Elongation factor P mediates a novel post-transcriptional regulatory pathway critical for bacterial virulence

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Bacterial pathogens detect and integrate multiple environmental signals to coordinate appropriate changes in gene expression including the selective expression of virulence factors, changes to metabolism and the activation of stress response systems. Mutations that abolish the ability of the pathogen to respond to external cues are typically attenuating. Here we discuss our recent discovery of a novel post-transcriptional regulatory pathway critical for Salmonella virulence and stress resistance. The enzymes PoxA and YjeK coordinately attach a unique \( \beta \)-amino acid onto a highly conserved lysine residue in the translation factor elongation factor P (EF-P). Strains in which EF-P is unmodified due to the absence of PoxA or YjeK are attenuated for virulence and display highly pleiotropic phenotypes, including hypersusceptibility to a wide range of unrelated antimicrobial compounds. Work from our laboratory and others now suggests that EF-P, previously thought to be essential, instead plays an ancillary role in translation by regulating the synthesis of a relatively limited subset of proteins. Other observations suggest that the eukaryotic homolog of EF-P, eIF5A, may illicit similar changes in the translation machinery during stress adaptation, indicating that the role of these factors in physiology may be broadly conserved.

Introduction

Stress adaptation pathways are necessarily multi-layered and complex in order to accurately integrate information from multiple environmental inputs into appropriate responses. Studies of virulence regulation have largely focused on transcriptional regulation including the roles of alternative sigma factors and two-component regulatory systems. Post-transcriptional regulation of stress adaptation and virulence pathways can be mediated by small RNAs, riboswitches, and nucleotide-derived second messengers like (p)pGpp, c-diGMP and cAMP. Additional levels of post-transcriptional regulation are provided by toxin-antitoxin systems that can cleave mRNA under conditions of stress and nutrient deprivation. A common assumption is that, aside from the stringent response, the prokaryotic ribosome is a relatively passive player in stress adaptation and that most post-transcriptional regulation occurs at the level of mRNA structure rather than through alterations of the ribosome itself. Only limited reports exist where the prokaryotic translational apparatus appears to play a direct role in selectively directing the expression of specific transcripts. This is despite an extensive body of work indicating that components of the eukaryotic translation apparatus, like eIF2 and eIF4, are modified under certain conditions to block global protein synthesis and promote translation of stress-response genes.

Our laboratories, in collaboration Dr. Ferric Fang at the University of Washington, recently identified a unique post-translational modification of a poorly understood bacterial elongation factor, EF-P, that is essential for Salmonella virulence and resistance to a variety of unrelated stressors including acid, detergents and antibiotics. As shown in Figure 1, the modification of EF-P is carried out by...
PoxA and YjeK are Critical for Salmonella Virulence and Stress Resistance

We first identified PoxA and YjeK during a screen for Salmonella mutants that were able to resist the cytostatic effects of S-nitroso-glutathione (GSNO), a compound that, in some aspects, mimics the nitrosative stress encountered by the bacteria within an activated macrophage. However, Salmonella \textit{poxA} and \textit{yjeK} mutants did not display enhanced resistance to other nitric oxide-generating compounds, indicating that they are not resistant to nitric oxide per se. Furthermore, rather than being hypervirulent as their GSNO-resistance might predict, the Salmonella \textit{poxA} and \textit{yjeK} mutants displayed profound virulence defects in a mouse infection model.\textsuperscript{5,13} PoxA was initially identified in 1982 in a screen for \textit{E. coli} mutants that displayed reduced pyruvate oxidase activity.\textsuperscript{16} The pyruvate oxidase enzyme was later found to be encoded by an unlinked gene, \textit{poxB}.\textsuperscript{17} PoxA appears to be critical for optimal expression of \textit{poxB} but its role appears to be post-translational given that mRNA levels in \textit{poxA} mutants are similar to wild-type. PoxA is an “orphan” member of the lysyl-tRNA synthetases (LysRS) family that shares a high degree of sequence and structural similarity with the catalytic domain of LysRS but lacks the canonical anticodon recognition domain.\textsuperscript{13} A number of residues critical for PoxA function in vivo map to residues that are known to be critical for aminocyl-tRNA synthetase (aaRS) activity.\textsuperscript{13} YjeK is a lysyl-2-3-aminomutase enzyme that catalyzes the conversion of L-lysine to R-β-lysine.\textsuperscript{18}

The enzymatic properties of the PoxA and YjeK proteins gave little clue as to their biological function. To address the role of these proteins in bacterial physiology, we performed a large-scale phenotypic analysis of \textit{poxA} and \textit{yjeK} mutants using a Biolog phenotype microarray,\textsuperscript{19} which measures bacterial viability under several different growth media and against several antimicrobial compounds by quantitating the turnover of a colorimetric tetrazolium redox dye. This assay revealed that \textit{poxA} and \textit{yjeK} mutant phenotypes are highly pleiotropic and almost identical for each one of the nearly 2000 conditions tested. Under nutrient-limiting conditions, the mutant strains exhibited enhanced respiration compared to wild-type Salmonella. This enhanced respiratory activity was particularly evident for the utilization of amino acids and dipeptides as nitrogen sources (in particular, those containing methionine or branched-chain amino acids) as well as for a number of inorganic and organic sources of phosphate or sulfur. In striking contrast,
the poxA and yjeK mutant strains are hypersusceptible to a broad range of external stressors, which is consistent with a more limited study on poxA mutants by Van Dyk and colleagues.\textsuperscript{20} Both mutants displayed enhanced susceptibility to antibiotics that fall into several distinct pharmacological and structural classes including antimicrobial peptides, detergents, lipophilic chelators, heavy metals and various inhibitors of cell wall synthesis, protein synthesis, RNA synthesis and DNA gyrase. Importantly, the large number of phenotypes shared by the yjeK and poxA mutants provided strong support that they function in a common pathway. This was further supported by our epistasis analysis that demonstrated that the phenotypes of the Salmonella poxA yjeK double mutant were no more or less severe than the poxA single mutant.

**PoxA and YjeK Modify EF-P**

The underlying cause of the pleiotropic phenotypes displayed by our mutants came to light through bioinformatic analysis indicating that both genes were closely linked with the efp gene, encoding EF-P.\textsuperscript{13,21} The poxA, yjeK and efp genes are linked, sometimes in the same operon, in a variety of Gram-negative bacteria. Genes that remain closely associated with one another despite large evolutionary distances often participate in a common pathway. EF-P was first identified in 1975 but neither its role in protein synthesis nor its function in cellular physiology are well understood.\textsuperscript{22} In an in vitro model of peptide bond synthesis, EF-P was found to stimulate the formation of a peptide bond between N-formyl-Met-tRNA and puromycin. Furthermore, the degree of stimulation was dependent on the amino acid acceptor, such that the stimulatory effect was the greatest for small, nonpolar amino acid acceptors such as glycine and leucine.\textsuperscript{23} The EF-P protein mimics a tRNA in both shape and size\textsuperscript{24} and a recent structure of EF-P bound to the 70S ribosome demonstrates that EF-P binds at a unique site between the peptidyl- and exit-tRNA sites where it interacts with ribosomal protein L1.\textsuperscript{25} This supported the general conclusion that EF-P plays a central role in protein translation, perhaps in the formation of the first peptide bond, despite the fact that the basic reactions of protein synthesis could be reconstituted in vitro in the absence of EF-P.

Earlier studies on EF-P purified from *E. coli* revealed that it carried a post-translational modification that had a mass consistent with that of lysine.\textsuperscript{26} We pursued the hypothesis that PoxA and YjeK may be responsible for modification of EF-P, perhaps with YjeK generating β-lysine that is subsequently ligated to EF-P by PoxA. In a reconstituted in vitro modification assay, we showed that EF-P becomes labeled with radioactive lysine in a PoxA-dependent manner.\textsuperscript{13} We also showed that this modification occurs at a specific lysine residue (Lys34) such that mutation of Lys34 to an alanine inhibits PoxA-mediated modification of EF-P and abolishes EF-P function in vivo, a finding corroborated by Yanagisawa et al.\textsuperscript{14} The recent crystal structure of EF-P in complex with PoxA showed that their interaction mimics that of an aaRS and its cognate tRNA, lending further support for the idea that the modification of EF-P by PoxA is analogous to the aminoacylation of tRNA by its cognate aaRS.\textsuperscript{14} YjeK enhanced the efficiency of the EF-P modification reaction, likely by generating in situ the preferred substrate for PoxA, β-lysine, from lysine.

**On the Role of EF-P in Bacterial Physiology**

The efp gene was previously reported to be essential for bacterial viability, consistent with the hypothesis that EF-P is critical for global protein synthesis.\textsuperscript{27,28} Systematic high-throughput deletion studies, however, have established that efp is not essential in either *E. coli* or *Salmonella typhi*.\textsuperscript{29-31} An earlier paper also demonstrated that EF-P is not essential in *Agrobacterium tumefaciens*.\textsuperscript{32} The Agrobacterium homolog of efp, chvH, was identified in a screen for mutants defective in plant virulence.\textsuperscript{33} *chvH* mutants exhibited reduced virulence gene expression and hypersusceptibility to detergents such as SDS and sodium deoxycholate, similar to what we observed for Salmonella poxA and yjeK mutants. Furthermore, the chvH mutant phenotypes could be rescued by complementation with the *E. coli* efp gene, supporting the hypothesis that chvH and efp are functional homologs. Together these findings support the hypothesis that EF-P does not play a role in global protein synthesis and, further, that its role in stress resistance and virulence is not limited to *E. coli* and *Salmonella* but may be universal in all bacterial species.

To assess the changes that might occur in response to EF-P modification, we assessed the protein expression patterns of wild-type, poxA and yjeK mutant strains. Our 2D-gel analysis of the mutant strains revealed that the changes in their soluble proteomes were relatively modest. Fewer than 90 spots differed in intensity, more than half of which appeared to be more abundant in the mutant strains. The majority of the downregulated proteins appear to have roles related to metabolism, such as nutrient acquisition and ATP generation. Interestingly a number of proteins belonging to the *Salmonella* pathogenicity island 1 (SPI-1) were upregulated in the poxA mutant strain. The upregulation of these proteins may be due to indirect effects in the poxA mutant strain. Given that the expression of relatively few proteins is affected by loss of PoxA or YjeK, it is highly plausible that modified EF-P does not act as a global translation factor, but rather as a posttranscriptional regulator of a specific subset of mRNAs. Our finding that most of the differentially expressed proteins are upregulated in the mutant strains provides important insight into the nature of EF-P regulation.

Given the pleiotropic nature of the *Salmonella* poxA and yjeK mutants, it is hard to tell at this point if there is a specific defect that directly attenuates virulence or if the virulence defect is caused by a combination of several factors. The detergent sensitivity observed in our *Salmonella* mutants as well as in the *Agrobacterium chvH* (efp) mutant, together with the hypersusceptibility to unrelated antimicrobial compounds, suggests that the underlying cause may involve changes in membrane biogenesis or permeability.

**Is There a Common Physiological Link with eIF5A?**

The functions of the eukaryotic homolog of EF-P, eIF5A, and its archaeal counterpart, aIF5A, are equally enigmatic. Interestingly, both eIF5A and aIF5A are modified at a highly conserved lysine residue to generate
the unique amino acid hypusine (Fig. 2). This residue maps to the same position that is modified in EF-P. However, despite the similarities between the eIF5A and EF-P modifications, the enzymes involved in the generation of hypusine bear no similarity to PoxA or YjeK.

Like EF-P, the cellular function of eIF5A remains poorly understood but the factor is likely to have an ancillary role in protein synthesis. Many eukaryotes encode multiple isoforms of eIF5A. For example, human and yeast have two isoforms whereas Arabidopsis has three. Intriguingly many of the phenotypes associated with eIF5A are involved with stress adaptation, cell cycle control and apoptosis. Following the induction of oxidative stress, eIF5A has also been shown to be essential for polysome disassembly and stress granule formation, most likely through its role in enhancing translation elongation. Recent research has shown that eIF5A may have rather direct effects on translation initiation. The solved structure of EF-P on the ribosome also revealed direct interactions between the modified region of EF-P and the 3′-end of initiator tRNA in the P-site, suggesting that EF-P may have rather direct effects on translation initiation. In addition to possible roles for EF-P in modulating ribosomal mRNA binding and initiation, a further effect to optimize the efficiency of translation elongation is also possible given that such a role has recently been demonstrated for eIF5A. Work is now needed to understand exactly how EF-P functions in translation, and how modification regulates its various possible activities.

The underlying reason for the virulence attenuation observed for both Salmonella and Agrobacterium will also be a central area of future inquiry. The apparent paradox that Salmonella poxA mutants are avirulent despite increased SPI-1 expression is likely explained by the stress resistance displayed by these mutants. Another possibility is that SPI-1 expression, which is rapidly shut down upon entry into macrophages, is not properly controlled in these mutants. We also do not currently know whether the lysyl-β-lysine modification of EF-P occurs constitutively or is only triggered under specific conditions. Given that EF-P appears to play a role in stress tolerance and virulence, it will be important to determine whether the modification system itself is regulated by those conditions. Alternatively, if EF-P is constitutively modified, there must exist additional inputs that determine the substrate specificity of EF-P-mediated regulation. All bacterial genomes sequenced to date encode at least one EF-P homolog, with many genomes containing additional paralogs. While many of the secondary EF-P paralogs lack the conserved lysine residue, there is a strong correlation between the presence of the lysine residue and whether or not the bacterium has PoxA and YjeK homologs. Ninety-seven percent of EF-P homologs from species with a PoxA and YjeK homolog have Lys34 compared to 70% of EF-P homologs from species lacking PoxA and YjeK. This raises the question of whether EF-P is modified in those species without a PoxA or YjeK enzyme, such as Pseudomonas. Since the modified lysine is a conserved feature in both the EF-P and eIF5A proteins, it will be
of interest to determine if and how lysyl modification is carried out in other bacterial species. The fact that many bacterial species have more than one EF-P paralog is reminiscent of the varied distribution of eIF5A among eukaryotes. Indeed, the two human isoforms of eIF5A appear to be differentially expressed and fulfill unique roles within the cell.35,48 Whether this is the case for bacterial EF-P paralogs remains to be determined and will be an important area for future research.

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