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1 ***Legionella* effectors explored with INSeq: new functional insights**

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14 **Keywords**

15 *Legionella pneumophila*, bacterial effectors, transposon insertion sequencing, virulence
16 factors

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35 **Abstract**

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

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

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

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

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

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

82 Shames and colleagues recently developed a new approach to define the contribution
83 of the many *L. pneumophila* effector proteins during infection using a high-throughput
84 transposon insertion sequencing technique called INSeq [6]. Guided by the principle of
85 signature tagged mutagenesis, a number of groups have previously developed multiplex
86 screening systems that combined random transposon mutagenesis and next generation
87 sequencing (TnSeq) [7]. These comprehensive approaches allow high-resolution functional
88 screening of whole genomes to identify key genes across various conditions *in vitro* or during
89 host colonization. However, a limitation of *in vivo* experiments is that during the
90 establishment of infection a considerable number of bacteria can be killed or removed
91 stochastically; thus one cannot distinguish whether the bacteria were eliminated by chance or
92 because they were indeed less fit. The approach of Shames *et al.* to circumvent such
93 bottlenecks was to reduce the complexity of the input pool, establishing an effector mutant
94 pool (EMP) library where 297 of the 315 genes predicted to encode effector proteins were
95 represented. Subsequently they used the INseq technique to study the effects of this EMP on
96 virulence phenotypes in amoeba, bone marrow-derived macrophages (BMDMs), and mice.
97 This comprehensive approach allowed them to cluster individual mutants in distinct
98 categories depending on their fitness phenotype. Mutants showing a universal defect, a
99 mammalian-, lung-, BMDM-, or amoeba-specific virulence defect were identified. Indeed,
100 effectors already known to play a role in *L. pneumophila* virulence were selected (*e.g. mavN*
101 [8]), confirming the power of the technique to identify proteins implicated in virulence.
102 Moreover, virulence phenotypes were detected for a large number of uncharacterized effector
103 mutants, opening new vistas on the characterization of secreted *L. pneumophila* proteins. Due
104 to the unbiased approach, effector proteins that contributed both positively and negatively to
105 bacterial fitness could also be identified.

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

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

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

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