Conserved features and major differences in the outer membrane protein composition of chlamydiae

Karin Aistleitner,1 Dorothea Anrather,2 Thomas Schott,1 Julia Klose,3 Monika Bright,3 Gustav Ammerer4 and Matthias Horn1*

Departments of 1Microbiology and Ecosystem Science, 2Mass Spectrometry Facility, Max F. Perutz Laboratories, 3Limnology and Oceanography, 4Department of Biochemistry and Cell Biology, Max F. Perutz Laboratories, University of Vienna, Vienna, Austria.

Summary

Chlamydiae are a highly successful group of obligate intracellular bacteria infecting a variety of eukaryotic hosts. Outer membrane proteins involved in attachment to and uptake into host cells, and cross-linking of these proteins via disulfide bonds are key features of the biphasic chlamydial developmental cycle. In this study, we used a consensus approach to predict outer membrane proteins in the genomes of members of three chlamydial families. By analysing outer membrane protein fractions of purified chlamydiae with highly sensitive mass spectrometry, we show that the protein composition differs strongly between these organisms. Large numbers of major outer membrane protein-like proteins are present at high abundance in the outer membrane of Simkania negevensis and Waddlia chondrophila, whereas yet uncharacterized putative porins dominate in Parachlamydia acanthamoebae. Simkania represents the first case of a chlamydia completely lacking stabilizing cysteine-rich proteins in its outer membrane. In agreement with this, and in contrast to Parachlamydia and Waddlia, the cellular integrity of Simkania is not impaired by conditions that reduce disulfide bonds of these proteins. The observed differences in the protein composition of the outer membrane among members of divergent chlamydial families suggest different stabilities of these organisms in the environment, probably due to adaptation to different niches or transmission routes.

Introduction

Chlamydiae are a group of obligate intracellular bacteria whose members colonize a variety of eukaryotic hosts, ranging from amoebae to insects, reptiles, birds and mammals, including humans (Horn, 2008). All chlamydiae switch between two morphologically and metabolically distinct stages during their developmental cycle: the smaller, infectious elementary body (EB) characterized by its condensed DNA, which represents the extracellular life stage, and the larger reticulate body (RB) that represents the intracellular and replicating life stage. The cycle starts with the adhesion of the EB to a host cell, followed by the uptake and differentiation into a RB inside a host-derived vacuole (Hackstadt et al., 1997). RBs divide several times before they re-differentiate into EBs and exit from the host cell (Abdelrahman and Belland, 2005; Hybiske and Stephens, 2007; Horn, 2008). Both developmental stages are well adapted to serve their designated purpose. EBs, which need to persist in the environment in order to infect new host cells, are extremely stable (Tamura and Higashi, 1963; Omsland et al., 2012) and have only limited metabolic capabilities (Haider et al., 2010; Omsland et al., 2012; Sixt et al., 2013), but are preloaded with effector proteins that are crucial for infection (Clifton et al., 2004). In contrast, RBs are very efficient in the acquisition of nutrients from the host cell to fuel their fast metabolic turnover during replication, but are not infectious and more fragile than EBs (Omsland et al., 2012). These differences in stability, infectivity and substrate uptake are linked not only to changes in the protein composition of the outer membrane, but also to changes in the redox state of the cysteine residues of these proteins during the progression of the developmental cycle (Hatch et al., 1984; Hatch, 1996). The importance of cysteine residues for chlamydial development is illustrated by the observation that cysteine deprivation inhibits re-differentiation of RBs to EBs (Allan et al., 1985).

Because of their significance for human health and the important role of outer membrane proteins (OMPs) for vaccine development (Hafner et al., 2014), the OMP composition of members of the Chlamydiaceae, comprising human pathogens like Chlamydia trachomatis and C. pneumoniae, has been extensively studied. The most
abundant protein in the outer membrane of the *Chlamydiaeae* is the major outer membrane protein (MOMP) which makes up  ~ 60% of the protein content of the outer membrane in EBs (Caldwell et al., 1981) and functions as porin (Bavoil et al., 1984). The permeability of the outer membrane is partly controlled by the oxidation state of the disulfide bridges between the cysteine residues of MOMP, with drastically enhanced pore-forming activity after their reduction (Bavoil et al., 1984). The remarkable rigidity of chlamydial EBs is thought to be the result of extensive disulfide bridge formation between two cysteine-rich OMPs, OmcA and OmcB, which represent together with MOMP the main constituents of the chlamydial outer membrane complex (COMC) (Hatch, 1996). These disulfide bridges are reduced after entry into the host cell accompanied by a decrease in the amount of OmcA and OmcB in RBs (Hatch et al., 1986; Newhall, 1987), leading to a more flexible and fragile life stage. Also, the redox state of certain proteins of the type three secretion system is linked to the developmental cycle and was suggested to regulate its activity (Betts-Hampikian and Fields, 2011).

A recent study that analysed the outer membrane composition of the amoeba symbiont *Protochlamydia amoebophila*, a member of the *Parachlamydiaceae*, showed that some, but not all of these main components, are conserved among chlamydiae (Heinz et al., 2009; 2010). Proteins homologous to OmcA and OmcB were also found at high abundance in *Protochlamydia*, suggesting a common mechanism for the stabilization of the cell wall in different chlamydial families. In contrast, no protein with similarity to MOMP was found in this organism (Heinz et al., 2010). Instead, a novel family of highly abundant porins represents the functional replacement of MOMP in *Protochlamydia* (Aistleitner et al., 2013). Sequencing and analyses of the genomes of members of other chlamydial families suggested an even higher diversity with respect to OMPs (Bertelli et al., 2010; Collingro et al., 2011).

In this study, we analysed the OMP composition of members of three chlamydial families originating from very different sources and showing different host ranges. *Parachlamydia acanthamoebae* was isolated from activated sludge (Collingro et al., 2005), *Waddlia chondrophi*a from an aborted bovine foetus (Rurangirwa et al., 1999) and *Simkania negevensis* was discovered in a contaminated mammalian cell culture (Kahane et al., 1993). We used a combination of *in silico* tools for the prediction of OMPs in the genomes of these organisms, experimentally enriched OMPs from purified chlamydial EBs and analysed these fractions by highly sensitive mass spectrometry. We show that there are major differences in their OMP composition and that *Simkania* lacks cysteine-rich proteins in its outer membrane, a feature so far assumed to be essential for all chlamydiae to stabilize their outer membrane.

**Results**

**A consensus approach for *in silico* prediction of chlamydial OMPs**

Most OMPs share features specific for this group of proteins and various *in silico* tools exist for their identification in genomes based on alternating hydrophobicity patterns, amino acid composition, N-terminal and C-terminal patterns, or homology detection. We used three subcellular localization (PSORTb, CELLO, SOSUI-GramN), three beta-barrel conformation (BOMP, MCMBB, TMBETADISC-RBF) and two lipoprotein (LIPO and LipoP) prediction tools to identify OMPs in the genomes of *Parachlamydia, Simkania* and *Waddlia* (Fig. S1). There were striking differences in the numbers of predicted OMPs for the different tools. For example, the beta-barrel prediction tool MCMBB predicted 724 proteins as beta-barrel proteins in the genome of *Parachlamydia*, in contrast to 92 proteins predicted by TMBETADISC-RBF and only 38 by BOMP (Fig. S1).

To increase the confidence of our predictions, we decided to establish a consensus prediction approach based on the combination of different tools (Fig. 1) as this has been shown to enhance the prediction performance (Shen and Burger, 2007; Horler et al., 2009; Goudenege et al., 2010; E-komon et al., 2012). We evaluated the prediction performance first by using an existing set of proteins of Gram-negative bacteria of known location (E-komon et al., 2012), and very stringent criteria were ideal for the identification of OMPs in this protein set (Table S1). However, the percentage of correct predictions dropped when a second, smaller test set consisting of only chlamydial proteins of known location (Table S2) was used, mainly due to a lower percentage of true positive predictions (Table S1). Based on the evaluation of prediction parameters with the chlamydial dataset, we chose the following criteria: at least two positive predictions by localization prediction tools or beta-barrel prediction tools for predicting OMPs; an additional prediction by a lipoprotein prediction tool for outer membrane lipoproteins; and at least one positive prediction by a lipoprotein prediction tool for lipoproteins in general (Fig. 1).

For a further evaluation of our consensus prediction approach, OMPs were predicted in the genomes of *C. trachomatis* and *Protochlamydia*, two organisms for which experimental data are available on the protein composition of the outer membrane. This showed a good agreement of *in silico* predictions with experimental data (Table S3).

Application of our *in silico* consensus approach for the prediction of OMPs to the genomes of *Parachlamydia,*...
Simkania and Waddlia lead to the identification of 64, 84 and 50 putative OMPs (including outer membrane lipoproteins) respectively (Table S4).

Optimization of the enrichment of OMPs from purified chlamydiae

Most OMPs make up only a small fraction of the overall protein content of a cell, and different methods exist for their enrichment. We evaluated three popular methods for the enrichment of OMPs by treating purified Simkania EBs with N-lauroylsarcosine (sarkosyl), n-octyl polyoxyethylene (n-POE) or Triton X-114. Band patterns were similar after treatment with sarkosyl and n-POE, but differed for Triton X-114 (Fig. 2). Mass spectrometry analysis of in-gel digests of proteins showed that sarkosyl and n-POE both worked well for the enrichment of OMPs – although Simkania might be more tightly associated with host organelles than other chlamydiae (Mehlitz et al., 2014) – as this class of proteins was strongly enriched and included the most abundant proteins for both treatments (Fig. 3). Although OMPs were also present in the Triton X-114-treated sample, cytoplasmic proteins and proteins of the amoeba host were much more abundant than for the other two detergents (Fig. 3). Fifty-three predicted OMPs were identified with all three detergents, but nine were only found after treatment with sarkosyl or n-POE (Fig. S2; Table S5). For Parachlamydia, the

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**Fig. 1.** Workflow for the prediction of outer membrane proteins in the proteomes of chlamydiae. A combination of in silico tools was used for the prediction of the subcellular location, possible beta-barrel conformation or the presence of lipoprotein signal peptides and transmembrane helices (TMH) of chlamydial proteins. The prediction performance was optimized by the calculation of statistical parameters for protein sets of known localization (Table S1). The list of putative outer membrane proteins was manually revised by taking into account probabilities calculated with the outer membrane prediction tool HHOMP and by searching the existing literature.

**Fig. 2.** Enrichment of outer membrane proteins of Parachlamydia, Simkania and Waddlia EBs using different detergents. Band patterns in SDS-PAGE gels after treatment with sarkosyl (lanes labelled S) differ between Simkania and the other two organisms, suggesting different protein compositions of the outer membrane of these organisms. Treatment of Simkania EBs with sarkosyl or n-POE (lanes labelled P) results in similar patterns in contrast to treatment with Triton X-114 (lane labelled T). Molecular mass of marker proteins in kDa is indicated on the left; M, marker; EB, purified EBs of the respective organism.
enrichment of OMPs was not as efficient as for *Simkania* (Fig. 3), but still a higher number of cell envelope proteins was identified in the sarkosyl-treated sample compared with n-POE (Fig. 3). Based on these results, we decided to use sarkosyl for the enrichment of OMPs in all further experiments.

After enrichment, OMPs must be effectively solubilized for further analysis, which is challenging for hydrophobic proteins. We compared two sample preparation procedures after sarkosyl treatment of purified *Simkania* and *Parachlamydia* cells: solubilization of proteins by the strong anionic detergent SDS followed by one-dimensional (1D) gel electrophoresis and in-gel digests and denaturation of proteins in urea followed by in-solution digests. Twenty-seven OMPs were detected only upon in-gel, but not upon in-solution digests for *Simkania*, including several MOMP-like proteins (Table S5). For both *Parachlamydia* and *Simkania*, OMPs were more frequently detected after in-gel digests compared with in-solution digests (Fig. 3). Based on these results, we decided to use the more sensitive in-gel digest in all further experiments.

### Enrichment of OMPs by sarkosyl

Treatment of purified EBs with sarkosyl resulted in the enrichment of cell envelopes devoid of cytoplasmic content for all three organisms as shown by transmission electron microscopy (Fig. 4). Envelopes of *Parachlamydia* and *Waddlia* maintained the shape of intact EBs, whereas envelopes of *Simkania* were more flexible in shape and often broken open (Fig. 4). 1D gel electrophoresis of the obtained fractions showed that the band patterns were strikingly different between *Simkania* and the other two organisms (Fig. 2). For *Simkania*, the complexity of the sample was substantially reduced after treatment with sarkosyl, resulting in only few bands at a molecular mass of ~40 kDa on SDS-PAGE gels. Proteins in specific bands were also enriched after treatment with sarkosyl for *Parachlamydia* and *Waddlia*, but overall band patterns were much more complex than those observed for *Simkania*. These differences in band patterns also corresponded to the number of proteins identified by mass spectrometry after in-gel digests of proteins (Table S6). For *Simkania*, 123 chlamydial proteins were identified in

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**Fig. 3.** Comparison of different extraction methods and digestion protocols for spectrometry-based detection of outer membrane proteins. The 50 most abundant proteins and their predicted locations are shown for each approach. Proteins were quantified and ranked by calculating the normalized spectral abundance factor (NSAF).

**Table S5.** Enrichment of OMPs by sarkosyl

| Protein Location | *Simkania* | *Parachlamydia* | *Waddlia* |
|------------------|------------|-----------------|-----------|
| Outer membrane   | 123        | 72              | 51        |
| Cytoplasm        | 48         | 34              | 27        |
| Lipoprotein      | 22         | 18              | 13        |
| Inner membrane   | 10         | 8               | 6         |
| Extracellular    | 5          | 3               | 3         |
| Periplasm        | 3          | 2               | 2         |
| Signal peptide   | 2          | 2               | 2         |
| Unknown          | 3          | 2               | 2         |
| Amoeba protein   | 1          | 1               | 1         |

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both biological replicates, and 65 of these were predicted
OMPs with an additional 13 being predicted lipoproteins.
For *Parachlamydia*, 825 chlamydial proteins were
detected, including 39 predicted OMPs and 15 lipopro-
ten. In fractions of *Waddlia*, 921 chlamydial proteins
were identified, 33 of them being predicted OMPs and 17
lipoproteins. These represent 58%, 76% and 65% of all
predicted OMPs of *Parachlamydia*, *Simkania* and
*Waddlia*, respectively, as well as all outer membrane lipo-
proteins predicted in the genomes of these organisms
(Fig. 5).

For all three chlamydiae, OMPs were the most abun-
dant proteins in the enriched fractions based on relative
quantification by the normalized spectral abundance
factor (NSAF) (Table S7). However, while all of the 20
most abundant proteins were either predicted OMPs or
lipoproteins for *Simkania*, this was only the case for five
and seven proteins for *Parachlamydia* and *Waddlia*. Other
OMPs detected for *Parachlamydia* and *Waddlia* were
present in much lower amounts, suggesting that these
proteins are either not abundant in EBs or that they were
not enriched by the applied method.
Many MOMP-like proteins dominate the outer membrane of Simkania and Waddlia

The most abundant protein in the outer membrane of the Chlamydiaceae is the porin MOMP (Caldwell et al., 1981). In the genomes of Simkania and Waddlia, large protein families with sequence similarity to MOMP are present (37 and 11 MOMP-like proteins respectively) (Bertelli et al., 2010; Collingro et al., 2011). Members of this rather divergent protein family share between 20% and 40% sequence identity with each other in Simkania and between 15% and 25% identity in Waddlia. Exceptions are pairs or triplets of MOMP-like proteins that are encoded adjacent to each other in the genomes, which show considerably higher sequence identity with each other (up to 81% sequence identity in Simkania and 35–80% identity in Waddlia). In our study, we detected all MOMP-like proteins in OMP fractions of the respective organism by mass spectrometry. This protein family clearly dominated the outer membrane of Simkania representing 17 of the 20 most abundant proteins in this organism (Table S7), and their predicted molecular mass of ∼40 kDa matched the dominant bands detected in OMP fractions on SDS-PAGE gels (Fig. 2). Only four MOMP-like proteins, which were encoded by adjacent genes, had a predicted lower molecular mass. Re-sequencing of the respective genome region revealed sequencing errors in the original genome sequence that wrongly introduced stop codons in both cases, and we found spectra matching the corrected parts of both full-length proteins in the database (Fig. S3). In fact, these two MOMP-like proteins were among the 20 most abundant proteins in outer membrane fractions of Simkania.

Proteins in bands of ∼34–38 kDa were also strongly enriched in sarkosyl-treated protein fractions of Waddlia compared with untreated EBs (Fig. 2). This molecular mass range matches MOMP-like proteins that were highly abundant in OMP fractions of Waddlia. For the majority of MOMP-like proteins (30 out of 37) of Simkania, but only for one protein of Waddlia, a beta-barrel conformation was predicted (Table S4), suggesting a possible role as porin in the outer membrane similar to MOMP (Bavoil et al., 1984). A single MOMP-like protein was also detected in OMP fractions of Parachlamydia, but at much lower abundance compared with the hypothetical proteins PUV_27500, PUV_11160 and PUV_07550, which were the most abundant proteins in OMP fractions of this organism. PUV_27500 is a homologue of the OMP pc0004 of Protochlamydia (Heinz et al., 2010) and the 76 kDa C. pneumoniae protein which was described as an EB surface antigen (Perez Melgosa et al., 1994). The function of all three hypothetical proteins is unknown, but a beta-barrel conformation was predicted by BOMP and TMBETADISC-RBF for PUV_27500 and by I-TASSER for PUV_07550, indicating a possible function as porins.

**Fig. 5.** Numbers of experimentally verified predicted outer membrane and lipoproteins of Parachlamydia, Simkania and Waddlia. More than half of all predicted outer membrane proteins (OMP) and all outer membrane lipoproteins (OML) were detected by mass spectrometry in outer membrane protein fractions of all three organisms. Only proteins that were identified in both biological replicates were considered. The percentage of predicted proteins that were detected by mass spectrometry is indicated. LP, lipoproteins.

**Autotransporters and secretion systems**

Autotransporters, which are classified as type V secretion systems (Henderson et al., 2000), are widely
distributed among Gram-negative bacteria and many of them have a role in virulence (Gripstra et al., 2013). *Simkania* is the only chlamydia besides the *Chlamydiaceae* that encodes proteins belonging to the polymorphic membrane protein family, autotransporters with an important role during attachment to the host in the *Chlamydiaceae* (Gomes et al., 2006). Three homologues of PmpB are encoded in the genome of *Simkania* and all are predicted to form beta-barrel structures in the outer membrane. Peptides corresponding to all three *Simkania* autotransporters were detected by mass spectrometry, and quantification based on the NSAF suggested that they are present in similar amounts in the outer membrane. In *Parachlamydia*, two proteins have highest similarity to autotransporters of *Proteobacteria*. For PUV_1000, formation of a beta-barrel structure and location in the outer membrane or extracellularly was predicted, similar to the Pmps of *Simkania*. PUV_3300 was predicted to form a beta-barrel by only one of three tools, but HHOMP predicted it with high confidence as OMP. However, both proteins were only present in low amounts in outer membrane fractions. A protein that is annotated as putative autotransporter in the genome of *Waddlia* (wcw_0271) (Bertelli et al., 2010) was also detected at low abundance by mass spectrometry. However, in contrast to the autotransporter of *Simkania* and *Parachlamydia*, neither a location in the outer membrane nor the formation of a beta-barrel structure was predicted for this protein with the *in silico* tools used in this study.

For all three organisms, structural components and chaperones of the type III secretion system were detected in OMP fractions (Table S8), in agreement with a previous study reporting the presence of these needle-like structures on EBs of *Simkania* and *Parachlamydia* (Pilhofer et al., 2014). *Simkania* encodes an additional type IV secretion system (Collingro et al., 2011), but no structural components of this apparatus were detected in outer membrane fractions.

*Simkania* lacks cysteine-rich OMPs

Cysteine-rich proteins play an important role in the stabilization of the chlamydial cell envelope and are major components of the COMC of *Protechlamydia* and members of the *Chlamydiaceae* (Hatch, 1996; Heinz et al., 2010). In agreement, homologues of the cysteine-rich protein OmcB were highly abundant in both organisms, with a cysteine content of 7.6% in *Parachlamydia* and 9.4% in *Waddlia*. In addition, also other abundant proteins in outer membrane fractions of both organisms showed cysteine contents higher than 3% (Table S7). Two homologous hypothetical proteins with a molecular mass of ~22 kDa (cysteine content 10.5% for PUV_11160 of *Parachlamydia*, and 6.9% for wcw_0272 of *Waddlia*) were particularly strongly enriched in outer membrane fractions of both organisms (Fig. 2). Although our *in silico* prediction approach could not clarify the location of these proteins, their high abundance in outer membrane fractions and the presence of a signal peptide suggest a location in the cell envelope.

No homologue of OmcB is encoded in the genome of *Simkania*, and in striking contrast to all other chlamydiae investigated so far, no detected OMP of *Simkania* showed a cysteine content higher than 1.8%. In fact, MOMP-like proteins, the most abundant protein component in the outer membrane of *Simkania*, show a percentage of cysteine ranging from 0% to 1.1%, far below the cysteine content of 3.5–5.3% of MOMP-like proteins of *Waddlia*. Overall, the average cysteine content of predicted OMPs of *Simkania* was 0.54%, comparable to *Escherichia coli* K-12 [0.51% based on OMP prediction by (Heinz et al., 2009)]. In contrast, OMPs of *Parachlamydia* and *Waddlia* encoded for an average of 1.3% and 2% cysteine respectively. The cysteine content of all proteins encoded in the genome was similar for all three chlamydiae (1.2% for *Simkania*, 1.4% for *Parachlamydia* and *Waddlia*) and comparable to *E. coli* K-12 or *Legionella pneumophila* (both 1.3%).

**Pinpointing the COMC of *Parachlamydia* and *Waddlia***

For *Parachlamydia* and *Waddlia*, high numbers of cytoplasmic proteins were detected among the 50 most abundant proteins in samples after sarkosyl treatment. To identify true components of the COMC in these organisms, we compared OMP fractions treated with or without the reducing agent dithiothreitol (DTT) by mass spectrometry. For each protein, an enrichment factor was calculated by dividing the percent spectral counts in the presence of DTT by the percent spectral counts in the absence of DTT. Many dominant OMPs of *Parachlamydia* and *Waddlia* were strongly depleted in the absence of DTT (Fig. 6; Table S9) demonstrating their participation in formation of the COMC. The cysteine-rich protein OmcB, a putative long-chain fatty acid transport protein, and eight of the 11 MOMP-like proteins were not detected at all in gels in the absence of DTT for *Waddlia*. The three MOMP-like proteins that were detected under both conditions were the most abundant OMPs under reducing conditions and were at least 57-fold enriched in the presence of DTT. In *Parachlamydia*, OmcB was enriched 126-fold under reducing conditions, and the cysteine-rich protein PUV_11160 was enriched more than 70-fold. In contrast, all MOMP-like proteins and all other components of the outer membrane except SNE_A20110 (probably involved in protein secretion) were present at similar levels in both *Simkania* samples.

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Stability of chlamydiae under reducing conditions

To test whether the observed differences in OMP composition also affect the stability of the organisms, purified Simkania, Parachlamydia and Waddlia cells were incubated in buffers of different osmolarities in the absence or presence of reducing agent. For Simkania, the integrity of cells decreased slowly for all incubations, and no differences associated with the presence of DTT were observed (Fig. 7). The presence of DTT had only a minor

Fig. 6. Abundant outer membrane proteins of Parachlamydia and Waddlia, but not of Simkania, have a high cysteine content and are enriched in SDS-PAGE gels in the presence of reducing agent. The percentage of cysteines per protein is plotted against fold enrichment in the presence versus absence of the reducing agent dithiothreitol (DTT). The fold enrichment was calculated by dividing the respective percent spectral counts determined by mass spectroscopy. The 50 most abundant proteins (based on quantification by NSAF) in the presence of reducing agent are shown.

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Fig. 7. The stability of Parachlamydia and Waddlia, but not of Simkania, is affected by reducing conditions. Changes in cell density of purified chlamydiae (measured as OD_{600}) during incubation in host-free buffers of different osmolarities in the presence (empty symbols) or absence (filled symbols) of DTT are shown. The cell density decreased rapidly for Parachlamydia or Waddlia under low osmolarity conditions in the presence of DTT. No differences were seen for incubations of Simkania in the presence or absence of reducing agent. PB, phosphate buffer; DTT, dithiothreitol.
influence during incubations under high osmolarity conditions for Parachlamydia and Waddlia. However, when these chlamydiae were incubated under low osmolarity conditions, cells lysed rapidly in the presence but not in the absence of DTT.

Discussion

Challenges in the prediction and enrichment of chlamydial OMPs

In this study, we characterized the protein composition of the outer membrane of members of three chlamydial families using a combination of in silico localization predictions and highly sensitive mass spectrometry analysis. The in silico analyses showed large discrepancies in the number and identity of predicted OMPs between the different tools. In general, predictions were more heterogeneous for chlamydia proteins compared with a published protein test set for Gram-negative bacteria that was used for a first evaluation of the prediction parameters. In comparison, also signal peptides were identified less frequently for predicted OMPs of chlamydiae than for OMPs in the test set (33–60% of chlamydia OMPs versus 70% of OMPs in the test set). One possibility is that this is caused by inaccurate gene predictions in chlamydial genomes. However, it more likely arises from the large phylogenetic distance of chlamydiae to the Proteobacteria, which are commonly used in the training of Gram-negative-specific versions of in silico prediction tools. Consistent with this, ambiguous in silico predictions were also encountered in a recent study that searched for OMPs in members of the chlamydial sister phyla Planctomycetes and Verrucomicrobia (Speth et al., 2012).

A major challenge in the preparation of outer membrane fractions is the minimization of contaminating proteins from other cellular compartments. Cellular subfractions represent rather an enrichment of proteins from the respective compartment than a sharp separation from other cellular compartments. Thus although OMPs can be enriched by treatment with detergents, co-purification of non-OMP is unavoidable (Lee et al., 2007; Walters and Mobley, 2009; Koßmehl et al., 2013; Cao and Bazemore-Walker, 2014), particularly when highly sensitive mass spectrometry methods are used. We indeed detected high numbers of low abundant proteins from all other cellular compartments in the outer membrane fractions, especially for Parachlamydia and Waddlia (Fig. 3).

The efficiency of the OMP enrichment differed largely between Simkania and the other two organisms. In Simkania, all but two of the 50 most abundant proteins were predicted to be associated with the outer membrane, and 63% of all detected proteins represented predicted OMPs or lipoproteins. This is a performance comparable to or better than achieved in other outer membrane proteome studies (Chung et al., 2007; Thein et al., 2010; Cao et al., 2012; Gessibauer et al., 2012). The same enrichment protocol was much less efficient for Parachlamydia and Waddlia and consistent with a recent study on immunogenic proteins of Waddlia (Lienard et al., 2014). In outer membrane fractions of these organisms, non-OMP such as ribosomal proteins, elongation factors and heat-shock proteins were detected in high amounts. Ribosomal proteins often are a major contaminant in outer membrane fractions due to their high abundance and hydrophobicity. In addition, protein aggregates and large complexes like GroEL can co-precipitate with membrane fractions during high-speed centrifugation (Thein et al., 2010). However, electron microscopy clearly showed a depletion of cytoplasmic contents after sarkosyl treatment for all three organisms similar to other studies (Fig. 4) (Heinz et al., 2010; Liu et al., 2010). The differences between Simkania versus Parachlamydia and Waddlia could result from lower abundance of OMPs in the latter organisms or from structural differences of the cell walls that hindered a better enrichment of OMPs.

Major differences in the OMP composition

We found remarkable differences in the most abundant protein components of the outer membrane not only between members of different chlamydial families, but also between members of the same family (Fig. 8). Parachlamydia and Protochlamydia, both members of the Parachlamydiaceae, differ from other chlamydiae in that they do not share MOMP or MOMP-like proteins as the most abundant protein and major porin in their cell envelope. In addition, the major porin also differs between the two Parachlamydiaceae, in contrast to the Chlamydiaceae which all share MOMP as the most abundant porin in their outer membrane (Raulston, 1995). PomS and PomT are the dominant OMPs in Protochlamydia (Heinz et al., 2010), and the function of PomS as porin has been demonstrated recently (Aistleitner et al., 2013). The outer membrane of Parachlamydia is dominated by three hypothetical proteins (PUV_27500, PUV_11160, PUV_07550), and for two of these a beta-barrel conformation has been predicted suggesting a function as porin. Interestingly, Parachlamydia encodes both a homologue of MOMP and member of the Pom family, but these proteins were only detected at low abundance, suggesting that they do not play a major role in the outer membrane of Parachlamydia.

In contrast to members of the Chlamydiaceae that encode a single copy of MOMP and the MOMP-homologue PorB (Kubo and Stephens, 2000), the outer membrane of Simkania and Waddlia is dominated by
large numbers of MOMP-like proteins. Of the 84 predicted OMPs of *Simkania*, 35 belonged to the family of MOMP-like proteins, and they account for more than half of all OMPs that were detected by mass spectrometry. In *Waddlia*, MOMP-like proteins represent a third of all detected OMPs. Expanded OMP families are also found in other bacteria, for example the Hop family of *Helicobacter pylori* (Alm et al., 2000) or the OMP-1/MSP2/P44 family of the obligate intracellular tick-borne pathogens *Anaplasma marginale* (Brayton et al., 2005) and *Ehrlichia chaffeensis* (Ohashi et al., 1998). All 22 full-length paralogues of the OMP-1/MSP2/P44 family were detected in a proteomic analysis of *E. chaffeensis* and *Anaplasma phagocytophilum* (Lin et al., 2011). Members of this protein family are differentially expressed between infections of vertebrate and invertebrate hosts (Unver et al., 2002; Noh et al., 2006) or at different temperatures (Unver et al., 2001). It was suggested that the porin activity of these proteins regulates nutrient uptake during intracellular development, for example by feeding the incomplete citric acid cycle in these organisms (Huang et al., 2007). The role of the individual members of the MOMP-like families of *Simkania* and *Waddlia* is unclear. However, similar to *E. chaffeensis*, expression levels could vary between different hosts or in the course of the developmental cycle.

For *C. trachomatis*, it was suggested that the folding of two loops towards the outer membrane surface by...
intramolecular disulfide bonds regulates the entrance of various molecules through the MOMP channel (Yen et al., 2005). As cysteine is completely or nearly absent in most MOMP-like proteins of Simkania, expression of different sets of proteins during the developmental cycle could substitute for the regulation via disulfide bridges. Differential expression of MOMP-like proteins in Simkania and Waddlia could thus help in the adaption to different osmolarity conditions inside and outside the host cell, confer host specificity during adhesion or help in the evasion of the immune system.

Diversification in the OMP composition can also be seen for autotransporters, a group of proteins that is associated with virulence in many Gram-negative bacteria. Autotransporters are represented by the Pmp family in the Chlamydiaceae, which are present in 9 (C. trachomatis) to 21 (C. pneumoniae) copies in their genomes. They are suggested to be involved in the adhesion to host cells (Crane et al., 2006) and to confer tissue specificity (Gomes et al., 2006). Pmps are remarkably absent from the outer membranes of other chlamydiae, with the exception of three PmpB homologues in Simkania. However, Parachlamydia and Waddlia both express putative autotransporters with highest similarity to proteobacterial autotransporter. These variations in proteins that are putatively involved in attachment likely represent an adaption to an environmental life style and might facilitate the attachment to different host cells.

The exception proves the rule – the cell wall of Simkania is not stabilized by disulfide-linked proteins

The cell envelope of all chlamydiae studied so far is enforced by the COMC, a mesh consisting of intra- and intermolecular cysteine cross-linked proteins (Hatch, 1996). Reduction and oxidation of the disulfide bonds of proteins in the COMC during the chlamydial developmental cycle strongly influence stability, permeability and probably also infectivity of chlamydiae (Bavoil et al., 1984; Hatch et al., 1984; Betts-Hampikian and Fields, 2011). Not all OMPs are necessarily components of the COMC. For example, PmpD can be extracted from intact EBs with gentle detergents in the absence of reducing agents, showing that it is not a true component of the COMC (Swanson et al., 2009; Liu et al., 2010). Strong depletion of several cysteine-rich proteins under non-reducing conditions indicated that these proteins are true components of the COMC in Parachlamydia and Waddlia. These included the cysteine-rich protein OmcB in both organisms and the MOMP-like proteins of Waddlia. All of these proteins share cysteine-rich clusters of CxCxC, CxxC, CC or CxxCxxC signature sequences that are essential in the cross-linking of chlamydial OMPs (Yen et al., 2005; Bertelli et al., 2010).

Simkania is the first member of the Chlamydiaceae that entirely lacks homologues of known chlamydial cysteine-rich proteins or other cysteine-rich proteins in its cell envelope. In contrast to MOMP or MOMP-like proteins of other chlamydiae, only two of the 35 MOMP-like proteins of Simkania (SNE_A02860 and SNE_A14850) encode two cysteines in close vicinity to each other. Although the genome of Simkania encodes some, mostly small proteins with high cysteine content, none of these proteins was detected by mass spectrometry. In addition, the stability of Simkania cells was not altered in the presence of reducing agent in contrast to Parachlamydia and Waddlia, strongly arguing against a stabilizing role of disulfide bridges in the Simkania cell envelope. It has been previously suggested that in contrast to other chlamydiae, both developmental stages of Simkania are infectious (Kahane et al., 2002), which might be a consequence of the lack of strongly cross-linked proteins in their outer membrane. The absence of stabilizing cysteine-rich proteins could also explain the earlier observation that Simkania EBs seem to be more flexible and are deformed inside tightly packed inclusions in contrast to Parachlamydia and Protochlamydia (Pilhofer et al., 2014).

The increased osmotic fragility of Parachlamydia and Waddlia in the presence of reducing agents is similar to that of C. trachomatis, where cysteine-rich proteins stabilize the cell wall (Hackstadt et al., 1985; Hatch et al., 1986). This suggests that Parachlamydia, Protochlamydia and Waddlia also stabilize their cell envelope through cross-linking of cysteine-rich proteins. Additional cysteine-rich proteins absent in the Chlamydiaceae (Heinz et al., 2010) may be used as an additional enforcement of the cell wall in those organisms. This is supported by the observed high stability of Parachlamydia under low osmolarity conditions up to 48 h (data not shown) in contrast to C. trachomatis and Simkania (Kahane et al., 2004). Interestingly, a higher stability was reported for Simkania in drinking water compared with C. trachomatis, raising the question how Simkania stabilizes its cell wall in the absence of cysteine-rich proteins. Peptidoglycan has recently been detected for the first time in some chlamydiae (Pilhofer et al., 2013; Liechti et al., 2014), but no evidence for the presence of this structure was found in Simkania.

In contrast to the Chlamydiaceae, Parachlamydia, Protochlamydia, Waddlia and Simkania infect free-living amoebae. This environmental transmission route and life style might require specific adaptations in the cell envelope and the ability to persist longer under host-free conditions. These differences contribute to the success of chlamydiae as obligate intracellular bacteria with a uniquely broad host spectrum. The original host of Simkania is not known since it was discovered as a cell culture contaminant. However, its atypical cell envelope
suggests a natural habitat with probably high osmolarity conditions where cysteine-rich proteins became dispensable.

**Experimental procedures**

*Cultivation, purification and transmission electron microscopy of chlamydiae*

*Acanthamoeba castellani* UWC1 infected with *P. acanthamoebae* UV7, *S. negevensis* or *W. chondrophila* 2032/99 were grown in trypticase soy broth with yeast extract (30 g l⁻¹ trypticase soy broth, 10 g l⁻¹ yeast extract, pH 7.2) at 20°C. Chlamydiae were purified from their amoeba hosts as previously described (Heinz et al., 2010) with some modifications. Infected amoebae were harvested and the pellet was resuspended in 6.5 ml sucrose-phosphate-glutamate buffer SPG g⁻¹ wet weight (SPG; 75 g l⁻¹ sucrose, 0.52 g l⁻¹ KH₂PO₄, 1.53 g l⁻¹ Na₂HPO₄ × 2H₂O, 0.75 g l⁻¹ glutamic acid). Complete, EDTA-free Protease Inhibitor Cocktail (Roche) was added and the host cells were broken using a Dounce homogenizer. Intact host cells and host debris were removed by centrifugation (300 × g, 10 min, 4°C) and filtration through 5 μm and 1.2 μm filters. Elementary bodies were enriched by sequential ultracentrifugation steps: first, the suspension was layered on 35% gastrografin and centrifuged at 55 000 × g for 45 min at 4°C. The pellet was resuspended in SPG. This was followed by two additional centrifugation steps using a sucrose/gastrografin gradient (50% sucrose overlaid with 30% gastrografin) and a gastrografin gradient (46% gastrografin overlaid with 40% gastrografin) (both 55 000 × g, 2 h, 4°C). Between the centrifugation steps, the suspension was homogenized using needles (diameter 0.90 and 0.45 mm). After the last centrifugation step, cell pellets representing enriched EBs were resuspended in SPG and either used directly or stored at −80°C until further use. For transmission electron microscopy, EBs or sarkosyl-treated samples (see Enrichment of chlamydial OMPs) were fixed with 2% glutaraldehyde in 10 mM phosphate buffer (pH 7.2) for 1 h at room temperature, rinsed in phosphate buffer 3 times for 10 min each, followed by fixation with 2% osmium tetroxide in 10 mM phosphate buffer. Samples were dehydrated in an ascending series of ethanol and embedded in low viscosity resin. Ultra-thin sections (70 nm) were cut using a Leica EM UC7 ultramicrotome, stained with 0.5% uranyl acetate and 3% lead citrate or 2.5% gadolinium and 3% lead citrate and imaged with a Zeiss EM 902 transmission electron microscope.

*Polymerase chain reaction and sequencing*

For re-sequencing of MOMP-like genes, DNA fragments spanning both adjacent genes were amplified with specific primer pairs (SNE_A08780_F, 5′-ATG AGA AAC TGG CTT ATT-3′/SNE_A08790_R, 5′-TTAGAAATCGAGGCACC-3′; and SNE_A02800_F, 5′-TTA GAA GTC AAA GCG GAA-3′/SNE_A02810_R, 5′-ATG TCT GGG CAA GGA ACTT-3′) and Sanger-sequenced using the BigDye Terminator Cycle Sequencing Kit v3.1 and an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions.

*Enrichment of chlamydial OMPs*

Three different detergents were tested for the enrichment of chlamydial OMPs: sarkosyl, which selectively solubilizes inner membrane proteins (Filip et al., 1973) leaving proteins of the chlamydial outer membrane to form an insoluble pellet (Caldwell et al., 1981); n-POE, which solubilizes OMPs; and Triton X-114 used for the enrichment of OMPs via phase separation (Arnold and Linke, 2008). For treatment with sarkosyl, purified chlamydiae were resuspended in 2% sarkosyl in phosphate-buffered saline (PBS) with protease inhibitor. The suspension was sonicated five times for 2 min. 5 mM MgCl₂ and 50 U benzonase (Novagen) were added followed by incubation at 37°C for 1 h with vigorous shaking. Samples were centrifuged (18 000 × g, 10 min) and the sarkosyl treatment was repeated for the pellet. The resulting sarkosyl-insoluble pellet was resuspended in protein loading buffer containing 400 mM DTT unless indicated otherwise. For treatment with n-POE, purified chlamydiae were resuspended in 100 μl POP5-buffer (0.087 g l⁻¹ EDTA, 5.94 g l⁻¹ NaCl, 300 mM Na₂PO₄, 0.5% n-POE; pH 6.5) with 100 mM DTT per 3 mg EBs (wet weight) and incubated for 1 h at 37°C on a rocking platform (Aistleitner et al., 2013). After removal of insoluble material (18 000 × g, 10 min, 4°C), ice-cold acetone was added to the supernatant and proteins were precipitated for 1 h at −20°C, followed by another centrifugation step (18 000 × g, 10 min, 4°C). The resulting pellet was resuspended in protein loading buffer. For treatment with Triton X-114, purified chlamydiae were resuspended in 250 μl ice-cold 2% Triton X-114 in phosphate buffer (pH 8.0), sonicated for 10 min and put on ice. Phases were separated by heating to 90°C in a water bath followed by centrifugation (400 × g, 10 min, 37°C). The aqueous upper phase was removed, 250 μl phosphate buffer was added to the lower phase and the extraction was repeated. Fifteen microlitre of the lower phase were mixed with 5 μl protein loading buffer. After addition of protein loading buffer, samples were heated to 95°C for 5 min and proteins were separated by 1D gel electrophoresis using either self-cast 12.5% SDS-PAGE gels or 10% Mini-PROTEAN TGX gels for samples subjected to mass spectrometry. Gels were stained with colloidal Coomassie brilliant blue and destained with water.

*In-gel digestion of protein fractions*

Each lane of the stained gel was cut into 10–14 sections, chopped, washed with 50 mM ammonium bicarbonate (ABC, pH 8.5), and dried with acetonitrile (ACN). Disulfide bonds were reduced with DTT (200 μl of 10 mM DTT for 30 min at 56°C). DTT was washed off and cysteines were alkylated by incubation with 100 μl 54 mM iodoacetamide for 20 min at room temperature in the dark. Gel pieces were dried with ACN, swollen in 10 ng μl⁻¹ trypsin (recombinant, proteomics grade, Roche) in 50 mM ABC and incubated over night at 37°C. The reaction was stopped by adding formic acid to a final concentration of approximately 1% and peptides were extracted by sonication.

*In-solution digestion of protein fractions*

The protein pellet was solubilized in 40 ml solubilization buffer (8 M urea, 100 mM Tris-HCl, pH 8.0) by sonication for
5 min. Reduction and alkylation were performed as described elsewhere (Cipak et al., 2013). Proteins were digested with LysC (Wako Chemicals; 1:25 of the estimated amount of protein) for 3 h at 30°C. Samples were diluted with 50 mM ABC to a concentration of 0.8 M urea followed by tryptic digestion (recombinant, proteomics grade, Roche; 1:30 of the estimated amount of protein) at 37°C overnight. Detergents were removed with Pierce Detergent Removal Spin Columns (Thermo Scientific) according to the manufacturer’s protocol. The eluates were acidified, desalted on STRATA-X columns (Phenomenex) and concentrated on the speed-vac.

Liquid chromatography-mass spectrometry/mass spectrometry analysis

Peptides were separated on an UltiMate 3000 nano high-performance liquid chromatography (HPLC) or an RSLC system (Dionex, Thermo Fisher Scientific). Digests were loaded on a trapping column (PepMap C18, 5 μm particle size, 300 μm i.d. × 5 mm, Thermo Fisher Scientific) equilibrated with 0.1% trifluoroacetic acid and separated on an analytical column (PepMap C18, 2 μm, 75 μm i.d. × 150–250 mm, Thermo Fisher Scientific) applying a 60–180 min linear gradient from 1.6% up to 32% ACN with 0.1% formic acid acid followed by a washing step with up to 72% ACN. The HPLC was directly coupled to an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) equipped with a nanoelectrospray ionization source (Proxeon, Thermo Fisher Scientific). The mass spectrometer was operated in the data-dependent mode: one full scan (resolution 60 000) with lock mass enabled was followed by maximal 12 MS/MS scans in the Linear Trap Quadrupole. The lock mass was set at the signal of polydimethyl cyclosiloxane at m/z 445.120025. Monoisotopic precursor selection was enabled and singly charged signals were excluded from fragmentation. The collision energy was set at 35%, Q-value at 0.25 and the activation time at 10 ms. Fragmented ions were put on an exclusion list for up to 120 s depending on the gradient length.

Interpretation of mass spectrometry data

Raw spectra were interpreted by Mascot 2.2.04 (Matrix Science) using Mascot Daemon 2.2.2. Spectra were searched against a database containing the sequences of Protochlamydia amoebophila UWE25 (Hom et al., 2004), W. chondrophila WSU 86-1044 (Bertelli et al., 2010), W. chondrophila 2032/99, Parachlamydia acanthamoebae UV7, Simkania negevensis (Collingro et al., 2011), Acanthamoeba castellani Neff (Clarke et al., 2013) and sequences of common contaminants. The parameters were set as following: the peptide tolerance was set to 5 ppm, MS/MS tolerance to 0.8 Da, carbamidomethylcysteine as static modification and oxidation of methionine as a variable modification. Trypsin was selected as the protease and two missed cleavages were allowed. Mascot results were loaded into Scaffold (Ver. 3.6.2; Proteome Software). Protein identifications were accepted when at least a minimum of two peptides were identified with a probability greater than 95% as assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003) resulting in a false discovery rate of 0.0% on the protein and peptide level. Relative protein abundance in one lane was determined over all gel sections by calculating the NSAF for proteins with a minimum of five spectra (Collier et al., 2010) if equal amounts of the gel pieces of one lane were loaded. For bands rich in protein, only a fraction of the corresponding digests were injected into the Liquid chromatography-mass spectrometry system in order to avoid overloading. The protein abundance in the corresponding lanes was calculated based on the total ion current and differences in the injected amount of the digests were adjusted by multiplication. Proteins were sorted according to their abundance and the mean rank between the two biological replicates was calculated. This showed that 32, 42 and 34 of the 50 most abundant proteins overlapped between both biological replicates in Parachlamydia, Simkania and Waddlia respectively.

Osmotic stability in the presence of reducing agent

Chlamydiae were purified from their host cells as previously described (Pilhofer et al., 2014). In brief, asynchronously infected amoebae were harvested and broken by vortexing with glass beads. Cell debris and intact amoebae were removed by centrifugation followed by two filtration steps (5 μm and 1.2 μm). The suspension was split into aliquots, spun down and the resulting pellets were resuspended in one of the following buffers either with or without 5 mM DTT: 10 mM phosphate buffer (pH 7.2), 10 mM phosphate buffer with 170 mM NaCl or 10 mM phosphate buffer with 480 mM NaCl. Cell suspensions were incubated at 37°C with gentle shaking. The optical density (OD600) was measured at 0, 10, 20, 40 and 60 min after start of the incubation. All measurements were done in three biological replicates.

In silico prediction of the subcellular localization of chlamydial proteins

For the prediction of OMPs in the genomes of Simkania, Parachlamydia and Waddlia, a combination of different prediction tools was used (Fig. 1). For Waddlia, genomes of both strain WSU 86-1044, representing a closed genome, and strain 2032/99, representing the strain used in the experimental part of this study and for which a nearly complete genome is available, were analysed, and a consensus list of OMPs was built. The subcellular localization of proteins was predicted using PSORTb v3.0 (Yu et al., 2010), CELLO (Yu et al., 2004) and SOSUI-GramN (Imai et al., 2008); beta-barrel OMPs were predicted using BOMP including the BLAST-option (Berven et al., 2004), MCMBB (Bagos et al., 2004) and TMBETADISC-RBF using PSSM features (Ou et al., 2008); lipoproteins were predicted using LIPO (Berven et al., 2006) and LipoP (Juncker et al., 2003); transmembrane helices were predicted using Phobius (Kall et al., 2007) and TMHMM v2.0 (Krogh et al., 2001). Signal peptides were predicted using SignalP 4.0 (Petersen et al., 2011). Criteria for the prediction of OMPs were evaluated using a dataset of 529 proteins of Gram-negative bacteria with known subcellular location (E-komon et al., 2012). Parameters were optimized by changing the number of positive predictions required for the assignment of a protein as
OMP and calculating the accuracy (percent of correct predictions), sensitivity (percent of true positive predictions), specificity (percent of true negative predictions) and the Matthews correlation coefficient (Table S1). Prediction parameters were further adjusted by using a test set of chlamydial proteins of known location, consisting of 24 OMPs and 25 proteins with other subcellular locations (Table S2). The optimized parameters were then used to predict OMPs, outer membrane lipoproteins and lipoproteins in the genomes of *Parachlamydia*, *Simkania*, *Waddlia*, *Protochlamydia* and *C. trachomatis* D/UW-3/CX. Positive results by a lipoprotein prediction tool overruled any beta-barrel prediction, since the prediction of lipoproteins was previously shown to be highly specific (Juncker et al., 2003). Whether a lipoprotein is attached to the inner or the outer membrane of Gram-negative bacteria depends on the presence of aspartic acid in position +2 relative to the cleavage site (Yamaguchi et al., 1988). However, this rule is not universal for all bacteria (Seydel et al., 1999; Lewenza et al., 2008). Since predictions by subcellular localization and lipoprotein prediction tools were contradictory for several proteins, we decided to annotate proteins only as outer or inner membrane lipoproteins if there was additional support by localization prediction tools and to annotate the others generally as ‘lipoprotein’.

Alpha-transmembrane helices are a feature characteristic for inner membrane proteins, and all proteins with more than one helix predicted by TMHMM or Phobius were removed from the list of OMPs. We allowed for one helix as signal peptides are sometimes incorrectly predicted as transmembrane helices by prediction tools. To further improve prediction results, proteins with an overall of at least two positive predictions were re-evaluated using HHOMP with a threshold of > 90% to predict a protein as OMP (Remmert et al., 2009) and the list of OMPs was manually revised. Structure predictions for selected proteins were performed using I-TASSER (Roy et al., 2010).

The subcellular localization of all non-OMPs in the genomes was predicted based on a consensus prediction by PSORTb, CELLO and SOSUI-GranM: if at least two of these tools agreed on the same subcellular localization, the protein was assigned to this compartment; otherwise, the localization was stated as ‘Unknown’.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Fig. S1. Comparison of outer membrane proteins predicted by different *in silico* tools. Venn diagrams indicating the numbers of outer membrane proteins predicted for *Simkania, Parachlamydia* and *Waddlia* by subcellular localization predictors (PSORTb, SOSUI-GramN and CELLO), predictors of beta-barrel conformation (BOMP, TMBDISC-RBF and MCMBB) and lipoproteins (LipoP and Lipo).

Fig. S2. Numbers of *Simkania* proteins identified by mass spectrometry using different detergents for the enrichment of outer membrane proteins. For all three detergents, a high number of non-outer membrane proteins was identified in addition to the enriched outer membrane proteins. Compared with sarkosyl, treatment with n-octyl polyoxyethylene (n-POE) and Triton X-114 (Triton) resulted in higher numbers of non-outer membrane proteins. Almost all predicted outer membrane proteins were identified in fractions obtained with either of the three detergents.

Fig. S3. Mass spectrometry analysis confirms the corrected sequences of two MOMP-like proteins. Two MOMP-like proteins were predicted in the genome to be split into two parts as result of sequencing errors introducing stop codons. Resequencing of these genes resulted in corrected sequences without stop codons, and spectra corresponding to the corrected sequences were found in both cases. Corrected parts of the sequence are indicated in orange; parts of the sequence for which spectra were identified by mass spectrometry are highlighted in grey. ‘new’ indicates the corrected sequence.

Table S1. Evaluation of prediction parameters for the consensus prediction of outer membrane proteins.

Table S2. Set of chlamydial proteins of known location used to evaluate the consensus prediction approach.

Table S3. Outer membrane proteins, outer membrane lipoproteins and lipoproteins predicted in the proteomes of *Protochlamydia amoebophila* UWE25 and *Chlamydia trachomatis* D/UW-3/CX.

Table S4. Outer membrane proteins, outer membrane lipoproteins and lipoproteins predicted in the proteomes of *Parachlamydia acanthamoebae*, *Simkania negevensis* and *Waddlia chondrophila* in the *in silico* consensus prediction approach.

Table S5. Outer membrane proteins of *Simkania* detected by mass spectrometry after extraction with sarkosyl, n-octyl polyoxyethylene (n-POE) or Triton X-114 (Triton).

Table S6. Outer membrane proteins, outer membrane lipoproteins and lipoproteins detected by mass spectrometry in outer membrane protein fractions of *Simkania, Parachlamydia* and *Waddlia* after treatment with sarkosyl.

Table S7. The 50 most abundant proteins detected in outer membrane protein fractions of *Parachlamydia, Simkania* and *Waddlia*.

Table S8. Structural components, chaperones and effectors of the type three secretion system of *Parachlamydia, Simkania* and *Waddlia* detected by mass spectrometry in this study.

Table S9. Enrichment of the 50 most abundant proteins (based on quantification by NSAF) of *Parachlamydia, Simkania* and *Waddlia* in the presence of reducing agent compared with the absence of reducing agent.

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