Characterization of UDP-Glucose Dehydrogenase and UDP-Glucose Pyrophosphorylase Mutants of *Proteus mirabilis*: Defectiveness in Polymyxin B Resistance, Swarming, and Virulence

Sin-Sien Jiang,¹ Tzu-Yi Lin,¹ Won-Bo Wang,² Ming-Che Liu,¹ Po-Ren Hsueh,³ and Shwu-Jen Liaw¹,3*

Department and Graduate Institute of Clinical Laboratory Sciences and Medical Biotechnology¹ and Graduate Institute of Microbiology,² College of Medicine, National Taiwan University, and Department of Laboratory Medicine, National Taiwan University Hospital,³ Taipei, Taiwan, Republic of China

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*Corresponding author. Mailing address: Department and Graduate Institute of Clinical Laboratory Sciences and Medical Biotechnology, College of Medicine, National Taiwan University, 10016 No. 1 Chang-Te Street, Taipei, Taiwan, R.O.C. Phone: 886-02-23123456, ext. 66911. Fax: 886-02-23711574. E-mail: sjliaw@ntu.edu.tw.

Proteus mirabilis is known to be highly resistant to the action of polymyxin B (PB). However, the mechanism underlying PB resistance is not clear. In this study, we used Tn5 transposon mutagenesis to identify genes that may affect PB resistance in *P. mirabilis*. Two genes, *ugd* and *galU*, which may encode UDP-glucose dehydrogenase (Ugd) and UDP-glucose pyrophosphorylase (GalU), respectively, were identified. Knockout mutants of *ugd* and *galU* were found to be extremely sensitive to PB, presumably because of alterations in lipopolysaccharide (LPS) structure and cell surface architecture in these mutants. These mutants were defective in swarming, expressed lower levels of virulence factor hemolysin, and had lower cell invasion ability. Complementation of the *ugd* or *galU* mutant with the full-length *ugd* or *galU* gene, respectively, led to the restoration of wild-type phenotypic traits. Interestingly, we found that the expression of Ugd and GalU was induced by PB through RppA, a putative response regulator of the bacterial two-component system that we identified previously. Mutation in either *ugd* or *galU* led to activation of RpoE, an extracytoplasmic function sigma factor that has been shown to be activated by protein misfolding and alterations in cell surface structure in other bacteria. Activation of RpoE or RpoE overexpression was found to cause inhibition of FlhDC and hemolysin expression. To our knowledge, this is the first report describing the roles and regulation of Ugd and GalU in *P. mirabilis.*
in LPS or capsular structures (16, 45, 57). UDP-glucose dehydrogenase (Ugd) is an enzyme that converts UDP-glucose into UDP-glucuronic acid (10). UDP-glucuronic acid is also necessary for the synthesis of EPS and LPS in many pathogenic bacteria (10, 21, 43, 53). Formation of these polysaccharides is critical to bacterial virulence (10, 28) because it enables the bacteria to evade attacks by host immune systems. Recent studies demonstrate that ugd mutation in Cryptococcus neoformans alters cell integrity and the mutant cells also become temperature sensitive and fail to grow in an animal model (17). Transcription of Salmonella ugd is controlled by three regulatory systems that respond to different signals (43, 44). The participation of multiple regulatory systems in the control of ugd expression suggests a role for the ugd gene product in a broad spectrum of environments. Till now, nothing has been known about the roles of gaU and ugd in P. mirabilis.

P. mirabilis is known to be highly resistant to the action of CAP, such as PB (40, 52). Although the detailed mechanisms underlying P. mirabilis resistance to PB are not clear, studies have shown that modification of LPS plays an important role in modulating CAP resistance in P. mirabilis (40, 52). Previously, we reported that RppA, a putative response regulator of the two-component system, can regulate PB susceptibility through modulating LPS modification in P. mirabilis (58). How RppA regulates LPS modification is not known. In this study, we used a Tn5 transposon mutagenesis approach to identify genes that may affect PB susceptibility in P. mirabilis. Two genes, ugd and gaU, whose products may be involved in LPS synthesis and modification were identified. Knockout mutants of these genes were found to be extremely sensitive to PB, presumably because of changes in LPS. These mutants also had lower ability to swarm and express virulence factors. More importantly, we found that the expression of these genes was under the control of RppA. To our knowledge, this is the first report describing the roles and regulation of Ugd and GaU in P. mirabilis.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth condition.** The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were routinely cultured at 37°C in Luria-Bertani (LB) medium. A medium, referred to as LSW-agar, was prepared to prevent the phenotypic expression of swarming motility and used for selecting Tn5-mutagenized clones (5).

**Transposon mutagenesis and identification of the mutated gene.** The mini-Tn5 transposon mutagenesis was performed as described previously (58). The Tn5-mutagenized cells were plated on the kanamycin (Km)-containing LSW-plates and then replica plated onto the LSW-plates containing 400 µg/ml PB. The matching colonies that grew on Km-containing plates but not on PB-containing plates were isolated. Chromosomal DNA was extracted from these PB-susceptible mutants and partially digested with AluI, and fragments over 4 kb were cloned into Smal-digested pGEM-4Z (Promega). Following the transformation of E. coli TOP10, Km-resistant Tn5-containing clones were selected. The nucleotide sequences of the cloned DNA fragments were determined and subjected to BLAST analysis at http://www.ncbi.nlm.nih.gov/. We then searched the sequence in the released genome sequence of P. mirabilis (http://www.sanger.ac.uk/) and cloned the ugd and gaU genes, including their promoter, by PCR TA cloning with primer pairs ugd-353F/ugd-compR and gaU-F1/gaU-R1, respectively (Table 2). The nucleotide sequence was determined step by step by using a 373A DNA sequencer (Applied Biosystems). P. mirabilis Ugd and GaU alignment with other Ugd and GaU proteins was performed using the DNAMAN software (version 4.15).

**Gene knockout by homologous recombination.** Full-length ugd, including its promoter region, was amplified by PCR using primers ugd-353F and ugd-compR (Table 2) and cloned into pGEM-T Easy (Promega) to generate pUgd. A streptomycin-resistant (Sm') cassette (14) was inserted into HindIII-digested pUGud to generate pUGud-Sm, which contains the Sm' cassette-inserted ugd gene. The DNA fragment containing the Sm' cassette-inserted ugd gene was cleaved from pUGud-Sm and ligated into Sall/SphI-digested pUT-mini-Tn5(Km)
to generate pUTugd-Sm. Gene inactivation mutagenesis by homologous recombination and confirmation of mutants with double-crossover events were performed as described previously (58).

Sequences flanking the galU gene were amplified by PCR using primer pairs Xbal-galU-up/FlgA-galU-promoter R and galU-down F/Xbal-galU-down R (Table 2), respectively, and cloned into pGEM-T Easy (Promega) to generate pGgalU-up and pGgalU-dn. pGgalU-up was digested with Sall/XbaI and the galU/upstream sequence-containing fragment was ligated to Sall/XbaI-digested pGmgly-udn to produce the pGgalU-updn plasmid, which contains the combined upstream and downstream sequences of the galU gene. A Km cassette was inserted into the XbaI-digested pGgalU-updn plasmid to generate pGgalU-updn-Km. The DNA fragment containing the combined upstream and downstream sequence of the galU gene inserted with the Km cassette was cleaved from pGgalU-updn-Km and ligated into Sall/SphI-digested pUT mini-Tn5(Km) (14) to generate pUTgalU-Km. Gene inactivation mutagenesis by homologous recombination and confirmation of mutants with double-crossover events were performed as described previously (58).

**Construction of the Ugd- and the GalU-complemented strains.** Full-length ugd and galU, including their promoter regions, were amplified by PCR using primer pairs ugd-353F/ugd-compR and galU promoter and coding region, with “galUR1” respectively, (Table 2) and cloned into pGEM-T Easy (Promega) to generate pGugd and pGgalU. DNA fragments containing full-length ugd and galU with their promoter regions were excised from pGugd and pGgalU, respectively, with Sall and SpfI. The DNA fragments were ligated into a Sall/SphI-digested low-copy plasmid, pACYC184, to generate the ugd and the galU complementation plasmids, pACYC184-ugd and pACYC184-galU. The pACYC184-ugd and pACYC184-galU plasmids were then transformed into the ugd knockout mutant and the galU knockout mutant, respectively, to generate the Ugd-complemented strain and the GalU-complemented strain.

**MIC assay.** In vitro determination of the MIC for PB was performed by the broth microdilution method according to the guidelines proposed by the Clinical and Laboratory Standard Institute (13). Stock solution of PB (40,960 µg/ml) prepared in sterile water was added to 96-well microtiter plates in 2-fold serial dilutions. Aliquots of bacterial culture (5 × 10^4 CFU/ml) were then dispensed into each well and incubated for 16 to 18 h. The MIC was defined as the lowest PB concentration at which no visible growth occurred. Determination of sodium dodecyl sulfate (SDS) susceptibility was performed as the MIC assay using SDS concentrations ranging from 62.5 to 4,000 µg/ml.

**Preparation and analysis of LPS.** LPS extraction and analysis were performed as described previously (23). A final bacterial concentration of about 10^8 cells/ml was used for atomic force microscopy (AFM) analysis. Precleaned slides were treated with 0.1% (wt/vol) poly-L-lysine and left to dry. A drop of bacterial suspension in distilled water (20 µl) was applied onto treated slides. After adsorption for 30 min, distilled water was added to remove the unabsorbed cells. AFM imaging was performed as described by Hsieh et al. (26). An SPI 3800 atomic force microscope (Seiko, Japan) was used.

**DNA uptake ability.** The overnight bacterial culture was diluted 100-fold and incubated at 37°C with vigorous shaking (225 rpm) for 2 h. Prior to imaging analysis, bacteria were gently washed with distilled water and were re suspended in water (250 µl). A final bacterial concentration of about 10^7 cells/ml was used for atomic force microscopy (AFM) analysis. Pre-cleaned slides were treated with 0.1% (wt/vol) poly-L-lysine and left to dry. A drop of bacterial suspension in distilled water (20 µl) was applied onto treated slides. After adsorption for 30 min, distilled water was added to remove the unabsorbed cells. AFM imaging was performed as described above.

**Swarming migration assay.** The swarming migration assay was performed as described previously (23, 34). Briefly, an overnight bacterial culture (5 µl) was inoculated centrally onto the surface of dry LB swarming agar (2% [wt/vol])

### Table 2. Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (′5′ to ′3′)</th>
<th>Gene amplified</th>
<th>Size of product (bp)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Km-l out</td>
<td>GAGCTCGAATTCGGCCTAG</td>
<td>—</td>
<td>—</td>
<td>For identification of transposon-inserted genes</td>
</tr>
<tr>
<td>Km-l O out</td>
<td>CCTGAGGGCATGCAAGTCCT</td>
<td>—</td>
<td>—</td>
<td>For identification of transposon-inserted genes</td>
</tr>
<tr>
<td>ugd-compR</td>
<td>CACGACGAGTCTGTGACTAAA</td>
<td>ugd promoter and coding region</td>
<td>1,562</td>
<td>For ugd cloning and complementation. Paired with “ugd-353F”</td>
</tr>
<tr>
<td>ugd-353F</td>
<td>GAATAGGAAAAGGACATTTAACAG</td>
<td>ugd promoter</td>
<td>353</td>
<td>For ugd reporter assay. Paired with “ugd-353F”</td>
</tr>
<tr>
<td>rpoE promoterF</td>
<td>CCAAGTCCAACTCTTGGGCA</td>
<td>rpoE promoter</td>
<td>323</td>
<td>For rpoE reporter assay</td>
</tr>
<tr>
<td>ugd real- timeF</td>
<td>TAGAGATGCACTCCTCAGGAA</td>
<td>ugd</td>
<td>133</td>
<td>For ugd real-time PCR. Paired with “ugd real-timeR”</td>
</tr>
<tr>
<td>ugd real-timeR</td>
<td>GAATAGGAAAAGGACATTTAACAG</td>
<td>ugd promoter and coding region</td>
<td>1,377</td>
<td>For galU real-time PCR. Paired with “galU real-timeR”</td>
</tr>
<tr>
<td>flhDC real-timeF</td>
<td>CGCATCAAGCCTGCAAGT</td>
<td>flhDC</td>
<td>90</td>
<td>For flhDC real-time PCR. Paired with “flhDC real-timeR”</td>
</tr>
<tr>
<td>flhDC real-timeR</td>
<td>GCCAGATGCGCAGGAAAGT</td>
<td>galU</td>
<td>131</td>
<td>For galU real-time PCR. Paired with “galU real-timeR”</td>
</tr>
<tr>
<td>galU real-timeR</td>
<td>TGGTGGTTAGTGTGTTGTC</td>
<td>galU promoter and coding region</td>
<td>1,028</td>
<td>For galU real-time PCR. Paired with “galU real-timeR”</td>
</tr>
<tr>
<td>galU1F</td>
<td>CTTGCCTGCTAAACGCCATCAT</td>
<td>galU promoter</td>
<td>331</td>
<td>For galU cloning and complementation. Paired with “galUR1”</td>
</tr>
<tr>
<td>galU-promoterF</td>
<td>TACTTCTACCCATTGAT</td>
<td>galU promoter</td>
<td>353</td>
<td>For galU cloning and complementation. Paired with “galU promoter R”</td>
</tr>
<tr>
<td>galU-promoterR</td>
<td>CTTTATGGAACACTTACACT</td>
<td>galU upstream</td>
<td>331</td>
<td>For galU reporter assay and galU knockout</td>
</tr>
<tr>
<td>Xbal-galU-up F</td>
<td>TCTACTATTTACCTGAGC</td>
<td>galU downstream</td>
<td>1,028</td>
<td>For galU knockout. Paired with “galU promoter R”</td>
</tr>
<tr>
<td>galU-down F</td>
<td>CAATTTGGGTGGTGGTTGAGG</td>
<td>galU downstream</td>
<td>1,028</td>
<td>For galU knockout. Paired with “Xbal-galU-down R”</td>
</tr>
<tr>
<td>Xbal-galU-down R</td>
<td>TCTAGAGATTGGCCTTGCAC</td>
<td>For galU knockout</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*—, not applicable.*
plates, which were then incubated at 37°C. The swarming migration distance was measured by following swarm fronts of the bacterial cells and recording progress over 60-min intervals.

Measurement of cell differentiation, flagellin level, and hemolysin activity. Overnight LB cultures of the wild-type and related strains were inoculated onto the surface of dry LB swarming agar plates, which were then incubated at 37°C. Preparation of cells for cell differentiation, hemolysin, and flagellin assays was performed as described previously (34, 36). Cell morphology was observed after Gram-staining and examined by light microscopy at a magnification of 1,000-fold under oil immersion with an Olympus BH2 microscope equipped with a graticule. Flagellin level and cell membrane-associated hemolysin activity were assayed as described previously (34, 36).

Cell invasion assay. The overnight culture was diluted 100-fold and incubated for 3 h before the cell invasion assay, which was performed according to the protocol of Liaw et al. (34), with some modifications. Briefly, human urothelial NTUB1 cells were grown and then infected at 37°C with 1 ml of a bacterial suspension containing 5 × 10^7 cells for 1.5 h. Urothelial cells were then washed twice with PBS and incubated at 37°C in 1 ml of RPMI 1640 medium containing streptomycin (250 μg/ml) for another 1.5 h. Cells were washed twice again and then lysed by incubation with 1 ml of lysis solution at 37°C for 30 min. Cell lyses were diluted serially in saline, and viable bacteria were counted by plating on LSW– agar plates. Cell invasion ability was assigned as the percentage of viable bacteria that survived the streptomycin treatment versus the total inoculum.

Real-time RT-PCR. To study the effect of type IV mutation and PB on the expression of ugd and GalU mRNA, overnight LB cultures of the wild-type, the type IV knockout mutant, and the type IV-complemented strains were washed with PBS, and cultured in LB medium (10% sucrose, 0.5 mM KCl, 7.5 mM (NH₄)₂SO₄, 1.0 mM KH₂PO₄, 0.2% glucose, 0.01% Casamino Acids, 0.1 mM Tris-HCl, pH 7.4), diluted to an optical density at 600 nm of 0.3 to 0.4 in N minimal medium without or with 1 μg/ml PB, and grown for 4 h at 37°C. Total RNA was extracted, and real-time reverse transcription (RT)-PCR was performed as described previously (58) to monitor the expression of ugd and GalU mRNA. The levels of ugd and galU mRNA were normalized against 18S rRNA. For determination of flhDC mRNA, cells were plated on the LB agar plates and incubated for 4 h before collecting all the cells on the plate with LB broth. The level of flhDC mRNA in the cells was then determined by real-time RT-PCR to investigate the effect of ugd and galU mutations or the effect of RpOE on the expression of flhDC mRNA.

Reporter assay. The 0.35-kb promoter region of ugd amplified by primers ugd-353F and ugd-353R was cloned into pGEM-T Easy to create pGEMugd. The xylE-containing yTA plasmid (27) was cut by SacI/PstI, and the xylE-containing fragment was ligated to the SacI/PstI-cleaved pGEMugd plasmid to create pGEMugd-xylE. The pGEMugd-xylE plasmid was cut with SmaI/SphI, and the ugd promoter-xylE fusion DNA fragment was ligated to EcoRV/SphI-cleaved pACYC184 to construct the ugd-xylE reporter plasmid. The galU-xylE reporter plasmid was constructed in the same way using galU promoter-F and galU-promoter R as primers. The ugd-xylE or galU-xylE reporter plasmid contains the wild-type strain and the type IV knockout mutant were grown overnight in LB broth with 2 μg/ml chloramphenicol, diluted 100-fold in the same medium, and grown again with or without PB, and then Xy/E activity was monitored as described previously (27) for 3 to 5 h at 1-h intervals. Briefly, a 1-ml cell suspension at each time point was centrifuged, resuspended in assay buffer, and subjected to kinetic assay of enzyme activities after adding the substrate (catechol). Meanwhile, the OD_{600} value of the cell suspension was recorded. One unit of enzyme activity was defined as the amount of enzyme that converts 1 nmol substrate per minute. The specific activity of the enzyme was determined in terms of units per OD_{600} unit. Each experiment was repeated three times. The specific activity of xylE (mole/min/liter/OD_{600}) was calculated as ΔOD_{600}/Δt × 10^3 × ε × X/OD_{600} × 1 × dilute factor (ε = 22,000 M⁻¹ cm⁻¹). The xylE-xylE reporter plasmid (containing the 0.343-kb promoter fragment) was constructed in the same way. xylE-xylE reporter activity in the wild type, the ugd knockout mutant, and the galU knockout mutant was monitored after cells were plated on the LB agar plates for 2 to 5 h to investigate the effect of ugd and GalU mutation on the expression of xylE mRNA.

Nucleotide sequence accession numbers. The nucleotide sequences of P. mirabilis N2 ugd and GalU have been deposited in the DDBJ/EMBL/GenBank databases under accession no. FJ865582 and GQ558626, respectively.

RESULTS

Isolation of PB-sensitive P. mirabilis mutants. To isolate PB-sensitive mutants of P. mirabilis, a mini-Tn5 transposon mutagenesis approach was performed as described previously (58), and the PB-containing LSW– plates were used. Two mutants, n2 and n5, which were over 20,480 and 10,240-fold, respectively, more sensitive to PB than the wild-type P. mirabilis N2, were isolated (MIC of 2 and 4 versus >40,960 μg/ml).

The nucleotide sequence was obtained from the cloned DNA fragment flanking the mini-Tn5 in the mutants n2 and n5. Through searching the released P. mirabilis genome sequence (http://www.sanger.ac.uk/) using the sequence we had obtained, we found that Tn5 was inserted into a gene which we named ugd (UDP glucose dehydrogenase) and galU (UDP-glucose pyrophosphorylase). The ugd gene is between cpsF (PMI13190, a capsule synthesis gene) and wbnF (PMI13188, a nucleotide sugar epimerase). The galU gene is between a putative wbnF gene (PMI1489) and a gene locus, PMI1491. The ugd and galU genes, with their respective upstream regions, were cloned and sequenced. Analysis of the upstream sequences of ugd and galU genes revealed putative binding sites for Salmonella RcsB and PhoP in the upstream region of ugd and only the putative RcsB site in that of galU gene, according to the report of Mouslim et al. (44).

The nucleotide sequence of ugd was found to be 97% identical to the corresponding sequence of the sequenced P. mirabilis strain HI4320. Ugd is homologous to S. enterica serovar Typhimurium Ugd (77% identity, 88% similarity), Escherichia coli Ugd (75% identity, 90% similarity), and Pseudomonas aeruginosa Ugd (27% identity, 48% similarity). The nucleotide sequence of galU was found to be 99% identical to the corresponding sequence of the sequenced P. mirabilis strain HI4320. GalU is homologous to S. enterica serovar Typhimurium GalU (76% identity, 87% similarity), E. coli GalU (75% identity, 86% similarity), and P. aeruginosa GalU (46% identity, 64% similarity).

The P. mirabilis ugd knockout and galU knockout mutants exhibit increased susceptibility to PB. To demonstrate the role of Ugd and GalU in regulating PB susceptibility, we sought to construct ugd and galU mutants through allelic exchange mutagenesis. The ugd (dU2) and galU (dG1) knockout mutants were constructed by homologous recombination using plasmid pUT (see Materials and Methods). Southern blot analysis indicated that the mutants contained a single disrupted ugd or galU gene and no wild-type allele (data not shown). The MICs of PB for the dU2c and dG1c strains were 40,960 μg/ml, those for the dU2 and dG1 mutants were 2 and 4 μg/ml, respectively. To further confirm that Ugd and GalU can affect PB susceptibility, we constructed the Ugd- and GalU-complemented strains dU2c and dG1c by transforming pACYC184-ugd and pACYC184-galU into the dU2 and dG1 mutants, respectively. We found that the MICs of PB for the dU2c and dG1c strains were >40,960 μg/ml. Together, these data suggest that both Ugd and GalU are determinants of PB resistance in P. mirabilis.

The P. mirabilis ugd knockout and galU knockout mutants have altered LPS profiles. LPS modification plays an important role in PB susceptibility in many Gram-negative bacteria, including Salmonella, Yersinia, Pseudomonas, E. coli, and P. mirabilis (40, 41, 50, 56). To investigate the underlying cause of PB sensitivity in the ugd knockout and the galU knockout mutants, we compared the LPS profile of the two knockout mutants (dU2 and dG1) with those of the wild-type strain (N2)
and the complemented strains (dU2c and dG1c). The LPS was extracted from equal amounts of these bacterial cells and was subjected to SDS-PAGE analysis. As shown in Fig. 1, while the LPS profiles of the wild-type and the complemented strains were similar, those of the ugd knockout and the galU knockout mutants were markedly different. In both knockout mutants, the O-antigen LPS ladder was lost, and there was a deficiency in the lower part of LPS bands (Fig. 1, compare lane 1 with lanes 2 and 4). These data indicate that the ugd and galU mutants have a defect in LPS in its outer membrane. To determine whether the two knockout mutants synthesized amounts of LPS similar to those of the wild-type and complemented strains, the LPS was extracted from equal amounts of these bacterial cells, and the concentration of LPS was determined (see Materials and Methods). As shown in Table 3, while the wild-type and the Ugd-complemented and the GalU-complemented strains synthesized similar amounts of LPS, the two knockout mutants synthesized much less LPS than the wild-type and complemented strains. Together, the above data indicate that the ugd knockout and the galU knockout mutants have undergone qualitative and quantitative changes in LPS and these changes may cause the ugd and galU mutants to become more sensitive to PB.

**Cellular surface architecture and cell permeability of the P. mirabilis ugd knockout and galU knockout mutants.** Since bacterial Ugd and GalU enzymes have been shown to be involved in the synthesis of different surface structures, we next studied the detailed cellular surface topography of the log-phase cells using AFM. A clear trend emerged, showing that the cell surface roughness and valley-to-peak distance of the ugd knockout mutant and the galU knockout mutant were increased compared to those of the wild-type cells (Table 3). These data suggest that Ugd and GalU are determinants of P. mirabilis cell surface topology and that defects in these two enzymes can lead to an aberrant cell surface topology. Consistent with this, we also found that the ugd knockout mutant and the galU knockout mutant were more sensitive to SDS and had an increased DNA uptake ability compared to the wild-type strain (Table 3). Together, these data indicate that Ugd and GalU proteins are involved in the maintenance of cell surface topology and cell permeability in *P. mirabilis*.

**The swarming behavior of the P. mirabilis ugd knockout and galU knockout mutants.** As described in the introduction, swarming and CAP susceptibility could be coregulated. We thus tested the swarming behavior of the ugd and galU mutants. We found that the ns2 and ns5 mutant strains, which we isolated originally from the Tn5 transposon mutagenesis, could not swarm on the LB swarming agar plate after 18-h incubation (data not shown). To further confirm the effect of ugd and galU mutations on swarming, we compared the swarming behavior of the wild-type strain (N2), the ugd knockout mutant (dU2), the galU knockout mutants (dG1), the Ugd-complemented strain (dU2c), and the GalU-complemented strain (dG1c). Figure 2A shows that the ugd knockout mutant and the galU knockout mutant could not swarm at all on LB swarming agar plates after 18-h incubation. Figure 2B shows that while the ugd knockout and the galU knockout mutants could not migrate after 11-h incubation, the Ugd-complemented strain exhibited migration ability similar to that of the wild-type strain. The GalU-complemented strain restored partial swarming ability of the wild-type strain. These data indicate that the integrity of the Ugd and GalU proteins is essential for maintenance of swarming ability and that changes in Ugd and GalU enzymatic activities may affect the swarming behavior of *P. mirabilis*.

Swarming migration in *P. mirabilis* involves the coordinated

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**Table 3. Phenotypic traits of the wild type (N2), the ugd knockout mutant (dU2), the Ugd-complemented strain (dU2c), the galU knockout mutant (dG1), and the GalU-complemented strain (dG1c)**

<table>
<thead>
<tr>
<th>Trait</th>
<th>N2</th>
<th>dU2</th>
<th>dU2c</th>
<th>dG1</th>
<th>dG1c</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS MIC (μg/ml)</td>
<td>2,000</td>
<td>500</td>
<td>2,000</td>
<td>500</td>
<td>2,000</td>
</tr>
<tr>
<td>Relative DNA uptake ability&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
<td>3 ± 0.3</td>
<td>1 ± 0.2</td>
<td>4.9 ± 0.8</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>Total LPS (mg/ml)</td>
<td>13.2 ± 1.2</td>
<td>5.0 ± 0.5</td>
<td>14.0 ± 0.9</td>
<td>4.8 ± 0.4</td>
<td>13.7 ± 0.9</td>
</tr>
<tr>
<td>Relative invasion ability&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1</td>
<td>0.2 ± 0.04</td>
<td>0.9 ± 0.09</td>
<td>0.3 ± 0.05</td>
<td>0.8 ± 0.04</td>
</tr>
<tr>
<td>AFM&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Valley to peak (nm)</td>
<td>186.70 ± 51.17</td>
<td>362.17 ± 127.74</td>
<td>ND</td>
<td>262.70 ± 48.94</td>
</tr>
<tr>
<td>Roughness (nm)</td>
<td>182.82 ± 55.13</td>
<td>343.23 ± 120.99</td>
<td>ND</td>
<td>262.65 ± 49.84</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> Relative ratio of DNA uptake ability. The DNA uptake ability of the wild type was set at 1.

<sup>b</sup> Relative ratio of cell invasion ability. The cell invasion ability of the wild type was set at 1.

<sup>c</sup> At least five cells in three 1-μm<sup>2</sup> areas from three independent experiments; each result is expressed as the mean and the standard deviation.
differentiation of short vegetative cells bearing a few peritrichous flagella into long multinucleate swarm cells with a much greater surface density of flagella (2, 34, 35). To further confirm that changes in Ugd and GalU enzymatic activities may affect the swarming behavior of \textit{P. mirabilis}, we measured the amount of flagellin synthesized in the \textit{ugd} knockout mutant (dU2), the \textit{galU} knockout mutant (dG1), and the wild-type strain (N2) during one differentiation-dedifferentiation cycle of the bacteria. As shown in Fig. 3A, the \textit{ugd} and \textit{galU} mutants synthesized lower levels of flagellin than did the wild-type strain at each time point. Consistent with this, we also found that the \textit{ugd} and \textit{galU} mutants synthesized a smaller amount of mRNA of \textit{flhDC}, which is a master regulator controlling the expression of flagellum genes (49), than did the wild-type strain (Fig. 3C). We also tested whether the differentiation of \textit{P. mirabilis} was affected by \textit{ugd} and \textit{galU} mutations. To this end, we compared the cell lengths of the \textit{ugd} knockout mutant (dU2) and the \textit{galU} knockout mutant (dG1) with that of the wild-type strain (N2) during one differentiation-dedifferentiation cycle of the bacteria. The \textit{ugd} and the \textit{galU} mutants

**FIG. 2.** (A) Swarming migration of wild-type \textit{P. mirabilis} (N2), the \textit{ugd} knockout mutant (dU2), and the \textit{galU} knockout mutant (dG1) on LB swarming plates. Aliquots (5 \(\mu\)l) of overnight culture were inoculated centrally onto the plates. The plates were incubated at 37°C, and the representative pictures were taken at 18 h after incubation. (B) The swarming migration of wild-type \textit{P. mirabilis} (N2), the \textit{ugd} knockout mutant (dU2), the Ugd-complemented strain (dU2c), the \textit{galU} knockout mutant (dG1), and the GalU-complemented strain (dG1c). Aliquots (5 \(\mu\)l) of overnight culture were inoculated centrally onto the LB swarming plates. The plates were incubated at 37°C, and the migration distance was measured hourly after inoculation. The data represent the averages of three results of independent experiments with standard deviations.

**FIG. 3.** (A) Flagellin level of wild-type \textit{P. mirabilis} (N2), the \textit{ugd} knockout mutant (dU2), and the \textit{galU} knockout mutant (dG1). The flagellin level was determined at different time points after seeding the strains on LB agar plates. The value obtained with the wild-type cells at 4 h postseeding was set at 100%, and all other values were expressed relative to this value. The data represent the averages of results of three independent experiments with standard deviations. (B) Microscopic observation of cell differentiation of wild-type \textit{P. mirabilis} (N2), the \textit{ugd} knockout mutant (dU2), and the \textit{galU} knockout mutant (dG1). Cells were Gram-stained and viewed under oil (magnification, \(\times 1,000\)). Three independent experiments were performed, and the representative pictures showing cell differentiation at 4, 5, and 6 h postseeding on LB agar plates are shown. The increase in cell length was taken as a sign of cell differentiation. (C) Expression of \textit{flhDC} mRNA in wild-type \textit{P. mirabilis} (N2), the \textit{ugd} knockout mutant (dU2), and the \textit{galU} knockout mutant (dG1). Total RNA was isolated from respective cells at 4 h postseeding on LB agar plates and was then subjected to real-time RT-PCR for the measurement of \textit{flhDC} mRNA. The value obtained with the wild-type cells was set at 1. The data represent the averages of results of three independent experiments with standard deviations.
formed shorter cells than did the wild type during the differentiation cycle (Fig. 3B), indicating that ugd and galU mutations can affect the differentiation of *P. mirabilis*. Together, these data further confirm that the enzymatic activities of Ugd and GalU are required for the maintenance of the swarming ability of *P. mirabilis*.

**Hemolysin expression and cell invasion ability of the *P. mirabilis* ugd knockout and galU knockout mutants.** Both swarming and CAP susceptibility are correlated with the expression of virulence factors in the bacterium (3, 18, 19, 35). We thus examined the expression of hemolysin during one differentiation-dedifferentiation cycle of the bacteria by measuring the cell membrane-associated hemolysin activity. As shown in Fig. 4, while the ugd knockout mutant and the galU knockout mutant expressed lower levels of hemolysin activity than did the wild-type strain, the complemented strains restored the wild-type hemolysin activity during the incubation period. The ability of the bacteria to invade human urothelial NTUB1 cells was also measured. As shown in Table 3, the ugd knockout mutant and the galU knockout mutant had a much lower cell invasion ability than the wild-type strain.

**Expression of Ugd and GalU is induced by PB through an RppA-dependent pathway.** It has been shown that the expression of Ugd is regulated by two-component signal transduction systems in *Salmonella* (43, 44). We figured that the expression of Ugd and GalU might be regulated by RppA, a putative response regulator of the two-component system, because the latter has been shown by us to be able to regulate PB susceptibility by modulating LPS modification in *P. mirabilis* (58). The expression of Ugd and GalU in wild-type *P. mirabilis* (N2), the *rppA* knockout mutant (dA10), and the RppA-complemented strain (dA10c) in the presence or absence of PB (1 μg/ml) was measured by real-time RT-PCR. We found that in the absence of PB, the expression levels of Ugd and GalU in all three strains were similar. Interestingly, when PB was added to the culture media, the expression of Ugd and GalU was induced in the wild-type and RppA-complemented strains but not in the *rppA* knockout mutant (Fig. 5A). These data suggest that PB can induce Ugd and GalU expression through the RppA-dependent pathway. To confirm this, the reporter assays using the ugd-xylE or galU-xylE transcriptional fusion constructs were performed. As shown in Fig. 5B, in the absence of PB, xylE expression (activity) levels driven by the ugd or galU promoter were about the same in the wild-type and the *rppA* mutant strains. However, in the presence of PB (1 μg/ml), xylE expression (activity) from both the ugd and galU promoters was induced in the wild type but not in the *rppA* mutant strain. Therefore, PB seems to be able to serve as a signal that can induce Ugd and GalU expression through the RppA-dependent pathway.

*ugd and galU* mutations cause the activation of RpoE, which in turn inhibits the expression of FlhDC and hemolysin. It has been shown that membrane stress/disruption causes the acti-
vation of RpoE, an extracytoplasmic function sigma factor which controls the expression of an array of genes that are involved in pathogenesis, the folding of outer membrane proteins, and the synthesis of cell envelope proteins, phospholipid, involved in pathogenesis, the folding of outer membrane pro-

vative phenotype of these two mutants of P. mirabilis. That the ugd or galU mutation can cause increased sensitivity to PB has been reported previously for Salmonella, E. coli, and Vibrio (21, 22, 45).

Previously we reported that RppA, a response regulator of the two-component system, can regulate PB sensitivity in P. mirabilis (58) and that the rppA-defective mutant exhibits an increased sensitivity to PB compared to the wild type. However, how RppA modulates PB sensitivity is not known. In this study, we found that the expression of Ugd and GalU could be stimulated in the presence of PB in the wild type but not in the rppA knockout mutant (Fig. 5). Since PB has been shown to be able to serve as a signal to activate RppA (58), we believe that, in the presence of PB, Ugd and GalU are induced by RppA, leading to modification of LPS and repulsion of PB. Moreover, our preliminary data indicate that RppA could bind to the galU promoter (data not shown). RppA can also induce the expression of pmrIp, a gene involved in an LPS modification, leading to PB resistance (29).

We found that both the ugd knockout and galU knockout mutants could not swarm at all after 11-h incubation on LB swarming agar plates (Fig. 2). Several lines of evidence highlight the importance of P. mirabilis Ugd and GalU in swarming motility. First, in comparison to the wild type, both ugd and galU mutants produced reduced amounts of flagellin and flhDC mRNA and exhibited impaired cell differentiation (Fig. 3). Second, both the P. mirabilis ugd and galU mutants had defective LPS, which has been shown to be essential for swarming migration (55). Third, we have found that both the P. mirabilis ugd and galU mutants expressed lower levels of flaA mRNA (data not shown). The products of the flaA and flaA genes are known to be involved in modulating the swarming behavior of P. mirabilis (12). That the galU mutation can affect swarming in P. mirabilis has also been reported previously (6).

Our data indicate that ugd and galU mutations not only affect PB sensitivity but also lead to lower expression of viru-

1. **Discussion**

In this study, we identified two genes, ugd and galU, that are involved in controlling PB resistance in P. mirabilis. In Salmonella enterica and E. coli, both ugd and galU have been shown to be involved in the production of 4-aminoarabinose (16, 20, 21, 48), which is necessary for modifying LPS to become more positively charged. Mutation in either ugd or galU can lead to an alteration in LPS structure in Salmonella, E. coli, Burkholderia, Aeromonas, and Vibrio (9, 21, 22, 45, 57). Here we also found that the ugd and galU mutants of P. mirabilis had altered LPS profiles and cell surface topologies (Fig. 1 and Table 3). Since alterations in LPS are known to affect resistance to CAP, including PB (15, 21, 22), we believe that LPS alterations caused by ugd and galU mutations contribute to the PB-sensitive phenotype of these two mutants of P. mirabilis. That the ugd or galU mutation can cause increased sensitivity to PB has been reported previously for Salmonella, E. coli, and Vibrio (21, 22, 45).

Previously we reported that RppA, a response regulator of the two-component system, can regulate PB sensitivity in P. mirabilis (58) and that the rppA-defective mutant exhibits an increased sensitivity to PB compared to the wild type. However, how RppA modulates PB sensitivity is not known. In this study, we found that the expression of Ugd and GalU could be stimulated in the presence of PB in the wild type but not in the rppA knockout mutant (Fig. 5). Since PB has been shown to be able to serve as a signal to activate RppA (58), we believe that, in the presence of PB, Ugd and GalU are induced by RppA, leading to modification of LPS and repulsion of PB. Moreover, our preliminary data indicate that RppA could bind to the galU promoter (data not shown). RppA can also induce the expression of pmrIp, a gene involved in an LPS modification, leading to PB resistance (29).

We found that both the ugd knockout and galU knockout mutants could not swarm at all after 11-h incubation on LB swarming agar plates (Fig. 2). Several lines of evidence highlight the importance of P. mirabilis Ugd and GalU in swarming motility. First, in comparison to the wild type, both ugd and galU mutants produced reduced amounts of flagellin and flhDC mRNA and exhibited impaired cell differentiation (Fig. 3). Second, both the P. mirabilis ugd and galU mutants had defective LPS, which has been shown to be essential for swarming migration (55). Third, we have found that both the P. mirabilis ugd and galU mutants expressed lower levels of flaA and flaA mRNA (data not shown). The products of the flaA and flaA genes are known to be involved in modulating the swarming behavior of P. mirabilis (12). That the galU mutation can affect swarming in P. mirabilis has also been reported previously (6).

Our data indicate that ugd and galU mutations not only affect PB sensitivity but also lead to lower expression of viru-
factors, lower cell invasion ability, and defective swarming phenotype (Fig. 2 and Table 3). How could mutation in the genes that are involved in synthesis of cell surface structure affect such a broad spectrum of phenotypic traits? Two possible mechanisms may explain this. First, cell surface alterations caused by \( \text{ugd} \) or \( \text{galU} \) mutation may activate two-component systems that in turn regulate swarming and virulence factor expression. The observation that bacterial two-component systems can sense and be regulated by alterations in the membrane has been reported previously. For instance, \textit{Serratia marcescens} RssA, a sensor kinase, has been suggested to be able to sense and be activated by alteration in membrane fluidity, leading to a change in swarming motility (32). Moreover, the RcsC-RcsD-RcsB two-component system is known to be activated by cationic amphipathic molecules that can insert into the lipid bilayer and perturb the bacterial membrane (38). It is possible that RppB, which is the membrane sensor kinase of the RppA-RppB two-component system, can sense and be activated by membrane perturbation caused by \( \text{ugd} \) or \( \text{galU} \) mutation. As we reported previously, activation of the RppA-RppB two-component system can lead to inhibition of swarming and virulence factor expression (58). Therefore, activation of the RppA-RppB two-component system can explain why \( \text{ugd} \) or \( \text{galU} \) mutation causes such a broad spectrum of effects. Our preliminary data indicated that the \( \text{ugd} \) or \( \text{galU} \) mutation led to an increase in RppA expression (data not shown). Since RppA is auto-induced by itself (58), these data support the hypothesis that the \( \text{ugd} \) or \( \text{galU} \) mutation can cause activation of the RppA-RppB two-component system and subsequent broad effects.

The alternative mechanism that may explain the broad effects of \( \text{ugd} \) and \( \text{galU} \) mutations is that cell surface alterations caused by these mutations lead to the activation of RpoE, an extracytoplasmic function sigma factor. RpoE regulates the expression of a large number of genes involved in stress response, the synthesis of surface structure, and pathogenesis (51). The activity of RpoE is negatively controlled by the anti-sigma factor RseA, and deletion of \( rseA \) leads to constitutively active RpoE (25, 51). Previous studies indicate that RpoE can be activated by protein misfolding, membrane disturbance, and changes in LPS (51, 54). In this study, we found that the promoter activity of RpoE was activated by mutation in \( \text{ugd} \) or \( \text{galU} \) (Fig. 6A), presumably because these two mutations caused alterations in LPS and the cell surface (Fig. 1 and Table 3). Moreover, we found that constitutively active RpoE or RpoE overexpression could decrease swarming (data not shown) and cause inhibition of FlhDC and hemolysin expression (Fig. 6). Therefore, activation of RpoE caused by \( \text{ugd} \) or \( \text{galU} \) mutation could explain why the \( \text{ugd} \) or \( \text{galU} \) mutant is defective in swarming and virulence factor expression. The observation that RpoE can negatively regulate FlhDC expression has been reported previously for \textit{Azotobacter} (33).

In conclusion, we have identified two genes, \( \text{ugd} \) and \( \text{galU} \), that are involved in the maintenance of cell surface architecture in \textit{P. mirabilis}. Mutation in either \( \text{ugd} \) or \( \text{galU} \) leads to increased PB sensitivity, defective swarming, and lower virulence factor expression. These data suggest that inhibition of Ugd and GalU enzymatic activities can make \textit{P. mirabilis}, which is inherently resistant to PB, become more vulnerable to PB treatment and less virulent. It is tempting to suggest that these two enzymes are potential targets for antibiotic drug development.

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