Deacidification by FhlA-dependent hydrogenase is involved in urease activity and urinary stone formation in uropathogenic *Proteus mirabilis*

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*Proteus mirabilis* is an important uropathogen, featured with urinary stone formation. Formate hydrogenlyase (FHL), consisting of formate dehydrogenase H and hydrogenase for converting proton to hydrogen, has been implicated in virulence. In this study, we investigated the role of *P. mirabilis* FHL hydrogenase and the FHL activator, FhlA. *fhlA* and *hyfG* (encoding hydrogenase large subunit) displayed a defect in acid resistance. *fhlA* and *hyfG* mutants displayed a delay in medium deacidification compared to wild-type and *ureC* mutant failed to deacidify the medium. In addition, loss of *fhlA* or *hyfG* decreased urease activity in the pH range of 5–8. The reduction of urease activities in *fhlA* and *hyfG* mutants subsided gradually over the pH range and disappeared at pH 9. Furthermore, mutation of *fhlA* or *hyfG* resulted in a decrease in urinary stone formation in synthetic urine. These indicate *fhlA*- and *hyf*-mediated deacidification affected urease activity and stone formation. Finally, *fhlA* and *hyfG* mutants exhibited attenuated colonization in mice. Altogether, we found expression of *fhlA* and *hyf* confers medium deacidification via facilitating urease activity, thereby urinary stone formation and mouse colonization. The link of acid resistance to urease activity provides a potential strategy for counteracting urinary tract infections by *P. mirabilis*.

Urinary tract infections (UTIs) are common infectious diseases, afflicting a large proportion of the human population. An estimated 40–50% of women will experience at least one UTI during their lifetime4,5. UTIs with usage of indwelling catheters and formation of urinary stones usually cause therapeutic failure3–5. Catheter-associated urinary tract infections (CAUTIs) are among the most common nosocomial infections, causing an enormous health and financial burden worldwide. In addition, the rise of antimicrobial resistance in uropathogens has complicated the treatment of UTIs6,8. Thus, new antibacterial strategies are open for further investigation.

Formate hydrogenlyase activator (FhlA) belongs to one of the RpoN-dependent enhancer binding proteins and is a master regulator of formate hydrogenlyase (FHL) which consists of formate dehydrogenase H (FDH), and hydrogenase multimers, connecting formate oxidation to proton reduction responsible for the H₂ production7–11. Hydrogenases are enzymes that catalyze the oxidation of molecular hydrogen (H₂) into protons or the reduction of protons to molecular hydrogen12. The hydrogenase of FHL belongs to [NiFe] hydrogenase involving in converting proton to hydrogen11,12. *Escherichia coli* could produce four distinct [NiFe]-hydrogenase isoenzymes including hydrogenase-1 (Hya), hydrogenase-2 (Hyb), hydrogenase-3 (Hyc) and hydrogenase-4 (Hyf) encoded by *hya*, *hyb*, *hyc* and *hyf* operon respectively11,13. The homologous Hyc and Hyf are energy-converting hydrogenases conserved in gammaproteobacteria such as uropathogenic *E. coli* (UPEC) and *Klebsiella pneumoniae*13. Hyc and Hyf are made of several components such as electron transfer proteins, membrane-anchored proteins and catalytic hydrogenase subunits11,14–15. The transcriptions of both *hyc* and *hyf* operons are activated by FhlA and are dependent on RpoN7–10. Taking *E. coli* as an example, Hyc and Hyf would couple with formate dehydrogenase to form FHL-1 and FHL-2, respectively. At slightly acidic pH 6.5, the production of H₂ was mostly dependent on FHL-1, while FHL-2 contributes to H₂ production at slight alkaline pH 7.516. The FHL-1 of *E coli* contributes to

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medium deacidification during mix acid fermentation and acid resistance in the anaerobic environment. In addition, hydrogenases of FHL may play a role in virulence by neutralizing hydroxyl free radicals (OH·), providing energy and maintaining acid–base homeostasis. In this regard, fhlA and FHL-2 genes have been shown to be induced in UPEC during urinary tract infections. Recently, fhlA and the genes encoding the components of FHL-2, were found to be likely to contribute to mouse colonization in uropathogenic Proteus mirabilis and urinary stone formation. Moreover, we confirmed the fitness defect of fhlA regulated hydrogenase gene expression accelerated medium deacidification, whereby facilitating urease activity for development of new therapeutic approaches to counteract UTIs caused by P. mirabilis. For the homologous genes of FhlA, FHL hydrogenase and formate dehydrogenase H (FDH-H encoded by fdhF), we searched the genome of P. mirabilis HI4320. No similar FHL hydrogenase genes in P. mirabilis were found in the upstream promoter region of fhlA from the genomic DNA of P. mirabilis HI4320 (Table 1) and the fhlA locus, fhlA and fdhF genes in P. mirabilis N2. To investigate the role of FhlA and FHL in uropathogenic P. mirabilis, we searched the genome of P. mirabilis HI4320 for the homologous genes of FhlA, FHL hydrogenase and formate dehydrogenase H (FDH-H encoded by fdhF). Only a putative FHL hydrogenase 4 operon (hyf) is present in P. mirabilis HI4320. No similar FHL hydrogenase 3 operon (hyc) was found in P. mirabilis HI4320. We amplified the entire DNA fragment (11,956 bp) containing hyfABCDEFGHIJhycI and the upstream region of hycI and fhlA gene locus sequencing. Paired with “fhlA_reporter assay. Paired with “fhlA_complementation. Paired with “fhlA_site_direct_R”

Table 1. Primers used in this study. The underlined sequences represent restriction endonuclease cutting sites.

| Primer | Sequence (5′–3′) | Description |
|--------|-----------------|-------------|
| fhlA_com_F | GGATCCATAAGAAATACGCGTTTGG | For fhlA complementation. Paired with “fhlA_com_R” |
| fhlA_com_R | GTCGACCTAAAGATCTCAGGAAATGGA | For fhlA gene locus sequencing. Paired with “fhlA_promoter_F” |
| fhlF_com_F | TCGGTTACGAGAAGTAAACG | For hyfF complementation. Paired with “hyfF_com_R” |
| fhlF_com_R | CGGCTGGCAGCATGCTCGGAGA | For fhlA gene locus sequencing. Paired with “fhlA_promoter_R” |
| rpoN_com_F | GAGCTATAGGATTCACTCGCATC | For rpoN complementation. Paired with “rpoN_com_R” |
| rpoN_com_R | CTGCAGACCATGTCTCTCTCTCTC | For fhlA gene locus sequencing. Paired with “fhlA_promoter_F” |
| hyfA_promoter_F | GCACTGAGATCTCAGGTTATG | For hyfA reporter assay. Paired with “hyfA_A_promoter” |
| hyfA_promoter_R | CTGCAGATCGGCGACATCATCGCAGA | For hyfA gene locus sequencing. Paired with “hyfA sites_direct_R” |
| fhlA_reporter_F | TTAGAAAATATAATCGCG | For fhlA reporter assay. Paired with “fhlA_A_complementation” |
| fhlA_reporter_R | TCTAGAATGACACCAAGGATTGC | For fhlA reporter assay. Paired with “fhlA_A_promoter” |
| fhlF_reporter_F | GCACTGAGGACACAGAATTCTCACA | For fhlF reporter assay. Paired with “fhlF_A_promoter_R” |
| fhlF_reporter_R | CTGCAGAAGCATTGCTCTGTGTCA | For fhlF gene locus sequencing. Paired with “fhlF_A_promoter_F” |
| hyF报告_F | CTAGCTCTTCGCGGCTCTCTTA | For hyfF operon genes locus sequencing. Paired with “hyfF_A_promoter” |
| hyF报告_R | CTAGCTCTTCGCGGCTCTCTTA | For fhlA gene locus sequencing. Paired with “fhlA_A_promoter_R” |
| fhlA_site_direct_F | CGGTTGCACACATCGCC | For fhlA strain construction. Paired with “fhlA_site_direct_R” |
| fhlA_site_direct_R | AAATAGCGCCTTATTACGTCG | For fhlA site construction. Paired with “fhlA_site_direct_R” |

Results

Counterparts of the FHL hydrogenase hyc locus, fhlA and fdhF genes in P. mirabilis N2. To

For fhlA gene locus sequencing. Paired with “fhlA_promoter_F”

For fhlA gene locus sequencing. Paired with “fhlA_A_promoter”

For fhlA strain construction. Paired with “fhlA_site_direct_R”

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Figure 1. *P. mirabilis* hyf locus and involvement of FHL-related genes in acid resistance. (a) (i) The *P. mirabilis* FHL hydrogenase gene locus (*hyfA-J-hycI*) corresponds to the well characterized *hyc* locus in *E. coli* K-12 MG1655 and UPEC CFT073 with corresponding genes in shadows. An amino acid sequence analysis of the *hyf* locus in *P. mirabilis* N2 and its counterparts in *E. coli* K-12 MG1655 and *E. coli* CFT073 was performed using position-specific iterative BLAST. The eight proteins of the *hyf* locus in *P. mirabilis* N2 are similar to the HycB, HycC, HycD, HycE, HycF, HycG, HycH and HycI in *E. coli* K-12 MG1655 and *E. coli* CFT073. The percent amino acid similarities between *P. mirabilis* N2 and *E. coli* K-12 MG1655 or *E. coli* CFT073 were shown below each gene. The number inside white arrow represents the amino acid length of each protein. (ii) The *P. mirabilis* *fhlA* and *fdhF* loci correspond to those of *E. coli* K-12 MG1655 and UPEC CFT073 with corresponding genes in shadows. An amino acid sequence analysis was carried out the same as above. The predicted amino acid sequences of *fhlA* and *fdhF* in *P. mirabilis* N2 are similar to FhlA and formate dehydrogenase-H respectively in *E. coli* K-12 MG1655 and *E. coli* CFT073. The percent amino acid similarities between *P. mirabilis* N2 and *E. coli* K-12 MG1655 or *E. coli* CFT073 were shown below each gene. The number inside white arrow represents the amino acid length of each protein. (b) The survival rate of the wild-type, mutants (*fhlA, hyfG* and *rpoN*), complemented strains (*fhlAc, hyfGc* and *rpoNc*) and *fhlAcSD* strain after exposure to acid (pH 3). Acid survival rate (expressed as percent) was calculated with the following formula: 100 × (CFU after acid treatment/CFU before acid treatment). Significant differences from the wild-type bacteria were determined by using the one-way ANOVA with Tukey’s multiple-comparison test (*P < 0.05, **P < 0.01). The data are averages and standard deviations of three independent experiments. wt, wild-type; *fhlA, fhlA* mutant; *hyfG, hyfG* mutant; *fhlAc, fhlA* complemented strain; *hyfGc, hyfG* complemented strain; *fhlAcSD, fhlA* mutant containing altered FhlA (F291I, T292S) in pGEM-T Easy; *rpoN, rpoN* mutant; *rpoNc, rpoN* complemented strain.
fhIA and hyf are involved in acid resistance. Knowing FhlA is the transcriptional activator for the expression of the genes encoding FHL components of *E. coli* and *E. coli* hyEc (encoding catalytic large subunit of hydrogenase 3) mutant has been shown to display reduced acid survival compared to the wild-type strain, we first investigated whether FhlA and Hyf hydrogenase affect acid resistance in *P. mirabilis* N2. We performed homologous recombination to construct kanamycin-resistant *fhIA* and *hyfG* mutants, followed by Southern blotting to verify the mutant clone. In view that the large subunit of hydrogenase is critical for hydrogenase activity, *hyfG* deletion stands for functional loss of Hyf activity in the study. There was no difference between the growth of the wild-type bacteria and respective mutants (Fig. 6c). We then tested the ability of the wild-type strain, *fhIA* and *hyfG* mutants and respective complemented strains to survive the acid exposure. We found that the survival rate of both *fhIA* and *hyfG* mutants was significantly lower than that of the wild-type strain and the respective complemented strains (Fig. 1b). The results indicate that FhlA and Hyf are involved in resistance to extreme acid exposure in *P. mirabilis*. FhlA was reported as an enhancer binding protein which could hydrolyze ATP to activate RpoN-dependent transcriptions through the RpoN-interacting motif, GAFTGA. Thus, we constructed the site-directed mutant *fhlAsd* strain (the *fhIA* mutant containing an altered FhlA with the conserved RpoN-interacting motif GAFTGA changing into GAISGA in the pGEM-T Easy vector) which exhibited no growth defect compared to the wild-type bacteria. Then we assessed the survival in acid using the *fhlAsd* strain, *fhIA* and the *rpoN*-complemented strain. The survival of *fhlAsd* strain and *rpoN* mutant was reduced relative to the wild-type and the *rpoN*-complemented strain. We concluded that FhlA (requiring the GAFTGA motif for RpoN-interaction), RpoN and HyfG contribute to the acid resistance (Fig. 1b).

**fhIA and fdhF are regulated by FhlA and induced by formate and anaerobiosis.** In *E. coli*, the transcription of both *fhIA* and *hyf* operons is regulated by FhlA and RpoN. To identify conserved promoter elements for FhlA-dependent transcriptions, we submitted position weight matrices of FhlA and RpoN from characterized FhlA- and RpoN-dependent promoters (Table 2) to the Regulatory Sequence Analysis Tools (RSAT) server. TGCGACGNNNNTGCA/T and the palindromic sequence TGA/TC-A/GAA/TA/GAT-GA/TCA were shown to be the conserved binding sites for FhlA and RpoN respectively. We found two putative FhlA and RpoN binding sequences (Fig. 2a). We therefore performed the reporter assay to examine if *fhIA*, *hyf* and *fdhF* sequences (Fig. 2a). We therefore performed the reporter assay to examine if *fhIA*, *hyf* and *fdhF* are regulated by FhlA and RpoN. We found *fhIA*, *hyf* and *fdhF* promoter activities were reduced in the *fhIA* and *rpoN* mutants relative to the wild-type and respective complemented strains at 3, 5, 7, and 24 h after incubation (Fig. 2b,c). In addition, the RpoN-interacting domain of FhlA is responsible for regulation of *fhIA* and *fdhF* promoter activities (Fig. 2b,c). According to the RpoN conserved regulatory sequence not in the *fhIA* gene direction, the reporter assay showed loss of *rpoN* or RpoN-dependent *fhIA* had no effect on *fhIA* promoter activity during the time period tested (Fig. 2d). It is known *fhIA*, *hyf* and *fhIA* could be induced by either formate or anaerobiosis in *E. coli*. We tested the effect of 30 mM formate or anaerobic condition on the promoter activities of *fhIA*, *fdhF* and *hyf* after incubation for 5 h. We found *P. mirabilis* *fhIA* and *hyf* were induced significantly by 30 mM formate and anaerobiosis respectively (Fig. 2f,g) while formate and anaerobiosis seemed not have a significant effect on *fhIA* promoter activity (Fig. 2e). The results of *fhIA* promoter activity contrasted with previous reports that showed formate and anaerobiosis could trigger expression of FhlA-regulated *hypABCDE*-*fhIA* in *E. coli*. Figure 2b–d showed the wild-type *P. mirabilis* displayed *fhIA*, *fdhF* and *fhIA* promoter activities under the aerobic condition at 3, 5, 7 and 24 h after incubation. In summary, promoter activities of *fdhF* and *hyf* were under the control of FhlA in response to formate and anaerobiosis.

**The deacidification of the medium requires fhIA and hyf.** It has been known that formate, a product of mixed-acid fermentation, can be converted to H$_2$ and CO$_2$ by the FHL complex, in a process consuming protons and resulting in medium deacidification, thereby increasing bacterial survival. We hypothesized that uropathogenic *P. mirabilis* could use the FHL system to consume protons, leading to deacidification of the environment. Therefore, we performed deacidification experiments using the synthetic urine at pH 4.8 (± 0.2) to test if loss of *hyfG* or *fhIA* affects the deacidification ability. The data showed both *hyfG* and *fhIA* mutants displayed a significant delay in deacidification, especially for *hyfG* mutant, compared to the wild-type and respective-complexion matrices of FhlA and RpoN binding sequences. These weight matrices based on 13 characterized FhlA-dependent promoters and 186 characterized RpoN-dependent promoters.

| Table 2. Position-weight matrices of FhlA and RpoN binding sequences. These weight matrices based on 13 characterized FhlA-dependent promoters and 186 characterized RpoN-dependent promoters. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| FhlA | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| A | 1 | 0 | 4 | 2 | 5 | 7 | 5 | 5 | 9 | 0 | 0 | 6 | 1 | 7 |
| C | 0 | 1 | 1 | 10 | 2 | 2 | 1 | 0 | 1 | 0 | 2 | 1 | 11 | 1 |
| G | 2 | 12 | 1 | 0 | 5 | 1 | 2 | 5 | 2 | 0 | 10 | 1 | 1 | 4 |
| T | 10 | 0 | 7 | 1 | 1 | 3 | 5 | 3 | 1 | 13 | 1 | 5 | 0 | 2 |
| RpoN | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| A | 12 | 2 | 0 | 12 | 139 | 11 | 55 | 51 | 46 | 44 | 38 | 13 | 4 | 1 | 9 | 76 |
| C | 14 | 0 | 0 | 147 | 23 | 122 | 17 | 48 | 64 | 42 | 62 | 22 | 18 | 2 | 173 | 5 |
| G | 10 | 184 | 186 | 6 | 18 | 10 | 103 | 69 | 36 | 35 | 43 | 15 | 10 | 181 | 1 | 17 |
| T | 150 | 0 | 0 | 21 | 6 | 43 | 11 | 18 | 40 | 65 | 43 | 136 | 154 | 2 | 3 | 88 |
Figure 2. The transcription regulations of hyf, fdhF and fhlA genes in *P. mirabilis*. (a) Schematic promoter region of fhlA, fdhF and hyf. FhlA and RpoN binding sites were predicted by Regulatory Sequence Analysis Tools24. Promoter activities of (b) hyf, (c) fdhF and (d) fhlA in wild-type, mutants (fhlA and rpoN), complemented strains (fhlAc and rpoNc) and fhlAcSD strain. The activity of XylE in the hyf-, fdhF- or fhlA-xyLE reporter plasmid-transformed bacterial strain was determined using the reporter assay at 3, 5, 7 and 24 h after incubation. (e–g) Inductivity of promoter activities of fhlA, fdhF and hyf in wild-type strain by formate or anaerobiosis. The overnight cultures of fhlA-, fdhF- or hyf-xyLE reporter plasmid-transformed wild-type strain were regrown for 5 h. The activity of XylE in the reporter plasmid-transformed wild-type strain was determined after treatment of formate or anaerobiosis for 30 min. nil, aerobic condition; formate, 30 mM formate; anaerobic, anaerobic condition. Significant differences from the wild-type bacteria were determined by using two-way ANOVA (in b–d) and by one-way ANOVA (in e–g) with Tukey’s multiple-comparison test (*P < 0.05; **P < 0.01; ***P < 0.001). The data are averages and standard deviations of three independent experiments. wt, wild-type; fhlA, fhlA mutant; fhlAc, fhlA complemented strain; fhlAcSD, fhlA mutant altered FhlA (F291I, T292S) in pGEM-T Easy; rpoN, rpoN mutant; rpoNc, rpoN complemented strain.
Type strain and mutants of hyfG essential process to deacidify synthetic urine medium for acidification was observed for wild-type and the mutants. The data suggest that urea hydrolyzed by urease is an hyfGase activity of rpoN urease activity of mutant still decreased relative to the wild-type bacteria (Fig. 4b). The results show that both urease activity and the involvement of fhlA activity to a certain level from pH 5 to 9 after urease activity relative to the wild-type bacteria in this pH range (Fig. 4b). The decreased relative to the wild-type, in contrast to that of rpoN.

Subsequently, we assessed the urinary stone formation using the same synthetic urine at pH 5.8. We found that in medium deacidification, we included the ureC (encoding urease subunit) mutant in deacidification assay (Fig. 3a). A similar reduction in deacidification ability of fhlA mutant was observed in the fhlAcSD mutant while rpoN mutant had a similar deacidification pattern to the wild-type strain (Fig. 3a).

Urease activity of P. mirabilis is pH-dependent and loss of hyf or fhlA gene affects urease activity and urinary stone formation. Urease can catalyze the hydrolysis of urea into ammonia, rising pH value and promoting urinary stone formation. The urease activity changes with different pH value in Helicobacter pylori, being activated by acidic pH down to pH 2.5 and 3 pH-dependent and loss of hyf or fhlA gene affects urease activity and urinary stone formation. To understand whether urease activity of P. mirabilis is affected by pH value, we monitored the urease activity in LB broth at different pH values and found that urease activity was very low at pH 4, increased gradually in the pH range from 5 to 9 and dropped suddenly at pH 10 (Fig. 4a). Knowing fhlA and hyfG contributed to acid resistance, we assessed the urease activity in wild-type strain, hyfG and fhlA mutants and respective complemented strains in the pH range from 5 to 9. The data showed that urease activity of hyfG and fhlA mutants were lower than the wild-type and respective complemented strains in the pH range from 5 to 8 (Fig. 4b). Likewise, fhlAcSD strain and rpoN mutant had reduced urease activity relative to the wild-type bacteria in this pH range (Fig. 4b). The rpoN mutant could restore the activity to a certain level from pH 5 to 9 after rpoN-complementation (Fig. 4b). Interestingly, we found the urease activity of hyfG, fhlA and fhlAcSD mutants was not different from that of the wild-type strain at pH 9 while urease activity of rpoN mutant still decreased relative to the wild-type bacteria (Fig. 4b). The results show that both urease activity and the involvement of fhlA and hyfG in the urease activity is pH-dependent in P. mirabilis. Subsequently, we assessed the urinary stone formation using the same synthetic urine at pH 5.8. We found that the ability of stone formation in fhlA and hyfG mutants was significantly lower than that of wild-type strain and respective complemented strains (Fig. 4c). The ability to form urinary stones of fhlAcSD mutant significantly decreased relative to the wild-type, in contrast to that of rpoN mutant being comparable to the wild-type strain.

To differentiate between direct proton consumption and modulation of urease activity for the role of hyf and fhlA in medium deacidification, we included the ureC in deacidification assay in synthetic urine. No medium deacidification but slight acidification by the ureC mutant was observed (Fig. 3a). In addition, using synthetic urine medium in which urea has been replace by an equivalent amount of NH4Cl, the medium deacidification did not occur in wild-type and mutants of hyfG, fhlA and ureC (Fig. 3b). Slight acidification was observed for wild-type and the mutants. The data suggest that urea hydrolyzed by urease is an essential process to deacidify synthetic urine medium for P. mirabilis and the role of hyfG and fhlA in medium deacidification is mainly dependent on modulation of urease activity. These results suggest that fhlA-regulated hyf expression could help P. mirabilis to deacidify the environment via facilitating urease activity and subsequent urinary stone formation.

fhlA-regulated hyf expression assists bacterial colonization in the mouse urinary tract. To confirm the finding that FHL and FhLA were responsible for mouse colonization in P. mirabilis, we investigated if fhlA and hyfG are associated with UTIs caused by P. mirabilis. The colonization ability was assessed in wild-type strain and mutants of hyfG and fhlA using the UTI mouse model. In the bladder, the colonization ability...
of fhlA and hyfG mutant were significantly lower than wild-type strain (Fig. 5a). Both fhlA and hyfG mutants exhibited a significantly low ability of colonization in the kidney relative to the wild-type strain (Fig. 5b).

**Loss of fhlA or hyf does not affect proton motive force-related motility, drug susceptibility and growth under carbonyl cyanide m-chlorophenyl hydrazone (CCCP).** Previous studies showed that Hyf complex is similar to respiratory NADH dehydrogenase complex I11,13,14 and the complex I was shown to provide the proton motive force (PMF)31, which could affect acid resistance, susceptibilities of polymyxin B and aminoglycosides, motility and growth under treatment of the PMF uncoupler32–37. We monitored the PMF-

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**Figure 4.** The pH-dependent urease activity and the effect of fhlA, hyfG or rpoN mutation on urease activity and stone formation in *P. mirabilis*. (a) The urease activity of wild-type bacteria regrown in LB for 5 h was determined by phenol-hypochlorite assay at pH 5–9. The relative urease activity is calculated with the following formula: OD̄₂₅ of test/OD̄₂₅ of wt at pH 7. Significant differences from the result at pH 7 were determined by using the Student’s *t* test (*P* < 0.05). (b) Urease activities of the wild-type, mutants (fhlA, hyfG and rpoN), complemented strains (fhlAc, hyfGc and rpoNc) and fhlAcSD strain regrown in LB for 5 h were measured at different pH values. The relative urease activity is calculated with the following formula: OD̄₂₅ of test/OD̄₂₅ of wt at pH 7. Significant differences from the wild-type bacteria at each pH were determined by using two-way ANOVA with Tukey’s multiple-comparison test (*P* < 0.05; **P** < 0.01). (c) Stone formation of the wild-type, mutants (fhlA, hyfG and rpoN), complemented strains (fhlAc, hyfGc and rpoNc) and fhlAcSD strain. Bacteria were regrown and adjusted to 2 × 10⁸ CFU/ml with synthetic urine and incubated for 1 h at 37 °C. The optical density at 630 nm of suspensions was measured as the intensity of stone formation. The relative urinary stone formation is calculated with the following formula: OD₆₃₀ of test/OD₆₃₀ of wt. Significant differences from the wild-type bacteria were determined by using one-way ANOVA with Tukey’s multiple-comparison test (*P* < 0.05; **P** < 0.01). The data are averages and standard deviations of three independent experiments. wt, wild-type; fhlA, fhlA mutant; hyfG, hyfG mutant; fhlAc, fhlA complemented strain; hyfGc, hyfG complemented strain; fhlAcSD, fhlA mutant containing altered FhlA (F291I, T292S) in pGEM-T Easy; rpoN, rpoN mutant; rpoNc, rpoN complemented strain.
related phenotypes including drug susceptibilities and motility in the wild-type bacteria and \( fhlA \) and \( hyfG \) mutants. Neither the MICs of the drugs (polymyxin B, streptomycin and gentamicin) used nor the swarming and swimming motility was different between wild-type strain and respective mutants (Table 3; Fig. 6a,b). We also examined the effect of CCCP, a PMF uncoupler, on growth of the wild-type, \( hyfG \) mutant and \( fhlA \) mutant. The growth curves of all strains showed no difference in the presence or the absence of CCCP (Fig. 6c). These results indicate that mutation of \( fhlA \) or \( hyfG \) did not affect the PMF-related phenotypes examined except acid resistance. It implies that the consumption of protons may be the main reason for FhlA-regulated hyf expression in acid resistance.

**Discussion**

To our knowledge, this study is the first to demonstrate that RpoN-dependent FhlA and hydrogenase Hyf participate in acid tolerance by medium deacidification and facilitate urease activity, urinary stone formation and mouse colonization. A typical characteristic of *P. mirabilis* UTIs is the development of urinary stones. *P. mirabilis* would rapidly invade bladder epithelium cells and start to form extracellular clusters within the bladder lumen adjacent to the urothelium38,39. The concentrated urease activity within extracellular clusters would cause mineral deposition that serves as an early step in urinary stone formation38. Urease decomposes urea to produce

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**Table 3.** Minimal inhibitory concentration of gentamicin (Gm), polymyxin B (PB) and streptomycin (Sm) in wild-type (wt) and mutants (\( hyfG \) and \( fhlA \)).

| Strain | MIC (μg/ml) | PB | Gm | Sm |
|--------|-------------|----|----|----|
| wt     | 50,000      | 8  | 32 |    |
| \( hyfG \) | 50,000      | 8  | 32 |    |
| \( fhlA \) | 50,000      | 8  | 32 |    |

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**Figure 5.** Colonization in mice by the wild-type *P. mirabilis*, \( hyfG \) mutant or \( fhlA \) mutant. ICR mice were inoculated transurethrally with bacteria at a dose of \( 1.5 \times 10^7 \) CFU per mouse. Bacterial loads (CFU) in the (a) bladders and (b) kidneys were determined on day 3 after inoculation. Horizontal bars indicate the average for each group, and the limit of detection was 100 CFU/g organ. Significant differences were determined using the Wilcoxon rank sum test (*\( P < 0.05 \); **\( P < 0.01 \)). wt, wild-type; \( hyfG \), \( hyfG \) mutant; \( fhlA \), \( fhlA \) mutant.
ammonia, damaging renal epithelial cells\(^{40}\) and facilitating the formation of infectious urinary stones, carbonate apatite and struvite\(^{38}\). \(P\). mirabilis extracellular clusters draw a massive neutrophil response and provide the environment to form biofilms\(^{39}\). The formation of extracellular clusters is critical to prevent clearance of bacteria by neutrophils and would influence the severity of infection\(^{38,39}\). In addition, medium deacidification has been shown to be involved in acid resistance of \(Salmonella\) enterica serovar Typhimurium\(^{41}\). We found FhlA and HyfG not only participated in medium deacidification and acid resistance (Figs. 1b, 3) but also affected urease activity and urinary stone formation in \(P\). mirabilis (Fig. 4b). We identified that urease activity was pH-dependent, increasing in the pH range from 5 to 9 (Fig. 4a) and was dependent on FhlA and Hyf in the pH range from 5 to 8 (Fig. 4b). We showed that FhlA and Hyf-mediated deacidification or urine alkalization assisted in urease activity, stone formation and mouse colonization (Figs. 3a, 4b,c, 5), suggesting that FhlA and Hyf play a role in \(P\). mirabilis virulence factor expression and virulence. In this regard, the data of transposon insertion-site sequencing (Tn-Seq) by Armbruster et al. have showed \(P\). mirabilis hyfB, hyfC, hyfD, hyfE, hyfF, fdhF, and fhlA are all likely to contribute to colonization in vivo\(^{21}\).

The published \(Proteus\) genomes including \(P\). mirabilis HI4320, \(P\). mirabilis BB2000, \(P\). hauseri, \(P\). penneri and \(P\). vulgaris contain only a hydrogenase 4-like operon but not the hydrogenase 3-like operon. It is likely that hydrogenase 4-like operon is the functional counterpart of hydrogenase 3-like operon for encoding the protein complex joining with FDH-H to form FHL in \(Proteus\) species. The facts that PCR produced a product of 4228 bp but not the predicted 524 bp using \(P\). mirabilis N2 genomic DNA as the template with primers annealing to the conserved region of hycD and hycE (data not shown) and \(P\). mirabilis hyfG mutant displayed reduced survival against acid exposure (Fig. 1b) as the hyc mutant\(^{18}\) reinforce the notion. Hyf belongs to group 4a hydrogenase and is highly conserved in gammaproteobacteria\(^{13}\). The key role of Hyf is to convert proton to hydrogen, namely deacidification\(^{1,11}\). The structure of Hyf is similar to respiratory complex I, indicating Hyf is probably involved in the formation of proton gradient and energy production\(^{13,19}\). We found hyfG and fhlA are required for the full extent of acid tolerance in \(P\). mirabilis (Fig. 1b). The dye DiOC\(_2\), commonly used to detect membrane proton gradient does not work in \(P\). mirabilis N2 as is the case for \(P\). mirabilis HI4320\(^{13}\). Instead, we determined other PMF-related phenotypes, motility and drug susceptibility, to know whether Hyf is involved in conservation of ammonia, damaging renal epithelial cells\(^{40}\) and facilitating the formation of infectious urinary stones, carbonate apatite and struvite\(^{38}\). \(P\). mirabilis extracellular clusters draw a massive neutrophil response and provide the environment to form biofilms\(^{39}\). The formation of extracellular clusters is critical to prevent clearance of bacteria by neutrophils and would influence the severity of infection\(^{38,39}\). In addition, medium deacidification has been shown to be involved in acid resistance of \(Salmonella\) enterica serovar Typhimurium\(^{41}\). We found FhlA and HyfG not only participated in medium deacidification and acid resistance (Figs. 1b, 3) but also affected urease activity and urinary stone formation in \(P\). mirabilis (Fig. 4b). We identified that urease activity was pH-dependent, increasing in the pH range from 5 to 9 (Fig. 4a) and was dependent on FhlA and Hyf in the pH range from 5 to 8 (Fig. 4b). We showed that FhlA and Hyf-mediated deacidification or urine alkalization assisted in urease activity, stone formation and mouse colonization (Figs. 3a, 4b,c, 5), suggesting that FhlA and Hyf play a role in \(P\). mirabilis virulence factor expression and virulence. In this regard, the data of transposon insertion-site sequencing (Tn-Seq) by Armbruster et al. have showed \(P\). mirabilis hyfB, hyfC, hyfD, hyfE, hyfF, fdhF, and fhlA are all likely to contribute to colonization in vivo\(^{21}\).

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the proton gradient and PMF. No difference of swarming motility, swimming and drug susceptibility between wild-type strain and *hyf* or *fhlA* mutant may indicate that the formation of PMF by FhlA-Hyf is negligible or compensable in these conditions. Although proton consumption should be the main reason for *fhlA* and *hyf*-involved acid tolerance. Possibly, it cannot be ruled out that the extent of PMF provided by FhlA-regulated Hyf may contribute to acid resistance of *P. mirabilis*. Recent studies showed that FHL-2 (FDH+Hyf) might couple with ATPase to generate PMF[42,43] and formate affects ATPase activity and changes the number of thiols[44]. Hyf activity is ATP-dependent, while the formate can increase the F$_{2}$F$_{1}$-ATPase activity[42]. It is worth noting that the effect of formate on ATPase activity disappeared in *hyf* mutant or under the respiratory condition[44]. In addition, the formate dehydrogenase-H activity was dependent on Hyf and F$_{2}$F$_{1}$-ATPase[46]. These studies indicate FHL-2 may contribute to regulation of formate-associated ATPase under fermentative condition. In this regard, our finding that *P. mirabilis* Hyf did not play an important role in generating PMF under aerobic condition may attribute to coupling of ATPase with FHL-2 only under fermentative or formate-rich conditions. Thus, it could not be ruled out that Hyf is involved in PMF formation under oxygen-limited condition during infection and contribute to virulence.

Unlike *fhlA* and *hyf* mutants, the urease activity of *rpoN* mutant still significantly decreased compared to the wild-type strain at pH 9 (Fig. 4b). Apparently, RpoN would have other regulatory pathways to affect urease activity at pH 9. In *P. mirabilis*, urea subunits and accessory proteins encoded by *ureDABCEF* operon has been shown to be regulated positively by UreR and negatively by H-NS in the transcription level[5]. Previous study showed that RpoN and its cognate enhancer binding protein NtrC regulated expression of urease gene operon indirectly through control *nac*, encoding a transcriptional factor Nac which could activate urease gene operon in *K. pneumoniae*[45]. We will investigate if other enhancer binding proteins such as NtrC, QseF and PspF are involved in the pathways of RpoN-regulated urease expression. In addition, we will examine whether UreR and H-NS are subject to the control of RpoN.

We found urinary stone formation ability of *rpoN* mutant was not impaired while *fhlA* mutant (lacking the enhancer binding protein, FhlA) was. In this regard, it was established that amino acids have effects on stone formation. For example, serine and asparagine could serve as the catalysts of this process[47]. Addition of asparagine enhancer binding protein, FhlA) was. In this regard, it was established that amino acids have effects on stone formation[48]. Previous reports showed formate and anaerobiosis could trigger transcription of FhlA-regulated *hypABCDEF*-*fhlA* operon in *E. coli*[42,43]. Two reasons could account for the discrepancy of *fhlA* expression in response to formate. First, In *E. coli*, *hypABCDEF*-*fhlA* constitutively express an operon subject to the control of FhlA, namely self-regulation of FhlA[49]. This is not the case for *P. mirabilis*, whose FhlA promoter is not dependent on RpoN and FhlA (Fig. 2a). Second, formate serves as a ligand to activate FhlA, thus leading to transcription of FhlA-dependent genes including *hypABCDEF*-fhlA operon in *E. coli*[50] but not *fhlA* in *P. mirabilis*. As for anaerobic induction of *fhlA*, there are an FNR binding site in the promoter region and an OxyS-interacting site in the 5’UTR of *fhlA* in *E. coli*[42]. FNR is a global regulatory protein that regulates gene expression in response to oxygen deprivation in *E. coli* and it was shown that FNR acts as an anaerobic activator of *hypABCDEF*-fhlA operon expression to control the expression of the hydrogenase maturation genes (Hyp) and FhlA. OxyS, a small regulatory RNA (sRNA) which is induced in response to the oxygen and oxidative stress in *E. coli*, inhibits FhlA translation by pairing with a short sequence overlapping the Shine-Dalgarno sequence, thereby blocking ribosome binding and translation[48]. Neither corresponding OxyS was found after searching for *P. mirabilis* sRNAs in the Bacterial Small Regulatory RNA Database (BSRD, https://kwanlab.bio.cuhk.edu.hk/BSRD/), nor FNR binding site was present in the *P. mirabilis* fhlA promoter region analyzed by RSAT. Therefore, unlike *E. coli*, fhlA which is regulated by OxyS sRNA and transcription factor FNR sensing oxygen availability, the *P. mirabilis* promoter of fhlA is unresponsive to anaerobiosis (Fig. 2e).

This study showed the proton-consuming acid resistance mechanism such as glutamate- and arginine-dependent acid resistance[51] may also contribute to urease activity in the presence of glutamate or arginine. Our preliminary data showed urease activity of wild-type *P. mirabilis* was induced by arginine and glutamate. What is the biological significance of the proton-consuming acid resistance of FHL? The different metabolites or signals would trigger different acid resistance system. Under stationary phase or the presence of extracellular glutamate, glutamate-dependent acid resistance would be important to bacterial acid response[52]. Likewise, arginine-dependent acid resistance is triggered by extracellular arginine[52]. However, FHL is the amino acid-dependent acid resistance induced by formate or anaerobiosis[53]. In this study, we found that *fhlA*, *hyf* and *fhlA* of FHL genes of wild-type *P. mirabilis* were expressed when cultured in the aerobic condition (Fig. 2b–d) and the expression of *fhlA* and *hyf* could also be induced by formate or anaerobiosis (Fig. 2g).

In this study, we showed FhlA-dependent *hyf* expression accelerates deacidification to facilitate urease activity and infectious urinary stone formation of *P. mirabilis* (Fig. 7) and subsequently mouse colonization. This is the first report investigating how *P. mirabilis* FHL-associated genes are involved in virulence and linking the ability of acid resistance to urease activity of *P. mirabilis*. The formate-induced FHL pathway provides a perspective for development of new approaches to counteract *P. mirabilis* UTIs. The prohibition against food producing formate (the pH above which calcium and magnesium phosphates come out of solution in urine) could form the basis of a strategy to prevent catheter encrustation in those with urinary tract colonization by urease-positive bacteria[53].
Materials and methods

Bacterial strains, plasmids, primers, reagents and growth conditions. The bacterial strains, plasmids and primers used in this study are listed in Tables 1 and 4. All chemicals were obtained from the Sigma-Aldrich unless otherwise indicated. Bacteria were routinely cultured in Luria–Bertani (LB) broth at 37°C with shaking at 200–250 rpm. The LSW− agar was used to prevent the phenotypic expression of swarming motility for selecting mutant clones and determining CFU. Ampicillin (100 μg/ml), chloramphenicol (40 μg/ml), kanamycin (100 μg/ml), tetracycline (20 μg/ml), streptomycin (100 μg/ml) was added to the medium as needed.

Synthetic urine was prepared according to a recipe previously described containing the following components (g/l): CaCl2·2H2O, 0.651; MgCl2·6H2O, 0.651; NaCl, 4.6; Na2SO4, 2.3; sodium citrate, 0.65; sodium oxalate, 0.02; KH2PO4, 2.8; KCl, 1.6; NH4Cl, 1.0; urea, 25.0; creatine, 1.1 and tryptic soy broth, 10.0. pH was adjusted to 5.8 and urine was sterilized by passing through a 0.2 μm pore-size filter.

The anaerobiosis was established and maintained in the anaerobic chamber (Whitley A35 anaerobic workstation).

Table 4. Strains and plasmids.

| Strain or plasmid | Genotype or relevant phenotype | Source or reference |
|-------------------|-------------------------------|---------------------|
| **P. mirabilis**  |                               |                     |
| *P. mirabilis* wt | wild-type strain; Tc<sup>+</sup> | Clinical isolate   |
| rpoN              | *P. mirabilis* N2; *rpoN* knock- | This study          |
| *fhlA*            | mutant; *fhlA* knockout mutant; |                     |
| *hyfG*            | *rpoN* mutant containing pGEM-T |                     |
| *fhlAc*           | *fhlA* knockout mutant; Kmr    |                     |
| *fhlAc<sub>SD</sub> | *fhlA* mutant containing pGEM-T | This study          |
| **Plasmids**      |                               |                     |
| pGEM-T Easy       | TA cloning vector; Amp<sup>+</sup> | Promega            |
| pGEM-T Easy-*rpoN*| *rpoN* sequence including its RBS; Amp<sup>+</sup> | This study          |
| pGEM-T Easy-*fhlA*| *fhlA* sequence including its RBS; Amp<sup>+</sup> | This study          |
| pGEM-T Easy-*hyfG*| *hyfG* sequence including its RBS; Amp<sup>+</sup> | This study          |
| pGEM-T Easy-*fhlA<sub>(F291I,T292S)</sub>*| *fhlA* mutant containing pGEM-T Easy-*fhlA* containing an altered FhlA with the conserved RpoN-interacting motif GAFTGA changing into GAISGA; Amp<sup>+</sup> | This study          |
| pACYC184-*fhlA-xylE*| *fhlA* reporter plasmid, pACYC184 containing intact *fhlA* promoter sequence before xylE; Cm<sup>+</sup> | This study          |
| pACYC184-*fdhF-xylE*| *fdhF* reporter plasmid, pACYC184 containing intact *fdhF* promoter sequence before xylE; Cm<sup>+</sup> | This study          |
| pACYC184-*hyf-xylE*| *hyf* reporter plasmid, pACYC184 containing intact *hyf* operon promoter sequence before xylE; Cm<sup>+</sup> | This study          |
Acid resistance assay. Acid resistance assay was performed as described by Wu et al.\textsuperscript{55} with some modifications. Overnight bacterial cultures were diluted 500-fold with fresh LB broth in centrifuge tubes and incubated at 37 °C with shaking at 220 rpm for 4 h. Cells were incubated in LB medium at pH 3.0 (± 0.1) for 2 h. Acid-treated cells and the untreated control (cells before the acid treatment) then were washed with phosphate-buffered saline (PBS), serially diluted in PBS and plated on LSW\textsuperscript{−} agar plates to determine the CFU. Acid survival rate (expressed as percent) was calculated with the following formula: 100 × (CFU after acid treatment/CFU before acid treatment).

Swarming assay. The swarming assay was performed on LB agar (tryptone, 10 g/l; yeast extract, 5 g/l; NaCl, 0.5 g/l; agar, 1.5%, w/v) plates as described previously.\textsuperscript{50} The swarming migration distance was monitored by following swarm fronts of the bacterial cells and recording progress every hour.

Swimming assay. The swimming assay was performed on LB agar (agar, 0.3%, w/v) plates as described previously.\textsuperscript{50} The swimming migration distance was recorded at 18 h after inoculation.

MIC assay. MICs of antibiotics for wild-type and mutant strains were determined by the broth microdilution method according to the guidelines proposed by the Clinical and Laboratory Standards Institute.

Growth curve analysis. Bacteria were grown overnight at 37 °C, the cultures were diluted to an initial optical density at 600 nm (OD\textsubscript{600}) of 0.01 in LB with or without 7.5 μM CCCP and then the OD\textsubscript{600} values were measured at 1-h intervals up to 8 h with a spectrophotometer.

Urease activity (phenol-hypochlorite) assay. Phenol-hypochlorite assay is based on the detection of ammonia released during urea hydrolysis. Ammonia can react with phenol-hypochlorite at high pH to form blue indophenol.\textsuperscript{56} Bacteria grown to log phase (5 h after subculture) were adjusted to 2 × 10\textsuperscript{8} CFU using LB and incubated statically in LB containing urea at 0.5 M for 2 h at 37 °C. Supernatants were collected (70 μl per well of 96-well microplates) for urease activity measurement after centrifugation at 12,000 rpm for 5 min. Urease activity was determined by measuring ammonia production after 2-h incubation in LB containing urea at 0.5 M. Briefly, 70 μl of phenol reagent (2% w/v phenol in 75% ethanol) and 70 μl of alkali reagent (0.28 M sodium hydroxide and 100 mM sodium hypochlorite) were added to each well. After one hour, the absorbance at 625 nm was measured using a microplate reader (SpectraMax M2, USA).

Urinary stone formation assay. Urinary stone formation assay was performed as described previously with some modifications. Wild-type and mutant strains were grown overnight in LB broth and diluted 100-fold in the same medium. After incubation for 5 h, bacteria were adjusted to 2 × 10\textsuperscript{8} CFU/ml with synthetic urine and incubated for another 1 h in the static condition at 37 °C. The optical density at 630 nm of well-suspended suspensions was measured as the intensity of stone formation.

UTI mouse model. The mouse model of UTIs was used as described previously.\textsuperscript{57} Six- to eight-week old female ICR mice were injected transurethrally with overnight cultures of bacteria at a dose of 1.5 × 10\textsuperscript{7} CFU per mouse. On day 3 after injection, mice were sacrificed and bladder and kidney samples were collected to determine the viable bacterial count. All animal experiments were performed in strict accordance to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Laboratory Animal Center (Taiwan), and the protocol was approved by the Institutional Animal Care and Use Committee of National Taiwan University College of Medicine.

Reporter assay. The reporter plasmid-transformed wild-type and mutant strains were grown overnight in LB broth with chloramphenicol (40 μg/ml) and diluted (100-fold) into 10 ml LB broth. Promoter activity was measured as described previously.\textsuperscript{58} For monitoring expression profiles, the XylE activity was monitored at 3, 5, 7 and 24 h after incubation. For signal induction, overnight bacterial cultures were regrown for 5 h and 30-min induction was performed in different conditions before the XylE activity was measured.

Deacidification assay. Overnight cultures were diluted 100-fold in fresh LB medium and incubated at 37 °C with shaking at 220 rpm for 5 h. Bacterial pellet was resuspended in synthetic urine (pH 4.8 ± 0.2). The pH value was monitored during 30–90 min at 10-min intervals by a pH meter (Jencomodel 6173, USA).

Statistical analysis. Statistical analyses were performed using GraphPad Prism software, version 6.01 or Microsoft Excel 2016. The specific statistical tests used are described in the figure legends.

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Author contributions

W.Y.L. performed experiments. W.Y.L. and S.J.L. performed data analysis. W.Y.L. and S.J.L. designed experiments. W.Y.L. and S.J.L. prepared the manuscript. W.Y.L. and S.J.L. wrote the paper.

Competing interests

The authors declare no competing interests.

Additional information

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