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Involvement of the GspAB Complex in Assembly of the Type II Secretion System Secretin of *Aeromonas* and *Vibrio* Species▽†

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The type II secretion system (T2SS) functions as a transport mechanism to translocate proteins from the periplasm to the extracellular environment. The ExeA homologue in *Aeromonas hydrophila*, $GspA_{Ah}$, is an ATPase that interacts with peptidoglycan and forms an inner membrane complex with the ExeB homologue ($GspB_{Ah}$). The complex may be required to generate space in the peptidoglycan mesh that is necessary for the transport and assembly of the megadalton-sized ExeD homologue ($GspD_{Ah}$) secretin multimer in the outer membrane. In this study, the requirement for GspAB in the assembly of the T2SS secretin in *Aeromonas* and *Vibrio* species was investigated. We have demonstrated a requirement for GspAB in T2SS assembly in *Aeromonas salmonicida*, similar to that previously observed in *A. hydrophila*. In the *Vibrionaceae* species *Vibrio cholerae*, *Vibrio vulnificus*, and *Vibrio parahaemolyticus*, *gspA* mutations significantly decreased assembly of the secretin multimer but had minimal effects on the secretion of T2SS substrates. The lack of effect on secretion of the mutant of *gspA* of *V. cholerae* ($gspA_{Vc}$) was explained by the finding that native secretin expression greatly exceeds the level needed for efficient secretion in *V. cholerae*. In cross-complementation experiments, secretin assembly and secretion in an *A. hydrophila gspA* mutant were partially restored by the expression of GspAB from *V. cholerae* in *trans*, further suggesting that $GspAB_{Vc}$ performs the same role in *Vibrio* species as $GspAB_{Ah}$ does in the aeromonads. These results indicate that the GspAB complex is functional in the assembly of the secretin in *Vibrio* species but that a redundancy of GspAB function may exist in this genus.

The type II secretion system (T2SS), also known as the main terminal branch of the general secretory pathway (GSP), is utilized by many Gram-negative bacteria to translocate folded proteins across the outer membrane from the periplasm to the extracellular milieu. This system is a major virulence factor, responsible for the translocation of a variety of proteins that mediate pathogenic effects, including the pore-forming toxin aerolysin of *Aeromonas hydrophila* (15, 16), cholera toxin of *Vibrio cholerae* (43), and heat-labile toxin (LT) of enterotoxigenic *Escherichia coli* (ETEC) (53).

Proteins destined for T2SS secretion are transported across the inner membrane via the Sec (38) and Tat (54) pathways into the periplasm. Once in the periplasm, the T2SS, a complex composed of 12 to 16 proteins that spans the periplasmic space, functions in extrusion of the folded protein substrate through the megadalton-sized outer membrane complex (the secretin), which is composed of 12 to 14 GspD monomers (4). Transport of the protein through the pore created by the secretin could involve extension and retraction of a pseudopilus structure composed of GspG to GspK that is anchored to an inner membrane platform complex composed of GspE, -F, -L, and -M (2, 19, 23, 37, 39). In this way, the pseudopilus may act as a piston to push folded proteins located in the periplasm through the pore of the secretin multimer. The energy required for this process is presumably provided by the ATPase function of GspE (7), and in some species, including the aeromonads, energy derived from the proton motive force is also required (24). Other members of the T2SS include the substrate specificity selector protein GspC (10), the small outer membrane pilotin lipoprotein GspS that is required for secretin assembly in some bacteria and functions in protecting the secretin from proteolytic degradation (13, 48), and a prepilin peptidase (GspO or provided by a type IV pilin assembly system) involved in proteolytic processing of prepilin subunits during assembly of the pseudopilus (51).

Members of the *Aeromonas* and *Vibrio* genera are significant human and animal pathogens. These species, classified as part of the same clade of gammaproteobacteria (55), include *Vibrio cholerae*, which is the causative agent of the gastrointestinal disease cholera, and the fish, amphibian, and opportunistic human pathogen *A. hydrophila*. The T2SS encoded within these species is composed of Gsp proteins GspC to -N, the prepilin peptidase TapD, and an additional set of proteins, GspA and GspB, but no identifiable GspS homologue (20, 21, 35). The GspA and GspB proteins encoded in *Aeromonas hydrophila* ($GspA_{Ah}$ and $GspB_{Ah}$) and *Vibrio cholerae* ($GspA_{Vc}$ and $GspB_{Vc}$) are alike, with GspA proteins being 40% identical and 54% similar and GspB proteins 27% identical and 44% similar. Interestingly, *Vibrio vulnificus* encodes a 718-amino-acid (aa) protein that contains a GspA

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domain within the N-terminal 530 aa of the protein and a GspB domain within the C-terminal 188 aa (see Fig. S1 and S2 in the supplemental material). The identification of a GspAB fusion protein is perhaps not surprising, since according to the "Rosetta Stone" hypothesis for evolution of protein interactions, a propensity exists for interacting pairs of proteins to evolve into one protein because fusion greatly increases the affinity of each protein for the other and is therefore thermodynamically favorable (27).

Previous studies of the 60-kDa ExeA homologue ($GspA_{Ah}$) and 25-kDa ExeB homologue ($GspB_{Ah}$) in *A. hydrophila* demonstrated the requirement for coordinated expression of both $GspA_{Ah}$ and $GspB_{Ah}$ such that deletion of either protein prevented detection of the other (20). $GspA_{Ah}$ and $GspB_{Ah}$ span the inner membrane once and form a large heteromultimeric complex (18, 20) that interacts with peptidoglycan (PG) (25). The $GspAB_{Ah}$ complex has been demonstrated to be required for the localization and multimerization of the GspD secretin multimer in the outer membrane of *A. hydrophila* (3). We have also shown that the $GspA_{Ah}$ cytoplasmic domain is a novel ATPase (46) and have confirmed the function of a peptidoglycan-binding site in the $GspA_{Ah}$ periplasmic domain as being required for the assembly of the secretin multimer (17, 25). Thus, our working hypothesis is that the GspAB complex in some way modifies or organizes the peptidoglycan to allow assembly of the GspD secretin, a function presumably necessitated by the 50-kDa size constraint imposed by the peptidoglycan mesh (9).

As described above, $GspAB_{Ah}$ is required for the transport and assembly of the secretin in *A. hydrophila.* In other bacteria with a functional T2SS, however, the role of the GspAB complex remains unclear. In some bacteria, no identifiable GspAB homologue is present; in other bacteria, a GspB but no GspA is present (8); and in others, GspAB homologs have been identified *in silico* but have not been studied with respect to their role in T2SS secretion or secretin assembly.

In this study, we sought to ascertain the involvement of the GspAB complex in the assembly of the T2SS secretin multimer in *Aeromonas salmonicida* and several *Vibrio* species. Previous studies have shown strong similarities between the T2SS systems of this genus, such that *A. hydrophila* and *V. cholerae* hybrid T2SS component proteins are often functional (42), and the genomes of all sequenced *Vibrio* species contain homologous *gspAB* genes. Similar to the results of previous studies of *A. hydrophila*, we found that GspAB was absolutely required in *A. salmonicida* for both assembly of the secretin multimer and secretion of T2SS substrates. Conversely, the presence of GspAB was not an absolute requirement for function of the T2SS in *V. cholerae*, *V. vulnificus*, and *Vibrio parahaemolyticus.* Insertional inactivation of *gspA* in these bacteria resulted in minor decreases in the secretion of T2SS substrates. However, a significant decrease in the amount of secretin multimer formed was observed for each of the *gspA* mutants, suggesting that the GspAB complex is involved in the assembly of the secretin in members of the *Vibrionaceae*. The minimal effect on secretion despite the clear role of GspAB in secretin assembly in *V. cholerae* was explained when the expression of $gspD_{Vc}$ in *trans* in a $gspD_{Vc}$ mutant demonstrated that native levels of secretin expression in this bacterium substantially exceed the required capacity for secretion. The expression of the *V. cholerae gspAB* genes in *trans* in *A. hydrophila gspAB* mutants resulted in partial complementation of both secretin assembly and secretion of the T2SS substrate aerolysin, whereas the expression of *V. cholerae gspAB* in wild-type *A. hydrophila* cells inhibited aerolysin secretion. These results suggest that, similar to the T2SS of aeromonads, GspAB of vibrios facilitates the assembly of the secretin; however, unlike the aeromonads, other unidentified factors may exist that provide redundancy for secretin assembly in the vibrios.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. The strains and plasmids used in this study are shown in Table 1. *A. salmonicida* was grown in Luria-Bertani (LB) medium with Davis salts (28) at 22°C until the mid-exponential phase of growth. *A. hydrophila* was grown in LB medium supplemented with 30 mM Na_2HPO_4, 30 mM K_2HPO_4, 16.5 mM NaH_2PO_4, 16.5 mM KH_2PO_4, 0.75 mM $(NH_4)_2SO_4$, 0.4 mM $MgSO_4$, pH 7.0, at 30°C until mid-log phase. *Vibrio* species were grown in LB at a temperature of 37°C for *V. cholerae* and 30°C for *V. vulnificus* and *V. parahaemolyticus* until the mid- to late logarithmic phase of growth. *E. coli* strains S17-1 and XL1-Blue were cultured in brain heart infusion (BHI) and LB, respectively. Antibiotics were used at the following concentrations: rifampin, 50 μg/ml; kanamycin (Kan), 50 μg/ml; chloramphenicol, 1.25 μg/ml; and streptomycin, 20 μg/ml.

Rifampin-resistant variants of *A. salmonicida* and each *Vibrio* strain were selected by spreading a 10-times-concentrated cell suspension from 1 ml of overnight saturated culture onto a BHI plate containing rifampin (100 μg/ml). Following overnight incubation at an appropriate temperature, rifampin-resistant colonies were selected and restreaked to confirm the resistant phenotype.

Enzyme assay of culture supernatants. Samples of whole-cell broth culture were taken and added to an equal volume of 2× sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.01% bromophenol blue, 10% β-mercaptoethanol) and heated for 5 min at 95°C. The remaining culture was centrifuged at 31,000 × *g* for 15 min, and 10 ml of supernatant was taken, filter sterilized, and stored at −20°C before being used to assay culture supernatant enzyme activity. When required, supernatant was concentrated approximately 10-fold by use of a Microcon-10 concentrator (10-kDa molecular-mass cutoff).

Lipase activity in culture supernatants was determined by assaying the release of *p*-nitrophenol (pNP) from *p*-nitrophenol caprylate (pNPC) (1), for which 100 μl of culture supernatant was added to 900 μl of substrate containing 100 mM Tris, pH 8.0, 0.2% Triton X-100, and 1 mM pNPC. The reaction mixture was incubated at room temperature for 30 min while the absorbance at 410 nm was measured at 5-min intervals. A unit of lipase activity equals 1 nmol pNPC hydrolyzed per minute. All enzymatic activities are expressed as units/ml supernatant per unit of optical density at 600 nm (OD_{600}) in the culture.

The amylase activity in supernatants from cultures of *Vibrio* species was determined according to the protocol that Jiang and Howard (21) used to assay the T2SS-dependent amylase activity in *A. hydrophila* culture supernatants. A 100-μl volume of supernatant was added to 250 μl 5% starch azure in 20 mM Na_2HPO_4 (pH 7), 50 mM NaCl, pH 7.0, and incubated at 37°C with vigorous shaking for 16 h. The reaction was stopped by the addition of 50 μl 2.5 M sodium acetate, the supernatant was collected by 5 min of centrifugation at 21,000 × *g*, and the absorbance at 595 nm was determined. Units are defined as the change in absorbance at 595 nm/hour.

Protease activity in culture supernatants was determined using azocasein as the substrate. A 100-μl volume of supernatant was incubated with 300 μl of 1% azocasein in 60 mM phosphate buffer (pH 7.2). The reaction mixture was incubated at 37°C with shaking for 2 h, stopped by the addition of 133 μl 30% trichloroacetic acid, and incubated on ice for 30 min. The sample was then centrifuged at 21,000 × *g* for 5 min, and 400 μl of supernatant was recovered. An equal volume of 1 M NaOH was added to the supernatant, and the absorbance at 450 nm was determined. Units of protease activity are expressed as the change in absorbance at 450 nm/hour.

To conduct the toxin B subunit secretion assay, the *etxB*-expressing plasmid pMMB68 that encodes the B-subunit of the heat-labile enterotoxin of *E. coli* was introduced into *V. cholerae* Bah2R and Bah2-*gspA* strains by conjugation. Strains were grown in M9 medium (28) supplemented with 0.2% glucose and 2% Casamino Acids. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.05 mM to induce the expression of EtxB, and the culture was grown for 1.5 h at 37°C. The amount of EtxB in culture supernatant and

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype/phenotype	Source
A. hydrophila strains		
Ah65	Wild type	Howard laboratory
C5.84	Ah65 *gspA*::Tn*5-751*	20
V. cholerae strains		
Bah2	E7946 derivative, Δ*tlc* Δ*attRS* Δ*ctx*Φ Δ*rtx*	34
Bah2-R	Rif^r mutant of Bah2	This work
Bah2-*gspA*	*gspA* mutant of Bah2-R	This work
TRH7000	El Tor O1 biotype, *thy* Hg^r *ctxAB*	32
TRH7000 *gspA*	*gspA* mutant of TRH7000	This work
TRH7000 *gspD*	*gspD* mutant of TRH7000, Kan^r	26
V. vulnificus strains		
V. vul. 67181283	Patient isolate	Provincial laboratory
VvR	Rif^r mutant of *V. vulnificus* 67181283	This work
Vv-*gspA*	*gspA*::Kan mutant of VvR	This work
V. parahaemolyticus strains		
V.para US32052027	Patient isolate	Provincial laboratory
VpR	Rif^r mutant of *V. parahaemolyticus* US32052027	This work
Vp-*gspA*	*gspA*::Kan mutant of VpR	This work
A. salmonicida strains		
As449	Wild type	22
AsR	As449 Rif^r	This work
As-*gspA*	AsR *gspA*::Kan	This work
E. coli strains		
SM10λ*pir*	*thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Kan^r	29
S17-1	*recA thi pro hsdR*, RP4::2-Tc::Mu::Kan Tn*7* λ*pir*	49
XL1-Blue	*recA1 lac endA1 gyrA96 thi-1 hsdR17 supE44 relA1*[F′ *proAB lacI*^{q}ZΔM15 Tn*10*]	6
Plasmids		
pMMB207	*tac* promoter, wide-host-range vector; Cm^r	30
pRJ31.1	2.5-kb BstXI fragment containing $gspAB_{Ah}$ in SmaI of pMMB207; Cm^r	20
pMMB/$gspAB_{Vc}$	5.6-kb EcoRI fragment containing $gspAB_{Vc}$ in EcoRI of pMMB207; Cm^r	This work
pMMB/$gspA_{Vc}$	Deletion of C-terminal half of $gspB_{Vc}$ from pMMB/$gspAB_{Vc}$; Cm^r	This work
pMMB/$gspD_{Vc}$	$gspD_{Vc}$ cloned into pMMB67; Ap^r	26
pMMB68	*etxB* under *Ptac* control; *Ptac mob*$^+$ Ap^r	41
pMRS101	*oriR*6K *sacBR pir* Ap^r St^r	44
pUC4K	*lacZ*α Ap^r Km^r	Amersham
pBluescript SK	*Plac lacZ*α f1 ColE1 Ap^r	Stratagene

whole-cell extract disrupted by sonication was determined by GM1 enzyme-linked immunosorbent assay (52) as described previously (32).

Aerolysin was assayed as described previously (47).

Marker exchange mutagenesis and complementation assays. Alleles $gspA_{Ah}$::Kan and $gspA_{Vc}$::Kan were introduced into *Aeromonas* and *Vibrio* species by marker exchange mutagenesis using the suicide vector pMRS101 (44). A 1,429-bp fragment of the *A. salmonicida gspA* gene ($gspA_{As}$) was amplified by PCR utilizing the primers US42 (ATCTCAACTACGGGTTGCAG) and US43 (CATCACATTGAGACGCAGCAG), blunt-end ligated into the EcoRV site of pBluescript II SK(+), and electroporated into *E. coli* XL-Blue. The aminoglycoside 3′-phosphotransferase gene conferring kanamycin resistance was excised from plasmid pUC4K via a HincII digestion and inserted into an EcoRV site located within *gspA* (nucleotide position 780) of a recombinant plasmid clone. The $gspA_{Ah}$::Kan gene was transferred from pSK into the suicide vector pMRS101 by ApaI/XbaI digestion. Recombinant plasmids were digested with NotI to remove the pBR322 origin of replication of the plasmid, self-ligated, and electroporated into *E. coli* SM10λpir by selecting for streptomycin and kanamycin resistance. The donor (SM10λpir containing pMRS101/*exeA*::Kan) and the recipient strain AsR were grown overnight, subcultured 1:10 in BHI broth without antibiotics, and incubated at 22°C for 1 h prior to conjugation. Five-hundred-microliter volumes of donor and recipient cells were mixed and collected by centrifugation. The pellet was resuspended in 100 μl BHI, applied to a pre-warmed BHI plate, and incubated for 3.5 h at 30°C. Half of the conjugation pool plate was scraped off, streaked onto LB containing kanamycin, rifampin, and sucrose (10%), and incubated at 22°C to directly screen for recombinant recipient colonies. The exchange of alleles was verified by PCR.

Marker exchange mutagenesis of *Vibrio* species was conducted as described above, except that 2,100- to 3,300-bp fragments containing all or part of *gspAB* were amplified from the following species using the indicated specific primer sets: UR70 (GAATTTGAGGTCAGCTATCCGA) and UR71 (GCATAAGCG GAATTCATCGCA) for *V. cholerae*, US30 (GTACAGCTGGCCGCTATCAT) and US31 (TGGTTGGGTTCAAAGCAAGT) for *V. parahaemolyticus* strain US32052027, and US32 (GCTGGGTTCAAGCAAAATTC) and US33 (TGAC ACATGGCGCAAAATAC) for *V. vulnificus* strain 67181283. The aminoglycoside phosphotransferase gene conferring kanamycin resistance was inserted into an EcoRV site located at positions 1106 and 1088 of the *V. vulnificus* and *V. parahaemolyticus gspA* genes, respectively, and into a SphI site located at position 1040 of *V. cholerae gspA*. *gspA*::Kan fragments were transferred into pMRS101 from pSK using BamHI/ApaI sites for *V. vulnificus* and *V. parahaemolyticus gspA*::Kan and BamHI/SalI for *V. cholerae gspA*::Kan.

The *A. hydrophila gspAB* clone pRJ31.1 was described previously (20). A 5.6-kb EcoRI fragment containing the entire *gspAB* operon of *V. cholerae*, including the presumed promoter, was cloned into pMMB207 to create plasmid pMMB/$gspAB_{Vc}$. The plasmids were conjugated into *V. cholerae* and *A. hydrophila* strains from *E. coli* S17-1 as described above for complementation assays.

Immunoblotting. GspD multimeric and monomeric protein forms were detected by immunoblot analysis of whole-cell samples taken from liquid culture. Samples were electrophoresed in a 3 to 8% SDS gradient PAGE gel (Bio-Rad)

TABLE 2. Enzymatic activities detected in culture supernatants of wild-type and *gspAB Aeromonas* and *Vibrio* species

Organism	Strain	Mean activity ± SD (%)[a]		
		Lipase[b]	Protease[c]	Amylase[d]
A. salmonicida	AsR	0.980 ± 0.072 (100)	NA	NA
	As-*gspA*	0.243 ± 0.051 (24.8)*	NA	NA
V. cholerae	Bah2-R	3.71 ± 0.042 (100)	3.08 ± 0.287 (100)	NA
	Bah2-*gspA*	3.74 ± 0.378 (101)†	2.28 ± 0.269 (74)*	NA
V. vulnificus	VvR	61.3 ± 6.74 (100)	0.221 ± 0.019 (100)	22.6 ± 2.20 (100)
	Vv-*gspA*	53.7 ± 6.97 (87.6)†	0.186 ± 0.011 (84.2)†	19.9 ± 1.51 (88.1)†
V. parahaemolyticus	VpR	0.276 ± 0.031 (100)	NA	19.8 ± 1.10 (100)
	Vp-*gspA*	0.211 ± 0.001 (76.4)*	NA	18.8 ± 1.03 (94.9)†

[a] The wild-type activity for each species is set as 100%. NA, no detectable activity; *, significantly different; †, not significantly different.
[b] Activity is expressed in μM/min/OD_{600} unit.
[c] Activity is expressed in A_{450}/hr/OD_{600} unit.
[d] Activity is expressed in A_{595}/hr/OD_{600} unit × 10^3.

until an 84-kDa protein standard was approximately 1 cm from the bottom of the gel and then transferred to polyvinylidene difluoride membrane. The amount of sample loaded was normalized such that 5 μl of a sample consisting of equal parts of 2× sample buffer and culture grown to an OD_{600} of 2.0 was loaded for electrophoresis, with the exception of the experiment whose results are shown in Fig. 2, in which samples were normalized according to the OD_{600} shown. Proteins were visualized using primary rabbit anti-$GspD_{Ah}$ (18) or rabbit anti-$GspD_{Vc}$ antiserum, a peroxidase-conjugated anti-rabbit IgG secondary antibody (Sigma), and a chemiluminescent substrate (GE Healthcare) detected with Hyperfilm (GE Healthcare). $GspD_{Vc}$ antibodies were raised in New Zealand White rabbits by using purified EpsD-His_6 monomers according to the method of Harlow and Lane (14).

Statistical analysis. The unpaired two-sided Student *t* test was used for all statistical analysis. Values were considered significantly different at *P* values of <0.05.

RESULTS

T2SS function and secretin assembly are abrogated in *A. salmonicida gspA* mutants. To investigate the importance of GspAB in the assembly of the secretin in *A. salmonicida*, we constructed a *gspA* mutant of As449 by marker exchange mutagenesis and determined the assembly and function of the T2SS by immunoblot detection of the GspD secretin and assay of culture supernatants for glycerophospholipid:cholesterol acyl transferase (GCAT) activity using the lipase assay. GCAT is a protein known to be secreted by the T2SS (5).

The GCAT assay results indicated that secretion was severely affected by the loss of $GspA_{As}$, as evidenced by the low lipase activity in culture supernatants from strain As-*gspA* in comparison to the lipase activity for strain AsR (Table 2). As expected from previous results in *A. hydrophila*, the loss of T2SS function in the *A. salmonicida gspA* mutant was accompanied and presumably caused by the failure to assemble the GspD secretin. The amount of secretin multimer from whole-cell samples was almost nonexistent in the *gspA* mutant compared to the amount in the wild type (Fig. 1). Although a small amount of multimer was evident in concentrated samples of the *gspA* strain, the amount was negligible in comparison to the amount in the wild type (Fig. 1, 3rd and 4th lanes). It should be noted that the failure to assemble the secretin multimer in *A. hydrophila gspA* mutants is accompanied by the accumulation of monomer GspD in the inner membrane (3), although in *A. salmonicida*, no accumulation of the monomer could be observed in the *gspA* mutant. This may result from degradation of the monomer when it cannot be assembled into the multimer in *A. salmonicida*. These results clearly demonstrate the essential requirement for GspAB in the assembly of the secretin in *A. salmonicida* and suggest that the absolute requirement for GspAB in secretin assembly is not specific to *A. hydrophila* but is a characteristic of the aeromonads.

Mutation of *gspA* has a minor effect on secretion of T2SS substrates in *Vibrio* species but significantly decreases GspD multimer formation. In order to examine the involvement of GspAB in secretin assembly in *Vibrio* species, the *gspAB* genes were cloned and *gspA* mutant strains constructed for each of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* by marker exchange mutagenesis. To investigate T2SS function in the *V. cholerae ctxAB* strain, we introduced a plasmid-encoded copy of the structurally similar cholera toxin B-subunit (CtxB) homologue heat-labile *E. coli* enterotoxin B subunit (EtxB) (50)

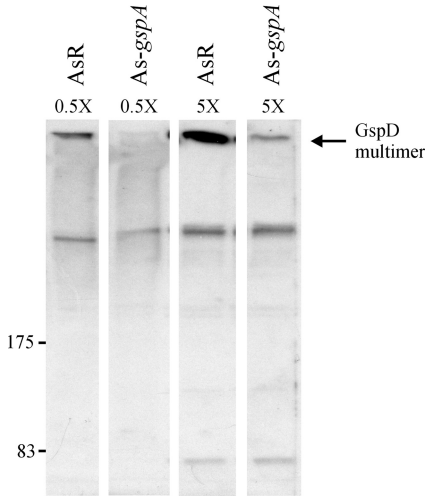

FIG. 1. In *A. salmonicida*, loss of $GspA_{As}$ abrogates assembly of the GspD secretin multimer. Anti-$GspD_{Ah}$ immunoblot analysis of 0.5- and 5-fold-concentrated whole-cell samples taken from AsR and As-*gspA* strains grown in liquid culture to an OD_{600} of 2.0 is shown. Locations of prestained standard protein markers (in kDa) and the $GspD_{As}$ secretin multimer are given.

into wild-type and *gspA* strains. EtxB has been extensively studied and has previously been shown to be secreted by the T2SS in *V. cholerae* (33).

In marked contrast to the requirement for GspAB in assembly of the secretin in *A. hydrophila* (3) and *A. salmonicida* (Fig. 1), mutation of *gspA* had a modest effect on T2SS secretion in *Vibrio* strains. The *V. cholerae gspA* mutant (Bah2-*gspA*) and the parent strain secreted equivalent amounts of the EtxB subunit (73% ± 8% [mean ± standard deviation] and 70% ± 4%, respectively; no significant difference). Similarly, as shown by the results in Table 2, the Bah2-*gspA* secreted equivalent amounts of lipase activity and 26% lower levels of protease activity compared to the wild type. Similar results were obtained for *V. cholerae* TRH7000 and TRH7000 *gspA* strains (data not shown).

Lipase, protease, and amylase activities in culture supernatants of the *V. vulnificus gspA* mutant (Vv-*gspA*) were not significantly different from those observed in supernatant from the wild-type culture (Table 2). Lipase and amylase activities were detected in *V. parahaemolyticus* culture supernatants, and the *gspA* mutant (Vp-*gspA*) secreted 24% less lipase activity than the wild type and an equivalent amount of amylase activity in comparison to the wild type.

Although mutation of *gspA* had a minimal effect on the secretion of T2SS substrates, a substantial reduction in the amount of assembled *gspD* secretin was observed in the *gspA* mutants of *V. cholerae* (Fig. 2A), *V. parahaemolyticus* (Fig. 2B), and *V. vulnificus* (Fig. 2C) compared to the amount in the wild type. Due to the difficulty in quantifying the relative differences in the amounts of protein by immunoblotting techniques, we provided multiple points for comparison by repeatedly sampling cultures of the *gspA* mutant and the wild type over the entire growth curve. In a manner similar to the secretin of *A. hydrophila* and *A. salmonicida*, the vast majority of the approximately 1-MDa assembled *Vibrio* secretin in wild-type cells is stable when heated in SDS-PAGE sample buffer. At each point in the growth curve, a smaller amount of assembled secretin and a greater amount of unassembled GspD monomer were observed in samples taken from the *gspA* mutant than in samples from the wild type (Fig. 2). This phenotype could be complemented in *V. cholerae*, since secretin levels increased in response to expression of $gspA_{Vc}$ in *trans*, but only if the complementing plasmid also encoded $gspB_{Vc}$ (Fig. 3). Apparently the *gspA* mutation also decreased the level of GspB in the cell, either through a direct polarity effect or, possibly, because GspB is unstable in the absence of GspA, as was previously found in *A. hydrophila* (18). In any case, this result confirmed the involvement of both GspA and GspB in secretin assembly.

The *V. cholerae* T2SS exhibits excess secretion capacity. The absence of $GspAB_{Vc}$ in *V. cholerae* resulted in a substantial decrease in secretin assembly (Fig. 2A) that was not accompanied by a concomitant decrease in lipase and protease activities in culture supernatants (Table 2). This result suggested that either the lipase and protease assayed in culture supernatant are not substrates of the T2SS or that wild-type levels of secretin are not required for efficient secretion of lipase and protease from the cell. The lipase and protease are indeed substrates of the T2SS, because these activities were nearly absent in supernatants from a culture of *V. cholerae* TRH7000 *gspD* (Fig. 4). In order to assay secretion capacity, the same

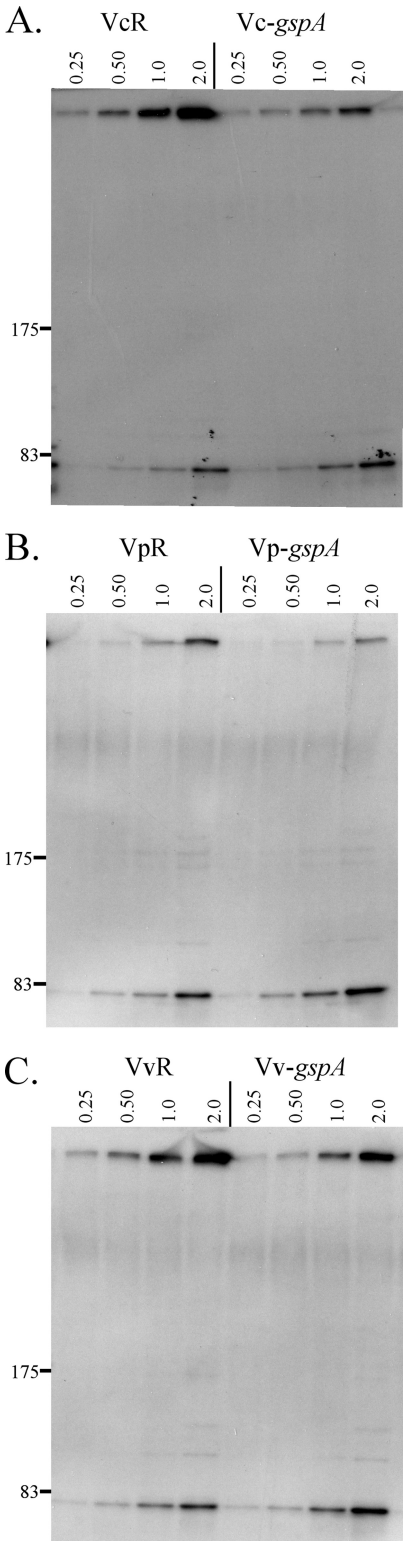

FIG. 2. Effect of *gspA* mutation on assembly of the GspD secretin in *Vibrio* species. Anti-$GspD_{Vc}$ immunoblot analysis of whole-cell samples taken from liquid culture at various stages of growth from early exponential phase to late exponential phase (OD_{600} is shown at the top of each lane) of wild-type (Bah2-R) and *gspA V. cholerae* (Bah2-*gspA*) (A), wild-type (VpR) and *gspA V. parahaemolyticus* (Vp-*gspA*) (B), and wild-type (VvR) and *gspA V. vulnificus* (Vv-*gspA*) (C) is shown. Locations of prestained standard protein markers (in kDa) are given.

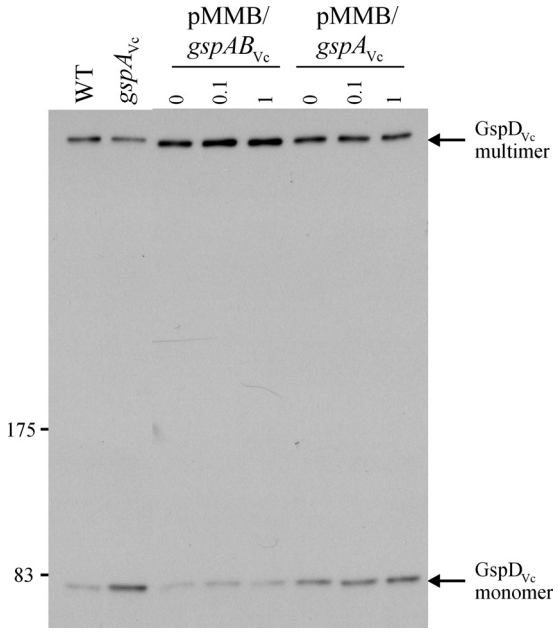

FIG. 3. Complementation of the partial secretin-negative phenotype of *V. cholerae* Bah2-*gspA* by expression of GspAB$_{Vc}$ or GspA$_{Vc}$ in *trans* from plasmids pMMB207/*gspAB*$_{Vc}$ and pMMB207/*gspA*$_{Vc}$. The cultures were grown to an OD_{600} of 2.0 in the absence or presence of IPTG (0.1 and 1.0 mM), and whole-cell samples were electrophoresed and immunoblotted with anti-GspD$_{Vc}$. Locations of standard protein markers (in kDa) are given. WT, wild type.

strain was complemented by the expression of *gspD* in *trans* at various levels of induction. The results, shown in Fig. 4, revealed that a relatively low level of secretin in comparison to the amount expressed in wild-type cells is sufficient for full secretion, since wild-type levels of lipase were present in supernatants from cells without induction (Fig. 4B) and wild-type levels of protease were present in supernatant from cells induced with 0.002 mM IPTG (Fig. 4C). In the absence of induction and at an induction level of 0.002 mM IPTG, much less secretin was observed in the complemented *gspD* strain than in wild-type cells (Fig. 4A). These data demonstrate that, at least under the growth conditions used in these experiments, there is a natural overexpression of the secretin relative to the amount of extracellular secretion, such that more secretin is assembled than is required for the secretion of substrates into the medium, a situation that may exist due to differential expression of genes that encode the T2SS and the substrates of the system.

***V. cholerae* GspAB partially complements the secretin assembly and secretion defects of *A. hydrophila gspA* mutants.** The secretion and secretin assembly results suggested that although *Vibrio* GspAB is not essential for the function of the T2SS, it is nevertheless involved in the assembly of the secretin. Another approach to elucidate the function of GspAB in *Vibrio* species would be to determine if GspAB$_{Vc}$ could complement *A. hydrophila gspA* mutants or otherwise interact with the T2SS of *A. hydrophila*. We investigated this possibility by introducing pMMB/*gspAB*$_{Vc}$ into Ah65 and C5.84 and analyzed the T2SS function and assembly. The recombinant plasmid contained the presumed *gspAB*$_{Vc}$ promoter and a vector-

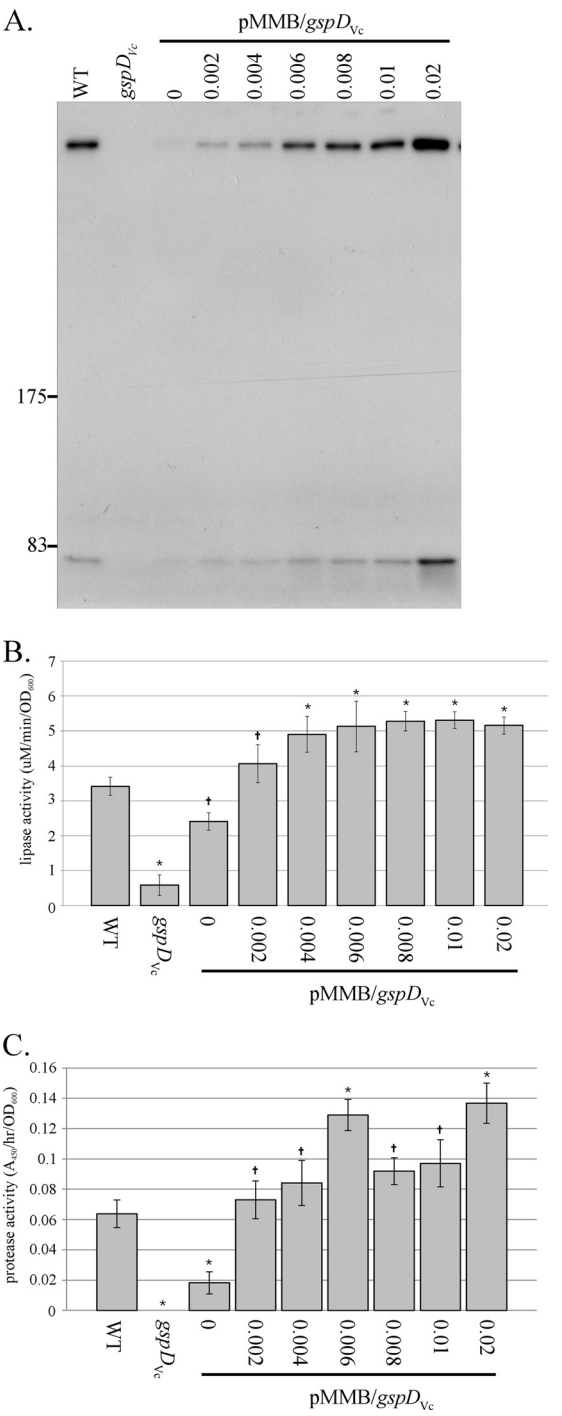

FIG. 4. Wild-type amount of GspD$_{Vc}$ secretin is not required for secretion of lipase and protease to wild-type levels. (A) The amounts of secretin multimer assembled in *Vibrio cholerae* TRH7000, the *gspD*$_{Vc}$ strain, and the *gspD*$_{Vc}$ strain expressing *gspD*$_{Vc}$ in *trans* (induced from plasmid pMMB/*gspD*$_{Vc}$ with 0 to 0.02 mM IPTG as indicated above the immunoblot) were assessed by immunoblot analysis with anti-GspD$_{Vc}$ antibody. Locations of standard protein markers (in kDa) are given. (B and C) The results of triplicate assays of lipase (B) and protease (C) activities in supernatant taken from cultures of TRH7000, the *gspD*$_{Vc}$ strain, and the *gspD*$_{Vc}$ strain expressing *gspD*$_{Vc}$ in *trans* (induced with 0 to 0.02 mM IPTG) are shown. Significant difference (†, nonsignificant; *, significant [$P < 0.05$]) was determined based on comparison of each value against activity observed in supernatant from wild-type culture. WT, wild type.

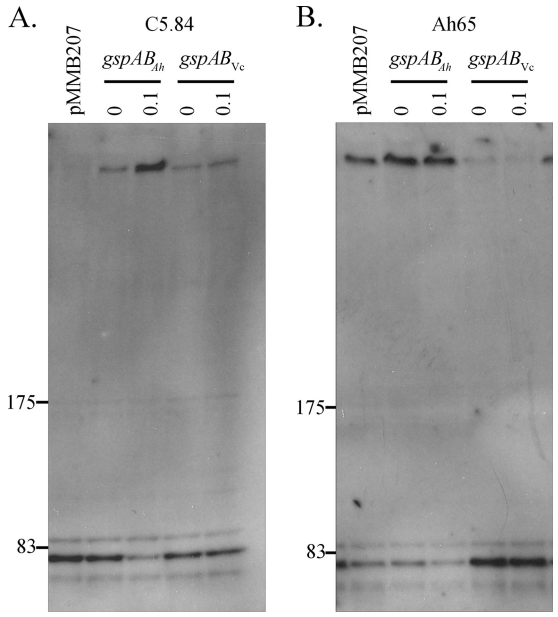

FIG. 5. Secretin assembly in *A. hydrophila gspAB* and the wild type upon expression of *V. cholerae gspAB* in *trans*. The amount of secretin multimer assembled upon induced expression (0.1 mM IPTG) of $gspAB_{Ah}$, $gspAB_{Vc}$, or neither (pMMB207) in C5.84 (A) and Ah65 (B) was assessed by anti-$GspD_{Ah}$ immunoblot analysis. Locations of prestained standard protein markers (in kDa) are given.

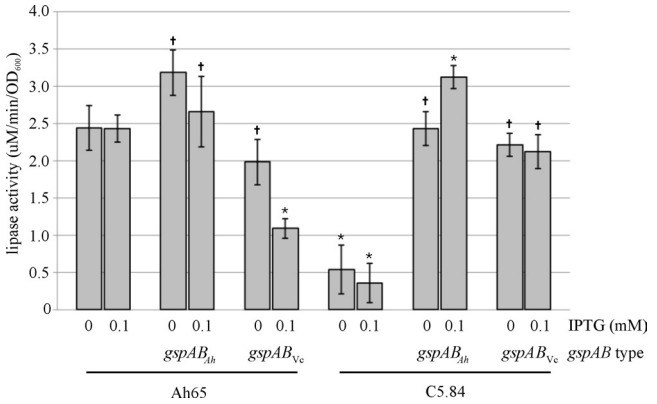

FIG. 6. Lipase activities in supernatants from cultures of wild-type and *gspA A. hydrophila* upon expression of $gspAB_{Ah}$ and $gspAB_{Vc}$ in *trans*. Lipase activities were assayed in supernatants taken from wild-type (Ah65) and *gspA* (C5.84) *A. hydrophila* cultures expressing $gspAB_{Ah}$ (from plasmid pRJ31.1) or $gspAB_{Vc}$ (from plasmid pMMB/$gspAB_{Vc}$) with or without induction with IPTG. Assays of culture supernatants were performed in triplicate, and the standard deviations are indicated. The results shown are representative of those obtained in multiple experiments. Significant difference (†, nonsignificant; *, significant [$P < 0.05$]) was calculated based on comparison of each value against lipase activity observed in wild-type (Ah65) culture.

encoded *tac* promoter to allow increased expression. The expression of $gspAB_{Vc}$ in *trans* partially complemented the secretin assembly defect of C5.84, as indicated by the reestablishment of secretin multimer assembly in C5.84 (pMMB/$gspAB_{Vc}$) (Fig. 5A). The amount of secretin multimer assembled was somewhat dependent upon the level of $gspAB_{Vc}$ expression, since induction with 0.1 mM IPTG resulted in a slightly greater amount of ExeD multimer formed. As previously demonstrated in the control experiment, the expression of $gspAB_{Ah}$ carried on plasmid pRJ31.1 in strain C5.84 completely restored secretin assembly (Fig. 5A) (3).

The C5.84 secretion-negative phenotype was nearly fully complemented by the expression of $gspAB_{Vc}$ in *trans*. As shown in Fig. 6, the lipase activity in supernatants from the uninduced C5.84(pMMB/$gspAB_{Vc}$) culture was 90% of the wild-type amount and did not increase with further induction. In the control experiment, wild-type $gspAB_{Ah}$ completely complemented the lipase secretion defect of the *gspA* mutant even without induction. Likewise, aerolysin secretion was partially restored in C5.84(pMMB/$gspAB_{Vc}$) and did not substantially increase with induction, as shown in Fig. 7. In the control experiment, the expression of $gspAB_{Ah}$ almost fully complemented the aerolysin secretion defect of C5.84 even without induction and, with induction, generated levels of aerolysin secretion greater than that observed in the wild type.

$GspAB_{Vc}$ confers a dominant negative effect on secretin multimer formation and secretion of T2SS substrates in wild-type *A. hydrophila*. Interestingly, the expression of $gspAB_{Vc}$ in wild-type *A. hydrophila* decreased the amount of assembled secretin multimer and increased the amount of GspD monomer as visualized by immunoblotting compared to the results for cells containing the vector only (Fig. 5B). This effect was not observed for Ah65(pRJ31.1) cells overexpressing the $gspAB_{Ah}$ genes, since the expression of $gspAB_{Ah}$ resulted in more secretin multimer and less unassembled monomer than were observed in the wild type. The expression of $gspAB_{Vc}$ in wild-type *A. hydrophila* also decreased the amount of aerolysin secreted, with a greater effect generated upon increased induction of $gspAB_{Vc}$ expression (Fig. 7). Likewise, the amount of lipase activity in the Ah65(pMMB/$gspAB_{Vc}$) culture supernatant was significantly decreased upon the induction of $gspAB_{Vc}$ with 0.1

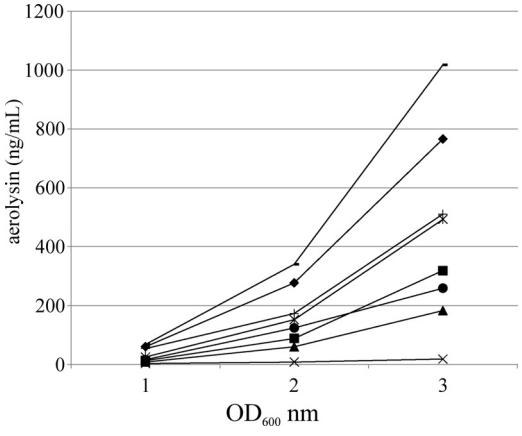

FIG. 7. The aerolysin secretion-negative phenotype of *A. hydrophila gspA* is partially complemented by expression of *V. cholerae gspAB* in *trans*. The amount of aerolysin secreted upon expression of $gspAB_{Vc}$ and $gspAB_{Ah}$ in wild-type (Ah65) and *gspA* (C5.84) *A. hydrophila* was determined at various stages of growth (indicated by the OD_{600}). The strains used were Ah65 (◆), Ah65 $gspAB_{Vc}$ (■), Ah65 $gspAB_{Vc}$ induced with 0.1 mM IPTG (▲), C5.84 (×), C5.84 $gspAB_{Vc}$ (∗), C5.84 $gspAB_{Vc}$ induced with 0.1 mM IPTG (●), C5.84 $gspAB_{Ah}$ (†), and C5.84 $gspAB_{Ah}$ induced with 0.1 mM IPTG (-). The results shown are representative of those obtained in multiple experiments.

mM IPTG, to 45% (Fig. 6) of the lipase activity in the Ah65 culture supernatant.

DISCUSSION

In Gram-negative bacteria that contain a T2SS, the presence of the *gspAB* operon in addition to the set of "core" *gsp* genes is not well conserved, therefore suggesting that the role of GspAB is not integral to the assembly of the T2SS. However, in some bacteria that contain *gspAB*, such as *A. hydrophila*, the GspAB complex is absolutely required for the construction of the T2SS (3), characterized by assembly of the megadalton-sized GspD secretin multimer in the outer membrane. The function of the $GspAB_{Ah}$ complex (46) may involve reorganization of the peptidoglycan meshwork to allow monomeric GspD subunits or the secretin multimer itself to pass through to reach the outer membrane or may possibly provide a scaffold for the assembly of the secretin. This activity is mediated at least in part by the C-terminal peptidoglycan-binding motif of $GspA_{Ah}$ (17, 25). In this study, we sought to determine if the requirement for the GspAB complex in the assembly of the secretin in *A. hydrophila* also applies to another species of *Aeromonas*, *A. salmonicida*, as well as to three *Vibrio* species, *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, by detecting the amount of assembled GspD secretin and by assaying the activity of T2SS substrates in culture supernatants isolated from wild-type strains and those without the GspAB complex.

We demonstrated a requirement for $GspA_{As}$ in the assembly of the secretin in *A. salmonicida*. A significant reduction in lipase activity (75%) was detected in the *gspA* mutant in comparison to the lipase activity in the wild type (Table 2), presumably caused by the lack of assembled GspD secretin multimer observed in the *gspA* strain (Fig. 1). This finding is consistent with that described by Ast et al. (3), where loss of the $GspAB_{Ah}$ complex abrogated both the secretion of aerolysin and the assembly of the secretin multimer in *A. hydrophila*. Together, these data suggest that GspAB is generally required for the assembly of the GspD secretin in *Aeromonas* species.

Surprisingly, we found that, contrary to the essential role of GspAB in the assembly of the secretin in the *Aeromonas* species *A. hydrophila* (3) and *A. salmonicida* (Fig. 1), GspAB is not an absolute requirement for the function of the T2SS in vibrios, because mutation of *gspA* (which also apparently inactivated *gspB*; see Fig. 3) did not prevent the secretion of T2SS substrates, including lipase, amylase, protease (Table 2), and EtxB (see Results) in *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. However, although the secretion of T2SS substrates remained largely unchanged in the *gspA* strain compared to the levels of secretion in wild-type strains, we did find significant reductions in the amounts of assembled secretin multimer in the absence of GspAB in *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* (Fig. 2). The dichotomy of the finding that substantial decreases in secretin multimer formation had only minimal effects on secretion can be explained by an apparent excess in T2SS secretion capacity at normal GspD levels, since minimal expression of *gspD* was shown to fully complement secretion in the *V. cholerae gspD* mutant (Fig. 4). In any case, the significant reduction in assembled secretin multimer in the absence of GspA suggested that the GspAB complexes of the *Vibrio* strains perform an important role in the assembly of their respective secretins.

In order to more definitively determine if GspAB performs the same role in vibrios as in aeromonads, we expressed a plasmid-carried copy of $gspAB_{Vc}$ in the *A. hydrophila gspAB* strain C5.84 and asked if $GspAB_{Vc}$ could complement the secretin assembly-negative and secretion-negative phenotypes of this strain. The expression of $GspAB_{Vc}$ reestablished the assembly of the $GspD_{Ah}$ secretin multimer (Fig. 5A) and the secretion of the T2SS substrates aerolysin (Fig. 7) and lipase (Fig. 6) in C5.84. Although complementation was not complete, this is perhaps not surprising given that $GspAB_{Ah}$ and $GspAB_{Vc}$ are 36% identical and 51% similar. Therefore, although $GspAB_{Vc}$ did not completely complement the *gspAB* phenotype of C5.84, its partial complementation did show that $GspAB_{Vc}$ and $GspAB_{Ah}$ likely perform the same function in their respective species.

Although similar enough for partial complementation of the $gspAB_{Ah}$ secretion- and secretin assembly-negative phenotypes of C5.84, $GspAB_{Vc}$ and $GspAB_{Ah}$ are apparently not sufficiently identical to function together as a heteromultimer composed of both complexes. When expressed in wild-type strain Ah65, $GspAB_{Vc}$ decreased the amount of assembled secretin (Fig. 5B) and the secretion of T2SS substrates (Fig. 6) in a dose-dependent manner. Since GspAB functions as a heteromultimeric complex (46), this phenotype was likely caused by a dominant negative effect of $GspAB_{Vc}$ whereby the assembly of an inactive heteromultimeric complex composed of $GspAB_{Ah}$ and $GspAB_{Vc}$ reduces the ability of the strain to assemble a functional secretin multimer and, thus, secrete T2SS substrates into the growth medium. The dominant negative effect of $GspAB_{Vc}$ expression is consistent with the ability to inactivate the function of a multimeric protein by the expression of a mutant subunit in *trans*, a hallmark of multisubunit proteins.

The difference in the requirement for GspAB in the assembly of the T2SS secretin in *Vibrio* and *Aeromonas* species suggests that a redundant GspAB function exists in the *Vibrio* genus that is absent in the *Aeromonas* genus. Although amino acid similarity studies of the *V. cholerae* proteome did not identify other obvious GspAB candidates, it is possible that a protein involved in peptidoglycan metabolism that is characterized as part of another periplasm-spanning system could perform the redundant function of $GspAB_{Vc}$. Alternatively, *Vibrio* species may encode a specific outer membrane determinant, such as lipopolysaccharide, or an outer membrane protein, such as a GspS pilot protein, that enables secretin assembly to exist in the absence of the PG-binding function of GspAB. For instance, a pilot protein could stabilize and localize the secretin to the outer membrane, thereby allowing partial secretin assembly in the outer membrane under conditions (absence of GspAB) that normally result in the degradation of GspD in other species, such as aeromonads.

The reason for the apparent lack of GspAB in a number of other species that contain a T2SS remains unclear but could involve one or a combination of factors, including an alternative peptidoglycan physiology or the presence of additional peptidoglycan-spanning systems. First, the difference between GspAB-containing Gram-negative bacteria and those that do not have the *gspAB* operon could be the nature of their peptidoglycan layer. Previous studies have suggested that the com-

position and organization of the peptidoglycan layer is identical in all Gram-negative bacteria (45); however, modifications of the glycan or amino acid components of the peptidoglycan of Gram-negative bacteria are species specific, particularly in the frequency of cross-links in peptidoglycan (11). Second, the involvement of peptidoglycan-binding proteins in the assembly of structures that traverse the peptidoglycan is well documented, with examples including FlgJ in the flagellar assembly of *Salmonella enterica* serovar Typhimurium (31), PtlE in type IV secretion of *Bordetella pertussis*, and IpgF in type III secretion in *Shigella sonnei* (56). A redundancy of function might exist in some species whereby a lytic transglycosylase or peptidoglycan-binding protein involved in remodeling the peptidoglycan layer for the assembly of one system could serve in that capacity for the assembly of the T2SS.

Identification of the putative GspAB-redundant function in *Vibrio* species may define the involvement of other proteins that perform roles in the assembly of the secretin and may also help to further elucidate the role of GspAB in the assembly of the T2SS.

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