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Transcription of N- and O-linked mannosyltransferase genes is modulated by the *pacC* gene in the human dermatophyte *Trichophyton rubrum*

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ABSTRACT

In fungi, ambient pH sensing involves the activation of the Pal/PacC signalling pathway. In the dermatophyte *Trichophyton rubrum*, pH-dependent secretion of keratinases, which are major virulence determinants, is affected by disruption of the *pacC* gene. Here, the transcription profiling of the genes coding for N- and O-linked mannosyltransferases, enzymes involved in protein glycosylation, was evaluated in *T. rubrum* in response to disruption of the *pacC* gene and growth in keratin, glucose, and glucose plus glycine. We show that transcription of these mannosyltransferase genes is affected by nutrients at acidic pH and by PacC.

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1. Introduction

Dermatophytes are the most common organisms infecting keratinized structures such as skin, hair, and nails, and their ability to degrade keratin is believed to be a major virulence factor [1,2]. A correlation between keratinolytic activity and pathogenesis has been proposed because dermatophytes secrete a battery of endo- and exoproteases during infection that degrade keratinized structures into oligopeptides and free amino acids for use as nutrients [3,4]. It is likely that proteases with optimal activity at both acidic and alkaline pHs are in

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regulation

sponse to the acidic pH of human skin, the pathogen de-represses the synthesis of non-specific keratinases and proteases that have optimal activity at acidic pH. They act on skin proteins, producing peptides that are hydrolyzed into amino acids, which are then used by the fungus as sources of carbon, nitrogen, and sulfur. The metabolism of some amino acids promotes the alkalinization of the host microenvironment, making it suitable for the action of keratinases with optimal activity at alkaline pH. The dermatophyte *Trichophyton rubrum* rapidly responds to pH changes by modulating the expression of genes, allowing the use of skin proteins over a wide pH range, thereby enabling the development of infection and persistence of the dermatophyte in host tissues [6,7] Moreover, inactivation of the *pacC* gene, a component of the pH signalling pathway in *T. rubrum*, reduces the activity of secreted keratinases [3], indicating that the *pacC* gene is somehow involved in the regulation of keratinolytic activity, and consequently in the virulence and pathogenicity of this organism.

Protein secretion from a eukaryotic cell requires movement through the endoplasmic reticulum (ER) and the Golgi apparatus. In the course of trafficking, the secreted proteins undergo glycosylation, which is the major post-translational molecular event [8–14]. In secreted proteins, the glycosyl groups are usually at-

threonine residues. During glycosylation, the oligosaccharide GlcNAc2Man9Glc3 is transferred to an Asn residue within the sequence Asn-XSer/Thr by an oligosaccharyltransferase), where X represents any amino acid except proline [15,16]. O-glycosylation occurs via several pathways. In higher eukaryotes, the main pathway utilizes sugar nucleotides and is located in the Golgi apparatus [17]. In yeasts, O-mannosylation begins in the ER lumen and, like N-glycosylation, it requires dolichol phosphate-activated sugar residues. The initial reaction is catalyzed by proteins from the evolutionarily conserved mannosyltransferase (Pmt) family [18,19]. Proteins secreted from yeast cells are usually heavily N- and/or O-glycosylated. In proteins that are glycosylated at both sites, it is not known whether N-glycosylation precedes O-mannosylation, or *vice versa* [20]. It is also unknown whether the O-mannosylation that takes place in the ER prevents N-glycosylation; however, there is some evidence for the opposite situation [21]. Altered glycosylation may affect the stability and half-life of proteins, thus changing their activities or affinities towards

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substrates [22,23].

Delineating the mechanisms underlying fungal adaptability to ambient variation is fundamental to an understanding of the mechanisms of pathogenicity and resistance to inhibitors in pathogenic organisms. This work was aimed at investigating the expression of genes encoding dolichyl-P-Man:Man(5)GlcNAc(2)-PP-dolichyl mannosyltransferase and an O-mannosyltransferase (referred to as the *N-man* and *O-man* genes, respectively) in the dermatophyte *T. rubrum* in response to nutrients, ambient pH, and disruption of the *pacC* gene. Our findings revealed a relationship between the expression of these two mannosyltransferase genes and the *pacC* gene in response to ambient pH and carbon source.

2. Materials and methods

2.1. Strains and growth conditions

T. rubrum clinical isolate H6 (ATCC MYA-3108) and a *pacC-1* mutant that carries a disrupted *pacC* gene, which were used throughout this study, were selected as previously described [3,24]. The H6 and *pacC-1* strains were cultivated on Sabouraud glucose agar for 15 days at 28 °C, and *pacC-1* cultures were supplemented with 450 μg/ml hygromycin. Mycelia were collected with a sterile spatula, vortexed in saline solution [0.9% (w/v) NaCl] with 0.01% (v/v) Tween, filtered through fiberglass to remove mycelial debris, and then centrifuged to recover the conidia. Then, 10^6 conidia were transferred to 50 ml of Sabouraud broth, and germination was carried out at 28 °C for 72 h on an orbital shaker at 180 rpm (control). After incubation, the final pH of the culture medium was measured with a pH meter, and the resulting mycelia were harvested by filtration through sterilized Whatman paper (Whatman International, Maidstone, UK), washed with sterilized water, and transferred to minimal medium (MM) [25] at pH 5.0 or pH 8.0, which was, in some cases (as indicated), buffered with 50 mM sodium citrate or 50 mM Tris–HCl, respectively. MM was supplemented with glucose (50 mM) or glucose plus glycine (50 mM each) and sodium nitrate (70 mM). MM was also supplemented with keratin from bovine hooves (2.5 g/l) as a nutrient source. All cultures were incubated at 28 °C for 3, 6, and 24 h with agitation.

2.2. RNA extraction and cDNA synthesis

Mycelia obtained from each culture were harvested by filtration, and total RNA was extracted from approximately 100 mg of frozen mycelium using TRIzol™ reagent, and treated with RNase-free DNase I (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. One microgram of DNase-treated RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The cDNA from 3 independent biological experiments was quantified spectrophotometrically, and stored at −80 °C until PCR amplification.

2.3. Real-time PCR

For quantitative real-time PCR analyses, the genes encoding dolichyl-P-Man:Man(5)GlcNAc(2)-PP-dolichyl mannosyltransferase (*N-man*, TERG_06338; GenBank: XM_003233297) and O-mannosyltransferase (*O-man*, TERG_06465; GenBank: XM_003233430) were amplified from cDNA using the following primers (5′–3′): TAAACGACAGTGGTATGCCG (*N-man*FWD) and TGTAGCCTGTTGGGTTCTCT (*N-man*REV); CCATGGGACGTGTATACTC (*O-man*FWD) and CGTCATCATAGCAACATTCAG (*O-man*REV). Reactions were performed in three independent experiments using SYBR green PCR master mix (Applied Biosystems, Foster City, CA), in the StepOne Plus Real-Time PCR system. A 12.5-μl reaction was set up using 50 ng of cDNA and 300 nM of each primer, and the PCR cycle was as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Melting curve analyses were performed after each cycling to exclude primer dimers and nonspecific PCR products. Relative transcript quantities were calculated using the $\Delta\Delta C_t$ method using the experimental condition that yielded the lowest C_t value (*N-man*, non-buffered H6 cultures in keratin, 6 h incubation) [26,27]. The *T. rubrum* α*-actin* (TERG_06637) and β*-tubulin* (TERG_07904) genes, which were used as endogenous reference genes, were amplified using the following oligonucleotides (sequence 5′–3′): AACGCCATCATGAAGTGT (*actin*FWD) and TCCTTCTGCATACGGTCAGA (*actin*REV); CCGTATGATGGCCACTTT (*tubulin*FWD) and CTGACCTGGGAAACGAAGAC (*tubulin*REV). Data normalization and analyses were performed using the GenEx 5 MultiD Analyses AB (www.multid.se). To confirm the identity of the genes analyzed in this work, as well as the endogenous reference genes, the PCR products were sequenced and analyzed by alignment with sequences retrieved from the Broad Institute Dermatophyte Comparative Database (http://www.broadinstitute.org/annotation/genome/dermatophyte_comparative/MultiHome.html).

3. Results

In vitro growth of the dermatophyte *T. rubrum* is dependent on the initial culture pH, with apparent optimal growth at pH 4.0–5.0, irrespective of carbon source (glucose, glycine, or protein). The initial pH of the *T. rubrum* cultures increased from 5.0 to a pH that ranged from 8.3 to 8.9 after 72–96 h of incubation in glycine or keratin. This effect was not observed when the fungus was cultivated with glucose as the carbon source, and the pH was maintained at approximately 5.0 [28,29]. Hydrolysis of keratin and other proteins releases amino acids, such as glycine, whose metabolism leads to the secretion of ammonia, thereby shifting the pH of the culture from acidic to alkaline [28]. Therefore, the metabolism of both glycine and keratin at pH 5.0 were alkalinizing events [28,29], even though the culture pH was still acidic after 24 h of incubation in glycine or keratin (Fig. 1). Moreover, disruption of the *pacC* gene in *T. rubrum* neither affects this pH shift (Fig. 1) [30] nor its growth on Sabouraud solid and in liquid media [3]. However, a marked decrease in the conidiation on Sabouraud solid medium and in the secretion of keratinolytic activity by the *pacC-1* mutant, which correlated with its reduced capacity to infect human nails *in vitro*, were observed as compared to the control strain [3]. No ambient pH changes were observed when the fungus was cultured in buffered media (data not shown). *T. rubrum* grows poorly in culture medium with a starting pH of 8.0, even though the culture becomes acidified after incubation with glucose as a carbon source (Fig. 1). Growth in glycine or keratin dropped the pH of the culture to approximately 7 after the first 3–6 h of incubation, which increased to approximately pH 7.5 after 24 h of incubation (Fig. 1). An attractive hypothesis is that *T. rubrum* senses the alkaline environment and then acidifies the culture medium to an ambient pH at which its growth is stimulated. Metabolism of glycine or keratin leads to the secretion of ammonia, which shifts the culture pH to alkaline pH values. Therefore, to better understand the transcription of both the *O-man* and *N-man* genes during the first 24 h of incubation, when the pH of the medium was still acidic in non-buffered cultures (Fig. 1), we estimated the expression of both genes by qRT-PCR in different culture conditions.

Transcription of the *O-man* and *N-man* genes in both the H6 and *pacC-1* mutant strains was affected differently by the carbon source, the culture pH, and the time of incubation (Fig. 2). At pH 5.0, *O-man* was preferentially transcribed in both buffered and non-buffered keratin cultures, whereas *N-man* was apparently preferentially transcribed in non-buffered glucose cultures. The expression of *N-man* decreased as incubation time increased at pH 5.0, but the expression decreased more consistently in buffered glucose cultures (Fig. 2). Disruption of the *pacC* gene decreased transcription of *O-man* in both glucose and keratin at pH 5.0; however, *O-man* expression was

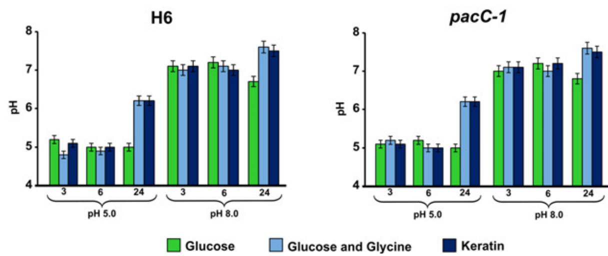

Fig. 1. Evaluation of pH changes during the cultivation of *Trichophyton rubrum* with glucose, glucose plus glycine, or keratin, as the carbon source, for 3, 6 and 24 h. The initial pH of the culture medium was 5.0 or 8.0. The bars in the columns represent the standard deviation of data obtained from three independent experiments.

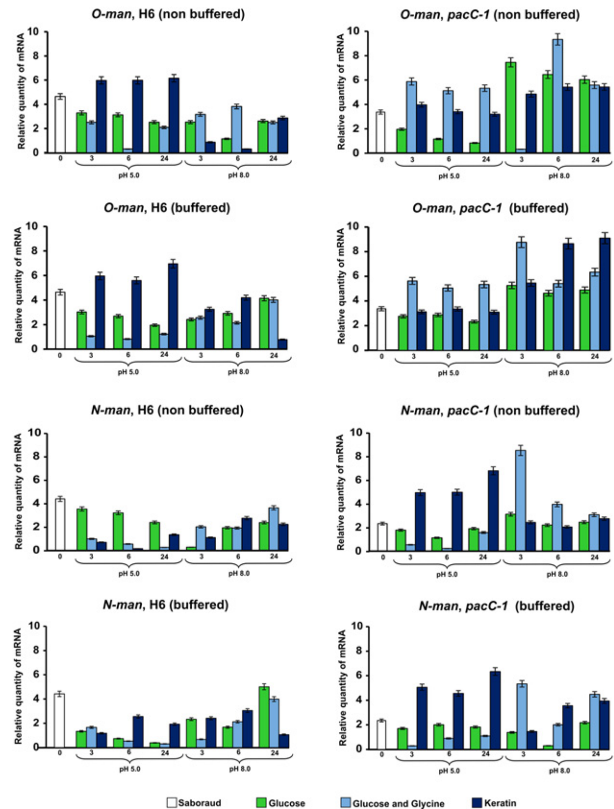

Fig. 2. Expression of mannosyltransferase genes (*O-man* and *N-man*) in *T. rubrum* by quantitative real-time RT-PCR. Strains H6 and *pacC-1* were cultured with glucose, glucose plus glycine, or keratin as the carbon source, for 3, 6, and 24 h. The initial pH of the culture medium was 5.0 or 8.0. The expression of the mannosyltransferase genes at 0 h is indicated (control). The bars in the columns represent the standard deviation of data obtained from three independent experiments.

enhanced in glucose plus glycine. Interestingly, the transcription of *N-man* in keratin cultures was enhanced in the *pacC-1* mutant at pH 5.0. Therefore, at acidic pH, transcription of the *O-man* and *N-man* genes was positively and negatively affected, respectively, by the *pacC* gene in keratine cultures (Fig. 2). Moreover, transcription of the *O-man* and *N-man* genes in buffered cultures indicated that the *O-man* gene was preferentially transcribed at acidic pH in the presence of keratin compared to glucose or glucose plus glycine as the carbon sources, whereas transcription of the *N-man* gene was almost the same in both acidic and alkaline pH in the presence of keratin, and these properties were affected by disruption of the *pacC* gene (Fig. 2). It is also worth noting that while the disruption of the *pacC* gene resulted in changed transcription of the *O-man* gene in the different conditions at pH 5.0, it also resulted in the opposite changes for growth in glucose and glucose plus glycine at pH 8.0. Thus, our results suggest that the product of the *pacC* gene negatively affect both the transcription of the *N-man* gene in keratin at pH 5.0 and the *O-man* gene at pH 8.0 in the presence of different nutrients (Fig. 2) i.e., the *pacC* gene is functional irrespective of the culture conditions assayed.

4. Discussion

It has been well documented that the dermatophyte *T. rubrum*, as well as other filamentous fungi, acidifies the culture medium and represses the secretion of proteases during growth in glucose as the sole carbon source [31,32]. Moreover, during growth in glycine or keratin, the culture medium is alkalinized; however, this change is dependent on the initial pH of the culture, with an apparent optimum at pH 4.0–5.0. Interestingly, glycine utilization is apparently not repressed by glucose, because alkalinization of the culture medium occurs with glucose and glycine as carbon sources [29,33]. However, it is worth noting that during the first 24 h of cultivation, the pH of the medium is still acidic (Fig. 1), implying that the metabolism of glycine or keratin, an alkalinizing event, occurs during the first 24 h of cultivation, exclusively in an acidic environment.

In the model fungi *Neurospora crassa* and *Aspergillus nidulans*, one of the metabolic responses to the pH of the culture medium is the pH-dependent glycosylation of secreted enzymes [34–37]. For example, the level of glycosylation of the Pho-2 alkaline phosphatase synthesized by *N. crassa* at alkaline pH differs from that synthesized at acidic pH, which is approximately 13% and 21% for the Pho-2 enzyme purified from mycelium grown at pH 5.4 and 7.8, respectively [37,38]. The loss of enzymatic activity observed for the Pi-repressible alkaline phosphatase secreted at acidic pH is probably because the glycosylation of this enzyme is lower than that secreted at alkaline pH. We have also provided evidence that glycosylation of secreted enzymes, as documented for the Pi-repressible phosphatases in *N. crassa* and *A. nidulans*, is PacC-dependent [35].

In *T. rubrum*, disruption of the *pacC* gene, as in the *pacC-1* mutant strain, resulted in decreased growth on human nails and decreased secretion of keratinolytic proteases in liquid medium when supplemented with keratin, which suggests that the keratinases secreted by *T. rubrum* are somehow regulated by the PacC protein [3]. PacC might be involved in the glycosylation of these keratinases through transcriptional modulation of O- and N-linked mannosyltransferases, a hypothesis supported by the results described here. Transcriptional profiling of both the *O-man* and *N-man* genes revealed a high level of complexity, because transcription of these genes was affected by nutrients, culture pH, and the functioning of the *pacC* gene. Disruption of the *pacC* gene increased the expression of *N-man* at pH 5.0 in keratin cultures. Moreover, if O-mannosylation precedes N-glycosylation in *T. rubrum*, as demonstrated in yeast [20], this physiological effect is dependent on the function of the *pacC* gene at acidic pH.

In conclusion, the genes encoding the O- and N-mannosyltransferases had different expression profiles, and the *O-man* gene was preferentially expressed at acidic pH when *T. rubrum* was grown on medium containing keratin. The balance between *N-man* and *O-man* expression levels in cultures at acidic pH may be under the control of the PacC transcription factor (in response to different carbon sources). Therefore, the product of the *pacC* gene of *T. rubrum* is functional at acidic pH. Moreover, transcription of the *N-man* and *O-man* genes might be required at different culture pHs for the glycosylation of transported proteins, according to the stage of infection, which suggests a possible role in cell adhesion and activation of signalling pathways regulating the production of enzymes that enable nutrient uptake for fungal development and maintenance in the host [39–41].

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