in yeast cells
was also ~2-fold for the wild type and the trend was also evident for the Δ *sreA* strain
250 (although the latter is not statistically significant and this may be in part due to the
low levels of expression). In contrast to the hyphal cells, there is a ~10-fold decrease
252 in *ftrA* levels in the Δ *sreA* strain compared to wild type under both low and high iron
conditions suggesting that SreA is required for full activation in yeast cells (Fig. 1B).

254
*Deletion of ftrA results in reduced growth of yeast cells but not hyphal
256 cells under low iron conditions.*

Both *fetC* and *ftrA* showed higher expression in *T. marneffei* yeast cells compared to
258 hyphal cells. To assess the requirement of the RIA system in these cell types the *ftrA*
gene was selected for deletion. Deletion of either gene is sufficient to inactivate RIA
260 in other systems but since FetC shows high conservation with other multicopper-
oxidases in *T. marneffei*, suggesting possible redundancy (Fig. S2), it was not selected
262 for deletion. A *ftrA* deletion strain was generated by transforming the *T. marneffei*
 Δ *ligD* *pyrG*⁻ strain with a construct containing 3.5 kb of 5' and 3' non-coding flanking
264 sequences of the *ftrA* gene and the *pyrG* selectable marker replacing the *ftrA* coding
region (Materials & Methods). To generate a complemented Δ *ftrA*[*ftrA*⁺] strain, a
266 Δ *ftrA* *pyrG*⁻ (G872) strain was generated by counterselection against the *pyrG*

selectable marker and this strain was transformed with the wild type copy of *ftrA* plus
268 flanking regions (4.98kb) using the *pyrG* marker (Materials & Methods). All *pyrG*⁺
transformants were confirmed by Southern blot hybridization, showing that the
270 $\Delta ftrA[ftrA^+]$ complemented strain contained at least one full-length copy of the wild
type gene at the native *ftrA* locus.

272
To test the effect of iron levels on growth of the $\Delta ftrA$ strain, conidia (1×10^6) from
274 the deletion and wild type strains were cultured at 25°C and 37°C under standard, low
and high iron conditions on solid and in liquid medium (Materials & Methods)(Fig
276 1C-F). Samples from the liquid medium cultures were harvested, dehydrated and the
dry mass recorded. At 25°C $\Delta ftrA$ showed no significant differences in growth rate in
278 solid or liquid medium compared to the wild type (*ftrA*⁺) (Fig. 1C & D). However, at
37°C $\Delta ftrA$ showed a significant reduction in growth in low iron solid and liquid
280 medium compared to wild type (*ftrA*⁺)(Fig. 1E & F). The $\Delta ftrA[ftrA^+]$ complement
strain showed growth consistent with wild type (not shown). These results suggest
282 that *ftrA* is required for full growth under low iron conditions *in vitro* at 37°C but not
at 25°C, perhaps due to an alternative uptake system active at 25°C or higher iron
284 uptake demands at 37°C.

286 To determine whether germination is affected in $\Delta ftrA$ and how the level of
exogenous iron affects this process, conidia (1×10^5) from the various strains were
288 grown at 25°C or 37°C under different levels of iron for 13 h. By 13 h the majority of
wild type conidia have germinated. No significant differences in germination rates
290 were observed between *ftrA*⁺, $\Delta ftrA$ and $\Delta ftrA[ftrA^+]$ at either temperature regardless
of exogenous iron levels. This showed that the growth rate differences noted
292 previously are not due to a delay in germination or viability (Fig. S3). To assess
whether deletion of *ftrA* affects growth inside host cells, LPS-activated J774 murine
294 macrophages grown in iron free medium were mixed with *T. marneffei* conidia ($1 \times$
 10^6), washed after 2 h to remove unphagocytosed conidia and incubated in iron free
296 medium for an additional 24 h at 37°C (Materials & Methods). Samples were
examined microscopically after calcofluor staining. The number of ungerminated
298 conidia, germlings, hyphae and yeast cells in at least 100 macrophages was recorded
for 3 independent experiments. Numerous yeast cells dividing by fission were
300 observed within macrophages 24 h post infection with wild type *T. marneffei*. There

was no difference between the wild type (*ftrA*⁺), deletion (Δ *ftrA*) and complemented
302 (Δ *ftrA*[*ftrA*⁺]) strains (Fig. S4). Given that yeast cells of the Δ *ftrA* strain grew poorly
under low iron conditions *in vitro*, and mammalian phagocytic cells represent an iron
304 restricted environment, this result is highly suggestive of the presence of an
alternative iron assimilation pathway in *T. marneffei* that is active inside host cells.

306

Siderophore biosynthetic genes are conserved and clustered in T.
308 *marneffei*.

The genes involved in the biosynthesis of siderophores have been identified in *A.*
310 *fumigatus* and their roles in the production of extracellular, scavenger siderophores
and/or intracellular, storage siderophores have been characterized (Haas, 2003).
312 Genes orthologous to all the *A. fumigatus* intracellular and extracellular siderophore
biosynthetic genes were identified in *T. marneffei*, with the exception of *sidG*. In *A.*
314 *fumigatus* SidG is a putative acetyltransferase responsible for conversion of fusarinine
C to TAFC, both extracellular siderophores. The *sidG* gene is conserved only in
316 TAFC-producing fungal species and its absence in *T. marneffei* is consistent with the
production of coprogen-siderophores and the absence of TAFC (see below).
318 Homologues of *sidG* are also absent from *T. stipitatus* and a taxonomically broad
range of other fungi including *C. immitis*, *Histoplasma capsulatum*, *Magnaporthe*
320 *oryzae*, *P. brasiliensis*, *Rhizopus oryzae* and *Uncinocarpus reesei* (*U. reesei*) (Table.
S1) Nevertheless, the conservation of both intracellular and extracellular siderophore
322 biosynthetic genes in *T. marneffei* underlines its potential to synthesise siderophores
for the scavenging and storage of iron.

324

T. marneffei produces intracellular and extracellular siderophores

326 To investigate whether *T. marneffei* produces siderophores and determine which
siderophores are produced, conidia (1×10^7) were cultured in low iron liquid medium
328 at 25°C or 37°C (Materials & Methods). Fungal pellet and medium supernatant
samples were iron-saturated and analysed by reversed-phase HPLC/MS using
330 photometric determination at 434 nm to detect ferri-siderophores. This analysis
identified putative siderophore peaks in both pellet (Fig. 2A) and supernatant samples
332 of *T. marneffei* cultures (Fig. 2B). Supernatant and pellet samples from *A. fumigatus*
cultures were also used as controls as the approximate siderophore retention times are

known (not shown). By comparison with these controls, the retention times for the compounds at absorbance 434 nm in pellet samples were indicative of the intracellular siderophores ferricrocin and ferrichrome and these were identified in both *T. marneffei* hyphal and yeast cell pellets (Fig. 2A). Extracellular siderophore coprogen B was detected in the supernatant samples from hyphal cultures but only trace amounts were evident in yeast cultures (Fig. 2B). It is important to note that the culturing conditions required for the production of yeast cells precluded accurate quantitation of siderophores.

342

T. marneffei has two *sidA* genes

Both *T. marneffei* and *T. stipitatus* have a paralogue of *sidA* (designated *sidX*). Examination of over 50 fungal species, including those in this study, showed that the ascomycetes *C. immitis*, *Pyrenophora tritici-repentis* and *P. nodorum* were the only others with at least one additional *sidA* paralogue (Haas *et al.*, 2008). Like the *C. immitis* *sidA* and paralogous gene, the *T. marneffei* *sidA* and *sidX* genes share little nucleotide identity and only 44% protein identity (not shown). While both predicted proteins contain a conserved K-oxygenase domain, SidA has a nicotinamide adenine dinucleotide (NAD) binding domain, while SidX has a pyridine nucleotide-disulphide oxidoreductase domain (Fig. S5). Comparing SidA and SidX to orthologues in other fungi, including fungi identified as having at least one *sidA* paralogue, clearly shows that SidA and SidX lie in separate clades (Fig. 3). Although distantly related, *C. immitis* and *T. marneffei* are mammalian pathogens and have both genes (Birren *et al.*, 2005). However, this feature is not strictly a pathogenicity trait as the non-pathogenic *T. stipitatus* also contains orthologues of both genes. These findings suggest that an ancient duplication of *sidA* or *sidX* has occurred followed by a subsequent partial/full loss of one or the other in most fungi examined. The relatedness analysis also shows that the previously characterised SidA proteins in *H. capsulatum*, *P. brasiliensis*, and *U. reesei* show higher similarity to *T. marneffei* SidX, rather than SidA (Fig. 3). Predictive domain conservation searches amongst these fungi (using the NCBI conserved domain database and CBS Cofactory v 1.0) revealed differences between the SidA and SidX clades. Absent in half the fungi in the SidA clade, all members of the SidX clade contain a pyridine nucleotide-disulphide oxidoreductase domain. The first enzymatic step of the siderophore biosynthetic pathway, the hydroxylation of L-

ornithine, is FAD-dependent. Four out of six fungi in the SidA clade contain a
368 Rossmann sequence domain with the potential to bind to both FAD and NADP. In the
SidX clade separate sites are present for FAD and NADP cofactor binding (Fig. S5).

370 Fungal siderophore biosynthesis genes are typically found in clusters (Eichhorn *et al.*,
372 2006, Hwang *et al.*, 2008, Johnson, 2008). In species with a *sidA* paralogue, including
T. marneffei, the paralogues are not clustered (Fig. S6). In *T. marneffei*, *sidA* is
374 clustered with *sidC*, which is involved in intracellular siderophore biosynthesis in *A.*
fumigatus. This arrangement is also found in *T. stipitatus* and the model organism
376 *Neurospora crassa*, but not in most other fungi including *A. fumigatus*. A second
cluster consists of *sidI*, necessary for extracellular siderophore biosynthesis (Yasmin
378 *et al.*, 2012), and *sidX* (Fig. S6). In contrast, in the *A. fumigatus* genome *sidI* is
clustered with *sidC*. In *T. stipitatus* the cluster containing genes *sidX* and *sidI* has
380 undergone an inversion event. In *T. marneffei* *sidD*, *sidF*, and *sidH*, predicted to be
involved in biosynthesis of extracellular siderophores, form the third cluster with the
382 ABC multidrug transporter encoding gene *sitT* and the putative siderophore
transporter encoding gene PMAA_002890 belonging to the major facilitator
384 superfamily (MFS)(Fig. S6). These results suggest species divergence in the
arrangement of these genes with no clear correlation between pathogenic and non-
386 pathogenic fungi. Despite this it is interesting to note that the clustering pattern in *T.*
marneffei suggests a functional division where *sidA* and *sidX* may have distinct roles
388 in intracellular and extracellular siderophore biosynthesis pathways, respectively.

390 *Putative extracellular siderophore biosynthesis genes are upregulated in* *yeast cells*

392 Microarray-based expression analysis has shown that the *sidF* and *sidD* genes are
upregulated during early yeast cell morphogenesis induced by switching the growth
394 temperature from 25°C to 37°C, (log2 fold-change increase of 0.62 (*p*-value of 0.162)
and 0.44 (*p*-value of 0.422), respectively) compared to early hyphal morphogenesis
396 induced by switching from 37°C to 25°C (log2 fold-change decrease of -1.99 (*p*-value
of 0.001) and -3.27 (*p*-value of 0.0001), respectively) and during yeast cell growth
398 compared to hyphal cell growth (log2 fold-change increase of 1.43 and 2.01,
respectively, *p*-value of 0.0001) (Pasricha *et al.*, 2013). RT-PCR on samples grown at

25°C and 37°C under standard iron conditions in BHI medium was performed for all identified siderophore-related genes (including the *sidA* paralogue, *sidX*, as well as MFS transporter encoding gene PMAA_002890 and putative siderophore transporter gene, *sitT*) to see if these trends hold true for these other genes. The results show that *sidA* and *sidC*, both predicted to be involved in intracellular siderophore biosynthesis were expressed in both hyphal and yeast cells, while all other genes were predominantly expressed in yeast cells (Fig. 4). It should be noted that there were two alternative transcripts detected for *sidA* and *sidH* and the difference between these is a differentially spliced intron. In both cases, intron retention would lead to premature termination and a truncated protein. Whether either of these truncated proteins has any function is unknown. The longer *sidA* transcript is of low abundance while the two *sidH* transcripts are similarly expressed. In the Δ *sreA* mutant the *sidC* transcript and the shorter *sidA* transcript showed dramatically reduced levels, in both hyphal and yeast cells suggesting they are positively regulated by SreA. The longer *sidA* transcript was strongly upregulated in Δ *sreA* yeast cells (Fig. 4). In contrast, all other genes showed increased expression in hyphal cells of the Δ *sreA* strain and maintained some level of expression in the yeast cells, with the exception of *sidT* that appears to require SreA. These general patterns of transcriptional regulation are similar to those seen for the RIA genes (Fig. 1A) and suggest that *sreA* plays a positive role in RIA-mediated uptake of iron and intracellular storage by siderophores in yeast cells. They also show that *sidA* and *sidC* are the only highly expressed iron acquisition/storage genes in hyphal cells and that positively regulates these genes while negatively regulating the other non-RIA and RIA genes. These results demonstrate that the differences in the expression of these genes show strong cell-type specific control and that the pathways are coordinately regulated.

The physical clustering and concordant gene expression patterns for *sidA* and *sidC* suggest they may play a role in a common pathway; putatively in intracellular siderophore biosynthesis. Similarly, the clustering and yeast-specific gene expression of all other genes coincides with their anticipated roles in extracellular siderophore production and suggests that the upregulation of extracellular siderophore biosynthesis genes is strongly influenced by cell type.

RT-PCR showed very clear differences in the regulation of the paralogous *sidA* and *sidX* genes. To investigate this more closely and examine the role of SreA in *sidA* and *sidX* expression, qRT-PCR was performed, comparing gene expression in wild type (*sreA*⁺) and Δ *sreA* strains for hyphal and yeast cells and under low and high iron conditions (Materials & Methods). Relative to the wild type (*sreA*⁺) hyphal cells, Δ *sreA* hyphal cells showed 9.9 times greater expression of *sidA* and 159.2 times greater expression of *sidX* under low iron conditions (Fig. 5). Similarly, relative to the wild type yeast cells, Δ *sreA* yeast cells showed 9.32 times greater expression of *sidA*, but only 13.22 times greater *sidX* expression (Fig. 5). High iron conditions led to unaltered *sidA* levels in the wild type strain but decreased *sidA* expression in Δ *sreA*. Expression of *sidX* in both wild type and Δ *sreA* strains was decreased under high iron condition (Fig. 5). Consistent with RT-PCR results, these findings highlight SreA as a negative regulator of *sidX* in hyphal cells. In addition, SreA appears to negatively regulate *sidA* expression in both hyphal and yeast cells under low iron conditions.

In *A. nidulans* and *A. fumigatus* SreA has been shown to regulate iron assimilation through a negative feedback loop with the CCAAT-binding bZIP transcriptional regulator HapX; SreA represses expression of *hapX* during iron sufficiency and HapX represses *sreA* expression under iron depleted conditions. Additionally in the absence of iron HapX function is repressed and SreA function activated posttranslationally (Haas *et al.*, 1999; Hortschansky *et al.*, 2007). The *T. marneffei* *hapX* orthologue contains the signature *sreA* target-gene binding motif in its 5' untranslated region, suggesting the potential for cross-regulation between these genes (data not shown). To investigate this further, *hapX* expression in wild type (*sreA*⁺) and Δ *sreA* strains for both hyphal and yeast cells and under low iron and high iron conditions were compared using qRT-PCR (Fig. 6). The results follow a similar trend to those of *sidX* and show that *hapX* expression is significantly increased in yeast cells compared to hyphal cells, and that *hapX* expression is increased in Δ *sreA* hyphal cells compared to wild type (*sreA*⁺), but not Δ *sreA* yeast cells. Additionally, *hapX* expression is slightly decreased in high iron conditions compared to low iron conditions.

The results suggest that SreA negatively regulates *hapX* expression in hyphal cells under low iron conditions in a similar manner to that in *A. nidulans* and *A. fumigatus*. However, *hapX* expression appears unaffected in Δ *sreA* yeast cells compared to wild

type (*sreA*⁺) as was noted for most of the non-RIA genes (Fig. 4 & 5).

468

Deletion of sidA and sidX leads to opposite effects under low iron growth
470 *conditions*

The paralogous *sidA* and *sidX* genes show diverging domain architecture and
472 differential expression patterns suggestive of diverging roles. To investigate these
roles in relation to siderophore biosynthesis and growth under different iron
474 conditions, two *T. marneffei* deletion strains were created. A *sidA* deletion construct
was generated containing 1.99 kb of 5' and 3' non-coding flanking sequences of the
476 *sidA* gene with the *barA* selectable marker replacing the *sidA* coding region. The *sidX*
deletion construct was generated containing 2.74 kb of 5' and 3' non-coding flanking
478 sequences of the *sidX* gene with the *barA* selectable marker replacing the *sidX* coding
region. *T. marneffei* strain $\Delta ligD$ *pyrG*⁺ was transformed with the linearised deletion
480 constructs, candidate transformants were screened for growth in the presence of
glufosinate and deletion of *sidA* and *sidX* was confirmed by Southern blot
482 hybridization (not shown). To confirm that any observed differences between the wild
type and the deletion strains were due to the deletion, complemented $\Delta sidX[sidX^+]$
484 and $\Delta sidA[sidA^+]$ strains were created by transforming the respective deletion strain
with the wild type allele targeted to the ectopic *pyrG* locus. Transformants confirmed
486 by Southern blot analysis showed integration of at least one full-length copy of the
plasmids at *pyrG* (not shown). Complemented $\Delta sidX[sidX^+]$ and $\Delta sidA[sidA^+]$ strains
488 showed phenotypes consistent with wild type for all phenotypes examined in this
study.

490

In order to assess the effects of varying the level of exogenous iron on the $\Delta sidA$ and
492 $\Delta sidX$ mutants, strains were grown under standard, low and high iron conditions. At
25°C during hyphal growth, the $\Delta sidA$ strain was unaffected by different iron levels,
494 growing as morphologically normal multinucleate hyphal cells comparable to the wild
type (*sidA*⁺)(Fig. 7A). Interestingly, in low iron liquid medium the $\Delta sidA$ hyphal cells
496 grew more rapidly than wild type, resulting in a higher dry weight after 2 days of
growth (Fig. 7C). In contrast to the $\Delta sidA$ strain at 25°C, the $\Delta sidX$ strain grew poorly
498 under low iron conditions in liquid medium (Fig. 7A), resulting in significantly
reduced biomass after 2 days (Fig. 7C). On low iron solid medium the $\Delta sidX$ strain

500 failed to produce a colony while the wild type was unaffected by iron levels (Fig. 7B).
Addition of iron to the medium resulted in recovery of growth for the $\Delta sidX$ strain
502 consistent with that of wild type ($sidX^+$) (Fig. 7B & 7C).

504 At 37°C during yeast cell growth, numerous $\Delta sidA$ cells had swollen apical tips when
grown in standard or low iron liquid medium, and this was more prominent under low
506 iron conditions, but burst apical tips were not evident (Fig. 8A). Despite this the
 $\Delta sidA$ strain showed an increase in dry weight compared to wild type ($sidA^+$) under
508 these conditions in liquid medium (Fig. 8C). No growth difference was detected under
different iron levels on solid medium (Fig. 8B). At 37°C the $\Delta sidX$ strain failed to
510 grow under low iron conditions in liquid or solid medium, similar to 25°C, whereas
growth was consistent with that of the wild type ($sidX^+$) under high iron conditions
512 (Fig. 8A & 8B). Morphogenesis and growth of yeast cells in macrophages for both the
 $\Delta sidA$ and $\Delta sidX$ mutants was consistent with that of wild type (Fig. S7 and Fig. S8).
514 Based on these results $sidX$ appears to be required for growth when iron is limiting,
regardless of cell type, and has a non-redundant role to $sidA$, while $sidA$, $sidX$ and/or
516 the RIA pathway appear be redundant for growth inside host cells.

518 *Deletion of $sidA$ and $sidX$ has opposite effects on conidial germination at* 37°C

520 Given the differences in growth rate observed between $\Delta sidA$ and $\Delta sidX$ strains under
low iron conditions, the rates of germination were assessed under different levels of
522 available exogenous iron. Conidia (1×10^5) from the $\Delta sidA$ and $\Delta sidX$ strains were
incubated at both 25°C and 37°C under standard iron, iron free, low and high iron
524 conditions for 13 h. At 25°C the $\Delta sidA$ strain germinated at a similar rate to wild type
($sidA^+$) regardless of the level of available iron (Fig. 9A), as was previously noted for
526 $\Delta ftrA$ strain (Fig. S3). In contrast, at 37°C the $\Delta sidA$ strain germinated slightly faster
than wild type ($sidA^+$) under both standard and low iron conditions, but showed
528 slightly reduced, but not statistically significant, germination under high iron
conditions (Fig. 9B).

530

At 25°C, the conidial germination rate for the $\Delta sidX$ strain is consistent with that of
532 the wild type ($sidX^+$) strain except under high iron conditions (Fig. 9A). This suggests

that $\Delta sidX$ conidia contain enough internal iron stores to germinate even under conditions of no exogenous iron in the medium. It also implies that the observed reduction in growth under low iron (Fig. 7) is not due to reduced conidial germination but indicative of an inability to grow as a result of a lack of available exogenous iron. In contrast, at 37°C the $\Delta sidX$ strain showed significantly reduced germination rates at 13 h, under all conditions with the exception of high iron medium (Fig. 9B). This differed from the germination of the wild type ($sidX^+$) which were unaffected by exogenous iron availability. Assuming that the process of germination is the same at both 25°C and 37°C, then the 37°C-specific reduction in germination of the $\Delta sidX$ strain under iron limited conditions suggests that there is a greater requirement for iron during germination at 37°C. These results are consistent with the lack of viability observed at 37°C under low iron conditions (Fig. 8).

Deletion of $sidA$ or $sidX$ has differential effects on siderophore levels.

The $sidA$ and $sidX$ paralogues have diverging domain architecture, gene expression and roles during growth under low iron conditions. Coupled with their genomic clustering, with respect to other siderophore biosynthetic genes, it is suggestive that they may play different roles in siderophore biosynthesis. To investigate this further, $\Delta sidA$ and $\Delta sidX$ conidia (1×10^7) were cultured in low iron liquid medium at 25°C or 37°C, and cell pellet and supernatant samples were prepared and subjected to HPLC/MS analysis (Materials & Methods). When samples from $\Delta sidA$ were examined, hyphal cells showed significant reduction in ferricrocin and ferrichrome (Fig. 10A) but no reduction in extracellular coprogen B (Fig. 10C). There were no differences in yeast cells (Fig. 10B). These results suggest that $sidA$ is important for intracellular, but not extracellular, siderophore production in hyphal cells.

In contrast, in samples from the $\Delta sidX$ strain, both hyphal and yeast cells showed a significant reduction in ferricrocin and ferrichrome (Fig. 10A & B), and hyphal cells also showed a reduction in coprogen B (Fig. 10C). This suggests that $sidX$ is important for both intracellular and extracellular siderophore production in hyphal cells. This is also likely to be the case in yeast cells, given the severe growth defect under low iron conditions, however extracellular siderophores were undetectable in

the yeast samples, likely due, in part, to rapid scavenging of exogenous iron by yeast
566 cells compared to hyphal cells.

568 *Disruption of high-affinity iron assimilation pathways highlights cross-regulation of the different assimilation systems.*

570 To examine the roles of *sidA* and *sidX* in siderophore biosynthesis further, and to
determine whether the absence of RIA results in increased expression of siderophore
572 biosynthetic genes and vice versa, the expression of *fetC* (involved in RIA), *sidC*
(involved in intracellular siderophore biosynthesis), *sidD* (involved in extracellular
574 siderophore biosynthesis) and *sidH* (links ergosterol biosynthesis to extracellular
siderophore biosynthesis) were compared in wild type, $\Delta ftrA$, $\Delta sidA$, $\Delta sidX$ hyphal
576 and yeast cells by qRT-PCR. All strains were cultured under low iron conditions
(Materials & Methods).

578

The results show that *fetC* expression was similar to wild type in hyphal cells of the
580 deletion strains (Fig 11A). In contrast, *fetC* expression was significantly increased in
 $\Delta sidA$ and $\Delta sidX$ yeast cells compared to wild type, but reduced in $\Delta ftrA$ yeast cells
582 (Fig. 11A). This suggests that in the absence of *sidA* or *sidX*, the RIA system is
upregulated in yeast cells. In contrast, while *sidC* expression was higher in wild type
584 yeast cells compared to hyphal cells, deletion of *ftrA*, *sidA* or *sidX* had no effect on
sidC expression in hyphal cells (Fig. 11B) while *sidC* expression shows a modest
586 increase in $\Delta sidX$ yeast cells but this was not statistically significant (Fig. 11B).

588 In numerous fungi such as *Alternaria brassicicola* and *Colletotrichum graminicola*
sidD orthologues are required for the biosynthesis of the extracellular siderophore,
590 coprogen B (Haas, 2014). In *T. marneffei* *sidD* was expressed in yeast cells, and
repressed in hyphal cells by SreA (Fig 4). Compared to wild type hyphal and yeast
592 cells, *sidD* expression is significantly increased in $\Delta ftrA$, $\Delta sidA$ and $\Delta sidX$ hyphal
cells, as well as $\Delta sidX$ yeast cells (Fig. 11C). Firstly, this suggests that extracellular
594 siderophore biosynthesis is upregulated when the RIA system is inactive in hyphal
cells under low iron conditions, which is presumably an iron starvation response.
596 Secondly, in the case of $\Delta sidA$ and $\Delta sidX$ strains, when siderophore production is
presumably inhibited, the upregulation of downstream extracellular siderophore

598 biosynthetic genes suggests that *sidA* and *sidX* do not have a strong posttranslational
regulatory effect on downstream genes and that the pathway(s) are predominately
600 controlled by a decline in incoming iron.

602 In *A. fumigatus*, the acyl-CoA ligase encoding gene *sidI* is required for the conversion
of mevalonate, originating from the ergosterol biosynthetic pathway, to mevalonyl-
604 CoA. This is further converted to anhydromevalonyl-CoA through the activity of
SidH, which is required for the biosynthesis of extracellular siderophores (Yasmin *et*
606 *al.*, 2012). Compared to wild type cells, *sidH* expression is increased in Δ *ftrA*, Δ *sidA*
and Δ *sidX* hyphal and yeast cells (Fig 11D). This may be the result of less internal
608 iron in these deletion strains compared to wild type. In *A. fumigatus* genes involved in
mevalonate production are upregulated under low iron conditions, promoting increased
610 extracellular siderophore production (Yasmin *et al.*, 2012).

612 Discussion

Nutrient acquisition and assimilation is fundamental to growth and survival for all
614 organisms.. It is integral to numerous energy producing cellular processes and a
number of key nutritional pathways require iron as a co-factor. For pathogens,
616 mammalian hosts have evolved mechanisms to restrict available iron in response to
infection. To counter this fungi, and other microbes, employ high-affinity iron uptake
618 mechanisms such as the reductive iron assimilation (RIA) system and/or the non-
reductive, siderophore-mediated iron assimilation (non-RIA) system (Haas, 2003).
620 Moreover, the availability of iron inside and outside of host cells differs so these
differences in iron availability presents different challenges for fungal pathogens
622 depending the specific niche they occupy in the host. In this study we have examined
the mechanisms used by a dimorphic fungal pathogen and how the various cell types
624 acquire iron, both inside and outside of host cells. In addition to the extrinsic factor of
iron availability, transcriptomic profiling has shown that genes involved in iron
626 consuming pathways are upregulated in yeast cells compared to hyphal cells (Pasricha
et al. 2013). For example, ergosterol biosynthesis is dependent on both oxygen and
628 iron and similar to *sidX*, the C-14 sterol reductase encoding gene *ergM* (orthologous
to *S. cerevisiae erg24*) is highly upregulated in yeast cells compared to hyphal cells,
630 with deletion resulting in severe growth impairments that is exacerbated at 37°C

(Pasricha *et al.* 2013). In addition, metabolomic profiling of *T. marneffei* hyphal and yeast cells has shown that respiratory pathways, such as the TCA cycle are significantly more active in yeast cells compared to hyphal cells, thus requiring higher levels of iron uptake (Pasricha *et al.* unpublished). Thus the challenge for *T. marneffei* is to overcome the extrinsic limitations and intrinsic requirements for iron. The data presented here, coupled with these other studies, clearly highlight the inherent differences in iron assimilation mechanisms used in the non-pathogenic hyphal cell type and the pathogenic yeast cell type to overcome these challenges

The RIA system is upregulated in yeast cells and contributes to yeast cell growth under low iron conditions

The uptake of ferrous iron by RIA involves its oxidation by a multicopper ferroxidase, such as FetC, and the subsequent transport to the cytosol by a permease such as FtrA. Unlike *S. cerevisiae*, both enzyme-encoding genes in the *T. marneffei* genome are clustered. Consistent with their interdependent role, *fetC* and *ftrA* show similar, coordinate expression patterns. Both genes are regulated by cell type, showing high expression in yeast cells (37°C) compared to hyphal cells (25°C) with a clear upregulation in response to iron limitation (Fig. 1A and B). Expression of *ftrA* is negatively regulated by *sreA* in hyphal cells (25°C) similar to repression of RIA gene expression by SreA orthologues in other fungi such as *C. neoformans* and *U. maydis* under iron sufficient conditions (An *et al.*, 1997a, Jung *et al.*, 2006). In contrast, SreA is required for high level expression of *ftrA* in yeast cells regardless of iron levels (Fig. 1B). Therefore in *T. marneffei* both cell type and iron availability act as signals for RIA gene expression and SreA appears to have opposing roles depending on the cell type..

Consistent with the expression data, deletion of *ftrA* in *T. marneffei*, resulting in a dysfunctional RIA system, led to reduced growth under low iron conditions in yeast cells (37°C) but not hyphal cells (25°C), and no other morphological effects that may suggest metabolic imbalance at either temperature regardless of iron levels were evident (Fig. 1C). The lack of effect in hyphal cells may be due to the upregulation of the extracellular siderophore biosynthetic gene *sidD* in $\Delta ftrA$ hyphal cells (Fig. 11C). We have shown that hyphal cells have the capacity to produce the extracellular

siderophore coprogen B for which *sidD* is required (Fig. 2C). In contrast, the reduction, but not absence, of yeast-specific growth under low iron conditions in liquid in the $\Delta ftrA$ strain and the lack of compensatory increased *sidD* expression would suggest that *ftrA*, and by extension RIA, is required for yeast cells to acquire enough iron to sustain full metabolic activity and growth (Fig. 1 and Fig. 11C). In *C. albicans*, which lacks siderophore biosynthetic genes, disruption of RIA by deletion of *ftr1* (*ftrA* orthologue) showed that it was required for growth under low iron conditions and systemic infection in mice (Ramanan *et al.*, 2000). In *A. fumigatus*, which possesses both RIA and non-RIA systems, disruption of *ftrA* did not affect its pathogenicity in mice (Schrettl *et al.*, 2004). Since *T. marneffei* $\Delta ftrA$ yeast cells display only impaired growth under low iron conditions and yeast cell growth is comparable to wild type when growing inside macrophages, it is clear that both RIA and non-RIA systems are at play in host cells (Fig. 1, Fig. 4 and see below).

sidA contributes to intracellular siderophore biosynthesis in T. marneffei

Free ferrous iron within the cell can lead to the formation of damaging free radicals and iron toxicity; as such acquired iron must be appropriately stored. Unlike plants, animals and bacteria that can store iron through chelation to ferritin-like molecules, most fungi use other mechanisms of iron storage to fulfill this function such as storage vacuoles and/or intracellular siderophores (Andrews, 1998, Arosio *et al.*, 2002, Haas *et al.*, 2008, Grundlinger *et al.*, 2013). In *A. fumigatus* *sidA* and *sidC* encode enzymes involved in the synthesis of intracellular siderophores. Unlike *A. fumigatus*, the *T. marneffei* genes are clustered in the genome and unlinked from other siderophore-related genes. The *sidA* and *sidC* genes display similar expression patterns, showing expression in both hyphal and yeast cells (Fig. 4).

HPLC/MS analysis from whole cell extracts of *T. marneffei* hyphal and yeast cells showed that these cells produce the intracellular hydroxamate-class of siderophores, ferricrocin and ferrichrome, under iron depleted conditions (Fig. 2A). Deletion of *sidA* resulted in undetectable ferricrocin and significantly reduced ferrichrome production in hyphal cells (Fig. 10A). Under low iron conditions $\Delta sidA$ hyphal cells showed an almost doubled growth rate compared to wild type (*sidA*⁺) and this difference was not evident under high iron conditions (Fig. 7C). In contrast, deletion of *sidA* had no

effect on the biosynthesis of the extracellular siderophore coprogen B in hyphal cells
698 (Fig. 10C). Therefore in hyphal cells loss of *sidA* leaves both extracellular siderophore
biosynthesis and the RIA system intact, but perturbing siderophore-chelated
700 intracellular storage affects growth and this may reflect the nexus between iron
homeostasis and metabolic flux in these cells. Based on what is known in other
702 systems, a lack of siderophore-chelated intracellular storage would be predicted to
trigger the activity of the transcriptional regulator *hapX*. This in turn might activate
704 genes involved in iron-dependent pathways such as the TCA cycle, resulting in
hyperactive growth under low iron conditions compared to wild type (Schrettl *et al.*,
706 2010).

708 In yeast cells, deletion of *sidA* showed no significant change in intracellular
siderophore production and almost no increase in growth compared to wild type (Fig.
710 8C) suggesting that *sidX* may be compensating for the loss of *sidA* in this cell type.
While the increase in growth observed for Δ *sidA* hyphal cells under low iron
712 conditions was not evident in yeast cells, the Δ *sidA* yeast cells did show significantly
increased germination rates compared to wild type yeast cells under low iron
714 conditions (Fig. 9). The basis for this remains unclear.

716 *sidA* paralogue, *sidX* is important for extracellular siderophore biosynthesis in *T. marneffei*

718 Genes involved in the biosynthesis of *A. fumigatus* extracellular siderophores (with
the exception of *sidG*) are conserved in *T. marneffei* (Table S2). The genes are
720 clustered within the genomes of both these fungi. The *sidA* paralogue in *T. marneffei*,
sidX, is absent in *A. fumigatus* and is clustered with the *sidI* orthologue in *T. marneffei*
722 (Fig. S6). The *sidI* gene encodes an acyl-CoA ligase which is involved in the
synthesis of mevalonate-CoA from mevalonate, a precursor of extracellular
724 siderophore synthesis in *A. fumigatus* (Yasmin *et al.*, 2012). This clustering suggested
that *sidX* may have a role in extracellular siderophore biosynthesis in *T. marneffei*,
726 which was supported by the HPLC/MS analysis, showing that *sidX* is important for
coprogen B production in hyphal cells at least (Fig. 10). In addition iron assimilation
728 genes *fetC* and *sidD* were both significantly upregulated in the Δ *sidX* strain, implying
a higher demand for iron uptake (Fig. 11).

730

Examination of SidX and SidA orthologues from *T. marneffei* and other species
732 revealed that they reside in separate clades suggestive of long divergence times (Fig.
3). In addition, it was found that while most members of the SidA clade contain one
734 site for both FAD and NADP binding, separate sites are present for FAD and NADP
cofactor binding in the SidX clade (Fig. S5). Typically it is important for
736 monooxygenases to avoid the uncoupling of O₂ activation (via NAD) and substrate
hydroxylation (via FAD) in order to avoid the unnecessary production of reducing
738 agents, resulting in H₂O₂. It is equally important, through the coupling of O₂
activation and FAD binding that hydroxylation of substrates is specific. In *A.*
740 *fumigatus* SidA, crucial residues have been shown to bind FAD covalently and to use
NADP (Frederick *et al.*, 2011). It will be interesting to investigate the implications of
742 varied FAD and NADP binding domains between the SidA and SidX clades.

744 The *sidX* gene is essential for *T. marneffei* growth *in vitro* under low iron conditions,
regardless of temperature but not for yeast morphogenesis and growth in macrophages
746 (Fig. 7, Fig. 8 and Fig. S8). This differs from other intracellular pathogens such as *H.*
capsulatum where *sid1* (orthologous to *T. marneffei sidX*) is required for growth in
748 low iron conditions and systemic infection (Hwang *et al.*, 2008). Deletion of *sidX*
results in a significant reduction of extracellular coprogen B suggesting scavenging of
750 ferric iron is impaired. This in turn may explain the consequential reduction in
intracellular siderophore production and an absence of proliferation under low iron
752 conditions (Fig. 7 and Fig. 10). In *A. fumigatus* disruption of extracellular siderophore
production through deletions of *sidD* and *sidF* results in reduced radial growth. This is
754 exacerbated when RIA is inhibited as well, showing that both pathways contribute to
iron acquisition (Schrettl, 2007). Although disruption of RIA results in reduced
756 growth of *T. marneffei* yeast cells, when iron is limiting it appears that *T. marneffei*
relies more heavily on extracellular siderophores, perhaps taken up directly as a
758 siderophore-iron complex by yeast cells, and this is consistent with the more severe
growth impairment observed in the *sidX* deletion strain. Thus, while *sidA* is
760 responsible for the first step of intracellular and extracellular siderophore biosynthesis
in *A. fumigatus* and many other fungi, these roles appear divided between *sidA* and
762 *sidX* in *T. marneffei* (Fig. 12).

764 The $\Delta sidX$ strain showed a reduced germination rate at 37°C compared to wild type
under standard iron to low iron conditions but not under high iron conditions. The fact
766 that wild type conidia can germinate in iron free medium suggests that the observed
reduction in germination of $\Delta sidX$ is the result of defects in iron storage during
768 conidial morphogenesis. This was consistent with HPLC/MS results that showed *sidX*
is important for intracellular siderophore production in yeast cells as well as
770 extracellular siderophore production. Amelioration of this defect under high iron
conditions suggests compensation by low affinity systems not examined here that
772 allow iron uptake.

774 In *T. marneffei*, putative extracellular siderophore biosynthetic genes, including *sidX*,
show yeast-specific gene expression under both low and standard iron growth
776 conditions (Fig. 4). This suggests that at the gene level, the biosynthesis of
extracellular siderophores is under cell type control. As a consequence of the growth
778 conditions required to generate yeast cells *in vitro* in *T. marneffei* extracellular
siderophores are not readily detected. It is possible that not only is extracellular
780 siderophore biosynthetic gene expression upregulated, but siderophore uptake is also
increased in yeast cells and therefore not evident at the metabolite level in
782 extracellular extracts. In contrast, production of coprogen B is prevalent in hyphal
cells. The production and prevalence of siderophores is likely to be temporally, as
784 well as spatially, controlled. In *A. nidulans* the extracellular siderophores fusigen and
TAFC are secreted at different times (Oberegger *et al.*, 2001). Secretion of fusigen
786 into iron free medium was at its optimal level at 24 h, but no longer detectable by 48 h,
while the level of TAFC reached its peak at 48 h followed by the gradual decrease
788 thereafter (Oberegger *et al.*, 2001).

790 *Transcriptional regulation of high-affinity iron assimilation by SreA*

Similar to other fungi, the expression of high-affinity iron assimilation genes in *T.*
792 *marneffei* is regulated by both exogenous iron levels and the transcriptional regulator
SreA (Fig 1, Fig 4 and Fig 5) (Chao *et al.*, 2008, Haas, 2003, Haas *et al.*, 2008,
794 Johnson, 2008 and Schrettl *et al.*, 2008). These studies have shown that SreA
typically acts as a repressor of high-affinity iron assimilation genes under high iron
796 conditions. Here we compared the regulation in hyphal and yeast cell types and

showed that SreA predominantly acts negatively in hyphal cells but has more gene-specific roles in yeast cells, including a positive role in the expression of *sidX* and *sidT*, involved in extracellular siderophore biosynthesis and transport, respectively. This departure from the well documented role of SreA orthologues in other systems may be a consequence of the selective pressure on the organism in the iron-restricted host environment, however why switching from negative to positive control of extracellular siderophore biosynthesis and uptake genes has occurred in *T. marneffei* remains open for speculation.

In *A. nidulans* and *A. fumigatus* SreA and HapX work together in a negative feedback loop to regulate iron assimilation. SreA represses high-affinity iron assimilation when iron is sufficient, while HapX represses iron-consuming pathways including mitochondrial metabolic pathways such as the TCA cycle when iron availability is low. Under this condition HapX has also been shown to activate iron acquisition, particularly extracellular siderophore biosynthesis (Hortschansky *et al.*, 2007, Schrettl *et al.*, 2010, Haas, 2012). It is through this tight regulation that the delicate balance between iron deficiency and iron toxicity is maintained in the cell. In *T. marneffei* hyphal cells, SreA was clearly required for the repression of *hapX* expression in both low and high iron conditions. This was not the case in yeast cells where SreA appears to have no role in *hapX* expression and only iron levels affect *hapX* expression levels. Given that SreA-mediated repression of high-affinity iron assimilation genes appeared alleviated for some genes in yeast cells it is possible that HapX, or an undiscovered regulator, may repress *sreA* expression and function in yeast cells.

The dynamics of iron assimilation and homeostasis is at its core conserved in fungal pathogens but also highly specialised for different types of fungal pathogens and their respective niches. *T. marneffei* represents a group of human pathogenic fungi which have the added complexity of occupying diverse niches through their capacity to generate, and alternate between, specialised cell types associated with these niches. *T. marneffei* uses both RIA and non-RIA systems for iron assimilation with a differential reliance on these for the pathogenic and non-pathogenic cell types. In addition, *T. marneffei* has, through gene duplication and diversification, paralogues of the key enzyme in siderophore biosynthesis that perform different function. Moreover, it is

clear from this work that assimilation of iron is very important in the pathogenic yeast cell type where it utilises both assimilation systems and this is consistent with the scarcity of available iron in the host and the altered metabolic flux that is evident in yeast cells. The next step will be to fully understand the individual contributions of *sidA* and *sidX* by examining their activities in an RIA-defective background and how the differences in their domain structure impart their specialised activities.

Materials and Methods

Molecular methods

For genomic DNA isolation, *T. marneffei* was grown in Synthetic Dextrose (SD) medium, plus ammonium (10 mM) for 4 days at 25°C and harvested through Miracloth filtration, blotted dry and snap frozen in liquid nitrogen. This was followed by genomic DNA extraction as previously described in (Borneman *et al.*, 2001). Genotypes were confirmed via Southern Blot Hybridisation. Approximately 2 µg of genomic DNA was separated by gel electrophoresis after digestion and depurinated by 15 min incubation in 0.1 M HCl. The DNA was then transferred to Hybond-N+ membrane (Amersham) using 0.4 M NaOH as per the manufacturer's instructions. Radiolabelled DNA probes were prepared using the random hexamer primers, [α -32P] dATP and Klenow fragment (Promega) (Sambrook *et al.*, 1989). Radiolabelled probes were hybridized to filters in formamide based hybridization solution (50% formamide, 4 x SSPE, 1% SDS, and dH₂O) overnight at 37°C. Filters were then washed for 0.5 h under low stringency (37°C, 2x SSC, 0.1% SDS) then under high stringency (68°C, 0.1x SSC, 0.1% SDS). Labelled filters were visualised using a phosphorimager.

Expression Profiling

T. marneffei was grown in 100 mL of liquid Brain heart infusion (BHI) medium (Oxoid) for 2 days at 25°C or 6 days (4 days followed by transfer of 10 mL of cells to fresh BHI medium and incubation for an additional 2 days) at 37°C. For low iron conditions 0.2 mM ferrous iron-specific chelator, bathophenanthrolinedisulfonic acid (BPS) and 6 µM FeSO₄ (after 42 h at 25°C or after 4 days and 42 h at 37°C) was added to BHI medium. For high iron conditions 0.2 mM BPS and 0.2 mM FeSO₄ (after 42 h at 25°C or after 4 days and 42 h at 37°C) was added to BHI medium. RNA isolation was performed as previously described (Borneman *et al.*, 2001). Both RT-PCR and qRT-PCR experiments were performed. Samples for RT-PCR experiments

were DNase treated using Ambion DNA-free kit and cDNA synthesis was completed
 866 using Reverse Transcription System (Promega) following manufacturer's instructions.
 RT-PCR was performed using Taq DNA polymerase and gene specific primers are
 868 listed (Table. S3). To optimise reactions, ensuring amplification was in the
 exponential phase, the cycle number for each gene varied slightly. β -tubulin (*benA*)
 870 was used as a loading control and no cDNA controls were also included.

872 Samples for qRT-PCR experiments were DNase treated using Ambion TURBO
 DNA-free kit and cDNA synthesis was completed using the iScriptTM cDNA
 874 synthesis kit (Bio-Rad). qRT-PCR was performed using SsoAdvancedTM Universal
 SYBR[®] Green Supermix (Bio-Rad) according to manufacturers instructions and gene
 876 specific primers used are listed (Table. S3). Samples were loaded onto MicroAmp[®]
 Optical 384-well reaction plates (Life technologies) in duplicate and run on the ABI
 878 Quant Studio 7 according to manufacturers instructions. Melting curve analysis was
 used to ensure there were no primer dimers. Negative controls included both a no-
 880 template control and a no cDNA control. Data were analysed by the threshold cycle
 method ($\Delta\Delta C_t$ method)(Yuan *et al.*, 2006) and normalised to *benA* abundance. All
 882 data are represented as fold induction relative to gene expression of wild-type hyphal
 cells except where stated. All experiments were carried out in triplicate. Statistical
 884 analyses, including unpaired t tests were performed using Prism software (GraphPad
 version 6).

886

Cloning and plasmid constructions

888 The *T. marneffei* *ftrA*, *sidX*, *sreA* and *sidA* genes, with approximately 2kb of flanking
 5' and 3' UTR sequences, were amplified from FRR2161 genomic DNA using
 890 flanking primer pairs detailed (Table S3). Products were ligated into pGemT Easy
 (Promega)(*ftrA*, *sidX* and *sreA*) or the *EcoCRI* site of pBluescript II SK+ (*sidA*),
 892 giving rise to pSP7465, pSP7466, pSP7467 and pSP7846, respectively (Table S4).
 Deletion constructs were created using one of two methods. Constructs $\Delta ftrA::pyrG^+$,
 894 $\Delta sidX::barA$ and $\Delta sreA::pyrG^+$ were generated by firstly performing inverse PCR on
 plasmids pSP7465 (primers QQ26 & QQ27), pSP7466 (primers QQ18 & QQ19) and
 896 pSP7467 (primers QQ24 & QQ25) respectively to remove the coding region of each
 gene. The selectable marker gene *pyrG*, removed from vector *pyrG* Blaster using
 898 *SmaI* and *EcoRV*, was then ligated into the *ftrA* and *sreA* PCR products to yield

pSP7544 and pSP7577. The selectable marker gene *barA*, digested from vector pMT1612 H3*ClasK*⁺ with *EcoRV*, was cloned into the *sidX* PCR product to yield pSP7543 (Table S4). The *T. marneffei* Δ *sida::barA* construct was generated using the GatewayTM system described for *T. marneffei* (Bugeja *et al.*, 2012). Briefly, inverse PCR was performed on pSP7846 using primers containing *attB1* and *attB2* overhangs (TT03 and TT04) to enable site-specific recombination with the *attP1* and *attP2* sites in the donor plasmid derivative pHW7771 that contains the *barA* selectable marker. Site-specific recombination in the presence of BP clonase yielded pSP7915. All constructs generated were linearized using gene-flanking primers originally used to clone the genes before transformation of *T. marneffei* strain Δ *ligD pyrG*⁻ (G816) according to methods previously described (Borneman *et al.*, 2001).

Complementation constructs *ftrA pyrG* (pSP7764), *sida pyrG* (pSP7914), *sidX pyrG* (pSP7913) and *sreA pyrG* (pSP7763) were generated by ligating *SmaI/EcoRV* digested pSP7465, pSP7466, pSP7467, respectively into the *SmaI/EcoRV* site of the *pyrG* containing vector pLS7413 (with the exception of pSP7914 which was ligated into pLS7804). This resulted in plasmids containing a fragment of the selectable marker *pyrG* and the respective gene with approximately 2kb of flanking DNA sequences (Table S2). Circular plasmids were transformed into *T. marneffei* mutant strains according to methods previously described (Borneman *et al.*, 2001).

Fungal strains and growth conditions

T. marneffei strains used in this study are listed in Table 1 and grown on Aspergillus-defined medium containing 1% glucose and 10 mM ammonium (ANM) (Cove, 1996). The *T. marneffei* type strain FRR2161 was kindly provided by Dr. J. Pitt (CSIRO Food Industries, Sydney). *T. marneffei* transformations were conducted using strain Δ *ligD pyrG*⁻ (G816) enabling targeted homologous recombination (Bugeja *et al.*, 2012). For iron level manipulation growth assays *T. marneffei* was grown in Synthetic Dextrose (SD) medium. A nitrogen source of ammonium (10 mM) was added to SD. Auxotrophic supplements were added as required. *T. marneffei* strains were grown on ANM plus uracil (5 mM) medium containing 1 mg ml⁻¹ 5-fluoroorotic acid (5-FOA) to select for loss of the *pyrG* selectable marker. Protoplast medium was used for the regeneration of *T. marneffei* protoplasts after DNA-mediated transformation (Borneman *et al.*, 2002).

934 *Iron-related Growth Assays*

Growth assays were performed using SD as the base medium with 10 mM (NH₄)₂SO₄ under optimal laboratory growth conditions. Standard iron conditions contained 200 µg/L ferric chloride in the yeast nitrogen base and traces of iron in the agar. Low iron conditions contained 0.2 mM BPS and 6 µM FeSO₄. High iron conditions contained 0.2 mM BPS and 0.2 mM FeSO₄. Conidia (20 µl) were inoculated onto each iron condition on solid medium in 12-well microtitre plates and incubated at 25°C (1 x 10³) or 37°C (1 x 10⁵) for 5 days. Conidia (1 x 10⁷) were also inoculated into liquid medium (4 mL) and incubated at 25°C for 2 days for hyphal growth or 37°C for 4 days, followed by a 1.5 mL transfer to fresh medium for an additional 2 days for yeast cell growth. Cells from the entire 4 ml cultures were pelleted at 8000 rpm for 10 min, the majority of the supernatant was removed and the pellets were resuspended in the remaining supernatant (~1 mL), transferred to pre-weighed 1.5 ml Eppendorf tubes and pelleted at maximum speed (RCF 13,982xg) for an additional 10 min. The supernatant was removed and pellets were dehydrated at 60°C overnight. From these desiccated cells the dry mass of each sample at both 25°C and 37°C was measured.

952 *Germination assays*

To determine the rate of *T. marneffei* germination, conidia (1 x 10⁶) were inoculated in triplicate into four different growth conditions (300 mL) and allowed to germinate at 25°C or 37°C for 13 h and 18 h. Media conditions were as follows; control (SD), zero iron (SD, 0.2 mM BPS), low iron (SD, 0.2 mM BPS, 6 µM FeSO₄), high iron (SD, 0.2 mM BPS, 0.2 mM FeSO₄) and all media were supplemented with (NH₄)₂SO₄ (10 mM) and uracil (10 mM) to eliminate any uracil auxotrophic effects. Samples were set up in 24-well microtitre plates and 100 cells were counted per well. Each counted cell was classified as either ungerminated (ungerminated conidium or isotropic growth) or germinated (polarised growth or germling). Quantification was assisted using ImageJ 1.45s (Rasband, 1997-2012).

964 *Siderophore isolation*

For growth at 25°C, *T. marneffei* conidia (1 x 10⁷) were cultured in liquid SD medium (100 mL). On day 3, 50 mL of culture was transferred to 60 mL fresh SD medium

plus 0.2 mM BPS for 7 h. At 5 h, 6 μ M FeSO₄ was added to the medium and
968 incubated for 2 h. For growth at 37°C *T. marneffei* conidia (1×10^7) were also cultured
in liquid SD medium (100 mL). However on day 5, 50 mL of culture was transferred
970 to 60 mL fresh SD medium plus 0.2 mM BPS for 7 h. At 5 h, 6 μ M FeSO₄ was added
to the medium and incubated for 2 h. Hyphal and yeast cells were transferred to two
972 50 mL Falcon tubes each and centrifuged at 8000 rpm for 10 min, separating
supernatant and pellet.

974
Freeze-dried samples of harvested hyphal and yeast cells and their respective growth
976 supernatants were examined by HPLC analysis to measure siderophore production.
For cell-associated siderophores, 50 mg of freeze-dried mycelium was homogenized
978 in a Mixer Mill 300 (Retsch) and resuspended in 50 mM sodium phosphate buffer (pH
6.5). After centrifugation, 1 mL of supernatant was applied to a XAD 16 Amberlite
980 column. For secreted siderophore 20 mL samples of freeze-dried *T. marneffei* growth
supernatant were resuspended in 5 ml of distilled H₂O centrifuged and applied to a
982 XAD 16 Amberlite column. Siderophores were iron saturated by addition of 100 μ l of
100 mM FeSO₄. The column was washed with 15 mL of distilled H₂O and bound
984 siderophores were eluted with 2 ml of methanol. Speed vacuum centrifugation was
used to remove any residual organic substances and samples were analysed by
986 reversed-phase HPLC (Konetschny-Rapp *et al.*, 1998). HPLC analysis was performed
in conjunction with mass-spectrometry to assign known siderophore types to
988 identified peaks.

990 *Macrophage isolation and infection*

Activated murine macrophage cells (J774) were grown in Dulbecco's Modified Eagle
992 Medium (DMEM)(Sigma) containing 4.5 g/L glucose with 10% fetal bovine serum, 4
mM L-glutamine and 1 x penicillin-streptomycin solution. Cells were passaged by
994 washing with phosphate buffer solution (PBS) and trypsinised with 10 x trypsin-
EDTA solution to allow detachment from the flask surface. Cells were then centrifuge
996 pelleted at 1800 rpm at 4°C.

998 J774 murine macrophages (1×10^5) were adhered to sterile coverslips in 6-well
microtitre plates and grown overnight at 37°C in iron free DMEM medium.
000 Macrophages were then activated with the addition of lipopolysaccharide (LPS)(0.1

002 $\mu\text{g ml}^{-1}$). Macrophages were infected with 1×10^6 *T. marneffei* conidia for 2 h to
allow phagocytosis of conidia. Coverslips were then gently washed twice with PBS to
remove free non-phagocytosed conidia, 2 mL of fresh iron free medium was added
004 and macrophages were incubated for 24 h.

006 *Microscopy*

For microscopic observation, *T. marneffei* cells were grown in liquid medium then
008 500 μL aliquots were transferred to 1.5 mL microfuge tubes containing 20 μL of 4%
paraformaldehyde in PME (50 mM piperazine-N, N' -bis(2-ethanesulfonic acid
010 (PIPES), 1 mM MgSO_4 , 20 mM EGTA pH 6.7) fixing solution. Samples were
incubated for 20 min at room temperature, and then pelleted by centrifugation for 5
012 min. The supernatant was removed and pellets were resuspended in 20 μL Tween 80.
Small aliquots (7 μL) were then placed onto slides and stained with fluorescent
014 brightener 28 (Calcofluor, CAL)(final concentration 0.014 mg ml^{-1}) and observed
microscopically.

016 *T. marneffei* infected J774 macrophages adhered on coverslips were fixed with 4%
018 paraformaldehyde in PME for 30 min at room temperature followed by two washes
with PBS. Cells were then stained with fluorescent brightener 28 (Calcofluor,
020 CAL)(final concentration 0.014 mg ml^{-1}) in Tween 80 and inspected microscopically.
The number of *T. marneffei* conidia, germings, hyphae and yeast cells in 100
022 macrophages were recorded. Experiments were performed as three biological repeats.

024 Slides were examined using differential interference contrast (DIC) and fluorescent
optics on a Reichart Jung Polyvar II microscope. Images were captured using a SPOT
026 CCD camera (Diagnostic Instruments) and adjusted for colour and contrast in Adobe
Photoshop™ 7.0.

028 *Bioinformatic Analysis*

030 Database searches were conducted using National Centre for Biotechnology
Information (NCBI) BLAST interface (<http://www.ncbi.nlm.nih.gov/>). Extensive
032 whole genome analyses of multiple fungi were carried out using custom scripts.
Multiple protein sequence alignments were completed using ClustalW and shaded

034 using MacBoxShade version 2.5 (Macintosh) or performed in MEGA version 5.0
 (Tamura *et al.*, 2011) in order to create neighbor joining relatedness trees,
 036 bootstrapped with 1000 replicates. Fungi examined included *A. fumigatus*, *A. nidulans*,
C. immitis, *C. neoformans*, *Fusarium oxysporum*, *H. capsulatum*, *M. oryzae*,
 038 *Metarhizium anisopliae*, *Neosartium fischeri*, *P. chrysogenum*, *P. brasiliensis*, *T.*
marneffeii, *P. tritici-repentis*, *R. oryzae*, *S. pombe*, *T. stipitatus* and *U. reesei* (unless
 040 otherwise specified). Rossmann sequence domains were identified using Center for
 biological sequence analysis (CBS) interface (<http://www.cbs.dtu.dk>). Genome
 042 synteny was examined using web Artemis comparison tool (webACT) (Abbott *et al.*,
 2008).

044

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238

240 Figure Legends

Figure 1. Characterisation of RIA in *T. marneffei*

242 A. Gene expression of *fetC* and *ftrA* was examined by RT PCR using gene-specific
primers and wild type (FRR2161) RNA extracted from hyphal (H) or yeast (Y) cells.
244 Hyphal cells were cultured at 25°C for two days and yeast cells were cultured at 37°C
for six days in standard iron conditions in BHI medium. For growth of wild type
246 under low iron conditions 0.2 mM ferrous iron-specific chelator,
bathophenanthrolinedisulfonic acid (BPS) was added to BHI medium. The *fetC* and
248 *ftrA* genes showed higher expression in yeast cells compared to hyphal cells under
both low and standard iron conditions. The *benA* gene was used as a loading control.
250 B. Transcript levels of *ftrA* in wild type (*sreA*⁺) and Δ *sreA* hyphal and yeast cells were
examined by qRT-PCR. Hyphal cells were cultured at 25°C for two days and yeast
252 cells were cultured at 37°C for six days in BHI medium. For growth under low iron
conditions 0.2 mM BPS and 6 μ M FeSO₄ was added to the medium for the last 8 h of
254 incubation while high iron conditions medium was supplemented with 0.2 mM BPS
and 0.2 mM FeSO₄. Transcript levels are presented relative to the *ftrA* expression of
256 wild type (*sreA*⁺) hyphal cells grown under low iron conditions. Transcript levels
were normalized to *benA*. Error bars represent the standard error of the mean (*
258 denotes $p < 0.05$).

C-F. *T. marneffei* conidia were cultured at 25°C (C, D) or 37°C (E, F) in standard, low
260 and high iron solid (C, E) and liquid (D, F) media. Wild type (*ftrA*⁺) and Δ *ftrA*
showed no growth differences on solid media at 25°C. Differences in dry weight of
262 Δ *ftrA* compared to wild type (*ftrA*⁺) showed no significant differences under all
growth conditions at 25°C. A reduction in Δ *ftrA* growth was observed on low iron
264 solid medium compared to wild type (*ftrA*⁺) at 37°C. Consistently, the dry weight of
 Δ *ftrA* was reduced under low iron conditions compared to wild type (*ftrA*⁺) at 37°C.
266 Error bars represent standard error of the mean.

268 Figure 2. *T. marneffei* produces siderophores

Wild type *T. marneffei* hyphal cells and yeast cells were cultured in low iron medium
270 at 25°C or 37°C, respectively. Cell pellet and growth supernatant samples were then
analysed by reverse-phase HPLC using photometric determination at 434 nm that
272 records only ferri-siderophores and the molecular mass of each siderophore was
determined using mass-spectrometry.

274 A. HPLC analysis of *T. marneffei* hyphal (blue) and yeast (red) cell pellets over a 25
min interval. *T. marneffei* hyphal and yeast cells contained a peak at retention time
276 19.27 min, identified as ferricrocin (asterisk) by mass spectrometry (molecular mass
771.25) and at 19.70 min ferrichrome (molecular mass 741.25) (circle).

278 B. HPLC analysis of *T. marneffei* hyphal (blue) and yeast (red) growth supernatant
over a 25 min interval. Hyphal supernatants contained a peak at retention time 20.5
280 min, identified as Coprogen B (molecular mass 780.30) (triangle), which was only
minimally detected in yeast supernatants.

282

Figure 3. *T. marneffei* SidA and its paralogue SidX reside in separate clades.

284 Multiple protein sequence alignment was constructed using ClustalW, and the
relatedness tree was created using the neighbour joining method (bootstrapped using
286 1000 replicates) in MEGA. Fungi represented are *A. fumigatus* (Af), *A. nidulans* (An),
C. immitis (Ci), *F. oxysporum* (Fo), *H. capsulatum* (Hc), *M. anisopliae* (Ma), *M.*
288 *oryzae* (Mo), *N. fischeri* (Ni), *P. brasiliensis* (Pa), *P. chrysogenum* (Pc), *T. marneffei*
(Pm), *P. tritici-repentis* Pt-1C-BFP (Pt), *S. pombe* (Sp), *T. stipitatus* (Ts) and *U.*
290 *reesei* (Ur) with the bacterium *Bacillus anthracis* (BA) as the outgroup. SidA and
SidX reside in separate clades, with *T. marneffei* SidX sharing a clade with *U. reesei*,
292 *C. immitis*, *P. brasiliensis* and *H. capsulatum* orthologues. *T. marneffei* proteins are
denoted with grey circles.

294

Figure 4. Regulation and expression of siderophore biosynthesis genes

296 The expression of candidate siderophore biosynthesis genes was examined by RT
PCR using gene-specific primers and RNA extracted from hyphal (H) or yeast (Y)
298 cells of the wild type (2161) or $\Delta sreA$ strains. Cells were cultured at 25°C for 2 days
in standard iron conditions. Both *sidA* and *sidC* were expressed in hyphal and yeast
300 cells while all other genes were expressed predominantly in yeast cells. *sidA* and *sidC*
expression appears reduced in $\Delta sreA$ hyphal cells compared to wild type hyphal cells,
302 while all other genes showed increased gene expression in $\Delta sreA$ hyphal cells
compared to wild type hyphal cells. *sitT* also showed reduced expression in $\Delta sreA$
304 yeast cells. *benA* was used as a loading control.

306 **Figure 5. Regulation and expression of *sidA* and *sidX* genes**

Transcript levels of *sidA* (A) and *sidX* (B) in wild type (*sreA*⁺) and $\Delta sreA$ hyphal and

yeast cells were examined by qRT-PCR. Expression was compared under low and high iron conditions. Transcript levels are presented relative to the expression of wild type (*sreA*⁺) hyphal cells grown under low iron conditions. Transcript levels were normalized to *benA*. Error bars represent the standard error of the mean (* denotes $p < 0.05$). The *sidA* and *sidX* transcript levels were greater in Δ *sreA* hyphal cells compared to wild type cells under low iron conditions while transcript levels were unaffected by the deletion of *sreA* in yeast cells. Expression of *sidX* was more sensitive to iron levels compared to *sidA* expression, but overall transcript levels were higher under low iron conditions compared to high iron conditions (with the exception of *sidA* expression in wild type hyphal cells).

Figure 6. Gene expression of *hapX* is influenced by *sreA* expression, cell-type and iron levels.

Transcript levels of *hapX* in wild type (*sreA*⁺) and Δ *sreA* hyphal and yeast cells were examined by qRT-PCR. Gene expression was compared under low and high iron conditions. Transcript levels are presented relative to the gene expression of wild type (*sreA*⁺) hyphal cells grown under low iron conditions. Transcript levels were normalized to *benA*. Error bars represent the standard error of the mean (* denotes $p < 0.05$). The *hapX* transcript levels were higher in yeast cells compared to hyphal cells. In addition in wild type yeast cells *hapX* expression is greater in low iron conditions compared to high iron conditions. Gene expression was greater in Δ *sreA* hyphal cells compared to wild type (*sreA*⁺) hyphal cells under low and high iron conditions while transcript levels were unaffected by the deletion of *sreA* in yeast cells.

Figure 7. Δ *sidA* and Δ *sidX* growth and morphology under varied iron conditions at 25°C.

T. marneffei wild type (*sidA*⁺ *sidX*⁺), Δ *sidA* and Δ *sidX* conidia were grown at 25°C in standard, low or high iron liquid and solid media.

A. Microscopic examination of Δ *sidA* hyphal cells showed that morphology was unaffected compared to wild type (*sidA*⁺) cells under all tested conditions. For the Δ *sidX* cells the morphology in standard iron conditions was also consistent with wild type, however under low iron conditions the Δ *sidX* hyphal cells were rarely detected and appear highly degraded compared to wild type. Cells were examined under differential interference contrast (DIC) or epifluorescence optics to observe calcofluor

342 (CAL) stained chitin. Scale bars, 20 μ M.

B. Growth of the wild type, Δ *sidA* and Δ *sidX* strains on solid medium with standard,
344 low or high iron at 25°C. All three strains showed little difference on standard and
high iron medium. The growth of Δ *sidA* strain appeared equivalent to the wild type
346 under low iron conditions. In contrast, the Δ *sidX* strain showed no growth under low
iron conditions.

348 C. Dry mass growth determination for the wild type, Δ *sidA* and Δ *sidX* strains in liquid
medium. There was no difference amongst the three strains for growth in standard and
350 high iron conditions. In contrast, the Δ *sidA* strain showed higher mass than wild type
under low iron conditions while the Δ *sidX* strain showed significantly lower mass
352 than wild type under low iron conditions. Error bars represent standard error of the
mean (* denotes $p < 0.05$).

354

**Figure 8. Δ *sidA* and Δ *sidX* growth and morphology under varied iron conditions
356 at 37°C.**

T. marneffei wild type (*sidA*⁺ *sidX*⁺), Δ *sidA* and Δ *sidX* conidia were grown at 37°C in
358 standard, low or high iron solid and liquid medium.

A. Microscopic examination of Δ *sidA* yeast cells showed that apical tips displayed
360 swelling under standard and low iron (more severe) conditions (indicated by arrows).
In standard iron conditions the morphology of Δ *sidX* cells was consistent with wild
362 type, while in low iron conditions Δ *sidX* cells were undetectable. Cells were
examined under differential interference contrast (DIC) or epifluorescence optics to
364 observe calcofluor (CAL) stained chitin. Scale bars, 20 μ M.

B. Growth of the wild type, Δ *sidA* and Δ *sidX* strains on solid medium with standard,
366 low or high iron at 37°C. All three strains showed little difference on standard and
high iron medium. The growth of Δ *sidA* strain appeared equivalent to the wild type
368 under low iron conditions. In contrast, the Δ *sidX* strain showed no growth under low
iron conditions.

370 C. Dry mass growth determination for the wild type, Δ *sidA* and Δ *sidX* strains in liquid
medium. There was no difference amongst the three strains for growth in standard and
372 high iron conditions. The mass of the Δ *sidA* strain was consistent with that of wild
type under low iron conditions while the Δ *sidX* strain showed no mass under low iron
374 conditions. Error bars represent standard error of the mean (* denotes $p < 0.05$).

Figure 9. Germination of $\Delta sidA$ and $\Delta sidX$ at 25°C and 37°C.

T. marneffei conidia were grown at 25°C (A) or 37°C (B) for 13 h under control (SD), zero iron (SD, 0.2 mM BPS), low iron (SD, 0.2 mM BPS, 6 μ M FeSO₄) or high iron (SD, 0.2 mM BPS, 0.2 mM FeSO₄) conditions. Cells were classified as being either ungerminated (spherical) or germinated (polarised or germling), with the number of germinated cells converted to a percentage of all fungal cells counted. Three biological repeats were performed. At 25°C the percentage of $\Delta sidA$ germlings was consistent with wild type under all conditions. At 37°C, $\Delta sidA$ displayed significantly increased germination compared to wild type when cultured in standard iron, zero iron and low iron conditions, and showed significantly decreased germling production under high iron conditions. At 25°C the percentage of $\Delta sidX$ germlings was consistent with wild type under all conditions. At 37°C, $\Delta sidX$ displayed significantly decreased germination compared to wild type when cultured under standard, zero iron and low iron conditions. Error bars represent standard errors of the mean (* denotes $p < 0.05$).

390

Figure 10. $\Delta sidA$ and $\Delta sidX$ siderophore biosynthesis.

Wild type ($sidA^+sidX^+$), $\Delta sidA$ and $\Delta sidX$ hyphal and yeast cells were examined by HPLC-MS. Cell pellet (A, B) and growth supernatant (C) samples were then analysed by reverse-phase HPLC using photometric determination at 434 nm and the molecular mass of detected intracellular and extracellular siderophores were confirmed using mass-spectrometry. Both $\Delta sidA$ and $\Delta sidX$ showed significantly less intracellular siderophores (ferricrocin and ferrichrome) in hyphal cells (A), while only the $\Delta sidX$ strain displayed significantly less intracellular siderophores in yeast cells (B). $\Delta sidX$ also showed significantly reduced coprogen B production by hyphal cells (C). Error bars represent standard errors of the mean (* denotes $p < 0.05$).

400

Figure 11. Expression of high affinity iron assimilation genes in $\Delta ftrA$, $\Delta sidX$ and $\Delta sidA$ hyphal and yeast cells.

Expression of *fetC* (A), *sidC* (B), *sidD* (C) and *sidH* (D) genes in wild type, $\Delta ftrA$, $\Delta sidX$ and $\Delta sidA$ hyphal and yeast cells cultured under low iron conditions was examined by qRT-PCR. Transcript levels were normalized to *benA* and are presented relative to the gene expression of wild type hyphal cells. Error bars represent the standard error of the mean (* denotes $p < 0.05$). Expression of *fetC* was upregulated in $\Delta sidX$ and $\Delta sidA$ yeast cells and downregulated in $\Delta ftrA$ yeast cells compared to wild

408

type cells. The expression of *sidC* in $\Delta ftrA$, $\Delta sidX$ and $\Delta sidA$ hyphal and yeast cells
410 was consistent to wild type levels. Expression of *sidD* was upregulated in $\Delta ftrA$,
 $\Delta sidX$ and $\Delta sidA$ hyphal cells compared to the wild type hyphal cells, but *sidD* was
412 significantly upregulated only $\Delta sidX$ yeast cells. Expression of *sidH* was upregulated
in $\Delta ftrA$, $\Delta sidX$ and $\Delta sidA$ hyphal and cells compared to wild type. Error bars are
414 standard error of the mean. The horizontal bars denote statistically significant
pairwise differences.

416

Figure 12. Proposed siderophore biosynthetic pathway of *T. marneffei*

418 The proposed siderophore biosynthetic pathway based on what is known in other
fungi, along with the enzymes and corresponding *T. marneffei* genes identified in the
420 genome. The position of the *sidA/sidX* orthologues are shown, as are the siderophores
coprogen B, ferricrocin and ferrichrome that were identified as synthesized by *T.*
422 *marneffei*. Data from this study suggests a clear role for *sidX* in extracellular
siderophore production and *sidA* in intracellular siderophore production. The presence
424 of *sidX* influences intracellular siderophore production (denoted by the dotted line),
however whether this is a consequence of no iron coming into the cell due to a lack of
426 extracellular siderophores or a direct role in intracellular siderophore biosynthesis is
yet to be delineated. Adapted from Haas (2012).

428

430

Table
Table 1. *T. marneffei* Strains

Name	Strain	Genotype	Origin
FRR2161	FRR2161 (ATCC18224)	Wild type	(Borneman, 2000)
G816	$\Delta ligD::pyrG^-$	$\Delta ligD$; $niaD$; $pyrG$	(Bugeja, 2012)
G809	$\Delta ligD::pyrG^+$	$\Delta ligD::pyrG^+$; $niaD$; $pyrG$	(Bugeja, 2012)
G871	$\Delta ftrA pyrG^-$	$\Delta ftrA$; $\Delta ligD$; $niaD$; $pyrG$	This study
G872	$\Delta ftrA::pyrG^+$	$\Delta ftrA::pyrG^+$; $\Delta ligD$; $niaD$	This study
G968	$\Delta ftrA::ftrA^+$	$\Delta ftrA::ftrA^+::pyrG^+$; $\Delta ligD$; $niaD$	This study
G967	$\Delta sidA::barA^+$ $pyrG^+$	$\Delta sidA::barA^+$; $\Delta ligD::pyrG^+$; $niaD$	This study
G1016	$\Delta sidA sidA^+$	$\Delta sidA::sidA^+;\Delta ligD$; $pyrG^+$; $niaD$	This study
G941	$\Delta sidX::barA^+$ $pyrG^-$	$\Delta sidX::barA^+$; $\Delta ligD$; $niaD$; $pyrG$	This study
G873	$\Delta sidX::barA^+$ $pyrG^+$	$\Delta sidX::barA^+$; $\Delta ligD::pyrG^+$ $niaD$	This study
G942	$\Delta sidX sidX^+$	$\Delta sidX::sidX^+;\Delta ligD$; $pyrG^+$; $niaD$	This study
G870	$\Delta sreA pyrG^-$	$\Delta sreA$; $\Delta ligD$; $niaD$; $pyrG$	This study
G869	$\Delta sreA pyrG^+$	$\Delta sreA::pyrG^+$; $\Delta ligD$; $niaD$	This study
G943	$\Delta sreA sreA^+$	$\Delta sreA;sreA^+::pyrG^+$; $\Delta ligD$; $niaD$	This study

432

434 Supporting Information Legends

436 Figure S1. Clustering of *T. marneffei* *fetC* and *ftrA*.

Comparison of the genomic locations of *fetC* and *ftrA* orthologues in *S. cerevisiae*, *T. marneffei*, *T. stipitatus* and *A. fumigatus*. *fetC* is indicated in green and *ftrA* in red. TSTA_014770 was misannotated in NCBI and has been corrected to acknowledge the presence of *T. stipitatus* *ftrA* (the first 1-375 bps of TSTA_04760), confirmed through bioinformatics analysis and Southern blot hybridisation to be orthologous to *T. marneffei* *ftrA*. An asterisk indicates this amendment. *fetC* and *ftrA* are clustered in the genomes of *T. marneffei*, *T. stipitatus* and *A. fumigatus*, but not in *S. cerevisiae*. In *S. cerevisiae* *FET3* lies on chromosome 13 and *FTR1* lies on chromosome 5.

446 Figure S2. Relatedness of *T. marneffei* FetC protein sequences to other fungi.

BLAST searches using *S. cerevisiae* Fet3 (YMRO58W, green) against *T. marneffei* and other fungi identified numerous homologues. A multiple protein sequence alignment was constructed and used to create the protein relatedness tree using the Neighbor-Joining method [1]. The bootstrap consensus tree inferred from 500 replicates [4] is shown. The analyses were conducted in MEGA6 [3]. Fungi examined include *T. marneffei* (PMAA), *T. stipitatus* (TSTA), *S. cerevisiae* (YM), *C. albicans* (Ca), *H. capsulatum* (HCBG), *A. fumigatus* (Afu), *A. nidulans* (ANID), *C. immitis* (CIMG), and *P. brasiliensis* (PAAG), *U. reesei* (URET), *M. anisopliae* (Ma), *M. oryzae* (MGG), *N. fischeri* (NFIA), *R. oryzae* (RO3G), *F. oxysporum* (FOXG), *S. pombe* (SPAC) and *P. chrysogenum* (Pc). Clades of the different classes of copper ion binding proteins defined as FetC, LccC laccases, Arb1, Arb2 and uncharacterised putative multicopper oxidase (MCO) orthologues are denoted. *T. marneffei* (red) and *T. stipitatus* (blue) proteins are marked with the *T. marneffei* FetC orthologue (PMAA_057450) marked by an asterisk. *A. nidulans*, *C. immitis* and *U. reesei* genomes did not contain a FetC orthologue.

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Figure S3. Δ *ftrA* germination rate is unaffected at 25°C and 37°C.

T. marneffei conidia (10^6) were grown at 25°C or 37°C for 13 h in 300 mL of the following liquid media; standard iron conditions control (SIC), zero iron (0.2 mM BPS), low iron (0.2 mM BPS, 6 μ M FeSO₄), high iron (0.2 mM BPS, 0.2 mM FeSO₄) All media were based on Synthetic Dextrose (SD) and supplemented with 10mM

(NH₄)₂SO₄ as a nitrogen source and 10 mM uracil to avoid any possible effects due to partial auxotrophy. Samples were set up in 24-well microtitre plates and at least 100 cells were counted per well in triplicate biological repeat experiments. Each recorded cell was classified as being either ungerminated (conidium or isotropic) or germinated (polarised or germling), with the number of each cell type converted to a percentage of all fungal cells counted. The percentage of $\Delta ftrA$ germlings was consistent with wild type ($ftrA^+$) and $\Delta ftrA[ftrA^+]$, unaffected by iron levels present in the medium. Error bars represent standard error of the mean (3 biological repeats).

Figure S4. *ftrA* is not essential for growth in macrophages.

LPS-activated J774 murine macrophages grown in iron free medium were infected with *T. marneffei* conidia (10⁶) and incubated for 24 h at 37°C. Cells were then fixed and stained with calcofluor. The experiment was performed in triplicate.

A. $\Delta ftrA$ morphology in macrophages. $\Delta ftrA$ conidia germinated into yeast cells, with yeast cells maintaining cell wall integrity, consistent with that of wild type ($ftrA^+$) and $\Delta ftrA[ftrA^+]$. Cells were examined under differential interference contrast (DIC) or epifluorescence optics to observe calcofluor (CAL) stained chitin. Scale bars, 20µM.

B. The number of conidia, germlings, hyphae and yeast cells in at least 100 macrophages for each strain were recorded and converted to a percentage of all fungal cells counted. $\Delta ftrA$ displayed no reduction in yeast cell production compared to wild type ($ftrA^+$) and $\Delta ftrA[ftrA^+]$. Error bars represent standard error of the mean.

Figure S5. Predicted protein-binding motifs of SidA and SidX orthologues

Comparison of the protein-binding motifs present in SidA (A) and SidX (B) fungal orthologues. Domain predictions are based on NCBI conserved domain analysis (cutoff <e-0.05). Rossmann sequence domains were identified using CBS analysis (predicted specific cofactors are based on neural network scores greater than 0.5). Fungi inspected include *A. fumigatus* (Afu), *A. nidulans* (ANID), *C. immitis* (CIMG), *H. capsulatum* (HCBG), *P. brasiliensis* (PAAG), *P. chrysogenum* (Pc), *T. marneffei* (PMAA), *T. stipitatus* (TSTA) and *U. reesei* (URET). SidA and SidX predicted protein-binding motifs (extracted from NCBI).

Figure S6. Gene clustering and expression of *T. marneffei* siderophore biosynthesis genes.

Comparison of the genomic locations of siderophore biosynthesis-related genes in *T. marneffei*, *T. stipitatus* and *A. fumigatus* using Artemis comparison tool (ACT release 8). Red and blue (inversions) lines between genomes denote regions of sequence similarity. Black dotted lines indicate where genome fragments have been concatenated. Unlike in *A. fumigatus*, in *T. marneffei* and *T. stipitatus*, *sidA* (red) and *sidC* (orange), predicted to be involved in intracellular siderophore biosynthesis, are clustered together. Major facilitator superfamily (MFS) transporter encoding gene (pink), *sidH* (dark purple), *sidF* (dark blue), *sitT* (light purple) and *sidD* (light blue), predicted to be involved in extracellular siderophore biosynthesis, make up a second gene cluster. Compared to the *T. marneffei* genome, this region of the *A. fumigatus* genome appears to have undergone an inversion event, and the third gene cluster consisting of *sidX* (light green) (absent in *A. fumigatus*) and *sidI* (dark green) appears to have undergone an inversion event in *T. stipitatus*.

Figure S7. Δ sidA produces a reduced number of yeast cells in macrophages.

LPS-activated J774 murine macrophages cultured in iron free medium were infected with *T. marneffei* conidia (10^6) and incubated for 24 h in 6-well microtitre plates at 37°C. Cells were then fixed and stained with fluorescent brightener, calcofluor. Experiment was performed in triplicate.

A. Δ sidA morphology in macrophages. Δ sidA conidia germinated into yeast cells, with all cell types maintaining cell wall integrity, consistent with wild type (*sidA*⁺). Cells were examined under differential interference contrast (DIC) or epifluorescence optics to observe calcofluor stained chitin (CAL). Scale bars, 20μM.

B. The number of conidia, germlings, hyphae and yeast cells in at least 100 macrophages for each strain were recorded and converted to a percentage of all fungal cells counted. Δ sidA displayed a significantly reduced number of yeast cells compared to wild type (*sidA*⁺) (p-value of 0.0314). Error bars represent standard error of the mean (*, $p < 0.05$).

Figure S8. *sidX* is not essential for growth in macrophages.

LPS-activated J774 murine macrophages cultured in iron free medium were infected with *T. marneffei* conidia (10^6) and incubated for 24 h in 6-well microtitre plates at 37°C. Cells were then fixed and stained with fluorescent brightener, calcofluor. Experiment was performed in triplicate.

536 A. $\Delta sidX$ morphology in macrophages. $\Delta sidX$ conidia germinated into yeast cells,
with all cell types maintaining cell wall integrity, consistent with wild type ($sidX^+$)
538 and $\Delta sidX[sidX^+]$. Cells were examined under differential interference contrast (DIC)
or epifluorescence optics to observe calcofluor stained chitin (CAL). Scale bars,
540 20 μ M.

B. The number of conidia, germlings, hyphae and yeast cells in at least 100
542 macrophages for each strain was recorded and converted to a percentage of all fungal
cells counted. $\Delta sidX$ displayed no reduction in yeast cell production compared to wild
544 type ($sidX^+$) and $\Delta sidX[sidX^+]$. Error bars represent standard error of the mean.

Iron acquisition gene synteny

59x44mm (300 x 300 DPI)

Accep

Figure 1

233x314mm (300 x 300 DPI)

Figure 2

113x76mm (300 x 300 DPI)

Accept

Figure 3

117x98mm (300 x 300 DPI)

Accepted

Figure 4

105x204mm (300 x 300 DPI)

Figure 5

74x33mm (300 x 300 DPI)

Accepted

Figure 6

56x32mm (300 x 300 DPI)

Accepted

Figure 7

150x134mm (300 x 300 DPI)

Accf

Figure 8

150x133mm (300 x 300 DPI)

Accf

Figure 9

154x162mm (300 x 300 DPI)

A

Figure 10

233x314mm (300 x 300 DPI)

Figure 11

166x238mm (300 x 300 DPI)

Figure 12

297x420mm (300 x 300 DPI)

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Author/s:

Pasricha, S;Schafferer, L;Lindner, H;Boyce, KJ;Haas, H;Andrianopoulos, A

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