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# Complementation of a manganese-dependent superoxide dismutase-deficient yeast strain with *Pneumocystis carinii sod2* gene

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ABSTRACT

Manganese-dependent superoxide dismutase (MnSOD) is one of the key enzymes involved in the cellular defense against oxidative stress. Previously, the *Pneumocystis carinii sod2* gene (*Pcsod2*) was isolated and characterized. Based on protein sequence comparison, *Pcsod2* was suggested to encode a putative MnSOD protein likely to be targeted into the mitochondrion. In this work, the *Pcsod2* was cloned and expressed as a recombinant protein in EG110 *Saccharomyces cerevisiae* strain lacking the MnSOD-coding gene (*Scsod2*) in order to investigate the function and subcellular localization of *P. carinii* MnSOD (PcMnSOD). The *Pcsod2* gene was amplified by PCR and cloned into the pYES2.1/V5-His-TOPO® expression vector. The recombinant construct was then transformed into EG110 strain. Once its expression had been induced, PcMnSOD was able to complement the growth defect of EG110 yeast cells that had been exposed to the redox-cycling compound menadione. N-term sequencing of the PcMnSOD protein allowed identifying the cleavage site of a mitochondrial targeting peptide. Immunecolocalization of PcMnSOD and yeast CoxIV further confirmed the mitochondrial localization of the PcMnSOD.

Heterologous expression of PcMnSOD in yeast indicates that *Pcsod2* encodes an active MnSOD, targeted to the yeast mitochondrion that allows the yeast cells to grow in the presence of reactive oxygen species (ROS).



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## Introduction

*Pneumocystis jirovecii* is an ubiquitous fungal microorganism that causes *Pneumocystis* pneumonia (PcP) in immunosuppressed patients, notably those who are HIV-positive. Despite the widespread use of Highly Active AntiRetroviral Therapy (HAART) and anti-*Pneumocystis* therapy, PcP remains the second most frequent opportunistic infection in AIDS patients (Antiretroviral Therapy Cohort Collaboration (ART-CC) *et al.*, 2009, Huang *et al.*, 2011). Nowadays, patients without HIV infection, such as transplant recipients and patients with primary or secondary immunodeficiencies such as haematological malignancies or solid tumours, account for the majority of PcP cases in industrialized countries (Catherinot *et al.* 2010). Besides active PcP, *Pneumocystis* organisms are also present in the lungs of immunocompetent hosts. This clinically silent state is defined as colonization or subclinical carriage (Morris *et al.* 2004a). Notably, the prevalence of lung colonization by *P. jirovecii* is increased in patients suffering from chronic obstructive pulmonary diseases (COPD) (reviewed in Morris & Norris 2012). Moreover, in COPD experimental model, *Pneumocystis* colonization seems to lead to chronic inflammation (Shipley *et al.* 2010) and to involve an irreversible decrease of pulmonary function (Kling *et al.* 2014). Thus, *Pneumocystis* carriage is suggested to play a role in the pathophysiology and progression of COPD (Morris *et al.* 2004b; Calderón *et al.* 2007) as well as in the impairment of pulmonary function among patients with active tuberculosis (To *et al.* 2013).

Although reactive oxygen species (ROS), including free radicals and peroxides, are now considered as major cell signalling molecules that participate in cell homoeostasis, they can lead to oxidative damage in cells or tissues when produced in excess (Haliwell & Gutteridge 2006; Nathan & Cunningham-Bussel 2013). They are generated as by-products of aerobic metabolism (Venditti *et al.* 2013) or by stimulated host immune effectors cells, such as neutrophils, monocytes and macrophages (Nathan & Cunningham-Bussel 2013; Hasenberg *et al.* 2013). Complex networks of ROS-detoxifying systems have been developed by pathogens to cope with this detrimental oxidative stress. These defense mechanisms include superoxide dismutases (SODs), catalases and glutathione peroxidases. In many organisms including pathogenic fungi, three main isoforms of SOD have been described depending on the cofactor metal: manganese – (MnSOD), iron – (FeSOD) and copper/zinc – (Cu/ZnSOD) (Fréalle *et al.* 2005). MnSODs are mostly mitochondrial metalloenzymes that detoxify the $O_2^{\cdot-}$ free radicals generated by mitochondrial respiration. Most of the fungal MnSODs play an essential role in homoeostasis and cell survival by protecting cells against oxidative stress (Longo *et al.* 1999; Hwang *et al.* 2003; Lambou *et al.* 2010) and for some of them, in virulence (Giles *et al.* 2005; Xie *et al.* 2012).

The *Pneumocystis* organisms inhabit the alveolar microenvironment where the phagocytes of the innate immune system protect the airways by releasing ROS (Hasenberg *et al.* 2013). *Pneumocystis* ROS-detoxifying pathways probably have an important role in the fungal development inside the lung. Moreover, as *Pneumocystis* organisms have an aerobic metabolism, these pathways may also control endogenous sources of ROS that could be produced intracellularly.

Previously, our group cloned, identified, and characterized the *Pcsod2* gene (originally named *Pneumocystis carinii sod gene*) (Denis *et al.* 1998). The deduced amino acid sequence was compared to those of related organisms and a putative MnSOD activity was inferred (Denis *et al.* 1996, 1998).

Since it is not possible to continuously culture *Pneumocystis* organisms or directly manipulate their genes, heterologous expression of *P. carinii* MnSOD protein in a MnSOD-deficient *Saccharomyces cerevisiae* strain was used in the present study to investigate the protein function and its cellular localization. Previously, this expression method was successfully applied to functional analysis of genes of *Pneumocystis* (Kottom & Limper 2004; Moukhlis *et al.* 2010). The purpose of the present study was to gain an understanding on PcMnSOD, the first-line enzyme of the antioxidant system of *P. carinii*.

## Materials and methods

### *Ethics statement*

All animal experiments performed in this work were conducted following the guidelines of the Pasteur Institute of Lille animal study board, which conforms to the Amsterdam Protocol on animal protection and welfare, and Directive 86/609/EEC on the Protection of Animals Used for Experimental and Other Scientific Purposes, updated in the Council of Europe's Appendix A (http://conventions.coe.int/Treaty/EN/Treaties/PDF/123-Arev.pdf) and in the strict accordance with the French law (nu 87-848 dated 19-10-1987) and the European Community's 1976 Amendment of Cruelty to Animals Act. The protocol was approved by the Ethics Committee for Experiments on Animals of the Nord-Pas-de-Calais region (approval number ECEA 022011) and was carried out by qualified personnel. All efforts were made to minimize animals suffering. Rat's euthanasia was made after isoflurane inhalation and performed by lethal dose of pentobarbital injection.

### *Source of* Pneumocystis carinii *organisms*

*Pneumocystis carinii* organisms were extracted from lungs of dexamethasone-treated athymic Lou nu/nu rats as described elsewhere (Martinez *et al.* 2013) and were used for extracting total RNA and synthetizing cDNA in order to amplify the *Pcsod2* coding sequence encoding the *P. carinii* manganese-cofactored superoxide dismutase (PcMnSOD).

### *Yeast strains*

The *Saccharomyces cerevisiae* strains used in this study are listed in Table 1.

The BY4742 haploid *S. cerevisiae* strain was purchased from the European *S. cerevisiae* Archive for Functional Analysis (EUROSCARF, Frankfurt, Germany) and was used as a source of genomic DNA for amplification by PCR of the *Scsod2* gene encoding the *S. cerevisiae* MnSOD (ScMnSOD), which will serve as positive control.