Identification of OmpA, a *Coxiella burnetii* Protein Involved in Host Cell Invasion, by Multi-Phenotypic High-Content Screening

Eric Martinez¹,²,³, Franck Cantet¹,²,³, Laura Fava¹,²,³, Isobel Norville⁴, Matteo Bonazzi¹,²,³*

¹ CNRS, UMR5236, CPBS, Montpellier, France, ² Université Montpellier 1, CPBS, Montpellier, France, ³ Université Montpellier 2, CPBS, Montpellier, France, ⁴ Defence Science and Technology Laboratory, Porton Down, United Kingdom

Abstract

*Coxiella burnetii* is the agent of the emerging zoonosis Q fever. This pathogen invades phagocytic and non-phagocytic cells and uses a Dot/Icm secretion system to co-opt the endocytic pathway for the biogenesis of an acidic parasitophorous vacuole where *Coxiella* replicates in large numbers. The study of the cell biology of *Coxiella* infections has been severely hampered by the obligate intracellular nature of this microbe, and *Coxiella* factors involved in host/pathogen interactions remain to date largely uncharacterized. Here we focus on the large-scale identification of *Coxiella* virulence determinants using transposon mutagenesis coupled to high-content multi-phenotypic screening. We have isolated over 3000 *C. burnetii* mutants, 1082 of which have been sequenced, annotated and screened. We have identified bacterial factors that regulate key steps of *Coxiella* infections: 1) internalization within host cells, 2) vacuole biogenesis/intracellular replication, and 3) protection of infected cells from apoptosis. Among these, we have investigated the role of Dot/Icm core proteins, determined the role of candidate *Coxiella* Dot/Icm substrates previously identified in silico and identified additional factors that play a relevant role in *Coxiella* pathogenesis. Importantly, we have identified CBU_1260 (OmpA) as the first *Coxiella* invasin. Mutations in ompA strongly decreased *Coxiella* internalization and replication within host cells; OmpA-coated beads adhered to and were internalized by non-phagocytic cells and the ectopic expression of OmpA in *E. coli* triggered its internalization within cells. Importantly, *Coxiella* internalization was efficiently inhibited by pretreating host cells with purified OmpA or by incubating *Coxiella* with a specific anti-OmpA antibody prior to host cell infection, suggesting the presence of a cognate receptor at the surface of host cells. In summary, we have developed multi-phenotypic assays for the study of host/pathogen interactions. By applying our methods to *Coxiella burnetii*, we have identified the first *Coxiella* protein involved in host cell invasion.

Introduction

*Coxiella burnetii* is an obligate intracellular Gram-negative bacterium responsible of the worldwide neglected zoonosis Q fever [1,2]. Acute forms of the disease are characterized by a febrile illness associated with severe headache, pneumonia and hepatitis. In a small percentage (2–5%) of cases, acute Q fever develops into a chronic infection that may lead to endocarditis and hepatitis. In a small percentage (2–5%) of cases, acute Q fever is associated with severe headache, pneumonia and a bacterium responsible of the worldwide neglected zoonosis Q fever. This pathogen invades phagocytic and non-phagocytic cells and *E. coli* adhered to and were internalized by non-phagocytic cells and the ectopic expression of OmpA in *E. coli* triggered its internalization within cells. Importantly, *Coxiella* internalization was efficiently inhibited by pretreating host cells with purified OmpA or by incubating *Coxiella* with a specific anti-OmpA antibody prior to host cell infection, suggesting the presence of a cognate receptor at the surface of host cells. In summary, we have developed multi-phenotypic assays for the study of host/pathogen interactions. By applying our methods to *Coxiella burnetii*, we have identified the first *Coxiella* protein involved in host cell invasion.

sources of infection, are extremely virulent. Phase II bacteria originate from spontaneous mutations after several in vitro passages of phase I organisms and present a truncated lipopolysaccharide (LPS) [8]. These non-reversible mutations result in a strong attenuation of virulence in vivo [9,10]. Phase II *Coxiella* organisms are internalized more efficiently than phase I organisms by both professional macrophages and non-phagocytic cells [9,11], however, once internalized, both antigenic phases replicate within host cells with similar kinetics. A phase II clone (Nine Mile phase II clone 4 or NMIIC4), which has been authorized for biosafety level 2 (BSL-2) manipulation, represents therefore an optimal model to study *Coxiella* infections [2,5]. In natural infections, *Coxiella* has a tropism for alveolar macrophages [1,2], however, infection of epithelial and endothelial cells has also been reported [12,13]. Indeed, in vitro, *Coxiella* invades and replicates in a wide variety of phagocytic and non-phagocytic cells [5]. *Coxiella* internalization within host cells is a passive, endocytic process, which involves the remodeling of the host cell actin cytoskeleton [14,15] and αvβ₃ integrins have been reported as *Coxiella* receptors in THP-1 cells [11]. However the *Coxiella* factors that mediate interactions with
host cell surfaces, as well as the bacterial host receptor on epithelial cells remain unknown. During the first 48 hours following internalization, bacteria reside into tight-fitting vacuoles, positive for early endosomal and autophagosomal markers [16]. As Coxiella-containing vacuoles mature along the endocytic pathway, the drop in vacuolar pH triggers the translocation of bacterial effector proteins by a Dot/Icm type 4b secretion system (T4SS) [17]. Effector translocation is essential for the biogenesis of a large parasitophorous vacuole (PV) that occupies the majority of the host cytoplasm [18,19]. Such large membranous structures are protected from apoptosis by a Dot/Icm-dependent mechanism [19,21–25]. Importantly, due to the obligate intracellular nature of Coxiella infections, mature Coxiella PVs are positive for lysosomal markers and contain active degradative enzymes [5,16]. Coxiella infections are not lytic and bacteria-filled PVs persist within infected cells, which are protected from apoptosis by a Dot/Icm-dependent mechanism [19,21–25]. Importantly, due to the obligatory intracellular nature of this pathogen, the microbial factors involved in host/pathogen interactions remain to date largely unknown. The homology between the T4SS of C. burnetii and L. pneumophila allowed the in silico identification of 354 candidate Coxiella effectors based on the presence of a conserved Dot/Icm regulatory motif (PmrA) [26–28], C-terminal translocation signals (E-block) [26–28], and eukaryotic-like domains [29–31]. Dot/Icm-dependent secretion has been validated for 108 of these using either Coxiella or Legionella as a surrogate host [18,26–31]. Recent advances in Coxiella axenic culture techniques [32] rendered this pathogen genetically tractable [33], allowing for the first time to couple bioinformatics analysis to morpho-functional assays and investigate the role of candidate Coxiella virulence determinants in intracellular replication [18,19,27]. To date, 20 Coxiella genes encoding Dot/Icm substrates have been mutated to investigate their role in Coxiella replication within the host [27]. Here we have set up new, integrative approaches that combine transposon mutagenesis with genomics, bioinformatics and fluorescence-based functional assays aiming at the large-scale identification of intracellular bacteria virulence factors. Our approach is designed for the simultaneous investigation of multiple key steps of Coxiella infections and is based on the identification and characterization of transposon-induced phenotypes. We have generated and isolated 3000 Coxiella transposon mutants, 1082 of which have been sequenced and screened in the present study. Our analysis revealed important insights into the functionality of the Coxiella Dot/Icm apparatus and revealed a variety of bacterial factors involved in 1) internalization within host cells, 2) PV biogenesis and intracellular replication, and 3) protection of the infected cell from apoptosis. By focusing our analysis on the early events of Coxiella infections we identified the first Coxiella invasin that plays an essential role in bacterial internalization by non-phagocytic cells.

Results

Generation of a bank of Phase II Coxiella mutants

To identify the Coxiella factors involved in host-pathogen interactions, we have undertaken the generation of a library of GFP-tagged bacterial mutants by transposon mutagenesis. We have modified the Himar1-based transposon system initially developed by Heinzen and colleagues [33,34], by inserting the enhanced green fluorescent protein (egfp) gene under the regulation of the Coxiella promoter P311, upstream of the chloramphenicol resistance cassette, thus generating pITR-CAT-ColE1-P311-GFP. To obtain stable mutants, Nine Mile Phase II clone 4 (NMIIIC4) Coxiella (hereafter referred to as wt Coxiella) were electroporated using a two-plasmid system, where the transposase is encoded by a suicide plasmid that is lost during bacterial replication [34]. The eGFP-tagged Coxiella mutants thus generated were isolated on ACCM-2 agar plates in the presence of chloramphenicol and further amplified for 7 days in liquid ACCM-2 supplemented with chloramphenicol. The final concentration of each bacterial culture was calculated using the Quant-iT PicoGreen dsDNA assay. Transposon insertion sites were identified by single-primer colony PCR followed by DNA sequencing. Using the primer SP3 we amplified DNA fragments including a 278 bp region upstream of the 3’ Inverted Terminal Repeat (ITR) of the inserted transposon (Fig. 1A). The amplified fragments were then sequenced using the transposon-specific primer P3, which recognizes a sequence in the 3’ region of the Chloramphenicol Acetyltransferase (CAT) gene (Fig. 1A). The obtained sequences were then aligned on the Coxiella burnetii RSA493 annotated genome using automated sequence analysis software. The genome of Coxiella burnetii RSA493 contains 1849 coding sequences (CDS), 1814 in the bacterial chromosome and 35 in the cryptic plasmid QpH1 [35]. To date we have isolated 3000 transposon mutants, 1082 of which have been sequenced, annotated and analyzed for this study (Fig. 1B). Transposon insertions were homogeneously distributed throughout the Coxiella chromosome and plasmid, with seven “hot spots” of preferential transposon insertion (identified and annotated from 1 to 7, Fig. 1B) and a large, 52 CDS region, upstream of hot spot n. 2, which remained non-mutated. Of note, region n.7 corresponds to the locus that hosts T4SS core genes (dot/icm genes) whereas the non-mutated region between CBU_0215 and CBU_0272 is enriched in genes encoding ribosomal proteins. Overall, 926 transposon insertions were found within Coxiella annotated CDS and 156 in intergenic regions of the Coxiella genome (excluding insertions within the first 100 bp upstream of a CDS; Fig. 1C). Frequency distribution analysis revealed that mutations occurred in 483 CDS on the Coxiella chromosome and 8 CDS on the QpH1 plasmid (corresponding to 26.6% and 22.8% of the total CDS present on chromosome and plasmid respectively; Fig. 1C). The mutated CDS were then clustered according to their predicted function based on the data available on the Pathosystems Resource Integration Center (PATRIC, www.patricbrc.org; Fig. 1D).

Author Summary

Infectious diseases are among the major causes of mortality worldwide. Pathogens’ invasion, colonization and persistence within their hosts depend on a tightly orchestrated cascade of events that are commonly referred to as host/pathogen interactions. These interactions are extremely diversified and every pathogen is characterized by its unique way of co-opting and manipulating host functions to its advantage. Understanding host/pathogen interactions is the key to face the threats imposed by infectious diseases and find alternative strategies to fight the emergence of multi-drug resistant pathogens. In this study, we have set up and validated a protocol for the rapid and unbiased identification of bacterial factors that regulate host/pathogen interactions. We have applied this method to the study of Coxiella burnetii, the etiological agent of the emerging zoonosis Q fever. We have isolated, sequenced and screened over 1000 bacterial mutations and identified genes important for Coxiella invasion and replication within host cells. Ultimately, we have characterized the first Coxiella invasin, which mediates bacterial internalization within non-phagocytic cells. Most importantly, our finding may lead to the development of a synthetic vaccine against Q fever.
High-content multi-phenotypic screen of *Coxiella* transposon mutants

Isolated transposon mutants were used to infect Vero cells at comparable multiplicities of infection (MOI). Non-infected Vero cells were used as negative control and cells infected with GFP-*Coxiella* (GFP-*Coxiella*) [33] were used as positive controls. Variations of GFP fluorescence associated with intracellular bacterial growth over 7 days of infection were monitored using a multimode micro-plate reader to obtain intracellular growth curves for all the *Coxiella* mutants screened (Fig. 2A). Seven days after infection, plates were imaged using an automated fluorescence microscope and images were analyzed using the automated image analysis software CellProfiler (www.cellprofiler.org). 9 morphological features were extrapolated from an average of 14000 cells for each condition (Fig. 2A). Nuclei features were used to assess the overall conditions of host cells and the potential cytotoxicity of *Coxiella* mutations. *Coxiella* features were used to score the intracellular replication of bacteria. Finally, the number of *Coxiella* colonies was divided by the number of host cell nuclei to estimate the efficiency of bacterial internalization within cells.

We validated our approach by comparing the growth curves and morphology of GFP-*Coxiella* to those of 6 mutants (Tn2384, Tn1948, Tn292, Tn2184, Tn514, Tn270) carrying independent transposon insertions in the gene CBU_1648, which encodes DotA, an essential component of the *Coxiella* Dot/Icm secretion system [36] (Fig. 2B). Axenic growth of the 6 *dotA* mutants was comparable to that of *wt Coxiella* (Fig. S1A). GFP signal analysis indicated that intracellular growth of GFP-*Coxiella* followed a typical growth curve, with bacterial replication clearly detectable from day 2 post-infection and increasing until day 7 post-infection (Fig. 2C). Morphological analysis of GFP-*Coxiella*-infected Vero cells reported bacterial colonies with an average area of 80.71 ± 4.65 microns² and an average number of *Coxiella* colonies/cell of 1.13 ± 0.12 (n = 21656; Fig. 2D). As expected, the GFP signal originated by all the *dotA* transposon mutants failed to

Figure 1. Generation of a bank of GFP-tagged *Coxiella* transposon mutants. (A). The transposable element used to generate the bank of *Coxiella* mutants contained the eGFP gene under the regulation of the strong *Coxiella* promoter p311 and the chloramphenicol acetyltransferase gene (CAT) under the regulation of the mild *Coxiella* promoter p1169, all flanked by Inverted Terminal Repeats (ITR). Arrows labeled SP3 and P3 indicate the site of annealing of the corresponding primers. (B). Sequenced transposon insertions were annotated on the *Coxiella* RSA493 chromosome (large circle) and QpH1 cryptic plasmid (small circle). Peaks indicate the site of insertion of each transposon and the height of the peak corresponds to the frequency of mutants isolated presenting a transposon insertion in a given site (1 inner circle corresponds to 2 insertions). Sites of preferential transposon insertions are labeled in red. (C). Pie charts indicating the frequency of transposon insertions in *Coxiella* coding sequences (CDS) or intergenic regions of the genome (left chart) and the total number of mutated CDS as compared to the total number of CDS in the chromosome (middle chart) and cryptic plasmid (right chart). (D). The mutated CDS were clustered according to their predicted functions as reported by the PathoSystems Resource Integration Center (PATRIC). doi:10.1371/journal.ppat.1004013.g001
Coxiella burnetii Virulence Determinants

A

Automated Microscopy and Image Analysis

- Nuclei Coxiiella
- COXIELLA COLONIES:
  - number
  - form factor
  - area
  - perimeter
  - GFP intensity
  - compactness

Plate reader

GFP intensity

7-days infection

B

C

D

- GFP-Coxiella
- Tn292
- Tn1948
- Tn2184
- Tn270
- Tn514
- Tn2384

Relative Fluorescence Intensity

Average Colony Area (µm²) | Average Colonies/Cell | n
--- | --- | ---
GFP-Coxiella | 80.71±4.65 | 1.13±0.12 | 21656
Tn292 | 30.25±4.92 | 2.91±0.72 | 16498
Tn1948 | 16.05±3.74 | 3.48±0.61 | 10718
Tn2184 | 13.37±3.43 | 1.44±0.38 | 16508
Tn270 | 5.86±1.85 | 0.17±0.08 | 19066
Tn514 | 32.03±4.83 | 3.00±0.25 | 14879
Tn2384 | 13.09±3.63 | 1.89±0.74 | 13876
increase significantly during the 7 days of infection (Fig. 2C). Accordingly, morphological analysis indicated a strong reduction in the average area of intracellular *Coxiella* colonies, which ranged from 5.86±1.85 to 32.03±4.83 microns² (n = 19066 and 14879 respectively, Fig. 2D). Interestingly, the majority of *dotA* mutations corresponded to an increased number of colonies/cell, which, in the case of mutant *Tn514*, reached 3.48±0.61 (Fig. 2D). This phenotype suggests that, in the absence of a functional secretion system, the *Coxiella*-containing vacuoles fail to coalesce to form the typical, large PV. Moreover, two independent transposon insertions in the *Dot/Icm* gene *icmV* (*Tn2445* and *Tn2214*), which is located in the same operon as *dotA*, produced a comparable, strong replication phenotype (data not shown).

The data derived from the multi-phenotypic analysis of *Coxiella* infections of host cells were mined to identify mutations that perturbed: 1) host cell invasion, 2) intracellular replication and 3) host cell survival. To screen for invasion and replication phenotypes, we plotted the average area of *Coxiella* colonies against the average number of colonies/cell (Fig. 3A). Statistical analysis was used to define regions in the resulting scatter plot corresponding to mild (−4<Z-score≤−2) and severe (Z-score≤−4) phenotypes. The 1082 analyzed *Coxiella* mutants were found in 3 well-defined clusters: one included mutants whose phenotype did not vary significantly from that of GFP-*Coxiella* (Fig. 3A green dots). A second cluster was clearly shifted towards a reduction in the size of *Coxiella* colonies and an increase in the average number of *Coxiella* colonies/cell, representative of mutations that affect *Coxiella* intracellular replication without affecting host cell invasion (Fig. 3A light and dark red dots). A third cluster was shifted towards a reduced number of colonies/cell, indicating mutations that affect host cell invasion (Fig. 3A light and dark blue dots). In parallel, the average area of *Coxiella* colonies was plotted against the total number of host cells surviving the 7 days of infection, to identify *Coxiella* genes that are potentially involved in the protection of the host cell from apoptosis (Fig. 3B). As above, statistical analysis was used to define regions corresponding to mild (−4<Z-score≤−2) and severe (Z-score≤−4) phenotypes. The vast majority of the mutants analyzed did not affect cell survival, regardless of bacterial replication within host cells (Fig. 3B green dots). 37 mutations mildly affected host cell survival (Fig. 3B light red dots), and 7 mutations were particularly detrimental to host cell survival (Fig. 3B dark red dots). The phenotypic data from mutations within CDS were integrated with the annotated transposon insertions in the *Coxiella* genome. We thus clustered the screened mutations according to the mutated CDS and assigned to each mutant a color-coded map based on the intensity of their replication (R), internalization (I) or cytotoxic (C) phenotype (Table S1). Intergenic transposon insertions were retained in a separate table (data not shown). Importantly, mutant *Tn1832* carries an intergenic transposon insertion and phenocopies *wt Coxiella* and GFP-*Coxiella* (Fig. S2). This mutant has been used in our validation experiments as additional control.

**Coxiella** factors required for intracellular replication

Intracellular replication of *Coxiella* relies on the functionality of a Dot/Icm T4SS, which is highly homologous to that of *L. pneumophila* [2,37–39]. The *Coxiella* genome contains 23 homologues to the 25 known *dot/icm* genes of the *Legionella* T4SS. Accordingly, *Legionella* has been used as a model organism to test the secretion of candidate *Coxiella* effector proteins. Recently, it has been reported that the *Coxiella* *dot/icm* genes *icmD, dotA, dotB* and *icmL* are essential for *Coxiella* replication within host cells, proving for the first time the functionality of the *Coxiella* T4SS [18,19,36]. The enrichment of transposon insertions in *dot/icm* genes (Fig. 1B, region n. 7), prompted us to analyze the phenotype of 38 *Coxiella* mutants carrying single transposon insertions in 16 *Dot/Icm* genes (Fig. 4A; Fig. S1). First, we followed the axenic growth of the 38 *Dot/Icm* transposon mutants and found it to be indistinguishable from that of *wt Coxiella* and of the control transposon mutant *Tn1832* (Fig. S1A). Multi-phenotypic analysis confirmed the previously reported observations that *icmD, dotA* and *icmL* are essential for *Coxiella* replication within host cells [18,19,36] (Fig. 4B, C; Fig. S1B). Moreover, we observed that 12 *dot/icm* genes (*dotA, dotB, icmV, E, D, G, J, N, C, P, K, X, L, I*) are essential for bacterial replication within the host, whereas mutations in *icmB* and *icmS* showed an intermediate phenotype, which corresponded to partial intracellular replication as assessed by morphological analysis (Fig. 4B, C; Fig. S1B). Of note, transposon insertions in *dot/icm* genes present in operons produced consistent phenotypes. Each mutation was then trans complemented by challenging host cells in combination with *wt Coxiella* as previously described [19]. The inergic mutant *Tn1832* was used as positive control. As illustrated in Figure 4C, mutations resulting in severe replication phenotypes were efficiently complemented by the presence of *wt Coxiella* in the PV occupied by *Dot/Icm* mutants (black bars).

We have also isolated and screened 63 transposon mutants in 31 CDS encoding predicted *Coxiella* *Dot/Icm* substrates (Fig. 3C). The products of 22 of these CDS have been previously reported to be positive for *Dot/Icm* secretion [18,26,27,29,30,31] (Fig. 3C). Our analysis indicated that mutations in 17 of these genes produced replication phenotypes (Fig. 3C, CDS in red), further suggesting that these genes encode *Coxiella* effectors. Interestingly, a transposon insertion in *CBU_1639* resulted in a strong cytotoxic phenotype (Fig. 3C DSS in blue), suggesting that this gene plays a role in the *Coxiella*-mediated inhibition of apoptosis.

The *Coxiella* genome encodes 4 two-component systems: PhoB-PhoR (*CBU_0367-CBU_0366*), GacA-GacS (*CBU_0712-CBU_0760*), QseB-QseC (*CBU_1227-CBU_1228*) and an Rsb-like system (*CBU_2005-CBU_2006*) [2,35]. In particular, the QseB-QseC system has been reported to be homologous to the *L. pneumophila* PmrA-PmrB system, a fundamental regulator of Dot/Icm secretion and its role has been indirectly confirmed [26,40]. Consistent with these observations and with our analysis on *Coxiella* Dot/Icm genes, 6 independent transposon insertions in
### Coxella burnetii Virulence Determinants

**A**

![Graph A](image1)

**B**

![Graph B](image2)

### Table: Coxella burnetii Virulence Determinants

| TnR | Locus Tag | Feature Type | Gene | SC | Candidate Feature | Deficit Domain | Ref |
|-----|-----------|--------------|------|----|-------------------|----------------|-----|
| 2359 | CBU_0002  | CDS          | dnaN | 110 | E-block           | N/A            | 28  |
| 1164 | CBU_0012  | Pseudogene   |       |     | E-block           | Yes            | 27/28|
| 3703 | CBU_0021  | CDS          | dnaA | 1694| E-block/PmrA     | Yes            | 27/28|
| 1809 | CBU_0044  | Pseudogene   |       |     | PmrA             | Yes            | 27  |
| 1995 | CBU_0072  | CDS          | anK  | 100 | ankyrin repeat    | Yes            | 26/30|
| 1719 | CBU_0077  | CDS          | PmrA | 375 | PmrA/Proximity to other CBU | Yes | 18/27|
| 357  | CBU_0133  | CDS          |      | 47  | E-block           | Yes            | 27/28|
| 4    | CBU_0144  | Pseudogene   | anK  | 100 | ankyrin repeat    | Yes/No         | 26/30|
| 259  | CBU_0352  | CDS          | ccdD | 457 | E-block           | N/A            | 28  |
| 1955 | CBU_0380  | CDS          |      | 1228| E-block           | Yes            | 27  |
| 1851 | CBU_0447  | CDS          | anK  | 290 | ankyrin repeat    | Yes            | 26/30|
| 1908 | CBU_0504  | CDS          |      | 302 | E-block           | N/A            | 28  |
| 830  | CBU_0623  | CDS          |      | 315 | E-block           | Yes            | 28  |
| 2451 | CBU_0675  | CDS          |      | 680 | E-block           | Yes            | 28  |
| 1637 | CBU_0675  | CDS          |      | 27  | E-block           | N/A            | 28  |
| 1504 | CBU_0675  | CDS          |      | 599 | PmrA             | Yes            | 27  |
| 1159 | CBU_0837  | CDS          |      | 161 | E-block           | Yes            | 27  |
| 2222 | CBU_1217  | CDS          |      | 341 | E-block           | Yes            | 26/30|
| 2340 | CBU_1292  | Pseudogene   |      | 351 | E-block           | N/A            | 28  |
| 584  | CBU_1292  | CDS          |      | 1   | E-block           | N/A            | 28  |
| 860  | CBU_1292  | Pseudogene   |      | 274 | E-block/PmrA     | Yes            | 26/30|
| 1959 | CBU_1525  | Pseudogene   |      | 156 | E-block           | Yes            | 27/28|
| 1298 | CBU_1525  | Pseudogene   |      | 181 | PmrA             | Yes            | 27  |
| 651  | CBU_1525  | Pseudogene   |      | 534 | E-block           | Yes            | 27  |
| 2243 | CBU_1525  | Pseudogene   |      | 111 | E-block           | N/A            | 26  |
| 1952 | CBU_1525  | CDS          |      | 960 | PmrA/CofA binding/E-block | Yes | Coiled Coil |
| 2222 | CBU_1898  | Pseudogene   |      | 4   | E-block           | Yes            | 27  |
| 952  | CBU_1898  | CDS          |      | 534 | E-block           | Yes            | 27  |
| 2055 | CBU_2076  | CDS          |      | 106 | E-block           | Yes            | 27  |
| 2055 | CBU_2076  | CDS          |      | 605 | Eukaryotic-like domain | Yes | Fix domain |
| 1693 | CBU_2076  | CDS          |      | 228 | Eukaryotic-like domain | Yes | Fix domain |
| 1877 | CBU_0023  | CDS          | cpeF | 641 | Conserved in plasmid | Yes | 31  |
Figure 3. Large-scale identification of *Coxiella* factors involved in host/pathogen interactions. (A). For each screened mutant, the average area (in microns$^2$) of *Coxiella* colonies was plotted against the relative number of colonies per cell to identify phenotypes of interest. Green dots represent phenotypes that deviate from wt *Coxiella* by a Z-score $>2$ (not significant). Pink and light blue dots represent replication and internalization phenotypes respectively, with a Z-score between $-2$ and $-4$ (mild phenotypes). Red and dark blue dots represent phenotypes with a Z-score $<-4$ (strong phenotypes). (B). For each screened mutant, the average area (in microns$^2$) of *Coxiella* colonies was plotted against the total number of cells (infected and not infected) that survived 7 days of infection to estimate the cytotoxicity of transposon insertions. Green dots represent phenotypes that deviate from wt *Coxiella* by a Z-score $>2$ (not significant). Pink dots represent cytotoxic phenotypes with a Z-score between $2$ and $4$ (mild phenotypes). Red dots represent cytotoxic phenotypes with a Z-score $<2$ or $>4$ (strong phenotypes). (C). Mutants presenting transposon insertions disrupting *Coxiella* genes previously identified as putative Dot/Icm substrates were clustered in rows according to the mutated gene (CDS) and their intracellular replication (R), internalization (I) and cytotoxic (C) phenotypes were illustrated. White squares represent non-significant phenotypes (Z-score $<2$). Pink squares represent mild phenotypes (Z-score between $2$ and $4$). Red squares represent strong phenotypes (Z-score $>4$). Mutant number (Tn), information on the feature (Feature Type), annotated CDS name (Gene), distance of the transposon insertion site from the CDS start codon (SC), feature that allowed the identification of the gene as a candidate Dot/Icm substrate (Candidate Feature), Dot/Icm-mediated secretion (Dot/Icm), identified protein domains (Domain) and reference to previous publications (Ref.) were integrated in the table. CDS codes in red indicate significant intracellular replication phenotypes; CDS codes in blue indicate significant cytotoxic phenotypes.

doi:10.1371/journal.ppat.1004013.g003

Figure 4. Role of Dot/Icm core proteins in *Coxiella* infections. (A). Schematic representation of the region of the *Coxiella* genome containing Dot/Icm genes. The genes that have been mutated in this study are represented in red. (B). Intracellular growth curves of *Coxiella* mutants containing transposon insertions in dot/icm genes calculated as variations of GFP fluorescence over 7 days of infection and compared to GFP-tagged wt *Coxiella* (Ctrl, dashed black line). Mutants in the same CDS are grouped by color. With the exception of Tn2098 (icmS), the growth curves of all Dot/Icm mutants were significantly different from that of GFP-Coxiella (P < 0.001, 2way ANOVA) from day 2 post-infection. The growth curve of Tn2098 (icmS) was significantly different from that of GFP-Coxiella (P < 0.001, 2way ANOVA) from day 3 post-infection. (C) Vero cells were incubated either with the Dot/Icm transposon mutants alone (white bars) or in combination with wt *Coxiella* (black bars) to complement the replication phenotypes observed. 7 days post-infection, the average area (in microns$^2$) was calculated using CellProfiler from an average of 6000 cells per condition. Values are mean ± standard deviation of triplicate samples (co-infection phenotypes were compared to their respective single infection condition. ns = non-significant; *=P<0.05; **=P<0.01; ***=P<0.001, 2way ANOVA).

doi:10.1371/journal.ppat.1004013.g004
Coxiella burnetii Virulence Determinants

Identification of a Coxella surface protein involved in host cell invasion

As for all obligate intracellular pathogens, Coxella invasion of host cells is a priming step of the infection. However, since the bacterial factors that mediate Coxella invasion of host cells remain unknown, we sought to identify bacterial factors whose mutations affect Coxella internalization. High-content screening identified 48 mutations in 37 Coxella CDS that resulted in a significant reduction in the number of infected cells after 7 days of infection (Fig. S5). Of these, 18 CDS involved in bacterial metabolism and transcription were excluded. Among the remaining 19 candidate CDS (Fig. S5, CDS boxed in red), we have identified 5 independent transposon insertions (Tn173, Tn298, Tn27, Tn907 and Tn749) in CBU_1260, all sharing a consistent, strong internalization phenotype (Fig. S5). CBU_1260 is a 747 bp CDS on the positive strand of the Coxella chromosome encoding a hypothetical protein of a predicted size of 23 kDa. Importantly, the gene is not part of an operon, indicating that the phenotype observed for the 5 mutants analyzed in this study was indeed due to the inactivation of CBU_1260 alone (Fig. 5A). Axenic growth of the 5 transposon mutants was comparable to that of wt Coxella and of the control transposon mutant Tn1832 (Fig. S6A). As expected, intracellular growth curve analysis of the 5 transposon mutants in CBU_1260 indicated that GFP fluorescence failed to increase during the 7 days of infection (Fig. 5B). Indeed, all 5 mutations in CBU_1260 reduced the number of cells presenting Coxella colonies at 7 days of infection by 60–70% compared to cells challenged with GFP-Coxella (Fig. 5C). We next used the online analysis software i-TASSER (http://zhanglab.ccmb.med.umich.edu/i-TASSER/) and Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/) to predict the structure of the hypothetical protein encoded by CBU_1260. Bioinformatics analysis predicted 8 transmembrane beta sheets forming a beta barrel domain, an N-terminal alpha helix and 4 unstructured loops (L1 to L4), exposed at the cell surface (Fig. 5A, D). This prediction was confirmed by analyzing the sequence of the protein using TMPred (http://www.ch.embnet.org/software/TMPRED_form) and the BetAware software [41]. Sequence analysis indicated that transposon insertions occurred in the distal part of the CDS, within the 4th and 6th beta sheets, with mutants Tn27 and Tn907 presenting insertions at the same site (Fig. 5A). The predicted transmembrane domain of CBU_1260 is typical of Outer Membrane Protein A (OmpA) family of proteins, which are found in several bacteria and mediate adhesion and/or internalization within host cells [42–50]. We therefore named the product of CBU_1260 OmpA. Coxiella encodes 3 hypothetical proteins that contain predicted OmpA-like domains: CBU_0307, CBU_0936 and CBU_1260 (ompA). Sequence alignment of these three hypothetical proteins showed high degree of homology at the level of the transmembrane domains and the N-terminal alpha helix (Fig. S7). However, little homology was observed at the level of the 4 unstructured loops of OmpA (Fig. S7). Accordingly, a transposon insertion in CBU_0307 produced a cytotoxic phenotype whereas 4 transposon insertions in CBU_0936 produced a replication phenotype (Table S1). An OmpA-specific antibody was then raised against the predicted extracellular domain of the protein. To this aim, a 15 amino acid peptide in predicted loop 1 (KKSGTSKVNFTGVTL) was used for its immunogenic potential as compared to peptides in the other loops. When tested by Western blot on lyastes from wt Coxiella and the control mutant Tn1832, the anti-OmpA antibody revealed a major band at the expected size of 23 kDa (Fig. 5E, arrow) and a faint, background band of lower molecular weight (Fig. 5E). When incubated on lysates from the 3 transposon mutants in CBU_1260, the anti-OmpA antibody only recognized the
Figure 5. Characterization of CBU_1260 (OmpA), a *Coxiella* protein involved in host cell invasion. (A): Schematic representation of the genomic context of CBU_1260 and sites of transposon insertion of 5 independent isolated mutants (Tn175, Tn208, Tn27, Tn907, Tn749). Black area indicates the position of the predicted N-terminal α-helix and grey areas indicate the position of predicted β-sheets. Predicted extracellular loops are indicated from L1 to L4. (B): Intracellular growth curves of *Coxiella* mutants containing transposon insertions in CBU_1260 measured as variation of GFP fluorescence over 7 days of infection and compared to GFP-tagged wt *Coxiella* (Ctrl). The growth curves of all CBU_1260 mutants were significantly different from that of GFP-Coxiella (P<0.001, 2way ANOVA) from day 2 post-infection. (C): For each CBU_1260 mutant analyzed, the total
The number of Coxiella colonies identified by automated image analysis was divided by the total number of cell nuclei and expressed as relative to GFP-tagged wt Coxiella (Ctrl) to derive the efficiency of host cell invasion. Values are mean ± standard deviation of triplicate samples where an average of 12,000 cells were analyzed per condition (values were compared with Ctrl condition. \( ** = P < 0.001 \), 2way ANOVA). (D). iTASSER-derived prediction the CBU_1260 product. The central core of the protein is composed of an \( \beta \)-sheet transmembrane barrel domain (light blue). The putative periplasmic domain is composed of a \( \alpha \)-helix (dark, whereas the predicted extracellular domain is composed of four (L1 to 4) unstructured loops. Representative Western blot of bacterial lysates from \textit{wt Coxella}, the control mutant \textit{Tn1832} and the 5 mutants presenting transposon insertions in \textit{CBU_1260}. Representative Western blot of \textit{wt Coxella} and mutant \textit{Tn208} subjected to membrane fractionation to assess the localization of OmpA (\( \alpha \)- insoluble fraction; SN/IM = supernatant and inner membrane fraction, OM = outer membrane fraction). Blots were revealed with an antibody raised against the first extracellular loop (L1) of OmpA (top panels) and with a polyclonal antibody against Coxella NMaterial (bottom panels) used as loading control. Cells were fixed in paraformaldehyde. Cells were then probed with an antibody against OmpA corresponding to the intracellular N-terminal alpha helix were used to increase the solubility of the protein. Red fluorescent latex beads were then coated with 100 \( \mu \)g/ml His-OmpA32-248 or GST as control and used to challenge A431 cells for 1 hour at 37°C. Unbound beads were then removed by rinsing cells in PBS and cells were fixed in paraformaldehyde. Cells were then probed with an anti-histidine antibody without permeabilization to differentially label adherent and internalized beads and with fluorescent phalloidin to define the cell perimeter and volume. Alternatively, after fixation samples were further processed for scanning electron microscopy. Confocal microscopy analysis of cross sections of cells incubated with His-OmpA32-248-coated beads revealed a fraction of beads adhering to the cell surface, hence positive for the anti-histidine staining, and another fraction within the cell volume (as defined by the actin labeling) and negative to the anti-histidine staining (Fig. 7A). Three-dimensional reconstruction of confocal sections coupled to surface rendering confirmed the presence of adhering and internalized beads (Fig. 7A). GST-coated beads failed to adhere to and invade A431 cells significantly (Fig. 7B). Scanning electron microscopy analysis corroborated these observations: several His-OmpA32-248-coated beads were adhering to the surface of A431 cells (Fig. 7C, green inset) whereas others were clearly covered by the cell plasma membrane (Fig. 7C, red inset). Very few GST-coated beads were observed at the surface of cells and none seemed to be internalized (data not shown), confirming our observations by fluorescence microscopy. We next assessed the capacity of OmpA to trigger the internalization of non-invasive bacteria by non-phagocytic cells using the gentamicin protection assay. To this aim \textit{E. coli} BL21-DE3 star pLysS were transformed with pET27b-OmpA, which allowed the IPTG-regulated expression and periplasmic targeting of full-length OmpA. The expression and outer membrane localization of OmpA were verified by Western blot using the OmpA-specific antibody on transformed \textit{E. coli} cultures induced overnight with IPTG and processed to separate the bacterial outer membranes from the inner membranes and cytoplasmic components (Fig. 7D). Non-induced, transformed bacteria were used as control. We could observe that IPTG-induced bacteria efficiently produced OmpA, which was enriched in the outer membrane fraction of \textit{E. coli} lysates (Fig. 7D). Importantly, induction of OmpA expression conferred \textit{E. coli} a 20-fold increase in invasiveness as compared to the non-induced bacteria (Fig. 7E). Next, we used the \textit{E. coli} ectopic expression approach to dissect the role of the 4 predicted...
extracellular loops of OmpA. By replacing each loop with a myc tag, we generated 4 OmpA mutants (OmpAΔL1, OmpAΔL2, OmpAΔL3, OmpAΔL4) that were used to test their capacity to confer invasiveness to E. coli in a gentamicin protection assay. Similar to wt OmpA, all mutated proteins were detected in the outer membrane fraction of induced E. coli (not shown). Interestingly, only the exchange of loop 1 with a myc tag significantly reduced E. coli internalization by non-phagocytic cells (Fig. 7E). Collectively, these data suggest that OmpA is necessary and sufficient to mediate Coxiella internalization within non-phagocytic cells and that loop 1 is primarily involved in interacting with a potential cognate receptor at the surface of host cells.

Blocking OmpA interactions with the host cell inhibits Coxiella internalization

To determine whether OmpA function requires the interaction with host cell surface factors, we sought to block candidate ligand/receptor interactions, either by saturating potential OmpA receptors at the surface of host cells or by masking OmpA at the surface of bacteria, prior to infection. In the first case, A431 cells were incubated with 100 µg/ml His-OmpA32-248 for 1 hour at 4°C prior to challenging with wt Coxiiella and the efficiency of bacterial internalization was determined by differential bacterial labeling. A431 cells incubated in the same conditions with GST or with buffer alone were used as controls. Indeed, pretreating A431 cells with His-OmpAΔ22-248 effectively inhibited wt Coxiiella internalization as compared to buffer- or GST-treated cells (Fig. 8A, B). Alternatively, wt Coxiiella were incubated with increasing concentrations (0.1, 1 and 5 µg/ml) of either anti-OmpA antibody or naive rabbit serum prior to infection and bacterial differential labeling was used to determine Coxiiella invasiveness in A431 cells. Confirming our previous observations, the pre-treatment of bacteria with the anti-OmpA antibody, but not naive rabbit serum, inhibited Coxiiella internalization in a concentration dependent manner (Fig. 8C, D). These observations suggest the presence of a receptor for OmpA at the surface of host cells, which remains to be identified, and that OmpA/receptor interactions are essential to mediate Coxiiella internalization within host cells.

Figure 6. Coxiiella OmpA is involved in host cell invasion and intracellular replication. A431 (A) and THP-1 (E) cells were incubated with wt Coxiiella, the control transposon mutant Tn1832 or the OmpA mutant Tn208. 60 minutes after infection (top panels) cells were fixed and labeled with an anti-Coxiiella antibody coupled to Alexa Fluor 555 (blue) and with Atto-647N phalloidin (red) prior to cell permeabilization. Internalized bacteria were detected by GFP fluorescence (green) in the case of Tn208 and Tn1832 whereas for wt Coxiiella infections, cells were permeabilized and bacteria were stained with the anti-Coxiiella antibody as above, coupled to Alexa Fluor 488 (green). Alternatively, cells were fixed 6 (A, bottom panels) or 5 (E, bottom panels) days after infection, DNA (red) was labeled with Hoechst 33258 and wt Coxiiella (green) with the specific antibody as above. The automated image analysis software CellProfiler was used to calculate the percentage of internalized bacteria (B and F), the number of colonies/cell (C and G) and the area (in microns²) of intracellular Coxiiella colonies identified for each condition (D and H). Values are means ± standard deviations of triplicate experiments where an average of 8000 bacteria (B and F) or 400 vacuoles (C, D, G, H) were analyzed for each condition (values were compared to wt Coxiiella infections. ns = non-significant; *** = P<0.001 2way ANOVA for B and F and t test for C, D, G, H). The difference between the percentage of internalized wt Coxiiella (or the control mutant Tn1832) and the Tn208 mutant was statistically significant in B (P<0.001, 2way ANOVA) and non-significant in F. Arrows indicate internalized bacteria (green); arrowheads indicate extracellular bacteria (green and blue). Scale bars 10 µm.

doi:10.1371/journal.ppat.1004013.g006
OmpA mutation attenuates Coxiella virulence in the in vivo model system Galleria mellonella

Larvae of the wax moth Galleria mellonella are an emerging, efficient model for the study of host/pathogen interactions in vivo. Like other insects, Galleria larvae present essential aspects of the innate immune response to microbial infections, which are conserved in mammals. In particular, insects possess humoral and cellular defense responses, the first including antimicrobial peptides (galiomycin, gallerimycin and lysozyme in the case of Galleria) and the latter consisting of specialized phagocytic cells, known as hemocytes or granulocytes [54–57]. Importantly, in the case of several bacterial pathogens, typical phenotypes observed in mammalian infection models were efficiently reproduced using Galleria [58–61]. It has been recently demonstrated that the Coxiella closely-related pathogen Legionella pneumophila invades Galleria hemocytes and replicates within large membranous compartments that present the same morphology and characteristics of Legionella-containing vacuoles generated during infection of macrophages and amoeba [62]. Infections by L. pneumophila result in severe damage to insect organs, which is accompanied by an immune response, including larval melanization and nodule formation [62]. Moreover, the role of bacterial virulence factors previously characterized in higher mammalian models is conserved during infections of Galleria mellonella [61,62]. Importantly, Galleria mellonella larvae are also susceptible to phase II Coxiella infections (Norville et al. Unpublished data). We thus investigated the phenotype associated with the OmpA mutation carried by Tn208 in the context of Galleria infections. Larvae were exposed to Coxiella by injecting $10^8$ bacteria (either wt Coxiella, Tn1832 or Tn208) in the upper right proleg and larvae were incubated at 37°C up to 300 h post-infection to determine survival rates. Alternatively, larvae were incubated up to 24 and 96 h prior to fixation in paraformaldehyde and processing for immunofluorescence. In all cases, larvae injected with PBS were used as a negative control. Larvae injected with PBS alone did not show any survival defect throughout the incubation time, whereas larvae infected with wt Coxiella or the control mutant Tn1832 died significantly faster compared to those infected with the OmpA mutant Tn208 (Fig. 9A). Immunofluorescence analysis revealed that at 96 h post-inoculation, wt Coxiella as well as the control mutant Tn1832 organisms triggered the formation of large, highly infected nodules of hemocytes (Fig. 9B). These were often juxtaposed to larval organs that also appeared severely infected and damaged (Fig. 9C, top panels). In contrast, when Galleria were challenged with the OmpA mutant Tn208 fewer nodules of smaller size were observed throughout the larvae and only a small fraction of these presented signs of infection (Fig. 9B). When these nodules were observed at higher magnification, we could detect small Tn208 colonies (Fig. 9C bottom panels) that were reminiscent of what we had previously observed in cultured macrophages. Our observations indicate that the OmpA-associated phenotypes observed in cultured cells can be reproduced during in vivo infections.

Discussion

Infection by bacterial pathogens depends on the subversion of host functions, which is tightly orchestrated by a battery of virulence proteins referred to as virulence factors. In the last decade, cellular microbiology has stressed the importance of studying pathogens in relation to their host, however, the effective identification of bacterial virulence determinants and the characterization of their diverse mechanisms of action requires the development of new high-throughput and high-content screens (HTS and HCS respectively) [63]. Here, we have set up protocols for the multi-phenotypic screen of bacterial factors that are involved in host cell invasion and colonization. Our approach integrates transposon mutagenesis, genomics, bioinformatics and fluorescence-based functional assays that have been adapted for the large-scale identification of virulence factors from virtually any intracellular bacterium. The advantage of our screening technique lies in the possibility of analyzing every bacterial mutations for multiple phenotypes, such as 1) internalization within the host/cell, 2) intracellular replication and 3) cytotoxicity, simultaneously. This analysis, integrated with the map of genome mutations, allows a global overview of bacterial genes involved in host/pathogen interactions. The emerging bacterial pathogen Coxiella burnetii is an excellent model system to apply our strategy. To date, very little is known about the bacterial factors that regulate Coxiella interactions with the host; however, the recent development of axenic culture techniques now allows genetic manipulation of this microbe and in-depth analysis of its virulence factors [32,64]. Moreover, previous in silico identification of putative Coxiella virulence factors [18,26–31], provides an excellent database to cross-reference bioinformatics analysis to our functional assays. Importantly, our assay allows the identification of Coxiella virulence factors on a whole-genome scale, based on the phenotypes associated with the random mutagenesis of Coxiella CDS.

Our aim being the generation of the first bank of C. burnetii transposon mutants, we chose to sequence all isolated mutants independently of their phenotype during infections. This has provided a global survey of the distribution of transposon insertions...
and allowed us to pinpoint also those genes, suspected to encode virulence factors, which failed to produce a phenotype during *Coxiella* colonization of host cells. Mapping of transposon insertions revealed an overall homogeneous distribution of mutations, with regions of preferential transposon insertion as well as other regions that remained non-mutated. Of note, the lack of transposon insertions in the region between CBU_0678 and CBU_0698 is expected, having used the annotated genome of *Coxiella* NMI (RSA493) to map transposon insertions in *Coxiella* NMII (RSA439) in which this region is missing. Interestingly however, we failed to isolate transposon mutants from the large region between CBU_0215 and CBU_0272, enriched in essential genes encoding ribosomal proteins. This suggests that non-mutated regions may have been targeted by the transposon but gene disruption was lethal for the bacterium. Non-mutated regions may be thus exploited to map essential genes in the *Coxiella* chromosome and plasmid. When analyzing transposon mutants exhibiting a strong replication phenotype, we have occasionally observed internalization phenotypes that were not consistently reproduced in all transposon mutants isolated for a given gene. We believe that this is due to a technical limitation of our screening technique, imposed by the extremely different sizes and associated fluorescence of *Coxiella*.

![Figure 8. Recombinant OmpA and anti-OmpA antibody perturb *Coxiella* interactions with the host cell surface.](A). Representative images of A431 cells pretreated with OmpA_{32-248} buffer or with 100 μg/ml His-tagged OmpA_{32-248} and incubated with wt *Coxiella* for 60 minutes. Cells were fixed and labeled with an anti-*Coxiella* antibody coupled to Alexa Fluor 555 (blue) and with Atto-647N phalloidin (red) prior to cell permeabilization. Internalized bacteria were detected by using the same anti-*Coxiella* antibody as above coupled to Alexa Fluor 488, on permeabilized cells (green). Arrowheads and arrows point at extracellular and intracellular bacteria, respectively. (B). The automated image analysis software CellProfiler was used to analyze cells pretreated with OmpA_{32-248} buffer, 100 μg/ml GST or 100 μg/ml His-tagged OmpA_{32-248} and incubated with wt *Coxiella* for 30, 45 and 60 minutes. For each condition, intracellular and extracellular bacteria were automatically identified, counted and the percentage of internalized bacteria was calculated. (C). Representative images of A431 cells incubated for 60 minutes with wt *Coxiella* pretreated with 1 μg/ml rabbit naive serum or anti-OmpA antibody. After fixation, cells were treated as in A. Arrowheads and arrows point at extracellular and intracellular bacteria, respectively. (D). CellProfiler was used to analyze cells incubated for 60 minutes with wt *Coxiella* pretreated with naive serum or anti-OmpA antibody at increasing concentrations as indicated. For each condition, intracellular and extracellular bacteria were automatically identified, counted and the percentage of internalized bacteria was calculated. In all cases, values are means ± standard deviations of triplicate experiments where an average of 6000 bacteria were analyzed for each condition. In B, the difference between the percentage of internalized wt *Coxiella* in cells treated with buffer alone (or with GST) and cells treated with recombinant OmpA was statistically significant from 45 min of infection (P<0.001, 2way ANOVA). In D, values were compared to naive serum condition. ns = non-significant; * = P<0.05; *** = P<0.001, 2way ANOVA. Scale bars 10 μm. doi:10.1371/journal.ppat.1004013.g008
colonies. In some cases the signal to noise ratio was very close to the threshold imposed to the image analysis software and resulted in colonies and/or bacteria that were not detected when images were segmented. Ongoing implementation of our automated analysis pipeline will allow the identification of these outlier phenotypes.

Independent studies have formally proven the essential role of the *Coxiella* Dot/Icm secretory apparatus by isolating mutants in 4 of the 23 *dot/icm* *Coxiella* genes (*dotA*, *dotB*, *icmD* and *icmL*). In all cases *dot/icm* mutants retained the capacity of invading host cells but failed to generate large PVs and replicate therein. The enrichment of transposon insertions in the region of the *Coxiella* genome hosting *dot/icm* core genes allowed us to validate our assays exploiting existing data and, more importantly, provided a comprehensive overview of the role of the different components of the *Coxiella* T4SS. Interestingly, we have identified mutations resulting in intermediate phenotypes that allowed partial bacterial replication. Of particular interest is a mutation in *icmS*, which confers a multivacuolar phenotype to mutants. The *icmS* gene encodes a chaperone protein that mediates the secretion of a subclass of bacterial effectors. The observation of a multivacuolar phenotype suggests that in *Coxiella*, IcmS may be involved in the secretion of effectors that mediate membrane fusion events required for the biogenesis of the PV. To facilitate the identification of putative IcmS substrates, a machine learning approach is currently being used to identify other transposon mutants that share the same multivacuolar phenotype.

To date, candidate *Coxiella* effectors have been identified by bioinformatics analysis based on conserved Dot/Icm regulatory motif (PmrA) [26,27], C-terminal translocation signals (E-block) [27,28], and eukaryotic-like domains. So far, 354 candidate effectors have been thus identified, however Dot/Icm-dependent translocation assays using either *Coxiella* or *Legionella* as a surrogate model, indicated that the majority of these might be false positives [18,26–28]. In addition, the lack of efficient methods for the genetic manipulation of *Coxiella* severely hampered the functional study of these putative effectors. In a recent study, transposon mutants were obtained from 20 *Coxiella* candidate effectors with 10 exhibiting a significant replication phenotype [27]. Here we report the entry, replication and cytotoxic phenotype of 63 transposon mutants in 31 previously identified *Coxiella* candidate Dot/Icm substrates. Indeed, some of these candidates play a role during infection whereas some others fail to produce a phenotype, stressing the importance of coupling high-content screens to *in silico* analysis to identify bacterial effectors. Moreover, further studies of other *Coxiella* genes sharing none of the features of Dot/Icm substrates, can be exploited to enrich existing databases for the bioinformatics-based identification of *Coxiella* effectors, thus creating a feedback loop that would significantly improve and accelerate the study of *Coxiella* pathogenesis.

A considerable number of transposon insertions were mapped outside *Coxiella* CDS. By excluding mutations that affected the first 100 bp upstream of annotated genes (to exclude mutations that...
might affect promoter regions of genes), we obtained a list of 151 intergenic transposon insertions. Interestingly, 71 of these resulted in a significant replication phenotype, suggesting that these non-coding regions of the *Coxiella* genome may play a role in host/pathogen interactions. sRNAs are emerging as regulators that enable pathogens to adapt their metabolic needs during infection and timely express virulence factors [65,66]. However, recent studies in other organisms revealed the existence of a number of putative sRNAs higher than initially expected, suggesting the presence of many non-functional sRNAs and complicating the identification of relevant sRNAs. The functional data obtained by our screening approach are being cross referenced with a list of putative *Coxiella* sRNAs identified by RNA-seq to facilitate the identification of *Coxiella* sRNAs that may coordinate host/pathogen interactions. Similarly, the interesting observation that a number of mutations in *Coxiella* CDS annotated as pseudogenes have an effect in host cell infection suggests that these genomic regions may have an important regulatory role.

Bacterial adhesion and invasion of host cells is a fundamental step of the infection by intracellular bacterial pathogens [67]. These processes can be active or passive depending on the nature of the pathogen. “Triggering” bacteria commonly use a type 3 secretion system (T3SS) to inject effectors across the host cell plasma membrane to trigger actin rearrangements and pathogen internalization by phagocytosis, whereas “zipping” bacteria use surface proteins that interact with cognate receptors at the surface of host cells [67]. This activates a ligand/receptor signaling cascade that leads to the internalization of large particles by an endocytosis-like mechanism [68–71]. The lack of a T3SS in *Coxiella* suggests that these organisms adhere to and invade host cells by a zipping mechanism. Indeed, it has been reported that *Coxiella* is passively internalized by host cells by a yet undefined mechanism, which is accompanied by the local rearrangement of the actin cytoskeleton [11,14,15]. α2β3 integrins have been shown to mediate *Coxiella* adhesion to THP-1 cells [11], however, the lack of these integrins at the surface of epithelial cells, which are effectively colonized by *Coxiella*, suggest the presence of additional/alternative receptors. Similarly, the *Coxiella* surface determinants for host cell adhesion and invasion remain to be defined. Here we have identified the product of CBU_1260 as the first *Coxiella* invasin. Predictive analysis on the primary sequence of CBU_1260 revealed the presence of 8 beta sheets forming an OmpA-like domain highly homologous to that identified and characterized in several other bacterial pathogens [49,50]. Examples are the OmpA proteins encoded by *E. coli* K1 [44,47], *Yersinia pestis* [45], *Francisella tularensis* [46], *Klebsiella pneumoniae* [48] and *Shigella flexneri* [72]. These outer membrane proteins are involved in bacterial adhesion and/or internalization within host cells, as well as in the NF-kB-mediated modulation of the immune response to infection, which is required for intracellular bacterial development. Importantly, OmpA-like proteins with similar functions in bacterial adhesion and internalization have been reported in other bacterial pathogens such as *Rickettsia conori*, *Anaplasma phagocytophilum* and *Ehrlichia chaffensis* [73–75], however these proteins share no structural homology with the OmpA proteins described above. In agreement with in silico predictions, membrane fractionation experiments performed in *Coxiella* as well as in *E. coli* ectopically expressing OmpA, showed that the protein is indeed enriched in the outer membrane fraction of bacterial lysates. Our multi-phenotypic analysis revealed that five independent transposon insertions that disrupted CBU_1260 sequence severely affected *Coxiella* internalization and replication within host cells. The internalization phenotype was specific of non-phagocytic cells, whereas OmpA mutants were still internalized by phagocytic cells.

This observation indicated that, in the absence of an active phagocytic process, OmpA is able to actively trigger *Coxiella* internalization by means of ligand/receptor interactions. Importantly, however, the intracellular replication of OmpA mutants was severely affected in both epithelial and macrophage cell lines. This phenotype is in line with a reported role of OmpA proteins in facilitating bacterial survival within host cells [46,48,51,53]. Importantly, OmpA-like proteins share conserved transmembrane domains but are characterized by extremely variable extracellular domains, which are unique to each pathogen, and confer specific functions [50]. OmpA was predicted to have 4 unstructured loops exposed at the bacterial surface. By replacing each loop with a myc tag, we have generated 4 OmpA mutants (OmpA ΔL1, ΔL2, ΔL3 and ΔL4) and showed that loop 1 is essential to confer invasiveness to *E. coli* ectopically expressing the OmpA mutants. Accordingly, a specific antibody against loop 1 effectively blocks OmpA function.

Bioinformatics analysis indicated the presence of 2 additional OmpA-like proteins in the *Coxiella* genome, CBU_0937 and CBU_0936, sharing with OmpA a good degree of homology at the level of the transmembrane OmpA-like domain but no significant homology in the 4 extracellular loops. In line with these observations, CBU_0937 and CBU_0936 failed to produce internalization phenotypes when mutated by transposon insertions. Finally, experiments aiming at blocking potential OmpA interactions with a cognate receptor, effectively blocked *Coxiella* internalization, indicating the presence of an interacting partner at the surface of host cells, which remains to be identified. Using *Galleria mellonella* larvae as a surrogate in vivo model system we could reproduce the OmpA mutant phenotypes observed in cultured cells. Indeed, only wt *Coxiella* and the control mutant Tn1832 were able to induce the formation of nodules that were abundantly colonized by *Coxiella* and disrupt the organization of *Galleria* peripheral organs. The OmpA mutant Tn208 induced a milder formation of nodules that presented few, isolated bacteria. Accordingly, larvae infected with the OmpA mutant survived *Coxiella* infections longer than those infected with the control mutants.

In summary, multi-phenotypic screening of host/pathogen interactions is an efficient method for the study of infectious diseases. Here we have applied this method to *Coxiella* infections and identified a bacterial protein that is essential for *Coxiella* internalization within non-phagocytic cells. Understanding how intracellular bacteria adhere to and invade their host is essential to 1) understand the cell biology of infection and identify the candidate targets of anti-infectious molecules and 2) to develop targeted vaccines. Of note, bacterial OmpA proteins are considered as new pathogen-associated molecular patterns (PAMPs) and are among the most immuno-dominant antigens in the outer membrane of Gram-negative bacteria [50,76]. Our laboratory currently investigates the possibility of using OmpA to develop a synthetic vaccine against Q fever.

**Materials and Methods**

**Bacterial strains, cell lines and growth conditions**

Strains used in this study are listed in Fig. S9. *Echerichia coli* strains were grown in Luria-Bertani (LB) medium supplemented with ampicillin (100 μg/ml), kanamycin (50 μg/ml) or chloramphenicol (30 μg/ml) as appropriate. *Coxiella burnetii* NMII and transposon mutants were grown in ACCM-2 [77] supplemented with kanamycin (340 μg/ml) or chloramphenicol (3 μg/ml) as appropriate in a humidified atmosphere of 5% CO₂ and 2.5% O₂ at 37°C. Cells were routinely maintained in RPMI (Vero, THP-1, J774 and RAW 264.7) or DMEM (A431 and HeLa), containing...
10% fetal calf serum (FCS) in a humidified atmosphere of 5% CO₂ at 37°C. For experiments, THP-1 were allowed to differentiate into macrophages for 2 days in the presence of 200 nM phorbol myristate acetate (PMA, Sigma).

**Antibodies and reagents**
Hoechst 33258, rabbit anti poly-His, anti mouse and anti-rabbit HRP-conjugated antibodies and Atto-647N phalloidin were purchased from Sigma. Rabbit anti *Coxiella* NMII antibodies were kindly provided by Robert Heinzen. Synthesis and production of the peptide KKS8TSKVNFTGVTL, as well as the generation of the cognate antibody in rabbit (named anti-OmpA in this study) were performed by Eurogentec, Belgium. Mouse and rabbit IgG conjugated to Alexa Fluor 488 and 555 as well as Prolong Gold antifade mounting reagent were purchased from Invitrogen. Paraformaldehyde was provided by Electronic Microscopy Sciences, PA.

**Plasmids**
Plasmids and primers used in this study are listed in Fig. S9. DNA sequences were amplified by PCR using Phusion polymerase (New England Biolabs) and gene-specific primers (Sigma). To create the plasmid pITR-CAT-ColE1-P311-GFP, the promoter of CBU_0311 (P311) was amplified from *Coxiella* RSA439 NMII genomic DNA using primers P311-XhoI-Fw and P311-Rv, GFP was amplified from pEGFP-N1 (Clontech) using primers EGFP-Fw and GFP-PITR-Rv, and P1169-CAT-ColE1 was amplified from plasmid pITR-CAT-ColE1 using primers GFP-PITR-Fw and XhoI-PITR-Rv. PCR fragments P311, GFP and P1169-CAT-ColE1 were fused by overlapping PCR. The resulting PCR product was digested with XhoI and ligated to obtain circular pITR-CAT-ColE1-P311-GFP. OmpA was amplified from *RSA439 NMII* genomic DNA using primers OmpA-BamHI-shift-Fw and OmpA-EcoRI-Rv and cloned into pET28a to obtain pET28a-OmpA-BamHI. OmpA was amplified from *Coxiella* RSA439 NMII genomic DNA using primers OmpA-BamHI-shift-Fw and OmpA-EcoRI-Rv and cloned into pET27b to obtain pET27b-OmpA. Plasmids pET27b-OmpA-L1, pET27b-OmpA-L12, pET27b-OmpA-L13 and pET27b-OmpA-L14 were generated by PCR using pET27b-OmpA as template and primer pairs loop1-myc-HindIII-Fw/loop1-myc-HindIII-Rv, loop2-myc-HindIII-Fw/loop2-myc-HindIII-Rv, loop3-myc-HindIII-Fw/loop3-myc-HindIII-Rv, loop4-myc-HindIII-Fw/loop4-myc-HindIII-Rv. The PCR products were digested with HindIII and ligated to obtain the corresponding plasmids.

**Generation of a bank of *Coxiella* transposon mutants**
*C. burnetii* RSA439 NMII organisms were electroporated with the pITR-CAT-ColE1-P311-GFP and pUC19:: Himar1C9 plasmids [34] using the following setup: 18 kV, 400 µF, 25 µF. Bacteria were then grown overnight in ACCM-2 supplemented with 1% FBS and the following day 3% BSA, 50 mM NH₄Cl in PBS, pH 7.4. Cells were then incubated in a humidified atmosphere of 5% CO₂ at 37°C. Unbound bacteria were removed after 1 h of incubation and cells were further incubated in fresh culture medium for 7 days. Plates were analyzed at a 24-hours interval using a TECAN Infinite 200 Pro operated by the Magellan software (TECAN) to monitor the variations of GFP fluorescence associated with the intracellular growth of *Coxiella*. Raw data were analyzed for background subtraction, normalization and quality control among triplicates using in-house developed methods. Seven days after infection, plates were fixed in 3% paraformaldehyde in PBS at room temperature for 30 minutes, rinsed in PBS and incubated in blocking solution (0.5% BSA, 50 mM NH₄Cl in PBS, pH 7.4). Cells were then incubated in Hoechst 33258 diluted 1:200 in blocking solution for 30 minutes at room temperature, rinsed and incubated in PBS. Images were acquired with an Arrayscan VTI Live epifluorescence automated microscope (G Billings) equipped with an ORCA ER CCD camera. 6 fields/well of triplicate 96-wells plates were imaged with a 20× objective in the GFP, DAPI and Bright-field channels. Images were then processed and analyzed using CellProfiler. Briefly, the GFP channel was subtracted from the corresponding DAPI channel to avoid false identification of large *Coxiella* colonies as host cell nuclei, images were thresholded using the Otsu global method and host cell nuclei as well as *Coxiella* colonies were identified and segmented. The number, form factor and fragmentation of *Coxiella* colonies were then calculated per object and per image. An average of 14000 cells per condition (infection with a given *Coxiella* mutant) were thus analyzed. Raw data were processed for background subtraction, normalization and quality control among the 6 fields per well and plate triplicates using in-house developed methods. Data were recorded in a relational database (FileMaker) that allowed clustering of phenotypes according to the annotated transposon insertions.
Immunofluorescence staining and microscopy

Cells were fixed in 3% paraformaldehyde in PBS at room temperature for 30 minutes, rinsed in PBS and incubated in blocking solution (0.5% BSA, 50 mM NH₄Cl in PBS, pH 7.4). When appropriate, 0.05% saponin was added to the blocking solution for cell permeabilization. Cells were then incubated with the primary antibodies diluted in blocking solution for 1 h at room temperature, rinsed five times in PBS and further incubated for 45 min with the secondary antibodies diluted in the blocking solution. Flourescent phalloidin was added to the secondary antibodies to label actin, where needed. After labeling, coverslips were mounted using Prolong Gold antifade mounting medium (Invitrogen) supplemented with Hoechst 33258 for DNA staining.  

For differential labeling, extracellular bacteria or beads were stained using specific antibodies without permeabilizing the cells. Intracellular bacteria or beads were visualized by green and red fluorescence, respectively. Alternatively, a second staining was performed after cellular permeabilization. Secondary antibody labeling using two different fluorochromes (before and after permeabilization) allowed discrimination between adherent extracellular bacteria/beads and those that have been internalized.  

Samples were analyzed with a Zeiss Axioimager Z1 epifluorescence microscope (Carl Zeiss) connected to a Coolsnap HQ2 CCD camera. Images were acquired alternatively with 63× or 40× oil immersion objectives and processed with MetaMorph (Universal Imaging Corp.). Image J and CellProfiler software were used for image analysis and quantifications. 3D reconstruction and surface rendering were performed using the OsiriX software.  

Complementation assay  

Transposon insertions in Dot/Icm core genes were complemented in trans as previously described [19]. Briefly, Vero cells grown on 96-wells plates were either challenged with the transposon mutants alone or in combination with wt Coxiella at a 1:1 ratio for a total MOI of 100. Bacterial contact with cells was promoted by centrifugation (10 min, 400 g, RT) and cells were incubated in a humidified atmosphere of 5% CO₂ at 37°C. Unbound bacteria were removed after 1 h of incubation and cells were further incubated in fresh culture medium for 7 days. Plates were then fixed in 3% paraformaldehyde in PBS at room temperature for 30 minutes, rinsed in PBS and incubated in blocking solution (0.5% BSA, 50 mM NH₄Cl in PBS, pH 7.4). Cells were then labeled with the anti-NMII antibody to detect wt Coxiella and with Hoechst 33258 as described above for DNA labeling. 96-well plates were imaged and analyzed essentially as described above for the mutant library screening protocol with the labeling. 96-wells plates were imaged and analyzed using an Arrayscan VTI Live epifluorescence automated microscope (Cellomics) equipped with an ORCA ER CCD camera. 10 fields/well were imaged with a 20× objective in the GFP (bacteria), TRITC (TUNEL), DAPI (nuclei) and Bright-field (cells) channels. Images were then processed and analyzed using CellProfiler. Briefly, the GFP channel was subtracted from the corresponding DAPI channel to avoid false identification of large Coxiella colonies as host cell nuclei, images were thresholded using the Otsu global method and host cell nuclei, Coxiella colonies and fragmented nuclei were identified and segmented. The percentage of fragmented nuclei over the total number of nuclei was then calculated on an average of 6000 cells per condition.  

Scanning electron microscopy

Cells were washed in PBS and fixed with 2.5% glutaraldehyde in Sorensen buffer, pH 7.2 for an hour at room temperature, followed by washing in Sorensen buffer. Fixed samples were dehydrated using a graded ethanol series (30–100%), followed by 10 minutes in graded Ethanol-Hexamethyldisilazane and finally Hexamethydisilazane alone. Subsequently, the samples were sputter coated with an approximative 10 nm thick gold film and then examined using a scanning electron microscope (Hitachi S4000, at CRIC, Montpellier France) using a lens detector with an acceleration voltage of 20 kV at calibrated magnifications.  

Immuno-histology

Galleria mellonella larvae were fixed overnight in paraformaldehyde 4% in PBS (pH 7.4). Larvae were then rinsed 3 times in PBS and cryo protected by successive incubations in PBS containing increasing concentrations of sucrose (10%, 20%, 30%). Samples were then frozen in isopentane at −80°C using a SnapFrost machine (Excilone). Consecutive 20 µm sections were then obtained from each sample using a Leica CM3050S cryostat. For indirect immuno-fluorescence, sections were permeabilized and blocked in PBS, 10% goat serum, 0.3% Triton X-100 for 1 h at room temperature. Samples were then incubated 48 hours at 4°C with the anti GFP antibody, then rinsed in PBS. Samples were then incubated with the appropriate secondary antibodies, Alexa 488 (Amersham) or with a GoTome-equipped Zeiss Axiomager Z1 epifluorescence microscope (Carl Zeiss) connected to a Coolsnap HQ2 CCD camera. Images were acquired alternatively with a 10× objective (EVOS) or with a 63× oil immersion objective (Axiomager Z1) and processed with Image J and *ImageVision (Carl Zeiss).*  

Protein expression and purification

Genes cloned into pET27b or pET28a vectors were expressed in *E. coli* BL21-DE3 star (pLysS) (Invitrogen). Bacterial cultures were grown at 25°C to mid-exponential phase (OD₆₀₀nm = 0.5) and were induced overnight with 400 µM isopropyl-β-D-thiogalactopyranoside (IPTG). For GST expression, *E. coli* XL1-blue were transformed with pGEX-4T1, grown at 37°C to mid-exponential phase (OD₆₀₀nm = 0.5) and induced for 4 h with 1 mM IPTG. Bacteria were harvested by centrifugation, resuspended in lysis buffer (20 mM Tris pH 8, 300 mM NaCl, 5% glycerol, complete anti-protease (Roche)) and lysed with BugBuster (Novagen) following the manufacturer’s recommendations. Lysates were then cleared by centrifugation (11 000 g, 20 min, 4°C). Proteins were purified by gravity flow using Ni²⁺ agarose His-select
resin column (Sigma) for His-tagged proteins or glutathione-
sepharose (Sigma) for GST. His-tagged and GST proteins were
euIed with lysis buffer supplemented with 250 mM imidazole or
25 mM reduced glutathione, respectively.

Membrane fractionation

For *Coxiella* membrane fractionation, 100 ml of wt *Coxiella* or
Tn208 mutant grown in ACCM-2 for 7 days were pelleted and
resuspended in 200 ml 20 mM Tris pH 8 containing 1 Complete
protease inhibitor (Roche). For *E. coli* membrane fractionation,
30 ml of IPTG-induced or non-induced *E. coli* BL21-DE3 star
pLysS pET27b-OmpA, pET27b-OmpAAL1, pET27b-OmpAAL2-
pET27b-OmpAAL3 or pET27b-OmpAAL4 were pelleted and
resuspended in 5 ml 20 mM Tris pH 8 containing 1 Complete
protease inhibitor (Roche). Bacteria were sonicated using a
Branson Sonifer S-450 (6 pulses of 20 s at 40% intensity) and
cleared by centrifugation at 10000 g for 5 min at 4°C. Inner
membrane proteins were extracted by incubation with sarcosyl
(0.5% final concentration) at RT for 15 min. Outer membrane
proteins were pelleted by ultracentrifugation (TLA-100, 12000 r.p.m.,
30 min, 4°C) and resuspended in 2× Laemml sample buffer. Insoluble, soluble/sarkosyl-solubilized and outer
membrane fractions were resolved by SDS-PAGE and analyzed
by Coomassie staining (Sigma) or immunoblotting with anti-
OmpA and anti NMII antibodies.

Preparation of protein-coated beads

0.5 μm fluorescent red sulfate-modified polystyrene beads
(Sigma) were washed three times with 25 mM MES pH 6.1
(MES buffer). The sulfate-modified beads (7.2 × 109) were
then mixed with either 100 μg/ml purified GST or His-OmpA32-248
and incubated at room temperature (RT) for 4 h. The GST- or
His-OmpA32-248-coated beads were then washed three times with
MES buffer and resuspended in MES buffer containing 1% BSA.

Internalization assays

For fluorescent beads internalization assay, 7 × 107 GST- or His-
OmpA32-248-coated beads in DMEM were applied to 1 × 104
A431 cells seeded onto glass coverslips in 24-well plates and contact
was promoted by centrifugation (10 min, 400 g, RT). Cells were
then incubated in a humidified atmosphere of 5% CO2 at 37
°C. Internalization assay described above. For antibody inhibition
experiments, 6 × 104 C. burnetii wt or pET27b-OmpA32-248-coated beads
were then washed three times with MES buffer and resuspended in MES buffer containing 1% BSA.

Figure S1 Role of Dot/Icm core proteins in *Coxiella*
infestions. (A) Axenic (ACCM-2) growth of the 38 Dot/Icm
transposon mutants isolated in this study, wt *Coxiella* (dashed black
line) and the control transposon mutant Tn1832 (black line)
were used as controls (Crls). Mutants in the same CDS are grouped by
color. (B) *Coxiella* mutants in dot/icm genes were clustered in rows,
according to the mutated gene and intracellular replication (R),
internalization (I) and cytotoxic (C) phenotypes were illustrated.
White squares represent non-significant phenotypes (Z-
score<−2). Pink squares represent mild phenotypes (Z-score
between −2 and −4). Red squares represent strong phenotypes
(Z-score≤−4). Tn: Mutant number; Locus Tag: CDS number;
Gene: gene name; PATRIC: accession number (PATRIC annotation);
Operon: operon number (DOOR annotation); Strand: sense vs.
antisense CDS; SC: transposon insertion site from CDS starting codon (bp).

(TIF)

Figure S2 Mutant Tn1832 carries an intergenic transpo-
son insertion that phenocopies wt *Coxiella* and GFP-
*Coxiella*. (A) Intergenic insertion sites of the miniTn7 transpo-
son carrying the egfp gene (top) used to generate GFP-*Coxiella*
and of the Himar1-based transposon in mutant Tn1832 (bottom). (B)
Intracellular growth curves of the Tn1832 mutant as compared to
GFP-*Coxiella* in Vero cells. (C) Representative images of Vero cells
infected with GFP-*Coxiella*, wt-*Coxiella* and the Tn1832 control
mutant. Colonies (green) are juxtaposed to nuclei of infected host
cells (red). The average area (in microns²) of colonies and the
number of colonies per cell were compared for the three strains.
Data were calculated using CellProfiler; values are means ±
standard deviations of triplicate samples; the total number of
analyzed cells is indicated in the right-most column of the table (n).
Scale bars 20 μm.

(TIF)

Figure S3 Coxiella pseudogenes mutated in this study.
Mutants presenting transposon insertions disrupting *Coxiella*
genes annotated as pseudogenes were clustered in rows according
to the mutated gene (CDS) and their intracellular replication (R),
internalization (I) and cytotoxic (C) phenotypes were illustrated.
White squares represent non-significant phenotypes (Z-
score<−2). Pink squares represent mild phenotypes (Z-score
between −2 and −4). Red squares represent strong phenotypes
(Z-score≤−4). Where available, information on the annotated
CDS name (Gene), feature (Feature), and domain (Domain) were
integrated in the table.

(TIF)

Figure S4 Characterization of the 7 cytotoxic mutants
isolated in this study. (A) Table indicating the CDS that were
mutated in the 7 cytotoxic mutants isolated. (B) HeLa cells were
either left non-infected (N.I) or inoculated with wt *Coxiella*,
the control transposon mutant Tn1832, the DotA transposon mutant
Tn207 (dotA::Tn6) and the 7 cytotoxic transposon mutants. 3 days
post-inoculation cells were either fixed (white bars, Ctrl) or treated
with staurosporine for 4 hours (black bars, STS) prior to fixation.
CellProfiler was used to calculate the percentage of fragmented
host cell nuclei as detected using the TUNEL assay. Values are means ± standard deviations of duplicate experiments where an average of 6000 cells were analyzed for each condition (values corresponding to untreated or staurosporine-treated cells were compared to their respective non-infected conditions. ns = non-significant; *** = P<0.001, 2way ANOVA). (TIF)

Figure S5 Identification of Coxiella mutations with a strong internalization phenotype. Mutants presenting transposon insertions with a strong internalization phenotype were clustered in rows according to the mutated gene (CDS) and their intracellular replication (R), internalization (I) and cytotoxic (C) phenotypes were illustrated. White squares represent non-significant phenotypes (Z-score ≥ −2). Pink squares represent mild phenotypes (Z-score between −2 and −4). Red squares represent strong phenotypes (Z-score ≤ −4). Where available, information on the annotated CDS name (Gene), feature (Feature), pathway (Pathway class) and domain (Motif/Domain) were integrated in the table. CDS putatively involved in bacterial metabolism were excluded and the remaining CDS were boxed in red. (TIF)

Figure S6 Characterization of CBU_1260 (OmpA) transposon mutants. (A), Axenic (ACC-M) growth of the 5 OmpA transposon mutants isolated in this study. (B), Coxiella (dashed black line) and the control transposon mutant Tn1832 (black line) were used as controls. (B), ompa was amplified with specific primers from mutant Tn208 and wt Coxiella. A sample without template was used as negative control (Negative). The shift in PCR product size corresponds to the transposon insertion in CBU_1260 (C). Mutant Tn208 genomic DNA was either left undigested or digested with BsaHI prior to migration on agarose gel and Southern blot analysis using a fluorescent GFP probe. The band size corresponds to the transposon insertion in CBU_1260. (black line) and the control transposon mutant Tn1832 (black line). A sample without template was used as negative control (Negative). The shift in PCR product size corresponds to the transposon insertion in CBU_1260 (C). Mutant Tn208 genomic DNA was either left undigested or digested with BsaHI prior to migration on agarose gel and Southern blot analysis using a fluorescent GFP probe. (TIF)

Figure S7 Sequence alignment of Coxiella OmpA-like transmembrane domain-containing proteins. The primary sequence of CBU_1260 (OmpA) was aligned to those of CBU_0307 and CBU_0936, two hypothetical proteins annotated as OmpA-like transmembrane domain-containing proteins. Light grey boxes indicate similarities, dark grey boxes indicate identity. (TIF)

Figure S8 Coxiella internalization by J774 and RAW macrophages and intracellular replication. J774 (top charts) and RAW (bottom charts) macrophages were incubated with Tn Coxiella, the control transposon mutant Tn1832 or the OmpA mutant Tn208 for the indicated time points. Cells were fixed and labeled with an anti-Coxiella antibody coupled to Alexa Fluor 555 and with Atto-647N phalloidin prior to cell permeabilization. Internalized bacteria were detected by GFP fluorescence in the case of Tn208 and Tn1832 whereas for Tn Coxiella infections, cells were permeabilized and bacteria were stained with the anti-Coxiella antibody as above, coupled to Alexa Fluor 488. Alternatively, cells were fixed at 5 days after infection; DNA was labeled with Hoechst 33258 and Tn Coxiella with the specific antibody as above. The automated image analysis software CellProfiler was used to calculate the percentage of internalized bacteria (A and D), the number of colonies/cell (B and E) and the area (in microns²) of intracellular Coxiella colonies (C and F) identified for each condition. Values are means ± standard deviations of triplicate experiments where an average of 8000 bacteria (A and D) or 400 vacuoles (B, C, E, F) were analyzed for each condition (values were compared to Tn Coxiella infections. ns = non-significant; *** = P<0.001 2way ANOVA for A and D and t test for B, C, E, F). (TIF)

Figure S9 Tables of strains, plasmids and primers used in this study. (TIF)

Table S1 Large-scale identification of Coxiella factors involved in host/pathogen interactions. All mutants screened in this study were clustered in rows according to the mutated gene (CDS) and their intracellular replication (R), internalization (I) and cytotoxic (C) phenotypes were illustrated. White squares represent non-significant phenotypes (Z-score ≥ −2). Pink squares represent mild phenotypes (Z-score between −2 and −4). Red squares represent strong phenotypes (Z-score ≤ −4). Tn: Mutant number; Locus Tag: CDS number; Feature type: CDS vs. pseudogene; Gene: gene name; Product: gene product; PATRIC ID: accession number (PATRIC annotation); Operon ID: operon number (DOOR annotation); Strand: sense vs. antisense CDS; Start: CDS starting codon position; Stop: CDS stop codon position; Length: CDS length (bp); GC: transposon insertion site from CDS starting codon (bp); Pathway name/class: metabolic pathways; Candidate Feature: presence of typical features of Dot/Icm substrates; Dot/Icm Translocation: previously reported data on Dot/Icm-mediated secretion; Domain: presence of eukaryotic-like domains; Reference: reference to previously reported studies on CDS. (XLSX)

Acknowledgments
The authors are grateful to: Dr. Robert Heinen for sharing C. burnetii strains, antibodies and tools and for helpful scientific discussions; Dr. Howard Shuman for sharing data and for scientific discussions; Chantal Cazevieille and Cécile Sanchez (Centre de Ressources en Imagerie Cellulaire, IUR, 641 Avenue du Doyen Gaston Giraud 34095 MONTPELLIER Cedex 5, Chantal Ripoll (Plateau Histologie, Réseau d’Histologie Experimenterale de Montpellier-RHEM-Institut des Neurosciences de Montpellier, INSMR 1051, 81, rue Auguste Fliche 34091 MONTPELLIER Cedex 13) and Virginie Georget and Sylvain DeRossi (Montpellier Rio Imaging-MRI, 1919 Route de Mende, 34293 MONTPELLIER) for their technical assistance and data analysis.

Author Contributions
Conceived and designed the experiments: EM MB FC IN. Performed the experiments: EM MB FC IN. Analyzed the data: EM MB FC LF IN. Performed the experiments: EM MB FC IN. Contributed reagents/materials/analysis tools: EM MB FC LF IN. Wrote the paper: EM MB.

References
1. Maurin M, Raoult D (1999) Q fever. Clin Microbiol Rev 12: 510–555.
2. Van Schaik EJ, Chen C, Mertens K, Weber MM, Samuel JE (2013) Molecular pathogenesis of the obligate intracellular bacterium Coxiella burnetii. Nat Rev Microbiol 11: 561–573. doi:10.1038/nrmicro3049.
3. Kazar J (2005) Coxiella burnetii infection. Ann N Y Acad Sci 1063: 105–114. doi:10.1196/annals.1355.018.
4. McCaul TF, Williams JC (1981) Developmental cycle of Coxiella burnetii: structure and morphogenesis of vegetative and sporogenic differentiations. J Bacteriol 147: 1063–1076.
5. Voth DE, Heinen RA (2007) Lounging in a lysosome: the intracellular lifestyle of Coxiella burnetii. Cell Microbiol 9: 829–840. doi:10.1111/j.1462-5822.2007.00901.x.
28. Lifshitz Z, Burstein D, Peeri M, Zusman T, Schwartz K, et al. (2013) Comparative virology of intracellular and intercellular lipopolysaccharide variants of Coxiella burnetii in the guinea pig model. Infect Immun 81: 1144–1150.

29. Ando H, Russell-Lodrigue KE, Zhang G, Samuel JE (2005) Comparative virology of phase I and II Coxiella burnetii in intracellular mouse models. Ann N Y Acad Sci 1063: 167–170. doi:10.1196/annals.1355.026.

30. Voth DE, Heinzen RA (2009) Sustained activation of Akt and Erk1/2 is required for intracellular replication of phase II Coxiella burnetii. Infect Immun 77: 205–213. doi:10.1128/IAI.01124-08.

31. Lührmann A, Roy CR (2007) Coxiella burnetii inhibits activation of host cell apoptosis through a mechanism that involves preventing cytochrome c release from mitochondria. Infect Immun 75: 5292–5298. doi:10.1128/IAI.00634-07.

32. Lührmann A, Noqueria CV, Carey KL, Roy CR (2010) Inhibition of programmed cell death by a Coxiella burnetii Icm/Dot effector protein. Proc Natl Acad Sci U S A 107: 18997–19001. doi:10.1073/pnas.1003810107.

33. Klingenberg I, Eckart RA, Berens C, Lührmann A (2012) The Coxiella burnetii type IV secretion system substrate CaeB inhibits intrinsic apoptosis at the mitochondrial level. Cell Microbiol [pub ahead of print]. doi:10.1111/j.1365-2958.2012.02306.x.

34. Chen C, Bansa S, Mertens K, Weber MM, Gorbatcheva I, et al. (2010) Large-scale identification and transcription of type IV secretion substrates by Coxiella burnetii. Proc Natl Acad Sci U S A 107: 21755–21760. doi:10.1073/pnas.1009547107.

35. Wehrer MM, Chen C, Rowin K, Mertens K, Galvan G, et al. (2013) Identification of Coxiella burnetii type IV secretion substrates required for intracellular replication and Coxiella-containing vacuole formation. J Bacteriol 195: 3914–3924. doi:10.1128/JB.00071-13.

36. Lifshtz Z, Burstein D, Peeri M, Zusman T, Schwartz K, et al. (2013) Computational modeling and experimental validation of the Legionella and Coxiella virulence-related type-IV secretion signal. Proc Natl Acad Sci U S A 110: E707–E715. doi:10.1073/pnas.1215271110.

37. Pan X, Lührmann A, Sato A, Laskowski-Arcz MA, Roy CR (2008) Ankyrin repeat proteins comprise a diverse family of bacterial type IV effectors. Science 320: 1653–1655. doi:10.1126/science.1156060.

38. Voth DE, Howe D, Beara PA, Vogel JP, Unsworth N, et al. (2009) The Coxiella burnetii Ankyrin Repeat Domain-Containing Protein Family Is Heterogeneous, with C-Terminal Truncations That Influence Dot/Icm-Mediated Secretion. J Bacteriol 191: 4142–4153. doi:10.1128/JB.01556-08.

39. Voth DE, Beara P A, Howe D, Sharma UM, Samolis G, et al. (2011) The Coxiella burnetii cysteine proteinase is enriched in genes encoding type IV secretion system substrates. J Bacteriol 193: 1495–1503. doi:10.1128/JB.01359-10.
57. Vogel H, Altincicek B, Glöckner G, Vilcinskas A (2011) A comprehensive transcriptome and immune-gene repertoire of the lepidopteran model host Galleria mellonella. BMC Genomics 12: 308. doi:10.1186/1471-2164-12-308.

58. Olsen RJ, Watkins ME, Cantu CC, Beres SB, Musser JM (2011) Virulence of serotype M3 Group A Streptococcus strains in wax worms (Galleria mellonella larvae). Virulence 2: 111–119. doi:10.4161/viru.2.2.14338.

59. Champion OL, Karlyshev A V, Senior NJ, Woodward M, La Ragione R, et al. (2010) Insect infection model for Campylobacter jejuni reveals that O-methyl phosphoramidate has insecticidal activity. J Infect Dis 201: 776–782. doi:10.1086/650494.

60. Bergin D, Reeves E (2005) Superoxide Production in Galleria mellonella Hemocytes: Identification of Proteins Homologous to the NADPH Oxidase Complex of Human Neutrophils. Infect Immun 73: 4161–70 doi:10.1128/IAI.73.7.4161.

61. Mukherjee K, Altincicek B, Hain T, Domann E, Vilcinskas A, et al. (2010) Galleria mellonella as a model system for studying Listeria pathogenesis. Appl Environ Microbiol 76: 310–317. doi:10.1128/AEM.01301-09.

62. Harding CR, Schroeder GN, Reynolds S, Kosta A, Collins JW, et al. (2012) Legionella pneumophila pathogenesis in the Galleria mellonella infection model. Infect Immun 80: 2780–2790. doi:10.1128/IAI.01302-12.

63. Brodin P, Christophe T (2011) High-content screening in infectious diseases. Curr Opin Chem Biol 15: 534–539. doi:10.1016/j.cbpa.2011.05.023.

64. Ormsland A, Heinen RA (2011) Life on the outside: the rescue of Coxiella burnetii from its host cell. Annu Rev Microbiol 65: 111–129. doi:10.1146/annurev-micro-090110-102927.

65. Papenfort K, Vogel J (2010) Regulatory RNA in bacterial pathogens. Cell Host Microbe 8: 116–127. doi:10.1016/j.chom.2010.06.008.

66. Toledo-Arana A, Repola F, Cossart P (2007) Small noncoding RNAs controlling pathogenesis. Curr Opin Microbiol 10: 102–108. doi:10.1016/j.mib.2007.03.004.

67. Pizarro-Cerdá J, Cossart P (2006) Bacterial adhesion and entry into host cells. Cell 124: 715–727. doi:10.1016/j.cell.2006.02.012.

68. Veiga E, Guttman J a, Bonazzi M, Bucourt E, Toledo-Arana A, et al. (2007) Invasive and adherent bacterial pathogens co-Opt host clathrin for infection. Cell Host Microbe 2: 340–351. doi:10.1016/j.chom.2007.10.001.

69. Bonazzi M, Vasudevan L, Mallet A, Sarche M, Sartori A, et al. (2011) Clathrin phosphorylation is required for actin recruitment at sites of bacterial adhesion and internalization. 195: 525–536. doi:10.1038/jcb.201105152.

70. Bonazzi M, Kühbacher A, Toledo-Arana A, Mallet A, Vasudevan L, et al. (2012) A common clathrin-mediated machinery co-ordinates cell-cell adhesion and bacterial internalization. Traffic 13: 1653–1666. doi:10.1111/tra.12009.

71. Pizarro-Cerdá J, Bonazzi M, Cossart P (2010) Clathrin-mediated endocytosis: what works for small, also works for big. Bioessays 32: 496–504. doi:10.1002/bies.200908172.

72. Porv D, Chakrabarti MK (2013) Outer membrane protein A (OmpA) from Shigella flexneri 2a: a promising subunit vaccine candidate. Vaccine 31: 3644–3650. doi:10.1016/j.vaccine.2013.05.100.

73. Hillman RD, Baktash YM, Martinez JJ (2013) OmpA-mediated rickettsial adherence to and invasion of human endothelial cells is dependent upon interaction with α2β1 integrin. Cell Microbiol 15: 727–741. doi:10.1111/cmi.12068.

74. Ojogun N, Kahlert A, Ragland SA, Troese MJ, Mastrominio JE, et al. (2012) Anaplasma phagocytophilum outer membrane protein A interacts with sialylated glycoproteins to promote infection of mammalian host cells. Infect Immun 80: 3748–3760. doi:10.1128/IAI.00654-12.

75. Popov VL, Yu XJ, Walker DH (2008) The 120 kDa outer membrane protein of Ehrlichia chaffeensis preferential expression on dense-core cells and gene expression in Escherichia coli associated with attachment and entry. Microb Pathog 28: 71–80. doi:10.1016/j.mpath.1999.0327.

76. Jeanmin P, Magistrrelli G, Goetsch L, Hazou J-F, Thieblemont N, et al. (2002) Outer membrane protein A (OmpA): a new pathogen-associated molecular pattern that interacts with antigen presenting cells-impact on vaccine strategies. Vaccine 20 Suppl 4: A23–7.

77. Ormsland A, Beare P a, Hill J, Cockrell DC, Howe D, et al. (2011) Isolation from animal tissue and genetic transformation of Coxiella burnetii are facilitated by an improved axenic growth medium. Appl Environ Microbiol 77: 3720–3725. doi:10.1128/AEM.02826-10.