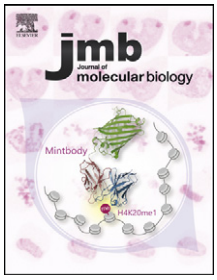

Unprecedented Abundance of Protein Tyrosine Phosphorylation Modulates *Shigella flexneri* Virulence

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Abstract

Evidence is accumulating that protein tyrosine phosphorylation plays a crucial role in the ability of important human bacterial pathogens to cause disease. While most works have concentrated on its role in the regulation of a major bacterial virulence factor, the polysaccharide capsule, recent studies have suggested a much broader role for this post-translational modification. This prompted us to investigate protein tyrosine phosphorylation in the human pathogen *Shigella flexneri*. We first completed a tyrosine phosphoproteome, identifying 905 unique tyrosine phosphorylation sites on at least 573 proteins (approximately 15% of all proteins). This is the most tyrosine-phosphorylated sites and proteins in a single bacterium identified to date, substantially more than the level seen in eukaryotic cells. Most had not previously been identified and included proteins encoded by the virulence plasmid, which is essential for *S. flexneri* to invade cells and cause disease. In order to investigate the function of these phosphorylation sites in important virulence factors, phosphomimetic and ablative mutations were constructed in the type 3 secretion system ATPase Spa47 and the master virulence regulator VirB. This revealed that tyrosine residues phosphorylated in our study are critical for Spa47 and VirB activity, and tyrosine phosphorylation likely regulates their functional activity and subsequently the virulence of this major human pathogen. This study suggests that tyrosine phosphorylation plays a critical role in regulating a wide variety of virulence factors in the human pathogen *S. flexneri* and serves as a base for future studies defining its complete role.

Introduction

Tyrosine phosphorylation has long been considered a critical post-translational regulatory mechanism in eukaryotes. However, only in recent times has it been shown that (i) tyrosine phosphorylation occurs in bacteria and (ii) that it is critical for virulence. Indeed, the protein tyrosine phosphatases and bacterial tyrosine kinases (BY-kinases) responsible are now recognized targets for the development of novel antimicrobials [1,2]. Much of the research done to date has focused on the role tyrosine phosphorylation plays in the regulation of capsular polysaccharide biosynthesis, a major virulence factor for a range of bacterial pathogens [3,4,5,6]. However, we and others have shown that tyrosine phosphorylation also regulates a variety of additional virulence factors in both Gram-positive and Gram-negative bacteria [3,7], suggesting a more significant role for tyrosine phosphorylation in the regulation of bacterial physiology and virulence.

Shigella flexneri is the principal bacterial causative agent of human bacillary dysentery, a bloody mucoid diarrhea that predominantly targets children under the age of five. In Asia, there are an estimated 125 million endemic cases annually resulting in 14,000 deaths [8], while the economic burden of infections in the USA is greater than $US 1 billion per annum [9]. Both management and treatment are complicated by the absence of a vaccine and ever-increasing resistance to all therapeutically useful antibiotics. Thus, a greater understanding of pathogenesis, and particularly the regulatory mechanisms that control it, is essential such that novel antimicrobial strategies can be developed.

The ability of *S. flexneri* to survive in harsh environmental conditions as it progresses down the esophagus, through the stomach and small intestine to the colon, is critical such that it can result in invasive disease. The pathogen possesses a large (≈220 kb) virulence plasmid (VP) that is essential for this invasive ability. Genes encoded on the VP are essential for disease and enable colonization and invasion of the intestinal epithelium, modulation of host responses, and regulation of gene expression. A critically encoded factor is the type 3 secretion system (T3SS), which is a needle-like structure that injects effector proteins directly into the host cell [10]. The major trigger inducing the expression of VP genes is a temperature shift to 37 °C after uptake by the host, although a number of additional factors influence VP gene expression including osmolarity, pH, iron, and anaerobiosis [11].

Hansen *et al.* [18] recently undertook a tyrosine phosphoproteomic study of *Escherichia coli*, identifying a total of 512 unique phosphotyrosine sites on 342 proteins. Until now, no study has investigated the role of tyrosine phosphorylation in *S. flexneri*. Here, we first undertook a similar tyrosine phosphoproteomic analysis in order to identify tyrosine-phosphorylated proteins. This led to the discovery of the most tyrosine-phosphorylated sites in any bacteria to date. Furthermore, we showed the functional effects of two of these residues, one in the master virulence regulator VirB [12] and the other in the T3SS ATPase Spa47 [13]. This is the first study to identify the fundamental role that tyrosine phosphorylation plays in the pathogenesis of *S. flexneri*, further cementing the importance of tyrosine phosphorylation in bacterial virulence in general.

Results

Tyrosine phosphorylation is highly prevalent in *S. flexneri*

To investigate the role of tyrosine phosphorylation in *S. flexneri* pathogenesis, we first identified the sites of protein tyrosine phosphorylation in *S. flexneri* 2457T serotype 2a [14]. Thus, we undertook a tyrosine phopshoproteome analysis using the post-translational modification scan (PTMScan) service from Cell Signaling Technology (S1 Dataset). This proprietary technique uses immunoaffinity enrichment to isolate tyrosine phosphorylation modifications, as described in the Materials and Methods. We have previously shown that expression of an active form of a *Strepcoccus pneumoniae* BY-kinase (CpsCD) in *E. coli* causes hyperphosphorylation [7]. Therefore, to maximize the discovery of tyrosine-phosphorylated proteins, we investigated a CpsCD expressing *S. flexneri* (2457T Hyper) in addition to 2457T.

The levels of phosphorylation were generally much higher in 2457T Hyper, suggesting that the presence of the BY-kinase led to increased specific phosphorylation. There was only a small difference in the number of tyrosine-phosphorylated proteins identified between the two strains; we detected 25 proteins that were solely phosphorylated in 2457T Hyper, while 6 proteins were solely tyrosine phosphorylated in the wild type (S1 Table), indicating that the hyperphosphorylation via CpsCD does not artificially inflate the number of phosphorylation sites.

The results described below involve discussion of the combined data, including all sites of tyrosine phosphorylation found across the two strains.

Our phosphoproteome analysis resulted in the identification of 905 unique tyrosine-phosphorylated sites in at least 573 proteins in *S. flexneri* 2457T [12.1% of all open reading frames (ORFs); S2 Table]. We were unable to apportion a number of peptides to one specific protein as a result of extensive protein homology between some proteins (e.g., insertion sequences). If all possible proteins were phosphorylated, this would lead to 708 proteins or 15% of all ORFs. Regardless, the level of tyrosine phosphorylation significantly exceeds reported tyrosine phosphorylation levels in eukaryotic cells [15].

Comparison with *E. coli* phosphotyrosine proteome

A recent study by Hansen *et al.* [18] identified 512 unique phosphotyrosine sites on 342 proteins in *E. coli* K12 and the human pathogen enterhemorrhagic *E. coli* O157:H7. Analysis of sequenced *Shigella* genomes has revealed that these pathogens are essentially members of the species *E. coli*, although it has lost a significant number of genes, rendering it more niche limited [16]. In addition, *Shigella* also possesses large (≈220 kb) VPs that are essential for virulence. Thus, due to the similarity between *S. flexneri* and *E. coli*, we analyzed the tyrosine phosphorylation sites in the two bacteria to identify conserved and convergent phosphoproteome features (S3 Table). Interestingly, there are significant differences between the two studies. The present study identified phosphorylation on 150 (or 49%) of homologous phosphorylated proteins from Hansen *et al.* [18]. However, in many instances, these phosphorylations were on different tyrosine residues. Indeed, comparison between the two studies has shown that only 109 tyrosine phosphorylation residues are conserved between the two studies. Thus, our study has identified a total of 796 new sites of tyrosine phosphorylation.

Phosphotyrosine motif sites

The large number of phosphorylation sites identified in *S. flexneri* allowed us to reliably predict