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A D, D-carboxypeptidase is required for *Vibrio cholerae* halotolerance

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Summary

The biological roles of low molecular weight penicillin-binding proteins (LMW PBP) have been difficult to discern in Gram-negative organisms. In *E. coli*, mutants lacking these proteins often have no phenotype, and cells lacking all 7 LMW PBPs remain viable. In contrast, we report here that *Vibrio cholerae* lacking DacA-1, a PBP5 homolog, displays slow growth, aberrant morphology, and altered peptidoglycan (PG) homeostasis in LB medium, as well as a profound plating defect. DacA-1 alone among *V. cholerae's* LMW PBPs is critical for bacterial growth; mutants lacking the related protein DacA-2 and/or homologs of PBP4 or PBP7 displayed normal growth and morphology. Remarkably, the growth and morphology of the *dacA-1* mutant were unimpaired in LB media containing reduced concentrations of NaCl (100 mM or less), and also within suckling mice, a model host for the study of cholera pathogenesis. PG from the *dacA-1* mutant contained elevated pentapeptide levels in standard and low salt media, and comparative analyses suggest that DacA-1 is *V. cholerae's* principal DD-carboxypeptidase. The basis for the *dacA-1* mutant's halosensitivity is unknown; nonetheless, the mutant's survival in biochemically uncharacterized environments (such as the suckling mouse intestine) can be used as a reporter of low Na^+ content.

Introduction

Peptidoglycan (PG) is the principal constituent of the bacterial cell wall, a complex and dynamic macromolecular structure that determines cell shape and promotes maintenance of cellular integrity in the face of environmental changes, such as alterations in osmolarity (Blaauwen et al., 2008). PG is composed of glycan strands that are linked to each other via peptide crossbridges. Biosynthesis of PG is a multistep process that begins in the cytoplasm, where precursor disaccharide pentapeptides are generated (Typas et al., 2011). After the

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precursors are ‘flipped’ outside the cytoplasmic membrane, they are assembled into the PG polymer by a diverse set of enzymes, the penicillin-binding proteins (PBPs) (Vollmer and Bertsche, 2008). These enzymes catalyze several distinct reactions but share the capacity to bind β-lactam rings, due to the resemblance of these rings to the enzymes' peptide substrates.

PBPs are typically divided into two broad groups – the high and low molecular weight PBPS (HMW and LMW, respectively) (Sauvage et al., 2008). HMW PBPs are bifunctional or monofunctional enzymes that catalyze transglycosylation and/or transpeptidation reactions. Transglycosylation links disaccharide PG precursors (inner membrane-anchored GlcNAc-MurNAc-pentapeptides) into the glycan strands that form the backbone of PG. Transpeptidation generates crosslinks between PG peptide sidechains, typically by linking the D-alanine in the fourth position of a donor pentapeptide (often L-Ala→D-Glu→*meso*-DAP→D-Ala→D-Ala in Gram-negative bacteria) to the third position *meso*-DAP of an acceptor peptide strand (Höltje, 1998). In *E. coli*, the activities of HMW PBPs have been fairly well defined, and 2 of the 5 (PBP2 and PBP3) are essential for cell elongation and cell division (Spratt, 1975). The enzymes with the highest synthetic activity - PBP1A and PBP1B - are individually dispensable, but cannot be disrupted simultaneously (Yousif et al., 1985; Dorr, Moll, et al., 2014).

In contrast, the biological roles for most LMW PBPs (of which *E. coli* contains at least 7) have been less well defined and are less pivotal (Ghosh et al., 2008). LMW PBPs lack transglycosylase activity, and have been shown to modify PG sidechains in a variety of ways. Most have been shown to be DD-carboxypeptidases (DD-CPases) that cleave the D-Ala→D-Ala bond in pentapeptides, leading to the release of the terminal D-Ala, and/or DD-endopeptidases, which can process various crosslinked peptides dependent on their specificity (van Heijenoort, 2011). In general, LMW PBPs are not essential for cell growth, and some bacterial species (e.g., *Helicobacter pylori*, *Caulobacter crescentus*) (Ghosh et al., 2008) do not contain obvious DD-CPase homologs. In *E. coli*, individual deletion of most LWM PBP genes does not impair cell growth or alter cell morphology (Denome et al., 1999). However, the absence of PBP5 (encoded by *dacA*), *E. coli*'s principal DD-CPase, does lead to subtle changes in cell shape under some conditions, although it does not impair cell proliferation (Nelson and Young, 2000). Deletion of multiple additional LMW PBPs in conjunction with *dacA* results in extensive morphological defects, such as branching; however, deletion of multiple LMW PBPs generally has no effect on cell morphology when PBP5 is present (Nelson and Young, 2001). Branching is thought to be a consequence of FtsZ mislocalization and associated aberrant placement of inert PG (L.-P. Potluri et al., 2012).

In wt *E. coli*, the fraction of pentapeptides is very low due to their rapid proteolytic degradation to tetrapeptides (Vollmer and Bertsche, 2008); however, in the absence of *dacA*, the pentapeptide content of *E. coli's* PG increases to ∼6%, consistent with PBP5's biochemical characterization as a DD-CPase (Santos et al., 2002). PBP5 can cleave the D-Ala→D-Ala bond both in monomeric and dimeric pentapeptides. It is thought to localize to areas of active PG synthesis, and to remove terminal D-Ala from newly synthesized PG strands, resulting in formation of monomeric and dimeric tetramers (M4 and D44 respectively) (L. Potluri et al., 2010). By regulating the availability of pentapeptides, PBP5

may influence the extent of PG crosslinking, as well as the frequency of reactions utilizing tetrapeptides and shorter peptide chains (Young, 2004). Tetrapeptides typically constitute the bulk (∼60%) of PG peptide subunits (Glauner et al., 1988), and are used as energy donors in several subsequent steps of PG processing and maturation (van Heijenoort, 2011).

In our previous studies of *Vibrio cholerae* cell wall biogenesis, we have found similarities, but also significant differences, between the HMW PBP-mediated processes underlying PG synthesis in *E. coli* and *V. cholerae*, the Gram-negative pathogen that causes cholera (Dorr et al., 2013; Dorr, Lam, et al., 2014; Dorr, Moll, et al., 2014; Moll et al., 2014). Here, we begin to investigate the roles of LMW PBPs in *Vibrio cholerae*. In particular, we focus on DacA-1 (one of *V. cholerae's* two PBP5 homologs), because we found that its absence markedly impairs *V. cholerae* growth and morphology in typical laboratory media. In contrast, the absence of the pathogen's other LMW PBP homologues does not have detectable effects on these phenotypes. The absence of DacA-1 has even more detrimental effects during *V. cholerae* growth in media with elevated salt concentrations. Surprisingly, however, DacA-1 was dispensable in salt-free media as well as in an animal model of infection, suggesting that the host environment during infection does not impose a high salt challenge on *V. cholerae*. Comparative analyses of PG from wild type *V. cholerae* and strains lacking various LMW PBPs indicate that DacA-1 is *V. cholerae's* principal DD-CPase, even in low salt media. Collectively, our data suggest that DacA-1's DD-CPase activity and its effects on PG structure are crucial for *V. cholerae*'s adaptation to salt stress, and that DacA-1 associated phenotypes can be used as reporters of the bacterial growth environment.

Results

LMW PBPs of V. cholerae

The *Vibrio cholerae* genome encodes four homologues of *E. coli* LMW PBPs (Fig. 1A). VC0632 is most similar to the bifunctional DD-peptidase PBP4 (encoded by the *dacB* gene), while VCA0870 is most similar to the DD-endopeptidase PBP7 (encoded by the *pbpG* gene), and we will refer to them here by the names of the genes encoding these homologs. Both VC0947 and VCA0270 are highly similar to the DD-CPase PBP5, and the encoding genes are annotated as *dacA*-1 and *dacA*-2, respectively. The protein sequences of DacA-1 and DacA-2 are 60% identical and the PBP5 active center (C. Chowdhury and Ghosh, 2011) is fully conserved between both proteins (Fig. S1). DacA-1, DacA-2 and PBPG all contain a Sec export sequence and a peptidase S11 domain, and DacA-1 and DacA-2 contain an additional C-terminal domain of unknown function that is present in PBP5 and homologues (PBP5_C domain in Fig. 1B). The C-termini of DacA-1 and DacA-2 contain predicted amphipathic helices; in *E. coli* PBP5, such a helix is required for anchoring the enzyme to the inner membrane (L. Potluri et al., 2010). DacB, on the other hand, contains a predicted transmembrane domain and a peptidase S13 domain.

DacA-1 is required for V. cholerae growth, colony forming ability and normal cell morphology

To determine the relative importance of these genes in *V. cholerae*, we generated mutants with single and multiple deletions of LMW PBP-encoding genes. Absence of *dacA-2*, *dacB*, and *pbpG* alone or in combination had no apparent effect on growth (based on culture optical density); however, the growth of the *dacA*-1 deletion mutant was markedly impaired, both in rich media (LB; Fig. 1C), and in minimal media such as M9 (Fig. S2A). Additionally, the mutant had a severe deficiency establishing colonies on solid media; for any given culture density, plating of wt and *dacA-1* cells resulted in ∼5000× fewer colonies of the mutant (Fig. S2B). Collectively, the slow growth in culture and the severe colony forming defect of the *dacA-1* mutant likely account for the classification of *dacA-1* as an essential gene in our recent genome-wide annotation of *V. cholerae* essential genes using a TnSeq-based approach (Chao et al., 2013).

In addition to its effects on *V. cholerae* growth, the absence of *dacA-1* had marked effects upon *V. cholerae* cell shape, while the absence of other LMW PBPs did not (Fig. 1D). In LB medium, cells of a Δ*dacA*-1 strain were enlarged as well as elongated, and formed aberrant poles and branches. Their morphological defects became more pronounced as their growth progressed (Fig. S2C). A comparable phenotype was observed upon depletion of DacA-1 in a strain in which its expression was controlled by an arabinose-inducible promoter ($P_{dacA\text{-}1}$: :P_{BAD}:*dacA-1*; *dacA-1 cond;* Fig. 1E). Cells of this strain appeared normal when grown in the presence of arabinose (Fig. 1E), providing further evidence that the observed morphological defects are due to the absence of DacA-1.

Overexpressed DacA-2 partially compensates for the absence of DacA-1

Given the sequence similarity of *dacA-1* and *dacA-2*, we wondered whether over-expression or deficiency of DacA-2 would alter the phenotype of the Δ*dacA-1* mutant. Notably, ectopic production of DacA-2 in DacA-1-deficient cells restored wt morphology (although it only partially compensated for their growth deficiency) (Fig. 1F and S2D). We were not able to generate a mutant lacking both *dacA-1* and *dacA-2*; however, strains in which one gene was deleted and the other was conditionally expressed showed an even greater growth deficiency than that of the *dacA-1* mutant under the non-inducing conditions (Fig. 1G). These results are consistent with the hypothesis, based on their sequence similarity, that DacA-1 and DacA-2 perform similar functions. Previous RNAseq-based transcriptome analyses revealed 7× higher transcript abundance for *dacA-1* (Mandlik et al., 2011); additionally, using chromosomal *lacZ* transcription reporter fusions for each locus, we observed ∼3× higher activity associated with the *dacA-1* promoter during exponential phase growth in LB (Fig. 1H). Consequently, the more severe phenotype associated with the absence of *dacA-1* may predominantly reflect its higher expression level, rather than the existence of functions that can only be performed by DacA-1, although we cannot rule out the possibility of unique functions for DacA-1 and DacA-2.

The absence of dacA-1 results in a global defect in PG homeostasis

To explore the influence of DacA-1 on *V. cholerae* PG synthesis and remodeling, we visualized wt and Δ*dacA*-1 cells grown in the presence of a fluorescent D-alanine analogue,

HADA, which is incorporated into PG (Kuru et al., 2012). Immediately following growth in HADA-supplemented media, both wt and Δ*dacA*-1 cells displayed similar and fairly even staining over the entire cell surface (Fig 2, time 0). Despite their elongation(indicative of a cell division defect), Δ*dacA-1* cells did not contain internal sites of enhanced HADA staining, such as have been seen in mutants lacking PG processing amidases, which fail to cleave PG at division septa (Moll et al., 2014). However, following 30 or 60 min outgrowth in the absence of HADA, the staining patterns of the two strains differed dramatically. Wild-type cells showed almost complete loss of the label along the cell body and at one pole, while the PG at the opposite pole retained the label through several generations (Fig. 2), consistent with the presence there (as described in *E. coli;* (de Pedro et al., 2003)) of inert PG. In contrast, staining decreased more slowly and uniformly in Δ*dacA*-1 cells, and a consistent imbalance between staining of polar PG and other regions was less evident. Thus, the absence of DacA-1 appears to markedly alter PG homeostasis throughout the cell, suggesting that either DacA-1 or processes that it regulates have a global impact on PG structure.

The ΔdacA-1 mutant contains elevated levels of monomeric and dimeric PG pentapeptides

To understand how DacA-1 (as well as other LMW PBPs) modifies PG at a more molecular level, we purified PG from mutants of interest and analyzed its composition (Fig. 3A, Table S1). Relative to the wt strain, PG from the Δ*dacA*-1 mutant contained markedly increased levels of M5 monomers, D4,5 dimers and total pentapeptides (Figs. 3A, 3B, S3A, and Table S1), suggesting that DacA-1 is *V. cholerae's* major DD-carboxypeptidase responsible for cleavage of the terminal D-Ala from pentapeptides. A corresponding decrease in the total amount of tetrapeptides and tripeptides was also detected in the Δ*dacA*-1 mutant (Figs. 3C, S3B). In contrast, the triple mutant Δ*dacA*-2 Δ*dacB* Δ*pbpG* showed only a slight increase in the pentapeptide fraction and no decrease in tetrapeptides or tripepeptides, consistent with these three enzymes having a minor influence on *V. cholerae* pentapeptide cleavage. We also observed a small but significant decrease in PG crosslinking and an ∼50% increase in average chain length (measured as decreased anhydro muropeptides) in the Δ*dacA*-1 mutant (Figs. 3D, 3E, and S3C). The latter observation suggests the possibility that pentapeptides may not be optimal substrates for lytic transglycosylases, which are thought to generate anhydro muropeptides, or that DacA-1 in some other manner alters the activity of lytic transglycosylases. We did not observe effects of the *dacA-2* deletion on PG composition, suggesting that under the conditions used to grow cultures for this experiment, DacA-2 has little PG modifying activity, or that its role is masked by DacA-1's action (Figs. 3, S3 and Table S1).

The ΔdacA-1 mutant has wt growth and morphology in salt-free media

As part of an unrelated project to characterize *V. cholerae's* halotolerance, we used transposon insertion site sequencing (TnSeq) to compare the pathogen's genetic requirements for growth in LB without added NaCl (LBSF) vs our standard LB (171 mM NaCl). As noted above, a *V. cholerae* transposon library grown in LB contained virtually no insertions in *dacA-1*, and the gene was classified as an essential gene by our HMM-based pipeline (Chao et al., 2013). Remarkably, in a library generated in LBSF medium, many reads mapped to *dacA-1*, suggesting that *dacA*-1 is not required for *V. cholerae* growth in

LBSF. In fact, *dacA-1* was among the most conditionally enriched genes in this comparison (relative read count ∼9× increased LBSF/LB, p value 1.27E-11; Fig. S4).

We further explored the importance of DacA-1 in *V. cholerae* halotolerance by culturing wt and Δ*dacA*-1 cells in media containing a range of salt concentrations. Notably, the mutant cells exhibited wild-type morphology in LBSF (Figs. 4A, 4B), and no growth deficiency was evident based on culture OD600 in this medium (Fig. S5A). Similarly, use of LBSF enabled cells lacking both DacA-1 and DacA-2 to grow at a normal rate and retain *V. cholerae's* typical rod shape (Figs. 4C and S5B). The plating deficiency of the Δ*dacA*-1 strain was also eliminated by use of salt free media (Fig. 4D) or LB containing up to 100 mM NaCl (Fig. 4E). Higher concentrations of NaCl resulted in formation of small (120-140 mM NaCl) or almost no (160mM) mutant colonies on plates (Fig. 4E), and increasing concentrations of NaCl induced increasingly aberrant cell morphology in liquid culture, leading to lysis (Fig. 4A). In contrast, wt *V. cholerae* exhibited normal cell morphology and growth kinetics even in LB containing 500mM NaCl (LBHS; Fig. 4D, 4G). Strikingly, growth of the Δ*dacA*-1 mutant appeared to be unimpaired on LBSF plates containing high concentrations of sucrose (up to 400 mM; Fig. 4F), and its morphology was markedly less impaired in LBSF supplemented with KCl (171 mM or 500 mM) than NaCl (Fig. 4H), suggesting that the *dacA-1* mutant is particularly sensitive to the presence of Na^+, rather than sensitive to all osmotic challenges.

To gain further insight into the impact of salt free media on the Δ*dacA*-1 strain, we compared the PG composition of wt and Δ*dacA*-1 *V. cholerae* cultured in LBSF. Similar to what we observed in LB medium, the most dramatic difference we observed between the PG compositions of the two samples was in their levels of pentapeptides. As in standard LB, PG from the Δ*dacA*-1 strain grown in LBSF contained at least 8 fold more pentapeptides than the wt strain (Table 1). Thus, although DacA-1 is not required for preservation of normal morphology in salt-free media, its activity appears to be maintained in wt cells under this growth condition. Notably, the accumulation of pentapeptide under both conditions suggests that pentapeptide accumulation *per se* is not detrimental to growth or morphology, and thus that the *dacA-1* mutant's halosensitivity likely reflects more complex changes in response to the lack of carboxypeptidase activity.

The precise amounts of other PG components also differed between the two strains; however, they shifted in similar ways in response to the new media. Both showed an increase of monomeric and a decrease of multimeric peptides in LBSF compared to LB, a moderate increase in Lpp-bound peptides, a decrease of crosslinked peptides, and an increase in chain length. Thus, salt sensitivity of Δ*dacA-1* is likely not caused by this enzyme's effects on overall PG architecture, but rather by its direct or indirect influence on another, unidentified factor (e.g. spatio-temporal organization of localized PG synthesis or degradation, or overall rates of PG synthesis and/or degradation).

DacA-1 is not required for V. cholerae intestinal colonization

Since robust growth of the Δ*dacA*-1 mutant occurred only in media containing 100 mM NaCl or less, we used this mutant as a reporter to investigate the ionic profile of the intestines of suckling mice, which are a well established model for studying *V. cholerae*

intestinal colonization. For these assays, exponential phase cultures of wt and Δ*dac-1 V. cholerae* grown in LBSF were orogastrically inoculated into 5 day old suckling mice, and approximately 25 hr post-infection, colony forming units in intestinal homogenates were enumerated using LBSF plates. We detected no statistically significant difference between recovery of wt and Δ*dac-1 V. cholerae* from infected animals (Fig. 5), suggesting that the absence of DacA-1 does not impair *V. cholerae* survival or growth in vivo. Given our knowledge of the mutant's growth in vitro, the in vivo result suggests that the murine intestine contains a relatively low (100 mM or less) level of NaCl.

Discussion

Biochemical characterization of PG from wt and *dacA-1 V. cholerae* supports the presumption that DacA-1 is a DD-CPase that cleaves the terminal D-Ala from pentapeptides in PG, as has been observed for its homolog in *E. coli*, PBP5. However, in stark contrast to its *E. coli* counterpart, *V. cholerae* lacking DacA-1 displays severe growth impairment and altered cell morphology when cultured in LB media or plated on LB agar. Our results indicate that DacA-1 is *V. cholerae's* principal D,D carboxypeptidase; in its absence, *V. cholerae* accumulates >8 fold more pentapeptides in PG, whereas the absence of the pathogen's remaining 3 LMW PBPS, either singly or in combination, has little or no effect on accumulation of pentapeptides, growth or morphology. *V. cholerae* is far more dependent upon DacA-1 than *E. coli* is upon any of its LMW PBPs, since *E. coli* lacking all of its LWM PBPs grows with near normal kinetics (Denome et al., 1999). Unexpectedly, we found that use of culture media with low (100mM NaCl) or no added salt enabled DacA-1-deficient *V. cholerae* to grow with wild type rate and morphology, while addition of extra salt exacerbated the defects of the mutant strain. These observations, coupled with our finding that DacA-1-deficient *V. cholerae* can readily colonize the intestines of suckling mice, suggests that intraintestinal Na^+ concentrations are not greater than 100mM.

Based on their homologies to *E. coli* proteins, we expect that three of *V. cholerae*'s LMW PBPs have DD-CPase activity (DacA-1, DacA-2, and DacB), and that DacB and PBPG have DD-endopeptidase activity. Despite the overlapping specificities of these proteins, only DacA-1 is required for normal growth under any of the conditions tested. DacA-2, the closest DacA-1 homolog, is expressed at lower levels than DacA1, and can at least partially complement a DacA-1 deficiency when overexpressed. The physiologic role of DacA-2 is not known, but it is possible that *V. cholerae* employs its second DD-CPase under as yet unidentified conditions where DacA-1 activity is insufficient to sustain normal PG metabolism.

The fact that DacA-1-deficient cells grown in LB are both longer and wider than wt cells is consistent with observations in *E. coli* that DacA contributes both to cell elongation and to cell division (Santos et al., 2002). *E. coli* DacA is thought to localize to sites of lateral and septal PG synthesis in a substrate-dependent manner (L. Potluri et al., 2010), and it has been proposed that increased levels of DacA can shift the balance between these two processes to promote cell division, potentially through a role in generating a PG substrate required by the cell division-specific PBP3 (Begg et al., 1990). Regulation of substrate availability is also a possible role of DacA in cell elongation. We have previously shown that an elongation-

specific endopeptidase, ShyA, has reduced cleavage activity on pentapeptide-containing PG (Dorr et al., 2013). Thus, it is possible that, at least in LB medium, accumulation of pentapeptides leads to reduced endopeptidase activity and thereby impairs cell elongation, which is concomitant with a cell width increase. In *E. coli*, the absence of *dacA-1* plus other LMW PBPs is also associated with mislocalization of FstZ and Z rings, which is thought to give rise to the branching observed in such mutants (L.-P. Potluri et al., 2012); however, the precise mechanism underlying this shape deficiency, and the relationship (if any) to pentapeptide accumulation, has not been ascertained.

Although pentapeptides are generally rare in *V. cholerae* PG, their accumulation in the *dacA-1* mutant is not intrinsically detrimental. The mutant maintains normal growth and cellular morphology under certain conditions (discussed below) despite a markedly elevated level of pentapeptides in its PG. Additionally, although pentapeptides are substrates for major transpeptidases, our data suggest that the abundance of pentapeptides is not necessarily a limiting factor in formation of PG crosslinks. We observed that the *V. cholerae dacA-1* mutant contains a reduced amount of crosslinks relative to the wt strain in both LB and LBSF, despite its markedly elevated level of pentapeptides.

Our discovery that use of salt-free media restores a normal growth profile to DacA-1-deficient *V. cholerae* is counterintuitive for several reasons. First, *V. cholerae* does not normally thrive in low salt media; indeed, in the absence of nutrients, it survives for less than a day in salt-free water, whereas it is viable for extended periods (> 70 days) in water containing added salt (C. J. Miller et al., 1982; 1984). The salt is required both as an osmoprotectant and (at lower levels) a specific need for Na^+ ions, and salinity is thought to be a crucial factor in environmental survival of *V. cholerae.* Thus, it might be expected that a “sick” strain such as the *dacA-1* mutant would fare worse in salt-free growth conditions. Second, given the reduced level of cross links in PG from the mutant strain, it might be expected to be less resistant to hypo-osmotic conditions and an increase in cellular turgor pressure than the wt strain, unless the observed increase in PG chain length compensates for the altered crosslinking. However, such predictions are somewhat premised on an expectation that PG structure will not be altered in response to environmental conditions. In fact, analyses in other organisms suggest that localization and expression of PBPs is altered in response to osmolarity (Piuri et al., 2005; Palomino et al., 2009; Hocking et al., 2012). In *V. cholerae*, growth in media containing no added salt, vs. growth in the presence of increasing salt levels, is associated with altered expression of numerous genes(Shikuma and Yildiz, 2009). In particular, we note that the absence of salt induces expression of VCA0870, *V. cholerae*'s homolog of the endopeptidase PBP7, probably due to altered expression of the salt responsive transcriptional regulator OscR. It is possible that altered expression of the putative endopeptidase facilitates adaptation of *V. cholerae* PG to the no salt milieu. However, since the *dacA-1* mutant has markedly elevated levels of pentapeptides in both LB and LBSF, it is likely that survival in LBSF is not due simply to the induction and compensatory activity of alternate DD-CPases.

Our biochemical analyses of PG support the hypothesis that the composition of *V. cholerae* PG is dependent upon the environment in which bacteria are grown. *V. cholerae* cultured in LBSF produced PG with high levels of monomers, longer average chain lengths, and a

reduced frequency of crosslinks than in PG from cells cultured in LB. All of these differences were also observed in the *dacA-1* mutant, suggesting that the mutant and wt strain respond similarly to the new environment. The reduced level of crosslinks in response to LBSF could be related to induction of VC0807/PBPG. It is also consistent with the concept of “smart” autolysins, which only cleave PG that is stressed, e.g., due to growth or some other cause of increased turgor pressure (Koch, 1990), although it has recently been demonstrated that incorporation of new PG subunits is not in fact dependent upon cellular turgor pressure (Rojas et al., 2014). We find it noteworthy that PG from LBSF cultures is less crosslinked than PG from LB cultures, despite the greater osmotic stress imposed on cells in LBSF. Our analyses suggest that increases in PG chain length, rather than in PG crosslinking, may be critical for enhancing PG resilience against osmotic challenges. Presumably, the presence of longer chains permits a greater number of muropeptides to be tethered to the PG mesh by a limited number of interpeptide bonds.

Our finding that *V. cholerae* lacking DacA-1 display a wt capacity to colonize the intestines of infant mice was somewhat unexpected, given the mutant's reduced viability under several in vitro growth conditions. Our in vitro data indicate that the *dacA-1* mutant has impaired growth in LB containing more than 100 mM NaCl, and is also sensitive to higher concentrations of KCl. It is possible that differences between the constituents of LB and the intestinal milieu might enable greater salt tolerance in vivo; however, a simpler explanation for the observed robust intestinal colonization by the *dacA-1* mutant is that the NaCl concentration in the intestinal environment is 100 mM or lower. Stool shed by cholera patients has been found to have an average Na^+ content of 88 mM (Molla et al., 1981); however, this has not necessarily been presumed to reflect the environment at the epithelium or in the lumen of the small intestine, where infecting *V. cholerae* reside (V. L. Miller and Mekalanos, 1988; Gupta and R. Chowdhury, 1997). Our in vitro characterization of the *dacA-1* mutant's NaCl sensitivity, coupled with the analyses of colonization, thus enabled indirect interrogation of the in vivo environment.

In conclusion, we have shown here that compared to the Gram-negative model organism *E. coli*, the human pathogen *V. cholerae* exhibits reduced redundancy in its LMW PBP repertoire. Most strikingly, a single carboxypeptidase, DacA-1, is required for normal growth and morphology in media containing moderate to high salt concentrations, although it is dispensable for growth in salt free media. Deciphering the underlying basis for the distinct requirements for DacA-1 in *V. cholerae* and *E. coli* should provide greater insight into the biological functions of DD-CPases and their interplay with other enzymes that mediate PG synthesis and maturation.

Material and Methods

Strains, media, and growth conditions

V. cholerae strains described in this study (Table S2) are derivatives of *V. cholerae* wild type El Tor strain C6706. *E. coli* DH5α λpir was used for general cloning purposes. *E. coli* SM10 λpir and MFDpir (Ferrieres et al., 2010) were used for conjugation.

Cells were grown at 37°C in Luria-Bertani (LB) medium (which contains 171 mm NaCl), salt-free LB medium (LBSF), LB medium containing 500 mM NaCl (LBHS), or M9 medium supplemented with 0.2% glucose (M9). Media were supplemented with 200 μg/ml streptomycin, 50 μg/ml kanamycin, 50 μg/ml carbenicillin, or 5 μg/ml (*V. cholerae*)/20 μg/ml (*E. coli)* chloramphenicol. For induction of genes under control of arabinose-inducible promoters, strains were grown in media supplemented with 0.2% L-arabinose. MFDpir was grown in the presence of 0.3 mM diaminopimelic acid.

For growth curves, at least 3 replicates per strain and condition were grown in 200 μl medium in a 100 well honeycomb plate inoculated 1:100 from exponentially growing pre-culture (OD600∼ 0.02) and analyzed in a Biotek growth plate reader at 10 min intervals. Data were analyzed using GraphPad PRISM® for Windows (GraphPad, San Diego, CA).

Construction of plasmids and strains

Plasmids and strains are described in Tables S2 and S3. Plasmids were generated with Gibson assembly (Gibson et al., 2009). In-frame deletions were introduced using sucrose-based counter selection with *sacB*-containing suicide vector pDS132 (Philippe et al., 2004). Proteins were overproduced by placing the respective gene under the control of the *araC* (P_{BAD}) promoter using vectors pBAD18-kan or pBAD33 (Guzman et al., 1995), and depleted by replacement of the native promoter with P_{BAD} using derivatives of pAM299. Transcriptional lacZ-reporter fusions of *E. coli lacZ* and the promoter of interest were integrated into the chromosome of *lacZ* C6706, resulting in generation of promoter merodiploid strains, using derivatives of pAM325.

β-galactosidase assays

V. cholerae strains expressing transcriptional *lacZ* reporter fusions were inoculated in triplicate from overnight cultures into 200μl LB medium to an OD600 of 0.005 and incubated at 37°C. 10μl of exponentially growing culture (OD600 0.2-0.4), were added to 95 μl Z-buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4) containing 50 mM sodium β-mercaptoethanesulfonate and 5% chloroform. Solutions were incubated 5 minutes at 30°C. After addition of 20 μl ortho-nitrophenyl-β-galactoside solution (4 mg/ml in Z-buffer), solutions were incubated at 30°C until turning yellow, and 50 μl 1M NaCl were added to stop the reaction. The A420 and the A550 of the stopped reaction mixture were recorded, and Miller units were then calculated by the following formula: 1,000*[A420-1.75*A550]/ [time(min)*volume (ml)*OD600] (J. H. Miller, 1992).

Microscopy

Unless otherwise stated, microscopy was performed using exponentially growing cells (OD600 ∼ 0.2-0.6). Bacteria were immobilized on pads containing 1% agarose, 10% LB, and 1× PBS, and supplemented with arabinose when noted. They were visualized using a Zeiss AxioImager.2 microscope equipped with a Plan Neofluor 100×/1.3 Oil Ph3 objective and a Hamamatsu Orca ER 1394 camera. Images were processed with ImageJ (Schneider et al., 2012).

Staining with 7-hydroxycoumarin-3-carboxylic acid coupled D-alanine (HADA) was essentially performed as described (Kuru et al., 2012). In short, cells were grown to exponential phase in LB medium and cultured in the presence of 100 μM HADA for 45 min at 37°C. Cells were washed twice in LB without HADA, and imaged as above.

Isolation of peptidoglycan and UPLC/HPLC analysis

Peptidoglycan of *V. cholerae* strains was isolated as described previously (Cava et al., 2011), starting with cells from 1 L culture in stationary phase. Samples were digested with pronase E (100 μg/ml) in 10 mM Tris-HCl, pH 7.5, 1 h at 60°C to remove Braun's lipoprotein. After addition of SDS to a final concentration of 1% (w/v), reactions were heat-inactivated and detergent was removed by washing in MilliQ water.

Purified peptidoglycan was re-suspended in 50 mM $NaPO_4$ buffer, pH 4.9, and treated with 100 μg/ml muramidase for 16 h at 37°C. Muramidase digestion was stopped by incubation in a boiling water bath and coagulated proteins were removed by 10 min centrifugation at 14,000 rpm. The supernatants were reduced by adding 150 μl 0.5 M sodium borate pH 9.5 and sodium borohydride to a final concentration of 10 mg/ml and incubating at RT for 30 min. Finally, samples were adjusted to pH 3.5 with phosphoric acid.

Muropeptides were separated in a linear gradient of 50 mM $NaPO_4$, pH 4.35 to 50 mM $NaPO_4$, pH 4.95, 15% methanol (v/v). Peptides were detected at A204 nm.

The relative amounts of the muropeptides were calculated as described by Glauner (Glauner, 1988). The values are the means of two independent experiments. Data were analyzed using PRISM. Variations of less than one percent were considered chance variation. Statistical significance was determined using an unpaired two-tailed t-test ($p<0.01$).

Transposon-insertion sequencing (TIS)

TIS was performed essentially as described (Chao et al., 2013), but using salt-free LB medium at all times. In brief, 500,000-600,000 transposon mutants were generated for each strain by conjugation with MFDpir containing the transposon delivery vector pSC189, and pooled genomic DNA fragments from the library of insertion mutants were analyzed on an Illumina MiSeq benchtop sequencer (Illumina, San Diego, CA). Insertion sites (which included 66% of TA sites) were identified as described (Chao et al., 2013), and significance was determined using ConArtist simulation-based normalization as described (Pritchard et al., 2014). Results were visualized using Artemis (Carver et al., 2012).

Protein sequence analyses

Protein sequences were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/). Protein primary sequence alignments were generated with ClustalOmega (Sievers et al., 2011) and edited with BioEdit 7.0.5 (Ibis Therapeutics, USA). Protein architectures were determined using SMART analyses (Letunic et al., 2012).

Mouse colonization assays

Assays were performed basically as described (Angelichio et al., 1999), except that cells for the innocula were grown to exponential phase (OD600 ~ 0.2) in LBSF at 37°, then diluted 1:100 in the same medium. Infant mice were gavaged with 50 μL of the diluted culture (containing ~5×10^6 cfu), then sacrificed after 25 hours. Dilutions of homogenized small intestines were plated on salt-free LB agar to enumerate cfu.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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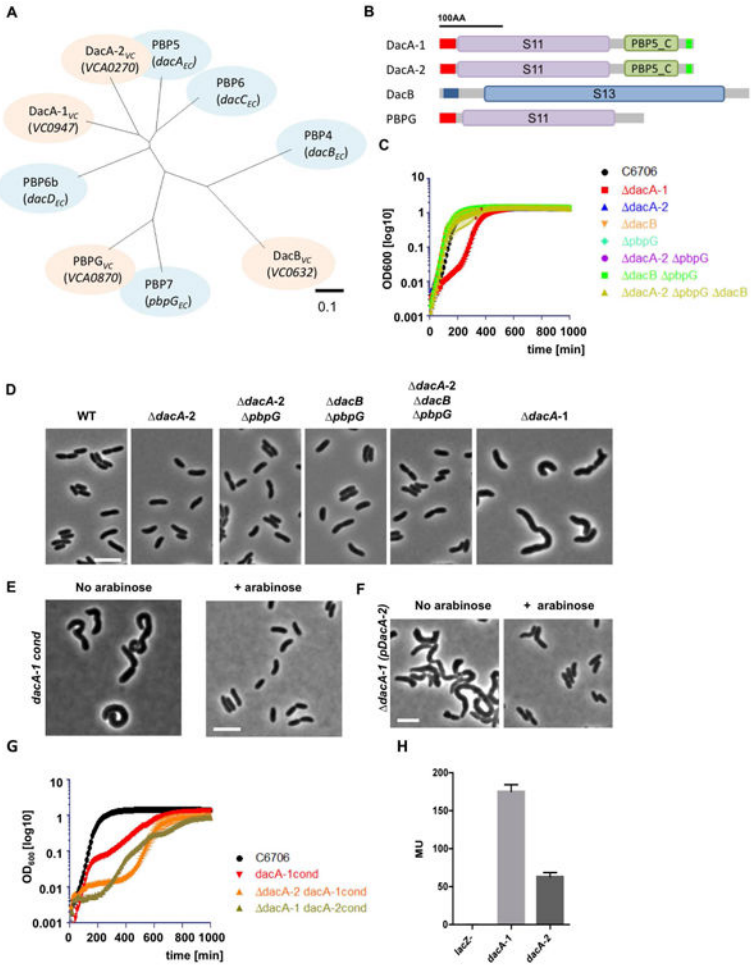

Fig. 1. DacA-1 is the principal LMW PBP of *V. cholerae* and is required for normal cell growth and shape

A. Unrooted left-stacked phylogram depicting distances between putative *V. cholerae* and *E. coli* LMW PBPs. Protein sequences were analyzed with ClustalOmega and the tree was edited with TreeView. B. Predicted domain structures of *V. cholerae* LMW PBPs. DacA-1 and DacA-2 contain a signal sequence (red), an S11 peptidase domain (S11) and a PBP5_C domain of unknown function (PBP5_C). DacB contains a transmembrane region (blue) and an S13 peptidase domain (S13). PBPG contains a signal sequence and an S11 peptidase domain (S11). C. Growth curves of LMW PBP mutants grown in LB medium. Values represent the mean and SEM of at least three replicates. D. Phase contrast images of cells grown to exponential phase in LB. Scale bars 5 μm. E. Phase contrast images of *V. cholerae* expressing *dacA-1* under the control of the arabinose-inducible promoter P_{BAD} (*dacA*-1 cond), grown to exponential phase in LB supplemented with 0.2% arabinose, washed, and subcultured in exponential phase in medium without inducer for 12 h to deplete DacA-1 (top) or grown in medium supplemented with 0.2% arabinose (bottom). F. Δ*dacA*-1 cells expressing ectopic *dacA*-2 under control of the P_{BAD} promoter were grown for 4h in medium supplemented with 0.4% arabinose or without arabinose. G. Growth curves for wt *V. cholerae* and strains expressing *dacA-1* or *dacA-2* under the control of the arabinose-inducible promoter P_{BAD} (*dacA-1* cond. or *dacA-2* cond., respectively). All strains were

precultured in media containing 0.2% arabinose, then diluted into LB without arabinose for measurement of OD600. H. β–gal activity from a *lacZ*-deficient control strain and from strains containing chromosomal *lacZ*-reporter fusions of the *dacA-1* and *dacA-2* promoters. Activity of the transcription reporter fusions was measured in log phase cultures in LB media. Columns depict the mean Miller units (MU) derived from 3 experiments with 3 replicates each +/− SEM.

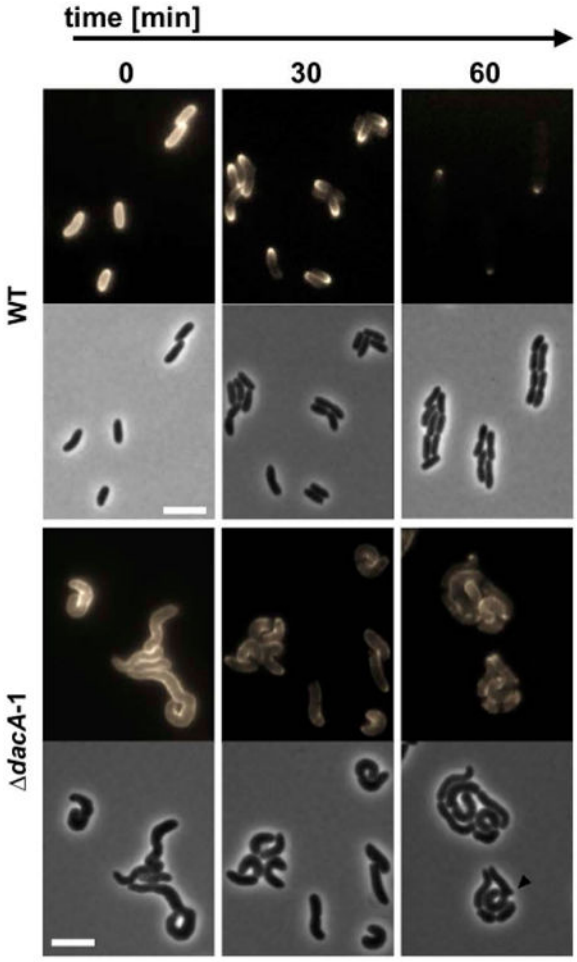

Fig. 2. Turnover of HADA, which stains PG, in wt and Δ*dacA*-1 cells

A. Wild-type and Δ*dacA-1* cells were stained with HADA for 30 min, washed, transferred to agarose pads without HADA, and visualized at different time points after wash. Scale bars 5 μm.

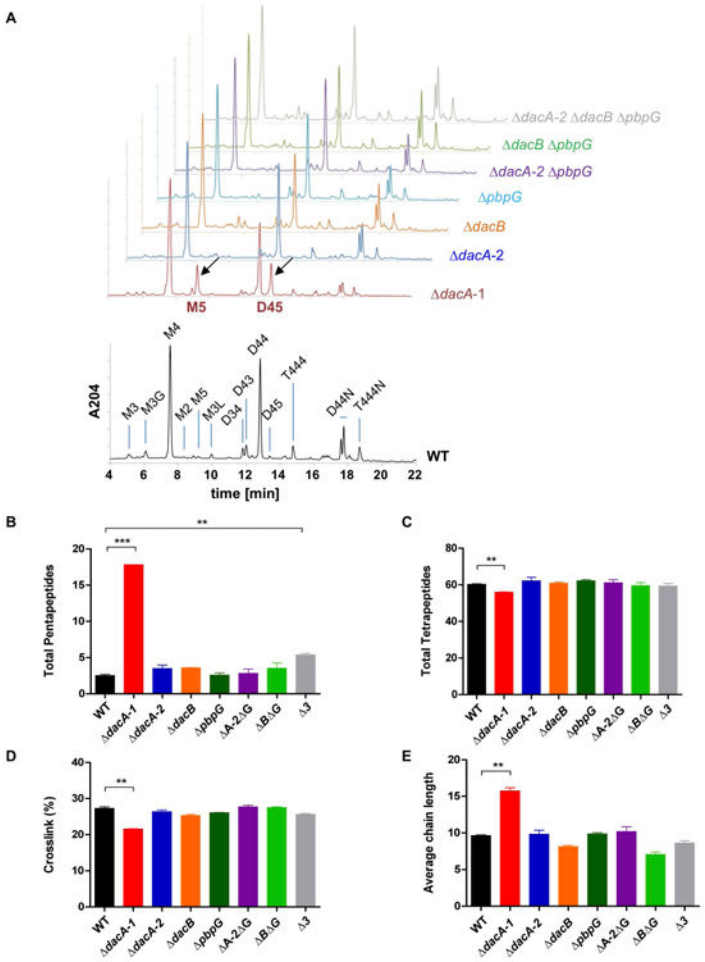

Fig. 3. Peptidoglycan composition of LMW PBP mutants

A. UPLC analysis of PG from wild-type (WT), Δ*dacA-1*, Δ*dacA-2*, Δ*dacB*, Δ*pbpG*, Δ*dacA-2*Δ*pbpG*, Δ*dacB*Δ*pbpG*, and Δ*dacA-2*Δ*dacB*Δ*pbpG (*Δ*3) V. cholerae*. Graphs were normalized by scaling the chromatograms relative to the maximum intensity measured in each run. Key peaks containing pentapeptides are marked with arrows. B, C. Comparison of the relative molar abundance of total pentapeptide (B) or tetrapeptide (C) moieties in the PG of the indicated strains. D. Crosslinking levels (%) in PG from the indicated strains. E. Average chain length based on the amount of anhydro-muropeptides. Relative amounts of muropeptides were calculated as described by Glauner (Glauner, 1988). The values are the means of two independent experiments. Error bars represent standard deviation of the mean. Stars indicate statistically significant differences based on unpaired t-test (**p£ 0.01, ***p£ 0.001).

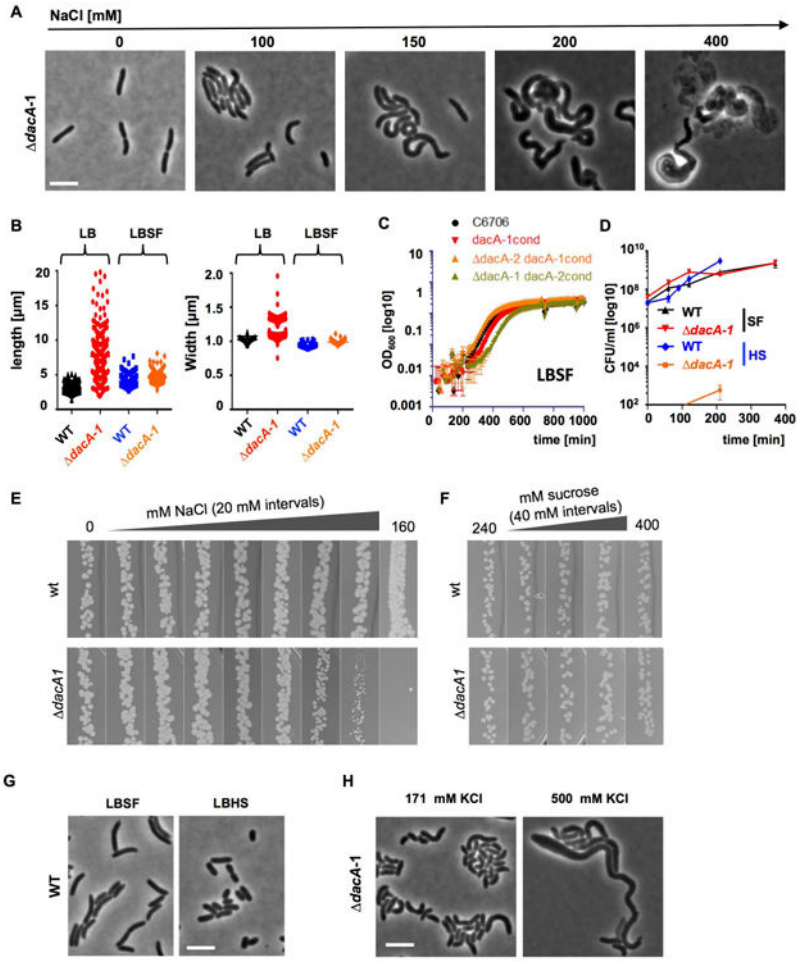

Fig. 4. The Δ*dacA*-1 phenotype is dependent on salt concentration

A. Phase contrast images of Δ*dacA*-1 cells grown to exponential phase in salt-free LB, then transferred to and grown for 4h in LB with different NaCl concentrations. B. Length and width of wt and *DdacA-1 V. cholerae* grown in LB or salt free LB (LBSF). At least 500 cells were measured for each condition using Microbe Tracker (Sliusarenko et al., 2011). Growth of wild type *V. cholerae* and the indicated conditional mutants in LBSF in the absence of inducer. Cells were precultured in LB containing arabinose. C. Growth curves of wt *V. cholerae* and strains lacking DacA-1 or DacA-1 and DacA-2 in LBSF lacking arabinose. Values represent the mean and SEM of at least three replicates. D. CFU of wild-type and Δ*dacA*-1 strains precultured in LBSF, then grown in LBSF or LB containing 500mM NaCl (LBHS), followed by plating on salt-free LB agar. E, F. Cultures were grown overnight in LBSF, then diluted in the same medium. For all conditions but 160mM NaCl, 20 ml of a 10^{-6} dilution was spotted on LB agar plates containing the indicated salt (E) or sucrose (F) concentrations. For plates containing 160 mM NaCl, 20 ml of a 10^{-5} dilution was spotted. G. Phase contrast images of wild-type cells grown as in (A). H. Phase contrast images of Δ*dacA*-1 cells grown in LBSF supplemented with 171 mM KCl or 500 mM KCl.

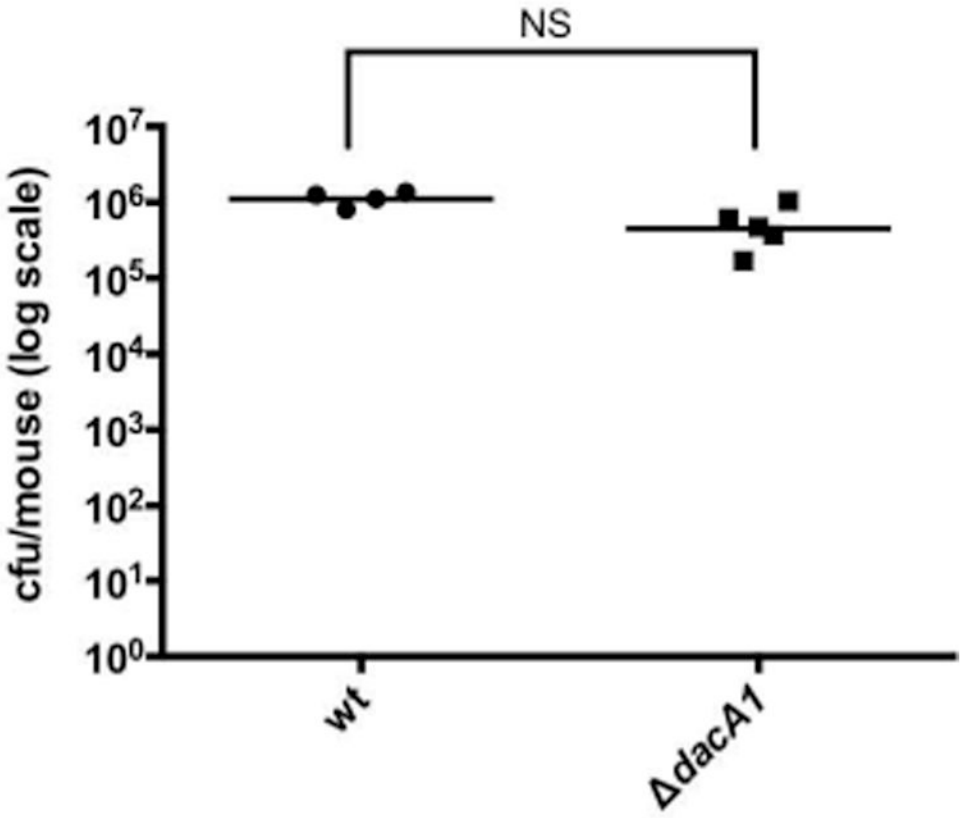

Fig. 5. Comparative analysis of wt and Δ*dacA*-1 colonization of infant mice

Suckling mice were orogastrically infected with ∼10^6 cfu of the indicated strain, and colonizing bacteria in intestinal homogenates were enumerated on salt free LB agar at ∼25 hr post infection.

Table 1

Analysis of PG composition[a] in wild-type and Δ*dacA*-1 *V. cholerae* grown in LB and LBSF

	wt		Δ*dacA-1*	
	LB	**LBSF**	**LB**	**LBSF**
Monomers	40.62	55.51	55.19	66.95
Dimers	26.10	21.17	21.12	15.96
Trimers	2.39	0.72	0.86	0.37
Tripeptides	5.78	12.56	2.71	5.08
Tetrapeptides	60.31	61.61	56.00	54.70
Pentapeptides	2.52	3.05	17.83	22.63
Anhydro peptides	10.38	4.86	6.35	3.50
Average chain length	9.64	20.87	15.75	28.53
Crosslink	27.30	21.53	21.55	16.15
LD-crosslink	1.57	0.51	0.71	0.31
DD-crosslink	25.73	21.02	20.84	15.84

[a]Relative amounts of muropeptides were calculated as described by Glauner (Glauner, 1988). The values are the means of two independent experiments. No significant differences were detected between the changes in PG in response to LBSF between the wt and Δ*dacA-1* strain.