



Defining the Role of the *Streptococcus agalactiae* Sht-Family Proteins in Zinc Acquisition and Complement Evasion

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ABSTRACT Streptococcus agalactiae is not only part of the human intestinal and urogenital microbiota but is also a leading cause of septicemia and meningitis in neonates. Its ability to cause disease depends upon the acquisition of nutrients from its environment, including the transition metal ion zinc. The primary zinc acquisition system of the pathogen is the Adc/Lmb ABC permease, which is essential for viability in zinc-restricted environments. Here, we show that in addition to the AdcCB transporter and the three zinc-binding proteins, Lmb, AdcA, and AdcAll, S. agalactiae zinc homeostasis also involves two streptococcal histidine triad (Sht) proteins. Sht and Shtll are required for zinc uptake via the Lmb and AdcAll proteins with apparent overlapping functionality and specificity. Both Sht-family proteins possess fivehistidine triad motifs with similar hierarchies of importance for Zn homeostasis. Independent of its contribution to zinc homeostasis, Sht has previously been reported to bind factor H leading to predictions of a contribution to complement evasion. Here, we investigated Shtll to ascertain whether it had similar properties. Analysis of recombinant Sht and Shtll reveals that both proteins have similar affinities for factor H binding. However, neither protein aided in resistance to complement in human blood. These findings challenge prior inferences regarding the in vivo role of the Sht proteins in resisting complement-mediated clearance.

IMPORTANCE This study examined the role of the two streptococcal histidine triad (Sht) proteins of *Streptococcus agalactiae* in zinc homeostasis and complement resistance. We showed that Sht and ShtII facilitate zinc homeostasis in conjunction with the metal-binding proteins Lmb and AdcAII. Here, we show that the Sht-family proteins are functionally redundant with overlapping roles in zinc uptake. Further, this work reveals that although the Sht-family proteins bind to factor H *in vitro* this did not influence survival in human blood.

KEYWORDS *Streptococcus agalactiae*, metal homeostasis, zinc transporter

First-row transition metals, such as manganese, iron, and zinc, are essential for the viability of all organisms. Metal ions contribute to essential cellular processes, serving as structural and/or catalytic cofactors in many key proteins (1). During infection, pathogenic bacteria acquire essential metal ions from the host environment. The essentiality of metal ion acquisition by invading pathogens is exploited in the host via metal sequestration mechanisms. These antimicrobial mechanisms, collectively referred to as nutritional immunity, have been extensively studied in the context of iron (2). Nevertheless, recent studies have revealed that the host also alters the abundance of other essential metal ions, including manganese and zinc (3). To subvert host restriction of zinc bioavailability, pathogenic bacteria use highly efficient zinc uptake pathways to acquire this metal. The most prevalent zinc import pathways in prokaryotes are the ATP

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FIG 1 *S. agalactiae* zinc acquisition. (A) Schematic of the Adc/Lmb system. The Adc permease comprises the ABC transporter AdcCB, three zinc-binding SBPs (Lmb, AdcA, and AdcAII), and the Sht and ShtII proteins. Cp, capsule; Cw, cell wall; Mb, membrane; Ct, cytoplasm. (B) Growth phenotypes of wild-type *S. agalactiae* A909 (WT) and $\Delta adcA$, $\Delta adcA \Delta sht \Delta shtII$, $\Delta Imb \Delta adcAII \Delta sht \Delta shtII$, $\Delta adcA \Delta Imb \Delta adcAII \Delta and \Delta \Delta Imb \Delta adcAII \Delta sht \Delta shtII$ mutant derivatives in zinc-restricted CDM. Growth was monitored by OD₆₀₀ measurements every 60 min for 12 h. The data are presented as mean OD₆₀₀ measurements \pm the standard deviations from three independent experiments.

binding cassette (ABC) transporters (4). It therefore follows that these metal ion acquisition mechanisms are crucial virulence factors of pathogenic bacteria.

Streptococcus agalactiae (group B streptococcus [GBS]) is not only a human commensal of the digestive and genital tracts but also an opportunistic pathogen that causes invasive infections, including septicemia, pneumonia, and meningitis in human neonates (5, 6). Zinc acquisition is essential to these processes since our prior studies have established that zinc is required for fundamental processes, including S. agalactiae growth and morphology (7). In S. agalactiae, zinc acquisition involves the AdcCB transporter, which is comprised of the integral membrane protein, AdcB, and the nucleotide binding domain, AdcC. The transporter functions in concert with three membrane tethered solute-binding protein (SBPs) that have specificity for zinc (Fig. 1A). These SBPs are AdcA, which has an unusual two-domain structure (8), and the canonical cluster A-I proteins AdcAll and Lmb (7, 9). The presence of multiple zinc-specific SBPs is an unusual feature largely unique to the streptococci, since most prokaryotes employ only a single protein. This appears to have arisen due to the ancient acquisition of a genetic element containing Lmb (or AdcAll) and a histidine triad (HT) protein in the streptococcal genus (10). Subsequent gene duplication events have led to the emergence of multiple copies of the histidine triad proteins and Lmb/AdcAll-type SBPs. To date, 25 streptococcal species encode histidine triad proteins, with gene copy numbers between one and four, and at least one Lmb/AdcAll-type SBP. The most extensively characterized examples are the pneumococcal histidine triad (Pht) proteins from Streptococcus pneumoniae, which encodes four copies (PhtA, PhtB, PhtD, and PhtE) and one AdcAll protein. Here, the Pht proteins are shown to be cell wall-associated proteins that contribute to zinc acquisition via AdcAll, albeit with overlapping functionality (8, 11, 12). PhtD is the most highly conserved Pht family protein across pneumococcal strains and contains five HT motifs, with each motif predicted to bind zinc (13-15). Recent studies have shown that the HT1 motif, which is closest to the cell membrane, is the most important for the contribution of PhtD to zinc acquisition (15). The mechanistic basis for how the Pht proteins contribute to pneumococcal zinc acquisition remains contentious, but the current consensus model proposes that Pht proteins bind the extracellular zinc via their HT motifs, with the zinc then transferred to the AdcAll-type SBP for cytoplasmic import via the AdcCB translocon (16). Whether the SBP recruits the metal ion from the Pht proteins directly or indirectly remains to be established. Irrespective of how zinc is acquired, the role of the Pht proteins in supporting AdcAll zinc import during in vitro growth and pneumococcal infection has been demonstrated (8, 11, 15).

To date, no histidine triad proteins have been characterized in *S. agalactiae*, although two candidate genes were identified in our previous work (7). These streptococcal histidine triad proteins, Sht and Shtll, share 52 and 49% identity with PhtD of *S. pneumoniae* (in 375 and 387 overlapping residues, respectively). The *sht* gene is contained within an operon that contains the *lmb* gene. This operon is present on a mobile genetic element that is found in almost all human *S. agalactiae* isolates (96.8%) but in only 26.7% of the animal isolates (7, 17). In contrast, Shtll is encoded in an operon with *adcAll*, which is present in all sequenced *S. agalactiae* strains. Although their contribution, if any, to zinc homeostasis remains to be determined, Sht has previously been implicated in aiding resistance of *S. agalactiae* to the innate immune system by facilitating factor H deposition, a regulator promoting inactivation of C3b (18). Despite the similarity between Sht and Shtll (47% protein identity in 403 overlapping residues), the role of Shtll in factor H binding remains to be determined.

Here, we examined the role of Sht and Shtll in *S. agalactiae* zinc acquisition via the Adc/Lmb components of the Adc permease. Our results reveal that Sht and Shtll both contribute to zinc uptake with the two proteins showing overlapping functionality and specificity. We further show that Sht and Shtll are able to bind factor H *in vitro*, but this functionality is not protective for survival in human blood. Collectively, these findings provide further knowledge regarding the roles of streptococcal histidine triad proteins in zinc acquisition and highlights that the capacity to bind factor H may not provide physiologically significant protection from complement-mediated clearance.

RESULTS

Sht and ShtII contribute to Lmb- and AdcAII-mediated zinc homeostasis. We hypothesized that the Sht and Shtll proteins contribute to S. agalactiae zinc homeostasis. To address this, we generated isogenic S. agalactiae deletion mutants of sht, shtll, or both sht and shtll, in isolation and in combination with the deletion of the zincrecruiting SBPs adcA, Imb, and adcAII. The phenotypic impact of these deletions was assessed by growth assays in a zinc-restricted chemically defined medium (CDM) in which a functional Adc permease was shown to be essential for bacterial viability (7). Under these conditions, the three SBPs can be considered to be redundant suppliers of zinc, with the deletion of adcA, Imb, and adcAII recquired to abolish growth (7). We observed that, in zinc-restricted CDM, the loss of one or both of the sht and shtll genes had no phenotypic impact (see Fig. S1 in the supplemental material). Similarly, the AdcA-only strain (S. agalactiae $\Delta lmb \Delta adcAll \Delta sht \Delta shtll$), showed wild-type growth, indicating that the two-domain SBP was necessary and sufficient for survival in zinc restricted media (Fig. 1B). However, growth was strongly impaired in the $\Delta adcA \Delta sht$ Δ shtll strain while the Δ adcA strain grew normally. These results suggest that the Sht and Shtll proteins were necessary for zinc acquisition via the Lmb and/or AdcAll proteins in the absence of AdcA. This growth deficiency was similar to that observed for the $\Delta adcA \Delta lmb \Delta adcAII$ mutant strain, which lacked all three SBPs or in the $\Delta adcA \Delta lmb$ ΔadcAll Δsht Δshtll mutant strain, which lacked all the cell surface components of the zinc acquisition machinery (Fig. 1B).

These results are consistent with prior studies from *S. pneumoniae*, which revealed distinct zinc acquisition mechanisms for pneumococcal AdcA and AdcAII (8, 16): one wherein AdcA facilitates zinc acquisition independent of accessory protein partners and a second in which AdcAII/Lmb are dependent upon one or both of the Sht-family proteins to facilitate zinc recruitment.

To complement the growth analyses, whole-cell metal ion accumulation was analyzed for wild-type *S. agalactiae* and the derived mutant strains. Here, we observed that zinc accumulation in the AdcA-only mutant strain ($\Delta lmb \ \Delta adcAll \ \Delta sht \ \Delta shtll$) was significantly reduced by comparison with the wild-type and the $\Delta adcA$ mutant strain (Fig. 2). This indicated that despite the lack of a growth phenotype, AdcA alone did not facilitate accumulation of the zinc to the same extent as wild-type bacteria or strains containing the Sht/Shtll and Lmb/AdcAll family of proteins. Further, as zinc accumulation was not significantly different between the AdcA-only mutant strain ($\Delta lmb \ \Delta sht$



FIG 2 Zn²⁺ content of *S. agalactiae* grown in zinc-restricted conditions Whole-cell Zn²⁺ accumulation of WT and mutant strains determined by ICP-MS. The data represent the means \pm the standard deviations from four biological replicates. The statistical significance of the differences in zinc concentrations compared to WT was determined by an unpaired Student *t* test (ns, not significant; *, *P* < 0.05).

 $\Delta adcAll \Delta shtll$) and the strain containing AdcA and both Sht proteins ($\Delta lmb \Delta adcAll$), these data are consistent with the Sht proteins not contributing to AdcA-mediated zinc homeostasis (Fig. 2). The impacts of these mutations were restricted to zinc, since analysis of other first-row transition metal ions, i.e., manganese, cobalt, nickel and copper, did not show significant altered metal ions abundances (Table S1).

Sht and ShtII both contribute to zinc homeostasis. Building on the above findings, we sought to assess the relative contributions of the Sht and ShtII proteins to zinc acquisition. This was addressed in the $\Delta adcA$ genetic background to remove the contribution of AdcA, which does not interact with the Sht proteins. We first examined the impact of deleting a single Sht-family gene. Phenotypic growth analyses comparing *S. agalactiae* $\Delta adcA$ Δsht and $\Delta adcA$ $\Delta shtII$ mutant strains showed no significant difference in growth rates (Fig. 3A). This indicates that either *sht* gene is sufficient for



FIG 3 Efficiency of the Sht-family proteins in zinc acquisition. (A) Growth of *S. agalactiae* A909 mutant strains in zinc-restricted CDM. The data are representative mean OD_{600} measurements \pm the standard deviations from three independent experiments. (B) Transcriptional profiling of *sht* (black bars) and *shtl* (gray bars) in *S. agalactiae* A909 strains during growth in zinc-restricted CDM to mid-exponential phase ($OD_{600} = 0.5$ phase). Transcription of each gene was normalized to *recA*. The results are presented as means \pm the standard deviations from three independent experiments. The asterisks indicate *P* values obtained using an unpaired Student *t* test to compare the level of expression of *sht* or *shtll* in the WT strain as a reference for each strain (nd, not detected; *, P < 0.05; **, P < 0.01).



FIG 4 Structural modeling of Lmb and AdcAlI. (A) Representation of the crystal structure of Lmb (3HJT; cyan) and the homology model of AdcAlI (green). The bound Zn^{2+} is shown as a gray sphere. The surface electrostatic potentials of AdcAlI (B) and Lmb (C) are shown in the same orientation as in panel A (right panel). Positive and negative potentials are shown in blue and red, respectively, colored continuously between -5 and 5 kT/e. The surface electrostatic potential was calculated using APBS (41).

growth with Lmb or AdcAll. Deletion of both *sht* genes abrogated bacterial growth, indicating that Lmb and AdcAll are unable to acquire sufficient zinc in the absence of these cell wall proteins (Fig. 3A). To complement this analysis, we examined the transcriptional responses in the *S. agalactiae* $\Delta adcA \Delta sht$ and $\Delta adcA \Delta shtll$ mutant strains. Here, we observed that in the mutant strains, the remaining Sht-family gene was significantly upregulated by ~3-fold (Fig. 3B). These findings indicate that a single Sht-family gene is sufficient for viability and growth. Nevertheless, the increased upregulation of the remaining Sht-family gene may show that an intracellular zinc deprivation is experienced by the mutant strains.

To confirm that the abrogated growth phenotype of the S. agalactiae $\Delta adcA \Delta sht$ Δ shtll strain was due to zinc starvation, we supplemented the growth medium with increasing concentrations of metal ions. Here, we observed the recovery of growth with zinc but not with other metal ions (Fig. S2A and B). We then exploited the compromised zinc homeostasis of the $\Delta adcA \Delta sht \Delta shtll$ strain to examine the relative efficiency of the Sht-family proteins to this process. This was addressed by ectopic expression of sht or shtll under the control of the constitutive promoter P_{Tet} (19). Transcriptional analyses of the Sht-family genes from pTCV-P_{Tet}::sht or pTCVP_{Tet}::shtll in S. agalactiae $\Delta adcA \Delta sht$ $\Delta shtll$ showed similar levels of expression (Fig. 3B). Phenotypic growth analyses of mutant strains containing the ectopic expression constructs in CDM showed growth comparable to the mutant $\Delta adcA$ strain (Fig. S3). This was in contrast to the parental strain, S. agalactiae $\Delta adcA \Delta sht \Delta shtll$, that was compromised for growth in this medium. Taken together, these findings indicate that a single Sht-family protein is necessary and sufficient to facilitate growth in zinc-limited media in the absence of AdcA. However, these findings also indicate that S. agalactiae experiences a greater level of intracellular zinc stress when only a single Sht-family protein is present, suggesting that two copies are optimally required.

Sht and ShtII have functionally redundant interactions with Lmb and AdcAII. A homology model of *S. agalactiae* AdcAII was generated based on the structure of *S. pneumoniae* AdcAII (Fig. 4). This allowed comparison of *S. agalactiae* Lmb (PDB 3HJT) and AdcAII, and the identification of any features that could suggest a preferential interaction with their respective Sht-family protein (i.e., Lmb-Sht versus AdcAII-ShtII). Structural comparisons of AdcAII with Lmb indicated that the two proteins had similar



FIG 5 Sht-family proteins aid in *S. agalactiae* zinc acquisition via either Lmb and/or AdcAII. (A) Growth of the Lmb-only strain (*S. agalactiae* $\Delta adcA$ $\Delta adcAII \Delta sht \Delta shtII$) complemented with the pTCV-P_{Tet} vector containing either *sht* or *shtII*. The strain $\Delta adcA \Delta adcAII \Delta sht \Delta shtII$ containing the empty vector (\otimes) was used as a negative control. (B) Growth of the AdcAII-only strain (*S. agalactiae* $\Delta adcA \Delta lmb \Delta sht \Delta shtII$) mutant (only AdcAII remains as the substrate binding protein) complemented with the pTCV-P_{Tet} vector containing either *sht* or *shtII*. The $\Delta adcA \Delta lmb \Delta sht \Delta shtII$ strain containing the empty vector (\otimes) was used as a negative control. Growth was monitored by OD₆₀₀ measurements every 60 min for 12 h. The data are representative mean OD₆₀₀ measurements from three independent experiments.

overall folds (C α root mean square deviation, 0.6 Å) with metal-binding sites buried ca. 10 to 15 Å beneath the molecular surface of the protein and occluded from solvent in the metal-bound state. Surface charge calculations revealed a negatively charged region at the interdomain cleft proximal to the metal-binding site in both Lmb and the model of AdcAll (Fig. 4). Collectively, these analyses did not reveal any apparent structural distinctions between Lmb and AdcAll that would otherwise suggest a structural basis for a preferential interaction with either Sht-family protein. To probe the veracity of our insights, we constructed different combinations of S. agalactiae deletion strains that expressed either Imb ($\Delta adcA \Delta adcAII \Delta sht \Delta shtII$) or $adcAII (\Delta adcA \Delta Imb \Delta sht$ Δ shtll) as the only cell surface component of the zinc homeostasis machinery. These mutant strains were incapable of growth in zinc-restricted CDM (Fig. 5). We then introduced the ectopic expression constructs for Sht (pTCV-P_{Tet}::sht) or Shtll (pTCVP_{Tet}:: shtll) into the mutant strains. Irrespective of the combination of Sht-family protein with Lmb or AdcAll, growth was restored in zinc-restricted CDM (Fig. 5). Taken together, these data show that there is no significant preference of SBP for Sht-family proteins, indicating that, in vitro, there is functional redundancy in this aspect of S. agalactiae zinc homeostatic machinery.

Role of the five-histidine triad motifs of Sht and Shtll proteins in zinc acquisition. Primary sequence comparisons of Sht-family proteins and PhtD revealed that the S. agalactiae proteins also contain five HT motifs (Fig. 6). The N-terminal portion of the proteins (residues 1 to 380) containing the three first HT motifs is the most conserved. Here, we sought to examine the respective contributions of the HT motifs to zinc acquisition. To address this, the three His residues (HXXHXH) of each HT motif in Sht and ShtII were replaced by three Phe residues. These mutations were shown to abrogate the zinc binding capacity of the HT motifs in S. pneumoniae PhtD while preserving steric bulk to ensure conformational fidelity (15). The sht and shtll HT mutant alleles (designated of shtAHT* or shtIIAHT*, where "*" represents the HT motif designated in Fig. 6) were introduced into the $\Delta adcA \Delta sht \Delta shtII$ strain via the pTCV-P_{Tet} vector. Phenotypic growth assays were then performed in zinc-limited CDM and compared to the $\Delta adcA \Delta sht \Delta shtll$ strain complemented with the respective parental allele (Fig. 6). In zinc-limited CDM, growth was completely abolished for the Δ HT1 and Δ HT2 Sht and ShtII strains. In contrast, growth was compromised for the Δ HT3 and Δ HT5 Sht and Sht strains, albeit to a lesser extent (Fig. 7). The Δ HT4 Sht and ShtII strains were the least affected, with growth of the shtllAHT4 strain indistinguishable from that of the parental strain (Fig. 7). Interestingly, both Sht and Shtll had identical patterns of behavior, despite significant sequence divergence with respect to HT4 and HT5. Supplementation of the CDM with 1 μ M zinc restored growth of all but the Δ HT1

	10	2	0 3	0 4	0 5	0 6	0 7	0 80	90	100
Sht	-MKKTYGYIG S	VAAILLATH	IGSYQLGKHH	MGLAT-KDNQ	IAYIDDSKGK	AKAPKTNK	TMDQISAEEG	ISAEQIVVKI	TDQGYVTSHG	DHYHYNGRV
ShtII PhtD	MNRKKTVIIS A	LSVALFGTG GSVAVLALS	VGAYQLGSYN VCSYELGREO	AQKSDNS AGOVKKESNR	VSYVKTDKSD VSYIDGDOAG	SKAQATAANK	TPDQISKEEG	ISAEQIVVKI INAEOIVIKI	TDDGYVTSHG	DHYHCYNGKV
									Н	T1
	110	120	130	140	150	160) 170	180	190	200
Sht	PYDAIISEEL L	MTDPNYREK	QSDVINEILD	GYVIRVNGNY	YVYLRPGSRR	KNIRTKQQIA	EQVARGTREA	RERGLAQVAH	LSREEVAA-V	NEARROGRYT
ShtII	PYDAIISEEL I	IKOPSYVEN	RADVINEVED	GYIIRVNGRY	YLYLKEGSKR	TNVRTKEQIQ	RORDEWSKGG	SKGESGKHSS	AKTQALSASV I	REARASCRYT
Enco	ELDALISTIC L	run run Ken	DODIVIGINO	UIVIRVDORI	I VI LED AALLA	DATATABUTA	W/W/Y	Kommino		- Man your I
	210	220	230	240	250	260	270	280	290	300
Sht	TDDGYIFSPT D	IIDDLGDAY	LVFHGNHYHY	IPKKDLSPSE	LAAAQAYWSQ	ROGRGARPSD	YRPTPAPGRR	KAPIPDVTP-	NPGQGHQ	PDNGGYHPAP
ShtII PhtD	TDDGYVFSPT D TDDGYIFNAS D	VIDDMGDAF IIEDTGDAY	LVFHGDHFHY	IPKADLSPSE IPKSDLSASE	LSAAQAYWNR LAAAOAYWNG	KTGRAGNSSK KOGSR	PSNSSSYIHA PSSSSSH	SAPSGNVSTG	RHANAPISIP	RVTHANHWSK RLSE-NH
			HT2							
	310	320	330	340	350	360	370	380	390	400
Sht	PRPNDASQNK H	QRDEF	KOKTERELLD	QLHRLDLKYR	HVEEDGLIFE	PTQVIKSNAF	GYVVEHCDHY	HIIPRSQLSP	LEMELAD	-RYLAGQTED
ShtII PhtD	PAGNHATAPK H	HAPTTKPIN HQN	QGENISSLLR	ELYARPLSER	HVESDGLVYD	PAQVNAFTAI PAQITSRTAN	GVSIHHGNHF GVAVFHGDHY	HFIHYKDMSP	LELEATRMVA	-EHRGHHIDA LRYRSNHWVP
	410	404	404		454		НТЗ	400	400	500
Sht	NDSGSEHSKP SI	DREVTHTFL	GHRIKAYGKG	LDGKPYDT	SDAYVESKES	IHSVDKSGVT	ARHGDHFHYI	GFGELEQYEL	DEVANWVKAK	GOADELAAAL
PhtD	DSRPEQPSPQ S	TPEPSPSPQ	PAPNPQPAPS	NPIDERLVKE	AVREVGDGYV	FEENGVPRYI	PAK-DLSAET	AAGIDSKLAK	QESLSHK	LGAKKTDLPS
							HT4		HT4	
	510	500		5.4/				-		
	510	520	530	540	55	0 56	57	580	590	600
Sht	510 . DQEQGREKPL FI	520 DTRKVSRKV	530 TKDGKVGYMM	540 PKDGKDYFYA	550 RDQLDLTQIA	0 56	0 57	0 580 DIVDTGI	590 EPRLAVDVSS	600
Sht ShtII PhtD	510 DOEOGREKPL FI SELKAAQEFL SO SDREFYNKAY D	520 DTKKVSRKV GKSEANQDK LLARIHODL	530 TRDGRVGYMM PRTGRTAQEI -LDNKGROVD	D 540 PKDGKDYFYA YEAIEPKAIV FEALDNLLER	S50 RDQLDLTQIA RPEDLLFGIA LKDVSSDKVK	0 56 	0 57 MLKDKKHYRY VIIHKDHYHY PIRHPERLGK	0 580 DIVDTGI VELKWFDE PNAQITYTDD	EPRLAVDVSS EKDLLADSDK EIOVAKLAGK	600 LPMHAGNATY TYSLEDYLAT YTTEDGYIFD
Sht ShtII PhtD	510 DQEQGREKPL FI SELKAAQEFL SO SDREFYNKAY D	520 DTKKVSRKV GKSEANQDK LLARIHQDL	D 530 TKDGKVGYMM PKTGKTAQEI -LDNKGRQVD) 540 PRDGRDYFYA YEAIEPKAIV FEALDNLLER	N 550 RDQLDLTQIA RPEDLLFGIA LKDVSSDKVK	0 560 FAEQEL QATDYKNGTF LVDDILAFLA	0 57/ MLKDKKHYRY VIEHKOHYHY PIRHPERLGK HT5	0 580 DIVDTGI VELKWFDE PNAQITYTDD	EPRLAVDVSS EKDLLADSDK EIQVAKLAGK	0 600 LPMHAGNATY TYSLEDYLAT YTTEDGYIFD
Sht ShtII PhtD	510 DOEQGRERPL P SELKAAQEFL SI SDREPYNKAY D 610	520 DTKKVSRKV GKSEANQDK LLARIHQDL 620 	D 530 TRDGRVGYMM PRTGRTAQEI -LDNRCRQVD 0 630) 54(PRDGRDYFYA YEAIEPKAIV FEALDNLLER) 64(D 550 RDQLDLTQIA RPEDLLFGIA LKDVSSDKVK D 650 	0 56 PAEQEL QATDYRNGTP LVDDILAFLA 0 66 	0 570 MLKDKKHYRY VIEHKDHYHY PIRHPERLGK HT5 0 670	0 580 DIVDTGI VELRWFDE PNAQITYTDD 0 680	D 590 EPRLAVDVSS EKDLLADSDK EIQVAKLAGK 0 690) 600 LPMHAGNATY TYSLEDYLAT YTTEDGYIFD) 700
Sht ShtII PhtD	510 	520) 530 TRDGRVGYMM PRTGRTAQEI -LDNRGRQVD) 630 -UVPYSWLTR) 54(PRDGRDYFYA YEAIEPKAIV FEALDNLLER) 64(D 550 RDQLDLTQIA RPEDLLFGIA LROVSSDRVR 0 650 	0 56 	0 57 MLKDKKHYRY VIHROBYHY PIRPERLGK HTS 0 67 VIPNVTPLDK	D 580 DIVDTGI VELKWFDE PNAQITYTDD D 680 RAGMPNWQII	D 590 EPRLAVDVSS EKDLLADSDK EIQVARLAGK D 690 HSAEEVQKAL	600 LPMHAGRATY TYSLEDYLAT YTTEDGYIFD 700 AEGRFATPDG
Sht ShtII PhtD Sht ShtII PhtD	510 	520 DTKKVSRKV GKSEANQDK LLARIHQDL 620 FVI:HIDHI YYMHPERR YVT:HIDHI YYMHPERR	S30 TRDGRVGYMM PKTGKTAQEI LDNKURQVD G30 G30 UVPYSWLTA PKVEGWG-RD WIRKDSLSE) 54(D 550 RDQLDLTQIA RPEDLLFGIA LKDVSSDKVK 0 650 	0 56 PAEQEL QATDYRNGTF LVDDILAFLA 0 66 SKPGHEESGS APTDNKSTSN PSTDHQDSGN	0 57 MLKDRKHYRY VIIHKDHYHY PIRHPERLGK HT5 0 67 UIPNVTPLDK SSDRNLSAAE TEAKGAEA	0 580 DIVDTGI VELKWFDE PNAQITYTDD 0 680 	D 590 EPRLAVDVSS EKDLLADSDK EIQVAKLAGK 0 690 HSAEEVQKAL VPLDRIAAHM VPLDRMPYNL	0 600 LPMHAGNATY TYSLEDYLAT YTTEDGYIFD 0 700
Sht ShtII PhtD Sht ShtII PhtD	510 	520 DTRKVSRKV GKSEANQDK LLARIHQDL 620 FVI:HIDHI YYMHPERR YVT:HIDHI YYMHPERR YVT:HMTS HI4 H720	D 530 TRDGRVGYMM PRTGRTAQEI -LDNRCRQVD 0 630 -UVPYSWLTR PRVEGWG-RD EVIRRDSLSE) 54(0 550 	0 56 PAEQEL QATDYRNGTF LVDDILAFLA 0 66 	0 570 MLKDRKHYRY VIERDPERLGK HTS 0 677 	0 580 DIVDTGI VELKWPDE PNAQITYTDD 0 680 	S90 S90 S90 S90 SPRLAVDVSS ERDLLADSDR EIQVARLAGR G90 SAEEVQRAL VPLDRIAAHM VPLDRMPYNL SAEVQRAL S	0 600
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FIG 6 Comparison of amino acid sequences of the Sht and ShtII proteins of *S. agalactiae* and the PhtD protein of *S. pneumoniae*. The signal sequences are highlighted in gray, and the identified histidine triad motifs (HT) are indicated by black boxes. The protein sequences were aligned using BioEdit software.

mutant strains. Further supplementation (10 μ M zinc) restored growth of all strains (Fig. 7). Collectively, these data suggest a hierarchy of the importance with respect to the Sht and Shtll HT motifs that follows the order of HT1 > HT2 > HT3 > HT5 > HT4.

Sht and Shtll bind factor H *in vitro*. Sht was first described as a factor H binding protein and a negative regulator of the alternative pathway of complement (18). Since Sht and Shtll share 43% of protein identity, we investigated whether the Shtll protein had the same ability to bind factor H. The factor H binding assay examined recombinant *S. agalactiae* A909 Sht, Shtll, and CcpA and *S. pneumoniae* PspC. CcpA is a cytoplasmic transcriptional regulator (i.e., negative control), whereas PspC is a pneumococcal surface protein that has been shown to interact strongly with factor H (i.e., positive control) (20).



FIG 7 Contribution of the Sht and Shtll HT sites to zinc acquisition. Growth of the *S. agalactiae* $\Delta adcA \Delta sht \Delta shtll$ mutant with the pTCV-P_{Tet} vector containing either wild-type *sht* or a mutant derivative, in which a single HT motif was mutated (A), or wild-type *shtll* or a mutant derivative, in which a single HT motif was mutated (B). The empty vector (\otimes) was used as a negative control. Bacteria were grown in zinc-restricted CDM, 1 μ M Zn²⁺, or 10 μ M Zn²⁺, as shown. Growth was monitored based on OD₆₀₀ measurements every 60 min for 18 h. The data are representative mean OD₆₀₀ measurements from three independent experiments.

Enzyme-linked immunosorbent assay (ELISA) analyses of inactivated serum containing factor H showed that both Sht and ShtIl bound factor H and to a similar extent (Fig. 8). Notably, Sht-family protein binding of factor H was significantly more than that observed for CcpA but weaker than PspC (Fig. 8). These data suggest that the Sht-family proteins may also serve a protective role for evading complement-mediated killing during *in vivo* infection.

Sht and Shtll do not provide resistance against complement in human blood. It has been proposed that the capacity of Sht to recruit factor H decreases C3b deposition on the surface of *S. agalactiae*, thereby contributing to evasion of bacterial lysis mediated by the membrane attack complex (18). However, direct experimental evidence remains lacking, and thus the relevance of the *in vivo* binding capacities of the Sht-family proteins for factor H is unclear. We examined the survival of *S. agalactiae*



FIG 8 Factor H binding by the Sht-family proteins. Factor H binding to the streptococcal proteins CcpA, Sht, Shtll, and PspC was determined by ELISA. The data are representative mean OD_{450} measurements \pm the standard deviations from three independent experiments. The asterisks indicate *P* values obtained using an unpaired Student *t* test to compare factor H binding by the Sht or Shtll proteins with the CcpA (negative control). *, *P* < 0.05; **, *P* < 0.01.



FIG 9 Expression and contribution of the Sht-family proteins in complement resistance. (A) Bacterial survival, determined as the percent CFU per milliliter relative to input of WT (white) and $\Delta sht \Delta shtll$ (black) mutant strains after 3 h of incubation in whole blood or heat-inactivated blood. The results represent means \pm the standard deviations of three independent experiments. (B and C) Transcription of *sht* (B) and *shtll* (C) after 90 min of incubation in zinc-restricted CDM (black), CDM supplemented with 10 μ M Zn²⁺ (white), or heat-inactivated serum (gray). Transcripts of each gene were normalized to *recA* with a reference value of 100 for *sht* and *shtll* transcripts after bacterial growth in zinc-restricted CDM. The results are means \pm the standard deviations from three independent experiments. The statistical significance of the differences was determined by a two-tailed unpaired Student *t* test comparing gene expression levels to the zinc-restricted CDM control (*, *P* < 0.05; **, *P* < 0.005).

A909 and the $\Delta sht \Delta sht I$ mutant strain in whole human blood and heat-inactivated blood. In whole blood, wild-type bacteria were nearly completely eliminated (\sim 20% survival) after 3 h. In contrast, survival of the wild-type strain was unimpeded in heat-inactivated blood, highlighting the contribution of complement-mediated killing (Fig. 9A). Further, loss of the Sht-family proteins did not affect the relative survival of the bacteria in whole blood (Fig. 9A). The same experiment was performed in human serum and provided the same results (Fig. S4). Taken together, these data indicate that the Sht proteins provide no apparent protection against complement-mediated killing under these conditions. Given the discrepancy in our observations by comparison to studies of Sht-family orthologs (18), we analyzed transcription of sht and shtll in human serum (Fig. 9B and C). Indeed, we have previously shown that the Imb-sht, adcAll-shtll, and adcA encoding genes were fully repressed as soon as bacteria are cultured in medium containing more than 10 μ M zinc, for example, in TH medium containing 20 μ M zinc (7). In serum, we observed that the Sht-family genes were also significantly repressed (Fig. 9B and C), most likely attributable to bioavailable zinc in the human serum (14 μ M in average [21]). Consequently, Sht and Shtll are unlikely to have been expressed at significant levels under the experimental conditions investigated.

DISCUSSION

In this study, we examined the role of the Sht-family proteins in zinc acquisition and complement resistance. We showed that Sht and Shtll facilitate zinc uptake via the Lmb and AdcAll, with no discernible preference or dependency on either SBP. This highlights two distinct routes of zinc acquisition in S. agalactiae mediated by either AdcA alone, or via Lmb/AdcAll in concert with the Sht-family proteins. These modes of zinc acquisition have previously been reported in S. pneumoniae and attributed to the distinct structural features of AdcA and AdcAll (8, 16). In S. agalactiae the zinc-specific SBPs all contain a common high-affinity metal-binding site, which is comprised of three histidine residues and a glutamic acid in Lmb and predicted by sequence alignments to be the same in AdcA and AdcAll (7, 22). However, AdcA also contains a short region enriched for histidine residues (residues 129 to 139) and a C-terminal extension (residues 319 to 502) that has homology to the periplasmic metal-binding protein ZinT. These accessory regions potentially aid in zinc acquisition for S. agalactiae AdcA, as has been proposed for orthologs in S. pneumoniae or S. pyogenes (7, 8, 16, 23). Further, these accessory domains provide a plausible explanation for the apparent lack of dependency of AdcA on Sht-family proteins to capture environmental zinc. The overlapping roles of these zinc acquisition systems in S. agalactiae raise the question of the

reason for the basis for the conservation of multiple zinc import pathways. Recently, it has been proposed that the Sht-family genes, which are frequently acquired as operons that also contain *Imb* and *adcAll* genes, were acquired prior to the emergence of the *Streptococcus* genus (10). As a consequence, maintenance or loss of Sht-family genes are presumed to arise from the competing selective pressures associated with their physiological function, such as zinc acquisition and evasion of host immune responses. The absence of such determinants provides a plausible explanation for the loss of the Sht-family proteins in nonpathogenic species, such as *Streptococcus thermophilus*, a nonpathogenic bacterium widely used in starter cultures for cheese and yogurt production and in oral streptococci. Notably, these bacteria encode only a single SBP, AdcA, and no Sht-family proteins. In contrast, pathogenic streptococci, such as *S. pneumoniae*, *S. pyogenes*, *S. suis*, and *S. agalactiae*, frequently encode one or more Sht-family proteins (7, 14, 24, 25). Consistent with these inferences, it has been shown in *S. pneumoniae* that, despite the overlapping functionality of AdcA and AdcAll *in vitro*, both proteins were required for optimal *in vivo* infection (26).

Gene duplication events have led to emergence of streptococcal strains with multiple copies of the Sht-family genes. In *S. agalactiae*, this has also led to the presence of the two additional SBPs, Lmb and AdcAll, whose genes are in operons with *sht* and *shtll*, respectively. Of the streptococci, *S. pneumoniae* contains the greatest number of Sht-family genes with four orthologs. Examination of their role in pneumo-coccal zinc homeostasis revealed that their roles were not completely redundant since strains containing only a single *pht* gene did not grow as effectively as strains containing two or more copies (12). Here, we have shown that in contrast to *S. pneumoniae*, a single *sht* gene was sufficient for growth under zinc-limiting conditions. Sht and Shtll also share a strong sequence similarity (43% identity) in contrast to the divergent sequences of the *S. pneumoniae* Pht proteins. Consistent with this observation, the highly similar SBPs, Lmb and AdcAll (58% sequence identity), can interact with either Sht-family protein to facilitate zinc homeostasis with equivalent levels of efficiency.

The Sht-family proteins contain five HT motifs, similar to the pneumococcal ortholog PhtD. Although the Pht proteins have been refractory to high-resolution structure determination of the full-length protein, truncated domains containing individual HT motifs (HT1, HT2, and HT3) have been revealed (13, 16, 27). Structural analyses have suggested that each of the HT motifs of PhtD adopt a similar fold, with an equivalent capacity for Zn^{2+} binding (13). Nonetheless, functional studies have shown that HT1 has the most critical role in zinc acquisition (15). Here, we examined the contribution of the HT motifs to Zn^{2+} acquisition by the Sht-family proteins. The HT1 motif was essential during growth in Zn²⁺-limited conditions, while the other HT mutants showed intermediate phenotypes. The HT4 motif was the only exception, with little to no apparent phenotypic impact associated with its loss. Sequence alignments show that motifs HT1 to -3 of Sht and Shtll align closely with the equivalent motifs in PhtD (Fig. 6). These data suggest that the amino-terminal portions of the proteins are structurally and functionally conserved between S. agalactiae and S. pneumoniae. However, further mutagenesis and structural analyses are required to assess the strength of this inference.

Despite the original description of Sht as a complement resistance protein (18), the role of ShtII in this process has remained unaddressed. Here, we showed that both of the Sht-family proteins contribute to bind factor H *in vitro*, albeit to a lesser extent than PspC. However, the Sht-family proteins were not protective in human blood, most likely due to their lack of expression. Confounding results have also been reported for the *S. pneumoniae* Pht proteins and their role in factor H recruitment (28, 29). However, the bioavailability of zinc can alter significantly over the course of infection, with serum levels decreasing as a component of the hypozincemic effect. Tissue abscesses caused by *Staphylococcus aureus* infection have also been shown to be devoid of detectable Zn^{2+} (30). As such, the *in vivo* role of the Sht proteins and their contribution to both zinc recruitment and resisting complement-mediated killing cannot be discounted and warrant further investigation.

The contribution of the Sht-family proteins to the virulence of *S. agalactiae* can be linked to the necessity for efficacious Zn^{2+} acquisition mechanisms during growth in human amniotic and cerebrospinal fluids, which are tightly restricted for Zn^{2+} abundance (zinc concentrations of about 1.5 and 2.3 μ M, respectively) (31–33). These two latter fluids are highly relevant for *S. agalactiae*, since it remains the main causative agent of neonatal meningitidis arising from the vertical transmission through contaminated amniotic fluid (5). Intriguingly, human-pathogenic *S. agalactiae* contains an additional *lmb-sht* operon, suggesting that the additional Zn^{2+} -recruiting machinery plays a role in the context of human colonization and/or infection. Further analysis of the zinc acquisition pathways of *S. agalactiae* during *in vivo* infection will elucidate the roles of the various components and their relative contribution to human disease.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. agalactiae* (GBS) and *E. coli* strains used in this study are listed in Table 1. *E. coli* XL1-Blue served as the host for recombinant plasmid pG+host1^{TS}, and *E. coli* BL21(DE3) was used for the expression of recombinant protein from plasmid pET28a (Table 1). *S. agalactiae* strains were cultured on 5% horse blood Trypticase soy agar plates (1.5% agar; bioMérieux) on Todd-Hewitt broth (TH) agar (Sigma-Aldrich) or in liquid TH medium at 37°C without agitation. Complemented mutant strains harboring pTCV-P_{Tet} plasmid were maintained in medium containing 10 μ g ml⁻¹ of erythromycin (Ery). *E. coli* strains harboring pG+host1^{TS} or pTCV-P_{Tet} plasmids were maintained in medium containing 150 μ g ml⁻¹ of Ery. *E. coli* strains harboring pFT28a plasmid were maintained in medium containing 150 μ g ml⁻¹ of Ery. *E. coli* strains harboring pFT28a plasmids were maintained in medium containing 150 μ g ml⁻¹ of Ery. *E. coli* strains harboring pFT28 plasmid were maintained in medium containing 150 μ g ml⁻¹ of Ery. *E. coli* strains harboring pFT28a plasmids were maintained in medium containing 150 μ g ml⁻¹ of Ery. *E. coli* strains harboring pFT28a plasmids were maintained in medium containing 150 μ g ml⁻¹ of Ery. *E. coli* strains harboring pFT28a plasmids were maintained in medium containing 150 μ g ml⁻¹ of Ery. *E. coli* strains harboring pFT28a plasmids were maintained in medium containing 25 μ g ml⁻¹ of ery. *E. coli* strains harboring pFT28a plasmids were maintained in medium containing 25 μ g ml⁻¹ of Ery. *E. coli* strains harboring pFT28a plasmids were maintained in medium containing 25 μ g ml⁻¹ of ery. *E. coli* strains harboring pFT28a plasmids

Construction of deletion mutants and complementation strains. Nonpolar deletion mutants strains of A909 *S. agalactiae* with a single or combined deletions of the entire coding sequences of the *lmb, adcA, adcAll, sht,* or *shtll* genes had been generated as previously described (7). PCR and DNA sequencing were used to confirm that the desired mutations had been introduced. The primers used to generate mutant strains are listed in Table 2. To complement the $\Delta sht \Delta shtll \Delta adcA$ mutant strain, the entire coding sequence of *sht* or *shtll* gene was amplified using Q5 high-fidelity DNA polymerase (New England BioLabs) and cloned into the pTCV-P_{Tet} vector using appropriate restriction sites as previously described (7). The cloned sequences into the pTCV-P_{Tet} vectors were confirmed by PCR and DNA sequencing. The oligonucleotides (Sigma-Aldrich) used for the complementation constructs are listed in Table 2.

Construction of HT motifs variants. Site-tagged mutagenesis was performed to obtain the five HT* motif variants of Sht and ShtII. The primers were designed with mutations to substitute phenylalanine residues in place of each histidine residue of the relevant triad (Table 2). For each construction, two DNA fragments were generated by PCR. The point mutations were obtained using oligonucleotides containing the designed mismatches and also carrying Bsal restriction sites, a type IIS restriction endonuclease, which cleaves after its restriction site, generating DNA fragments with tetranucleotide cohesive ends (Table 2). After digestion with Bsal (1 h, 37°C; New England BioLabs), the two fragments were purified with a NucleoSpin PCR cleanup kit (Macherey-Nagel) and ligated using the sticky-end instant ligase (New England BioLabs), seamlessly fusing the fragments together without adding any additional nucleotides. The resulting fragments were reamplified by PCR using the external oligonucleotides OAH248 and OAH284 for *sht* and OAH249 and OAH241 for *shtIl* and cloned in the pTCV-P_{Tet} vector (Tables 1 and 2).

GBS culture in chemically defined medium. *S. agalactiae* strains (Table 1) were cultured in zinc-restricted chemically defined medium (CDM) as previously described (7). Briefly, *S. agalactiae* was grown in TH until it reached stationary phase and was then inoculated, at an optical density at 600 nm (OD₆₀₀) of 0.005, into zinc-restricted CDM supplemented with 0.2% (w/vol) glucose [D-(+)-glucose; Sigma-Aldrich] and 500 μ M EDTA and grown overnight. *S. agalactiae* was then inoculated at an OD₆₀₀ of 0.005 into zinc-restricted CDM supplemented with 1% glucose (w/vol), 500 μ M EDTA, and ZnSO₄ concentrations as specified. *S. agalactiae* strains were then grown at 37°C for 12 h in microtiter plates (Greiner Bio-One; Cellstar) with OD₆₀₀ measurements recorded using an Eon spectrophotometer (BioTek).

Whole-cell metal ion accumulation analyses. Whole-cell metal ion accumulation was determined by inductively coupled plasma-mass spectrometry (ICP-MS) essentially as previously described (34, 35). Briefly, *S. agalactiae* strains were grown to mid-log phase (OD₆₀₀ of 0.4 to 0.5) into CDM plus 1% glucose plus 500 μ M EDTA. Cells were harvested by centrifugation, washed twice with PBS plus 5 mM EDTA, and then washed twice with PBS prior to desiccation at 95°C for 18 h. Metal ions were released by treatment with 0.5 ml of 35% HNO₃ at 95°C for 60 min. Samples were then diluted to a final concentration of 3.5% HNO₃ and metal content determined on an Agilent 8900 QQQ ICP-MS. The data represent four biological replicates, and the statistical difference was assessed using an unpaired Student *t* test (GraphPad Prism 7.0c).

RNA extraction. For analysis of the *sht* and *shtll* expression in CDM and in serum, cells were grown in zinc-restricted CDM until reaching an OD₆₀₀ of 0.5 and resuspended either in zinc-restricted CDM, in CDM supplemented with 100 μ M zinc, or in inactivated serum (90 min). Cells were harvested (10 ml), and the pellets were frozen and stored at -80° C. For all other analyses, CDM-grown cells were harvested to mid-exponential phase (OD₆₀₀ = 0.5). Bacteria were mechanically lysed by glass beads in a FastPrep-24 instrument, and total RNA was extracted using a phenol/TRIzol-based purification method as previously

TABLE 1 Bacterial strains and	plasmids	used i	in this	study
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Strain or plasmid	Genotype or description	Source or reference
Strains	Strains	
Escherichia coli		
XL1-Blue	endA1 gyrA96 (Nal') thi-1 recA1 relA1 lac glnV44 hsdR17(r _K [−] m _K ⁺) F′ [::Tn10 (Tet') proAB ⁺ lacl ^q ZΔM15]	Stratagene
BL21 codon $+$ (DE3)-RIL	F^- dcm ompT hsdS(r_B^- m _B ⁻) gal [malB ⁺] K-12 (λ^{s})	Novagen
Streptococcus agalactiae		-
A909 (WT)	Isolated from a septic human neonate in 1934	42
∆ <i>lmb</i> ∆ <i>adcAll</i> strain	Isogenic Imb (sak_1319) and adcAll (sak_1898) deletion double mutant of A909	7
$\Delta adcA$ strain	Isogenic adcA (sak_0685) deletion mutant of A909	7
$\Delta adcA \Delta lmb \Delta adcAll$ strain	Isogenic adcA, Imb, and adcAII deletion triple mutant of A909	7
Δsht strain	Isogenic sht (sak_1318) deletion mutant of A909	This study
$\Delta shtll$ strain	Isogenic shtll (sak_1897) deletion mutant of A909	This study
Δ sht Δ shtll strain	Isogenic sht and shtll deletion double mutant of A909	This study
$\Delta adcA \Delta sht$ strain	Isogenic adcA and sht deletion double mutant of A909	This study
$\Delta adcA \Delta shtll$ strain	Isogenic <i>adcA</i> and <i>shtll</i> deletion double mutant of A909	This study
$\Delta adcA \Delta sht \Delta shtll strain$	Isogenic <i>adcA</i> , <i>sht</i> , and <i>shtll</i> deletion triple mutant of A909	This study
ΔadcA Δsht Δshtll/pTCV-P _{Tet} ::sht strain	sht (sak_1318) plasmid complementation of A909 $\Delta adcA \Delta sht \Delta shtll$	This study
ΔadcA Δsht Δshtll/pTCV-P _{Tet} ::sht ΔHT1 strain	sht containing a point mutation in the HT1 motif plasmid complementation of A909 $\Delta adcA \Delta sht \Delta shtll$	This study
ΔadcA Δsht Δshtll/pTCV-P _{Tet} ::sht ΔHT2 strain	sht containing a point mutation in the HT2 motif plasmid complementation of A909 $\Delta adcA \Delta sht \Delta shtll$	This study
$\Delta adcA \Delta sht \Delta shtll/pTCV-P_{Tet}$::sht $\Delta HT3$ strain	sht containing a point mutation in the HT3 motif plasmid complementation of A909 $\Delta adcA \Delta sht \Delta shtll$	This study
$\Delta adcA \Delta sht \Delta sht II/pTCV-P_{Tet}$::sht $\Delta HT4$ strain	sht containing a point mutation in the HT4 motif plasmid complementation of A909 $\Delta adcA \Delta sht \Delta shtll$	This study
$\Delta adcA \Delta sht \Delta sht ll/pTCV-P_{Tet}$::sht $\Delta HT5$ strain	sht containing a point mutation in the HT5 motif plasmid complementation of A909 $\Delta adcA \Delta sht \Delta shtll$	This study
ΔadcA Δsht Δshtll/pTCV-P _{Tet} ::shtll strain	shtll (sak_1897) plasmid complementation of A909 Δ adcA Δ sht Δ shtll	This study
$\Delta adcA \Delta sht \Delta sht II/pTCV-P_{Tet}$::shtll $\Delta HT1$ strain	shtll containing a point mutation in the HT1 motif plasmid complementation of A909 $\Delta adcA \Delta sht \Delta shtll$	This study
$\Delta adcA \Delta sht \Delta sht II/pTCV-P_{Tet}$::shtll $\Delta HT2$ strain	shtll containing a point mutation in the HT2 motif plasmid complementation of A909 ΔadcA Δsht Δshtll	This study
$\Delta adcA \Delta sht \Delta sht ll/pTCV-P_{Tet}$::shtll $\Delta HT3$ strain	shtll containing a point mutation in the HT3 motif plasmid complementation of A909 NadcA Asht Ashtll	This study
$\Delta adcA \Delta sht \Delta sht II/pTCV-P_{Tet}$::shtll $\Delta HT4$ strain	<i>Shtll</i> containing a point mutation in the HT4 motif plasmid complementation of A909	This study
$\Delta adcA \Delta sht \Delta sht ll/pTCV-P_{Tet}$::shtll $\Delta HT5$ strain	<i>htll</i> containing a point mutation in the HT5 motif plasmid complementation of A909	This study
$\Delta lmb \Delta sht \Delta adcAll \Delta shtll strain$	lsogenic <i>lmb, sht, adcAll,</i> and <i>shtll</i> deletion guadruple mutant of A909	This study
$\Delta adcA \Delta lmb \Delta sht \Delta adcAll \Delta sht ll strain$	Isogenic adcA. Imb. sht. adcAll shtll deletion guintuple mutant of A909	This study
ΔadcA ΔadcAll Δshtll Δsht strain	Isogenic <i>adcA</i> , <i>adcAll</i> , <i>shtll</i> , and <i>sht</i> deletion guadruple mutant of A909	This study
$\Delta adcA \Delta adcAll \Delta shtll \Delta sht/pTCV-P_{Tax}$	sht plasmid complementation of A909 $\Delta adcA \Delta adcA II \Delta sht II \Delta sht$	This study
ΔadcA ΔadcAll Δshtll Δsht/pTCV-P _{Tat} ::shtll strain	shtll plasmid complementation of A909 $\Delta adcA \Delta adcAll \Delta shtll \Delta sht$	This study
$\Delta adcA \Delta lmb \Delta sht \Delta shtll strain$	Isogenic adcA, Imb, sht, and shtII deletion quadruple mutant of A909	This study
ΔadcA Δlmb Δsht Δshtll/pTCV-P _{Tet} ::sht strain	sht plasmid complementation of A909 $\Delta adcA \Delta lmb \Delta sht \Delta sht II$	This study
$\Delta adcA \Delta lmb \Delta sht \Delta sht ll/pTCV-P_{Tet}$::shtll strain	shtll plasmid complementation of A909 $\Delta adcA \Delta lmb \Delta sht \Delta shtll$	This study
Plasmids		
pG+host1 ^{TS}	Replication-thermosensitive shuttle (TS) plasmid; Ery ^r	43
pTCV-P _{Tet}	Mob ⁺ (IncP); <i>oriR</i> pACYC184; <i>oriR</i> pAM_1; complementation vector, promoter P _{Tet}	19
pTCV-P _{Tet} ::sht	sht complementation vector, promoter P _{Tet}	This study
pTCV-P _{Tet} :: <i>sht</i> ∆HT1	sht containing a point mutation in the HT1 motif complementation vector, promoter P_{Tet}	This study
pTCV-P _{Tet} :: <i>sht</i> ∆HT2	sht containing a point mutation in the HT2 motif complementation vector, promoter P_{Tet}	This study
pTCV-P _{Tet} ::: <i>sht</i> ∆HT3	sht containing a point mutation in the HT3 motif complementation vector, promoter P_{Tet}	This study
pTCV-P _{Tet} :: <i>sht</i> ∆HT4	sht containing a point mutation in the HT4 motif complementation vector, promoter P_{Tet}	This study
pTCV-P _{Tet} :: <i>sht</i> ∆HT5	sht containing a point mutation in the HT5 motif complementation vector, promoter P_{Tet}	This study
pTCV-P _{Tet} ::shtll	shtll complementation vector, promoter P _{Tet}	This study
pTCV-P _{Tet} :: <i>shtll</i> ΔHT1	shtll containing a point mutation in the HT1 motif complementation vector, promoter P_{Tet}	This study
pTCV-P _{Tet} :: <i>shtll</i> ΔHT2	shtll containing a point mutation in the HT2 motif complementation vector, promoter P_{Tet}	This study
pTCV-P _{Tet} ::shtllΔHT3	shtll containing a point mutation in the HT3 motif complementation vector, promoter P_{Tet}	This study
pTCV-P _{Tet} ::shtllΔHT4	shtll containing a point mutation in the HT4 motif complementation vector, promoter P_{Tet}	This study
pTCV-P _{Tet} ::shtllΔH15 pET28a	sntil containing a point mutation in the H15 motif complementation vector, promoter P _{Tet} Vector for expression of His-tagged proteins; Kan ^r	EMD
nET28asht	pFT28a containing the sht gene of S <i>agalactice</i> A909 in the EcoRI/Sall sites	Biosciences
pET28a::sht/l	pET28a containing the shtll gene of S. agalactiae A909 in the Econi/Sail sites	This study
pET28a::pspC	pET28a containing a part of the <i>pspC</i> gene of <i>S. pneumoniae</i> D39 in the EcoRI/Sall sites	This study
pET28a::ccpA	pET28a containing the <i>ccpA</i> gene of <i>S. agalactiae</i> A909 in the EcoRI/Sall sites	This study

TABLE 2 Oligonucleotides used in this study

Primes used for deletion of sht OAH 103 OAH 124 OAH 125 OAH 125 OAH 125 OAH 25 OAH 25 OAH 25 OAH 25 OAH 25 OAH 25 OAH 25 OAH 25 OAH 25 OAH 26 OAH 26 OAH 26 OAH 27 OAH 26 OAH 26 OAH 27 OAH 26 OAH 27 OAH 26 OAH 26 OAH 27 OAH 26 OAH 26 OAH 27 OAH 26 OAH 27 OAH 26 OAH 27 OAH 26 OAH 27 OAH 26 OAH 27 OAH 26 OAH 26 OAH 27 OAH 26 OAH 27 OAH 26 OAH 27 OAH 26 OAH 26 OAH 27 OAH 26 OAH 26 OAH 26 OAH 27 OAH 26 OAH 26 OAH 26 OAH 27 OAH 26 OAH 26 OAH 26 OAH 26 OAH 27 OAH 26 OAH 26 O	Function and primer	Sequence (5′–3′) ^a
OAH 107 TITGTTGGTACCCCATTCACTACTTACTC OAH 108 CAACAACCATGGCCATTACACTACCTTACTC OAH 109 CAACAACCATGGCCTTAACCAAACAAACCATGCCTTACTC OAH 109 CAACAACCATGGCCTTAACCAAACAAAACCTCGCAATAATACTCCTTACTC OAH 123 ATGCTGGGAACACCTGGCAATATACTCGCTTAATACAAAGCAAAACCAAACAAA	Primers used for deletion of sht	
OAH 108 ATAGTERCEATGEACTAATACTECTTACTTC OAH 109 CAACACATGECTTAATACCATATACTECTC OAH 110 ATGTERCEATGEACTAATACACAAAGAGATETC OAH 110 ATGTERCEATGEACTAATACACAAAGAGATETC Primers used for deletion of shu! ACTCTAGAATTCCTGTAATGAAAGGTTCAAAAG OAH 123 CTAGCACCCTGCCTCCTTCATTAATACAAGGTTCAATAAAGAGTTCAATAAAAGAGTTCAATAAAAGAGTTAATAAAAGAGTTGAATAAAAAGGTTAATAA	OAH 107	TTTGTT GGTACC CCATTCACATACCTTAGAAGC
OAH 109 CAACACCATGCCTTACCAACAGAGATCC OAH 109 CAACAACCATGCCTTACCAAGATATGCCTGAAAATCCCAAGAGATCC OAH 123 ATGCTCCCCGGGGAACACCTGCAAGATATGCCCAAGAGATAGCCCAAGAGATAAGACTCAAAAGA OAH 124 CTACGAGGTACCCTCGCGCCCTCTGCTTATATAA OAH 125 CTACGAGGTACCCCCGCGCCCTCTGCTTATATAAAAGAGAGAAA Primers used for deletion of <i>Imb</i> CGCCTGGAGTCCCCCCGCCCCCCCCCCCCTGGAGACAAAA Primers used for deletion of <i>adcAll</i> CGCCTGGAGTCCCCCCCGCCCCCCCGGAGACCCCCCCCCC	OAH 108	ATATGT CCATGG CACTAATAATCTCCTTTACTTC
OAH 110 ATGTCTCCCGGGGGAACACCTGCAGATAATGCCTG Primers used for deletion of shtl ACTCTAGAATTCCTGTAATTGAAGCTTCAAAAG OAH 124 TTTACGGGTACCCTCGCATCTTTATGATA OAH 125 CTAGCAGGGCATAATGCCCTCGCAGTTACTTTATATA OAH 126 CTAGCAGGGCATAATGCAGGGCGTACTGTTCC OAH 25 CTCCTTACTTGAAGAGGCGCTACTGTTCC OAH 26 CTCCTTTACTTGAAGAGGCGTTAATGCAGGGGATAATGCCAGGG OAH 27 CTCCTTTACTTGAAGCGTTGAAGTAAGCGAGAGGAG Primers used for deletion of adcAll AAAATCGAATTCCAACGTGTTAATAAAAGGAAGG OAH 28 CTCATGGGATCCCGTATGTTATATAAACCTCC OAH 29 TAGGTACCGGATGTAAGTAAAGGAAGGAC Primers used for deletion of adcA AAAATCGAATTCCAACGTGTTAATAAACCTCC OAH 20 TTAAGGTACCCATGTTATATAAAGGAAGGAC Primers used for deletion of adcA AATCCTGGTACCCCAGCTCGTAATCTAAACCTCC OAH 28 TTCCTGGGATCCCGTCAGTGTTATATAAACGACG OAH 77 CATCAAGGCTCGATGTTATTATAAAGAAAGGACG OAH 78 CATCCAGGATCCCCCACGTCTGTAGCAGCAGCGCCCCCAGGTGTTGTATAACCTCC OAH 78 TTTTTGCGGGATACCGAGGTCCCTCCAGGGTGTTTTGCAGAAGAAACATATGGT OAH 28 TTTTTGCGGGATACTGGAGGAAAATTCGGATGGCGCCCTAGGGT OAH 28 TTTTTGCGGGATACTGGAGGGCCCCCCAGGGTGAGGTAAGCCCATGGGCGCCCCCCGGGGTACGGTTTTTTAAAGAAGGGCCCCCCCC	OAH 109	CAACAA CCATGG CCTTAACCAAAAGAAGATCTC
Primers used for deletion of shill OAH 123 OAH 123 OAH 123 OAH 123 OAH 125 CTGCACACCCGGCTCCTCGTCATTCTTATATA OAH 125 Primers used for deletion of <i>imb</i> OAH 24 CTCCTGGAATTCCTGGAAGCGCCTACTCGTCC CAAACCCCGGGCTACTCGTCCCCCAATT OAH 25 OAH 25 OAH 26 OAH 27 Primers used for deletion of <i>adcAll</i> OAH 26 OAH 27 Primers used for deletion of <i>adcAll</i> OAH 28 OAH 29 Primers used for deletion of <i>adcAll</i> OAH 29 Primers used for deletion of <i>adcAll</i> OAH 20 CTCCTTGCGGGACCCAACTCGACGCTACTCGCCACTCCCCAATT CACTAGGAAGAAAAAAGCGCCTCACTCGCACTCCCCAATT CACTAGGAAGACTCCAACGCGGGTCAACTCGACACTCCCCCAATT CACTAGGAAGACTCCAACGCGGCTCACTCGCACCTCCCCAATT CACTAGGAACACTCGCAAGTGGCTAATGAAGAAGAAGACGC OAH 29 Primers used for deletion of <i>adcA</i> OAH 80 OAH 72 Primers used for complementation of <i>sht</i> OAH 28 Primers used for fT site directed mutagenesis of <i>sht</i> OAH 28 OAH 29 Primers used for thill tite-directed mutagenesis of <i>shtl</i> OAH 29 OAH 29 OAH 24 OAH 29 TTGCTCGGAAATCCACGCAACTCGAAGTGCACTCAAGGGCACTCAAGGCGCACCCCAAGGCCGCACTCGCGAAGTGCGCCAAGGCGCACACCCCAAGGCGCCGCACTGCGAAGTGCGCCAAGGCGCACATTGCCCCAAGGCGCCCATAGGCGCGCGACTGCGCAAGTGGCGCAAGGCGCCATAGGCG OAH 29 Primers used for complementation of <i>sht</i> OAH 28 TTGCTCGGAAATCAAAGGCGGTACTGCGAAGTGCGCCAAGTGGCGCCAATTGCCC OAH 29 OAH 29 TTGCTCGGAAATCAAAGTTGCAAGTGCGCAAGTGGCGCAATTGCCCAAGGCGCGATTGCGCGAAGTGGGCGCAATTGCCCCAAGGCGCGATTGCGCGAATTGCCCCAAGGCGGCGAATTGCCCCAAGGCGGCGAATTGCCCCAAGGCGGGAAAAATTGCCCCAAGGCGGAATTGCCCCAAGGCGGAATTGCCCCAAGGCGGATTGCGCGGAAGGCGCGAATTGCCCCAAGGCGGATTGCGCGGAAGGCGCGAATTGCCCCAAGGCGGATTGGCGCGGAATGGCGCGAATTGCCCCAAGGCGGATTGGCGCGGAACGCGCGAATGGCCAATTGCCCCAAGGCGGATTGGCGCGGAACGCGCGAATGGCGCGAATGGCGCTTTGCGGGAAAAGTTGCCCCAAGGCGGATTGGCCGGAAGGCGCGAATGGCCAATTGCCCCAAGGCGGATTGGCCGGAAGGCGCGAATGGCCAATTGCCCAAAGCTTGCGCGAATTGCCCAAAGCGCGAATTGCCCCAAAGCTTGCCCAAAGCTTACATGGCCGAATGGCCAATTGCCCCAAAGCTTGCCCAAAGCTTACATGGCG OAH 29 TTGCTCGGGGAACAAAGCTTGCCCAAAGCTTACATGGAAGGCGCGAATGGCCAATTGCCCCAAAGCTTGCCCAAAGCTTGCCCAAAGCTTGCCCAAAGCTTGCCCCAAAGCTTGCCCCAAAGCTTGCCCCAAAGCTTGCCCCAAAGCTTGCCCCAAAGCTTGCCCCAAAGCTTGCCCCAAAGCTTGCCCCAAAGCTTGCCCCAAAGCTTGCCCCAAAGC	OAH 110	ATGTCT CCCGGG GGAACACCTGCAGATAATGCCTG
In other 123 to detector of Julia ACTCHAGANTECTGTAATGAAGCTCAAAAG OAH 123 CONCENTRATION OF A CONCENTRATIO	Primars used for deletion of shill	
OH 123 ALCOMANI CONTRACTOR CONTRACT AND		
DH 12* ITACGRITACCCLOSCUTTATICLATIANA DH 125 CTACCAGRITACCCLOSCUTTATICLATIANA Primers used for deletion of <i>imb</i> AGGCTGGAATTCTGGAAGGGGGCTACTGTTCC DH 24 AGGCTGGAATTCTGGAAGGGGGCTACTGTTCC DH 25 CTACTAGAAWAAGGGTTGAAGTAAGGAAGAA Primers used for deletion of <i>adcAll</i> CTCCTTTATTCAAGGACCCCACTTGGAGAC DH 25 TACTATGAAWAAGGGTTGAAGTAAGGAAGGAGATTATTAGTGAAG OAH 20 TAAGGTACCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAG		
OH 125 CLINGLAUGURCLOANTIANTANAGASTICAGATA Primers used for deletion of <i>lmb</i> AGGCTGGAATTCTGGAAGGCGCCTGTTCC OH 24 AGGCTGGAATTCTGGAAGGCGCCTGTTCC OH 25 CTCCTTTACTCTGAAGGCGCTACTGTGCAAGGCGCAATTAGGTGAAG Primers used for deletion of <i>adcAll</i> AAATCGAATTCCAACGTAGTTACCTGGAAGCAGATTAGGTGAAG OAH 25 CTCCTTTACTCCAACGTAGTTAGCTGGAAGCAGATTATAGTGAAG Primers used for deletion of <i>adcAll</i> AAAATCGAATTCCAACGTAGTTAAACCTCC OAH 62 TTAGGTACCCAGCGTACCTATGCAAGCAGAC Primers used for deletion of <i>adcA</i> AATCGTGGTCCCGCAGTCTTAGCAAGCAGACC OAH 63 TTAGGTACCCGAGCTCTAGCAGACCACAC OAH 76 ATAAAGGTTGAAAATTCTTTCTCCCATTGCTAGCAAGCACC OAH 77 CATCCAAGGTCCCGTCCGTTAGCTAGCAGAAACATATGGT OAH 78 CATCCAAGGTCCCGTCCGTCGTTTGCTAAGCAAGAAACATAGGT OAH 78 CATCCAAGGTCCCGTCCGTCGGAAGAAAACATAGGT OAH 284 TTTTTGGAACATTGGAGAAAACATGGGCAACATGGG OAH 284 TTGTCTGCGAAATTAAAAATTGAAAGGGTCAAAAGGG OAH 284 TGGTCTCGGGAAATTAAAAAAAGGGTCCAAAGGC OAH 284 TGGTCTCGGGAAATTAAAAAAAAGGGTCCAAAGGC OAH 284 TGGTCTCGGTGAAGAAAAAATTGAAGGCGCGCGCGCTGCGAAGGAAAGATAACATGAAGGC OAH 284 TGGTCTCGGAAGAAAAAAAAGGGTCCTAAGGCTCAAAGGC OAH 284 TGGTCTCGGAAGAAAAAAAAGGGTGCGTAAAAGGC OAH 284 TGGTCTCGGAAGAAAAAAAAAGGCGGAAAGGAATGAACACTGAAGGGAAAACAATGAATTGAGGG		
Primers used for deletion of <i>lmb</i> Primers used for deletion of <i>lmb</i> Primers used for deletion of <i>lmb</i> Primers used for deletion of <i>adcAll</i> CriceTTTACTTCAACCCTTTTTCCAACCCTCTCTCATT ACTATGAAAAAAAGGGGTGAAGGAAGGAAGGAAGGAGGGGAAGGAGG	OAH 125 OAH 126	
Primers used for deletion of <i>lmb</i> OAH 24 AGGCTGGAATTCTGGAAGGCGCTACTGTTCC OAH 25 CTCCTTTACTTCAACCCCTTTTTCATAGGCACTGTGTCC OAH 25 TACTAGGAAAAAAGGGTGTAAGGAAGGAGGAGGAGGAGGGACGGAAGGGGAAGGGGGAAGGGGAAGGGGATAAAAAGGGGTGGGAAGGGGATAGGAAGGGGATAAGGGGATAACGGAAGGGGATAGGGAGGG		
OAH 24 AGGCTOGAATTCTGGAAGGCGCTACTGTCC OAH 25 CTCCTTACTACAACCCTTTTCCAAGTCACTCCAATT OAH 26 TACTATGAAAAAAGGGTGTAAGTCAACGCAATTAGTGAAG OAH 27 GGCTTGGGATCCAAGTCAAGCTAAGTCAAGTCAAGTCAA	Primers used for deletion of Imb	
OAH 25 CTCCTTTACTTCAACCCTTTTTCATAGTACTCCCCAATT OAH 25 TACTATGAAAAAGGGTGTGAAGTAAAAGGGATTATTATGGAAG OAH 27 GGCTTGGGATCCAGCTGAGCTAAAGGGAGCAATTAGTGAAG Primers used for deletion of adcAll AAAATCGAATTCCAACGTGTTAATCAAGGAGATTATTATGAGAGG OAH 62 TAAGGTACCTCGGTATCTAAGGAAGAGGG OAH 63 TAAGGTACCTCGGTACTCTAGGTAGATTATATAAAGAAGGAGG OAH 63 TAAGGTACCTCGGTACTCAGGTAGTTAATAAAAGGGG OAH 84 TACTAGGTAGCTGGTACTCACGCAGCCTCAGCAGCACC OAH 86 AATCGTGGTACCGCAGCTCTAGGAGATCCACAC OAH 87 CATCAAGGATCCCTGGTACTCCCCAGCGATCCACAC OAH 87 CATCAAGGATCCCTTAGCAGATTCCCCCAGC OAH 87 CATCAGGGTACCCCCGCAGCTCTAGGAGATCCACAC OAH 86 AATCGTGGAGGTACTCGCAAGCTCTAGCAGATTCCCCACAC OAH 87 CATCAGGGTACTCCCGAGCTCTAGGAGATACCAATGGC OAH 88 TITTTGGAGCGGGGGTACTCGTGACGCCGAGGTACTCACACC OAH 284 TITTTGGAGCTGCGAGATATAAAAGGTCCCCAATAGGGT OAH 284 TITCTGCGAGATATAAAAGGTTCCCAAAGGGTCCCCTATGGGGCTCCCTATGGAGGTCCCCTATGGAGGTCCCCTATGGAGGTCCCCTATGGAGGTCCCCTATGGAGGTCCCCTATGGAGGTCCCCTATGGAGGTCCCCTATGGAGGTCCCCTATGGAGGTCCCCTATGGAGGTCCCCTATGGAGGTCCCCCAAAGGAGATTGCCCCCGAATGCGAATTACCAAAGGGCTGCCCCAAGGGATTGCCCCCCGAAGGGATTGCCCCCCGAAGGGATTGCCCCCGAAGGGATTGCCCCCGAAGGGATTGCCCCCCGAAGGGATTGCCCCCCGAAGGGATTGCCCCCCGAAGGGATTGCCCCCCCGAAGGGATTGCCCCCAAGGGAGTGCCCCCCAAAGGGATTGCCCCCCCTATAGGGATGCCCCCCCGAAGGGATTGCCCC	OAH 24	AGGCTG GAATTC TGGAAGGCGCTACTGTTCC
OAH 26 OAH 27 OAH 27 GGCTTGGATCCAGCTGGATCAAGCTAGAGCAGATATTAGTGAAG GGCTTGGATCCAGCTGGTAAGTAGAGGAGGAGATTATTAGTGAAG GGATCGCGATCGCGATCGCTGGTAAGTAGCAAGTG OAH 40 OAH 40 OAH 42 OAH 43 OAH 43 OAH 43 OAH 43 OAH 43 OAH 43 OAH 44 OAH 43 OAH 44 OAH 43 OAH 44 OAH 43 OAH 44 OAH 43 OAH 44 OAH 43 OAH 44 OAH 44 OAH 44 OAH 44 OAH 45 OAH 45 OAH 45 OAH 46 OAH 46 OAH 47 OAH 46 OAH 46 OAH 46 OAH 47 OAH 48 OAH 46 OAH 47 OAH 48 OAH 47 OAH 48 OAH 28 OAH 28 O	OAH 25	CTCCTTTACTTCAACCCTTTTTTCATAGTACCTCCTCAATT
OAH 27 GGCTTGGGATCCAGCTAGCTCACTTGGAGAC Primers used for deletion of adcAll AAAATCGGATTCCAACGTGTTAATCAAAGGAAGTG OAH 40 TAAGGTACCTCCGTATCCTTTTCATTAAAAGAAAGGAC OAH 43 TACTAAGGATCCATCCCGGTATCCTTTCATTAAAAGAAAG	OAH 26	TACTATGAAAAAAGGGTTGAAGTAAAGGAGATTATTAGTGAAG
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OAH 63 TTAGGATACTTAGGTAGTTATATAAAAAGACGC OAH 63 TACTAAGGATCCATCTCCCAAGGATCTATATAAAAAGACGC OAH 83 TACTAAGGATCCATCTCCCCAAGGATCCACCA OAH 86 OATCGTGGTACCGACGCCAAGATCCACCAC OAH 87 ATGCTGGTACCGCGCAGCTCTACCAAGATCCACCAC OAH 87 ATGCAAGCTTCCAAGGATCCCACGGCATTATATATTATAAAAGATGATATCGG OAH 76 ATGAAGCTTCCAAGGATCCCGTCCAATGACTAGAAAACATATGGT OAH 284 TTITTGGCAGAAAAACTTTGGCAAGAAAACATATGGT Primers used for complementation of sht TTICTGCAAAAAAACTTTGGCAGGATACTGTGAAGAAAACATATGGT OAH 284 TTITGTGCAAAAAAACTTGGCCCAAAGGGAAAGTTCCCTAAGGGGTACGTGGAGGAAGTTCCTTATGAGCG OAH 284 TTTGTCGCGGAAAATAAAAAGTCACCGAATGAGGTACATAGCC OAH 284 TGGTCTCGGAAAATAAAAAGTCACCGAATGAGGTCCCATAGCC OAH 284 TGGTCTCGGGGAAAATAAAAAGTGCCAATAGCC OAH 284 TGGTCTCGGCGGAAAATAAAAAATTGCCAAAGGG OAH 284 TGGTCTCGGCGGAAAATTGAAAAGTGCCCAATAGCC OAH 284 TGGTCTCGGCGGAAAATTAAAAATTGCCAAAGGG OAH 284 TGGTCTCGGGGAAAATTGAAAAGTTGCCAAAGGG OAH 284 TGGTCTCGGGGAAAATTGCCAAAGGGGAAAGTTGCCAAAGGG OAH 284 TGGTCTCGGGGAAAATTGCCAAAGGGGAAAGATTGCCAAAGGG OAH 285 TGGTCTCGGGGAAAATTGAAAAACCCAAATGCCCAATGGGG OAH 284 <td< td=""><td></td><td>ΤΔΔGGTΔCCTCCGTΔTCCTTTTCΔTTΔΔΔCCTCC</td></td<>		ΤΔΔ GGTΔCC TCCGTΔTCCTTTTCΔTTΔΔΔCCTCC
OAH 33 TAGGIACCIAGIAGIAGIAGIAGIAGIAGIAGIAGIAGIAGIAGIAGI		
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OAH 86 AATCGTGGATACCGAGCTCAGCAGATCCACAC OAH 76 ATAAAGCTTGAATTCTTGCCATTTTTTCTTCCC OAH 77 ATGAAGCTTGATAATATTTAAAAGATGATATCGG OAH 87 CATCCAGGATCCCGTCCAGTTGTTTTCTTAGATAC Primers used for complementation of sht TITTTGGAGAGAAAACTTTGAGAGAAAACATATGGT OAH 284 TITTTGGCAGAAAAACTTTGAGCAGAAAACATATGGT Primers used for HT site-directed mutagenesis of sht GGTCTCGAAAAATAAAAGTCACCGAAATGAGGCCACATAGCC OAH235 HT1 TGGTCTCGGAAAATAAAAGTCACCGAAATGAGGCCACATAGCC OAH236 HT1 TGGTCTCGTTTTTTTACAATGGGAAAATCAAAGGC OAH235 HT2 TGGTCTCGGGAATATAAAAATAAAAGTCCCCAAAAGG OAH236 HT3 TGGTCTCGGCAAAATAAAAAAAAAGACTCTCCAAAAGG OAH254 HT3 TGGTCTCGTCGCAAAATAAAAAAAAAAAAGCCTTGCAAAAGG OAH254 HT3 TGGTCTCGTCAGAAAATAAAAAAAAAACATCACAAAGG OAH254 HT3 TGGTCTCGTCAGAAAATAAAAAAATCACAAAAGG OAH254 HT3 TGGTCTCGTCAGAAAATAAAAAAATCACAAAAGGC OAH254 HT3 TGGTCTCGTCAGAAAAAAAAAAAAATCACAAAAGGC OAH254 HT3 TGGTCTCGTCAGAAAAAAAAAAAAAAAAAAAAAGCGGT OAH254 HT4 TGGTCTCGTCATATAAAAATAAAAAATCACAAAATGGGATAAC OAH254 HT3 TGGTCTCGCAAAAAAAATCACAAAAATGGAACAGTT OAH254 HT3 TGGTCTCGTCATATAGAAAATCACAAAAATGGGATAAC OAH254 HT4 TGGTCTCGTCATATAAAAATTGAAAAAAAACAGGTT OAH254 HT5 TGGTCTCGCAAAAGTAAAAATGCACAAAAAATGCCAAAGGTAAAAAAATGCCCAAAAGTAAA	Primers used for deletion of <i>adcA</i>	
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OAH 87 CATCCAGGATCCCGTCCAGTTGTTTCTTAGATAC Primers used for complementation of sht TTTTTAGATCTGGAAGAAAACATTAGGT OAH 248 TTTTTGCAGAAAAACTTGGAGCAATTGGCAAAGATTATGGT OAH 284 TTTGTGCAGAAAAACTTGGACAATTGGCAAAGTTAGGGTCACATAGGGT OAH235 HT1 TGGTCTCGAAAAAAACTTGGGAAAGTCACCAAAGTTTTTTAAGGGT OAH236 HT1 TGGTCTCGAAAATAAAAGTCACCGAATGAGGTCACATAGCC OAH236 HT2 TGGTCTCGGAAATAAAAAGTGCACCAAAGGG OAH251 HT2 TGGTCTCGGGAATATAAAAATGAAATTACCAAAAGG OAH254 HT3 TGGTCTCGGGAATATAAAAATAAAAATCCCCAAAGGG OAH255 HT3 TGGTCTCGTCGCAAAAAAAATTGCCAAAGGC OAH262 HT5 TGGTCTCGTCAAAAAAAACTTTGCAGAAAAAATCCACGAAAGACCATTAAAAAGCC OAH263 HT5 TGGTCTCGCGAAATAAAAAACTTGGACACCTGAAC OAH263 HT5 TGGTCTCGCGAAAAAAATTGGAAAAAATCCATGAACAAGTTACCATAAC OAH263 HT5 TGGTCTCGCGAAACAAAAATTGCCAAAAAACTTTAAGGAATAAAAACCATTAAAAAACAGTT OAH264 HT3 TTTTTGGAAAGAAAAATTGCCAAAAAACTTTGAGCAATTAAAAAAACCAGTT OAH264 HT3 TTGGTCTCGGAAGTAAAAAAATTGCCAAAAAGTTTCCTCAAAAGAAGTTACCTAAAAAAACTTTGCCAAAAGATTTTTTAATTAA	OAH 77	ATG AAGCTT CATTAATATTTAAAAGATGATATCGG
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OAH236 HT1 TGGTCTCGTTTTTTTTACAATGGGAAAGTTCCTTATGATGCG OAH250 HT2 TGGTCTCGGGAATATAAAAAATTAGAAAATTACCAAAAGG OAH251 HT2 TGGTCTCGTCCGTAAAAAGGATTGTCTCCAAGTG OAH254 HT3 TGGTCTCGTCCTAAAAAGGATTGCTCCCAAGAGGC OAH255 HT3 TGGTCTCGTCCCAAGAAGTCAGTTATCACCTC OAH259 HT4 TGGTCTCGTCGTAGAAGAATTGCCGAATTGAAC OAH262 HT5 TGGTCTCGCCGGAACGACGACAAGATCAATAAAATGGAAC OAH263 HT5 TGGTCTCGTCCGTCCTATTAGACGTGTGACGCGCGCGATCAG Primers used for complementation of <i>shtll</i> TGGTCTCGCGGAGGTACTATGAATCGTAAAAAAAACAGTT OAH241 TTTTGGATCCGGAGGTACTATGAACGAGAGTTACAATAAAAAAACAGTT OAH241 TTGGTCTCGAAAAGTTACCAAAGAAGATGCCCAAAGGTACTATGAACGAGAGTTACATAACC OAH2421 TGGTCTCGGAAAGTAAAAGGACTTCCCAAAAGAAGTTACATAACC OAH237 HT1 TGGTCTCGGAAAGTAAAAGATTGCCCAAAAGAAGTTACATAACC OAH238 HT1 TGGTCTCGGAAAGGTAAAAGAAGTGCCATATGAAGCC OAH238 HT1 TGGTCTCGGAAAGTAAAAGAAGTGCCAAAGGAGGCAATAGAGCC OAH238 HT1 TGGTCTCGGAAAGTAAAAGAAGTGCCAAAGGGAAGTGCC OAH238 HT1 TGGTCTCGGAAAGGAAAAAAAAAAAAAAAAACCC OAH238 HT1 TGGTCTCGGAGAGAAAAAAAAAAAAAAAAAACCC OAH238 HT1 TGGTCTCGGAGAGAAAAAAAAAAAAAAAAAAACCC OAH238 HT1 TGGTCTCGGAAGCAAAAGGAGAAAAAAAAAAAAACCC	OAH235 HT1	TGGTCTCGA <u>AA</u> ATAA <u>AA</u> GTCACCG <u>AA</u> TGAGGTCACATAGCC
OAH250 HT2TGGTCTCGGAATATAAAAATAGAAATTACCAAAAGGOAH251 HT2TGGTCTCGTCCTAAAAAGGATTTGTCCCAAGAGGOAH254 HT3TGGTCTCGTCCTAAAAAGGATTTGTCCCAAAGGCOAH255 HT3TGGTCTCGTCCCAAAAATTAACCTCOAH258 HT4TGGTCTCGTCCCAAGAAGTCAGTTATCACCTCOAH259 HT4TGGTCTCGTCGTAGAAGAACTCAACAACOAH262 HT5TGGTCTCGCGGAACGACAAAGGATAAAAATCAATAAATGGAATAACOAH263 HT5TGGTCTCGTCCGTCGTATATAGGATTAGGAACTTGAACOAH263 HT5TGGTCTCGCGGAACGACAAAGGATCAAAAATCAATAAATGGGATAACOAH263 HT5TGGTCTCGCGGAGGTACTATGAAACAGGATOAH241TTTTTGGATCCGGAGGTACTATGAATCGTAAAAAAACAGTTOAH237 HT1TGGTCTCGCAAAAGGTAAAAGGAAGTTACATAAACOAH238 HT1TGGTCTCGGGAAAGTAAAAATCGCCAAAAGAGTCCCOAH238 HT1TGGTCTCGGGAATATAAAAGAAAGAGGCCCOAH252 HT2TGGTCTCGGGAATATAAAAGAAAAATGCGCCAAATGGOAH252 HT3TGGTCTCGGAACGAAAAAAATTACCCGAAAGGAGGTCCCAAATGGOAH253 HT2TGGTCTCGGAAAGAAAAAAATTACCCGAAAGGAGGACCCOAH257 HT3TGGTCTCGGAACAAAAAATTAACCGAAACGGAATAGAAAACOAH256 HT3TGGTCTCGGAACAAAAAATTACCGAAAGAAAAACOAH260 HT4TGGTCTCGGGTACCCAAAAGGAATCAAAAAACOAH260 HT4TGGTCTCGGCGAACCCAAAAGCAATCAAAAGGAATGGCAATGGAATGG	OAH236 HT1	TGGTCTCG TTTTTTTACAATGGGAAAGTTCCTTATGATGCG
OAH251 HT2TGGTCTCGTTCCTAAAAAGGATTTGTCTCCAAGTGOAH254 HT3TGGTCTCGGGGATAATAAAAATAAAAAATCTCCAAAAGGCOAH255 HT3TGGTCTCGTCGTCCCAAGAAGTCAGTTATCACCTCOAH258 HT4TGGTCTCGTCGTCGTAAAAAAATCTCCCGAATTTACCTGTAOAH259 HT4TGGTCTCGTCGTCATATAGGAATTTGGACGAACTTGAACOAH262 HT5TGGTCTCGCGGAACGACAAAGATAAAATCAATAAAATGGGAATAACOAH263 HT5TGGTCTCGCGGAACGACCAAAGATAAAATCGAAACAGTTPrimers used for complementation of shtllTTTTTGGATCCCGGAGGTACTATGAATCGTAAAAAAACAGTTOAH 249TTTTTGGATCCGGAGGTACTATGAACGGAAGTTACATAAAAACAGTTOAH 241TTTGCATGCAAAAAACTTTGAGCAATTGCTCAAAGTTTTTTTATTCACTPrimers used for HT site-directed mutagenesis of shtllTGGTCTCGAAAGGTAAAAAGTCTCCCAAAAGAAGTTACATAAACCOAH237 HT1TGGTCTCGAAAGGTAATAAAAGGAAGTTACATAACCOAH238 HT1TGGTCTCGGAATATGAAAAGAAGTGCCCATATGATGCCOAH252 HT2TGGTCTCGGGATATAAAAAGAAAAATCGCCAAATGGOAH254 HT3TGGTCTCGAAGAAAAAATTACCCAAATGGGAATAGGCOAH256 HT3TGGTCTCGAAGAAAAAATTACCCGAACGGAATAGGAAAGCOAH256 HT3TGGTCTCGAGAAAAAAATTACCCGAACGGAATAGAAACOAH260 HT4TGGTCTCGGTACCCAAAACTCAAAAGGAAATGCCCAAATGGAATGGCAATGGCAATAGAAACC	OAH250 HT2	TGGTCTCG GGAATATAA <u>AA</u> ATAG <u>AA</u> ATTACCA <u>AA</u> AGG
OAH254 HT3TGGTCTCGGGGATAATAAAAATCACAAAAGCOAH255 HT3TGGTCTCGTCCCAAGAAGCACAGTTATCACCTCOAH258 HT4TGGTCTCGTCCCAAGAAGCAAAAATCTCCGAATTTAGCTGTAOAH259 HT4TGGTCTCGTCGAAGAAAAATCCCGAAGCACACOAH262 HT5TGGTCTCGCGGAACGACCAACGACGACGACGACGACGACGACGACG	OAH251 HT2	TGGTCTCGTTCCTAAAAAGGATTTGTCTCCAAGTG
OAH255 HT3TGGTCTCGTCCCAAGAAGTCAGTTATCACCTCOAH258 HT4TGGTCTCGTCGTAGAAGAAAAATCTCCCGAATTTAGCTGTAOAH259 HT4TGGTCTCGTCTATATAGGAATTGGAGAACTTGAACOAH262 HT5TGGTCTCGCGGAACGACAAAGATAAAATCAATAAAATGGGATAACOAH263 HT5TGGTCTCGCCGTATTCATGGTTGACGCGCGATCAGPrimers used for complementation of shtllTTTTTGGATCCGGAGGTACTATGAATCGTAAAAAAACAGTTOAH 249TTTTTGGATCCGGAGGTACTATGAATCGTAAAAAAAACAGTTOAH 241TTTGCCATGCAAAAACTTTGAGCAAATTGCTCAAAGTTTTTTTT	OAH254 HT3	TGGTCTCG GGGATAATA <u>AA</u> ATAA <u>AA</u> ATCTCCA <u>AA</u> AGGC
OAH258 HT4TGGTCTCGTAGAAGAAAAAATTCTCCGAATTTAGCTGTAOAH259 HT4TGGTCTCGTCATATAGGAATTAGGAACTTGAACOAH262 HT5TGGTCTCGCGGAACGACAAAGATAAAATCAATAAATGGGATAACOAH263 HT5TGGTCTCGCGGAACGACCAAAGATAAAATCAATAAATGGGATAACPrimers used for complementation of sht/lTTTTTGGATCCGGAGGTACTATGAATCGTAAAAAAACAGTTOAH 249TTTTTGGATCCGGAGGTACTATGAATCGTAAAAAAAACAGTTOAH 241TTTGCATGCAAAAAACTTTGAGCAATTTGCTCAAAGTTTTTTTATTTCACTPrimers used for HT site-directed mutagenesis of sht/lTGGTCTCGAAAGGTAAAAAGGTTACATAACCOAH237 HT1TGGTCTCGAAAGTAAAAGGTCCCCAAAAGGTTACATAACCOAH238 HT1TGGTCTCGGATATTAAAAGGAAAGAGGCCATATGATGCCOAH252 HT2TGGTCTCGGGATATAAAAAGACGCCAATGGGOAH253 HT2TGGTCTCGAAGAGCGAAATAAAATCCCCCAAAGGCGAATTACCCCAAAGCGAAATGGAACGOAH256 HT3TGGTCTCGAAGAAAAAAATCACCCGAACGGAACGGAATAGAAAACOAH250 HT4TGGTCTCGAGAGAAAAAAATTACCCAAACGAACGCGAATAGGAAAGGCCAATAGAAACAACGCCAAAGGCGAATAGGAAAGCCCAAAGGCCAATAGGAAAGAACCCCCAAAGCCGAATAGGAAACGCCAATAGGAAACGCCAATAGGAAACGCCAAAGGCCGAATAGAAACAACCCCCAAAACGCCAATAGCAAACAACCCCCAAAACGCCAATAGCCAAAGCCCAAAGCCCAAAGCCCAATGGAATAGGAAACGCCAATAGGAAACGCCAATAGGAAACGCCAATAGGAAACGCCAATAGCAAACGCCAATAGGAAACGCCAATAGGAACGCAATAGGAAACGCCAATAGGAAACGCCAATAGGAAACGCCAATAGGAAACGCCAATAGGAACGCAATAGGAAACGCCAATGGAATAGGCCCAAGCCCAAAAGCCCAATGGAACGCCAATAGGAACGCCAATAGGAACGCCAATAGGAACGCCAATGGAATAGGCCCCCAAAACGCCAATGCCCAAAGCCCCAAAACGCCAATGCCCCCAAACGCCCAAAAGCCCCAAAAGCCCCAAAAGGAACGCAATAGGAATAGGCCCCCAAACGCCCAATGCCCCAAACGCCAATGGAATGGCCCCCAAACGCCCAAAAGGTCCCCCAAAAGGAACGCCAATAGGAATGGCCCCAAAGGCCCCAAAAGGTCCCCAAAAGGTAATGGCCCCCAAAAGGAACGCCAATGGAATGGCCCCCAAAAGGCCCCAAAAGGAACGCCAATGGAATGGCAATGGCCCCCAAAAGGCCCCAAAAGGTCCCCAAAAGGAACGCCAATGCAATGGCAATGGCAATGCCCCAAAAGGAACGCCCAAAAGGTCCCCAAAAGGAATGCCCCCAAAAGGTCCCCAAAAGGAACGCCAATGGAATGGCAA	OAH255 HT3	TGGTCTCGTCCCAAGAAGTCAGTTATCACCTC
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OAH262 HT5 TGGTCTCGCGGAACGACAAAGATAAAATCAATAAATGGGATAAC OAH263 HT5 TGGTCTCGCCGTATTCATGGTTGACGCGCGATCAG Primers used for complementation of sht// TTTTTGGATCCGGAGGTACTATGAATCGTAAAAAAACAGTT OAH 249 TTTTTGGATCCGGAGGTACTATGAATCGTAAAAAAAACAGTT OAH 241 TTTGCATGCAAAAAACTTTGAGCAATTTGCTCAAAGTTTTTTTATTTCACT Primers used for HT site-directed mutagenesis of sht// TGGTCTCGAAAAGTTACATAAGAAGTTACATAACC OAH237 HT1 TGGTCTCGGATATGAAAAGAGTCCCAAAAGGAAGTTACATAACC OAH238 HT1 TGGTCTCGGGATATAAAAAGAAAAAAGGCCATATGATGCC OAH252 HT2 TGGTCTCGGGATATAAAAAAAAAAAAACGCCAAATGGG OAH253 HT2 TGGTCTCGGAAAAAAAAAAAAAAAAAAAACCGAAAATGGCCAAAATGGC OAH257 HT3 TGGTCTCGAGAAAAAAAAATCCCGAAAGGAAAAAACC OAH257 HT3 TGGTCTCGGGTACCCAAAACTCAAAATGTCCCC OAH260 HT4 TGGTCTCGGGATACCCCAAAACTCAAAATGTCACCAAATGGCAATGGAATGG	OAH259 HT4	TGGTCTCGTCTATATAGGATTTGGAGAACTTGAAC
OAH263 HT5 TGTCTCGCGTATTCATGGTTGACGCGCGATCAG Primers used for complementation of shtll TTTTTGGATCCGGAGGTACTATGAATCGTAAAAAAACAGTT OAH 249 TTTTGCATGCAAAAAACTTTGAGCACGTAAAAAAAACAGTT OAH 241 TTTGCATGCAAAAAACTTTGAGCAAATTGCTCAAAGTTTTTTTATTTCACT TCTGCTAGTGTTTTAATATC TCGTCTCGAAAGGTAAAAAAGAAGTTACATAACC Primers used for HT site-directed mutagenesis of shtll TGGTCTCGAAAGGTAAAAAGGAAGTTACATAACC OAH237 HT1 TGGTCTCGAAAGGTAAAAAGGAAGTTACATAACC OAH238 HT1 TGGTCTCGGATATAAAAGGAAAGTGCCATATGATGCC OAH252 HT2 TGGTCTCGGGATATAAAAGAAAAAAAGCGCAAATGG OAH253 HT2 TGGTCTCGAAAAGCTGAAAAAAAAATGCCCAAATGG OAH256 HT3 TGGTCTCGAGAAGAAAAAAATTACCGAACGGAATAGAAAC OAH257 HT3 TGGTCTCGGGTACCCAAAACTAAAGGAAATGGCAATGGCAATGGCAATGGAAAGC OAH260 HT4 TGGTCTCGGGTACCCAAAAAGTCAAATGGCAATGGAATG	OAH262 HT5	
Primers used for complementation of <i>shtll</i> OAH 249 OAH 241 TTTG GATCC GGAGGTACTATGAATCGTAAAAAAACAGTT TTTG CATGC AAAAACTTTGAGCAATTGCTCAAAGTTTTTTATTTCACT TCTGCTAGTGTTTAATATC Primers used for HT site-directed mutagenesis of <i>shtll</i> OAH237 HT1 OAH237 HT1 OAH238 HT1 OAH238 HT1 OAH252 HT2 OAH253 HT2 OAH254 HT3 OAH257 HT3 OAH257 HT3 OAH256 HT3 OAH257 HT3 OAH250 HT4 TGGTCTCGAAAACTCAAAAGTAAAAAATGGCAATGGAATG	OAH263 HT5	TGGTCTCGTCCGTATTCATGGTTGACGCGCGATCAG
OAH 249 TTTTTGGATCCGGAGGTACTATGAATCGTAAAAAAACAGTT OAH 241 TTTGCATGCAAAAACTTTGAGCAATTGCTCAAAGTTTTTTATTTCACT TCTGCTAGTGTTTTAATATC TCTGCTAGTGTTTTAATATC Primers used for HT site-directed mutagenesis of sht/l GAH237 HT1 OAH237 HT1 TGGTCTCGAAAGTACAAAGGTCCCCAAAAGAAGTTACATAAACC OAH238 HT1 TGGTCTCGCTTTTATTACAATGGGAAAGTGCCATATGATGCC OAH252 HT2 TGGTCTCGGATATAAAAGAAAAAATCGCCAAATGG OAH253 HT2 TGGTCTCGAAAGCTGATTATACCTCA OAH256 HT3 TGGTCTCGAGAAAAATTACCGAACGGAATAGAAAC OAH257 HT3 TGGTCTCGGGTACCCAAACTCAAATGTCCCC OAH260 HT4 TGGTCTCGGGTACCCAAACTCAAATGTCATAAATGGAATG	Drimory used for complementation of chtll	
OAH 241 TTTGCATGCAAAAACTTTGAGCAATTGCTCAAAGATTTTTTTATTTCACT Primers used for HT site-directed mutagenesis of sht/l TGGTCTCGAAAGTAAAAAGGTCTCCAAAGAAGTTACATAACC OAH237 HT1 TGGTCTCGAAAGTAAAAAGGTCTCCCAAAGAAGTTACATAACC OAH238 HT1 TGGTCTCGCTTTTATTACAATGGGAAAGTGCCATATGATGCC OAH252 HT2 TGGTCTCGGATAAAAAAAACGCCCAAATGG OAH256 HT3 TGGTCTCGACGAAAGTACAAAAAATCGCCAAATGGAAAC OAH257 HT3 TGGTCTCGGGTACCCAAAAGTACCAAATGTCCCC OAH260 HT4 TGGTCTCGGGTACCCAAAACTCAAAATGCAAAAGAAAC	$\cap \Delta H$ 249	ΤΤΤΤΤΤ GGATCC GGAGGTACTATGAATCGTAAAAAAAACAGTT
Primers used for HT site-directed mutagenesis of shtll TCTGCTAGTGTTTTAATATC OAH237 HT1 TGGTCTCGAAAGTAAAAGTCTCCAAAAGAAGTTACATAACC OAH238 HT1 TGGTCTCGGGATATAAAAGAAGTGCCATATGATGCC OAH252 HT2 TGGTCTCGGGATATAAAAGAAAAAATCGCCAAATGG OAH253 HT2 TGGTCTCGAGAAGAAGAAAAAAATTACCCAAACGAAAGTGAAAG OAH256 HT3 TGGTCTCGAGAAAGAAAAAAATTACCGAACGGAATAGAAAC OAH257 HT3 TGGTCTCGGGATATAAAAGAAAAAATTGTCTCC OAH260 HT4 TGGTCTCGGGTACCCAAAACTCAAATGTCATAAATGGAATG	OAH 249 OAH 241	TTT GCATGC AAAAACTTTGAGCAATTTGCTCAAAGTTTTTTTTTT
Primers used for HT site-directed mutagenesis of sht/l TGGTCTCGAAAGTAAAAGTCTCCCAAAAGAAGTTACATAACC OAH237 HT1 TGGTCTCGAAAGTAAAAGACTCCCAAAAGAAGTACATAACC OAH238 HT1 TGGTCTCGCTTTTATTACAATGGGAAAGTGCCATATGATGCC OAH252 HT2 TGGTCTCGGGATATAAAAGAAAATCGCCAAATGG OAH253 HT2 TGGTCTCGAGAAAGTGCCAATGAACG OAH256 HT3 TGGTCTCGAGAAAGTACCGAACGGAATAGAAAC OAH257 HT3 TGGTCTCGGGTACCCAAAACTTACCGAACGGAATAGAAAC OAH260 HT4 TGGTCTCGGGTACCCAAAACTCAAATGCCAAATGGAATG		TCTGCTAGTGTTTTAATATC
Finite's deel for H1 site-directed mutagenesis of situit OAH237 HT1 TGGTCTCGAAAGTAAAAGTCTCCAAAAGAAGTTACATAACC OAH238 HT1 TGGTCTCGCTTTTATTACAATGGGAAAGTGCCATATGATGCC OAH252 HT2 TGGTCTCGGGATATAAAAGAAAAATCGCCAAATGG OAH253 HT2 TGGTCTCGAGAAAGTACCAAATGGCAAATGACC OAH256 HT3 TGGTCTCGAGAAAAAAATTACCGAACGGAATAGAAAC OAH257 HT3 TGGTCTCGGGTACCCAAACTCAAATGTCATAAATGGAATG OAH260 HT4 TGGTCTCGGGTACCCAAAACTCAAATGTCATAAATGGAATG	Drimors used for HT site directed mutagenesis of shill	
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OAH238 HT1 IGGTCTCGCTTTATTACAATGGGGAAAGTGCCATATGATGCC OAH252 HT2 TGGTCTCGGGATATAAAAGAAAATCGCCAAATGG OAH253 HT2 TGGTCTCGAGAAGGAAAGTGCCATATGATGG OAH256 HT3 TGGTCTCGAGAAGAAAATCCGAACGGAATAGAAAC OAH257 HT3 TGGTCTCGGTACCCAAAACTCAAATGGCAAATGGCAATGGAATG OAH260 HT4 TGGTCTCGGGTACCCAAAACTCAAATGGAATG		
OAH252 H12TGGTCTCGGGATATAAAAGAAAAAATCGCCAAATGGOAH253 HT2TGGTCTCGATCCGATCGATTTATCTCCATCAOAH256 HT3TGGTCTCGAGAAGAAAAAATTACCGAACGGAATAGAAACOAH257 HT3TGGTCTCGTTCTTTATTCACTATAAGGATATGTCTCCOAH260 HT4TGGTCTCGGTACCCAAAACTCAAATGGAATG		
OAH253 H12 TGGTCTCGATCCGATAGCTGATTTATCTCCATCA OAH256 HT3 TGGTCTCGAGAAGAAAAATTACCGAACGGAATAGAAAC OAH257 HT3 TGGTCTCGTTCTTTATTCACTATAAGGATATGTCTCC OAH260 HT4 TGGTCTCGGGTACCCAAAACTCAAATGTCATAAATGGAATG	UAH252 H12	
OAH256 HT3 TGGTCTCGAGAAGAAAAAATTACCGAACGGAATAGAAAC OAH257 HT3 TGGTCTCGTTCTTATTCACTATAAGGATATGTCTCC OAH260 HT4 TGGTCTCGGGTACCCAAAACTCAAATGTCATAAATGGAATG	UAH253 HT2	IGGICICGAICCCGAAAGCIGAITTATCTCCATCA
OAH257 HT3 TGGTCTG <u>T</u> CTTTATTCACTATAAGGATATGTCTCC OAH260 HT4 TGGTCTCG <u>G</u> GTACCCAAAACTCAAATGTCATAAATGGAATG	OAH256 HT3	TGGTCTCG AG <u>AA</u> GAAA <u>AA</u> ATTACCG <u>AA</u> CGGAATAGAAAC
OAH260 HT4 TGGTCTCGGGTACCCAAAACTCAAATGTCATAAATGGAATG	OAH257 HT3	TGGTCTCGTTCTTTATTCACTATAAGGATATGTCTCC
	OAH260 HT4	TGGTCTCG GGTACCCAAAACTCAAATGTCATAAATGGAATG
OAH261 HT4 TGGTCTCG TACCAAAGAAAGATTTATCAGAGTCGG	OAH261 HT4	TGGTCTCG TACCAAAGAAAGATTTATCAGAGTCGG
OAH264 HT5 TGGTCTCG CCACATAA <u>AA</u> GTAA <u>AA</u> ATCTTTA <u>AA</u> AGGAATTAC	OAH264 HT5	TGGTCTCGCCACATAA <u>AA</u> GTAA <u>AA</u> ATCTTTA <u>AA</u> AGGAATTAC
OAH265 HT5 TGGTCTCGTCCGTATTCATGGTTGACGCGCGATCAGAT	OAH265 HT5	TGGTCTCG TCCGTATTCATGGTTGACGCGCGATCAGAT

(Continued on next page)

TABLE 2 (Continued)

Function and primer	Sequence $(5'-3')^a$		
Primers used for recombinant protein expression			
OAH 173 Sht	CATATT GAATTC TACCAACTTGGTAAGCATCATATGGGT		
OAH 174 Sht	ATCTTC GTCGAC TTA AGGGTTTATTTGTTGAAGTGT		
OAH 175 Shtll	TACCAA GAATTC AGCTATAATGCCCAAAAATCAGAC		
OAH 176 Shtll	TCA ACT GTCGAC TTATTTCACTTCTGCTAG		
OLM 186 CcpA	AAAATC GGATCC ATACAGATGATACGATTACGATTA		
OLM 181 CcpA	ACTCTA GAATTC ATTATTTGTTGTGCACGTTTAAC		
OAH 287 PspC	CTTGTT GGATCC AGTGTGCTTCATGCGACA		
OAH 288 PspC	AGCTTG GTCGAC ATCTGTTTTTCTGCTTTTGGTTG		
Primers used for gRT-PCR			
OAH223 sht	TATCCATGTCGTTCCGTATTCATGGTT		
OAH192 sht	CTTAGACCATACATCCGGACG		
OAH224 shtll	TGCACCCAGAAAAACGTCCTAAAGTTG		
OAH194 shtll	GGTGCAGGACTTGGTTTATCT		
OLM321 recA	CTGGTGGTCGTGCTTTGAAA		
OLM322 recA	TATGCTCACCAGTCCCCTTG		

^aAdded restriction site sequences are indicated in boldface. Nucleotides targeting histidine residues for mutation to phenylalanine to generate HT variants are underlined.

described (36). The concentration and purity of RNA were assessed with a NanoDrop Lite spectrophotomer (Thermo Fisher Scientific) with subsequent treatment using DNase (Turbo DNA-free DNase; Ambion). The absence of DNA contamination was confirmed by PCR using 50 ng of the purified RNA.

Reverse transcription and qRT-PCR. RNA was reverse transcribed by using an iScript cDNA synthesis kit (Bio-Rad), according to the manufacturer's instructions. Primers (Table 2) were selected with Primer3web software (http://bioinfo.ut.ee/primer3/) in order to design 100- to 200-bp amplicons. Quantitative reverse-transcriptase PCRs (qRT-PCRs) were performed in a 20-µl reaction volume containing 40 ng of cDNA, 1 µl of gene-specific primers (10 µM), and 10 µl of LightCycler 480 SYBR Green I mix (Roche). PCR amplification, detection, and analysis were performed with a LightCycler 480 PCR detection system and LightCycler 480 software (Roche). PCR conditions included an initial denaturation step at 95°C for 2 min, followed by a 45-cycle amplification (95°C for 5 s and 60°C for 20 s). The specificity of the amplified product and the absence of primer dimer formation were verified by generating a melting curve (65 to 95°C). The crossing point (C_p) was defined for each sample. The expression levels of the tested genes were normalized using the *recA* (primers OLM321 and OLM322) gene of *S. agalactiae* as an internal standard whose transcript level did not vary under our experimental conditions. Each assay was performed in duplicate and repeated with a least three independent RNA samples.

Purification of recombinant Sht, Shtll, CcpA, and PspC proteins. S. agalactiae A909 DNA was used to amplify the sht, shtll, and ccpA genes by PCR, and the S. pneumoniae D39 DNA was used to amplify the pspC genes with the Q5 high-fidelity DNA polymerase (New England BioLabs) using the OAH173-174, OAH175-176, OLM186-181, and OAH287-288 primers, respectively (Table 2). For Sht and Shtll, the primers were designed to remove the predicted sequence signal of each proteins (residues 1 to 23 for Sht and residues 1 to 27 for Shtll). For PspC, only the adhesive fraction of the protein was expressed (residues 33 to 445) (20). PCR amplifications were cloned into pET28a(+) vector (EMD Biosciences) in E. coli BL21 codon + (DE3)-RIL (Novagen) for high-level expression and addition of an amino-terminal $6 \times$ His tag. The derived expression constructs are listed in Table 1. Protein expression was conducted in E. coli BL21(DE3). Here, the expression construct-containing strains were grown in LB broth to an OD₆₀₀ of 0.5 at 37°C, with shaking at 200 rpm in an orbital shaking incubator. Protein expression was induced by addition of IPTG (isopropyl- β -D-thiogalactopyranoside), and the cells were then grown for a further 5 h at 37°C at 200 rpm. Postinduction, the cells were pelleted at $10,000 \times q$, and the supernatant was decanted. The cells were then resuspended in 1 ml of lysis buffer (20 mM Tris [pH 8.0], 300 mM NaCl, 10% [wt/vol] glycerol) and lysed mechanically with glass beads in a FastPrep-24 instrument. Recombinant proteins were then purified by immobilized metal affinity chromatography (His-Select nickel affinity gel; Sigma-Aldrich) and eluted under native conditions according to the manufacturer's instructions. Eluates were analyzed by SDS-PAGE and Coomassie blue staining to assess protein purity, as described by Laemmli (37).

Homology modeling. The homology model of AdcAll was constructed using the SwissModel webserver (38), using *S. pneumoniae* AdcAll (PDB 3CX3) as a template. The resulting model of AdcAll was energy minimized in SwissPDBViewer (39) using the inbuilt 43B1 vacuum force field (40). Surface electrostatic potentials for the Lmb and AdcAll structures were calculated using APBS (41).

Factor H binding assays. A 96-well microtiter plate (Sarstedt) was coated with 1 μ g of the purified recombinant proteins (PspC, Sht, ShtII, or CcpA) overnight at 4°C. The wells were washed with 200 μ l of PBS and then blocked with 200 μ l of PBS containing 5% of skim milk (w/vol) for 1 h at room temperature. Human serum, provided by healthy donors of the Etablissement Français du Sang (EFS Centre Atlantique, France), was inactivated by treatment at 56°C for 30 min and used as a source of factor H. Then, 200 μ l of inactivated serum was added to the wells for 2 h, followed by incubation at 37°C. The wells were then washed with PBS and incubated with 200 μ l of a mouse antibody against factor H (1:2,000 [vol/vol]; Sigma-Aldrich) in PBS for 1 h at 37°C. After the wells were washed, 100 μ l of peroxidase-conjugated

anti-mouse IgG (1:2,000 [vol/vol]; Sigma-Aldrich) in 0.05 M carbonate-bicarbonate buffer (pH 9.6) was added, and the plate was incubated for 1 h at room temperature. After a final wash, 200 μ l of Sigma Fast OPD substrate (Sigma-Aldrich) was added, and the absorbance at 450 nm was read using an Eon spectrophotometer (BioTek). The readings were background corrected by subtracting the absorbance of wells coated with bovine serum albumin. All assays were repeated three times in duplicate.

Complement resistance assays. Human whole blood or serum was provided by healthy donors of the Etablissement Français du Sang who were not taking medication. Blood and serum were then used directly or heat inactivated by treatment at 56°C for 30 min. Bacterial strains, both wild-type (WT) and mutant strains, were grown for 8 h in TH broth and then inoculated at an OD₆₀₀ of 0.005 into zinc-restricted CDM and grown for a further 12 h. The cells were harvested by centrifugation at 12,000 × *g*, washed, and resuspended in PBS. Bacteria were then inoculated into 1 ml of blood or heat-inactivated blood at a concentration of 10⁶ CFU ml⁻¹. The samples were gently mixed by rotation at 37°C for 3 h. Bacterial counts were performed after resuspension in blood (T_0) or after incubation (T_3) by dilution plating on TH agar. The survival rate represents the ratio between the number of cells at T_3 and T_0 . The data represent the means of three independent experiments.

Statistical analyses. The data represent means \pm the standard errors of the mean. Statistical analyses were performed using a two-tailed unpaired Student *t* test. A probability value of <0.05 was considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB .00757-18.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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