Outer Membrane Vesicles as a Candidate Vaccine against Edwardsiellosis

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Abstract
Infection with *Edwardsiella tarda*, a Gram-negative bacterium, causes high morbidity and mortality in both marine and freshwater fish. Outer membrane vesicles (OMVs) released from Gram-negative bacteria are known to play important roles in bacterial pathogenesis and host immune responses, but no such roles for *E. tarda* OMVs have yet been described. In the present study, we investigated the proteomic composition of OMVs and the immunostimulatory effect of OMVs in a natural host, as well as the efficacy of OMVs when used as a vaccine against *E. tarda* infection. A total of 74 proteins, from diverse subcellular fractions, were identified in OMVs. These included a variety of important virulence factors, such as hemolysins, OmpA, porin, GAPDH, EseB, EseC, EseD, EvpC, EvpP, lipoprotein, flagellin, and fimbrial protein. When OMVs were administrated to olive flounder, significant induction of mRNAs encoding IL-1β, IL-6, TNFα, and IFNγ was observed, compared with the levels seen in fish injected with formalin-killed *E. tarda*. In a vaccine trial, olive flounder given OMVs were more effectively protected (p<0.0001) than were control fish. Investigation of OMVs may be useful not only for understanding the pathogenesis of *E. tarda* but also in development of an effective vaccine against Edwardsiellosis.

Introduction
Outer membrane vesicles (OMVs) are spherical blebs of average diameter 10–300 nm that are naturally released from Gram-negative bacteria into the environment [1]. Although the budding mechanisms are unclear, it has been shown that OMVs are continuously produced during growth of various Gram-negative bacteria including *Escherichia coli*, *Heliocobacter pylori*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, *Shigella flexneri*, and *Vibrio cholerae* [2–7]. Such vesicles are known to contain lipopolysaccharide (LPS), lipoproteins, outer membrane, periplasmic, and cytoplasmic proteins, DNA, and RNA [1,8–10], and have been suggested to be involved in exclusion of competing bacteria, conveyance of proteins or genetic material to other bacteria, and presentation of virulence factors to the host [1].

*Edwardsiella tarda* is the causative agent of Edwardsiellosis in a variety of cultured freshwater and marine fish, including channel catfish *Ictalurus punctatus*, olive flounder *Paralichthys olivaceus*, Japanese eel *Anguilla japonica*, red sea bream *Pogus major*, mullet *Mugil cephalus*, and turbot *Scophthalmus maximus* [11–16]. Edwardsiellosis has been implicated in the mass mortality of olive flounder, which is the main mariculture species of South Korea. Typical clinical symptoms of *E. tarda* infection in olive flounder are exophthalmia, enlargement of the spleen, malodorous ascites, and rectal hernia [17].

A number of studies have shown that vaccination using outer membrane proteins results in development of protective effects against *E. tarda* infection [18–21]. In addition, the outer membrane proteins of *E. tarda* include several important virulence factors that play key roles in pathogenicity [17]. Virulence factors of *E. tarda* that have been investigated include dermatotoxin, hemolysins, catalase, outer membrane proteins, EseDs, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [19,22–24].

Previous proteomic studies indicated that both outer membrane proteins and LPS in OMVs might play roles as pathogen-associated molecular patterns (PAMPs) delivered to the host innate immune system, and could thus elicit immune responses [25,26]. Because OMVs have antigenic properties, such vesicles have been investigated as useful candidate vaccines against Gram-negative bacterial infections [27–29]. For example, a *Neisseria meningitidis* serogroup B vaccine was successfully developed using derived OMVs; 55 million doses have been administered to date [30].

However, none of OMV protein composition, antigenicity, or vaccine efficacy has been studied in bacteria pathogenic for fish, especially the bacterium *E. tarda*. Thus, we investigated the possibility of using OMVs released from *E. tarda* as a vaccine against Edwardsiellosis, based on both a proteomic study and cytokine induction assays.

Results
TEM examination of OMVs
Numerous ovoid-to-round-shaped blebs were evident on the surface of ED45 cells when thin sections were stained to show OMVs (Figure 1-A). The supernatant concentrate contained
OMVs that were round in shape, ranged from 10–40 nm in diameter, and contained electron-dense substances (Figure 1-B). Because cell debris and pili were observed upon negative staining, OMVs suspended in PBS were purified by discontinuous sucrose gradient centrifugation prior to protein analysis and in vivo immunogenicity testing. After centrifugation, two clear white bands were evident in the centrifugation tube, and the materials therein were examined by TEM (Figure 1-C). The upper band appeared to contain cell debris or aggregates, whereas the lower band was mainly OMVs, at a density of 1.185 g/ml. All further experiments were performed using these purified OMVs.

**Protein profiling of OMVs**

Purified OMVs were loaded onto 1D SDS-PAGE gels and proteins were visualized, after electrophoresis, using a silver staining method, and compared with proteins in WCL, PP, and OMP fractions. OMV proteins of molecular size 68, 31.5, 30, 24, 19, 14.5, and 14 kDa (Figure 2) were similar to those in the OMP fraction, but the 17-, 37-, and 54-kDa bands of OMVs were absent from the OMP sample. To identify the protein components of OMVs, the proteins were separated by 12.5% (w/v) SDS-PAGE and the gels were cut into 12 slices. Proteins in individual slices were analyzed by LC-ESI-MS/MS; acquired peptide mass data were analyzed using the MASCOT Daemon interface. A total of 74 proteins were identified in the *E. tarda* database (Table 1), and each slice contained at least 5 of these proteins (data not shown). Proteins were categorized into 15 different orthologous groups using the COG approach (Figure 3), indicating that the identified proteins were involved in both cellular processing and signaling (COG groups M, O, and N; 33% of proteins); information storage and processing (COG groups L, K, and J; 25% of proteins), and metabolism (COG groups I, H, G, F, E, and C; 21% of proteins). In total, 5% of proteins fell into poorly characterized categories (COG groups S and R), whereas 16% could not be identified in any COG grouping. The subcellular locations of the identified proteins were predicted using the PSORTb algorithm; this exercise suggested that 37 proteins were localized in the cytoplasmic space, 6 in the inner membrane, 16 in the outer membrane, and 9 in the extracellular space. The locations of six proteins could not be identified.
Table 1. Proteins of OMVs of *Edwardsiella tarda* identified by LC-ESI-MS/MS.

| Accession number | Description | Mascot score | PI | MW (Da) | Protein matches | Functional category | Subcellular localization |
|------------------|-------------|--------------|----|---------|-----------------|----------------------|--------------------------|
| gi|2244627 | Hemolysin | 288 | 5.99 | 165447 | 34 | R | outer membrane |
| gi|291091829 | Lipoprotein | 131 | 9.67 | 33041 | 10 | M | outer membrane |
| gi|267985457 | long-chain fatty acid transport protein | 429 | 6.45 | 47578 | 15 | I | outer membrane |
| gi|267984860 | murein lipoprotein | 224 | 8.93 | 8384 | 9 | M | outer membrane |
| gi|267985939 | nucleoside-specific channel-forming protein Tsx | 50 | 5.62 | 32786 | 2 | M | outer membrane |
| gi|267984912 | OmpA/MotB domain protein | 44 | 9.62 | 17648 | 19 | M | outer membrane |
| gi|267984281 | outer membrane protein A | 1482 | 7.66 | 38034 | 105 | M | outer membrane |
| gi|253720368 | outer membrane protein A precursor | 1447 | 7.66 | 38048 | 104 | M | outer membrane |
| gi|291089413 | peptidoglycan-associated lipoprotein | 52 | 5.93 | 18793 | 10 | M | outer membrane |
| gi|267985578 | peptidoglycan-associated outer membrane lipoprotein | 89 | 5.92 | 18770 | 7 | M | outer membrane |
| gi|267989327 | putative hemolysin precursor | 288 | 5.9 | 167296 | 29 | R | outer membrane |
| gi|267983035 | putative outer membrane lipoprotein | 38 | 10.01 | 22250 | 2 | M | outer membrane |
| gi|267984253 | putative outer membrane porin F protein | 303 | 5.03 | 40058 | 11 | M | outer membrane |
| gi|73532672 | putative virulence-related membrane protein | 95 | 9.4 | 19858 | 5 | - | outer membrane |
| gi|26795681 | virulence-related outer membrane protein 910 | 8.89 | 20476 | 34 | - | outer membrane |
| gi|291091795 | lysophospholipid transporter LplT | 36 | 9.08 | 34123 | 10 | R | cytoplasmic membrane |
| gi|291089401 | protein YdcF | 36 | 5.28 | 28546 | 4 | - | cytoplasmic membrane |
| gi|291089679 | putative inner membrane protein | 65 | 5.19 | 19700 | 2 | S | cytoplasmic membrane |
| gi|291090200 | transcriptional regulator, LysR family | 33 | 6.46 | 35207 | 4 | - | cytoplasmic membrane |
| gi|291089298 | twin arginine regulator-targeting protein translocase TatA | 42 | 9.3 | 9330 | 21 | K | cytoplasmic membrane |
| gi|267985130 | ABC transporter-related protein | 32 | 9.43 | 28704 | 33 | N | cytoplasmic membrane |
| gi|26796212 | 30S ribosomal protein S13 | 146 | 10.58 | 13283 | 6 | J | cytoplasmic |
| gi|267985854 | 30S ribosomal protein S16 | 63 | 8.36 | 6755 | 4 | J | cytoplasmic |
| gi|267983753 | 30S ribosomal protein S2 | 37 | 6.61 | 26281 | 10 | J | cytoplasmic |
| gi|26796226 | 30S ribosomal protein S3 | 44 | 10.27 | 25980 | 9 | J | cytoplasmic |
| gi|26798384 | 30S ribosomal protein S6 | 51 | 5.29 | 15270 | 12 | J | cytoplasmic |
| gi|26796235 | 30S ribosomal protein S7 | 120 | 10.3 | 17609 | 12 | J | cytoplasmic |
| gi|267983534 | 30S ribosomal protein S9 | 96 | 10.94 | 14834 | 6 | J | cytoplasmic |
| gi|267983533 | 50S ribosomal protein L13 | 33 | 9.91 | 16049 | 15 | J | cytoplasmic |
| gi|291091309 | 6,7-dimethyl-8-ribityllumazine synthase | 76 | 5.32 | 16195 | 5 | H | cytoplasmic |
| gi|267983456 | altronate hydrolase | 31 | 5.78 | 54172 | 22 | G | cytoplasmic |
| gi|291091008 | asparagine-tRNA ligase | 39 | 5.16 | 52655 | 7 | J | cytoplasmic |
| gi|267984254 | asparaginyl-tRNA synthetase | 39 | 5.1 | 52605 | 7 | J | cytoplasmic |
| gi|291092134 | aspartate ammonia-lyase | 50 | 5.32 | 53103 | 7 | E | cytoplasmic |
| gi|291092137 | chaperonin GroL | 836 | 4.84 | 57456 | 65 | O | cytoplasmic |
| gi|267985752 | cobyrinic acid ac-diamide synthase | 32 | 5.12 | 30638 | 6 | H | cytoplasmic |
| gi|267983856 | conserved hypothetical protein | 36 | 10.34 | 13945 | 2 | - | cytoplasmic |
| gi|22121758 | Cpn60 | 54 | 4.46 | 19369 | 6 | O | cytoplasmic |
| gi|267986210 | DNA-directed RNA polymerase subunit alpha | 68 | 5.03 | 36725 | 12 | K | cytoplasmic |
| gi|55981975 | EseD | 166 | 5.34 | 21101 | 6 | - | cytoplasmic |
**Table 1.** Cont.

| Accession number | Description                                                                 | Mascot scorePI | MW (Da) | Protein matches | Functional category | Subcellular localization |
|------------------|------------------------------------------------------------------------------|----------------|---------|----------------|----------------------|--------------------------|
| gi|291089872 | formate acetyltransferase                                                       | 35 | 5.71 | 85456 | 13 | C | cytoplasmic |
| gi|267985188 | formate acetyltransferase 1                                                    | 35 | 5.65 | 85471 | 13 | C | cytoplasmic |
| gi|222457929 | glyceraldehyde-3-phosphate dehydrogenase                                      | 146 | 6.6 | 35684 | 11 | G | cytoplasmic |
| gi|224382175 | heat shock protein 60                                                          | 54 | 4.41 | 17870 | 5 | O | cytoplasmic |
| gi|267985045 | hypothetical protein ETAE_2037                                                  | 104 | 9.3 | 20384 | 10 | - | cytoplasmic |
| gi|291092198 | lysine decarboxylase                                                           | 70 | 5.63 | 81254 | 15 | E | cytoplasmic |
| gi|267983773 | lysine decarboxylase 1                                                         | 327 | 5.53 | 81313 | 33 | E | cytoplasmic |
| gi|291091450 | lysine decarboxylase, inducible                                                | 235 | 5.53 | 81368 | 34 | E | cytoplasmic |
| gi|291089681 | O-succinylbenzoate-CoA ligase                                                  | 39 | 8.62 | 50218 | 7 | IQ | cytoplasmic |
| gi|267985017 | polypeptide-transport-associated domain protein                                | 396 | 6.17 | 27889 | 24 | - | cytoplasmic |
| gi|267983427 | polynucleotide-associated nucleotidyldtransferase                             | 130 | 5.2 | 76425 | 7 | J | cytoplasmic |
| gi|267985748 | putative integrase                                                             | 32 | 9.73 | 44853 | 8 | L | cytoplasmic |
| gi|267983991 | riboflavín synthase beta-chain                                                 | 76 | 5.65 | 16208 | 8 | H | cytoplasmic |
| gi|267984414 | ribose-phosphate pyrophosphokinase                                             | 143 | 5.25 | 34557 | 9 | FE | cytoplasmic |
| gi|291088840 | ribosomal protein 519                                                          | 42 | 10.42 | 10380 | 6 | J | cytoplasmic |
| gi|291091495 | ribosomal protein 52                                                           | 37 | 6.33 | 26830 | 13 | J | cytoplasmic |
| gi|291089791 | site-specific recombinase, phage integrase family                              | 32 | 9.7 | 44682 | 9 | L | cytoplasmic |
| gi|73532652 | type III secretion system effector protein D                                   | 53 | 5.11 | 13695 | 4 | N | cytoplasmic |
| gi|74474901 | antigenic protein Et 46                                                        | 55 | 5.2 | 43774 | 19 | H | extracellular |
| gi|256599595 | Chain A, Structure Of A Type Six Secretion System Protein                      | 290 | - | 18043 | 16 | - | extracellular |
| gi|61743025 | EseC                                                                          | 597 | 6.15 | 53717 | 42 | O | extracellular |
| gi|40287638 | EvpC                                                                          | 308 | 5.71 | 18143 | 19 | K | extracellular |
| gi|38016008 | fimbrial protein                                                               | 281 | 9.14 | 38109 | 12 | - | extracellular |
| gi|117307392 | flagella (i) hook associated protein HAP2                                       | 95 | 6.62 | 50267 | 33 | C | extracellular |
| gi|24306148 | Flagellin                                                                     | 55 | 4.88 | 43722 | 18 | C | extracellular |
| gi|27808146 | major fimbrial subunit protein                                                  | 2000 | 7.71 | 18473 | 78 | G | extracellular |
| gi|267983886 | type III secretion system effector protein C                                   | 597 | 6.08 | 50911 | 37 | O | extracellular |
| gi|61743023 | EseB                                                                          | 287 | 5.51 | 21777 | 12 | - | unknown |
| gi|237861343 | EvpP                                                                          | 165 | 9.38 | 20991 | 11 | E | unknown |
| gi|291092160 | N-acetylmuramoyl-L-alanine amidase                                             | 47 | 10.81 | 60479 | 15 | E | unknown |
| gi|267985878 | outer membrane lipoprotein                                                     | 217 | 9.56 | 33180 | 11 | E | unknown |
| gi|291088780 | pseudouridine synthase, RluA family                                            | 35 | 9.11 | 7339 | 4 | IQ | unknown |
| gi|267983367 | putative N-acetylmuramoyl-L-alanine amidase                                    | 47 | 10.56 | 59093 | 12 | - | unknown |

*Theoretical protein charge.
 bTheoretical protein molecular mass.
 cFunctional categorization based on COGs. The abbreviations are shown in Figure 3. '-' indicates 'Not in COGs'.
 dSubcellular localization was predicted using the PSORTb v3.0 algorithm.

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**Immune responses in olive flounder**

Based on the proteomic data, QRT-PCR was conducted to measure mRNA expression levels of IL-1β, IL-6, TNFα, and IFNγ in olive flounder injected with FKC or OMVs, to investigate whether OMVs could elicit host immune responses. After intraperitoneal injection of OMVs or FKC, kidneys of injected fish were sampled at 0, 3, 7, and 12 hpi; and 1 and 5 dpi (Figure 4). Fish injected with OMVs showed increased cytokine levels compared with those measured at 0 hours. IL-1β and IL-6 expression levels were induced 320- and 515-fold at 5 hpi, and these levels were maintained to 5 dpi. TNFα and IFNγ synthesis levels increased 4.8- and 7.6-fold at 3 hpi,
compared with zero time measurements. Fish injected with FKC showed differences in IL-1β and IL-6 expression levels (compared with controls) at early time points, but neither the TNFα nor IFNγ synthesis level was distinct from that of 0-hour control values. When expression levels of IL-1β, IL-6, TNFα, and IFNγ in fish injected with OMVs were compared with those in fish injected with FKC, the cytokine levels of OMV-injected fish were significantly higher, at early time points, than in fish injected with FKC. The level of IL-1β expression in fish injected with OMVs differed significantly from that in fish injected with FKC, at 3, 7, and 12 hpi, whereas IL-6 levels were significantly different at 3 and 7 hpi. TNFα and IFNγ expression levels in fish injected with OMVs differed, at 3 hpi, from those in fish injected with FKC.

Figure 3. Functional classification of OMVs according to COG functional categories. The pie chart shows the numbers and percentages of identified proteins in each COG grouping. Individual protein assignment to COGs is shown in Table 1.
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Figure 4. Relative induction of IL-1β (A), IL-6 (B), TNFα (C), and IFNγ (D) in olive flounder injected with OMVs or FKC, estimated using quantitative real-time PCR. OMV: group injected with outer membrane vesicles; FKC: group injected with formalin-killed ED45. *P<0.05, **P<0.01, and ***P<0.001. Bars indicate standard deviations. N=4.
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mRNA TLR22 (toll-like receptor 22) and TLR2 expression levels, which are pattern recognition receptors (PRRs) in olive flounder, were measured to determine whether the innate immune response could be elicited by OMVs [31]. TLR22 levels in fish injected with OMVs were significantly higher than in fish injected with FKC. However, TLR2 levels did not differ (Figure 5).

Protective effects of OMV injection into olive flounder

OMVs were administered to olive flounder as a candidate vaccine against *E. tarda* infection; control fish were injected with PBS (Figure 6). Fish injected with FKC were used as positive controls. After acclimatization of fish for 28 days, 1.1 × 10^5 CFU/ml ED45 was used as an intraperitoneal challenge, and survival rates were recorded over 31 days. Control fish died from 6 dpi and all fish were dead at 11 dpi. OMV-vaccinated fish showed some mortality at 5 dpi; however, 70% of fish survived until 31 dpi. FKC-injected fish had a survival rate of 65%. OMV showed an RPS of 70%; this differed significantly from that of control (PBS) (p < 0.0001).

Discussion

Several Gram-negative bacteria produce OMVs [1]. In the present study, *E. tarda* was also shown to produce round OMVs, 10–40 nm in diameter, when grown in a liquid medium. Preparation of pure OMVs from bacterial supernatants is essential when studying the roles played by OMVs in both bacteria and their hosts [9,10]. Recently, pure OMVs from bacterial supernatants have been obtained by filtration followed by density gradient ultracentrifugation [8,25]. In the present study, homogenously sized *E. tarda* OMVs were separated from contaminants using such methods. Flagella, pili, and aggregated proteins were present in OMV samples before sucrose density gradient purification, but were removed during this step.

Two representative proteomic analysis methods have been suggested for the study of OMVs. Of these, the first is two-dimensional electrophoresis (2-DE) followed by matrix-associated laser desorption/ionization time-of-flight (MALDI-TOF) spectrometry, and the other is 1D SDS-PAGE followed by LC-ESI-MS/MS [10,25,26]. 2-DE is most commonly used to establish proteomic maps of bacteria, but limitations of the technique include poor separation of high-molecular weight and hydrophobic proteins [32]. However, 1D SDS-PAGE in combination with LC-ESI-MS/MS was effective in analysis of hydrophobic outer membrane proteins contained in OMVs [6]. In the present work, a total of 74 proteins were identified, 16 of which were characteristic in the outer membrane, as indicated by the PSORTb algorithm. However, a proteomic survey of the *E. tarda*...
outer membrane identified only 21 proteins by 2-DE analysis, and only 1 protein was confined to this membrane [33]. Therefore, 1D SDS-PAGE coupled with LC-ESI-MS/MS is more sensitive when used to analyze proteins of outer membranes or OMVs.

Some reports have suggested that OMV proteomes consist mainly of outer membrane and periplasmic proteins [34], but other proteomic studies found that OMVs contained proteins of various origin, including all of cytoplasmic, inner membrane, outer membrane, and periplasmic proteins [9,25,35]. By 1D SDS-PAGE, a 17-kDa band evident in OMVs could not be observed in outer membrane or periplasmic protein fractions. The PSORTb algorithm also indicated that proteins of OMVs originated not only from the outer membrane but also from cytoplasmic and extracellular compartments. These findings, together with those of previous proteomic studies, indicate that the OMV proteome includes proteins varying in subcellular origin, and other components.

LC-ESI-MS/MS analysis of OMVs showed that several proteins involved in pathogenesis, including hemolysin, outer membrane protein A (OmpA), GAPDH, EseB, EseC, EseD, ExpC, ExpP, lipoprotein, flagellin, and fimbral protein, were present. Hemolysin is known to be required for cellular invasion and cytotoxicity [22]; hemolysin-negative E. tarda mutants could not invade human epithelial cell lines [36]. An ompA-negative E. coli strain invaded brain microvascular endothelial cells only with difficulty [37,38]. GAPDH in the outer membrane is highly antigenic, and is considered to be a strong vaccine candidate to counter not only Gram-negative but also Gram-positive bacterial infections [19,39]. EseB, EseC, EseD, ExpC, and ExpP are essential for E. tarda pathogenesis; these proteins are located on the cell surface and are responsible for pore formation [21,40,41]. Thus, OMVs released from E. tarda contain numerous virulence factors that may be important both for bacterial survival and to enhance immunogenicity in the natural host.

QRT-PCR demonstrated that the genes encoding all of IL-1β, IL-6, TNFα, and IFNγ were induced in fish injected with OMVs of E. tarda, compared with fish injected with FKC, especially at early post-injection time points. Similarly, mice injected with OMVs of Bordetella pertussis showed upregulation of mRNAs encoding IL-6 and TNFα, compared with the levels seen in animals injected with formalin-killed B. pertussis [27]. It is known that the primary host immune response is mediated by the proinflammatory cytokines IL-1β, IL-6, and TNFα [42]. Expression of such cytokines following injection of E. tarda OMVs indicates that the OMVs may initiate a proinflammatory cytokine cascade, including both recruitment and activation of macrophages and stimulation of an adaptive immune response. IFNγ, a Th1 cytokine, is also upregulated in fish injected with OMVs, and is known to enhance cell-mediated immunity and antigen presentation to macrophages [42]. Such observations may indicate that OMVs trigger an elevated host immune response, thus provoking host adaptive immunity, even though protein levels in OMVs are lower than those in FKC.

Innate immunity is an important first line of defense against invading pathogens, and recognition of PAMPs is achieved principally by PRRs [43]. In the present study, virulence factors, including lipoprotein, flagellin, and peptidoglycan, contained within OMVs of E. tarda, may have played roles as PAMPs, thus interacting with the innate immune system. In addition, OMVs are known to possess LPS and bacterial DNA that function as ligands for host PRRs [44,45]. OMVs have been reported to be recognized by TLR2 and TLR9, both of which participate in IL-6 production via myeloid differentiation factor 88 (MyD88)-dependent pathways [45]. In the present study, TLR2 was not induced after injection with OMVs (compared with what was seen in positive control fish), whereas, in contrast, the TLR22 level was significantly increased. Therefore, the PRRs of olive flounder also participate in recognition of OMVs but the teleost detection mechanism for OMVs may differ from that of mammals.

The proteomic data, and the demonstrated immunostimulatory effects of OMVs from E. tarda, showed that OMVs exhibited high protective efficacy against E. tarda infection of olive flounder, with an RPS of 70% (p<0.0001). Other reports have described successful inhibition of E. tarda infection in laboratory experiments using various antigen preparations including formalin-killed and ghost cells, recombinant proteins, and outer membrane fractions [18–21]. However, these advances have not been translated into successful commercial vaccines. Although OMVs were no more effective as a vaccine than was FKC, vaccines of the latter type sometimes function poorly [46]. The OMV work of the present study offers a new insight into vaccine formulation. Thus, an acellular vaccine, or the use of an acellular materials as a vaccine adjuvant, may be helpful in development of an effective vaccine protecting against Edwardsiella in fish.

In conclusion, OMVs naturally released from E. tarda contained various important virulence factors originating from diverse subcellular locations. Such vesicles were able to induce synthesis of several proinflammatory cytokines, and may stimulate the host innate immune system, thus serving as PAMPs. To demonstrate vaccine efficacy, we injected olive flounder with OMVs, and found that such fish were protected to a significantly higher extent than were control fish. The present study on OMVs may lead to development of more effective vaccines, and may enhance our understanding of the roles of OMVs in hosts.

Materials and Methods

Bacterial strain and growth conditions

ED45, a virulent E. tarda strain isolated from the spleen of infected olive flounder, was used in the present study; the LD50 value was 1.2×10⁷ CFU/ml (data not shown). ED45 was cultured on Tryptone Soya Agar (TSA; Oxoid, Hampshire, England) or in Tryptone Soya Broth (TSB; Oxoid), supplemented with 2% (v/v) NaCl (termed the TSA-2 and TSB-2 media, respectively). ED45 was grown on TSA-2 at 25°C for 36 h; a single colony was inoculated into 25 ml TSB-2, and the culture grown with shaking to a cell density of 1×10⁸ CFU/ml. Next, 20 ml of this culture was added to 4 l TSB-2 and growth proceeded in a shaking incubator at 25°C for 15 h. After centrifugation at 5,000 xg for 20 min, the supernatant was used for isolation of OMVs.

OMV preparation

OMVs were collected and purified from the supernatant as described previously, with several modifications [25,47]. The supernatant containing OMVs were filtered through a 0.45 μm pore-sized hollow fiber cartridge and concentrated using a hollow fiber membrane module system (GE Healthcare, Uppsala, Sweden). The concentrated suspension was added to 4 l TSB-2 and growth proceeded in a shaking incubator at 25°C for 1 h. After centrifugation at 5,000 xg for 20 min, the supernatant was used for isolation of OMVs.

Pelleted OMVs were suspended in phosphate-buffered saline (PBS; pH 7.2), layered onto a 30–60% (w/v) discontinuous sucrose gradient, and centrifuged at 200,000 xg for 20 h at 4°C in an XL-90 ultracentrifuge running a SW41 Ti rotor (Beckman Coulter, Palo Alto, CA).

Pelleted OMVs were resuspended in phosphate-buffered saline (PBS; pH 7.2), layered onto a 30–60% (w/v) discontinuous sucrose gradient, and centrifuged at 200,000 xg for 20 h at 4°C in an XL-90 ultracentrifuge running an SW41 Ti rotor, to obtain purified OMVs. Two clear bands were visualized, and the sucrose density of each band was measured by refractometry (Figure 1).
After washing material from both bands (collected separately) with a 20-fold dilution of PBS, the materials were centrifuged at 150,000×g for 3 h at 4°C in a XL-90 ultracentrifuge. The final pellets were resuspended in PBS and protein concentrations were determined using a non-interfering assay (G-Biosciences, St. Louis, MO). The final preparations were stored at −80°C prior to use.

**Transmission electron microscopy (TEM)**

ED45 cells were grown in TSB-2 at 25°C until an OD600 value of 1.0 was attained, centrifuged at 5,000×g for 30 min, and washed three times with PBS prior to preparation of ultrathin sections. After centrifugation at 5,000×g for 20 min, pellets were pre-fixed in 2% (v/v) paraformaldehyde for 3 h, washed, and fixed in 4% (w/v) osmium tetroxide for 2 h. After washing with PBS, the fixed pellets were dehydrated in a series of 70–100% (v/v) ethanol baths and embedded in epoxy resin. Sections were cut using a diamond blade (Diatome, Bieł, Switzerland) fitted to an ULTRACUT UCT (Leica, Vienna, Austria), mounted on carbon-coated copper grids, and stained with 3% (w/v) uranyl acetate and lead citrate. To visualize OMVs after negative staining, samples were placed on 400- mesh carbon-coated grids for 2 min, washed with deionized sterile water (dd H2O), and negatively stained with 3% (w/v) uranyl acetate for 30 sec. All TEM images were acquired using a Tecnai 12 (FEI, Hillsboro, OR) operating at an acceleration voltage of 120 kV.

**Preparation of whole cell lysates, periplasmic proteins, and outer membrane proteins**

Periplasmic proteins (PPs) and outer membrane proteins (OMPs) were purified as described previously [48]. Whole cell lysates (WCLs) of ED45 were obtained from cells grown in TSB-2 at 25°C, pelleted at 5,000×g for 30 min, and washed with PBS. Pelleted cell density was adjusted to 4 g/ml in 20% (w/v) sucrose and lysozyme (600 U/g cells). After incubation on ice for 40 min, 0.5 M MgCl2 (0.16 ml/g cells) was added and spheroplasts were removed by centrifugation (9,500×g for 20 min). The supernatant containing PP was stored at −80°C until use. Spheroplasts were resuspended in ice-cold 10 mM Tris-HCl (pH 8.0) and sonicated for purification of OMPs. Following centrifugation at 8,000×g for 5 min to remove cells, the supernatant was centrifuged at 40,000×g for 1 h to pellet cell membrane material. Pellets were washed in 10 mM Tris-HCl (pH 8.0), resuspended in dd H2O, and freeze-thawed. The membranes were incubated in 0.5% (w/v) Sarkosyl (sodium N-lauroylsarcosinate; Sigma, St. Louis, MO) at 25°C for 20 min and OMP pellets were purified by centrifugation at 40,000×g for 1 h. Purified OMPs were resuspended in 10 mM Tris-HCl (pH 8.0) and stored at −80°C until use. Protein concentration was determined using a non-interfering protein assay (G-Biosciences, Hercules, CA).

**Electrophoresis and in-gel digestion**

SDS-PAGE was performed using a 13% (w/v) acrylamide separating gel, according to the method of Laemmli [49]. Briefly, each resuspended WCL, PP, OMP, and OMV protein sample was mixed with 5×sample buffer (5:1 v/v ratio of buffer to sample). The buffer contained 60 mM Tris-HCl, 25% (v/v) glycerol, 2% (w/v) SDS, 14.4 mM β-mercaptoethanol, and 0.1% (w/v) bromphenol blue. Samples were boiled for 10 min, cooled on ice, and centrifuged at 16,000×g for 20 min. Supernatants were subjected to SDS-PAGE analysis. Five microgram amounts of WCL, PP, OMP, and OMV proteins were loaded and proteins were detected by silver staining [50]. To achieve in-gel digestion, 20 μg amounts of OMVs were electrophoresed on a 12.5% (w/v) separating gel and stained with Bio-safe Coomassie G-250 (Bio-Rad, Hercules, CA). After cutting the gel into 12 slices, each slice was desiccated with a 500 μl amount of 40% (v/v) ethanol in 75 mM ammonium bicarbonate (ABC) and treated with DTT (0.0039 g dithiothreitol in 5 ml 25 mM ABC) and IAA (0.0509 g iodoacetamide in 5 ml 25mM ABC) solutions. Each gel slice was dehydrated using 300 μl acetonitrile (ACN) for 30 min at 37°C, dried, and used for in-gel digestion employing 20 ng/ml of sequencing-grade modified trypsin (Promega, Madison, WI) at 37°C overnight. Tryptic peptides were extracted into 30 μl 0.1% (v/v) formic acid and the solutions were sonicated for 10 min.

**LC-ESI-MS/MS and data analysis**

LC-ESI-MS/MS (liquid chromatography electrospray ionization tandem mass spectrometry) analysis was carried out using a Thermo Finnigan Proteome X workstation with an LTQ linear ion trap and a MS apparatus equipped with NSI sources (Thermo Electron, San Jose, CA). Twelve micro liter amounts of peptide mixtures were injected and loaded onto peptide trap cartridges (Agilent, Palo Alto, CA). Trapped peptides were eluted onto a 10 cm-long reverse-phase PicoFrit column packed in-house with 5 μm C18 resin of pore size 300 Å, and the peptides were next separated on an RP column using gradient elution. The mobile phase solutions were H2O and ACN, both containing 0.1% (v/v) formic acid, and delivered at a constant flow rate of 0.2 μl/min. The gradient commenced with 2.0% (v/v) ACN, rising linearly to 60% (v/v) ACN over 50 min, next increasing to 80% (v/v) ACN over the next 5 min, with a change to 100% H2O for the final 15 min. A data-dependent acquisition mode (m/z 300–1,800) was enabled, and each survey MS scan was followed by five MS/MS scans with the 30 sec dynamic exclusion option set. The spray voltage was 1.9 kV and the ion transfer tube temperature 195°C. The normalized collision energy was set to 35%.

The mzData file of tandem mass spectra was used to search the E. tarda database of NCBI downloaded on 9 July 2010, using the Mascot Deamcon interface (Version 2.2.2, Matrix Science Inc., London, UK) supported by the Korea Basic Science Institute (KBSI; Yusung-gu, Daejeon, Korea). The peptide tolerance of the parent ion was adjusted to be 1 Da and the MS/MS tolerance was 0.8 Da. During analysis, carbamidomethyl (C) modification was fixed whereas oxidation modification (M) was not. One missed cleavage was permitted, and peptide charges were set at 2+ and 3+. Individual ion scores of more than 15 (p<0.05) were considered reliable and were subsequently used.

Functional annotations of OMV proteins were performed using the “clusters of orthologous groups” (COGs) functional classification (http://www.ncbi.nih.gov/COG) [51]. The subcellular localization of identified proteins was predicted using PSORTb version 3.0 (http://www.psort.org) [52].

**Fish, and immunization with OMVs or FKC**

Naïve olive flounders (with an approximate weight of 55 g) were purchased from a commercial fish farm in Korea and acclimatized for 2 weeks at 21°C in aerated seawater. Formalin-killed E. tarda cells (FKC) were prepared by addition of 1% (v/v) formalin to an ED45 culture, followed by adjustment to an OD600 value of 1.0 using PBS. OMVs were prepared as described above and used after confirmation that no colonies formed when OMVs were plated onto TSA-2. Aliquots (0.1 ml) of FKC (1.2×106 CFU/ml, 344 μg), or 10 μg amounts of OMVs, were intraperitoneally injected into olive flounder. The administered dosage was determined based on previous studies on immunization with
OMVs originating from several gram negative bacteria [25,27,33,34]. To evaluate expression of immune response-related molecules in fish, the kidneys of immunized olive flounder were dissected 0, 3, 7, and 12 hours post-infection (hpi), and 1.5 days post-infection (dpi), and stored in “RNA-later” solution (Ambion, Austin, TX) until all samples were available (N = 4).

All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Gyeongsang National University, Jinju, Republic of Korea (Approval Number: GNU-LA-17).

Quantitative real-time PCR

Total RNA was isolated from kidney samples using the TRIzol reagent (Invitrogen, Scotland, UK) according to the manufacturer’s protocol. After purification of 1 μg aliquots of mRNA using deoxyribonuclease I (Invitrogen), cDNAs were synthesized with the assistance of a high-capacity cDNA reverse transcriptase kit (Applied Biosystems, Foster city, CA) according to the manufacturer’s protocol. cDNA samples were diluted 1:4 (v/v) into nuclease-free water prior to amplification using quantitative real-time PCR (QRT-PCR). Primers were designed employing the Primer Express software of Real-Time PCR version 3.0 (Applied Biosystems). Primer names, sequences, and GenBank accession numbers are listed in Table 2. QRT-PCR was achieved using StepOne-Plus Real-Time PCR (Applied Biosystems) with a Fast-Start Universal SYBR Green Master Mix (Roche, Indianapolis, IN), according to the manufacturers’ instructions. The amplification steps consisted of initiation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 sec each followed by annealing at 60°C for 1 min, with next a melting curve analysis step featuring 1 cycle at 95°C for 15 sec, a hold at 60°C for 1 min, and a further hold at 95°C for 15 sec, to confirm that only a single amplicon was present. All reactions were performed in duplicate using olive flounder β-actin mRNA as an internal control. The relative standard curve method was applied to calculate standard errors of the mean (SEM). In statistical analysis, Student’s t-test was used to determine differences between tests using FKCC and OMV; p<0.05 was viewed as significant.

Fish vaccination and challenge

Before vaccination, 10 naïve olive flounders in each group were intraperitoneally injected with ED45 (1.2×10^9 CFU/ml) and mortality was recorded over the next 28 days. A group of fish injected with PBS served as controls. After acclimatization for 28 days, fish were challenged with 100 μl amounts of ED45 (1.1×10^9 CFU/ml) by intraperitoneal injection, and mortality was recorded over the following 31 days. Vaccine efficacy was calculated as relative percentage survival (RPS = [1 minus vaccine group mortality/control group mortality]×100) [55]. In statistical analysis, Fisher’s exact test was applied to compare survival differences between vaccinated and control groups at the p<0.0001 level.

Table 2. Oligonucleotide sequences used in the present study.

| Gene   | Accession number | Primer name | Sequences (5'→3') |
|--------|------------------|-------------|-------------------|
| β-actin | AU050773         | WCUP090-F   | CTGCCCTCACCTCCAAGAG | |
|         | WCUP091-F        | CTCAGTGTCACCGAGTTG    | |
| IL-1β   | AB070835         | WCUP279-F   | ATGGAATCCCCAGAAGATG    | |
| IL-6    | DQ884914         | WCUP281-F   | CCGAACACCTACATGGTCT    | |
| TNFα    | AB040448         | WCUP282-F   | GTCCGCGGGTTTTCTGTTA    | |
| IFNγ    | AB435093         | WCUP284-F   | TCAGAAGGATGAAAAAACA    | |
| TLR2    | AB109394         | WCUP784-F   | CTTGCTGTGTTTGAGCACA    | |
| TLR4    | WCUP785-F        | GGAGCAGCCGCTTCCACA    | |
|         | WCUP780-R        | TTTTTCCACTGCTTCA    | |

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Author Contributions

Conceived and designed the experiments: SBP JH TA TSJ. Performed the experiments: SBP SWN ISC. Wrote the paper: SBP TA TSJ.

References

1. Kuehn MJ, Kesty NC (2005) Bacterial outer membrane vesicles and the host-pathogen interaction. Genes Dev 19: 2645–2655.
2. Beveridge TJ (1999) Structures of gram-negative cell walls and their derived membrane vesicles. J Bacteriol 181: 4723–4733.
3. Devos JW, Gächter JE (1975) Release of endotoxin in the form of cell wall blebs during in vitro growth of Neisseria meningitidis. J Exp Med 158: 1156–1167.
4. Hoekstra D, van der Laan JW, de Leij L, Witholt B (1976) Release of outer membrane vesicles from normally growing Escherichia coli. Biochim Biophys Acta 455: 859–899.
5. Fiocca R, Neechi V, Sommi P, Ricci V, Telford J, et al. (1999) Release of Helicobacter pylori vacuolating cytotoxin by both a specific secretion pathway and budding of outer membrane vesicles. uptake of released toxin and vesicles by gastric epithelium. J Pathol 188: 220–226.
6. Kadupuluvuma JI, Beveridge TJ (1999) Membrane vesicles derived from Pseudomonas aeruginosa and Shigella flexneri can be integrated into the surfaces of other gram-negative bacteria. Microbiology 145: 2051–2068.
7. Kondo K, Takade A, Amako K (1993) Release of the outer membrane vesicles from Vibrio cholerae and Vibrio parahaemolyticus. Microbiol Immunol 37: 149–152.
8. Nevo M, Derouen C, Mesner P, Guinea J, Mercade E (2006) Characterization of outer membrane vesicles released by the psychrotolerant bacterium Pseudoalteromonas antarctica NF3. Environ Microbiol 8: 1525–1533.

9. Wai SN, Lindmark B, Soderblom T, Takade A, Westermark M, et al. (2003) Vesicle-mediated export and assembly of pore-forming oligomers of the enterobacterial ChYa cytotoxin. Cell 115: 25–35.
10. Bauman SJ, Kuehn MJ (2006) Purification of outer membrane vesicles from Pseudomonas aeruginosa and their activation of an IL-8 response. Microb Infect 8: 2400–2408.
11. Meyer FP, Bullock GL (1975) Edwardsiella tarda, a new pathogen of channel catfish (Ictalurus punctatus). Appl Microbiol 25: 155–156.
12. Egusa S (1976) Some bacterial diseases of freshwater fishes in Japan. Fish Pathol 10: 103–114.
13. Nakatsugawa T (1983) Edwardsiella tarda isolated from cultured young flounder. Fish Pathol 18: 99–101.
14. Kusuda R, Toyoshima T, Iwamura Y, Sako H (1976) Edwardsiella tarda from cultured young turbot (Scophthalmus maximus) reared in a sea farm in the bay of biscoy. Bull Jap Soc Sci Fish 42: 271–275.
15. Nougayrede PH, Vuillaume A, Vigneulle M, Faivre B, Luengo S, et al. (1994) First isolation of Edwardsiella tarda from cultured tank (Bacillus pumilus). Appl Environ Microbiol 60: 149–152.
16. Herman RL, Bullock GL (1986) Pathology caused by the bacterium Edwardsiella tarda in striped bass. Trans Am Fish Soc 115: 232–235.
17. Mohanty BR, Sahoo PK (2007) Edwardsiellosis in fish: A brief review. J Biosa 32: 1331–1344.
35. Galka F, Wai SN, Kusch H, Engelmann S, Hecker M, et al. (2008) Proteomic
33. Kumar G, Sharma P, Rathore G, Bisht D, Sengupta U (2010) Proteomic
32. Wu CC, Yates JR (2003) The application of mass spectrometry to membrane
31. Hirono I, Takami M, Miyata M, Miyazaki T, Han HJ, et al. (2004)
30. Holst J, Martin D, Arnold R, Huergo CC, Oster P, et al. (2009) Properties and
29. van den Dobbelsteen GPJM, van Dijken HH, Pillai S, van Alphen L (2007)
28. Schild S, Nelson EJ, Bishop AL, Camilli A (2009) Characterization of Vibrio
27. Roberts R, Moreno G, Bottero D, Gaillard ME, Fingermann M, et al. (2008)
26. Post DM, Zhang D, Eastvold JS, Teghanemt A, Gibson BW, et al. (2005)
25. Lee EY, Bang JY, Park GW, Choi DS, Kang JS, et al. (2007) Global proteomic
24. Ullah MA, Arai T (1983) Pathological activities of the naturally occurring strains
20. Kwon SR, Nam YK, Kim SK, Kim KH (2006) Protection of tilapia
19. Kawai K, Liu Y, Ohnishi K, Oshima S (2004) A conserved 37 kDa outer
18. Castro N, Toranzo AE, Nunes S, Magariños B (2008) Development of an
effective Edwardsiella tarda vaccine for cultured turbot (Scophthalmus maximus). Fish Shellfish Immun 25: 208–212.
17. Kawanishi Y, Sato T, Ohno S, Nishimura S, Shimizu K, et al. (2006) Characterization of outer
membrane vesicles released from Edwardsiella tarda and their use as an effective vaccine candidate. Mar Biotechnol Doi: 10.1007/s10126-009-9235-5.
16. Kwon SR, Nam YK, Kim SK, Kim KH (2006) Protection of tilapia
15. Kwon SR, Nam YK, Kim SK, Kim KH (2006) Protection of tilapia
14. Kawanishi Y, Sato T, Ohno S, Nishimura S, Shimizu K, et al. (2006) Characterization of outer
membrane vesicles released from Edwardsiella tarda and their use as an effective vaccine candidate. Mar Biotechnol Doi: 10.1007/s10126-009-9235-5.
13. Kawanishi Y, Sato T, Ohno S, Nishimura S, Shimizu K, et al. (2006) Characterization of outer
membrane vesicles released from Edwardsiella tarda and their use as an effective vaccine candidate. Mar Biotechnol Doi: 10.1007/s10126-009-9235-5.
12. Kawanishi Y, Sato T, Ohno S, Nishimura S, Shimizu K, et al. (2006) Characterization of outer
membrane vesicles released from Edwardsiella tarda and their use as an effective vaccine candidate. Mar Biotechnol Doi: 10.1007/s10126-009-9235-5.
11. Kawanishi Y, Sato T, Ohno S, Nishimura S, Shimizu K, et al. (2006) Characterization of outer
membrane vesicles released from Edwardsiella tarda and their use as an effective vaccine candidate. Mar Biotechnol Doi: 10.1007/s10126-009-9235-5.
10. Kawanishi Y, Sato T, Ohno S, Nishimura S, Shimizu K, et al. (2006) Characterization of outer
membrane vesicles released from Edwardsiella tarda and their use as an effective vaccine candidate. Mar Biotechnol Doi: 10.1007/s10126-009-9235-5.
9. Kawanishi Y, Sato T, Ohno S, Nishimura S, Shimizu K, et al. (2006) Characterization of outer
membrane vesicles released from Edwardsiella tarda and their use as an effective vaccine candidate. Mar Biotechnol Doi: 10.1007/s10126-009-9235-5.
8. Kawanishi Y, Sato T, Ohno S, Nishimura S, Shimizu K, et al. (2006) Characterization of outer
membrane vesicles released from Edwardsiella tarda and their use as an effective vaccine candidate. Mar Biotechnol Doi: 10.1007/s10126-009-9235-5.
7. Kawanishi Y, Sato T, Ohno S, Nishimura S, Shimizu K, et al. (2006) Characterization of outer
membrane vesicles released from Edwardsiella tarda and their use as an effective vaccine candidate. Mar Biotechnol Doi: 10.1007/s10126-009-9235-5.
6. Kawanishi Y, Sato T, Ohno S, Nishimura S, Shimizu K, et al. (2006) Characterization of outer
membrane vesicles released from Edwardsiella tarda and their use as an effective vaccine candidate. Mar Biotechnol Doi: 10.1007/s10126-009-9235-5.
5. Kawanishi Y, Sato T, Ohno S, Nishimura S, Shimizu K, et al. (2006) Characterization of outer
membrane vesicles released from Edwardsiella tarda and their use as an effective vaccine candidate. Mar Biotechnol Doi: 10.1007/s10126-009-9235-5.
4. Kawanishi Y, Sato T, Ohno S, Nishimura S, Shimizu K, et al. (2006) Characterization of outer
membrane vesicles released from Edwardsiella tarda and their use as an effective vaccine candidate. Mar Biotechnol Doi: 10.1007/s10126-009-9235-5.
3. Kawanishi Y, Sato T, Ohno S, Nishimura S, Shimizu K, et al. (2006) Characterization of outer
membrane vesicles released from Edwardsiella tarda and their use as an effective vaccine candidate. Mar Biotechnol Doi: 10.1007/s10126-009-9235-5.
2. Kawanishi Y, Sato T, Ohno S, Nishimura S, Shimizu K, et al. (2006) Characterization of outer
membrane vesicles released from Edwardsiella tarda and their use as an effective vaccine candidate. Mar Biotechnol Doi: 10.1007/s10126-009-9235-5.
1. Kawanishi Y, Sato T, Ohno S, Nishimura S, Shimizu K, et al. (2006) Characterization of outer
membrane vesicles released from Edwardsiella tarda and their use as an effective vaccine candidate. Mar Biotechnol Doi: 10.1007/s10126-009-9235-5.