

Short communication

MOLECULAR CHARACTERIZATION OF THE *niaD* AND *pyrG* GENES FROM *Penicillium camemberti*, AND THEIR USE AS TRANSFORMATION MARKERS

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Abstract: Genetic manipulation of the filamentous fungus *Penicillium camemberti* has been limited by a lack of suitable genetics tools for this fungus. In particular, there is no available homologous transformation system. In this study, the nitrate reductase (*niaD*) and orotidine-5'-monophosphate decarboxylase (*pyrG*) genes from *Penicillium camemberti* were characterized, and their suitability as metabolic molecular markers for transformation was evaluated. The genes were amplified using PCR-related techniques, and sequenced. The *niaD* gene is flanked by the nitrite reductase (*niaA*) gene in a divergent arrangement, being part of the putative nitrate assimilation cluster in *P. camemberti*. *pyrG* presents several polymorphisms compared with a previously sequenced *pyrG* gene from another *P. camemberti* strain, but almost all are silent mutations. Southern blot assays indicate that one copy of each gene is present in *P. camemberti*. Northern blot assays showed that the *pyrG* gene is expressed in minimal and rich media, and the *niaD* gene is expressed in nitrate, but not in reduced nitrogen sources. The functionality of the two genes as transformation markers was established by transforming *A. nidulans pyrG*- and *niaD*-deficient strains. Higher transformation efficiencies were obtained with

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Abbreviations used: dCTP – deoxycytidine triphosphate; Moco – molybdenum cofactor; *niaD* – nitrate reductase-encoding gene; *niaA* – nitrite reductase-encoding gene; OMPD – orotidine-5'-monophosphate decarboxylase; ORF – open reading frame; *pyrG* – orotidine-5'-monophosphate decarboxylase-encoding gene

a *pyrG*-containing plasmid. This is the first study yielding a molecular and functional characterization of *P. camemberti* genes that would be useful as molecular markers for transformation, opening the way for the future development of a non-antibiotic genetic transformation system for this fungus.

Key words: *Penicillium camemberti*, *niaD* gene, *pyrG* gene, Transformation, *Aspergillus nidulans*

INTRODUCTION

The filamentous fungus *Penicillium camemberti*, which is the ripening agent for Camembert cheese, could be a good candidate for genetic improvement, or a good model for industrial enzyme production, especially for enzymes used in the food industry [1]. However, despite its biotechnological potential, not much progress has been made in the study of its molecular genetics. Other than reports on the genes encoding mono- and diacyl glycerol lipase, β -tubulin, or those involved in conidiation and the fungus' surface properties [2-4], there have been no papers on molecular or functional studies of the genes coding for structural proteins and enzymes in *P. camemberti*. Therefore, there are no available homologous metabolic markers for transformation of this fungus. Although dominant antibiotic markers are available for fungi, the permanent expression of antibiotic resistance genes is a current concern in the case of food-related organisms such as *P. camemberti*. For this reason, metabolic markers are preferred.

The most frequently used metabolic markers for fungal transformation are the *niaD* and *pyrG* genes. *niaD* encodes for a NADPH-dependent nitrate reductase that reduces nitrate to nitrite [5], and *pyrG* encodes for orotidine-5'-monophosphate decarboxylase (OMPD), an enzyme that catalyzes the synthesis of uridine 5'-monophosphate from orotidine 5'-monophosphate [6]. It has been described that mutants lacking *pyrG* are not able to grow in a medium lacking uridine, but this disability can be restored by the transformation of the fungus with a functional copy of the *pyrG* gene [7, 8]. In the case of *niaD*-deficient mutants, they are unable to grow in a medium containing nitrate as the sole nitrogen source, but the transformation of the fungus with a functional copy of the *niaD* gene restores this ability [9, 10].

To take advantage of the biotechnological potential of *P. camemberti*, it is necessary to gain insight into the genes that could be useful as biotechnological tools. Therefore, we characterized the *niaD* and *pyrG* genes from *P. camemberti*, and used them as transformation markers using *Aspergillus nidulans* as the host. Our results open the way for the future development of a non-antibiotic genetic transformation system for *P. camemberti*.

MATERIALS AND METHODS

Strains, culture media and genomic DNA isolation

P. camemberti strain CECT 2267 was kept on Power agar plates [11]. The *A. nidulans* A691 *niaD*-deficient strain was kept on Power medium plus nitrite and biotin. The *A. nidulans* A722 *pyrG*-deficient strain was kept on YEG (2% glucose, 0.5% yeast extract) agar plus uridine. Both *Aspergillus* strains were obtained from the Fungal Genetics Stock Center (Kansas, USA). Genomic DNA from *P. camemberti* was isolated according to Bainbridge *et al.* [12].

Sequencing of the *niaD* and *pyrG* genes and their flanking genomic regions

From a multiple alignment of several fungal *niaD* genes, the degenerate primers NIAD1FW (5'-CC(AC)AGTGA(CT)AACTGGTA-3') and NIAD2RV (5'-AC(AG)GACCAGTACAT(AG)TC-3') were designed, and used for PCR with *P. camemberti* genomic DNA as the template, obtaining a *niaD*-specific DNA fragment (597 bp), which was then sequenced. The rest of the 5' end of *niaD* and its promoter part of the divergent *niiA* gene were amplified as a single PCR product (1,267 bp) using the specific primer BEGNIAD (5'-ACTAATTTCCCAATCCGG-3') and the degenerate primer BEGNIIA (5'-CC(AG)AT(GC)AC(GA)AC(GA)AT(AG)TCGTA-3'), designed from a multiple alignment of fungal *niiA* genes. Finally, a 1,657-bp sequence containing the complete 3' end of *niaD* and its 3' UTR sequence was amplified by two consecutive reactions using the Universal Genome Walker kit (Clontech). The library construction, primer design and PCR conditions were according to the manufacturer's instructions.

For *pyrG* gene amplification, the degenerate primers PDpyrG1 (5'-ACAT (CT) GCCGTGAT (CT) AAA-3') and PDpyrG2 (5'-GA (AT) CCCTT (GC) GAGGTCAT-3') were designed and used for PCR as above, obtaining a specific *pyrG* DNA fragment (319 bp), which was sequenced. The rest of the sequences upstream and downstream from this segment were amplified using the Genome Walker libraries as above.

Amplified DNA segments were cloned and sequenced, obtaining a 4,127-bp sequence comprising the whole *niaD* open reading frame (ORF) plus its adjacent regions, and a 2,307-bp sequence comprising the whole *pyrG* and its upstream and downstream non-coding regions. Both sequences have been deposited in Genbank (Accession numbers FJ211612 (*niaD*) and EU784664 (*pyrG*)).

Southern and Northern blot analysis

Forty micrograms of genomic DNA was digested with restriction enzymes, electrophoresed and blotted onto a Hybond N⁺ nitrocellulose membrane (Amersham Pharmacia Biotech) by capillarity transfer, and UV-cross-linked to the membrane.

For the *niaD* Northern blot assay, *P. camemberti* was grown in Czapek minimal medium (3% sucrose, 0.05% K₂HPO₄, 0.05% MgSO₄ x 7H₂O, 0.001% FeSO₄ x 7H₂O) supplemented with the different nitrogen sources (25 mM of glutamine,

urea or ammonium chloride). The incubation was at 28°C for 96 hours and at 220 r.p.m. After 96 hours, the mycelia were collected by filtering, and washed with 0.9% NaCl. To induce the transcription of nitrate reductase, mycelia from the glutamine-containing medium were transferred to Czapek minimal medium containing 25 mM sodium nitrate, incubated for the indicated times (see Fig. 1C), collected, and washed again.

For the *pyrG* Northern blot assay, *P. camemberti* was grown in Czapek minimal medium containing nitrate as the nitrogen source, or in CM complete medium (2% glucose, 0.5% yeast extract, 0.5% malt extract), in each case for 24 hours at 220 r.p.m and 28°C. The mycelia were collected and washed as above. Once collected, the mycelia were frozen in liquid nitrogen, ground in a mortar, and then the total RNA was extracted using the RNeasy Plant Minikit (Qiagen) according to the manufacturer's instructions. Twenty micrograms of each sample of extracted total RNA was electrophoresed and blotted onto a Hybond N⁺ nylon membrane (Amersham Pharmacia Biotech). The pre-hybridization and hybridization steps for the Southern and Northern blot assays were carried out according to Sambrook and Russell [13]. As probes, an 870-bp fragment containing almost the whole *pyrG* sequence, and a 597-bp fragment containing part of the *niaD* gene were used. The probes were amplified by PCR and labeled with [α -³²P]-dCTP using the Decalabel DNA labeling kit (Fermentas). The membranes were washed [13], and then analyzed with a PhosphorImagerTM.

Plasmid construction and transformation of the *niaD* and *pyrG* *A. nidulans* strains

A 3,129-bp sequence, including the *niaD* gene, its promoter and 3' non-coding sequence, was amplified by PCR using the primers CNIADFW (5'-CATATC GTAGTCCATCCAGT-3') and CNIADRV (5'-AACCAGTGTCTTTGA GCATC-3'). It was then cloned into the T&A Cloning Vector (RBC Bioscience) yielding the plasmid pYESNIAD. The complete sequence of *pyrG*, including its promoter and 3' non-coding sequence, was amplified by PCR using the primers PForw (5'-TGCGCAAGGAGGCCAAGGTG-3') and PRever (5'-GATATA GGTAATCGGGCCGC-3'), and cloned into the T&A Cloning Vector yielding the plasmid pAsw-pyrG. The transformations of the suitable *A. nidulans* strains with these plasmids were carried out as described by Yelton *et al.* [14], using 20 mg/ml Lysing Enzymes (Sigma). The transformed protoplasts were plated on a suitable medium containing 1.2 M sorbitol as the osmotic stabilizer.

RESULTS

The analysis of the *niaD* gene and its deduced protein

The *niaD* gene is 2,946 bp long, is interrupted by six introns (60, 54, 72, 50, 56 and 53 bp in length), and encodes a deduced protein of 864 amino acids that presents a high degree of identity to other fungal nitrate reductases. The domain structure of *P. camemberti* nitrate reductase is similar to that of other eukaryotic

nitrate reductases, containing a molybdenum cofactor (Moco) domain, heme-binding domain, FAD domain and NADPH-binding domain. According to a multiple alignment with other fungal nitrate reductases, all the conserved residues important to catalytic activity and substrate binding are present in the deduced protein [15].

In a divergent orientation from *niaD*, and separated by an intergenic promoter (see below), a 225-bp sequence belonging to the partial *niiA* ORF was found. This sequence is interrupted by an intron (49 bp long) and encodes for the first 58 amino acids of a deduced protein with high similarity to fungal nitrite reductases.

Finally, several binding sites for elements that have been described as important regulators in the nitrogen metabolism are found in the 649-bp intergenic promoter that separates the divergent genes *niaD* and *niiA*. Nine GATA sequences, involved in the binding of the positive broad regulator AREA [16, 17], and three consensus sequences for the binding of the specific regulatory protein NirA [18, 19] are present in this promoter. The presence of these binding sites suggests that the expression of *niaD* could be modulated by the nitrogen source in *P. camemberti* (see below).

The analysis of the *pyrG* gene and its deduced protein

A *pyrG* sequence from another *P. camemberti* strain (U-150) was previously deposited in Genbank (accession number AB100244), so we tried to amplify the *pyrG* gene from our strain (CECT 2267) using specific primers designed from that sequence, albeit unsuccessfully. This suggested that important differences must exist at the nucleotide level between the *P. camemberti pyrG* genes from U-150 and CECT 2267. This was checked once the *pyrG* from *P. camemberti* CECT 2267 had been sequenced. This gene is 890 bp long, contains one intron (59 bp) and shows 24 nucleotide variations when it is compared to the *pyrG* sequence from strain U-150 (Tab. 1). Three mutations were located in the intron and 21 mutations were found in the exon sequences. All but two of these mutations were silent. Thus, the protein sequence deduced from our *pyrG* gene

Tab. 1. Nucleotide polymorphisms in the *pyrG* genes from the *P. camemberti* strains CECT 2267 and U-150. The numbering starts at the first nucleotide at the ATG start codon. The differences located in the intron region are in bold. The differences resulting in amino acid substitution are underlined.

Change of nucleotide	Position of the change
C→T	24, 84, 440, 581, 641, 656, 701
T→C	27, 33, 168, 174, 179 , 572, 647, 686
C→G	105, 404
A→G	135, <u>588</u>
G→C	<u>456</u> , 626
G→A	749, 848
A→G	815

corresponds to a 276-amino acid long polypeptide that is almost identical to the OMPD protein deduced from strain U-150, especially in the region that includes the signature motif (see Discussion). Neither of the two differences at the amino acid level (E133Q and T177A) is in a critical amino acid. Several other nucleotide differences were detected in the promoter and 3' non-coding sequences (data not shown).

Molecular characterization of *niaD* and *pyrG*

The copy numbers of *pyrG* and *niaD* in *P. camemberti* were determined by Southern blot (Fig. 1A and B). The results obtained agree with the presence of only one copy of these genes in *P. camemberti*. As was suggested above, the results of the Northern blot assays indicate that *niaD* is differentially expressed

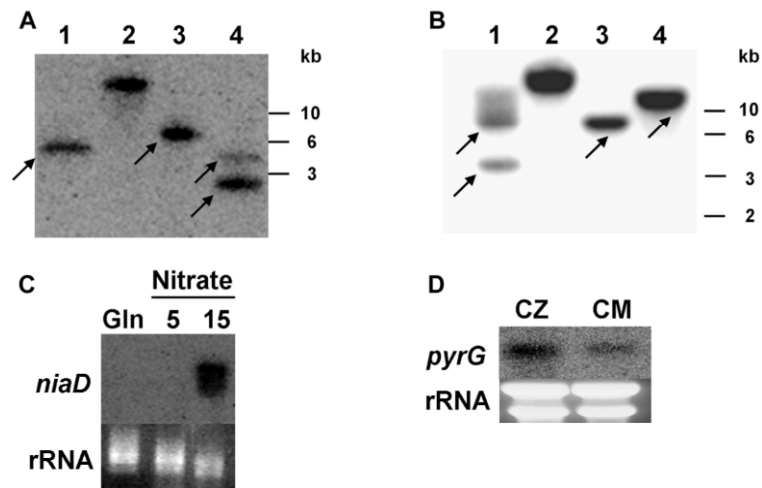


Fig. 1. Molecular characterization of *niaD* and *pyrG* genes from *P. camemberti*. A – The Southern blot assay was applied to determine the *niaD* copy number in *P. camemberti*. Genomic DNA from strain CECT 2267 was cut with *Hind*III (lane 1), *Sac*I (lane 3) and *Eco*RI (lane 4). *Eco*RI cut in the region covered by the probe, while the other enzymes did not cut in this region. Hybridized fragments are indicated with arrows. Lane 2 contains undigested control DNA. The sizes of the standard used (GeneRuler DNA Ladder Mix, Fermentas) are indicated (kb). B – The Southern blot assay was applied to determine the *pyrG* copy number in *P. camemberti*. Genomic DNA was cut with *Pst*I (lane 1), *Sca*I (lane 3) and *Bgl*II (lane 4). *Pst*I cut in the gene, while *Sca*I and *Bgl*II did not. Hybridized fragments are indicated with arrows. Lane 2 contains undigested control DNA. The standard used is the same as indicated above. C – The Northern blot assay was performed for *niaD* expression. Total RNA was extracted from mycelia after growth in the presence of glutamine for 96 hours (lane Gln), or from mycelia grown in glutamine and then transferred to a medium containing sodium nitrate for 5 minutes (lane 5) or for 15 minutes (lane 15). Ribosomal RNA (rRNA) is shown as a loading control. D – The Northern blot assay of the total RNA extracted from mycelia after growth in minimal Czapek (CZ) or CM complete medium, and hybridized with a *pyrG* probe. Ribosomal RNA (rRNA) is shown as a loading control.

in *P. camemberti*, depending on the nitrogen source used. When the fungus is grown in urea, ammonium or glutamine, its expression is not detected (data not shown), but if the mycelium is transferred from glutamine to a nitrate-containing medium, its expression was observed after 15 minutes (Fig. 1C). With respect to the transcriptional expression of *pyrG*, Fig. 1D shows that this gene is expressed in both minimal (Czapek) and rich (CM) media, suggesting that *pyrG* expression is independent of the medium used.

The *niaD* and *pyrG* genes from *P. camemberti* are suitable as metabolic markers for fungal transformation

To prove that the genes obtained were suitable as transformation markers, DNA segments containing the *niaD* and the *pyrG* genes from *P. camemberti*, including their promoters and 3' untranslated sequences, were used for the complementation of suitable *A. nidulans* strains. Protoplasts from the *A. nidulans niaD*⁻ and *pyrG*⁻ strains were transformed, respectively with the plasmids pYESNIAD or pAsw-pyrG. After 3 to 5 days, several transformant colonies were obtained. Some randomly selected transformants were replica-plated on a suitable medium, demonstrating that these plasmids were able to complement a *niaD* and *pyrG* deficiency in *A. nidulans* strains (Fig. 2). The transformation efficiencies obtained with pAsw-pyrG (10 ± 1 transformants/ μg DNA per 10^7 protoplasts) were higher than with pYESNIAD (3 ± 1 transformants/ μg DNA per 10^7 protoplasts).

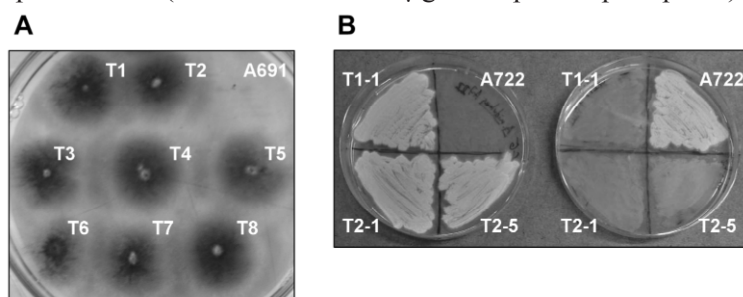


Fig. 2. Complementation of *A. nidulans* strains with plasmids containing *niaD* and *pyrG* genes from *P. camemberti*. A – *A. nidulans* A691 (*niaD*⁻) was transformed with pYESNIAD, and some transformants (T1 to T8) were grown on Czapek-biotin agar containing nitrate as the sole nitrogen source, demonstrating *niaD* complementation by pYESNIAD. As a control, *A. nidulans* A691 is shown. B – Agar plates showing the complementation of *A. nidulans* A722 *pyrG* deficiency by pAsw-pyrG. Left plate: *A. nidulans* A722 was transformed with pAsw-pyrG, and some transformants (T1-1, T2-1 and T2-5) were grown on YEG agar without uridine. As a control, *A. nidulans* A722 is shown. Right plate: The same transformants were not able to grow on plates containing 5'-fluoro-orotic acid (1 mg/ml), which is metabolized to 5'-fluoro-orotidin monophosphate and, finally, to 5'-fluoro-uridin monophosphate (5'-fluoro UMP) by functional OMPD. 5'-fluoro UMP is highly toxic, thus inhibiting the growth of fungi with functional *pyrG* genes. The control *A. nidulans* A722 (WT) can grow on this compound because it lacks a functional *pyrG* gene. The plate on the right contains 1% uridine to support the growth of *A. nidulans* A722.

DISCUSSION

The unavailability of homologous metabolic and non-antibiotic selection markers is one of the main limitations on the genetic improvement of and biotechnological applications for *P. camemberti*. The *niaD* and *pyrG* genes characterized in this study could be excellent tools to overcome these limitations.

The *niaD* gene is divergently clustered with the *niiA* gene, suggesting that, like other filamentous fungi, *P. camemberti* contains a nitrate assimilation cluster. The genomic array for the *niaD* and *niiA* genes is variable, depending on the specific organism analyzed. Recently, Slot and Hibbett [20] summarized the genomic array of nitrate assimilation genes from 15 whole genome sequences available for filamentous fungi. They showed that the *niaD-niiA* divergent array is maintained in the *Aspergillus* sp. and *Trichoderma reesei* genomes, but not in most of the fungal species analyzed, where these genes were not clustered; or that the arrangement presented was the *niaD-niiA* genes clustered but transcribed in the same direction. In the genus *Penicillium*, the genomic array of these genes has been published only for *P. chrysogenum*, showing the same divergent array described here [21].

The *pyrG* genes from the *P. camemberti* strains CECT 2267 and U-150 show a high genetic variability. However, most of the nucleotide differences between the two *pyrG* genes represent neutral mutations [22], which do not lead to amino acid substitution. Thus, we found much less polymorphism at the protein level. We noticed that nucleotide mutations are absent in regions that encode for protein segments important for activity. Thus, while nucleotide mutations seem to be randomly distributed along the gene, there is a large invariant region downstream of the intron (comprising nucleotides 217 to 403), which encodes for a highly conserved region that includes the signature motif DXKXXDIXXT and most of the residues involved in catalysis in OMPDs, the protein product of *pyrG* [23]. This suggests that nucleotide substitutions in this region are less tolerated than in the rest of the gene, reflecting a strong selective pressure against mutations that alter the properties of the enzyme.

To obtain *pyrG*- or *niaD*-deficient strains for complementation in *P. camemberti* in future studies, rounds of random mutagenesis or gene replacement of the host genes must be done, but these strategies have low probabilities to succeed if the host contains more than one copy of the genes. Our data suggests that *P. camemberti* CECT 2267 has only one copy of *pyrG* and *niaD*. In addition, we found that *pyrG* is expressed independently of the medium used, while *niaD* expression was detected in the presence of nitrate but not when urea, ammonium or glutamine was used. Similar results were described for the *P. chrysogenum niaD* gene [24]. In contrast to *P. camemberti* and *P. chrysogenum*, a putative basal expression of the *niaD* gene was found via RT-PCR in *P. griseoroseum* using similar conditions to those described here [10]. These differences suggest

that nitrate reductases could be differentially expressed in *Penicillia*, which deserves more detailed study in the future.

The successful transformation of *A. nidulans* strains with the pAsw-pyrG and pYESNIAD plasmids, respectively complementing *pyrG* and *niaD* deficiency, demonstrated that these genes, isolated from *P. camemberti*, are useful as metabolic markers. However, they show differences in transformation efficiencies. Higher efficiencies obtained with pAsw-pyrG could reflect the particular transformation competence of each *A. nidulans* strain used. On the other hand, since the *pyrG* gene is smaller than the *niaD* gene, pAsw-pyrG could be more easily taken up by the cell than pYESNIAD due to its size. While the transformation marker genes are usually functional in heterologous fungi with close phylogenetic relationships, it has been observed that higher efficiencies are obtained for homologous transformation compared to heterologous transformation [7]. Thus, it is expected that these plasmids will work even better in *P. camemberti* than in *A. nidulans*.

Summarizing, since no metabolic transformation system has been developed for any cheese-ripening fungi, including *P. camemberti*, our results open the way to the future development of metabolic complementation and non-antibiotic transformation systems in this fungus based on the *pyrG* or the *niaD* genes.

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