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Differential Requirement for PBP1a and PBP1b in *In Vivo* and *In Vitro* Fitness of *Vibrio cholerae*

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We investigated the roles of the *Vibrio cholerae* high-molecular-weight bifunctional penicillin binding proteins, PBP1a and PBP1b, in the fitness of this enteric pathogen. Using a screen for synthetic lethality, we found that the *V. cholerae* PBP1a and PBP1b proteins, like their *Escherichia coli* homologues, are each essential in the absence of the other and in the absence of the other's putative activator, the outer membrane lipoproteins LpoA and LpoB, respectively. Comparative analyses of *V. cholerae* mutants suggest that PBP1a/LpoA of *V. cholerae* play a more prominent role in generating and/or maintaining the pathogen's cell wall than PBP1b/LpoB. *V. cholerae* lacking PBP1b or LpoB exhibited wild-type growth under all conditions tested. In contrast, *V. cholerae* lacking PBP1a or LpoA exhibited growth deficiencies in minimal medium, in the presence of deoxycholate and bile, and in competition assays with wild-type cells both *in vitro* and in the infant mouse small intestine. PBP1a pathway mutants are particularly impaired in stationary phase, which renders them sensitive to a product(s) present in supernatants from stationary-phase wild-type cells. The marked competitive defect of the PBP1a pathway mutants *in vivo* was largely absent when exponential-phase cells rather than stationary-phase cells were used to inoculate suckling mice. Thus, at least for *V. cholerae* PBP1a pathway mutants, the growth phase of the inoculum is a key modulator of infectivity.

The main component of the bacterial cell wall, peptidoglycan (PG), is an intricate mesh of polysaccharide chains cross-linked by short peptide bridges. The periplasmic assembly of this complex polymer from [N-acetylglucosamine–N-acetylmuramic acid]pentapeptide subunits is facilitated by extracytoplasmic enzymes known as penicillin binding proteins (PBPs). These enzymes carry out two main reactions: transglycosylation (i.e., polymerization of glycan subunits) and transpeptidation (i.e., cross-linking the peptide side chains between polymerized glycan strands). In *Escherichia coli*—the focus of most studies of Gram-negative cell wall synthesis—two principal high-molecular-weight (HMW) PBPs with both transglycosylase and transpeptidase activity play a pivotal role in PG synthesis. *E. coli* PBP1a and PBP1b appear to be largely interchangeable, and mutants lacking one of the two proteins have at most mild phenotypes under standard growth conditions (1–3). However, cells lacking both proteins are not viable, and PBP1a and PBP1b are termed synthetically lethal. The activity of each PBP1 enzyme is strictly dependent on the presence of a specific outer membrane activator, either LpoA (for PBP1a) or LpoB (for PBP1b), and mutations in either *lpo* locus are consequently also synthetically lethal with a mutation of the noncognate *pbp1* locus (4, 5). It has been proposed that PBP1a may contribute preferentially to cell elongation whereas PBP1b may play a more prominent role in cell division (3, 6); however, the viability of the individual mutants clearly demonstrates that each enzyme can contribute to both processes.

We have previously shown that disruption of the PBP1a-encoding locus (*mrcA*) of *Vibrio cholerae* causes the bacteria to lose their characteristic rod shape in stationary phase and that the aberrant morphology of *V. cholerae* $\Delta mrcA$ is due at least in part to accumulation of noncanonical D-amino acids (DAA) in culture supernatants (7). Notably, DAA do not induce morphological changes in *V. cholerae* lacking *mrcB*, suggesting that in *V. cholerae*,

PBP1a and PBP1b may have distinct roles. However, the physiological roles for these two enzymes remain largely undefined.

Here, we have investigated the contribution of these bifunctional PBPs to *V. cholerae* fitness, both *in vitro* and in an animal model of infection. As in *E. coli*, the activity of *V. cholerae* PBP1a and PBP1b appears to depend upon specific outer membrane lipoproteins. Additionally, our analyses revealed that the absence of PBP1a/LpoA impairs *V. cholerae* growth/survival under a variety of conditions, whereas the absence of PBP1b/LpoB does not have notable effects. PBP1a-deficient cells appear to be particularly vulnerable in stationary phase at high densities, and stationary-phase mutant cells (but not exponentially growing cultures) displayed marked attenuation in the infant mouse model of infection.

MATERIALS AND METHODS

Media and growth conditions. Cells were grown in 3 ml LB broth supplemented with streptomycin at 37°C unless otherwise indicated. Growth curve analyses were conducted in a Biotek growth plate reader using 200- μ l cultures in 100-well honeycomb plates, and growth curves were analyzed using the program GrowthRates (8).

All antibiotics and bile/deoxycholate were purchased from Sigma-

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Aldrich. Antibiotics and supplements were routinely used at the following concentrations: carbenicillin, 50 µg/ml; D-methionine, 5 mM; and streptomycin, 200 µg/ml.

Strain construction. All *Vibrio cholerae* strains are derivatives of El Tor N16961. All cloning procedures were conducted using isothermal assembly (9). Deletion mutants were generated utilizing the *sacB*-containing suicide vector pCVD442 (10). In short, homology regions flanking the gene of interest were cloned into a suicide vector (pCVD442). Single-crossover mutants were generated by mating *E. coli* SM10λpir carrying pCVD442 containing the relevant flanking regions with a *V. cholerae* (streptomycin-resistant) recipient for 6 h on agar plates at 37°C and subsequent selection on streptomycin/carbenicillin plates. The suicide vector was then counterselected by streaking on agar plates containing 10% sucrose and incubating at 30°C overnight.

The *E. coli* *ponA* and *ponB* mutants were constructed using a lambda red system as described previously (11). All *V. cholerae* deletions are complete (start codon to stop codon) deletions without antibiotic resistance replacement, except for $\Delta mrcB$, which is a replacement of the *mrcB* *orf* with a kanamycin resistance cassette.

TnSeq. Transposon insertion sequencing (TnSeq) was performed as described previously (12). Briefly, *V. cholerae* was subjected to saturating transposon mutagenesis to generate duplicate libraries of ~200,000 transposon insertion mutants for each strain. Genomic DNA was harvested from these mutants using a Promega genomic DNA extraction kit and fragmented to 200 to 800 bp using NEB fragmentase. Fragments were then blunted (NEB blunting enzyme mix), A-tailed (using *Taq* polymerase), and ligated to adaptors. Fragments with adaptors were PCR amplified with primers targeting the transposon and the adaptor sequence to enrich for transposon junctions. Illumina barcode sequences were then added via a second PCR, and the resulting 200- to 400-bp product was gel purified and sequenced on an Illumina MiSeq benchtop sequencer. Data analysis was performed as described previously (12).

MIC assays. MIC assays were conducted using an adaptation of a standard methodology with exponential-phase cultures (13). In short, 2× dilution series of the antimicrobial agents were generated in 50 µl of LB in 96-well plates. Then, to each well was added 50 µl of a culture prepared by diluting an overnight culture 1,000-fold into fresh LB broth, growing it for 1 h at 37°C, and again diluting it 1,000-fold into fresh medium. The plates were then incubated standing for 24 h at 37°C.

Competition experiments. Mutant strains were competed against a *lacZ*-negative derivative of wild-type (wt) *V. cholerae* N16961. For *in vivo* competitions, 5-day-old suckling mice (Charles River) were orally inoculated with 50 µl of a culture (either an overnight culture or a culture first grown to an optical density at 600 nm [OD₆₀₀] of 0.1; see Results for details) diluted to OD₆₀₀ = 0.002, corresponding to a total inoculum of ~2 × 10⁵ CFU. Mice were sacrificed after 24 h, and the small intestine was homogenized using a tissue grinder. Dilutions of the homogenate were then plated on agar plates containing streptomycin and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) to distinguish *lacZ*-negative wild-type and *lacZ*-positive (*lacZ*⁺) mutant strains. For *in vitro* competitions, the inoculum used for the *in vivo* assay was added to 3 ml of LB broth and the mixture was incubated for 24 h shaking at 37°C.

Plasmolysis assay. Plasmolysis analysis as a proxy for cell fractionation was carried out as described previously (14). In short, cells carrying plasmids encoding IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible, C-terminal mCherry fusions to LpoA and LpoB were grown in LB supplemented with kanamycin and 200 µM IPTG to an OD₆₀₀ of ~0.6, pelleted, resuspended at a 10× concentration in 20 mM Tris–15% sucrose–0.02% sodium azide, incubated at room temperature for 15 min, and then imaged.

Imaging and analysis. Cells were grown to exponential phase (OD₆₀₀ of ~0.3) and imaged on 0.8% agarose pads–10% LB–phosphate-buffered saline (PBS). Cell length was determined using MicrobeTracker (15).

Phylogenetic trees. Phylogenetic analysis was conducted using ClustalW and Treeview (16, 17).

RESULTS

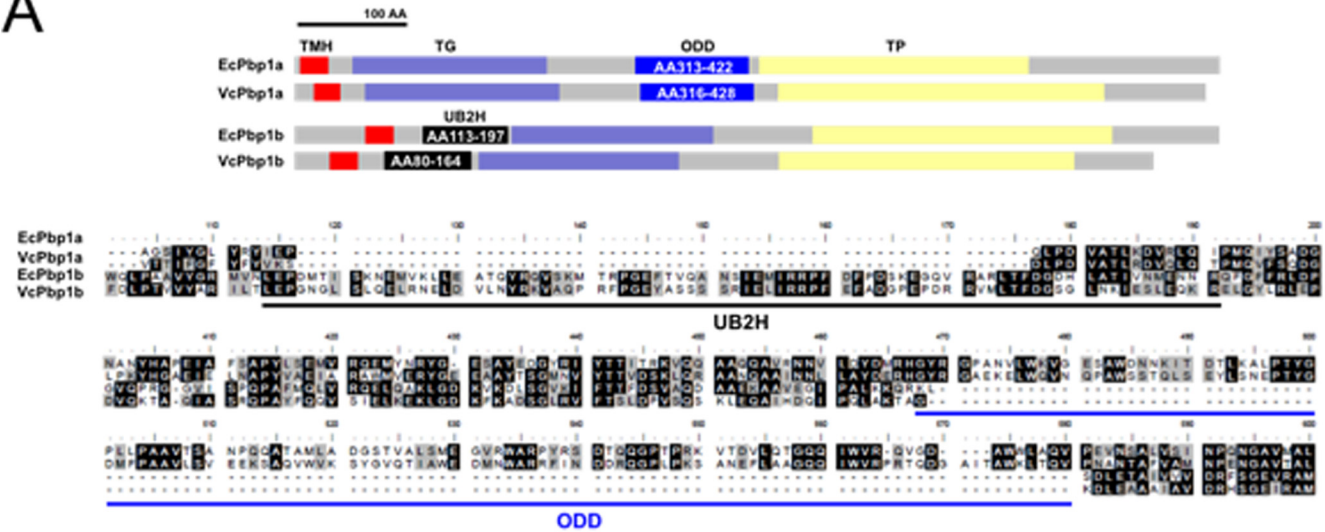
PBP1a and PBP1b appear to act in concert with lipoproteins.

Bioinformatic analysis revealed that *V. cholerae* encodes homologues of two principal bifunctional HMW PBPs of *E. coli*, PBP1a and PBP1b, and that homologous pairs group together in a phylogenetic tree (see Fig. S1A in the supplemental material). All four enzymes contain predicted transmembrane helices as well as transglycosylase and transpeptidase domains. The *V. cholerae* PBP1a and PBP1b sequences, like the corresponding *E. coli* sequences, also contain ODD (PBP1a) or UB2H (PBP1b) domains (Fig. 1A). These ODD and UB2H domains are the sites of interactions between *E. coli* PBP1a and PBP1b and their respective lipoprotein activators, LpoA and LpoB (4, 18), suggesting that *V. cholerae* also encodes homologues of LpoA and LpoB. BLAST searches identified fairly distant *V. cholerae* homologues: VC0581 (32% identical to LpoA over the entire protein sequence, bit score 157) and VC1894 (29% identical to LpoB over the entire protein sequence, bit score 64) (see Fig. S1A and B). Like their putative *E. coli* homologues, both VC0581 and VC1894 are predicted to be lipoproteins (<http://www.lipopredict.cdac.in/jsp/index.jsp>) and contain an outer membrane sorting signal (cys-ser [19]), and both appear (based on a plasmolysis assay) to localize to the outer membrane (see Fig. S2).

To begin to assess functional and genetic relationships between the *V. cholerae* PBPs and their putative lipoprotein activators, we carried out transposon mutagenesis-based screens for mutations that are synthetically lethal in the $\Delta mrcA$ (coding for PBP1a) and $\Delta VC0581$ backgrounds. We recently determined that transposon insertion mutants in *mrcA*, *mrcB* (coding for PBP1b), VC0581, and VC1894 are present with the expected frequency in an insertion library derived from wild-type *V. cholerae* (12), indicating that these genes are not individually essential for *V. cholerae* growth, at least under the conditions used for library construction (LB agar). In marked contrast, almost no mutants with insertions in *mrcB* or VC1894 were apparent when transposon insertion libraries were constructed in either the $\Delta mrcA$ or $\Delta VC0581$ background (Fig. 1B), suggesting that both *mrcB* and VC1894 are required for *V. cholerae* growth in the absence of *mrcA* or VC0581. Thus, our data are consistent with the supposition that *V. cholerae* PBP1a/VC0581 and PBP1b/VC1894 pairs operate in parallel, functionally redundant pathways and that, as in *E. coli*, VC0581 (henceforth LpoA) and VC1894 (henceforth LpoB) act as PBP1a and PBP1b activators, respectively.

PBP1a and PBP1b are not entirely redundant. To further explore the contributions of PBP1a/LpoA- and PBP1b/LpoB-dependent processes to *V. cholerae* growth, we monitored the growth of the four mutants in three different media (LB medium, M9 minimal medium with glucose, and M9 minimal medium with glucose and Casamino Acids). Under all three conditions, growth of the $\Delta mrcB$ and $\Delta lpoB$ mutants was indistinguishable from that of the wild-type strain (Fig. 2A and B). In contrast, while the $\Delta mrcA$ and $\Delta lpoA$ mutants grew at a rate very similar to that of the wild type in LB, they exhibited reduced growth rates in minimal media, suggesting that PBP1a/LpoA play a more prominent role in *V. cholerae* cell wall synthesis than do PBP1b/LpoB, at least under a subset of growth conditions. We sometimes also observed a longer lag phase for the $\Delta mrcA$ and $\Delta lpoA$ mutants (e.g., in Fig. 2, LB); however, the lag times differed considerably between experiments, and their biological significance is unknown. The re-

A



B

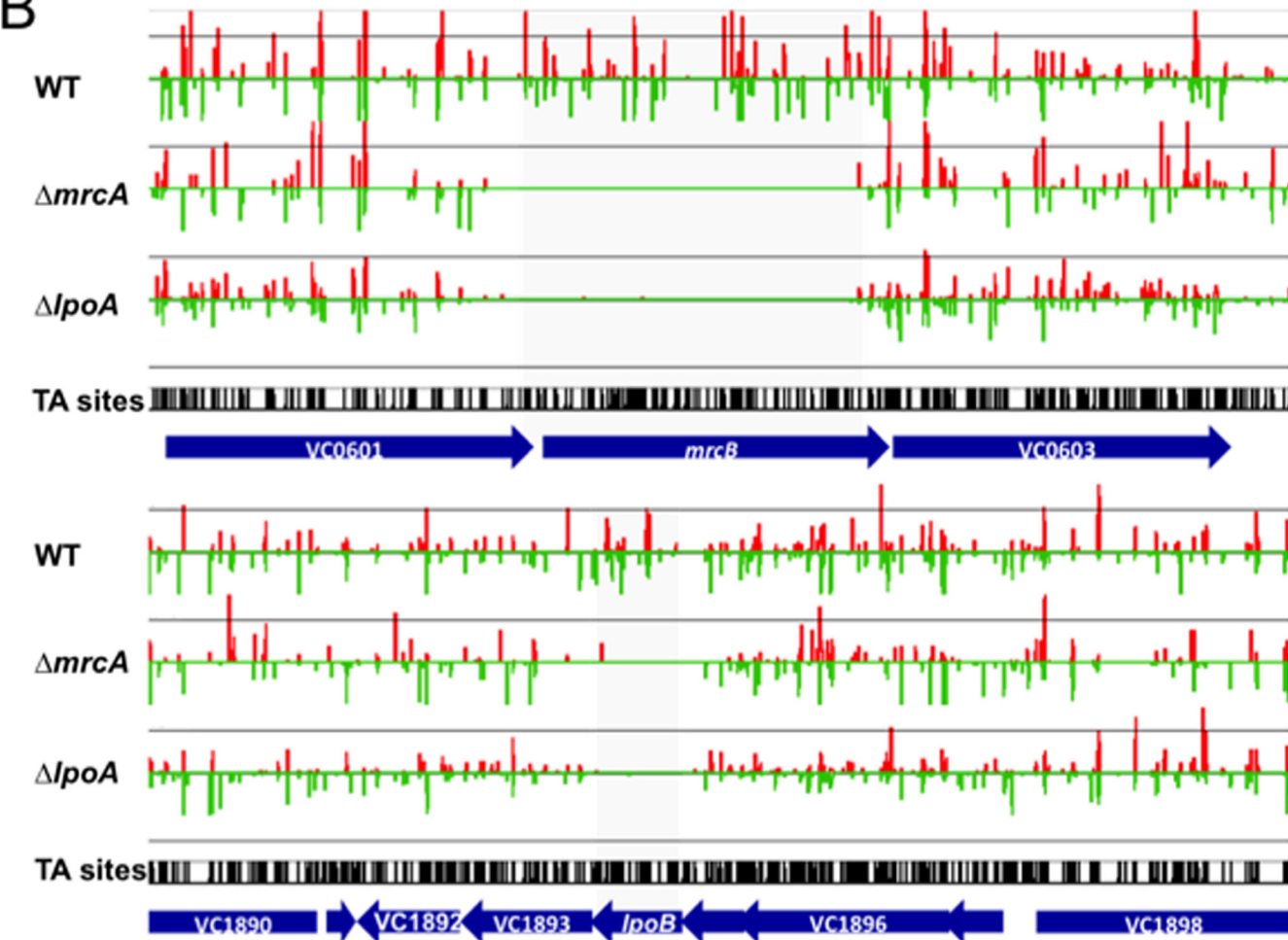


FIG 1 Genetic wiring of bifunctional PBPs in *V. cholerae*. (A) Schematic and partial alignment of bifunctional PBPs of *V. cholerae* and *E. coli*. TMH = transmembrane helix, TG = transglycosylase domain, TP = transpeptidase domain, UB2H = LpoB binding domain, ODD = LpoA binding domain. (B) Artemis screenshots of transposon insertion sequencing (TnSeq) results. Red and green bars represent histograms of read depth; the two colors represent the two different transposon orientations. TA sites (shown in black) are potential transposon insertion sites.

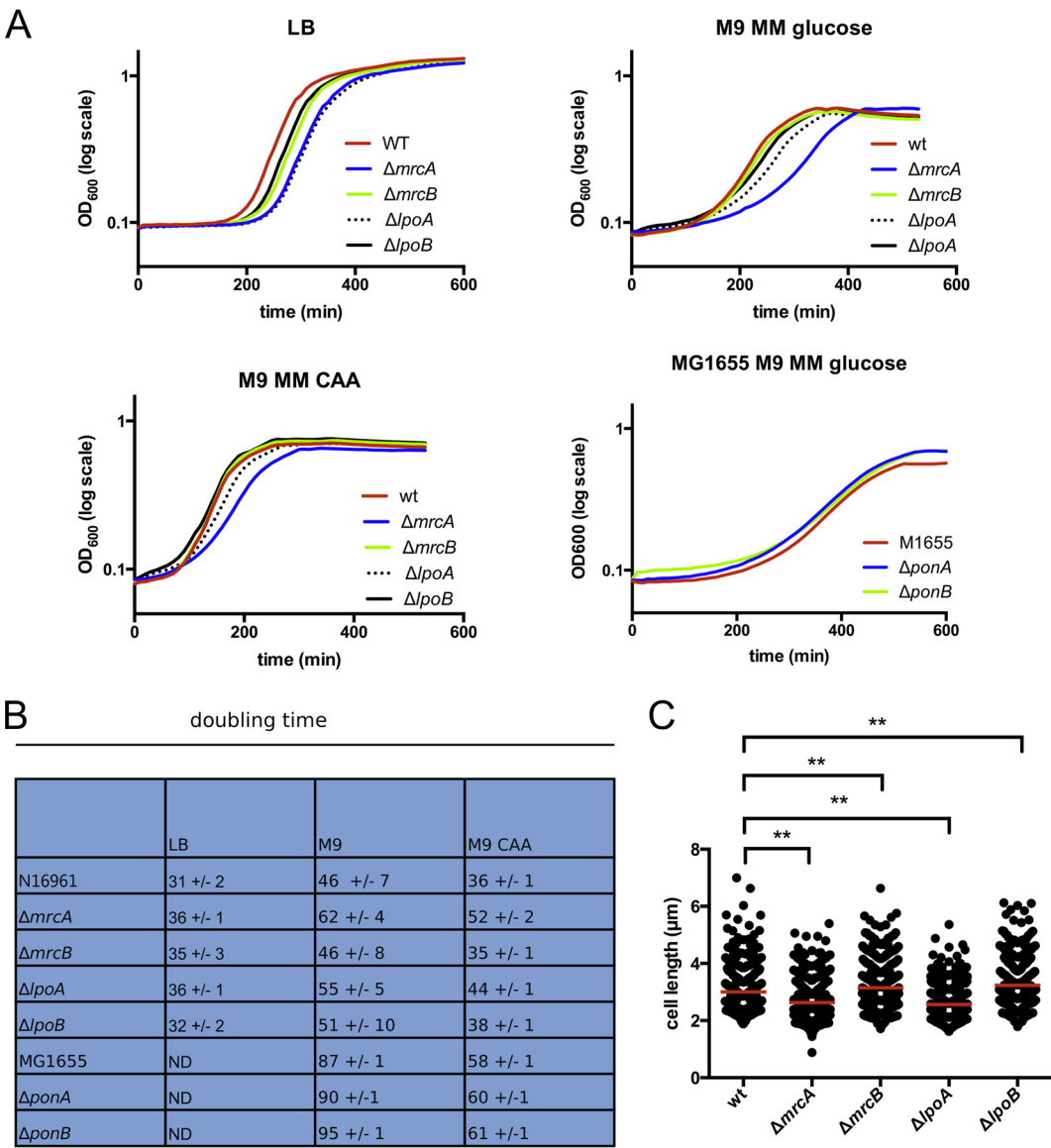


FIG 2 Growth phenotypes of PBP mutants *in vitro*. (A) Growth curves of PBP mutants in rich medium, minimal medium (MM), and minimal medium supplemented with 0.02% Casamino Acids (CAA). Data are means of triplicates and representative of the results of at least three different experiments. WT or wt, wild type. (B) Doubling times $\pm$ standard deviations for different growth conditions were calculated using the program GrowthRates. Values represent means of the results of two experiments done in biological triplicates. ND = not determined. (C) The cell length distribution was measured from levels in LB in exponential phase using MicrobeTracker (** = $P < 0.01$, two-tailed t test).

duced growth rate of *V. cholerae* PBP1a pathway mutants is not evident in equivalent *E. coli* mutants; instead, mutants lacking either PBP1a (*ponA*) or PBP1b (*ponB*) grew as well as wild-type *E. coli* in minimal media with or without Casamino Acids (Fig. 2A and B). Thus, there may be more functional equivalence of PBP1a and PBP1b pathways in *E. coli* than in *V. cholerae*.

Finally, we measured the length and width distributions of cells grown in LB medium in exponential phase. While the cell widths were the same in the wild type and all mutants (data not shown), the length distributions differed significantly. $\Delta mrcA$ and $\Delta lpoA$ mutant cells were significantly shorter than wild-type cells, suggesting a slight elongation defect, while $\Delta mrcB$ and $\Delta lpoB$ cells were slightly longer than the wild-type cells (Fig. 2C), pointing to a division defect. These findings imply that PBP1a may be more

important for cell elongation whereas PBP1b may be more important for cell division and are consistent with the proposed different functions of PBP1a (interacts with PBP2, which is essential for elongation) and PBP1B (interacts with PBP3, which is essential for division) in *E. coli* (2).

Mutations in penicillin binding proteins are often associated with an altered susceptibility to antibiotics that target cell wall synthesis (1, 20). Consequently, we measured MICs of select antibiotics for wild-type and PBP1-deficient *V. cholerae*. MICs of penicillin G and ticarcillin (beta-lactam antibiotics that inhibit all PBPs) were markedly lower for the $\Delta mrcA$ and $\Delta lpoA$ mutants than for the wild-type strain and the $\Delta mrcB$ and $\Delta lpoB$ mutants, which displayed equivalent levels of sensitivity to these agents (Fig. 3A and B). Additionally, the $\Delta mrcA$ and $\Delta lpoA$ mutants were

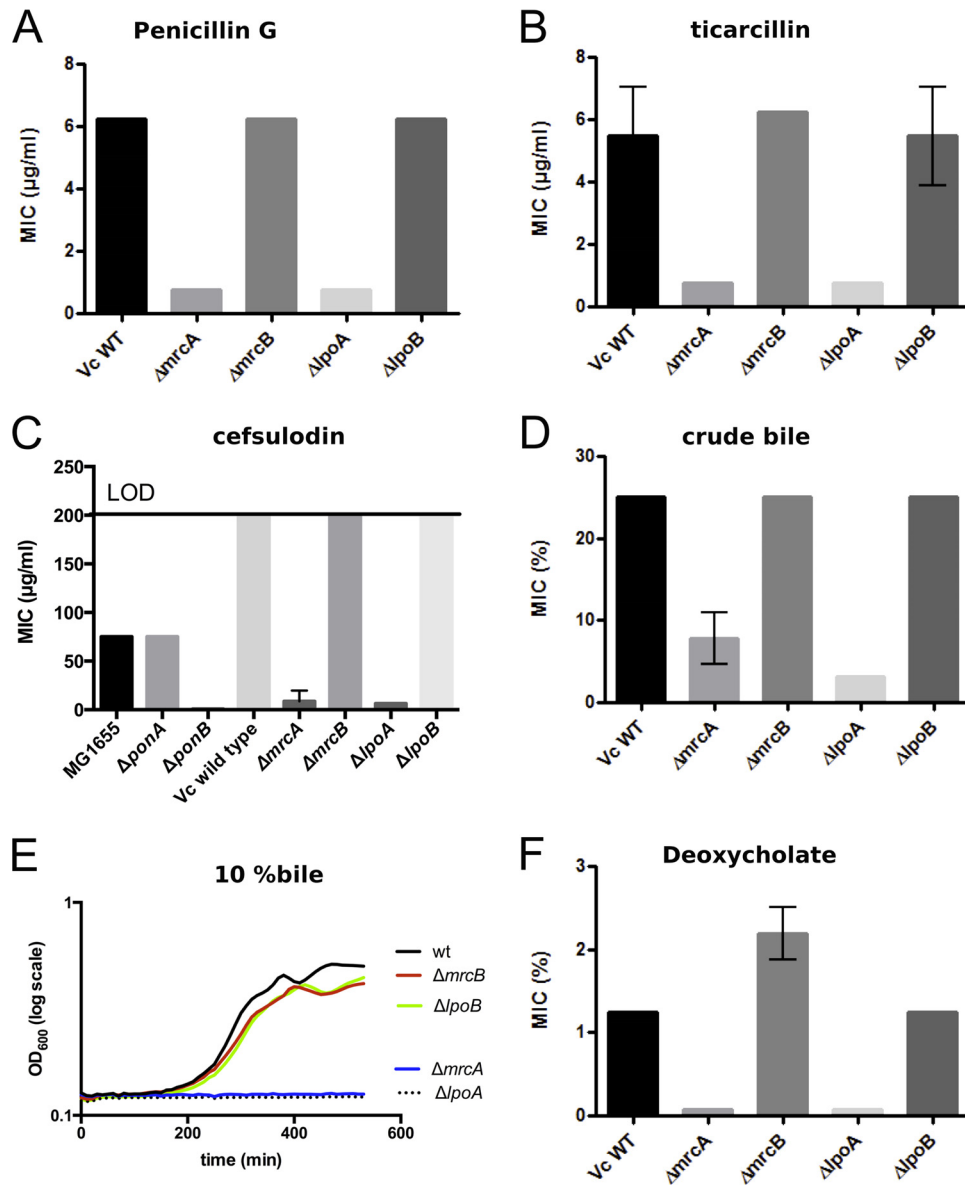


FIG 3 PBP1a pathway mutants exhibit enhanced sensitivity to bile and antibiotics targeting cell wall synthesis. Exponential-phase cells of the indicated *V. cholerae* (Vc) strains were used to determine MICs for the indicated substances. All assays were done at least 3 times, and the error bars represent standard deviations; absence of an error bar indicates that the exact same value was obtained in three repetitions. The growth curves in 10% bile represent the means of the results from 4 biological replicates. LOD = limit of detection.

highly susceptible to the cephalosporin cefsulodin (Fig. 3C), while the wild-type, $\Delta mrcB$, and $\Delta lpoB$ strains were resistant (up to at least 200 $\mu\text{g/ml}$ cefsulodin). These results suggest that cefsulodin exclusively inhibits PBP1b in *V. cholerae*. In contrast, for *E. coli*, the cefsulodin MIC was the same for wild-type and $\Delta ponA$ strains but was drastically reduced in the $\Delta ponB$ mutant (Fig. 3C) (similar to the observations in reference 21), suggesting that low concentrations of cefsulodin preferentially inhibit PBP1a in *E. coli* but that additional enzymes are inhibited at higher concentrations of the drug.

We also tested the susceptibility of the PBP1a/LpoA and PBP1b/LpoB mutants to bile, a membrane-acting agent that *V. cholerae* encounters during growth in the host intestine (Fig. 3D and E). Notably, MICs of crude bile were 5- to 10-fold lower for

the PBP1a pathway mutants than for wild-type cells or PBP1b pathway mutants. An even greater reduction was observed in the MIC of deoxycholate (Fig. 3F), a secondary bile acid that is generated in the intestinal tract. These data suggest that disruption of the PBP1a pathway, but not the PBP1b pathway, results in a compromised cell envelope that is more susceptible to cell stressors.

PBP1a and LpoA mutants are strongly outcompeted by the wild type *in vitro* and *in vivo*. We also investigated the importance of PBP1a- and PBP1b-mediated processes for *V. cholerae* survival/growth in the suckling mouse intestine, a well-established animal model of *V. cholerae* infection (22). For these experiments, each PBP1a/PBP1b or LpoA/LpoB mutant was coinoculated along with the wild-type strain into infant mice, and the relative abundances of the wild-type and mutant strains within the intestines of

infected animals were analyzed ~24 h postinfection. For comparison, complementary competitive analyses were performed *in vitro* (in LB) for each inoculum. For the PBP1b- and LpoB-deficient strains, no difference between the mutant and wt strains with respect to intestinal colonization or *in vitro* growth was detected; mutants had competitive indices (C.I.) (mutant/wt output ratio divided by input ratio) of ~1 both *in vivo* and *in vitro* (Fig. 4A and B). In contrast, the $\Delta mrcA$ and $\Delta lpoA$ strains were markedly (~50 $\times$ to ~1,000 $\times$) outcompeted *in vivo* (Fig. 4A). However, this relative growth deficiency was matched by a comparable deficiency *in vitro* (Fig. 4B), which could be partially complemented by ectopic expression of the deleted *orf* (Fig. 4C). The latter finding confirms the importance of PBP1a/LpoA deficiency as the basis for the reduced competitive indices of the mutants and suggests that optimal fitness is likely dependent upon expression of PBP1a/LpoA at proper physiological levels.

The competitive defects for the $\Delta mrcA$ and $\Delta lpoA$ strains *in vitro* were not expected, given our observation that the wt and mutant strains exhibited comparable growth kinetics in LB (Fig. 2). This result suggested the possibility that competitive analyses might provide additional insight into the roles and significance of PBP1a/LpoA and prompted additional *in vitro* analyses of the growth of PBP1a pathway mutants.

The competitive disadvantage of PBP1a mutants is most severe in the stationary phase of growth. As noted above, *V. cholerae* bacteria lacking PBP1a were previously found to lose their rod shape (becoming spherical) in stationary phase but not in log phase, raising the possibility that the observed competitive defect of these mutants might not be manifested during all stages of growth. To explore this possibility, we performed time course competitive assays for the $\Delta mrcA$ mutant versus the wild-type strain under normal growth conditions and also monitored the fitness of these strains in cocultures that were repeatedly diluted and thereby maintained in exponential-phase growth. As observed in growth studies of individual strains (Fig. 2), in exponential-phase cocultures, the growth of the $\Delta mrcA$ mutant was similar to that of the wild-type strain. A slight growth deficiency was evident over the course of the 12-h experiment (Fig. 5A); however, this subtle defect was insufficient to account for the results observed in overnight cultures and *in vivo* competition assays (Fig. 4A and B). In contrast, in cocultures that were allowed to proceed into stationary phase, the $\Delta mrcA$ mutant showed a strikingly different pattern of survival. It exhibited a mild (~5- to ~10-fold) competitive disadvantage as cells entered stationary phase (~2 to 10 h) and a >10,000-fold relative deficiency in viable cells after 24 h (Fig. 5B). For most of this period (4 to 24 h), the number of wt cells in cocultures remained static (Fig. 5B). In parallel analyses of individually cultured wt and $\Delta mrcA$ cells, a dramatic decline in the abundance of the mutant strain was not observed, although after 24 h mutant cells were ~5-fold less abundant than wt cells (Fig. 5B). Collectively, these analyses suggest that PBP1a has some influence on stationary-phase survival even in the absence of competition but that the stationary-phase survival of PBP1a-deficient cells is particularly impaired by the presence of wild-type cells.

Wild-type stationary-phase culture supernatants inhibit the growth and viability of $\Delta mrcA$ *V. cholerae*. PBP1a pathway mutants are highly susceptible to growth inhibition caused by D-amino acids, which induce sphere formation in these mutants (7) (see Fig. S3 in the supplemental material). D-Amino acids accumulate in *V. cholerae* culture media as cells enter stationary

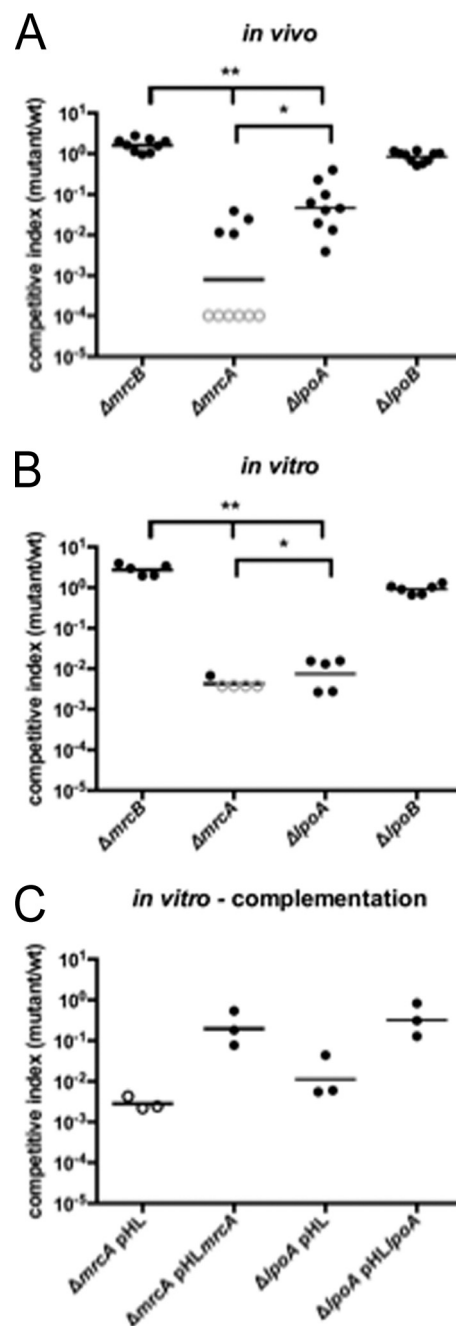


FIG 4 PBP1a pathway mutants are outcompeted *in vivo* and *in vitro*. (A and B) Diluted stationary-phase cultures of the indicated mutants and wt were competed against a *lacZ*-negative derivative of the wild-type strain in suckling mice (A, *in vivo*) or LB (B, *in vitro*). Competitive index data represent the output ratio (mutant CFU/wild-type CFU) divided by the input ratio. The horizontal lines represent geometric means; asterisks indicate significance levels (* = $P < 0.05$, ** = $P < 0.01$, Wilcoxon signed-rank test); open circles represent cases where no CFU were recovered and indicate the lower limit of detection. (C) Complementation of PBP1a pathway competitive defect. Cells carrying empty vector or ectopically expressing the deleted *orf* were treated as described above.

phase, largely due to the activity of a periplasmic amino acid racemase, BsrV (7, 23). Therefore, we assessed whether BsrV is required for the $\Delta mrcA$ mutant's reduced viability in stationary-phase competition assays. In competition assays with a $\Delta bsrV$

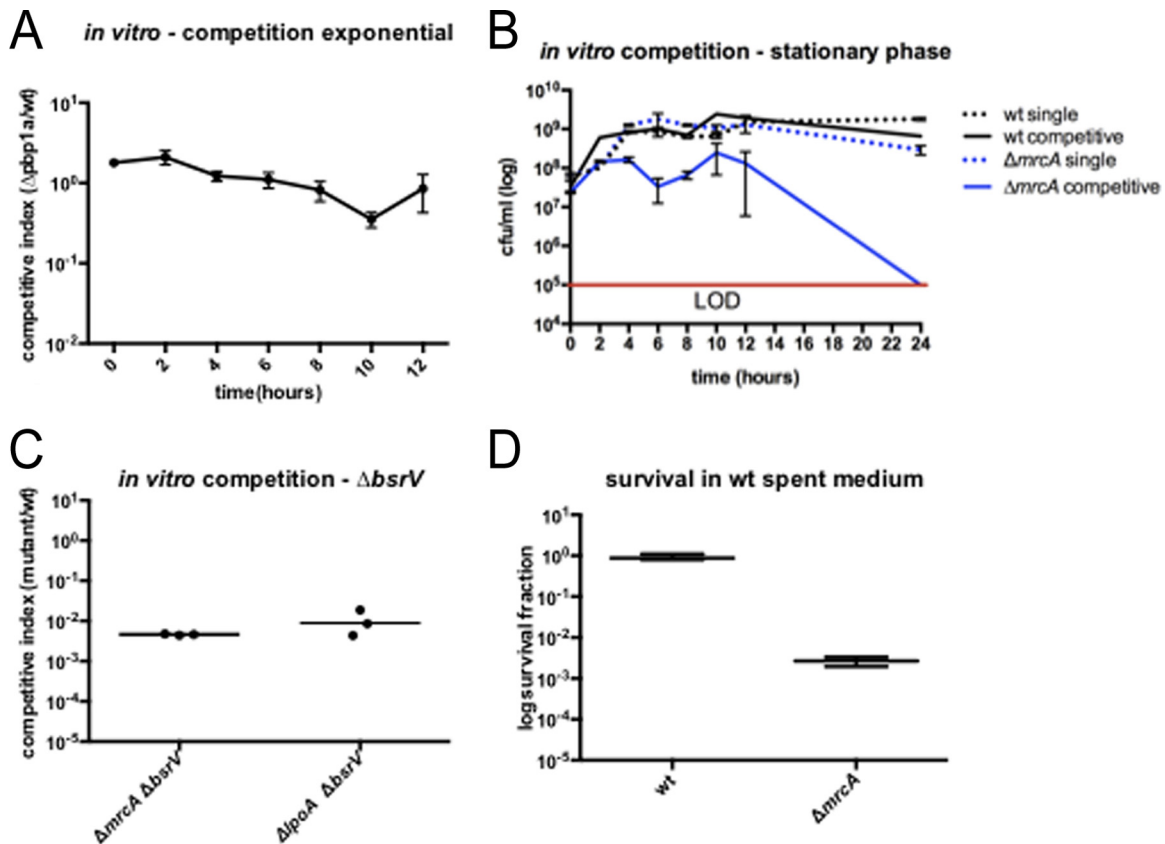


FIG 5 Growth-phase-dependent competitive defect of PBP1a pathway mutants. (A) Time course competition of the $\Delta mrcA$ mutant versus wild-type (*lacZ*-negative) *V. cholerae* in exponential phase. Mutant and wild-type strains were inoculated into LB at equal numbers and maintained at an OD_{600} ranging from ~ 0.1 to ~ 0.2 through hourly redilution (0 to 12 h). (B) Time course competition of the $\Delta mrcA$ mutant versus wild-type (*lacZ*-negative) *V. cholerae* in stationary phase. The mutant and the wild type were grown separately to $OD_{600} = 0.1$, rediluted to $OD_{600} = 0.002$, grown for an additional 2 h (T_0), and then grown to stationary phase either alone or in competition. LOD = limit of detection. (C) *In vitro* competition between the $\Delta bsrV$ mutant and its strain $\Delta mrcA$ and $\Delta lpoA$ derivatives. (D) Survival of the wild-type and $\Delta mrcA$ strains in spent medium. Cultures were grown overnight in LB and pelleted. Supernatants were removed and filter sterilized (= spent medium). Cells were then resuspended in a volume to achieve stationary-phase cell density ($\sim 2 \times 10^9$ CFU/ml) and incubated for 12 h. Log percent survival values represent CFU/ml after 12 h divided by CFU/ml at t_0 .

mutant, a $\Delta mrcA \Delta bsrV$ strain had a competitive index of 0.001 (Fig. 5C), which was indistinguishable from that of the $\Delta mrcA$ mutant grown with the wild-type strain. Thus, the absence of BsrV did not restore the viability of the $\Delta mrcA$ mutant in competition assays allowed to reach stationary phase. The absence of BsrV also had no effect on the survival of the $\Delta lpoA$ mutant in the *in vitro* competition assay (Fig. 5C) or in *in vivo* competition (see Fig. S4). These data suggest that the impaired growth/survival of PBP1a pathway mutants during stationary phase is not due to the products of BsrV and, thus, that additional attributes of stationary-phase cultures likely impair the viability of these mutants.

To further explore whether culture supernatants contain a factor(s) that inhibits the growth or viability of the PBP1a pathway mutants, we tested whether mutants could grow in spent media (filtered stationary-phase culture supernatants) from either wt or BsrV-deficient *V. cholerae*. Cells from stationary-phase cell cultures were pelleted and then resuspended at a lower cell density in spent media supplemented with glucose. These analyses revealed that glucose-fortified spent media from wt or $\Delta bsrV$ cultures permitted growth (i.e., an increase in culture density) of wt, $\Delta mrcB$, and $\Delta lpoB$ cells but not growth of $\Delta mrcA$ cells (see Fig. S5 in the supplemental material). Growth was also markedly impaired for

$\Delta lpoA$ cells, although some increase in culture OD_{600} was observed. Like their BsrV⁺ counterparts, $\Delta bsrV$ derivatives of PBP1a pathway mutants also became spherical in prolonged stationary phase (see Fig. S6). These data provide support for the hypothesis that spent medium contains a factor(s) other than DAA produced by BsrV that can disrupt the growth and morphology of PBP1a pathway mutants. Finally, we observed that resuspension of stationary-phase $\Delta mrcA$ *V. cholerae* at high density in spent media from wt cells resulted in a dramatic ($\sim 1,000$ -fold) loss of viability after 12 h (Fig. 5D). In contrast, no loss of viability was observed for wt cells assayed in parallel. Thus, it appears that wt cells release a factor into culture supernatants that markedly reduces the survival of PBP1a-deficient cells at high cell densities. The identity of this factor(s), as well as the parameters that govern its activity, remains to be determined.

The *in vivo* competitive disadvantage of PBP1a pathway mutants depends on the growth phase of the inoculum. Since the *in vitro* competitive disadvantage of PBP1a pathway mutants was largely restricted to stationary phase, we investigated whether the competitive deficiency of this strain *in vivo* was linked to use of stationary-phase (overnight) cultures as inocula (which is our standard protocol for such assays). We repeated the *in vivo* com-

petitions using inocula derived from early exponential-phase cultures (OD_{600} of ~ 0.1) and found that the competitive index for the $\Delta mrcA$ mutant was markedly higher using log-phase inocula ($\sim 10^{-1}$, rather than $\sim 10^{-3}$ with inocula from overnight cultures) (Fig. 6A). Use of log-phase cultures also increased the competitive index for the $\Delta lpoA$ mutant to $\sim 10^{-1}$. These relatively small deficiencies may be more indicative of the slightly reduced growth rates of these mutants than of a specific requirement for the PBP1a pathway in *in vivo* growth. In support of this idea, we observed that mice infected with log-phase cultures of the $\Delta mrcA$ and $\Delta lpoA$ mutants were colonized to the same extent as were mice infected with wt bacteria (10^6 to 10^7 CFU) (Fig. 6B). In contrast, when overnight cultures were used to infect mice, colonization by the mutants was 30- to 100-fold lower than that by the wt strain (Fig. 6C). Collectively, these results suggest that the absence of the PBP1a pathway *in vivo* is not particularly detrimental to *V. cholerae* growth in this environment; however, its absence likely impairs initial survival within the infant mice of stationary-phase cells, since PBP1a-deficient cells appear to be particularly vulnerable during this phase of their growth cycle.

DISCUSSION

Our study of the roles of the two *V. cholerae* high-molecular-weight bifunctional penicillin binding proteins, PBP1a and PBP1b, in the fitness of this enteric pathogen yielded several unexpected findings. Like *E. coli* PBP1a and PBP1b, *V. cholerae* PBP1a and PBP1b work in concert with lipoproteins LpoA and LpoB, respectively, and the phenotypes of PBP1a and PBP1b mutants generally mirrored those of the cognate lipoprotein mutants. PBP1a/LpoA proteins appear to play a more prominent role in generating or maintaining the cell wall of *V. cholerae*. We found that mutants lacking PBP1b or LpoB exhibited wild-type growth under all conditions tested. In contrast, mutants lacking PBP1a or LpoA exhibited growth deficiencies in minimal medium, in the presence of bile, and in competition assays with wild-type cells (both *in vitro* and in the infant mouse small intestine). The vulnerability of the PBP1a pathway mutants is particularly prominent in stationary-phase cells, which are sensitive to a product(s) present in stationary-phase supernatants from wt cells. The marked competitive defect of the PBP1a pathway mutants *in vivo* was mostly eliminated when exponential-phase cells rather than stationary-phase cells were used to inoculate suckling mice. Thus, at least for *V. cholerae* PBP1a pathway mutants, growth phase is a key modulator of infectivity.

The two bifunctional PBPs of *E. coli* are often referred to as functionally redundant, although some differences in the roles of the two enzymes have been described (2). PBP1a is thought to be mainly involved in cell wall synthesis during elongation (3), while PBP1b localizes to the septum, where it interacts with the division-specific PBP3 and is thought to mediate septal PG synthesis (6, 18). *E. coli* lacking PBP1b (but not lacking PBP1a) is deficient in long-term (several-day) stationary-phase survival in rich medium under conditions of competition against wt cells (24) and in biofilm formation (25) and is hypersensitive to cefsulodin (26) and D-amino acids (our unpublished observations). Intriguingly, all of these phenotypes are similar to those we observed for the *V. cholerae* $\Delta mrcA$ mutant (this study and our unpublished results for biofilm formation). Thus, although *V. cholerae* seems to rely more heavily on its PBP1a pathway than *E. coli* relies on its PBP1b pathway, the biological roles of *V. cholerae* PBP1a and *E. coli* PBP1b

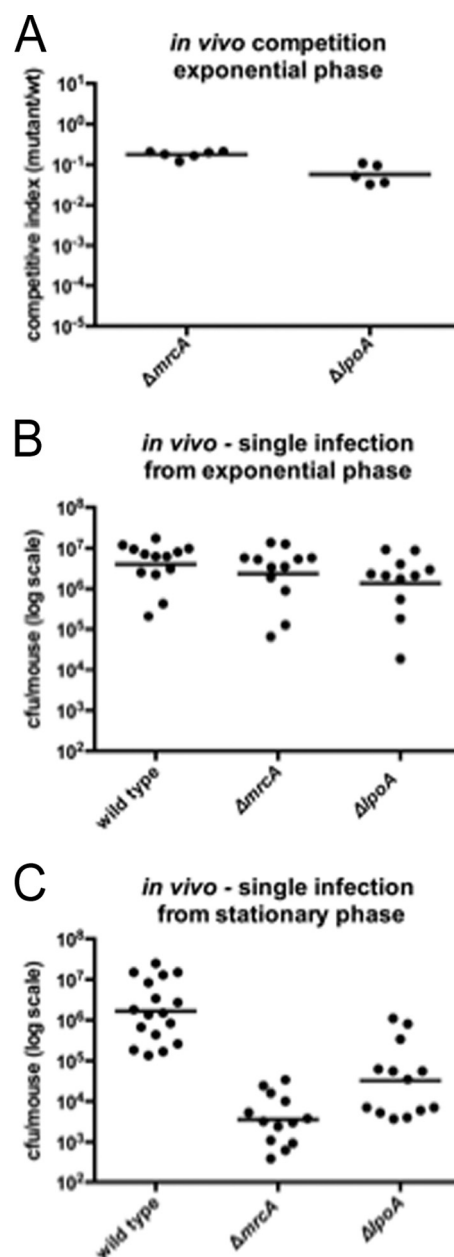


FIG 6 The competitive defect of PBP1a pathway mutants depends on the growth phase of the inoculum. (A) Strains were grown separately to $OD_{600} = \sim 0.1$ and then rediluted to $OD_{600} = 0.002$ and competed against a likewise-treated *lacZ*-negative derivative of the wild type in suckling mice. Competitive index data represent the output ratio (mutant strain CFU/wild-type strain CFU) divided by the input ratio. Horizontal lines are geometric means. The difference between strain $\Delta mrcA$ and strain $\Delta lpoA$ is not statistically significant. (B and C) Wild-type and PBP1a pathway mutant strains were inoculated noncompetitively from cultures pregrown to $OD_{600} = 0.002$ (B) or from stationary-phase cultures directly diluted to $OD_{600} = 0.002$ (C).

appear to be similar. It is possible that differences in the relative expression levels of PBP1a and PBP1b pathway proteins in the two organisms account for these observations; however, there is currently limited knowledge regarding the expression and regulation of these proteins in either organism.

Beta-lactam antibiotics and cephalosporins inhibit bifunc-

tional PBPs by covalently binding to the active site within the enzymes' transpeptidase domains, which these drugs can enter as a consequence of their structural similarity to the natural PBP substrate (27). *V. cholerae* N16961 is resistant to cefsulodin (our unpublished observations), but the molecular basis for this resistance is not known. We found that PBP1a pathway mutants are highly susceptible to this cephalosporin antibiotic, suggesting that it inhibits PBP1b exclusively. Though it is well established that beta-lactam and cephalosporin antibiotics have differing affinities for different PBPs (28–30), such a stark difference is unusual. Similarly, we found that penicillin G and ticarcillin, broad-spectrum inhibitors of PBPs, were effective against PBP1a pathway mutants at lower concentrations than those that are effective against the wild type or the PBP1b pathway mutants. PBP transpeptidase architecture is necessarily highly conserved, since these enzymes process similar substrates (31). However, our results suggest that *V. cholerae* PBP1a and PBP1b might have inherent differences in their abilities to recognize/bind to their substrates, which might explain some of the apparent differences between their roles.

In addition to their heightened sensitivity to antibiotics that disrupt cell wall synthesis, PBP1a pathway mutants are also hypersensitive to bile and to deoxycholate (a secondary bile acid that is typically generated in the intestine). Bile acids act as natural detergents which can solubilize fats and disrupt cell membranes, and most bile-sensitive mutants have been found to have deficits in membrane integrity (32). Bile can also induce oxidative stress, protein denaturation, and DNA damage (32, 33), and previous analyses have suggested that bile regulates *V. cholerae* motility *in vivo* and that it modulates temporal and spatial expression of *V. cholerae* virulence factors during infection (34, 35). It is not entirely clear why disruption of the PBP1a pathway reduces the resistance of *V. cholerae* to bile. Analyses of LpoA and LpoB activity in *E. coli* revealed that targeting of LpoA (but not LpoB) to the outer membrane is critical for its activity (4); thus, we might expect that cells relying on LpoA (i.e., PBP1b pathway mutants) would be more sensitive to outer membrane disruption. Results from our *in vivo* assays suggest that the levels of intraintestinal bile are insufficient to restrict growth of PBP1a/LpoA-deficient *V. cholerae*, although it is possible that bile contributes to the poor survival of stationary-phase mutants *in vivo*.

Our analyses also revealed that the PBP1a pathway of *V. cholerae* is particularly important to the survival of the bacterium in stationary phase. In addition to the previously reported stationary-phase cell shape defect, mutants exhibit a slight reduction in stationary-phase culture densities ($\sim 5\times$ fewer CFU) and have restricted survival and growth in spent medium. Our data suggest that spent medium contains a factor(s) that is specifically toxic to the PBP1a pathway mutants. It is possible that this factor (e.g., an inhibitor of the PBP1b pathway) accumulates in stationary-phase culture supernatants and is absent during exponential-phase growth; alternatively, it may be present throughout the growth cycle but detrimental only under a subset of conditions (e.g., in the absence of cell division). We presume that factors released into the media of stationary-phase wt cells account for the reduced fitness of the $\Delta mrcA$ mutant in competitive assays. Characterization of the factor(s) impairing growth and survival of the $\Delta mrcA$ mutant in stationary phase will be the subject of future investigations.

The magnitude of the colonization defect of the PBP1a mutant was dependent on the growth phase of the culture used as the inoculum. When stationary-phase cultures were used to inoculate

suckling mice, the mutant was highly attenuated in its capacity to colonize the small intestine (C.I. = ~ 0.001). In contrast, when exponential-phase cultures were used to inoculate the mice, there was only a modest colonization defect in competition assays versus the wt (C.I. ~ 0.2) and there was no discernible defect in single-infection assays. The absence of a severe colonization defect with the exponential-phase *mrcA* mutant inocula suggests that *V. cholerae* does not have sustained exposure to concentrations of bile that exceed the MIC of the mutant ($\sim 10\%$ for crude bile). In addition, the relatively minor colonization defect of the *mrcA* mutant may also suggest that the infant mouse gut is not limiting for nutrients (since the growth of this mutant is attenuated in minimal medium). However, the latter conclusion must be tempered by our limited knowledge of *V. cholerae* metabolism during its growth in the host intestine. Finally, the precise reasons why the stationary-phase mutant cells are particularly defective at survival/growth in the host environment are not known but may include enhanced susceptibility to killing by host factors. Additional processes required for emergence from stationary phase by PBP1a pathway mutants (e.g., recovery of normal cell morphology) may also contribute to the reduced fitness of the mutants. Regardless of the reasons for the pronounced colonization defect of the stationary-phase PBP1a-deficient cells, the growth-phase-dependent differences in the outcomes of the intestinal colonization experiments provide an important cautionary tale regarding the profound influence that growth-phase-dependent effects can exert on infectivity.

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