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Conditional Mutation of an Essential Putative Glycoprotease Eliminates Autolysis in *Staphylococcus aureus*▽

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Our previous studies demonstrated that a putative *Staphylococcus aureus* glycoprotease (Gcp) is essential for bacterial survival, indicating that Gcp may be a novel target for developing antibacterial agents. However, the biological function of Gcp is unclear. In order to elucidate the reason that Gcp is required for growth, we examined the role of Gcp in bacterial autolysis, which is an important biological process for bacterial growth. Using both a *spacp*-regulated *gcp* expression strain and a TetR-regulated *gcp* antisense expression strain, we found that the down-regulation of *gcp* expression can effectively inhibit Triton X-100-induced lysis, eliminate penicillin- and vancomycin-caused cell lysis, and dramatically increase tolerance to hydrolases. Moreover, we determined whether resistance to lysis is due to a defect in murein hydrolase activity by using a zymogram analysis. The results showed that the cell lysate of a down-regulated *gcp* expression mutant displayed several bands of decreased murein hydrolytic activity. Furthermore, we explored the potential mechanism of Gcp's involvement in autolysis and demonstrated that Gcp may function independently from several key autolysins (Atl, LytM, and LytN) and regulators (ArlRS, Mgr/Rat, and CidA). Taken together, the above results indicate that the essential Gcp is involved in the modification of substrates of murein hydrolases as well as in the regulation of expression and/or activity of some murein hydrolases, which, in turn, may play important roles in bacterial viability.

Staphylococcus aureus is a major animal and human pathogen that causes a wide range of infections (23). The emergence of multidrug-resistant staphylococcal isolates, especially methicillin-resistant *S. aureus*, is generating enormous public health concern and highlights an urgent need for new, alternative agents for treating multidrug-resistant pathogens. Our previous studies demonstrated that a putative glycoprotease (Gcp) is essential for the viability of *S. aureus* and indicated that Gcp may be a potential target for developing novel antibacterial agents (49).

Various glycoprotease homologues have been found in many gram-positive and gram-negative pathogens, including *Bacillus anthracis*, *Streptococcus pyogenes*, *Pasteurella haemolytica* A1 (31), and *Escherichia coli* (29), which have >42% amino acid identity (49). Glycoproteases have a variety of functions. The first discovered glycoprotease of *P. haemolytica* A1 is highly specific for O-glycosylated glycoproteins (1). The Gcp homolog in *E. coli* may be involved in the modulation of a macromolecular operon (29). However, in the cyanobacterium *Synechocystis* sp., mutation of the glycoprotease gene results in a reduction of salt tolerance and alters pigmentation and cyanophycin accumulation (50). For *S. aureus*, although we demonstrated that Gcp is required for bacterial survival, Gcp function and the reasons that Gcp is required for growth are still unclear.

Our preliminary microarray analysis indicated that the down-regulation of *gcp* expression may affect the expression of genes associated with bacterial autolysis (unpublished data). Therefore, we predicted that Gcp may be involved in modulating autolysis of *S. aureus*. Bacterial autolysis plays important physiological roles in cell separation and ongoing peptidoglycan remodeling (6, 25). It has been demonstrated that peptidoglycan (murein) hydrolases are involved in the lysis of bacteria by hydrolyzing either the glycan or the peptide moieties of peptidoglycan in the gram-positive cell wall (38). The major murein hydrolases in staphylococci have been revealed, including *N*-acetyl muramidase, *N*-acetyl glucosaminidase, *N*-acetylmuramyl-L-alanine amidase, transglycosylases, and endopeptidase (36, 39, 40, 43, 44). Autolysin (Atl) is a bifunctional protein containing glucosaminidase and amidase domains, which are separated by proteolytic processing to generate two extracellular lytic enzymes, a 51-kDa glucosaminidase and a 62-kDa amidase, which cleave MurNAc(1-4)GlcNAc and GlcNAc(1-4)MurNAc, respectively (30, 41). More importantly, these murein hydrolases are also associated with other important biological processes in cell division and growth, including cell wall biosynthesis, daughter cell separation, and peptidoglycan recycling (2, 4, 16, 47, 48).

Because murein hydrolases are important for the maintenance of bacterial cell integrity and growth, their activity must be tightly controlled. In *S. aureus*, the expression of the murein hydrolases is coordinately regulated at the transcriptional level by different regulators, including different two-component signal transduction systems. The two-component system *lytSR* is

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involved in the repression of peptidoglycan hydrolases, as the mutation of *lytS* increases hydrolysis and autolysis (8). The *lytSR* system positively regulates the expression of *lrgA* and *lrgB*, whose products are similar to bacteriophage murein hydrolase transporter proteins (known as holins) that are able to inhibit murein hydrolases (9, 15). Another two-component system, *arlRS*, has a negative effect on autolysis (12). Our recent data indicate that the *arlRS* system may function through positive regulation of *lytSR*, *lrgA*, and *lrgB* expression (22). In addition, some murein hydrolase activities are repressed by transcriptional regulators, including *sar* (13) and *rat* (also known as *mgr*) (17), but are positively regulated by *agr* (13) and the *cidAB* operon (37). Moreover, Clp protease activity seems to have a positive impact on the expression of regulators related to murein hydrolases, as the mutation of *clpP* downregulates the expression of *lytSR*, *lrgAB*, *arlRS*, and *rat* (27). On the other hand, the activities of some murein hydrolases are mediated at the posttranslational level, including substrate modification, selective transport, interaction with lipoteichoic acids, etc. (5, 10, 25, 45).

In this study, we report that the essential putative glycoprotease appears to be involved in modifying the substrate (peptidoglycan) of murein hydrolases as well as in modulating the expression and/or activity of some murein hydrolases. Conditional mutation of *gcp* had a lethal effect on bacterial viability and dramatically reduced lysis induced by Triton X-100, penicillin, and vancomycin. Based on our results, we propose that Gcp functions as an important modulator involved in the cell wall biosynthesis pathway associated with the basic physiological process of cell autolysis in *S. aureus*, which, in turn, may play important roles in bacterial survival.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *S. aureus* strains used in this study are listed in Table 1. The bacterial cells were incubated in Trypticase soy broth (TSB) at 37°C, with shaking, unless stated otherwise. *E. coli* cells were grown in Luria-Bertani (LB) medium.

Construction of TetR-regulated *gcp* antisense expression strains. In order to examine the effect of Gcp on autolysis in the wild-type *S. aureus* isolate, the TetR-regulated *gcp* antisense expression vector, pYH4/gcp-as (49), and the control vector, pYH4, were electroporated into strain WCUH29 as described previously (18), resulting in strains WCUH29/gcp-as and WCUH29/pYH4, respectively. In order to determine the effect of the *arlRS* regulator on Gcp function, we utilized the same method and introduced the TetR-regulated *gcp* antisense expression vector, pYH4/gcp-as, into the *arlRS* null mutant and its parent strain, 15981 (46), resulting in strains ΔarlRS/gcp-as and 15981/gcp-as, respectively.

Triton X-100-induced autolysis assays. Autolysis assays were performed as previously described (12). RN4220/Pspac-gcp cells were grown in TSB containing 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and appropriate antibiotics at 37°C, with shaking, to an optical density at 600 nm (OD_{600}) of 1.2 to 1.3. WCUH29/pYH4 and WCUH29/gcp-as cells were grown in TSB containing 5 μg/ml of erythromycin (Erm) at 37°C, with shaking, to an OD_{600} of 1.2 to 1.3. The bacterial cultures were then diluted 1:100 with fresh TSB containing 1 M NaCl, with or without inducer (1 mM IPTG for RN4220/Pspac-gcp and 500 ng/ml anhydrotetracycline [ATc] for WCUH29/pYH4 and WCUH29/gcp-as), and incubated to an OD_{580} of 0.6 to 0.8 at 37°C. The bacterial cells were harvested by centrifugation at 4,000 × *g* and resuspended in the same volume of buffer containing 50 mM Tris-HCl (pH 7.5) and 0.1% Triton X-100. The bacterial cells were then incubated at 30°C with shaking, and the changes in OD_{580} were measured. Results were normalized to the OD_{580} at time zero (OD_0), i.e., percent lysis at time $t = [(OD_0 - \text{OD at time } t)/OD_0] \times 100$. All experiments were repeated at least three times.

Penicillin and vancomycin tolerance assay. To assess the sensitivity of the *gcp* conditional mutants to penicillin and vancomycin, the mutants were incubated in TSB in the presence of the inducer IPTG or the antisense inducer ATc. RN4220/Pspac-gcp cells were grown in TSB containing 1 mM IPTG and appropriate antibiotics at 37°C, with shaking, to an OD_{600} of 1.2 to 1.3. WCUH29/pYH4 and WCUH29/gcp-as cells were grown in TSB containing 5 μg/ml Erm at 37°C, with shaking, to an OD_{600} of 1.2 to 1.3. The bacterial cultures were then inoculated at 1% with fresh TSB in the absence or presence of inducer (1 mM IPTG for RN4220/Pspac-gcp and 500 ng/ml ATc for WCUH29/pYH4 and WCUH29/*gcp-as*) and grown at 37°C, with shaking, to reach exponential phase (OD_{600} of ~0.5). Penicillin G was added to a final concentration of 8 μg/ml (20× MIC), and vancomycin was added to a final concentration of 16 μg/ml (1× MIC). Cultures were incubated continuously, and the OD_{600} values for cultures were measured every hour for 8 h.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
S. aureus strains		
RN4220	Laboratory strain; *rsbU*	19
RN6390	Parental strain of KB350	33
KB350	RN6390 *cidA*::Erm Erm^r	37
WCUH29	Clinical human isolate; $rsbU^+$	18
15981	Clinical human isolate	46
ΔarlRS	15981 with deletion of *arlRS*	46
RN4220/Pspac-gcp	*spacp*-regulated *gcp* mutant	49
RN4220/gcp-as	TetR-regulated *gcp* antisense mutant	49
WCUH29/gcp-as	TetR-regulated *gcp* antisense mutant	This study
ΔarlRS/gcp-as	TetR-regulated *gcp* antisense mutant	This study
Plasmids		
pYH4	Shuttle vector; Erm^r	49
pYH4/gcp-as	Shuttle vector with *gcp* antisense construct; Erm^r	49
pSB2025	Shuttle vector containing *luxABCDE*; Cm^r	34
pCY806	pYH4/gcp-as with promoterless *luxABCDE*; Erm^r	This study
pLZ106	pCY806 with *cidA* promoter-*lux* reporter fusion; Erm^r	This study

Hydrolase-induced autolysis assay. The bacterial cell's susceptibility to extracellular hydrolases was determined as described previously (12). Briefly, the *spacp*-regulated *gcp* mutant was grown in TSB, with or without the inducer IPTG, at 37°C with shaking to reach the exponential phase (OD_{600} of ~0.5). The bacterial cells were heat killed at 121°C for 15 min, collected by centrifugation, and washed with H_2O. The heat-killed cells were resuspended in fresh, filter-sterilized supernatants of overnight cultures of RN4220. The OD_{600} of resuspended dead cells was adjusted to 0.5, and the dead cells were incubated at 37°C with shaking. The OD_{600} was measured every 30 min.

Zymographic analysis. In order to detect the presence of extracellular and intracellular murein hydrolases, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)-based zymographic analyses were performed as described previously (17). Briefly, various strains were grown in TSB, with or without inducers, for 16 h at 37°C with shaking. The extracellular murein hydrolases were isolated from the cultures by centrifugation at 10,000 × *g* for 15 min at 4°C. The supernatants were collected, filter sterilized, and concentrated 100-fold by ethanol precipitation overnight at 4°C. To obtain intracellular and cell wall-associated murein hydrolases, the bacterial cell pellets were washed with phosphate-buffered saline (PBS) and resuspended with PBS. The bacterial cells were lysed with lysostaphin, and the total proteins were evaluated by running the samples in 10% SDS-PAGE. The concentration of total proteins in each sample was determined by using the Bradford assay (Pierce Biotech) according to the manufacturer's instructions. A total of 10 μg of proteins from each sample was resolved in a 10% SDS-PAGE gel containing 0.2% autoclaved and lyophilized *S. aureus* RN4220 wet cells. After electrophoresis, gels were washed with water and incubated overnight in 25 mM Tris-Cl, pH 7.0, containing 1% Triton X-100 at 37°C to allow hydrolysis of the embedded bacterial cells. After incubation, gels were scanned (HP Scanjet 4570c). The zones of hydrolysis appeared as white

TABLE 2. Primers used in this study

N315 open reading frame	Gene	Primer orientation	Primer sequence (5′–3′)[a]
SA1854	*gcp*	Forward	TTGGCGCGCCGTTTAAAGCTATTCAA
		Reverse	GGGATAGTTAATTGAATGTC
SA1248	*arlR*	Forward	TGACAAAGTTGCTGGGCTTGATTAC
		Reverse	TGTGGCTGACGACGTAAAATTGC
SA0252	*lrgA*	Forward	TGAAACAACAAAAAGACGCATCAAAACCAG
		Reverse	ACTTCGCCTAACTTAACAGCACCAG
SA0250	*lytS*	Forward	GCATGGTTCTATCGTCGGTACATTG
		Reverse	ACTTACTTTGCGTTTCGGCTTCAC
SA2329	*cidA*	Forward	GTCTTTTTCTTCATACCGTCAGT
		Reverse	TCATTCATAAGCGTCTACACCT
SA0641	*rat*/*mgr*	Forward	CGCAGATGATTATATGCAAAAC
		Reverse	CGACAGCATCTTGCCAAGTC
SA0905	*atl*	Forward	GCTGGTTATAGTTTAGTTGATGATG
		Reverse	GGTTGTGCTGAAGCGCTAAAAG
SA0265	*lytM*	Forward	GCAGGAGATAACAATGACTACAC
		Reverse	TTACTTGCTGATCCACCATTTTG
SA1090	*lytN*	Forward	AGCTGAACCTGGGGACTTAG
		Reverse	CAACTTTATGTGCAACCTCTGC
SA1206	*femA*	Forward	TCATCGATTACAGACGAAGACAC
		Reverse	TCTTTTAGTTTAGACGGCGCAACC
	16S rRNA	Forward	CTGTTGCACATCTTGACGGTA
		Reverse	TCAGCGTCAGTTACAGACCA
	lux	Forward	TA**GAATTC GCGGCCGC GGCCGGCC AGTACT** AGGTAGG TAATAGGAGGACTCTCTATGAAATTTGGAAACTT (EcoRI, NotI, FseI, and ScaI, respectively)
		Reverse	TA**GAATTC**TCAACTATCAACGCTTCGGTTAAGCT (EcoRI)
SA2329	*cidA* promoter	Forward	AA**GCGGCCGC**CCACTTTGCCAGCTGATCATCA (NotI)
		Reverse	AC**AGTACT**AAATATGTCTAAAATGTTACAATAACT (ScaI)
SA0905	*atl* promoter	Forward	AA**GCGGCCGC**TGATTTTGTTAGCATGTGGAGGAA (NotI)
		Reverse	TT**AGTACT**TGTGCGTATTTAACCAATTTGTA (ScaI)

[a] Restriction sites are indicated in bold, with the corresponding enzyme(s) shown in parentheses after the sequence.

bands in the gels, but black bands indicate regions of murein hydrolase activity in the figures.

RNA isolation and purification. Overnight cultures of *S. aureus* were inoculated in 1% TSB and grown to the mid-exponential (3 h) phase of growth. Cells were harvested by centrifugation, and the RNAs were isolated with an RNAPrep kit (Promega, Madison, WI). Contaminating DNA was removed with a DNA-*free* kit (Ambion), and the RNA yield was determined spectrophotometrically at 260 nm.

Quantitative real-time RT-PCR analysis. In order to examine whether down-regulation of *gcp* expression has any impact on the expression of genes involved in autolysis, we employed quantitative real-time reverse transcription-PCR (RT-PCR) to compare the RNA levels, as described previously (22). The first-strand cDNAs were synthesized using reverse transcriptase with a SuperScript III Platinum two-step qRT-PCR kit (Invitrogen). For each RNA sample, we performed duplicate reverse transcription reactions, as well as a control reaction without reverse transcriptase, in order to determine the levels of DNA contamination. PCRs were set up in triplicate by using SYBR green PCR master mix (Stratagene). Real-time sequence-specific detection and relative quantitation were performed with the Stratagene Mx3000P real-time PCR system. Gene-specific primers were designed to yield ~100-bp specific products (Table 2). Relative quantification of the product was calculated using the comparative cycle threshold method, as described for the Stratagene Mx3000P system. The housekeeping 16S rRNA gene was used as an endogenous control (22). All samples were analyzed in triplicate and normalized against 16S rRNA gene expression. The experiments were repeated at least three times.

Construction of *cidA* promoter-*lux* reporter fusion system. In order to confirm whether the conditional mutation of *gcp* has an impact on *cidA* expression, we created a *cidA* promoter-*lux* reporter fusion system by using a TetR-regulated *gcp* antisense expression vector (49). First, the *luxABCD* genes were obtained from pSB2025 by a PCR using the primers listed in Table 1. They were digested with EcoRI and ligated into the EcoRI site of pYH4/gcp-as vector, which resulted in plasmid pCY806. The *cidA* promoter region was amplified by a PCR using the primers listed in Table 1, digested with NotI and ScaI, and ligated into the NotI and ScaI sites of pCY806, which resulted in plasmid pLZ106, containing a *cidA* promoter-*lux* reporter fusion system. The resulting plasmid, pLZ106, was purified and electroporated into *S. aureus* WCUH29, resulting in strain WCUH29/pLZ106. *lux* expression was monitored with a Chiron luminometer.

Scanning electron microscopy. For scanning electron microscopy, staphylococci were grown overnight in TSB on polystyrene chamber slides at 37°C. After the medium was decanted, the slides were washed three times with 1× PBS, mounted on aluminum stubs, and shadowed with gold. For visualization, a scanning electron microscope (Zeiss DSM962) was used at 15 kV.

RESULTS

Conditional mutation of *gcp* results in bacteriostatic phenotype. Using a *spacp*-regulated *gcp* mutant and a TetR-regulated *gcp* antisense mutant, we demonstrated that the down-regulation of *gcp* expression can have a lethal effect on bacterial growth (49). To investigate the reason that Gcp is essential for survival, we first examined whether the down-regulation of Gcp is bactericidal or bacteriostatic by using the *spacp*-regulated *gcp* expression strain. The results showed that in the absence of the inducer IPTG, no growth was detected during a 30-h period of incubation; in contrast, in the presence of IPTG, the *spacp*-regulated *gcp* expression strain reached the early log phase and stationary phase of growth at 8 and 12 h, respectively (Fig. 1). To test whether the addition of the inducer IPTG was able to revive the bacteria that were originally inoculated and grown in TSB without IPTG, we added different concentrations of IPTG to the 24-h culture and incubated the culture. The capacity of bacterial growth was recovered in a dose-dependent manner after the addition of the inducer (Fig.

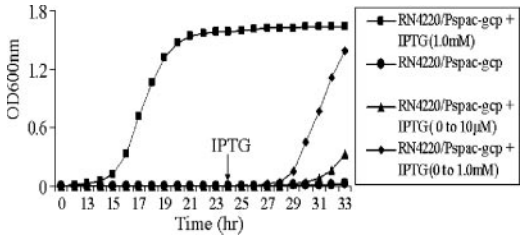

FIG. 1. Growth curves for the *spacp*-regulated *gcp* mutant. The *spacp*-regulated *gcp* mutant was incubated overnight in TSB in the presence of 1 mM IPTG. Bacteria were diluted in fresh TSB and incubated in TSB, with or without IPTG, at 37°C with shaking. Different concentrations of IPTG were added to the bacterial cultures without IPTG 24 h after incubation. The bacterial cultures were continuously incubated at 37°C, with shaking, and the OD_{600} of the cultures was measured every hour.

1). Interestingly, after 32 h of incubation without the inducer IPTG, no cell lysis was observed (Fig. 1). In addition, we counted viable CFU from the 24-h culture without inducer to further confirm the impact of *gcp* on bacterial survival. The results showed that with IPTG induction, the bacteria were still viable (data not shown). Taken together, the above data indicate that the down-regulation of Gcp leads to a bacteriostatic effect.

To determine whether the down-regulation of Gcp has any morphological effect on bacterial cells, we performed light microscopic and electronic microscopic assays. No obvious morphological changes, including the size and appearance of the cell surface, were observed during the down-regulation of *gcp* expression (data not shown).

Down-regulation of *gcp* expression decreases Triton X-100-induced autolysis. Our preliminary microarray data showed that the down-regulation of *gcp* expression has some impact on the transcription of *arlRS* and *cidA*, which are both involved in the regulation of bacterial autolysis (12, 37). Therefore, we hypothesized that Gcp may be involved in the modulation of autolysis and/or cell division pathways, which, in turn, may play important roles in bacterial viability. To test our hypothesis, we first examined the effect of Gcp on susceptibility to cell lysis induced by a nonionic detergent, Triton X-100, using the *spacp*-regulated *gcp* mutant strain. *S. aureus* strains were grown in TSB containing 1 M NaCl and resuspended in a medium containing 0.1% Triton X-100. In the presence of IPTG, >50% of the *spacp*-regulated *gcp* expression cells harvested from a mid-exponential-phase culture lysed within 3 h at 30°C in the presence of 0.1% Triton X-100 (Fig. 2A). Moreover, Triton X-100-induced cell lysis significantly increased in the presence of IPTG, in a dose-dependent manner (Fig. 2A). In contrast, fewer than 20% of the *spacp*-regulated *gcp* mutant cells lysed within 3 h in the absence of IPTG (Fig. 2A).

To further confirm the effect of Gcp on autolysis, we transformed the *gcp* antisense expression vector into wild-type *S. aureus* WCUH29 and examined the Triton X-100-induced autolysis. The results showed that >70% of the control bacterial cells, including the control strain carrying the parent vector pYH4, with or without inducer, and the *gcp* antisense strain without the inducer, lysed within 3 h (Fig. 2B). In contrast, only 15% of the *gcp* antisense mutant cells lysed within 3 h with the

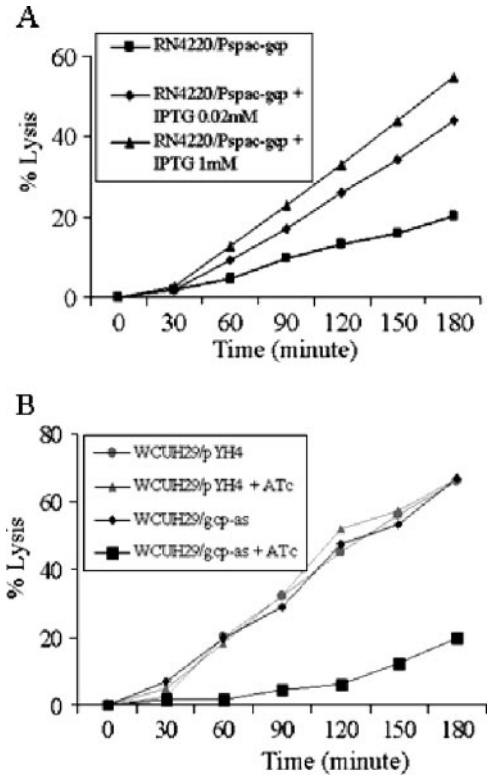

FIG. 2. Triton X-100-induced autolysis of conditional *gcp* mutants. (A) *spacp*-regulated *gcp* mutant RN4220/Pspac-gcp; (B) *gcp* antisense mutant WCUH29/gcp-as. The indicated strains and the control, WCUH29/pYH4, were grown in TSB in the presence of different concentrations of IPTG (1 mM) or ATc (0.5 μg/ml). Results were normalized to the OD_{580} at time zero (OD_0). The percent lysis was determined as follows: percent lysis at time $t = [(OD_0 - OD_t)/OD_0] \times 100$. The experiments were repeated at least three times. Each figure represents the results of one experiment.

induction of *gcp* antisense RNA (Fig. 2B). Taken together, the above results indicate that Gcp is involved in cell autolysis.

Down-regulation of *gcp* expression increases bacterial tolerance to penicillin- and vancomycin-induced autolysis. The β-lactam moiety of penicillin binds to the transpeptidase to inhibit the transpeptidation reaction linking the pentaglycine bridge between two peptidoglycan chains (14). We examined the effect of Gcp on penicillin-induced cell lysis in the presence of 8 μg/ml of penicillin (20× MIC), using the *spacp*-regulated *gcp* mutant as described previously (17). In the presence of the inducer IPTG (1 mM), penicillin-induced cell lysis significantly increased in a dose-dependent manner (Fig. 3A). In contrast, in the absence of IPTG, the *spacp*-regulated *gcp* mutant cells were significantly resistant to penicillin-induced lysis (Fig. 3A). To further confirm the importance of Gcp in cell autolysis, we determined the effect of Gcp on penicillin-induced cell lysis by using the *gcp* antisense mutant created in the wild-type *S. aureus* isolate WCUH29. In the presence of ATc induction, the *gcp* antisense mutant was more resistant to penicillin-induced autolysis than either the mutant without ATc induction or the control strain, whether or not ATc was present in the culture (Fig. 3B). Moreover, the antisense effects shown in Fig. 3B

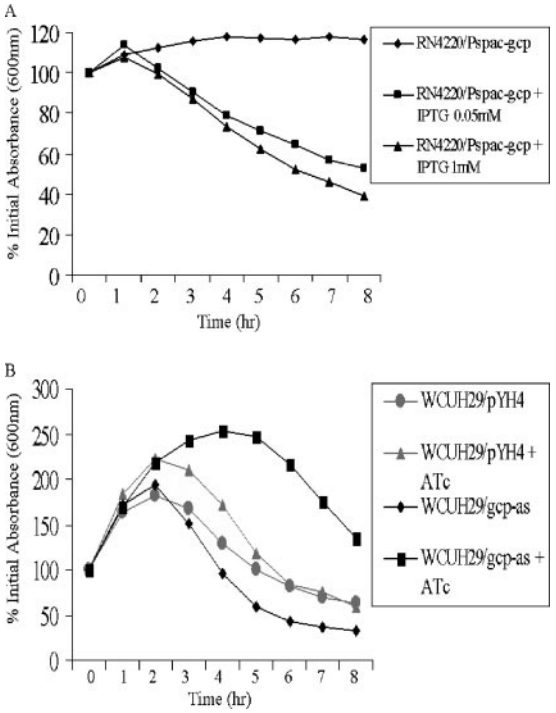

FIG. 3. Effects of penicillin G on the growth of conditional *gcp* mutants. (A) *spacp*-regulated *gcp* mutant RN4220/Pspac-gcp; (B) *gcp* antisense mutant WCUH29/gcp-as. The indicated strains and the control, WCUH29/pYH4, were grown in TSB in the presence of different concentrations of inducer (either IPTG or ATc). Penicillin (20× MIC) was added to the exponential-phase cultures at a final concentration of 8 μg/ml. The bacterial cultures were continuously incubated, and the OD_{600} values for the cultures were measured every hour for 8 h.

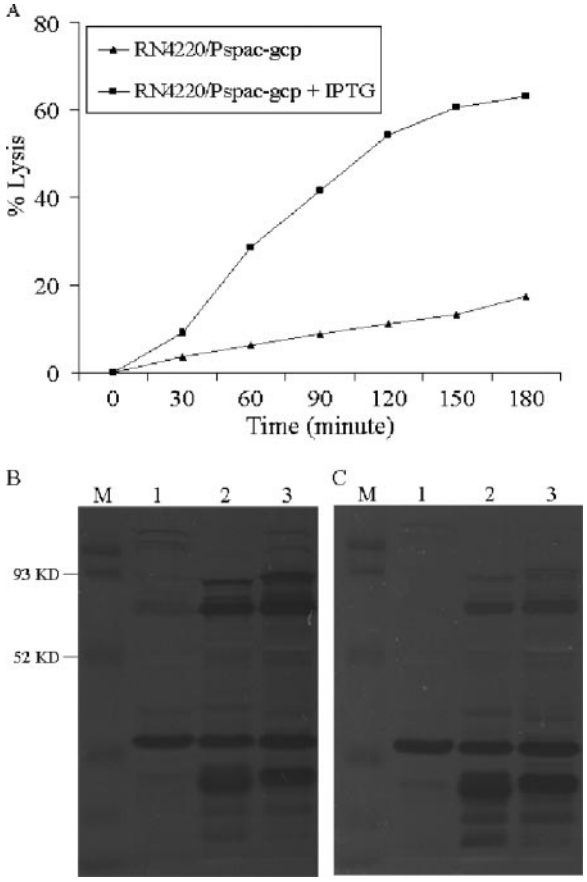

FIG. 4. Effect of down-regulation of *gcp* expression on hydrolase activity. The *spacp*-regulated *gcp* mutant was grown in TSB, with or without the inducer IPTG (1 mM), and the bacterial cells were heat killed, collected by centrifugation, and washed with H_2O. (A) The heat-killed cells were resuspended with fresh filter-sterilized supernatants of overnight cultures of RN4220. The optical density of resuspended dead cells was adjusted to 0.5 at 600 nm, and the dead cells were incubated at 37°C with shaking. The OD_{600} was measured every 30 min. (B and C) Zymogram analysis of the *spacp*-regulated *gcp* mutant. Equal amounts (10 μg) of proteins prepared from the supernatants of different cultures of *S. aureus* strains were loaded and separated in 10% SDS-PAGE gels containing 0.2% heat-killed cells harvested from culture with 1 mM IPTG (B) or without IPTG (C). Lytic bands appeared as dark zones after scanning. M, protein molecular size marker.

resulted from the strong down-regulation of endogenous *gcp* expression (data not shown).

To further determine whether Gcp is involved in the cell wall biosynthesis pathway, we examined the impact of Gcp on bacterial susceptibility to vancomycin by using the *spacp*-regulated *gcp* mutant. Vancomycin is a glycopeptide antibacterial agent that prevents the incorporation of *N*-acetylmuramic acid–peptide and *N*-acetylglucosamine–peptide subunits into the peptidoglycan matrix and inhibits peptidoglycan synthesis by binding to D-Ala-D-Ala (3). Bacterial autolysis plays a role in vancomycin-mediated killing in *S. aureus* (7). The results showed that the down-regulation of *gcp* expression increased bacterial tolerance to vancomycin-induced cell lysis (data not shown). Taken together, the above results indicate that Gcp may be involved in the cell wall peptidoglycan biosynthesis pathway.

Down-regulation of *gcp* expression increases bacterial resistance to hydrolases. In order to further investigate Gcp's association with cell wall synthesis, we examined the effect of Gcp on susceptibility to extracellular murein hydrolases. Bacterial cells were collected from log-phase (OD_{600} of ~0.5) cultures of the *spacp*-regulated *gcp* mutant, with or without IPTG, and then killed. The susceptibility to hydrolases was analyzed using the heat-killed bacterial cells as described previously (12). The results showed that of the heat-killed cells from the culture with IPTG that were incubated with the supernatant collected from the culture of wild-type *S. aureus*, >60% lysed within 3 h (Fig. 4A). In contrast, <20% of the heat-killed cells from the culture without IPTG were lysed (Fig. 4A). We then performed zymographic analysis to compare the sensitivities to hydrolases, as described previously (17). Similarly, the heat-killed cells from the culture with IPTG were more sensitive to different hydrolases, especially to hydrolases of between 52 and 93 kDa (Fig. 4B and C). Collectively, these data demonstrate that Gcp is involved in the modification of the cell wall peptidoglycan biosynthesis pathway.

Down-regulation of *gcp* expression inhibits the expression and/or activity of autolysins. Finally, we determined whether resistant lysis resulted from a defect in murein hydrolase activity, using a zymogram analysis as described previously (17). No significant difference in zymographic patterns was observed for exported hydrolases that were isolated from the supernatants of stationary-phase cultures of either the control strain

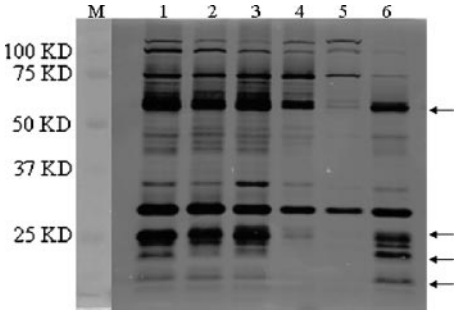

FIG. 5. Zymogram analysis of regulated *gcp* mutants. Equal amounts (10 μg) of proteins prepared from the supernatants of the *gcp* mutants were loaded and separated in 10% SDS-PAGE gels containing *S. aureus* RN4220 cells (0.2% [wet weight] heat-killed cells). Lytic bands appeared as clear zones on an opaque background but showed dark zones after scanning. Lane 1, RN4220/pYH4; lane 2, RN4220/pYH4 with ATc (500 ng/ml); lane 3, RN4220/gcp-as; lane 4, RN4220/gcp-as with ATc (500 ng/ml); lane 5, RN4220/Pspac-gcp without IPTG; lane 6, RN4220/Pspac-gcp with IPTG (1 mM). M, protein molecular size marker.

carrying the parent vector pYH4, with or without the inducer ATc, or the *gcp* antisense strain without inducer (Fig. 5, lanes 1 to 3). In contrast, using the exported hydrolases isolated from the supernatants of stationary-phase cultures of the *gcp* antisense mutant in the presence of the inducer ATc, the zymogram displayed several bands of reduced murein hydrolytic activity (Fig. 5, lane 4 [major changes are indicated by arrows]). This result was further confirmed by using the *spacp*-regulated *gcp* mutant (Fig. 5, lane 5 [without IPTG]).

To explore the possibility that the decreased activities of hydrolases resulted from the impact of down-regulated Gcp on a translocation enzyme, we performed zymogram analyses using whole-cell lysates and examined the activity of the intracellular or cell wall-associated hydrolases. No obvious changes in intracellular hydrolase activity were detected after the down-regulation of *gcp* expression, using both the *spacp*-regulated *gcp* mutant and the *gcp* antisense mutant, compared to controls (data not shown). This suggests that Gcp is not involved in mediating the translocation of murein hydrolases.

Down-regulation of Gcp has different effects on the expression of genes associated with autolysis. To determine the mechanism by which Gcp is involved in bacterial autolysis, we examined the impact of Gcp on the expression of genes associated with autolysis in *S. aureus* by using quantitative RT-PCR. Several regulators, including LrgAB, LytSR, ArlRS, and Rat (Mgr), are repressors of autolytic activity in *S. aureus*, as the mutation of any these regulators has been shown to increase autolysis in *S. aureus* (8, 12, 15, 17, 22). In contrast, the *cidAB* operon is a positive regulator, since the mutation of *cidA* decreases autolysis in *S. aureus* (37). When *gcp* was down-regulated using the *spacp*-regulated *gcp* expression strain, we detected no significant changes in the expression of many genes, including *rat*, *atl*, *lytM*, *lytN*, and *femA*, whereas we observed an ~2-fold increase in *lrgA* expression, a slight increase in *arlR* expression, and a >2-fold decrease in *cidA* expression (Table 3). Similar results were obtained using the regulated *gcp* antisense mutant (data not shown).

To further confirm the effect of Gcp on *cidA* expression, we

TABLE 3. Real-time RT-PCR analysis of gene expression in mid-log phase of growth, using the *spacp*-regulated *gcp* expression strain

N315 open reading frame	Gene	Fold change[a]
SA1854	*gcp*	−16.1
SA1248	*arlR*	1.1
SA0252	*lrgA*	1.9
SA0250	*lytS*	NC
SA2329	*cidA*	−2.1
SA0641	*rat/mgr*	NC
SA0905	*atl*	NC
SA0265	*lytM*	NC
SA1090	*lytN*	NC
SA1206	*femA*	NC

[a] Negative numbers represent genes that were down-regulated without the inducer IPTG (1 mM). NC, no change.

constructed a transcriptional *cidA* promoter-*lux* reporter system by using the regulated *gcp* antisense expression vector. Bioluminescence activity was detected using a luminometer. No light signal was detected in the controls (the regulated *gcp* antisense strain carrying a promoterless *lux* reporter, with or without inducer). In contrast, in the presence of inducer, the luciferase activity significantly decreased in the log-phase cultures compared to that without *gcp* antisense expression (Fig. 6). However, no change in luciferase activity was detected during the down-regulation of *gcp* expression using an *atl* promoter-*lux* reporter system (data not shown).

To investigate the possibility that Gcp may function through the regulation of *cidA*, we compared the autolysis profiles of the *cidA* null mutant and the *spacp*-regulated *gcp* mutant. The magnitudes of increased tolerance to lysis induced by Triton X-100, penicillin, and vancomycin were clearly different between the down-regulated *gcp* expression strain and the *cidA* null mutant (data not shown). In addition, zymographic patterns also showed obvious differences between the down-regulated *gcp* mutant and the *cidA* null mutant (data not shown). These data indicate that despite the fact that Gcp positively affects *cidA* expression, Gcp and CidA may function differently in the regulation of cell autolysis.

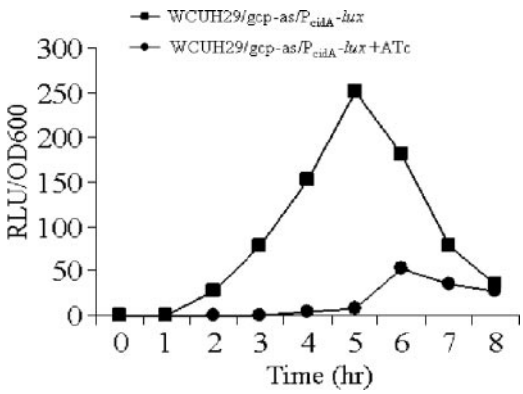

FIG. 6. Expression of *lux* driven by the *cidA* promoter in the *gcp* antisense expression strain WCUH29/gcp-as. Promoter activation was represented as the mean light intensity/OD_{600} ratio from triplicate readings at different times during growth. RLU, relative light units.

Mutation of *arlRS* has no impact on the role of Gcp in the regulation of bacterial autolysis. The two-component system *arlRS* is involved in the negative regulation of autolysis in *S. aureus* (12), and our data above indicate that Gcp positively affects bacterial autolysis. To investigate whether *arlRS* has any impact on the role of Gcp in the regulation of autolysis, we introduced the regulated *gcp* antisense expression vector into an *arlRS* deletion mutant. The down-regulation of *gcp* expression significantly inhibited growth and penicillin-induced autolysis of the *arlRS* null mutant, similar to the case with the parental control (data not shown). In addition, the effects of the mutation in *arlRS* on bacterial susceptibilities to penicillin and hydrolase activities were not complementary to those of the down-regulated *gcp* mutant (data not shown). The above data indicate that mutation of *arlRS* has no influence on the function of Gcp in the regulation of autolysis.

DISCUSSION

The putative glycoprotease (Gcp) is a potential target for a new class of antibacterial agents because it is essential for bacterial survival. However, very little is known about the biological function of Gcp in *S. aureus*. A better understanding of the function of Gcp will allow us to elucidate the reason that Gcp is essential for bacterial survival and enable us to more comprehensively evaluate it as a drug target. It has been reported that murein hydrolases are important for bacterial growth because murein hydrolases target cell wall peptidoglycan during cell division and remodeling of the cell wall (28). In this study, we have demonstrated that Gcp is a critical mediator involved in the modification of cell wall biosynthesis and/or cell division as well as in the regulation of the activities of different murein hydrolases in *S. aureus*.

Our results showed that the down-regulation of *gcp* expression had a lethal effect on growth and dramatically increased the cell's resistance to autolysis induced by detergent and penicillin. It has been reported that cell wall modification and a decrease in autolysis can be produced with protein synthesis inhibitors, including tetracycline and chloramphenicol (20), and we reasoned that a Gcp-specific inhibitor should have a similar effect on autolysis and should not be used together with other cell wall-active antibacterial agents. However, using the MIC assay, we found that the down-regulation of *gcp* expression had no significant impact on bacterial susceptibility to penicillin, vancomycin, and other antibiotics (data not shown). Therefore, the Gcp-specific inhibitor may be a more potent antibacterial agent for the treatment of infections caused by methicillin- and vancomycin-resistant *S. aureus*.

Bacterial cells regulate autolysis through various mechanisms, including by modifying cell wall peptidoglycan (the substrate of murein hydrolases) and regulating the expression and activity of murein hydrolases (8, 15, 17, 26, 37). Our results clearly indicate that Gcp is associated with the modification of cell wall peptidoglycan synthesis, because the consequences of down-regulating *gcp* expression include increased bacterial tolerance to detergent-, penicillin-, and vancomycin-induced lysis. We confirmed a similar effect on autolysis by using both a *spacp*-regulated *gcp* mutant (which was created in the *rsbU* library strain RN4220) and a TetR-regulated *gcp* antisense expression strain (which was constructed in the *rsbU*$^+$ wild-type strain WCUH29). We also found that the down-regulation of *gcp* expression led the heat-killed bacterial cells to be resistant to exported murein hydrolases (Fig. 4). On the other hand, our data show that Gcp in *S. aureus* also partially contributes to the positive regulation of different murein hydrolases. When *gcp* was down-regulated, the activities of certain murein hydrolases (size ranges of ~60 kDa and 20 to 25 kDa) were either decreased or abolished in the conditional *gcp* mutants (Fig. 5). We now have studies in progress to investigate the cell wall composition when Gcp is depleted and to identify which hydrolases are affected by Gcp. In addition, we found an increased tolerance to lysis induced by penicillin and vancomycin after the down-regulation of *gcp* expression, suggesting that Gcp may play important roles in bacterial persistence after the bacterial population is exposed to antibiotics, although the mechanism of bacterial persistence is still unclear (21).

Since Gcp is essential for viability, we predicted that Gcp may be involved in the modulation of autolysins. In *S. aureus*, Atl is an important cell wall-associated autolysin involved in cell separation, cell lysis, and release of cell wall material at the cell surface, and it is essential for penicillin-induced autolysis (11, 40, 43). In addition, glycylglycine endopeptidase (LytM) and cell wall hydrolase (LytN) are two major secreted enzymes involved in cell lysis (35, 42). After down-regulating *gcp* expression, we found no significant impact on the expression of Atl, LytM, and LytN, and no difference in hydrolytic activity was observed in the size range of ~37 kDa, the molecular mass of Gcp in *S. aureus*. However, we did find that the down-regulation of *gcp* expression inhibited several extracellular hydrolase activities (Fig. 5). These data indicate that Gcp is likely associated with extracellular hydrolase activity distinct from those of Atl, LytM, and LytN. However, we cannot rule out the possibility of the posttranslational regulation of hydrolase activity by Gcp.

In order to further understand the mechanisms of Gcp involvement in autolysis, we explored whether the impact of Gcp on autolysis may occur through the *arlRS*-regulated *lytSR* and *lrgAB* pathway. The two-component regulatory system LytSR negatively modulates the expression of murein hydrolases and positively regulates the expression of the *lrgAB* operon (9). The *lrgA* gene product, LrgA, seems to be analogous to an antiholin, which negatively controls extracellular murein hydrolase activity by inhibiting the transport of murein hydrolase across cell membranes (15). Our recent findings indicate that another two-component signal system, ArlSR, positively regulates *lytSR* expression and may be indirectly involved in mediating autolysis (22). Our studies have demonstrated that Gcp negatively affects the expression of *lrgA*, which is consistent with the decreased autolytic activities in the down-regulation of *gcp* expression mutants. However, we found that the mutation of the *arlRS* regulator had no influence on the growth-deficient phenotype and elevated tolerance to penicillin-induced lysis of the conditional *gcp* mutant. Another important global regulator, Mgr/Rat, has been demonstrated to negatively modulate autolytic activity by differentiated regulation of the expression of *lytSR*, *lrgAB*, *arlRS*, *lytM*, *lytN*, and *cidA* (17, 24), but our studies showed that the down-regulation of *gcp* expression had no obvious effect on *rat* expression. Taken together, these findings suggest that Gcp involved in the modulation of autolysis may function independently from *arlRS* and *rat* regulons.

The *cidAB* operon encodes the holin-like counterpart of the *lrgAB* operon and is a positive regulator of cell autolysis in *S. aureus* (37). The results reported here showed that Gcp positively affects *cidA* expression at the transcriptional level and led us to predict that Gcp's involvement in autolysis may be through the modulation of the *cidAB* operon. However, we found that the conditional *gcp* mutants displayed completely different magnitudes of resistance to autolysis and showed distinct zymographic patterns of murein hydrolases compared to the *cidA* mutant. Moreover, our studies indicate that Gcp plays critical roles in controlling cell lysis in the log phase of growth, whereas *cidA* has less of an effect on cell lysis during this period of growth, which is consistent with a previous report that a mutation in *cidA* inhibits cell lysis in the stationary phase but has only a minimal influence on survival (32). Thus, Gcp's involvement in the regulation of autolysis may be independent of the *cidAB* regulon. Moreover, a bioinformatic analysis of Gcp motifs indicated that Gcp is not a DNA-binding protein, because it has no helix-turn-helix DNA-binding structure (data not shown). These data suggest that Gcp may indirectly influence *cidA* expression.

Taking these results collectively, Gcp's involvement in the modification of cell wall biosynthesis is likely an important reason that it is essential for bacterial survival. We now have studies in progress to investigate the mechanism of Gcp's involvement in cell wall biosynthesis, using comprehensive genomic and proteomic approaches. The outcome of this research will provide additional insights into the complex mechanisms of bacterial cell autolysis as well as help us to elucidate the biological function of this essential glycoprotease in *S. aureus*.

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