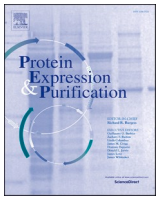
Protein Expression and Purification 215 (2024) 106409

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep



Research Article

Recombinant expression of *Yersinia ruckeri* outer membrane proteins in *Escherichia coli* extracellular vesicles

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ARTICLE INFO

Keywords:
Outer membrane proteins
OmpA
OmpF
Recombinant expression
Extracellular vesicles
Gram-negative bacteria
Outer membrane vesicles
OMVs

ABSTRACT

The secretion of extracellular vesicles (EVs) is a common process in Gram-negative bacteria and can be exploited for biotechnological applications. EVs pose a self-adjuvanting, non-replicative vaccine platform, where membrane and antigens are presented to the host immune system in a non-infectious fashion. The secreted quantity of EVs varies between Gram-negative bacterial species and is comparatively high in the model bacterium *E. coli*. The outer membrane proteins OmpA and OmpF of the fish pathogen *Y. ruckeri* have been proposed as vaccine candidates to prevent enteric redmouth disease in aquaculture. In this work, *Y.ruckeri* OmpA or OmpF were expressed in *E. coli* and recombinant EVs were isolated. To avoid competition between endogenous *E. coli* OmpA or OmpF, *Y. ruckeri* OmpA and OmpF were expressed in *E. coli* strains lacking *ompA*, *ompF*, and in a quadruple knockout strain where the four major outer membrane protein genes *ompA*, *ompC*, *ompF* and *lamB* were removed. *Y.ruckeri* OmpA and OmpF were successfully expressed in EVs derived from the *E. coli* mutants as verified by SDS-PAGE, heat modifiability and proteomic analysis using mass-spectrometry. Transmission electron microscopy revealed the presence of EVs in all *E. coli* strains, and increased EV concentrations were detected when expressing *Y. ruckeri* OmpA or OmpF in recombinant EVs compared to empty vector controls as verified by nanoparticle tracking analysis. These results show that *E. coli* can be utilized as a vector for production of EVs expressing outer membrane antigens from *Y. ruckeri*.

1. Introduction

The secretion of extracellular vesicles (EVs) is a described mechanism in all domains of life [1,2]. EVs, originally believed to be vessels containing cellular waste products, have been shown to be involved in many cellular processes, such as intercellular communication [3], horizontal gene transfer [3,4] and host-pathogen interaction [5]. Bioengineering of EVs for various purposes is an emerging field and EV research is getting increasing attention for its biomedical applications and biomarker potential [5–8].

This study is focusing on EVs derived from Gram-negative bacteria. These spherical bodies vary in size range from 50 to 250 nm and their secretion is described as a bulging of the outer membrane [9,10] and are therefore often also referred to as outer membrane vesicles (OMVs). However, EVs with double membranes are readily observed [11,12] and the term EV might be better suited to include all types of vesicles derived from Gram-negative bacteria. The quantity of secreted EVs varies among bacterial species and strains and can be altered by environmental factors, for example by change of growth medium or temperature as well as by chemical treatment [9,10]. Deletion of membrane bound proteins has also been shown to impact EV secretion [13–15]. EVs display features of the parent bacterium and contain RNAs, DNAs and proteins as cargo as well as lipopolysaccharides (LPS), outer membrane proteins (OMP) and virulence factors [2,16]. Due to their non-replicative, immunogenic and self-adjuvanting properties, EVs are considered potential vaccine candidates and are already part of vaccine formulations used against meningitis in humans [17–20].

Continuous vaccine development is not only required to prevent disease outbreaks caused by human pathogens. The growing aquaculture industry worldwide is in need of efficient vaccines against fish

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https://doi.org/10.1016/j.pep.2023.106409
Received 25 August 2023; Received in revised form 11 November 2023; Accepted 25 November 2023
Available online 30 November 2023

Table 1
Overview of strains and plasmid constructs used in the study.

Expression background strains from Ref. [50]	Plasmid constructs	Recombinant EV (rEV)
E. coli ΔompA	pASK-IBA2 $ompA_{Y.r}$ (this study)	*ΔompA* $OmpA_{Y.r}$
E. coli ΔBACF		*ΔBACF* $OmpA_{Y.r}$
E. coli ΔompF	pASK-IBA2 $ompF_{Y.r}$ (this study)	*ΔompF* $OmpF_{Y.r}$
E. coli ΔBACF		*ΔBACF* $OmpF_{Y.r}$
E. coli ΔompA	pASK-IBA2 empty (Original plasmid)	*ΔompA* empty
E. coli ΔompF		*ΔompF* empty
E. coli ΔBACF		*ΔBACF* empty

pathogens [21–23]. Cultured fish are routinely vaccinated against different bacterial and viral diseases. Currently, the majority of licensed vaccines against bacterial fish pathogens are based on whole-cell formulations, containing formalin inactivated bacteria [22,23]. As for human vaccines, there are also other types of vaccine formulations for use in fish, such as live-attenuated or subunit vaccines [23,24]. The administration is in most cases performed by intraperitoneal injection of each fish. Alternative application routes of vaccines such as coated food or bath submergence are the subject of ongoing research but do not seem to promote comparable vaccine efficacy [22,24]. To date, there is no EV-based vaccine licensed for use in fish, despite promising studies on EVs derived from the Gram-negative species *Edwardsiella* spp. against edwardsiellosis in olive flounder (*Paralichthys olivaceus*) [25] and against infections caused by *Piscirickettsia salmonis* in a zebrafish model [26].

The Gram-negative fish pathogen *Yersinia ruckeri* causes the disease yersiniosis, also called enteric red mouth (ERM) disease, and is mainly infecting farmed and wild salmonid fish [27,28]. Disease outbreaks are reported in cultured Rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) worldwide [29,30]. The disease poses a risk for fish welfare as well as leading to economic losses in the aquaculture industry [29,31]. Interestingly, geographically separated virulent clonal clusters of different biotypes and host specificity have been identified, leading to regional differences in disease causing strains [31]. Effective vaccines against ERM exist based on inactivated cells. However, potential changes like reported loss of motility in *Y. ruckeri* strains specific to Norwegian aquaculture or increasing diversity of strains infecting Atlantic salmon in Scotland, pose the risk of vaccine evasion [32,33]. Thus, continuous development on versatile vaccine candidates should be explored to readily adjust vaccine formulations against new infectious strains. Here, an EV based vaccine formulation is an option [34].

For the purpose of vaccination, outer membrane protein A (OmpA) and OmpF in *Yersinia* sp. have previously been described as promising antigens in the form of a recombinant subunit vaccine, eliciting immunogenic host reactions [35,36]. OMPs in Gram-negative bacteria are associated with several properties including membrane biogenesis, transport and signaling across the cellular membrane [37]. These proteins consist of a transmembrane β-barrel as well as periplasmic and extracellular loops [37]. Many OMPs are known to play a role in host-pathogen interactions and in host immune reactions and can therefore be potential antigens for vaccine development [38–42]. Different *Y. ruckeri* strains, isolated from Rainbow trout or Atlantic salmon, were found to have variable amino acids in the extracellular loops of OmpA and OmpF [43]. The variable regions have been proposed to have an impact on virulence, host-specificity and potentially host evasion. The diversification of those surface-localized regions is presumably driven by virulence-related selection [43].

For vaccine production high EV yields must be achieved which can be difficult due to varying vesiculation rates in bacterial strains [13]. Additionally, for other pathogens, EVs are reported to be part of the bacterial pathogenesis [44,45]. Rather than being an immune stimulant, the EVs dampen the host immune response to the pathogen [44–46]. To avoid these pathogen specific EV complications in vaccine development a recombinant EV (rEV) approach may be taken, and have successfully been performed for other pathogens previously [7,47–49].

In this study, we expressed *Y. ruckeri* OmpA and OmpF in *omp* knockout strains of *Escherichia coli* BL21 Gold (DE3) [50]. We successfully isolated EVs from *E. coli* and verified the presence of *Y. ruckeri* OmpA and OmpF in the derived recombinant EVs (rEVs).

2. Material and methods

2.1. Bacterial strains and growth conditions

Protein expression was performed in *E. coli* BL21 Gold (DE3) with deletions of one or several genes encoding OMPs; *ΔompA* or *ΔompF* or *ΔlamB ΔompA ΔompC ΔompF* (further referred to as *ΔBACF*) [50]. These strains were grown in low-salt-lysogeny broth (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) at 30 °C with 150 rpm agitation (MaxQ 6000 shaker, Thermo Scientific). The media was supplemented with 100 μg/mL ampicillin (Sigma-Aldrich; Cat. nr. A9518) for plasmid maintenance. *E. coli* DH5α chemically competent cells, used for plasmid propagation and isolation, were grown at 37 °C and 150 rpm in lysogeny broth (LB) (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl). *Y. ruckeri ompA* and *ompF* genes were amplified by PCR from *Y. ruckeri* strain NHV_3758 (NZ_CP023184), an isolate from Atlantic salmon (*Salmo salar*) in Norway in 1987 [51]. Primers used for PCR amplification and the *Y. ruckeri* gene accession numbers are listed in Suppl. Table 1. *Y. ruckeri* was grown in LB at 27 °C and 110 rpm.

2.2. Generation of plasmids

The expression plasmid pASK-IBA2 (BioTAGnology GmbH), encoding an *E. coli* OmpA signal peptide and a C-terminal Strep-Tactin affinity tag, was used as an empty vector control and as backbone to generate the plasmid construct used in this study. The *E. coli* OmpA signal peptide and a C-terminal Strep-Tactin affinity tag were removed during cloning and replaced by the respective endogenous *Y. ruckeri* signal peptide and coding region for *ompA* or *ompF*. Gibson cloning [52] was used to generate the two plasmids, pASK-IBA2-$ompA_{Y.r}$ and pASK-IBA2-$ompF_{Y.r}$ (Suppl. Figs. 1 and 2). Phusion HF polymerase (NEB, Cat nr. M0530) and the Gibson assembly Master Mix (NEB, Cat nr. E2611) were used to assemble the constructs using primers containing the respective up- and downstream sequences complementary to the neighboring target sequence (Suppl. Table 1). Plasmids and colonies were verified by PCR and sequencing of PCR products. An overview of expression strains and naming of the resulting rEVs is given in Table 1.

2.3. Protein expression

For protein expression a 5 mL preculture (30 °C, 150 rpm, low-salt-lysogeny broth with 100 μg/ml ampicillin) was incubated overnight. The next day, the preculture was used to inoculate 200 mL of low-salt-lysogeny broth (including 100 μg/ml ampicillin) to an OD_{600} 0.05. The 200 mL culture was incubated (30 °C, 150 rpm) until an OD_{600} ~0.5–0.6 was reached. Anhydrotetracycline (Abcam, Cat. nr. ab145350) (200 μg/L end concentration) was added to induce protein expression and the culture was incubated for an additional 4 h.

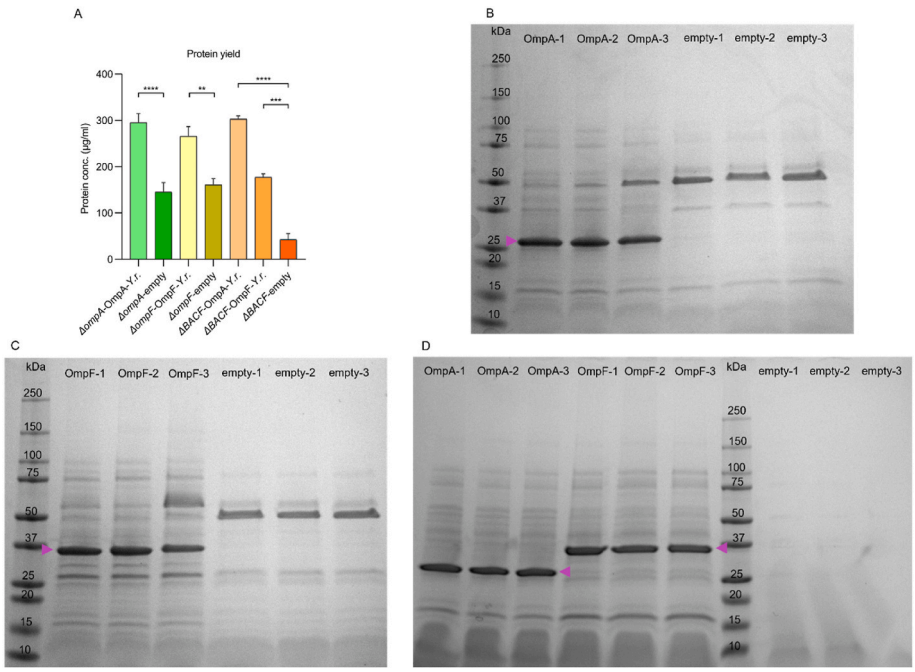

Fig. 1. Protein concentration and protein pattern in biological replicates of isolated rEVs from *E. coli* BL21 Gold mutants expressing OmpA or OmpF from *Y. ruckeri*. (A) Averaged protein concentration of three biological replicates (1–3) of rEVs, measured using BCA assay. (B, C, D) Protein patterns of three biological replicates of rEVs, visualized using SDS-PAGE. rEVs isolated from (B) *E. coli* BL21 Gold *ΔompA* $OmpA_{Y.r}$ or empty vector control, (C) BL21 *ΔompF* $OmpF_{Y.r}$ or empty vector control, (D) BL21 *ΔBACF* $OmpA_{Y.r}$. $OmpF_{Y.r}$ or empty vector control. All three replicates of the rEVs containing either $OmpA_{Y.r}$ or $OmpF_{Y.r}$ show the desired protein band (indicated by purple arrowhead) while the band is lacking in the empty vector controls. Comparable protein patterns between the biological replicates can be observed. The samples were boiled at 95 °C for 5 min and the gels were loaded with 3.5 µg of total protein per sample (except for *ΔBACF* empty vector control).

2.4. EV isolation

EVs were isolated 4 h after induction of protein expression using differential centrifugation. First, bacteria were pelleted (Hettick Rotina 420R; 4200×*g*; 4 °C for 30 min) and subsequently, the supernatant was filtered through a 0.45 µm filter (PES membrane, VWR, Cat. nr.514-0341). The filtrate was ultracentrifuged to collect and pellet EVs (Sorvall Discovery 100; 100,000×*g*; 4 °C; 2 h). After the first centrifugation round, the pellets were washed with Phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4; pH 7.4) and pooled in one centrifugation tube. Finally, after the second round of centrifugation, the EV containing pellet was resuspended in 500 µl of PBS and filtered through a 0.45 µm filter (Pall Corporation; Cat. nr. 4654) once more. Aliquoted samples were stored at −80 °C until further characterization.

2.5. Characterization of rEVs

2.5.1. Transmission electron microscopy (TEM)

TEM was performed on isolated vesicles using negative staining. Formvar coated copper grids (200–300 mesh) were placed on a drop of vesicle sample for one to 5 min. The grids were then washed by placing them on five consecutive drops of H_2O for ten to 20 s per drop. Finally, the grids were placed on a drop of 1 % of uranyl acetate for 30 s. A JEOL JEM-1400 Plus transmission electron microscope (TEM) was used for imaging.

2.5.2. SDS-PAGE

Protein concentration of rEV samples was determined with a BCA assay (Pierce BCA Protein Assay Kit, Thermo Scientific, Cat. nr. 23225) following the suppliers manual. 5x Laemmli (10 % SDS, 50 % Glycerol, 250 mM Tris-HCL, bromophenol blue) was added to the sample to a 1x concentration with 5 % 2-Mercaptoethanol (Gibco, Cat. nr. 31350) freshly added to the sample preparation. The samples were either directly loaded on an SDS-gel (folded protein control; non-boiled) or boiled at 95 °C for either 5 min or 30 min. Proteins were separated using SDS-PAGE (Mini-PROTEAN TGX 4–20 %, BioRad; Cat. nr.4561094; Tris/Glycine buffer); Precision Plus Protein Standard (BioRad; Cat. nr. 161–0374) was used and 3.5 µg of protein per sample was loaded on each gel. Protein bands were visualized with Coomassie Blue stain (Bio-Safe Coomassie Blue G-250 Stain, BioRad; Cat. nr. 1610786). The SDS-gels were washed in dH_2O three times before Coomassie staining was performed for 30 min. The gels were destained in dH_2O overnight. Imaging of stained SDS-PAGE gels were performed in a GelDoc XR+ (BioRad).

2.5.3. Nanoparticle tracking analysis (NTA)

The average particle size and count were measured using a NanoSight NS500 instrument (Malvern Panalytical) equipped with a sCMOS camera, a 488 nm laser, and a syringe-pump for steady sample injection. rEVs samples were mixed by vortexing and diluted in PBS (0.02 µm filtered) (50–5000 times dilution, Suppl. Fig. 5) to obtain a concentration of 10^7-10^8 particles/ml for the measurement. For positive control, the instrument was tested with 100 nm polystyrene beads in the beginning of every measurement session. The samples were video analyzed in technical triplicates (three videos of 60 s, 1499 total frames, infusion rate of 20, camera level at 15, detection threshold of 3, viscosity set to water) using the NTA 3.4 software (Malvern Panalytical).

2.5.4. Mass spectrometry

Coomassie G-250 stained gel pieces were in-gel digested with 0.2 µg trypsin GOLD (Promega) for 16 h at 37 °C. The digestion was stopped by adding 5 µL 50 % formic acid and the generated peptides were purified using a 10 µL OMIX C18 micro-SPE pipette tip (Agilent) and dried using a Speed Vac concentrator (Concentrator Plus, Eppendorf). The samples were analyzed by LC-MS using a timsTOF Pro (Bruker Daltonik) which

was coupled online to a nanoElute nanoflow liquid chromatography system (Bruker Daltonik) via a CaptiveSpray nanoelectrospray ion source. The dried peptides were dissolved in 4 μL 0.1 % formic acid and 2 μL of sample was injected. The peptides were separated on a reversed phase C18 column (25 cm × 75 μm, 1.5 μm, PepSep (Bruker Daltonics)). Mobile phase A contained water with 0.1 % formic acid, and acetonitrile with 0.1 % formic acid was used as mobile phase B. The peptides were separated by a gradient from 0 to 35 % mobile phase B over 60 min at a flow rate of 300 nl/min at a column temperature of 50 °C. MS acquisition was performed in DDA-PASEF mode. The capillary voltage was set to 1.5 kV with a mass range of 100–1700 *m/z*. The number of PASEF ranges was set to 20 with a total cycle time of 1.16 s, charge up to 5, target intensity of 20,000, intensity threshold of 1.750, and active exclusion with release after 0.4 min. An inversed reduced TIMS mobility (1/k0) of 0.85–1.40 Vs/cm2 was used with a range time of 100 ms, an accumulation time of 100 ms, a duty cycle of 100 %, and a ramp rate of 9.51 Hz. Precursors for data-dependent acquisition were fragmented with an ion mobility-dependent collision energy, which was linearly increased from 20 to 59 eV. The LC/MS data were searched against the *E. coli* Uniprot (42.281 entries) and *Yersinia ruckeri* NHV_3758 (19.205 entries) database, using Mascot 2.7.0.1 The following parameters were used: digestion enzyme, trypsin; maximum missed cleavage, 1; fragment ion mass error tolerance, 0.03 Da; parent ion error tolerance, 15 ppm. Oxidation of methionine, propionamide formation of cysteines, and acetylation of the N-terminus were specified as variable modifications. Scaffold 5.1.2 (Proteome Software Inc.) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0 % probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at a false-discovery rate better than 1 %.

2.6. Statistical analysis

Data analysis was performed using GraphPad PRISM 8 (Dotmatics). Data are shown as mean of biological replicates ± standard error of mean (SEM). Statistical analysis was performed using One-way ANOVA with Sidak's multiple comparison test, comparing the recombinant protein expressing sample with its respective empty vector control. Differences were considered significant with a p value < 0.05 (*), <0.005 (**), <0.0005 (***), <0.0001 (****). Scaffold 5 (Proteome Software Inc.) was used to analyze and view Mass-spectrometry data results.

3. Results and discussion

We expressed *Y. ruckeri* OmpA or OmpF recombinantly in three different *E. coli* membrane protein knockout backgrounds, to avoid competition for space in the outer membrane. *Y. ruckeri* OmpA was expressed in *E. coli ΔompA* and *E. coli ΔBACF* background and *Y. ruckeri* OmpF was expressed in the *E. coli ΔompF* and E. coli *ΔBACF* background. The rEVs from each strain were subsequently isolated. The different rEV preparations (Table 1) were each isolated in three biological replicates including the controls consisting of the respective mutant background containing the pASK-IBA2 plasmid vector without an insert (empty vector control).

3.1. $OmpA_{Y.r}$ and $OmpF_{Y.r}$ protein expression in E. coli rEVs

Starting with 200 mL of expression culture, rEVs were successfully isolated using differential centrifugation, including filtration steps and ultracentrifugation with a final resuspension of rEV samples in 500 μL of PBS. The total protein concentrations were measured using a BCA assay and the average ranged from as little as 43 μg/mL for the *ΔBACF* empty vector controls up to 303 μg/mL for the *ΔBACF* $OmpA_{Y.r}$ sample (Fig. 1A). In the case of *ΔBACF* mutant the protein yield was seven times higher in the *ΔBACF* $OmpA_{Y.r}$ samples compared to the *ΔBACF* empty vector control, while the protein yield for *ΔBACF* $OmpF_{Y.r}$ was four times higher than measured in the *ΔBACF* empty vector sample (Fig. 1A). The reason for the nearly twice as high protein yield of *ΔBACF* $OmpA_{Y.r}$ compared to *ΔBACF* $OmpF_{Y.r}$ remains to be investigated. For the *ΔompA* background, the *ΔompA* $OmpA_{Y.r}$ had two times higher protein yield compared to the *ΔompA* empty vector control sample. The least difference between protein yield was detected between the *ΔompF* $OmpF_{Y.r}$ and its control with a 1.6 times higher protein yield in the *ΔompF* $OmpF_{Y.r}$ preparation.

Generally, the rEV preparations, isolated from expressing backgrounds, namely *ΔompA* $OmpA_{Y.r}$, *ΔompF* $OmpF_{Y.r}$, *ΔBACF* $OmpA_{Y.r}$ and *ΔBACF* $OmpF_{Y.r}$ had significantly higher total protein yield compared to their respective empty vector controls. This was seen even though the cultures were grown, and rEVs were isolated under the same conditions. One explanation for these results could be an increased vesiculation under stressful conditions. It is well established that stress can lead to hypervesiculation in certain bacterial strains [13,14]. Stress can be caused by environmental factors such as change of temperature, addition of antibiotics or stress in form of cellular stress, such as membrane protein deletions or accumulation of misfolded proteins [13,14]. In this study, the overexpression of $OmpA/F_{Y.r}$, could cause cellular stress for the bacterial cells by increased protein production and load. The increased vesiculation rate, induced by protein overexpression, leads accordingly to higher total protein content in the rEV samples isolated from protein expressing bacteria compared to the empty vector controls. Especially the isolation of empty vector EVs from the BL21 *ΔBACF* background resulted in low protein yield. This might be explained by the overall lack of OMPs in the outer membrane and thereby in the vesicles, which affects the molecular weight and physical properties such as the density of the rEVs so that they might simply not be sufficiently pelleted after 2 h centrifugation at 100,000×*g*. Even though the protein concentration was too low to visualize the *ΔBACF* empty rEVs with Coomassie staining, the vesicles were successfully visualized using TEM (Fig. 4), demonstrating that the preparation was successful, and confirming that this strain has only low amounts of protein in its outer membrane and any vesicles derived from it [50].

To check for successful expression and presence of $OmpA_{Y.r}$ or $OmpF_{Y.r}$, in the rEVs, three biological replicates were analyzed with help of SDS-PAGE, with normalized total protein amount set to 3.5 μg and loaded on the gel for each sample (Fig. 1B, C, D). The overall protein patterns among the replicates are qualitatively comparable by visual assessment. This indicates that the protein content is similar among the three rEV replicates and the $OmpA_{Y.r}$ and $OmpF_{Y.r}$ expression and rEV isolation is reproducible. The expressed $OmpA_{Y.r}$ and $OmpF_{Y.r}$, present in the isolated rEVs are indicated by purple arrowhead in Fig. 1B, C, D. The three biological replicates of the empty vector controls did not contain any *Y. ruckeri* OMP, which was verified by Mass spectrometry (data not shown). Both $OmpA_{Y.r}$ and $OmpF_{Y.r}$ proteins bands migrate through the SDS-gel at a different apparent weight than expected from the predicted molecular weight of 38 and 40 kDa, respectively. Therefore, the successful expression of $OmpA_{Y.r}$ and $OmpF_{Y.r}$, was verified by Mass spectrometry. Bands were excised from the SDS-gels and the identified peptides were found to span nearly the whole protein sequences of $OmpA_{Y.r}$ (95 %) and $OmpF_{Y.r}$ (88 %) (Suppl Fig. 3). The corresponding signal peptides were also identified for both proteins which most likely originate from not fully processed proteins that co-purify with the EVs. Due to the overexpression of $OmpA_{Y.r}$ and $OmpF_{Y.r}$, it is possible that not all translated protein will be processed, translocated, and folded in the outer membrane. Several bottlenecks in this process are posed by transport systems, translocating proteins through the inner membrane into the periplasm, proper folding and integration into the outer membrane [53–55]. There are, however, no visible unprocessed band on the fully denaturing gels (Fig. 1) and we therefore assume that in all proteins present in the outer membrane, the signal peptide has been cleaved off properly-. To demonstrate that

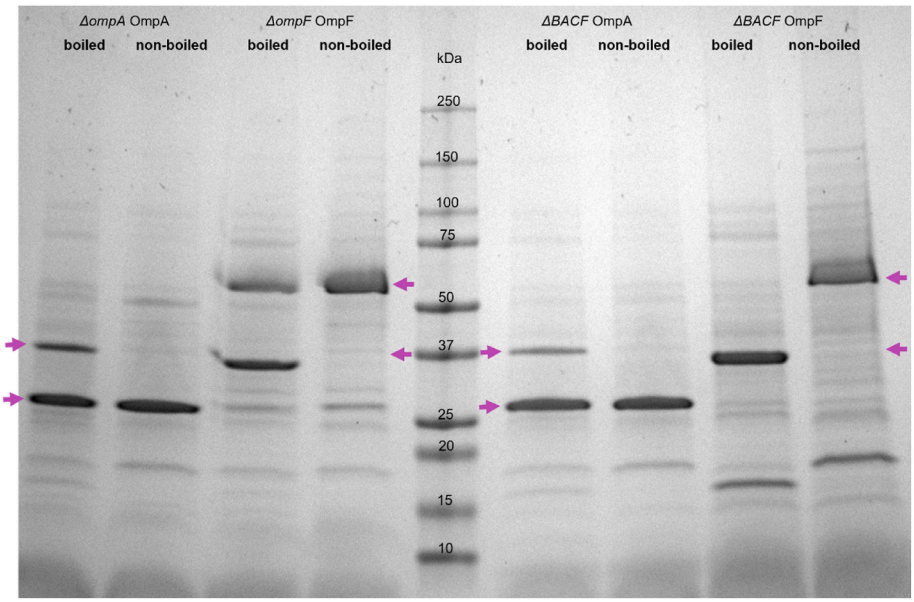

Fig. 2. Heat modifiability visualized by change in protein pattern of rEVs using SDS-PAGE. Samples loaded were either boiled at 95 °C for 30 min or non-boiled. rEVs were isolated from *E. coliΔompA* expressing $OmpA_{Y.r}$, *E. coliΔompF* expressing $OmpF_{Y.r}$, *E. coliΔBACF* expressing $OmpA_{Y.r}$, and *E. coliΔBACF* expressing $OmpF_{Y.r}$. Both rEV samples containing $OmpA_{Y.r}$ show a band around 26 kDa, while the boiled samples show an additional band at around 37 kDa that corresponds to the expected molecular weight (indicated by arrows on the left). The $OmpF_{Y.r}$ expressing samples show a shift in molecular weight of the expression band. Boiled samples show the band around 37 kDa while the non-boiled samples present a band >50 kDa (indicated by arrows on the right). The gel were loaded with 3,5 μg of total protein per sample.

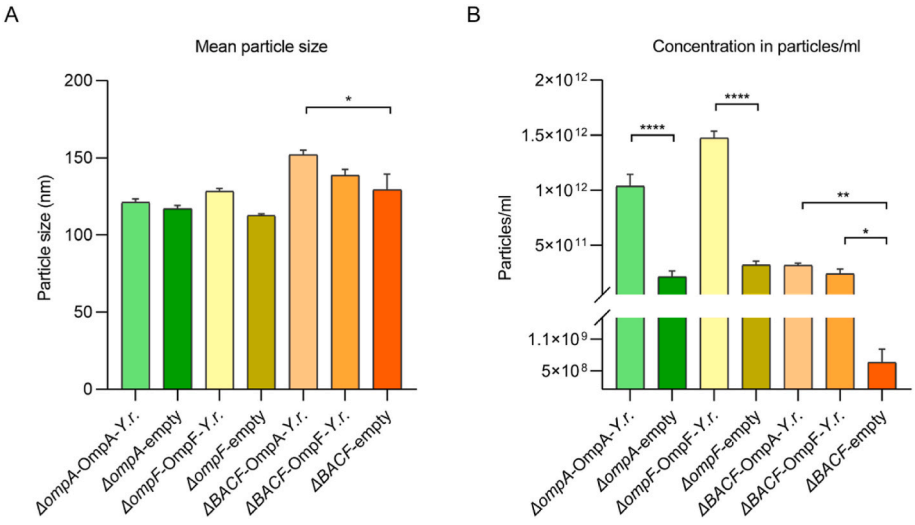

Fig. 3. Particle size and concentration of rEVs isolated from either $OmpA_{Y.r}$/ $OmpF_{Y.r}$ expressing background or empty vector controls. NTA measurements were performed on all three biological replicates and three videos were taken per sample. The mean particle size (A) is plotted showing standard error of mean (SEM). The averaged concentration in particles/mL between biological replicates was corrected for the dilution factor and was plotted (B).

$OmpA_{Y.r}$ and $OmpF_{Y.r}$ are properly inserted into the outer membrane and folded, their heat modifiability was investigated.

3.2. Heat modifiability verified by typical shift in apparent molecular weight on gel

Heat modifiability has been established as a verification of properly folded β-barrel proteins [56–59]. Without heat treatment, OMPs remain in their folded and potentially oligomeric structure when separated with SDS-PAGE, while heat-treated OMPs will denature, unfold and be visible at their monomeric and proper molecular weight [56–59]. Therefore, the differential migration of boiled versus non-boiled OMP samples is an established method to confirm the presence of folded protein in the outer membrane.

In order to demonstrate that $OmpA_{Y.r}$ and $OmpF_{Y.r}$ were folded and properly inserted into the outer membrane, protein migration was compared in boiled (95 °C, 30 min) and non-boiled rEV samples (Fig. 2, only one replicate shown). All isolated rEV replicate samples show a shift in apparent molecular weight when comparing the boiled versus non-boiled sample preparations, confirming the presence of correctly folded $OmpA/F_{Y.r}$ (Suppl. Fig. 4). This was independent of the *E. coli* mutant backgrounds. $OmpA_{Y.r}$ isolated from either the single-knockout background or the *ΔBACF* background showed a band size at the predicted molecular weight of the monomer (~38 kDa) in addition to the previously detected band around 26 kDa when boiled for 30 min. The respective non-boiled samples show only the band at the apparent

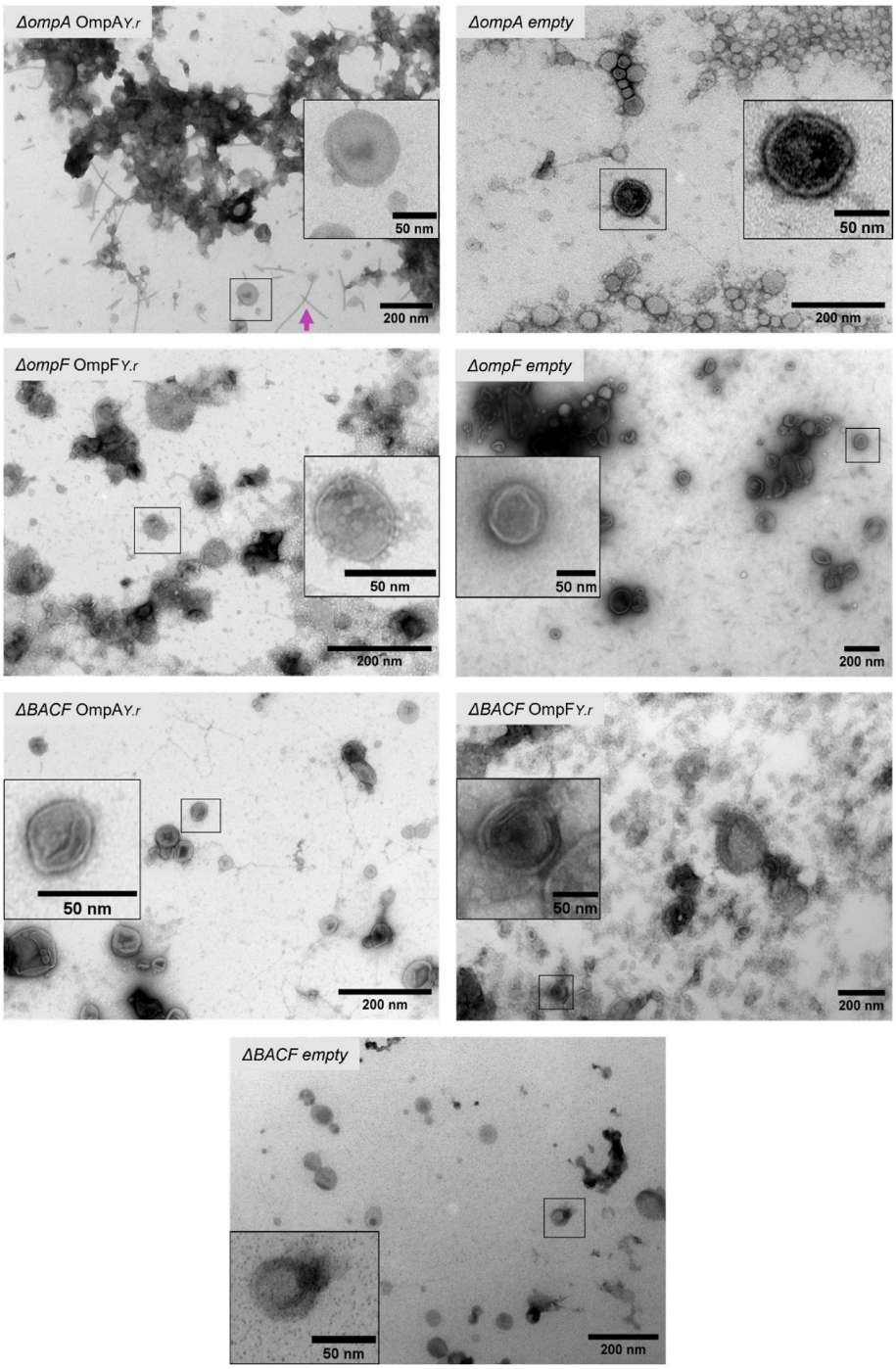

Fig. 4. TEM images of negative stained rEV samples. Images show typical EV shapes in all preparations. Purple arrow indicates flagella-like structures. Scale bars indicate 200 nm for large images and 50 nm for inserts.

molecular weight around 26 kDa. Comparable band shifts for OmpA from different *Y. ruckeri* isolates have been reported by Ref. [32,32]). Conversely to $OmpA_{Y.r.}$, the non-boiled $OmpF_{Y.r}$ samples show a protein band at an apparent weight above 50 kDa, compared to the boiled samples with a band at the predicted molecular weight of the $OmpF_{Y.r}$ monomer (~40 kDa) (Fig. 2). The remaining fractions of folded $OmpA_{Y.r}$ and $OmpF_{Y.r.}$ demonstrate the stability of the proteins even after heat treatment.

3.3. NTA measurements show varying rEV concentration

EV quantity and size distribution was measured by NTA [60–62]. NTA analysis of EV samples is part of the recommendations of "minimal information for studies of extracellular vesicles" (MISEV) published by the International Society for Extracellular Vesicles, in their effort to implement standardized analysis and reporting methods in the EV research field [63].

We evaluated the average rEV size to determine if overexpression of recombinant protein would impact the size of the isolated rEVs compared to their empty vector controls. The mean particle sizes ranged from an averaged size of 113 nm in the *ΔompF* empty control to 152 nm in the *ΔBACF* $OmpA_{Y.r}$ sample (Fig. 3A).

The mean particle sizes (Fig. 3A) between the respective samples and empty vector controls did not show statistically significant differences,

except for the *ΔBACF* $OmpA_{Y.r}$ compared to the *ΔBACF* empty vector control. A similar trend is seen in the mode particle size (Suppl. Fig. 5). The mean diameters of the EVs isolated from *ΔBACF* $OmpA_{Y.r}$ was 152 nm while the *ΔBACF* empty vector was 129 nm, accounting for a difference of 16.7 % in average size. For the *E. coli ΔompA* and *ΔompF* background the induction of protein expression and higher vesiculation rate has no significant impact on the rEV size.

When investigating the rEV concentration in the preparations by NTA analysis, the quantity ranges from an average of 6.6 x 10^8 particles/mL in the *ΔBACF* empty vector control to 1.4 x 10^{12} particles/mL in the *ΔompF* $OmpF_{Y.r}$ sample (Fig. 3B). Overall, the rEV preparations containing the overexpressed proteins have a significantly higher rEV concentration compared to their empty vector controls. The difference between *ΔBACF* $OmpF_{Y.r}$ and *ΔBACF* $OmpA_{Y.r}$ compared to the *ΔBACF* empty vector control is notable as these vesicle preparations contain 4.8 x 10^2 and 3.6 x 10^2 more particles/mL, respectively. The preparation from *ΔompA* $OmpA_{Y.r}$ compared to *ΔompA* empty vector control contained around one order of magnitude more particles/mL (1 x 10^{12} vs. 2.1 x 10^{11}). Similarly, the *ΔompF* $OmpF_{Y.r}$ preparations contained an average of 1.5 x 10^{12} particles/mL compared to the *ΔompF* empty vector control with an average 3.2 x 10^{11} particles/mL.

Notably, the *ΔBACF* empty vector control has a very low rEV concentration which can be explained by the overall lack of OMPs in the outer membrane that may change the EV weight and thereby the sedimentation rate, as previously discussed. The *ΔBACF* empty vector control also contains the least homogenous EV population as revealed in the finite track length adjustment (FTLA) graph (Suppl. Fig. 6). The FTLA graph visualizes the concentration distribution per size of the three technical replicates during a measurement of one sample. For all other rEV samples, the FTLA shows mostly one peak and a generally normally distributed graph, indicating a quite homogenous rEV population in the samples (Suppl. Fig. 6).

3.4. TEM shows typical EV appearance of the isolated E. coli rEVs

To visualize the rEV preparations, TEM imaging with negative stain was performed. The TEM analysis visualized a typical cup shaped morphology of EVs (Fig. 4). The rEVs tend to cluster together, which was especially apparent for the *ΔompA* $OmpA_{Y.r}$ preparation. In the same preparation flagella-like structures can be seen as these co-sedimented during the ultracentrifugation procedure. The co-isolation of flagella, although not intended, might improve the self-adjuvanting properties of rEV preparation when administered as vaccines as flagellin is shown to have immunogenic properties [64]. It remains to be investigated if this self-adjuvanting properties would be beneficial and lead to a better efficacy or if a host would rather develop, non-desired, specific immunity against *E. coli* flagella. Additional purification steps such as size exclusion chromatography or density gradient centrifugation could help to avoid the presence of flagella in the preparations.

As mentioned before, the TEM analysis revealed the presence of EVs also in the *ΔBACF* empty vector preparations EVs even though they were barely detectable by Coomassie stain SDS-PAGE analysis (Fig. 1).

4. Conclusion

In this study we show the successful expression of *Y. ruckeri* OmpA or OmpF in different *E. coli* BL21 Gold knockout backgrounds (*ΔompA*, *ΔompF*, *ΔBACF*) and the presence of the correctly folded $OmpA_{Y.r}$ and $OmpF_{Y.r}$ in the membrane of isolated rEVs. We verified, by using SDS-PAGE, that the expression and isolation of rEVs is reproducible by isolating three qualitatively comparable biological replicates. rEVs isolated from $OmpA_{Y.r}$ or $OmpF_{Y.r}$ expressing backgrounds had a significantly higher particle/mL concentration than EV preparations from their empty vector controls. rEV preparations derived from the *ΔompA* and *ΔompF* background resulted in higher rEV concentrations than those derived from the *ΔBACF* background. The size of the rEVs was however only significantly affected in the *ΔBACF* $OmpA_{Y.r}$ sample compared to the *ΔBACF* empty vector control. TEM imaging visualized typical characteristics of EVs in all preparations.

By recombinant expression of these OMPs and consecutive isolation of secreted rEVs, the antigenic properties of the OMPs and the self-adjuvanting properties of EVs can be exploited simultaneously. Future studies will investigate the potential of these rEVs to elicit a protective immune reaction in the host.

Due to the fact that there is sequence variability in the surface-exposed loops of *Y. ruckeri* OmpA and OmpF between different virulent *Y. ruckeri* strains, it is possible that a single version of OmpA or OmpF is not sufficient for protectivity. In this case, the presentation of multiple different variants, or of a recombinant gene product with multiple, different antigens would be desirable andcould potentially give protection against several infectious strains at once.

Funding

This work was financially supported by the University of Oslo, and the Research Council of Norway (RCN) Milepæl program [Grant No. 313638] to VM and HCWL and RCN grants 302723 and 294605 to DL.

Author contribution

VM; AS; EM; DL; HCWL; EBT; NR *Data curation;* VM *Formal analysis;* DL, AS, VM, NR, HCWL Funding *acquisition;* HCWL, DL *Investigation;* VM, EM, EBT, NR, HCWL, *Methodology;* DL, AS, VM *Project administration;* DL, VM, HCWL *Resources;* HCWL, DL, *Supervision;* DL, AS, HCWL, VM *Visualization;* VM, EM, NR, HCWL, *Roles/Writing - original draft;* VM *Writing - review & editing*; DL, AS, HCWL.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

We would like to thank Reidun Øvstebø and Anne-Marie Siebke Trøseid from the Department of Medical Biochemistry at Oslo University Hospital for giving us access and support to perform the NTA instrument in their laboratory. We thank Bernd Thiede at the Proteomics Core Facility, Department of Biosciences, University of Oslo, for performing the Mass spectrometry-based proteomic analyses. This facility is a member of the National Network of Advanced Proteomics Infrastructure (NAPI), which is funded by the Research Council of Norway INFRASTRUKTUR-program (project number: 295910). Further, we would like to thank Antje Hofgaard at the Electron microscopy laboratory at the Department of Biosciences, University of Oslo, for her help with TEM imaging.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pep.2023.106409.

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