the FEBS Journal

High diversity of polyketide synthase genes and the melanin biosynthesis gene cluster in *Penicillium marneffei*

Patrick C. Y. Woo[1,2,3], Emily W. T. Tam[3], Ken T. K. Chong[3], James J. Cai[3], Edward T. K. Tung[3], Antonio H. Y. Ngan[3], Susanna K. P. Lau[1,2,3] and Kwok-Yung Yuen[1,2,3]

1 State Key Laboratory of Emerging Infectious Diseases, Hong Kong, China
2 Research Centre of Infection and Immunology, The University of Hong Kong, China
3 Department of Microbiology, The University of Hong Kong, China

Keywords
genome; melanin; *Penicillium marneffei*; phylogeny; polyketide synthase

Correspondence
K.-Y. Yuen, State Key Laboratory of Emerging Infectious Diseases, Department of Microbiology, The University of Hong Kong, University Pathology Building, Queen Mary Hospital, Hong Kong, China
Fax: (852) 28551241
Tel: (852) 22554892
E-mail: hkumicro@hkucc.hku.hk

Database
Nucleotide sequence data of the 23 putative PKS genes and two putative PKS-NRPS genes are available in the EMBL/GenBank/DDBJ databases under the accession numbers HM070045–HM070069

(Received 14 April 2010, revised 1 June 2010, accepted 13 July 2010)

doi:10.1111/j.1742-4658.2010.07776.x

Despite the unique phenotypic properties and clinical importance of *Penicillium marneffei*, the polyketide synthase genes in its genome have never been characterized. Twenty-three putative polyketide synthase genes and two putative polyketide synthase nonribosomal peptide-synthase hybrid genes were identified in the *P. marneffei* genome, a diversity much higher than found in other pathogenic thermal dimorphic fungi, such as *Histoplasma capsulatum* (one polyketide synthase gene) and *Coccidioides immitis* (10 polyketide synthase genes). These genes were evenly distributed on the phylogenetic tree with polyketide synthase genes of *Aspergillus* and other fungi, indicating that the high diversity was not a result of lineage-specific gene expansion through recent gene duplication. The melanin-biosynthesis gene cluster had gene order and orientations identical to those in the *Talaromyces stipitatus* (a teleomorph of *Penicillium emmonsii*) genome. Phylogenetically, all six genes of the melanin-biosynthesis gene cluster in *P. marneffei* were also most closely related to those in *T. stipitatus*, with high bootstrap supports. The polyketide synthase gene of the melanin-biosynthesis gene cluster (*alb1*) in *P. marneffei* was knocked down, which was accompanied by loss of melanin pigment production and reduced ornamentation in conidia. The survival of mice challenged with the *alb1* knockdown mutant was significantly better than those challenged with wild-type *P. marneffei* ($P < 0.005$). The sterilizing doses of hydrogen peroxide, leading to a 50% reduction in survival of conidia, were 11 min for wild-type *P. marneffei* and 6 min for the *alb1* knockdown mutant of *P. marneffei*, implying that the melanin-biosynthesis gene cluster contributed to virulence through decreased susceptibility to killing by hydrogen peroxide.

Introduction

Penicillium marneffei is the most important thermal dimorphic fungus causing respiratory, skin and systemic mycosis in China and Southeast Asia [1–4]. After the discovery of *P. marneffei* in 1956, only 18 cases of human disease caused by *P. marneffei* were reported until 1985 [5]. The appearance of the HIV pandemic, especially in China and Southeast Asian countries, saw the emergence of the infection as an important opportunistic mycosis in HIV-positive patients. About 8% of patients with acquired immune-deficiency

Abbreviations
ACP, acyl carrier protein; AIDS, acquired immune-deficiency syndrome; AT, acyltransferase; KS, ketosynthase; NRPS, nonribosomal peptide synthase; PKS, polyketide synthase.

syndrome (AIDS) in Hong Kong are infected with *P. marneffei* [6]. In northern Thailand, penicilliosis is the third most common indicator disease of AIDS, after tuberculosis and cryptococcosis [2]. Besides HIV-positive patients, *P. marneffei* infections have been reported in other immunocompromised patients, such as transplant recipients, patients with systemic lupus erythematosus and patients on corticosteroid therapy [7–10].

Polyketides are a diverse group of secondary metabolites produced by microorganisms. These secondary metabolites include bioactive compounds that are non-essential, but which confer survival advantages to the microbes. Some of the best-known secondary metabolites include pigments, antibiotics and mycotoxins. Polyketides are synthesized by complex enzymatic systems called polyketide synthases (PKS). In close proximity to the PKS genes additional genes are present that encode modifying enzymes which form biosynthetic clusters. The availability of increasing numbers of genome sequences has enabled us to predict the variability of metabolites and even some specific secondary metabolites produced by microorganisms, making rational design of experiments to confirm the predictions possible. In 2002, the complete genome-sequencing project of *P. marneffei* was started. Currently, a 6 × coverage of the genome has been completed. Based on the genome sequence, we assembled the complete mitochondrial genome sequence, analyzed the phylogeny that was predicted for the presence of the sexual cycle and developed a highly discriminative multilocus sequence-typing scheme for *P. marneffei* [11–13]. In this study, we explored the diversity of PKS genes in the genome of *P. marneffei* and analyzed their phylogenies. In addition, the melanin-biosynthesis gene cluster, which contains an important PKS gene (*alb1*) of *P. marneffei*, was also characterized.

Results

PKS genes in the *P. marneffei* genome

Using the PKS domains of *Aspergillus* sp. in a BLAST search of our *P. marneffei* genome database, 23 putative PKS genes and two putative PKS nonribosomal peptide synthase (PKS–NRPS) hybrid genes (*pks2* and *pks8*) were identified in the *P. marneffei* genome (Fig. 1). Among the 23 putative PKS genes, 21 contained the ketosynthase (KS), acyltransferase (AT) and acyl carrier protein (ACP) domains, typical of PKS genes, whereas ACP was not present in the other two PKS genes (*pks13* and *pks25*), implying that they may be pseudogenes. These 21 putative PKS genes with KS, AT and ACP domains were grouped into 18 clusters, with three clusters having two PKS genes (*pks11* and *pks12*, *pks16* and *pks17*, and *pks20* and *pks21*). Twelve of these 21 PKS genes were of the nonreducing type and the remaining nine were of the highly reducing type, which contained dehydrogenase and ketoreductase domains in addition to the KS, AT and ACP domains. Eight of these nine PKS genes of the highly reducing type also contained the enoylreductase domain. For the three clusters with two PKS genes, two contained one nonreducing and one reducing PKS, and the other contained two reducing PKS. As for the two putative PKS–NRPS hybrid genes, the PKS modules were of the highly reducing type, with dehydrogenase and ketoreductase domains, and the full NRPS modules that contained the condensation, adenylation, thiolation and thiolester reductase domains.

Phylogenetic analysis showed that the two PKS–NRPS hybrid genes were clustered with the PKS–NRPS hybrid genes of other fungi, the 12 nonreducing PKS genes were clustered with the nonreducing PKS genes of other fungi and the nine highly reducing PKS genes were clustered with the highly reducing PKS genes of other fungi (data not shown).

Melanin-biosynthesis gene cluster in the *P. marneffei* genome

The melanin-biosynthesis gene cluster spanned a 25.3-kb region in the *P. marneffei* genome (Fig. 2). Both the gene order and the orientation of the genes were identical to those in the *Talaromyces stipitatus* (teleomorph of *Penicillium emmonsii*) genome. However, the *arb2* and *alb1* (PKS 4 in Fig. 1) genes in the *P. marneffei* genome were separated by five ORFs that encoded hypothetical proteins, but the *arb2* and *alb1* genes in the *T. stipitatus* genome were not separated by any other ORF (Fig. 2). Phylogenetically, all six genes of the melanin-biosynthesis gene cluster in *P. marneffei* were also most closely related to those in *T. stipitatus*, with high bootstrap supports (Fig. 3).

Knockdown of the PKS gene (*alb1*) in the melanin-biosynthesis gene cluster

RNA interference is expected to trigger the post-transcriptional degradation of targeted mRNA. The degree of silencing level of the knockdown mutant of *P. marneffei* was analyzed by real-time RT-PCR using the actin gene for normalization. The transcription level of the *alb1* knockdown mutant was 8.6% of that in the wild-type gene. A loss of the melanin pigment

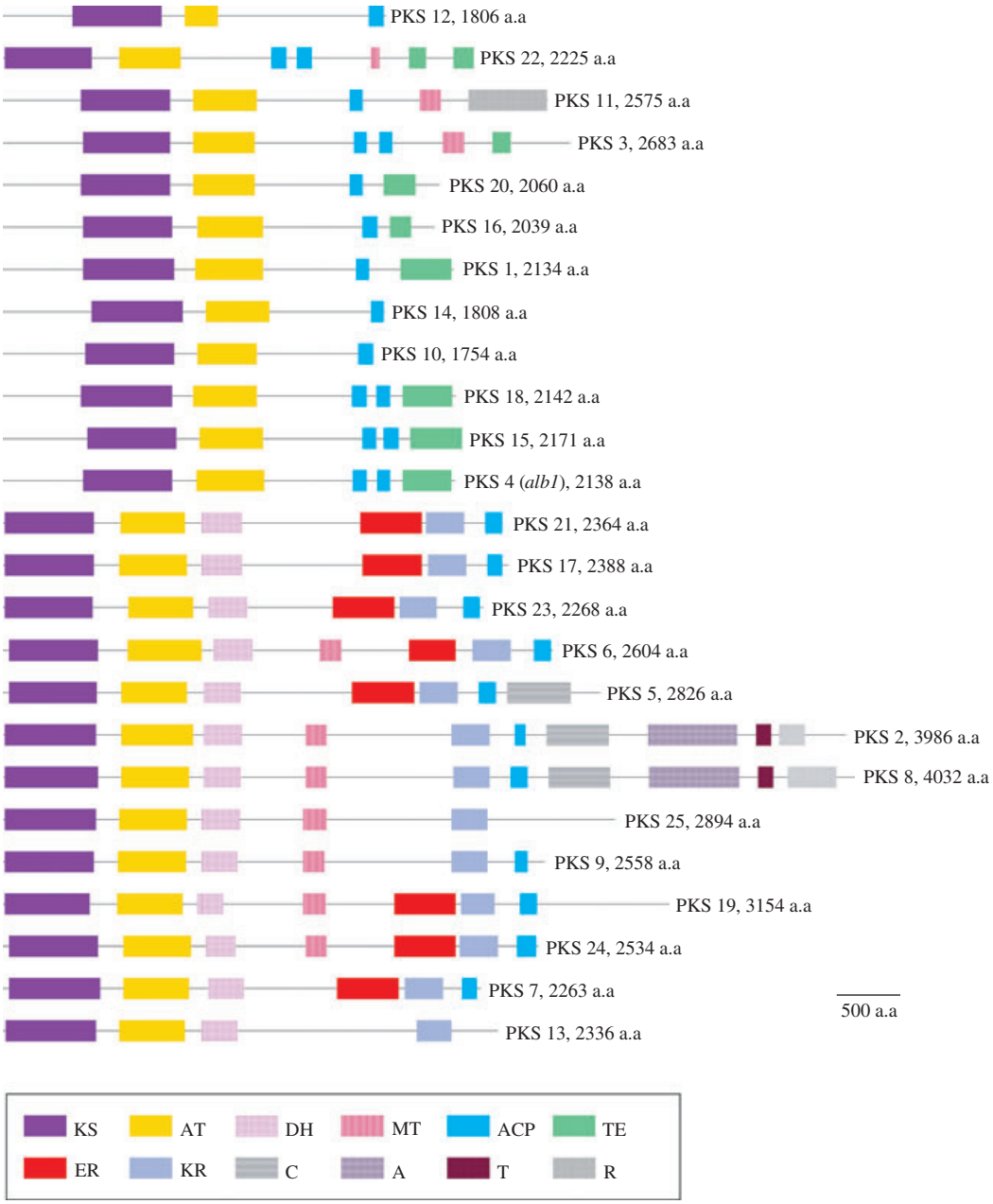

Fig. 1. Domain structures of predicted PKS genes in the *Penicillium marneffei* genome. Schematic representation of the domains of the 23 putative PKS genes and two putative PKS-nonribosomal peptide synthase hybrid genes in the *P. marneffei* genome. A, adenylation; ACP, acyl carrier protein; AT, acyltransferase; C, condensation; ER, enoylreductase; DH, dehydratase; KR, ketoreductase; KS, ketosynthase; MT, methyltransferase; R, thiolester reductase; T, thiolation; TE, thioesterase.

was observed in the spores of the *alb1* knockdown mutant.

Scanning electron microscopy

Scanning electron microscopic studies showed that the conidia of wild-type *P. marneffei* displayed an ornamented surface, whereas the conidia of its *alb1* knockdown mutant showed a reduced degree of ornamentation (Fig. 4).

Animal experiments

The survival of mice after intravenous challenge with wild-type *P. marneffei* or its *alb1* knockdown mutant on day 60 is summarized in Fig. 5. The survival rate

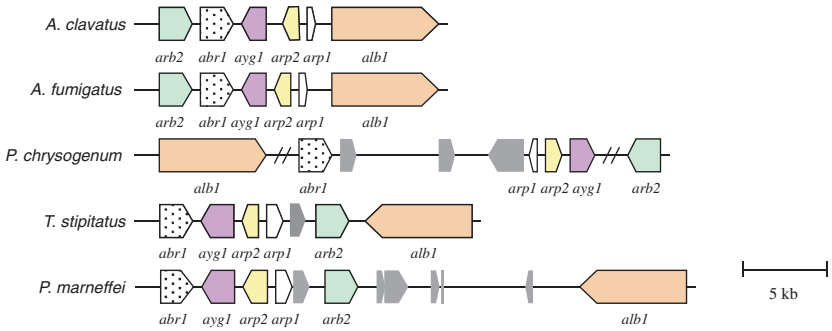

Fig. 2. Map of the melanin-biosynthesis gene cluster in *Penicillium marneffei* and closely related fungi. *abr1*, conidial pigment biosynthesis oxidase; *alb1*, conidial pigment polyketide synthase; *arb2*, conidial pigment biosynthesis oxidase; *arp1*, conidial pigment biosynthesis scytalone dehydratase; *arp2*, conidial pigment biosynthesis 1,3,6,8-tetrahydroxynaphthalene reductase; *ayg1*, conidial pigment biosynthesis protein.

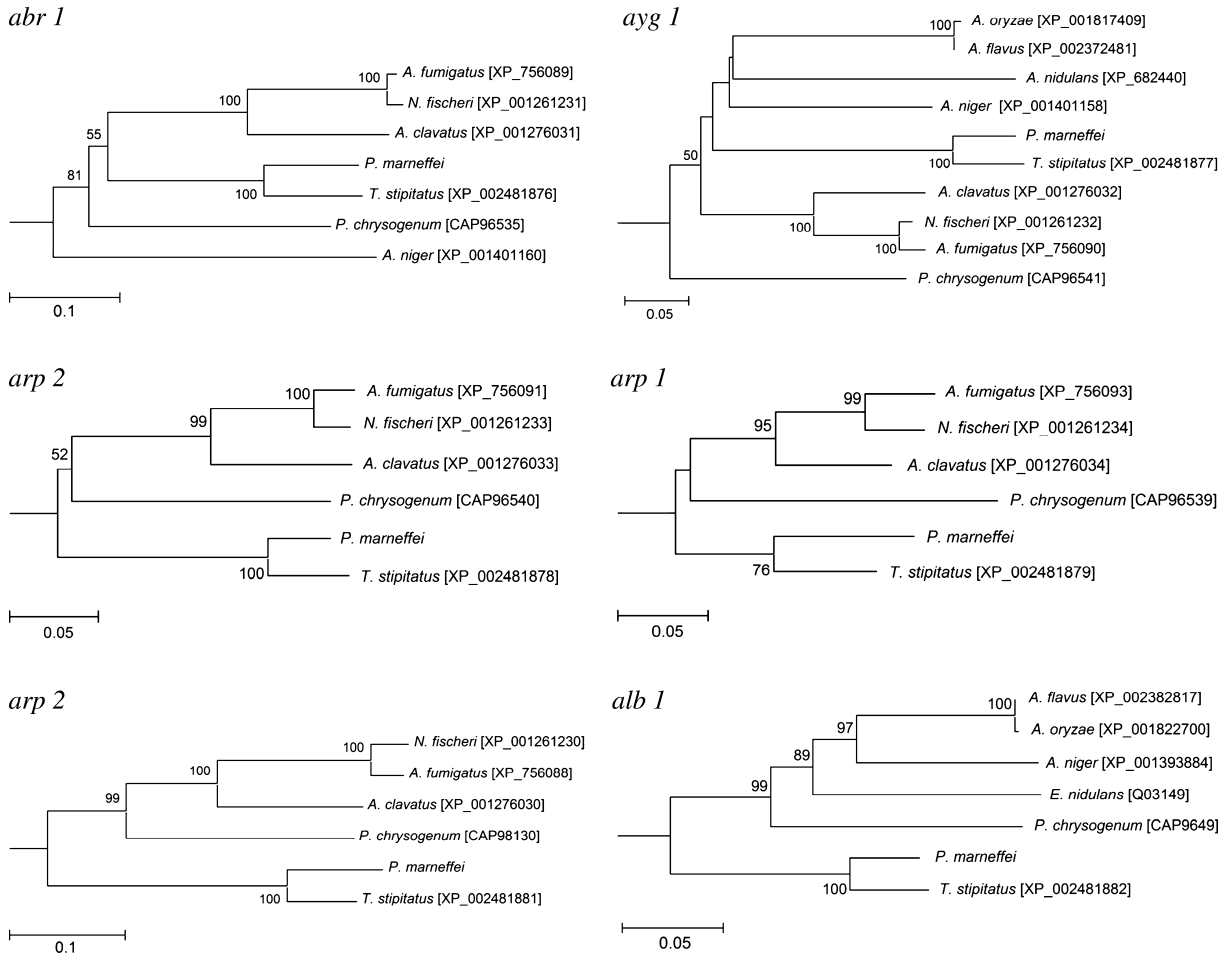

Fig. 3. Phylogenetic analysis of six proteins encoded in the melanin-biosynthesis gene cluster in *Penicillium marneffei*. The trees were constructed by the neighbor–joining method using Poisson correction and rooted using the *brown1*, *arb2*, scytalone dehydratase, tetrahydroxynaphthalene reductase, *ayg1* and *ywa1* genes in *Neurospora crassa* (GenBank:CAD70788), *Microsporum canis* (GenBank:EEQ35720), *Magnaporthe grisea* (GenBank:XP_359718), *N. crassa* (GenBank: XP_959252), *Pyrenophora tritici-repentis* (GenBank:XP_001936839) and *M. canis* (GenBank:EEQ32235). Bootstrap values were calculated from 1000 trees. For *abr1* and *arb2*, the scale bars indicate the estimated number of substitutions per 10 amino acids. For *ayg1*, *arp1*, *arp2* and *alb1*, the scale bar indicates the estimated number of substitutions per 20 amino acids. All names and accession numbers are given as cited in the GenBank database. *abr1*, conidial pigment biosynthesis oxidase; *alb1*, conidial pigment polyketide synthase; *arb2*, conidial pigment biosynthesis oxidase; *arp1*, conidial pigment biosynthesis scytalone dehydratase; *arp2*, conidial pigment biosynthesis 1,3,6,8-tetrahydroxynaphthalene reductase; *ayg1*, conidial pigment biosynthesis protein.

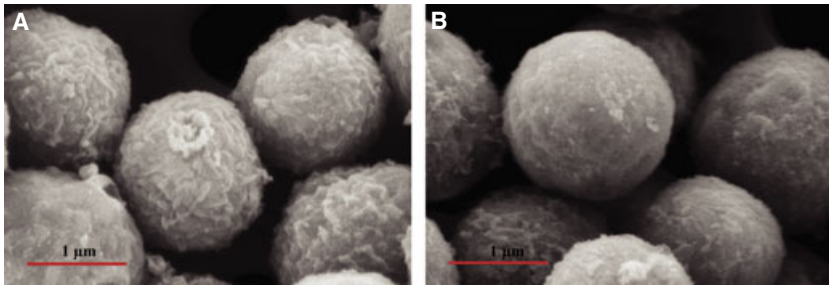

Fig. 4. Scanning electron micrographs of conidia of (A) wild-type *Penicillium marneffei* and (B) its *alb1* knockdown mutant. The conidia of wild-type *P. marneffei* showed an ornamented surface, whereas the conidia of its *alb1* knockdown mutant showed a reduced degree of ornamentation.

of mice challenged with the *alb1* knockdown mutant was significantly higher than those challenged with wild-type *P. marneffei* ($P < 0.005$).

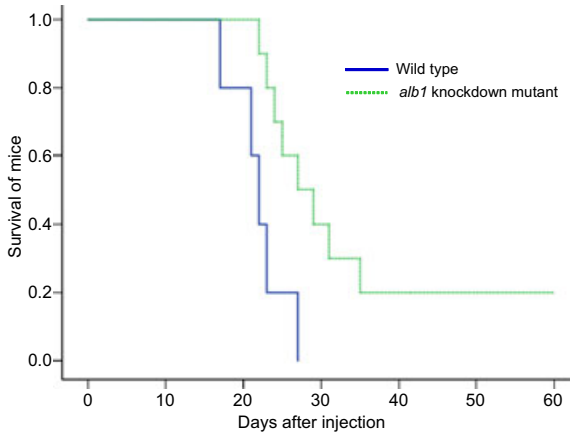

Fig. 5. Survival of BALB/c mice challenged with wild-type *Penicillium marneffei* or its *alb1* knockdown mutant. Groups of 10 BALB/c mice were challenged intravenously with 8×10^6 spores. Survival was recorded daily for 60 days.

Susceptibility to killing with hydrogen peroxide

The relative survival of *P. marneffei* conidia capable of forming visible colonies was calculated and plotted as a function of time of incubation in 25 mM hydrogen peroxide (Fig. 6A). The sterilizing doses leading to a 50% reduction in survival of the conidia were 11 min for wild-type *P. marneffei* and 6 min for the *alb1* knockdown mutant, respectively.

Susceptibility to killing by UV light

No difference was observed between the survival rates of wild-type *P. marneffei* and its *alb1* knockdown mutant when exposed to different doses of UV light (Fig. 6B).

Discussion

A high diversity of PKS genes is present in the *P. marneffei* genome. Twenty-five PKS genes are found in the *P. marneffei* genome; this is a much higher number of PKS genes than found in other pathogenic thermal dimorphic fungi, such as *Histoplasma capsulatum* and

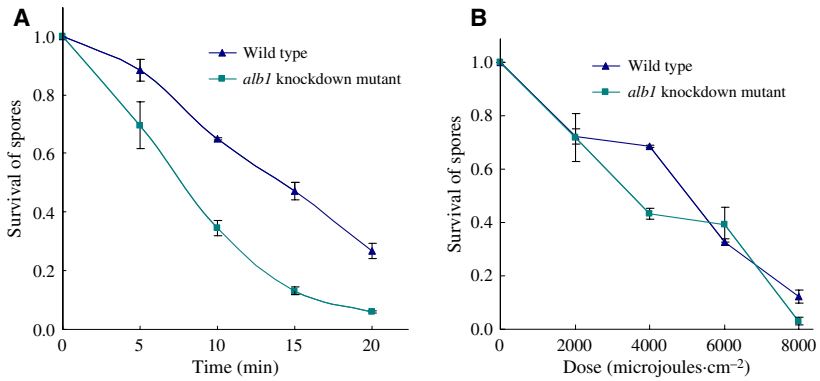

Fig. 6. Susceptibility of wild-type *Penicillium marneffei* and its *alb1* knockdown mutant to hydrogen peroxide and UV radiation. Conidia of wild-type and the *alb1* knockdown mutant of *P. marneffei* were subjected to killing by (A) hydrogen peroxide for 5, 10, 15 and 20 min and (B) UV light radiation at 2000, 4000, 6000 and 8000 microjoules per cm^2. Aliquots were plated on Sabouraud dextrose agar in duplicate to monitor cell viability. Data represent means spore survival ± SEM.

Coccidioides immitis, which have only one and 10 PKS genes, respectively [14]. The abundance of PKS genes implies the richness of polyketides that could potentially be produced by *P. marneffei*. These polyketides are involved in pigment production, virulence, toxin production, aerial hyphae formation, conidiation and antibiotic production, which may help the fungus to adapt to different environmental niches as well as cause disease. This is in line with the distinct phenotypic characteristics of *P. marneffei*, as it is thermal dimorphic, produces a variety of pigments (including the diffusible red pigment produced by its mycelial form) and is a major pathogen in HIV-positive patients in China and other parts of Southeast Asia. Although the *P. marneffei* genome contains a diversity of PKS genes, they were relatively evenly distributed on the phylogenetic tree with PKS genes of *Aspergillus* and other fungi (data not shown). This implies that the PKS genes of *P. marneffei* were not generated as a result of lineage-specific gene expansion through recent gene duplication.

The evolution of the melanin biosynthesis gene cluster in *P. marneffei* paralleled the evolution of the fungus. It has been documented, by immunofluorescence microscopy using melanin-binding mAb for labeling, that both the conidia and yeast cells of *P. marneffei* produce melanin [15]. Furthermore, *P. marneffei*-infected mice developed a significant antibody response against the fungus [15]. In the present study, all six genes of the melanin-biosynthesis gene cluster in *P. marneffei* were shown to be most closely related to the corresponding genes in *T. stipitatus* (Fig. 3), a nonpathogenic heterotrophic and saprophytic fungus commonly found in soil, dung and decaying plant material, which secretes a variety of hydrolytic enzymes to mobilize nutrients. Moreover, the gene orders and orientations were identical in both fungi. As the six genes are all closely related to other phylogenetically closely related fungi of mitosporic *Trichocomaceae*, which includes other *Penicillium* species and *Aspergillus* species (Fig. 3), the melanin-biosynthesis gene cluster was probably acquired by the common ancestor of this group of fungi, and subsequent divergence and gene re-arrangement resulted in the different gene orders and orientations of the individual genes in the different fungi. The series of five ORFs encoding hypothetical proteins located between the *arb2* and *alb1* genes were probably acquired after *P. marneffei* had evolved into a distinct species, as they were not present in the *T. stipitatus* genome.

The melanin-biosynthesis gene cluster in *P. marneffei* contributed to virulence probably through decreased susceptibility to killing by hydrogen peroxide. As a proof-of-concept study, we demonstrated the feasibility of knocking down and characterizing the PKS gene (*alb1*) of the melanin-biosynthesis gene cluster in *P. marneffei*. The plasmid used for knocking down genes in other ascomycetes, such as *Magnaporthe oryzae, Colletotrichum lagenarium* and *Bipolaris oryzae* [16,17], was able to achieve high efficiency in knocking down the *alb1* gene in *P. marneffei*, achieving an *alb1* transcript level of less than 10%. The *alb1* knockdown mutant of *P. marneffei* was associated with phenotypic changes, including loss of pigment, a decrease in virulence (Fig. 5) and a decrease in resistance to killing by hydrogen peroxide (Fig. 6A). This is in line with the observations in some other fungi, such as *Sporothrix schenckii, Aspergillus fumigatus* and *Paracoccidioides brasiliensis*, which found a decreased resistance to killing by hydrogen peroxide; the ability to mask pathogen-associated molecular patterns and induction of the cytokine response were thought to be the reasons for their decreased virulence when the corresponding dihydroxynaphthalene-like melanin pigment was lost [18–21]. It is notable that some fungi, such as *T. stipitatus*, also possess the melanin-biosynthesis gene cluster, yet are not pathogenic. This is because resistance to killing by hydrogen peroxide is just one of the steps, among many other traits, that lead to pathogenicity, such as the size of conidia, the ability to grow at 37 °C, adhesion to the host, evasion from host defense, the secretion of hydrolytic enzymes and toxin production, which may also be encoded by other PKS genes. Knocking down and characterizing other PKS genes in the genome, as well as genes in close proximity, and comparing the liquid chromatography/NMR/MS profiles of the wild-type and PKS knockdown mutants will reveal the chemical structure of the secondary metabolite associated with each PKS cluster as well as its biosynthetic pathway, and examining the knockdown mutants in the mouse model will reveal their relative contribution to the pathogenicity of the fungus.

Materials and methods

Strain and DNA extraction for genome sequencing

P. marneffei strain PM1 was isolated from an HIV-negative patient, suffering from culture-documented penicilliosis, in Hong Kong. The yeast form of PM1 was used throughout the DNA-sequencing experiments. Genomic DNA was prepared from *P. marneffei* grown at 37 °C. A single colony of the fungus grown on Sabouraud dextrose agar at 37 °C was inoculated into yeast peptone broth and incubated in a shaker at 30 °C for 3 days. Cells were cooled on ice for 10 min, harvested by centrifugation at 2000 ***g*** for 10 min, washed twice and resuspended in ice-cold 50 mM EDTA (pH 7.5). Then, 20 mg of novazym per mL to the cell

mixture was added and incubated at 37 °C for 1 h followed by digestion in a mixture of 1 mg proteinase K per mL of the culture, 1% *N*-lauroylsarcosine and 0.5 M EDTA (pH 9.5), at 50 °C for 2 h. Genomic DNA was then extracted with phenol, followed by extraction with phenol/chloroform and finally precipitated and washed in ethanol. After digestion with RNase A, a second ethanol precipitation was followed by washing with 70% ethanol; the pellet was air-dried and dissolved in 500 μL of TE buffer (10 mM Tris/HCl, 1.0 mM EDTA; pH 8.0).

Library construction and sequence assembly

A genomic DNA library was made in pUC18 using inserts of 3.0–5.0 kb in size. DNA inserts were prepared by physical shearing using the sonication method. One end of 315 580 clones, representing a 6 × coverage of the *P. marneffei* genome, were sequenced. The Phred/Phrap/Consed software package was used for base calling and sequence assembly [22–24].

Identification of PKS genes and the melanin-biosynthesis gene cluster in the *P. marneffei* genome

As *P. marneffei* and *Aspergillus* species are phylogenetically closely related [11], putative PKS of *P. marneffei* were identified by using the PKS domains of *A. fumigatus*, *Aspergillus nidulans*, *Aspergillus flavus*, *Aspergillus terreus*, *Aspergillus ochraceus*, *Aspergillus parasiticus*, *Aspergillus usami*, *Aspergillus oryzae*, *Aspergillus niger* and *Aspergillus clavatus* to BLASTP against our *P. marneffei* genome database. Consensus sequences of domains were retrieved from the Conserved Domains Database of NCBI and PFAM (http://pfam.sanger.ac.uk/search?tab=searchSequenceBlock). Multiple alignments of homologous sequences were visually inspected and confirmed.

PKS genes and melanin-biosynthesis gene clusters in other fungi and phylogenetic analysis

To perform phylogenetic analysis on the putative PKS enzymes in *P. marneffei*, PKS enzymes and PKS-like enzymes from representative fungal species with assembled genome sequences were retrieved from precomputed results in Secondary Metabolism Region Finder (SMURF) (http://www.jcvi.org/smurf). To perform phylogenetic analysis on the putative *alb1*, *abr1*, *arb2*, *arp1*, *arp2* and *ayg1* genes, these genes were used as queries to BLASTP against the GenBank database. The corresponding genes in *P. chrysogenum*, *T. stipitatus*, *A. fumigatus* and *A. clavatus* were manually annotated. Phylogenetic trees were constructed using the neighbor-joining method with Mega 4.0. Eight-hundred and thirty nine amino acid positions of the KS domains of the putative PKS enzymes, 732 amino acid positions of the KS and AT domains of the *alb1* gene and 538, 601, 157, 273 and 433 amino acid positions of the *abr1*, *arb2*, *arp1*, *arp2* and *ayg1* genes, respectively, in the melanin-biosynthesis gene clusters were used for analysis.

Knockdown of a PKS gene (*alb1*) in the melanin-biosynthesis gene cluster

DNA extraction was performed using the DNeasy Plant Mini Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). The extracted DNA was eluted in 50 μL of AE buffer (10 mM Tris/HCl, 0.5 mM EDTA; pH 9.0), the resultant mixture was diluted 10 × and 1 μL of the diluted extract was used for PCR.

Plasmid construction was performed according to a published protocol, with modifications [16]. Plasmid pSilent-1 [17], obtained from the Fungal Genetics Stock Center, was used to construct the pPW1302 plasmid for knocking down *alb1*. First, the internal *alb1* fragment (sense) was amplified using primers LPW9506 5′-CCGCTCGAGCCAAACCAC TCAGAGTAGCC-3′ and LPW9507 5′-CCCAAGCTTGG GACCCTGGTAGAGGAGATTCC-3′ (Invitrogen, Carlsbad, CA, USA). The PCR mixture (25 μL) contained *P. marneffei* DNA, PCR buffer [10 mM Tris/HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$ and 0.01% gelatin], 200 μM of each deoxynucleoside triphosphate and 1.0 U of *Taq* polymerase (Applied Biosystems, Foster City, CA, USA). The mixtures were amplified for 32 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 40 s, and a final extension at 72 °C for 10 min, in an automated thermal cycler (Applied Biosystems). The PCR product was purified using the QIAquick Gel Extraction kit (QIAgen), digested with *Xho*I and *Hin*dIII, and cloned into the *Xho*I/*Hin*dIII site of the pSilent-1 plasmid, resulting in pPW1302-1. Second, the internal *alb1* fragment (antisense) was amplified with primers LPW9508 5′-GGGGTACCCCAAACCACTCAGAGTAG-CC-3′ and LPW9509 5′-GAAGATCTACCCTGGTA-GAGGAGATTCC-3′ (Invitrogen), using the PCR conditions described above. This amplified fragment was purified as described above, digested with *Bgl*II and *Kpn*I, and cloned into the *Bgl*II/*Kpn*I site of the pPW1302-1, resulting in pPW1302. The wild-type *P. marneffei* strain PM1 was transformed with linearized pPW1302, using 200 μg ml^{-1} of hygromycin for selection.

Real-time quantitative RT-PCR

Total RNA was extracted using RiboPure-Yeast (Ambion, Foster City, CA, USA). The RNA was eluted in 70 μL of RNase-free water and was used as the template for real-time RT-PCR. Reverse transcription was performed using the SuperScript III kit (Invitrogen). Real-time RT-PCR assays were performed, as described previously [25], for the *alb1* fragment using primers LPW9508 and LPW9509, and

for actin using primers LPW8614 (5′-CAYACY TTCTACAAYGARCTCC-3′) and LPW8615 (5′-KGCVA RRATRGAACCACC-3′) for normalization. cDNA was amplified in a LightCycler 2.0 (Roche, Basel, Switzerland) in 20-μL reaction mixtures containing FastStart DNA Master SYBR Green I Mix reagent kit (Roche), 2 μL of cDNA, 2 mM $MgCl_2$ and 0.5 mM primers, at 95 °C for 10 min followed by 50 cycles of 95 °C for 10 s, 57 °C (55 °C for the actin gene) for 5 s and 72 °C for 23 s (36 s for the actin gene).

Scanning electron microscopy

Spores of wild-type and *alb1* knockdown *P. marneffei* were washed twice using Milli-Q water. A suspension of the spores was settled onto a polycarbonate membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK), with a 5 μm pore size, for 5 min. The membrane was fixed in 2.5% glutaraldehyde (w/v) for 1 h and washed once in 0.1 M sodium cacodylate buffer. Fixed material was dehydrated through a 20% increment of ethanol concentration every 15 min from 30% to 90%, followed by two subsequent dehydration steps, of 15 min each, in absolute ethanol. Dehydrated material in absolute ethanol was critical-point-dried in a BAL-TEC CPD O30 Critical Point Drier using carbon dioxide as the drying agent. Critical dried material was mounted onto an aluminum stub and coated with palladium in the BAL-TEC SCD 005 SEM coating system. Coated material was examined in a Leica Cambridge Stereoscan 440 scanning electron microscope operating at 12 kV, and the specimen stage was tilted at 0°.

Animal experiments

BALB/c (H-2^d) mice (6–8 weeks of age; 18–22 g in weight) were obtained from the Laboratory Animal Unit, The University of Hong Kong [26]. The experimental protocols were approved by the Committee on the Use of Live Animals in Teaching and Research, The University of Hong Kong, in accordance with the Guidelines laid down by the NIH in the USA regarding the care and use of animals for experimental procedures. The number of animals used was kept to the minimum number that still ensured statistical significance of survival differences between the experimental groups. Mice were housed in cages, under standard conditions with regulated day length, temperature and humidity, and were given pelleted food and tap water *ad libitum*. Ten mice were challenged intravenously with 8×10^6 spores of wild-type *P. marneffei* and another 10 mice were challenged with the same amount of spores from *alb1* knockdown *P. marneffei*. Survival of the mice was recorded daily for 60 days and analyzed using the Kaplan–Meier method and the Log-rank test. $P < 0.05$ was regarded as statistically significant. The experiment was performed in duplicate.

Susceptibility to killing by hydrogen peroxide

Conidial suspensions of wild-type and *alb1* knockdown *P. marneffei* were adjusted to 4×10^3 cells·mL^{-1} in 100 mM potassium phosphate buffer (pH 7.0) containing 25 mM hydrogen peroxide [18]. At 5-min intervals, aliquots were taken, diluted in 100 mM potassium phosphate buffer and plated onto Sabouraud dextrose agar plates. The experiment was performed in triplicate.

Susceptibility to killing by UV light

Conidial suspensions of wild-type and *alb1* knockdown *P. marneffei* were adjusted to 4×10^3 cells·mL^{-1}. Appropriate dilutions of cells were plated on Sabouraud dextrose agar plates and exposed to UV light (254 nm) generated in a Crosslinker (UVP, Upland, CA, USA) at various energy settings. Percentage survival was determined by comparing the number of colonies on irradiated plates with those on nonirradiated plates [18]. The experiment was performed in triplicate.

Acknowledgements

This work was partly supported by the Research Fund for the Control of Infectious Diseases (Commissioned study) of the Health, Welfare and Food Bureau of the Hong Kong SAR Government, Research Grant Council Grant, University Development Fund, Committee for Conference and Research Grant, Providence Foundation Limited in memory of the late Dr Lui Hac Minh, HKU Award for CAE Membership, HKU Medical Faculty Award for CAE Membership, Dr Hector T. G. Ma. We thank the Fungal Genetics Stock Center for providing the pSilent-1 vector.

References

1 Hsueh PR, Teng LJ, Hung CC, Hsu JH, Yang PC, Ho SW & Luh KT (2000) Molecular evidence for strain dissemination of *Penicillium marneffei*: an emerging pathogen in Taiwan. *J Infect Dis* **181**, 1706–1712.
2 Supparatpinyo K, Khamwan C, Baosoung V, Nelson KE & Sirisanthana T (1994) Disseminated *Penicillium marneffei* infection in southeast Asia. *Lancet* **344**, 110–113.
3 Wong SS, Siau H & Yuen KY (1999) Penicilliosis marneffei – West meets East. *J Med Microbiol* **48**, 973–975.
4 Yuen KY, Wong SS, Tsang DN & Chau PY (1994) Serodiagnosis of *Penicillium marneffei* infection. *Lancet* **344**, 444–445.
5 Deng ZL & Connor DH (1985) Progressive disseminated penicilliosis caused by *Penicillium marneffei*. Report of eight cases and differentiation of the causative organism from Histoplasma capsulatum. *Am J Clin Pathol* **84**, 323–327.

6 Low K & Lee SS (2002) The pattern of AIDS Reporting and the implications on HIV surveillance. *Public Health Epidemiol Bull* **11**, 41–49.
7 Lo CY, Chan DT, Yuen KY, Li FK & Cheng KP (1995) *Penicillium marneffei* infection in a patient with SLE. *Lupus* **4**, 229–231.
8 Wang JL, Hung CC, Chang SC, Chueh SC & La MK (2003) Disseminated *Penicillium marneffei* infection in a renal-transplant recipient successfully treated with liposomal amphotericin B. *Transplantation* **76**, 1136–1137.
9 Wong SS, Woo PC & Yuen KY (2001) *Candida tropicalis* and *Penicillium marneffei* mixed fungaemia in a patient with Waldenstrom's macroglobulinaemia. *Eur J Clin Microbiol Infect Dis* **20**, 132–135.
10 Woo PC, Lau SK, Lau CC, Chong KT, Hui WT, Wong SS & Yuen KY (2005) *Penicillium marneffei* fungaemia in an allogeneic bone marrow transplant recipient. *Bone Marrow Transplant* **35**, 831–833.
11 Woo PC, Zhen H, Cai JJ, Yu J, Lau SK, Wang J, Teng JL, Wong SS, Tse RH, Chen R *et al.* (2003) The mitochondrial genome of the thermal dimorphic fungus *Penicillium marneffei* is more closely related to those of molds than yeasts. *FEBS Lett* **555**, 469–477.
12 Woo PC, Chong KT, Tse H, Cai JJ, Lau CC, Zhou AC, Lau SK & Yuen KY (2006) Genomic and experimental evidence for a potential sexual cycle in the pathogenic thermal dimorphic fungus *Penicillium marneffei*. *FEBS Lett* **580**, 3409–3416.
13 Woo PC, Lau CC, Chong KT, Tse H, Tsang DN, Lee RA, Tse CW, Que TL, Chung LM, Ngan AH *et al.* (2007) MP1 homologue-based multilocus sequence system for typing the pathogenic fungus *Penicillium marneffei*: a novel approach using lineage-specific genes. *J Clin Microbiol* **45**, 3647–3654.
14 Collemare J, Billard A, Bohnert HU & Lebrun MH (2008) Biosynthesis of secondary metabolites in the rice blast fungus *Magnaporthe grisea*: the role of hybrid PKS-NRPS in pathogenicity. *Mycol Res* **112**, 207–215.
15 Youngchim S, Hay RJ & Hamilton AJ (2005) Melanization of *Penicillium marneffei* in vitro and in vivo. *Microbiology* **151**, 291–299.
16 Moriwaki A, Ueno M, Arase S & Kihara J (2007) RNA-mediated gene silencing in the phytopathogenic fungus *Bipolaris oryzae*. *FEMS Microbiol Lett* **269**, 85–89.
17 Nakayashiki H, Hanada S, Nguyen BQ, Kadotani N, Tosa Y & Mayama S (2005) RNA silencing as a tool for exploring gene function in ascomycete fungi. *Fungal Genet Biol* **42**, 275–283.
18 Romero-Martinez R, Wheeler M, Guerrero-Plata A, Rico G & Torres-Guerrero H (2000) Biosynthesis and functions of melanin in *Sporothrix schenckii*. *Infect Immun* **68**, 3696–3703.
19 Tsai HF, Chang YC, Washburn RG, Wheeler MH & Kwon-Chung KJ (1998) The developmentally regulated *alb1* gene of *Aspergillus fumigatus*: its role in modulation of conidial morphology and virulence. *J Bacteriol* **180**, 3031–3038.
20 da Silva MB, Thomaz L, Marques AF, Svidzinski AE, Nosanchuk JD, Casadevall A, Travassos LR & Taborda CP (2009) Resistance of melanized yeast cells of *Paracoccidioides brasiliensis* to antimicrobial oxidants and inhibition of phagocytosis using carbohydrates and monoclonal antibody to CD18. *Mem Inst Oswaldo Cruz* **104**, 644–648.
21 Chai LY, Netea MG, Sugui J, Vonk AG, van de Sande WW, Warris A, Kwon-Chung KJ & Jan Kullberg B (2009) Aspergillus fumigatus conidial melanin modulates host cytokine response. *Immunobiology*, doi:10.1016/j.imbio.2009.10.002. [Epub ahead of print].
22 Ewing B & Green P (1998) Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Res* **8**, 186–194.
23 Ewing B, Hillier L, Wendl MC & Green P (1998) Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res* **8**, 175–185.
24 Gordon D, Abajian C & Green P (1998) Consed: a graphical tool for sequence finishing. *Genome Res* **8**, 195–202.
25 Woo PC, Tung ET, Chan KH, Lau CC, Lau SK & Yuen KY (2010) Cytokine profiles induced by the novel swine-origin influenza A/H1N1 virus: implications for treatment strategies. *J Infect Dis* **201**, 346–353.
26 Wong LP, Woo PC, Wu AY & Yuen KY (2002) DNA immunization using a secreted cell wall antigen Mp1p is protective against *Penicillium marneffei* infection. *Vaccine* **20**, 2878–2886.