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Metabolic Phenotyping of the *Yersinia* High-Pathogenicity Island that Regulates Central Carbon Metabolism

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Abstract

The high-pathogenicity island (HPI) is an important determinant of the pathogenicity of pathogenic *Yersinia* microbes. The HPI carries a cluster of virulence genes that chiefly account for the biosynthesis, transportation and regulation of a virulence-associated siderophore, yersiniabactin. This siderophore is also present in uropathogenic *E. coli* (UTI89) but not in non-uropathogenic *E. coli*. We sought to perform metabolic phenotyping and to understand how the presence of the HPI influences central carbon metabolism, which remains poorly understood, by combining targeted metabolomics with a genetic approach. Unexpectedly, our results revealed that uropathogenic *E. coli* (UPEC) with an HPI had superior metabolic homeostasis to non-UPEC K12 strain without an HPI, thereby allowing UPEC with an HPI to flexibly adapt to a variety of growth environments. In this study, we elucidate the unrecognized regulatory effects of the HPI virulence genes on central carbon metabolism, in addition to their roles in directing yersiniabactin. These regulatory effects may be implicated in differentiating UPEC from non-UPEC.

Keywords: high-pathogenicity island, metabolic phenotyping, central carbon metabolism, metabolomics, uropathogenic *Escherichia coli*

Urinary tract infections (UTIs) are common, uncomfortable and, in some cases, even life threatening. Most UTIs are caused by uropathogenic *Escherichia coli* (UPEC) (1). UPEC are characterized by distinct bacterial properties, products, or structures that are referred to as virulence factors because they help the organism to overcome host defenses and to colonize or invade the urinary tract (2). The high-pathogenicity island (HPI) is a typical virulence factor (**Supplementary Fig. 1a**) that is essential for the high pathogenicity of UPEC. The HPI carries a cluster of genes involved in the synthesis, transport and regulation of the siderophore yersiniabactin, which is important for conferring a highly pathogenic phenotype (3-5). Essentially, the core function of the HPI is to acquire iron; its critical importance for *in vivo* bacterial growth and dissemination (6) supports the idea that the ability to acquire iron is crucial for bacterial growth during an infection. However, it remains unclear whether there are differences in the metabolic phenotypes of UPEC and non-UPEC due to regulatory differences in central carbon metabolism (CCM) that depend on the HPI (**Supplementary Fig. 1b**). CCM is a fundamental biological process that is responsible for the production of accessible energy and for the creation of the primary building blocks of biomolecules; it is directed primarily by glycolysis and the tricarboxylic acid (TCA) cycle (7), which are important for the pathogenesis of a diverse range of bacteria associated with infectious diseases (8-10). CCM uses a complex series of enzymatic steps to convert sugars into metabolic precursors (small-molecule metabolites). These precursors are then used to produce the entire biomass of the cell, the necessary primary metabolites for energy synthesis and storage (11), and the secondary metabolites that often contribute to diverse biological functions in different biochemical environments. Metabolomics is a simple way to monitor and capture the expression profile and activity of CCM and its associated secondary metabolome. It is designed to qualitatively and quantitatively determine all

of the biochemically relevant small metabolites in a diverse biological matrix (12). More importantly, those metabolites are incorporated into CCM, and the whole metabolome represents the end products of gene expression patterns that direct the biochemistry inside a cell, a tissue, or a host system (13, 14). A metabolomic assay determines the molecular link between a genotype and a functional phenotype (15) and permits the simultaneous investigation of the environmental and genetic factors that influence CCM in a bacterial population. In this study, we combined metabolomics with a genetic approach to take a snapshot of the metabolic differences between UPEC and non-UPEC under different culture conditions and in the presence of different genetic mutations. We sought to determine the metabolic phenotype of the HPI with respect to primary metabolism in UPEC.

Establishment of a reliable liquid chromatography/mass spectrometry-based metabolomic profiling method allowed us to precisely detect forty-two hydrophilic CCM metabolites in bacterial cells using the multiple monitoring mode (MRM). We also developed another LC-MS method in MRM mode to detect ferric yersiniabactin and (^{13}C)-ferric yersiniabactin (as an internal standard). (new-3,16, 17) (**Fig. 1a, Supplementary Fig. 1c, Supplementary Table 1 and Supplemental methods**).

First, we determined the expression of yersiniabactin via the established LC-MS method in UPEC (+HPI; UTI89) and non-UPEC (-HPI) strains grown under various culture conditions. The data revealed that the biosynthesis of yersiniabactin in UTI89 varied significantly according to the culture environment and showed preferred growth adaptability (**Supplementary Figs. 2 and 3**). Most of the HPI genes were dependent and associated with yersiniabactin production as

except in the complete starvation environment with M63 minimal medium (without 0.2% glycerol) (**Fig. 1b-f**). There was a complete deficiency of yersiniabactin biosynthesis in the non-UPEC strain MG1655 **(Fig. 1b)**, as the genome of this strain lacks the HPI that carries the cluster of virulence genes required for yersiniabactin synthesis **(Fig. 1c).** To elucidate the metabolic phenotype of the HPI with respect to central carbon metabolism and thereby to mechanistically differentiate UPEC from non-UPEC, we used an established metabolomic profiling method to characterize the CCM differences between UPEC (UTI89) and non-UPEC (MG1655) that correspond to the presence or absence of the HPI. A heatmap illustration revealed distinct metabolic differences between the UPEC and non-UPEC strains when incubated in a rich medium (LB broth)(**Supplementary Fig. 4a**). The differences, which were mainly caused by the differing genome structure due to the presence or absence of the HPI, were further illustrated by an OPLS-DA analysis, which was characterized by a noticeable classification of these two *E. coli* strains into different groups (**Supplementary Fig. 4b**) due to differences in CCM expression. Our results showed a high level of activity for amino acid metabolism and for the TCA cycle in UPEC (UTI89); however, the activity levels of the pentose phosphate pathway and of glycolysis were significantly lower than in non-UPEC (MG1655) (**Supplementary Fig. 4c**). The non-UPEC strain MG1655 was characterized by significantly higher expression of Ribose 5-phosphate (R 5-P), Xylulose 5-phosphate (X 5-P) and Fructose 6-phosphate (F 6-P), and most of the analyzed amino acids were found at a high level in the UPEC strain UTI89 (**Supplementary Fig. 4d**). These results revealed that CCM in the bacteria could include the critical components that are required for conferring virulence on pathogenic microbes. The results also suggest that gluconeogenesis and the TCA cycle are required for optimum *E. coli* fitness during a urinary

tract infection, whereas glycolysis, both the non-oxidative and oxidative branches of the pentose phosphate pathway, and the Entner-Doudoroff pathway are dispensable *in vivo* (18).

In addition, we examined the effect of nutrient levels on CCM in UPEC and non-UPEC strains by growing the strains in M63 minimal medium (0.2% glycerol), M63 minimal medium without glycerol but with 0.2% glucose, or M63 minimal medium without glycerol. Significant metabolic differences between MG1655 and UTI89 were revealed by growth at a variety of nutritional levels in the culture media (**Supplementary Fig. 5-7**). The presence of both carbon sources (0.2% glycerol or 0.2% glucose) induced the production of extracellular yersiniabactin and increased CCM (**Fig. 1b, Supplementary Fig. 5-6**). However, the activities of glycolysis, the pentose phosphate pathway and amino acid metabolism were significantly reduced in UTI89 compared with MG1655 (**Supplementary Fig. 5d-e**, **Supplementary Fig. 6d-e**) (cutoff of VIP value >1, *P* value >0.05); these results are nearly the opposite of the results observed for the strains when grown in LB broth. In UT189, the TCA cycle, pyruvate synthesis, and certain amino acids were significantly lower than in MG1655 when a carbon source (0.2% glycerol) was withheld from the M63 minimal medium (**Supplementary Fig. 7d -e**). In short, the above results suggest that UTI89 displays metabolic homeostasis and the flexibility to respond to a changing culture environment (19) by harmonizing the intake and consumption of nutrient molecules and an energy source. There are two possible reasons for this phenomenon. First, UTI89 might modulate metabolic expression to minimize energy/nutrient utilization in a low-nutrient environment. Second, UT189 may make efficient use of nutrient molecules to metabolize more amino acids, resulting in increased protein synthesis and secretion, thereby supporting the virulence of UPEC. A large number of secreted proteins play important pathogenic roles during infections caused by many Gram-negative bacteria (20). Energy optimization would foster more

energy production in a high-nutrient environment; it is possible that metabolic homeostasis is partially linked to the presence of the HPI in UTI89, given that the HPI encodes a siderophore (yersiniabactin) that is necessary for the high pathogenicity seen in UPEC (3, 4, 5, 6).

Furthermore, to clarify the metabolic phenotype of HPI-mediated CCM regulation, we used metabolomic profiling to investigate the effects of the HPI virulence genes on the CCM of UPEC with respect to the expression of yersiniabactin. As a general strategy, we mutated several HPI genes in the wild-type UT189 strain to elucidate their regulatory effects. The mutated HPI genes included *ybtS, ybtX, ybtQ, ybtP, ybtA, irp1, ybtU, ybtT, ybtE,* and *fyuA* (**Supplementary Fig. 1a**). Our metabolomic analyses revealed the differing effects of each virulence gene on the synthesis, transport and regulation of extracellular yersiniabactin (**Fig. 1d- f**) (4). Different culture conditions, including LB broth, fresh urine and M63 minimal medium, were observed to exert an effect on the expression of yersiniabactin that was directly regulated by the HPI virulence genes. We observed a near-total loss of yersiniabactin production in LB broth except in the presence of membrane receptor mutants of *ybtQ* and *ybtP*, which showed a substantially high level of yersiniabactin production. Additionally, there was a relatively low level of yersiniabactin produced by wild-type UPEC UTI89 bearing an *fyuA* mutation (**Fig. 1g- h**). In contrast, we observed high expression of yersiniabactin when the bacteria were incubated with fresh urine or M63 minimal medium. The absence of the yersiniabactin siderophore reduced the bacterial fitness *in vivo*, and yersiniabactin is required for optimal fitness during urinary tract infections (21). Our results showed that mutations of *ybtS, ybtE,* and *ybtU* resulted in a complete block in yersiniabactin synthesis. A reduced level of yersiniabactin was observed in a strain bearing *ybtT* and *irp1* mutations (22), supporting the idea that the presence of the HPI enhances the fitness and adaptability of UPEC by targeting yersiniabactin and CCM. The notion that the HPI can enhance

fitness is also supported by the superior growth phenotype of wild-type UPEC (UTI89) compared with the growth of the same strain carrying HPI gene mutations (**Supplementary Figs. 8-9**).

Next, we sought to study the effects of the HPI virulence genes on CCM (**Fig. 2a- c**). We showed that substantial modifications in amino acid metabolism, the TCA cycle, glycolysis, the pentose phosphate pathway, and energy charge and redox associated metabolites (**Fig. 2d-e**) were correlated with the presence or absence of each HPI virulence gene. The effects of each gene on CCM are distinguishable, thus permitting the clarification of how each HPI virulence gene regulates CCM by targeting a primary metabolic pathway. The metabolic phenotypes associated with HPI-mediated regulation of CCM were elucidated by mapping the HPI virulence genes to the individual metabolic pathways they target. For example, it was clear that mutations of *ybtS, ybtX*, and *ybtQ* significantly decreased the expression levels of genes involved in redox reactions, energy charge (AMP), the glycolysis pathway and the TCA cycle, while slightly perturbing amino acid metabolism (**Fig. 2d- e**). These perturbations resulted in decreased energy production and an unbalanced growth environment (oxidation rate and pH), which could then decrease the fitness and pathogenicity of UPEC *in vivo*. UPEC pathogenicity and fitness may be correlated with the function of *ybtS*, which governs the biosynthesis of yersiniabactin, which is transported by the protein encoded by *ybtQ* (3, 5) to maintain normal growth. However, the function of *ybtX* is not yet clear. Our results revealed similar regulatory effects of *ybtS and ybtQ* on CCM. We will focus on the functional characterization of *ybtX* in a future study.

The same metabolomic method was used to analyze the effects of the UT189 HPI mutants when grown in M63 medium or fresh urine. The results showed that the regulatory effects of the HPI virulence genes on CCM in M63 culture medium were similar to those in the LB broth

culture environment (**Supplementary Fig. 10**), as revealed by the significant modifications in amino acid metabolism and glycolysis (**Supplementary Fig. 10d-e**). However, comparison of CCM in the UT189 wild-type and HPI mutants grown in fresh urine suggested that the HPI virulence genes had more significant effects on redox and oxidative stress-associated metabolites (**Supplementary Fig. 11**). Such alterations in redox and oxidative stress pathways could lead to higher oxidative stress and a lower-energy state that would impair the fitness of mutant UPEC bacteria.

The same analytical strategy was then used to profile the metabolites in conditioned media derived from the post-culture supernatants of UPEC UTI89 grown in fresh-urine based culture media. This experiment was designed to identify the small molecules in fresh urine that act as the main energy source facilitating the growth of UPEC. Our results showed that the amino acid levels in fresh urine were substantially decreased after UPEC UTI89 culture. The higher levels of certain amino acids after culture with HPI gene mutants, which did not occur after culture with wild-type UPEC UTI89, clearly revealed modulation by HPI virulence genes (**Supplementary Fig. 12**). Taken together, our results suggest that UPEC grown in urine can use amino acids as an energy source (18), and that HPI virulence genes contribute to the differing effects on targeted small-molecule metabolites.

The metabolic phenotyping results obtained while evaluating HPI gene-related differences in CCM revealed a marked flexibility in the homeostatic metabolic response of UPEC, likely increasing its growth and fitness over a range of culture conditions (18). This metabolic homeostasis may be partially attributable to the presence of the HPI. Moreover, the metabolic phenotyping of HPI genes that regulate CCM was systematically mapped by directly assessing the interaction of HPI genes with CCM and by assessing the biosynthesis, transport and uptake

of the siderophore yersiniabactin. Our results demonstrate the recognizable effects of HPI virulence genes on CCM and the energetic advantage gained by amino acid metabolism when UPEC strains are grown in urine. Together, these data suggest that the presence of the HPI is critical for maintaining the fitness and favorable growth of UPEC strain UTI89 by positively regulating CCM.

This study is the first to combine metabolomic analysis with a genetic approach to better understand the biochemical roles of HPI genes on CCM. Metabolic phenotyping and metabolomic assays revealed novel functions and roles for the virulence genes in the HPI of UPEC, thereby providing a basis for the pathogenicity of UPEC that is absent in non-UPEC strains. Our future studies will take a metabolic perspective to elucidate the global functions and roles of HPI genes as they relate to the high pathogenicity of UPEC by tracking metabolic phenotypes in a clinical setting.

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The authors declare no conflicts of interest.

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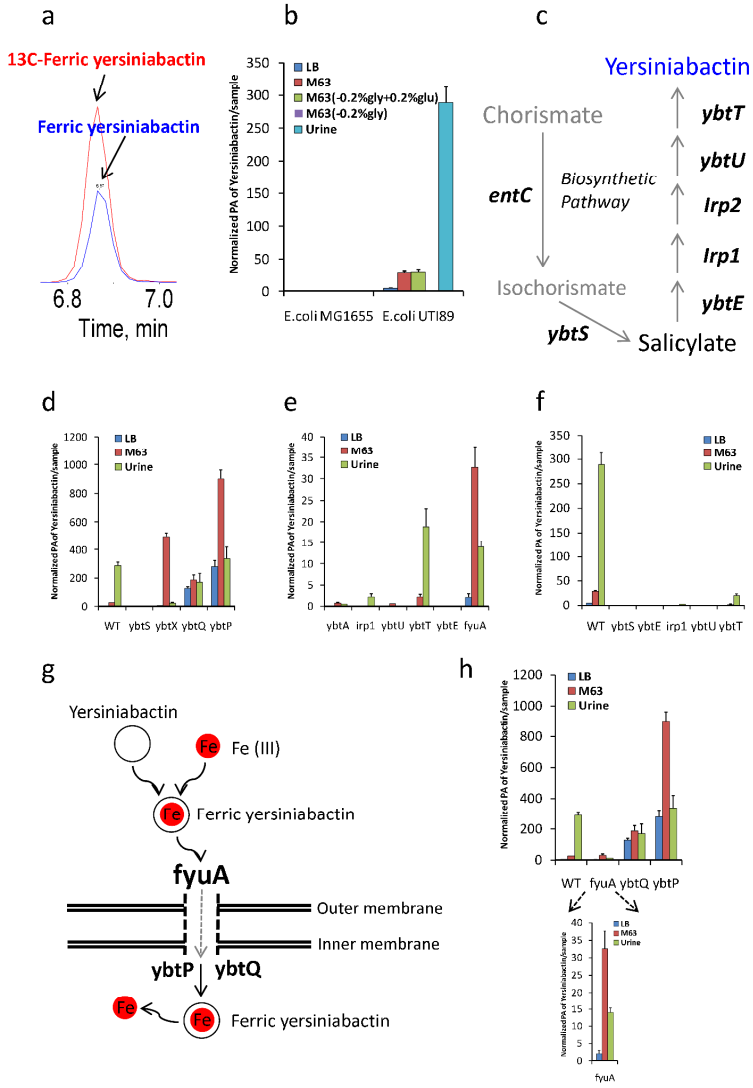

Figure 1 Determination of yersiniabactin levels in different culture environments by LC-MS in multiple reaction-monitoring mode. (a) LC-MS profiles of ferric yersiniabactin and ^{13}C-labeled ferric yersiniabactin (internal standard). (b) Incubation of non-UPEC (MG1655) and UPEC (UTI89) with LB broth, M63 minimal medium, M63 medium without 0.2% glycerol but with 0.2% glucose, M63 minimal medium without 0.2% glycerol, and pooled fresh urine. The results show that yersiniabactin is produced by UTI89 except when incubated with M63 minimal medium without 0.2% glycerol, whereas yersiniabactin is completely absent from MG1655 because of the HPI deficiency in its genome structure. Interestingly, the urine-based culture system contributes to a comparatively high level of yersiniabactin. (c) *In vivo* biosynthetic scheme of yersiniabactin encoded by HPI cluster genes. (d)(e) Incubation of UPEC (UTI89) in LB broth, M63 minimal medium, or fresh pooled urine shows the different effects of HPI virulence genes on the expression of yersiniabactin in different culture environments. (f) Graph highlighting the phenotypic effects of typical yersiniabactin biosynthetic genes on the production of yersiniabactin (g) Scheme showing the transport process of yersiniabactin from the extracellular to the intracellular environment via the outer membrane receptor encoded by *fyuA* and the inner membrane receptors encoded by *ybtP* and *ybtQ*. (h) The effects on the expression of extracellular yersiniabactin of mutations in the genes encoding the outer and inner membrane receptors (*fyuA, ybtP*, and *ybtQ*).

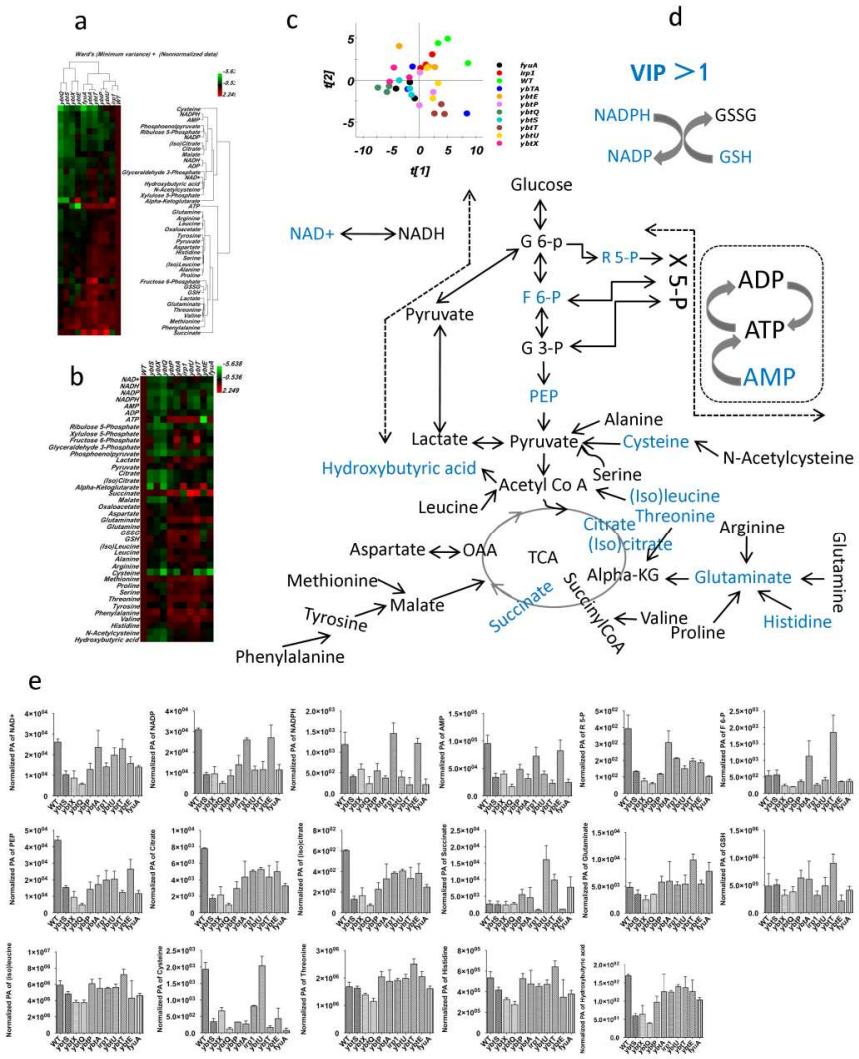

Figure 2 High-pathogenicity island genes influence the CCM of UPEC (UTI89) when incubated in LB broth. (a)(b) Unsupervised heatmap of the relative levels of hydrophilic metabolites in CCM are clustered by hierarchical clustering analysis (HCA) and sequenced by metabolic pathway (the detailed protocol is shown in the online supplementary methods). (c) Supervised OPLS-DA scatter plot of the relative levels of hydrophilic metabolites in CCM; R2Y: 0.86, Q2: 0. 91. (d) The metabolites whose changed levels can distinguish UTI89 strains with genetic mutations from wild-type UTI89 are highlighted in blue for the relevant metabolic pathways, with a cutoff of VIP > 1. (e)Histograms of expression levels of of the metabolites that are differentially expressed among the strains.

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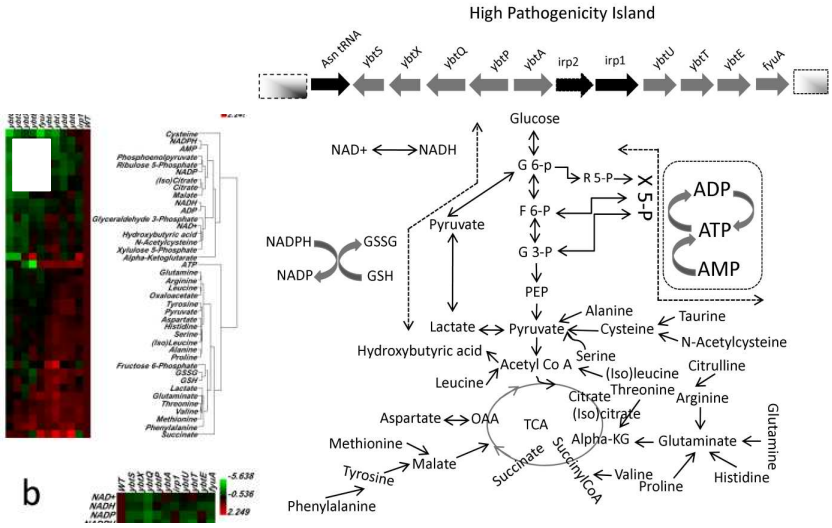

HPI Regulates Central Carbon Metabolism

The regulatory effects of the HPI virulence genes on central carbon metabolism differentiate UPEC from non-UPEC.