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PBP1a/LpoA but Not PBP1b/LpoB Are Involved in Regulation of the Major β-Lactamase Gene *blaA* in *Shewanella oneidensis*

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β-Lactamase production is one of the most important strategies for Gram-negative bacteria to combat β-lactam antibiotics. Studies of the regulation of β-lactamase expression have largely been focused on the class C β-lactamase AmpC, whose induction by β-lactams requires LysR-type regulator AmpR and permease AmpG-dependent peptidoglycan recycling intermediates. In *Shewanella*, which is ubiquitous in aquatic environments and is a reservoir for antibiotic resistance, production of the class D β-lactamase BlaA confers bacteria with natural resistance to many β-lactams. Expression of the *blaA* gene in the genus representative *Shewanella oneidensis* is distinct from the AmpC paradigm because of the lack of an AmpR homologue and the presence of an additional AmpG-independent regulatory pathway. In this study, using transposon mutagenesis, we identify proteins that are involved in *blaA* regulation. Inactivation of *mrcA* and *lpoA*, which encode penicillin binding protein 1a (PBP1a) and its lipoprotein cofactor, LpoA, respectively, drastically enhances *blaA* expression in the absence of β-lactams. Although PBP1b and its cognate, LpoB, also exist in *S. oneidensis*, their roles in *blaA* induction are dispensable. We further show that the *mrcA*-mediated *blaA* expression is independent of AmpG.

Beta-lactams are the most widely used antibiotics for treatment of bacterial infections. They mimic the dipeptide D-Ala-D-Ala and bind covalently to the serine active center of penicillin binding proteins (PBPs), resulting in inactivation of the latter, which are responsible for peptidoglycan synthesis and hydrolysis (1, 2). As one of the major approaches that evolved in bacteria to cope with the threat of β-lactams, β-lactamases are produced to directly degrade this group of antibiotics (3, 4). A variety of β-lactamases have been isolated, and their biochemical characteristics are intensively studied. Among them, some are found to be induced by β-lactams, but studies of the underlying regulatory mechanisms are largely focused on the class C β-lactamase AmpC in several Gram-negative bacteria, including *Enterobacter cloacae*, *Citrobacter freundii*, and *Pseudomonas aeruginosa* (5–13). Because of the discrepancy of the locations between β-lactam action and β-lactamase production, intermediate molecular signals must be produced to guide AmpC induction. Many studies have demonstrated that these molecular cues are derived from peptidoglycan recycling and that multiple proteins are involved in their production. AmpG is an inner membrane-bound permease that is responsible for the transportation of signal precursors, and its loss results in enhanced sensitivity to β-lactams because of the interruption of signal transduction (12–16). AmpR, a LysR family transcriptional regulator, alternates its conformation by binding to two different ligands to activate or repress the transcription of *ampC* (7, 17–21).

As the primary targets for β-lactams, PBPs are no doubt potential candidates to sense the stress from antibiotics in the periplasm. PBPs are classified into two main categories: high-molecular-weight PBPs (HMW PBPs) and low-molecular-weight PBPs (LMW PBPs) (22, 23). In total, five HMW PBPs have been identified in *Escherichia coli*, and they belong to either class A or class B. Class A PBPs (PBP1a, PBP1b, and PBP1c) are bifunctional enzymes containing both glycosyltransferase (GTase) and transpeptidase (TPase) domains, whereas class B PBPs (PBP2 and PBP3) consist of a TPase domain only and thus are monofunctional. In *E. coli*, PBP1s are organized into multienzyme peptidoglycan-synthesizing complexes with other components. PBP1a interacts with PBP2 for cell elongation, while PBP1b interacts with PBP3 and FtsZ for cell division (24–26). Loss of PBP1a reduces both the growth rate in minimal medium and the cell diameter (24), but depletion of PBP1b results in a hypersensitivity to some β-lactams (27). Despite these phenotypic differences, removal of both PBP1s together causes synthetic lethality, implying that there is a functional complementation between them (27, 28). Importantly, the functionality of PBP1a and PBP1b requires the outer membrane lipoproteins LpoA and LpoB, respectively, as essential cofactors. Each lipoprotein forms a complex with its cognate PBP and stimulates both the GTase and TPase activities (27–29).

Shewanella species are gammaproteobacteria that are widely distributed in various habitats and renowned for the ability to use a diverse range of electron acceptors for respiration (30, 31). The members of the genus are regarded as emerging human pathogens and as a reservoir for antibiotic resistance, given that a number of β-lactamases and Qnr-type determinants have been isolated from them (32–37). The representative species *Shewanella oneidensis* possesses seven genes that are predicted to encode β-lactamases (38, 39). The Amber class D β-lactamase BlaA (SO0837), also

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named OXA-54, confers on the bacterium a natural resistance to many β-lactams, while others were found to be dispensable by a mutational analysis (39). BlaA, along with its homologues from other *Shewanella* members, is proposed to be the progenitor of the carbapenem-hydrolyzing enzyme oxacillinase, which has been acquired by the clinically relevant Gram-negative species *Acinetobacter baumannii* and *Klebsiella pneumoniae* (34, 37, 40). Our previous studies showed that the *blaA* gene can be induced by ampicillin at high (>12.5 μg/ml) but not low (<5 μg/ml) levels, and its induction is also intimately linked to peptidoglycan recycling (39, 41). However, a homologue of AmpR is absent in *S. oneidensis*, and the impacts of major peptidoglycan recycling enzymes on *blaA* expression are distinct from the *ampR-ampC* paradigm. In particular, the loss of AmpG increases *blaA* expression, in contrast to the effect observed in previously studied cases (41).

The goal of the work reported here was to identify proteins involved in regulation of the *blaA* gene in *S. oneidensis*. We found that the loss of PBP1a/LpoA results in enhanced expression by designing and performing a colony color-based transposon mutagenesis assay. We then showed that PBP1b and LpoB had no effect on the expression of *blaA*. In addition, we showed that elevated *blaA* expression due to PBP1a depletion was independent of AmpG.

MATERIALS AND METHODS

Bacterial stains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Information for all of the primers used in this study is available upon request. For genetic manipulation purposes, *S. oneidensis* and *E. coli* were cultivated aerobically in Luria-Bertani (LB) medium (Difco, Detroit, MI) at 30°C and 37°C, respectively. Where needed, the growth medium was supplemented with chemicals at the following concentrations: 2,6-diaminopimelic acid, 0.3 mM; ampicillin (AMP), 100 μg/ml; kanamycin (Km), 50 μg/ml; and gentamicin (Gm), 15 μg/ml. All chemicals were acquired from Sigma-Aldrich (Shanghai, China) unless otherwise noted.

In-frame mutagenesis and complementation. In-frame deletion strains of *S. oneidensis* were constructed by the *att*-based fusion PCR method (42). In brief, two fragments flanking the targeted gene were amplified by PCR with primers containing *attB* and gene-specific sequences and then were joined by a second round of PCR (fusion PCR). The fusion fragments were introduced into plasmid pHGM01 by site-specific recombination, using BP Clonase (Invitrogen), and then transformed into *E. coli* WM3064. The resulting mutagenesis vectors were transferred from *E. coli* into the correct *S. oneidensis* strains via conjugation. Integration of the mutagenesis constructs into the chromosome was selected by resistance to gentamicin and verified by PCR. The correct transconjugants were grown in LB broth in the absence of NaCl and plated onto LB medium supplemented with 10% sucrose. Gentamicin-sensitive and sucrose-resistant colonies were screened by PCR for deletion of the targeted gene. The deletion mutations were then verified by sequencing.

Plasmid pHG101 was used for genetic complementation of mutants (43). A fragment containing the gene of interest and its native promoter was amplified by PCR and cloned into pHG101. To control the levels of gene expression, the *araBAD* promoter (P_{BAD}) along with *araC* was ligated into pHG101 to construct the arabinose-inducible plasmid pHGC02. The gene of interest, generated by PCR, was introduced into pHGC02 and verified by sequencing, and the resulting vectors were transferred into the relevant strains via conjugation.

Transposon mutagenesis. Plasmid pHGT02, derived from the widely used mariner transposon-containing plasmid pFAC (44), was used to perform transposon mutagenesis. As pFAC contains an AMP resistance (Ap^r) gene that interferes with screening, it was digested by ApaLI and then self-ligated to remove the Ap^r gene, giving pHGT02. The resulting plasmid was transferred into *S. oneidensis* via conjugation, and transconjugants were plated onto medium containing Gm and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). Mutants with a blue colony phenotype were selected for further studies. The locations of the transposon insertion in the selected mutants were determined by arbitrarily primed PCR (AP-PCR) as described previously (45).

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
E. coli strains		
DH5α	Host strain for plasmids	Lab stock
WM3064	Donor strain for conjugation; Δ*dapA*	W. Metcalf, UIUC
S. oneidensis strains		
MR-1	Wild type	Lab stock
HG0280	Δ*mrcA* strain derived from MR-1	This study
HG0300	Δ*lpoA* strain derived from MR-1	This study
HG0633	Δ*mrcB* strain derived from MR-1	This study
HG0837	Δ*blaA* strain derived from MR-1	39
HG1060	Δ*lpoB* strain derived from MR-1	This study
HG3814	Δ*ampG* strain derived from MR-1	41
HG0280-0837	Δ*mrcA* Δ*blaA* strain derived from MR-1	This study
HG0300-0837	Δ*lpoA* Δ*blaA* strain derived from MR-1	This study
HG0280-3814	Δ*mrcA* Δ*ampG* strain derived from MR-1	This study
WT/P_{blaA}-*lacZ*	Wild type harboring the P_{blaA}-*lacZ* fusion	This study
Plasmids		
pHGM01	Ap^r Gm^r Cm^r; suicide vector	42
pHG101	Promoterless vector for complementation	43
pHGC02	pHG101 harboring the P_{BAD} promoter	This study
pFAC	Mariner-based transposon vector	44
pHGT02	pFAC removing the Ap^r gene	This study
pHGEI01-P_{blaA}	pHGEI01 containing the *blaA* promoter	41

Synthetic lethality test. To identify synthetically lethal interactions between PBP1 and Lpo proteins, a conditional Δ*mrcA* Δ*lpoA* double-deletion mutant was constructed. The mutagenesis vector for the *lpoA* gene, obtained by constructing an *lpoA* in-frame deletion strain, was introduced into the Δ*mrcA* strain by conjugation. pHGC02-*mrcA* was introduced after chromosomal integration of the mutagenesis construct. The remaining steps of the in-frame deletion procedure were carried out in the presence of arabinose. For the synthetic lethality test, late-logarithmic-phase cultures (optical density at 600 nm [OD_{600}] ≈ 0.6) were used to prepare a decimal dilution series. Three microliters of each dilution was placed on an LB medium plate supplemented with or without 0.002% (vol/vol) arabinose. The plates were incubated for 18 h at 30°C and then photographed.

Antibiotic susceptibility assays. The MICs of the antibiotics were determined by the broth microdilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI) (46). Antibiotics used in the susceptibility assay were AMP, cefotaxime (CTX), and imipenem (IPM). All MICs were determined at least in triplicate.

Promoter activity assay. The activity of the P_{blaA} promoter was assayed by use of a markerless integrative *lacZ* reporter system as described

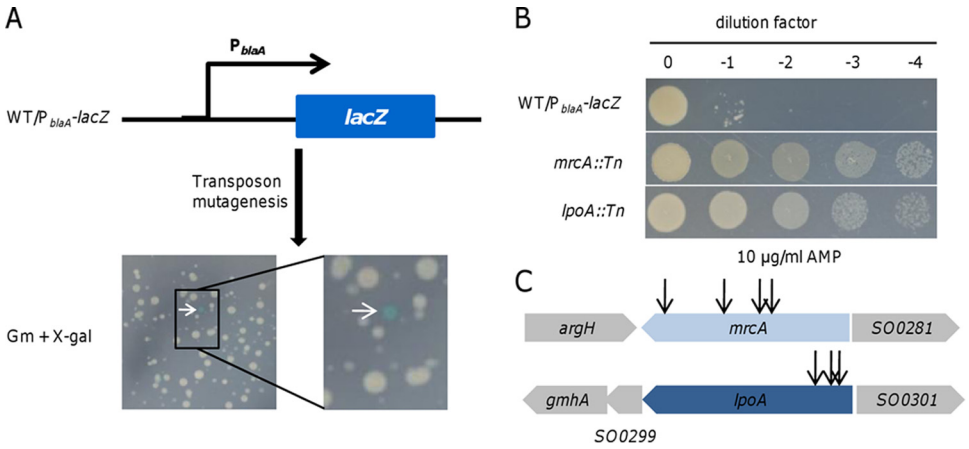

FIG 1 Screening for mutants with enhanced *blaA* expression by transposon mutagenesis. (A) Colonies of transposon mutants grown on a Gm plate harboring X-Gal. The arrow indicates a blue colony with enhanced *blaA* expression. (B) AMP susceptibility assay of transposon mutants. (C) Relative insertional sites of transposon mutants with hyperresistance to AMP.

previously (41). The P_{blaA}-*lacZ* fusion was integrated into the degenerated *nrfCD* locus as described before (47). Cells grown with or without AMP for 3 h were harvested, and their β-galactosidase activity was determined by monitoring color development at 420 nm as described previously (43).

BlaA β-lactamase activity assay. The specific BlaA β-lactamase activity was measured directly by nitrocefin hydrolysis as described previously (41). BlaA β-lactamase activity was expressed in nanomoles of nitrocefin hydrolyzed per minute per milligram of protein. All experiments were performed in triplicate, and the results presented are averages for the three experiments.

Statistical analyses. Experimental values were subjected to statistical analyses and are presented as means ± standard deviations. Student's *t* test was performed for pairwise comparisons of groups.

RESULTS

Screening for mutants with enhanced *blaA* expression. We have previously shown that expression of the *blaA* gene is substantially induced by AMP at high (>12.5 μg/ml) but not low (<5 μg/ml) levels in *S. oneidensis* (39, 41). We speculated that an *S. oneidensis* strain harboring the *E. coli lacZ* gene under the control of the *blaA* promoter would form white and blue colonies on X-Gal plates in the presence of AMP at different levels. To this end, a P_{blaA}-*lacZ* fusion was integrated into the chromosome of the wild type at the degenerated *nrfCD* locus (WT/P_{blaA}-*lacZ*) by homologous recombination (39, 47). As expected, blue colonies appeared on X-Gal plates supplemented with AMP at 50 but not 2.5 μg/ml (Fig. 1A; see Fig. S1 in the supplemental material). Therefore, the expression of *blaA* in the fusion strain was mirrored by the colony color.

To identify genes involved in regulating *blaA* expression, pHGT02, a mariner-based transposon vector, was used to generate a random mutant library in the background of the WT/P_{blaA}-*lacZ* strain. pHGT02 was derived from pFAC by removing the AMP resistance gene, which interferes with screening (44). Apart from their application for construction of transposon insertion libraries, these vectors can also be used to unveil cryptic operons because of an embedded promoter in the transposable sequence (47, 48).

The resulting library was plated on selective medium containing X-Gal (Fig. 1A). A total of ~20,000 colonies were screened, and 14 isolates with a blue colony phenotype were obtained. An antibiotic susceptibility assay revealed that eight of these mutants were hyperresistant to AMP, a phenotype consistent with enhanced expression of the *blaA* gene (Fig. 1B). The remaining six, with modest increases in resistance, were unstable, reverting to white after subculturing. Four of the highly resistant mutants had transposon insertions that mapped to the coding region of the *mrcA* gene, which encodes PBP1a according to the genome annotation (Fig. 1C). Interestingly, insertion sites of three mutants were identified to be within the *lppC* gene (Fig. 1C), whose product is predicted to be an outer membrane lipoprotein homologous to *E. coli* LpoA. Given that inactivation of *mrcA* and *lppC* caused similar phenotypes against AMP, it seems probable that PBP1a and LppC participate in the same physiological processes by the same mechanism as that for *E. coli* PBP1a/LpoA. To be consistent, we renamed the *S. oneidensis lppC* gene *lpoA*.

The last of the highly resistant isolates had a transposon insertion that mapped to the initiation codon of the *mdoH* gene, from an operon responsible for the biosynthesis of membrane-derived oligosaccharides (MDOs) (49). Experiments designed to identify the role of MDOs in *blaA* regulation are under way.

Loss of PBP1a and LpoA increases *blaA* expression. To further evaluate the effects of PBP1a and LpoA on the β-lactam resistance of *S. oneidensis*, we deleted their coding genes from the genome individually. Both the Δ*mrcA* and Δ*lpoA* strains displayed significant defects when grown in LB (Fig. 2A). The generation times of these two mutants increased 62% and 30%, respectively, relative to that of the wild type. Not surprisingly, the resistance of both the Δ*mrcA* and Δ*lpoA* strains to tested β-lactams was strongly increased (Table 2). Compared to the wild type, the Δ*lpoA* strain had 8-, 4-, and 2-fold increases in resistance to AMP, IPM, and CTX, respectively. The Δ*mrcA* strain had additional 2- to 4-fold increases in resistance to all tested β-lactams.

To confirm the observed phenotypes of the Δ*mrcA* and Δ*lpoA* strains described above, genetic complementation was carried out with the multicopy plasmid pHG101 harboring a copy of *mrcA* or *lpoA* under the control of its native promoter (43). The growth defect of these two mutants was fully corrected by expression of the corresponding genes in *trans* (Fig. 2A). However, although the arrangement reduced the β-lactam resistance of the Δ*lpoA* strain, it failed to do so with the Δ*mrcA* strain (see Fig. S2 in the supple-

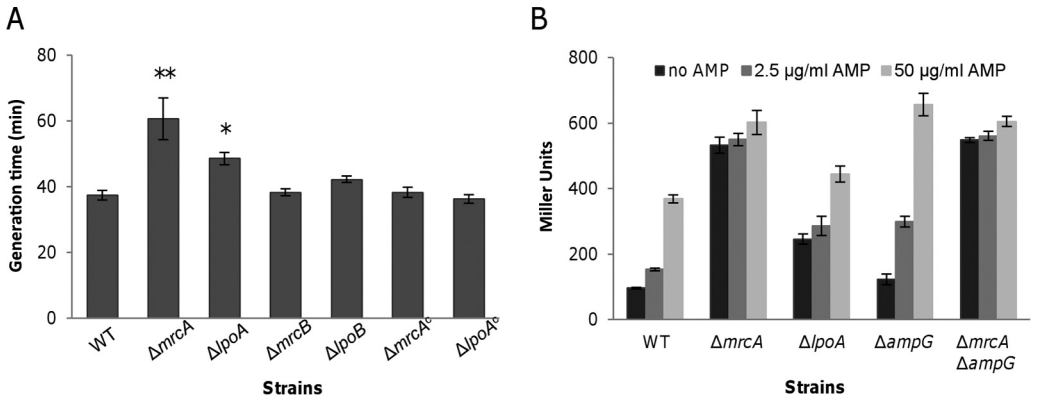

FIG 2 PBP1a and LpoA play important roles in cell growth and *blaA* expression. (A) PBP1a pathway mutants have impaired cell growth. The generation times for different strains were calculated from the OD_{600} values. Δ*mrcA*^{c} and Δ*lpoA*^{c} represent the Δ*mrcA* and Δ*lpoA* strains, respectively, complemented with pHG101 in *trans*. Results are averages for at least three replicates, and the error bars represent standard deviations (SD) (* and **, $P < 0.05$ and 0.01, respectively; two-tailed *t* test). (B) Enhanced β-lactam resistance in *mrcA* and *lpoA* mutants is dependent on *blaA* expression. P_{blaA} promoter activities were determined by measuring β-galactosidase activity (in Miller units) by use of the P_{blaA}-*lacZ* reporter system in different strains in the absence and presence of AMP at 50 μg/ml and 2.5 μg/ml. Results are averages for at least three replicates, and the error bars represent SD.

mental material). Since genes within the vector are overexpressed (43, 50, 51), we supposed that this result was due to an excess of PBP1a, presumably by trapping a larger amount of β-lactams. To keep *mrcA* and *lpoA* expression at proper levels, the *araBAD* promoter (P_{BAD}) along with *araC* was ligated into pHG101 to construct a tightly regulated plasmid, pHGC02. In the absence of arabinose, the resistance to β-lactams of both the Δ*mrcA* and Δ*lpoA* strains carrying the respective genes within pHGC02 for complementation was unaffected (Table 2). When arabinose was added to the cultures, the β-lactam MICs of these two mutants with proper complementation vectors were reduced 2- to 4-fold (Table 2). These results indicate that the phenotypes observed for the Δ*mrcA* and Δ*lpoA* strains were due to the intended mutations.

To validate that the elevated resistance to tested β-lactams of the Δ*mrcA* and Δ*lpoA* strains was due to enhanced expression of the *blaA* gene, a markerless integrative *lacZ* reporter system and nitrocefin hydrolysis were employed to determine the activity of the *blaA* promoter (P_{blaA}) and the BlaA β-lactamase activity, respectively (41). The expression levels of β-galactosidase driven by the *blaA* promoter in the Δ*mrcA* and Δ*lpoA* strains were substantially higher than that in the wild type under all tested conditions (Fig. 2B). In particular, expression of *lacZ* in the Δ*mrcA* strain was constant at high levels, independently of AMP, suggesting that the *blaA* gene is constitutively expressed in the absence of PBP1a (Fig. 2B). Consistently, the basal levels of BlaA activity in the Δ*mrcA* strain were approximately equal to that induced by AMP at 200 μg/ml, which was ~2-fold higher than the induced level in the wild type (Table 2). The Δ*lpoA* strain also had drastically increased basal levels of BlaA activity, i.e., approximately 6-fold higher than that of the wild type. Unlike that in the Δ*mrcA* strain, BlaA activity in the Δ*lpoA* strain could be induced by 200 μg/ml AMP, with an ~3.4-fold increase compared to the level without induction (Table 2). Moreover, expression of *mrcA* and *lpoA* under the control of P_{BAD} significantly decreased the basal levels of BlaA activity in the Δ*mrcA* and Δ*lpoA* strains, respectively (Table 2). Importantly, deletion of *blaA* from either the Δ*mrcA* or Δ*lpoA* strain completely disabled growth in the presence of β-lactams (Table 2). Collectively, these data indicate that inactivation of PBP1a or LpoA raises the level of *blaA* expression.

TABLE 2 MICs of β-lactams and specific activities of BlaA in wild-type *S. oneidensis* and its derivative strains

Strain[a]	MIC (μg/ml)[b]			Mean β-lactamase activity[c] ± SD	
	AMP	CTX	IPM	No inducer	Inducer
WT	4	0.02	0.5	12 ± 0.8	125 ± 7
Δ*mrcA*	128	0.08	4	258 ± 22	228 ± 21
Δ*lpoA*	32	0.04	2	71 ± 11	241 ± 23
Δ*mrcB*	4	0.02	0.5	13 ± 0.8	116 ± 7.9
Δ*lpoB*	4	0.02	0.5	13 ± 1.2	128 ± 13
Δ*ampG*	128	0.08	2	29 ± 4	238 ± 5.1
Δ*blaA*	<0.5	<0.005	<0.06	2 ± 1	0
Δ*mrcA*/*mrcA*	128	0.08	4	−	−
Δ*mrcA*/*mrcA* + Ara	32	0.04	1	44 ± 2.3	227 ± 7.1
Δ*lpoA*/*lpoA*	32	0.04	2	−	−
Δ*lpoA*/*lpoA* + Ara	8	0.02	0.5	19 ± 0.1	174 ± 17
Δ*mrcA* Δ*blaA*	<0.5	<0.005	<0.06	3 ± 0.5	0
Δ*lpoA* Δ*blaA*	<0.5	<0.005	<0.06	3 ± 0.8	0
Δ*mrcA* Δ*ampG*	128	0.08	2	216 ± 35	236 ± 18

[a] Δ*mrcA*/*mrcA* and Δ*lpoA*/*lpoA* represent the Δ*mrcA* and Δ*lpoA* strains, respectively, complemented with pHGC02 harboring a copy of the corresponding *S. oneidensis* genes in *trans*. Ara, arabinose.
[b] AMP, ampicillin; CTX, cefotaxime; IPM, imipenem.
[c] Nanomoles of nitrocefin hydrolyzed per minute per milligram of protein. Induction was carried out with 200 μg/ml ampicillin for 2 h. −, not tested.

Loss of PBP1b has no effect on *blaA* expression. Like many other Gram-negative bacteria, *S. oneidensis* possesses three major class A PBPs: PBP1a, PBP1b, and PBP1c. The *mrcB* gene (encoding PBP1b) was not revealed in our screening, although transposon events in *mrcA* occurred multiple times. Given that these two genes are similar in length and that the number of total interruptions is sufficiently large to cover the entire genome, it is conceivable that PBP1b differs from PBP1a in its role in regulating *blaA* expression.

To test the involvement of PBP1b in the process, a strain devoid of the *mrcB* gene was constructed. In contrast to the Δ*mrcA* strain, neither growth nor the MICs for AMP, CTX, and IPM were affected in this strain (Fig. 2A and Table 2). Consistently, both basal and induced levels of BlaA activity in the Δ*mrcB* strain were

similar to those in the wild type (Table 2). These results indicate that PBP1a and PBP1b differ from each other in mediating *blaA* expression in *S. oneidensis*.

SO1060 (LpoB) is likely the lipoprotein cofactor for PBP1b. Similar to LpoA for PBP1a, the outer membrane lipoprotein LpoB is required for PBP1b function in *E. coli* (27, 28). Typas et al. (28) proposed that LpoA and PBP1a are evolutionarily limited to gammaproteobacteria but that LpoB and its docking domain (UB2H) in PBP1b are further restricted to enterobacteria. However, a recent study demonstrated that *Vibrio cholerae* contains both LpoB and the UB2H domain, indicating that LpoB is present in bacteria beyond enterobacteria (52). *S. oneidensis*, a gammaproteobacterium, possesses the UB2H domain in PBP1b (residues 73 to 157) (see Fig. S3 in the supplemental material), implying that a homologue of LpoB may have evolved in this bacterium.

To identify the lipoprotein cofactor for PBP1b, we performed a BLAST search using *E. coli* LpoB, revealing a single putative homologue, SO1060 (E value, 3e−13). While the protein sequence of SO1060 shares only 49% similarity and 28% identity with that of *E. coli* LpoB (59% coverage), the identity with *V. cholerae* LpoB (95% coverage) is 67%. In addition, SO1060 is predicted to be a lipoprotein, as it contains a lipobox motif with the consensus sequence L(A/S)(G/A)C (see Fig. S3) (27, 53).

To determine whether SO1060 is the outer membrane lipoprotein that works with PBP1b, we deleted the gene and characterized the resulting mutant. Neither growth nor the MICs for β-lactams were affected in the strain, a scenario observed with the Δ*mrcB* strain (Fig. 2A and Table 2). Subsequently, we made attempts to delete the *SO1060* gene from the Δ*mrcA*, Δ*lpoA*, and Δ*mrcB* strains. As we expected, the Δ*mrcB* Δ*SO1060* strain, but not the Δ*mrcA* Δ*SO1060* or Δ*lpoA* Δ*SO1060* strain, was successfully obtained. The phenotypes of the Δ*mrcB* Δ*SO1060* strain with respect to growth and β-lactam resistance were identical to those of the wild-type, Δ*mrcB*, and Δ*SO1060* strains (data not shown). These results indicate that the *SO1060* gene likely encodes the counterpart of *E. coli* LpoB, as it could not be inactivated simultaneously with *mrcA* or *lpoA*.

To further confirm that SO1060 is essential for PBP1b function, we employed the pBAD inducible system to construct synthetically lethal mutants. The *lpoA*, *mrcB*, and *SO1060* genes were deleted from a Δ*mrcA* strain which carried a copy of the gene within pHGC02 in *trans*. In parallel, *mrcA*, *mrcB*, and *SO1060* were deleted from the Δ*lpoA* strain by the same strategy. In total, six strains were obtained, and all grew normally in LB supplemented with arabinose (Fig. 3). In the absence of arabinose, normal growth was observed with the Δ*mrcA* strain lacking LpoA and the Δ*lpoA* strain without PBP1a. In contrast, the loss of PBP1b or SO1060 upon *mrcA* depletion caused synthetic lethality. Similar results were obtained with the Δ*lpoA* strain. These results indicate that, similar to LpoA as the essential cofactor for PBP1a, SO1060 is required for the function of PBP1b. Overall, we concluded that SO1060 is the lipoprotein cofactor for PBP1b, and we thus designated its coding gene *lpoB*.

PBP1a and AmpG mediate *blaA* expression via independent pathways. It has been reported that inactivation of *mrcA* elevates the basal expression of L1 and L2 β-lactamases in *Stenotrophomonas maltophilia*, which is dependent on AmpR and AmpG (54). To assess whether PBP1a-mediated *blaA* expression is dependent on AmpG in *S. oneidensis*, we constructed a double mutant lacking *mrcA* and *ampG* for β-lactam susceptibility testing (Table 2). The

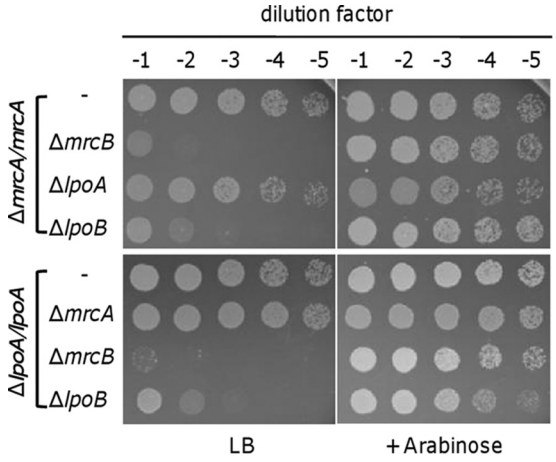

FIG 3 Synthetic lethality assay for PBP1a/LpoA and PBP1b/LpoB. Δ*mrcA*/*mrcA* and Δ*lpoA*/*lpoA* represent the Δ*mrcA* and Δ*lpoA* strains, respectively, complemented with pHGC02 harboring a copy of the corresponding *S. oneidensis* genes in *trans*. Cells from the late logarithmic phase ($OD_{600} \approx 0.6$) were used to prepare a decimal dilution series. Three microliters of each dilution was placed on an LB plate supplemented with or without 0.002% arabinose.

Δ*mrcA*, Δ*ampG*, and Δ*mrcA* Δ*ampG* strains displayed similar levels of resistance to all tested β-lactams. However, differences between the Δ*mrcA* and Δ*ampG* strains were observed by direct analyses of BlaA activity and *blaA* expression (Fig. 2B and Table 2). The basal levels of BlaA activity were constitutively robust in the Δ*mrcA* strain, contrasting with the relatively low but inducible levels in the Δ*ampG* strain. Similar results were obtained in the P_{blaA} activity assay. Importantly, the BlaA activities in the Δ*mrcA* Δ*ampG* strain were comparable to those in the Δ*mrcA* strain (Table 2), indicating that PBP1a-mediated *blaA* expression is independent of AmpG.

DISCUSSION

Regulation of the *ampC* β-lactamase gene has been studied most intensively in the *Enterobacteriaceae* and *P. aeruginosa* (5–11), in which the LysR-type transcriptional regulator AmpR and peptidoglycan recycling are required. However, this paradigm is not applicable to the class D β-lactamase gene *blaA* in *S. oneidensis*, which lacks an AmpR homologue (40, 41). As a result, the loss of AmpG on expression of these two β-lactamase genes in the respective bacteria elicits contrasting effects. More importantly, the *blaA* gene in the *S. oneidensis ampG* mutant remains inducible, implicating the presence of a parallel regulatory pathway for *blaA* induction that is independent of AmpG (41). Here we present evidence to demonstrate that the targets of β-lactams play an important role in *blaA* expression. Using transposon mutagenesis, we found that inactivation of PBP1a or its lipoprotein cofactor LpoA substantially elevates *blaA* expression, conferring the bacterium with hyperresistance to many β-lactams. Although the complex of PBP1b and LpoB is functionally redundant, to some extent, with that of PBP1a/LpoA for peptidoglycan synthesis, the roles of the former in *blaA* expression are dispensable.

In *E. coli*, PBP1a and PBP1b are bifunctional enzymes containing both GTase and TPase domains, and the outer membrane lipoproteins LpoA and LpoB are essential cofactors for PBP1a and PBP1b function, respectively (27, 28). It has been shown that these cofactors improve both the GTase and TPase activities of their

cognate PBPs (29). Although LpoB was initially predicted to be limited to enterobacteria (28), a homologue of LpoB was recently identified in *V. cholerae* (52), expanding the scope of bacteria that adopt this mechanism for regulation of PBP1s. In line with this, the present study demonstrated that LpoA and LpoB also exist in *S. oneidensis*. Like *E. coli* LpoA and LpoB, *S. oneidensis* counterparts are essential for their cognate PBP function. At least one of the PBP1/Lpo complexes is required for cell survival.

Although PBP1a/LpoA and PBP1b/LpoB are synthetically lethal and thus largely complement each other, *S. oneidensis* strains lacking each complex display somewhat different phenotypes. It is evident that the physiological roles of PBP1a/LpoA are more profound than those of PBP1b/LpoB, as growth is significantly affected by the loss of the former but not the latter. This difference coincides with those observed with the homologous protein pairs studied in other Gram-negative bacteria (24, 52). Additionally, the sensitivities of the *E. coli* PBP1b/LpoB and *V. cholerae* PBP1a/LpoA mutants to β-lactams are substantially increased, which may result from the high affinity between β-lactams and the remaining PBP1 protein (52, 55).

In *S. oneidensis*, the removal of PBP1a/LpoA leads to enhanced resistance to β-lactams, a scenario also observed in *S. maltophilia* (54, 56, 57). However, the regulatory mechanisms of PBP1a on β-lactamase expression utilized by these two microorganisms are certainly different. The regulation in *S. maltophilia* is dependent on AmpG and AmpR (54). Although both the *S. oneidensis* Δ*ampG* and Δ*mrcA* strains have increased resistance to β-lactams, the *blaA* gene in the former remains inducible by AMP, but not that in the latter (41). Moreover, it appears that PBP1a functionally overwhelms AmpG in basal expression of *blaA* and that the pathways involving these two proteins have no additive effect, because the *blaA* expression in the Δ*mrcA* Δ*ampG* strain is comparable to that in the *mrcA* single mutant strain. Given that *S. oneidensis* lacks AmpR and its PBP1a/LpoA-mediated *blaA* expression is independent of AmpG, it is undoubted that the mechanisms by which PBP1a and LpoA regulate β-lactamase expression are novel.

Generally, strains lacking PBP1 or its cognate Lpo display identical phenotypes (27, 28, 52). Nevertheless, our data showed a significant difference between *S. oneidensis* PBP1a- and LpoA-deficient mutants with respect to *blaA* expression. Compared to the strain lacking PBP1a, the Δ*lpoA* strain has a lower basal level of *blaA* expression and remains inducible by AMP. Given that LpoA increases the GTase and TPase activities of PBP1a (29), it is reasonable to assume that the enzymatic activities of PBP1a account for the elevated BlaA production. In the absence of LpoA, PBP1a retains a share of the GTase and TPase activities, thus leading to an unsaturated basal level of *blaA* expression.

Except for PBP1, LMW PBPs are also involved in the regulation of β-lactamase expression in Gram-negative bacteria. Compared to the mysterious effects of PBP1a, loss of PBP4 (DacB) triggers overexpression of the β-lactamase AmpC mediated by the CreBC (BlrAB) two-component system in *P. aeruginosa* and *Aeromonas* spp. (58, 59). In *S. oneidensis*, our previous results demonstrate that inactivation of PBP5 (DacA) decreases the induced level of *blaA* expression via an unknown mechanism (39). Although the homologues of AmpR and CreBC are absent in *S. oneidensis*, it cannot be ruled out that other regulators are involved in the PBP1a-mediated *blaA* expression pathway.

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