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Proteomic characterization of the outer membrane vesicle of the halophilic marine bacterium *Novosphingobium pentaromativorans* US6-1[§]

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***Novosphingobium pentaromativorans* US6-1 is a Gram-negative halophilic marine bacterium able to utilize several polycyclic aromatic hydrocarbons such as phenanthrene, pyrene, and benzo[a]pyrene. In this study, using transmission electron microscopy, we confirmed that *N. pentaromativorans* US6-1 produces outer membrane vesicles (OMVs). *N. pentaromativorans* OMVs (hereafter OMV_{Novo}) are spherical in shape, and the average diameter of OMV_{Novo} is 25–70 nm. Proteomic analysis revealed that outer membrane proteins and periplasmic proteins of *N. pentaromativorans* are the major protein components of OMV_{Novo}. Comparative proteomic analysis with the membrane-associated protein fraction and correlation analysis demonstrated that the outer membrane proteins of OMV_{Novo} originated from the membrane-associated protein fraction. To the best of our knowledge, this study is the first to characterize OMV purified from halophilic marine bacteria.**

***Keywords*:** *Novosphingobium pentaromativorans*, outer membrane vesicle, proteomics

Introduction

Extracellular membrane vesicles are spherical buds of membrane released from cells or microorganisms. In Gram-negative bacteria, extracellular membrane vesciles are called outer membrane vesicles (OMVs). Among the OMV-producing Gram-negative bacteria, pathogenic bacteria (*Escherichia coli*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Neisseria meningitidis*) have been major targets for OMV studies (Choi *et al.*, 2014; Kim *et al.*, 2014; Lee *et al.*, 2016) . The reason for focusing on these bacteria is that their OMV components [outer membrane proteins, lipoproteins, and lipopolysaccharides (LPS)] and their cargo proteins were considered as virulence factors or immunogens on the host or agents of inflammatory responses. However, functions of OMV are not restricted to pathogenicity. Recently, environmental Gram-negative bacteria and marine bacteria also have been reported to produce OMVs (or MVs) and the presence of OMVs in their natural habitats has been verified (Choi *et al.*, 2014). *P. putida* K2440 is a soil bacterium capable of utilizing various aromatic compounds as major carbon and energy sources (Nelson *et al.*, 2002). OMVs of *P. putida* KT2440 were purified and characterized by proteomic analysis. OMVs of *P. putida* KT2440 had lower cytotoxic effects on cultured mammalian cells than other Gram-negative pathogenic bacteria, even though these OMVs also have LPS. However, the OMV of *P. putida* KT2440, cultured in benzoate, carries benzoate degrading enzymes suggesting possible biodegradation by OMV in natural habitats (Choi *et al.*, 2014). Because the dominant marine cyanobacterium, *Prochlorococcus*, was reported to constantly release extracellular vesicles into the environment (Biller *et al.*, 2014), it has been suggested that extracellular vesicles of this strain could significantly affect other marine microorganisms in the marine ecosystem. These effects include changes in nutrient supply, increased gene transfer, and anti-viral protection. In this study, we confirmed OMVs are produced by the marine bacterium *Novosphingobium pentaromativorans* US6-1. *N. pentaromativorans* US6-1 is a halophilic marine bacterium which was isolated from muddy sediment of Ulsan Bay of South Korea. This strain possesses biodegradation activity for polycyclic aromatic hydrocarbons (Sohn *et al.*, 2004) which was confirmed by genomic and comparative proteomic analyses (Luo *et al.*, 2012; Yun *et al.*, 2014). The purpose of this study was to perform proteomic analysis of an OMV of *N. pentaromativorans* US6-1) (OMV_{Novo}), which can be used to elucidate this bacterium's biological functions. OMV_{Novo} has similar proteomic properties to other previously reported OMVs but also has unique properties. To our knowledge, this is the first proteomic characterization of OMV originating from halophilic marine bacteria.

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Materials and Methods

Bacteria culture

N. pentaromativorans US6-1 was pre-cultured according to a previous method (Sohn *et al.*, 2004). Bacteria were cultured in marine broth (BD) until they reached an OD_{600} of 0.4–0.6 at 30°C. The cultured bacteria were aseptically harvested by centrifugation at 10,000 × *g* for 30 min and washed with phosphate-buffered saline twice to remove cell debris. Harvested bacteria were transferred to fresh Bushnell-Hass broth (BD) containing *p*-hydroxybenzoate (PHB)(50 ppm) and NaCl (513.3 mM). Initial OD_{600} of inoculated cells was adjusted to 0.4. Bacteria were cultured by shaking (180 rpm) until they reached the exponential phase (OD_{600} = 0.7) at 30°C.

Purification of OMVs

The OMV_{Novo} (OMV of *N. pentaromativorans* US6-1) was purified with cell culture media using a modification of a previously published method (Choi *et al.*, 2014). The bacterial cells were removed by centrifugation at 10,000 × *g* for 30 min and the supernatant was vacuum-filtered through a 0.45 μm filter to remove any residual cells and debris. The OMVs were ultrafiltered and concentrated with a QuixStand benchtop system (GE Healthcare) using a 0.22 μm filter and a 500 kDa hollow fiber membrane (GE Healthcare). The concentrated OMVs were precipitated by ultracentrifugation at 150,000 × *g* for 3 h at 4°C, and then the pellets containing OMVs were suspended in 1.0–2.0 ml of 20 mM Tris-HCl (pH 8.0). The OMV solution was stored at -80°C until further analysis.

Transmission electron microscopy analysis of *N. pentaromativorans* US6-1 and purified OMVs

For transmission electron microscopy (TEM) micrographs of OMVs produced by *N. pentaromativorans* US6-1, the pellets obtained from cultured US6-1 were fixed with 2.5% glutaraldehyde in a 0.1 M phosphate buffer. Without destroying the pellet within the block, the samples were post-fixed with 1% osmium tetroxide in the same buffer. Before embedding them in epoxy resin, the samples were dehydrated in an ethanol gradient and treated with propylene oxide. For resin infiltration, propylene oxide and epoxy resin were used in the ratios of 2:1, 1:1, 1:2, and 1.5:0.5. As the final step, the samples embedded with 100% epoxy resin were placed at room temperature for 3 h and then polymerized at 70°C for overnight. Thin 80 nm thick sections of the polymerized samples were sliced using an ultra-microtome and were post-stained with both 2% uranyl acetate and lead citrate. Each sliced US6-1 sample was placed on carbon-film 200-mesh copper grids and visualized on an FEI Tecnai F20 electron microscope operated at 200 kV. Purified OMV_{Novo} solutions were diluted with 10 volumes of 20 mM Tris-HCl (pH 8.0), applied to 400-mesh copper grids, and stained with 2% uranyl acetate. The purified OMV_{Novo} was visualized on an FEI Tecnai G2 Spirit Bio-TEM operated at 120 kV.

Preparation of the membrane-associated protein fraction

The membrane-associated protein fraction was prepared by the sodium carbonate precipitation method (Molloy *et al.*, 2000; Choi *et al.*, 2014). Harvested bacteria were suspended in 20 mM Tris-HCl buffer (pH 8.0) and disrupted several times in a French pressure cell (SLM AMINCO) at 20,000 lb/in^2. Residual bacteria and cell debris were removed by centrifugation at 5,000 × *g* for 20 min. The supernatants were dissolved with sodium carbonate solution in ice until the final concentration of the solution reached 100 mM and were then centrifuged at 150,000 × *g* for 1 h. The pellets contained the membrane-associated protein fraction and were dissolved in 20 mM Tris-HCl (pH: 8.0).

SDS-PAGE and in-gel digestion

Purified OMV_{Novo} and membrane-associated proteins were incubated in a denaturation buffer (25 mM ammonium bicarbonate and 2% SDS) at room temperature for 1 h. Pelleted cell debris was discarded by centrifugation at 18,000 rpm for 10 min. Quantification of the protein were determined using the modified bicinchoninic acid assay (Thermo Scientific). The protein components of the OMV_{Novo} and membrane fractions (approximately 10 μg) were separated on 12% SDS polyacrylamide gel. In-gel digestion was performed as previously described (Choi *et al.*, 2014). Sliced gels were destained with a destaining solution (50% acetonitrile and 10 mM ammonium bicarbonate). The gels then were rinsed with distilled water followed by 100% acetonitrile. Remaining proteins in the sliced gels were sequentially treated with a reducing solution (10 mM dithiothreitol and 100 mM ammonium bicarbonate) and an alkylation solution (55 mM iodoacetamide) to break disulfide bonds in the sample proteins. After the gels were washed with distilled water, tryptic digestion was performed in 50 mM ammonium bicarbonate at 37°C for 12–16 h. For optimal digestion, the final trypsin concentration was adjusted to 10 ng/ml. Extraction of the tryptic peptides was performed in an extraction solution [50 mM ammonium bicarbonate and 50% acetonitrile containing 5% trifluoroacetyl acid (TFA)]. The resulting peptide extracts were pooled and lyophilized. The tryptic peptides were dissolved in 0.5% TFA prior to further fractionation using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Protein identification with LC-MS/MS

Digested peptides were analyzed according to a modification of a previously described method using a linear trap quadrupole (LTQ) mass spectrometer (Moon *et al.*, 2012). Concentrated tryptic peptides were eluted from the column and directed onto a 10 cm × 75 μm I.D. C18 reverse phase column (PROXEON) at a flow rate of 300 nl/min. Peptides were eluted by a gradient of 0–65% acetonitrile for 80 min. All MS and MS/MS spectra were acquired in a data-dependent mode with an LTQ-Velos ESI Ion Trap mass spectrometer (Thermo Scientific). For protein identification, MS/MS spectra were analyzed by MASCOT (Matrix Science, http://www.matrix science.com). Mass tolerance of parent ion or fragment ion was 0.8 Da. Cabamidomethylation of cysteine and oxidation of methionine were considered in MS/MS analysis as variable modifications of tryptic peptides. The genome

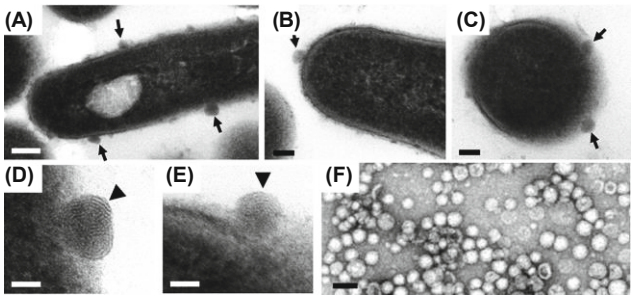

Fig. 1. TEM images of OMVs (OMV_{Novo}) induced by cultured *N. pentaromativorans* US6-1 and purified OMVs. (A–C) OMV_{Novo} (black arrows) budding out from cultured *N. pentaromativorans* US6-1. (D–E) Components of OMV_{Novo} constituted in lattice form (black arrowheads). (F) Purified OMV_{Novo} from cultured US6-1. Bar in A is 200 nm, bars in B, C, and F are 100 nm, and bars in D, E are 50 nm.

sequence of *N. pentaromativorans* US6-1 (GI: 359402640) was downloaded from National Center for Biotechnology Information and used as the database for protein identification. The MS/MS data were filtered according to a false discovery rate (FDR) criteria of 1%.

Bioinformatic analysis for the characterization of identified proteins by proteomic methods

The subcellular location of the identified proteins was predicted by Cello (ver. 2.0; http://cello.life.nctu.edu.tw/) (Yu *et al.*, 2006). Prediction of transmembrane topology was performed using Phobius (http://phobius.sbc.su.se/) (Käll *et al.*, 2004). Spearman correlation coefficients and scatter plots of mol% of each protein were calculated with R (http://www.r-project.org) (Choi *et al.*, 2014).

Table 1. Summary of proteomic analysis by 1DE-LC-MS/MS

Cello[a]	Outer membrane vesicles (OMVs)		Membrane associated protein fraction (MAF)	
	No	Percentage (%)[b]	No	Percentage (%)[b]
Outer Membrane	54	65.17	55	30.91
Extracellular	9	2.67	5	4.55
Periplasmic	86	25.12	70	31.85
Cytoplasmic	34	6.83	115	28.13
Inner Membrane	4	0.21	37	4.56
Total	187	100	282	100

[a] Prediction of cellular location by CELLO v.2.5: http://cello.life.nctu.edu.tw/
[b] Summarization of average by MACOT

Results and Discussion

OMV production by marine bacterium *N. pentaromativorans* US6-1

N. pentaromativorans US6-1 cultured in Bushnell-Haas broth containing *p*-hydroxybenzoate (50 ppm) and NaCl (513.3 mM) reached exponential phase after cultivation for 24 h. Cultured bacteria in this stage were used for TEM analysis. The bacteria embedded with 100% epoxy resin were sliced using an ultra-microtome into 80 nm thicknesses and visualized on an FEI Tecnai F20 electron microscope operated at 200 kV. As shown in Fig. 1A–C, the process of OMV_{Novo} budding (black arrows) was examined. The OMV_{Novo} are spherical in shape with an average diameter of approximately 70 nm. A magnified view of OMV_{Novo} attached to the bacteria demonstrates that the OMV_{Novo} are homogeneous in size (70 nm) and have a uniform round shape (Fig. 1D and E). In addition, the protein components in OMV_{Novo} were aligned into a lattice formation (black arrowheads in Fig.

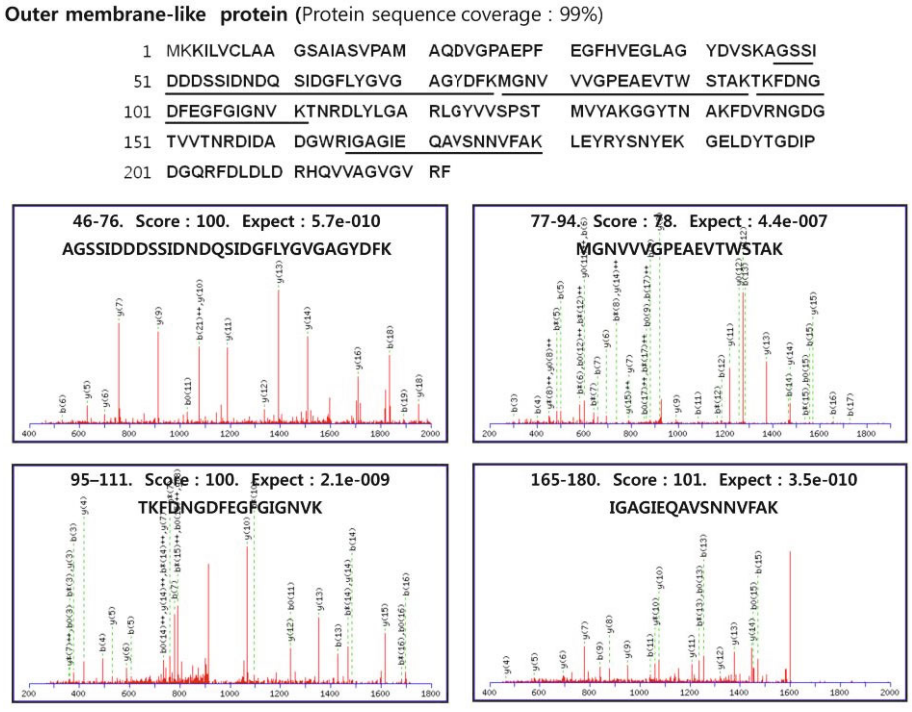

Fig. 2. MS/MS analysis of 23.8 kDa outer membrane-like protein. MS/MS analysis using Mascot confirmed 99% amino acid sequence of this protein. Four spectra of underlined peptide fragments were shown.

Table 2. COG categories of major protein components of outer membrane vesicle of *Novosphingobium pentaromativorans* US6-1

COG category/ Accessions	Description (conserved domain of hypothetical protein)	Predicted localization[a]	Mol%
Cell wall/membrane/envelope biogenesis			
WP_007014739.1	membrane protein (Outer membrane-like protein)	Outer Membrane	49.98
WP_038575953.1	flagellar motor protein MotB	Extracellular	1.42
WP_007011976.1	structural protein MipA	Outer Membrane	1.32
WP_013831521.1	OmpA/MotB protein	Outer Membrane	1.04
WP_007011933.1	peptidoglycan-associated lipoprotein	Periplasmic	0.96
WP_007013102.1	OmpA/MotB	Periplasmic	0.69
WP_007012756.1	membrane protein	Periplasmic	0.36
WP_038575344.1	lytic transglycosylase	Periplasmic	0.32
Coenzyme transport and metabolism			
WP_007015966.1	TonB-dependent receptor	Outer Membrane	1.78
WP_007011575.1	TonB-dependent receptor	Outer Membrane	0.72
WP_038575474.1	TonB-dependent receptor	Outer Membrane	0.44
WP_007014195.1	TonB-dependent receptor	Outer Membrane	0.42
WP_007011824.1	TonB-dependent receptor	Outer Membrane	0.31
WP_007011760.1	TonB-dependent receptor	Outer Membrane	0.63
Posttranslational modification, protein turnover, chaperones			
WP_038577625.1	protease	Periplasmic	2.99
WP_038576792.1	peptidase M48	Outer Membrane	0.86
WP_007011314.1	peptidyl-prolyl cis-trans isomerase	Periplasmic	0.57
WP_007011815.1	peptidase M28	Periplasmic	0.36
WP_038577482.1	peptidase S41	Cytoplasmic	0.34
Lipid transport and metabolism			
WP_038576121.1	aromatic hydrocarbon degradation protein	Outer Membrane	0.34
Amino acid transport and metabolism			
WP_007015276.1	ABC transporter substrate-binding protein	Outer Membrane	0.34
Intracellular trafficking, secretion, and vesicular transport			
WP_007011934.1	translocation protein TolB	Outer Membrane	1.16
Defense mechanisms			
WP_038575422.1	peroxiredoxin	Periplasmic	2.14
BAM complex			
WP_052117945.1	outer membrane protein assembly factor BamA	Outer Membrane	0.12
WP_013831912.1	pyrrolo-quinoline quinone, partial (BamB)	Outer Membrane	0.20
WP_007014754.1	transporter (BamD)	Periplasmic	0.47
WP_038577105.1	hypothetical protein (BamE)	Periplasmic	0.12
Unknown			
WP_007012280.1	hypothetical protein (VWA domain-containing protein)	Periplasmic	0.35
WP_007014806.1	hypothetical protein (OM_channels superfamily)	Periplasmic	4.30
WP_038576381.1	hypothetical protein (OM_channels superfamily)	Outer Membrane	2.45
WP_007011837.1	hypothetical protein	Cytoplasmic	1.73
WP_007015216.1	hypothetical protein	Periplasmic	1.38
WP_007014809.1	hypothetical protein (oxidative stress defense protein)	Periplasmic	1.16
WP_007012831.1	hypothetical protein	Cytoplasmic	0.81
WP_007015007.1	twin-arginine translocation pathway signal	Cytoplasmic	0.70
WP_007015052.1	hypothetical protein (DUF4197 superfamily)	Cytoplasmic	0.62
WP_007013695.1	hypothetical protein (DUF1134 superfamily)	Extracellular	0.61
WP_007011801.1	hypothetical protein	Periplasmic	0.50
WP_007015039.1	hypothetical protein (Entericidin superfamily)	Cytoplasmic	0.49
WP_051010007.1	hypothetical protein	Periplasmic	0.49
WP_038577529.1	class A beta-lactamase	Cytoplasmic	0.45
WP_007013809.1	membrane protein	Periplasmic	0.44
WP_007013157.1	hypothetical protein (periplasmic TolA-binding protein)	Periplasmic	0.44
WP_007011725.1	hypothetical protein (Gly-zipper_OmpA superfamily)	Periplasmic	0.37
WP_038576145.1	lipoprotein transmembrane	Extracellular	0.33

[a] Localization prediction of identified proteins were performed by Cello program.

1D and E) which indicates that the OMV_{Novo} carries some bacterial proteins. TEM visualization of lattices in bio-field may evaluate the identification of some proteins that can self-assemble into well-ordered one- and two-dimensional arrays (Jun *et al.*, 2011). The presence of the purified OMV_{Novo} was also confirmed by TEM (Fig. 1F). The purified OMVs had an appearance similar to that of OMVs budding from US6-1 and had a diameter of 20–70 nm.

Purification of *N. pentaromativorans* US6-1 OMV (OMV_{Novo})

OMV_{Novo} was purified from Bushnell-Haas culture media according to the previously described procedure by using ultrafiltration and centrifugation (Choi *et al.*, 2014). The amount of OMV_{Novo} protein obtained from a 1-l Bushnell-Haas broth culture was approximately 1.02 ± 0.10 mg. TEM analysis of purified OMV_{Novo} confirmed that OMV_{Novo} was spherical in shape similar to OMVs of other Gram-negative bacteria, and the size of OMV_{Novo} was homogeneous in the diameter range of 25–70 nm (Fig. 1B). TEM analysis showed that OMV_{Novo} was successfully purified and could undergo proteomic analysis.

Proteomic characterization of *N. pentaromativorans* US6-1 OMVs

Purified OMV_{Novo} (approximately 10 μg) was directly applied to SDS-PAGE for proteomic analysis. SDS-PAGE of purified OMV samples before proteomic analysis has two purposes: one is to remove remaining salts and contaminants and the other is to fractionate the protein components of OMV according to molecular size before tryptic digestion. Shotgun proteomics using MS/MS analysis revealed that about 189 protein components of OMV_{Novo} were identified and semi-quantified (Table 1). The identified proteins were categorized according to cellular location and COG (Supplementary data Table S1). Among the identified proteins, the amount of surface proteins (outer membrane proteins and periplasmic proteins) accounted for about 90% of total proteins of OMV_{Novo} (Supplementary data Table S1). In particular, the 23.8 kDa outer membrane-like protein (Accession No. WP_007014739.1) is the most abundant protein, accounting for more than 50% of the total protein present (Fig. 2 and Supplementary data Table 1). Abundance of this protein was also confirmed on the SDS-PAGE of purified OMV_{Novo} (Supplementary data Fig. S1). Based on its abundance, the 23.8 kDa outer membrane-like protein is considered to be a

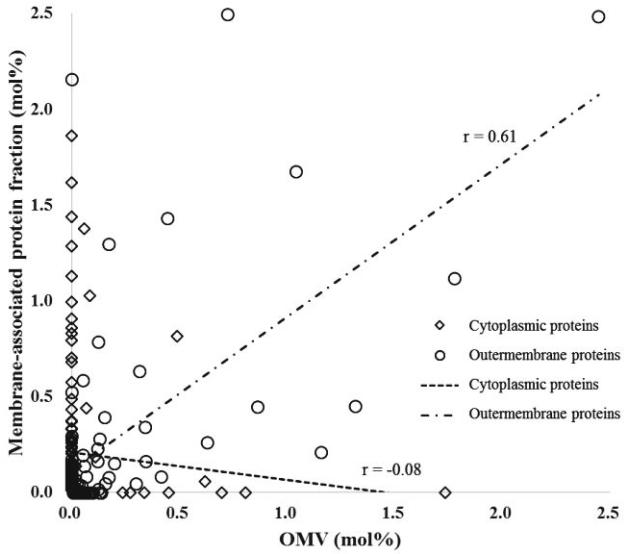

Fig. 3. Correlation scatter plots between OMV and membrane-associated protein fractions. Correlation values of two protein groups (cytoplasmic proteins and outer-membrane proteins) in two fraction were calculated and compared by using Spearman correlation analysis. Spearman correlation analysis is a statistic method measuring rank correlation of induced proteins between two variable conditions. Correlation values of two cytoplasmic protein groups and two outer membrane protein groups are -0.08 and 0.61, respectively. 0.61 means more than half of outer membrane proteins of OMVNovo were closely related with outer membrane proteins of membrane-associated protein fractions.

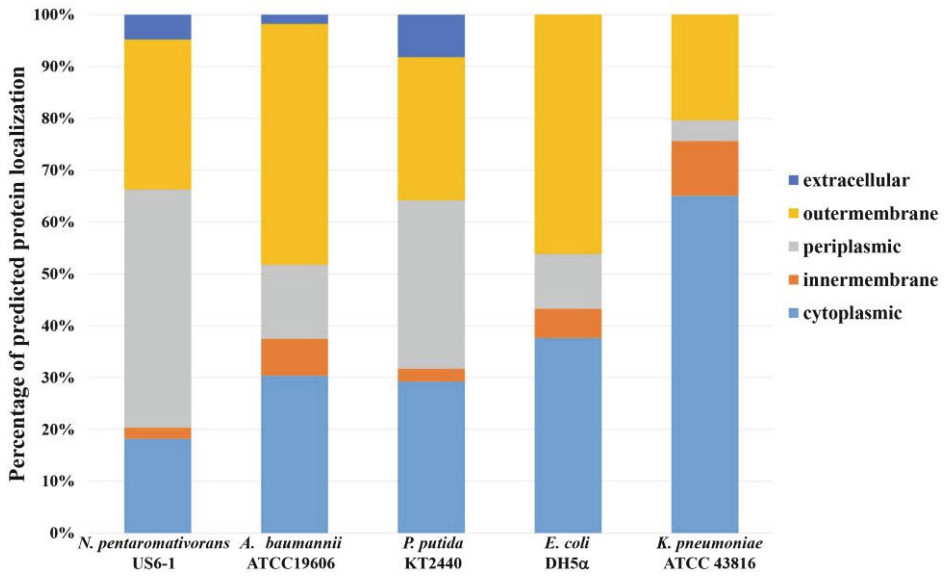

Fig. 4. Comparative analysis of protein components of OMVs prepared from representative Gram-negative bacteria according to cellular distribution. Proteomic data used in this study were extracted from proteomic studies of corresponding bacteria (Lee *et al.*, 2007; Jin *et al.*, 2011; Choi *et al.*, 2014; Cahill *et al.*, 2015).

major OMV_{Novo} structural protein. Another high copy number protein is the β-barrel–assembly machinery (BAM) complex (BamABDE) (Table 2). Identification of this complete protein complex is significant because the BAM complex is responsible for the folding and insertion of nascent β-barrel OMPs (e.g. the 23.8 kDa outer membrane-like protein) in the outer membrane (Han *et al.*, 2016). Peptidyl-prolyl cis-trans isomerase (Accession No. WP_007011314.1) and protease (Accession No. WP_03877625.1) were homologous with SurA and DegP, which have been implicated in guiding OMPs through the periplasm (Krojer *et al.*, 2008; Workman *et al.*, 2012) Additionally, OM channel proteins, OmpA/MotB proteins, and TonB-dependent receptors were identified as major proteins. Abundant OMV_{Novo} proteins are listed in Table 2.

Comparative analysis of *N. pentaromativorans* US6-1 OMVs

The proteomic analysis of the membrane-associated protein fraction of *N. pentaromativorans* US6-1 was also performed, and 282 proteins were identified and semi-quantified (Table 1). In general, OMVs of Gram-negative bacteria budded from the cell wall. To elucidate the relationship between OMV_{Novo} and the membrane-associated protein fraction of *N. pentaromativorans* US6-1, comparative proteomic analysis was performed. The results revealed that each subcellular fraction of the two samples (OMV_{Novo} and the membrane-associated protein fraction) has different protein populations except for the outer membrane fraction (Table 1). To perform a more accurate comparative analysis of the identified proteins, correlation analysis was performed (Fig. 3). This Spearman correlation analysis confirmed that only the outer membrane fractions of the two samples were significantly correlated ($r_S = 0.61$). This result suggests that outer membrane proteins of OMV_{Novo} should originate from the membrane-associated protein fraction. However, inner membrane proteins or cytoplasmic proteins of the membrane-associated protein fraction were not transferred into OMV_{Novo} ($r_S < -0.08$). We also performed comparative proteomic analysis of OMV_{Novo} with other representative OMVs of Gram-negative bacteria that are previously reported (Fig. 4). The low ratio of cytoplasmic proteins of OMV_{Novo} (18.2% of the total protein number; 6.80% of the total protein amount) is a notable result. This result suggests that OMV_{Novo} may not be a carrier of the cytoplasmic protein cargo, one of the major functions of OMV in Gram-negative bacteria. The culture condition used in this study, Bushnell-Haas broth containing *p*-hydroxybenzoate as the sole carbon source, creates a nutrient-restricted condition. Therefore, this minimal culture condition may contribute to the lower ratio of cytoplasmic proteins.

Conclusion

OMV_{Novo} was discovered and its protein components were characterized by proteomic analysis. Comparative proteomics showed that OMV_{Novo} was unique in having a high portion of outer membrane proteins and a low portion of cytoplasmic proteins. In particular, 23.8 kDa outer membrane-like proteins are highly abundant structural proteins in *N. pentaromativorans*. We assume that the differential proteome of OMV_{Novo} reflects the natural environments of *N. pentaromativorans* such as high salts and low nutrients. Based on our results, the function of OMV_{Novo} has not been clearly identified. However, we hypothesize that there is a possibility that the role of OMV_{Novo} may be related to survival or adaptation of the bacterium to its environment. To elucidate the detailed roles of the protein components of OMV_{Novo}, further biochemical and bioinformatic study of *N. pentaromativorans* is needed under different culture or natural conditions.

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