INSTITUT PASTEUR

Research in Microbiology 161 (2010) 869–875

www.elsevier.com/locate/resmic

Identification of outer membrane proteins of *Vibrio parahaemolyticus* and *Vibrio alginolyticus* altered in response to γ-irradiation or long-term starvation

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Received 1 June 2010; accepted 30 August 2010
Available online 28 October 2010

Abstract

Vibrio parahaemolyticus and *Vibrio alginolyticus* were subjected to γ-irradiation (0.5 kGy) or starvation by incubation for 8 months in seawater to study modifications in their outer membrane protein patterns. After treatment, outer membrane protein profiles of starved or γ-irradiated bacteria were found to be altered when analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Altered proteins were identified by mass spectrometry (MS and MS/MS) and analyses revealed that OmpU can be considered a starvation stress-induced protein. In addition, expression of OtnA, OmpW, OmpA and peptidoglycan-associated lipoprotein decreased to non-detectable levels in starved cells. Furthermore, MltA-interacting protein MipA appeared under γ-irradiation or starvation conditions. Thus, it can be considered to be a γ-irradiation, long-term starvation stress protein in some vibrios.

Keywords: Vibrio; Starvation; γ-Irradiation; Alteration; Outer membrane proteins; Mass spectrometry

1. Introduction

Vibrio, a food-borne pathogen, can sense and respond to changes in its external environment (Ben Kahla et al., 2008). The ability of *Vibrio* spp. to sense and respond effectively to changes in the environment is crucial for their survival (Morita, 1997). One such environmental parameter is the nutrient quantity in the extracellular medium. In general, microorganisms do not respond to nutrient deprivation or starvation by simply arresting all metabolic activities and stopping growth. Instead, they carry out starvation-induced activities that may include production of degradative enzymes and stress proteins (Siegele and Kolter, 1992). During nutrient deficiency, *Vibrio* spp. can survive for a long time by sequential changes in cell physiology and gradual changes in morphology (Morita, 1997). Srinivasan and Kjellberg (1998) found that, under starvation in natural environments, the response of vibrios indicated that the life cycle of bacteria broadly consisted of two major phases. The transition between these two major phases involves dramatic changes in gene expression, physiology and morphology.

Recently, gamma irradiation technology has shown positive effects in preventing decay by sterilizing microorganisms and improving the safety and shelf-stability of food products without compromising the nutritional or sensory quality (Lee et al., 2006). Andrews et al. (2003) reported that naturally incurred *Vibrio vulnificus* in oysters was reduced to non-detectable levels with ^{60}cobalt gamma radiation treatment at

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doi:10.1016/j.resmic.2010.10.009

0.75 kGy. Treatment of oysters inoculated with *Vibrio parahaemolyticus* O3:K6 with 1.0–1.5 kGy also reduced contamination to non-detectable levels. Gamma irradiation causes damage to the DNA of cells (Villavicencio et al., 2004; Lee and Levin, 2008). The types of DNA damage experienced may be: i) direct physical effects of ionizing radiation with primary free radicals, and indirect biochemical effects from reactive oxygen species resulting in double-strand breaks; ii) single-strand breaks; and iii) base pair substituting mutations due to the conversion of pyrimidine bases to 5-(hydroxymethyl) uracil, 5-formyluracil, 5-hydroxycytosine, and 5-hydroxyuracil (Jung, 1997; Lydersen and Pettijohn, 1997; Min et al., 2000)

In Gram-negative bacteria, the outer membrane plays an important role in infection and pathogenicity toward the host (Tsolis, 2002). In the outer membrane, proteins play a crucial role during many cellular and physiological processes (Qian et al., 2008). Outer membrane proteins (OMPs) play a key role in adaptation to changes of external environments due to their location at the outermost area of the cell (Xu et al., 2005). It has been shown that when bacteria are transferred to a new environment, the synthesis of their OMPs changes (Kustos et al., 2007). In this way, Wu et al. (2006) demonstrated that OmpW and OmpV are required for environmental salt regulation in *Photobacterium damsela*. Furthermore, the altered osmolarity of the culture medium caused changes in the OMP patterns of *Vibrio alginolyticus* (Xu et al., 2005) and *V. parahaemolyticus* (Xu et al., 2004).

The aim of this work was to study modifications in the OMPs profiles of starved or gamma-irradiated *V. parahaemolyticus* and *V. alginolyticus* strains. OMPs profiles were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In order to identify altered OMPs, mass spectrometry (MS and MS/MS) was used.

2. Materials and methods

2.1. Bacterial strains

Six *Vibrio* strains were used in this study, including three reference strains: *V. alginolyticus* ATCC 33787 (S1), *V. alginolyticus* ATCC 17749 (S2) and *V. parahaemolyticus* ATCC 17802 (S5). In addition, *V. parahaemolyticus* strain (S6) isolated from the Calich estuary (Alghero, Italy) and two *V. alginolyticus* strains (S3 and S4) isolated, respectively, from the internal organs of aquacultured-diseased gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) in a Tunisian aquaculture farm (Ben Abdallah et al., 2009) were included in this work.

2.2. Starvation stress

V. alginolyticus and *V. parahaemolyticus* were grown at 30 °C in tryptic soy broth (Pronadisa, Spain) supplement with 1% NaCl (TSB 1%) for 24 h. The natural seawater (100 ml) was sterilized by membrane filtration (pore size, 0.22 μm; Millipore Corp., Bedford, MA, USA) and autoclaving (121 °C for 20 min). *Vibrio* cells were washed three times (13,000 rpm for 10 min at 20 °C) and then suspended in 10 ml of sterilized seawater. The microcosms (100 ml) were inoculated with these suspensions (10^9 Colony Forming Unit (CFU)/ml) and then incubated at room temperature (22–25 °C) for 8 months.

2.3. Gamma irradiation treatment

V. alginolyticus and *V. parahaemolyticus* tested strains were cultivated at 30 °C in TSB 1% with shaking (150 rpm). The cultures of *Vibrio* strains grown to late log phase (O.D.600 = 0.6) were divided into 1 ml aliquots without change of broth and were exposed, in triplicate, on ice to a ^{60}cobalt γ-irradiator (point source, AECL, IR-79, Technopole, Sidi Thabet, Tunisia). The applied dose was 0.5 kGy. The control cells were introduced in ice during the time of irradiation. The irradiated and non-irradiated *Vibrio* were diluted 50-fold by using 50 ml of fresh medium and then harvested after 24 h of incubation at 30 °C with shaking (150 rpm).

2.4. Outer membrane protein extraction

OMPs of *V. alginolyticus* and *V. parahaemolyticus,* before and after irradiation, were prepared according to the method described previously (Sabri et al., 2000). Briefly, the bacterial cells were harvested by centrifugation at 4000 × *g* for 15 min at 4 °C. The cells were then washed three times in 40 ml of sterile saline water (0.9% NaCl) and then resuspended in 5 ml sterile saline water. Cells were disrupted by intermittent sonic oscillation in ice bath (350 W, 10 min × 3). Unbroken cells and cellular debris were removed by centrifugation at 5000 × *g* for 20 min. Supernatant was collected and was further centrifuged at 100 000 × *g* for 40 min at 4 °C. The pellet was resuspended in 10 ml of 2% (w/v) sodium lauryl sarcosinate (Sigma, St Louis, MO) and incubated at room temperature for 1 h, followed by centrifugation at 100 000 × *g* for 40 min at 4 °C. The resulting pellet was resuspended in 200 μl of sterile saline water. The concentration of the OMPs in the final preparation was determined using the Bradford Kit (Sigma).

2.5. SDS-PAGE

OMPs of starved (2 μg) and γ-irradiated (1 μg) *V. alginolyticus* and *V. parahaemolyticus* were analyzed in triplicate by SDS-PAGE (Laemmli, 1970) with 15% acrylamide in the separating gel and 5% acrylamide in the stacking gel. After separation, the proteins were visualized according to standard procedures by staining with Coomassie brilliant blue G250 (Sigma).

2.6. Protein identification by MS

After staining with colloidal Coomassie blue, 1D gel bands were manually excised from gels and collected in a 96-well plate. Destaining, reduction, alkylation and trypsin digestion of the proteins followed by peptide extraction were carried out