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Attaching and effacing pathogens modulate host mitochondrial structure and function

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Abstract

Enteropathogenic and enterohemorrhagic *Escherichia coli* (EPEC and EHEC) are human enteric pathogens that contribute significantly to morbidity and mortality worldwide. These extracellular pathogens attach intimately to intestinal epithelial cells and cause signature lesions by effacing the brush border microvilli, a property they share with other "attaching and effacing" (A/E) bacteria, including the murine pathogen *Citrobacter rodentium*. A/E pathogens use a specialized apparatus called a type III secretion system (T3SS) to deliver specific proteins directly into the host cytosol and modify host cell behavior. The T3SS is essential for colonization and pathogenesis, and mutants lacking this apparatus fail to cause disease. Thus, deciphering effector-induced host cell modifications is critical for understanding A/E bacterial pathogenesis. Several of the ~20–45 effector proteins delivered into the host cell modify disparate mitochondrial properties, some via direct interactions with the mitochondria and/or mitochondrial proteins. In vitro studies have uncovered the mechanistic basis for the actions of some of these effectors, including their mitochondrial targeting, interaction partners, and consequent impacts on mitochondrial morphology, oxidative phosphorylation and ROS production, disruption of membrane potential, and intrinsic apoptosis. In vivo studies, mostly relying on the *C. rodentium*/mouse model, have been used to validate a subset of the in vitro observations; additionally, animal studies reveal broad changes to intestinal physiology that are likely accompanied by mitochondrial alterations, but the mechanistic underpinnings remain undefined. This chapter provides an overview of A/E pathogen-induced host alterations and pathogenesis, specifically focusing on mitochondria-targeted effects.

1. Introduction

1.1 Attaching and effacing pathogens

The group of bacteria designated as Attaching and Effacing (A/E) pathogens adhere intimately to the apical side of intestinal epithelial cells and efface the brush-border

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Conflicts of interest

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microvilli (Wales et al., 2005). This group includes the human pathogens enteropathogenic and enterohemorrhagic *Escherichia coli* (EPEC and EHEC), the poorly-studied zoonotic agent, *Escherichia albertii*, the murine pathogen *C. rodentium*, rabbit EPEC (REPEC), rabbit diarrheagenic *E. coli* (RDEC), and the porcine pathogen PEPEC.

EPEC, a major cause of diarrhea in children in developing countries, has also been increasingly identified in stool samples from symptomatic patients in developed countries (Carlino et al., 2020; Pearson et al., 2016). EHEC strains asymptomatically colonize cattle and have been associated with foodborne human infection outbreaks in developed countries (Kolodziejek et al., 2022). EHEC strains express Shiga toxins that can contribute to bloody diarrhea and hemorrhagic colitis which, in some instances, may progress to hemolytic uremic syndrome, acute kidney failure, and thrombocytopenia. The mouse pathogen *C. rodentium* harbors many homologs of EPEC and EHEC effectors and has been used extensively to model EPEC and EHEC human infections (Caballero-Flores et al., 2021; Mullineaux-Sanders et al., 2019). Instead of causing overt diarrhea like EPEC or EHEC, however, *C. rodentium* infection results in inflammation and transmissible colonic crypt hyperplasia, replicating many of the histological and pathological features of human inflammatory bowel diseases (Collins et al., 2014).

A/E bacteria are typically considered to be extracellular pathogens that are restricted to the intestinal niche. All A/E pathogens harbor a pathogenicity island called the locus of enterocyte effacement (LEE) which encodes a type III secretion system (T3SS) (Riebisch and Mühlen, 2020). The T3SS straddles the inner and outer bacterial membranes and extends into a tube-like “translocon” that inserts into host cell membranes. This highly specialized apparatus hierarchically delivers bacterial “effector” proteins directly into the host cytosol. The secreted proteins orchestrate diverse changes of epithelial cell structures and organelles and modify host cell functions to facilitate bacterial colonization and cause disease. Different A/E pathogens harbor unique effector repertoires as well as additional virulence factors and, consequently, may trigger distinct host cell modifications and subsequent pathophysiological changes. Such differences may occur even at the strain level. For instance, while the region corresponding to *espO*, the gene encoding the effector protein EspO, is a pseudogene in the best studied “prototype” strain of EPEC, about 25% of sequenced clinical isolates harbored the homologs *espO1, espO2*, or both (Chattejee et al., 2021).

Mitochondria are prominent targets for bacterial pathogens since these organelles play a central role in cell energetics, reactive oxygen species generation, production of biomolecules, stress responses, cell signaling, innate immune responses, and cell death (Khan et al., 2020; Nandi et al., 2021). This chapter will focus on select A/E effector molecules that interact with mitochondrial components and/or affect mitochondrial physiology. Of the various T3-secreted A/E effectors that are targeted to host mitochondria, Map, EspF and NleB1 have canonical mitochondrial targeting sequences (MTS), while NleF and EspJ have non-canonical MTS; EspZ, EspO, EspT, and Tir lack an obvious MTS, but have been observed in association with the organelle (Chattejee et al., 2021; Malish et al., 2003; Nandi et al., 2021). Map, EspF, EspZ, and NleF have been shown to directly interact with various mitochondrial proteins (Nougayrede et al., 2007; Pallett et al., 2014;

Papatheodorou et al., 2006; Roxas et al., 2022; Shames et al., 2011). It is not known if EspH localizes to the mitochondria, but we recently demonstrated its ability to inhibit mitochondrial fusion in epithelial cells (Roxas et al., 2022).

2. Role of mitochondria in the intestinal epithelium

The primary targets for A/E bacteria are the intestinal epithelial cells (IEC) that form a highly absorptive, single-layered, polarized, simple epithelium that is resistant to mechanical, chemical, and biological insults (Gehart and Clevers, 2019). The intestinal epithelium is organized into crypts and villi and undergoes complete renewal every 3–5 days. Crypt-localized pluripotent Lgr5+ stem cells respond to R-spondins, which are agonists of the canonical Wnt/β-catenin pathway and generate transit amplifying cells that further proliferate and subsequently differentiate into six different IEC subtypes. The differentiated cells move upwards, and constantly replace the older apoptotic cells that are shed near the villus tip. During these transitions, cells exhibit distinct metabolic profiles mirrored in mitochondrial activity changes (Rath et al., 2018). Crypt cells depend primarily on glycolysis for ATP production, while cells maturing and differentiating into enterocytes rely on oxidative phosphorylation (OXPHOS) (Rath et al., 2018). During OXPHOS, reducing equivalents in NADH and FADH are sequentially transferred along the electron transport chain, culminating in the reduction of molecular oxygen by cytochrome *c* oxidase on the inner mitochondrial membrane. The resulting pH gradient across the mitochondrial inner membrane drives cellular ATP generation by ATP synthase (Wilson, 2017).

Junctional complexes between gut epithelial cells perform two critical functions—they maintain the apical-basal polarity of the monolayer (fence function) and regulate the passive flow of water and molecules along the paracellular pathway (barrier function) (Roxas and Viswanathan, 2018). The fence function is critical for directional transport of nutrients and water, and the secretion of antimicrobial peptides and enzymes into the lumen, while epithelial barrier function regulates paracellular transport and curtails infiltration of microbes and microbial products from the lumen into the serosal side (Roxas and Viswanathan, 2018).

Maintenance of the barrier is energy intensive and relies substantially on mitochondrial OXPHOS (Li et al., 2022). OXPHOS inhibition and consequent ATP depletion was shown to disrupt barrier function and increase permeability (Janssen Duijghuijsen et al., 2017), while mouse mitochondrial polymorphisms potentiating OXPHOS and ATP generation protected against experimental colitis (Bär et al., 2013). Additionally, increased mitochondrial reactive oxygen species (mtROS) production has been implicated in barrier dysfunction in the context of both dextran sodium sulfate (DSS)-induced colitis and high fat diet-induced obesity models (Guerbette et al., 2022). DSS-induced OXPHOS inhibition and mtROS increase can result in internalization of the tight junction protein (TJ) occludin and TJ-associated protein ZO-1, and compromise barrier function. It is also known that mtROS-mediated cytoskeletal rearrangements perturb interactions with ZO-1 and Occludin (Gangwar et al., 2017). A/E pathogens disrupt intestinal epithelial barrier function in vitro and in vivo (Roxas and Viswanathan, 2018), but a potential contribution of mitochondrial alterations in this process remains to be established. Beyond energetics, ATP and ROS

generated in the mitochondria can act as signaling molecules that dictate cell fates and coordinate cellular metabolism, stress responses, wound repair, immunity, and apoptosis.

3. A/E pathogens and host cell mitochondria—In vivo studies

In vivo studies of A/E pathogens have primarily relied on the mouse/*C. rodentium* infection model (Collins et al., 2014). *C. rodentium* infection results in inflammation and transmissible colonic crypt hyperplasia and a self-limiting disease in mouse strains like C57BL/6 and Swiss Webster, while susceptible mouse strains like FVB, AKR/J and C3H/HeJ develop severe disease and succumb due to dehydration. This differential susceptibility was linked to a chromosomal locus Cri1, which encodes R-spondin-2 (*rspo2*) (Papapietro et al., 2013). Kang et al. used RNA sequencing technology to assess conserved and dissimilar responses to *C. rodentium* infection in susceptible and resistant congenic mice (Kang et al., 2018). The *rspo2* gene was strongly and continuously induced during infection in susceptible, but not resistant, mice (Papapietro et al., 2013).

Total and activated β-catenin levels were higher, and Wnt target genes were upregulated, in *C. rodentium*-infected susceptible mice, but not in resistant mice (Papapietro et al., 2013). Under physiologic conditions, there is a gradient of Wnt signaling along the crypt-villus axis. Strong Wnt signaling, as in the Lgr5+ stem cells, promotes stemness and prevents differentiation (Flanagan et al., 2018). Consistent with the inhibitory effect of strong Wnt signaling on differentiation, infected mice had substantially fewer goblet cells and decreased markers for terminally differentiated enterocytes, including Slc26a3 and Car4, which are critical for intestinal exchange of chloride and bicarbonate ions, respectively. Slc26a3 mutations in humans have been associated with congenital chloride-losing diarrhea (Papapietro et al., 2013; van de Wetering et al., 2002; van den Brink et al., 2004). Thus, heightened Rspo2 stimulation and Wnt signaling in *C. rodentium*-infected susceptible mice leads to generation of poorly differentiated colonic epithelium and fatality through loss of proper intestinal function.

Wnt signaling and mitochondria are involved in a bi-directional regulatory interplay. Increased Wnt signaling leads to elevated mitochondrial biogenesis and metabolism (Delgado-Deida et al., 2020). In colorectal cancer cells, Wnt signaling is associated with a switch from OXPHOS to aerobic glycolysis (Warburg effect) via upregulation of glycolytic enzymes as well as suppression of cytochrome C subunits. Mitochondria, in turn, stabilize Wnt signaling through ATP-dependent maintenance of endoplasmic reticulum homeostasis. Additionally, in conditions of mitochondrial stress, the mitochondrial phosphatase Pgam5 dephosphorylates and stabilizes β-catenin to replenish the mitochondrial pool via cell-intrinsic Wnt signaling (Bernkopf et al., 2018).

Berger et al. used global proteomics and targeted metabolomics and lipidomics on colonic intestinal epithelial cells isolated from *C. rodentium*-infected C57BL/6 mice (Berger et al., 2017). Their data suggest that *C. rodentium* infection inhibits β-oxidation and mitochondrial ATP biogenesis: relative to control uninfected mice, IECs from *C. rodentium*-infected mice had (a) lower abundance of mitochondrial transporters that supply substrates for the tricarboxylic acid (TCA) cycle, (b) decreased abundance of Tfam, the nuclear-

encoded mitochondrial transcription factor A which regulates expression of mitochondrial β-oxidation genes, (c) reduced amounts of high-molecular weight cardiolipins, the inner membrane lipids that are required for establishing the electrochemical gradient needed for ATP production, consistent with (d) lower abundance of cardiolipin maturation enzymes and increased levels of immature cardiolipins and, likely (e) higher oxygen levels on the apical surface. On the other hand, glycolytic enzymes were either unchanged or increased in abundance following *C. rodentium* infection. Xu et al. also demonstrated increased glycolytic enzymes in the colons of mice infected with wildtype, but not an isogenic NleB-deficient, *C. rodentium* strain (Xu et al., 2018). How alterations in IEC bioenergetics specifically benefit *C. rodentium* remains to be established. One indirect benefit appears to be an alteration of the intestinal niche to favor bacterial colonization. Consistent with elevated luminal oxygen and fecal cholesterol levels in *C. rodentium*-infected mice, colonic microbiota analyses showed increased abundance of bacteria capable of using oxygen and cholesterol relative to control uninfected mice (Berger et al., 2017).

4. A/E pathogens and host cell mitochondria—Mechanistic studies

For in vitro mechanistic studies on A/E pathogen-induced host mitochondria alterations, investigators have mostly relied on EPEC or *C. rodentium* infection of cultured epithelial cells. The effectors EspZ, Map, EspF, EspJ, NleB1, and NleF localize to the mitochondria (Nandi et al., 2021; Pallett et al., 2014; Shames et al., 2011), and, of these, EspZ, Map, and NleF have been shown to interact with mitochondrial proteins (Fig. 1). Additionally, EspH and Tir primarily localize near the plasma membrane and are known to impact mitochondrial biology (Malish et al., 2003; Roxas et al., 2022). The following section discusses the various host mitochondrial phenotypes influenced by A/E pathogen effectors, and this is summarized in Fig. 2.

4.1 A/E pathogen impacts on mitochondrial morphology

Mitochondria morphology and function are inextricably linked, and altered mitochondrial shape can be both the cause and consequence of disease states. Early histological studies on intestinal biopsies from EPEC-infected infants showed mitochondrial enlargement and distortion (Rothbaum et al., 1983). Fused, toroidal mitochondria were seen in EPEC-infected TC7 cells (sub-clone of Caco-2 colonic adenocarcinoma cells), but not in cells infected with a T3SS-deficient strain (Dean et al., 2013). Similar changes were noted in colonic epithelia of *C. rodentium*-infected mice (Ma et al., 2006).

Mitochondrial morphology, function, and positioning within the cell are dynamically regulated (Friedman and Nunnari, 2014). Cellular cues, including stress, regulate mitochondrial shape via the processes of fusion and fission (Hu et al., 2017). Fusion results in the formation of a cell-wide network and is mediated by three large GTPases, the outer membrane Mitofusin 1 and Mitofusin 2 (MFN1, MFN2), and the inner membrane Optic Atrophy 1 (OPA1). Fusion can mitigate the impact of stress by combining the contents of partially damaged mitochondria, and is optimal for ATP synthesis (Pagliuso et al., 2018).

Fission facilitates organelle distribution during mitosis, and also serves as a form of quality control to demarcate and dispose of damaged mitochondria. The defective mitochondria

are enclosed in a double membrane, and eliminated via a lysosome-dependent pathway called mitophagy (Pagliuso et al., 2018). Fission largely relies on the highly-regulated large GTPase, dynamin-related protein 1 (DRP1). During fission, DRP1 is recruited to the mitochondria surface (by outer mitochondrial membrane proteins MFF, FIS1, MID49 or MID51), where it multimerizes along a ring; DRP1 GTPase activity then drives organelle constriction and separation (Ji et al. ,2015). Inhibition of fission was previously shown to result in hyperfused, and in some instances, donut-shaped, mitochondria (Xu et al., 2018).

Balanced and dynamic regulation of mitochondrial morphology in response to cell physiological states is critical for normal function, and excessive shift towards either fission or fusion can be detrimental. Mitochondria with distorted morphology have been seen in tissues from patients with intestinal inflammation including IBD, and in animal models of gastrointestinal diseases (Mancini et al., 2020; Söderholm et al., 2002).

We recently demonstrated upregulation of the mitochondrial protein FIS1 in EPEC-infected Caco-2_{BBe} intestinal epithelial cells and showed that this was dependent on the effector protein EspH (Roxas et al., 2022). FIS1 has been implicated in fission via its ability to recruit cytosolic DRP1 to the mitochondrial surface (James et al., 2003; Roxas et al., 2022), and inhibit fusion by interacting with MFN1 and MFN2 (Yu et al., 2019). Despite increased FIS1 abundance, however, WT EPEC-infected cells displayed enlarged mitochondria in the initial stages of infection (3 h). FIS1 abundance was significantly lower in cells infected with an isogenic strain lacking *espH* (Δ*espH*) relative to WT EPEC-infected cells, and these cells had hyperfused mitochondria.

We also showed that the early-secreted protein EspZ interacts with FIS1, and the two proteins co-localize to the mitochondria. Infection of epithelial cells with an *espZ*-deficient strain (Δ*espZ*) resulted in a marked increase in mitochondrial fragmentation and mitophagy (mitochondria encased within double membranes, and recruitment of the lysosomal marker LC3 to the organelle) (Roxas et al., 2022). Relative to WT-infected cells, there was a robust increase in DRP1 recruitment to the mitochondria in Δ*espZ*-infected cells, consistent with a model whereby EspZ sequesters FIS1 and limits mitochondrial fission during EPEC infection. Transfected cells expressing EspZ were protected against mitophagy induced by the uncoupler carbonyl cyanide m-chlorophenylhydrazine (CCCP), and FIS1-depletion curtailed Δ*espZ*-induced mitochondrial fragmentation and mitophagy (Roxas et al., 2022).

Recently, Li et al. demonstrated a potential role for Map in mitochondrial fragmentation in EPEC-infected MAF-T mammary epithelial cells (Li et al., 2022). Their data suggested that WT EPEC, but not a Map-deficient derivative, could increase DRP1 abundance, decrease DRP1 phosphorylation at serine 637 (which curtails recruitment to the mitochondria), and reduce the levels of fusion proteins (especially MFN1), which can collectively contribute to fragmentation. Interestingly, we observed clustering of the mitochondrial network around the nucleus in EPEC infected cells; Ma et al. made a similar observation with WT *C. rodentium*-infected, but not Δ*map*-infected, epithelial cells (Ma et al., 2006). Perinuclear mitochondrial re-distribution has been associated with oxidative, proteasomal inhibition, and hypoxia stresses, though the physiological significance remains unknown (Al-Mehdi et al., 2012; Bauer and Richter-Landsberg, 2006; Hallmann et al., 2004; Tsushima et al., 2020).

The effector EspH inhibits RhoGTPases by sequestering RhoGEFS and/or via stimulation of RhoGAP (Dong et al., 2010; Ramachandran et al., 2022; Wong et al., 2012), while Map activates the RhoGTPase Cdc42 (Alto et al., 2006; Huang et al., 2009), interacts with the ezrin/radixin/moesin (ERM)-binding phosphoprotein 50 (EBP50)(Simpson et al., 2006), and activates the sheddase activity of metalloproteinase domain-containing protein 10 (ADAM10) (Ramachandran et al., 2020). It remains to be established if any of these activities of EspH and Map contribute to their ability to promote mitochondrial fission. Collectively, these studies suggest that EPEC effectors like EspH and Map contribute to mitochondrial network alterations, fragmentation, and mitophagy in epithelial cells, and these impacts are curtailed/delayed by EspZ directly via sequestration of FIS1.

EspZ has two transmembrane domains and integrates itself into the plasma membrane in a hairpin loop formation. The extracellular loop of EspZ has been implicated in limiting the translocation of other effectors into host cells (“rheostat” function), though the precise mechanisms remain undefined (Berger et al., 2012). Thus, EspZ may also indirectly delay mitochondrial toxicity by curtailing the premature translocation of other effectors via the T3SS (Berger et al., 2012).

4.2 A/E pathogen effectors and ATP production

Relative to control-uninfected cells, WT *C. rodentium* infection decreased the activity of the TCA cycle enzyme succinate dehydrogenase (an indirect measure of ATP producing capacity), while Δ*map*-infected cells showed an intermediate phenotype (Ma et al., 2006). As discussed above, proteomic and metabolomic analyses of the intestines of *C. rodentium*-infected mice also suggested decreased TCA cycle enzymes and β-oxidation (Berger et al., 2017). In parallel, there was marked oxygen depletion (presumably via β-oxidation in host cells) close to intestinal epithelial cells of Δ*map*-infected, but not WT *C. rodentium*-infected mice (Berger et al., 2017).

Alongside the inhibition of β-oxidation, colonic cells of *C. rodentium*-infected mice exhibit increased abundance of several glycolytic enzymes and a greater reliance on glycolysis, and the effector protein NleB has been implicated in this shift (Xu et al., 2018). Together with heightened Wnt signaling, this shift in cellular energetics is consistent with impaired differentiation of the colonic epithelium. NleB is a glycosyltransferase that can transfer *N*-acetylglucosamine to conserved arginine residues in target host proteins (Gao et al., 2013; Li et al., 2013; Pearson et al., 2013). Xu et al. demonstrated NleB-dependent GlcNAcylation of a conserved arginine (Arg 18) in HIF-1α, a global regulator of oxygen homeostasis, in EPEC- and *C. rodentium*-infected host cells. This resulted in activation of HIF-1α transcription and consequent upregulation of various genes involved in glucose metabolism. Interestingly, NleB has a canonical MTS, and it has been hypothesized to modify pro-apoptotic mitochondrial proteins, but this remains to be established (Nandi et al., 2021).

ATP may have a role beyond cellular energetics in the context of infections. In a series of studies, Crane et al. demonstrated that EPEC infection stimulated the release of ATP from host cells, which was eventually hydrolyzed to adenosine by the enzyme ecto-5′-nucleotidase (Crane and Shulgina, 2009; Crane et al., 2002, 2005, 2006, 2007).

Adenosine is a secretagogue which, via its interaction with apical A2b receptors on intestinal epithelial cells, can stimulate copious fluid secretion and contribute to diarrhea. It was demonstrated that ecto-5′-nucleotidase inhibitors could inhibit EPEC-induced chloride secretory responses. In addition, adenosine stimulated EPEC growth and altered the pattern of its adherence to host cells (Crane et al., 2007).

4.3 A/E pathogens and ROS production

Premature oxidation of electrons along the electron transport chain can result in the formation of reactive oxygen species (ROS) (Cabiscol et al., 2000; Crowley and Vallance, 2020). Up to 5% of the oxygen consumed by the mitochondria is converted to ROS, making it the primary source of these molecules within the cell (Srinivasan et al., 2017). ROS can promote cellular damage and are therefore rapidly eliminated via antioxidant defenses. However, the anti-microbial and pro-inflammatory properties of ROS can also curtail pathogen proliferation. *C. rodentium* was shown to increase ROS production in infected mice, and in in-vitro studies on mouse macrophages (Ahmed et al., 2022; Bording-Jorgensen et al., 2017). In mouse macrophages, exogenous ATP increased *C. rodentium* clearance by activating the NLRP3 inflammasome pathway at least partially in a ROS-dependent manner (Bording-Jorgensen et al., 2017). As discussed earlier, *C. rodentium* inhibits Krebs' cycle and OXPHOS, and shifts host cells reliance on aerobic glycolysis for ATP generation, and treating mice with a glycolysis inhibitor decreased *C. rodentium*-induced colitis and enhanced survival of the infected animals (Ahmed et al., 2022; Berger et al., 2017; Carson et al., 2020; Lopez et al., 2016). Thus, while increased ROS production may represent a host response to infection, *C. rodentium*-dependent switching ofhost cells to glycolysis may limit antimicrobial ROS production and favor pathogen colonization.

The zoonotic A/E pathogen EHEC uses a type VI secretion system (T6SS) to deliver a Mn-containing catalase KatN into host cells (Wan et al., 2017). By neutralizing intracellular ROS in macrophages, KatN helped EHEC survive within macrophages and evade the immune system. Furthermore, the authors demonstrated that KatN enhanced EHEC survival in BALB/c mice.

4.4 A/E pathogen effectors implicated in membrane potential alteration, cytochrome C release and host cell death

A/E pathogens elaborate proteins that promote (EspF, EspH, Map, EspC, Cif), as well as those that limit (EspZ, NleH1, NleB1, NleF), host cell death (Eng and Pearson, 2021). Host cell death following infection exhibits features of both apoptosis and necrosis. These cells exhibit loss of mitochondrial membrane potential ($\Delta\psi m$), release of cytochrome C from the mitochondria to the cytosol, and activation of caspases (Crane et al., 1999); caspase inhibitors, however, are not effective in inhibiting EPEC-induced host cell death.

Work from multiple groups reveals that the mitochondria-localized protein EspZ preserves $\Delta\psi m$, limits cytochrome C release and caspase activation, and delays the death of EPEC-infected host cells (Roxas et al., 2012, 2022; Shames et al., 2011; Wilbur et al., 2015). Relative to WT EPEC, Δ*espZ* infection resulted in increased death of host epithelial cells, but this was substantially curtailed in FIS1-depleted epithelial cells, implicating a role

for EspZ-dependent fission inhibition in cytoprotection. This is further supported by the observation that EspH, which is primarily responsible for EPEC-induced increase in FIS1 abundance and fission in epithelial cells, also promotes Δψm loss and causes host cell death (Roxas et al., 2022).

Map has a canonical mitochondrial targeting signal and, with the aid of the mitochondrial translocase of outer membrane apparatus proteins TOM40 and TOM22, an intact membrane potential (Δψm), and the mitochondrial heat-shock protein 70 (mtHSP70), enters the mitochondrial matrix (Papatheodorou et al., 2006). Consistent with its ability to promote mitochondrial fragmentation, Ramachandran et al. showed Map-dependent loss of Δψm, loss of calcium homeostasis, and apoptosis in HeLa cells (Ramachandran et al., 2020). While Ma et al. demonstrated altered mitochondrial morphology and loss of respiratory function, they did not observe apoptosis (via TUNEL staining) in *C. rodentium*-infected mice.

The effector EspF has an N-terminal MLS and enters mitochondria through the translocase of outer membrane (TOM) import apparatus (Nougayrède and Donnenberg, 2004). EspF, but not a mitochondrial entry-deficient mutant (EspF-L16E) (Nagai et al., 2005), caused Δψm loss in EPEC-infected epithelial cells (Nagai et al., 2005; Nougayrède and Donnenberg, 2004; Ramachandran et al., 2020). EspF-induced Δψm loss was accompanied by increased cytochrome *c* release from the mitochondria, caspase activation, and intrinsic apoptosis. EspF was shown to interact with ATP-binding cassette transporter, ABCF2, a caspase inhibitor that blocks intrinsic apoptosis (Nougayrède and Donnenberg, 2004).

A small fraction (~11%) of the effector EspO localizes to the mitochondria, though it primarily localizes to focal adhesins (Chatterjee et al., 2021). EspO interacts with HAX-1, an anti-apoptotic protein that is generally bound to the cytosolic face of the mitochondria and the endoplasmic reticulum. Transfected HeLa cells expressing EspO were protected against staurosporine-induced caspase-3 cleavage, while this was not observed in HAX-1-depleted cells. HAX-1 interaction and apoptosis inhibition were independent of EspO's ability to bind to integrin-linked kinase (Morita-Ishihara et al., 2013). The prototype *C. rodentium* andEHEC strains encode 1 and 2 copies of *espO*, respectively; while the gene is absent in the prototype EPEC strain, it is present in some sequenced clinical EPEC strains.

Extrinsic apoptosis inhibition may also be mediated via NleB-dependent glycosylation of death-domain-containing host proteins including TRADD, FADD, TNFR1, and RIPK1 (Li et al., 2013; Pearson et al., 2013; Xue et al., 2020).

Finally, EspT, EspJ and Tir may have functions that influence the mitochondria. Ectopically expressed EspT localized to the mitochondria, but no mitochondria-associated phenotypes have been attributed to this effector (Bulgin et al., 2009). EspJ was shown to localize to the mitochondria via an N-terminal non-canonical targeting sequence. Its function remains to be established, but it appears not to be involved in regulation of host cell death (Kurushima et al., 2010). One early study demonstrated localization of the effector Tir to the mitochondria, and a pro-apoptotic phenotype was observed in transfected cells. There is currently no evidence to suggest such a role in the context of an infection (Malish et al., 2003).

Apart from the above Type III effectors, the Type V-secreted EPEC protein EspC, also contributes to Δψm disruption, cytochrome C release, and caspases (9, 3, and 7) activation (Serapio-Palacios and Navarro-Garcia, 2016). These effects were partly mediated via EspC-dependent reduction in expression of the antiapoptotic protein Bcl-2, and the recruitment of pro-apoptotic protein Bax to the mitochondria.

5. Relevance of A/E pathogen mitochondrial impacts to virulence and pathogenesis

While many effector proteins mediate marked effects on various epithelial cell pathways and alter host cell physiology in vitro, most *C. rodentium* single effector deletions do not result in obvious in vivo colonization defects (Sanchez-Garrido et al., 2022). Thus, of the 31 *C. rodentium* T3SS effectors, only deletions in Tir, EspZ, NleA and NleB, respectively, resulted in a colonization defect (Ruano-Gallego et al., 2021). Based on their extensive analysis of isogenic *C. rodentium* strains engineered to express different combinations of effectors, Ruano-Gallego et al. proposed a new paradigm whereby effectors, rather than acting individually, form flexible “intracellular networks” that coordinate to alter host cell properties. In such a network, deletion of effectors that occupy essential “nodes” would result in a colonization defect, while it may require combinatorial loss of more than one “non-nodal” effector to result in a similar defect. The investigators employed the term “context-dependent essentiality” for such effectors.

Although some of the effectors that are targeted to, or that impact, mitochondria have been implicated in colonization and/or virulence in various animal models, there is very little data to specifically implicate a role for mitochondria in pathogenesis (especially because many effectors also target other host cell pathways). EspZ was shown to be critical for colonization and virulence of both *C. rodentium* and REPEC (Deng et al., 2005; Ruano-Gallego et al., 2021; Wilbur et al., 2015). In an infant rabbit model of EHEC infection, it was demonstrated that mutants lacking EspH, despite colonizing at levels comparable to the parent strain, were impaired for bacterial persistence and for inducing robust disease symptoms (Ritchie and Waldor, 2005). A similar persistence defect of an EspH-deficient EPEC strain was observed in a mouse colonization model (Roxas et al., 2018).

There is some variability in the literature regarding the impact of EspF on *C. rodentium* colonization and pathogenesis, with some studies showing marginal or no colonization defect of a Δ*espF* mutant relative to the parent strain (Deng et al., 2005; Mundy et al., 2004; Ruano-Gallego et al., 2021), and others implicating a key role for this protein (Nagai et al., 2005). Interestingly, one study in susceptible C3H/HeJ mice showed that *C. rodentium* strains lacking EspF, as well a strain expressing a targeted EspF mutant (L16E) that is impaired for mitochondrial localization, caused less severe pathology and were less lethal (0% and 20% mortality, respectively) compared to the parent WT strain (100% mortality) (Nagai et al., 2005). The effector Map was shown to localize to mitochondria in *C. rodentium* infected mice (Ma et al., 2006), and Map-deficient strains exhibited a colonization defect, and caused less severe symptoms compared to the parent WT strain (Ma et al., 2006; Mundy et al., 2004; Nguyen et al., 2015; Simpson et al., 2006). Finally,

mice infected with a *C. rodentium* mutant lacking EspO displayed a decrease in intestinal epithelial cell proliferation and altered IEC signaling, and reduced neutrophil recruitment (Berger et al., 2018).

6. Caveats and considerations

The studies discussed above unequivocally demonstrate that A/E pathogens trigger mitochondrial alterations, and also highlight numerous gaps in our understanding of the underlying processes or the relevance of these changes to pathogenesis. Some caveats and considerations are worth noting regarding the current state of the field. Although A/E bacteria have been observed within host cells following infection, they are usually considered to be extracellular pathogens, and it is unclear how they benefit from altering host mitochondrial biology. In comparison, the energetics and survival imperatives of intracellular pathogens like *Salmonella*, and consequently their impacts on the mitochondria, are more readily appreciated. Second, EPEC and EHEC are primarily human pathogens, and do not induce overt disease symptoms in mice. The murine pathogen *C. rodentium* shares many of the virulence-associated effector molecules of EPEC and EHEC, and is used to model human infections. These organisms, however, also harbor unique effector proteins, and their cellular and pathogenic impacts differ substantially. Use of additional model systems, such as rabbit infections with REPEC, may provide more specific insight into EPEC/EHEC-dependent mitochondrial changes. Third, many studies provide only a “snapshot” of events occurring at specific stages of infection. It is, however, well recognized that there is temporal regulation of effector expression, and there is a hierarchy of effector secretion. This may be a partial explanation for the pathogens harboring effectors with apparently opposing actions. Thus, EspZ may limit fission early in the infection process, while EspH- and Map-induced fission may dominate later in infection. Apart from expression and secretion hierarchy, other parameters such as effector half-life within host cells likely influence the kinetics of host cell changes. Some of these questions are technically challenging to address since effector proteins are delivered in relatively small amounts into host cells. Indeed, one caveat to many effector localization studies is that they have typically relied on transfection and ectopic expression in epithelial cells. Finally, studies on the cellular impacts of these pathogens, including mitochondrial alterations, have primarily focused on individual secreted proteins, their host cell interacting partners, and the consequences ofthose interactions. The major outstanding questions in the field relate to how the interactions and impacts ofdifferent effector proteins integrate to modify host mitochondria (and cell physiology) in a temporal fashion, and how such changes contribute to A/E bacterial virulence and pathogenesis.

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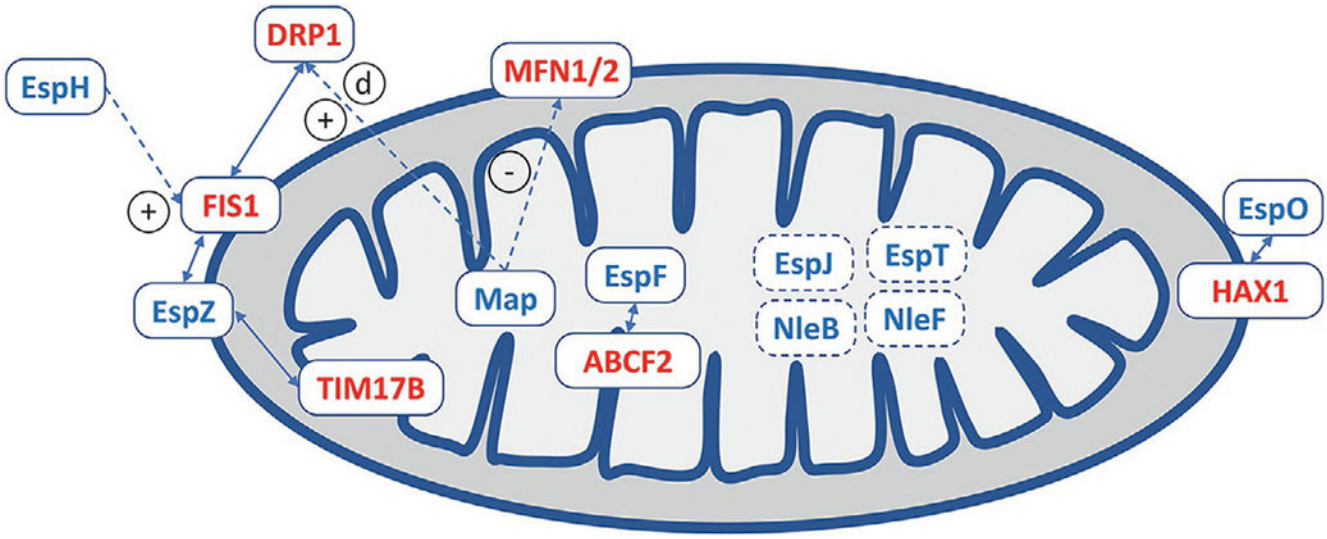

Fig. 1.

A/E pathogen effectors (blue font) that have defined, or predicted (dashed border), mitochondrial association or impacts. Host cell proteins are indicated in red font. Interactions are shown by double-ended arrows. Effector-dependent increase (+), decrease (+), and dephosphorylation (d) are indicated. For the effectors, only mitochondrial interaction partners are shown; several of these effectors also localize to other parts of the cell or interact with non-mitochondrial proteins—these aspects are not shown. EspO is absent in the prototype EPEC strain (E2348/69).

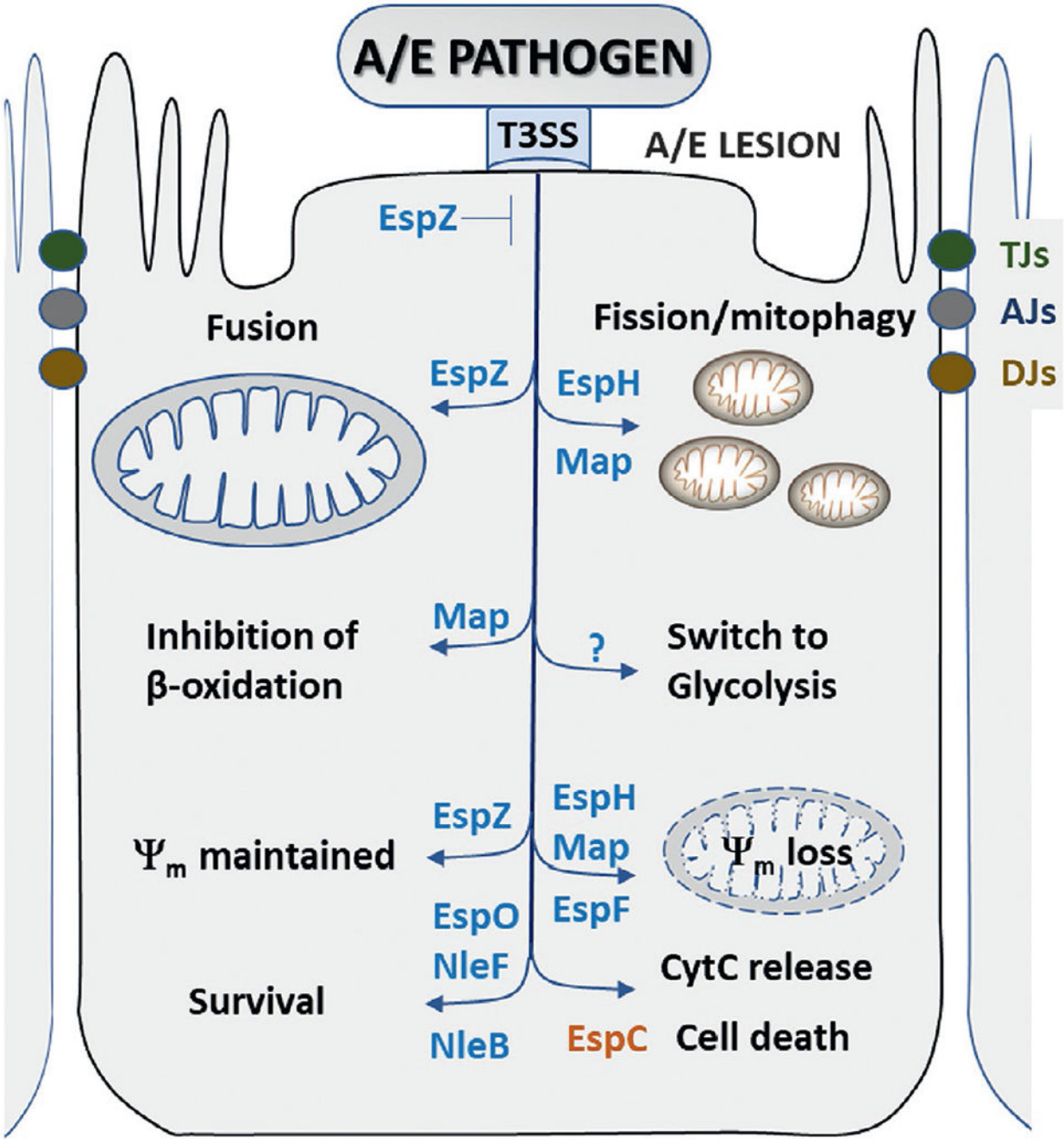

Fig. 2.

A/E pathogen-induced host cell alterations, with a focus on mitochondrial changes. Effectors (blue) delivered via the T3SS dynamically alter mitochondrial physiology, cellular energetics, and cell survival. EspC is delivered via a type 5 secretion system. Mitochondrial dynamics can influence junctional complexes, depicted as TJs (tight junctions), AJs (adherens junctions) and DJs (desmosomal junctions). EspO is absent in the prototype EPEC strain (E2348/69).