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# Effect of phosphatidylcholine on the level expression of *plc* genes of *Aspergillus fumigatus* by real time PCR method and investigation of these genes using bioinformatics analysis

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**ABSTRACT**

**Background and Objectives:** Phosphlipases are a group of enzymes that breakdown phosphatidylcholine (phospholipids) molecules producing second products. These produced products have a divers role in the cell like signal transduction and digestion in humans. In this research the effect of phosphatidylcholine on the expression of *plc* genes of *A. fumigatus* was studied. The *plc* genes of this fungus were also interrogated using bioinformatics studies.

**Materials and Methods:** Real-time PCR was performed to study the expression of *plc* genes and these genes were interrogated using bioinformatics studies.

**Results:** There was more significant expression for all three *plc* genes when *A. fumigatus* was grown on the presence of phosphatidylcholine in the medium. The sequence of *plc* genes of *A. fumigatus* was also interrogated using bioinformatics analysis and their relationship with the other microorganisms was investigated.

**Conclusion:** Real-time PCR revealed that *afplc*1*, afplc2* and *afplc3* were up-regulated in the presence of phosphatidylcholine. In this study we suggest either the *plc'*s of *A. fumigatus* were present in an ancestral genome and have become lost in some lineages, or that they have been acquired from other organisms by horizontal gene transfer. We also found that *plc's* of this fungus appeared to be more closely related to the plant *plc*'s than the bacterial *plc*'s.

**Keywords:** *Aspergillus fumigatus*, phospholipase C, *plc* gene, gene expression, phosphatidylcholine

## INTRODUCTION

Fungi are the cause of a number of infections in man and animals ranging from relatively superficial and benign infections through to systemic life-threatening diseases (1). Immunocompromised patients are particularly vulnerable to fungal infection (2). The opportunistic fungal pathogen *Aspergillus fumigatus* in immunocompromised patients, causes aspergillosis and it has been reported as the most common cause of invasive aspergillosis (IA) in patients with leukaemia or patients who have undergone organ transplantation (3). *A. fumigatus* is thermo tolerant, saprophytic fungus which is frequently found on a wide variety of dead organic material (4). Its conidia is the most ubiquitous in the atmosphere and it has a range from 2.5 – 3 µm in diameter and because of their small size they are able to enter the alveoli of the lung (2, 5, 6).

The membrane-associated phosphoinositide-specific phospholipase C's are known to be involved in eukaryotes in intracellular signalling, specifically hydrolyzing phosphatidylinositol - 4,5 - bisphosphate

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to liberate the protein kinase C activator diacylglycerol and the intracellular calcium mobilizing agent inositol 1,4,5 - trisphosphate (7-12). In contrast, many bacterial species are known to actively secrete PLC enzyme and some are important pathogenic determinants including those of *Clostridium perfringens* , *Listeria monocytogenes*, *Bacillus cereus*, *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* (13-18).

Previously the role of phospholipase B (PLB) have been reported in *A. fumigatus*. In a study by Shen *et al*. (2004) they reported two secreted PLB enzymes and probably account for the extracellular phospholipase activity (19). In other studies a partial of phopholpiase B1 and B2 genes (*plb1* and *plb2*) was cloned and sequenced and the role of phospholipase B3 was confirmed in the pathogenicity of *A. fumigatus* (20-23). The role of phospholipase D as a growth and photogenic factor in microorganisms was confirmed in another study too (24).

The aim of this study was to find out the expression of *plc* gene of *A. fumigatus* and to undertake a bioinformatics analysis of the available genomic DNA sequence of *A. fumigatus* to attempt to identity potential candidate genes encoding secreted PLC(s) which would account for the observed extracellular activity (25).

## MATERIALS AND METHODS

**Strain, media and culture condition.** *A. fumigatus* (ATCC 90240) were cultured on Vogels (Vogel 1956) chloramphenicol agar at 37˚C up to 24 h with constant shaking (200 rpm) containing 1% (w/v) glucose with or without 0.5% (w/v) phosphatidylcholine (Sigma).

Spore suspension was serially diluted to 10-4, 10-5 and 10-6 spores/ml-1 and plated into Petri dishes and incubated overnight at 37˚C. For liquid cultures, 50 ml of Vogel's media with or without phosphatidylcholine, were distributed into 250 ml Erlenmeyer flasks and inoculated with 0.1 ml of a 1x 108/ml-1 spore suspension and incubated with shaking (250 rpm) at 37˚C up to 24 h.

**RNA extraction and Primers for *afplc* genes.** The RNA extracted by RNeasy Mini Kit from Qiagen, UK (http://www.qiagen.com/). The sequences of primers used in the study are as follows:

*plc1* 5'-CGGCGAGGTCATCAACTACT - *Tm = 59.4˚C*

and 5'-AAGTGCCGGACGTCAGATAG - *Tm = 59.4˚C* , *plc2* 5'-CAGGTCGAGCAGAAGGGTA - *Tm=59.4˚C* and5'-GGGGTAAAGGGCTCAAAGTC - *Tm = 59.4˚C*

*plc3* 5'-CGGACGCTCTCTTCTTCAAC - *Tm = 59.4˚C* and 5'-ATGGAGTTGGTGTCGAGGTC - *Tm = 59.4˚C,*

*Actin* 5'-TGCTCCTCCTGAGCGTAAAT - *Tm= 59.9˚C* and 5'-ACATCTGCTGGAAGGTGGAC - *Tm = 60.0˚C*

**Expression of *afplcs* and cDNA synthesis.** iQSYBER Green Kit (from BioRad, UK) was used for making cDNA from RNA and the real-time PCR reactions were prepared according to the manufacturer's protocol. *actin* gene was used as reference gene. cDNA was made using cDNA Kit from Qiagen.

**Comparative $C_T$ method, statistical and bioinformatics analysis.** The comparative CT method was used to measure the level of expression in the target gene by real-time PCR. CT values for gene are normalised against *actin* (reference housekeeping gene) to give the normalised ΔCT value.

$\Delta C_T = C_{T\ Target} - C_{T\ Reference}$

Standard deviation for the $\Delta C_T$ values were calculated using the following equation: (26)

$SD\Delta C_T = (SD\ C_{T\ Target}^{\ 2} - SD\ C_{T\ Reference}^{\ 2})^{1/2}$

To compare the relative expression of a gene grown on phosphatidylcholine compared to absence of

phosphatidylcholine, the $\Delta C_T$ value of the gene grown on lecithin is first subtracted from the $\Delta C_T$ value of the gene grown on absence of lecithin to give the $\Delta\Delta C_T$ value.

$\Delta\Delta C_T = \Delta CT_{\ absence\ of}$ phosphatidylcholine - $\Delta C_T$ phosphatidylcholine

The fold difference in gene expression when grown on phosphatidylcholine compared to absence of phosphatidylcholine is then calculated using the formula below: (26).

Fold change in gene expression = $2^{-\Delta\Delta C_T}$ . As the $\Delta C_T$ phosphatidylcholine value is subtracted from the $\Delta C_T$ absence of phosphatidylcholine value, the standard deviation of $\Delta\Delta C_T$ is the same as the standard deviation of $\Delta C_T$ absence of phosphatidylcholine. For comparing the statistical significance of the fold changes, the student t test was used to compare the $\Delta C_T$ absence of phosphatidylcholine and $\Delta C_T$ phosphatidylcholine values of each gene. DNA sequences were submitted for analysis through

the Basic Local Alignment Search Tool (BLAST). BLAST was accessed through the National Centre for Biotechnology Information (NCBI) or through the Bioinformatics Centre, University of Kyoto and used to search for sequence similarities within sequences held on the BLAST database.

## RESULTS

**Identification of phospholipase C genes from the *A. fumigatus* genome.** Publicly available protein sequences encoding prokaryotic *plc*'s were obtained from the NCBI database and used to interrogate the *A. fumigatus* database using tblastn to search for similarities against the DNA sequence translated in all 6 frames. Three separate regions were identified in different contiguous DNA sequences from *A. fumigatus* with homology to prokaryotic *plc* sequences. These sequences along with an additional 2 Kbp upstream and downstream were analysed using the Genefinder program which had been trained using *A. niger* protein sequences, to identify the full length sequences of the three putative *plc's* sequences. Three sequences including putative introns were identified and the predicted coding sequences translated using Expasy software. The first, *afplc1*encoded a 1302 bp gene, the second gene, *afplc2* encoded a 1421 bp gene and the third, *afplc3* encoded a 1429bp gene. A summary of the three genes identified is shown in Table 1. Programme rpsblast was used to search the *plc's* sequences for conserved domains. All three genes contained a single conserved phosphoesterase domain (pfam PF04185, interpro IPR007312) associated with *plc* and all three contained a pre-pro sequence identified using SignalP software with probabilities >0.999 indicating that all three are secreted extracellular enzymes. When the translated sequences were used to interrogate the public NCBI database, all three sequences gave the highest positive hits to *plc's* from plants and prokaryotes. Further searches with the *plc* amino acid sequences against the *A. fumigatus* database revealed no further *plc* homologues.

**Identification and comparison of *plc* genes from other fungi.** BlastP and tblastn searches using the translated *plc's* sequences identified from *A. fumigatus* revealed homologues were present in *A. nidulans*, *A. oryzae* and *A. niger* with only a single homologue present in *Trichoderma reesei* and *Gibberella zeae*. No extracellular *plc* sequences were present in any of the other sequenced genomes which included *N. crassa, U. maydis, M. grisea* and *C. cinereus, C. albicans, S. cerevisia , P. chrysosorium, C. globosum, C. dubliniensis, S. pombe, C. neoformans serotype A* and *C. immitis* amongst others. All predicted *plc*'s contained a single phosphoesterase domain (pfam PF04185, interpro IPR007312) typically found in all *plc*'s. To investigate the relatedness of the plc sequences from *A. fumigatus, A. nidulans* and *A. oryzae*, the translated sequences were compared both by multiple alignment and by ClustalW from which a phylogenetic phenogram was constructed created by the nearest joining method publicly available from the European Molecular Biology Network. From the multiple alignments, several domains were present that had identical amino acid sequences and several regions of amino acid conservation were found amongst the phospholipases that were not shared by *A. oryzae* Q2UQU5, *G. zeae* Q4HUX2 or *T. reesei*. Overall, there was little homology at the N and C termini of the phospholipases , with *A. oryzae* Q2UQU5, *G. zeae* Q4HUX2 and *T. reesei* having extended C-termini compared to the other sequences. When the phylogenetic relationship was examined, one orthologue of *A. fumigatus plc1* and *plc3* were found in both *A. nidulans* (Q5AT34 and Q5BAU4) and *A. oryzae* (Q2TYX2 and Q2UDQ1) respectively. For *A. fumigatus plc2*, one orthologue was found in *A. nidulans* (Q5AV19) but not in *A. oryzae*. By contrast, two *A. oryzae* sequences (Q2UQU5 and Q2UL66) did not appear to have orthologues in either *A. fumigatus* or *A. nidulans* (Fig. 1).

**Comparison of phospholipase C genes from fungi and other organisms.** A phylogenetic analysis of all available *plc* protein sequences from bacteria, plants and the Aspergilli was constructed using ClustalW version 1.7 and a phenogram created by the nearest joining method publicly available from the European Molecular Biology Network and the results shown in Fig. 2. To test the reliability of this tree, bootstrapping was undertaken (1000 samples) and all branches were deemed reliable. The *plc* proteins clustered into three main groups, the fungi, the bacteria and the plants. All of the Aspergillus *plc*'s sequences showed highest levels of homology to plant sequences with the notable exception of one gene from *A. oryzae* (Q2UQU5) and the *plc* from *G. zeae* (Q4HUX2) and *T. reesei* (TRTRES) that showed higher levels of homology to bacterial *plc*. However, these sequences lack a C-terminal tandem repeat domain of unknown function

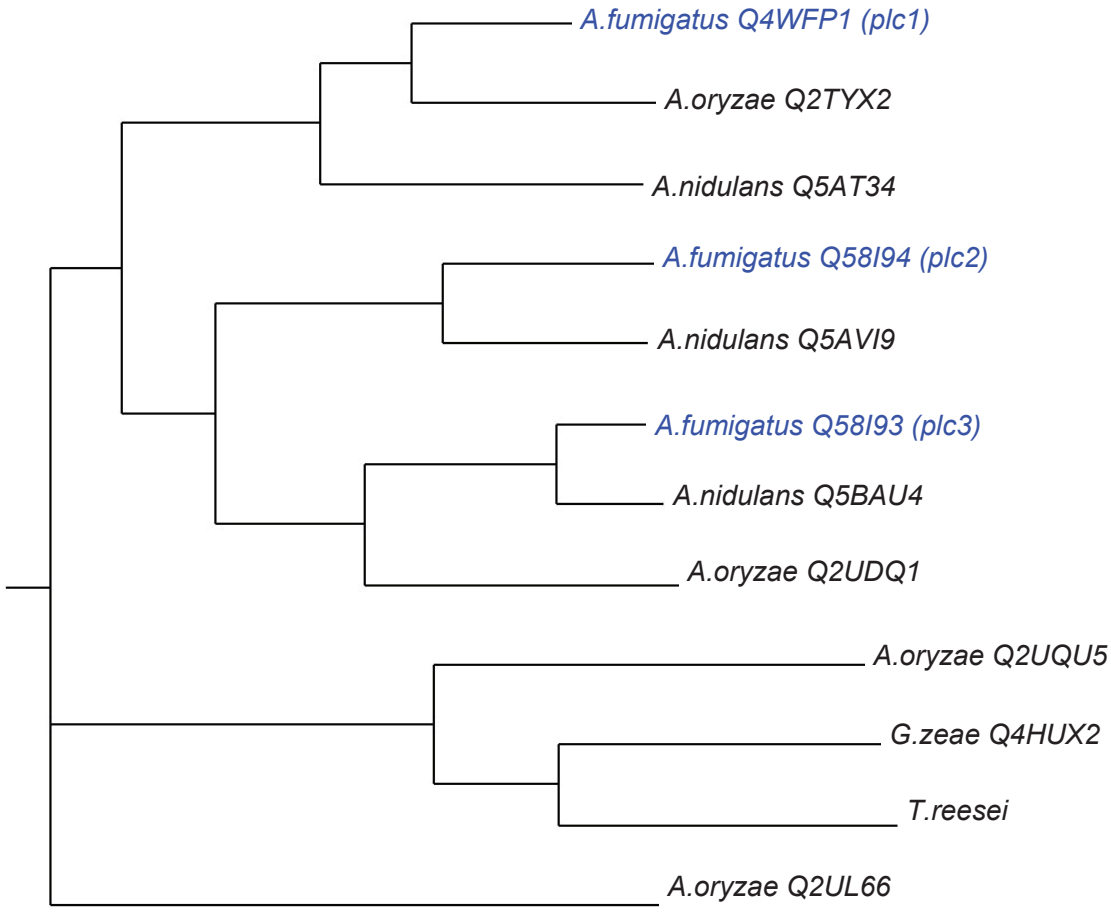


**Fig. 1.** Phylogenic relationship between the protein sequences of plc's from *A. fumigatus, A. nidulans, A. oryzae, T. reesei* and *G. zeae.*

(pfam 05506) which is found in the bacterial non-haemolytic PLC family (27).

Fungal *plc*'s are in red, plant *plc*'s in green and bacterial *plc*'s in blue. Codes represent SwissProt accession numbers. *T.reesei* was derived from genome sequence and no accession number was available.

**Expression of *plc* genes by real-iime PCR.** Expression of *plc genes of A. fumigatus* was measured by real time PCR using cDNA and primers which mentioned in material and method section (Table 2). The expression was determined using mRNA from *A. fumigatus* mid-log phase cultures grown at 37˚C. Results are pooled

**Table 1.** Summary of the properties of three putative secreted plc genes identified from the *A. fumigatus* genomic DNA.

| | ***Plc*1 (Q4WFP1)** | ***Plc*2 (Q58I94)** | ***Plc*3 (Q58I93)** |
|---|---|---|---|
| Genomic length (bp) | 1,302 | 1,421 | 1,429 |
| CDS length (bp) | 1,302 | 1,371 | 1,383 |
| Number of introns | 0 | 1 | 1 |
| Intron length (bp) | NA | 50 | 46 |
| Amino acid length | 433 | 456 | 460 |
| Mw (Kd) | 47.7 | 49.9 | 50.3 |
| PI | 4.86 | 6.12 | 4.94 |
| Pre-pro splice site | AG/AAP | ASA/IP | AAA/AA |
| % identity to PLC1 | NA | 42.6 | 42.3 |
| % identity to PLC2 | 42.6 | NA | 48.5 |
| % identity to PLC3 | 42.3 | 48.5 | NA |

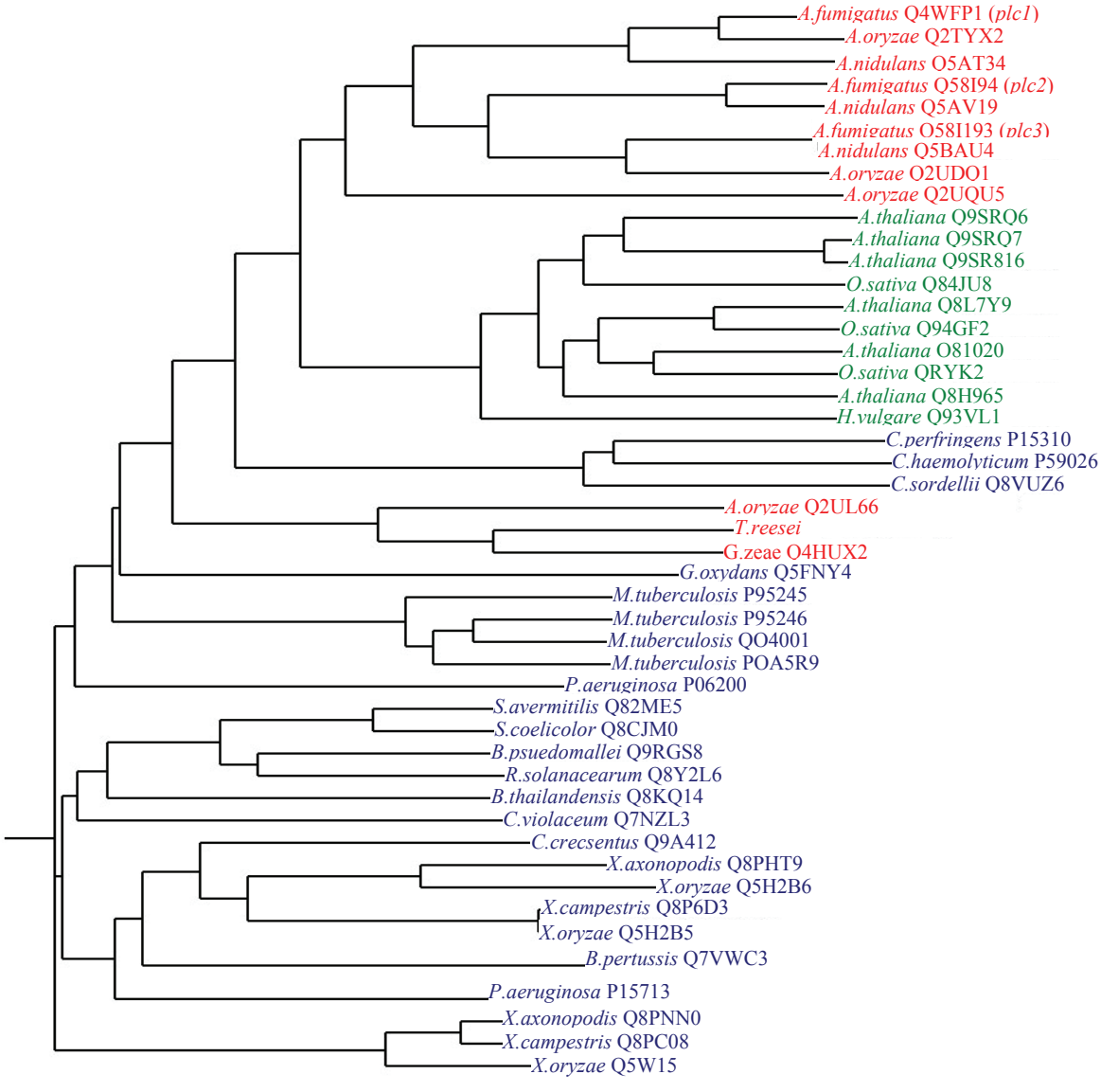


**Fig. 2.** Phylogenic relationship between the protein sequences of plc's from fungi, plants and bacteria.

from two independent experiments each with at least five replicates normalized to *actin* gene.

## DISCUSSION

Real-time PCR (called also quantitative or kinetic PCR) is used to quantify gene expression and to confirm differential expression of genes detected by array technology. This technique is able to measure the abundance of particular DNA or RNA sequences in clinical and industrial samples and is an effective and powerful technique for accurately quantifying

**Table 2.** Influence of phosphatidylcholine on the expression levels of plc genes of *A. fumigatus*.

| Gene | CT without phospholipids factor | ΔCT without phospholipids factor | CT with phospholipids | ΔCT with phospholipids | ΔΔCT | Fold change |
|---|---|---|---|---|---|---|
| ***Plc1*** | 34 | 13 | 22 | 1 | 12 | 4196• |
| ***Plc2*** | 33 | 12 | 23 | 2 | 10 | 1024• |
| ***Plc3*** | 35 | 14 | 25 | 4 | 10 | 1024• |

• Significant ($P < 0.05$) change in gene expression (t-test).

gene expression (28, 29).

As shown in Table 2, *afplc1*, *afplc2* and *afplc3* are expressed and are up-regulated by phosphatidylcholine. As the main site of infection of *A. fumigatus* is through the inhalation of spores which lodge in the lung and are therefore exposed to a phospholipid rich environment, it would appear that many of the extracellular phospholipases are likely to be up-regulated following inhalation. However, real time PCR on infected lung tissue will need to be performed to confirm if any are up-regulated *in vitro*. PLC is known to be a major pathogenicity factor in some bacterial pathogens where it causes cell lysis and tissue damage (30) although the *A. fumigatus plc*'s appear to lack the hydrophobic motif associated with haemolytic phospholipases. In filamentous fungi, it is usually the case that genes encoding extracellular hydrolases are normally not expressed or expressed at low levels in the absence of the substrate (31, 32). In this case *plc1*, *plc2* and *plc3* were all expressed at high levels in the presence of phosphatidylcholine. Moreover, a previous study examining a range of *A. fumigatus* isolates isolated from patients and the environment showed that clinical isolates produced significantly higher levels of PLC enzymes compared to environmental isolates (33). As orthologues to the secreted phospholipases are present in other *Aspergillus* species, differences in levels of expression rather than the presence of the genes may account for the differences in the levels of pathogenicity and quantifying secreted phospholipase activity amongst other *Aspergillus* species compared to *A. fumigatus* would be valuable. Although it is clear that the genome of *A. fumigatus* encodes a number of putative secreted phospholipases C that are likely to play a role in colonising the lung however it is unclear as to whether any are important in the pathogenicity of the organism, how damaging they may be to the lung surface and underlying cells, or to how critical they are in nutrient acquisition and therefore in allowing the fungus to proliferate in the lung.

As phosphatidylcholine is the major phospholipid in human lung, the presence of phospholipids may trigger the rapid extension and proliferation of hyphae which may play an important role in lung colonisation and in escaping macrophage engulfment.

Interrogations of the un-annotated genome sequence from TIGR revealed the presence of three *plc* genes which all contained pre-pro secretion signals and are therefore probably secreted into the medium.

Three PLC protein sequences from *A. fumigatus* were used in a tblastn search for orthologous sequences. While orthologues were found in *A. nidulans, A. oryzae, G. zeae* and *T. reesei*, *plc* orthologues are not universally present in the fungi and are absent from a taxonomically diverse group including no extracellular *plc* sequences were present in any of the other sequenced genomes which included *N. crassa, U. maydis, M. grisea and C. cinereus, C. albicans, S. cerevisia , P. chrysosorium, C. globosum, C. dubliniensis, S. pombe, C. neoformans serotype A* and *C. immitis* amongst others. Moreover, the number of genes encoding *plc* also varied. For example, while orthologues of all three *A. fumigatus plc*s were present in *A. nidulans*, *A. oryzae* contained four *plc* genes with only two being orthologues of *afplc1* and *afplc2*. Moreover, *G. zeae* and *T. reesei* contained only one *plc* gene which was not an orthologue of any of the *A. nidulans* or *A. fumigatus plc*s, but appeared to be more related to one of the *A. oryzae plc* genes (Fig. 2).

Thus it appears that the *plc*s are not universally distributed throughout the filamentous fungi and that their phylogeny is complex. This suggests either that the *plc*s were present in an ancestral genome and have become lost in some lineages, or that they have been acquired from other organisms by horizontal gene transfer. The acquisition of new genetic material by horizontal gene transfer is thought to be an important process in speciation, exploitation of new habitats and in maintaining organism vitality (34-36). Most of the evidence for horizontal gene transfer is between bacteria, particularly where cell densities are high such as in the rumen of animals (37) and evidence for horizontal gene transfer in the fungi has been scant by comparison (38). However, as more genome sequences are becoming available, evidence for such processes occurring in fungi is growing. For example, there is evidence that many of the glycosyl hydrolases produced by the rumen fungi may have been acquired by horizontal gene transfer from an anaerobic bacterium since most cluster phylogenetically with bacterial glycosyl hydrolases rather than with other fungal glycosyl hydrolases (39). More recently, evidence from phylogenetic analysis has also strongly suggested that a number of other genes have a prokaryotic origin including pea pathogenicity genes in *Nectria haematococca* and glucuronidase in some fungal soil isolates (40, 41). When the fungal *plc*'s were aligned with available full length *plc*'s from plants and bacteria, the fungal *plc*'s with three exceptions, clustered

together and appeared to be more closely related to the plant *plc*'s than the bacterial *plc*'s. The exceptions were the single *plc* present in *T. reesei* and *G. zeae* and one of the four *plc*s from *A. oryzae* which clustered together and were clearly more closely related to the bacterial *plc*'s than the plant *plc*'s (Fig. 2). Nonetheless this preliminary analysis clearly demonstrates that some fungi appear to have *plc*'s from an origin which is different to the other fungal *plc*'s and may have been acquired by horizontal gene transfer from a bacterial host. When compared to the bacterial *plc*'s, none of the fungal *plc*'s contain a hydrophobic region in the C-terminus commonly found associated with the haemolytic class of *plc*'s, suggesting that like the non-haemolytic bacterial *plc*'s, they may not be involved directly in cell lysis. Thus, for *A. fumigatus*, the extracellular *plc*'s may play a role in nutrient acquisition rather than in cell damage and lysis *per se*, particularly in the phospholipid rich environment of the lung (42). PLC activity has been inferred from a previous study by analysing the phospholipid breakdown products by FAB-MS (25). The majority of extracellular enzymes produced by filamentous fungi are growth related (43) although there are a number of exceptions, for example cellobiohydrolase which is produced on entering the stationary phase (44). PLC therefore appears to be constitutive, although extracellular phospholipid did appear to act as an inducer.

In order to elucidate the role of the *plc* genes in the infection process, each gene would need to be disrupted and tested in an animal model. Triple disruptants would need to be created to remove all extracellular PLC activity. Disrupted strains would be better tested through an inhalation model as it is the main route of infection and it is predicted that phospholipase activity may be important due to the phospholipid rich environment of the lung.

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