Molecular biology, genetics and biotechnology

Use of 2-D electrophoresis and ESI mass spectrometry techniques to characterize Fusobacterium nucleatum proteins up-regulated after oxidative stress

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1. Introduction

Although the oral cavity is an overtly aerobic environment, most of the components of the associated microbial ecosystem are anaerobic bacteria. In the oro-pharynx, anaerobes outnumber their aerobic counterpart in a ratio of 10:1–100:1 [2]. The Gram-negative obligate anaerobic bacterium Fusobacterium from Fusobacteriaceae family is naturally found in the oral microbiota of healthy humans [1]. The Fusobacterium nucleatum is one of the dominant species of the 500–700 or more bacteria that coexist in the oral cavity of healthy human beings and is one of the most common species in human infections. It is associated with periodontal diseases and other clinical infections in and around the oro-pharynx and in non-oral sites, including peritonsillar abscesses, otitis and sinusitis, skin ulcers, bacteremia, liver abscesses, endocarditis, and pleuropulmonary infections [3,4].

It has been shown that F. nucleatum cannot grow under aerobic conditions but may exhibit a high degree of aerotolerance [5]. During the initial stages of infection, when anaerobic bacteria are shifted from anaerobic to aerobic conditions, oxidative stress is unavoidable and an adaptive response for survival may occur. This adaptive response to oxidative stress could figure as an important virulence factor that allows a successful resistance to oxygenated environments and host’s immune oxidative burst [6,7]. It may also interfere with other microorganism abilities that could increase its virulence, as we have recently demonstrated in conventional mouse model [8], and alter its biochemical and physiological profile, that may lead to the misidentification of the organism when phenotypic methods are employed [9].

Although over the past decade there have been great advances in the genomic and proteomic of microorganisms little is known regarding the mechanisms of bacterial oxidative stress adaptive response and the influence of this adaptation on the relationship between the microorganisms and their host. Current tools for proteome analyses include 2-dimensional electrophoresis (2-DE) gel and a range of techniques that focus on the use of mass spectrometry (MS). For microbes, such analyses are aimed at complete
proteome display, protein expression profiling and biomarker discovery [10].

The ability of MS to identify ever smaller amounts of protein from increasingly complex mixtures is a primary driving force in proteomics. Initial proteomics efforts relied on protein separation by 2-DE, with subsequent mass spectrometry identification of protein spots. The rapid developments in MS have shifted the balance to direct mass spectrometry analysis, and further developments will increase sensitivity and data handling. One of the techniques of MS is the electrospray ionization (ESI), which enables analyses of a wide range of compounds including proteins, nucleic acids and synthetic polymers [11].

In this study, we aimed to use both 2-DE and Electrospray Ionization Mass Spectrometry (ESI-MS) to characterize proteins up-regulated in Enucleatum subsp. nucleatum ATCC 25586, after oxidative stress by atmospheric oxygen exposure.

2. Materials and methods

2.1. Obtention of F. nucleatum strain to oxidative stress by atmospheric oxygen exposure

An oxidative stress adapted strain of F. nucleatum derived from the F. nucleatum subsp. nucleatum ATCC 25586 was obtained after molecular oxygen exposure, as already described [8]. Briefly, a 300 ml volume of Brain Heart Infusion Broth (BHI) was inoculated with nearly 10% of an overnight culture of F. nucleatum wild-type strain (wt-strain) and the resulting bacterial suspension was grown with nearly 10% of an overnight culture of a 300 ml volume of Brain Heart Infusion Broth (BHIB) was inoculated molecular oxygen exposure, as already described [8]. Briefly, atmospheric oxygen exposure was regulated in acids and synthetic polymers [11].

Three random different protein spots which were up-regulated after oxidative stress in F. nucleatum aero-strain were manually excised from the polycrylamide gel and processed for ESI-MS, as follows: each sample was washed 3 times in 400 μl of 50% acetonitrile (ACN)/25 mM l−1 NH4, pH 8, for 15 min, to remove the excess of Coomassie Blue. Then, the spots were soaked in 100% ACN for 5 min to dehydrate the samples. The ACN was removed and the spots were dried in Speed-Vacuum for 20–30 min. After that, each sample was re-hydrated with 15 μl of cold trypsin solution (Tryptsin sequencing grade, 10 μg ml−1 in 25 mM l−1 NH4) (Sigma-Aldrich CO., St. Louis, Missouri, USA), and placed on a 37° C water bath for 16–24 h. The tryptic peptides were extracted using Zip Tip pipette tip (C18 resin; P10, Millipore Corporation, Bedford, MA, USA), with ACN/trifluoroacetic acid for concentrating and purifying the peptides. The samples were frozen until MS analysis. ESI-Q/TOF-MS analyses were performed by using a Q-TOF MicroTM instrument (Micromass-Waters, Manchester, UK) equipped with an ESI source operated in positive ion mode, as described previously [15]. The capillary voltage was 2.5–3.0 kV and sample cone voltages were 40–60 V. Mass spectrometer calibrations were achieved by using a standard mixture contained in the 100–2000 m/z range. Samples were introduced into the mass spectrometer by means of a spray pump with a flow rate of 1–5 μl/min using an electro spray source. Obtained mass spectra were analyzed with the MassLynxTM 4.0 software. Swiss-Prot.2005.01.06 and NCBI.blnr.2005.01.06 databases were used for protein identification through the search engine of MS-Fit in the Protein Prospector v.4.05 package (http://prospector.ucsf.edu Protein Prospector, San Francisco, CA, USA). The following parameters were used in all searches: digest used, trypsin; maximum number of missed cleavages allowed = 3; peptide mass tolerance = 50 ppm; minimum peptides required to match = 4; and the monoisotopic mass values of observation peaks were used to match the calculated monoisotopic fragment masses for protein identification. Possible covalent modifications considered in the search procedure were carbamidomethylation of cysteine, conversion of peptide N-terminal glutamine to pyroglutamate, oxidation of methionine, and acetylation of protein N-terminus.

2.2. Two-dimensional electrophoresis (2-DE) gel analysis

The wt- and aero-strain had its protein content displayed after 2-DE, carried out as follows: aliquots of 4 ml of each cell suspension were centrifuged and solubilized in 100 μl of lysis buffer [7 M urea, 2 M thiourea, 4% 3-[(3-chloramidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS), 40 mM dithiothreitol (DTT), 2% IPG Buffer (pH 4–7), 40 mM Tris base and phenylmethanesulphonyl fluoride – PMSF (GE Healthcare)]. Crude cell extracts were obtained by mechanical disruption with glass beads in a cell blender followed by centrifugation for 30 min at 4°C, and then protein content was measured (2-D Quant Kit; Amersham Pharmacia Biotech, Piscataway, NY, USA). The crude protein extracts were submitted to two-dimensional (2-D) gel electrophoresis, as described before [12,13]. Briefly, the first dimension electrophoresis was carried out using an immobilized pH gradient (IPG) strips (14 cm, pH gradient from 4.0 to 7.0, Immobiline DryStrip Kit; Amersham Pharmacia Biotech), which were loaded with 300 μg of crude cell extract and electrophoresed for 20 h, at 20°C, 1 mA and 5 W, using a gradient programming electrophoresis power supply EPP 3500 XL (Amersham Pharmacia Biotech), as follows: 100 V, 0.01 h; 100 V, 4 h; 300 V, 0.01 h; 300 V, 4.5 h; 2000 V, 5 h and 2000 V, 6.5 h. After IEF, each strip was incubated for 15 min in 10 ml of 50 mM Tris–HCl buffer, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% bromophenol blue and 125 mM dithiothreitol (DTT), followed by a second incubation step in the same buffer solution, except for DTT, which was replaced by 125 mM iodoacetamide. The second dimension electrophoresis was carried out in a 12% SDS-PAGE column, according to established procedures [14]. After 2-D electrophoresis, the gels were stained with Coomassie Blue R250 (Sigma-Aldrich CO., St. Louis, Missouri, USA) for analysis. The experiments were performed in duplicate at least three times and the differentially regulated proteins were identified through manual visual inspection for presence or absence of spots. Only those protein spots that changed in every gel were considered.

2.3. Spot handling and mass spectrometry

This study reports the use of techniques such as 2-D electrophoresis (2-DE) and electrospray ionization (ESI), to identify proteins after oxidative stress in F. nucleatum. Besides more than 25 proteins identified after oxidative stress in F. nucleatum, the ability of MS to identify ever smaller amounts of protein after oxidative stress in F. nucleatum was confirmed. Besides more than 25 proteins identified after oxidative stress in F. nucleatum, the ability of MS to identify ever smaller amounts of protein after oxidative stress in F. nucleatum was confirmed.
differences were seen overall, as previously published [9], in this study three different proteins which were highly up-regulated by oxidative stress in F. nucleatum subsp. nucleatum ATCC 25586 were identified. The experiments were reproduced at least three times and the results shown are typical (Fig. 1). As the F. nucleatum genome is already sequenced [4], these three proteins were found in database search (Swiss-Prot.2005.01.06 and NCBInr.2005.01.06), by homology (Table 1).

Considering the analyses of F. nucleatum genome, the ORF for glutamate synthase was not found, although a NAD-specific glutamate dehydrogenase, which converts 2-oxoglutarate and ammonia to l-glutamate, is present. This compound is important for pyrimidine synthesis. The protein phosphoglycerate kinase belongs to the phosphoglycerate kinase family. Also known as 3-phosphoglycerate 1-phosphotransferase, this major enzyme in glycolysis catalyzes the reversible conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate, generating one molecule of ATP. The Acyl-CoA dehydrogenase, is an oxidoreductase, and acts as an electron transport [4]. It is well known that adaptation to adverse conditions in the environment of a bacterium requires the ability to respond rapidly. This response to environmental changes involves activation of existing enzymes and enhanced rates of transcription of genes, resulting in enhanced levels of proteins, which might act as defensive mechanisms [16].

Since there is no available data on the oxidative stress response regarding F. nucleatum metabolic pathways, it is difficult to address specific comments on the role of the identified proteins. However, as reported in the literature for other bacteria, the microbial response to stress conditions may involve a global regulation which may be related to different environmental stimuli. Under this global control, several proteins may play a role in order to promote metabolic and structural shifts to facilitate the cellular adaptation to the situation [6].

Regarding the glutamate dehydrogenase (GDH), it has been proposed that the excess glutamate that accumulates in response to osmotic stress is synthesized by GDH, and may also occur after other stress conditions, such as acid stress. Pre-exposure to low pH not only provides protection against an otherwise lethal pH, but also enhances survival during their challenges such as heat, ethanol and oxidative stress. It is accepted that GDH and other glutamate system proteins are genetically under control of a sigma global regulator [16,17].

According to some authors, Acyl-CoA dehydrogenase (AcDH) plays important role in the beta oxidation of fatty acids which

Table 1
<table>
<thead>
<tr>
<th>Spot</th>
<th>Score</th>
<th>MW (Da)/pI</th>
<th>Matched (%)</th>
<th>Cov (%)</th>
<th>Protein</th>
<th>Description</th>
<th>Biological process</th>
<th>Swiss-Prot/TrEMBLb</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>1550</td>
<td>48,244/6.4</td>
<td>17/107 (15)</td>
<td>39</td>
<td>GDH</td>
<td>NAD-specific glutamate dehydrogenase</td>
<td>Amino acid metabolic process</td>
<td>Q8RG30_FUSNN</td>
</tr>
<tr>
<td>02</td>
<td>151</td>
<td>42,836/5.6</td>
<td>11/27 (40)</td>
<td>19</td>
<td>PGK</td>
<td>Phospoglycerate kinase</td>
<td>Glycolysis</td>
<td>PGK_FUSNN</td>
</tr>
<tr>
<td>03</td>
<td>80.7</td>
<td>41,207/5.2</td>
<td>14/82 (17)</td>
<td>31</td>
<td>AcDH</td>
<td>Acyl-CoA dehydrogenase, short-chain specific</td>
<td>Oxidation, reduction</td>
<td>Q8RING_FUSNN</td>
</tr>
</tbody>
</table>

a Sequence covered.
b Accession.
enables the utilization of medium- and long-chain fatty acids as carbon source in bacterial metabolism during starvation stress response conditions. It is accepted that the induction of this starvation stress protection system in other bacteria makes the cells more resistant to a number of other environmental stresses, such as oxidative stress [18].

In this study, phosphoglycerate kinase (PGK) was also found to be up-regulated after oxidative stress by molecular oxygen exposure in *E. nucleatum*. Considering its role in the energy metabolism, it is difficult to address its protective effects during molecular oxygen exposure by the anaerobic bacterium. However, PGK was found to be up-regulated by other authors as molecular target of oxygen exposure by the anaerobic bacterium. This work represents one of the first studies using genetic and physiological approaches to understand specific proteins in disease. This work did have other significant effects on the organisms, once some other systems might be involved in this phenomenon.

Thus, by using ESI-Q/TOF-MS, in addition to 2-DE, the opportunity exists to gain a more holistic view of the bacterial proteome of human pathogens, to achieve a better understanding of species diversity, to identify new biomarkers and to elucidate the role of specific proteins in disease. This work represents one of the first studies using genetic and physiological approaches to understand the phenomenon of oxidative stress in *E. nucleatum*. Advances were made related to the changing patterns of gene/protein expression in the adapted strains; however, there is still much to be undertaken regarding the precise mechanisms of oxidative stress protection and how they might evolve in the environment of the host.

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References