Bis-molybdopterin guanine dinucleotide modulates hemolysin expression under anaerobiosis and contributes to fitness in vivo in uropathogenic Escherichia coli

Xinyang Zhang1 | Dongyan Huang2,3 | Zihui Zhao1 | Xuwang Cai3 | Wentong Cai1 | Ganwu Li1,3

Abstract
Uropathogenic Escherichia coli (UPEC) is the primary causative agent of urinary tract infections (UTIs). Successful urinary tract colonization requires appropriate expression of virulence factors in response to host environmental cues, such as limited oxygen and iron availability. Hemolysin is a pore-forming toxin, and its expression correlates with the severity of UPEC infection. Previously, we showed that hemolysin expression is enhanced under anaerobic conditions; however, the genetic basis and regulatory mechanisms involved remain undefined. Here, a transposon-based forward screen identified bis-molybdopterin guanine dinucleotide cofactor (bis-MGD) biosynthesis as an important factor for a full transcription of hemolysin under anaerobiosis but not under aerobiosis. bis-MGD positively influences hemolysin transcription via c3566-c3568, an operon immediately upstream of and cotranscribed with hlyCABD. Furthermore, suppressor mutation analysis identified the nitrogen regulator NtrC as a direct repressor of c3566-c3568-hlyCABD expression, and intact bis-MGD biosynthesis downregulated ntrC expression, thus at least partially explaining the positive role of bis-MGD in modulating hemolysin expression. Finally, bis-MGD is involved in hemolysin-mediated uroepithelial cell death and contributes to the competitive fitness of UPEC in a murine model of UTI. Collectively, our data establish that bis-MGD biosynthesis plays a crucial role in UPEC fitness in vivo, thus providing a potential target for combatting UTIs.

Keywords
colonization, hemolysin, molybdenum cofactor, uropathogenic Escherichia coli, virulence regulation
1 | INTRODUCTION

Urinary tract infections (UTIs), which are among the most common infections in humans, constitute a serious health concern worldwide, potentially affecting millions of people each year (Barber et al., 2013). Uropathogenic *Escherichia coli* (UPEC) is the primary etiological agent of all UTIs (Foxman, 2002). Bacteria can enter the urinary tract when the periurethral area is contaminated by UPEC which may have colonized gastrointestinal tract. UTIs usually begin with cystitis, an infection of the bladder; later on, the bacterium may ascend the ureter to cause pyelonephritis, an infection of the kidney. In some severe cases, UPEC enters the bloodstream, resulting in bacteremia and even deadly urosepsis (Foxman, 2010; Ikaheimo et al., 1994; Kalra & Raizada, 2009).

UPEC employs an arsenal of virulence factors (VFs) to successfully establish infection, but there is no core set of VFs shared by all UPECs, suggesting that there is heterogeneity in this type of pathogen (Klein & Hultgren, 2020). VFs carried by UPEC can be categorized into several classes, namely, adhesins, iron receptors, immune evasion factors, toxins, and metabolic traits (Subashchandrabose & Mobley, 2015). Hemolysin is a prototype pore-forming toxin that is expressed by approximately 50% of UPEC isolates (Cook & Ewins, 1975; Marrs et al., 2002). It is associated with severe cases of UTIs, including pyelonephritis and urosepsis (Bien et al., 2012). The *hlyCABD* operon is responsible for the hemolysis phenotype (Hacker & Hughes, 1985). HlyC encodes an acyltransferase that posttranslationally modifies HlyA by adding acyl groups to Lys564 and Lys690 residues. Modified HlyA, in turn, matures and exhibits cytotoxic and lytic activities (Stanley et al., 1994). HlyB and HlyD, together with a genetically unlinked ToLC, form a type I secretion apparatus that achieves the secretion of HlyA across the inner and outer membranes (Wandersman & Delepelaire, 1990). Hemolysin executes diverse functions at a range of concentrations. At high concentrations, hemolysin can form pores on membranes of various host cell types, including red blood cells, epithelial cells, and leukocytes, leading to cell lysis (MV Murthy et al., 2018; Russo et al., 2005; Wiles & Mulvey, 2013), whereas at low (sublytic) concentrations, it can either alter cell functions or induce cell death pathways. For example, sublytic doses of hemolysin produced by UPEC strain UTI89 can activate host proteases, especially mesotrypsin, which leads to the degradation of paxillin and subsequently damages the host cell membrane (Dhakal & Mulvey, 2012). Hemolysin can also cause cell death via the pyroptosis, apoptosis, and necrosis pathways, depending on the UPEC isolate and host cell type (Nagamatsu et al., 2015; Russo et al., 2005).

To date, it has been revealed that the expression of hemolysin is regulated by multiple regulatory proteins, including the positive regulators OrhK-OrhR (Gu et al., 2021), BarA-UvrY (Tomenius et al., 2006), and FNR (Barbieri et al., 2014) and the negative regulators CpxR (Nagamatsu et al., 2015), H-NS (Nhu et al., 2019), and RfaH (Leeds & Welch, 1996). Notably, the overproduction of HlyA reduces UPEC virulence in a murine model of UTI, and CpxR fine-tunes hlyA expression to optimize UPEC virulence (Nagamatsu et al., 2015).

Additionally, hlyA expression responds to environmental cues such as oxidative stressors and temperature (Mourino et al., 1994). Using the TraDIS method, Nguyen Thi Khanh Nhu et al identified several previously unknown factors impacting hemolysin activities, including LPS core biosynthesis and DnaKJ chaperones (Nhu et al., 2019). Therefore, because of the diverse nature of its cytotoxic effects, hemolysin needs to be properly regulated.

During infection, UPEC unavoidably encounters oxygen-limiting conditions likely because: (i) the oxygen tension in the bladder and renal medulla is low, possibly due to the heavy oxygen consumption by local tissues (Wang et al., 2008); (ii) during infection, oxygen is consumed by cells and cannot be supplied to UPEC in a timely manner (Melican et al., 2008); and (iii) when adopting an intracellular lifestyle, UPEC persists in biofilm-like communities, which are characterized by an oxygen gradient (Beebout et al., 2019). Our lab has shown that hemolysin expression and activities are enhanced under anaerobic conditions (Barbieri et al., 2014 and Figure 1a), but an understanding of the underlying regulatory mechanisms is lacking. Thus, we began this study with a transposon (Tn)-based forward screen to identify genetic factors involved in hemolysin production under anaerobiosis. We then revealed that *bis*-molybdopterin guanine dinucleotide cofactor (bis-MGD) is important for hemolysin expression under anaerobic conditions but not under aerobic conditions. The mechanism used by *bis*-MGD to control hemolysin production was further investigated by comparative transcriptomics and suppressor mutation analysis. Finally, we explored the role of *bis*-MGD biosynthesis in UPEC virulence in cell culture and murine UTI models.

2 | RESULTS

2.1 | Identification of genetic factors involved in hemolysis under anaerobic conditions

Our lab has shown that the transcription of *hlyA* and hemolytic activity is significantly higher under anaerobiosis than under aerobic conditions (Barbieri et al., 2014). In agreement with the liquid suspension assay, in a blood agar assay, we observed that the hemolysis halo is larger in size and more translucent under anaerobiosis than under aerobic; moreover, this hemolytic phenotype was dependent on *hlyA* (Figure 1a). Taking advantage of this remarkable hemolytic phenotype under anaerobiosis, we screened for genetic factors that contribute to UPEC hemolysis under anaerobic conditions. A mini-Tn5 transposon-based random mutagenesis library was prepared and plated onto blood agar plates, and mutants, showing impaired hemolytic halos (smaller in size or less translucent) were selected and subjected to arbitrary PCR for Tn insertion site mapping.

In total, approximately 50,000 mutants were screened, yielding 36 mutants with impaired hemolytic halos and 1 mutant with an enhanced hemolytic halo. Mapping analysis revealed that Tn insertions localized in 16 genes. Table 1 lists all identified genes in which insertions led to a substantial change in hemolysis. Nine
FIGURE 1 Identification of genetic factors involved in hemolysis under anaerobic conditions. (a) Uropathogenic Escherichia coli hemolytic activities are enhanced under anaerobic conditions. Bacteria streaked on blood agar plates were allowed to grow at 37°C for 18–24 hr under aerobic and anaerobic conditions. The ΔhlyA deletion mutant was used as a negative control. A hemolytic halo with a larger size or greater translucence indicates a stronger hemolytic activity. Halos were compared among colonies with similar size. Quantification of hemolysis was performed by densitometry using ImageJ, and hemolysis of the wild type under aerobiosis was set as 1. Means of halos derived from 10 colonies were indicated below each graph. (b) Hemolysis of various deletion mutants of CFT073 under anaerobic conditions. A transposon-based genetic screen was performed to identify genetic factors involved in hemolysis under anaerobic conditions. Single-gene deletion mutants of genes with previously unreported roles in hemolysis were constructed. Hemolysis of wild-type CFT073 and its various mutants was evaluated as described above. Data are representative of three independent experiments.

| Disrupted gene locus | No. of inserts | Hemolysis score | Gene product |
|----------------------|---------------|----------------|-------------|
| Wild type            | +             | Not applicable |
| hlyC                 | 3             | −              | A cyltransferase HlyC |
| hlyA                 | 8             | −              | Hemolysin A |
| hlyB                 | 1             | −              | ATP-binding protein HlyB |
| hlyD                 | 4             | −              | Membrane fusion protein HlyD |
| orhR                 | 2             | ±              | Response regulator OrhR |
| orhK                 | 1             | ±              | Histidine kinase OrhK |
| Hns                  | 1             | ++             | Histone-like nucleoid structuring protein H-NS |
| Fnr                  | 2             | ±              | Fumarate and Nitrate reductase Regulatory protein FNR |
| c0866                | 2             | ±              | Molybdopterin biosynthesis converting factor subunit 1 MoaD |
| c0911                | 1             | ±              | Biosynthesis of Mo cofactors Moeb |
| c4801                | 3             | ±              | Molybdenum cofactor guanylyltransferase MobA |
| c4895                | 1             | −              | Putative 5′-nucleotidase/2′,3′-cyclic phosphodiesterase or related esterase, ushA-like protein |
| c5261                | 1             | ±              | Purine ribonucleotide biosynthesis PurA |
| c3051                | 2             | ±              | Fe-S cluster biosynthesis chaperone protein HscA |
| c3567                | 2             | ±              | Molybdopterin-containing membrane protein in electron transfer chain C3567 |
| c4786                | 3             | ±              | Twin-arginine translocator subunit TatB |

*Hemolysis score indicates: ++, very strong; +, strong; ±, weak; −, very weak.*
of these genes are known factors involved in hemolysin production, including hns, hlyCABD, orhK- orhR, c3567, and fnr, indicating the robustness of our genetic screen. In contrast, the other seven genes have not been previously reported to have roles in hemolysis. These genes include moaD, moeB, mobA, purA, hscA, tatB, and c4895. We then constructed targeted gene deletion mutants and confirmed that moaD, moeB, mobA, purA, hscA, and tatB positively influenced hemolysis (Figure 1b), in line with the Tn mutant hemolysis results.

2.2 Bis-MGD is required for full hemolysis under anaerobiosis

We noticed that several genes involved in molybdenum cofactor (Moco) biosynthesis contributed to hemolysis under anaerobiosis. Molybdenum, a redox-active transition metal, forms the active site of molybdoenzymes, which perform key reactions in nitrogen, carbon, and sulfur metabolism in nature (Iobbi-Nivol & Leimkuhler, 2013). A great majority of molybdoenzymes contain molybdenum in the form of Moco or its derivatives, for example, bis-MGD and molydbioterin cytosine dinucleotide cofactor (MCD). Molybdoenzymes include DMSO reductase family enzymes (containing bis-MGD) and xanthine oxidase family enzymes (containing MCD), which are oxidoerductases that mediate electron transfer (Iobbi-Nivol & Leimkuhler, 2013). A schematic depicting the Moco biosynthesis pathway is shown in Figure 2a.

To delineate the roles of reaction steps in the Moco biosynthetic pathway other than those involving moaD and moeB and mobA in hemolysis, we generated various deletion mutants and subjected them to hemolytic analysis on blood agar under anaerobic conditions. The results of the hemolysis assay showed that deletion of moaABC or moeA substantially reduced hemolysis on blood agar, whereas inactivation of mogA or mocA did not affect hemolysis (Figure 2b). Furthermore, we tested whether mobA contributed to hemolysis under aerobic conditions, and our results showed that deletion of mobA did not influence hemolysis under aerobiosis (Figure 2c). In the presence of high concentrations of molybdenum, MogA is dispensable for Moco biosynthesis; MoeA alone can perform molybdenum ligation to MPT. MocA is responsible for converting Moco to MCD (Iobbi-Nivol & Leimkuhler, 2013). Taken together, these data suggest that bis-MGD is required for full hemolysis under anaerobiosis but not under aerobiosis.

2.3 Impaired Bis-MGD biosynthesis reduces hlyCABD transcription and the production of hemolysin

We then tested whether defective bis-MGD biosynthesis affects hlyCABD expression at the transcription level. First, a growth kinetic assay demonstrated that the mobA mutant grew slightly slower than the wild type, and it could not reach wild-type levels of cell density at the stationary phase (Figure 3a). RNA was extracted from ΔmobA mutant and wild-type cells grown on blood agar under anaerobiosis, followed by qPCR analysis of transcript levels. As shown in Figure 3b, the transcript levels of hlyCABD were reduced 4- to 16-fold in the mobA mutant compared with the wild type. Furthermore, in an immunoblotting assay, the production of hemolysin was substantially decreased in the mobA mutant relative to the wild type, and as expected, the introduction of a plasmid carrying the mobA locus to the mobA mutant restored hemolysin production (Figure 3c). Together, these results demonstrate that bis-MGD contributes to the transcription and translation of hlyCABD genes.
Then, we tried to explore whether deletion of *mobA* affects the expression of other major virulence factors. We found that transcription of *chuA* (encoding an iron receptor) and *tsh* (encoding a serine protease autotransporter) was only slightly influenced by *mobA* mutation, whereas the transcription of *fimH* (encoding type 1 pilus adhesin) and *pic* (encoding a serine protease autotransporter) did not respond to *mobA* deletion (Figure 3d). Thus, it is likely that bis-MGD plays a major role specifically in the expression of hemolysin.

To test whether the role of bis-MGD in hemolysin expression and hemolysis can be applied to cystitis isolate of UPEC, we constructed a *mobA* mutant strain of UPEC isolate UTI89 and compared the hemolysin expression and hemolysis of the mutant to that of its parental strain. Figure 3e demonstrates that deletion of *mobA* in UTI89 abolished HlyA production and hemolysis on blood agar. Altogether, these data suggest that the contribution of bis-MGD to hemolysin expression and hemolysis is not strain-specific and may be applicable to all hemolysin-positive UPEC strains.

### 2.4 | RNA-Seq analysis revealed extensive transcriptomic changes due to *mobA* deletion

To potentially identify factors that mediate the modulation of hemolysin expression by bis-MGD, RNA-seq analysis was used to compare the transcriptomes of the wild-type and Δ*mobA* mutant. With a false discovery rate <0.05 and fold change ≥2, 592 genes (Figure 4a, volcano plot) were differentially expressed in the *mobA* mutant compared with the wild type; of these genes, 367 genes were downregulated and 225 were upregulated. Among the top enriched KEGG pathways were metabolic pathways, including nitrogen metabolism, arginine biosynthesis and amino acid metabolism, and the tricarboxylic acid (TCA) cycle (Figure 4b). A complete set of differentially expressed genes (DEGs) can be found in Table S1, while some DEGs relevant to this study are indicated on the volcano plot, including nitrate reductase genes *nar* and *nap*, nitrite reductase genes *nir* and *nrf*, HlyA, and its upstream operon c3566-c3568. In line
with our qPCR analysis, hlyCABD was dramatically downregulated in the ΔmobA mutant compared with the wild-type strain. Additionally, the transcription levels of selected DEGs were further validated by qPCR, and the results were mostly consistent with the RNA-seq data (Figure 4c). Interestingly, a few genes encoding molybdoenzymes (nitrate reductase, formate dehydrogenase, etc.) were greatly induced in response to mobA deletion. Altogether, these data demonstrate that disrupted bis-MGD biosynthesis leads to extensive transcriptomic changes and that bis-MGD is important for maintaining metabolic homeostasis under anaerobic conditions.

2.5 Bis-MGD modulates hemolysin expression primarily via c3566–c3568

Previously, we showed that the c3566–c3568 operon upstream of hlyCABD is cotranscribed with hlyCABD, and the transcription of the seven genes is regulated by TCS orhK–orhR (Gu et al., 2021). Remarkably, we found that the c3566–c3568 operon was downregulated >8-fold due to mobA deletion (Figure 4c). To further validate the effects of mobA deletion on c3566–c3568 expression, we constructed a plasmid carrying the promoter region of c3566 fused to the lacZ reporter gene (P_{c3566-lacZ}), transformed the P_{c3566-lacZ} plasmid into LMP10 (CFT073ΔlacZYA), LMP10ΔmobA and LMP10ΔmobA complemented strains, and then tested the β-galactosidase activities in these strains. The LMP10ΔmobA mutant exhibited severely reduced β-galactosidase activities, while the complemented strain displayed comparable β-galactosidase activities as to the wild type (Figure 5a). In contrast, a P_{bla-lacZ} fusion driven by the constitutively expressed promoter P_{bla} showed similar β-galactosidase activities among different strains. These results indicate that bis-MGD is important for the full expression of c3566–c3568. Given that bis-MGD contributes to c3566–c3568 and hlyCABD expression and that c3566–c3568 and hlyCABD are transcriptionally linked, we hypothesized that bis-MGD modulates hemolysin expression through c3566–c3568. To test this hypothesis, we first generated a P_{c3566-hlyC-lacZ} plasmid construct containing a lacZ gene fused to the P_{c3566} promoter region and the coding regions of c3566 to hlyC as well as

![Figure 4](image-url)
part of hlyA, and then tested the β-galactosidase activities in LMP10, LMP10ΔmobA, and LMP10ΔmobA complemented strains. The results showed that deletion of mobA greatly reduced the expression of P<sub>pc3566</sub>-lacZ, suggesting that the entire transcriptionally linked region is subjected to modulation by mobA (Figure 5a). Further, we constructed a mutant strain ΔP3566::P1.0, in which P<sub>pc3566</sub> was replaced by a synthetic strong promoter P1.0 on the chromosome, and another mutant ΔP3566::P1.0ΔmobA, in which mobA was deleted from ΔP3566::P1.0. First, we affirmed that removal of P<sub>pc3566</sub> did not affect the expression of the upstream orhK–orhR and that P1.0-driven c3566–c3568 and hlyCABD were expressed as normal (data not shown). When we compared the expression levels of c3566–c3568 and hlyCABD in ΔP3566::P1.0ΔmobA to those in ΔP3566::P1.0, we found that the loss of mobA did not significantly alter the transcription of these genes (Figure 5b), suggesting that bis-MGD impacts hlyCABD expression primarily via c3566–c3568.

### 2.6 Loss of mobA upregulates ntrC, which encodes a direct repressor of c3566–c3568–hlyCABD expression

In an attempt to understand the mechanism by which bis-MGD modulates c3566–c3568–hlyCABD expression, we sought to identify suppressor mutations of mobA that would lead to enhanced production of hemolysin in mobA mutants. A Tn insertion mutant library based on ΔmobA was generated and plated onto blood agar to identify mutants with stronger hemolysis. In total, approximately 100,000 mutants were screened, giving rise to 33 mutants with enhanced hemolytic halos, that is, halos that were larger in size and/or more translucent. Further mapping analysis revealed that Tn insertions localized in eight genes (Table 2).

---

**TABLE 2** Disrupted genes by Tn insertions led to enhanced hemolysis of the ΔmobA mutant under anaerobic conditions

| Disrupted gene locus | No. of inserts | Hemolysis score<sup>a</sup> | Gene product |
|----------------------|---------------|-----------------------------|--------------|
| ΔmobA                | NA<sup>b</sup> | ±                           | bis-MGD biosynthesis |
| trxB                 | 5             | ++                          | Thioredoxin reductase |
| bipA                 | 8             | ++                          | Ribosome-associated GTPase |
| narX                 | 1             | +                           | Nitrate sensory histidine kinase |
| rpoN                 | 3             | ++                          | RNA polymerase sigma 54 factor |
| ntrC                 | 5             | ++                          | Nitrogen regulation response regulator |
| glnD                 | 8             | +                           | Uridylyl removing enzyme |
| mutM                 | 1             | +                           | Formamidopyrimidine-DNA glycosylase |
| nlpI                 | 2             | +                           | Outer membrane lipoprotein |

<sup>a</sup>Hemolysis score indicates: ++, very strong; +, strong; ±, weak.  
<sup>b</sup>NA, not applicable.
for pathway illustration). Among these, Tn mutants of ntrC and rpoN produced very strong hemolysis (Figure 6b). NtrC is a σF (RpoN)-dependent transcriptional regulator; thus, we hypothesized that NtrC was the terminal player acting to influence gene expression. We therefore generated a ΔntrC single deletion mutant as well as a ΔmobAΔntrC double deletion mutant. A qPCR analysis indeed showed that the loss of ntrC in the ΔmobA mutant led to upregulation of c3566-c3568-hlyCABD expression but to levels lower than those in the wild type (Figure 6c). Similarly, the loss of ntrC in the ΔmobA mutant led to greater production of HlyA but again to levels lower than those in the wild type (Figure 6d). Furthermore, we tested whether the deletion of ntrC in the wild-type background affected hemolysin expression, and we found that the loss of ntrC resulted in an increase in c3566-c3568-hlyCABD transcription and HlyA production (Figure 6c, d). Moreover, a liquid hemolysis assay demonstrated that inactivation of ntrC in both wild-type and ΔmobA background resulted in higher hemolytic activities (Figure 6e). These results strongly suggest that NtrC is a repressor of hemolysin production and hemolysis. We thus suspected that mobA deletion may lead to upregulation of ntrC and subsequent repression of c3566-c3568-hlyCABD. Indeed, we found that ntrC expression was increased by ~2-fold due to mobA deletion (Figure 6f).

To examine whether NtrC directly represses the expression of c3566-c3568-hlyCABD, an electrophoretic mobility shift assay (EMSA) was performed. Figure 6g shows that the purified His-NTC protein shifted the P_c3566 probe but not a negative control probe as the protein concentration increased, suggesting that NtrC can directly bind to the c3566 promoter region. Taken all together, our data suggest that defective bis-MGD biosynthesis led to increased expression of ntrC, and NtrC directly repressed c3566-c3568-hlyCABD expression, at least partially accounting for reduced c3566-c3568-hlyCABD expression in mobA mutant.

2.7 Bis-MGD contributes to hemolysin-mediated uroepithelial cell death and fitness in vivo in UPEC

To assess the role of bis-MGD in hemolysin-mediated uroepithelial cell death, cell morphological evaluation and a CCK-8-based quantitative cell viability assay were performed. At 2 h after infection, wild-type CFT073 caused obvious rounding and lifting of T24 bladder cells, whereas cells infected with the ΔhlyA mutant remained healthy, with noticeable cell clustering and spindle-like shapes similar to those of uninfected cells. On the other hand, cells infected with the ΔmobA mutant appeared mostly healthy, resembling the cells challenged with the ΔhlyA mutant (Figure 7a). These results suggest that bis-MGD is involved in causing uroepithelial cell death. To quantify the observations above, we infected cells with various strains at different MOIs and MOIs of 10 and 100 for different lengths of time and then subjected the cells to a CCK-8-based assay. At an MOI of 10 and 4 hpi, ~35% of cells infected with WT (wild type) remained viable, whereas approximately 95% of cells infected with the ΔmobA or ΔhlyA mutant remained viable. Complementation of the ΔmobA or ΔhlyA mutant with the corresponding locus in trans dramatically enhanced cell death. Moreover, a plasmid carrying the hlyCABD (pHlyCABD) locus can rescue cytotoxic effects of ΔmobA mutant. Similar trends were also observed with an MOI of 100 at 3 hpi (Figure 7b). Therefore, these data indicate that bis-MGD contributes to hemolysin-mediated uroepithelial cell death under anaerobiosis.

To evaluate the role of bis-MGD biosynthesis in UPEC fitness in vivo, a murine cochallenge model of ascending UTI was used. Mice were inoculated with the WT/ΔmobA or WT/ΔhlyA cocultures, and the relative competitive index was determined based on the bacterial load of each strain in the tissue at 48 hpi. As shown in Figure 7c, the ΔmobA mutant was significantly outcompeted by the WT in the bladder (p < .05), while the ΔhlyA mutant and the WT colonized the tissue at similar levels. These results demonstrate that the bis-MGD biosynthesis is an important fitness factor for UPEC colonization in vivo and that this role of bis-MGD is likely independent of hlyA.

3 DISCUSSION

During UTI, UPEC inevitably encounters low-oxygen environments, such as the renal medulla (Melcan et al., 2008; Subashchandrabose et al., 2014; Wang et al., 2008). To achieve optimal fitness and/or virulence, UPEC must exhibit adaptive responses by altering the expression of its virulence factors, for example, the upregulation of type 1 fimbria (Subashchandrabose & Mobley, 2015). In this study, we initially showed that under anaerobic conditions, UPEC increases the expression of the pore-forming toxin hemolysin and also enhances hemolysis on blood agar (Figure 1a). Using a Tn-based screening approach, we identified multiple genes important for wild-type levels of hemolysis under anaerobic conditions. Similarly, Nguyen Thi Khanh Nhu et al. performed a Tn-based genome-wide screen on the highly hemolytic ExPEC strain 56SEC to define the genetic basis underlying hemolysis under general laboratory (aerobic) conditions (Nhu et al., 2019). Comparing their study with this work, a few shared factors, for example, hlyCABD, hns, c3567, were identified; in contrast, there are some contributing factors that are specific to each study, for instance, repressor AcrR, ribonuclease E, and LPS core biosynthesis and DnaKJ in their study and FNR, bis-MGD, Tat, and HscA in this study. Collectively, our study and the previous study add to the growing list of factors modulating hemolytic activities in UPEC, which greatly further our understanding of HlyA-mediated hemolysis.

Using a systemic deletion analysis, we show that bis-MGD is an important factor for wild-type levels of hemolysin expression under anaerobic conditions but not under aerobic conditions. Bis-MGD is primarily incorporated into DMSO reductase (DMSOR)-family enzymes, including nitrate reductase A and Z (NarGHI and NarZYV), periplasmic nitrate reductase (NapABCgh), TMAO reductase (TorAC and TorZY), DMSO reductase (DmsABC), formate dehydrogenase N, O, and H (FdnGHI, FdoGHI, and FdhF), and biotin/methionine sulfoxide reductase (BisC) (Ezraty et al., 2005).
Defective bis-MGD biosynthesis due to mobA deletion dramatically impacts the activities of DMSOR molybdoenzymes in multiple bacterial pathogens (Dhouib et al., 2015; Tombline et al., 2013; Williams et al., 2015), and these enzymes play a key role in the bacterial respiratory chain as terminal reductases for anaerobic energy generation as well as in donating electrons to the respiratory chain (Unden et al., 2014). In addition, the ΔmobA mutant produced small colonies compared with the wild type (Figure 1), and small colony variants arise frequently due to impaired electron transport and energy production (Basta et al., 2017; McNamara & Proctor, 2000; Ramiro et al., 2016). Together, these results suggest that energy generation defects are the major consequence of mobA deletion. Indeed, previous reports indicate that energy generation defects are one of the two major physiological impacts caused by dysfunctional DMSOR molybdoenzymes (Zhong et al., 2020). Our RNA-seq analysis identified that GlnA, an indicator of nitrogen availability, was upregulated in the mobA mutant (Table S1), suggesting nitrogen limitation. Ammonium assimilation and amino acid biosynthesis require substantial ATP input (Chubukov et al., 2014). Thus, we propose a model in which defective bis-MGD biosynthesis results in deficiencies in energy production, which leads to limited nitrogen availability, and this further signals to NtrC, which is upregulated to augment the Ntr response. Similar to CpxR, as a negative regulator of hemolysin, NtrC may function to fine-tune hemolysin expression, as overproduction of hemolysin can be a disadvantage for UPEC colonization (Nagamatsu et al., 2015). It is noteworthy that deletion of ntrC in the ΔmobA mutant did not restore hlyA expression to wild-type levels, suggesting that other mechanisms besides NtrC may be involved. Importantly, nitrogen-limiting conditions also induce guanosine tetraphosphate (ppGpp)-mediated stringent response (Brown et al., 2014) and altered expression of hns (Brandi et al., 2020), and thus other regulatory factors could underlie reduced hlyA expression in the ΔmobA mutant. This possibility certainly warrants future research.

In addition to previously known regulators of hemolysis, our Tn screening also identified another three previously unreported factors that contribute to hemolysis under anaerobiosis, namely, tatB, hscA, and purA. TatB is a component of the twin-arginine translocation system, which usually comprises three essential components, TatA, TatB, and TatC (Palmer & Berks, 2012). The Tat system is present in many bacterial and archaeanal species, and it transports folded proteins, usually containing cofactors, across the cytoplasmic membrane (Frobel et al., 2012). Protein substrates of Tat in E. coli include a number of Moco- and Fe–S cluster-containing enzymes, such as NapA and FdnG. Notably, a great majority of DMSOR enzyme complexes have a subunit that is targeted by the Tat pathway (Palmer et al., 2010). A faulty Tat system fails to properly translocate its substrates, thereby leading to dysfunction of the substrates, such as nitrate reductase and TMAO reductase activities. HscA (heat shock cognate protein A) is a member of the Hsp70 family of chaperones and is required for the assembly of Fe–S clusters (Seaton & Vickery, 1994; Takahashi & Nakamura, 1999). An absence of HscA results in defective Fe–S cluster assembly, impeding the activity of Fe–S cluster-containing enzymes such as nitrate reductase, FDH–N and FDH–O, under anaerobic conditions (Jaroschinsky et al., 2017). Because bis-MGD, Tat, and HscA affect the activities of a shared set of enzymes, we speculate that these factors affect hemolysis via a similar mechanism. In support of this notion, inactivation of hscA or tatB dramatically reduced c3566–c3568–hlyCABD expression at the transcriptional level in anaerobiosis but not in aerobiosis (Figure S2). To assess whether individual major DMSOR members have roles in anaerobic hemolysis, we constructed a number of mutants with individual bis-MGD-containing enzyme genes deleted, followed by examining the hemolysis of these mutants along with the wild type. We found that all of the mutants displayed similar hemolysis compared with the wild type (Figure S3), indicating that no single-enzyme mutant can recapitulate the phenotype of mobA mutant. These suggest that defects in a combination of multiple
DMSOR enzymes due to mobA mutation were responsible for impaired hemolysis.

Given their prevalence and importance in bacterial physiology, it is unsurprising that bis-MGD and/or its associated enzymes are implicated in fitness in vivo or virulence in bacterial pathogens. For example, *Mycobacterium tuberculosis* encounters hypoxic environments during infection, and the persistence of *M. tuberculosis* in the lungs of guinea pigs is severely affected due to mobA deletion (Williams et al., 2015). Single molybdoenzymes may also contribute to virulence. For instance, FDH-N promotes *E. coli* fitness in a mouse colitis model, and FdhA supports the virulence of *C. jejuni* in cell and animal models (Kassem et al., 2012; Pryjma et al., 2012). We show that the ΔmobA mutant is less fit than the wild type during colonization of the murine urinary tract, whereas hlyA does not contribute to in vivo fitness in the mouse model. These results suggest that bis-MGD affects fitness through other factors, likely bis-MGD-containing enzymes. The reduced fitness of the ΔmobA mutant may be attributed to two factors: (1) impaired energy generation and (2) dysfunction of single DMSOR enzymes. Reports have shown that the molybdoenzyme genes *fdnI* and *nirD* are highly upregulated in the urine of infected mice (Snyder et al., 2004) and that *fdhF* is transcribed at high levels
during human UTI, which contributes to fitness in a murine model of UTI (Subashchandrabose et al., 2014). FDH could influence virulence via two mechanisms: (1) supporting energy generation by respiration or fermentative growth (Unden et al., 2014) and (2) controlling formate concentrations, where formate is a signal that modulates virulence (Koestler et al., 2018). Therefore, our study described the importance of bis-MGD biosynthesis in virulence regulation and pathogenesis.

In summary, the present work defined the set of genes implicated in UPEC hemolysis under anaerobiosis. Furthermore, we discovered a negative regulator of hemolysin expression, NtrC, and then elucidated the mechanism used by bis-MGD to maintain optimal hemolysin expression. Defects in bis-MGD biosynthesis extensively impact the transcriptome and UPEC fitness in vivo. Given that DMSOR occurs only in prokaryotes (e.g., dissimilatory nitrate reductase and formate dehydrogenase; Zhong et al., 2020), our study has provided potential drug targets for treating UPEC infections.

4 | EXPERIMENTAL PROCEDURES

4.1 | Bacterial strains and culture conditions

Strains and plasmids used in this study are listed in Table S2. UPEC strains CFT073 and UTI89 as well as their derivatives were grown on tryptic soy agar (TSA) or in tryptic soy broth (TSB) (Difco) for expression studies. Aerobic growth was achieved by shaking in the air at 180 rpm, and anaerobic growth by incubating in a sealed jar with MGC AnaeroPack pouch C1 (Mitsubishi Gas Chemical Company, Japan) and an indicator. For genetic
manipulations, all E. coli strains were grown routinely in a lysogenic broth (LB) medium. For growth curve assays, media were equilibrated overnight in an anaerobic jar before inoculation; and samples were taken and OD$_{600}$ was measured according to previously described (Cai et al., 2013). When needed, selective antibiotics and IPTG were added at the following concentrations: ampicillin (Amp), 100 µg/ml; kanamycin (Kan), 50 µg/ml; chloramphenicol (Chl), 25 µg/ml; nalidixic acid, 25 µg/ml; and IPTG, 1 mM.

### 4.2 | Recombinant DNA techniques

Polymerase chain reaction (PCR), electroporation, and DNA agarose gel electrophoresis were performed according to Sambrook and Russell (2001) unless otherwise indicated. All restriction and DNA-modifying enzymes were purchased from New England Biolabs or ThermoFisher Scientific and used following the suppliers’ recommendations. Recombinant plasmids, PCR products, and restriction fragments were purified using MiniBEST DNA Fragment purification kit orMiniElute gel extraction kit (Takara) as recommended by the supplier. Deletion mutants were constructed using the Lambda-Red recombinase system described by Datsenko and Wanner (2000). The ΔmobA complemented strain was generated by transforming into the ΔmobA mutant a pGEN-mcs (Lane et al., 2007) variant carrying the mobA locus driven by its native promoter. For the construction of promoter-lacZ transcripcional fusion plasmid, the P$_{c3566}$ and P$_{mob}$ promoter regions were PCR amplified and cloned into pCJ112 vector (Cai et al., 2017) using MultiS one-step cloning kit (Vazyme, China). The resulting recombinant plasmid was transformed into CFT073 and its variants, and the transformants were selected on LB agar supplemented with kanamycin. All constructs were confirmed by DNA sequencing (Comate Bioscience Company, China) to ensure sequence fidelity. Oligonucleotides used in this study are listed in Table S3.

### 4.3 | Transposon mutagenesis to screen for mutants with altered hemolysis

To screen for genetic factors involved in hemolysis under anaerobic conditions, a Tn (pUTmini-Tn5km2, KmR) library based on the wild-type background was constructed according to previously reported (Zhang et al., 2019). Aliquots containing ~300–400 mutants were plated onto blood agar plates supplemented with nalidixic acid and kanamycin to select for mutants with altered hemolysis. For suppressor mutant identification, a Tn (Mariner Tn in pSC137, CmR, Chiang & Mekalanos, 2000) library based on the ΔmobA background was constructed. Aliquots containing ~300–400 mutants were plated onto blood agar plates supplemented with nalidixic acid and chloramphenicol to select for mutants with enhanced hemolysis halo. Candidates were picked and streak-purified on blood agar to confirm altered hemolysis. Arbitrary PCR was used to map Tn insertion sites (Zhang et al., 2019).

### 4.4 | RNA isolation and quantitative real-time reverse transcription PCR (QPCR)

Wild-type CFT073 and its various derivatives were streaked on TSA plates and allowed to grow for 18 hr in an anaerobic jar, and then bacteria were collected by scraping off the plates and immediately stabilized in RNAlater reagent (Ambion). RNA was extracted using an SV Total RNA Isolation Kit (Promega). Three biological replicates of each sample were prepared. The concentration of RNA was determined using an Implen NanoPhotometer NP80 (Implen, Germany). RNA samples were treated to remove genomic DNA and reverse transcribed to cDNA using a PrimeScript RT reagent Kit with gDNA Eraser (Clontech).

For qPCR, melting curve analyses were performed after each reaction to ensure amplification specificity. Differences (n-fold) in transcripts were calculated using the relative comparison method, and amplification efficacies of each primer set were verified as described by Schmittgen et al. (2000). RNA levels were normalized using the housekeeping gene rpoB as an endogenous control (Skyberg et al., 2008). qPCR was performed with an Applied Biosystem using TB Green™ Premix Ex Taq™II Tli RNaseH Plus (Takara) according to the manufacturer’s instructions (Li et al., 2011).

### 4.5 | SDS-PAGE and Western Blot

CFT073 and its derivative strains were grown on TSA plates at 37°C for 18 hr in an anaerobic jar. Two to three colonies were picked up by inoculation loops and resuspended in ice-cold PBS, followed by an OD$_{600}$ normalization. Proper dilutions of boiled samples were separated on 10% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Merck) as previously described (Gu et al., 2021). Blots were probed with a polyclonal antibody against HlyA (Gu et al., 2021) and a horse radish peroxidase-conjugate antirabbit IgG secondary antibody (CST, 70745) using the ECL detection reagents or KPL DyLight 800-labeled antibody (SeraCare, 5230-0346). Antibodies against GroEL (Abcam, ab90522) or RpoB (Abcam, ab191598) were used as loading controls. Membranes were viewed by an Azure500 imager (Azure Biosystems, CA).

### 4.6 | Transcriptomics by RNA sequencing (RNA-Seq)

RNA-seq analysis was performed using a standard protocol with minor modifications (Li et al., 2021). Briefly, bacterial culturing and RNA extractions were carried out as described above, and then the quality and concentrations were determined by an Agilent 2,100 Bioanalyzer (Agilent Technologies) and NanoDrop system (Thermo Fisher Scientific Inc.), respectively. One microgram of high-quality RNA extractions were carried out as described above, and then the quality and concentrations were determined by an Agilent 2,100 Bioanalyzer (Agilent Technologies) and NanoDrop system (Thermo Fisher Scientific Inc.), respectively. One microgram of high-quality...
RNA (A260/A280 ratio > 2.0 and RIN value >7.0) was used for each NextGen sequencing library, which was constructed according to the manufacturer’s protocol (NEBNext Ultra Directional RNA Library Prep Kit for Illumina). Ribosomal RNA was depleted from total RNA using the Ribo-Zero rRNA Removal Kit for Bacteria (Illumina). Sequencing of the libraries was performed using a 2 × 150 paired-end (PE) configuration on an Illumina HiSeq platform according to the manufacturer’s instructions (Illumina, CA), and image analysis and base calling were conducted using HiSeq Control Software (HCS) + OLB + GAPipeline-1.6 (Illumina) on an HiSeq system. The sequences were processed and analyzed, and raw reads were assessed by fastQC and further treated by Cutadapt (version 1.9.1). Clean reads were then aligned to the CFT073 genome (GenBank accession: NC_004431.1) using bowtie2 (version V 0.6.1). Differential gene expression analysis was then performed using DESeq2 (V1.9.3) with R version 3.3.2 following a standard workflow. All genes with a \(|\log_2(\text{fold-change})| > 1\) and a Benjamini-Hochberg adjusted p-value (q-value) < 0.05 were considered differentially expressed. Table S1 lists all DEGs in the mutant compared with the wild type, and raw data are available at the National Microbiology Data Center (Accession: NMDC40009706).

4.7 | β-Galactosidase assays

Bacteria from glycerol stock were streaked on TSA plates with appropriate antibiotics and incubated at 37°C for 18–24 hr in an anaerobic jar. Bacteria were scraped off the plates in ice-cold Z buffer. The resulting bacterial suspensions were OD_{540} normalized and assayed for β-galactosidase activity using ortho-Nitrophenyl-β-galactoside (ONPG) as a substrate as described previously (Miller, 1972).

4.8 | Hemolysis assay

Hemolysis on blood agar was evaluated as described before (Velasco et al., 2018). UPEC CFT073 was streaked on blood agar plates containing 10% defibrinated sheep blood, and the plates were incubated at 37°C for 18–24 hr under aerobic and anaerobic conditions. A hemolytic halo with larger size or higher translucence indicates stronger hemolytic activities and vice versa. Hemolysis assay in liquid was performed as previously described (Tsou & Zhu, 2010) but with minor modifications. Bacteria were cultured in TSB medium at 37°C for 24 hr in an anaerobic jar, and then culture supernatants were collected by centrifugation and filtering after OD_{600} normalization. Sheep blood (Luqiao, China) was prepared by washing 3x with PBS. Culture supernatants (245 μl) were combined with 5 μl of washed sheep blood in the presence of 10 mM CaCl₂ and incubated for 1 hr at 37°C statically. TSB was used in place of culture supernatant as a negative control, and 1% Triton X-100 was used as a positive control. After incubation, samples were centrifuged, and hemoglobin release was determined by measuring the optical density at 540 nm (OD_{540}). Percent hemolysis was calculated by setting the positive control as 100% hemolysis.

4.9 | Electrophoretic mobility shift assay (EMSA)

EMSA was carried out to study the binding of protein to DNA probes as previously reported (Cai et al., 2017). Briefly, recombinant protein His_{6}-NtrC was expressed using pET28 prokaryotic expression system (Novagen), purified to homogeneity using NTA Spin Columns (QIAGEN), and dialyzed against the binding buffer (10 mM Tris [pH 7.5], 1 mM EDTA, 1 mM dithiothreitol, 50 mM KCl, 50 mM MgCl₂, 1 μg/ml bovine serum albumin [NEB]). DNA probes were PCR amplified using specific primers and gel purified. EMSAs were performed by adding increasing the amounts of purified fusion protein to the DNA probe (10 ng) in the binding buffer and incubating for 30 min at room temperature. Reaction mixtures were then subjected to electrophoresis on a 6% polyacrylamide gel in 0.5 × TBE buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA, pH 8.0) on ice for 30 min at 100 V plus 60 min at 150 V. The gel was stained in 0.5 × TBE buffer containing 1 × SYBR Gold nucleic acid staining solution for 15 min. Gels were then visualized and photographed using ChampGel 7,000 Imager (SageCreation).

4.10 | Cell morphology assay

T24 bladder epithelial cells (ATCC HTB-4) were infected with various bacterial strains in 24-well plates at an MOI of 10 at 37°C, and uninfected cells were used as controls. At 2.5–3.5 hpi, cell monolayers were visualized with an Axiocor 40C inverted optical microscope at 20× magnification (Carl Zeiss).

4.11 | Cell viability assay

T24 bladder epithelial cells were cultured in 96-well plates to 90% confluence, with each well containing 100 μl McCoy’s 5a medium with 10% heat-inactivated fetal bovine serum (FBS). Bacteria were grown in TSB at 37°C in an anaerobic jar for 24 hr, followed by washing with PBS and resuspending in McCoy’s 5a medium. One hundred microliters of normalized bacterial culture was added to a monolayer of T24 cells for infection, achieving an MOI of 10 or 100. Plates were then incubated at 37°C in an anaerobic jar for desired times. Next, the cell medium was removed completely and then replaced with 100 μl fresh medium containing Cell Counting Kit-8 (CCK-8) solution. Cells were allowed to stain for 2–3 hr, before OD_{450} was determined with an ELx800 microplate reader (Bio-TEK). The mean OD_{450} of five replicate wells was used to calculate the percentage of cell viability: percentage of cell viability = (OD_{treatment} − OD_{blank})/(OD_{uninfected control} − OD_{blank}) × 100%. Of note, after infection, bacterial CFUs were measured, and the
4.12 | Experimental UTIs

Mouse infection studies were performed according to the methods of Johnson et al. (2005). Overnight grown CFT073 and its mutant strains were pelleted and resuspended in sterile PBS, mixed in equal numbers, and adjusted to make challenge inocula. Female CBA/J mice (6-10 weeks of age) were anesthetized and inoculated via transurethral catheterization with a 20 μL (2 x 10^5 CFU) challenge inocula per mouse. To determine the initial CFU/ml, dilutions of each inoculum were plated onto LB plates with and without chloramphenicol. After 48 hr, the mice were euthanized and the bladder and kidneys were aseptically removed, weighed, and homogenized in tubes containing PBS. Dilutions of the homogenized tissue were then plated onto duplicate LB plates with and without chloramphenicol or plates with different antibiotics to determine the bacterial concentration (CFU/g) of tissue. After overnight incubation, distinct colonies on plates were enumerated. The numbers of colonies on selective plates were subtracted from those on LB plates to obtain the number of wild-type bacteria. A group of 10 mice for each dual-strain challenge were used to determine alterations in fitness. The competition assay was performed twice and shown are the combined data. For statistical analysis, a two-tailed Wilcoxon matched-pairs test was used (Prism software, CA) and the threshold for statistical significance was a p value < .05.

4.13 | Statistical analysis

One-way ANOVA was used to analyze the differences between multiple strains, and all other binary comparisons were analyzed by Student’s t-test (GraphPad 9.0, Prism). A p value of <.05 was considered to be statistically significant.

ETHICS STATEMENT

Handling and Care of mice were performed according to the Beijing Administration Guidelines for the Use of Laboratory Animals. The entire protocol with respect to animal experiments was approved by the Review Board of Harbin Veterinary Research Institute and by the Animal Care and Use Committee of Heilongjiang Province (SYXK(H)2006–032). All efforts were made to minimize the suffering of animals (Yan et al., 2020).

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China Young Scholars Project (31902242). The funders played no roles in study design, data collection and interpretation, or submission for publication.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

GL and WC conceived and designed the experiments. WC, DH, ZZ, XC, and XZ performed the experiments. All authors participated in the discussion of the results. WC and GL provided the resources and the funding. GL and WC wrote and reviewed the paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in National Microbiology Data Center (Accession: NMDC40009706).

ORCID

Wentong Cai https://orcid.org/0000-0002-2282-302X
Ganwu Li https://orcid.org/0000-0002-6370-9573

REFERENCES

Barber, A.E., Norton, J.P., Spivak, A.M. & Mulvey, M.A. (2013) Urinary tract infections: current and emerging management strategies. Clinical Infectious Diseases, 57, 719–724. https://doi.org/10.1093/cid/cit284

Barbieri, N.L., Nicholson, B., Hussein, A., Cai, W., Wannemuehler, Y.M., Dell’Anna, G. et al. (2014) FNR regulates expression of important virulence factors contributing to pathogenicity of uropathogenic Escherichia coli. Infection and Immunity, 82, 5086–5098.

Basta, D.W., Bergkessel, M. & Newman, D.K. (2017) Identification of fitness determinants during energy-limited growth arrest in Pseudomonas aeruginosa. Mbio, 8, e01170-17.

Beebout, C.J., Eberly, A.R., Werby, S.H., Reasoner, S.A., Brannon, J.R., De, S. et al. (2019) Respiratory heterogeneity shapes biofilm formation and host colonization in uropathogenic Escherichia coli. Mbio, 10, e02400-18. https://doi.org/10.1128/mBio.02400-18

Bien, J., Sokolova, O. & Bozko, P. (2012) Role of uropathogenic Escherichia coli virulence factors in development of urinary tract infection and kidney damage. International Journal of Nephrology, 2012, 681473.

Brandi, A., Giangrossi, M., Fabbretti, A. & Falconi, M. (2020) The hns gene of Escherichia coli is transcriptionally down-regulated by (p)pGpp. Microorganisms, 8, 1558. https://doi.org/10.3390/microorganisms8101558

Brown, D.R., Barton, G., Pan, Z., Buck, M. & Wigneshwararaj, S. (2014) Nitrogen stress response and stringent response are coupled in Escherichia coli. Nature Communications, 5, 4115. https://doi.org/10.1038/ncomms5115

Cai, W., Cai, X., Yang, Y., Yan, S. & Zhang, H. (2017) Transcriptional control of dual transporters involved in alpha-Ketoglutarate utilization reveals their distinct roles in uropathogenic Escherichia coli. Frontiers in Microbiology, 8, 275.

Cai, W., Wannemuehler, Y., Dell’Anna, G., Nicholson, B., Barbieri, N.L., Kariyawasam, S. et al. (2013) A novel two-component signaling system facilitates uropathogenic Escherichia coli’s ability to exploit abundant host metabolites. PLoS Path, 9, e1003428. https://doi.org/10.1371/journal.ppat.1003428

Chiang, S.L. & Mekalanos, J.J. (2000) Construction of a Vibrio cholerae vaccine candidate using transposon delivery and FLP recombinase-mediated excision. Infection and Immunity, 68, 6391–6397.

Chubukov, V., Gerosa, L., Kochanowski, K. & Sauer, U. (2014) Coordination of microbial metabolism. Nature Reviews Microbiology, 12, 327-340. https://doi.org/10.1038/nrmicro3238

Cooke, E.M. & Ewins, S.P. (1975) Properties of strains of Escherichia coli isolated from a variety of sources. Journal of Medical Microbiology, 8, 107–111. https://doi.org/10.1099/00222615-8-1-107

Datsenko, K.A. & Wanner, B.L. (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proceedings of the National Academy of Sciences USA, 97, 6640–6645. https://doi.org/10.1073/pnas.120163297
Dhakal, B.K. & Mulvey, M.A. (2012) The UPEC pore-forming toxin alpha-hemolysin triggers proteolysis of host proteins to disrupt cell adhesion, inflammatory, and survival pathways. Cell Host & Microbe, 11, 58–69.

Dhouib, R., Pg Othman, D.S., Essilfie, A.T., Hansbro, P.M., Hanson, J.O., McEwan, A.G. et al. (2015) Maturation of molybdoenzymes and its influence on the pathogenesis of non-typeable Haemophilus influenzae. Frontiers in Microbiology, 6, 1219. https://doi.org/10.3389/fmicb.2015.01219

Ezraty, B., Bos, J., Barras, F. & Aussel, L. (2005) Methionine sulfoxide reduction and assimilation in Escherichia coli: New role for the biotin sulfoxide reductase Bsc. Journal of Bacteriology, 187, 231–237.

Frobel, J., Rose, P. & Muller, M. (2012) Twin-arginine-dependent translocation of folded proteins. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences, 367, 1029–1046. https://doi.org/10.1098/rstb.2011.0202

Gu, H., Cai, X., Zhang, X., Luo, J., Zhang, X., Cai, W. & Li, G. (2021) A bacterial “shield and sword”: a previously uncharacterized two-component system protects uropathogenic Escherichia coli from host-derived oxidative insults and promotes hemolysin-mediated host cell cytoptryosis. bioRxiv. 2021.04.2021.440293.

Hacker, J. & Hughes, C. (1985) Genetics of Escherichia coli hemolysin. Current Topics in Microbiology and Immunology, 118, 139–162.

Ikehaire, R., Siitonen, A., Karkkainen, U., Mustonen, J., Heiskanen, T. & Makela, P.H. (1994) Community-acquired pneumonia in adults: characteristics of E. coli isolates in bacteremic and non-bacteremic patients. Scandinavian Journal of Infectious Diseases, 26, 289–296. https://doi.org/10.1080/0365549409117797

Ilobi-Nivol, C. & Leimkuhler, S. (2013) Molybdenum enzymes, their maturation and molybdenum cofactor biosynthesis in Escherichia coli. Biochimica Et Biophysica Acta, 1827, 1086–1101. https://doi.org/10.1016/j.bbabio.2012.11.007

Jaroschinsky, M., Pinske, C. & Gary Sawers, R. (2017) Differential effects of isc operon mutations on the biosynthesis and activity of key anaerobic metalloenzymes in Escherichia coli. Microbiology (Reading), 163, 878–890. https://doi.org/10.1099/mic.0.000481

Johnson, J.R., Jelacic, S., Schoening, L.M., Clabots, C., Shaikh, N., Mobley, H.L. et al. (2005) The IrgA homologue adhesin Iha is an Escherichia coli virulence factor in murine urinary tract infection. Infection and Immunology, 73, 965–971.

Kalra, O.P. & Raizada, A. (2009) Approach to a patient with urosepsis. Journal of Global Infectious Diseases, 1, 57–63. https://doi.org/10.4103/0974-777X.52984

Kassem, I.I., Khatri, M., Esseli, M.A., Sanad, Y.M., Saif, Y.M., Olson, J.W. et al. (2012) Respiratory proteins contribute differentially to Campylobacter jejuni’s survival and in vitro interaction with hosts’ intestinal cells. BMC Microbiology, 12, 258. https://doi.org/10.1186/1471-2180-12-258

Klein, R.D. & Hultgren, S.J. (2020) Urinary tract infections: microbial pathogenesis, host-pathogen interactions and new treatment strategies. Nature Reviews Microbiology, 18, 211–226. https://doi.org/10.1038/s41579-020-0324-0

Koestler, B.J., Fisher, C.R. & Payne, S.M. (2018) Formate promotes shigaella intercellular spread and virulence gene expression. mBio, 9, e01777-18.

Lane, M.C., Alteri, C.J., Smith, S.N. & Mobley, H.L. (2007) Expression of flagella is coincident with uropathogenic Escherichia coli ascension to the upper urinary tract. Proceedings of the National Academy of Sciences USA, 104, 16669–16674. https://doi.org/10.1073/pnas.0607898104

Leeds, J.A. & Welch, R.A. (1996) RsfA enhances elongation of Escherichia coli hlyCABD mRNA. Journal of Bacteriology, 178, 1850–1857. https://doi.org/10.1128/ jb.178.7.1850-1857.1996

Li, G., Tivendale, K.A., Liu, P., Feng, Y., Wannemuehler, Y., Cai, W. et al. (2011) Transcriptome analysis of avian pathogenic Escherichia coli O1 in chicken serum reveals adaptive responses to systemic infection. Infection and Immunity, 79, 1951–1960. https://doi.org/10.1128/IAI.01230-10

Li, H., Hu, S., Yan, X., Yang, Y., Liu, W., Bu, Z. et al. (2021) An extracytoplasmic function (ECF) sigma/anti-sigma factor system regulates hypochlorous acid resistance and impacts expression of the type IV secretion system in Brucella melitensis. Journal of Bacteriology, e00127-21.

Marrs, C.F., Zhang, L., Tallman, P., Manning, S.D., Somsel, P., Raz, P. et al. (2002) Variations in 10 putative uropathogen virulence genes among urinary, faecal and peri-urethral Escherichia coli. Journal of Medical Microbiology, 51, 138–142.

McNamara, P.J. & Proctor, R.A. (2000) Staphylococcus aureus small colony variants, electron transport and persistent infections. International Journal of Antimicrobial Agents, 14, 117–122. https://doi.org/10.1016/S0924-8579(99)00170-3

Melican, K., Boekel, J., Mansson, L.E., Sondoval, R.M., Tanner, G.A., Kallskog, O. et al. (2008) Bacterial infection-mediated mucus signalling induces local renal ischaemia as a defence against sepsis. Cellular Microbiology, 10, 1987–1998. https://doi.org/10.1111/j.1462-5822.2008.01182.x

Miller, J.H. (1972) Experiments in molecular genetics. Long Island, NY: Cold Spring Harbor Laboratory.

Mourino, M., Munoa, F., Balsalobre, C., Diaz, P., Madrid, C. & Juarez, A. (1994) Environmental regulation of alpha-haemolysin expression in Escherichia coli. Microbial Pathogenesis, 16, 249–259.

MV Murthy, A., Phan, M.D., Peters, K.M., Nhu, N.T.K., Welch, R.A., Ulett, G.C. et al. (2018) Regulation of hemolysin in uropathogenic Escherichia coli fine-tunes killing of human macrophages. Virulence, 9, 967–980.

Nagamatsu, K., Hannan, T.J., Guest, R.L., Kostakioti, M., Hadjifrangiskou, M., Binkley, J. et al. (2015) Dysregulation of Escherichia coli alpha-haemolysin expression alters the course of acute and persistent urinary tract infection. Proceedings of the National Academy of Sciences of the United States of America, 112, E871–E880.

Nhu, N.T.K., Phan, M.D., Forde, B.M., Murthy, A.M.V., Peters, K.M., Day, C.J. et al. (2019) Complex multilevel control of hemolysin production and assimilation in Brucella melitensis. Infection and Immunology, 79, 1729–1738.

Palmer, T. & Berks, B.C. (2012) The twin-arginine translocation (Tat) protein export pathway. Nature Reviews Microbiology, 10, 483–496. https://doi.org/10.1038/nrmicro2814

Palmer, T., Sargent, F. & Berks, B.C. (2010) The tat protein export pathway. EcoSal Plus, 4. https://doi.org/10.1128/ecosalplus.4.3.2

Pryjma, M., Apel, D., Huynh, S., Parker, C.T. & Gaynor, E.C. (2012) FdhTU-modulated formate dehydrogenase expression and electron donor availability enhance recovery of Campylobacter jejuni following host cell infection. Journal of Bacteriology, 194, 3803–3813.

Ramiro, R.S., Costa, H. & Gordo, I. (2016) Macrophage adaptation leads to parallel evolution of genetically diverse Escherichia coli small-colony variants with increased fitness in vivo and antibiotic collateral sensitivity. Evolutionary Applications, 9, 994–1004.

Russo, T.A., Davidson, B.A., Genagon, S.A., Warholic, N.M., MacDonald, U., Pawlikci, P.D. et al. (2005) E-coli virulence factor hemolysin induces neutrophil apoptosis and necrosis/lysis in vitro and necrosis/lisis and lung injury in a rat pneumonia model. American Journal of Physiology-Lung Cellular and Molecular Physiology, 289, L207–L216.
Sambrook, J. & Russell, D.W. (2001) Molecular cloning: a laboratory manual. Long Island, NY: Cold Spring Harbor Laboratory Press.

Schmittgen, T.D., Zakrzewski, B.A., Mills, A.G., Gorn, V., Singer, M.J. & Reed, M.W. (2000) Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods. *Analytical Biochemistry*, 285, 194–204. https://doi.org/10.1006/abio.2000.4753

Seaton, B.L. & Vickery, L.E. (1994) A gene encoding a DnaK/hsp70 homolog in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America*, 91, 2066–2070. https://doi.org/10.1073/pnas.91.6.2066

Skyberg, J.A., Johnson, T.J. & Nolan, L.K. (2008) Mutational and transcriptional analyses of an avian pathogenic *Escherichia coli* CoIV plasmid. *BMC Microbiology*, 8, 24. https://doi.org/10.1186/1471-2180-8-24

Snyder, J.A., Haugen, B.J., Buckles, E.L., Lockatell, C.V., Johnson, D.E., Donnenberg, M.S. et al. (2004) Transcriptome of uropathogenic *Escherichia coli* during urinary tract infection. *Infection and Immunity*, 72, 6373–6381.

Stanley, P., Packman, L.C., Koronakis, V. & Hughes, C. (1994) Fatty acylation of two internal lysine residues required for the toxic activity of *Escherichia coli* hemolysin. *Science*, 266, 1992–1996. https://doi.org/10.1126/science.7801126

Subashchandrabose, S., Hazen, T.H., Brumbaugh, A.R., Himpsl, S.D., Smith, S.N., Ernst, R.D. et al. (2014) Host-specific induction of *Escherichia coli* fitness genes during human urinary tract infection. *Proceedings of the National Academy of Sciences of the United States of America*, 111, 18327–18332.

Subashchandrabose, S. & Mobley, H.L.T. (2015) Virulence and fitness determinants of uropathogenic *Escherichia coli*. *Microbiology Spectrum*, 3, UTI-0015-2012.

Takahashi, Y. & Nakamura, M. (1999) Functional assignment of the ORF2-iscS-iscU-iscA-hscB-hscA-fdx-ORF3 gene cluster involved in the assembly of Fe-S clusters in *Escherichia coli*. *Journal of Biochemistry*, 126, 917–926.

Tombline, G., Schwingel, J.M., Lapek, J.D. Jr, Friedman, A.E., Darrah, T., Maguire, M. et al. (2013) *Pseudomonas aeruginosa* PA1006 is a persulfide-modified protein that is critical for molybdenum homeostasis. *PLoS One*, 8, e55593. https://doi.org/10.1371/journal.pone.0055593

Tomenius, H., Pernestig, A.K., Jonas, K., Georgellis, D., Mollyb, R., Normark, S. et al. (2006) The *Escherichia coli* BarA-UvrY two-component system is a virulence determinant in the urinary tract. *BMC Microbiology*, 6, 27. https://doi.org/10.1186/1471-2180-6-27

Tsou, A.M. & Zhu, J. (2010) Quorum sensing negatively regulates hemolysin transcription and posttranslationally in *Vibrio cholerae*. *Infection and Immunity*, 78, 461–467.

Unden, G., Steinmetz, P.A. & Degreif-Dunnwald, P. (2014) The aerobic and anaerobic respiratory chain of *Escherichia coli* and *Salmonella enterica*: enzymes and energetics. *Ecosal Plus*, 3. https://doi.org/10.1128/ecosalplus.3.2.2

Velasco, E., Wang, S., Sanet, M., Fernandez-Vazquez, J., Jove, D., Glaria, E. et al. (2018) A new role for zinc limitation in bacterial pathogenicity: modulation of alpha-hemolysin from uropathogenic *Escherichia coli*. *Scientific Reports*, 8, 6535.

Wandersman, C. & Delepelaire, P. (1990) TolC, an *Escherichia coli* outer membrane protein required for hemolysin secretion. *Proceedings of the National Academy of Sciences of the United States of America*, 87, 4776–4780. https://doi.org/10.1073/pnas.87.12.4776

Wang, Z.J., Joe, B.N., Coakley, F.V., Zaharchuk, G., Busse, R. & Yeh, B.M. (2008) Urinary oxygen tension measurement in humans using magnetic resonance imaging. *Academic Radiology*, 15, 1467–1473. https://doi.org/10.1016/j.acra.2008.04.013

Wiles, T.J. & Mulvey, M.A. (2013) The RTX pore-forming toxin alpha-hemolysin of uropathogenic *Escherichia coli*: progress and perspectives. *Future Microbiology*, 8, 73–84.

Williams, M.J., Shanley, C.A., Zilavy, A., Peixoto, B., Manca, C., Kaplan, G. et al. (2015) bis-Molybdopterin guanine dinucleotide is required for persistence of *Mycobacterium tuberculosis* in guinea pigs. *Infection and Immunity*, 83, 544–550.

Yan, X., Hu, S., Yang, Y., Xu, D., Li, H., Liu, W. et al. (2020) The twin-arginine translocation system is important for stress resistance and virulence of *Brucella melitensis*. *Infection and immunity*, 88, e00389-20.

Zhang, H., Chen, X., Nolan, L.K., Zhang, W. & Li, G. (2019) Identification of host adaptation genes in extraintestinal pathogenic *Escherichia coli* during infection in different hosts. *Infection and Immunity*, 87, e00666-19.

Zhong, Q.F., Kobe, B. & Kappler, U. (2020) Molybdenum enzymes and how they support virulence in pathogenic bacteria. *Frontiers in Microbiology*, 11, 615860.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of the article at the publisher’s website.

How to cite this article: Zhang, X., Huang, D., Zhao, Z., Cai, X., Cai, W. & Li, G. (2021) Bis-molybdopterin guanine dinucleotide modulates hemolysin expression under anaerobiosis and contributes to fitness in vivo in uropathogenic *Escherichia coli*. *Molecular Microbiology*, 116, 1216–1231. [https://doi.org/10.1111/mmi.14809](https://doi.org/10.1111/mmi.14809)