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Co-cultivation of antifungal *Lactobacillus plantarum* MiLAB 393 and *Aspergillus nidulans*, evaluation of effects on fungal growth and protein expression

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Abstract

The fungal inhibitory effects of strain *Lactobacillus plantarum* MiLAB 393, producing broad-spectrum antifungal compounds, were evaluated. A co-cultivation method was set up to monitor effects on fungal growth and protein expression of growing *Aspergillus nidulans* with *L. plantarum* MiLAB 393. The effects of inhibitory metabolites produced by *L. plantarum* MiLAB 393, cyclo(L-Phe–L-Pro), lactic acid and 3-phenyllactic acid, were also investigated by addition of pure compounds to the growth medium of *A. nidulans*. The co-cultivation strongly affected the morphology of the fungal mycelium and decreased the biomass to 36% of control. Co-cultivation with *Lactobacillus coryniformis* MiLAB 123 gave only marginal morphological changes and minor biomass reduction, suggesting specific effects of *L. plantarum* MiLAB 393. The amount of several *A. nidulans*-proteins was increased during co-cultivation and by all of the inhibiting substances. This study shows that the growth of *A. nidulans* is inhibited during co-cultivation with *L. plantarum* MiLAB 393 and that the expression of fungal proteins is altered.

Keywords: Antifungal activity; Two dimensional gel electrophoresis; 3-Phenyl lactic acid; Cyclic dipeptide

1. Introduction

Lactic acid bacteria (LAB) are known to produce antimicrobial metabolites and have a long tradition as starter cultures for different fermented food and feed. Production of metabolites such as organic acids, ethanol, hydrogen peroxide and diacetyl are associated with the preserving and inhibitory effect of this group of bacteria [1]. Production of bacteriocins, ribosomally synthesized antimicrobial peptides, is also important for the antibacterial properties of these organisms [2]. LAB coexist with filamentous fungi and yeasts in many ecosystems, e.g., in various fermented foods. They can also inhibit fungal growth and have a potential as biopreservatives [3].

LAB produce low molecular weight compounds [4], peptides [5], and proteins [6] with antifungal properties. Presently, not much is known about how these substances affect the fungal growth. We have earlier identified the antifungal compounds cyclo(L-Phe–L-Pro), cyclo(L-Phe–*trans*-4-OH-L-Pro) and 3-phenyllactic acid from *Lactobacillus plantarum* MiLAB 393 [7]. In this study we have investigated the effects of co-cultivation with *L. plantarum* MiLAB 393 on the

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biomass, the morphology and protein expression of the model fungus *Aspergillus nidulans*. In addition, the effects of three of the known inhibiting substances, cyclo(L-Phe–L-Pro), 3-phenyllactic acid and lactic acid were investigated.

2. Material and methods

2.1. Cultures and media

The strains *L. plantarum* MiLAB 393 and *L. coryniformis coryniformis* MiLAB 123 were grown on MRS agar (Oxoid Ltd, Basingstoke, England) at 30 °C in anaerobic jars under $CO_2 + N_2$ atmosphere (GasPak System, BBL, Cockeysville, MD, USA). The cultures were stored anaerobically on MRS agar plates at 5 °C, or for long-term storage at −70 °C in a 15% glycerol salt solution (0.82 g K_2HPO_4, 0.18 g KH_2PO_4, 0.59 g Na-citrate and 0.25 g $MgSO_4 \cdot 7\,H_2O$ per liter). Liquid cultures were grown in modified MRS-medium (mMRS) without acetate and Tween 80 (10 g bacteriological peptone, 8 g meat extract, 4 g yeast extract, 2 g K_2HPO_4, 2 g $(NH_4)_3$citrate, 0.2 g $MgSO_4 \cdot 7\,H_2O$, 0.05 g $MnSO_4 \cdot 4\,H_2O$ and 20 g glucose per liter). Buffered cultures were prepared by phosphate-citrate buffering of 2× concentrated mMRS to a final pH of 4.0 or 5.9 (35 mM Na_2HPO_4/32 mM citric acid and 60 mM Na_2HPO_4/20 mM citric acid, respectively) in the final mMRS.

The strain *A. nidulans* J283 (FGSC, #A4 wt, Kansas City, KS) was grown on malt extract agar (MEA) slants (Oxoid), at 25 °C until sporulation, then stored at 4 °C. Spore inocula were prepared by collecting conidia by vigorously shaking the slants with sterile peptone water (0.2% [w/v]).

Both mould (conidia) and bacterial cell concentrations were determined using a haemocytometer, and adjusted to 10^6 (cells) per ml of sterile peptone water.

2.2. Co-cultivation

Strains *A. nidulans* J283 and *L. plantarum* MiLAB 393 (or *L. coryniformis* MiLAB 123) were co-cultivated in a similar way as described by Janisiewicz et al. [8]. The strains were cultured in a 6-well cell culture plate with a cell culture insert (transparent PET membrane, 0.4 μm pore size, 1.6×10^6 pores cm^{-2}, BD Falcon™, BD Biosciences, Bedford, USA), (Fig. 1). *A. nidulans* was inoculated in the well to a concentration of 10^4 spores ml^{-1} in a total volume of 2 ml. *L. plantarum* was inoculated in the culture insert at a concentration of 10^5 cells ml^{-1} in a volume of 1.5 ml. The cultures were grown in mMRS-broth. *A. nidulans* controls were grown in wells without cell inserts, but with all other conditions comparable to those of the co-cultivation. The plates were incubated at 30 °C with gentle agitation. Mycelia were collected after 30–32 h of growth.

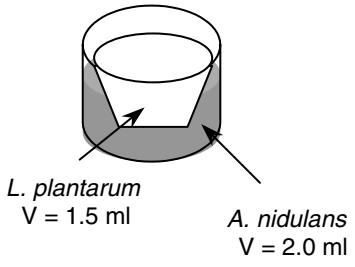

Fig. 1. Schematic figure of co-cultivation *A. nidulans* grown in the cell culture insert and *L. plantarum* grown in the cell. Separated by transparent PET membrane (0.4 μm pore size).

2.3. Growth in presence of antifungal substances

A. nidulans was grown in presence of three different antifungal substances produced by *L. plantarum* MiLAB 393: 100 mM lactic acid (VWR International, England), pH 4.0, 20 mM 3-phenyl lactic acid (Sigma-Aldrich Chemie, Germany), pH 5.0 and 10 mg ml^{-1} cyclo(L-Phe–L-Pro) (Bachem AG, Switzerland), pH 6.5. The cultures were grown in mMRS-broth, in the cell culture plates, at 30 °C with gentle agitation. Mycelia were collected after 30–48 h of growth.

2.4. Evaluation of growth

The growth of *A. nidulans* was evaluated by light microscopy of the harvested mycelia and by determining the dry weight of the mycelia when the fungus was grown alone, or in the presence of *L. plantarum* MiLAB 393 and *L. coryniformis* MiLAB 123. Mycelia were transferred to pre-dried and pre-weighed filter papers and the samples were dried at 80 °C for 24 h before weight determination. The fungal biomass was calculated as the mean value of three independent samples.

2.5. Two-dimensional gel electrophoresis

Mycelia were collected after co-cultivation in mMRS-broth as described above or after growth in presence of one of the following inhibitory substances: cyclo(L-Phe–L-Pro), lactic acid or 3-phenyllactic acid. Protein extracts were prepared from freeze-dried mycelia by TCA–urea extraction as previously described [9]. The relative amount of protein in the final extracts was determined by Bradford assay (Bio-Rad, Hercules, CA, USA).

Proteins were separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) as previously described [9]. In the first dimension, proteins were separated using Immobiline dry strip, pH 3–10 (Amersham Biosciences AB, Uppsala, Sweden). Second dimension separations were done on 15% polyacrylamid gels and

proteins were visualized by silver staining. Analysis of gel images were performed using the PDQuest 7.0.1 software (Bio-Rad Laboratories, Hercules, CA, USA). Selected up- or down-regulated proteins were excised from the gels, and amino acid sequences were determined by tandem electrospray MS ([10], Q-toft, Maslynx Software, Micromass). Peptide sequences were compared to *A. nidulans* database at Broad Institute (http://www.broad.mit.edu) using BLAST, and to other DNA and protein entries using the NCBI BLAST server (http://www.ncbi.nlm.nih.gov/). Searches for possible signal peptide cleavage sites were done by using the Signal P 3.0 algorithm [11] (http://www.cbs.dtu.dk/services/SignalP/).

3. Results

3.1. Co-cultivation of L. plantarum MiLAB 393 and A. nidulans

A. nidulans mycelia were examined in light microscope after co-cultivation and compared to mycelia grown without *L. plantarum* MiLAB 393. The co-cultivation mycelium was clearly affected by the presence of the antifungal LAB strain, visible both as inhibited growth and changed morphology, e.g., increased vacuolization, disturbed branching and swollen hyphal tips (Fig. 2). The mycelial dry weight in the co-cultivation was 36% (range 30–46%) of the dry weight of the control.

We wanted to ensure that the observed morphology and biomass changes were not a non-specific effect found after co-cultivation with any LAB. Thus *A. nidulans* was co-cultivated with *L. coryniformis* strain MiLAB 123, which has been found to be less effective in inhibiting fungi [12]. Co-cultivation with *L. coryniformis* MiLAB 123 did not affect the mycelium morphology to the same extent as *L. plantarum* MiLAB 393 (Fig. 2). The mycelial dry weight in the co-cultivation with strain *L. coryniformis* MiLAB 123 was 76% (range 71–85%) of the dry weight of the control.

3.2. Protein abundances are altered in A. nidulans

The molecular responses of the fungus were monitored as changes at the protein level using Two-Dimensional gel electrophoresis (2D-PAGE). 2D-PAGE

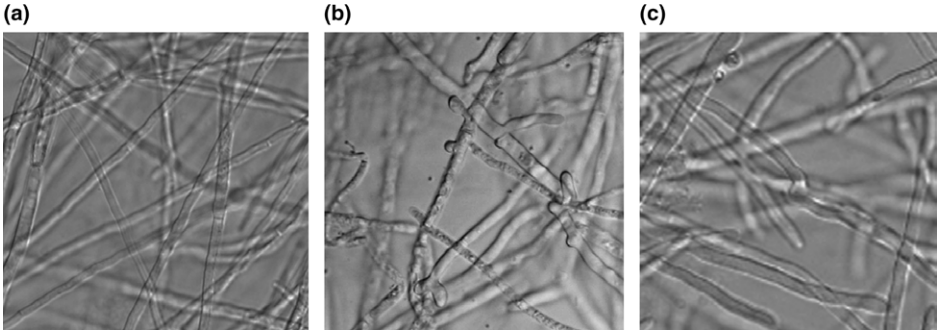

Fig. 2. Effects on *A. nidulans* mycelial growth. (a) Control: *A. nidulans* grown in mMRS broth. (b) *A. nidulans* co-cultivated with *L . plantarum* MiLAB 393 in mMRS broth. (c) *A. nidulans* co-cultivated with *L. coryniformis* MiLAB 123.

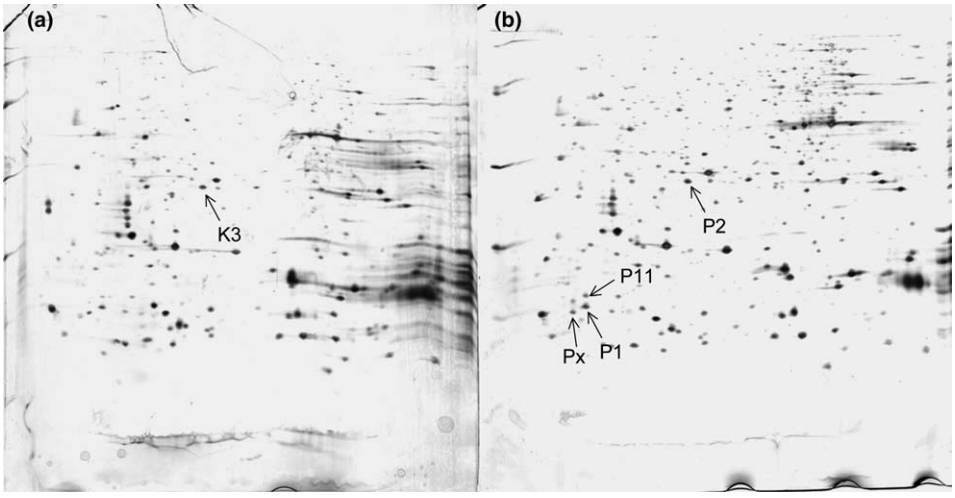

Fig. 3. 2D gels from mycelium protein extracts, control (a) and co-cultivation (b). Protein changes P1, Px, P11 and P2/K3 are marked on the gels.

separations of mycelium protein extracts from co-cultivation of MiLAB 393 and *A. nidulans* J283, revealed a group of clearly up-regulated proteins (Px, P1 and P11), and one protein, P2/K3, possibly up-regulated or alternatively shifted in the first dimension, (Fig. 3). This pattern of change in protein expression was identified in three separate experiments. The spots (P1, P11, Px and P2/K3) were selected for determination of amino acid sequences.

To investigate if the alterations observed after co-cultivation could be attributed to any of the antifungal substances produced by *L. plantarum* MiLAB 393, *A. nidulans* was grown in media containing one of three inhibitory substances. To ensure that the antifungal substance affected growth of the fungus at the concentrations used, we performed dry weight determinations after growth in presence of the substances. The mycelial dry weights, compared to the control, were 87% for lactic acid at 100 mM, 60% for cyclo(L-Phe–L-Pro) at 10 mg ml^{-1} and 102% in presence of phenyl lactic acid at 20 mM. Phenyl lactic acid clearly increased the length of the lag phase (data not shown) at this concentration, but did not reduce the final dry weight.

2D-PAGE separations of protein extract from *A. nidulans* grown in presence of cyclo(L-Phe–L-Pro), 3-phenyllactic acid and lactic acid revealed different alterations in protein expression compared to the control. The position of protein P2/K3 was shifted in the presence of lactic acid in a similar way as by co-cultivation. Phenyl lactic acid did not alter the location of this protein, whereas cyclo(L-Phe–L-Pro) shifted the protein in the opposite direction (Fig. 4). To ensure that the shift in protein migration of P2/K3 in the first dimension was not related to pH differences only, *A. nidulans* was cultivated in buffered media at pH 4.0 and 5.9, representing the highest and lowest start pH of cultivations. Proteins were extracted from the mycelia of the buffered cultivations for 2D-PAGE analysis. The buffered cultivations did not alter protein P2/K3 compared to the control (data not shown). This indicates that the change in the proteome is an effect of the antifungal compounds and not caused by a pH change only.

The group of proteins up-regulated during co-cultivation (Px, P1 and P11) was also up-regulated by all of the three inhibitory substances, but the degree of up-regulation was dependent on the substance present. The cyclic dipeptide cyclo(L-Phe–L-Pro) had the most predominant effect, with protein P1 up to eight times up-regulated compared to the co-cultivation.

3.3. Protein sequences

We excised some of the protein spots for identification by tandem electrospray MS. This also provides a

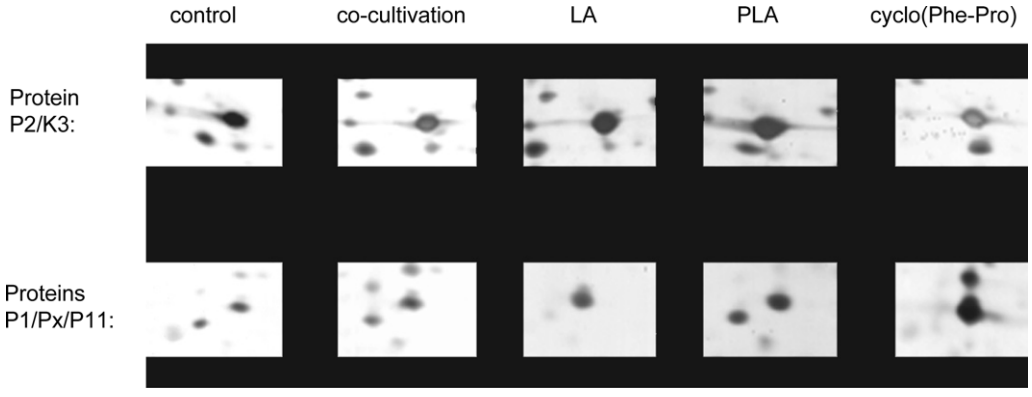

Fig. 4. Shift and up-regulation of proteins in the 2D-gels. A comparison of proteins P2/K3 and P1/Px/P11 at the different growth conditions, control, co-cultivation, lactic acid (LA), 3-phenyllactic acid (PLA) and cyclo(L-Phe–L-Pro).

Table 1
Summary of proteins and peptides identified in this study

Protein spot	Peptide sequences determined by MS	Protein	Accession Number (Swiss-Prot/TrEMBL)
P_1	KVAAQEGISAFDAMAK; QLLEPPAELR; SVPLGYVHIADDG; GVARAYDENESVID; EEDHLHAVFDAVDGR	LbuA	P84515
Px	QLLEPPAELR; SVIDYVPLTNDQL		
P11	KVAAQEGISAFDAMAK; QLLEPPAELR; SVPLGYVHIADDG; GVARAYDENE; SVIDYPLTNDQL; EEDHLHAVFDAVDGR		
P2	IVSCDGGGGPLGHPR; KSLPTSSYPLEPTGAAEEVNENQR	LbsA	P84514
K3	IVSCDGGGGPLGHPR; KSLPTSSYPLEPTGAAEEVNENQR		

possibility to reveal if some spots correspond to the same protein, i.e., to identify putative post-translational modifications. The obtained peptides from proteins P1, Px and P11 contained the same amino acid sequences (Table 1), suggesting that this group of proteins are the same protein, but with different modifications. The sequencing of protein P2/K3 confirmed that the same amino acid sequences were present in P2 and K3 (Table 1). Thus the shift in protein migration is due to post-translational modifications.

The corresponding DNA sequences from the peptides of the P1-group and of the protein P2/K3 were found in the *A. nidulans* genome database (ID EAA65324 and ID EAA59671.1, respectively), but with a partially different reading frame in protein P1 compared to that in the database (confirmed by peptide sequencing). Unfortunately, none of these DNA sequences corresponded to annotated proteins. Nucleotide sequences with high similarity to both the P1 and the P2/K3 DNA-sequences, were also found in the non-completed genome database for *Aspergillus fumigatus* (at http://www.tigr.org/). The complete DNA and protein sequences were deduced and blasted against other databases. No similar sequences were found in any other organism for the P1-protein. But the protein is likely to be translocated through the membrane since the first 18 residues appears to be a signal peptide (probability of 1.0 according to Signal P 3.0). The protein identified in the P1-group was named LbuA (*L*acto*b*acillus *u*p-regulated).

The P2/K3 sequence showed high similarity (64% identity) to a protein annotated as NADH-ubiquinone oxidoreductase in *Paracoccidioides brasiliensis* (ID AAN87885.1, unpublished). Similarities to other unknown or hypothetical proteins in various organisms were also found. NADH–ubiquinone oxidoreductase (also called Complex I) is the major entry point for electrons in the respiratory chain [13] and contains several different subunits. Several entries representing NADH–ubiquinone oxidoreductase chain sequences from *A. nidulans* or other *Aspergillus* sp. are present in the database, but protein LbsA was not similar to any of those. The P2/K3 protein was named LbsA (*L*acto*b*acillus *s*hifted). The protein sequence data reported in this paper will appear in the Swiss-Prot and TrEMBL knowledgebase under the accession numbers P84514 and P84515.

4. Discussion

The co-cultivation system employed in this study is useful to study interactions between organisms able to grow in the same substrate. We have identified morphological changes and growth inhibition of *A. nidulans* both in the presence of *L. plantarum* MiLAB 393 and its pure metabolites. *A. nidulans* was selected as model organism since the genome is available for sequence searches. In order to find potential primary or secondary targets for the antifungal compounds we decided to monitor changes at the protein level. Proteomics has earlier been shown to be suitable for studying responses at the protein level during microbial interactions [14].

In this study we identified two groups of proteins that were affected by the *L. plantarum* MiLAB 393. The position of one protein (LbsA) on the 2D-PAGE is changed upon co-cultivation. LbsA showed high similarity to a protein sequence, annotated as NADH–ubiquinone oxidoreductase in *P. brasiliensis*. Since NADH–ubiquinone oxidoreductase consists of several subunits one would expect that additional proteins would be up regulated on the gel if LbsA corresponded to one of these subunits. Hence, the function of LbsA could not be established from this still unpublished database entry. Another protein, present in four different spots, (LbuA) was up-regulated in the co-culture and by all of the tested compounds. MS analysis was not able to distinguish these spots from each other. A possible hypothesis is that the synthesis of this protein is increased in the fungus when encountering the bacterium or the fungal metabolites, and that the protein is modified to multiple forms after translation. Another explanation is that the protein has been partially degraded during preparation. However, this is less likely as most proteins were present as discrete spots on the 2D-PAGE gels. The presence or absence of the putative signal peptide cannot entirely explain the multiple forms of LbuA. Although the corresponding sequences were found in the *A. nidulans* genome, none of them were annotated.

Lavermicocca et al. [15], reported the growth delaying and growth inhibitory activity of PLA to be increased at lower pH and in presence of lactic acid. This could explain why changes in protein expression during co-cultivation do not seem to be related just to one of the inhibitory substances, but rather to a combination of them. The diketopiperazine cyclo(L-Leu–L-Pro), a cyclic dipeptide structurally related to cyclo(L-Phe–L-Pro), inhibits aflatoxin production in *Aspergillus flavus* and also affects growth of this fungus [16]. This further indicates that this type of LAB produced substances not only reduce fungal growth, but also influences other important biological features.

Further studies have to be done to determine the function of the proteins identified in this study. The rational approach for these studies is to disrupt the genes encoding LbsA and LbuA and allow the mutant strains to encounter *L. plantarum* MiLAB 393.

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