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Legionella pneumophila multiplication is enhanced by chronic AMPK signalling in mitochondrially diseased Dictyostelium cells

Lisa Francione¹, Paige K. Smith¹, Sandra L. Accari¹, Philip E. Taylor², Paul B. Bokko¹, Salvatore Bozzaro³, Peter L. Beech² and Paul R. Fisher¹,*

SUMMARY

Human patients with mitochondrial diseases are more susceptible to bacterial infections, particularly of the respiratory tract. To investigate the susceptibility of mitochondrially diseased cells to an intracellular bacterial respiratory pathogen, we exploited the advantages of Dictyostelium discoideum as an established model for mitochondrial disease and for Legionella pneumophila pathogenesis. Legionella infection of macrophages involves recruitment of mitochondria to the Legionella-containing phagosome. We confirm here that this also occurs in Dictyostelium and investigate the effect of mitochondrial dysfunction on host cell susceptibility to Legionella. In mitochondrially diseased Dictyostelium strains, the pathogen was taken up at normal rates, but it grew faster and reached counts that were twofold higher than in the wild-type host. We reported previously that other mitochondrial disease phenotypes for Dictyostelium are the result of the activity of an energy-sensing cellular alarm protein, AMP-activated protein kinase (AMPK). Here, we show that the increased ability of mitochondrially diseased cells to support Legionella proliferation is suppressed by antisense-inhibiting expression of the catalytic AMPKα subunit. Conversely, mitochondrial dysfunction is phenocopied, and intracellular Legionella growth is enhanced, by overexpressing an active form of AMPKα in otherwise normal cells. These results indicate that AMPK signalling in response to mitochondrial dysfunction enhances Legionella proliferation in host cells.

INTRODUCTION

Mitochondrial diseases are complex, degenerative disorders resulting from various mitochondrial DNA or nuclear gene mutations. Depending on which tissues are affected most severely, the phenotypic outcomes are diverse and can include diabetes, blindness, deafness, stroke-like episodes, epilepsy, ataxia, muscle weakness, exercise intolerance and kidney disease (Wallace et al., 1988; Goto et al., 1992; Tattuch and Robinson, 1993; Mackey et al., 1996; Geromel et al., 2001; Rossignol et al., 2003; McKenzie et al., 2004). A number of reports suggest that mitochondrial disease patients are more susceptible to recurrent bacterial infections, particularly of the respiratory tract (Lachawan et al., 2000; Edmonds et al., 2002; Katsanos et al., 2002; Ogawa et al., 2003; Edmonds, 2004; Wortmann et al., 2006). However, the mechanisms that underlie such increased susceptibility have not been studied and are not understood. Here, we use the established Dictyostelium discoideum model for mitochondrial disease (Wilczynska et al., 1997; Kotsifas et al., 2002; Chida et al., 2004; Torija et al., 2006; Barth et al., 2007) and for Legionella pneumophila pathogenesis (Hägge et al., 2000; Solomon et al., 2000; Skriwan et al., 2002; Hilbi et al., 2007) to show that intracellular proliferation of the bacterial pathogen is supported better by mitochondrially diseased cells than by healthy cells.

In humans, L. pneumophila infection can lead ultimately to a severe pneumonia termed Legionnaires’ disease (Fields, 1996).

¹Department of Microbiology, La Trobe University, VIC 3086, Australia
²Centre for Cellular and Molecular Biology, Deakin University, Burwood, VIC 3125, Australia
³Department of Clinical and Biological Sciences, University of Turin, Ospedale S. Luigi, 10043 Orbassano, Italy
⁴Author for correspondence (e-mail: p.fisher@latrobe.edu.au)

The disease process can be initiated by human inhalation of contaminated water aerosols that contain L. pneumophila growing inside amoebae, enabling entry of the bacteria into the lungs where they invade alveolar macrophages (Fiore et al., 1998; Molmeret et al., 2005). When L. pneumophila infects host phagocytes, it avoids phagosome-lysosome fusion and subverts host cellular pathways to facilitate its own multiplication inside specialized Legionella-containing vesicles (LCVs) within the host cell (Horwitz and Silverstein, 1980; Horwitz, 1983a; Horwitz and Maxfield, 1984). The molecular mechanisms by which it achieves this are essential for pathogenesis and are similar in phagocytic human cells and protozoa, including the social amoeba Dictyostelium discoideum (Albert-Weissenberger et al., 2007; Hilbi et al., 2007). Because of this and its genetic tractability, Dictyostelium has become a favoured model in which to study the interactions between Legionella and its host (Hägge et al., 2000; Solomon et al., 2000; Skriwan et al., 2002; Fajardo et al., 2004; Farbrother et al., 2006; Peracino et al., 2006; Weber et al., 2006).

In one of the earliest such interactions in human monocytes and macrophages, host cell endoplasmic reticulum (ER) membranes and mitochondria are recruited to the vicinity of the phagosome (Horwitz, 1983b; Swanson and Isberg, 1995; Tilney et al., 2001). In the case of Dictyostelium, ER recruitment is well established (Fajardo et al., 2004; Lu and Clarke, 2005) but mitochondrial recruitment to the phagosome has not been reported; however, published electron micrographs of Legionella-containing cells from 30 minutes (Otto et al., 2004) and 3 hours after infection (Farbrother et al., 2006) suggest strongly that mitochondrial recruitment does occur. Here, we confirm, by laser scanning confocal and electron microscopy, that mitochondria are
recruited to LCVs within the first 30 minutes of Legionella infection in Dictyostelium.

Recruitment of mitochondria to the phagosome is not the only interaction between Legionella and the mitochondria of its host. A recent microarray study revealed that Dictyostelium genes encoding mitochondrial proteins, including components of the respiratory electron transport chain, are upregulated within 3 hours of infection (Farbrother et al., 2006). The upregulation of mitochondrial biogenesis and the phagosomal recruitment of mitochondria suggest that mitochondria may provide nutritional or other support for the pathogen and that, to this end, Legionella subverts the host pathways regulating mitochondrial biogenesis and subcellular localization.

One of the key regulators of mitochondrial biogenesis and energy production in mammalian cells, and in Dictyostelium, is the energy-sensing protein kinase AMPK (AMP-activated protein kinase) (Hardie and Sakamoto, 2006; Bokko et al., 2007). AMPK is activated by AMP and inhibited by ATP so that it functions as a highly sensitive sensor of cellular energy status. In its activated form, AMPK phosphorylates target proteins and switches metabolism from anabolic to catabolic pathways. This is achieved by stimulating such processes as the uptake of glucose, fatty acid oxidation and mitochondrial biogenesis, and by inhibiting ATP-consuming processes, such as progression through the cell cycle and associated protein synthesis. In this way, AMPK maintains cellular energy homeostasis during periods of energy stress (Zhou et al., 2001; Hawley et al., 2003; Kahn et al., 2005; Hardie and Sakamoto, 2006; Kukidome et al., 2006).

Chronic cellular energy stress is characteristic of mitochondrial diseases, an eclectic group of poorly understood genetic disorders that impair the ATP-generating capacity of the mitochondria. In mitochondrial disease, AMPK is expected to be activated chronically because the ATP-generating capacity of the mitochondria is compromised permanently. We reported recently that, in the Dictyostelium model for mitochondrial disease, this chronic AMPK signalling causes diverse cytopathological outcomes (Bokko et al., 2007). Since mitochondrial biogenesis is stimulated by AMPK, and since Legionella infection upregulates both mitochondrial biogenesis and AMPK, we hypothesized that the chronic activation of AMPK in mitochondrially diseased cells might enhance the ability of Legionella to multiply within them. If so, mitochondrially diseased cells should support Legionella growth better than healthy cells because of chronic AMPK signalling. Here, we report molecular genetic evidence that this is so.

RESULTS
Mitochondria are recruited to LCVs in Dictyostelium

It has been established that the *L. pneumophila* Corby strain can infect and proliferate in *D. discoideum* in a manner that is analogous to its growth in macrophages (Solomon et al., 2000; Hägele et al., 2000). Macrophage and monocyte mitochondria are recruited to the Legionella phagosome soon after infection (Horwitz, 1983b; Tilney et al., 2001), but similar recruitment of the mitochondria has not been reported in Dictyostelium. To determine whether Dictyostelium mitochondria are recruited to LCVs, we infected cells of a transformant of the parental Dictyostelium strain AX2 that expressed a mitochondrially targeted green fluorescent protein (GFP) (Ahmed et al., 2006). The GFP was targeted to the mitochondria by fusion with the N-terminal 150 amino acids from the essential mitochondrial matrix protein, chaperonin 60. The Legionella strain was a transformant of the Corby strain expressing a rapidly maturing Discosoma red fluorescent protein (DsRed) variant (Mampel et al., 2006). Within 30 minutes of infection, GFP-labelled mitochondria were associated closely with the Legionella
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was caused either by disruption of the mitochondrial large subunit ribosomal RNA gene rnl (Wilczynska et al., 1997), or by antisense inhibition of chaperonin 60 (Kotsifas et al., 2002). We therefore examined whether mitochondrially diseased cells exhibit altered susceptibility to Legionella. To do this, we fixed infected cells at different stages of infection. We therefore examined whether mitochondrially diseased cells exhibit altered susceptibility to Legionella. To do this, we fixed infected cells at different stages of infection. We therefore examined whether mitochondrially diseased cells exhibit altered susceptibility to Legionella. To do this, we fixed infected cells at different stages of infection. We therefore examined whether mitochondrially diseased cells exhibit altered susceptibility to Legionella. To do this, we fixed infected cells at different stages of infection. We therefore examined whether mitochondrially diseased cells exhibit altered susceptibility to Legionella. To do this, we fixed infected cells at different stages of infection.

At no stage of the infection was there a mass recruitment of mitochondria, but instead the Legionella appear to recruit a small number of mitochondria that become very closely associated, possibly attached as in macrophages, to the LCVs (Table 1). Of the LCVs that we examined inside infected cells by laser confocal microscopy, all had at least one closely associated mitochondrion, with most LCVs having two or three, and some having as many as five mitochondria in close association. Three-dimensional (3D) reconstructions revealed these associations to be extensive and intimate, in that they appeared to involve large areas of the mitochondrial and LCV surfaces being closely apposed to one another. This was confirmed by electron microscopy of thin sections of infected cells, as illustrated by Fig. 2, which shows an example from a cell at 1-hour post-infection. Two mitochondria are seen with parts of their outer membrane closely apposed to the membrane of the LCV. This close apposition of the membranes is reminiscent of that observed in the early stages of Legionella infection of human cells (Horwitz, 1983b).

Mitochondrially diseased Dictyostelium strains support greater L. pneumophila growth

The recruitment of mitochondria to the LCVs suggests that mitochondria may play a supportive role for Legionella in the early stages of infection. We therefore examined whether mitochondrially diseased cells exhibit altered susceptibility to Legionella. To do this, we fixed infected cells at different stages of infection. We therefore examined whether mitochondrially diseased cells exhibit altered susceptibility to Legionella. To do this, we fixed infected cells at different stages of infection. We therefore examined whether mitochondrially diseased cells exhibit altered susceptibility to Legionella. To do this, we fixed infected cells at different stages of infection. We therefore examined whether mitochondrially diseased cells exhibit altered susceptibility to Legionella. To do this, we fixed infected cells at different stages of infection. We therefore examined whether mitochondrially diseased cells exhibit altered susceptibility to Legionella. 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mitochondrial disease phenotypes in Dictyostelium (Kotsifas et al., 2002; Bokko et al., 2007), a correlation exists between copy number and susceptibility to Legionella infection in the chaperonin 60 antisense-inhibited cells (Fig. 4). The \( R^2 \) value indicates that this correlation explains about 40% of the variance in the Legionella counts on day 5 of the infection.

The increased susceptibility of mitochondrially diseased strains to Legionella is not the result of increased rates of infection

The increased proliferation of Legionella in mitochondrially diseased Dictyostelium cells could have been the result of an increased susceptibility to initial infection. However, we reported recently that although mitochondrial dysfunction retards growth in Dictyostelium, it has no effect on the rate of nutrient uptake by pinocytosis or by phagocytosis of bacterial prey (such as \textit{Escherichia coli}) (Bokko et al., 2007). We therefore anticipated that the initial uptake of Legionella by Dictyostelium cells would also be unaffected and so would not explain the enhanced proliferation that we observed. To verify whether this was true, we assayed the rate of Legionella uptake by wild-type strains and by representative strains in which mitochondrial dysfunction was elicited by chaperonin 60 antisense inhibition. We found that the rate of Legionella uptake was unaltered in the mitochondrially diseased strains during the first 2 hours of infection (Fig. 5).

The increased susceptibility of mitochondrially diseased Dictyostelium strains is caused by chronic AMPK signalling

The mitochondrial disease phenotypes reported previously for Dictyostelium, including impaired phototaxis, thermotaxis, growth and multicellular development, are the result of the activity of an energy-sensing cellular alarm protein, AMPK (Bokko et al., 2007). This was shown genetically by overexpressing a truncated active subunit (AMPK\(\alpha\Tilde{T}\)) and by antisense-inhibiting native AMPK\(\alpha\) expression in mitochondrially diseased cells. The diverse phenotypic outcomes of mitochondrial dysfunction were all phenocopied by AMPK\(\alpha\Tilde{T}\) overexpression in otherwise healthy cells, whereas antisense inhibition of AMPK expression in mitochondrially diseased cells suppressed all aberrant phenotypes. Phagocytosis and pinocytosis rates were unaffected by the levels of active AMPK.

To determine whether AMPK activation was similarly responsible for the increased susceptibility of mitochondrially diseased cells to Legionella, we infected AMPK\(\alpha\Tilde{T}\)-overexpressing cells, AMPK\(\alpha\) antisense-inhibited cells, and mitochondrially diseased cells (chaperonin 60 antisense inhibition) in which AMPK\(\alpha\) expression was antisense inhibited. Fig. 6A shows representative growth curves for Legionella infections in strains belonging to these different categories. AMPK\(\alpha\Tilde{T}\) hyperexpression (HPF437) caused a significant increase in the susceptibility of Legionella.
Fig. 4. Effect of chaperonin 60 antisense construct levels on susceptibility to Legionella. Legionella viable counts from day 5 are plotted. Each circle represents an independent infection assay of a specific Dictyostelium strain containing the indicated number of copies of the chaperonin 60 antisense construct. The previously adopted convention of assigning negative values to copy numbers for antisense constructs was followed (Bokko et al., 2007). Copy numbers of zero represent the wild-type strain (AX2).

Fig. 5. Effect of mitochondrial dysfunction on the rate of Legionella uptake. The uptake of Legionella during a 2-hour period of infection was measured for the parental strain (AX2) and for representative mitochondrially diseased strains (in which mitochondrial dysfunction was elicited by antisense-inhibited chaperonin 60 expression). The copy numbers for the chaperonin 60 antisense construct were 162 (HPF413), 112 (HPF414) and 93 (HPF416). The Legionella uptake was based on viable counts of internalized bacteria, normalized against the count for AX2 after 2 hours of infection in the same experiment. Error bars are standard errors of the mean from three independent experiments. The inset shows a representative time course of infection of AX2.

Dictyostelium to Legionella and thus phenocopied the effects of mitochondrial disease (HPF602). Conversely, when expression of the native AMPKα was antisense inhibited in otherwise healthy cells (HPF461), there was a slight reduction, or no significant difference, in Legionella proliferation in comparison to infections of wild-type host cells (AX2). Legionella growth was enhanced in host cells with a mitochondrial dysfunction resulting from chaperonin 60 antisense inhibition (HPF602), but this phenotype was suppressed by concurrent antisense inhibition of AMPKα expression (HPF505). These results suggest that AMPK signalling is responsible for the enhanced ability of mitochondrially diseased host cells to support Legionella proliferation.

To verify that increased levels of active AMPK cause increased proliferation of L. pneumophila in host cells, we examined the correlation between Legionella proliferation and the copy numbers of the antisense-inhibition and overexpression constructs. Fig. 6B shows that Legionella growth was enhanced in a copy-number-dependent manner by overexpression of active AMPKαT and slightly inhibited or unaffected by AMPKα antisense inhibition. Whereas mitochondrial dysfunction caused increased Legionella proliferation in otherwise healthy cells, the susceptibility to Legionella was restored to normal in all mitochondrially diseased strains in which AMPKαT expression was antisense inhibited (red squares). This genetic suppression is analogous to that observed in these strains in relation to the other mitochondrial disease phenotypes (Bokko et al., 2007). We conclude that the increased susceptibility to L. pneumophila that are associated with mitochondrial disease in D. discoideum is mediated by AMPK signalling.

DISCUSSION

During the process of L. pneumophila infection in macrophages and monocytes, phagosomes containing Legionella interact with mitochondria for approximately 1 hour and then associate with ribosomes after about 4 hours (Horwitz, 1983b; Tilney et al., 2001). Our results confirm that this early association between mitochondria and L. pneumophila exists also in Dictyostelium. Within 30 minutes of infection, every single LCV had between one and five closely associated mitochondria. Our experiments do not show whether this association requires active pathogen functions. In future experiments, it would be of interest to use killed Legionella and nonpathogenic mutants to determine whether specific pathogen genes are required for association of the mitochondria with the LCV.

The association between the mitochondria and the Legionella continued throughout the first few hours of the infection in the form of a close apposition between each LCV and a small number of mitochondria. However, by 24 hours of infection, the mitochondrial GFP and the Legionella DsRed signal appeared to be colocalized and the DsRed signal no longer showed the sharp, discrete outline of the bacteria. We do not know the basis for this apparent colocalization, which has not been reported previously in any Legionella host; however, there are several possibilities that could be the subject of future investigation. One is a fusion between the mitochondria and the LCV, releasing the contents of the mitochondrial matrix into the lumen of the LCV. However, this would not explain the loss of the bacterial DsRed outline. A second possibility is that at least some LCVs with attached mitochondria have fused with lysosomes where they are undergoing degradation. Although this would be consistent with the altered appearance of the DsRed fluorescence, it cannot be the case that every LCV
undergoes such a fate since the Legionella would not then proliferate subsequently as they do. Furthermore, DsRed fluorescence is pH insensitive from pH 4.5 to 12 (Baird et al., 2000), whereas the GFP fluorescence should fade dramatically in the acidic environment of the lysosome (Kneen et al., 1998). An obvious loss of the GFP signal relative to the DsRed signal after 24 hours of infection was not apparent in our experiments. A third possibility is that the DsRed has been exported from the Legionella into the mitochondria, perhaps along with some of the other pathogen proteins, through the bacterial Dot/Icm protein secretion system. This would explain the change in appearance of the DsRed fluorescence.

Our findings have also demonstrated that mitochondrially diseased D. discoideum cells can support the growth of L. pneumophila better than the wild-type host strain AX2. This was observed both for strains in which chaperonin 60 expression was antisense inhibited (Kotsifas et al., 2002) and strains in which the mitochondrial rnl gene had been disrupted in a subpopulation of the mitochondrial genomes (Wilczynska et al., 1997). In the former case, the intracellular proliferation of Legionella increased with the copy number of the chaperonin 60 antisense-inhibition construct, that is, as the severity of the resulting mitochondrial dysfunction increased.

We reported recently that chronic AMPK signalling is responsible for diverse cytopathologies in mitochondrially diseased Dictyostelium cells, where it impairs cell proliferation, multicellular morphogenesis and photosensory signal transduction (Bokko et al., 2007). The results presented here demonstrate that AMPK signalling in response to mitochondrial dysfunction likewise causes increased susceptibility to Legionella. The intracellular growth of Legionella increased with the copy number of the chaperonin 60 antisense-inhibition construct, that is, as the severity of the resulting mitochondrial dysfunction increased.

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antisense inhibition made only a small difference to Legionella proliferation in otherwise healthy cells.

In mammalian cells, AMPK is not only activated by various stressors, but the expression of AMPK subunits is also upregulated by such stresses, including hypoxia, osmotic stress and hyperbaric stress (Tian et al., 2001; Fraser et al., 2005; Laderoute et al., 2006). AMPK activity has been shown to cause mitochondrial proliferation both in mammalian cells (Bergeron et al., 2001; Zong et al., 2002) and in Dictyostelium (Bokko et al., 2007). Intriguingly, a recent report has shown that Dictyostelium genes encoding both AMPK and mitochondrial proteins, including components of the respiratory electron transport chain, are upregulated during the first few hours of Legionella infection (Farbrother et al., 2006). Although this increased expression need not be reflected in a proportionate increase in AMPK activity, it nonetheless suggests that there is a homeostatic host response directed at maintaining normal intracellular ATP levels. Our results indicate that proliferation of the pathogen would be enhanced as a consequence.

Legionella proliferation in macrophages and in D. discoideum relies on the pathogen avoiding the endolysosomal pathway, which it does by inhibiting fusion of the phagosome with lysosomes (Roy et al., 1998; Wiater et al., 1994; Solomon et al., 2000). However, an integrated view of the signalling pathways and interactions between Legionella proteins and host proteins during Legionella pathogenesis is yet to be established. Numerous interactions may occur between mitochondria, AMPK and L. pneumophila virulence factors during pathogenesis. For example, it was reported recently that one of the secreted virulence factors produced by L. pneumophila, Lpg1905, is a novel ecto-nucleoside triphosphate diphosphohydrolase (ecto-NTPDase or apyrase) (Sansom et al., 2007). Lpg1905 exhibited ATPase and ADPase activity and contributed to the ability of L. pneumophila to infect and replicate in macrophages, epithelial cells and amoebae. Lpg1905 may act as a virulence factor by regulating extracellular or intracellular levels of ATP, depending on whether secretion occurs before or after Legionella uptake by the host cell (Sansom et al., 2007; Sansom et al., 2008). An intracellular site of action seems probable if apyrase secretion accompanies that of other virulence factors that are known to be secreted within the host cell after pathogen uptake. Since AMPK is activated by AMP and inhibited by ATP, secretion of apyrase into the host cell by invading Legionella could result in AMPK activation. Our results show that this would in turn facilitate Legionella proliferation within the host cell. It is possible that AMPK also facilitates intracellular replication of other microbial pathogens, such as Mycobacterium spp., which also secrete ecto-ATPases (Zaborina et al., 1999).

Although AMPK activation provides a possible mechanism by which the Legionella apyrase Lpg1905 contributes to virulence, this ecto-NTPDase could also work by preventing purinergic P2X receptor activation within the host tissue (Sansom et al., 2007). Extracellular ATP promotes fusion of the phagosome and lysosome, and has been shown to stimulate killing of intracellular Mycobacterium spp. in infected human macrophages (Fairbairn et al., 2001; Kusner and Barton, 2001). This action of ATP requires the purinergic P2X receptor, an ATP-activated Ca2+ channel, but the mechanisms involved are still unclear (Ferrari et al., 2006; Coutinho-Silva et al., 2007). The Dictyostelium genome has recently been reported to encode five different P2X receptors (Fountain et al., 2007), at least one of which could be a plasma membrane Ca2+ channel that is responsible for purinergic Ca2+ responses (Ludlow et al., 2008). Another P2X receptor (P2X4) is localized within the contractile vacuolar membrane in Dictyostelium, but can conduct Ca2+ ions across the plasma membrane of mammalian cells (Fountain et al., 2007). Phagolysosome fusion requires a transient local elevation of cytosolic Ca2+ (Vieira et al., 2002; Worth et al., 2003; Stockinger et al., 2006) and so could be inhibited by intracellular or extracellular ATP depletion, preventing ATP-mediated activation of P2X receptors in the vacuolar, lysosomal or plasma membrane. It will be important in future work to determine whether AMPK activation or P2X receptor inactivity is the means by which Legionella apyrase contributes to virulence.

Whether or not AMPK is the downstream target of Legionella ecto-NTPDase, our results show that mitochondrialy diseased cells are more permissive for replication of the pathogen because of chronic AMPK signalling. Consistent with this, various lines of evidence suggest that mitochondrial disease in humans results in increased susceptibility to infection. Neurodegeneration in mitochondrial disease is correlated clinically with an increased incidence of upper respiratory tract infections and other common infections (Naviaux, 1999). Other reports have indicated that children and adults with mitochondrial disorders have a high risk of recurrent infection (Edmonds et al., 2002; Edmonds, 2004; Wortmann et al., 2006). Furthermore, recurrent infection has been reported in individuals with specific mitochondrial diseases such as Kearns-Sayre syndrome (Katsanos et al., 2002) and Pearson syndrome (Lacbawan et al., 2000).

Genetic defects leading to mitochondrial dysfunction are thus among the many associations that have been reported over the years between human genes and vulnerability/susceptibility to specific infectious diseases (reviewed by Casanova and Abel, 2007). Our results show that host cell susceptibility to Legionella is elevated in an AMPK-dependent manner by energy stress arising from genetic mitochondrial dysfunction. Since AMPK is activated by a variety of other stressors, including oxidative stress, ischemia and osmotic stress (Hardie and Hawley, 2001; Hardie, 2004; Kahn et al., 2005; Hardie and Sakamoto, 2006), it is possible that cellular stress in a range of different circumstances may elevate pathogen replication not only in Legionnaires’ disease, but also in other microbial diseases.

**METHODS**

**Dictyostelium strains and culture conditions**

All experiments were conducted with D. discoideum parental strain AX2 and transformants created from this strain (Wilczynska et al., 1997; Kotsifas et al., 2002; Bokko et al., 2007). The various strains belonged to five categories: (1) mutants in which the mitochondrial rnl gene was disrupted in a subset of the mitochondria: HPF266-270 (Wilczynska et al., 1997); (2) transformants expressing a chaperonin 60 (hspA) antisense RNA: HPF405-416, HPF601-612, or its corresponding sense RNA control: HPF417-418 (Kotsifas et al., 2002; Bokko et al., 2007); (3) transformants expressing an AMPKα (snfA) antisense RNA: HPF455-465, or its corresponding sense RNA control: HPF466-468 (Bokko et al., 2007); (4) transformants hyperexpressing a truncated active form of the catalytic α subunit of AMPK (AMPKαT): HPF432-445 (Bokko et al., 2007); and (5) the hspA and
snfA antisense and sense constructs in each of the four possible combinations: HPF501-512, hspa/snfA double antisense; HPF551-553, hspa/snfA double sense; HPF581-586, hspa antisense/snfA sense; and HPF576-580, hspa sense/snfA antisense (Bokko et al., 2007). The copy numbers of the sense, antisense and overexpression constructs were determined previously by quantitative Southern blotting (Bokko et al., 2007). The presence of the snfA sense construct or the hspa sense construct had no effect on the phenotypes examined in this or in previous work (Bokko et al., 2007). For confocal microscopy, we used an AX2 transformant (HPF614) expressing GFP that was targeted to the mitochondria by N-terminal fusion with the first 150 amino acids of chaperonin 60 (Ahmed et al., 2006).

Cells grown axenically were cultured in HL5 liquid medium supplemented with 100 μg/ml ampicillin, 20 μg/ml streptomycin and 10 μg/ml tetracycline. Strains were also grown on bacterial lawns prepared from Klebsiella aerogenes on standard medium (SM) agar. As a selective marker, 20 μg/ml of G-418 was added to the growth media for all transformants during subculturing. However, for phenotypic studies, antibiotics were excluded from the media to prevent any possible associated effects.

Legionella strains and culture

The L. pneumophila strains used were derivatives of the pathogenic Corby strain (Mampel et al., 2006). Legionella were grown on ACES [N-(2-acetamido)-2-aminoethanesulfonic acid]-buffered charcoal yeast extract agar (BCYE) supplemented with 5 μg/ml chloramphenicol at 37°C with 5% CO₂ for 3 days (Wintermeyer et al., 1995). For storage, the bacteria were frozen in sterile distilled water in 200 μl aliquots and placed at –70°C. For confocal microscopy and Legionella uptake assays, we used the Corby strain transformed with a plasmid expressing a variant of DsRed, DsRed-Express, which lacks the minor green fluorescence of wild-type DsRed (Mampel et al., 2006).

Infection assay

The intracellular growth of the L. pneumophila in the mitochondrially diseased Dictyostelium strains was quantitated using a growth assay modified from Hägele et al. (Hägele et al., 2000) and Otto et al. (Otto et al., 2004). Dictyostelium amoebae were grown to a density of 1-2×10⁶ cells/ml in axenic medium in shaken flasks. Cells were harvested by performing a 3-minute spin at 600 × g and washed twice in Sorensen 1×C buffer (17 mM KH₂PO₄/Na₂PO₄, 50 μM CaCl₂, pH 6.0) before finally being resuspended in modified broth (MB) medium (0.7% yeast extract, 1.4% proteose peptone, 0.062% Na₂HPO₄·2H₂O, 0.049% KH₂PO₄, pH 6.9) at a density of 5×10⁵ cells/ml. For each strain, 10⁵ cells were inoculated into each of five wells of a 96-well tissue culture plate. The cells in these wells were used for assaying Legionella viable counts at five time points: 0 hours, 24 hours, 48 hours, 72 hours and 96 hours. Cells were allowed to adhere for 30 minutes at 21°C before being infected with Legionella.

L. pneumophila were harvested after growth for 72 hours on BCYE plates, resuspended in water and used to infect D. discoideum at a multiplicity of infection (MOI) of approximately 1:1. The required concentration of bacteria was determined by assuming that an OD₆₀₀ of 1 is equivalent to 10⁹ bacteria/ml. To initiate infection at the first time point, adherence between Dictyostelium and Legionella was achieved by centrifuging the bacterial suspension onto the attached amoebae for 10 minutes at 600 × g.

At each assay time point, the cells were resuspended, then transfected into a microcentrifuge tube, pelleted for 8 minutes at 16,000 × g in a microcentrifuge, and vortexed vigorously for 15 seconds. A dilution series of the harvested bacteria was then prepared from 10⁻¹ to 10⁻⁴ to measure the colony-forming units (c.f.u.) on BCYE plates incubated at 37°C with 5% CO₂ for 72-96 hours. For the time points ranging from 24 to 96 hours, the infected amoebae were incubated at 25°C.

Statistical techniques

To compare Legionella proliferation in the wild-type strain with the mitochondrially diseased strain, the two sample t-test assuming unequal variances and the Kruskal-Wallis nonparametric test for differences in location were employed. Standard regression and correlation analyses were carried out for data relating Legionella proliferation to the number of copies of plasmid constructs per cellular genome. The coefficient of variation (R²) was determined for fits to linear, exponential, logarithmic, power or polynomial models, as appropriate. R² is equivalent to the square of the Pearson product-moment r, which was used to determine the significance probability for correlations. The significance of all correlations was also tested using the nonparametric Kendall rank r and, in all cases, gave the same outcome at a significance level of P<0.01.

Confocal microscopy

Dictyostelium cultures were grown in culture flasks to a density of 1-2×10⁶ cells/ml in HL5. Cells were washed twice in Sorensen buffer and resuspended to a final density of 5×10⁵ cells/ml in 12 mM phosphate buffer (pH 6.5) or, for the 24-hour time points, LoFlo medium. 10⁶ cells were added to each sterile coverslip in a Costar well and allowed to attach for 0.5 hours. L. pneumophila was harvested and resuspended, as for the infection assay, and added at an MOI of either 1:1 or 10:1 to the coverslips. Costar plates were centrifuged at 1370 × g for 10 minutes to allow Legionella attachment to cells. Coverslips were then removed at 0-, 0.5-, 1-, 1.5- and 24-hour intervals and washed twice in phosphate buffer (12 mM Na₂HPO₄, 12 mM NaH₂PO₄, pH 6.5). Cells were flattened and fixed under a layer of 1% agarose in phosphate buffer containing 3.7% paraformaldehyde for 30 minutes. After fixation, the coverslips were washed three times using PBS, blotted dry, mounted on glass slides using 10 μl of 90% glycerol in PBS solution, and sealed.

Confocal microscopy was performed with a Leica TCS SP2 confocal scanning laser microscope with a DM IRE2 inverted microscope using a 63× multi-immersion objective lens. Samples were excited with laser settings of 76% of 488 nm and 89% of 543 nm, and emissions were captured at 500-556 nm and 580-680 nm for wtGFP and DsRed-Express detection, respectively. A z-series of up to 30 focal plane images were captured for each cell, with 8 to 16 line averaging. Image stacks were compressed, filtered, spliced and 3D-animated using the Leica confocal software.

Electron microscopy

Infected cells were fixed with 2.5% glutaraldehyde in phosphate buffer; infiltrated with acetone and ethanol; and embedded in Spurr’s epoxy resin. Sections, 80 nm thick, were stained with uranyl
TRANSLATIONAL IMPACT

Clinical issue
Mitochondrial diseases are an eclectic family of genetic disorders that compromise mitochondrial energy production. In many cases, the precise genetic defect causing mitochondrial dysfunction is known, but the downstream pathological outcomes for the mitochondrialy diseased cell remain poorly understood. Patients experience a variety of symptoms affecting the central nervous system, muscles, heart and other tissues, and are also more susceptible to infections, particularly of the respiratory tract. The basis for this susceptibility has not been studied and is not understood.

Results
To understand the influence of mitochondrial disease on susceptibility to microbial pathogens, this study examines Legionella infections in Dictyostelium amoebae. The authors show that, early during infection, Legionella-containing vacuoles associate with host cell mitochondria in Dictyostelium, as in human macrophages. For the first time, they show that this early association is followed by colocalization of proteins from the Legionella and the host cell mitochondria, suggesting that proteins are actively exported from Legionella into the host mitochondria. Cells with affected mitochondria show enhanced intracellular proliferation of Legionella, which is independent of pathogen uptake. This study also shows that the enhanced proliferation of Legionella inside mitochondrialy diseased cells is dependent on chronic AMP-activated protein kinase (AMPK) signalling. AMPK is activated when cellular energy consumption outstrips production. The activated kinase inhibits many cellular energy-consuming activities and stimulates mitochondrial biogenesis and ATP production. The enzyme homeostatically regulates cellular energy status in a healthy cell, but in a mitochondrialy diseased cell, chronic hyperactivity contributes to pathology. These results are consistent with previous data showing that mitochondrial dysfunction in Dictyostelium induces cytopathology by chronically activating AMPK. Importantly, Legionella multiplication is stimulated by hyperexpression of an active form of AMPK, which mimics the effects of mitochondrial dysfunction on this process. Conversely, genetic inhibition of AMPK expression suppresses the Legionella growth characteristic in mitochondrialy diseased host cells.

Implications and future directions
This is the first report that Legionella-containing vacuoles recruit mitochondria in infected Dictyostelium cells. Future work should determine whether this association requires active pathogen functions, as it does in other hosts, and whether pathogen proteins are exported into the mitochondria. It is not known why human mitochondrial disease patients are more susceptible to microbial infections, but this study reveals that mitochondrialy diseased Dictyostelium cells are more supportive of Legionella proliferation than healthy cells. This is at least partially because of chronic AMPK signalling. Future research should determine whether this is true also for human and mouse macrophages and whether, in the mouse, AMPK signalling induces susceptibility to respiratory disease from Legionella infections. Enhanced Legionella proliferation is just one of several diverse cytopathological outcomes of AMPK activity in Dictyostelium mitochondrial disease. Thus, AMPK signalling pathways may provide drug targets for the management of these currently untreatable genetic disorders.

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Legionella uptake assay
Dictyostelium cultures growing in HL5 medium in conical flasks were harvested through centrifugation for 1 minute at 250 × g, washed twice in Sorensen phosphate buffer and resuspended in MB medium at 5 × 10⁶ cells/ml. Aliquots of 200 μl were transferred to wells in a 96-well Costar plate and allowed to settle for 30 minutes.

L. pneumophila DsRed growing on BCYE agar containing chloramphenicol (5 μg/ml) were harvested in distilled water and resuspended in MB medium at 1 × 10⁶ cells/ml (based on OD₅₅⁰). Aliquots of 100 μl were added to each Costar well containing Dictyostelium cells (i.e. at an MOI of 1). Costar plates were then spun at 1370 × g for 10 minutes at 21°C after which Dictyostelium cells were allowed to consume the L. pneumophila. At each time point, gentamicin sulphate (50 μg/ml) was added to the wells to kill extracellular L. pneumophila. The gentamicin killing was shown to be completely effective in separate control experiments without Dictyostelium. Following 30 minutes of gentamicin treatment, cells were resuspended, harvested at 13,600 × g for 7 minutes, washed twice in Sorensen phosphate buffer, and lysed in 0.02% saponin with vigorous vortexing to release the intracelular Legionella. Viable counts of the released Legionella were performed in duplicate using BCYE agar plates incubated at 37°C with 5% CO₂ for 3-4 days.

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COMPETING INTERESTS
The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS
L.F. conducted most of the experiments and drafted the manuscript; P.K.S. and S.L.A. were recipients of Australian Postgraduate Research Awards; P.B.B. isolated the AMPK antisense and overexpression strains; S.B. supervised and provided materials for some of the Legionella infection assays, while hosting L.F. in his laboratory for part of the work. P.R.F. conceived and supervised the project, contributed to the data analysis and preparation of the figures, and drafted parts of the manuscript.

SUPPLEMENTARY MATERIAL
Supplementary material for this article is available at http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.003319/-/DC1

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REFERENCES

Legionella and mitochondrial disease

acetate and Reynold’s lead citrate before viewing with a Philips CM120 BioTWIN TEM. Images were digitally captured and formatted with Photoshop software.

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Legionella and mitochondrial disease


