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Sample preparation and further proteomic investigation of the inhibitory activity of pyridinium oximes to Gram-positive and Gram-negative food pathogens

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ABSTRACT

The antimicrobial activity of quaternary ammonium salts of pyridinium oxime against four food pathogenic bacteria, the Gram positive *Bacillus subtilis* and *Listeria monocytogenes*, as well as the Gram negative *Escherichia coli* and *Yersinia enterocolitica*, were evaluated. Changes of proteome in these bacteria grown under stress conditions were identified. By the application of a new method for sample preparation, followed by both in-gel and in-solution digestion and LC–MS/MS, both characterization and comparison of proteomes of these food pathogens were achieved. It was shown in all investigated bacteria that some of the proteins of key importance for protein turnover and bacterial metabolism are down regulated. Some stress proteins involved in protein folding and degradation were up regulated. Most of both up- and down-regulated proteins belong to the group of proteins with high abundance. Flagellin is the only protein of lower abundance that was found to be down regulated in two strains, *B. subtilis* and *E. coli*. The presented results give the better view into the proteome of food pathogens, and pave the way for further investigation of their virulence, pathogenicity and detection of biomarkers for tracing the ways and sources of food contamination.

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1. Introduction

Bacterial pathogens are frequently responsible for both food spoilage and food-borne illnesses that cause enormous commercial and health damage around the world. Outbreak of food-borne diseases has always been a severe health risk in developing countries. However, food-borne diseases are still a problem in industrial countries, too. Consequently, protection against spoilage as well as prevention of food-borne diseases is a task of enormous economic and social importance (Giacometti, Buretic, Tomljenovic, & Josic, 2012; Havelaar et al., 2010).

Pathogenic Gram-positive bacteria *Bacillus subtilis* and *Listeria monocytogenes*, as well as Gram-negative *Escherichia coli* and *Yersinia enterocolitica*, are important microorganisms involved in food spoilage and human food-borne infections. These bacteria are also often used as model microorganisms when bacterial food contamination is experimentally investigated (Cairns & Payne, 2009; Costa et al., 2009; Tompkin, 2002; Valerio, De Bellis, Lonigro, Visconti, & Lavermicocca, 2008; Xanthopoulos, Tzanetakis, & Litopoulou-Tzanetaki, 2010). *B. subtilis* is a sporogenic bacterium that contaminates flour, bread and yeast, and survives baking temperatures. Germinated spores are capable of deteriorating bread texture and cause ropy bread spoilage (Valerio et al., 2008). *L. monocytogenes* is a pathogenic bacterium that relatively frequently contaminates food products, in particular cheese and ready-to-eat meat-containing food products. Depending of the host's susceptibility, it can cause mild gastroenteritis or severe infections of the blood stream and/or the central nervous system, and abortion (Cairns & Payne, 2009). This bacterium is capable of surviving a broad range of temperatures during food production and storage, and contamination with this microorganism is of primary concern in processed food products (Tompkin, 2002). *E. coli* is the most common inhabitant of the intestinal tract of humans and animals, and can be easily disseminated in different ecosystems through the food chain and water. It is considered as an indicator of fecal contamination of food (Costa et al., 2009). The enteric bacterium *Y. enterocolitica* is an important food-borne pathogenic organism. Outbreaks of human infections associated with consumption of raw fruits and vegetables have increased in recent years since this species is capable of surviving and multiplying in food under refrigeration

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temperatures (Xanthopoulos et al., 2010). *E. coli* and *Y. enterocolitica* are of greatest current concern in fresh products, while *L. monocytogenes* contamination is of primary concern in processed products (Tompkin, 2002). *B. subtilis* has been frequently used as a model organism for sporogenic food pathogens (Budde, Steil, Scharf, Völker, & Bremer, 2006).

Various fundamental changes in an organism's cellular physiology are caused by stress exposure. Stress responses are of particular importance to microorganisms because their habitats are subjected to continuous changes of temperature, osmotic pressure, and availability of nutrients (Capozzi, Fiocco, Amodio, Gallone, & Spano, 2009). Pathogen survival and growth on food-produce is influenced by a number of interdependent factors such as storage temperature, product type/ combinations, minimal processing operations (e.g. slicing, shredding, washing and decontamination treatments), mild processing methods (like high pressure, pulsed electric field processing, cold plasma, advanced heating), package atmosphere and competition from the natural microflora present on food-produce (Capozzi et al., 2009). Antimicrobial washing by using different disinfectants and adding natural antimicrobials agents is another important factor that diminish populations of microorganisms on fresh produce and in processed food (Park, Hung, Doyle, Ezeike, & Kim, 2001).

Proteomic, together with metabolomic and genomic, methods are increasingly used for determination of stress factors important for survival of microorganisms during food processing (Josic & Kovač, 2008). den Besten, Mols, Moezelaar, Zwietering, and Abee (2009) performed a thorough proteomic analysis of *B. cereus* cells grown under differnt stress conditions. In this and a following study, several cellular factors for bacterial stress adaptive behavior at transcriptome, proteome and metabolome level were investigated, and potential candidate biomarkers to stress response were identified (Abee, Wels, de Been, & den Besten, 2011). The investigations by use of a wide variety of enviromental changes were extended to the other bacteria including *B. subtilis*, *L. monocytogenes* and *E. coli* (Allen, Lepp, McKellar, & Griffiths, 2008; Petersohn et al., 2001; van der Veen et al., 2007). This approach leads to prediction of microbial performance using cellular biomarkers for the early detection of food pathogens and to the control of their adaptive behavior that results in enhanced resistance. Interestingly, for *Y. enterocolitica*, only a few proteomic investigations regarding behavior of this microorganism under stress conditions have been performed (Vanlint, Rutten, Michiels, & Aertsen, 2012).

Pyridinium oximes have been firstly demonstrated to be potent re-activators of organophosphate-inhibited acetylcholinesterase (AChE). There are many commonly used re-activators of inhibited AChE such as pralidoxime, trimedoxime, obidoxime and the oxime HI-6 (Kassa, 2002; Kassa, Bajgar, Kuča, Musílek, & Karasová, 2008). Pyridoxal oxime, a derivative of vitamin B_6, can be used for the synthesis of compounds structurally similar to common antidotes. Derivatives of pyridoxal oximes have been tested as re-activators of AChE inhibited by the neural poisons sarin, soman, tabun, paraoxon and VX (Gaso-Sokac, Katalinić, Kovarik, Busić, & Kovac, 2010).

Quaternary ammonium salts of heterocyclic bases of dimethylpyridine have been evaluated for antimicrobial activity against Gram positive and Gram negative microorganisms (Hameed et al., 1994). Pyridinium oximes were also tested against a number of microorganisms having lower pathogenicity (Berger & Knodel, 2007). Quaternary salts exhibit antimicrobial properties by adsorption to the cell wall of microorganisms, and also bind to other cellular components causing disruption of several biochemical processes which lead to the inhibition of bacterial growth and cell death (Bharate & Thompson, 2010). It makes them suitable as model substances for proteomic studies in order to find changes in both cell surface and intracellular proteins in four investigated food pathogens.

Methods for cellular destruction, fractionation and identification of separated proteins were developed, and changes in the bacterial proteomes, grown under these stress conditions, were identified.

2. Experimental section

Solvents and reagents for the synthesis of pyridinium oximes were purchased from Thermo Fisher Scientific (Fair Lawn, NJ, USA), Fluka and Sigma-Aldrich (Taufkirchen, Germany), and used without further purification. IR spectra were measured on a Paragon 500 FT-IR spectrophotometer with KBr pellets. ^{1}H NMR and ^{13}C NMR spectra were measured on a Varian XL-GEM 300 spectrophotometer in DMSO-d_6 solutions, and chemical shifts are reported in δ values downfield from TMS as an internal standard. Mass spectra were recorded using an API2000 LC/MS/MS System from Applied Biosystems/MDS SCIEX. Melting points were determined with a capillary Stuart Melting Point Apparatus SMP3. The purities of compounds were determined by ^{1}H NMR, ^{13}C NMR and elemental analysis.

2.1. Preparation of the new oximes **2**, **3**

Preparation of the new oximes **2**, **3** are shown in Fig. 1 and Reference (Gaso-Sokac, Katalinić, Kovarik, Busić, & Kovac, 2010).

Quaternary salts **2**, **3**, were converted by anion exchange reaction (metathesis) from bromides into chlorides **4**, **5,** according to Reference (Gaso-Sokac et al., 2010b) and Fig. 2.

2.2. Bacteria and antibacterial testing

Bacterial strains of Gram negative *E. coli* and *Y. enterocolitica* as well as Gram positive *B. subtilis* and *L. monocytogenes* were isolated from patients, identified according to the API system (bioMérieux, Craponne, France), and maintained on Tryptic Glucose Yeast Agar (Biolife, Milan, Italy) at 4 °C. Before experiments, cultures were revived by 2 consecutive 24 hour inoculations and incubations at 37 °C, except *Y. enterocolitica*, which was incubated at 25 °C.

Determination of the minimal bactericidal and inhibitory concentrations of the oximes was performed in Brain Heart Infusion Broth (BHI; Biolife) (Kim & Fung, 2004). The tested compounds were dissolved in a combination of DMSO (4 mL) and 10% Tween 80 (6 mL), and sterilized by filtration (0.22 μm size of pores). Establishment of minimal inhibitory and bactericidal concentrations was performed by the macro broth method according to Morcello, Mizer and Granato (2003) and Barbour et al. (2004). Briefly, the tested compound was serially diluted through series of tubes with 0.5 mL BHI broth and inoculated with a 0.1 mL suspension of 1×10^6 CFU mL^{-1}, and incubated at 37 °C (25 °C for *Y. enterocolitica*) for 24 h. After incubation, to assess bactericidal activity, growth was transferred to tubes with BHI broth without inhibitory compounds and incubated for 24 h at 37 °C (25 °C for *Y. enterocolitica*). After incubation, in tubes where no growth was observed, the corresponding concentration of compound was bactericidal, while the minimal inhibitory concentration was recorded from the tube where bacterial growth occurred.

2.3. Selective extraction of proteins from bacterial cells compatible with MS techniques

A sequential protein extraction method compatible with MS analysis was established and optimized in order to fractionate proteins from cell specimens. After ultrasonication or mechanical destruction of bacterial cells by use of 0.1 mm silica spheres in Lysing Matrix B tubes (MP Biomedicals, Solon, OH, USA) in a Mini-Beadbeater-1 high-energy cell disrupter (Biospec Products, Beatlesville, OK, USA), the homogenates were subjected to sequential extraction using the ReadyPrep Extraction Kit (BioRad, Hercules, CA, USA). The ReadyPrep kit provides three necessary reagent solutions of increasingly stronger solubilizing power that differ in their detergent and chaotropic agent concentrations: Solution 1 (40 mM Tris base) extracts only the most soluble, cytosolic proteins, Solution 2 (8 M urea, 4% (w/v) CHAPS, 40 mM Tris, 0.2% (w/v) Bio-Lyte 3/10 ampholyte and 2 mM reducing agent tributyl phosphine (TBP)) extracts proteins of intermediate solubility, and Solution 3 (5 M urea, 2 M

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