NEW TECHNOLOGIES: METHODS AND APPLICATIONS

Engineering of obligate intracellular bacteria: progress, challenges and paradigms

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Abstract | It is estimated that approximately one billion people are at risk of infection with obligate intracellular bacteria, but little is known about the underlying mechanisms that govern their life cycles. The difficulty in studying *Chlamydia* spp., *Coxiella* spp., *Rickettsia* spp., *Anaplasma* spp., *Ehrlichia* spp. and *Orientia* spp. is, in part, due to their genetic intractability. Recently, genetic tools have been developed; however, optimizing the genomic manipulation of obligate intracellular bacteria remains challenging. In this Review, we describe the progress in, as well as the constraints that hinder, the systematic development of a genetic toolbox for obligate intracellular bacteria. We highlight how the use of genetically manipulated pathogens has facilitated a better understanding of microbial pathogenesis and immunity, and how the engineering of obligate intracellular bacteria could enable the discovery of novel signalling circuits in host–pathogen interactions.

Bacteria have historically been divided into two distinct groups: extracellular bacteria, which exist as free-living organisms in their environmental niches, and intracellular bacteria, which infect and replicate inside host cells. Facultative intracellular bacteria, including *Salmonella* spp., *Francisella* spp., *Legionella pneumophila*, *Listeria monocytogenes*, *Yersinia* spp. and many others, can replicate in either niche. Conversely, obligate intracellular bacteria, including *Chlamydia* spp., members of the order Rickettsiales (*Anaplasma* spp., *Ehrlichia* spp., *Rickettsia* spp. and *Orientia* spp.) and *Coxiella burnetii*, generally require a host cell for replication (BOX 1).

Pathogenic obligate intracellular bacteria pose a substantial public health threat. In this Review, we describe, in detail, the best-studied and clinically relevant pathogens (TABLE 1), which include *Coxiella* spp., pathogenic *Chlamydia* spp. and arthropod-transmitted members of the Rickettsiales. For decades, facultative intracellular bacteria, such as *Salmonella* spp., *Francisella* spp. and *L. pneumophila*, have been used as models to study host–pathogen interactions[1], whereas obligate intracellular bacteria have been underappreciated, despite their requirement to silently establish a replicative niche and remodel the host environment. By infecting host cells, cell biologists and immunologists can uncover novel signalling cascades that have remained uncharacterized, in part, owing to the historical focus on facultative intracellular bacteria[2]. Thus, obligate intracellular bacteria provide an unparalleled opportunity to discover novel principles of both pathogen and host biology.

Compared with facultative intracellular bacteria, relatively little is known about the underlying principles of bacterial physiology, host–pathogen interactions and the mechanisms that govern infection by obligate intracellular bacteria. Among the reasons for this discrepancy is the difficulty in studying bacterial signalling networks *in vivo*. For arthropod-transmitted members of the Rickettsiales, a lack of a basic understanding of vector physiology, immune response and microbial interactions also remains a major hurdle. More than 20 years have passed since the first attempt to transform an obligate intracellular bacterium[3] (FIG. 1). The refinement of genetic manipulation methods for all obligate intracellular bacteria has proven exceedingly difficult, mostly because these microorganisms only proliferate inside of host cells. Their limited genetic toolbox often precludes the sophisticated structure–function analyses that are routinely carried out in facultative intracellular bacteria and hinders progress in public health and basic science. In this Review article, we provide an overview of the advances in, and challenges of, genetically engineering obligate intracellular bacteria. We also highlight

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Transformation
A technique that induces bacteria to take up exogenous DNA molecules, usually through chemical or electrical methods.

Polyamidoamine dendrimers
(PAMAM dendrimers). Highly branched polymers that can be used to deliver small molecules or DNA to cells.

examples in which the use of genetically manipulated pathogens has improved our understanding of microbial pathogenesis and immunity. Finally, we provide an outlook on the most pressing scientific issues that could be addressed in regard to host–pathogen interactions using engineered obligate intracellular bacteria.

Genetic tools: methods and limitations

Until recently, the intracellular lifestyle of obligate intracellular bacteria has precluded the development of practical genetic tools. Prior to 2009, when an axenic medium that supports extracellular growth was developed, genetic tools for *C. burnetii* were limited[4] (FIG. 1). Sophisticated genetic tools were rapidly developed once extracellular growth was possible, owing to the faster growth rate and ease of selecting clonal transformants[5]. *Chlamydia* spp. and members of the Rickettsiales must still be cultured and manipulated intracellularly, which poses substantial technical hurdles. In addition, obligate intracellular bacteria often grow slower than facultative intracellular bacteria. For example, *Rickettsia prowazekii* has a replication time of 8–12 h, which is 2–3 times longer than *L. pneumophila*[6]. Obligate intracellular bacteria must also be purified from host cells before most transformation methods can be applied, because chemical reagents typically cannot cross both host and pathogen membranes. The purification steps are laborious, inefficient and tend to damage bacteria, which might render them non-infectious. As mutants must be selected and propagated in host cells, mutating genes that are essential for cell invasion and survival remains problematic. Last, complementation of a mutated gene with the wild-type gene under the control of its own promoter remains a major bottleneck that has only been overcome for three obligate intracellular bacteria: *C. burnetii*[7], *Chlamydia trachomatis* serovar L2 (REFS 8,9) and *Rickettsia parkeri*[10]. Despite these difficulties, there has been substantial progress in the development of genetic tools for obligate intracellular bacteria (BOX 2).

Transformation methods. The first step in almost all methods of genetic manipulation is transformation. Three transformation methods have been reported: chemical transformation, electroporation and polyamidoamine dendrimers (PAMAM dendrimers) mixed with a plasmid vector (FIG. 2a). Chemical transformation in a solution of calcium chloride has been used routinely to transform *C. trachomatis* serovar L2 since the development of a groundbreaking standard protocol in 2011 (REF. 11) (FIG. 1). Other obligate intracellular bacteria are transformed through the electroporation of cell-free bacteria[3,5,12–15]. Following transformation, bacteria are mixed with host cells that they must infect to survive. As chemical transformation or electroporation requires cell-free infectious bacteria, efficiency and the rate of mutant recovery would probably be improved if obligate intracellular bacteria could be manipulated within the host cell. Thus, attempts have been made to transform *Chlamydia* spp. and *Anaplasma phagocytophilum* with PAMAM dendrimers (FIG. 2a).

In proof-of-principle experiments, complexes comprising plasmid DNA and PAMAM dendrimers were added directly to infected host cell monolayers to reintroduce the cloned *C. trachomatis* plasmid into two plasmid-free strains, *C. trachomatis* serovar L2 (25667R)[16] and *Chlamydia pneumoniae* AR-39 (REF. 17). These chlamydial transformants were stable over several passages. In addition, fluorescein isothiocyanate-conjugated (FITC) dendrimers were evaluated for their ability to transform RF/6A primate endothelial cells infected with *A. phagocytophilum*; indeed, vacuoles that contained fluorescent *A. phagocytophilum* indicated that the dendrimers penetrated the host cell and the vacuole. In subsequent experiments, intracellular *A. phagocytophilum* was transformed to express GFP that was optimized for excitation by ultraviolet light (GFP*uv*) through dendrimers that were complexed with the transforming plasmid that integrated into the bacterial genome. However, these transformants were only retained for a few passages in medium that contained antibiotics, possibly owing to the disruption of essential genes[18]. Although dendrimer-enabled transformation still warrants further technical development, it could enable the introduction of markers that can be used to readily identify individual mutants in a pool, thus enabling the high-throughput screening of more recalcitrant species (for example, *A. phagocytophilum*). Currently, dendrimers are not routinely used to transform obligate intracellular bacteria, despite the attractive possibility to transform these bacteria inside host cells.

Shuttle vectors. Shuttle vectors are important genetic tools because they can be easily manipulated in *Escherichia coli* and maintained for the ectopic expression of genes under investigation in *C. trachomatis, Rickettsia* spp. and *C. burnetii*. *C. trachomatis* shuttle

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Allelic exchange
A common method that is used to knock in, knock out or otherwise mutagenize a DNA segment that relies on homologous recombination between the wild-type gene and an exogenous DNA construct.

Fluorescence-reported allelic exchange mutagenesis
(FRAEM). A method for allelic exchange in *Chlamydia trachomatis* serovar L2 that can be monitored byobserving fluorescent chlamydial inclusions.

vectors were derived from the endogenous 7.5 kb plasmid of *C. trachomatis* serovar LGV L2 (REF. 19). This plasmid (pL2) encodes eight genes that have diverse poorly understood roles in the infection cycle of *C. trachomatis*[11,20]. A pL2 variant, pSW2, contains a deletion in the first coding sequence[20]. These plasmids were used to generate the shuttle vectors p2TK2-SW2 (pSW2 parent)[21] and pBOMB4 (pL2 parent)[22] by fusing them to an *E. coli* origin of replication and β-lactamase (*bla*) for selection (FIG. 2b). p2TK2-SW2 and pBOMB4 are used for the routine ectopic expression of proteins in *C. trachomatis* and have been modified to include fluorescent markers, multiple cloning sites and/or tetracycline-inducible promoters[21–25]. Other modified versions of the pSW2 plasmid include the vector pL2dest, which was generated to enable the direct testing of potential secreted proteins with the TEM β-lactamase reporter assay[26]. Until recently, it was challenging to directly manipulate the chlamydial genome through allelic exchange because the transformed bacterium retained both the targeted and wild-type gene on a stably maintained plasmid[27]. A technical breakthrough occurred when a suicide vector was developed. The pSU6 plasmid enabled fluorescence-reported allelic exchange mutagenesis (FRAEM) in *C. trachomatis* by putting *pgp6*, which is required for plasmid maintenance, under the control of a tetracycline-induced promoter[27] (FIG. 2b). Once tetracycline is removed from the medium, *pgp6* is no longer expressed and the plasmid is not maintained. The pSU6 suicide vector enabled routine allelic exchange, which was previously limited to small DNA segments and a few selection markers in *Chlamydia psittaci*[28]. The utility of the pSU6 suicide vector was illustrated by the replacement of tryptophan synthase alpha subunit (*trpA*), which is involved in tryptophan biosynthesis, and three type III secretion system (T3SS) effectors with a *gfp* and *bla* cassette[27]. For each of the mutants, exclusively GFP-fluorescing inclusions were observed in the next passage following the removal of tetracycline, which indicates the loss of the plasmid backbone (which encodes red-fluorescent mCherry) and the integration of the allelic exchange cassette into the chlamydial genome[27]. As the pSU6 suicide vector was only recently developed, it has not yet been widely adopted by researchers in the *Chlamydia* field; however, the potential to generate targeted gene knockouts in *C. trachomatis* serovar L2 through allelic exchange is an exciting development[24].

Shuttle vectors for *Rickettsia* spp. were derived from three endogenous plasmids of the spotted fever group (SFG) *Rickettsia amblyommatis*, namely pRAM18, pRAM23 and pRAM32, although only pRAM18 has been used extensively[29]. These shuttle vectors contain a rifampicin resistance cassette, a fluorescent marker,

Box 1 | **Lifecycle of obligate intracellular bacteria**

The life cycles of *Anaplasma* spp. and *Ehrlichia* spp. (see the figure, part **a**) and *Chlamydia* spp., (see the figure, part **b**) are biphasic and characterized by cyclical infectious and replicative forms. Eukaryotic host cells internalize the infectious particles (step 1), termed dense core cells (for *Anaplasma* spp. and *Ehrlichia* spp.) and elementary bodies (for *Chlamydia* spp.). Once inside the host cell, the elementary body differentiates into the replicative morphotype (step 2), known as the reticulate cell (for *Anaplasma* spp. and *Ehrlichia* spp.) and reticulate body (for *Chlamydia* spp.). Reticulate bodies replicate inside a vacuole (step 3), in which they eventually differentiate back to the elementary body morphotype and exit the host cell through extrusion, lysis or possibly other unknown mechanisms (step 4)[58,69]. The life cycles of members of the Rickettsiaceae feature cytosolic replication (see the figure, parts **c**,**d**)[66]. Members of the Rickettsiaceae induce their uptake by host cells (step 1) and, once internalized, they must escape the phagosome (step 2) before replication. Next, cytosolic bacteria replicate and redistribute themselves intracellularly (step 3). To complete their life cycles, *Rickettsia* spp. lyse the host cell (step 4) or infect neighbours through intercellular spread (step 5), whereas *Orientia* spp. bud from the host cell (step 4, figure part **d**)[66,95]. *Coxiella burnetii* (see the figure, part **e**) is phagocytosed by the host (step 1). The phagosome matures into the *Coxiella*-containing vacuole (step 2), in which the bacteria differentiate into the large cell variant and replicate (step 3). Last, the bacteria differentiate back into the infectious small cell variant and lyse the cell (step 4)[96,97].

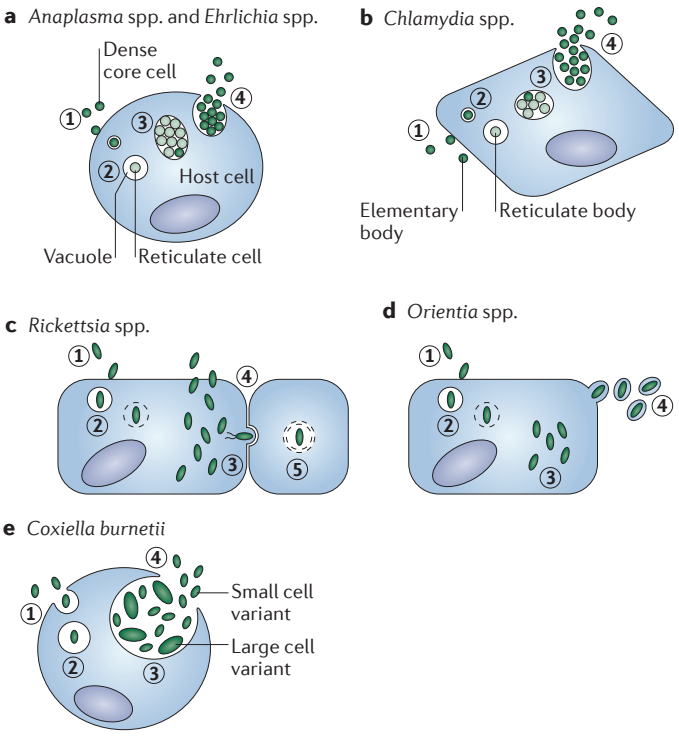


Table 1 | **Disease pathogenesis of selected obligate intracellular bacteria**

	Coxiella burnetii	*Chlamydia trachomatis*	*Anaplasma phagocytophilum*	*Ehrlichia chaffeensis*	*Rickettsia* spp.	*Orientia tsutsugamushi*
Disease	Q fever[97]	Genital infection and trachoma[58]	Anaplasmosis[69]	Ehrlichiosis[69]	Spotted fever and typhus[66]	Scrub typhus[98]
Clinical presentation	Mild-to-severe pneumonia and hepatitis; it may progress to chronic infection[97]	• Genital: 70% have no symptoms[99] • Trachoma: conjunctival inflammation[100]	Asymptomatic-to-severe fever, malaise, leukopenia and increased liver enzymes[69]	Mild-to-severe fever, malaise, leukopenia, increased liver enzymes[69] and occasional CNS symptoms[101]	Fever, respiratory and CNS symptoms, and organ failure[66]	Fever, disseminated intravascular coagulation and organ failure[98]
Distribution	Global[97]	Global[58]	The Americas, Europe and Asia[102]	The Americas and Asia[69]	Global[103]	Asia, Oceania and Chile[98,104]
Epidemiology	Ubiquitous in animals; potential for outbreaks among agricultural workers[97]	130 million new genital infections annually[105] and 40 million people with active trachoma (230 million at risk)[106]	2,600 reported cases annually in the United States[107] with increasing incidence[108] but limited global estimates	3 cases per million people annually in the United States; increasing incidence[108] but limited global estimates	Historically devastating outbreaks, global estimates limited and new species constantly emerging[109,110]	1 million infections per year; 1 billion people at risk[109]
Transmission	Inhalation[38]	Contact with infected fluids[58]	Tick vector[69]	Tick vector[69]	Arthropod vector[66]	Mite vector[98]
Major mammalian host cell	Alveolar macrophage[38]	Epithelial cells[58]	Granulocytes and endothelial cells[69]	Monocytes and macrophages[69]	Endothelial cells[66]	Multiple[98]
Organs affected	• Acute: the lungs • Chronic: the heart and liver[97]	Genital tract and eyes[58]	Inflammatory lesions in organs and liver damage[72]	Multiple[101]	Multiple[66]	Multiple[98]
Notes	Highly virulent[97]; lung endothelium and epithelium are minor targets of infection[97]	Serovars A–C cause trachoma and serovars D–K cause genital infection[58]	Lacks LPS and peptidoglycan[69]; macrophages are minor targets of infection[72]; antigenic variation[69]	Lacks LPS and peptidoglycan; antigenic variation[69]	SFG, typhus group, transitional and ancestral groups[66]	~40% of genome contains repeated sequences[85,111]
Genetic tractability	Genetically tractable; axenic medium[7]	Serovar L2 is genetically tractable[24]	Transposon library[13]	Transposon library[15]	Mutagenesis[41,42,49,53] and shuttle vectors[29,30]	No reports

CNS, central nervous system; LPS, lipopolysaccharide; SFG, spotted fever group.

portions of standard *E. coli* vectors that confer antibiotic resistance and regions of the endogenous *R. amblyommatis* plasmids that contain *dnaA* and *parA*[29], which are required for the replication and maintenance of DNA (FIG. 2b). The pRAM18dRGA and pRAM32dRGA plasmids were transformed into the SFG *Rickettsia conorii*, *R. parkeri*, *Rickettsia montanensis*, *Rickettsia monacensis* and *R. amblyommatis*, the typhus group *R. prowazekii*[30] and *Rickettsia typhi*[31], and the ancestral group *Rickettsia bellii*. With the exception of *R. amblyommatis*, all of the bacteria acquired a fluorescent signal, which indicated successful transformation and expression from the shuttle vector[29]. Failure to establish pRAM18dRGA in the parent *R. amblyommatis* confirms that plasmids that share the same partitioning system are incompatible in rickettsiae[32].

A multiple cloning site was inserted into pRAM-18dRGA, which facilitated the ligation of a gene that encodes the red fluorescent protein mCherry under the control of an *Anaplasma marginale* promoter (yielding pRAM18dRGA[AmTrCh]). Both *R. montanensis* and *R. conorii* were transformed with this shuttle vector and acquired red fluorescence, which demonstrates the functionality of this construct[29,33]. Interestingly, *R. conorii* transformed with pRAM18dRGA[AmTrCh] was maintained in cell culture and within an experimentally infected animal without the administration of antibiotics[34], which indicates that the plasmids were stable for the duration of these experiments in the absence of antibiotic selection. Furthermore, *rickA* from *R. monacensis* was overexpressed in *R. bellii* from the multiple cloning site of the shuttle vector, which resulted in significant changes in adhesion to cells and intracellular motility[35]. Finally, pRAM18dRGA[Sca4] complemented surface cell antigen 4 (*sca4*) in an *R. parkeri* mutant[10].

Shuttle vectors for *C. burnetii* were created following the establishment of the axenic medium. Two reporter plasmids that enabled for β-lactamase–gene fusions, pCBTEM[36] and pJB-CAT-BlaM[37], were independently generated. *C. burnetii* uses the Dot/Icm type IV secretion system to remodel its intracellular niche (Supplementary information S1 (figure)), and detailed knowledge of substrates was gained through TEM β-lactamase reporter assays[38]. The pJB-CAT plasmid backbone was used to generate other shuttle vectors that enabled the ectopic expression of tagged proteins in *C. burnetii* and complementation of mutated genes[7]. Although the copy numbers of shuttle vectors are difficult to control

Dot/Icm type IV secretion system
A set of bacterial proteins that inject effector molecules into the eukaryotic host cytosol to remodel the intracellular niche.

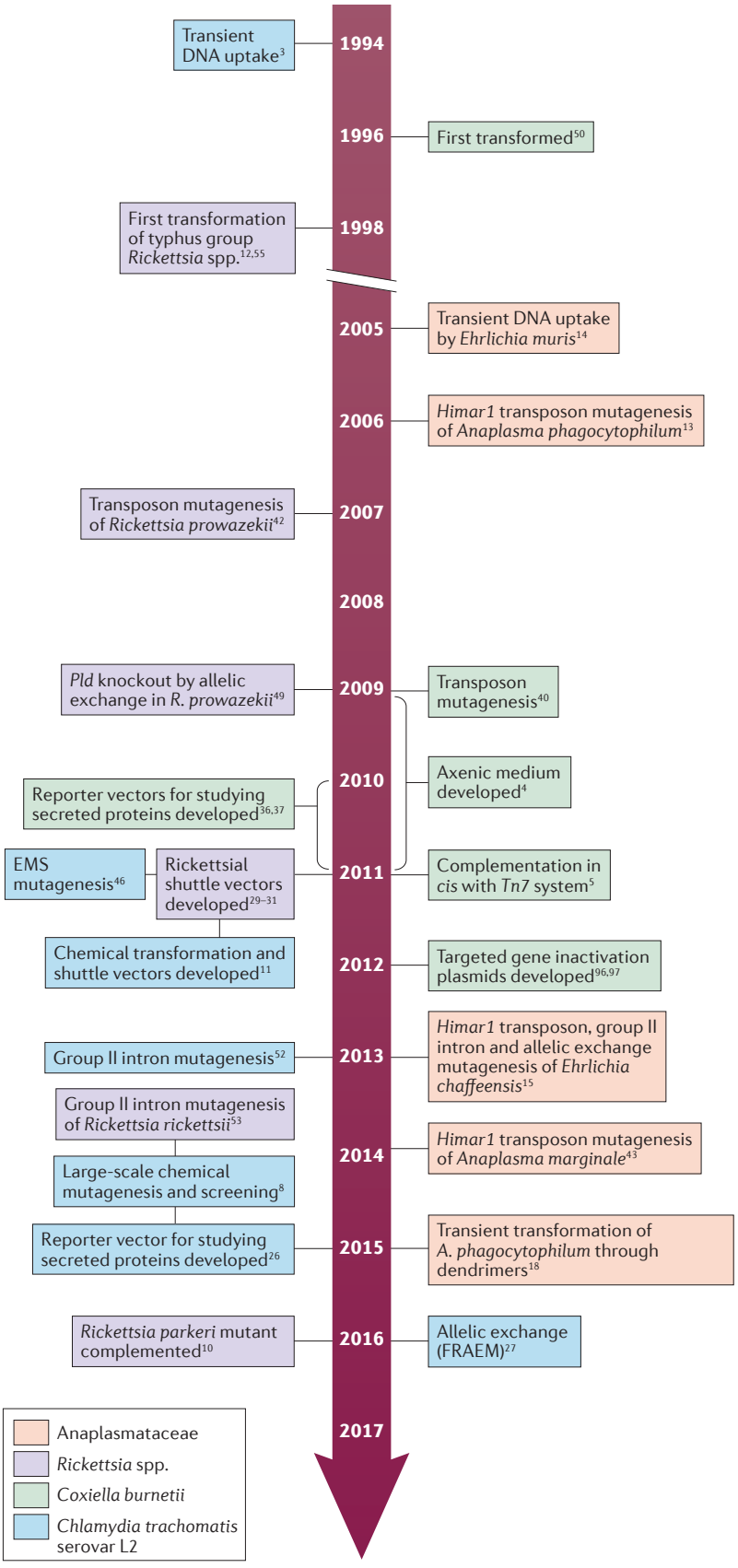


Figure 1 | **Advances in the genetic manipulation of obligate intracellular bacteria.** EMS, ethyl methanesulfonate; FRAEM, fluorescence-reported allelic exchange mutagenesis; *Pld*, phospholipase D.

and may cause polar effects, they provide a convenient method to examine gene function directly in obligate intracellular bacteria.

Mutagenesis. Random mutagenesis methods include transposon insertion and chemical mutagenesis (FIG. 2c). *Himar1* is a mariner transposon that is derived from a transposable element isolated from the stable fly *Haematobia irritans*[39] that has been successfully used to mutagenize various organisms, including several obligate intracellular bacteria, such as *C. burnetii*[40], *Rickettsia spp.*[41,42], *Anaplasma* spp.[13,43] and *Ehrlichia chaffeensis*[15]. The *Himar1* transposase system encodes the transposase and the transposon on one or two suicide plasmids that are electroporated into host cell-free bacteria[13,42,44]. The *Himar1* transposase randomly integrates the transposon, which contains an antibiotic resistance cassette and fluorescent markers flanked by repeats, into AT dinucleotide sites in the bacterial genome through a cut-and-paste mechanism[13]. Genetic markers in the transposon facilitate selection, visual detection and the identification of insertion loci in the bacterial genome. Transposase systems have also been used to complement *C. burnetii* mutations in *cis*[7].

Chemical mutagenesis provides an alternative to the *Himar1* transposon system, but without selection or detection markers. To date, only *Chlamydia abortus* (formerly *C. psittaci* var. *ovis*) and *C. trachomatis* have been successfully mutagenized using DNA-alkylating agents to generate libraries[8,45–47]. Chemical mutagenesis does not require bacterial transformation, and obligate intracellular bacteria do not need to be purified from the host cell before application of the mutagen. Another advantage of chemical mutagenesis is that hypomorphic mutations, which decrease gene function instead of completely inactivating it, can be generated, enabling the characterization of genes that are involved in essential functions[25]. Linking mutations to observed phenotypes can be laborious because it is technically demanding to identify, select and recover mutants in the absence of molecular tags[24]. Nonetheless, as *Chlamydia* spp. are naturally competent, carrying out linkage analysis can be carried out using lateral gene transfer[24,25]. Co-infection with a rifampicin-resistant mutant and a spectinomycin-resistant wild-type strain produces recombinant mutants that are resistant to both antibiotics. The recombinant clones are then genotyped and assessed for phenotype[48]. Repeated rounds of co-infection, phenotypic screening and genotyping are carried out until a single mutation can be linked to a phenotype. Other strategies involve using temperature-sensitive mutants to carry out genetic mapping, which does not require the generation of antibiotic-resistant strains of *C. trachomatis*[47]. Libraries of mutant *C. trachomatis* strains obtained through ethyl methanesulfonate mutagenesis (EMS mutagenesis) have been screened using various approaches, including temperature, forward genetics and reverse genetics[46–48]. Although linking phenotypes to mutations is tedious, chemical mutagenesis has proven to be an important technique for elucidating the function of genes in *C. trachomatis* serovar L2. For example, EMS mutagenesis has

Mariner transposon
An abundant class II transposable element first discovered in *Drosophila* spp. that integrates into a wide range of genomes.

Ethyl methanesulfonate mutagenesis
(EMS mutagenesis). A technique in which a DNA-alkylating agent (EMS) is applied to a population of cells to create a library of strains that contain random mutations.

Mobile group II introns
Mobile bacterial ribozymes that self-splice, reverse transcribe the spliced RNA into DNA, and then integrate the DNA into the bacterial chromosome.

led to the characterization of chlamydial metabolic features and the identification of an effector that is responsible for mediating the assembly of filamentous actin (F-actin) around the inclusion[8].

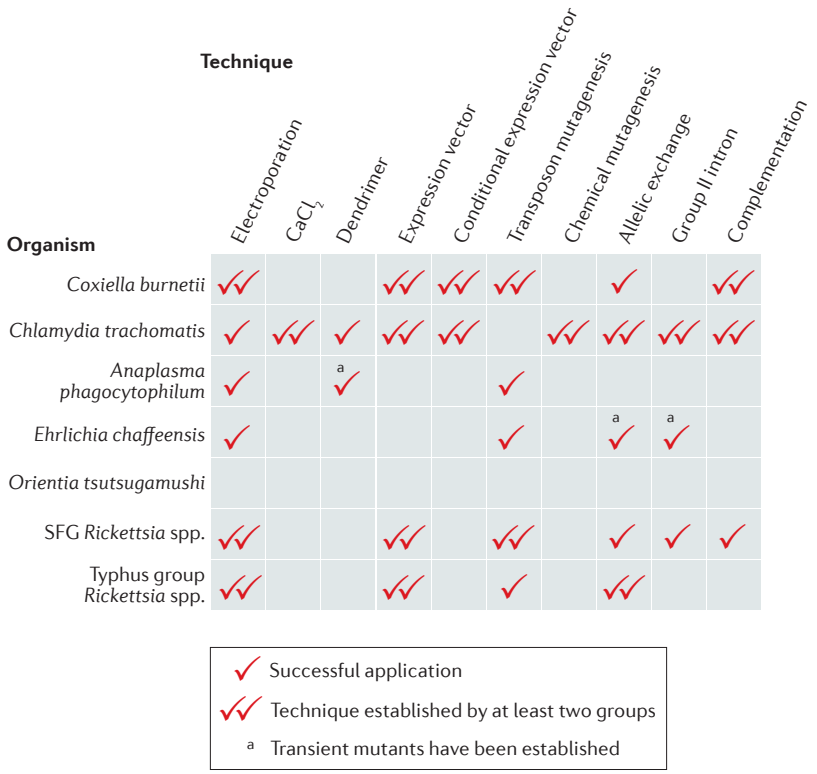
Targeted mutagenesis includes allelic exchange and mobile group II introns. Allelic exchange is routinely used to generate mutants in facultative intracellular bacteria and extracellular bacteria; only recently has this technique been successfully applied to obligate intracellular bacteria (FIG. 3a). Early attempts to mutagenize *Chlamydia* spp. resulted in allelic exchange of the ribosomal RNA (rRNA) operon with a synthetic 16S rRNA gene that contained point mutations, which rendered the mutant bacterium resistant to spectinomycin and kasugamycin[28]. This technique has only been successful in *C. psittaci* and has been limited to replacing small gene segments[28]. The recent development of the pSU6 suicide vector facilitated allelic exchange in *C. trachomatis* serovar L2 (REF. 27) (FIG. 2b).

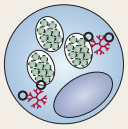
Allelic exchange has also been carried out in *R. prowazekii* to inactivate the phospholipase D gene (*pld*), which is a putative virulence factor[49]. The *pld* ORF and surrounding bases were cloned into the pBluescript SKII$^+$ plasmid and an internal segment was replaced with a rifampicin resistance cassette[49]. Transformation was achieved using a linear DNA fragment that was amplified from the construct, as it was less likely to result in the insertion of the entire plasmid into the rickettsial genome[49]. The resulting Δ*pld R. prowazekii* mutant strain was used to determine whether the disruption of *pld* leads to attenuated virulence in *R. prowazekii*[49]. In *E. chaffeensis*, two types of recombinant linear DNA segment that targeted a hypothetical protein were generated. One segment was prepared to introduce an antibiotic resistance cassette to disrupt the gene, which resulted in an increase in the size of the bacterial genome. To avoid polar effects, a second recombinant segment was created to remove a segment of the disrupted gene, such that the resulting construct was approximately the size of the wild-type gene. Mutants were detected independently of the recombination methods, but they persisted for only a few days in culture[15]. Last, allelic exchange was used to transform *C. burnetii* to ampicillin resistance[50], but this technique is not widely used in the *Coxiella* field, perhaps, in part, owing to the success of other genetic tools.

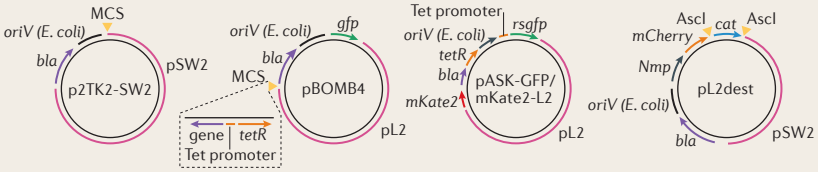
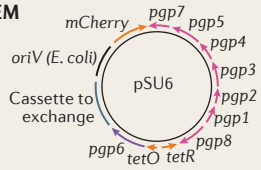
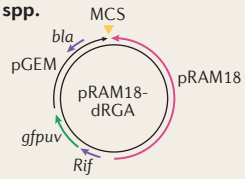
Mobile group II introns facilitate the selective integration of DNA that is reverse-transcribed from RNA into specific sites in the bacterial genome[51]. The selectivity of insertion is conferred through the sequence of the intron RNA, and commercial systems, such as TargeTron, have been successfully used to genetically modify *E. chaffeensis*, *C. trachomatis* serovar L2 and *Rickettsia* spp. through insertional mutagenesis (FIG. 3a). In *E. chaffeensis*, TargeTron constructs were assembled for six different genes, including those that encode outer membrane proteins[15]. Unfortunately, the resulting mutants were only detectable for less than a week and stable mutant lines could not be selected. By contrast, TargeTron constructs were used to mutate the *C. trachomatis* serovar L2 effector inclusion membrane protein A (*incA*)[52], which

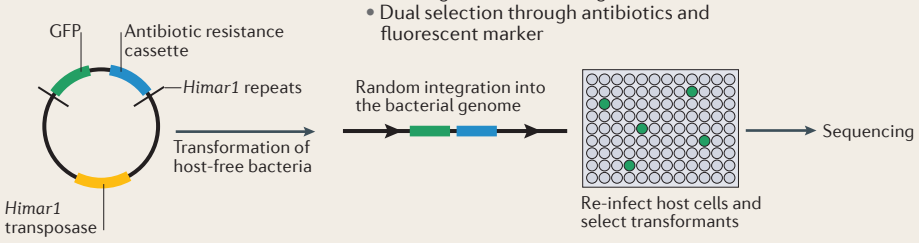
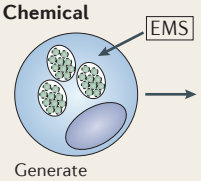
Box 2 | Genetic tools for obligate intracellular bacteria

Although all obligate intracellular bacteria adopt intracellular lifestyles, they are not equally amenable to genetic manipulation. *Coxiella burnetii* is technically no longer an obligate intracellular bacterium owing to the development of an axenic medium that enabled its host-free cultivation in the laboratory[5]. Most genetic tools have been developed for *Chlamydia trachomatis* serovar L2, which is now considered to be genetically tractable[24]. However, genome manipulation of clinically relevant *C. trachomatis* serovars and other *Chlamydia* spp. remains challenging. In the order Rickettsiales, *Rickettsia* spp. (spotted fever group (SFG) and typhus group) have the most sophisticated genetic tools available, and a recent article was the first to report genetic complementation[10]. Fewer genetic tools have been developed for members of the Anaplasmataceae family (*Anaplasma* spp. and *Ehrlichia* spp.), and no successful genetic manipulation has been reported for *Orientia tsutsugamushi* (in the family Rickettsiaceae). The differential ability to genetically manipulate obligate intracellular bacteria may be due, in part, to the biological differences between species (for example, the ability to form plaques or the presence of endogenous plasmids). A lack of adoption of some genetic tools may be caused by the small size of the scientific community that researches obligate intracellular bacteria, which limits the number of groups that contribute to the development and refinement of genomic techniques. In the figure, one tick indicates successful application of a genetic tool (columns) for each major species (rows). Two ticks indicate that the technique has been published by at least two independent research groups.



a Transformation methods		Advantages	Disadvantages
Chemical		• Simple • Inexpensive • Rapid	• Protocol established only for *C. trachomatis* L serovars • Requires antibiotic selection
Electroporation		• Rapid • Successful for many obligate intracellular bacteria • Widely adopted	• May damage bacteria • Requires bacterial isolation and reinfection • Requires antibiotic selection
PAMAM dendrimers		• Can penetrate both host and bacterial membranes • Less damaging than chemical or electrical methods • Does not require bacterial isolation and reinfection	• Requires antibiotic selection for some bacteria (for example, *A. phagocytophilum*) • Not reproduced by independent laboratories

b Expression vectors		Advantages	Disadvantages
***C. trachomatis* shuttle vectors**		• Enables ectopic expression of proteins in *C. trachomatis* • Conditional expression possible • Widely used for *C. trachomatis* L2	• Plasmid is maintained indefinitely
FRAEM		• Enables allelic exchange in *C. trachomatis* • Can be used to cure the endogenous plasmid from *C. trachomatis* serovar L2 • Mutants present after one passage	• Cannot mutate essential genes for the *C. trachomatis* life cycle
***Rickettsia* spp. shuttle vectors**		• Maintained in SFG *Rickettsia* spp., typhus group *Rickettsia* spp. and ancestral group *R. bellii* • pRAM18dRGA[MCS] plasmid has been widely used to express proteins in several rickettsiae	• Plasmids are not maintained in *R. amblyommatis*

c Random mutagenesis		Advantages	Disadvantages
***Himar1* transposon**		• Stable mutants • *Himar1* transposon randomly integrates into the bacterial genome, enabling the generation of comprehensive mutant libraries with coverage over the entire genome • Dual selection through antibiotics and fluorescent marker	• Mutagenesis of essential genes not possible • Slow and tedious • Technically demanding
Chemical		• Modification of bacteria in host cells • Rapid and practical library generation • Flexibility for forward or reverse screening • Characterization of essential genes through the generation of hypomorphic mutations • Wide adoption for *C. trachomatis* L2	• Clonal isolation of some mutants tedious, if not impossible • Screening can be tedious • Requires linking genotype to phenotype • Generating isogenic control strains difficult, if not impossible

is thought to promote the fusion of inclusions inside cells that are infected with *C. trachomatis*[52]. Recently, *Chlamydia* promoter of cell survival (CpoS) was disrupted by TargeTron, confirming the phenotype of a chemically mutagenized strain[9]. In another example, a premature stop codon and a rifampicin resistance cassette were introduced to inactivate outer membrane protein A (*ompA*) in virulent *Rickettsia rickettsii* str. Sheila Smith[53]. There are only a few cases of the successful application of TargeTron constructs to generate stable targeted mutants of obligate intracellular bacteria. This could be attributed to the proprietary TargeTron technology or the availability of other tools (for example, allelic exchange).

Recovery and selection. Three methods are currently used to distinguish mutants from the wild-type bacteria: antibiotic selection, fluorescence-activated cell sorting (FACS) and laser microdissection (FIG. 3b). Antibiotic selection is a standard technique that enables the survival of only those bacteria that acquired a resistance cassette that encodes factors that promote survival when exposed to xenobiotics. Only clinically irrelevant antibiotics are permitted for use by regulatory agencies, owing to the potential for the escape of resistant strains into the environment. These regulations further limit the small range of antibiotics that are effective against obligate intracellular bacteria. Moreover, different species, strains, biovars and serovars may be differentially susceptible to antibiotics[24], and resistance can develop after continued exposure in cell culture. Cells infected with labelled *C. trachomatis* and *Rickettsia* spp. that express a fluorescent protein have been sorted by FACS[33,54–56]. Similarly, single cells that are infected with labelled *C. trachomatis* have been successfully isolated through laser microdissection[57]. However, the technical demands and cost of specialized equipment, coupled with biosafety limitations (for example, maintenance of cell sorters in biosafety level 3 laboratories), prevent these techniques from being widely used. Although the selection of transformants can be tedious and potentially expensive, a major hurdle to overcome is the isolation of clonal mutants. Micromanipulation of cells that are infected with *C. burnetii* has been used to extract single *Coxiella*-containing vacuoles, which can then infect sterile host cells or be cultivated in axenic medium[5,40]. As there are no axenic media available for most obligate intracellular bacteria, simple clone picking and expansion are not possible. Some strains of *Chlamydia* spp. and *Rickettsia* spp. can be plaque purified to obtain clonal transformants, but not all obligate intracellular bacteria form visible plaques. Therefore, this method is not widely applicable[5]. Alternatively, clonal transformants can be recovered through time-consuming limiting dilution[5] (FIG. 3b).

Figure 2 | **Transformation methods, expression vectors and random mutagenesis.** **a** | Chemical transformation with calcium chloride is used to transform *Chlamydia trachomatis* serovar L2. Electroporation is primarily used to transform members of the order Rickettsiales[13,15,33]. Plasmids complexed with polyamidoamine (PAMAM) dendrimers provide an alternative method to transform obligate intracellular bacteria. **b** | Shuttle vectors have been developed for *C. trachomatis* and *Rickettsia* spp. In both cases, portions of endogenous plasmids (pSW2 and pL2 for *C. trachomatis*, and pRAM18 for *Rickettsia* spp.) are fused to *Escherichia coli* plasmid backbones to enable replication. The shuttle vectors include *E. coli* origins of replication (*oriV*), fluorescent markers (GFP and GFP optimized for excitation by ultraviolet light (GFP*uv*) or red fluorescent protein (mCherry)), antibiotic selection genes (β-lactamase (*bla*), chloramphenicol (*cat*), spectinomycin (*aadA*) and rifampicin resistance (*rif*)) and multiple cloning sites (MCS)[21,22,29]. Two chlamydial shuttle vectors, pBOMB4 and pASK-GFP/mKate2-L2, can be modified to include a tetracycline-inducible promoter for conditional gene expression[22,23]. The shuttle vector pL2dest enables the expression of proteins that are fused with a β-lactamase reporter to study protein secretion[26]. The fluorescence-reported allelic exchange mutagenesis (FRAEM) vector pSU6 enables allelic exchange in *C. trachomatis* by behaving as a suicide vector in the absence of tetracycline[27]. **c** | The *Himar1* transposase randomly inserts the transposon into the bacterial genome. Successful transformants are selected with antibiotics and the expression of a fluorescent protein[13]. The mutants are selected and sequenced to determine insertion sites. In chemical mutagenesis, mutations are caused by DNA-alkylating agents (for example, ethyl methanesulfonate (EMS)). Mutants are selected, pooled and subjected to forward or reverse genetics screens[8]. *A. phagocytophilum*, *Anaplasma phagocytophilum*; *R. amblyommatis*, *Rickettsia amblyommatis*; *R. bellii*, *Rickettsia bellii*; SFG, spotted fever group; *tetO*, tetracycline operator; *tetR*, tetracycline repressor.

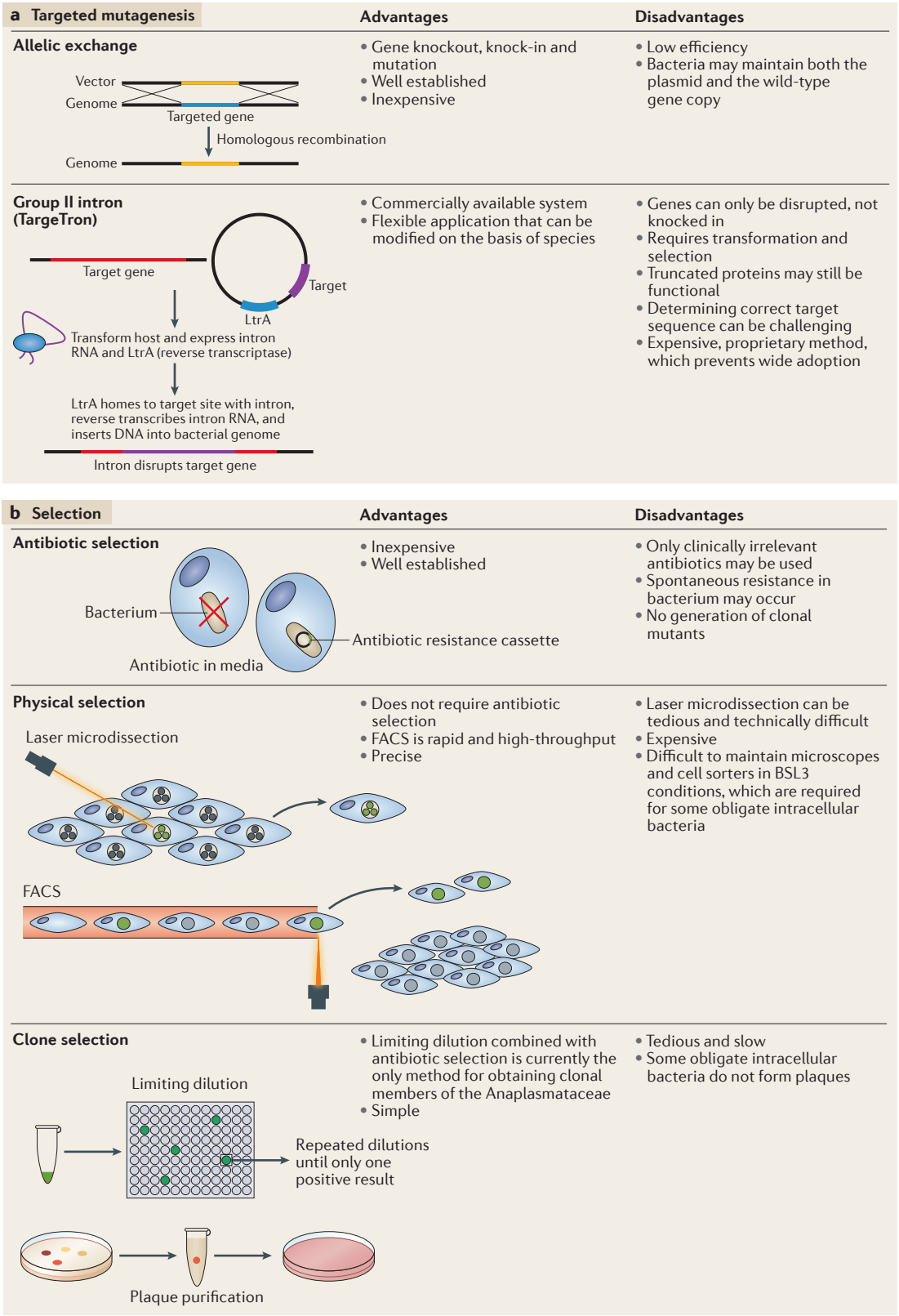
New functional insights

Chlamydia trachomatis *effectors.* *Chlamydia* spp. reside in a membrane-bound inclusion that becomes remodelled by integral membrane proteins (Supplementary information S1 (figure)). These inclusion proteins (Incs) are secreted by the T3SS and regulate membrane trafficking, cell signalling and cytoskeletal rearrangements to promote the formation and maintenance of inclusions[58]. *C. trachomatis* has more than 50 putative Inc proteins, some of which are expressed at unique stages of the developmental cycle[59].

Despite the importance of Incs for infection with *Chlamydia* spp., detailed knowledge at the mechanistic level was limited until recently, owing to the genetic intractability of these microorganisms. Recently, a mutant library of approximately 1,000 EMS-mutagenized isolates of *C. trachomatis* was generated[8]. A microscopy-based forward-genetics screen was used to identify two mutants that had abnormal F-actin assembly, as deficits in this function were likely due to mutations in proteins that control inclusion maturation. The sequencing of one mutant (strain M407) and genetic linkage analysis led to the identification of a nonsense mutation in CTL0184 (C307T/Q103*). The assembly of F-actin was restored in strain M407 complemented with the p2TK2-SW2 shuttle vector that encoded wild-type CTL0184 under the control of its endogenous promoter.

CTL0184 was subsequently investigated and renamed inclusion membrane protein for actin assembly (InaC; FIG. 4a). Co-immunoprecipitation assays identified two classes of host molecule, 14-3-3 scaffolding proteins and ADP-ribosylation factors (ADP-ribosylation factor 1 (ARF1), ARF4 and ARF5), as InaC interacting partners. InaC and F-actin assembly were shown to be required for the redistribution of the Golgi body, which is thought to be a source of lipids for inclusion remodelling[58]. ARFs are Golgi-associated GTPases that regulate membrane trafficking[60]. However, the InaC-mediated enlistment of ARFs was not important

for lipid acquisition in the cell culture system[8], and the mechanism by which InaC regulates the assembly of F-actin and Golgi distribution together with ARFs and 14-3-3 proteins remains to be identified. Other intracellular pathogens, such as *L. pneumophila* and typhus group *Rickettsia* spp., recruit host ARFs through their RalF effector proteins[61–63]. *L. pneumophila* uses ARF1 to control the delivery of endoplasmic reticulum



Necrosis
A type of inflammatory cell death that occurs spontaneously after damage to a cell.

Apoptosis
A mode of non-inflammatory programmed cell death.

Stimulator of interferon genes
(STING). An endoplasmic reticulum-associated cytosolic intracellular pattern recognition molecule that senses cyclic dinucleotides and induces the production of type I interferons.

Vinculin
A mammalian cytoskeletal protein that anchors the cell membrane to the actin cytoskeleton.

Granulocytic anaplasmosis
A mild-to-severe tick-borne infectious disease caused by *Anaplasma phagocytophilum*, which infects neutrophils and myeloid cells, that is characterized by fever, thrombocytopenia, leukopenia and liver damage.

(ER)-derived vesicles to the maturing *Legionella*-containing vacuole[63]. Interestingly, the typhus group *Rickettsia* species *R. prowazekii*[61] and *R. typhi* hijack ARFs to manipulate the rearrangement of the actin cytoskeleton at the plasma membrane, which has been shown to lead to pathogen uptake in *R. typhi*[62]. Thus, it is likely that *C. trachomatis*, similarly to other intracellular pathogens, uses ARFs to manipulate host vesicle trafficking and actin cytoskeleton dynamics during the establishment of the inclusion.

The Inc protein CTL0481, which was renamed *Chlamydia* promoter of survival (CpoS), was also recently identified in a forward genetic screen for *C. trachomatis* mutants that enhanced the death of infected cells[9]. Loss-of-function mutations in *cpoS* led tothe induction of necrosis and apoptosis midway through the infection cycle, which correlated with an exaggerated type I interferon (IFN) response. In agreement with this, complementation of the chemically mutagenized strain with a p2TK2-SW2-derived plasmid that encodes *cpoS* did not elicit host cell death. Furthermore, inactivation of *cpoS* using a TargeTron construct in a wild-type strain of *C. trachomatis* serovar L2 induced cell death. Unexpectedly, the release of type I IFNs was uncoupled from cell death, although the cyclic dinucleotide sensor stimulator of interferon genes (STING) was required. The interacting partners of CpoS included multiple RAB proteins that control ER-to-Golgi protein trafficking, which suggests that it might modulate membrane trafficking at the inclusion[9]. Last, as STING and calcium flux are suggested to be linked[64,65], and the depletion of ER calcium stores partially rescued the cell death phenotype of CpoS-deficient mutants, CpoS-mediated STING and cell death inhibition may be attributed to calcium homeostasis[9].

Rickettsial dissemination, A.* phagocytophilum *moonlighting and* E. chaffeensis *growth. SFG rickettsiae are cytosolic obligate intracellular bacteria that cause vascular and endothelial tissue damage[66] (TABLE 1; Supplementary information S1 (figure)). They disseminate intracellularly in the nutrient-rich host cytosol and move from cell to cell without entering the extracellular environment[10]. Largely on the basis of electron microscopy and studies in *L. monocytogenes*, it was assumed that SFG rickettsiae co-opted host actin with a single bacterial effector to propel themselves through plasma membranes into a recipient cell, in which they quickly escaped into the cytoplasm[10]. However, *R. parkeri* mutants that are defective in actin-based motility revealed that *R. parkeri* uses two different actin-polymerizing proteins, RickA and Sca2, which are not used by other motile intracellular bacteria[41]. This, together with basic phenotypic differences with the *L. monocytogenes* model (such as a shorter time in protrusions, a lack of actin tails and a quicker transition from protrusion to vesicle), suggests an alternative mechanism for intercellular spread[10].

A *Himar1* mutagenesis library of *R. parkeri* was screened for mutants that formed small plaques, as these were likely to be deficient in some aspect of intercellular spread[10,41] (FIG. 4b). One mutant had a transposon insertion in *sca4*, which led to the production of a truncated Sca4 protein[10]. Sca4 was predicted to be secreted[67] and shown to bind to vinculin[68], but its role in infection remained unclear. A series of traction force microscopy experiments showed that Sca4 decreased intercellular tension to enable *R. parkeri* to spread into the donor cell independently of actin-based motility[10] (FIG. 4b). Importantly, normal intercellular spread of *sca4*::*Himar1* was restored after complementation with the pRAM18dRGA[Sca4] expression vector[10].

A. phagocytophilum is a tick-borne pathogen that causes granulocytic anaplasmosis in humans (HGA) and other mammals[69] (TABLE 1; Supplementary information S1 (figure)). *Himar1* transposon mutagenesis enabled the generation of a mutant library of *A. phagocytophilum* to study disease pathogenesis[13]. Library screening led to the identification of an *A. phagocytophilum* mutant that contains a single transposon insertion in the dihydrolipoamide dehydrogenase 1 (*lpda1*) gene[70] (FIG. 4c), which encodes a metabolic enzyme that has been implicated to have alternate functions in virulence[71]. In a murine model of infection, the *A. phagocytophilum* mutant *lpda1*::*Himar1* induced several clinical abnormalities, including more pronounced peripheral blood neutropenia and higher erythrocyte counts[70]. Increased levels of IFNγ, splenomegaly and splenic extramedullary haematopoiesis were observed in mice that were infected with the *lpda1*::*Himar1* mutant strain but not with the wild-type bacterium. Transient infection of macrophages by the *lpda1*::*Himar1 A. phagocytophilum* strain correlated with enhanced nuclear factor-κB (NF-κB) activity, which led to an increase in the production of reactive oxygen species (ROS) and the release of pro-inflammatory cytokines[70]. Thus, the normal expression of LpdA1 in wild-type bacteria may prevent enhanced immune activation in macrophages, which are increasingly being recognized as the immune cells that are responsible for the cytokine storm that is characteristic of *A. phagocytophilum* infection[72]. These findings suggest that protein moonlighting, a phenomenon whereby a protein carries out more than one function[71], may be used as a strategy of immune evasion by *A. phagocytophilum*.

Transposon-based mutagenesis of *E. chaffeensis*, an emerging tick-borne pathogen, has become valuable for vaccine development (TABLE 1; Supplementary information S1 (figure)). This approach was used to create

◀ Figure 3 | **Targeted mutagenesis and selection. a** | Targeted mutagenesis enables the alteration of specific bacterial genes. Allelic exchange can be used to introduce point mutations, and to insert and delete specific genes. Group II intron technology (TargeTron) enables introns to be specifically inserted into bacterial genes to disrupt gene function through LtrA, a multifunctional protein derived from *Lactococcus lactis*, which reverse transcribes and splices the intron and cleaves the recipient DNA for intron insertion[51]. TargeTrons have been successfully applied to generate transient mutants of *Ehrlichia chaffeensis*[15] and stable mutants of *Chlamydia trachomatis* and *Rickettsia rickettsii*[52,53]. **b** | Two methods are currently used to distinguish mutants from wild-type bacteria: antibiotic selection and physical selection. Mutants can be physically separated from wild-type bacteria by fluorescence-activated cell sorting (FACS) and laser microdissection. One final step is obtaining clonal mutants, which can be isolated by limiting dilution, or, in some cases, plaque purification[5]. BSL3, biological safety level 3.

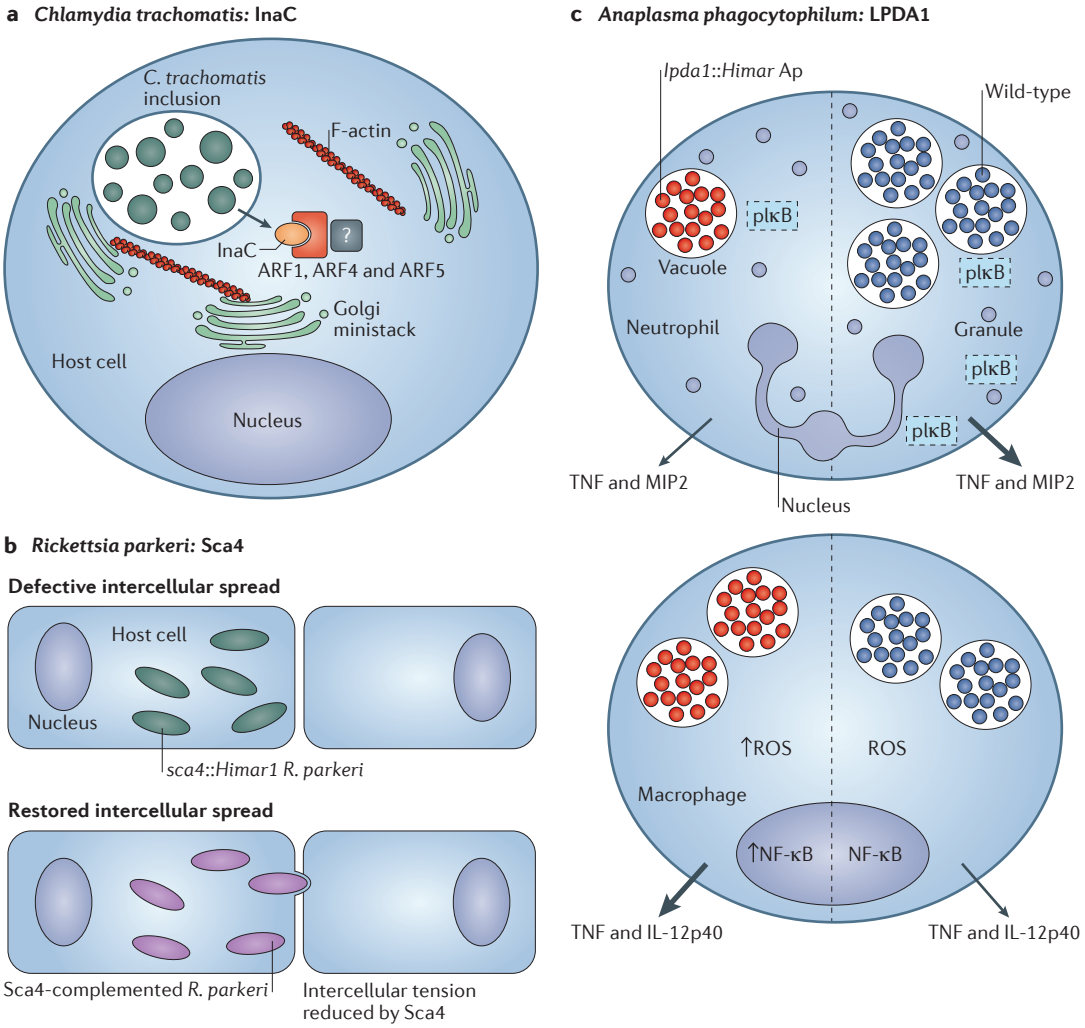


Figure 4 | **Discoveries in microbial pathogenesis facilitated by genetic tools. a** | Chemical mutagenesis of *Chlamydia trachomatis* led to the identification of inclusion membrane protein for actin assembly (InaC), which recruits filamentous actin (F-actin) and induces Golgi redistribution around the inclusion in manner that is dependent on ADP ribosylation factor 1 (ARF1), ARF4 and ARF5 (REF. 8). **b** | *Rickettsia parkeri* disseminates intercellularly through a mechanism that is not used by facultative intracellular bacteria. Screening of a *Himar1* transposon mutagenesis library of *R. parkeri* identified a mutant deficient in intercellular spread named surface cell antigen 4 (*sca4*)::*Himar1* (REF. 10). The *sca4*::*Himar1* mutant was complemented by pRAM18dRGA[Sca4], which restored intercellular spread. Sca4 was found to inhibit vinculin binding toα-catenin (not shown), thereby reducing intercellular force transduction and enabling the intercellular spread of *R. parkeri*. **c** | *Himar1* transposon mutagenesis was used to generate an *Anaplasma phagocytophilum* mutant library. One mutant exhibited a single transposon insertion in the dihydrolipoamide dehydrogenase 1 (*lpda1*) gene. Compared with wild-type infection, the *lpda1*::*Himar1 A. phagocytophilum* mutant infected neutrophils less well , and activated nuclear factor-κB (NF-κB) poorly (as measured by decreased phosphorylated inhibitory subunit of NF-κB (plκB)), and elicited the production of less tumour necrosis factor (TNF) and macrophage inflammatory protein 2 (MIP2; also known as CXCL2) in neutrophils[70]. Transient infection of macrophages by the *lpda1*::*Himar1 A. phagocytophilum* strain correlated with enhanced nuclear NF-κB activity, which led to the increased production of reactive oxygen species (ROS) and the release of pro-inflammatory cytokines[70]. Mice that were infected with the *lpda1*::*Himar1* mutant or wild-type *A. phagocytophilum* HZ exhibited differences in immunopathology and cell-specific outcomes, which suggests that LPDA1 may have a role in inhibiting excessive host immune activation[70]. Arrow size correlates with relative quantity of cytokines secreted. IL-12p40, interleukin-12 subunit p40.

mutations in four genes that are predicted to encode membrane proteins (*Ech*_0230, *Ech*_0379, *Ech*_0601 and *Ech*_0660)[15]. With the exception of *Ech*_0601, all mutations caused attenuation of the growth of the pathogen in both the reservoir (deer) and incidental (dog) hosts. Conversely, no effect on bacterial growth was observed in the tick vector *Amblyomma americanum*[73]. Two clonally-purified attenuated mutants that had insertions in the *Ech*_0379 and *Ech*_0660 genes provided protection against wild-type infection in both deer and dogs[74]. Thus, refining genetic tools for practical application in obligate intracellular bacteria could accelerate vaccine development and protect vulnerable populations.

Correcting virulence factor paradigms. Classified as a select agent, *R. prowazekii* is considered a potential bioweapon and no vaccine against *R. prowazekii* exists in the United States; hence, research into the virulence of *R. prowazekii* that leads to an effective vaccine is vital to protect public health[49]. Comparative genomics approaches that examined the differences between virulent and avirulent strains of *R. prowazekii* identified several putative virulence genes[75]. One example was *pld*, which, when expressed in a surrogate system, enabled *Salmonella enterica* subsp. *enterica* serovar Typhimurium to escape the phagosome[76]. Several years later, allelic exchange was used to replace wild-type *pld* of *R. prowazekii* with a mutated copy[49]. No differences in growth kinetics, rickettsial burden or ability to escape the phagosome were observed between the virulent wild-type *R. prowazekii* str. Madrid Evir and the *pld* mutant *in vitro*[49]. Nevertheless, in a guinea pig infection model, the Δ*pld* mutant was attenuated compared with the virulent *R. prowazekii* strain. Guinea pigs that were infected with the Δ*pld* mutant lost less weight and were afebrile compared with animals infected with virulent *R. prowazekii*[49].

OmpA of *R. rickettsii* was considered a major virulence factor before the advent of genetic tools[66,77]. The original study relied on purified OmpA, which was shown to block the attachment of *R. rickettsii* when pre-incubated with host cells. Selective degradation and denaturation of OmpA also prevented *R. rickettsii* from adhering to host cells[77]. Uncertainties in regard to the function of OmpA remained, and group II intron technology was recently used to insert a 5′ premature stop codon into *ompA* of the highly virulent *R. rickettsii* str. Sheila Smith[53]. Unexpectedly, no differences in virulence between the wild-type and mutant strains were detected in either cell culture or the guinea pig infection model[53], refuting the notion that OmpA is required for, or enhances, the invasion and disease pathogenesis of *R. rickettsii*.

Advances in genetic tools have aided in clarifying the function of *Chlamydia* protease-like activity factor (CPAF). Following its identification, CPAF was shown to cleave host transcription factors that control the expression of major histocompatibility complex (MHC) class I and class II[78], which ultimately leads to evasion of the immune system and undetected infection with *Chlamydia* spp.[78]. Subsequent studies reported that many other host proteins were cleaved by CPAF[79]. However, as genetic tools were unavailable, recombinant and ectopically expressed CPAF were used for validation of these substrates[79]. Thirteen years after the initial discovery of CPAF, a *C. trachomatis* mutant with defects in CPAF expression was generated[80]. Using this mutant, several of the phenotypes ascribed to CPAF were shown to occur independently of CPAF. Specifically, CPAF did not affect Golgi fragmentation, NF-κB activation, inhibition of apoptosis and protection from reinfection[80]. CPAF was described to cleave vimentin, which is an intermediate filament that surrounds the inclusion, and nuclear lamin-associated protein 1 (LAP1; also known as TOR1AIP1) following the disruption of the membrane[80]. CPAF was also required for the optimal development of elementary bodies[80] and has unanticipated roles in infection, including in the regulation of T3SS effectors and bacterial survival in the lower genital tract[81,82]. Despite these new findings, other questions in regard to the role of CPAF in infection remain; for example, whether CPAF is secreted into the host cytosol or is sequestered in its active form in the inclusion[83]. Overall, genetic tools have enabled the chlamydial and rickettsial fields to correct artefactual results, which benefits researchers and the public alike, as new therapeutics and vaccines against obligate pathogens are desperately needed.

Concluding remarks

Much has been achieved in the genetic engineering of obligate intracellular bacteria since the first transformation of *C. trachomatis*[3] (FIG. 1). The genetic toolbox for these organisms has grown to include transformation protocols; rickettsial, chlamydial and *Coxiella* shuttle vectors; random mutagenesis systems; and the ability to generate targeted knockouts through allelic exchange, group II intron technology and FRAEM (FIGS 2,3). Despite these advances, limitations still impede the complete application of genetic tools. Chiefly among these is the lack of axenic media, which enables host-free growth of obligate intracellular bacteria. Challenges due to the intracellular lifestyle of obligate intracellular bacteria, tedious selection methods and the inability to efficiently obtain clonal populations limit the practicality of genetic manipulation. Routine complementation of mutagenized genes by expressing the wild-type gene under the control of its own promoter is still technically demanding. Nonetheless, the future of genetic manipulation of obligate intracellular bacteria is promising, as highlighted using the example of *C. burnetii*.

C. burnetii was strictly classified as an obligate intracellular bacterium until the formulation of an axenic medium that enabled its host-free cultivation in the laboratory[4]. This landmark development was facilitated through advances in genome sequencing and systematic refinements to the growth medium. No defect in infectivity has been shown for *C. burnetii* extracellular growth compared with host-cell cultivated bacteria[4]. *C. burnetii* grows much faster in axenic medium than in host cells and forms colonies, which enables selection and the subsequent expansion of clonal populations. Instead of several months, transformation experiments now take less than three weeks to complete, which has accelerated the development of genetic tools available to modify the genome of *C. burnetii*[4,5] (FIG. 1). These genetic tools and the study of the metabolic requirements of *C. burnetii* have provided important insights into the composition and availability of nutrients in the parasitophorous vacuole.

One promising avenue of research is the rickettsial type IV secretion system (T4SS). Members of the Rickettsiales encode a *vir*-type P-type IV secretion system (*rvh*) and use this molecular scaffold, in part, to remodel the host environment[84–86]. The functional characterization of *rvh*, including the identification of substrates, has been hampered by the inability to easily generate

Rvh-deficient species in the Rickettsiales. Duplications and repeats in *rvh* seem to have persisted despite evolutionary pressure towards genome reduction; thus, these features may be evidence of an alternative function or, conversely, a regulatory circuit[86]. We anticipate that mechanistic characterization of *rvh*, aided by mutagenesis using the TargeTron system or random approaches, will shed light onto rickettsial pathogenesis, virulence and host–pathogen interactions.

Another key question in the biology of obligate intracellular bacteria is how *Chlamydia* spp. and members of the Anaplasmataceae establish and protect their intracellular niches. Inc proteins that are secreted by *Chlamydia* spp. mediate these processes[58]. The functional relevance of each Inc protein, how they are regulated and in what manner they contribute to the establishment of this unique organelle, remain poorly understood. Although few Anaplasmataceae effectors have been characterized, some encode ankyrin repeat motifs that are common in eukaryotes and may have implications for virulence[87]. Ankyrin repeats mediate protein–protein interactions, provide scaffolding for signalling pathways and localize molecules to subcellular compartments in eukaryotes[88]. Ankyrin-repeat containing proteins (Anks) in *A. phagocytophilum* and *E. chaffeensis* were shown to mediate host epigenetic changes to promote bacterial survival[69,89]. Elucidating the function of conserved Anks in obligate intracellular bacteria can provide insight into host signalling pathways. Furthermore, characterizing mediators of inclusion development through genomic manipulation of obligate intracellular bacteria will reveal basic cell biology principles and 'druggable' targets.

Iron is an essential and limiting nutrient for both hosts and pathogens[90]. Remarkably, little is known about iron homeostasis in obligate intracellular bacteria, including sources, mechanisms of uptake and regulation during colonization of the mammalian host and the arthropod vector. *A. marginale*, a bovine pathogen that is transmitted by hard ticks, parasitizes erythrocytes. No iron regulatory elements, enzyme systems to degrade haem, surface receptors or siderophores have been identified in the genome of *A. marginale*[91]. Only a single orthologue to a bacterial transferrin (ferric binding protein A (FbpA)), a permease (FbpB), and a transporter that is thought to supply energy through ATP hydrolysis (FbpC) have been found[92]. Unlike similar iron transport systems, these genes are not encoded in a single operon, but rather are dispersed throughout the genome of *A. marginale*[93,94]. How this minimal machinery is used for iron uptake and homeostasis is unknown, but routine targeted genetic manipulation could improve our understanding of bacterial iron acquisition.

The development of novel genetic tools, especially for members of the Rickettsiales, will be crucial to discover novel paradigms in microbial pathogenesis and immunity. As mutants of *Chlamydia* spp., *Rickettsia* spp., *Orientia* spp., *Anaplasma* spp., *Ehrlichia* spp. and *Coxiella* spp. are characterized, the research community will learn more about the fascinating lifestyles of these organisms. Although formerly thought of as just an exotic group of bacteria, the unfolding story of genetic engineering in obligate intracellular bacteria highlights the importance of intellectual curiosity, ingenuity and persistence to overcome scientific obstacles.

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