Legionella Effectors Explored with INSeq: New Functional Insights

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Keywords
Legionella pneumophila, bacterial effectors, transposon insertion sequencing, virulence factors

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Abstract

Legionella pneumophila secretes over 300 effector proteins that manipulate host cells. This multiplicity of effectors hampers the characterization of their individual roles. Shames *et al.* report a new approach to solve the enigma of *Legionella* effector function by using INSeq to analyse effector functions in the context of infection.

Understanding how bacterial pathogens modulate host pathways and hence counteract host defence and cause disease is fundamental in our fight against infectious diseases. The intracellular pathogen *Legionella pneumophila,* the causative agent of Legionnaires' disease, is a unique model to study pathogen manipulation of host pathways during infection, with its sophisticated arsenal of molecular weapons that are secreted into the host cell to counteract host defences.

L. pneumophila is primarily an environmental bacterium that replicates within protozoan hosts (amoeba). When *Legionella*-contaminated aerosols are inhaled the bacterium can replicate within alveolar macrophages and cause an atypical pneumonia in susceptible individuals. *L. pneumophila* utilizes the same processes to parasitize human or protozoan cells as the *L. pneumophila* life cycle has been shown to be identical in all host cell types examined: the bacterium is internalized in a membrane-bound compartment called the *Legionella* containing vacuole (LCV) that escapes from lysosomal degradation, with the LCV remodeled through recruitment of host endoplasmic reticulum-derived vesicles into a protective vacuole for bacterial replication [1].

All key steps of the establishment of the LCV and the ability to replicate intracellularly are dependent on a functional Dot/Icm type IV secretion system (T4SS). This highly conserved machinery is essential for virulence as it secretes over 300 effector proteins into the host cell [2]. Genome sequencing has revealed a considerable plasticity and diversity in the effector repertoires encoded by different *L. pneumophila* strains, suggesting that this diversity allows adaptation to the different hosts *L. pneumophila* can parasitize [3]. Uniquely, among the over 300 T4SS effectors are an abundance of proteins with high similarity to eukaryotic proteins or proteins encoding eukaryotic-like domains, suggesting that molecular mimicry of eukaryotic proteins is a main virulence strategy of *L. pneumophila* [4]. Thus, the broad spectrum of tools employed for bacterial survival and replication in eukaryotic cells seems to have evolved during co-evolution of *L. pneumophila* and its protozoan hosts by horizontal gene transfer among domains of life, allowing this bacterium to parasitize a wide variety of hosts.

Although the T4SS is strictly necessary to initiate and sustain intracellular replication of *L. pneumophila*, the contribution of individual effectors remains minor [5]. Indeed, after the first excitement of scientists that *L. pneumophila* may encode as many functions as effectors, it soon became evident that high functional redundancy was present, and that most of the effectors did not show a virulence phenotype in the standard infection models used.

Furthermore, although a few effectors analyzed individually have been shown to play a role in intracellular replication of *L. pneumophila*, an efficient method to systematically assess the contribution of each effector was missing.

Shames and colleagues recently developed a new approach to define the contribution of the many *L. pneumophila* effector proteins during infection using a high-throughput transposon insertion sequencing technique called INSeq [6]. Guided by the principle of signature tagged mutagenesis, a number of groups have previously developed multiplex screening systems that combined random transposon mutagenesis and next generation sequencing (TnSeq) [7]. These comprehensive approaches allow high-resolution functional screening of whole genomes to identify key genes across various conditions *in vitro* or during host colonization. However, a limitation of *in vivo* experiments is that during the establishment of infection a considerable number of bacteria can be killed or removed stochastically; thus one cannot distinguish whether the bacteria were eliminated by chance or because they were indeed less fit. The approach of Shames *et al.* to circumvent such bottlenecks was to reduce the complexity of the input pool, establishing an effector mutant pool (EMP) library where 297 of the 315 genes predicted to encode effector proteins were represented. Subsequently they used the INseq technique to study the effects of this EMP on virulence phenotypes in amoeba, bone marrow-derived macrophages (BMDMs), and mice. This comprehensive approach allowed them to cluster individual mutants in distinct categories depending on their fitness phenotype. Mutants showing a universal defect, a mammalian-, lung-, BMDM-, or amoeba-specific virulence defect were identified. Indeed, effectors already known to play a role in *L. pneumophila* virulence were selected (*e.g. mavN* [8]), confirming the power of the technique to identify proteins implicated in virulence. Moreover, virulence phenotypes were detected for a large number of uncharacterized effector mutants, opening new vistas on the characterization of secreted *L. pneumophila* proteins. Due to the unbiased approach, effector proteins that contributed both positively and negatively to bacterial fitness could also be identified.

Another strength of the study was that the effector mutants were analyzed in three different host systems, which opened the possibility of evaluating the contribution of individual effectors in specific host contexts. An example is LegC4, an effector that, when deleted, conferred a competitive advantage on the mutant during *in vivo* murine infections, but not for *ex vivo* cellular infections. This was likely due to a role of LegC4 in stimulating pro-inflammatory responses *in vivo* that drive enhanced host control of the infection.

Furthermore, new metaeffectors were identified. First described by Kubori and colleagues [9], metaffectors are defined as effectors that act on another effector to modulate its function in the host cell. The characterization of metaeffectors is an important step in understanding *L. pneumophila*-host interactions as uncovering their spatiotemporal regulation provides insight on how the bacterium modulates diverse host cell functions at discrete stages of infection. Shames and colleagues showed that Lpg2505 is a metaeffector that regulates the previously characterized effector SidI [10]. Interestingly, as with all metaeffectors identified to date, *lpg2505* constitutes an operon with *sidI*, and suppresses SidI toxicity to prevent host damage that would limit *L. pneumophila* replication efficiency.

In summary, INSeq analyses of a defined effector mutant pool in three different infection models allowed virulence phenotypes to be assigned to multiple effectors for which functions were previously unknown. Furthermore, effectors having distinct impact in different host models, or metaeffectors and effectors that act negatively on fitness can be identified. This new approach to identifying previously cryptic effector functions can now be applied across multiple bacterial pathogens and infection systems, opening the door to systematic deconvolution of host-pathogen interactions.

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