The C-Terminal Domain of the Novel Essential Protein Gcp Is Critical for Interaction with Another Essential Protein YeaZ of Staphylococcus aureus

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Abstract

Previous studies have demonstrated that the novel protein Gcp is essential for the viability of various bacterial species including Staphylococcus aureus; however, the reason why it is required for bacterial growth remains unclear. In order to explore the potential mechanisms of this essentiality, we performed RT-PCR analysis and revealed that the gcp gene (sa1854) was co-transcribed with sa1855, yeaZ (sa1856) and sa1857 genes, indicating these genes are located in the same operon. Furthermore, we demonstrated that Gcp interacts with YeaZ using a yeast two-hybrid (Y2H) system and in vitro pull down assays. To characterize the Gcp-YeaZ interaction, we performed alanine scanning mutagenesis on the residues of C-terminal segment of Gcp. We found that the mutations of the C-terminal Y317-F322 region abolished the interaction of Gcp and YeaZ, and the mutations of the D324-N329 and S332-Y336 regions alleviated Gcp binding to YeaZ. More importantly, we demonstrated that these key regions of Gcp are also necessary for the bacterial survival since these mutated Gcp could not complement the deletion of endogenous Gcp. Taken together, our data suggest that the interaction of Gcp and YeaZ may contribute to the essentiality of Gcp for S. aureus survival. Our findings provide new insights into the potential mechanisms and biological functions of this novel essential protein.

Introduction

The prevalence of methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant S. aureus (VRSA) has caused serious public health concerns worldwide [1,2]. The limited options of antibiotics for the treatment of infections associated with MRSA and/or VRSA highlight an urgent need for the development of novel potent antimicrobial agents. Bacterial essential proteins are potential targets for the development of new classes of antibiotics [3,4]; however, the biological function of many essential proteins is still unclear. The characterization and validation of functional unknown essential proteins are therefore of great importance to assess their suitability as targets for the development of the novel antibiotics.

Our previous studies have indicated that a novel essential protein Gcp is a potential target for the development of new classes of antibacterial agent against MRSA and/or VRSA [5,6]. Gcp is homologous to the Gcp first identified in Mannheimia haemolytica (formerly Pasteurella haemolytica) and specifically cleaves O-sialoglycosylated protein [7]. Gcp homologs are ubiquitous in all three kingdoms of life, with the exception of the endosymbiotic bacteria Carnobacterium munitum and Salmo salar, which possess highly reduced genomes [8,9]; however, these Gcp homologs do not exhibit glycoprotease activity. It has been revealed that these Gcp homologs are required for cell viability of many bacterial species examined to date, including S. aureus [10], Streptococcus pneumoniae [10], Escherichia coli [11], Bacillus subtilis [12,13], Francisella novicida [14], Pseudomonas aeruginosa [15], and Mycoplasma genitalium [16]. Additionally, the protein is important for eukaryotes, such as Saccharomyces cerevisiae [17] and Arabidopsis thaliana [18]. We have demonstrated that S. aureus Gcp plays an important role in the process of bacterial autolysis, suggesting a potential role in the cell wall biosynthesis pathway [6], however, the reason why Gcp is required for bacterial viability remains elusive.

Structural analysis of Gcp homologs shows that Gcp belongs to the ASKHA (acetyl and sugar kinases, HSP70 and actin) superfamily [18–22]. A conserved metal binding motif HXEXH is inserted within the HSP70 (heat-shock protein70)-actin-like fold (HALF), suggesting a metal binding ability and an ATP dependent protease activity [18]. The crystal structure analysis of the Gcp Pyrococcus abyssi ortholog, Pa-Kae1, has revealed that Pa-Kae1 is multifunctional, binding iron and ATP, as well as possessing DNA binding and apurinic endonuclease activity [22], whereas the Gcp homolog in yeast, Kae1 (kinase-associated endopeptidase 1), is a component of the KEOPS/EKC (kinase, endopeptidase and other proteins of small size/endopeptidase-like and kinase associated to transcriptional chromatin) complex that are required for telomere maintenance and transcription of essential eukaryotic genes [21,23,24]. Collectively, the above data indicate that Gcp homologs may possess different functions among different species.

Recently, it has been reported that in E. coli, an essential protein YeaZ binds to YigD (the Gcp homolog in E. coli) and YjeE, which
are both essential for E. coli growth [25,26]. YeaZ is a bacterial specific member of the ASKHA superfamily. It is also essential for growth of a variety of bacterial species [3,10–15], such as S. aureus [10] and E. coli [11]. Crystal structural analyses reveal that the E. coli [27], Salmonella typhiurium and Thermotoga maritme YeaZs possess a HALF fold, but lack the metal binding motif and the ATP binding site [28,29]. This suggests that YeaZ may bind to a nucleotide through an interaction with a small molecule ligand or a partner protein to adopt an active conformation [28]. In addition, EYeaZ can specifically cleave YgdD [26], but SYeaZ does not exhibit such an enzymatic activity [28].

Sequence alignment analysis revealed that S. aureus Gcp, YeaZ, and SA1857 proteins are the homologs of YgdD, YeaZ, and YjeE in E. coli, respectively [26], however, whether these proteins interact with each other in S. aureus is currently unknown. In this study, we utilized the genetic and biochemical approaches and demonstrated that the staphylococcal Gcp binds to YeaZ. Importantly, we have identified the key domains of Gcp that are important for Gcp-YeaZ interaction, as well as critical for Gcp’s essentiality for S. aureus growth.

Results

Both gcp and yeaZ genes are located in the same operon

Genomic DNA sequence alignment for S. aureus revealed that the sequences of sa1857, yeaz (sa1856), sa1855, and gcp (sa1854) are sequentially overlapped (Fig. 1A). It was revealed that the first 47 bps of yeaz overlap with the last 47 bps of sa1857; the first 28 bps of sa1855 overlap with the last 28 bps of yeaz and the first 8 bps of gcp overlap with the last 8 bps of sa1855. This data suggests that the four genes might be co-transcribed from a common promoter. To examine this possibility, we performed RT-PCR using the forward primers P1for, P2for, and P3for specifically binding to sa1855, yeaz and sa1855, respectively, and a common reverse primer P4rev specifically binding to gcp. The RT negative controls using total RNA as a template did not yield a PCR product (Fig. 1B, lane 2, 5, 8). In contrast, when cDNA was used as a template, PCR products of 1.4 kb, 800 bp, and 400 bp were obtained, corresponding to the expected sizes (Fig. 1B), indicating that the genes are co-transcribed from at lease one common promoter.

Staphylococcal Gcp interacts with YeaZ

In E. coli the Gcp homolog, YgdD, interacts with YeaZ [25,26]; in S. aureus, sa1857, yeaz, sa1855, and gcp genes are localized to the same operon. These led us to hypothesize that in S. aureus Gcp may interact with YeaZ, SA1855, and/or SA1857 and function coordinately. To test this hypothesis, we utilized a yeast two-hybrid system by fusing Gcp, YeaZ, SA1855 and SA1857 separately with the GAL4 activation domain (GAD) and the GAL4 DNA binding domain (GBD), respectively. Two different fusion plasmids were co-transformed into the yeast PJ9-4A competent cells. The results showed that the negative controls carrying pGAD-gcp/pGBD, or pGAD/pGBD empty vectors did not grow in the media lacking His/Leu/Trp, and media lacking Ade/Leu/Trp (Fig. 2A). In contrast, yeast cells carrying pGAD-gcp/pGBD-yeaz grew normally on the above selective media, indicating a possible binding interaction between Gcp and YeaZ (Fig. 2A). No other interactions were revealed among these four proteins (data not shown). In addition, yeast cells carrying either pGAD-yeaz/pGBD-gcp or pGAD/pGBD-gcp grew in the above selective media, suggesting that GBD-Gcp fusion may interact with the activation domain leading to an auto-activation of the reporter (data not shown).

To further confirm the interaction between staphylococcal Gcp and YeaZ, we performed in vitro pull down assays using the purified His-tagged Gcp and GST-YeaZ fusion proteins. The GST tag was used to immobilize YeaZ on glutathione Sepharose resin. Western blot analysis showed that the YeaZ bound resin was able to retain the refolded Gcp, whereas the control resin alone could not retain the Gcp (Fig. 2B), indicating that the recombinant staphylococcal Gcp specifically binds to the recombinant staphylococcal YeaZ.

The overexpression of Gcp in E. coli resulted in the recombinant protein forming inclusion bodies, thus the recombinant protein was purified under denaturing conditions, and refolded by a series of dialysis. Sequence analysis of Gcp revealed four cysteine residues in the C-terminus, thus it is possible for the purified recombinant Gcp molecules to form intermolecular disulfide bonds, which would result in malfunctioning Gcp polymers. To rule out this possibility, we examined the integrity of unfolded and refolded recombinant Gcp proteins by SDS-PAGE under non-reducing condition and did not reveal any oligomers of Gcp (data not shown).

C-terminal Y317–Y336 segment is crucial for the staphylococcal Gcp binding to YeaZ

In order to identify which domains of staphylococcal Gcp are important for the Gcp-YeaZ interaction, we created nine different truncated Gcp mutants by PCR (Fig. 3A). These truncated Gcp
segments were fused with the activation domain of pGAD, respectively, and the resulting recombinant plasmids were utilized to conduct Y2H analyses with pGBD-yeaZ. The results showed that the yeast cells carrying Gcpgseg1 mutant (E337–E341 truncated Gcp) and YeaZ grew on both SC-His, -Leu, -Trp simplifying synthetic complete (SC-His, -Leu, -Trp) plates with 3-amino-1,2,4,-triazole (3-AT) and SC plates lacking adenine, leucine, and tryptophan drop out synthetic complete (SC-Ade, -Leu, -Trp). Gcp was fused with the activation domain and YeaZ was fused to binding domain. The minus signs indicate empty vector controls. (B) In vitro immunoprecipitation analysis of interaction between Gcp and YeaZ. His-tagged Gcp and GST-tagged YeaZ were purified from E. coli. Gcp and YeaZ were incubated with Glutathione Sepharose 4B beads together or separately. Western blotting was carried out with rabbit anti-Gcp serum to detect Gcp. Lane 1: purified Gcp protein was loaded as a positive control.

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Figure 2. The determination of Gcp binding to YeaZ. (A) Yeast two hybrid analysis of interaction between Gcp and YeaZ on histidine, leucine, and tryptophan drop out synthetic complete (SC-His, -Leu, -Trp) plates with 3-amino-1,2,4,-triazole (3-AT) and SC plates lacking adenine, leucine, and tryptophan (SC-Ade, -Leu, -Trp). Gcp was fused with the activation domain and YeaZ was fused to binding domain. The minus signs indicate empty vector controls. (B) In vitro immunoprecipitation analysis of interaction between Gcp and YeaZ. His-tagged Gcp and GST-tagged YeaZ were purified from E. coli. Gcp and YeaZ were incubated with Glutathione Sepharose 4B beads together or separately. Western blotting was carried out with rabbit anti-Gcp serum to detect Gcp. Lane 1: purified Gcp protein was loaded as a positive control.

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We have previously demonstrated that Gcp is essential for S. aureus growth [5,6], but the molecular mechanisms of Gcp’s essentiality remain elusive. The discovery that Gcp interacts with YeaZ led us to hypothesize that their interaction is important for Gcp’s essentiality. In order to examine this hypothesis, we first created the Gcp complementary system (Fig. 5A). Then, we examined the complementary effect of gcp expression in trans by measuring the bacterial growth during the depletion of endogenous Gcp. The results showed that the growth of the staphylococcal Gcp complementary strain was IPTG-independent, indicating the gcp expression in trans complements the depletion of endogenous Gcp (Fig. 5B), whereas the growth of the parental control was IPTG-dependent (Fig. 5C). These data further demonstrated the requirement of Gcp for bacterial viability in culture and the feasibility of the Gcp complementary system.

Next, we determined whether the identified segment and regions that are crucial for Gcp interaction with YeaZ are also required for growth by examining the complementary effect of the mutated Gcp genes. The results showed that the growth of the Pspac-regulated gcp expression strain carrying complementary Gcpgseg1 was independent of IPTG (Fig. 5D), indicating that the expression of Gcpgseg1 in trans can complement Gcp and the C-terminal region E337–E341 is dispensable for Gcp’s essentiality. In contrast, the growth of the Pspac-regulated gcp expression strain carrying complementary Gcpgseg2 was still dependent on IPTG (Fig. 5E). To determine which domain is critical for growth, we examined the complementary effect of Gcp317-322A, Gcp324-329A and Gcp332-336A, and found that the Pspac-regulated gcp expression strains carrying Gcp317-322A (Fig. 5F), Gcp324-329A (Fig. 5G), or Gcp332-336A (Fig. 5H) mutant exhibited IPTG dependent growth, indicating that these mutated Gcp are unable to complement the depletion of endogenous Gcp. These data suggest that the three domains, Y317–Y336, are likely critical for Gcp’s essentiality.

Staphylococcal Gcp alanine mutants cannot complement wild type Gcp for bacterial growth

No O-sialoglycoprotein endopeptidase activity was detected for the recombinant staphylococcal Gcp and YeaZ proteins

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To determine whether the novel essential proteins Gcp and YeaZ possess an O-sialoglycoprotein endopeptidase activity, the purified recombinant Gcp and YeaZ were utilized to treat O-sialoglycoprotein, glycoporphin A, according to the manufacturer’s instruction. The degradation of glycoporphin A protein was detected by SDS-PAGE and western blot using a monoclonal
or absence of Mg\(^{2+}\), Mn\(^{2+}\), Ca\(^{2+}\), Zn\(^{2+}\) (Fig. 6B).

**Discussion**

In the present study, we employed genetic and biochemical approaches and demonstrated that in the _gcp_ operon of _S. aureus_, the essential _Gcp_ protein interacts with another essential protein _YeaZ_. More importantly, we identified key domains of _Gcp_ that are not only required for _Gcp_ binding to _YeaZ_, but also play important roles in _Gcp_'s essentiality for bacterial growth. Our results suggest that the interaction of _Gcp_ and _YeaZ_ may contribute to the essentiality of _Gcp_. In addition, our results showed that there was neither O-sialoglycoprotein endopeptidase activity nor _Gcp_ specific protease activity detected for the purified recombinant staphylococcal _Gcp_ and/or _YeaZ_, suggesting that the staphylococcal _Gcp_ and _YeaZ_ may function differently compared to their homologs in _M. haemolytica_ and _E. coli_, respectively. These findings may provide new insights into the molecular mechanisms and biological function of the essential protein, _Gcp_.

The identification of the staphylococcal _Gcp-YeaZ_ interaction is consistent with the previous report that the _Gcp_ homolog in _E. coli_, YigD, binds to _YeaZ_ the homolog of SA1856 [25,26]. However, in _E. coli_ another essential protein, YjeE, also interacts with _YeaZ_, suggesting that _YjeE_ may function as a regulator to modulate _YeaZ-YigD_ interaction, and that the _YjeE-YeaZ-YigD_ network may be involved in an essential cellular process [26]. In contrast, in _S. aureus_ there is no report indicating that the homolog of _YjeE_, SA1857, is indispensable for bacterial viability [10]. We are in the process of examining the requirement of SA1857 and SA1855 for the growth of _S. aureus_. Furthermore, in our _Y2H_ studies we found no evidence that SA1857 and SA1855 interacts with either _Gcp_ or _YeaZ_. Although the staphylococcal _YeaZ_ and _Gcp_ proteins have approximately 26% and 42% identity compared to _E. coli_ _YeaZ_ and _YigD_ sequences, respectively, our studies indicate that _Gcp_ and _YeaZ_ likely function differently or interact with different partner proteins compared to their homologs in _E. coli_. We observed that in _Y2H_ assays the growth of yeast cells on SC-His plates seems to be faster than on SC-Ade plates. This is likely due to the stringent growth difference between SC-Ade and SC-His selective medium [33]. Although we unveiled that the mutation of Y317-F322 region eliminated the ability of _Gcp_ to bind _YeaZ_, and the mutations of D324-N329 and S332-Y336 regions alleviated the capacity of _Gcp_ to bind _YeaZ_, the attenuated binding ability may be attributable to the altered tertiary conformation of _Gcp_. To determine the potential impact of topology for mutated _Gcp_ proteins is beyond scope of the present study. However, we are currently working to further characterize and define the interaction of _Gcp_ and _YeaZ_ and identify the critical residue(s) for _Gcp_ to bind _YeaZ_ and vice versa.

In both yeast and archaea, Kae1p (Gcp homolog) directly interacts with a Bud32p kinase, inhibiting the kinase activity, which is required both for transcription and the telomere homoeostatic function of the endopeptidase-like kinase chromatin-associated (EKC)/kinase, endopeptidase, and other peptidases of small size (KEOPS) in yeast cells [21,23,24]. In eukaryotes, the _Gcp_ homolog is Qri7 in yeast and OSGEPL1 (O-sialoglycoprotein endopeptidase) in worm and human. Qri7 and OSGEPL1 anchor to the mitochondrial inner membrane and are essential for the maintenance of mitochondrial genome [18,34]. Qri7 is able to complement the deletion of _E. coli_ YigD (homolog of _Gcp_), whereas Kae1 fails to do so, suggesting the functional similarity between Qri7 and YigD [34]. Although bacteria lack a Bud32 homolog, _YeaZ_ may substitute for Bud32 to form a functional complex with _Gcp_. Our complementation experiments established that the mutations of _Gcp_ that disrupt the _Gcp-YeaZ_ interaction could not complement the deletion of endogenous _Gcp_. We also observed that the deletion of the C-terminal residues, E337–E341, slightly impaired the ability of _Gcp_ to interact with _YeaZ_ (Fig. 3B); and consequently, this truncated _Gcp_ (Gcpsseg1) delayed its complementary effect on bacterial growth (longer lag-phase of growth). These data suggest that the staphylococcal _Gcp-YeaZ_ interaction may play an important role in _Gcp_’s essentiality; thus the interruption of _Gcp-YeaZ_ interaction may be utilized as a novel mode of action for developing new classes antibacterial agents, partially against MRSA or VRSA caused infections. It is known that bacterial autolysis is important for bacterial cell division and growth. Previously, we have demonstrated that the staphylococcal _Gcp_ is a critical modulator of bacterial autolysis [6]; however, the mechanism of regulation of autolysis by _Gcp_ remains to be determined; it is necessary to explore the potential role of the _Gcp-YeaZ_ interaction in the process of autolysis.
Our purified recombinant staphylococcal Gcp and YeaZ did not appear to have an O-sialoglycoprotein endopeptidase activity against glycoporphin A. We also employed an alternative approach to examine the potential glycoprotease activity by measuring cell associated ligand, PSGL1 (P-selectin glycoprotein ligand 1), using FACS. Neither the purified recombinant staphylococcal Gcp, YeaZ, nor the concentrated supernatants of \textit{S. aureus} culture appeared to have the glycoprotease activity (data not shown). Our results are in agreement with previous reports that YgjD (homolog of Gcp in \textit{E. coli}) and Kae1 (homolog of Gcp in \textit{Pyrococcus abyssi}) do not exhibit any endopeptidase activity [20,26]. In addition, \textit{E. coli} YeaZ is able to specifically cleave YgjD (the homolog of Gcp), whereas consistent with \textit{S. typhimurium} YeaZ [28] our purified soluble recombinant staphylococcal YeaZ did not exhibit such a protease activity. The lack of protease activity of purified recombinant Gcp and YeaZ may result from potential modifications during expression and purification process. However, it is likely that the Gcp and YeaZ homologs may have different biological functions among different species, because sequence alignment analysis revealed that the staphylococcal Gcp does not possess any \textit{E. coli} YeaZ cleavage sites, including K171- L172 and F195-V196 residues that were identified in YgjD [26]. In addition, we cannot exclude the possibility that the staphylococcal Gcp and YeaZ proteins may possess a specific proteolytic activity against a substrate in a process that is critical for the viability of \textit{S. aureus}.

In conclusion, we demonstrated within the four novel proteins encoded by the gcp operon, the essential Gcp interacts with another essential protein YeaZ of \textit{S. aureus}. Moreover, we identified the C-terminal Y317-F322, D324-N329, and S332-Y336 regions to be important for Gcp to bind YeaZ, as well as for Gcp’s essentiality, whereas the C-terminal E337-E344 region is dispensable for the staphylococcal Gcp-YeaZ interaction and Gcp’s essentiality. These data suggest that the interaction of Gcp and YeaZ may at least partially contribute to the essentiality of Gcp for \textit{S. aureus} growth. Our findings provide new insights into the potential mechanisms and biological function of the novel essential protein, Gcp, as well as potential novel targets for the development of new classes antibacterial agents.

Materials and Methods

Bacterial strain and culture medium

The bacterial strains and plasmids used in the study are listed in Table 1. \textit{E. coli} strain, DH10B, was used as a host for recombinant plasmid construction and amplification. Luria-Bertani (LB) liquid medium and LB-agar plates were used for the growth and maintenance of \textit{E. coli}. The \textit{E. coli} strain BL21(DE3) was used as a host strain for the expression of recombinant Gcp and YeaZ. Ampicillin was used at 100 μg/ml in LB media for the selection of \textit{E. coli} carrying the plasmid pGEX4T-1, pMY1107, or Yeast two-hybrid (Y2H) vectors pGAD-G1 and pGBD-C1. Kanamycin was used at 50 μg/ml in LB media for the selection of \textit{E. coli} carrying plasmid pET24b. A \textit{Saccharomyces cerevisiae} strain, PJ69-4A, was used in Y2H studies [33]. The PJ69-4A strain has three reporter genes for a positive Y2H interaction: \textit{HIS3} driven by \textit{GAL1} promoter, \textit{ADE2} by the \textit{GAL2} promoter, and \textit{lacZ} by the \textit{GAL7} promoter. YEPD (1% yeast extract, 2% peptone, 2% dextrose, pH 6.0) and synthetic complete (SC) liquid medium and plates were used for \textit{S. cerevisiae} growth. Where indicated, certain components were dropped out from the SC media. A \textit{Pspac}-regulated \textit{gcp} mutant of \textit{S. aureus} was constructed as previously described [5], which was used as a host cell for complementation experiments. \textit{S. aureus} was grown in Tryptic Soy Broth (TSB) containing erythromycin (5 μg/ml) and different concentrations of isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37°C with shaking.

RNA purification and RT-PCR analysis

Overnight cultures of \textit{S. aureus} were used at 1% inoculum in TSB medium and grown to the medium-exponential phase of growth. Cells were harvested by centrifugation, and the total RNA...
was isolated by using the RNAprep kit (Promega, MI). Contaminating DNA was removed with a TURBO DNA-free Kit (Ambion), and the RNA yield was determined spectrophotometrically at 260 nm. The cDNA was synthesized by employing RNA as a template using the Promega-reverse transcription kit. Co-transcription of gcp operon was determined by RT-PCR using primers listed in Table 2.

Examination of protein interactions using the Y2H system

To examine possible protein-protein interaction, we employed a yeast two-hybrid (Y2H) technology as described [33,35]. The staphylococcal gcp, sa1855, yeaZ and sa1857 genes were amplified by PCR using the primers listed in Table 1. PCR products were purified, cloned into EcoRI and BglII sites of pGAD-C1 two-hybrid vector containing GAL4 transcription activation domain (GAL4AD) and BamHI and SalI sites of pGBD-C1 vector containing GAL4 DNA-binding domain (GAL4BD), respectively. The yeast strain PJ69-4A was co-transformed with the two fusion plasmids using the high efficiency LiAc-PEG method [35]. The above strains were incubated overnight in TSB in the presence of appropriate antibiotics and 0.2 mM IPTG. Bacteria were diluted and incubated in fresh TSB containing appropriate antibiotics and different concentration of IPTG at 37°C with shaking. The cell growth was monitored by measuring OD600 nm, every 15 min for 18 h with 1 min mixing before each reading.

Cloning, expression and purification of recombinant Gcp-His tag fusion protein and GST-YeaZ fusion proteins

The staphylococcal gcp and yeaZ genes were obtained by PCR using the primers listed in Table 2 and cloned into NdeI and Xhol sites of pET21b and BamHI and SalI sites of pGEXKT-1, respectively. The recombinant plasmids were transformed into E. coli strain BL21 (DE3), respectively. An approximately 500 ml culture of each strain in LB medium was incubated aerobically at 37°C to an optical density (OD) at 600 nm of 0.6. The recombinant protein expression was subsequently induced by the addition of 1 mM IPTG. After 5 h of incubation at 37°C, cells were harvested. For the purification of Gcp-His fusion proteins, the cell pellet was collected and resuspended in the binding buffer (300 mM NaCl, 50 mM NaH2PO4, 8 M urea, 10 mM imidazole, pH 8.0) with the addition of 1 mg/ml lysozyme and 1 mM PMSF at room temperature for 1 h. The cells were lysed by sonication on ice; after centrifugation at 25,000 g for 20 min at 4°C, the supernatant was collected and applied onto Ni-NTA resin (Qiagen) under denaturing condition according to the manufacturer’s instructions. The purified recombinant Gcp proteins were refolded by dialysis using a series of dialysis buffers (300 mM NaCl, 50 mM NaH2PO4, 10% glycerol with 4 M, 2 M, 1 M, 0 M urea, final buffer 150 mM NaCl, 20 mM NaH2PO4, pH 7.3). For the purification of GST-YeaZ proteins, the cell pellet was resuspended in the binding buffer (150 mM NaCl, 20 mM NaH2PO4, pH 7.3) containing 1 mg/ml lysozyme and 1 mM PMSF at room temperature for 1 h. The cells were lysed by sonication on ice; after centrifugation at 25,000 g for 20 min at 4°C, the supernatant was collected and mixed with Glutathione Sepharose 4B beads (GE Healthcare) according to the manufacturer’s instructions. The purified recombinant proteins were confirmed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Coomassie bright blue staining; the protein concentration was measured using the BCA™ Protein Assay kit (Thermo Scientific).

In vitro pull down assay

Purified GST-YeaZ fusion protein was mixed with Glutathione Sepharose 4B resin at room temperature and allowed to bind for 30 min. A total of 15 μl of resin bound GST-YeaZ was used by incubating with the bacterial lysate (1 μg of purified Gcp) and bacterial extract (1 μg of recombinant Gcp) for 1 h at 4°C. After centrifugation, the supernatant was collected and analyzed using SDS-PAGE and Western blot.
protein was incubated with the molar equivalents of purified Gcp-His tag protein for 2 h at room temperature, washed 6 times with phosphate buffer solution using the equivalent volume of 100 times the bead bed volume, and eluted by mixing with 2× the bead bed volume of elution buffer containing 10 mM reduced glutathione. The elution fraction was subjected to SDS-PAGE and standard western blotting using rabbit anti-Gcp serum.

Table 1. Strains and plasmids used in this study.

| Strain or plasmid | Description | Reference |
|-------------------|-------------|-----------|
| Strains           |             |           |
| RN4220            | Laboratory strain; rsbU<sup>+</sup> | [39] |
| WCUH29            | Clinical human isolate; rsbU<sup>+</sup> | [40] |
| JRN0105           | JRN0105 with plasmid pXL108 | This study |
| JRN0110           | JRN0105 with plasmid pMY1107 | This study |
| JRN0210           | JRN0105 with plasmid pLT109 | This study |
| JRN0310           | JRN0105 with plasmid pLT209 | This study |
| JRN0410           | JRN0105 with plasmid pLT309 | This study |
| JRN0510           | JRN0105 with plasmid pLT409 | This study |
| JRN0610           | JRN0105 with plasmid pLT509 | This study |
| PJ69-4A           | MATa trp1-901 leu2-3, 112 ura3-52 his3-200 gpd1::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ | [33] |
| Plasmids          |             |           |
| pGEX4T-1          | Overexpression vector, can be used to add GST tag to the N terminal of protein of interest | GE Healthcare |
| pET-24b           | Overexpression vector, can be used to add His tag to the N or C terminal of protein of interest | Novagen |
| pGAD-C1/pGBD-C1   | Y2H vector carrying GAL4 transcription activation/DNA binding domain | [33] |
| pGAD/gcp          | pGAD-C1 carrying gcp | This study |
| pGAD/gcpg1        | pGAD-C1 carrying gcpg1 mutant | This study |
| pGAD/gcpg2        | pGAD-C1 carrying gcpg2 mutant | This study |
| pGAD/gcpg3        | pGAD-C1 carrying gcpg3 mutant | This study |
| pGAD/gcpg4        | pGAD-C1 carrying gcpg4 mutant | This study |
| pGAD/gcpg5        | pGAD-C1 carrying gcpg5 mutant | This study |
| pGAD/gcpg6        | pGAD-C1 carrying gcpg6 mutant | This study |
| pGAD/gcpg7        | pGAD-C1 carrying gcpg7 mutant | This study |
| pGAD/gcpg8        | pGAD-C1 carrying gcpg8 mutant | This study |
| pGAD/gcpg9        | pGAD-C1 carrying gcpg9 mutant | This study |
| pGAD/gcp1-1       | pGAD-C1 carrying gcpl-1 mutant | This study |
| pGAD/gcp1-2       | pGAD-C1 carrying gcpl-2 mutant | This study |
| pGAD/gcp1-3       | pGAD-C1 carrying gcpl-3 mutant | This study |
| pGAD/sa1855       | pGAD-C1 carrying sa1855 | This study |
| pGAD/yeaZ         | pGAD-C1 carrying yeaZ | This study |
| pGAD/sa1857       | pGAD-C1 carrying sa1857 | This study |
| pGBD/gcp          | pGBD-C1 carrying gcp | This study |
| pGBD/sa1855       | pGBD-C1 carrying sa1855 | This study |
| pGBD/yeaZ         | pGBD-C1 carrying yeaZ | This study |
| pGBD/sa1857       | pGBD-C1 carrying sa1857 | This study |
| pMY1107           | pFF40 inserted with TetR regulation region | This study |
| pXL108            | pMY1107 carrying gcp | This study |
| pLT109            | pMY1107 carrying gcpg1 mutant | This study |
| pLT209            | pMY1107 carrying gcpg2 mutant | This study |
| pLT309            | pMY1107 carrying gcpl-1 mutant | This study |
| pLT409            | pMY1107 carrying gcpl-2 mutant | This study |
| pLT509            | pMY1107 carrying gcpl-3 mutant | This study |

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Table 2. Primers used in this study.

| Name       | Sequence                                                                 |
|------------|--------------------------------------------------------------------------|
| P1for      | CTGATGAAAGATTAGGTTTGTAGA                                                 |
| P2for      | TTAAGGCTAAATTACAGGTAAGATG                                                |
| P3for      | TTACGAATCAAGCTCAAATTACAACAG                                               |
| P4rev      | ACTAAGGCTTGGTATTTGTTGTGA                                                 |
| gcfor1     | GGATCCGCGGCGGCGGCCTGGCTGCTGCTGTTAGGATTTAATTAAATATAG                     |
| gcrev1     | TTAGTTCTTCTTCCGCAAAACTCTCTCTCA                                           |
| gcseg1     | TTAGTTCTTCTTCCGCAAAACTCTCTCTCA                                           |
| gcprev2    | TTAGTTCTTCTTCCGCAAAACTCTCTCTCA                                           |
| gcseg3     | TTAGTTCTTCTTCCGCAAAACTCTCTCTCA                                           |
| gcseg4     | TTAGTTCTTCTTCCGCAAAACTCTCTCTCA                                           |
| gcseg5     | TTAGTTCTTCTTCCGCAAAACTCTCTCTCA                                           |
| gcseg6     | TTAGTTCTTCTTCCGCAAAACTCTCTCTCA                                           |
| gcseg7     | TTAGTTCTTCTTCCGCAAAACTCTCTCTCA                                           |
| gcseg8     | TTAGTTCTTCTTCCGCAAAACTCTCTCTCA                                           |
| gcseg9     | TTAGTTCTTCTTCCGCAAAACTCTCTCTCA                                           |
| gcseg10    | TTAGTTCTTCTTCCGCAAAACTCTCTCTCA                                           |
| gcseg11    | TTAGTTCTTCTTCCGCAAAACTCTCTCTCA                                           |
| gcseg12    | TTAGTTCTTCTTCCGCAAAACTCTCTCTCA                                           |
| gcseg13    | TTAGTTCTTCTTCCGCAAAACTCTCTCTCA                                           |

Alanine scanning mutagenesis

To identify the domains of Gcp important for binding to YeaZ, we performed alanine scanning mutagenesis assays. Five to six amino acids of alanine per stretch replaced the native amino acids and were generated by PCR using the primers listed in Table 2 as previously described [36]. In brief, six pairs of primers were designed, including Gcpfor1 with Gcp1-1revSacII, Gcp1-2revSacII, and Gcp1-3revSacII (Table 2). Three different 5' end segments and 3' end segments of gp were amplified by PCR, ligated to SacII site, and reformed mutated gcp1-1, gcp1-2, and gcp1-3, respectively. These mutated genes encode three different C-terminal alanine stretch mutants, Gcp317-322A, Gcp324-329A, and Gcp332-336A, respectively.

Construction of complementary plasmid and characterization of bacteria growth

In order to examine whether the expression of wild type or the constructed alanine mutants of Gcp in trans are able to complement the depletion of endogenous Gcp, we created a complementary plasmid using a shuttle vector, pFF40, which expresses the lacI gene [37]. A TetR regulatory cassette, obtained by PCR, was inserted into the SacII site of pFF40 and the new plasmid was designated as pMY1107. The wild type and alanine mutant gcp genes were obtained by PCR using the primers listed in Table 2 and cloned into the Pml and AscI sites of pMY1107, resulting in plasmids pXL108, pLT109, pLT209, pLT309, pLT409, and pLT509. The recombinant plasmids were electrophorated into the Ppsac-regulated gcp S. aureus mutant [5]. The growth curves of transformed S. aureus were obtained using an automated microtiter plate format on SpectraMaxPlus384 spectrophotometer (Molecular Devices). S. aureus strains were incubated in TSB with appropriate antibiotics and different concentrations of IPTG (0, 5, 20, 50 and 100 μM) at 37°C. Cell growth was monitored by measuring OD600 nm every 15 min with 1 min mixing before each reading.

Enzymatic activity analysis

To identify the domains of essential Gcp and YeaZ possessing any O-sialoglycoprotein endopeptidase activity or protease activity, purified recombinant Gcp and YeaZ proteins were utilized to treat O-sialoglycoprotein glycoporphin A (Sigma) as described [38]. The degradation of glycoporphin A protein was detected by SDS-PAGE and western blotting using a monoclonal antibody against glycoporphin A. To examine the effect of different ions, including magnesium, calcium, and zinc on the potential enzymatic activity, the purified recombinant Gcp and/or YeaZ was incubated with glycoporphin A in individual reaction buffers containing different concentrations of Mg2+, Mn2+, Ca2+, or Zn2+ at 37°C for 3 h.

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Author Contributions

Conceived and designed the experiments: TL BW YJ. Performed the experiments: TL XL JY MY LZ BW. Analyzed the data: TL BW YJ. Wrote the paper: TL YJ.

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