Identification of Outer Membrane Proteins Altered in Response to UVC-Radiation in *Vibrio parahaemolyticus* and *Vibrio alginolyticus*

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Received: 4 June 2012 / Accepted: 15 August 2012 / Published online: 4 September 2012
 Association of Microbiologists of India 2012

Abstract *Vibrio parahaemolyticus* and *V. alginolyticus*, marine foodborne pathogens, were treated with UVC-radiation (240 J/m²) to evaluate alterations in their outer membrane protein profiles. Outer membrane protein patterns of UVC-irradiated bacteria were found altered when analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Altered proteins were identified by mass spectrometry (MS and MS/MS) and analysis revealed that OmpW, OmpA, Long-chain fatty acid transport protein, Outer membrane receptor protein, Putative uncharacterized protein VP0167, Maltoporin (IamB), Polar flagellin B/D, Agglutination protein Peptidoglycan-associated lipoprotein and MltA-interacting protein MipA were appeared, thereby they can be considered as UVC-stress proteins in some vibrios. In addition, expression of OmpK decreased to non-detectable level. Furthermore, we observed a decrease or an increase in the expression level of other outer membrane proteins.

Keywords *Vibrio* · UVC · Alteration · Outer membrane proteins · Mass spectrometry

Introduction

Vibrio, a food-borne pathogen, can sense and respond to changes in their external environment [1]. The ability of *Vibrio* spp. to sense and respond effectively to changes in the environment is crucial for their survival [2]. Bacteria in nature have cellular physiological systems to protect cells from various environmental stresses such as ultraviolet irradiation [3]. Marine bacteria, such as *Vibrio alginolyticus* and *V. parahaemolyticus*, are exposed to solar UV radiation daily. The response of marine bacteria to UVA irradiation is considered important because approximately 95 % of UV radiation is UVA (320–400 nm). On the other hand, bacteria are also exposed to UVC (254 nm) radiation used as a disinfection method to kill bacteria and prevent food poisoning in humans [3].

Ultraviolet-C (UV-C) radiation has been suggested as one of the successful disinfection practices for water treatment. Therefore, UV-sterilization has become a practical solution for safe disinfection of water [4]. The effectiveness of UV radiation in biological inactivation arises primarily from the fact that DNA molecules absorb UV photons between 200 and 300 nm, with peak absorption at 254 nm [5]. This absorption creates damage in the DNA by altering the nucleotide base pairing, thereby creating new linkages between adjacent nucleotides on the same DNA strand [6]. Several investigators have determined the effects of UV radiation on the inhibition of growth [7] and damage of protein, RNA, and DNA for *Vibrio* [8, 9]. All these effects have been related directly or indirectly to genomic damage arising out of primary photochemical changes in nucleic acids.

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In Gram-negative pathogenic bacteria like *Vibrio*, the outer membrane plays an important role in the infection and pathogenicity to host [10]. In the outer membrane, proteins have a crucial role during many cellular and physiological processes [11]. OMPs play a key role in the adaptation to changes of external environments, due to their location at the outmost area of the cell [12]. Several works have shown that when bacteria are transferred to a new environment, the synthesis of their OMPs change [13]. Several alterations were observed in the OMPs profiles of *V. alginolyticus* and *V. parahaemolyticus* under starvation conditions [1]. Furthermore, the altered osmolarity of the culturing medium caused changes in the OMP patterns of *V. alginolyticus* [12] and *V. parahaemolyticus* [14]. In *Escherichia coli* OmpF is highly expressed at low osmolarity and suppressed at high osmolarity, while OmpC is totally the opposite [15]. More and more similar regulations have been found in other bacteria, such as major outer membrane protein and Omp50 in *Campylobacter jejuni* [16], and OmpK35 and OmpK36 in *Klebsiella pneumoniae* [17].

The aim of this work was to study the effect of UVC-radiation on the OMPs profiles of *V. alginolyticus* and *V. parahaemolyticus*. OMPs pattern were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). To identify the altered OMPs mass spectrometry (MS and MS/MS) was used.

Materials and Methods

Bacterial Strains

Six *Vibrio* strains were used in this study including three reference strains: *Vibrio 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 Tunisian aquaculture farm [18], were included in this work.

UVC Treatment

V. alginolyticus and *V. parahaemolyticus* were cultivated at 30 °C in Tryptic soy broth 1 % NaCl (TSB 1 %) with shaking (150 rpm). Then, the cultures of *Vibrio* (OD₆₀₀ = 0.6) were diluted and spread on Tryptic soy agar 1 % NaCl (TSA 1 %, Pronadisa, Spain) in glass Petri dishes. After 18 h of incubation at 30 °C, bacterial colonies that appeared on the plates were exposed, in triplicate, to UV

radiation according to the method described previously [19]. The plates with even bacterial growth were covered with a piece of glass for non-UV treatment (untreated bacteria). The plates (covered and non-covered) were exposed to a 4-W UV lamp with a wavelength of 254 nm. The applied dose was 240 Joules/m². After exposure, 250 ml Erlenmeyer flasks containing 100 ml of TSB 1 % were inoculated with a loopful of colonies from control and UV treated bacteria. All flasks were kept in at 30 °C for 18 h with a shaking.

OMP Extraction

OMPs of *V. alginolyticus* and *V. parahaemolyticus*, before and after irradiation, were prepared according to the method described previously [6]. Briefly, the bacterial cells were harvested by centrifugation at 4,000×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 5,000×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).

SDS-PAGE

OMPs (3 µg) of UVC irradiated and untreated *V. alginolyticus* and *V. parahaemolyticus* were analyzed in triplicate by SDS-PAGE [20] 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).

Protein Identification by Mass Spectrometry

After staining with Coomassie brilliant blue G250 (Sigma), 1D gel bands were manually excised from gels and collected in a 96-well plate. Destaining, reduction, alkylation, trypsin digestion of the proteins followed by peptide extraction were carried out with the Progest Investigator (Genomic Solutions, Ann Arbor, MI, USA). After the desalting step (C18-µZipTip, Millipore) peptides were eluted directly using the ProMS Investigator, (Genomic

Solutions, Ann Arbor, MI, USA) onto a 96-well stainless steel MALDI target plate (Applied Biosystems/MDS SCIEX, Framingham, MA, USA) with 0.5 µl of CHCA matrix (2.5 mg/ml in 70 % ACN/30 % H₂O/0.1 % TFA).

MS and MS/MS Analysis

Raw data for protein identification were obtained on the 4800 MALDI TOF/TOF Analyzer (Applied Biosystems/MDS SCIEX, Framingham, MA, USA) and were analyzed by GPS Explorer 3.6 software (Applied Biosystems/MDS SCIEX, Framingham, MA, USA). For positive-ion reflector mode spectra 3000 laser shots were averaged. For MS calibration, autolysis peaks of trypsin ($[M+H]^+ = 842.5100$ and $2,211.1046$) were used as internal calibrators. Monoisotopic peak masses were automatically determined within the mass range 800–4,000 m/z with a signal to noise ratio minimum set to 30. Up to twelve of the most intense ion signals were selected as precursors for MS/MS acquisition excluding common trypsin autolysis peaks and matrix ion signals. In MS/MS positive ion mode, 4000 spectra were averaged, collision energy was 2 kV, collision gas was air and default calibration was set using the Glu1-Fibrino-peptide B ($[M+H]^+ = 1570.6696$) spotted onto fourteen positions of the MALDI target. Combined PMF and MS/MS queries were performed using the MASCOT search engine 2.1 (Matrix Science Ltd., UK) embedded into GPS-Explorer Software 3.6 (Applied Biosystems/MDS SCIEX, Framingham, MA, USA) on the UNIPROT database (downloaded 2009 06 25; 9064751 sequences; 2941541906 residues) with the following parameter settings: 50 ppm peptide mass accuracy, trypsin cleavage, one missed cleavage allowed, carbamidomethylation set as fixed modification, oxidation of methionine was allowed as

variable modification, MS/MS fragment tolerance was set to 0.3 Da. Protein hits with MASCOT Protein score ≥ 82 and a GPS Explorer Protein confidence index = 95 % were used for further manual validation.

Results

Effect of UVC Irradiation on OMPs

Outer membrane proteins of *V. alginolyticus* and *V. parahaemolyticus* cells were analyzed by SDS-PAGE (Fig. 1). Before their treatment with UV-C, each *Vibrio* strain presents its specific OMPs profile. In addition, almost 15 bands were detected in each profile containing from three to six major OMPs. After UV-C irradiation, several OMPs were to be found altered. These alterations were manifested by the appearance or disappearance of proteins as well as by a modification in the expression level of certain proteins. Indeed, as shown in Fig. 1, we observed alterations of 5 bands in *V. alginolyticus* (S2), 6 bands in *V. alginolyticus* (S1, S3 and S4), 7 bands in *V. parahaemolyticus* (S5) and 9 bands in *V. parahaemolyticus* (S6).

OMPs Identification

Altered OMPs of UV-C irradiated bacteria were identified using mass spectrometry (Table 1). Our results showed that OmpU was induced in *V. alginolyticus* S1 and S2, whereas its expression level was decreased in *V. alginolyticus* (S3 and S4) and *V. parahaemolyticus* (S5). For *V. parahaemolyticus* (S6), we noted that OmpU was appeared while outer membrane protein (ompU) became less abundant. OmpW was

Fig. 1 Outer membrane proteins of *V. alginolyticus* and *V. parahaemolyticus* cells exposed to UVC radiation. *M*: high-range rainbow (Amersham, Little Chalfont, Buckinghamshire, UK); *S1*, *S2*, *S3* and

S4: *V. alginolyticus* strains; *S5* and *S6*: *V. parahaemolyticus* strains; *n*: strain non irradiated; *i*: strain irradiated

Table 1 List of altered OMPs of UVC-irradiated *V. alginolyticus* and *V. parahaemolyticus* identified using MS–MS/MS

Strain	Band number	Identified protein	Accession number (Uniprot)	Mascot score	Molecular mass (Da)	Expression level
S1	01	Outer membrane channel protein TolC	Q1V4T2 Q1V4T2_VIBAL	94	47,846.3	+
	02	Outer membrane protein OmpU	Q1V395 Q1V395_VIBAL	360	37,067.4	–
	03	Putative outer membrane protein	Q1V592 Q1V592_VIBAL	331	37,991.1	+
	04	Outer membrane protein OmpK	Q1VF49 Q1VF49_VIBAL	493	29,310	+
	05	Peptidoglycan-associated lipoprotein	Q1V5X7 Q1V5X7_VIBAL	180	18,740.4	+
		Outer membrane protein W (fragment)	P83151 OMPW_VIBAL	149	2,095.1	+
	32	Agglutination protein	Q1V422 Q1V422_VIBAL	709	47,190.7	+
S2	06	Outer membrane channel protein TolC	Q1V4T2 Q1V4T2_VIBAL	384	47,846.3	–
	07	Porin, putative	Q1VA77 Q1VA77_VIBAL	522	39,676	+
	08	Outer membrane protein OmpU	Q1V395 Q1V395_VIBAL	360	37,067.4	–
	09	Outer membrane protein OmpA	Q1VAZ6 Q1VAZ6_VIBAL	260	36,182.2	+
	10	Outer membrane protein	A7JZ07 A7JZ07_9VIBR	198	36,074.2	+
S3	11	Outer membrane protein OmpU	Q1V395 Q1V395_VIBAL	213	37,067.4	–
	12	Outer membrane protein OmpA	Q1VAZ6 Q1VAZ6_VIBAL	908	36,182.2	+
	13	Outer membrane protein	A7JZ07 A7JZ07_9VIBR	231	36,074.2	+
	33	Porin, putative	Q1VA77 Q1VA77_VIBAL	615	39,676	–
	34	Outer membrane channel protein TolC	Q1V4T2 Q1V4T2_VIBAL	227	47,846.3	–
S4	39	Vitamin B12 receptor	Q1V4E1 Q1V4E1_VIBAL	1,080	68,061.3	–
	14	Outer membrane protein OmpU	Q1V395 Q1V395_VIBAL	237	37,067.4	–
	15	Outer membrane protein	A7JZ07 A7JZ07_9 VIBR	406	36,074.2	+
	16	Outer membrane protein OmpA	Q1VAZ6 Q1VAZ6_VIBAL	260	36,182.2	+
	35	Outer membrane protein OmpA	A7K5D9 A7K5D9_9VIBR	551	34,468	–
	36	Outer membrane protein W (Fragment)	P83151 OMPW_VBAL	162	2,095.1	+
	37	MltA-interacting protein MipA	A7JZF9 A7JZF9_9VIBR	131	31,026.2	+
S5	17	Maltoporin=lamB	C3W4N2 C3W4N2_VIBPA	437	46,349.8	–
	18	Polar flagellin B/D	Q56702 FLAB_VIBPA	905	40,149	+
	19	Outer membrane protein (ompU)	Q4ZIM7 Q4ZIM7_VIBPA	324	36,189.9	–
	20	Putative outer membrane porin protein	Q87QZ0 Q87QZ0_VIBPA	782	39,489	+
	21	Putative outer membrane protein	Q87JT2 Q87JT2_VIBPA	144	38,007.2	+
	22	Outer membrane protein K=OmpK	B6DVI5 B6DVI5_VIBPA	668	30,280.5	+
	23	Outer membrane protein W=OmpW	A6B1W5 A6B1W5_VIBPA	246	23,509.8	+
S6	24	Outer membrane protein (ompU)	Q4ZIM7 Q4ZIM7_VIBPA	516	36,189.9	–
	25	Putative outer membrane protein OmpA	Q87JK1 Q87JK1_VIBPA	156	35,645.2	+
	26	Outer membrane receptor protein	Q1VAM1 Q1VAM1_VIBL	1,380	93,865.3	+
	27	Maltoporin=lamB	C3W4N2 C3W4N2_VIBPA	313	46,349.8	+
		Outer membrane protein TolC	Q87SJ8 Q87SJ8_VIBPA	148	47,954.4	+
	28	Porin, putative	Q1VA77 Q1VA77_VIBAL	448	39,676	+
		Long-chain fatty acid transport protein	Q1V8C7 Q1V8C7_VIBAL	94	45,059.6	
	29	Outer membrane protein OmpU	Q1VCV6 Q1VCV6_VIBAL	186	35,837.3	+
	30	Outer membrane protein=ompK	Q4PNS4 Q4PNS4_VIBAL	431	31,311.9	+
		Putative uncharacterized protein VP0167	Q87TA3 Q87TA3_VIBPA	224	28,406.5	+
	31	Outer membrane protein W	A6B1W5 A6B1W5_VIBPA	145	2,095.1	+
	38	Outer membrane protein ompK	P51002 IOMPK2_VIBPA	235	29,448.1	–

S1: *V. alginolyticus* ATCC 33787, S2: *V. alginolyticus* ATCC 17749, S3: *V. alginolyticus* (*Sparus aurata*), S4: *V. alginolyticus* (*Dicentrarchus labrax*), S5: *V. parahaemolyticus* ATCC 17802, S6: *V. parahaemolyticus* (Calich estuary). + up-regulated; – down-regulated

appeared in *V. alginolyticus* (S1 and S4) and *V. parahaemolyticus* (S5 and S6). The expression level of OmpK was increased in *V. alginolyticus* (S4) and *V. parahaemolyticus* (S5), whereas this protein became non detectable in *V. parahaemolyticus* (S6). After irradiation, we observed that OmpA was appeared in *V. alginolyticus* (S2 and S3), however this protein persist in *V. alginolyticus* S4 but with a lower molecular weight compared to non-treated strain. In addition to the modification already cited, we also noted that Outer membrane channel protein (TolC) was appeared in *V. parahaemolyticus* (S6), whereas its abundance was decreased in *V. alginolyticus* (S2 and S3) and was increased in *V. alginolyticus* (S1). Further, Outer membrane protein was induced in irradiated in *V. alginolyticus* (S2, S3 and S4), Putative outer membrane protein became more abundant in *V. alginolyticus* (S1) and *V. parahaemolyticus* (S5). Porin putative was appeared in *V. parahaemolyticus* (S6), whereas its expression level was decreased in *V. alginolyticus* (S3) and was increased in *V. alginolyticus* (S2). In irradiated *V. parahaemolyticus* (S6) we noted that Long-chain fatty acid transport protein, Outer membrane receptor protein, Putative uncharacterized protein VP0167 and Maltoporin (IamB) were appeared while Putative outer membrane protein OmpA became more abundant. Similar results have been observed in irradiated *V. parahaemolyticus* ATCC 17802 (S5), which respond to UV radiation by the appearance of Polar flagellin B/D and by increasing the expression of Putative outer membrane porin protein. For *V. alginolyticus* (S1) we observed that Agglutination protein and Peptidoglycan-associated lipoprotein were appeared after irradiation, while MltA-interacting protein MipA was appeared in *V. alginolyticus* (S4). Furthermore, Vitamin B12 receptor and Maltoporin (IamB) were decreased in *V. alginolyticus* (S1) and *V. parahaemolyticus* (S6), respectively.

Discussion

V. alginolyticus and *V. parahaemolyticus* respond to environmental stress by modifying the rate of synthesis of certain proteins [1]. Alterations observed in the OMPs profiles of UV-C irradiated *V. alginolyticus* and *V. parahaemolyticus* were manifested by the appearance of proteins as well as by modifications in the expression level of certain proteins. After the beginning of an adverse effect, such as irradiation, the synthetic of proteins are inhibited and cells division is interrupted. In parallel, the expression of several proteins increases; these are the so-called stress proteins [21]. Furthermore, in this work OmpW, OmpA, Long-chain fatty acid transport protein, Outer membrane receptor protein, Putative uncharacterized protein VP0167, Maltoporin (IamB), Polar flagellin B/D, Agglutination protein Peptidoglycan-associated lipoprotein and MltA-

interacting protein MipA were appeared, thereby they can be considered as UVC-stress proteins in some vibrios species. OMPs have been produced, probably, to stimulate the bacterial cells to enter an exponential growth phase in a shorter time and grow at a faster rate, this mechanism is important for bacterial population to survive under UV radiation. In addition, Outer membrane protein, Putative outer membrane protein, Putative outer membrane porin protein and Putative outer membrane protein OmpA can be regarded as UV-C inducible proteins since these proteins became more abundant in some tested *Vibrio* strains. The new and induced OMPs can protect *V. alginolyticus* and *V. parahaemolyticus* against UV-C radiation. These results are in accordance with those reported by Nicholson et al. [22] who demonstrate that UV radiation induces the synthesis of several proteins in *Phormidium Zaminosum*. Bockrath and Hanawalt [23] showed that RecA protein was induced in UV irradiated *E. coli*. However, Vitamin B12 receptor and Maltoporin (IamB) are down-regulated by UV-C radiation. Additionally, the expression levels of OmpU, OmpK, Outer membrane channel protein (TolC) and Porin putative are variable under UV-C radiation. According to Miller et al. [24] UV radiation can cause indirect damage to DNA by generating reactive oxygen species that then damage bases, break strands, and cross-link DNA and proteins. Such degradation of proteins observed in this work could be due to any of the mechanisms proposed for protein inactivation by UV radiation [25]. These include (i) splitting of disulfide bonds of cystine or photooxidation of tyrosine, thereby changing the tertiary structure of proteins, or (ii) ring cleavage in the aromatic.

Generally, environmental changes influence the expression of OMPs and a rise in temperature may induce significant changes in the OMP expression of *E. coli* [26] and *Borrelia burgdorferi* [27]. Acidic pH induced the expression of new proteins on the surface of *Yersinia pestis* [28] and OMP expression in *B. burgdorferi* was also altered at different pH values [29]. The alterations observed in the OMP patterns of UV-irradiated *V. alginolyticus* and *V. parahaemolyticus* may testify the existence of certain modification of resistance toward some antibiotics. Indeed, Mushtaq et al. [30] described the loss of OprD protein from the OMPs of *P. aeruginosa* strains, which causes increased resistance against carbapenems. Chen and Livermore [31] demonstrated the resistance to β -lactam antibiotics owing to the lack of OmpC and OmpF in the OMPs of *E. coli* and *Klebsiella pneumoniae*.

Many vibrios are pathogenic for humans and/or marine vertebrates and invertebrates [32]. OMPs, whose production is often regulated by environmental cues, play important roles in bacterial pathogenesis by enhancing the adaptability of the pathogens to various environments. Thus, OMPs contribute to the virulence of bacteria and play essential

roles in bacterial adaptation to host niches, which are usually hostile to invading pathogens [33]. The various alterations observed in the OMP profiles of UV-C irradiated *Vibrio* reflect the stability of these virulence factors under stress conditions. In addition, alterations observed between examined vibrios cells are variable. This can be in relation to the genomic plasticity of the tested strains.

In summary, this work showed for the first time alterations in the OMPs profiles of *V. alginolyticus* and *V. parahaemolyticus* cells under UV-C radiation. These modifications were manifested by the appearance of proteins as well as by a decrease and/or an increase in the expression level of certain proteins. In addition one OMP becomes non detectable while others were appeared. This allows us to define UVC stress proteins capable to protect these bacteria against UV radiation.

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