Transcriptional Down-Regulation and rRNA Cleavage in Dictyostelium discoideum Mitochondria during Legionella pneumophila Infection

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Abstract

Bacterial pathogens employ a variety of survival strategies when they invade eukaryotic cells. The amoeba Dictyostelium discoideum is used as a model host to study the pathogenic mechanisms that Legionella pneumophila, the causative agent of Legionnaire’s disease, uses to kill eukaryotic cells. Here we show that the infection of D. discoideum by L. pneumophila results in a decrease in mitochondrial messenger RNAs, beginning more than 8 hours prior to detectable host cell death. These changes can be mimicked by hydrogen peroxide treatment, but not by other cytotoxic agents. The mitochondrial large subunit ribosomal RNA (LSU rRNA) is also cleaved at three specific sites during the course of infection. Two LSU rRNA fragments appear first, followed by smaller fragments produced by additional cleavage events. The initial LSU rRNA cleavage site is predicted to be on the surface of the large subunit of the mitochondrial ribosome, while two secondary sites map to the predicted interface with the small subunit. No LSU rRNA cleavage was observed after exposure of D. discoideum to hydrogen peroxide, or other cytotoxic chemicals that kill cells in a variety of ways. Functional L. pneumophila type II and type IV secretion systems are required for the cleavage, establishing a correlation between the pathogenesis of L. pneumophila and D. discoideum LSU rRNA destruction. LSU rRNA cleavage was not observed in L. pneumophila infections of Acanthamoeba castellanii or human U937 cells, suggesting that L. pneumophila uses distinct mechanisms to interrupt metabolism in different hosts. Thus, L. pneumophila infection of D. discoideum results in dramatic decrease of mitochondrial RNAs, and in the specific cleavage of mitochondrial rRNA. The predicted location of the cleavage sites on the mitochondrial ribosome suggests that rRNA destruction is initiated by a specific sequence of events. These findings suggest that L. pneumophila specifically disrupts mitochondrial protein synthesis in D. discoideum during the course of infection.

Introduction

Legionella pneumophila is an intracellular gram-negative bacterium that causes Legionnaires’ disease, a severe form of pneumonia [1,2,3]. The human disease is associated with inhalation of contaminated aerosols, usually emanating from air conditioning units, where amoebal species have also been found to contain the pathogen [4,5,6]. The bacterium attacks the human alveolar macrophage cells when inhaled. Following uptake into target cells, the bacterium occupies a phagosomal compartment that fails to acidify and to fuse with lysosome, therefore, escaping the normal degradation pathway [7,8]. Shortly after internalization, the L. pneumophila containing vacuole is surrounded by host mitochondria and small vesicles derived from the endoplasmic reticulum (ER) [7,9,10]. As the infection progresses ER membrane surrounds the vacuole and the bacterium starts to multiply, eventually lysing the host cell [11]. None of these cellular hallmarks is found in hosts that have engulfed dead bacteria or avirulent strains of the bacteria [7].

Two bacterial secretion systems have been associated with the pathogenesis of L. pneumophila. The bacteria use these secretion machines to transfer effector molecules, including proteins, into the host cell and also for the horizontal transfer of virulence genes between bacterial species [12]. The Dot/Icm complex, a type IV secretion apparatus, is essential for intracellular growth and pathogenesis of L. pneumophila [13,14]. This secretion apparatus also promotes uptake and allows the establishment of the replication vacuole [15]. An Lsp system, a type II secretion complex, is also indispensable for pathogenesis in amoeba [16,17].

Dictyostelium discoideum is a social amoeba found in the soil that has unicellular and multicellular life cycle phases [18]. Upon starvation, it develops into a fruiting body and forms spores. In nature, D. discoideum cells are proficient phagocytes that feed on soil bacteria. Thus, D. discoideum and other amoebae are likely to serve as the natural hosts for L. pneumophila and as such they may act as reservoirs of bacteria that pose additional risks of exposure to human populations [19,20]. It has been demonstrated that L. pneumophila can infect D. discoideum amoebae in a manner similar to the way they infect human cells so L. pneumophila infection of D.
discoideum has been suggested to be a good model for studying host-interactions with this pathogen [21,22].

As in all eukaryotes, D. discoideum’s mitochondria carry their own genome that encodes RNAs and proteins for mitochondrial ribosomes as well as proteins for energy production [23]. In D. discoideum, disruption of the large subunit ribosomal RNA in a subpopulation of mitochondria results in defective chemotaxis and phototaxis, but not abnormal cell growth [24]. Intriguingly, physical contacts established between the nascent L. pneumophila containing phagosomes and host mitochondria were also observed using electron microscopy, but the potential functional relevance of this observation has not been established [9]. We report a new means by which L. pneumophila may disrupt D. discoideum cell physiology by causing a severe reduction the level of mitochondrial messenger RNAs and by destruction of the mitochondrial large subunit ribosomal RNA (LSU rRNA).

Materials and Methods

Cell culture

Legionella pneumophila bacteria were maintained on solid agar plates BCYE (buffered charcoal yeast extract). They were inoculated and grown in AYE rich media for 24 hours prior to infection [25,26]. Dictyostelium discoideum wild type strain AX4 was maintained axenically, at 22°C, in shaken liquid culture (HL-5 media) or on solid SM agar plates in association with Klebsiella aerogenes as a food source [27].

Acanthamoeba castellani (ATCC 30234) was maintained as adherent cells in PYG media in 75 cm² tissue culture flasks at 35°C [28]. Human U937 cells (ATCC CRL-1593.2) were maintained as non-adherent cells in RPMI 1640 media supplemented with 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 10% fetal bovine serum and 50 μg/ml gentamicin, in 75-cm² tissue culture flasks, at 37°C. Macrophage-like cells were induced from U937 cells by adding 10⁻⁸ M phorbol-12-myristate-13-acetate (PMA, Sigma). Cells were allowed to differentiate for 48 hours prior to use in infection experiments [29].

Legionella pneumophila infections

Exponentially growing D. discoideum cells were harvested from HL-5 shaken culture (2-4x10⁶/ml). The cells were washed twice with phosphate buffered saline (PBS). Aliquots of 10⁵ cells, were suspended in 5 ml modified HL-5 media (without addition of glucose, pH adjusted to 6.9), distributed to 60-mm culture dishes, and incubated at 26°C overnight to allow the cells to attach and adjust to the new media [21]. L. pneumophila cells used for infection were harvested at a post exponential OD₆₀₀>3.0, because the cells at this growth stage have the maximum infectivity [30]. The bacteria were washed twice in PBS buffer to eliminate any soluble extracellular factors and resuspended in PBS at the concentration needed to achieve the desired multiplicity of infection (MOI). Infections were initiated by adding 100 μl of bacterial suspension into equilibrated D. discoideum cultures. In control experiments, green fluorescent protein expressing L. pneumophila cells were used to follow the uptake of the bacteria by the amoebae. At various times, amoebae were harvested and washed repeated to remove bacteria and then examined by fluorescence microscopy. At MOI = 10, >90 percent of the amoebae had at least one L. pneumophila inside after 4 hours, and 100 percent contained L. pneumophila after 12 hours.

The infections of Acanthamoeba castellani and human U937 cells were conducted in a similar way. Adherent cells were tapped off the bottom of 75-cm² stock culture flasks, washed in PBS, and resuspended in appropriate infection media and allowed to reattach to the bottom of 60-mm culture dishes. The infection of A. castellani was assayed at 25°C or 37°C with PYG media deprived of glucose or with non-nutrient Ac buffer, as described [28]. The infection of human U937 cells was conducted at 37°C in RPMI 1640 media without antibiotics.

D. discoideum cell viability during L. pneumophila infection was assessed from triplicate samples by diluting cells from the infection media and allowing them to recover on SM agar plates inoculated with Klebsiella aerogenes bacteria as a food source. D. discoideum colonies emanating from single cells (CFU) were scored after four days of incubation at 22°C. Total RNA from infected cells was prepared for each time-point by harvesting the culture from the entire culture dish and dissolving the cell pellet in 1 ml TRIZOL reagent (Invitrogen).

Chemical treatments

Various types of cellular stress were induced in D. discoideum AX4 cells that were prepared in the same way as for L. pneumophila infection, as described above. The chemicals (Sigma) were added directly into cell culture. Camptothecin and etoposide were applied at 100 μM; staurosporine was used at 1 μM; H₂O₂ was at 1 mM; G418 was used at 100 μg/ml; chloramphenicol was used at 200 μg/ml. All the treatments were carried out at 26°C, the same temperature used for L. pneumophila infection. Total RNA was collected at various times after the addition of the chemicals and examined for cleavage of LSU rRNA by northern blot analyses.

Molecular analyses of LSU rRNA

The oligonucleotides used to study mitochondrial RNAs are listed in (Table 1). The D. discoideum mitochondrial LSU rRNA (GenBank NC_000895) was amplified by reverse transcription polymerase chain reaction (RT-PCR) from a preparation of total RNA. In some experiments, RNA was purified from isolated mitochondria as described [31]. The antisense primer Dd5’-2863 was used to amplify the first strand cDNA, which then served as template for primers Dd5’-144 and Dd3’-2518 to amplify the entire mitochondrial LSU rRNA. The resulting product was

| Table 1. Oligonucleotide primers used to detect and map rRNA cleavage events. |
|-----------------------------------------------|
| Oligonucleotide primer | Sequence (5’ - 3’) |
|------------------------|--------------------|
| Dd3’-2863              | atacgtcatccatatattagt|
| Dd5’-144               | aagaagaagccagtagtaagtgaac|
| Dd3’-2518 (GP1)        | tagataggggaaacacgttccag|
| Dd3’-2243 (GP2)        | gcacatcatgccattaccatcacc|
| Dd3’-2169 (GP3)        | tctttagaagttaccgcaccacctc|
| Dd3’-1391 (GP1)        | tgcagctcagctatatgggaaac|
| Dd3’-1351 (GP2)        | tcgctgtaaccctaatccaagttacttcc|
| Dd3’-1271 (GP3)        | cttcatacagcacttttaatccga|
| Ac5’-73                | TTTCTGGCCCTAATCGAGC|
| Ac5’-2679              | TACGGCTAAAACCCTTGATTACCA|
| Hs5’-18                | acccactccatccctactacccagaca|
| Hs5’-1545              | gggtggtgtggttataatactaaag|

Dd, Dictyostelium discoideum; Ac, Acanthamoeba castellani; Hs, Homo sapiens. doi:10.1371/journal.pone.0005706.0001
digested with CdeI and HaeIII to generate four DNA fragments used as hybridization probes that correspond to segments of the rRNA. The approximate locations of the cleavage sites were determined by northern blot analysis using these four D. discoideum DNA probes and were estimated from the rRNA fragment lengths and the known length of the LSU rRNA. This information was used to design primers to map the cleavage sites precisely using the rapid amplification of cDNA ends (RACE) technique. The A. castellanii mitochondrial LSU rRNA (GenBank NC_001637) probe was amplified from its genomic DNA using primers Ac5'-73 and Ac3'-2679 as described previously. The human mitochondrial LSU rRNA (GenBank NC_001807) probe was amplified from human genomic DNA using primers Hs5'-18 and Hs3'-1545 as described.

Antisense primers Dd3'-2243 and Dd3'-2169 were used as gene specific primers (GSP) to run the 5' RACE, modified from the manufacturer's protocol (Invitrogen). Briefly, the first strand cDNA was amplified by GSP1 Dd3'-2518 with Superscript III reverse transcriptase (Invitrogen) at 55°C, followed by capping the 5' end of the cDNA with dCTP using terminal deoxynucleotidyl transferase. An anchor primer with a polyG tail (Invitrogen) and a nested GSP2 Dd3'-2243 were used to PCR amplify the capped cDNA. A universal anchor primer without polyG tail (Invitrogen) and the third GSP3 Dd3'-2169 were used to run nested PCR to confirm the presence of the required cDNA species. The PCR products were TOPO cloned into TA vectors and sequenced. The rRNA primary cleavage sites and the 3' side secondary cleavage sites were determined by aligning the cDNA sequences next to polyC tails to the original rRNA sequences. After the primary cleavage site was determined, another set of primers GSP1 Dd3'-1391, GSP2 Dd3'-1351 and GSP3 Dd3'-1271 were designed to the 5' side of the primary cleavage site. The 3' side secondary cleavage site was determined in a similar way that the other two cleavage sites were resolved.

The secondary structure of D. discoideum mitochondrial LSU rRNA was derived from comparative prediction modeling [32]. The high-resolution ribosome crystal structure of the halobacterium Haloarcula marismortui was used as a reference model to map D. discoideum mitochondrial LSU rRNA structure in three-dimensions, using PDB 1FFK coordinates [33,34]. RasMol software was used to manipulate and render the three-dimensional structures [35].

Results
Mitochondrial mRNA levels decrease during L. pneumophila

In the course of carrying out transcriptional profiling of D. discoideum during infection with L. pneumophila we observed a decrease in the steady-state levels of a number of mRNAs that are related to mitochondrial function (data not shown). Given the reported association of L. pneumophila-containing phagosomes with the host cell mitochondria, we examined this finding more closely. We assessed the steady-state mRNA levels of four mitochondria-encoded genes, each of which code for one subunit of each of the four complexes of the electron transport chain [23]. These four genes; nad3 (NADH dehydrogenase subunit), atp6 (ATP synthase, Fα subunit), cytB (cytochrome b), and cox3 (cytochrome c oxidase subunit) are transcribed from the mitochondrial genome as part of four distinct polycistronic primary transcripts [36]. After infection with the virulent L. pneumophila strain J32r, we observed two modes of mRNA decline, nad3 and atp6 levels decreased dramatically within the first 4 h of infection, while cytB and cox3 levels declined more gradually throughout the infection (Figure 1A). Exposure of cells to an avirulent L. pneumophila strain (iceT mutant) or Klebsiella aerogenes (a bacterium commonly used as a laboratory food source for D. discoideum) had no detectable effect on transcript levels. In these experiments, cell viability began to decrease rapidly after 8 h, so the decline in nad3 and atp6 mRNAs appears to precede cell killing.

We next tested whether the decrease in mitochondrial transcripts could result from any form of cellular stress by treating cells with several toxins known to kill cells in different ways. The pattern of transcript decrease observed with L. pneumophila infection was closely reproduced by treatment with hydrogen peroxide, but not by treatment with the aminoglycoside G418, or the topoisomerase II inhibitor etoposide (Figure 1B). The oxidative stress induced by hydrogen peroxide is known to lead to mitochondrial damage, so this suggests that L. pneumophila infection leads specifically to a rapid disruption of mitochondrial function by inducing oxidative stress, or conditions that resemble oxidative stress.

Cleavage of mitochondrial LSU rRNA during L. pneumophila infection

D. discoideum mitochondrial LSU rRNA is typically used as a normalization control in northern blots, but when we probed for the LSU rRNA it appeared to be cleaved during L. pneumophila infection. Using the LSU rRNA 5' end as probe, smaller fragments appeared in RNA samples isolated from infected D. discoideum cultures, and these fragments accumulated as the infection progressed as judged by the increase in band intensities (Figure 2A). A larger fragment appeared to accumulate earlier in the course of infection than the smaller fragments, suggesting a specific and sequential cleavage, with a primary cleavage followed by secondary cleavage events. The cleavage did not occur when cells were exposed to avirulent strains L. pneumophila (Figure 2A; and see below). We also demonstrated by northern blot analysis that cytoplasmic rRNAs remained intact during infection by probing the same RNA samples that contained cleaved mitochondrial LSU rRNA (data not shown). The cleavage of the mitochondrial LSU rRNA also correlated with decreased survival of D. discoideum during infection (Figure 2B). The presence of detectable cleavage at 8 hours post infection, before any detectable loss in cell viability, suggests that LSU rRNA cleavage precedes cell killing. Contrary to the similar effect that virulent L. pneumophila and hydrogen peroxide had on mitochondrial mRNA levels, hydrogen peroxide treatment did not induce LSU rRNA cleavage in cells (Figure 2C).

LSU rRNA cleavage is not caused by other forms of cytotoxic stress

It has been observed that virulent L. pneumophila infection induces host cell apoptosis in mammalian cells [37,38,39]. To begin to test whether the observed cleavage of mitochondrial LSU rRNA is a host cell response to any stress, or caused by L. pneumophila directly, we induced stress in D. discoideum cells by a variety of chemical treatments and assessed mitochondrial rRNA integrity on northern blots. Camptothecin and etoposide are apoptosis inducers that poison eukaryotic DNA topoisomerases I and II, respectively [40,41]. Staurosporine is a potent protein kinase C inhibitor [42] while the antibiotics G418 and chloramphenicol inhibit eukaryotic cytoplasmic and mitochondrial ribosomal protein synthesis, respectively, and hydrogen peroxide induces oxidative stress in cells. These treatments caused D. discoideum cell death but none of them led to the mitochondrial LSU rRNA cleavage observed during L. pneumophila infection (Table 2). These results suggest that the observed rRNA cleavage is caused by L. pneumophila infection rather than a general host cell stress response.
LSU rRNA cleavage requires both the type II (Lsp) and type IV (Dot/Icm) secretion systems

All of the L. pneumophila pathogenic strains that have been sequenced so far carry type IV Dot/Icm and type II Lsp secretion systems that are believed to be required for pathogenesis [43,44]. Most of the bacteria’s pathogenic functions reported so far require the integrity of one or the other of these secretion systems [14,16,17,45,46]. We tested which of these two L. pneumophila secretion pathways are required for the rRNA cleavage to take place. The icmT and dotA genes reside within two different gene clusters that encode the core components of the type IV secretion machinery [13,47,48]. The lspF and lspG genes are clustered together and encode the core of the type II machinery, while the lspDE genes encode another component of the type II secretion machinery [43]. The type IV mutants, icmT and dotA, as well as the type II mutants lspF, lspG and lspDE were tested for their ability to induce rRNA cleavage. None of these mutants were observed to induce cleavage of the mitochondrial rRNA after mixing them with D. discoideum (Table 3). These results indicate that both the type IV and the type II secretion systems must be functional for D. discoideum mitochondrial LSU rRNA cleavage to occur.

We also tested the pathogenicity of these type IV and type II mutants on D. discoideum. As expected, none of these L. pneumophila mutants killed D. discoideum, as their parental wild type strains did, which corroborates the findings using other amoebae as hosts (Table 3). Since we could have observed some LSU rRNA cleavage without death of the Dictyostelium cells, but did not, a strict correlation exists between the pathogenesis of L. pneumophila and the cleavage of D. discoideum’s mitochondrial LSU rRNA.

We attempted to identify possible L. pneumophila enzymes responsible for the rRNA cleavage by using a candidate gene approach. We inspected all predicted ribonuclease genes in the genomic sequence of L. pneumophila and excluded genes predicted to encode ribonucleases with defined functions (10). We found a single predicted T2 ribonuclease with an RNase core domain plus uncharacterized domains that we hypothesized might be involved in determining substrate specificity (GenBank AE017354). A deletion mutant of the gene encoding this protein, provided by the laboratory of Ralph Isberg, was assayed for LSU rRNA cleavage activity upon infection of D. discoideum. Mitochondrial LSU rRNA cleavage was observed, just as in wild type L. pneumophila infection, indicating that this protein is not responsible for the rRNA cleavage activity (data not shown).

**Determination of the LSU rRNA cleavage sites**

We used RACE (Rapid Amplification of cDNA Ends) to map the specific cleavage sites on the rRNA [49]. In order to design the RACE primers we mapped the approximate locations of the
cleavage sites by northern blot analyses using segments of RT-PCR-amplified mitochondrial LSU rRNA as probes (Figure 3A and 3B). Two sets of primers were designed for the RACE reactions that bordered the approximate locations of the cleavage sites. We carried out RACE reactions on samples of total RNA collected from cells that had been infected with virulent and avirulent L. pneumophila. Using two different RACE primers two fragments and the full-length copy of the rRNA were easily detectable, but only from cells infected with virulent L. pneumophila (Figure 3C). The RACE products representing sub-fragments of the copied and amplified rRNA were cloned into plasmids and the cleavage sites were then determined by sequencing the inserts (Figure 3C). The sequencing of the RACE products revealed the precise locations of one major and one minor cleavage site on the D. discoideum mitochondrial LSU rRNA (Figure 3A). With the major cleavage site mapped, RACE primers were designed that lay to the 5-prime side of that site and used to amplify the other minor fragment (Figure 3D).

The northern blot data indicates that the LSU rRNA is cleaved into discrete fragments and the unambiguous ends identified by sequencing each of the RACE products strongly suggests that the fragmentation results from cleavage at discrete sites. The major cleavage site mapped between nucleotides 1533 and 1534 (Figure 3A). A minor cleavage site on the 5’ side of the major site mapped between nucleotides 862 and 865 and another minor cleavage site on the 3’ side of the major site mapped between nucleotides 1878 and 1879. Because the eukaryotic mitochondrial ribosomes are thought to have shared a common ancestor with archaeabacterial ribosomes, we mapped the cleavage sites onto the predicted secondary structure of the D. discoideum mitochondrial LSU rRNA based on the halobacteria Haloarcula marismortui rRNA structure [33,50] (Figure 4A). When mapped onto the corresponding regions of the H. marismortui rRNA high-resolution crystal structure, the major cleavage site is predicted to be on the surface of the ribosome (Figure 4B). This is consistent with the expectation that the initial cleavage site is accessible on the intact ribosome similar to the sarcin/ricin loop, a distinct site that is subject to cleavage by those toxins (Figure 4B and 4C). The minor cleavage site at base 1878 is predicted to be in domain IV of the LSU rRNA (Figure 4A), where essential functional contacts are made between the ribosome’s large and small subunits (Figure 4B and 4C). The minor cleavage site at base 862 and is predicted to be deep within the ribosomal structure (Figure 4C).

No detectable rRNA cleavage in Acanthamoeba castellanii or human U937 cells

We tested whether LSU rRNA cleavage occurs in other cells infected with L. pneumophila. Acanthamoeba castellanii and human U937 cells are two widely used models of L. pneumophila infection [28,29]. We infected these cells using established protocols and assayed for host cell viability and mitochondrial LSU rRNA integrity, but we did not observe any cleavage events during either of these infections (Figure 5). This result with human cells is not surprising since the entire structural domain where the primary cleavage site is located in D. discoideum LSU rRNA has been lost in human LSU rRNA [32,51]. However, in A. castellanii, the major cleavage site and the minor site at base 1878 are conserved, whereas the minor cleavage site at base 832 is within a loop that is unique in D. discoideum [32].

Discussion

We have described major changes in the stability of D. discoideum’s mitochondrial RNAs during L. pneumophila infection. The LSU rRNAs of the mitochondrial ribosomes are cleaved at specific sites and there is a decrease in the steady-state level of each of the mitochondrial mRNAs that we examined. Some mRNA levels drop to undetectable levels within four hours of the start of the infection while other mRNAs decrease more gradually. Thus, some mRNA levels decrease well before cell death is detectable after 8 hours. We found that we could reproduce this pattern of mRNA loss by treatment with hydrogen peroxide, but not by treatments with other toxic agents. The production of hydrogen
peroxide and other reactive oxygen species (ROS) by the mitochondria and the protective responses to ROS toxicity are well described [52]. The incomplete reduction of oxygen by the electron transport chain results in ROS and this causes reduction in the flux through the tricarboxylic acid cycle through the reversible unfolding of redox-sensitive enzymes such as aconitase [53] and, eventually, loss of mitochondrial mRNAs [54]. Thus, L. pneumophila infection appears to induce an effect on host cell mitochondria reminiscent of an oxidative stress response.

Our results suggest that infection results in a complete shut down of mitochondrial gene expression. Although we did not test the expression of every gene encoded by the mitochondrial genome (mtDNA), the four genes that we did test are transcribed initially as part of four polycistronic transcripts that comprise about half of the entire coding capacity of the mtDNA [36]. If the decrease in mRNAs that we observed resulted from transcriptional initiation, it is reasonable to assume that the mRNA encoding all of the proteins that come from those transcripts decrease during infection. Farbrother, et al., recently reported alterations in the transcriptome that occur during infection under similar conditions [55]. Using cDNA hybridization to oligonucleotide microarrays, they reported that the mRNA for genes such as cox3 increase slightly during progression of the infection, whereas our northern analyses clearly indicate that cox3 mRNA decreases quite dramatically. We cannot account for this discrepancy.

In addition to the effect on mRNAs, we also found that L. pneumophila infection results in cleavage of the mitochondrial LSU rRNA, an effect that could not be mimicked by treatment with hydrogen peroxide or any other cytotoxic agents that we tested. Hydrogen peroxide has been reported to cause mitochondrial rRNA destruction in other systems but we saw no evidence of this in Dictyostelium [56]. If the specific cleavage and destruction of LSU rRNA were an intrinsic response of D. discoideum to particular forms of stress, we would have expected to observe it under different conditions. We tested six different chemical agents that induce stress by different means and eventually kill cells, but we never observed LSU rRNA cleavage (Table 2). Thus, we favor the notion that L. pneumophila induces the cleavage directly, either by secretion of a ricin-like RNase or by specific alteration a host cell enzyme.

The identification of the protein(s) responsible for the LSU rRNA cleavage, or the selection of a D. discoideum mutant fully resistant to the rRNA cleavage during infection, are required to further characterize the rRNA cleavage mechanism and its relationship to L. pneumophila pathogenesis. We demonstrated a requirement for both the type II and the type IV secretion systems of L. pneumophila in mitochondrial LSU rRNA cleavage. The simplest interpretation of this result is that the protein(s) responsible for cleavage are bacterial cargo of the type II or the type IV systems. However, since both secretion systems are required for productive L. pneumophila infection, it is equally plausible that the progression of cellular pathogenesis is required to set up the proper environment for rRNA cleavage, independent of whether or not the cleavage is carried out by a bacterial enzyme.

### Table 2. Chemical-induced stress and mitochondrial rRNA integrity.

| Chemical reagent   | Expected Target     | Concentration used | LSU rRNA cleavage |
|--------------------|---------------------|--------------------|------------------|
| Camptothecin       | DNA topoisomerase I | 100 µM             | Not detected     |
| Etoposide          | DNA topoisomerase I | 100 µM             | Not detected     |
| Staurosporine      | PKC inhibitor       | 1 µM               | Not detected     |
| H₂O₂               | Oxidative stress    | 1 mM               | Not detected     |
| G418               | Cytoplasmic ribosome| 100 µg/ml           | Not detected     |
| Chloramphenicol    | Mitochondrial ribosome| 200 µg/ml         | Not detected     |

1 Treating were for 20 hours prior to isolation of total RNA.
2 Integrity of the LSU rRNA was determined by northern blot analyses as described in Figure 2.

### Table 3. Effect of L. pneumophila type II and type IV secretion mutants on D. discoideum mitochondrial rRNA cleavage.

| Strain     | Phenotype | Source     | Human toxicity | Amoebae toxicity | Cleave rRNA |
|------------|-----------|------------|----------------|------------------|-------------|
| JR32       | Wild type | [47]       | Yes            | Yes              | Yes         |
| icmT       | Type IV   | [47]       | No             | No               | No          |
| LP02(thy+ dot+) | Wild type | [14]       | Yes            | Yes              | Yes         |
| LP03(thy+dotA+) | Type IV   | [14]       | No             | No               | No          |
| GL84(thy+dotA+)/pDOT1(thy+dotA+) | Type IV rescue | Isberg, R. | Yes            | Yes              | Yes         |
| 130b       | Wild type | Ciacciotti, N. | Yes            | Yes              | Yes         |
| NU275/pSP   | Type II   | [45]       | Yes            | No               | No          |
| NU259/pSPG  | Type II   | [17]       | Yes            | No               | No          |
| NU258/pSPDE | Type II   | [17]       | Yes            | No               | No          |

D. discoideum AX4 cells were infected by various type IV or type II secretion mutants of L. pneumophila and their parental strains. The human toxicity data are from the cited references. The D. discoideum cell viability was determined by plating for viable cells in an assay for colony forming unit and mitochondrial LSU rRNA integrity was assessed by northern blot analyses as described in Figure 2 (data not shown).

doi:10.1371/journal.pone.0005706.t002
We attempted to identify the *L. pneumophila* protein responsible for the rRNA cleavage using a candidate gene approach. We examined all of the predicted protein products of the *L. pneumophila* genome for those containing any known RNase or ricin/sarcin domains [43]. The best candidate that we identified is a T2 RNase family enzyme that contains additional amino acid sequences that are not found in other “housekeeping” ribonucleases. An *L. pneumophila* deletion mutant in this enzyme is still capable of...
Figure 4. Location of the cleavage sites on the *D. discoideum* mitochondrial LSU rRNA structure. (A) Predicted secondary structure of *D. discoideum* mitochondrial LSU rRNA showing the locations of the cleavage sites (red arrows). For clarity, the rRNA is split between domains III and IV and the thick dashed line indicates the covalent linkage between the two halves. Lines indicate predicted basepairs in the three dimensional structure between bases that widely separated in the primary sequence. The domains are labeled in roman numerals near where their secondary structure emanates from the center. (B) LSU rRNA cleavage sites (red) mapped onto the three-dimensional structure of the *Haloarcula marismortui* mitochondrial ribosome (pdb 1ffk). The LSU rRNA (grey line), the 5S rRNA (blue line) and ribosomal proteins (yellow) are shown. The rRNA sarcin/ricin loop is indicated (green). The points of contact with the small subunit (purple) in domain IV of the rRNA secondary structure are where essential ribosomal functions are carried out and where the cleavage site at base 1878 is located (red). (C) Predicted structure in B rotated 60 degrees counterclockwise on the vertical axis.

doi:10.1371/journal.pone.0005706.g004
inducing LSU rRNA cleavage in D. discoideum. Considering that the presumed initial cleavage site on the surface of the ribosome has not been previously recognized as a site of attack by ribonuclease toxins, it is possible that a new family of rRNA nucleases carries out the rRNA cleavage.

The sequential appearance of the LSU rRNA fragments during L. pneumophila infection is suggestive of a regulated destruction of the mitochondrial ribosomes. We mapped the locations of the cleavage events onto the predicted structure of the D. discoideum LSU rRNA based on high-resolution structures of archaeobacterial ribosomes. The first cleavage event is predicted to occur on the surface of the ribosome while secondary events are predicted to occur within the ribosomal structure. We can hypothesize two scenarios that would produce the observed order of cleavage. The mitochondrial LSU rRNA based on high-resolution structures of archaebacterial rRNA cleavage in infected cells does not lead to degradation of the bacteria. Horwitz et al. reported an association between L. pneumophila containing phagosomes and host cell mitochondria soon after the internalization of the bacteria, which were extended by the demonstration of physical contacts between the two compartments [9,58]. Although these observations have not been further explored as a potential pathogenic mechanism, we have now shown that L. pneumophila infection of D. discoideum results in the destruction of the mitochondrial large subunit ribosomal RNA (LSU rRNA). This suggests that the host cell mitochondria are a target of modulation by L. pneumophila during infection. Since the destruction of the mitochondrial rRNA correlates temporally with host cell death, we are now in a position to explore how compromised mitochondrial protein synthesis might contribute to a productive L. pneumophila infection and the demise of the host cell.

**Acknowledgments**

We thank Howard Shuman, Ralph Isberg and Nicholas Cianciotto for providing L. pneumophila strains and Tracy Chen and Ralph Isberg for providing the RNAse mutant. We also thank Gad Shaulsky and Nancy Van Dreissche for initial transcriptional profiling of Legionella infection.

**Author Contributions**

Conceived and designed the experiments: CZ AK. Performed the experiments: CZ. Analyzed the data: CZ AK. Wrote the paper: CZ AK.
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