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Research Article

RESEARCH ARTICLE

OmpA family proteins and Pmp-like autotransporter: new adhesins of *Waddlia chondrophila*

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One sentence summary: In this work, the *Waddlia* molecules involved in the adhesion of *Waddlia* to host cells have been identified, demonstrating the importance of OmpA-like proteins in adhesion as well as a Pmp-like protein.

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ABSTRACT

Waddlia chondrophila is a obligate intracellular bacterium belonging to the *Chlamydiales* order, a clade that also includes the well-known classical *Chlamydia* responsible for a number of severe human and animal diseases. *Waddlia* is an emerging pathogen associated with adverse pregnancy outcomes in humans and abortion in ruminants. Adhesion to the host cell is an essential prerequisite for survival of every strict intracellular bacteria and, in classical *Chlamydia*, this step is partially mediated by polymorphic outer membrane proteins (Pmps), a family of highly diverse autotransporters that represent about 15% of the bacterial coding capacity. *Waddlia chondrophila* genome however only encodes one putative Pmp-like protein. Using a proteomic approach, we identified several bacterial proteins potentially implicated in the adhesion process and we characterized their expression during the replication cycle of the bacteria. In addition, we demonstrated that the *Waddlia* Pmp-like autotransporter as well as OmpA2 and OmpA3, two members of the extended *Waddlia* OmpA protein family, exhibit adhesive properties on epithelial cells. We hypothesize that the large diversity of the OmpA protein family is linked to the wide host range of these bacteria that are able to enter and multiply in various host cells ranging from protozoa to mammalian and fish cells.

Keywords: intracellular bacteria; adhesins; autotransporter; chlamydia; virulence

INTRODUCTION

Waddlia chondrophila is an strict intracellular bacterium belonging to the *Chlamydiales* order. This clade is currently divided in nine family-level lineages (Stride *et al.* 2013) which, according to a recent report by Lagkouvardos *et al.* (2014), largely underestimates the huge diversity of these bacteria, especially in the marine environment. Bacteria belonging to the *Chlamydiales* order are strictly intracellular; some represent harmless environmental species multiplying in amoebae whereas others are important human and animal pathogens. *Chlamydia trachomatis*, for example, causes urogenital infections associated with infertility (Baud, Regan and Greub 2008) and *C. pneumoniae* is a recognized agent of pneumonia (Hahn *et al.* 2002). Other members of the *Chlamydiaceae* family, such as *C. abortus* or *C. psittaci*, are well-known animal pathogens associated with important economic repercussions and having a significant zoonotic potential (Everett 2000).

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Waddlia chondrophila was isolated twice, in two different locations, from aborted bovine foetuses. Abortion was also observed after inoculation of these bacteria to bovines (Dilbeck *et al.* 1990; Henning *et al.* 2002; Dilbeck-Robertson *et al.* 2003). Moreover, a strong correlation was observed between anti-*Waddlia* antibodies and abortion in ruminants (Dilbeck-Robertson *et al.* 2003) and, more recently, *Waddlia* DNA was detected in vaginal swabs of 8% of 150 cows suffering from abortion (Barkallah *et al.* 2014). In humans, *W. chondrophila* seropositivity is associated with adverse pregnancy outcomes such as miscarriage or ectopic pregnancy (Baud *et al.* 2007, 2014; Hornung *et al.* 2015). Moreover, *Waddlia* has been detected by qPCR and immunohistochemistry in vaginal swabs and placentas of women suffering from miscarriage (Baud *et al.* 2011, 2014). In addition, these bacteria were also detected in clinical samples of patients with community-acquired pneumonia or bronchiolitis, which suggest their implication in lower respiratory tract infections (Haider *et al.* 2008; Goy *et al.* 2009).

Waddlia, like all other members of the *Chlamydiales* order studied so far, undergo a biphasic replication cycle that involves an infectious form able to adhere to and enter into the host cell, the elementary body (EB) and a replicative form, the reticulate body (RB), which is strictly intracellular and divides by binary fission. When bacteria encounter stress conditions such as antibiotic treatment or nutrient starvation, a third form, called an aberrant body (AB) can also be observed. Thus, ABs have been observed when *Waddlia* was grown in endometrial cells in a low nutrient medium (Kebbi-Beghdadi, Cisse and Greub 2011) and when it was exposed to penicillin derivatives and to phosphomycin (Jacquier *et al.* 2014). ABs correspond to a dormant form of the bacteria that can revert to infectious forms when conditions improve and that are associated with long-term persistence in the host organism (Beatty, Morrison and Byrne 1994; Hogan *et al.* 2004).

For obligate intracellular bacteria such as member of the *Chlamydiales* order, attachment and entry in the host cell are essential requirements for survival and replication. In *Chlamydia*, several bacterial proteins are implicated in adhesion. These include two important constituents of the chlamydial membrane, the major outer membrane protein (MOMP) (Su *et al.* 1990, 1996; Swanson and Kuo 1994) and OmcB, a cysteine-rich protein thought to play a role in the stabilization of the cell envelope by disulfide bonds (Hatch 1996; Stephens *et al.* 2001). OmcB binds to heparan sulfate-like glycosaminoglycans (GAGs) on the surface of host cells via basic amino acids located in its N-terminal region (Zhang and Stephens 1992; Fadel and Eley 2007; Moelleken and Hegemann 2008; Fechtner *et al.* 2013), this interaction being probably the first step in the attachment process. GroEL1 and heat shock proteins (HSP) 70 are also localized on the surface of infectious EBs and play a role in the establishment of infection (Raulston *et al.* 2002; Wuppermann *et al.* 2008).

In addition, chlamydial adhesion also relies on a specific family of highly diverse autotransporters, the polymorphic outer membrane proteins (Pmps) (Longbottom *et al.* 1996, 1998; Grimwood, Olinger and Stephens 2001). As many as 21 different Pmps, classified in six subtypes, have been identified in *C. pneumoniae*. They exhibit little similarity in amino acid sequences but all contain multiple repeats of the tetrapeptide motifs GGA(ILV) and FxxN. Pmp6, Pmp20 and Pmp21, representing three of the six Pmp subtypes, were shown to be implicated in *C. pneumoniae* adhesion to human epithelial cells precisely via their repeated motifs (Mölleken, Schmidt and Hegemann 2010). The epidermal growth factor receptor on the host cell was recently identified as the receptor for the *C. pneumoniae* adhesin and invasin Pmp21 (Mölleken, Becker and Hegemann 2013). The *C. trachomatis* Pmp family comprises nine members (PmpA to PmpI), also classified in the same six subtypes, that all mediate adhesion to human epithelial and endothelial cells (Becker and Hegemann 2014).

We have previously demonstrated that *W. chondrophila* is able to enter and multiply in various host cells ranging from mammalian epithelial cells and macrophages to fish and insect cells and to protists (Goy and Greub 2009; Croxatto and Greub 2010; Kebbi-Beghdadi, Batista and Greub 2011; Kebbi-Beghdadi, Cisse and Greub 2011; Kebbi-Beghdadi *et al.* in preparation). Nothing is known about the molecules implicated in the adhesion of *W. chondrophila* to its host cell but OmcB and HSP60 (GroEL) are present in the *Waddlia* genome (Bertelli *et al.* 2010). However, *Waddlia* OmcB lacks the XBBXBX sequence present in the N-terminal region of all *Chlamydia* OmcB and that was shown to be crucial for adhesion (Moelleken and Hegemann 2008; Fechtner *et al.* 2013). A more detailed analysis of this genome also revealed the presence of a family of 11 genes coding for putative porins with a β-barrel structure. These OmpA proteins could represent the functional homologs of MOMP, a major chlamydial protein that is notably absent in the *Waddlia* genome. Furthermore, one putative autotransporter protein has also been described (gene wcw_0271) that displays a predicted overall structure somehow similar to the chlamydial Pmps with an N-terminal leader sequence followed by a passenger domain and a β-barrel C-terminal domain. In addition, this *Waddlia* Pmp-like protein also harbors six tetrapeptide repeats identical to those implicated in adhesion of the chlamydial Pmps.

In the present study, we identified, using a proteomic approach, the *Waddlia* molecules implicated in adhesion and we characterized their transcriptional and protein expression profiles. We also evaluated *in vivo* on epithelial cells the adhesion potential of these molecules and of the putative Pmp-like protein, knowing that understanding this crucial step of host–pathogen interactions is a major stone on the way towards efficient diagnostic and treatments of this emerging pathogen.

MATERIAL AND METHODS

Cell culture and bacterial strains

Human lung carcinoma cell line A549 (ATCC CCL-185), Vero cells derived from kidney epithelial cells of the African Green Monkey (ATCC CCL-81) and the human endometrial adenocarcinoma Ishikawa cell line were cultivated as described in Kebbi-Beghdadi, Cisse and Greub (2011). HEp-2 cells (ATCC CCL-23) were routinely maintained in high glucose Dulbecco's modified minimal essential medium (DMEM; Gibco, Life Technologies, Zug, Switzerland) supplemented with 10% foetal calf serum (Biochrom AG, Berlin, Germany), 1% non-essential amino acids and 1% vitamins (Gibco, Life Technologies). *Waddlia chondrophila* strain ATCC VR-1470 was grown at 32°C within *Acanthamoeba castellanii* strain ATCC 30010 in 25 or 75 cm^2 cell culture flasks (Corning, New York, USA) with peptone-yeast extract-glucose broth (Jacquier *et al.* 2013). EBs were purified as described elsewhere (Greub and Raoult 2002). For infection of epithelial cells, bacteria were recovered from a 4 day-old amoebal co-culture and filtered through a 5-μm filter (Millipore, Carrigtwohill, Ireland) to eliminate trophozoites and cysts.

Infection procedure

Epithelial cells were harvested from Corning culture flasks with 0.25% trypsin (Sigma-Aldrich, Buchs, Switzerland), washed with

fresh medium and seeded, 1 day before infection, at 2–5 $\times$ 10^5 cells per well in 24-wells microplates (Corning) or at 4 $\times$ 10^6 cells per 25 cm^2 flasks for RNA extraction and total protein preparation. Cells were infected with a 1/2000 dilution of bacteria (MOI 0.1–1). Plates were then centrifuged at 1790 g for 10 min at room temperature. After 15 min of incubation at 37°C in the presence of 5% CO_2, cells were washed with fresh medium to remove non-internalized bacteria and were then incubated for different periods of time at 37°C in a 5% CO_2 atmosphere.

Outer membrane proteins extraction and 2D gel electrophoresis

Purified EBs were incubated for 1 h at 37°C in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and 2% sodium lauroyl sarcosinate (sarkosyl) (Fluka BioChemika, Buchs, Switzerland). Detergent-insoluble complexes were pelleted by ultracentrifugation at 100 000 g for 1 h. Outer membrane proteins were solubilized by a 10-min incubation at 56°C in 0.125 M Tris-HCl (pH6.8), 0.33 M urea and 50 mM DTT. 100 μg of sarkosyl-insoluble proteins were resuspended in 37.5 mM Tris, 7 M urea, 2 M Thiourea, 50 mM DTT, 4% CHAPS, 0.5% Triton X-100 and 1% ASB and separated by 2D gel electrophoresis as described by Centeno *et al.* (2007). Proteins were visualized by Coomassie Blue staining or transferred to nitrocellulose for overlay assays.

Biotinylation of epithelial cell membrane proteins

Confluent monolayer of A549, Vero and Ishikawa cells in 75 cm^2 flasks were washed three times with ice-cold PBS and then scraped with a rubber policeman. 2.5 $\times$ 10^7 cells were incubated for 1 h at 37°C with 10 mg ml^{-1} EZ-link sulfo-NHS-LC-biotin (Pierce, Thermo Fisher Scientific, Lausanne, Switzerland) in PBS, washed three times with PBS, 100 mM glycin (Biosolve Chimie, Dieuze, France) and lysed by sonication in PBS. Cellular debris was removed by 15-min centrifugation at 13 000 rpm, 4°C and biotinylated proteins were kept at −80°C.

Overlay assays

Nitrocellulose membranes were blocked overnight in PBS, 0.05% Tween 20, 1% BSA and washed three times for 15 min in PBS, 0.05% Tween 20, 0.1% BSA. Membranes were incubated for 1.5 h at 4°C with biotinylated epithelial cells diluted 1/1000 in PBS, 0.05% Tween 20, 0.1% BSA. After three washing steps of 15 min with PBS, 0.05% Tween 20, 0.1% BSA, membranes were incubated during 1.5 h with horseradish peroxidase-labeled streptavidin (Amersham, GE Healthcare, Glattbrugg, Switzerland) diluted 1/10 000 in PBS, 0.05% Tween 20, 5% BSA. The membranes were then washed four times for 15 min with PBS, 0.05% Tween 20 and reactive proteins were detected with a chemiluminescence-based kit (Lite-Ablot, Euroclone, Milano, Italy).

Mass spectrometry

For identification of proteins, Coomassie Blue-stained spots were excised from 2D gels and transferred to special 96-well plates (Perkin Elmer Life Sciences, Schwerzenbach, Switzerland). MALDI-MS-MS analysis was performed on a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA) as described in Kebbi-Beghdadi *et al.* (2012). Briefly, in-gel proteolytic cleavage with trypsin was performed in an automated workstation, and digests were resuspended in alpha-cyano-hydroxycinnamic acid matrix before being deposited in duplicate on a target plate. After MALDI-TOF MS analysis, the 10 most intense ion signals were selected for MS/MS analysis. Non-interpreted peptide tandem mass spectra were used for direct interrogation of *W. chondrophila* open reading frames. With the parameters used, the threshold for statistical significance ($P < 0.05$) corresponded to a MASCOT score of 17, but only scores greater than 30 were considered. When proteins could not be identified by MALDI-TOF MS analysis, spots were reanalyzed by LC-MS followed by the same identification procedure as described above.

Purification of recombinant putative adhesins, *Escherichia coli* subcellular localization and antibody production

The *Waddlia* genes wcw_0063 (OmpA2), wcw_0122 (Pal) and wcw_0272 were cloned in pET200-D-TOPO (Invitrogen) as full-length proteins and expressed in *E. coli* BL21-DE3 after induction with 1 mM Isopropyl-β-D-thiogalactopyranoside (IPTG, Axon Lab, Baden-Dättwil, Switzerland). The same plasmid was used to express the C-terminal part of wcw_0271 (Pmp-like protein) (aa 893–1400) and of wcw_1162 (OmcB) (aa 240–520). Gene wcw_1343 (GroEL1) was cloned in pBAD and expressed following induction with 0.02% arabinose. All proteins were purified on Ni-NTA agarose (Qiagen, Hombrechtikon, Switzerland) under denaturing conditions with 6 M urea (except for GroEL1, non-denaturing conditions), following the manufacturer's instructions. Due to major problems in the purification of OmpA2, the production of this protein was successfully outsourced to GeneCust (Dudelange, Luxembourg). A total of 180 μg of purified proteins were used to immunize three mice with the classical protocol for custom polyclonal antibodies of Eurogentec (Liège, Belgium). Anti-GroEL antibodies are in-house mouse antibodies directed against the *Simkania negevensis* GroEL that shares 72% amino acids identity with the *Waddlia* protein and were obtained in the frame of another project. For the subcellular localization in *E. coli*, BL21-DE3 expressing the different proteins was lysed by sonication (six burst of 10 s at maximum power) in 20 mM Tris-HCl pH 8.0 and Halt protease inhibitor. Cytoplasmic proteins were recovered by centrifugation at 10 000 g for 5 min and the pellet corresponding to membrane proteins was resuspended in 0.5% sodium lauroyl sarcosinate (sarkosyl). After 15-min incubation at room temperature, inner (sarkosyl-soluble) and outer (sarkosyl-insoluble) proteins were separated by ultracentrifugation at 32 000 g during 30 min at 4°C. Sarkosyl-insoluble proteins were solubilized by a 10-min incubation at 56°C in loading buffer containing 50 mM DTT prior to separation by SDS PAGE.

Immunofluorescence and confocal microscopy

At 16 h post-infection (pi), cells cultivated on glass coverslips were fixed with ice-cold methanol for 5 min, washed three times with PBS and incubated overnight in blocking solution (PBS, 0.01% NaN_3, 1% BSA) at 4°C. Coverslips were incubated for 2 h at room temperature with polyclonal mouse antibodies diluted 1/10 (anti-GroEL 1/100) in PBS, 0.1% saponin, 1% BSA. After three washing steps in PBS, 0.1% saponin, coverslips were incubated for 1 h at room temperature with a 1/500 dilution of AlexaFluor 488-conjugated goat anti-mouse (Invitrogen) and a 1/1000 dilution of DAPI (dilactate, D3571, Molecular Probes, OR, USA). After washing twice with PBS 0.1% saponin, once with PBS and once with deionized water, the coverslips were mounted onto glass slides using Mowiol (Sigma-Aldrich). Cells were observed

under an epifluorescent microscope (Axioplan 2, Zeiss, Feldbach, Switzerland) and a confocal microscope (AxioPlan 2 LSM 510, Zeiss).

Western blot

At different time points after infection, HEp-2 cells were harvested by scraping the flask with a rubber policeman and the bacteria were lysed by a 2-h incubation at 37°C in 10 mM EDTA pH 9.5. Membrane and cytoplasmic proteins were separated by 1-h ultracentrifugation at 100 000 g at 4°C (Everett and Hatch 1995) and further solubilized in loading buffer prior to their separation by SDS PAGE and transfer on nitrocellulose membranes. Membranes were blocked by 2-h incubation with 5% non-fat dry milk in Tris-buffered saline with 0.05% Tween 20 (TBS), washed three times with TBS, 0.5% milk and incubated overnight at 4°C with mouse anti-adhesins sera (see above) diluted 1/100 in TBS, 0.5% milk or with mouse anti-His antibody diluted 1/3000 (Sigma-Aldrich). After three subsequent washes with TBS, 0.5% milk, membranes were probed with horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad, Cressier, Switzerland) diluted 1/3000 and washed three times with TBS. Immunoreactive spots were detected with a home-made chemiluminescence reagent containing coumaric acid and luminol in Tris buffer.

Gene expression

At different time points after infection, HEp-2 cells were harvested in TRIzol (Ambion^{R}, Life Technologies) by scraping the flask with a rubber policeman and RNA extracted as described in Chomczynski and Mackey (1995). cDNA was produced using random primers and the GoScript Reverse Transcription kit (Promega, Dübendorf, Switzerland). Quantitative PCR was performed on total cDNA using I Taq SYBRGreen technology (Bio-Rad), 4 μl of 1/25 cDNA sample and 300 nM of the following primers: 16S for: 5′ggcccttgggtcgtaaagttct3′/16S rev: 5′cggagttagccggtgcttct3′; wcw_0063 for: 5′tcaggcttccgcatcggtgt3′/wcw_0063 rev: 5′gagctgccgtctttgctgcg3′; wcw_0122 for: 5′agggg tccggactcttgccg3′/wcw_0122 rev: 5′ggatgatagcgcggctctgcc3′; wcw_0271 for: 5′ttcagcgcaaaggggagcc3′/wcw_0271 rev: 5′acag cgcgctccggttcaaa3′; wcw_0272 for: 5′tcctgccgatgaaggatgccct3′/wcw_0272 rev: 5′gcgcggcactgcgattgttg3′; wcw_1162 for: 5′ggggtgct ccaaccgtagcg3′/wcw_1162 rev: 5′ttgcatgccggctcacagca3′; wcw_1343 for: 5′acgaccgcgaaaaattgcaa3′/wcw_1343 rev: 5′gaatgccctcttccactgct3′; cycling conditions were 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Amplification and detection of PCR products were performed with the StepOne Real-Time PCR System (Applied Biosystems). qRT-PCR results were analyzed using the 16S rRNA gene as the endogenous control (Croxatto *et al.* 2013) and 40 h pi as the reference time point. Fold change values are normalized (maximum value = 100%, minimum value = 0%).

Adhesion assays

Escherichia coli BL21-DE3 were induced during 2.5 h with 1 mM IPTG (Axon Lab) or 0.02% arabinose to express the proteins of interest. They were incubated for 45 min at room temperature with 50 μg ml^{-1} fluorescein 5(6) isothiocyanate (FITC, Sigma-Aldrich), washed three times in PBS and diluted in cell culture medium to about 1×10^7 bacteria ml^{-1}. Bacteria were applied to HEp-2 cells seeded the day before at 5×10^5 cells per well in 24-wells microplates (Corning) on glass coverslips. Cells and bacteria were centrifuged for 10 min at 1790 g and incubated for 30 min at 37°C, 5% CO_2. Cells were then washed carefully three times with PBS and fixed for 10 min at room temperature in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA). Nucleic acids were stained by a 15-min incubation in DAPI 1/1000 (Molecular Probes). After washing twice with PBS and once with deionized water, the coverslips were mounted onto glass slides using Mowiol (Sigma-Aldrich). Cells were observed under an epifluorescent microscope (Axioplan 2, Zeiss, magnification 1000×) and the number of FITC-stained bacteria in 10 fields (=about 500 HEp-2 cells) were counted. Since the number of bacteria alive after the induction step can vary according to the expressed protein, the *E. coli* inoculum applied to HEp-2 cells was simultaneously plated on LB agar containing the appropriate antibiotic. CFU were counted and results of the adhesion assay were normalized to the number of CFU. Statistical analysis was performed using ordinary one-way ANOVA non-corrected for multiple comparisons (uncorrected Fisher's LSD test) on GraphPad Prism version 6.04 for Windows (GraphPad Software, La Jolla, CA, USA).

Yeast adhesion assays were performed as previously described (Mölleken, Schmidt and Hegemann 2010) except that incubation was performed during 1 h at 37°C.

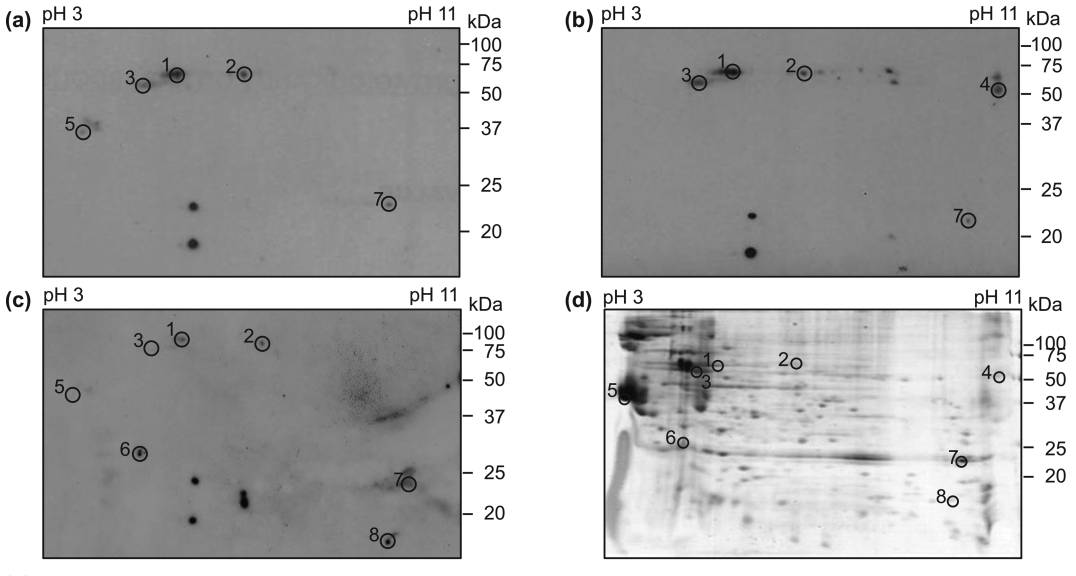
Binding assay with recombinant soluble protein

Binding of soluble recombinant proteins was assessed by overlaying confluent HEp-2 cells with 250 μl of culture medium containing the soluble recombinant protein of interest at various concentrations (10–160 μg ml^{-1}) and incubating for 30 min at 4°C or 1 h at 37°C. After extensive washing, cells were detached, pelleted for 5 min at 1000 × g and resuspended in 100 μl HBSS. Bound recombinant protein was then quantified by immunoblotting using anti-His antibody. A total of 3 μg of purified soluble recombinant protein was used as positive control in the Western blot and HEp-2 cells incubated without protein as negative control. Mouse anti-6His was used at a 1:2000 dilution and mouse anti-actin at a 1:3000 dilution. Secondary antibody (anti-mouse-AP) was used at 1:7500.

RESULTS

Identification of putative adhesins

To identify waddlial surface proteins that interact with eukaryotic cells, an enriched outer membrane protein fraction was obtained from EBs by sarkosyl extraction (Everett and Hatch 1995; Lienard *et al.* 2014). Proteins were separated by two- dimensional polyacrylamide gel electrophoresis (2D gels), transferred to nitrocellulose and probed in an overlay assay with biotinylated proteins of Vero, lung and endometrial cells, three cell lines that were previously shown to be permissive for this bacterium (Kebbi-Beghdadi, Cisse and Greub 2011). As depicted on Fig. 1a–c, eight protein spots could be detected that were not present in the negative control performed with unbiotinylated proteins. The Coomassie Blue-stained 2D gel obtained with the same *W. chondrophila* enriched membrane fraction (Fig. 1d) allowed the identification by MALDI-TOF MS of the proteins spots excised from the gel (Fig. 1e). Spots #1–3, detected with biotinylated proteins of the three cell lines, were identified as GroEL, a protein implicated in adhesion in *C. pneumoniae* (Wuppermann *et al.* 2008) while spot #7, also present on the three overlay assays, corresponded to hypothetical protein wcw_0272.



(e)

Spot	MS score	Gene number	Protein name
1	272	wcw_1343	**Chaperonin GroEL**
2	1308	wcw_1283	Pyruvate kinase
	1256	wcw_1849	**Chaperonin GroEL**
3	360	wcw_1396	**Molecular chaperone GroEL**
	74	wcw_0584	Elongation factor Tu
4	439	wcw_1162	**Putative 60 kDa cysteine-rich outer membrane protein (OmcB)**
	354	wcw_0064	Putative membrane protein (OmpA3)
5	356	wcw_0063	**Putative membrane protein (OmpA2)**
	150	wcw_0065	Putative membrane protein (OmpA4)
6	393	wcw_0330	Inorganic pyrophosphatase
	382	wcw_0272	**Hypothetical protein***
7	131	wcw_0272	**Hypothetical protein***
8	118	wcw_0122	**Putative peptidoglycan-associated lipoprotein precursor (Pal)**

*located immediately next to a putative Pmp-like protein (wcw_0271).

Figure 1. Overlay assays with epithelial cells. *Waddlia* membrane proteins separated by 2DGE were probed with biotinylated proteins of Vero cells (panel **a**), human pneumocytes (panel **b**) or human endometrial cells (panel **c**). Coomassie Blue staining is shown in panel **d**. Results of the MS analysis are presented in panel **e**. When more than one protein was identified in the same spot, only those with the two highest MS scores are presented. Putative adhesins indicated in bold were further characterized.

OmcB, a major component of chlamydial membrane with adhesive properties (Zhang and Stephens 1992; Fadel and Eley 2007; Moelleken and Hegemann 2008; Fechtner *et al.* 2013), was retrieved in spot #4 and two members of the OmpA protein family, described in 2010 by Bertelli *et al.* (2010) as a large family of 11 porins with β-barrel structure, were identified in spot #5. Spots #6 and #8 were only detected in the overlay assay with endometrial cells (Fig. 1c) and were identified as hypothetical protein wcw_0272 and peptidoglycan-associated lipoprotein precursor (Pal), respectively.

Interestingly, a putative autotransporter protein that might belong to the Chlamydia-specific family of Pmps (Bertelli *et al.* 2010) is located on the *Waddlia* genome immediately next to the hypothetical protein wcw_0272 identified in spots #6 and #7. Since Pmps are well-characterized adhesins in *Chlamydia* (Wehrl *et al.* 2004; Mölleken, Schmidt and Hegemann 2010; Becker and Hegemann 2014), the proximity of these two proteins on the genome could be more than a coincidence, and consequently, the Pmp-like protein wcw_0271 was selected for further characterization in addition to the five putative *W. chondrophila* adhesins identified by the proteomic approach described above (OmpA2, retrieved in spot #5,was chosen as representative of the OmpA family proteins).

Transcriptional profile of the putative adhesins during the course of a replication cycle

The transcriptional expression of the genes encoding the six putative adhesins was assessed by qRT-PCR at different time points during the course of an infection of HEp-2 cells by *W. chondrophila*. According to microscope observations, EBs differentiate into RBs as early as 3 h pi in this cell line. RBs multiply exponentially between 8 and 32 h and then asynchronously redifferentiate into infectious EBs that are released by host cell lysis. The replication cycle is completed in 48 h and reinfection of new host cells can be observed at this time point (data not shown). Interestingly, the expression profiles displayed on Fig. 2 revealed that the six putative adhesin genes can be clustered in four groups. Those expressed early during the replication cycle, such as *groEL* and *pal* (Fig. 2a), whose transcripts are detected 3 h pi, reach a maximum level of expression 8 or 16 h pi and drastically decrease at the end of the cycle. In contrast, *wcw_0272* is clearly a late gene, being only expressed when RBs start to redifferentiate into EBs and the number of transcripts staying high until the end of the replication cycle (Fig. 2b). The *omcB* and Pmp-like protein genes are mid-late genes with a peak of expression during the replicative phase, at 24 h pi and 16 h pi, respectively. The *ompA2* gene displays a different profile, transcripts being

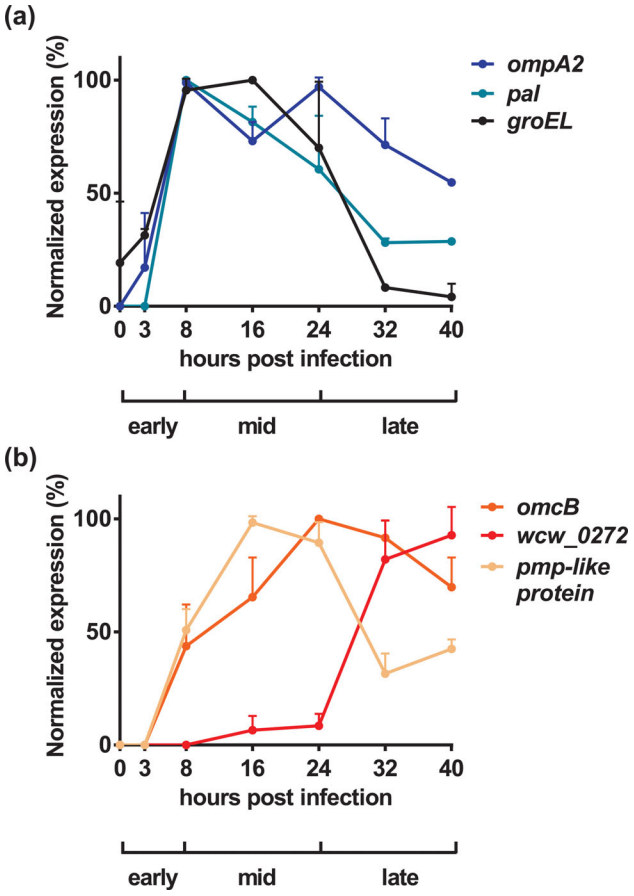


Figure 2. Temporal transcriptional expression of the putative adhesin genes. Transcriptional expression of genes coding for putative adhesins was analyzed by qRT-PCR. Early and early-mid genes are presented in panel **a**, while mid-late and late genes are presented in panel **b**. Results are the means and SD of three independent experiments.

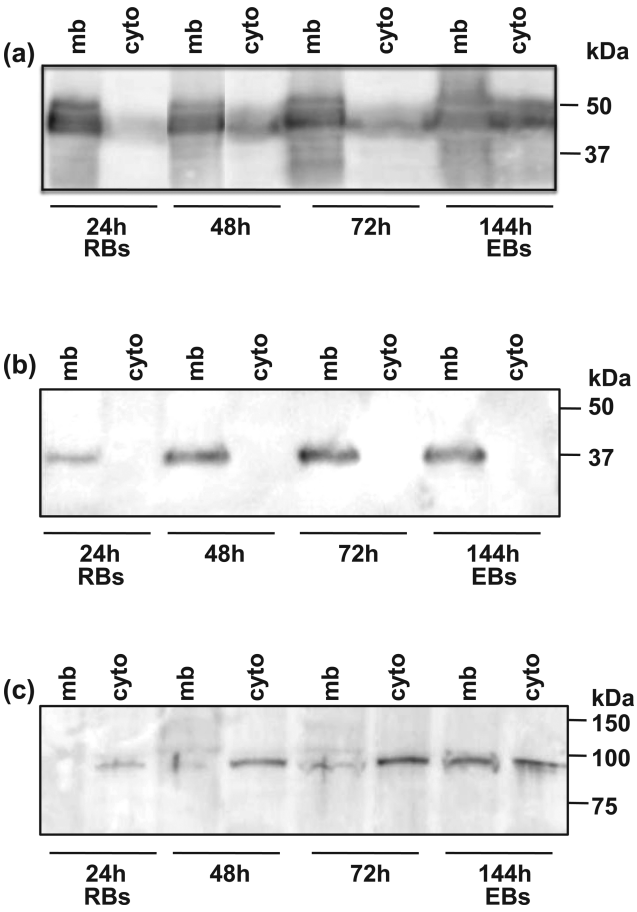


Figure 3. Protein expression during the replication cycle. Detection of OmcB (**a**), OmpA2 (**b**) and Pmp-like protein (**c**) in the membrane or cytoplasmic fractions of *Waddlia*-infected HEp-2 cells at different time points following infection.

detected already 3 h pi and their level staying high all through the replicative stage of the bacteria and only slightly decreasing when RBs re-differentiate into EBs. The high level of *ompA2* gene transcription during most of the developmental cycle of the bacteria might reflect important roles played by this protein and most probably by all members of the OmpA protein family during *Waddlia* life cycle. The nucleotide sequence identity between *ompA* genes wcw_0063, wcw_0064 and wcw_0065 is about 80% and this qRT-PCR directed against *ompA2* transcripts (wcw_0063) probably also recognize *ompA3* and *ompA4* transcripts.

Protein expression analyzed by immunofluorescence and Western blot

The six putative adhesins were expressed in *E. coli* with a 6His tag, either as full-length proteins or, if expression levels were too low, as truncated molecules (OmcB aa 240–520, Pmp-like protein aa 893–1400). Five of them were purified on Ni-NTA agarose and polyclonal mouse antisera against these proteins were produced. Hypothetical protein wcw_0272 could not be purified despite extensive efforts and is therefore absent from experiments requiring antibodies (immunofluorescence and Western blot).

As depicted in Fig. 3, antibodies against OmcB, OmpA2 and Pmp-like protein recognized their target protein by Western blot in a lysate of HEp-2 cells infected with *Waddlia* and analyzed at different time points after infection. The antibodies raised against the C-terminal part of OmcB recognized the full-length protein (about 50 kDa) in the membrane fraction of RBs and EBs (Fig. 3a). In EBs, the protein seems to be also present in the cytoplasmic fraction. Due to high amino acid sequence similarity between OmpA2 and OmpA3 or OmpA4 (74.9 and 72.6% identity), the polyclonal antibodies produced against OmpA2 probably also recognize the two other proteins whose molecular weights are identical. These proteins are detected very clearly in RBs and EBs, exclusively in the membrane fraction (Fig. 3b). Antibodies directed against aa 893–1400 of the Pmp-like protein recognized a protein of about 100 kDa only present in the cytosolic fraction of RBs but in both membrane and cytosolic fractions of EBs.

The anti-GroEL antibody strongly reacted with the homologous eukaryotic protein, thus hampering the localization of the bacterial protein. Finally, the peptidoglycan-associated lipoprotein (Pal) could not be detected by Western blot in *Waddlia*-infected cells despite the fact that the antibodies were fully able to recognize the purified recombinant protein (data not shown). This negative result is likely to be due to an overall low amount of the protein or to a damaging effect of the alkaline lysis protocol on Pal epitopes.

As shown in Fig. 4, OmcB, OmpA2, Pmp-like protein, Pal and GroEL could all be detected at the surface of RBs (16 h pi) by immunofluorescence. They could also be detected at the surface of EBs but only if disulfide bridges were reduced by DTT treatment.

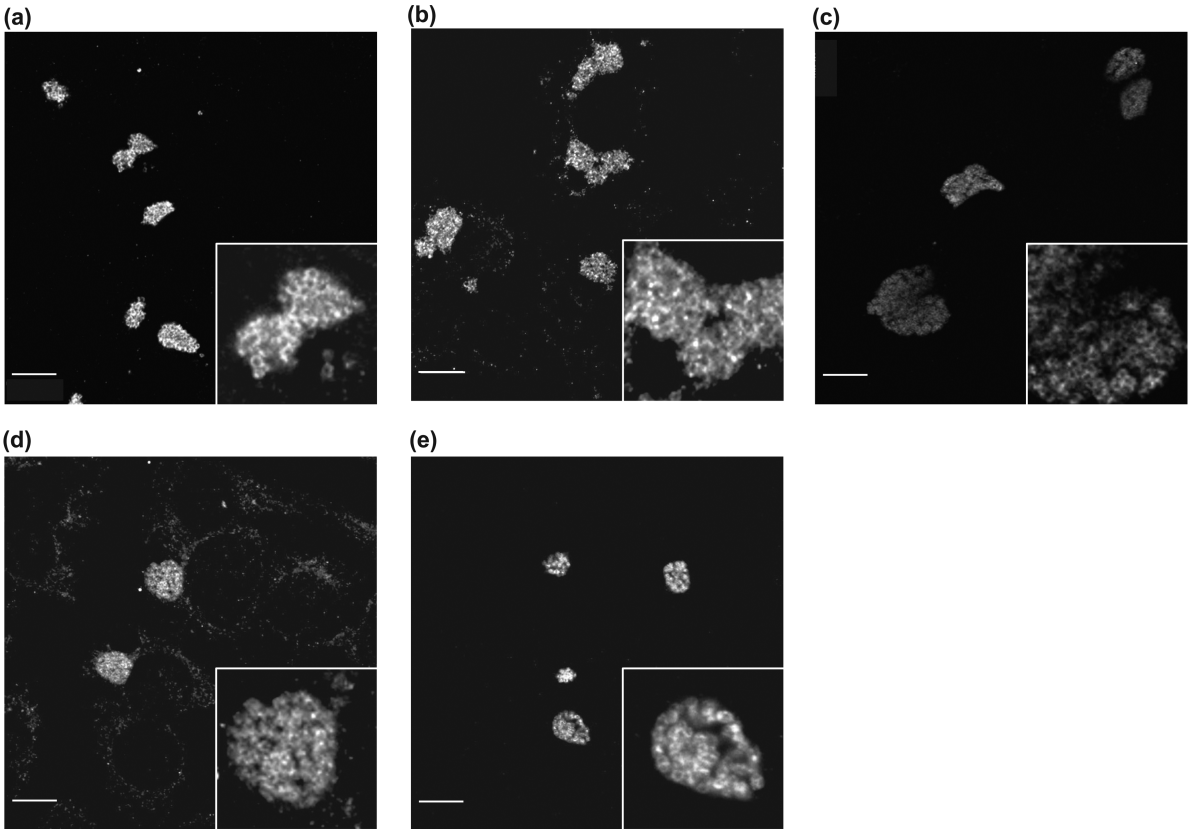


Figure 4. Localization of putative adhesins by immunofluorescence. *Waddlia*-containing vacuoles in HEp-2 cells 16 h pi. Bacteria are stained with polyclonal antibodies against OmcB (panel **a**), OmpA2 (panel **b**), Pmp-like protein (panel **c**), Pal (panel **d**) or GroEL (panel **e**). Bar: 10 μm. Inset magnification: 2.7×.

Adhesion assays

To assess the adhesive properties of the five proteins identified in the first part of this work, we incubated HEp-2 cells with a non-invasive strain of *E. coli* expressing these putative adhesins on their surface and reported the number of adherent bacteria per 100 cells. The *E. coli* surface expression of the proteins of interest was confirmed by microimmunofluorescence and by Western blot on sarkosyl-extracted proteins (i.e. outer membrane proteins) using anti-His antibodies (data not shown). Since OmcB could not be expressed in *E. coli* as a full-length protein (see above), the adhesion assay was performed with its C-terminal part (aa 240–520). As depicted in Fig. 5a, *E. coli* expressing OmpA2 were able to adhere to HEp-2 cells about 10 times more efficiently than the control strain (*P* value <0.01). Strains expressing the four other putative adhesins showed adhesion properties not statistically different from the negative control. To further investigate the adhesion potential of members of the OmpA family, *E. coli* expressing OmpA3 were also tested in the same adhesion assay (Fig. 5b). These bacteria were able to adhere to epithelial cells about twice more efficiently than OmpA2 expressing *E. coli*, 20 times more bacteria being attached to HEp-2 cells than with the reference strain (*P* value <0.0001).

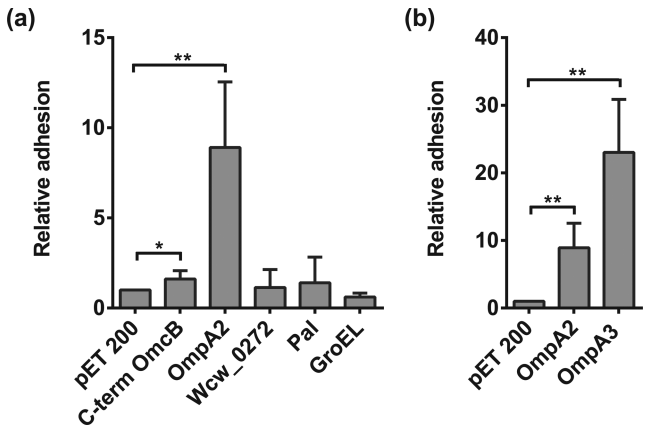


Figure 5. *Escherichia coli* adhesion assay. (**a**) Adhesion of *E. coli* strains expressing five putative adhesins to HEp-2 cells. The number of adherent bacteria per 100 cells was normalized to wild-type *E. coli* (negative control pET200 = 1). Results are the means and SD of at least two independent experiments performed in duplicates. $^{*}P < 0.05$, $^{**}P < 0.01$. (**b**) Adhesion of OmpA2 and OmpA3 expressing *E. coli* to HEp-2 cells. Results are the means and SD of at least three independent experiments performed in duplicates. $^{**}P < 0.01$.

As shown in Fig. 6a, the Pmp-like protein of *W. chondrophila* displays similar structural features with the *C. pneumoniae* Pmp21, with an N-terminal signal sequence followed by a passenger domain and a C-terminal β-barrel domain. In addition, its amino acid sequence includes six repeats of the FxxN motif present in 17 locations in the Pmp21 sequence. Because of these similarities, the adhesive properties of the Pmp-like protein were assessed with the yeast adhesion assay previously used to report the adhesion properties of the *Chlamydial* Pmps (Mölleken, Schmidt and Hegemann 2010; Mölleken, Becker and Hegemann 2013; Becker and Hegemann 2014). Briefly, yeast cells expressing the surface protein Aga2 either alone or fused to part of the passenger domain of *C. pneumoniae* Pmp21 (Pmp21D: aa 942–1145)

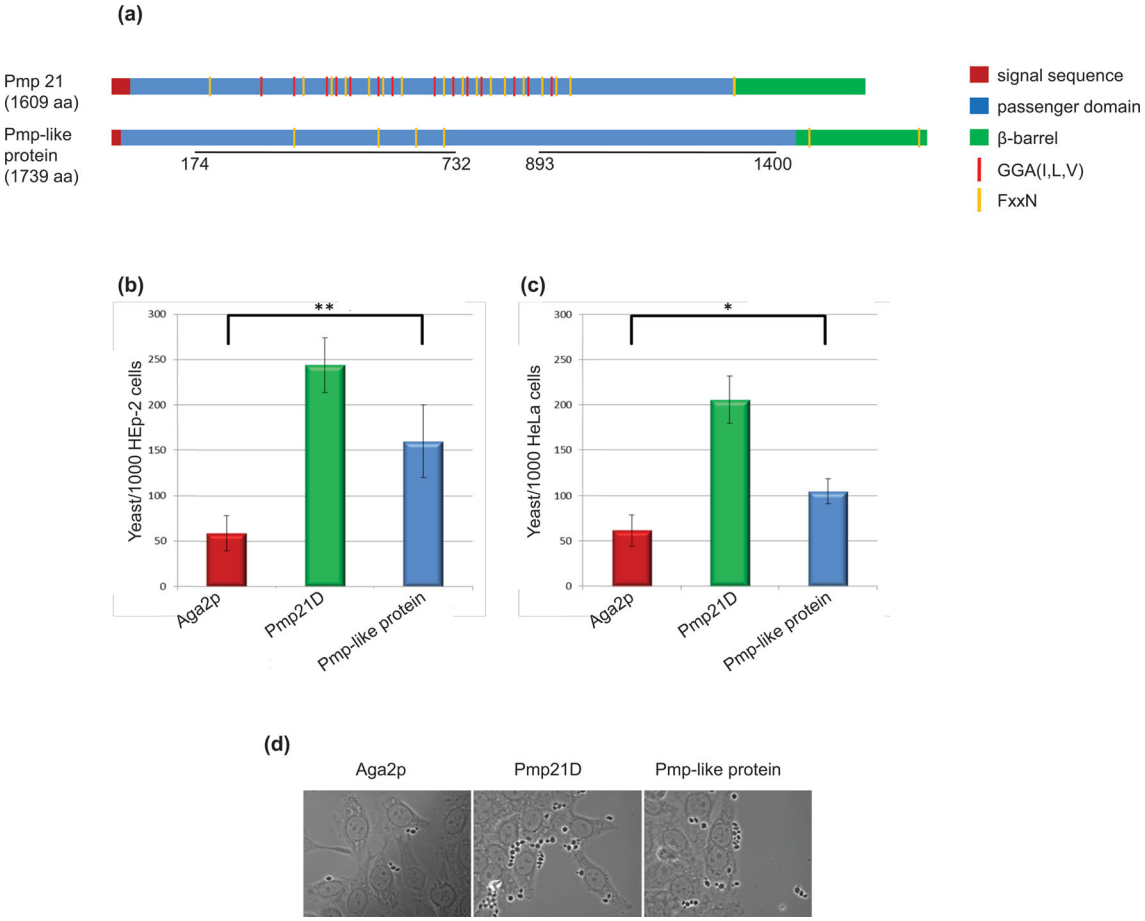


Figure 6. Structural features and adhesive properties of Pmp-like protein. (**a**) Schematic representation of *C. pneumoniae* Pmp21 and *W. chondrophila* Pmp-like protein with their signal sequence in red, passenger domain in blue and β-barrel domain in green. Multiple repeats of the tetrapeptides GGA(LIV) or FxxN are represented as red and yellow bars, respectively. The passenger domain segment used in the yeast adhesion assay and in the soluble recombinant adhesion assay (aa 174–732) and the segment used to raise antibodies (aa 893–1400) are indicated. (**b**) Adhesion of yeast cells expressing Aga2 alone (negative control), Aga2 fused to aa 942–1145 of *C. pneumoniae* Pmp21 (Pmp21D; positive control) or Aga2 fused to aa 174–732 of *Waddlia* Pmp-like protein on HEp-2 cells. Results are the mean +/− SD of six independent experiments. $^{**}P < 0.01$. (**c**) Adhesion of yeast cells expressing Aga2 alone (negative control), Aga2 fused to aa 942–1145 of *C. pneumoniae* Pmp21 (Pmp21D; positive control) or Aga2 fused to aa 174–732 of *Waddlia* Pmp-like protein on HeLa cells. Results are the mean +/− SD of three independent experiments. $^{*}P < 0.05$. (**d**) Micrographs of HEp-2 cells incubated with yeast cells expressing the indicated proteins on their surface (magnification 630×).

(Mölleken, Schmidt and Hegemann 2010) and of *Waddlia* Pmp-like protein (aa 174–732) were applied to confluent HEp-2 (Fig. 6b) or HeLa cells (Fig. 6c), and the number of yeast cells attached were counted under the microscope. As depicted in Fig. 6b–d, yeast cells expressing Aga2 fused to the passenger domain of *Waddlia* Pmp-like protein adhered to human cells three times (HEp-2 cells) or two times (HeLa cells) more efficiently than the negative control expressing Aga2 alone. Adhesion of yeast cells expressing Aga2 fused to the passenger domain of Pmp21 (positive control) was respectively five times and four times more efficient than adhesion of the negative control.

Binding of a soluble recombinant fragment of *Waddlia* Pmp-like protein to HEp-2 cells

Binding of soluble recombinant proteins was assessed by overlaying confluent HEp-2 cells with culture medium containing various concentrations of either a soluble fragment of *Waddlia* Pmp-like protein (aa 174–732, see Fig. 6a) or, as a positive control, a soluble fragment of *C. pneumoniae* Pmp21 (Pmp21M) (Mölleken, Becker and Hegemann 2013). Incubation was performed either during 30 min at 4°C or during 1 h at 37°C, and bound recombinant proteins were quantified by immunoblotting using anti-His antibodies.

As shown in Fig. 7, the soluble fragment of *Waddlia* Pmp-like protein bound to HEp-2 cells with higher efficiency at 37°C than at 4°C (Fig. 7a and b). A similar result was obtained with a soluble fragment of *C. pneumoniae* Pmp21 except that binding already occurs with lower concentrations of the soluble protein (Fig. 7c and d). In contrast, no binding can be detected when cells are incubated with a His-tagged Glutathione-S-transferase (Fig. 7e).

Altogether, results obtained in the yeast adhesion assay and in the soluble protein binding assay clearly indicated that *Waddlia* Pmp-like protein is able to mediate adhesion to epithelial cells similarly to what is observed for *Chlamydia* Pmps.

DISCUSSION

In this study, we used a proteomic approach conjugated to classical overlay assays to detect putative adhesins of *W. chondrophila*. These experiments performed on a membrane protein-enriched fraction of the bacteria revealed eight protein spots that specifically reacted with lysates of three different eukaryotic host cells, simian kidney epithelial cells (Vero), human

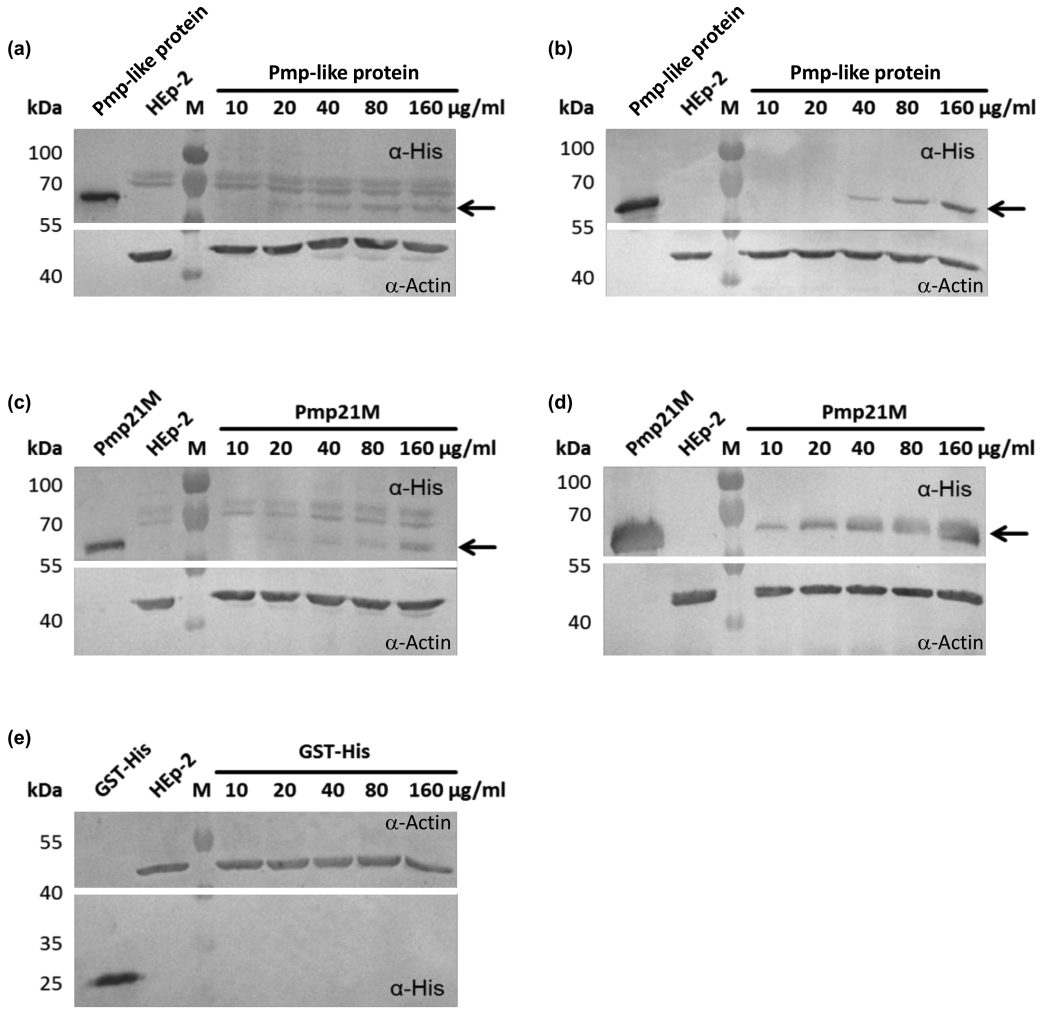


Figure 7. Binding of a soluble recombinant fragment of the Pmp-like protein to HEp-2 cells. Various concentrations of a His-tagged soluble recombinant fragment of the Waddlia Pmp-like protein (panel **a** and **b**), a His-tagged soluble fragment of *C. pneumoniae* Pmp21 (Pmp21M) (panel **c** and **d**) or a His-tagged GST were incubated with HEp-2 cells either during 30 min at 4°C (panels a, c and **e**) or during 1 h at 37°C (panels b and d). Bound recombinant proteins were visualized by Western blot using an anti-His antibody to detect the protein of interest and an anti-actin antibody as internal control. Purified proteins were loaded in the first well as positive control and HEp-2 cells not incubated with proteins were loaded in the second well as negative control.

pneumocytes (A549) and human endometrial cells (Ishikawa). Interestingly, some reactive protein spots are common to the three cell lines but two of them seem to be specific for Ishikawa cells and one is only detected by the pneumocytes lysate. This result indicates that some of the molecules implicated in adhesion vary according to the host cell, which, when considering the large diversity of eukaryotic hosts able to support entry and replication of these bacteria, probably reflect the existence of multiple adhesion and entry pathways for *Waddlia*. MS analysis of these eight reactive spots led to the clear identification of five proteins, among which are OmcB and GroEL, two proteins already described as important adhesins in *Chlamydia* (Moelleken and Hegemann 2008; Wuppermann *et al.* 2008; Fechtner *et al.* 2013). Their retrieval in this analysis proved that our approach indeed identified molecules interacting with the host cell.

A striking feature of the MS identification is the presence in two reactive spots of several members of the OmpA protein family. This family, first described by Bertelli *et al.* (2010), contains 11 cystein-rich proteins with a putative β-barrel structure (Bertelli *et al.* 2010). A recently published study revealed that proteins of this family are very abundant in the outer membrane fraction of *Waddlia* and are immunogenic (Lienard *et al.* 2014). Results presented here suggest a strong implication of at least two members of this protein family, OmpA2 and OmpA3, in adhesion. Thus, *Waddlia* can probably rely on the large diversity of this protein family not only to avoid host immune surveillance but also to adhere to and enter a wide variety of eukaryotic cells. In the *Chlamydiales* order, such an extended family of OmpA proteins has also been reported for *S. negevensis* (Aistleitner *et al.* 2015) and this diversity again correlates with a wide host range for this bacteria that was first isolated as a contaminant of mammalian cell culture (Kahane, Metzer and Friedman 1995; Kahane *et al.* 1999) and further demonstrated to grow in amoebae (Kahane *et al.* 2001; Knab *et al.* 2011) as well as in insect cells (Sixt *et al.* 2012). Similarly, the recently discovered *Chlamydiales* bacteria, *Estrella lausannensis*, also possess an extended OmpA protein family (Bertelli *et al.* 2015) and displays a wide host range that includes mammalian, fish and insect cells as well as multiple amoebae (Kebbi-Beghdadi, Batista and Greub 2011; Lienard *et al.* 2011; Rusconi *et al.* in press, Kebbi-Beghdadi and Greub 2014). Analysis of the transcription profile of *ompA2* gene during the bacterial replication cycle revealed that transcripts are produced

all through the cycle. Expression starts early, when EBs differentiate into RBs, and stays high during the replication phase and even when RBs redifferentiate into EBs. In addition, OmpA proteins can be detected in the bacterial membranes in EBs and in RBs. This continuous expression of OmpA transcripts and proteins throughout the life cycle of *Waddlia* suggest that these proteins play crucial roles in the biology and pathogenesis of the bacteria.

Our screening for proteins interacting with host cells revealed the implication of hypothetical protein Wcw_0272, a cystein-rich protein present in the outer membrane of EBs (Lienard *et al.* 2014; Aistleitner *et al.* 2015). Analysis of the transcriptional profile of this gene indicated a late expression, at the stage of RBs to EBs redifferentiation, a result that is consistent with a role of this protein in adhesion. Interestingly, a homolog of Wcw_0272 (Pah_c050o014/PUV_11160), very abundant in the outer membrane of *Parachlamydia acanthamoebae* (Aistleitner *et al.* 2015), is also implicated in adhesion (C. Kebbi, unpublished data). Furthermore, protein Wcw_0272 is located on the genome immediately next to Wcw_0271, a putative Pmp-like protein displaying some structural similarities with chlamydial Pmps. This large family of autotransporters has been extensively studied and shown to be implicated in adhesion to epithelial and endothelial cells (Mölleken, Schmidt and Hegemann 2010; Mölleken, Becker and Hegemann 2013; Becker and Hegemann 2014). The *Waddlia* Pmp-like protein was detected by immunofluorescence at the surface of the bacteria and by Western blot as a single band of about 100 kDa in lysates of infected cells. This band probably represents a cleaved form of the protein whose full length predicted molecular weight is 150 kDa. Autotransporters are usually able to cleave their N-terminus (Henderson and Lam 2001; Vandahl *et al.* 2002), and indeed numerous cleaved forms of Pmps have been observed in *Chlamydia* (Grimwood, Olinger and Stephens 2001; Vandahl *et al.* 2001; Montigiani *et al.* 2002). Since polyclonal antibodies against Pmp-like protein have been obtained using a fraction of the protein (aa 893–1400), other undetected cleaved forms could exist.

Chlamydial Pmps are very diverse and display little similarity in their amino acid sequences; however, they all contain between 4 and 17 repeats of the tetrapeptide GGA(LIV) and between 4 and 19 repeats of the FxxN sequence, two motifs that were shown to be essential for the adhesion function of these proteins (Mölleken, Schmidt and Hegemann 2010; Becker and Hegemann 2014). *Waddlia* Pmp-like protein contains six repeats of the tetrapeptide motif FxxN but none of the GGA(ILV) motif. However, results obtained in the yeast adhesion assay with a protein fragment harboring four FxxN motifs are conclusive and definitely prove the adhesive properties of this region. Moreover, the same fragment expressed as a soluble recombinant protein was demonstrated to exhibit increased adhesion properties on HEp-2 cells at 37°C than at 4°C. Since *W. chondrophila* is able to enter and efficiently multiply at 25°C in amoebae, that could represent its natural reservoir, and at 25 or 28°C in fish and insect cell lines, respectively (Kebbi-Beghdadi, Batista and Greub 2011; Kebbi-Beghdadi *et al.* in preparation), it would be interesting to compare the adhesion properties of the soluble fragment at different temperatures and to determine the most suitable temperature for Pmp-like mediated binding to host cells. The Pmp-like protein was not retrieved among the 50 (respectively 40) most abundant proteins in the outer membrane protein fraction of *Waddlia* in two independent studies (Lienard *et al.* 2014; Aistleitner *et al.* 2015).This low abundance conjugated with a large size that hampers its resolution by 2D gel electrophoresis could explain why it was not detected in the overlay assay.

Negative results obtained in the *E. coli* adhesion assay with all putative adhesins identified in the overlay assay except the OmpA2 and OmpA3 are puzzling. However, for OmcB they can be explained since the fragment used in this experiment is the C-terminal part of the protein (aa 240–520) and since it was demonstrated in *Chlamydia* that the N-terminal part of the protein is surface exposed and binds to heparan sulfate-like GAGs (Moelleken and Hegemann 2008; Fechtner *et al.* 2013). We could not confirm a role in adhesion for the peptidoglycan-associated lipoprotein (Pal) but this protein was recently shown to be involved in cell division (Jacquier *et al.* submitted). Negative results obtained with GroEL and protein Wcw_0272 might be explained by assay limitations due to overexpression in an heterologous system that might induce misfolding or abnormal presentation of the protein binding sites at the cell surface.

In the end, results obtained in this study revealed that the putative Pmp-like protein Wcw_0271, the only representative in *W. chondrophila* of the very large chlamydial Pmp family, is implicated in adhesion together with several members of the extended OmpA protein family that could represent functional counterparts of the chlamydial MOMP. Thus, contrarily to classical *Chlamydia* that exhibit a single MOMP and various Pmps, *W. chondrophila* express a single Pmp-like protein but various OmpA proteins, acting as adhesins and likely explaining the wide host range of this strict intracellular bacteria as well as the high diversity of targeted cell lines.

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Conflict of interest. None declared.

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