The ZrfC alkaline zinc transporter is required for Aspergillus fumigatus virulence and its growth in the presence of the Zn/Mn-chelating protein calprotectin

Jorge Amich,1† Rocío Vicentefranqueira,1 Emilia Mellado,2 Ana Ruiz-Carmuega,1,2 Fernando Leal1 and José Antonio Calera1*  

1 Instituto de Biología Funcional y Genómica (IBFG), centro mixto del Consejo Superior de Investigaciones Científicas y Universidad de Salamanca, Edificio IBFG, Lab. P1.10. C/Zacarías González n°2, 37007 Salamanca, Spain. 

2 Centro Nacional de Microbiología, Instituto de Salud Carlos III, Servicio de Micología, Ctra. Majadahonda-Pozuelo Km 2, 28220 Majadahonda, Madrid, Spain.

Summary

Aspergillus fumigatus can invade the lungs of immunocompromised individuals causing a life-threatening disease called invasive pulmonary aspergillosis (IPA). To grow in the lungs, A. fumigatus obtains from the host all nutrients, including zinc. In living tissues, however, most zinc is tightly bound to zinc-binding proteins. Moreover, during infection the bioavailability of zinc can be further decreased by calprotectin, an antimicrobial Zn/Mn-chelating protein that is released by neutrophils in abscesses. Nevertheless, A. fumigatus manages to uptake zinc from and grow within the lungs of susceptible individuals. Thus, in this study we investigated the role of the zrfA, zrfB and zrfC genes, encoding plasma membrane zinc transporters, in A. fumigatus virulence. We showed that zrfC is essential for virulence in the absence of zrfA and zrfB, which contribute to fungal pathogenesis to a lesser extent than zrfC and are dispensable for virulence in the presence of zrfC. The special ability of ZrfC to scavenge and uptake zinc efficiently from lung tissue depended on its N-terminus, which is absent in the ZrfA and ZrfB transporters. In addition, under Zn- and/or Mn-limiting conditions zrfC enables A. fumigatus to grow in the presence of calprotectin, which is detected in fungal abscesses of non-leucopenic animals. This study extends our knowledge about the pathobiology of A. fumigatus and suggests that fungal zinc uptake could be a promising target for new antifungals.

Introduction

Aspergillus fumigatus is a saprophytic filamentous fungus that can invade the lungs of immunocompromised individuals and cause invasive pulmonary aspergillosis (IPA) (Kousha et al., 2011). This fungus exhibits several traits that enable it to grow within living tissues of immunosuppressed patients and cause disease (Tekaia and Latge, 2005; Askew, 2008; Dagenais and Keller, 2009), including its ability to obtain essential cations such as iron and zinc from the hostile environment provided by the host (Calera and Haas, 2009).

The concentration of readily available zinc (i.e. free Zn2+ ions) is strongly dependent on the environmental pH as it tends to form complexes with other compounds as pH increases (Sandrin and Maier, 2003). Therefore, the concentration of free Zn2+ ions in living tissues at the physiological pH (~ 7.3–7.4) is very low because most zinc is tightly bound to zinc-binding proteins both inside the cells and in extracellular fluids. For instance, the average concentration of total zinc in the human serum is around 15 μM (equivalent to ~ 1 μg g−1 of serum) (Iyengar and Woittiez, 1988). However, most zinc in serum is bound to plasma zinc-binding proteins such as albumin and α2-macroglobulin (Foote and Delves, 1984). Thus, the average concentration of free Zn2+ in serum is 0.08 μM (~ 5 ng g−1) (Foote and Delves, 1988), which is about 150-fold lower than the minimal concentration required for A. fumigatus to grow optimally in a defined liquid medium (J. Amich and R. Vicentefranqueira, unpubl. data).

The average concentration of total zinc in human lungs is around 12 μg g−1 of lung tissue (Lech and Sadlik, 2011), but the concentration of free Zn2+ in the lungs has not been reported. Nevertheless, given that lungs have an
extensive network of vessels and capillaries, the concentration of free Zn\(^{2+}\) in the extracellular fluid of the lungs should be similar to that in serum. In addition, the zinc availability during fungal invasion could be reduced further in lung abscesses by calprotectin, which is a Zn/Mn-chelating protein released by neutrophils that is able to inhibit microbial growth by sequestering Zn\(^{2+}\) and Mn\(^{2+}\) (Kormdörfer et al., 2007; Corbin et al., 2008; Hayden et al., 2013). Thus far, it has not been reported whether calprotectin is able to inhibit the growth of *A. fumigatus* although it can inhibit the growth of other fungi such as *Candida albicans* (Steinbakk et al., 1990; Sohnle et al., 1996; Urban et al., 2009) and *A. nidulans* (Bianchi et al., 2011). In sum, both healthy and infected lung tissues provide a slightly alkaline zinc-limiting environment that prevents any sustained microbial growth unless microorganisms have developed some mechanisms to circumvent this nutritional restriction at the pH of the lungs.

The adaptation of *A. fumigatus* to grow in the lungs under alkaline zinc-limiting conditions is co-regulated by the ZafA and PacC transcription factors, which are essential for *Aspergillus* virulence (Bignell et al., 2005; Moreno et al., 2007). PacC adjusts to the environmental pH the induction of zinc uptake by ZafA under zinc-limiting conditions (Moreno et al., 2007; Amich et al., 2009; 2010). Thus, when ΔzafA conidia germinate on the lung mucous membrane produce short germ tubes that stop growing soon afterwards because they cannot express any zinc uptake system (Moreno et al., 2007).

The most obvious downstream target genes of ZafA that primarily would determine the virulence of *A. fumigatus* should be those involved in zinc uptake through the plasma membrane. In eukaryotic cells the transport of zinc from the extracellular space (or organellar lumen) into the cytoplasm relies on proteins belonging to the Zrt-, Irt-like Protein (ZIP) family of zinc transporters (Gaither and Eide, 2001). Six ZIP transporters of the eight encoded by the *A. fumigatus* genome (zrfA, zrfB, zrfC, zrfD, zrfE and zrfH) are localized presumably at the plasma membrane (Calera and Haas, 2009). The transcription levels of the zrfD, zrfE and zrfH genes are similar in acidic and alkaline media regardless of zinc availability and are not regulated by ZafA (J. Amich and R. VicenteFranqueira, unpubl. data). In contrast, the expression of the zrfA, zrfB and zrfC genes is upregulated by ZafA only under zinc-limiting conditions (VicenteFranqueira et al., 2005; Amich et al., 2009; 2010). Therefore, the capacity of *A. fumigatus* hyphae to scavenge and uptake zinc from the lung mucosa and abscesses could rely on the function of these genes, which hence might be critical for fungal virulence.

In this work we investigated the role of the zrfA, zrfB and zrfC genes in the pathobiology of *A. fumigatus*. We found that ZrfC was the major zinc transporter devoted to scavenge and uptake zinc from living tissues. This capacity of ZrfC was largely dependent on its N-terminus, which is absent in the ZrfA and ZrfB transporters. Besides, under Zn- and/or Mn-limiting conditions zrfC enables *A. fumigatus* to overcome the Zn/Mn-chelating capacity of calprotectin, which is readily detected in fungal abscesses of non-leucopenic animals. These findings suggested that zrfC might confer *A. fumigatus* the capacity to grow in the alkaline zinc-limiting environment provided by the lung tissue and/or abscesses of immunosuppressed individuals with IPA.

**Results**

**ZrfC is the major zinc transporter used by *A. fumigatus* to obtain zinc from host tissue**

The ZafA regulator induces the transcription of the zrfA and zrfB genes at the highest level under acidic zinc-limiting conditions, whereas under neutral and alkaline zinc-limiting conditions their expression is remarkably reduced (VicenteFranqueira et al., 2005). In spite of this, the expression level of both genes in alkaline zinc-limiting conditions could be high enough to play a role in pathogenesis. In contrast, ZafA induces the zrfC transcription exclusively under alkaline and extreme zinc-limiting conditions (Amich et al., 2010). To determine the relevance of all these genes in the pathobiology of *A. fumigatus*, the survival of non-leucopenic mice (NL-mice), was analysed in parallel with that of leucopenic mice (L-mice) inoculated with the wild-type, ΔzrfC, ΔzrfABC or ΔzrfABCΔzrfC strains using 10⁵ conidia per mouse (c m⁻¹) (Fig. 1). In both murine models of IPA, the survival rates of mice inoculated with the ΔzrfC mutant (45% in NL-mice and 23.8% in L-mice) were significantly higher than those of mice inoculated with the wild-type (AF54 > AF14), i.e. the deletion of zrfC significantly reduced the virulence of *A. fumigatus* (P < 0.0001). This reduction in virulence further increased in a ΔzrfAB background. Thus, the survival rates of NL- and L-mice inoculated with a ΔzrfABC strain were 100% and 76.9% respectively. In both murine models of IPA the virulence of the ΔzrfABC strain was restored at the wild-type level upon reintroduction of the zrfC gene (i.e. a ΔzrfABCΔzrfC strain showed a wild-type virulence). In concordance with this, a ΔzrfAB strain exhibited a wild-type virulence similar to that of a ΔzrfABCΔzrfC strain, as shown in an independent biossay in L-mice (Fig. S1). All these results indicated that the zrfC gene was essential for virulence in the absence of the zrfA and zrfB genes, whereas these genes were dispensable for virulence in the presence of zrfC. Therefore, zrfA and zrfB have an accessory role in virulence to that of zrfC. Intriguingly, the deletion of zrfC seemed to reduce
fungal virulence at a larger extent in NL- than in L-mice, which suggests that NL-mice somehow pose a more restricted environment regarding zinc availability.

Histological examinations of lung sections from L-mice inoculated with the wild-type and ΔzrfABC [zrfC] strains revealed a high degree of invasion around the lung airways, with hyphae that spread throughout the lung parenchyma and angioinvasion (Fig. 2, right panels). The lung tissue surrounding the affected areas was characterized by oedema and intra-alveolar haemorrhage. The great structural disorganization of the lung tissue caused by the fungal overgrowth might explain the sudden decrease in survival of L-mice inoculated with wild-type and ΔzrfABC [zrfC] strains between 4 and 5 days post infection. In contrast, the lung sections of L-mice inoculated with the ΔzrfC strain showed a paucity of conspicuous oedematous and necrotized areas with a low degree of fungal invasion and tissue damage. The lungs of L-mice inoculated with the ΔzrfABC strain showed a very low degree of fungal invasion. Nevertheless, germinated ΔzrfABC conidia were readily observed on the epithelia of the lung airways and within macrophages. The histopathological findings and the fungal burden of L-mice inoculated with each A. fumigatus strain correlated well with the survival rates (Table 1).

Unlike L-mice, lung sections from NL-mice inoculated with the wild-type, ΔzrfC or ΔzrfABC [zrfC] strain showed well defined infectious foci infiltrated with neutrophils and the absence of angioinvasion (Fig. 2, left panels). The infectious foci produced by the ΔzrfC strain showed a degree of fungal growth and neutrophil infiltration much lower than those produced by the wild-type and ΔzrfABC [zrfC] strains. Mice sacrificed after 15 days post infection with the ΔzrfABC strain showed weak signs of an inflammatory response and very few germinated conidia. The low fungal burden of the ΔzrfABC strain in the lungs is unlikely to be due to a defect in germination as the zrfA, zrfB and zrfC genes were not required for germination in vitro under alkaline zinc-limiting conditions (Fig. S2). However, the ΔzrfC and ΔzrfABC strains showed a defect in their capacities to grow onto the same media without a zinc supplement and solidified with agar, i.e. the SDN alkaline zinc-limiting and the BSD50 bovine serum agar media (Fig. S3). Taken these results together, we concluded that the low fungal burden of the ΔzrfC and ΔzrfABC strains in the lungs of immunosuppressed animals arises from a zinc shortage caused by a defect in zinc uptake during fungal growth, which slows down the growth of the ΔzrfC mutant and stops that of the ΔzrfABC germ tubes, as reported previously for a ΔzafA mutant (Moreno et al., 2007). In summary, the histopathological analysis of lungs from both L- and NL-mice were consistent with survival...
Fig. 2. Histopathological features of IPA in immunosuppressed mice. Sections of infected lungs from non-leucopenic mice (NL-mice, left panels) and leucopenic mice (L-mice, right panels) inoculated with the wild-type, $\Delta zrfC$, $\Delta zrfABC$ and $\Delta zrfABC[zrfC]$ strains. The yellow arrowhead indicates a conidium germinating inside a macrophage. NL-mice inoculated with the wild-type, $\Delta zrfC$ and the $\Delta zrfABC[zrfC]$ revertant strain were euthanized after 7 days post inoculation and after 15 days that inoculated with the $\Delta zrfABC$ strains. L-mice inoculated with the wild-type and revertant strains were euthanized after 4 days post inoculation. L-mice inoculated with the $\Delta zrfC$ and $\Delta zrfABC$ strains were euthanized after 6 days post inoculation. One lung from each euthanized mice was immediately excised and processed for histology. Tissues sections were stained with the GMS-HE stain. Hyphae appear as black filaments. Pictures were taken with a 20× objective (except that for L-mice infected with the $\Delta zrfABC$ strain that was taken with a 100× objective). Bars = 50 μm.

© 2013 John Wiley & Sons Ltd, Cellular Microbiology, 16, 548–564
The correlations between survival and fungal burden were \( r^2 = 0.959 \) (for CFU, \( P = 0.0006 \)) and \( r^2 = 0.708 \) (for GM, \( P = 0.035 \)). Survival is that after 15 days post-infection.

data and the relevance of the zrfA, zrfB and zrfC genes in the virulence of \( A. \) fumigatus.

The N-terminus of ZrfC is required for zinc uptake from the lung tissue

The zrfC gene plays a role more relevant for \( A. \) fumigatus virulence than the zrfA and zrfB genes. However, this was not unexpected because under alkaline zinc-limiting culture conditions (similar to that provided by the lung tissue) the expression of zrfC is higher than that of zrfA and zrfB (Amich et al., 2009; 2010). Thus, to investigate if the transcriptional profiles of these genes explained their different relevance in virulence, we exchanged the coding sequences between the zrfC and zrfB genes and analysed the growth capacity of fungal strains expressing the chimerical zrfB\(^{\Delta} \rightarrow\) zrfC\(^{\Delta} \) and zrfC\(^{\Delta} \rightarrow\) zrfB\(^{\Delta} \) genes in a \( \Delta zrfABC \) background (Fig. 3A). The transcription of these chimerical genes occurred as expected, i.e. zrfC expressed at its highest level under acidic conditions and at a lesser extent under alkaline conditions whereas zrfB only expressed in the alkaline medium (Fig. 3B). The expression of zrfB\(^{\Delta} \) driven by zrfC\(^{\Delta} \) and the expression of zrfC\(^{\Delta} \) driven by zrfB\(^{\Delta} \) did not enhance the fungal growth capacity under acidic zinc-limiting conditions (Fig. 3C). The growth capacity of a strain that expresses zrfC\(^{\Delta} \) driven by zrfB\(^{\Delta} \) was reduced drastically under alkaline zinc-limiting conditions compared with that of a strain expressing the wild-type zrfC gene (Fig. 3C). In contrast, the growth ability of a strain that expresses zrfB\(^{\Delta} \) driven by zrfC\(^{\Delta} \) was enhanced slightly under alkaline conditions compared with that of a strain that expresses the wild-type zrfB gene (Fig. 3C). These results indicated that zrfC\(^{\Delta} \) cannot promote the zinc uptake from acidic media whereas zrfB\(^{\Delta} \) can do so from alkaline media, although less efficiently than from acidic media. Besides, this is in perfect agreement with functional complementation studies of these proteins in a yeast model (Vicentefranqueira et al., 2005; Amich et al., 2010). Therefore, these results predicted that the transcriptional profiles of the zrfB and zrfC genes per se would not be sufficient to explain the profuse fungal growth capacity in the alkaline zinc-limiting environment of the lungs and, hence, in virulence. Consequently, the importance of zrfC in virulence should be based on structural features of the ZrfC protein that would enable ZrfC to scavenge and uptake \( \text{Zn}^{2+} \) specifically under alkaline and extreme zinc-limiting conditions.

The most obvious structural difference between ZrfC and ZrfA/ZrfB is that the former has a long N-terminus (\(~\)200 residues) with four putative zinc-binding motifs that extends towards the extracellular side of the membrane, which is absent in the ZrfA and ZrfB proteins (Amich et al., 2010). In addition, the growth ability under alkaline zinc-limiting conditions (i.e. with a 0–1 \( \mu \text{M Zn}^{2+} \) supplement) of a strain that expresses the zrfC\(^{\Delta} \) gene (i.e. a mutated version of the zrfC gene that encodes a ZrfC protein without its N-terminus) was more similar to the growth capacity of a strain that expresses zrfB\(^{\Delta} \) driven by the same promoter (i.e. zrfC\(^{\Delta} \)) than to that of a strain expressing the wild-type zrfC gene (Fig. S4). This indicated that the zinc uptake capacity of the ZrfC\(^{\Delta} \) protein might be more similar to that of the ZrfB protein than to the zinc uptake capacity of the wild-type ZrfC protein and, consequently, that the capacity of ZrfC to enhance both fungal growth under alkaline zinc-limiting conditions and virulence might largely depend on its N-terminus. Therefore, to investigate the role of the N-terminus of ZrfC in virulence, we inoculated leucopenic mice with a \( \Delta zrfABC[zrfC^{\Delta}] \) strain using our standard inoculum (i.e. \( 10^5 \text{ c m}^{-1} \)) and a low-dose inoculum (\( 10^4 \text{ c m}^{-1} \)) (Smith et al., 1993) (Fig. 4A). At first the use of a low-dose inoculum was primarily intended to detect any unexpected subtle differences in virulence between the \( \Delta zrfABC[zrfC] \) and \( \Delta zrfABC[zrfC^{\Delta}] \) strains. Nevertheless, as expected the survival rate of L-mice inoculated with the \( \Delta zrfABC[zrfC^{\Delta}] \) strain was significantly higher than that of mice inoculated with the \( \Delta zrfABC[zrfC] \) strain (AF791 \( > \) AF731, \( P = 0.008 \)). Interestingly, the survival rate of L-mice inoculated with a \( \Delta zrfABC[zrfC^{\Delta}] \) strain was similar to that of L-mice inoculated with a \( \Delta zrfC \) strain for each inoculum size (i.e. \(~\)50% survival for L-mice.
Fig. 3. Construction and phenotypic analysis of strains with exchanged coding sequences between the zrfB and zrfC genes. (A) Schematic representation of the construction of the derivative uridine–uracil-prototrophic PyrG⁺ A. fumigatus strains that harbour either the coding sequence of zrfB under the control of the zrfC promoter (AF771, zrfC→zrfB) or the coding sequence of zrfC under the control of the zrfB promoter (AF781, zrfB→zrfC) inserted between the AFUA_2G08360 (pyrG) and AFUA_2G08350 loci of the AF2511 uridine–uracil-auxotrophic pyrG1 strain. (B) Transcriptional analysis by Northern blot of the zrfB and zrfC genes in the strains AF771 and AF781 cultured in the acidic (SDA–Zn, pH 4.5) and alkaline (SDN–Zn, pH 7.5) zinc-limiting media for 20 h at 37°C. The expression of these genes was also analysed in the AF761 ΔzrfABC[zrfB] and AF731 ΔzrfABC[zrfC] strains as controls. The 5′-UTR regions of zrfB and zrfC have approximately 268 and 32 nucleotides, respectively (Vicentefranqueira et al., 2005; Amich et al., 2010). The size of transcripts is indicated in kb. The size of the zrfB transcript synthesized by the AF771 strain was lower than the wild-type zrfB transcript synthesized by AF761 because the former has the 5′-UTR region of zrfC which is ∼236 nucleotides shorter than the 5′-UTR of zrfB. In contrast, the size of the zrfC transcript synthesized by the AF781 strain was bigger than the wild-type zrfC transcript synthesized by AF731 because the former carries the 5′-UTR region of zrfB, which is ∼236 nucleotides longer than the 5′-UTR of zrfC. (C) Growth capacity of the AF771 and AF781 strains cultured on acidic (SDAE–Zn, pH 4.5) and alkaline (SDNE–Zn, pH 7.5) agar media supplemented with increasing amounts of Zn²⁺ (0–1000 μM). The AF761 and AF731 were included as controls. It could be possible that the 5′-UTR region of zrfB and/or zrfC in the chimerical zrfB→zrfC and zrfC→zrfB genes somehow interfere with the translation of the ZrfC and ZrfB proteins, respectively. However, this is unlikely as these 5′-UTRs lack alternative AUGs or any other known feature that could interfere with translation. The SDAE and SDNE agar plates inoculated with conidia were incubated at 37°C for 5 and 3 days, respectively, before pictures were taken.
Fig. 4. Function of the N-terminus of ZrFC in virulence. (A) Survival of leucopenic mice (L-mice) inoculated with $10^4$ conidia per mouse (open black symbols) of the wild-type, ΔzrfC, ΔzrfABC, ΔzrfABC[zrfC] and ΔzrfABC[zrfCΔN] strains and $10^5$ c m$^{-1}$ of the ΔzrfABC[zrfCΔN] strain (closed red symbols). The survival of L-mice inoculated with $10^5$ c m$^{-1}$ of the ΔzrfC strain (closed blue symbols) showed in Fig. 1 was also represented here for comparison purposes. Each survival curve represents the combined results of two independent experiments (20 mice per strain). Survival curves were created with the Prism 4.0 Software, using the product-limit method of Kaplan–Meier, and were compared using the log-rank test. No statistically significant differences were observed between the survival of L-mice inoculated with $10^4$ c m$^{-1}$ of the wild-type and ΔzrfABC[zrfC] revertant strain (AF14 ≈ AF731, $P = 0.838$), between the survival of L-mice inoculated with $10^5$ c m$^{-1}$ of the ΔzrfC and ΔzrfABC[zrfCΔN] strain (AF54 ≈ AF791, $P = 0.925$) and between the survival of L-mice inoculated with $10^5$ c m$^{-1}$ of the ΔzrfC and ΔzrfABC[zrfCΔN] strain (AF54 ≈ AF731, $P = 0.791$). In contrast, highly statistically significant differences were observed between the survival of L-mice inoculated with $10^4$ c m$^{-1}$ of the ΔzrfC and revertant or wild-type strain (AF54 > AF14 and AF54 > AF731, $P = 0.001$), between the survival of L-mice inoculated with the ΔzrfABC and ΔzrfC or ΔzrfABC[zrfCΔN] strain (AF721 > AF54 and AF721 > AF791, $P < 0.003$) and between the survival rate of L-mice inoculated with the ΔzrfABC[zrfCΔN] and ΔzrfABC[zrfC] strain (AF791 > AF731, $P = 0.008$).

(B) Histopathological features of lung sections from infected L-mice inoculated with $10^5$ c m$^{-1}$ of the wild-type, ΔzrfC, ΔzrfABC[zrfCΔN] and ΔzrfABC[zrfC] strains. All mice were euthanized after 5 days post inoculation. Tissue sections were stained with GMS-HE. Pictures were taken with a 10× objective. Bar = 50 μm.
Zinc and virulence in Aspergillus fumigatus

inoculated with 10⁴ c m⁻¹ and ~ 20% for L-mice inoculated with 10⁵ c m⁻¹). Accordingly, the lung sections from L-mice inoculated with a low-dose inoculum of the ΔzrfABC strain appeared healthy whereas that from L-mice inoculated with the ΔzrfC and ΔzrfABC[ΔzrfCΔ] strains showed a few number of conspicuous oedematous areas with a lower degree of fungal invasion and tissue damage than mice killed by the wild-type and ΔzrfABC[ΔzrfCΔ] strains (Fig. 4B). In summary, these results indicated that ZrfC with its N-terminus has lost its ability to scavenge and uptake zinc from living tissue as efficiently as the native ZrfC protein.

In addition, the nearly identical survival rates of L-mice inoculated with the ΔzrfC and ΔzrfABC[ΔzrfCΔ] strains pointed out that the zinc uptake activity of ZrfC without its N-terminus (in the ΔzrfABC[ΔzrfCΔ] strain) should equal that of the ZrfA and ZrfB together (in the ΔzrfC strain).

zrfC enables A. fumigatus to overcome the effect of calprotectin under Mn- and/or Zn-limiting conditions

Calprotectin is a Zn/Mn-chelating protein with antimicrobial properties that is released by infiltrated neutrophils in abscesses (Corbin et al., 2008). As shown before, NL-mice inoculated with the wild-type, ΔzrfC or ΔzrfABC[ΔzrfCΔ] strain showed well defined infectious foci infiltrated with neutrophils whereas L-mice did not show obvious neutrophil infiltrations (Fig. 2). Thus, it would be expected that the amount of calprotectin released by neutrophils around or within the infectious foci of L-mice were lower than that released in fungal abscesses of NL-mice. Indeed, we showed by immunocytochemistry (using an antibody raised against the calgranulin A subunit of mouse calprotectin) that calprotectin was detected in patches that localized co-ordinately with fungal abscesses in lung sections of NL-mice infected with a ΔzrfABC[ΔzrfCΔ] strain (Fig. 5, left I panels). In contrast, calprotectin was localized in scattered neutrophils throughout the whole lung sections of infected L-mice (Fig. 5, right I panels). As expected, the lung sections of non-inoculated NL-mice showed an amount of calprotectin (observed within neutrophils) > 10-fold higher than the non-inoculated L-mice (P > 0.0001) (Fig. 5, NI panels). The comparison of the increase rates in the amount of calprotectin between lung sections of non-inoculated and inoculated mice revealed surprisingly that the lung calprotectin content increases at a lesser extent in infected NL-mice (~ 12-fold) than in infected L-mice (~ 28-fold). Nevertheless, the gross amount of calprotectin in lung sections of NL-mice was significantly higher than that of L-mice infected with a ΔzrfABC[ΔzrfCΔ] strain (~ 5.6-fold; P = 0.0002) (Fig. 5). Therefore, given that the ΔzrfC and ΔzrfABC strains were less virulent in NL-mice than in L-mice and that the zinc transporter ZrfC is required for A. fumigatus to grow in living tissues, we wondered whether the zrfC gene enables A. fumigatus to grow in the presence of calprotectin (as it might occur in the fungal abscesses of NL-mice). Thus, we performed microculture-based bioassays for all strains in an alkaline Mn-replete but Zn-limiting medium (pH 7.2) in the presence of 2.0 μM recombinant human calprotectin (rhCP) to investigate the effect of calprotectin on the growth of A. fumigatus (Fig. 6A). Under these cultures conditions calprotectin inhibited significantly the growth capacity of the ΔzrfC strain (P = 0.0092), which showed a 2.6-fold reduction compared with the growth capacity in the absence of calprotectin (Fig. 6A, left panel). The growth capacity of the ΔzrfABC strain was not inhibited by calprotectin and was similar to that of the ΔzrfC strain in presence of calprotectin. The addition of a Zn²⁺ supplement not only increased the growth capacity of all strains but also suppressed the effect of calprotectin on the growth of the ΔzrfC strain (Fig. 6A, right panel). This indicated that the zrfC gene (but not the zrfA and zrfB genes) was able to counteract completely the inhibition of fungal growth by calprotectin under Zn-limiting conditions.

Additionally, given that calprotectin also binds manganese (Hayden et al., 2013), we also tested whether the zrfC gene was able to counteract the inhibition of fungal growth by calprotectin under Mn- and Zn-limiting conditions (Fig. 6B). Thus, we observed that calprotectin reduced by 2.2-fold the growth capacity of the wild-type strain and nearly abrogated the fungal growth capacity of a ΔzrfC null mutant (Fig. 6B, left panel). The addition of Zn²⁺ increased the overall growth capacity of all strains and suppressed completely the antifungal effect of calprotectin against the wild-type and ΔzrfABC[ΔzrfCΔ] strains (Fig. 6B, right panel). In contrast, a Zn²⁺ supplement did not restored completely the growth capacity of the ΔzrfC strain. This unexpected finding suggested that zrfC might play also a role in overcoming the effect of calprotectin under Mn-limiting conditions. However, under zinc-limiting conditions the ΔzrfC strain grew identical on both Mn-limiting and Mn-replete agar (Fig. S5). Therefore, if the ZrfC transporter is involved in manganese uptake under Zn-limiting conditions its overall contribution to this process must be very low. Lastly, we observed that the addition of Zn²⁺ and Mn²⁺ to ΔzrfC microcultures after 60 h of incubation in the presence of calprotectin restored the growth capacity of the ΔzrfC strain as in the absence of rhCP (P = 0.001). This indicated that calprotectin had a fungistatic effect against this strain (data not shown).

In summary, under the Mn- and Zn-limiting conditions used in our experiments the growth capacity of a wild-type strain of A. fumigatus was reduced about 50% in the presence of calprotectin compared with in the absence of calprotectin. However, the zrfC gene (but not zrfA and zrfB) enables A. fumigatus to overcome partially the static effect of calprotectin on fungal growth under
Fig. 5. Immunolocalization and quantification of calprotectin in lungs of immunosuppressed mice. Non-inoculated (NI panels), non-leucopenic and leucopenic mice (NL- and L-mice) and NL- and L-mice inoculated (I panels) with a ΔzfABCΔzfC strain (10⁵ c m⁻¹) were sacrificed after 4 days post infection. Consecutive lung sections were stained with the GMS-HE stain and immunocytochemically with a goat antibody against the A subunit of mouse calprotectin (α-CalA). The insets in the NI panels show in more detail the immunostained calprotectin in neutrophils that are not observed at low magnification. Lung sections from mice infected with the ΔzfABCΔzfC strain were also stained immunocytochemically with a goat isotype antibody as a control for the α-CalA antibody specificity. The per cent area of immunostained calprotectin (ISCP), which can be located in patches and/or within scattered neutrophils, was measured in the lung sections of five mice per condition using the ImageJ software. The per cent areas of ISCP in NL- and L-mice were represented and analysed statistically with the Prism 4.0 Software using the two-tailed, unpaired t-test. Only significant differences were indicated with asterisks (***P < 0.001, ****P < 0.0001). The increase ratios between the average per cent area measured in lung sections of inoculated (I) and non-inoculated mice (NI) immunostained with the α-CalA antibody are compiled in a little table. Pictures were taken with a 4× objective except those in the insets that were taken with a 20× objective. Bar = 1 mm (in the inset = 100 μm).
Mn- and Zn-limiting conditions, but completely under either Mn- or Zn-limiting conditions.

Discussion

One of the most critical aspects for pathogens to grow in a susceptible organism and cause disease is their ability to uptake nutrients from the host tissues. In this regard, we reported for the first time that the regulation of zinc homeostasis by the ZafA transcriptional regulator was essential for virulence in the pathogenic fungus *A. fumigatus* (Moreno *et al.*, 2007). In addition, in a previous work we anticipated that the *zrfC* gene of *A. fumigatus*, which encodes a new prototype of ZIP transporters exclusively distributed among fungi (Amich *et al.*, 2010), could be the main responsible for zinc uptake from host tissues. This assumption was based on two facts: (i) the *zrfC* gene is upregulated by the essential-for-virulence ZafA transcriptional activator exclusively under alkaline zinc-limiting conditions (i.e. that provided by host tissues); (ii) ZrfC has a long N-terminus towards the extracellular side of the plasma membrane that could be used for

© 2013 John Wiley & Sons Ltd, *Cellular Microbiology*, 16, 548–564
scavenging Zn$^{2+}$ from the lungs. In addition, the zrfA and zrfB genes could play a role in A. fumigatus virulence as they both are also upregulated by ZafA under alkaline zinc-limiting conditions, although at a lesser extent than in acidic zinc-limiting media (Vicentefranqueira et al., 2005). Nevertheless, the relevance of ZafA target genes that encode specific zinc transporters in fungal virulence remained to be proven. Therefore, we analysed the role of these genes in fungal virulence using mice subjected to both a leucopenic and non-leucopenic immunosuppressive regime that resemble those most frequently administered to patients at a highest risk of IPA. In addition, we also intended to ascertain whether the role of these genes in virulence was influenced by the immunosuppressive regime, as it may determine the pathobiology of A. fumigatus (Spikes et al., 2008; Kwon-Chung and Sugui, 2009) and the development, pathogenesis and histopathology of IPA (Berenguer et al., 1995; Balloy et al., 2005; Stephens-Romero et al., 2005). However, both murine models of IPA essentially raised the same conclusion about the role of these genes in the pathobiology of A. fumigatus, i.e. ZrfC is the major ZIP transporter used by A. fumigatus to uptake zinc from the lungs whereas ZrfA and ZrfB have an accessory although relevant role to fulfil the nutritional requirement of A. fumigatus growing in the lungs and, hence, in virulence. This conclusion is supported by the fact that the deletion of the zrfA and zrfB genes is compensated completely by zrfC (in the ΔzrfAB and ΔzrfABC[ΔzrfC] strains) whereas the deletion of zrfC is compensated partially by zrfA and zrfB (in a ΔzrfC strain). Indeed, the expression of the ZrfC transporter, which is adapted to function under alkaline conditions (Amich et al., 2010), might easily overcome the lack of the ZrfA and ZrfB transporters in the former strains. In contrast, it would be expected that the expression of the ZrfA and ZrfB transporters, which are adapted to uptake zinc from acidic media more efficiently than from alkaline media (Amich et al., 2010), is largely insufficient to compensate the absence of ZrfC in a ΔzrfC strain. However, a ΔzrfC strain is more virulent than expected if the zrfA and zrfB genes are dispensable for virulence, as shown. In this regard, we hypothesized that the attenuated virulence of a ΔzrfC strain could be due to an increase in the transcription level of zrfA and/or zrfB as part of a compensatory mechanism to alleviate the zinc shortage of this strain growing under the alkaline and extreme zinc-limiting conditions provided by the lung tissue. To test this hypothesis we measured and compared the transcription level of the zrfA and zrfB genes between the ΔzrfC and wild-type strain both grown in vitro under alkaline and extreme zinc-limiting conditions (Fig. S6). Interestingly, we showed that in a ΔzrfC background the expression of zrfA and zrfB increases approximately by 3.5- and 2-fold, respectively, compared with a wild-type strain. This finding is consistent with the notion that the attenuated virulence of a ΔzrfC strain might be due to the overexpression of their zrfA and zrfB genes when growing in the zinc-limiting environment of the lungs.

The special ability of ZrfC to function under alkaline zinc-limiting conditions largely depends on its N-terminus, which is absent in the acidic zinc transporters (ZrfA and ZrfB). Indeed, the ZrfCΔN protein enhances cell growth in a yeast model at a similar extent than the ZrfA and ZrfB proteins under alkaline zinc-limiting conditions (Amich et al., 2010). In addition, the N-terminus of ZrfC is required for virulence, which is consistent with the notion that the N-terminus of ZrfC could function in Zn$^{2+}$ scavenging from host tissues. Moreover, it could function in Zn$^{2+}$ scavenging in concert with other proteins such as Aspf2, which is an immunodominant antigen secreted by A. fumigatus (Banerjee et al., 1998; Segurado et al., 1999) that could bind zinc and whose expression is also upregulated by ZafA (Amich et al., 2010). In this regard, a similar mode of action has been proposed for the Pra1 and Zrt1 proteins of Candida albicans (Citiulo et al., 2012), which are the Aspf2 and ZrfC orthologues respectively. However, the deletion of zrfC does not influence the aspf2 expression and the deletion of aspf2 has no impact either on the zrfC expression or A. fumigatus virulence (J. Amich, unpubl. data). Thus, further investigations will be required to establish the relevance of a putative ZrfC-Aspf2 interaction in the ZrfC and/or Aspf2 functionality and its connection to the pathobiology of A. fumigatus.

Calprotectin is a heterodimeric protein synthesized constitutively at high levels by neutrophils (Striz and Trebichavsky, 2004). It binds two metal ions (either 2 Zn$^{2+}$ or 1 Zn$^{2+}$ plus 1 Mn$^{2+}$) with high affinity (Körndörfer et al., 2007; Hayden et al., 2013), which may deplete zinc and manganese in culture media and, in turn, inhibit microbial growth in vitro (Steinbakk et al., 1990; Miyasaki et al., 1993; Sohnie et al., 1996; 2000; Corbin et al., 2008; Bianchi et al., 2011; Liu et al., 2012). To inhibit microbial growth in vivo calprotectin is released in abscesses by neutrophils through the formation of neutrophil extracellular traps (NETs) (Fuchs et al., 2007; Urban et al., 2009). Like most pathogens, Aspergillus also triggers the release of NETs by neutrophils both in vitro and in vivo (Bruns et al., 2010; McCormick et al., 2010; Bianchi et al., 2011). However, so far it has not been proven whether calprotectin inhibits the growth of A. fumigatus as it does that of A. nidulans (Bianchi et al., 2011). Here we show that calprotectin does inhibit the growth capacity of A. fumigatus in vitro and that zrfC enables the fungus to overcome the effect of calprotectin under Zn- and/or Mn-limiting conditions. However, there are some striking differences between the effect of calprotectin on the growth of A. fumigatus and that
Zinc and virulence in Aspergillus fumigatus

Table 2. Aspergillus fumigatus strains used in this study.

| Strain | Detailed genotype | Short genotype | Reference |
|--------|-------------------|----------------|-----------|
| AF14   | wild-type         | wt             | Vicentefranqueira et al, 2005 |
| AF15   | ΔzfA::neo ΔzrfB::hisG pyrG1 (PyrG–) | ΔzfA             | Vicentefranqueira et al, 2005 |
| AF48*  | ΔzfA::neo ΔzrfB::hisG | ΔzfA             | This study |
| AF52   | ΔzrfC::lac pyrG1 (PyrG–) | ΔzrfC             | Amich et al, 2010 |
| AF54*  | ΔzrfC::lac | ΔzrfC             | This study |
| AF2511 | ΔzrfA::neo ΔzrfB::hisG ΔzrfC::lac pyrG1 (PyrG–) | ΔzrfABC         | Amich et al, 2010 |
| AF721* | ΔzrfA::neo ΔzrfB::hisG ΔzrfC::lac | ΔzrfABC          | This study |
| AF731* | ΔzrfA::neo ΔzrfB::hisG ΔzrfC::lac | ΔzrfABC ΔzrfC | Amich et al, 2010 |
| AF791* | ΔzrfA::neo ΔzrfB::hisG ΔzrfC::lac [ΔzfC::lac] | ΔzrfABC ΔzrfC | Amich et al, 2010 |
| AF761* | ΔzrfA::neo ΔzrfB::hisG ΔzrfC::lac [ΔzfB::lac] | ΔzrfABC ΔzrfB | Amich et al, 2010 |
| AF771* | ΔzrfA::neo ΔzrfB::hisG ΔzrfC::lac [ΔzfC::lac] | ΔzrfABC(CΔzfB) | This study |
| AF781* | ΔzrfA::neo ΔzrfB::hisG ΔzrfC::lac [ΔzfB::lac] | ΔzrfABC(BΔzfC) | This study |
| AF171* | ΔzrfA::hisG | ΔzrfA    | Moreno et al, 2007 |

a. Mutant strains labeled with an asterisk are isogenic to the wild-type strain for the pyrG gene.
b. Genes in brackets were reintroduced into the mutant strains labeled with an asterisk are isogenic to the wild-type strain for the pyrG gene.

to counteract the increase in ROS production under the long extracellular N-terminus similar to ZrfC, have been noted that some ZIP proteins of the LZT family (Taylor and Nicholson, 2003), also characterized by having a shorter N-terminus with one less putative zinc-binding motif than the ZrfC protein from A. fumigatus (data not shown). However, it is also possible that the different susceptibility of these fungi to calprotectin could be explained by the distinct culture conditions and/or experimental procedures used for the in vitro bioassays.

An unexpected finding of this work is that the ZrfC transporter could play also a role in manganese uptake under Mn-limiting conditions. In this regard, it is worth noting that some ZIP proteins of the LZT family (Taylor and Nicholson, 2003), also characterized by having a long extracellular N-terminus similar to ZrfC, have been reported to uptake both Zn2+ and Mn2+ (Himeno et al., 2009). Moreover, given the importance of Mn2+ for oxidative stress management, it is tempting to speculate about a role of ZrfC in manganese uptake as a mechanism to counteract the increase in ROS production under the extreme Zn-limiting conditions imposed by calprotectin (Jukubovic et al., 2002; Kehl-Fie et al., 2011).

Many different physiological aspects from both the host and fungus determine the development and pathophysiology of IPA. For instance, NL-mice show an exacerbated and sustained inflammatory response to A. fumigatus hyphae compared with L-mice (Balloy et al., 2005). This response may prevent fungal invasion in NL-mice, e.g. through the formation of NETs (Bruns et al., 2010; McCormick et al., 2010), but causes a respiratory distress that may lead to death (Balloy et al., 2005). We have observed that, in terms of survival, the deletion of zrfC seemed to reduce fungal virulence at a larger extent in NL- than in L-mice. This observation led us to postulate that calprotectin released by neutrophils in fungal abscesses of NL-mice may create extremely Zn- and Mn-limiting microenvironments. In these conditions, the fungus could manage to grow using the ZrfC protein for Zn2+ scavenging and uptake by overcoming the Zn/Mn-chelating activity of calprotectin. In contrast, the low amount (or complete absence) of calprotectin released in the infectious foci of L-mice would allow the fungus to use ZrfC to uptake zinc more efficiently resulting in a profuse and extended fungal growth. The demonstration of this hypothesis will surely improve our understanding of the IPA pathogenesis. In either case, our findings about the mechanisms used by A. fumigatus to obtain zinc from the host expands our knowledge about the pathobiology of A. fumigatus and might anticipate the development of new antifungal therapies to treat IPA based on inhibiting fungal zinc uptake.

Experimental procedures

Strains, growth media and culture conditions

All fungal strains used in this work are listed in Table 2. All strains were routinely grown in PDA complex medium (20 g l−1 potato dextrose agar, 20 g l−1 sucrose, 2.5 g l−1 MgSO4–7H2O, 1 ml l−1 Hutmér’s trace element solution 1000×) (Amich et al., 2009).

The acidic and alkaline zinc-limiting liquid (SDA–Zn, pH 4.5 and SDN–Zn, pH 7.5) and agar (SDAE–Zn, pH 4.5 and SDNE–Zn, pH 7.5) media were prepared as described previously (Amich et al., 2010). The alkaline BSD10 and BSD50 media (pH 7.5) are complex media than contains 0.1 g l−1 dextrose and 10% or 50% (v/v) bovine serum (Cat. No. 16170-078, Invitrogen). The BSD plates were solidified with 2% agar. The BSD media are not...
zinc-limiting media as bovine serum usually contains > 10 μM zinc (Yokus and Cakir, 2006). Agar media were spotted with 10^3 conidia per strain. Liquid media were inoculated to a density of 5 × 10^5 spores ml^-1 and incubated at 37°C with shaking at 200 rpm.

The fungal growth capacity in microcultures was tested in vitro in the liquid synthetic dextrose nitrate–Zn–Mn medium (SDN–Zn–Mn, pH 7.2) (20 g l^-1 dextrose, 3 g l^-1 NaNO3, 1 g l^-1 KH2PO4, 0.5 g l^-1 MgSO4–7H2O, 5 ml l^-1 vitamin solution 100x [RPML-1640, Sigma], 1.0 ml l^-1 YNB–Zn–Mn trace element solution 1000x [50 g l^-1 NaCl, 14.7 g l^-1 CaCl2–2H2O, 0.5 g l^-1 BOH, 0.1 g l^-1 IK, 1.4 g l^-1 FeSO4–7H2O, 0.4 g l^-1 CuSO4–5H2O, 0.72 g l^-1 Na2MoO4–2H2O]). Media were supplemented with Zn^2+ and/or Mn^2+ using sterile stock solutions of 1.0 mM ZnSO4–7H2O or MnSO4–H2O in ultrapure water. The concentration of total zinc and total manganese in the liquid SDN–Zn–Mn medium was 1.15 ± 0.3 μM and 0.8 ± 0.1 μM, respectively, as determined by inductively coupled mass spectrometry in a Perkin-Elmer Elan 6000 ICP-MS instrument using mineralized samples of this medium and the appropriate standards for calibration.

**Standard molecular biology procedures**

DNA manipulations were performed following standard molecular biology protocols (Sambrook and Russell, 2001). The transforming DNA used to construct the AF771 and AF781 strains was obtained from plasmids pZRF28 and pZRF310 respectively. The pZRF28 plasmid was designed and constructed to reintroduce the coding sequence of zrfB under the control of the zrfC promoter at the pyrG locus of the A. fumigatus AF2511 strain. More precisely, the chimerical zrfCChim–zrfBChim gene was targeted at few nucleotides downstream from the pyrG3′–622 mutant gene encodes a PyrG protein in A. niger (Moreno et al., 2007). To generate the AF54, AF48 and AF721 strains respectively, these strains were transformed with a DNA fragment that carries the zrfC gene to generate a prototrophic and isogenic zrfC-reconstituted strain (zrfABC[zrfC]), as described previously (Amich et al., 2010). The prototrophic and isogenic zrfB-reconstituted strain (zrfABC[zrfB], AF761) and the zrfC13–622-reconstituted strain (zrfABC[zrfC]13–622, AF791) were constructed as described previously (Amich et al., 2010). The zrfC13–622 mutant gene encodes a ZnC protein without its N-terminus (ZnC13–622). The AF771 and AF781 strains were constructed respectively by reintroducing the coding sequence of zrfB driven by the zrfC promoter (zrfC → zrfB) and the coding sequence of zrfC driven by the zrfB promoter (zrfB → zrfC) at the pyrG locus of the AF2511 strain by using the same strategy to construct the AF731, AF761 and AF791 strains. Genomic DNA obtained from several independent PyrG isolates of these strains was analysed by Southern blot to verify that these strains harboured the correct integration event at the expected locus.

**Expression, purification and reconstitution of calprotectin**

Human calprotectin (hCP) is made up of two proteins (calgranulin A and B) associated non-covalently (Korndörfer et al., 2007). To obtain a biologically functional recombinant hCP (rhCP), the hCalA and hCalB polypeptides were expressed in Escherichia coli (BL21-DE3) from plasmids pET28-rhCalA and pTAC2-rhCalB. The proteins were purified by affinity to a Ni-NTA resin, concentrated with Amicon Ultra-15 3K units and dialysed step-wise against the renaturation buffer [100 mM glycine (pH 7.5), 1 mM EDTA, 1 mM EGTA, 2 mM DTT]. The purity of the proteins was > 98% as assessed by SDS-PAGE.

The rhCP was reconstituted following a denaturing/renaturing step protocol to obtain an heterodimer with identical biophysical properties as calprotectin purified directly from PMNs (Vogl et al., 2006). The rhCP solution (28.5 μM) was stored in aliquots at −20°C in calprotectin buffer [20 mM Tris–HCl (pH 7.5), 50 μM EDTA, 50 μM EGTA, 0.2 mM DTT].
Bioassay of the effect of calprotectin on fungal growth in microcultures

Microcultures were set up in 20 ml screw-top glass vials. The vials were washed thoroughly with ultrapure water and sterilized at 170°C. To set up a microculture 1.0 ml of the SDN–/−/Zn medium, 0.36 ml of ultrapure sterile water and 0.14 ml of either calprotectin buffer or reconstituted 28.5 μM rhCP solution were added and mixed gently. Each vial was inoculated with 10^6 conidia. For each strain and culture condition three vials were inoculated and incubated unshaken for 60 h in a humid atmosphere at 37°C. The mycelium was harvested by filtration through GFC filters, dried at 70°C and weighted.

Virulence assays

The animals were housed, cared for and used for experimentation in accordance with the current European (Directive 2010/63/EU of the European Parliament and of the Council) and Spanish (BOE-A-2005–17344) animal welfare regulations. All animal experiments were performed according to the CBA PA45_2011-v2 protocol approved by the ‘Comité de Ética para Investigación y Bienestar Animal’ from the Instituto de Salud Carlos III (ISCIII, Spain). This committee did not raise any concerns and approved our study.

SPF-CD1 male mice (~25 g each) were grouped in cohorts of 10 mice. Spores were harvested from PDA plates and washed twice with a saline buffer plus 0.01% Tween-20. Two different immunosuppressive regimens were used: (i) a cyclophosphamide/cortisone-based treatment, which causes a depletion of circulating white blood cells (i.e. a leucopenic regime), and (ii) a corticosteroid-based treatment (i.e. a non-leucopenic regime). Mice subjected to the leucopenic regime were immunosuppressed on days −3 and −1 before inoculation with cyclophosphamide (150 mg kg⁻¹) and cortisone 21-acetate (112 mg kg⁻¹). Afterwards only cyclophosphamide (150 mg kg⁻¹) was administered on day +3, +6, +9 and +12 after inoculation. Mice subjected to the non-leucopenic regimen were immunosuppressed on days −3 and −1 with cortisone 21-acetate (380 mg kg⁻¹). Later on day +3 it was administered one extra dose of cortisone (190 mg kg⁻¹). Before infection, each mouse was anesthetized with 0.1 ml of a mix of ketamine plus xylazine. Each mouse was inoculated intranasally with 30 μl of a suspension with 6.5 × 10^6 conidia ml⁻¹ (or 6.5 × 10^5 conidia ml⁻¹) to ensure that during the instillation process each mice is inoculated with no less than 10^5 conidia (or 10^4 conidia). Non-inoculated control immunosuppressed mice only received saline buffer. Mice were euthanized to avoid suffering when they developed symptoms of severe respiratory distress.

Histology and immunocytochemistry

One lung from each mouse was fixed in 10 ml of 10% neutral buffered formalin for 6 h at room temperature and then transferred to 70% ethanol. Each lung was embedded in paraffin and cut into 4 μm thick sections that were stained with the Grocott’s methenamine-silver nitrate (GMS) procedure, followed by a counterstain with H&E (GMS-HE stain) as described elsewhere (Huppert et al., 1978). To detect calprotectin was used an indirect immunocytochemical method with the avidin–biotin–peroxidase complex procedure (Hsu et al., 1981), using a goat anti-mouse calgranulin A antibody (C1033-50E US Biologicals) or a goat isotype control (Thermo Scientific, Cat. 31871) (both at a 1:500 dilution) as the primary antibody and a biotinylated rabbit anti-goat antibody as the secondary antibody.

High quality images of immunostained lung sections taken with a 4× objective were analysed using the ImageJ 1.47v software (http://imagej.nih.gov/ij/) to measure the per cent area of immunostained calprotectin. The green channel of split images, which provided the best separation, was used for the threshold process. The lower and upper threshold levels were set respectively at the values of 0 and 130 for all images.

Determination of fungal burden

One lung from each mouse was weighed, homogenized in PBS and filtered through 100 μm Nylon filters. Each homogenate was used to determine the fungal burden by two different procedures: counting the colony-forming units on PDA plates and measuring the amount of galactomannan per gram of lung tissue using a quantitative GM enzyme immunoassay (PlateliaTM GM-EIA kit) according to the manufacturer’s instructions (Sheppard et al., 2006).

Acknowledgements

We wish to thank Belinda Rodriguez for her excellent technical assistance, Thomas Vogl (Institute of Immunology, Muenster, Germany) and Constantin F. Urban (Molecular Biology Department, Umeå University, Sweden) for providing plasmids for the expression of calprotectin and Mercedes Garayoa (Center for Cancer Research, Salamanca, Spain) for the meticulous examination of lung sections under the light microscope and photographic report. This work was supported by the Ministerio de Ciencia e Innovación (Spain) through grant BFU2010–22172/BMC to J.A.C. and the ERA-NET Pathogenomics grant BFU2008–04709-EBMC to E.M. ARC was supported through the EYE/962/2010 training program in scientific research funded by the Junta de Castilla y León (Spain).

References

Amich, J., Leal, F., and Calera, J.A. (2009) Repression of the acid ZrfA/ZrfB zinc-uptake system of Aspergillus fumigatus mediated by PacC under neutral, zinc-limiting conditions. Int Microbiol 12: 39–47.

Amich, J., Vicentefranqueira, R., Leal, F., and Calera, J.A. (2010) Aspergillus fumigatus survival in alkaline and extreme zinc-limiting environments relies on the induction of a zinc homeostasis system encoded by the zrfC and aspf2 genes. Eukaryot Cell 9: 424–437.

Askew, D.S. (2008) Aspergillus fumigatus: virulence genes in a street-smart mold. Curr Opin Microbiol 11: 331–337.

Balloy, V., Huerrer, M., Latge, J.P., and Chignard, M. (2005) Differences in patterns of infection and inflammation for corticosteroid treatment and chemotherapy in experimental invasive pulmonary aspergillosis. Infect Immun 73: 494–503.

Banerjee, B., Greenberger, P.A., Fink, J.N., and Kurup, V.P. (1998) Immunological characterization of Asp f 2, a major
allergen from *Aspergillus fumigatus* associated with allergic bronchopulmonary aspergillosis. *Infect Immun* **66**: 5175–5182.

Berenguer, J., Allende, M.C., Lee, J.W., Garrett, K., Lyman, C., Ali, N.M., *et al.* (1995) Pathogenesis of pulmonary aspergillosis. Granulocytopenia versus cyclosporine and methylprednisolone-induced immunosuppression. *Am J Respir Crit Care Med* **152**: 1079–1086.

Bianchi, M., Niemiec, M.J., Siler, U., Urban, C.F., and Eide, D.J. (2001) Eukaryotic zinc transporters in cadmium and manganese transport in mammalian cells. *J Cell Biol* **152**: 679–686.

Bignell, E., Negrete-Urtasun, S., Calcagno, A.M., Haynes, K., Arst, H.N., Jr, and Rogers, T. (2005) The *Aspergillus* pH-responsive transcription factor PacC regulates virulence. *Mol Microbiol* **55**: 1072–1084.

Bruns, S., Kniemeyer, O., Hasenberg, M., Alimanianda, V., Nietzsche, S., Thywissen, A., *et al.* (2010) Production of extracellular traps against *Aspergillus fumigatus in vitro* and in infected lung tissue is dependent on invading neutrophils and influenced by hydrophobin RodA. *PLoS Pathog* **6**: e1000873.

Calera, J.A., and Haas, H. (2009) Cations (Zn, Fe). In *Aspergillus fumigatus and Aspergillosis*. Latgé, J.P., and Steinbach, W.J. (eds). Washington, DC: ASM press, pp. 107–129.

Citiulo, F., Jacobsen, I.D., Miramon, P., Schild, L., Brunke, S., Zipfel, P., *et al.* (2012) *Candida albicans* scavenges host zinc via Pra1 during endothelial invasion. *PLoS Pathog* **8**: e1002777.

Corbin, B.D., Seeley, E.H., Raab, A., Feldmann, J., Miller, M.R., Torres, V.J., *et al.* (2008) Metal chelation and inhibition of bacterial growth in tissue abscesses. *Science* **319**: 962–965.

Dagenais, T.R., and Keller, N.P. (2009) Pathogenesis of *Aspergillus fumigatus* in Invasive Aspergillosis. *Clin Microbiol Rev* **22**: 447–465.

Foote, J.W., and Delves, H.T. (1984) Albumin bound and alpha 2-macroglobulin bound zinc concentrations in the sera of healthy adults. *J Clin Pathol* **37**: 1050–1054.

Foote, J.W., and Delves, H.T. (1988) Determination of non-protein-bound zinc in human serum using ultrafiltration and atomic absorption spectrometry with electrothermal atomization. *Analyst* **113**: 911–915.

Fuchs, T.A., Abed, U., Goosmann, C., Hurwitz, R., Schulze, I., Wahn, V., *et al.* (2007) Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol* **176**: 231–241.

Gaithler, L.A., and Eide, D.J. (2001) Eukaryotic zinc transporters and their regulation. *Biometals* **14**: 251–270.

Hayden, J.A., Brophy, M.B., Cunden, L.S., and Nolan, E.M. (2013) High-affinity manganese coordination by human calprotectin is calcium-dependent and requires the histidine-rich site formed at the dimer interface. *J Am Chem Soc* **135**: 775–787.

Himeno, S., Yanagiya, T., and Fujishiro, H. (2009) The role of zinc transporters in cadmium and manganese transport in mammalian cells. *Biochimie* **91**: 1218–1222.

Hsu, S.M., Raine, L., and Fanger, H. (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem Cytochem* **29**: 577–580.

Huppert, M., Oliver, D.J., and Sun, S.H. (1978) Combined methenamine-silver nitrate and hematoxylin & eosin stain for fungi in tissues. *J Clin Microbiol* **8**: 598–603.

Iyengar, V., and Wottiez, J. (1988) Trace elements in human clinical specimens: evaluation of literature data to identify reference values. *Clin Chem* **34**: 474–481.

Jakubovics, N.S., Smith, A.W., and Jenkinson, H.F. (2002) Oxidative stress tolerance is manganese (Mn(2+)) regulated in *Streptococcus gordonii*. *Microbiology* **148**: 3255–3263.

Kehl-Fie, T.E., Chitayat, S., Hood, M.I., Damo, S., Restrepo, N., Garcia, C., *et al.* (2011) Nutrient metal sequestration by calprotectin inhibits bacterial superoxide defense, enhancing neutrophil killing of *Staphylococcus aureus*. *Cell Host Microbe* **10**: 158–164.

Komhdörfer, I.P., Brueckner, F., and Skerra, A. (2007) The crystal structure of the human (S100A8/S100A9)2 heterotetramer, calprotectin, illustrates how conformational changes of interacting alpha-helices can determine specific association of two EF-hand proteins. *J Mol Biol* **370**: 887–898.

Kousha, M., Tadi, R., and Soubani, A.O. (2011) Pulmonary aspergillosis: a clinical review. *Eur Respir Rev* **20**: 156–174.

Kwon-Chung, K.J., and Sugui, J.A. (2009) What do we know about the role of glio-toxin in the pathobiology of *Aspergillus fumigatus*? *Med Mycol* **47** (Suppl. 1): S97–S103.

Lech, T., and Sadlik, J.K. (2011) Zinc in postmortem body tissues and fluids. *Biol Trace Elem Res* **142**: 11–17.

Liu, J.Z., Jellbauer, S., Poe, A.J., Ton, V., Pesciaroli, M., Kehl-Fie, T.E., *et al.* (2012) Zinc sequestration by the neutrophil protein calprotectin enhances *Salmonella* growth in the inflamed gut. *Cell Host Microbe* **11**: 227–239.

McCormick, A., Heesemann, L., Wagener, J., Marcos, V., Hartl, D., Loeffler, J., *et al.* (2010) NETs formed by human neutrophils inhibit growth of the pathogenic mold *Aspergillus fumigatus*. *Microbes Infect* **12**: 928–936.

Miyasaki, K.T., Bodeau, A.L., Murthy, A.R., and Lehrer, R.I. (1993) *In vitro* antimicrobial activity of the human neutrophil cytotoxic S100 protein complex, calprotectin, against *Capnocytophaga sputigena*. *J Dent Res* **72**: 517–523.

Moreno, M.A., Ibrahim-Granet, O., Vicentefranqueira, R., Amich, J., Ave, P., Leal, F., *et al.* (2007) The regulation of zinc homeostasis by the ZafA transcriptional activator is essential for *Aspergillus fumigatus* virulence. *Mol Microbiol* **64**: 1182–1197.

Sambrook, J., and Russell, D.W. (2001) *Molecular Cloning. A laboratory manual*. Cold Spring, NY: Cold Spring Harbor Laboratory Press.

Sandrin, T.R., and Maier, R.M. (2003) Impact of metals on the biodegradation of organic pollutants. *Environ Health Perspect* **111**: 1093–1101.

Segurado, M., López-Aragon, R., Calera, J.A., Fernández-Abalos, J.M., and Leal, F. (1999) Zinc-regulated biosynthesis of immunodominant antigens from *Aspergillus* spp. *Infect Immun* **67**: 2377–2382.
Sheppard, D.C., Marr, K.A., Fredricks, D.N., Chiang, L.Y., Doedt, T., and Filler, S.G. (2006) Comparison of three methodologies for the determination of pulmonary fungal burden in experimental murine aspergillosis. Clin Microbiol Infect 12: 376–380.

Smith, J.M., Davies, J.E., and Holden, D.W. (1993) Construction and pathogenicity of Aspergillus fumigatus mutants that do not produce the ribotoxin restrictocin. Mol Microbiol 9: 1071–1077.

Sohnle, P.G., Hahn, B.L., and Santhanagopalan, V. (1996) Inhibition of Candida albicans growth by calprotectin in the absence of direct contact with the organisms. J Infect Dis 174: 1369–1372.

Sohnle, P.G., Hunter, M.J., Hahn, B., and Chazin, W.J. (2000) Zinc-reversible antimicrobial activity of recombinant calprotectin (migration inhibitory factor-related proteins 8 and 14). J Infect Dis 182: 1272–1275.

Spikes, S., Xu, R., Nguyen, C.K., Chamilos, G., Kontoyiannis, D.P., Jacobson, R.H., et al. (2008) Gliotoxin production in Aspergillus fumigatus contributes to host-specific differences in virulence. J Infect Dis 197: 479–486.

Steinbakk, M., Naess-Andresen, C.F., Lingaas, E., Dale, I., Brandtzaeg, P., and Fagerhol, M.K. (1990) Antimicrobial actions of calcium binding leucocyte L1 protein, calprotectin. Lancet 336: 763–765.

Stephens-Romero, S.D., Mednick, A.J., and Feldmesser, M. (2005) The pathogenesis of fatal outcome in murine pulmonary aspergillosis depends on the neutrophil depletion strategy. Infect Immun 73: 114–125.

Striz, I., and Trebivcksky, I. (2004) Calprotectin – a pleiotropic molecule in acute and chronic inflammation. Physiol Res 53: 245–253.

Taylor, K.M., and Nicholson, R.I. (2003) The LZT proteins; the pleiotropic molecule in acute and chronic inflammation. Physiol Res 53: 245–253.

Taylor, K.M., and Nicholson, R.I. (2003) The LZT proteins; the pleiotropic molecule in acute and chronic inflammation. Physiol Res 53: 245–253.

Tekaia, F., and Latge, J.P. (2005) Aspergillus fumigatus: saprophyte or pathogen? Curr Opin Microbiol 8: 385–392.

Urban, C.F., Ermert, D., Schmid, M., Abu-Abed, U., Goosmann, C., Nacken, W., et al. (2009) Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans. PLoS Pathog 5: e1000639.

Vencenftfrenauer, R., Moreno, M.A., Leal, F., and Calera, J.A. (2005) The zrfA and zrfB genes of Aspergillus fumigatus encode the zinc transporter proteins of a zinc uptake system induced in an acid, zinc-depleted environment. Eukaryot Cell 4: 837–848.

Vogl, T., Leukert, N., Barczyk, K., Strupat, K., and Roth, J. (2006) Biophysical characterization of S100A8 and S100A9 in the absence and presence of bivalent cations. Biochim Biophys Acta 1763: 1298–1306.

Weidner, G., d’Enfert, C., Koch, A., Mol, P.C., and Brakhage, A.A. (1998) Development of a homologous transformation system for the human pathogenic fungus Aspergillus fumigatus based on the pyrG gene encoding orotidine 5’-monophosphate decarboxylase. Curr Genet 33: 378–385.

Yokus, B., and Cakir, U.D. (2006) Seasonal and physiological variations in serum chemistry and mineral concentrations in cattle. Biol Trace Elem Res 109: 255–266.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Fig. S1. Virulence of a ΔzrfAΔzrfB strain. Survival of leucopenic mice inoculated with 10^5 c m^-1 of the ΔzrfAB and ΔzrfABCzrfC strains. Each survival curve represents the combined results of two independent experiments (20 mice per strain). Survival curves were created with the Prism 4.0 Software, using the product-limit method of Kaplan–Meier, and were compared using the log-rank test. No statistically significant differences were observed between mice survival (AF48 = AF731, P = 0.485).

Fig. S2. Germination rate of conidia in vitro. The germination rates of the wild-type, ΔzrfC, ΔzrfABC and ΔzrfABCzrfC conidia were measured both in the alkaline SDN zinc-limiting medium (pH 7.5) and in the BSD50 medium (pH 7.5). The germination rate of ΔzrfA conidia, which have a defect in germination under acidic zinc-limiting conditions (Moreno et al., 2007), was also measured in these media. Conidial germination was followed by microscopic observations of culture samples taken after 5, 6, 7, 8, 9 and 10 h of incubation in liquid media inoculated to a density of 5 × 10^6 spores ml^-1 and incubated at 37°C with shaking at 200 rpm. It was considered that a conidium had germinated when the length of the germ tube reached at least half of the diameter of a swollen conidium. To obtain more accurate counts of germinated conidia, aggregates were dispersed by sonication before they were counted on a haemocytometer. No changes in the germination rates were observed after 8 h. All strains exhibited germination rates > 95% in the BSD50 medium, which provided a good control for conidial viability. The germination rates were expressed as the mean ± standard error of the mean (SEM) from three independent experiments, each with three independent counts for each strain and time-point. Graphs were created and data analyzed statistically using Prism 4.0 software, using a two-way ANOVA test followed by a Bonferroni post-test with the wild-type germination rate as reference. Asterisks indicate a significance of P < 0.05 (*) or P < 0.001 (**); ns, not significant. The box at the bottom of the figure shows the colour code used to identify the fungal strain.

Fig. S3. Growth of fungal strains used in virulence studies.

A. Growth of the wild-type, ΔzrfC, ΔzrfAB, ΔzrfABC, ΔzrfABCzrfC and ΔzrfABCzrfC^BC strains onto acidic (SDAE–Zn, pH 4.5) and alkaline (SDNE–Zn, pH 7.5) agar supplemented with increasing amounts of Zn^2+ (0–1000 μM). All plates were spotted with 10^3 conidia per strain. The BSD10 and SDNE agar plates were incubated for three days before pictures were taken. The BSD50 and SDAE agar plates were incubated for five days.

B. Growth of the same strains onto alkaline BSD10 and BSD50 agar (pH 7.5) and in these media with a supplement of 100 and 1000 μM Zn^2+. All plates were spotted with 10^3 conidia per strain. The BSD10 and SDNE agar plates were incubated for three days at 37°C before pictures were taken. The BSD50 and SDAE agar plates were incubated for five days.

Fig. S4. Comparison of the effect of the zrfB and zrfC^BC genes on fungal growth under alkaline zinc-limiting conditions. The ΔzrfABCzrfC^BC and ΔzrfABCzrfC^BC^BC strains were cultured onto alkaline agar (SDNE–Zn, pH 7.5) supplemented with increasing amounts of Zn^2+ (0–1000 μM). Note that the zrfC promoter drives the expression of both the zrfC^BC and zrfB coding sequences in AF791 and AF771, respectively. The ΔzrfABCzrfC and ΔzrfABCzrfB strains were included as controls. The SDNE agar plates were spotted with 10^3 conidia per strain and incubated at 37°C in a humid atmosphere for three days, respectively, before pictures were taken.
Fig. S5. Effect of manganese on the growth ability of a ΔzrfC mutant depending on zinc availability. The ΔzrfC and wild-type strains were cultured onto alkaline manganese/zinc-limiting agar (i.e. SDN–Mn–Zn plus 250 μM EDTA and solidified with 2% agar, pH 7.2) supplemented with increasing amounts of Zn²⁺ (0–1000 μM) and 2 μM Mn²⁺ (+ Mn) or without Mn²⁺ (−Mn). Plates were spotted with 10⁵ conidia per strain and incubated at 37°C in a humid atmosphere for three days before pictures were taken. As shown, the growth capacity of a ΔzrfC strain is not affected apparently under Mn-limiting conditions regardless of zinc availability.

Fig. S6. Relative expression measured by quantitative real-time PCR (qRT-PCR) of zrfA and zrfB in a ΔzrfC strain and of zrfC in a ΔzrfAΔzrfB strain compared to a wild-type strain. All strains were cultured for 20 h at 37°C in the alkaline SDN zinc-limiting medium without a zinc supplement (− Zn) and supplemented with 2 and 100 μM Zn²⁺. Total RNA was purified with the RNeasy Plant kit (Qiagen) and treated with DNase I RNase-free (Invitrogen) to remove genomic DNA. DNase-treated RNA (12 μg) was used to synthesize cDNA at 50°C for 90 min using the SuperScript III RNase H–Reverse Transcriptase (Invitrogen) and oligo dT₁₅ (Roche) as primers. qRT-PCR was done using the SsoAdvanced SYBR Green supermix (Bio-Rad) and the primers ZRFA-D and ZRFA-R for zrfA, ZRFB-D and ZRFB-R for zrfB, and ZRFC-D and ZRFC-R for zrfC (Table S1). The CFX96 Touch Real-Time PCR Detection System (Bio-Rad) was used with the following program: initial denaturation at 95°C for 8 min following by 40 cycles of 95°C for 15 s, 59°C for 30 s and 72°C for 10 s. The expression of the zrfA, zrfB and zrfC genes was measured in triplicate for two independent cDNA samples from each strain and culture condition. Amplification specificity was determined by melting curve analysis. Data were normalized using the gdpA and tubA genes as internal references (Table S1). The 2⁻ΔΔCt method was used to measure the relative expression level of the genes in the AF48 (ΔzrfAB) and AF54 (ΔzrfC) strains compared to that in the AF14 (wild-type) strain. Data were represented and analyzed statistically with the Prism 4.0 Software. The reference expression level for all genes in the wild-type strain is indicated by a dotted line. As expected, the expression of zrfA and zrfB in the ΔzrfAB strain and zrfC in the ΔzrfC strain were not detected by qRT-PCR. Nevertheless, near-zero relative expression values have been represented to allow the readers to observe their places in the graph (#). Statistical analysis was performed using a two-way ANOVA test followed by a Bonferroni post-test with the wild-type expression level as reference. Significant differences were detected for the expression of both zrfA and zrfB between the wild-type and ΔzrfC strains (AF14 < AF54, P = 0.001).

Table S1. Oligonucleotides used in quantitative real-time PCR (qRT-PCR).