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Characterization of *Staphylococcus aureus* Cardiolipin Synthases 1 and 2 and Their Contribution to Accumulation of Cardiolipin in Stationary Phase and within Phagocytes[▽]

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In many bacteria, including *Staphylococcus aureus*, progression from the logarithmic to the stationary phase is accompanied by conversion of most of bacterial membrane phosphatidylglycerol (PG) to cardiolipin (CL). Phagocytosis of *S. aureus* by human neutrophils also induces the conversion of most bacterial PG to CL. The genome of all sequenced strains of *S. aureus* contains two open reading frames (ORFs) predicting proteins encoded with ~30% identity to the principal CL synthase (*cls*) of *Escherichia coli*. To test whether these ORFs (*cls1* and *cls2*) encode cardiolipin synthases and contribute to CL accumulation in *S. aureus*, we expressed these proteins in a *cls* strain of *E. coli* and created isogenic single and double mutants in *S. aureus*. The expression of either Cls1 or Cls2 in CL-deficient *E. coli* resulted in CL accumulation in the stationary phase. *S. aureus* with deletion of both *cls1* and *cls2* showed no detectable CL accumulation in the stationary phase or after phagocytosis by neutrophils. CL accumulation in the stationary phase was due almost solely to Cls2, whereas both Cls1 and Cls2 contributed to CL accumulation following phagocytosis by neutrophils. Differences in the relative contributions of Cls1 and Cls2 to CL accumulation under different triggering conditions suggest differences in the role and regulation of these two enzymes.

Staphylococcus aureus infections are associated with significant morbidity and mortality in community and health care settings. These infections are associated with a tremendous economic burden (14.5 billion dollars in the United States alone in 2003 [20]) and are further complicated by the increasing frequency of antibiotic resistance. The burden of disease from methicillin-resistant *Staphylococcus aureus* (MRSA) infections is growing and leading to worse patient outcomes. Approximately 100,000 invasive MRSA infections occurred in 2005 in the United States alone (20), leading to ca. 19,000 deaths, even more than that due to HIV infection.

Interactions of *S. aureus* with human hosts do not always lead to disease. In many individuals, *S. aureus* is part of the normal microbial flora (e.g., in the anterior nares), usually without significant pathological consequences (25). However, when the skin or a mucosal barrier is breached, colonizing *S. aureus* can penetrate across epithelial barriers to cause an array of local and more invasive infections, including skin and soft tissue infections, necrotizing pneumonia, osteomyelitis, sepsis, and infectious endocarditis (25). Once inside the host, invading *S. aureus* is challenged by professional phagocytes and by locally and systemically mobilized secreted antimicrobial compounds, including the group IIA phospholipase A_2 (7, 11, 51, 52). *S. aureus* can adapt to the hostile host conditions by initiating expression of a variety of secreted and cell envelope-associated virulence factors (4, 30) and by adopting new physical and metabolic states (e.g., biofilms, endocardial vegetations, and small colony variants) that are associated with long-term bacterial persistence (9, 27, 28, 32, 36). What specific metabolic changes are needed for long-term survival are largely unknown.

Among the adaptive changes in the chemical composition of the cell envelope that have been well documented is a change in membrane phospholipid composition. In actively growing *S. aureus*, the predominant phospholipid species is phosphatidylglycerol (PG), whereas in stationary growth phase the predominant phospholipid is cardiolipin (CL) with a corresponding decline in PG content (23, 44). Similar bacterial phospholipid changes have been observed under conditions of osmotic stress, energy deprivation, or after phagocytosis by polymorphonuclear leukocytes (PMN) (7, 21, 24, 43, 44).

The synthesis of CL in bacteria is catalyzed by cardiolipin synthase, which catalyzes condensation of two PG molecules to yield CL and glycerol (43, 45). Cardiolipin synthase belongs to the phospholipase D class of enzymes and shares the same key residues forming the catalytic site of these enzymes (40). In *E. coli* and many other bacterial species, multiple genes are present that encode proteins with cardiolipin synthase activity and/or share homology with the principal *E. coli* cardiolipin synthase. In many cases, the relative role and meaning of the apparent multiplicity of cardiolipin synthases are not clear. For example, in *E. coli*, the principal cardiolipin synthase is encoded by the *cls* gene (Gen Bank accession number U15986) and is responsible for nearly all CL production *in vivo*. The activity of a second cardiolipin synthase, encoded by the f413 gene, has to date only been demonstrated *in vitro* (13, 31, 45).

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Similarly, three *cls* homologues have been identified in *Bacillus subtilis*, but to date only one of these genes has been shown to encode active cardiolipin synthase under the test conditions explored (12, 24, 37). Cardiolipin synthase activity has also been demonstrated in partially purified *S. aureus* membranes (43), but the genes encoding active CL synthases and the proteins responsible for CL accumulation in *S. aureus* were not known when the present study was initiated.

Accordingly, we have sought here to identify the gene(s) in *S. aureus* encoding active cardiolipin synthases and to establish the role of these enzymes in bacterial CL accumulation in the stationary phase and in bacteria ingested by neutrophils. We identified two genes encoding cardiolipin synthase that together accounted for essentially all net CL formation under these conditions and provide evidence suggesting that the regulation of these two enzymes is different.

MATERIALS AND METHODS

Materials. Clinical grade dextran T500 (molecular mass, 500,000 Da) was purchased from Pharmacosmos A/S (Holbaek, Denmark). Sterile endotoxin-free H_2O and 0.9% sterile endotoxin-free sodium chloride for patient use were purchased from Baxter Healthcare (Deerfield, IL). Ficoll-Paque Plus was purchased from GE Healthcare (formally Amersham Biosciences), Piscataway, NJ. HEPES, Hanks balanced salt solution (HBSS), and Dulbecco phosphate-buffered saline, with or without divalent cations, were obtained from Mediatech Cellgro (Manassas, VA). Chloroform, methanol, glacial acetic acid, and the HEMA-3 staining kit were purchased from Fisher Scientific. Endotoxin-free human serum albumin (25% [wt/vol]) was obtained from Talecris Biotherapeutics (Research Triangle Park, NC). Tryptic soy broth (TSB) and Bacto agar were purchased from BD Biosciences (Bedford, MA). [1-^{14}C]palmitic acid (57 mCi/mmol) was purchased from Amersham Life Science, bovine serum albumin (BSA) was purchased from Boehringer Mannheim (Indianapolis, IN), and HP-TLC (high-performance thin-layer chromatography) plates were purchased from Merck (Germany). Purified lipid standards for PG and CL—1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] and tetraoleoyl cardiolipin, respectively—were purchased from Avanti-Polar Lipids (Alabaster, AL). DNA and RNA isolations were carried out using Qiagen kits (Valencia, CA). Lysostaphin was purchased from Sigma-Aldrich (St. Louis, MO). Mechanical disruption of *S. aureus* was carried out in FastPrep24 using blue matrix tubes (MP Biomedicals, Solon, OH). *Taq* polymerase, deoxynucleoside triphosphates, BP clonase, and RNasin were purchased from Invitrogen (Carlsbad, CA), and Phusion DNA polymerase was obtained from Finnzymes (Woburn, MA). Avian myeloblastosis virus reverse transcriptase (AMV-RT) and random hexamers were obtained from Roche (Palo Alto, CA). Sybr green PCR master mix was obtained from Applied Biosystems (Foster City, CA).

Construction of a *cls* deletion. Markerless chromosomal deletions of *cls1* and/or *cls2* were constructed in both *S. aureus* SA113 (18) and LAC (USA300-0114 MRSA [50]) using the pKOR1 plasmid for allelic replacement as described by Bae and Schneewind (1). Briefly, flanking regions of *cls1* were amplified by using the primer pairs TK161/TK162 and TK163/TK164 and of *cls2* by using the primer pairs TK149/TK151 and TK150/TK152 (Table 1). The two PCR products were joined by overlap extension (49) and moved into pKOR1 using the Gateway cloning system (Invitrogen). The resulting plasmid was transformed into SA113 or LAC, and the gene deletions were constructed as described previously (26). Gene deletions were confirmed by PCR using primers annealing to flanking regions upstream and downstream of the deleted gene. To complement the deleted genes (*cls1* or *cls2*) in *trans*, each structural gene, together with the upstream DNA region containing putative promoter sites, was amplified by PCR using the primer pairs TK167/TK168 for *cls1* and TK169/TK170 for *cls2*. The resulting PCR product was digested by KpnI and SacI and ligated into the *E. coli*/*S. aureus* shuttle vector, pUC19/pC194. The resulting plasmids, pTKOcls1 and pTKOcls2 (Table 2), were introduced into *S. aureus* SA113 and LAC by electroporation (39).

Radiolabeling of *S. aureus* lipids during bacterial growth. Bacterial lipids were radiolabeled during growth in subculture as previously described (8). Briefly, bacteria from overnight cultures in TSB were diluted ~200-fold in fresh TSB supplemented with 1 μCi of [1-^{14}C]palmitic acid/ml and 0.01% BSA and subcultured at 37°C to either mid-logarithmic phase (3 h) or to stationary phase (24 h). At the time of harvesting of the bacteria, BSA was added to a final concentration of 0.5% (wt/vol), and the bacteria were washed to remove any remaining bacterium-associated radiolabeled free fatty acid. The washed bacteria were resuspended to the desired concentration in the desired medium and used promptly, as described below.

Neutrophil isolation. PMN from normal healthy volunteers were purified from peripheral blood as described earlier (7). Donor consent was obtained from each individual according to the protocol approved by the Institutional Review Board for Human Subjects at the University of Iowa. Isolated PMN were resuspended in sterile endotoxin-free HBSS without divalent cations, counted, and diluted to a final density of no greater than 2×10^7/ml. The PMN purity was ≥95%, as judged by microscopic examination of the cell suspension after staining with the HEMA-3 kit. PMN were kept at room temperature for no longer than 1 h before use in subsequent assays.

Phagocytosis. *S. aureus* (5×10^7/ml) and PMN (1×10^7/ml) were separately incubated in HBSS containing divalent cations, 1% human serum albumin (HSA), and 10% pooled human serum at 37°C for 10 (PMN) or 20 (bacteria) min and then mixed 1:1 (vol/vol) to achieve a multiplicity of infection (MOI) of 5. Incubations were carried out in 5-ml polypropylene round-bottom tubes with shaking in a 37°C water bath. After a 30-min coincubation of bacteria and PMN, the samples were gently spun ($500 \times g$) for 5 min in a Savant high-speed tabletop centrifuge to pellet the PMN and associated bacteria. The supernatant containing extracellular bacteria was discarded. The cell pellet was resuspended in the original volume of HBSS containing divalent cations supplemented with 1% HSA and 10% pooled human serum and further incubated at 37°C for specified intervals before assays of bacterial RNA and lipids, as described below. For all experiments with PMN, mid-logarithmic-phase *S. aureus* was used that was harvested after 3 h of metabolic labeling with [1-^{14}C]palmitic acid. Bacterial uptake by PMN was quantified both by measurement of bacterial radioactivity recovered in PMN and in extracellular medium after sedimentation of PMN (see above) and by light microscopic evaluation of stained smears of resuspended PMN as previously described (6, 15) and confirmed in selected samples by fluorescence microscopy using green fluorescent protein (GFP)-expressing bacteria (6).

Assay of bleaching of bacterial cytosolic GFP in *S. aureus* ingested by neutrophils. *S. aureus* (both wild type [wt] and *cls1 cls2* mutant) carrying pCM29 encoding super bright sGFP (33) was grown and incubated with PMN as described above except that extracellular bacteria were removed 10 min after incubation with neutrophils by centrifugation at $500 \times g$ for 5 min. At subsequent time points, the fluorescence of *S. aureus*-PMN suspensions was measured by flow cytometry as described previously (33). PMN without *S. aureus* were analyzed and gated as an GFP-*S. aureus*-negative control. PMN outside this gate were considered GFP positive. For each sample the mean fluorescent index (MFI) was calculated by multiplying the geometric mean of the fluorescence by the percentage of cells in the sample that were GFP positive (i.e., PMN containing ingested and still fluorescent *S. aureus*) At each time point, the MFI was calculated as a percentage of the MFI of the sample recovered and analyzed immediately after removal of the extracellular bacteria.

Characterization of ^{14}C-labeled bacterial (*S. aureus*) phospholipids after incubation of [1-^{14}C]palmitate-labeled *S. aureus* with neutrophils. After incubation of [1-^{14}C]palmitate-labeled *S. aureus* with neutrophils, cell suspensions containing 1-^{14}C-labeled *S. aureus* and neutrophils were extracted via a modified Bligh-Dyer method (13, 32). Briefly, whole-cell suspensions (1 volume) were mixed with 6 volumes of chloroform-methanol (1:2 [vol/vol]) and stored at 4°C until analyzed. Samples were spun at $1,900 \times g$ for 10 min, and the supernatant was transferred to a fresh glass tube. Then, 3 volumes of 50 mM potassium chloride and 2 volumes of chloroform were added to the recovered supernatant. The samples were vortexed and spun for 10 min at $1,900 \times g$ to separate the upper water-methanol phase from the lower chloroform phase. The chloroform phase was removed and saved. The remaining aqueous phase was mixed with 4 volumes of chloroform, vortexed, and spun for 10 min at $1,900 \times g$. The recovered chloroform phase was combined with the previously recovered chloroform phase, dried under a stream of nitrogen, resuspended in chloroform-methanol (2:1 [vol/vol]), and applied to an HP-TLC plate. Lipids were resolved either in one dimension using either (i) chloroform-methanol-acetic acid (65:25:10 [vol/vol/vol]) or (ii) chloroform-methanol-petroleum ether-acetic acid (40:20:30:10 [vol/vol/vol/vol]) (38) or in two dimensions, using chloroform-methanol-acetic acid-water (first dimension, 65:25:4:1 [vol/vol/vol/vol]; second dimension, 80:18:12:5 [vol/vol/vol/vol] [21]). The resolved ^{14}C-labeled lipids were visualized by exposure of the HP-TLC plates to tritium storage phosphor screens and analyzed by using an Amersham Typhoon 9410 variable mode imager (Amersham Biosciences). Quantification was done by using ImageQuant software from Molecular Dynamics. Bacterial [^{14}C]PG and [^{14}C]CL were identified by their comigra-

TABLE 1. Primers used in this study

Primer	Sequence (5′–3′)[a]	Description	Source or reference
TK161	*GGGGACAAGTTTGTACAAAAAAGCAGGCT*GCCCA ACTGGTTGCGAATCTAACA	Forward primer for amplification of DNA region upstream of *cls1* with attB1 sites	This study
TK162	CCTCTATAATCGAGACTCCTTACAAATAAAA**AGT CTTTTCTCCTATAAAGAAAGGCAC**	Reverse primer with overlapping ends for amplification of DNA region upstream of *cls1*	This study
TK163	**GTGCCTTTCTTTATAGGAGAAAAGACT**TTTATTTGT AAGGAGTCTCGATTATAGAG	Forward primer with overlapping ends for amplification of DNA region downstream of *cls1*	This study
TK164	*GGGGACCACTTTGTACAAGAAAGCTGGGT*CGGA AACCACAATCCACCTAATACTGC	Reverse primer with attB2 for amplification of DNA region downstream of *cls1*	This study
TK149	*GGGGACAAGTTTGTACAAAAAAGCAGGCT*ATGGT GGTAGTGCGCTATGGAGTA	Forward primer with attB1 sites for amplification of DNA region upstream of *cls2*	This study
TK151	AAAGTTACACTCCTCATATTTCTAT**TTGAAAC CTCC CATCGAAAATC**	Reverse primer with overlapping ends for amplification of DNA region upstream of *cls2*	This study
TK150	**GATTTTCGATGGGAGGTTTCAA**ATAGAAATATGA GGAGTGTAACTTT	Forward primer with overlapping ends for amplification of DNA region downstream of *cls2*	This study
TK152	*GGGGACCACTTTGTACAAGAAAGCTGGGT*GCTCA GCATCAGCACTTGGTTTGT	Reverse primer with attB2 ends for amplification of DNA region downstream of *cls2*	This study
TK167	ACGTAGGTACCTTTAATAAGATAAACCAATTTC AAAACTAGTTCG	Forward primer for amplification of *cls1* with the KpnI restriction site	This study
TK168	AGTAGAGCTCGGCAAAATAAAAAGAGCCTCTA TAATCGAGACTCCTTACAAATAAAT	Reverse primer for amplification of *cls1* with the SacI restriction site	This study
TK169	ACGTAGGTACCAGATGCTGGCAAAGTATTAAT AATGTCTAA	Forward primer for amplification of *cls2* with the KpnI restriction site	This study
TK170	TATGTGAGCTCGCATTAAAGTTACACTCCTCAT ATTTCTAT	Reverse primer for amplification of *cls2* with the SacI restriction site	This study
TK180	CACAGAGCAGCAAAAGCGTTAG	Forward primer for *cls1* real-time RT-PCR	This study
TK181	CGCTTGCGAATTCCAGTCTAA	Reverse primer for *cls1* real-time RT-PCR	This study
TK182	GTGGAACAATTGGCGTTCAA	Forward primer for *cls2* real-time RT-PCR	This study
TK183	CCCATTCTTCGTCAGGACCA	Reverse primer for *cls2* real-time RT-PCR	This study
TK23	AGCCGACCTGAGAGGGTGA	Forward primer for 16S rRNA real-time RT-PCR	22
TK24	TCTGGACCGTGTCTCAGTTCC	Reverse primer for 16S rRNA real-time RT-PCR	22
1317 F	GGGGATTTAAATGGATCCAATTAAACTTAAAAC	Forward primer for cloning of Cls1 in pRB473 with the BamHI restriction site	This study
1317 R	AATCGAGGATCCTTACAAATAAATTATAAAATT GGCG	Reverse primer for cloning of Cls1 in pRB473 with the BamHI restriction site	This study
2088 F	CAGTCATAGCATGCACTCCTTCATTTACATTCC	Forward primer for cloning of Cls2 in pRB473 with the SphI restriction site	This study
2088 R	TTGATGAATAGGATCCATATATTTTTTGGC	Reverse primer for cloning of Cls2 in pRB473 with the BamHI restriction site	This study

[a] Italics are used to indicate the attB1 and attB2 sites, boldfacing indicates overlapping ends, and underlining indicates restriction sites.

tion with purified PG and CL standards. The recovery of total ^{14}C-labeled lipids (in counts per minute [cpm]) was similar from each of the bacterial strains studied, both before and after incubation with neutrophils.

Characterization of unlabeled and [^{14}C]palmitate-labeled *S. aureus* phospholipids after growth in TSB. Lipids of *S. aureus* harvested after growth in TSB were extracted and resolved by each of the various TLC systems described above. For visualization of total lipids from either ([1-^{14}C]palmitate-labeled or unlabeled bacteria, TLC plates were sprayed with 3% cupric acetate–8% phosphoric acid and charred on a hot plate (120°C). Approximately 10 to 20 μg of total phospholipid was applied, corresponding to 1×10^9 to 2×10^9 bacteria. Images and quantification of charred samples were performed using the Kodak GelLogic system and accompanying software.

Real-time reverse transcription-PCR (RT-PCR). RNA was isolated from *S. aureus* (5×10^7) after incubation of bacteria alone, with 10% serum, or incuba-

TABLE 2. Plasmids used in this study

Plasmid	Description	Restriction sites	Source or reference
pDB81	pUC19/pC194 *E. coli* and *S. aureus* shuttle vector		D. J. Bartels
pKORI	Original plasmid knockout vector	AttB/AttP	1
pKOR*cls1*	*cls1* knockout vector		This study
pKOR*cls2*	*cls2* knockout vector		This study
pTKO*cls1*	pDB81 with *cls1* (SAOUHSC_1310) including 460 bp upstream of translation start codon	KpnI/SacI	This study
pTKO*cls2*	pDB81 with *cls2* (SAOUHSC_2323) including 738 bp upstream of translation start codon	KpnI/SacI	This study
pRB474	*E. coli*/*S. aureus* shuttle vector		35
pRB474*cls1*	pRB473 with *cls1* (SAV1317)	BamHI/BamHI	This study
pRB474*cls2*	pRB473 with *cls2* (SAV2088)	BamHI/SphI	This study
pCM29	sGFP expression under constitutive *sarA*P1 promoter		33

tion of the serum-pretreated (opsonized) bacteria plus PMN as described above. After sedimentation of the bacteria associated with PMN or bacteria incubated alone (with or without preopsonization by 10% serum), the cell pellets were resuspended in 700 μl of RLT (Qiagen) and transferred to 2-ml tubes filled with 0.1-mm silica matrix (MP Bio/Blue matrix). The bacteria were disrupted by using a FastPrep24 homogenizer for 45 s at the highest speed. The tubes were then cooled and spun at 14,000 rpm at 4°C. The recovered supernatant (ca. 350 μl) was transferred to a new tube and spun again to remove any remaining particulate matter before isolating RNA according to the RNeasy protocol (Qiagen). Contaminating DNA in the samples was removed by Turbo DNase according to the manufacturer's protocol (Ambion). Total RNA was reverse transcribed by using AMV-RT (Roche), 20 ng random hexamers (Roche)/μl, and 20 U of RNase inhibitor (Roche) at 40°C for 1 h. The PCR included Sybr green (Applied Biosystems), 200 nM concentrations of each forward and reverse primer (Table 1), and cDNA equivalent to 4 ng of input RNA. The reactions were performed with an ABI Prism 7000 detection system (Applied Biosystems). In each set of analyses, standard curves for each primer pair used were analyzed, together with the experimental samples, to facilitate quantification of the amplicons.

Isolation and analysis of *E. coli* lipids. *E. coli* was grown in LB medium to stationary phase (18 to 20 h). After the various bacterial cultures were adjusted to the same optical density, bacterial polar lipids were extracted with chloroform-methanol by the Bligh-Dyer procedure (2), vacuum dried, and dissolved in chloroform-methanol (2:1 [vol/vol]). The concentrated polar lipid extracts were spotted onto HP-TLC plates (Merck, Darmstadt, Germany) by using a Linomat 5 sample application unit (Camag, Berlin, Germany) and developed with chloroform-methanol-acetic acid (65:25:10 [vol/vol/vol]). Lipid species containing phosphate groups were stained with molybdenum blue spray (Sigma).

RESULTS

Cloning and activity of *S. aureus cls1* and *cls2* in *E. coli*. The gene encoding the principal cardiolipin synthase in *E. coli* was used to screen the genome of *S. aureus* NCTC 8325 and other strains available at NCBI for homologous open reading frames (ORFs). Two ORFs were identified in *S. aureus* NCTC 8325, annotated SAOUHC_01310 and SAOUHC_2323, that we named *cls1* and *cls2*, respectively. Each of the homologs is present in each of the *S. aureus* strains in the genomic database (e.g., in *S. aureus* MU50 genes SAV1317 and SAV2088 encode for the *cls1* and *cls2* homologs, respectively). The putative *S. aureus* cardiolipin synthases encoded by *cls1* and *cls2* display ~30% amino acid identity (~50% similarity) to the *E. coli* cardiolipin synthase. *S. aureus* Cls1 and Cls2 share 53% amino acid identity and are predicted by HMMTOP and TopPred 2 software (47, 48; http://mobyle.pasteur.fr/cgi-bin/portal.py?form=toppred) (Fig. 1B) to have two similar transmembrane-spanning helices (TMH, residues 15 to 38 and residues 48 to 70; Fig. 1B). Cls1 and Cls2 also have two predicted phospholipase D domains, a characteristic of all prokaryotic cardiolipin synthases (45), residing within a region of the protein (residues 227 to 252 and residues 403 to 434) predicted to be outside the cell membrane (Fig. 1B). To investigate whether *cls1* and *cls2* actually encode cardiolipin synthases, we cloned individually *S. aureus cls1* and *cls2*, together with their putative promoter regions, into an *E. coli* plasmid expression vector pRB474 and used these plasmids to transform the CL-deficient *E. coli* SD9 (42) that lacks both *cls* (cardiolipin synthase) and *pss-1* (phosphatidylserine synthase) (42). After overnight growth in LB to stationary phase, bacterial lipids were extracted and analyzed as described in Materials and Methods. As expected, CL was present in the wt strain (*E. coli* DH5α) but not detectable in *E. coli* SD9pRB474 vector control (Fig. 2). In contrast, significant amounts of CL were present in transformants of *E. coli* SD9 containing plasmids encoding *cls1* (pRB474*cls1*) or *cls2* (pRB474*cls2*). These findings strongly

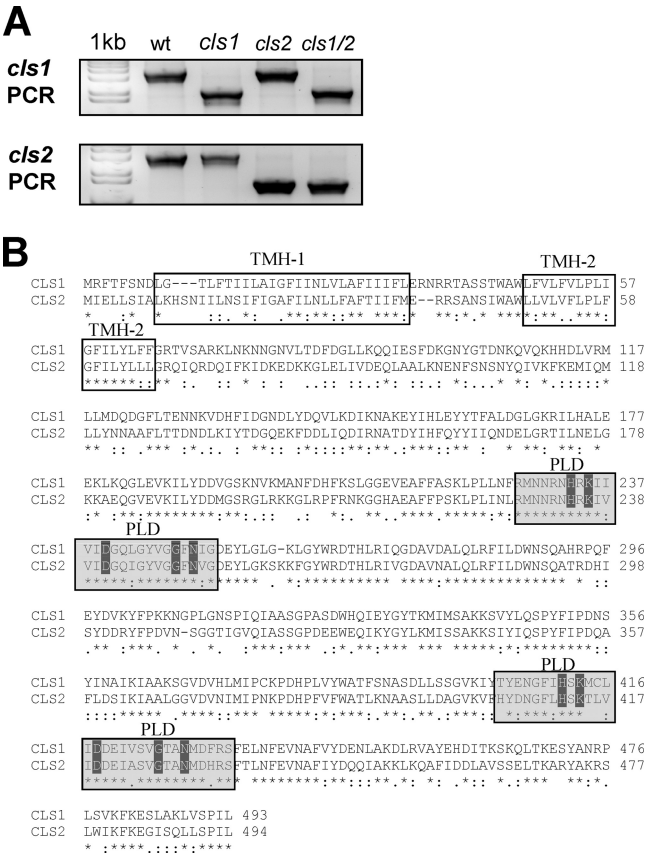

FIG. 1. (A) Evidence of chromosomal deletion of *cls1* and/or *cls2* in *S. aureus cls1*, *cls2*, and *cls1 cls2* mutants by PCR. Primer pairs annealing upstream and downstream of the *cls1* (TK161/164; upper line) and *cls2* (TK149/152; lower line) structural genes were used to confirm *cls1* and *cls2* chromosomal deletions. The source of genomic DNA used as a template is depicted above each PCR product. wt, wild type; *cls1*, *S. aureus cls1*; *cls2*, *S. aureus cls2*; *cls1*/2, *S. aureus cls1 cls2*. (B) Amino acid sequence alignment of *S. aureus* Cls1 and Cls2 using CLUSTAL W. Asterisks denote sites of amino acid identity; colons, sites of amino acid similarity. Boxed regions correspond to predicted transmembrane helices (TMH-1 and TMH-2), using HMMTOP 2.0 and TopPredII secondary structure prediction software, and conserved domains of the active site [HxK(x)4D(x)6GSxN, shaded amino acids] of the phospholipase D (PLD) family, identified by NCBI Blastp.

suggest that both ORFs in *S. aureus*—*cls1* and *cls2*—encode for functional cardiolipin synthases (Fig. 2).

CL accumulation in log and stationary phases of wt and mutant (*cls*) *S. aureus*. To test the role of *cls1* and/or *cls2* in accumulation of CL by *S. aureus*, we constructed isogenic mutants in both *S. aureus* SA113 and the USA300 LAC strain in which *cls1* and/or *cls2* were disrupted. Successful disruption of these genes was confirmed by PCR, using primers annealing outside the coding regions of these genes in wt and mutant SA113 (Fig. 1A). The same analyses and observations were made with wt and mutant strains of *S. aureus* LAC (data not shown). We initially compared the lipid content of wt and double mutant (*cls1 cls2*) *S. aureus* harvested after growth in TSB with or without [1-^{14}C]palmitate at 37°C for 3 or 24 h. Growth in TSB of the wt and each of the mutant strains was essentially the same (not shown). Bacteria harvested at 3 h were in mid-log phase, whereas bacteria harvested at 24 h were

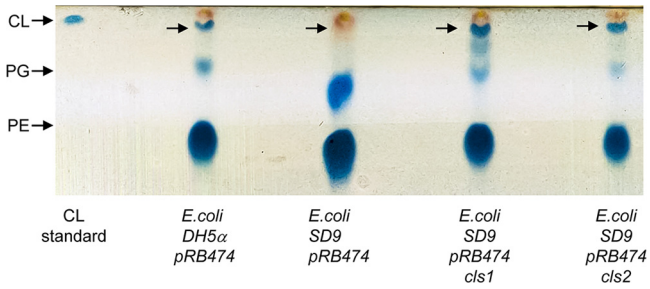

FIG. 2. *S. aureus cls1* and *cls2* induce accumulation of CL in CL-deficient *E. coli* (SD9). *S. aureus cls1* and *cls2* were cloned into the pRB474 vector and expressed in CL-deficient *E. coli* SD9. After overnight incubation, the bacteria were harvested, and lipids were extracted and analyzed as described in Materials and Methods. The migration of PE (phosphatidylethanolamine), PG, and CL in the strains tested, as determined by comigration of purified standards (only CL is shown), is indicated by arrows. The results shown are representative of three independent experiments.

in stationary phase. Bulk and metabolically ([1-^{14}C]palmitate)-labeled phospholipids were resolved in three different TLC systems (Fig. 3) and visualized by charring or phosphorimage analysis (Fig. 3). The identities of bacterial PG and CL were confirmed by their comigration with purified PG and CL standards in each of these TLC systems (Fig. 3). Using the same TLC-based separation (solvent system chloroform-methanol-acetic acid, 65:25:10 [vol/vol/vol]), Tsai et al. (46) have confirmed the identity of CL by mass spectrometry. As shown in Fig. 3, the relative levels of CL to PG were substantially increased in wt *S. aureus* harvested from stationary (versus the log phases) (i.e., harvested after 24 h versus after 3 h of culture in TSB), a finding consistent with previous observations made in *S. aureus* and a number of other bacterial species (3, 10, 14, 15, 19, 24, 29, 34). In contrast, no accumulation of CL was observed in the *cls1 cls2* double mutants of *S. aureus* LAC (Fig. 3A to C) and SA113 (Fig. 4) after 24 h of culture in TSB even when a 25-fold-higher load of lipids was applied (Fig. 3D).

The above findings demonstrate a critical role of Cls1 and Cls2 in CL accumulation by stationary phase *S. aureus.* To define the relative roles of Cls1 and Cls2 in this lipid adaptive response, the lipid profiles of isogenic single-mutant (*cls1* or *cls2*) *S. aureus* and double-mutant (*cls1 cls2*) bacteria complemented with either *cls1* or *cls2* were compared to wt and *cls1 cls2 S. aureus* (Fig. 4). The close similarity in the profiles of the bulk and metabolically [1-^{14}C]palmitate-labeled lipids (Fig. 3) permitted the use of the metabolically labeled bacteria for more sensitive quantification of bacterial PG and CL. Both *cls1* and *cls2 S. aureus* showed decreased CL levels in log and stationary phases, but the effects of disruption of *cls2* were much more dramatic, with virtually no increased CL accumulation in stationary phase observed in *cls2* bacteria (Fig. 4). Complementation studies in the double-mutant (*cls1 cls2*) strain by introduction of wt *cls1* or *cls2* in a high-copy-number plasmid (pTKcls1 and pTKcls2, respectively) confirmed that the absence of CL accumulation in *cls1 cls2 S. aureus* was due to disruption of these two genes and that, in both log and stationary phases, Cls2 contributed much more to bacterial CL accumulation than did Cls1 (Fig. 4).

Bacterial CL accumulation after phagocytosis of wt and *cls* mutant *S. aureus* by human neutrophils. Accumulation of CL in *S. aureus* ingested by human neutrophils (PMN) has been previously observed (7, 16). To investigate the roles of Cls1 and Cls2 in accumulation of CL after phagocytosis of *S. aureus* by PMN, we analyzed the ^{14}C-labeled lipids of [1-^{14}C]palmitate-prelabeled bacteria before and after ingestion of *S. aureus* by PMN. The use of metabolically prelabeled bacteria permitted the unambiguous detection and quantification of bacterial PG and CL in the presence of the other unlabeled, nonbacterial (serum, PMN) lipids. Preopsonization of the wt and each of the mutant *S. aureus* strains with 10% pooled human serum had no appreciable effect on labeled bacterial lipids of the mid-log bacteria; PG remained the predominant labeled bacterial phospholipid and exceeded the levels of labeled CL by at least 4-fold (data not shown). Bacterial uptake by PMN of all of the strains tested was essentially the same: at an MOI of 5 cocci/PMN, >90% of each of the strains were ingested within 30 min, as judged both by assay of PMN-associated radiolabeled bacteria and by light microscopy (7, 16, 41; data not shown). As shown previously (7, 16), phagocytosis of wt *S. aureus* was followed by a roughly 10-fold increase in the relative levels of bacterial CL to PG (Fig. 4, open versus black bars). Changes in bacterial CL/PG were triggered within 30 min of phagocytosis but took 1 to 2 h for maximum changes to be manifest. This lipid change was modestly reduced in *cls1 S. aureus* and reduced to a greater but incomplete extent in *cls2 S. aureus*. Disruption of both *cls1* and *cls2* was needed to eliminate the PG to CL conversion during and after phagocytosis of *S. aureus* by PMN (Fig. 4). Consistent with that finding, complementation of *cls1 cls2* with either *cls1* or *cls2* permitted substantial or virtually complete restoration of CL accumulation in *S. aureus* ingested by PMN, similar to that seen in *cls2* and *cls1 S. aureus*, respectively (Fig. 4).

Fate of wt and *cls* double mutant *S. aureus* within PMN. The absence of detectable conversion of bacterial PG to CL in *cls1 cls2 S. aureus* ingested by PMN made it possible to examine how this lipid adaptation influences the fate of *S. aureus* within human PMN. For this purpose, we measured the loss of fluorescence in bacteria expressing GFP after phagocytosis by PMN (33). The chlorination of GFP by the HOCl produced by the neutrophil results in ablation of the fluorescence of the chromophore (6). Whereas the overall extent of bleaching of cytoplasmic GFP is closely similar to the percentage of ingested *S. aureus* killed within PMN (33, 41), the kinetics of bleaching are somewhat slower than that of bacterial killing and therefore bleaching of bacterial cytosolic GFP might be more affected by the progressive accumulation of CL in wt *S. aureus*. However, as shown in Fig. 5, the rate and extent of GFP bleaching was essentially the same in ingested wt and *cls1 cls2 S. aureus*, as judged by flow cytometry. The uptake peaked at 10 min and was the same for wt and the double mutant (data not shown). The rate and extent of killing of wt and *cls1 cls2 S. aureus* by PMN were also the same (data not shown). These findings indicate that the conversion by *S. aureus* of PG to CL did not protect against neutrophil killing and myeloperoxidase-hydrogen peroxide-chloride-dependent bacterial alterations. However, induced CL accumulation could impact the subsequent fate of the remaining intracellular viable *S. aureus* and undigested bacterial phospholipids (see the Discussion).

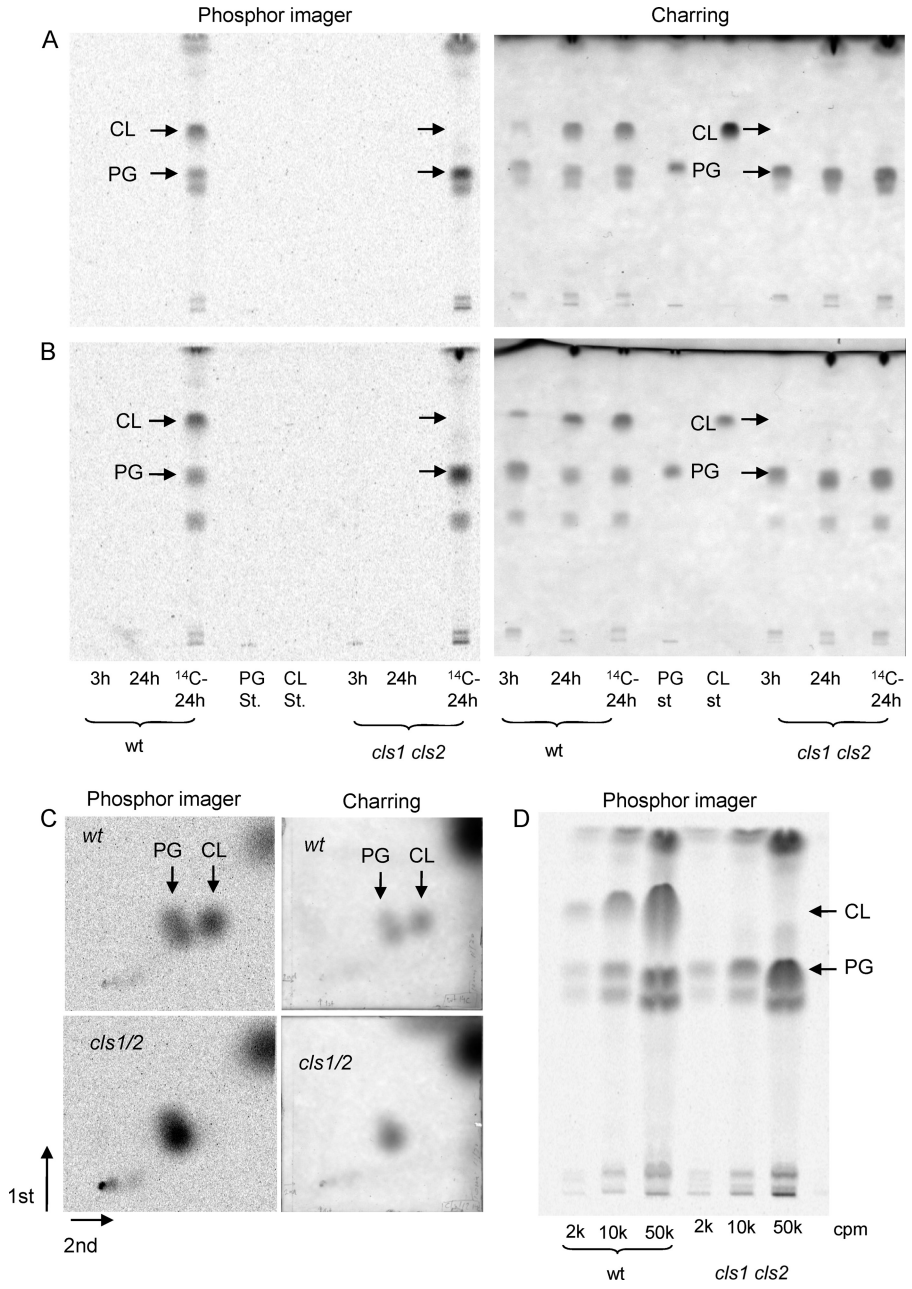

FIG. 3. Lipid profiles of *S. aureus* LAC (wt and *cls1 cls2* double mutant) as determined by TLC. (A) *S. aureus* strains were grown with or without [1-^{14}C]palmitic acid in TSB. Bacteria were harvested at 3 h (exponential phase; panels A and B) or at 24 h (stationary phase; panels A to D) of growth in TSB at 37°C, and lipids were extracted and resolved by one-dimensional (A, B, and D) or two-dimensional (C) TLC using chloroform-methanol-acetic acid (65:25:10 [vol/vol/vol]) (A), chloroform-methanol-petroleum ether-acetic acid (40:20:30:10 [vol/vol/vol/vol]) (B), or chloroform-methanol-acetic acid-water (65:25:4:1 [vol/vol/vol/vol] in the first dimension and 80:18:12:5 [vol/vol/vol/vol] in the second dimension) (C). (D) Increasing amounts (cpm) of lipids extracted from *S. aureus* LAC wt and the *cls1 cls2* double-mutant strains after 24 h of growth in TSB were evaluated by TLC and resolved in one dimension using the chloroform-methanol-acetic acid (65:25:10 [vol/vol/vol]) solvent system. Purified lipid standards (PG and CL) were used to identify bacterial PG and CL in each of the solvent systems used. Total lipids were stained with 3% cupric acetate and 8% phosphoric acid and visualized after charring (A to C; right panels). ^{14}C-labeled lipids were visualized after exposure to phosphorimager screen (A to C, left panels, and D). The arrows denote the migration of PG and CL. The chromatograms are representative of three or more experiments with strains derived from both *S. aureus* SA113 and LAC.

Differences in the regulation of *cls1* and *cls2* mRNA levels. The findings described above strongly suggest a greater role of Cls2 versus Cls1 in bacterial CL accumulation both in the stationary phase and after phagocytosis by human PMN. This difference could reflect differences in *cls1* versus *cls2* gene expression, differences in Cls1 versus Cls2 activity, or both. To determine whether there were differences in levels of *cls1* versus *cls2* mRNA under these experimental conditions, we measured *cls1* and *cls2* mRNA in wt *S. aureus* (SA113) by quantitative RT-PCR after culture in TSB and before and after

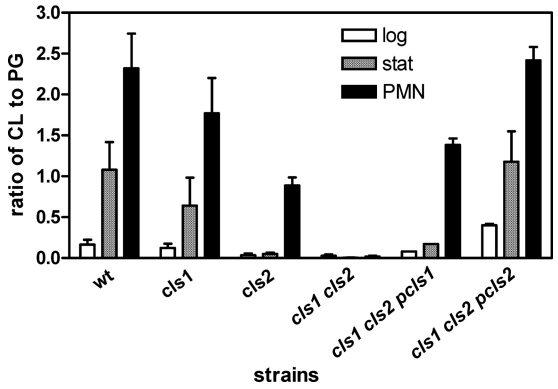

FIG. 4. Ratio of [^{14}C]CL to [^{14}C]PG in wt and mutant *S. aureus* after subculture in TSB for 3 h (log) and 24 h (stat) or 2 h after phagocytosis of log-phase bacteria by PMN. [1-^{14}C]palmitate-labeled PG and CL were resolved by TLC in two dimensions (chloroform-methanol-acetic acid-water (65:25:4:1 [vol/vol/vol/vol] in the first dimension and 80:18:12:5 [vol/vol/vol/vol] in the second dimension) and quantified by image analysis as described in Materials and Methods. The results shown represent the means ± the standard errors of the mean (SEM) of three to eight individual experiments with wt and mutant *S. aureus* SA113. Similar results were observed with wt and mutant *S. aureus* LAC. pcls1 and pcls2 refer to pTKOcls1 and pTKOcls2, respectively.

phagocytosis by PMN (Fig. 6). In early logarithmic growth (3 h), *cls2* mRNA levels were ~2.5-fold higher than that of *cls1* (not shown). *cls2* mRNA levels declined ca. 2- to 4-fold during the next several hours of culture in TSB but rebounded to the earlier (3 h) levels after 24 h culture in TSB. In contrast, the levels of *cls1* mRNA remained virtually constant throughout the 24-h culture period in TSB (Fig. 6A). *cls1* versus *cls2* mRNA levels were also affected differently by phagocytosis by PMN: higher *cls1* mRNA levels were temporarily induced shortly after phagocytosis, whereas *cls2* mRNA levels declined ~5-fold (Fig. 6B). These findings indicate different regulation of the synthesis and/or turnover of *cls1* versus *cls2* mRNA.

DISCUSSION

Our findings demonstrate the existence of two ORFs in *S. aureus*, denoted *cls1* and *cls2*, that each encode proteins that contribute to CL accumulation in *S. aureus*. We have demonstrated roles for both *cls1* and *cls2* in CL accumulation by: (i) creation of isogenic single and double mutants, which showed reduced CL accumulation in logarithmic and stationary phases of growth or after phagocytosis by PMN, and (ii) expression of *cls1* or *cls2* in either CL-deficient *E. coli* or a *cls1 cls2* double mutant of *S. aureus* with restoration of bacterial CL accumulation under those conditions (Fig. 2 to 4). The presence of the same ORFs in each of the *S. aureus* strains for which complete genomic information is available indicates that these proteins are conserved elements of *S. aureus* biochemical machinery.

Both in the stationary phase and after ingestion by neutrophils, Cls2 displayed a much more prominent role than Cls1 in CL accumulation by *S. aureus* (Fig. 3 and 4). This was apparent both by the extent of induction of CL accumulation in single mutants (*cls1* versus *cls2*) and the extent of restoration of CL accumulation by *cls1* versus *cls2* complementation of the *cls1 cls2* double mutant (Fig. 4). These findings confirm and complement the recent findings of Tsai et al. (46) that were published while our study was in review. Of note, in each of the conditions in which Cls1, as well as Cls2, contributed to CL accumulation (i.e., during phagocytosis [Fig. 4] or during culture of the bacteria in high concentrations [15 to 25%] of NaCl, at pH 5, or under anaerobiosis [46]), there was a greater overall accumulation of CL and higher CL/PG ratio, suggesting that Cls1 may be needed and activated when more extensive conversion of PG to CL is required. In the case of long exposures of *S. aureus* to high salinity, activation of either Cls1 or Cls2 appeared to have adaptive value since either single mutant but not the *cls1 cls2* double mutant showed near-normal long-term survival in 15 to 25% NaCl (46).

Together, our findings and those of Tsai et al. (46) clearly establish Cls1 and Cls2 as key elements of the PG→CL adaptive response of *S. aureus.* It should be noted, however, that neither study directly demonstrated the cardiolipin synthase activity of Cls1 or Cls2, i.e., the catalytic conversion of 2 PG to CL plus glycerol. Nevertheless, several considerations argue strongly that Cls1 and Cls2 are cardiolipin synthases: (i) the homology of the proteins encoded by *cls1* and *cls2* to bona fide prokaryotic cardiolipin synthases; (ii) the reduction in bacterial PG that paralleled accumulation of CL in *S. aureus* (Fig. 3A to

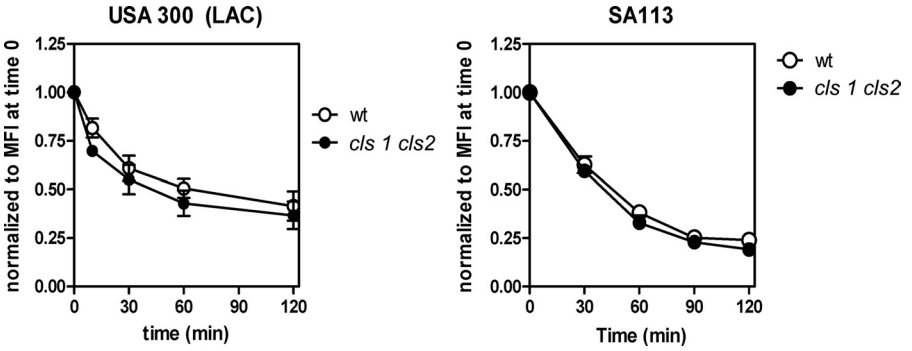

FIG. 5. Bleaching of sGFP-*S. aureus* after phagocytosis by PMN. wt and the double *cls1 cls2* knockout *S. aureus* USA300 LAC (left) and *S. aureus* SA113 (right) expressing sGFP were opsonized and incubated with PMN for 10 min before extracellular bacteria were removed (time zero). The mean fluorescence index (MFI) of gated PMN population harboring sGFP expressing *S. aureus* was assessed at the indicated times by flow cytometry as described in Materials and Methods. The MFIs of samples at each time point are expressed as the percent MFI of the time zero sample for each strain. The data shown represent means ± the SEM of three (USA300 LAC) or five (SA113) independent experiments.

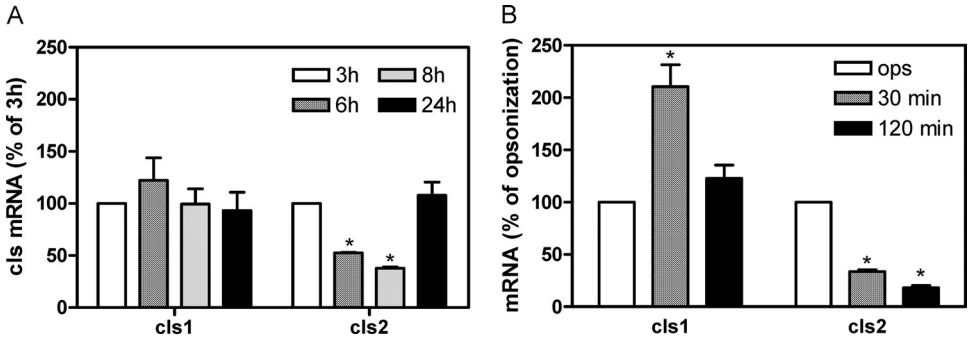

FIG. 6. Levels of *cls1* and *cls2* mRNA in wt *S. aureus* SA113 during growth in TSB (A) and after phagocytosis by PMNs (B). Overnight cultures of *S. aureus* were diluted 100-fold in fresh TSB medium and, after the indicated times in subculture, the levels of *cls1* and *cls2* mRNA were measured by real-time RT-PCR as described in Materials and Methods, expressed relative to 16S rRNA, and normalized to the 3-h sample. *S. aureus* harvested after 3 h of subculture in TSB were opsonized by incubation in the presence of 10% human serum for 20 min at 37°C and then mixed with PMN at MOI of 5. Samples for the assay of *cls1* and *cls2* mRNA were taken before (0 min) and 30 and 120 min after phagocytosis by PMN. The data shown represent means ± the SEM of triplicate determinations from three independent experiments. *, Significant differences as determined by Student *t* test ($P < 0.05$).

D), a finding consistent with the conversion of PG to CL; (iii) the accumulation of CL in CL-deficient *E. coli* carrying plasmids encoding *cls1* or *cls2* (Fig. 2); (iv) the virtual absence of CL accumulation in the *cls1 cls2* double mutant of *S. aureus*; and (v) the fact that in other bacterial species where the biochemical basis of the conversion of PG to CL has been characterized, it was mediated by cardiolipin synthases.

How the various conditions that induce PG→CL conversion in *S. aureus* (e.g., high salinity, hypertonicity, exposure to organic solvents, inhibitors of glycolysis and oxidative phosphorylation, nutrient deprivation, growth stasis, and phagocytosis) affect cardiolipin synthase activity is unknown. Our comparative analyses of *cls1* versus *cls2* mRNA under conditions favoring or not favoring CL accumulation (Fig. 6) suggest that neither the induction of Cls2-dependent CL accumulation in the stationary phase and during phagocytosis nor the greater contribution of Cls2 versus Cls1 under these conditions is regulated by modulating cardiolipin synthase transcript levels. One exception could be the elevation of *cls1* mRNA levels observed during the phagocytosis of *S. aureus* that seems to parallel the greater contribution of Cls1 to CL accumulation in ingested *S. aureus* (Fig. 4). More definitive conclusions, however, must await the development of specific antibodies for Cls1 and Cls2 to permit the measurement of Cls1 and Cls2 protein levels and assays of Cls1 and Cls2 enzyme activity. The relatively acute effects of exposure to organic solvents, hypersalinity, energy poisons, and phagocytes seem most compatible with regulation of CL accumulation occurring being either by allosteric alterations of cardiolipin synthase, alterations of the physical presentation of PG, or both.

The different regulation of *cls1* and *cls2*, at least as manifested by contrasting regulation of mRNA levels, suggests that Cls1 and Cls2 are designed to function in *S. aureus* CL metabolism under different circumstances. Why that would be is not yet clear. Studies in *E. coli* have revealed two different proteins, each with cardiolipin synthase activity, that differ in their ability to degrade CL and thus potentially differ in the extent to which they may assist the bacteria to reverse the effects of CL accumulation when extracellular conditions change (13). CL may also be needed in specialized membrane sites of growing bacteria for initiating DNA replication and protein secretion (5, 17, 53). Thus, diverse proteins may participate in bacterial CL metabolism as needed both for constitutive functions of growing bacteria and stress responses of nongrowing bacteria.

The significance of CL accumulation in nongrowing and ingested *S. aureus* is not known. The growth of *S. aureus* ingested by human neutrophils is rapidly arrested and the majority of the ingested bacteria is killed within 15 to 30 min, whereas most of conversion of bacterial PG to CL takes place 1 to 2 h after phagocytosis (7, 16, 41). Thus, it is not surprising that uptake, killing, and myeloperoxidase-H_2O_2-chloride-dependent bleaching of cytosolic GFP of wt and *cls1 cls2* mutant *S. aureus* by neutrophils were essentially the same. However, neutrophils neither fully eradicate viable ingested *S. aureus* nor, in the absence of mobilized extracellular group IIA phospholipase A_2, digest bacterial phospholipids (7, 16, 41). Therefore, the consequences of the conversion of *S. aureus* PG to CL after phagocytosis by neutrophils may not bear on the intracellular fate of *S. aureus*, as our findings indicate, but rather on subsequent interactions between remaining viable *S. aureus* and bacterial phospholipids with surrounding host tissue, including cellular and extracellular defenses mobilized later in the immune response (51). Studies to examine these possibilities are in progress.

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