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FTIR spectroscopy structural analysis of the interaction between *Lactobacillus kefir* S-layers and metal ions

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ABSTRACT

FTIR spectroscopy was used to structurally characterize the interaction of S-layer proteins extracted from two strains of *Lactobacillus kefir* (the aggregating CIDCA 8348 and the non-aggregating JCM 5818) with metal ions (Cd^{+2}, Zn^{+2}, Pb^{+2} and Ni^{+2}). The infrared spectra indicate that the metal/protein interaction occurs mainly through the carboxylate groups of the side chains of Asp and Glut residues, with some contribution of the NH groups belonging to the peptide backbone. The frequency separation between the νCOO^- anti-symmetric and symmetric stretching vibrations in the spectra of the S-layers in presence of the metal ions was found to be *ca.* 190 cm^{-1} for S-layer CIDCA 8348 and *ca.* 170 cm^{-1} for JCM 5818, denoting an unidentate coordination in both cases. Changes in the secondary structures of the S-layers induced by the interaction with the metal ions were also noticed: a general trend to increase the amount of β-sheet structures and to reduce the amount of α-helices was observed. These changes allow the proteins to adjust their structure to the presence of the metal ions at minimum energy expense, and accordingly, these adjustments were found to be more important for the bigger ions.

1. Introduction

In general, food and water represent the main sources of exposure to metals. Toxic metals are not degradable and tend to accumulate in the exposed organisms causing serious health effects. Therefore, their removal represents an important task for the care of both human and animal health and the environment. Bioremediation (the use of biological agents to remove or neutralize contaminants) is a very important tool for the removal of such toxics. In particular, the use of inactivated microorganisms as adsorbents (biosorption) has been suggested as an effective and economical way to remove heavy metals from water. Several genera of microorganisms have been used for such purposes [1–7]. Among lactic acid bacteria, different species of lactobacilli have been successfully used in the removal of lead and cadmium [8]. In spite of that, no bacterial structures have been conclusively assigned as responsible for the metal removal. To understand the molecular mechanisms involved in biosorption, a deep insight on the interaction of bacterial superficial structures with metals is necessary.

In several species of microorganisms, the S-layer represents the most outer structure covering the cell envelope, being attached to the peptidoglycan or the outer membrane by non-covalent bonds. Most of the S-layers are composed of protein monomers assembled in two-dimensional arrays with regularly arranged pores of identical size that enable the exchange of ions and small molecules between the living cells and their environment. The ability of S-layers to bind metal ions has attracted the attention due to the potential application in bioremediation and for the generation of metal nanoclusters that might receive different practical uses. For example, the uranium-mining waste pile isolate *Bacillus sphaericus* JG-A12 was found to be capable of selective and reversible binding of large amounts of different metals [4], functioning as a barrier for toxic heavy metal ions in the environment. S-layer extracts from the same microorganism, incubated in an Au^{3+} solution in the presence of reducing agents such as molecular H_2, were able to form gold nanoparticles regularly distributed according to the pores of the protein lattice [9].

In spite of these evidences, the available information regarding the molecular basis of the S-layer/metal ions interaction and its consequences on the secondary structure of the S-layer proteins is still very scarce [9,10]. In contrast with many proteins that interact with metals through a low number of highly specific binding sites that are conformationally regulated by metal/protein interactions, S-layer proteins bear a high number of metal binding sites, each one exhibiting low specificity, thus being capable of binding a large variety of different metals [4,10,11]. In the interaction between Pd^{2+} and the S-layer of *B. sphaericus* JG-A12, a predominant chelation/bridging Pd^{2+} coordination to the side chain carboxylate groups of Asp and Glu residues, and a possible additional

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participation of nitrogen through side chains (Lys, Asn) rather than through the peptide backbone has been suggested [10].

In the present investigation, the interaction between the S-layers from two strains of *Lactobacillus kefir* and Cd^{+2}, Zn^{+2}, Pb^{+2} and Ni^{+2} was addressed by FTIR spectroscopy. The characterization of the S-layer/metal interaction at a molecular level was undertaken and the impact of this interaction on the secondary structure of the S-layer proteins was evaluated.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Two strains of *L. kefir*, JCM 5818 and CIDCA 8348, were used in this work. They were cultured in de Man, Rogosa and Sharpe (MRS) broth [12] (Biokar Diagnostics, Beauvais, France) at 30 °C for 48 h. Microorganisms were harvested and washed twice with milli Q water and the pellets obtained were suspended in 1 mL of $Pb(NO_3)_2$, $Cd(NO_3)_2$, $Ni(NO_3)_2{\cdot}6H_2O$ or $Zn(NO_3)_2{\cdot}6H_2O$ 0.3 mM solution. Microorganisms non-treated with metal ions were used as controls. The suspensions were further incubated for 24 h at 30 °C. After that, they were centrifuged at 6600*g* for 4 min, and the pellets were used for transmission electronic microscopy determinations.

2.2. Electron microscopy

Samples for transmission electron microscopy (TEM) were prepared according to Vidgrén et al. [13]. Cells were fixed in 20 g glutaraldehyde/L (PBS buffer, pH = 7.2) for 15 h, at room temperature. Fixed cells were collected by centrifugation and washed three times with phosphate buffer. All samples were post-fixed with phosphate-buffered 10 g osmium tetroxide/L and dehydrated for 2 h. Samples were included in Epon 812 and sliced with a Sorvall MT 2B ultramicrotome. A JEOL 1200-EXII transmission electron microscope (Jeol Ltd., Japan) was operated at 85 kV.

2.3. S-layer proteins preparation

Bacterial cells were harvested at stationary phase, collected by centrifugation (10,000 *g* at 10 °C for 10 min), washed twice with PBS (pH = 7), and resuspended to an $OD_{550\ nm}$ of 10 in the same buffer. Cells were mixed with 5 M LiCl (J.T. Baker, Mallinckrodt Baker S.A., Mexico) in a proportion of 4 mL of solution per mL of bacterial suspension. The mixture was incubated in a shaking incubator (Environ Shaker, Lab-line Instruments Inc., Melrose Park, IL, USA) at 200 rpm and 37 °C for 60 min to extract non-covalently bound proteins. Then, the mixture was centrifuged (12,000 *g* at 10 °C for 15 min). The supernatant containing the S-layer protein was concentrated by ultrafiltration in an Amicon stirred cell 8050 (Millipore Corporation, US) equipped with a regenerated cellulose membrane (Ultracell PLGC04310, MWCO 10,000, Millipore Corporation, US), and dialyzed against bidistilled water at 4 °C for 24 h using a cellulose membrane (SpectraPor membrane tube, MWCO 6000–8000, Spectrum Medical Industries, California, US). Taking advantage of the capacity of the S-layers to aggregate in the absence of chaotropic agents, the dialyzed extracts were centrifuged 4 min at 6600*g* and the pellets were then suspended in 1 mL of $Pb(NO_3)_2$, $Cd(NO_3)_2$, $Ni(NO_3)_2{\cdot}6H_2O$ or $Zn(NO_3)_2{\cdot}6H_2O$ 0.3 mM solution. S-layers non-treated with metal ions were used as controls. The suspensions were further incubated for 24 h at 30 °C. After that, they were centrifuged at 6600 *g* for 4 min and the pellets were used for the FTIR determinations.

2.4. FTIR spectroscopy

Thirty microliter of the S-layer pellets were put on a CaF_2 window and further dried at 45 °C for 15 min to get a transparent film, which was directly used for FTIR experiments. The FTIR spectra of the S-layers were also recorded on KBr pellets to check for eventual effects of adsorption and the results obtained were found to be similar to those obtained in CaF_2 (though of general worst quality, due to degradation of the optical material), indicating that adsorption, if taking place, is negligible. Note also that in the present study we are mostly interested in the differences observed in the spectra in the presence of the metals selected for the analysis, and, because the spectra of the proteins in the absence of these metals were systematically used as controls, possible undesired effects such as those due to an eventual adsorption were eliminated from the spectra analysis.

FTIR spectra were recorded in the 4000–500 cm^{-1} range in transmission mode in a system continuously purged under dry nitrogen in order to eliminate spectral contributions of atmospheric water vapor and CO_2. The IR spectra collected at room temperature were obtained co-adding 128 scans with 4 cm^{-1} spectral resolution. All spectra were recorded in a BOMEM MB40 spectrometer (ABB Ltd., Zurich) with a Zn/Se beam splitter and a DTGS detector.

2.5. Data analysis of FTIR spectra

The recorded infrared spectra were analyzed using the OMNIC suit of programs (Nicolet Instrument Co., Madison, WI). Whenever necessary, residual contributions due to atmospheric water vapor and CO_2 were eliminated by subtraction of the corresponding spectra from the registered samples' spectra. The resultant spectra were smoothed with a seven-point (13 cm^{-1}) Savitzky–Golay function.

Inverted second derivative spectra were used to estimate the number and position of individual elements composing Amide I band (1600–1720 cm^{-1}), and this information was taken into account to fit Amide I bands in protein spectra with Gaussian band profiles, using the program OMNIC (Nicolet Instrument Co., USA).

The assignment of protein secondary structures to the principal Amide I frequencies was as previously described in literature [14–17].

3. Results and discussion

According to previous studies carried out in our group, *L. kefir* strains have different surface properties. Among them, aggregation ability is perhaps one of the most important differential characteristic among strains, because of its putative role in some bacterial properties, such as adhesion to intestinal cells, biofilm formation, and pathogen inhibition [18–20]. Two *L. kefir* strains, CIDCA 8348 and JCM 5818, were here selected to investigate the bacteria/metal interaction. Considering that biosorption involves bacterial superficial molecules and that the structure of these molecules ultimately determines the aggregation properties of the whole microorganisms, the selection of these two strains has been made on the basis of their differential pattern of superficial properties (aggregation and co-aggregation abilities) [18,20] (Table 1) and recognition by antibodies [23]. In these strains, S-layer represents the most outer structure covering the cell envelope and occupies *ca.* 90% of the outer surface [21]. Therefore, the interaction between the metal ions and the bacteria must essentially involve the S-layer.

Transmission electron micrographs of the two selected *L. kefir* strains before and after the treatment with metal ions clearly showed the presence of deposited metal on the bacterial S-layer surface. Fig. 1 shows the images obtained using lead ion. Similar results were observed after treatment with the other metal ions used in this work (not shown). This observation reinforced the interest in studying the interaction between the metal ions and the S-layer under well-controlled conditions. Therefore, purified S-layers from