Knockout of *ykb*, a putative glycosyltransferase, leads to vancomycin resistance in *Bacillus subtilis*

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Running Head: Role of *ykb* in *B. subtilis* vancomycin resistance

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ABSTRACT

Vancomycin resistance of gram-positive bacteria poses a serious health concern around the world. In this study, we searched for vancomycin-resistant mutants from a gene deletion library of a model gram-positive bacterium, *Bacillus subtilis*, to elucidate the mechanism of vancomycin resistance. We found that knockout of *ykcB*, a glycosyltransferase that is expected to utilize C55-P-glucose to glycosylate cell surface components, caused vancomycin resistance in *B. subtilis*. Knockout of *ykcB* altered the susceptibility to multiple antibiotics, including sensitization to β-lactams, and increased the pathogenicity to silkworms. Furthermore, the *ykcB*-knockout mutant had: i) an increased content of diglucosyl diacylglycerol, a glycolipid that shares a precursor with C55-P-glucose, ii) a decreased amount of lipoteichoic acid, and iii) decreased biofilm formation ability. These phenotypes and vancomycin resistance were abolished by knockout of *ykcC*, a *ykcB*-operon partner involved in C55-P-glucose synthesis. Overexpression of *ykcC* enhanced vancomycin resistance in both wild-type *B. subtilis* and the *ykcB*-knockout mutant. These findings suggest that *ykcB* deficiency induces structural changes of cell surface molecules depending on the *ykcC* function, leading to resistance to vancomycin, decreased biofilm formation ability, and increased pathogenicity to silkworms.
Although vancomycin is effective against gram-positive bacteria, vancomycin-resistant bacteria is a major public health concern. While the vancomycin resistance mechanisms of clinically important bacteria such as *Staphylococcus aureus*, *Enterococcus faecium*, and *Streptococcus pneumoniae* are well-studied, they remain unclear in other gram-positive bacteria. In the present study, we searched for vancomycin-resistant mutants from a gene deletion library of a model gram-positive bacterium, *Bacillus subtilis*, and found that knockout of a putative glycosyltransferase, *ykcB*, caused vancomycin resistance in *B. subtilis*. Notably, unlike the previously reported vancomycin-resistant bacterial strains, *ykcB*-deficient *B. subtilis* exhibited increased virulence while maintaining its growth rate. Our results broaden the fundamental understanding of vancomycin-resistance mechanisms in gram-positive bacteria.
INTRODUCTION

Antibiotics are widely used to treat bacterial infections, but the emergence of antibiotic-resistant bacteria is now a common and intractable problem. Vancomycin is a glycopeptide antibiotic that inhibits the polymerization of peptidoglycans in the cell wall of gram-positive bacteria by binding D-alanyl D-alanine residues. The emergence of vancomycin-resistant strains of Staphylococcus aureus, Streptococcus pneumoniae, and Enterococcus faecium poses a serious problem (1). In particular, vancomycin is one of the few effective antibiotics against methicillin-resistant S. aureus (MRSA), a bacterium resistant to many antibiotics, including β-lactams. Several cases of vancomycin-resistant MRSA have been reported (2). Understanding the mechanisms of vancomycin resistance is critical toward the development of new, effective antibiotics.

Vancomycin-resistant S. aureus is classified into 2 types: vancomycin-resistant S. aureus (VRSA) and vancomycin intermediate-resistant S. aureus (VISA) (2). While the vancomycin MIC of vancomycin-sensitive S. aureus is typically 0.5–2 μg/mL, the MICs for VRSA and VISA are ≥16 μg/ml and 4–8 μg/ml, respectively (3). The vancomycin resistance of VRSA is caused by acquisition of the E. faecium vanA gene, which encodes D-alanyl D-lactate ligase and changes the peptidoglycan structure (4). On the other hand, the vancomycin resistance of VISA is caused by gene mutations that result in cell wall thickening, such as rpoB (5), graS (6), walK (7), and sdrC (8). VRSA and VISA have reduced growth rates compared with vancomycin-sensitive S. aureus (8-11). The attenuation of virulence in VISA was demonstrated in a mouse model of sepsis and a Galleria mellonella infection model (12-14). VISA also has reduced ability to form biofilm, which is suggested to correlate with both the decreased virulence and vancomycin resistance (15, 16). While the molecular mechanisms underlying
vancomycin resistance in clinically important bacteria, including *S. aureus*, have been studied, they remain unclear in other gram-positive bacteria.

In the present study, we searched for vancomycin-resistant mutants from a gene deletion library of a model gram-positive bacterium, *Bacillus subtilis*, and identified that knockout of *ykcB*, a putative glycosyltransferase, caused vancomycin resistance. *ykcB* is proposed to act cooperatively with *ykcC*, another glycosyltransferase, and *yngA*, a flippase on the plasma membrane (17). After *ykcC* produces the lipid phosphate carrier C55-P-glucose from UDP-glucose on the cytoplasmic side of the plasma membrane, C55-P-glucose is flipped to the outer surface of the plasma membrane by the function of *yngA*, and finally *ykcB* transfers a glucose from C55-P-glucose to some cell surface components (17). Here, we report that *ykcB* deficiency induces structural changes of the cell surface, such as a decreased amount of lipoteichoic acid, in a *ykcC*-dependent manner, leading to resistance to vancomycin.

**RESULTS**

**Knockout of *ykcB* changes antibiotic susceptibility and increases virulence in silkworms**

We searched for vancomycin-resistant strains among 3967 strains of the *B. subtilis* gene deletion library (18) and identified 23 strains with higher resistance to vancomycin than the parent strain (Table 1). Among the vancomycin-resistant strains, the *ykcB*-knockout mutant (Δ*ykcB*) exhibited the highest vancomycin resistance (Fig. 1A). To confirm that the vancomycin resistance was induced by *ykcB* knockout, we examined whether *ykcB* expression cancelled the vancomycin resistance of Δ*ykcB*. Introduction of *ykcB* into the *amyE* locus decreased vancomycin resistance in Δ*ykcB*, whereas introduction of empty
vector into the amyE locus did not affect vancomycin resistance (Fig. 1B). These results suggest that loss of ykcB function leads to vancomycin resistance in *B. subtilis*.

We examined the sensitivity of ΔykcB to antibiotics other than vancomycin. ΔykcB became sensitive to the cell wall synthesis inhibitors ampicillin, oxacillin, and ceftazidime, and the DNA synthesis inhibitor levofloxacin (Fig. 1C). On the other hand, ΔykcB became resistant to the protein synthesis inhibitor chloramphenicol and showed no change in sensitivity to tetracycline (Fig. 1C). These results indicate that ykcB deficiency alters the susceptibility to various antibiotics.

As vancomycin-resistant *S. aureus* strains are known to have attenuated pathogenicity (12-14), we investigated the pathogenicity of ΔykcB using the silkworm infection model. Contrary to our expectation, silkworms injected with ΔykcB died earlier than the parent strain, indicating increased virulence of ΔykcB (Fig. 1D). ΔykcB showed the same growth rate as the parent strain in nutrient medium (Fig. 1E), ruling out the possibility that the change in pathogenicity against silkworms depends on the bacterial growth rate.

**Vancomycin resistance in ΔykcB is cancelled by the knockout of ykcC**

To reveal more details of the mechanism of vancomycin resistance by ykcB knockout, we deleted the chromosomal region around the ykcB gene because ykcB and ykcC form an operon and are thought to be functionally related (19). Knockout of mhqA and ykcC, which respectively locate upstream and downstream of ykcB, did not lead to vancomycin resistance (Fig. 2A, 2B). Deletions of the intergenic region between mhqA and ykcB (delA and delB) caused slightly higher vancomycin resistance than that of the parent strain, but lower than that of ΔykcB (Fig. 2A, 2C). Deletions in the ykcB coding region (delC, delD, delE, and delF) caused vancomycin resistance comparable to that of ΔykcB (Fig. 2A, 2C).
Introduction of a stop codon mutation in the \( ykcB \) gene in the \( delA \) background \((\text{delA/}\text{ykcBstop})\) caused a vancomycin-resistant phenotype to the same extent as \( \Delta ykcB \), but introduction of a stop codon mutation into both the \( ykcB \) and \( ykcC \) genes in the \( delA \) background \((\text{delA/}\text{ykcBstop/}\text{ykcCstop})\) did not cause a vancomycin-resistant phenotype, indicating that \( ykcC \) knockout cancelled the effect of \( ykcB \) knockout (Fig. 2A, 2C). These findings suggest that \( ykcB \) deficiency confers vancomycin resistance to \( B. \ subtilis \) in the presence of \( ykcC \).

**Knockout of \( ykcB \) increases the amount of diglucosyl diacylglycerol**

According to the UniProt database, YkcB is predicted to be a glycosyltransferase with 14 transmembrane domains that belongs to the glycosyltransferase 39 family. In addition, it is assumed that YkcB utilizes C55-P-glucose, which is synthesized from UDP-glucose by YkcC, to glycosylate some cell surface molecules (17). Therefore, we hypothesized that \( ykcB \) knockout leads to the accumulation of C55-P-glucose and UDP-glucose, which results in an increased amount of diglucosyl diacylglycerol produced from UDP-glucose. \( \Delta ykcB \) and the \( \text{delA/}\text{ykcBstop} \) mutants had increased amounts of diglucosyl diacylglycerol compared with the parent strain, indicating that the \( ykcB \) knockout increases the amount of diglucosyl diacylglycerol (Fig. 3A, 3B). In contrast, the \( ykcC \) knockout and the \( \text{delA/}\text{ykcBstop/}\text{ykcCstop} \) mutants did not have increased amounts of diglucosyl diacylglycerol (Fig. 3A, 3B), indicating that the \( ykcB \) knockout increases the amount of diglucosyl diacylglycerol in a \( ykcC \)-dependent manner.

To determine whether diglucosyl diacylglycerol contributes to vancomycin resistance in \( B. \ subtilis \), we examined the effect of knocking out the \( ugpP \) gene, which encodes a diglucosyl diacylglycerol synthetase. As expected, the \( ugpP \) knockout mutant did not
produce diglucosyl diacylglycerol (Fig. 3C). The ugpP knockout mutant showed vancomycin sensitivity indistinguishable from that of the parent strain (Fig. 3D). Therefore, diglucosyl diacylglycerol does not contribute to vancomycin resistance.

Knockout of ykcB decreases the amount of lipoteichoic acid and attenuates biofilm-forming ability

Based on the observation that ΔykcB exhibits altered sensitivity to antibiotics, increased killing activity against silkworms, and an increased amount of diglucosyl diacylglycerol, we hypothesized that knockout of ykcB alters the amount of lipoteichoic acid or changes biofilm formation, both of which have important roles in antibiotic resistance and virulence. ΔykcB and the delA/ykcBstop mutants had decreased amounts of lipoteichoic acid (Fig. 4A, 4B). ΔykcB and the delA/ykcBstop mutants formed less biofilm than the parent strain and the delA mutant, respectively (Fig. 5A, 5B). These findings suggest that the ykcB knockout decreases the amount of lipoteichoic acid, and decreases biofilm forming ability. The delA/ykcBstop/ykcCstop mutant did not have a decreased amount of lipoteichoic acid or decreased biofilm formation (Fig. 4A, 4B, 5A, 5B), indicating that ykcC knockout cancelled the phenotypic changes caused by ykcB knockout.

Overexpression of ykcC increases vancomycin resistance

Because ykcC knockout cancelled the vancomycin resistance caused by the ykcB knockout, expression of ykcC is hypothesized to have a positive role in vancomycin resistance. To evaluate this hypothesis, we transformed the parent and ΔykcB strains with a multicopy plasmid encoding FLAG-tagged ykcC under the ykcBC native promoter. Western blot analysis revealed that the expression of FLAG-tagged ykcC was higher in
$\Delta ykcB$ than in the parent strain (Fig. 6A), suggesting that positive feedback triggered by
$ykcB$ knockout upregulates the $ykcBC$ promoter. The parent and $\Delta ykcB$ strains
transformed with FLAG-tagged $ykcC$ exhibited higher vancomycin resistance than those
transformed with an empty vector (Fig. 6B). In addition, the $\Delta ykcB$ strain transformed
with FLAG-tagged $ykcC$ exhibited slightly higher vancomycin resistance than the parent
strain transformed with FLAG-tagged $ykcC$ (Fig. 6B). These findings suggest that $ykcC$
confers vancomycin resistance to $B.\ subtilis$ in an expression-dependent manner.

**DISCUSSION**

The findings of the present study revealed that knockout of $ykcB$, a putative
glycosyltransferase gene, confers $B.\ subtilis$ resistance against vancomycin in a $ykcC$-
dependent manner. Knockout of $ykcB$ also leads to bacterial sensitivity to beta-lactams,
decreases the amount of lipoteichoic acids, attenuates biofilm formation, and increases
silkworm-killing activity. This study is the first to reveal that knockout of a specific gene
leads to vancomycin resistance in $B.\ subtilis$.

The $ykcC$ knockout mutant did not exhibit the same phenotypes as the $ykcB$ knockout
mutant. In addition, in the $ykcC$-stop codon mutant background, the stop codon mutation
of $ykcB$ led to no phenotypic changes. Therefore, the $ykcC$ gene is required for the
phenotypic changes triggered by $ykcB$ knockout. Furthermore, overexpression of $ykcC$
increases vancomycin resistance in the $B.\ subtilis$ parent strain and the $ykcB$ knockout
strain. These findings suggest that expression of $ykcC$ as well as knockout of $ykcB$
increases the amount of some biologic molecule that leads to vancomycin resistance (Fig.
7). A previous study predicted that YkcC catalyzes UDP-glucose to C55-P-glucose and
YkcB transfers glucose from C55-P-glucose to some cell surface molecule (17) (Fig. 7).
Considering this prediction and the findings of the present study, C55-P-glucose might accumulate in the \( ykb \)-knockout mutant and lead to various phenotypic changes, including vancomycin resistance (Fig. 7). C55-P-glucose is utilized as a sugar donor by YfhO to glycosylate lipoteichoic acid (17, 20). C55-P acts as an anchor to synthesize wall teichoic acid and peptidoglycan (21). Therefore, accumulation of C55-P-glucose might alter the structures of peptidoglycan, lipoteichoic acid, and wall teichoic acids, which may underlie the phenotypic changes observed in the \( ykb \)-knockout mutant.

In the \( ykb \)-knockout mutant, the amount of diglucosyl diacylglycerol was increased. We speculate that the accumulation of C55-P-glucose in the \( ykb \)-knockout mutant increases UDP-glucose, a precursor of C55-P-glucose, and the increase in UDP-glucose leads to an increase in diglucosyl diacylglycerol (Fig. 7). Because knockout of \( ugp \), a synthetase gene of diglucosyl diacylglycerol, did not alter vancomycin resistance (Fig. 3D), the increased amount of diglucosyl diacylglycerol in the \( ykb \)-knockout mutant does not contribute to the vancomycin resistance. In addition, in the \( ykC \)-knockout mutant and the \( ykb \text{stop}/ykC \text{stop} \) mutant, diglucosyl diacylglycerol was not increased. In the absence of \( YkC \), UDP-glucose might be utilized for molecules other than diglucosyl diacylglycerol, which would prevent the accumulation of diglucosyl diacylglycerol (Fig. 7).

The \( ykb \)-knockout mutant was resistant to vancomycin, but sensitive to beta-lactams. In VISA, a vancomycin resistance phenotype is accompanied by a beta-lactam sensitive phenotype, referred to as a "seesaw phenomenon" (22, 23). Thus, the beta-lactam sensitivity of the \( ykb \)-knockout mutant of B. subtilis is consistent with that of VISA. The amounts of penicillin-binding protein 2 or phosphatidylglycerols are proposed to contribute to the beta-lactam sensitivity of VISA (24, 25). Further investigation is needed...
to examine penicillin-binding protein 2 and phosphatidylglycerols in the ykB-knockout B. subtilis mutant. This study demonstrated that the ykB-knockout mutant has increased silkworm killing activity. In our previous study, Escherichia coli mutant strains resistant to vancomycin also showed resistance to antimicrobial peptides and increased silkworm-killing activity (26-28). The B. subtilis ykB-knockout mutant might have resistance against silkworm antimicrobial peptides. In addition, a lipoteichoic acid synthetase gene knockout mutations in Staphylococcus aureus exhibits increased virulence against Drosophila melanogaster (29), leading to the proposal that lipoteichoic acid is a target molecule of Draper-dependent phagocytosis and lipoteichoic-deficient mutant bacteria escape the phagocytosis (29). The ykcB-knockout mutant might escape phagocytosis by silkworm immune cells because the ykcB-knockout mutant has little lipoteichoic acid. In conclusion, this study identified that knockout of ykb leads to vancomycin resistance in B. subtilis. The ykb-knockout mutant exhibited increased virulence in silkworms, in contrast to VISA. Molecular investigation of vancomycin resistance using B. subtilis, a model gram-positive bacterium, is important to understand the conserved mechanism of vancomycin resistance between bacterial species.

**MATERIALS and METHODS**

**Bacterial strains and culture conditions**

B. subtilis 168 trpC2 and its mutant strains were aerobically cultured in LB broth at 37°C. B. subtilis mutant strains carrying an erythromycin resistance gene were grown on LB plates containing erythromycin (1 µg/ml) and the colonies were aerobically cultured in LB broth without antibiotics at 37°C. B. subtilis strains transformed with pHY300PLK or
pDR110 were cultured in LB broth containing tetracycline (30 µg/ml) or spectinomycin (50 µg/ml). Bacterial strains and plasmids used in this study are listed in Table 2.

Screening of vancomycin-resistant strains
The BKE library (18) was cultured in LB broth using a 96-well microplate at 37°C and the bacterial culture was spotted onto LB plates with or without vancomycin (0.45 µg/ml) using a replicator. The plates were incubated overnight at 37°C and mutant strains whose colonies appeared on vancomycin-containing plates were searched. The experiments were repeated and the strains were judged as vancomycin resistant when they formed colonies on vancomycin-containing plates in 2 experiments.

Silkworm killing assay
Third instar silkworms were purchased from Ehime Sansyu (Ehime, Japan) and raised to fifth instar larvae by feeding them an artificial diet (Silkmate 2S; Nihon Nosan Kogyo Co., Kanagawa, Japan) at 27°C (30-32). The fifth instar hatched silkworms were fed an antibiotic-free artificial diet (Sysmex Co., Hyogo, Japan) for 1 day and used for infection experiments. B. subtilis overnight culture was diluted 5-fold with 0.9% saline and 0.05 ml was injected into the silkworm hemolymph using a tuberculin syringe equipped with 27-gauge needle. The OD₆₀₀ values of B. subtilis overnight cultures were measured to confirm that the injected bacterial numbers were the same between strains.

Genetic manipulation
1) Construction of pDR110-ykcB
Genomic DNA of *B. subtilis* 168 trpC2 was isolated using a QIAamp DNA blood minikit (Qiagen). A DNA fragment containing the *ykcB* gene was amplified by PCR using a 168 trpC2 genomic DNA as a template and oligonucleotide primers (Table 3). The amplified DNA fragments were inserted into SphI and SalI sites in pDR110, resulting in pDR110-ykcB. Double crossover recombination of pDR110 or pDR110-ykcB at the *amyE* locus was confirmed by PCR using oligonucleotide primers (Table 3) and template genomic DNA from a spectinomycin-resistant colony.

2) **Construction of pHY300PLK-ykcC-FLAG**

Two DNA fragments containing the promoter region of the *ykcBC* and *ykcC* ORF were amplified by PCR using oligonucleotide primers (Table 3) and the template genomic DNA of 168 trpC2. The 2 DNA fragments were connected by recombinant PCR and inserted into HindIII and EcoRI sites of pHY300PLK, resulting in pHY300PLK-ykcC-FLAG.

3) **Transformation by electroporation**

Because the *ykcB* mutant did not have natural competency, we performed an electroporation to transform the *ykcB* mutant. *B. subtilis* overnight culture (1 ml) was inoculated into 100 ml of LB broth and aerobically cultured at 37°C until the OD$_{600}$ reached 1.5. The culture was cooled on ice for 10 min and centrifuged at 3000 g for 10 min at 4°C. The bacterial pellet was suspended in ice-cold water. The washing procedure using ice-cold water was repeated 3 times and the bacterial pellet was suspended in 1 ml of 30% polyethylene glycol 6000. The bacterial suspension was frozen in liquid nitrogen and stored at -80°C. The frozen cells (100 µl) were thawed and mixed with plasmid DNA.
(200 ng). Electroporation (25 µF, 2500 V, 400 Ω) was performed in a 2-mm cuvette using the Gene Pulser Xcell Electroporation System (BioRad). After electroporation, the cells were immediately mixed with 2 ml SOC medium and incubated at 37°C for 90 min. The cells were spread onto LB plates containing appropriate selective antibiotics and incubated overnight at 37°C.

4) Construction of chromosome deletion mutant by natural transformation

Targeting cassettes were constructed according to the previously described method (18) with minor modification. A DNA fragment containing the erythromycin resistance marker was amplified by PCR using oligonucleotide primers (Table 3) and a template genomic DNA from the ykcB mutant (BKE12880). The upstream and downstream DNA regions of the targeting chromosome locus were amplified by PCR using oligonucleotide primers (Table 3, Table 4) and a template genomic DNA from 168 trpC2. The 3 DNA fragments comprising the upstream and downstream regions and the erythromycin-resistance gene were mixed in an equal molar ratio and connected by PCR overlap extension using KOD FXneo DNA polymerase (Toyobo, Osaka, Japan). The connected DNA fragment was used for transformation without purification.

Competent cells for natural transformation were prepared according to the previous method (33) with minor modification. B. subtilis 168 trpC2 overnight culture (50 µl) was inoculated into 5 ml of SPI medium (0.2% ammonium sulfate, 1.4% dipotassium hydrogen phosphate, 0.6% potassium dihydrogen phosphate, 0.1% trisodium citrate dihydrate, 0.02% magnesium sulfate heptahydrate, 0.5% glucose, 0.02% casamino acids, 0.1% yeast extract, 50 µg/ml L-leucine, 50 µg/ml L-methionine) and aerobically cultured at 37°C for 4.5 hour. Glycerol was added to the bacterial culture to a final concentration
of 12.5%, frozen in a liquid nitrogen, and stored in a -80°C freezer. The frozen cells were
thawed in a 37°C water bath and a 7.5-fold amount of SPII medium (0.2% ammonium
sulfate, 1.4% dipotassium hydrogen phosphate, 0.6% potassium dihydrogen phosphate,
0.1% trisodium citrate dihydrate, 0.02% magnesium sulfate heptahydrate, 0.5% glucose,
5 mM magnesium chloride, 0.02% yeast extract, 5 µg/ml L-leucine, 5 µg/ml L-
methionine) was added, and then the cells were aerobically cultured at 37°C for 90 min.
A 50-µl amount of the cells was mixed with a targeting cassette and incubated at 37°C
for 30 min. After adding 100 µl of LB broth to the cells, they were further incubated at
37°C for 60 min. The cells were spread onto LB plates containing 1 µg/ml erythromycin
and incubated overnight at 37°C. The desired chromosomal deletion was confirmed by
PCR.

5) Construction of mutant strains carrying the stop codon mutation
A DNA fragment carrying the mhqA-ykcBC region and the erythromycin resistance gene
was amplified by PCR using primer pairs (Table 3, ykcB-F2-Sall, 5pR-BglII) and
template genomic DNA from the delA mutant. The DNA fragment was inserted into SalI
and BglII sites of pGEM-3Z. Using the plasmid as a template, a thermal cycling reaction
was performed using oligonucleotide primers to introduce the ykcB stop codon (Table 3).
The reaction solution was digested with DpnI, and then used to transform the E. coli
JM109 strain. A plasmid carrying the ykcB stop codon was purified from the E. coli
colonies. Using the plasmid as a template, a thermal cycling reaction was performed using
oligonucleotide primers to introduce the ykcC stop codon (Table 3), and the reaction
solution was processed as described above. Plasmids carrying the ykcB stop codon and
the ykcC stop codon were purified from the E. coli colonies. These 2 plasmids were
digested with SaI and BglII and used for transformation of 168 \textit{trpC2}. After transformation, genomic DNA was isolated from the erythromycin-resistant colonies and the desired stop codon mutations were confirmed by Sanger sequencing.

\textbf{Evaluation of antibiotic resistance}

Autoclaved LB agar medium was mixed with antibiotic solutions and poured into a square dish (Eiken Chemical). \textit{B. subtilis} overnight cultures were serially diluted 10-fold with LB broth in a 96-well microplate and 5 µl of the diluted bacterial solutions were spotted onto LB plates with or without antibiotics using an 8-channel Pipetman. The plates were incubated overnight at 37˚C and photographed using a digital camera.

\textbf{Biofilm forming assay}

\textit{B. subtilis} overnight culture (20 µl) was inoculated into 2 ml of LB broth containing 1M NaCl in a glass tube and incubated for 2 days at 37˚C. The bacterial culture containing biofilms was poured onto a Kimwipe placed on a Kimtowel (Nippon Paper Cresia, Tokyo, Japan). MilliQ water (2 ml) was added to the KimWipe, which was vortexed to detach the biofilms. The OD$_{600}$ value of the solution was measured.

\textbf{Lipid extraction and TLC assay}

\textit{B. subtilis} overnight culture (1 ml) was added to 100 ml of LB broth and aerobically cultured at 37˚C for 24 h; then, 40 ml of the bacterial culture was centrifuged at 10,400 g for 10 min at 4˚C. The bacterial pellet was suspended with 1 ml of milliQ water and the lipids were extracted using the Bligh and Dyer method (34). The lipid fraction was evaporated by a centrifuge evaporator and the lipids were dissolved with 500 µl of
chloroform:methanol (1:1 v/v). The sample was spotted onto TLC Silica gel 60 F₂₅₄ (Merck) and the plate was developed in chloroform:methanol:water (65:25:4 v/v). Sugars were visualized by spraying a coloring agent (10.5 ml 15% 1-naphthol in ethanol, 40.5 ml ethanol, 6.5 ml sulfuric acid, and 4 ml water) and heating at 115°C.

Western blot analysis

FLAG-tagged YkcC was detected according to a previous method (28) with minor modifications. B. subtilis overnight cultures was centrifuged at 21,400 g for 2 min and the bacterial pellet was frozen in liquid nitrogen. The bacterial pellet was thawed in buffer (50 mM Tris-HCl pH 7.8, 2 mM EDTA, 0.5 mM dithiothreitol, 0.4 mg/ml lysozyme) and subjected to freeze-thawing 2 times. TritonX-100 was added to the sample to produce a final concentration of 0.1% and the sample was incubated at 37°C for 30 min. An equal volume of 2x Laemmli sample buffer with 350 mM dithiothreitol was added to the sample and the sample was heated at 95°C for 3 min. The sample was centrifuged at 21500 g for 15 min, and the supernatant was electrophoresed in a 12% sodium dodecyl sulfate-polyacrylamide gel. Anti-DYKDDDDK (anti-FLAG) antibody (Wako, Japan) diluted 1:3000 in Canget signal solution 1 (Toyobo, Japan) was used as a first antibody solution. Anti-mouse IgG conjugated with horseradish peroxidase (HRP; Promega, Japan) diluted 1:3000 in Canget signal solution 2 (Toyobo, Japan) was used as a second antibody solution.

For detection of lipoteichoic acid, a previously described method (35) was used with modifications. B. subtilis overnight culture (50 µl) was inoculated to 5 ml of LB broth and aerobically cultured at 37°C for 24 h. The culture was centrifuged at 10,400 g for 10 min and the bacterial pellet was suspended in a 1.5x Laemmlie sample buffer. The sample
was boiled for 40 min and centrifuged at 10,400g for 10 min. The supernatants were
electrophoresed in a 15% polyacrylamide gel and transferred to a nitrocellulose
membrane (0.2 µm, Trans-Blot Transfer Transfer Medium, BioRad). The membrane was treated
with 1:1000 anti- lipoteichoic acid antibody (clone 55, Hycult Biotech, Uden, The
Netherlands) and washed 3 times with phosphate buffered saline. The membrane was
treated with anti-mouse IgG HRP conjugate (Promega) and washed 3 times with
phosphate buffered saline. The membrane was reacted with HRP substrate (Western
Lightning, Perkin Elmer) and the signals were detected using ImageQuant LAS 4000
(Fujifilm, Tokyo, Japan). The band intensity was measured by Image J software (36).

Statistical analysis
Survival curves of silkworms were analyzed by the log-rank test. The amounts of
lipoteichoic acid and diglucosyl diacylglycerol were analyzed by Dunnett's multiple
comparisons test. The amount of biofilm was analyzed by Tukey's multiple comparisons
test. The statistical analysis was performed using Prism 9 (GraphPad Software).

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Table 1. Gene knockout mutants resistant to vancomycin

| ID       | Gene  | Product                                                                 |
|----------|-------|-------------------------------------------------------------------------|
| BKE09500 | yhdK  | Probable anti-sigma-M factor YhdK                                       |
| BKE10520 | glcP  | Glucose/mannose transporter GlcP                                         |
| BKE10930 | yitB  | Adenosine 5’-phosphosulfate reductase 2                                 |
| BKE10950 | yitD  | Phosphosulfolactate synthase                                             |
| BKE12880 | ykcB  | Putative mannosyltransferase YkcB                                        |
| BKE13610 | mtnB  | Methylthioribulose-1-phosphate dehydratase                               |
| BKE13750 | queF  | NADPH-dependent 7-cyano-7-deazaguanine reductase                         |
| BKE00250 | xpaC  | Anti-sigma-G factor Gin                                                  |
| BKE13950 | mcpC  | Methyl-accepting chemotaxis protein McpC                                 |
| BKE17050 | mutL  | DNA mismatch repair protein MutL                                         |
| BKE00850 | mcsB  | Protein-arginine kinase                                                  |
| BKE23830 | yqjL  | Uncharacterized protein YqjL                                             |
| BKE24770 | mgsR  | Regulatory protein MgsR                                                  |
| BKE01570 | ybaN  | Probable polysaccharide deacetylase PdaB                                 |
| BKE26610 | yrkA  | UPF0053 protein YrkA                                                     |
| BKE30190 | bioI  | Biotin biosynthesis cytochrome P450                                       |
| BKE30500 | ytpB  | Tetrateryl-beta-curcumene synthase                                       |
| BKE31290 | yugT  | Probable oligo-1,6-glucosidase 3                                         |
| BKE32840 | fadN  | Probable 3-hydroxyacyl-CoA dehydrogenase                                 |
| BKE33222 | rsoA  | Sigma-O factor regulatory protein RsoA                                   |
| BKE37200 | ywjD  | UV DNA damage endonuclease                                               |
| BKE02860 | adcC  | High-affinity zinc uptake system ATP-binding protein ZnuC                |
| BKE35910 | rbsR  | Ribose operon repressor                                                 |
### Table 2. List of bacterial strains and plasmids used

| Strain or plasmid | Genotypes or characteristics | Source or reference |
|-------------------|------------------------------|---------------------|
| **Strains**       |                              |                     |
| B. subtilis       |                              |                     |
| 168               | trpC2                        | BGSC                |
| BKE12880          | trpC2 ΔykcB; Erm'^r           | NBRP (18)           |
| BKE12870          | trpC2 ΔmhqA; Erm'^r           | NBRP (18)           |
| BKE12890          | trpC2 ΔykcC; Erm'^r           | NBRP (18)           |
| BKE21920          | trpC2 ΔugtP; Erm'^r           | NBRP (18)           |
| DelA              | trpC2 delA; Erm'^r            | This study          |
| DelB              | trpC2 delB; Erm'^r            | This study          |
| DelC              | trpC2 delC; Erm'^r            | This study          |
| DelD              | trpC2 delD; Erm'^r            | This study          |
| DelE              | trpC2 delE; Erm'^r            | This study          |
| DelF              | trpC2 delF; Erm'^r            | This study          |
| DELP1000          | trpC2 delA; Erm'^r, ykcB Y71Stop | This study        |
| DELP1001          | trpC2 delA; Erm'^r, ykcB Y71Stop, ykcC L55Stop | This study    |
| **E. coli**       |                              |                     |
| JM109             | Host strain for cloning       | Takara Bio          |
| **Plasmids**      |                              |                     |
| pH300PLK          | A shuttle plasmid, Amp'^r, Tet'^r | Takara Bio        |
| pH300PLK-ykcC-FLAG | pH300PLK with FLAG-tagged ykcC | This study         |
| pGEM-3Z           | Cloning vector, Amp'^r         | Promega             |
| pDR110            | An integration vector, Amp'^r, Spc'^r | BGSC              |
| pDR110-ykcB       | pDR110 with ykcB, Amp'^r, Spc'^r | BGSC              |

rm: erythromycin, Amp: ampicillin, Tet: tetracyclin, Spc: spectinomycin.
| Primers to construct pDR110-ykcB | Sequence |
|----------------------------------|----------|
| ykb\_compl\_F\_SalI             | GGAGTCGACGGGACATAAGGAGGAACTACTATGGAAAAAGAAAA |
| ykb\_compl\_R                   | GCAGCATGCTATTTCATAGCATATGGTTTCGATAATGTT    |

| Primers to construct chromosomal deletion mutants | Sequence |
|--------------------------------------------------|----------|
| Ab-F                                             | CAGGGGAGAAAAGGAGAGGAGGAAAGGCAAGGA |
| Ab-R                                             | CGAGGCTCTCTGACTGCGCCGCATATCTGCTCCT |
| ykbB-F2-Sall                                    | GTCGTCAGCGCTGACAGGATGGAAGCA |
| UP1-mhqA-ykbB-inter-R                           | CTCTCTCTTCTCGCGCCTCCCCATTCACGACG |
| UP4-mhqA-ykbB-inter-F                           | GACATGACAGGAAGCCCGCGTAATGGAATG |
| ykbB-F2-Sall                                    | GTCGTCAGCGCTGACAGGATGGAAGCA |
| 3pR-BglIII                                     | AGAAGATCTGGCAAACCTCGGTGATTC |
| 3pR-BglIII                                     | AGAAGATCTGGCAAAGGGGTGGATGG |
| UP4-mhqA-ykbB-inter-F2                         | GCACTGACAGGAAGCCCGCGTAATGGAATG |
| UP4-ykbB-int-F4                                 | GCAGTGCAGGAGGCTGCTCTTTCCTCATTTTG |
| UP4-ykbB-int-F3                                 | GCAGTGCAGGAGGCTGCTCTTTCCTCATTTTG |
| UP4-ykbB-int-F2                                 | GCAGTGCAGGAGGCTGCTCTTTCCTCATTTTG |
| YkbB-int-R                                      | CAGCATGCAAGGAAGCATCAGCAGAAGCATAA  |
| UP1-ykbB-int-R                                  | CTCTCTCTTCTCGCGGCTACGAGCATCAGCAGAAGCATAA |

| Primers to introduce stop codon mutation | Sequence |
|-----------------------------------------|----------|
| YkbB-Stop-F                             | GTAGATAAACCCGCTGTATTACATAACAAAACCTCAAAGCATCAGCAGC |
| YkbB-Stop-R                             | TCGCTGATGTTGATGTTGATGTTGATGTAACAGGGCCTGTTATCATC |
| YkcC-Stop-F                             | AAAGACCGCAATGCAATTGCAATTTAAGAGGAAGCAGCGCGGATAC |
| YkcC-Stop-R                             | TCAGATCAAATCGCGGCTGCTCTTCTTTAAAATCTCAATAATCTGGCTCCT |

| Primers to construct pHY300PLK-ykcC | Sequence |
|-----------------------------------|----------|
| ykbBProHdIII-F                    | AAAACGCTTTTGCCCAAAGGCTCTGTTTTCATGCAGGGAACAAAAC |
| ykbBPro-R                        | TCACTCAGCATGTTGTTTTTTGCTCAACCTTGAAA |
| ykbC-F                            | ACTACATCTGCTGATGAAATTGAGGCGGAAACATG |
| ykbCFLEcI-R                      | TTTTTTTTTAAACAGGGAATTTTCTTTTGCTATCGCTGCTCTTTCTTGATCTGACATGCTGCTC |

| Primers to confirm the replacement of \textit{amyE} locus | Sequence |
|----------------------------------------------------------|----------|
| amyE-F                                                   | TACAGCAACGTGATCAAAA |
| amyE-R                                                   | CTGGGTGCTCGTTACAACAT |
| Strain | Primers |
|--------|---------|
| DelA   | ykcB-F2-Sall, UP1-mhqA-ykcB-inter-R, UP4-mhqA-ykcB-inter-F, 3pR-BglII |
| DelB   | ykcB-F2-Sall, UP1-mhqA-ykcB-inter-R, UP4-mhqA-ykcB-inter-F2, 3pR-BglII |
| DelC   | ykcB-F2-Sall, UP1-mhqA-ykcB-inter-R, UP4-ykcB-int-F4, 3pR-BglII |
| DelD   | ykcB-F2-Sall, UP1-mhqA-ykcB-inter-R, UP4-ykcB-int-F3, 3pR-BglII |
| DelE   | ykcB-F2-Sall, UP1-mhqA-ykcB-inter-R, UP4-ykcB-int-F2, 3pR-BglII |
| DelF   | ykcB-F2-Sall, UP1-ykcB-int-R, UP4-ykcB-int-F2, 3pR-BglII |

Table 4. Primer sets used for constructing chromosomal deletion mutants
**FIGURE LEGENDS**

**Figure 1** Knockout of ykcB alters sensitivity to antibiotics and increases silkworm killing activity.

A. Overnight cultures of the parent strain (Parent) and ykcB knockout mutant (ΔykcB) were serially diluted 10-fold and spotted onto LB plates supplemented with or without vancomycin (0.3 µg/ml). The plates were incubated overnight at 37°C.

B. The parent strain transformed with empty vector (Parent/Pspank) and the ykcB knockout mutant transformed with empty vector (ΔykcB/Pspank) or a vector encoding ykcB (ΔykcB/Pspank-ykcB) were aerobically cultured overnight in the presence of 1 mM IPTG. The overnight cultures were serially diluted 10-fold and spotted onto LB plates supplemented with 1 mM IPTG and vancomycin or 1 mM IPTG alone. The plates were incubated overnight at 37°C.

C. Overnight cultures of the parent strain (Parent) and the ykcB knockout mutant (ΔykcB) were serially diluted 10-fold and spotted onto LB plates supplemented with or without ampicillin, oxacillin, ceftazidime, levofloxacin, chloramphenicol, or tetracycline. The plates were incubated overnight at 37°C.

D. The silkworm killing activity of the parent strain (Parent) and ykcB knockout mutant (ΔykcB) was examined. Silkworms (n=20) were injected with *B. subtilis* cells (8 x 10^6 CFU) and silkworm survival was monitored. Asterisk indicates log-rank test p-value less than 0.05.

E. The parent strain (Parent) and ykcB knockout mutant (ΔykcB) were aerobically cultured in LB broth and the OD_{600} values of the cultures were measured.
Figure 2 Knockout of ykcB leads to vancomycin resistance in a ykcC-dependent manner.

A. Schematic representation of the ykcB flanking region is shown. The magenta box represents the chromosome region replaced with the erythromycin resistance gene in the gene knockout mutants or the chromosomal deletion mutants. Black arrowhead indicates the position at which the stop codon mutation was introduced.

B. Overnight cultures of the parent strain (Parent), ykcB knockout mutant (ΔykcB), mhqA knockout mutant (ΔmhqA), and ykcC knockout mutant (ΔykcC) were serially diluted 10-fold and spotted onto LB plates supplemented with or without vancomycin. The plates were incubated overnight at 37°C.

C. Overnight cultures of the parent strain (Parent), chromosomal deletion mutants, and stop codon mutants were serially diluted 10-fold and spotted onto LB plates supplemented with or without vancomycin. The plates were incubated overnight at 37°C.

Figure 3 Knockout of ykcB leads to the accumulation of diglucosyl diacylglycerol in a ykcC-dependent manner

A. The B. subtilis parent strain (Parent), ykcB knockout mutant (ΔykcB), ykcC knockout mutant (ΔykcC), delA mutant (delA), delA/ykcBstop mutant (delA/ykcBstop), and delA/ykcBstop/ykcCstop mutant (delA/ykcBstop/ykcCstop) were cultured for 24 h and total lipids were extracted. Diglucosyl diacylglycerol was analyzed by TLC.

B. The signal intensities of diglucosyl diacylglycerol in A were measured. Data are presented as means ± SD from 5 independent experiments. Stars indicate Dunnett's multiple comparisons p value less than 0.05.
C. The *B. subtilis* parent strain (Parent), *ykcB* knockout mutant (Δ*ykcB*), and *ugtP* knockout mutant (Δ*ugtP*) were cultured for 24 h and total lipids were extracted. Diglucosyl diacylglycerol was analyzed by TLC.

D. Overnight bacterial cultures used in C were serially diluted 10-fold and spotted onto LB plates supplemented with or without vancomycin. The plates were incubated overnight at 37˚C.

**Figure 4** Knockout of *ykcB* decreases the amount of lipoteichoic acid in a *ykcC*-dependent manner.

A. The *B. subtilis* parent strain (Parent), *ykcB* knockout mutant (Δ*ykcB*), *ykcC* knockout mutant (Δ*ykcC*), *delA* mutant (Δ*delA*), *delA/ykcB*stop mutant (Δ*delA/ykcB*stop), and *delA/ykcB*stop/*ykcC*stop mutant (Δ*delA/ykcB*stop/*ykcC*stop) were cultured for 24 h and the lipoteichoic acids were extracted. Lipoteichoic acids were detected by Western blot analysis.

B. The band intensities of lipoteichoic acids in A were measured. Data are presented as means ± SD from five independent experiments. Stars indicate Dunnett’s multiple comparisons p value less than 0.05.

**Figure 5** Knockout of *ykcB* decreases biofilm formation in a *ykcC*-dependent manner.

A. The *B. subtilis* parent strain (Parent), *ykcB* knockout mutant (Δ*ykcB*), *ykcC* knockout mutant (Δ*ykcC*), *delA* mutant (Δ*delA*), *delA/ykcB*stop mutant (Δ*delA/ykcB*stop), and *delA/ykcB*stop/*ykcC*stop mutant (Δ*delA/ykcB*stop/*ykcC*stop) were cultured for 2 days in glass tubes without shaking. The water surface areas were photographed.
The amount of biofilm in A was measured. Data are presented as means ± SD from 8 independent experiments. Stars indicate Tukey's multiple comparisons test p value less than 0.05.

**Figure 6** Overexpression of ykcC enhances vancomycin resistance in B. subtilis.

A. The B. subtilis parent strain or the ykcB knockout mutant transformed with an empty vector (pHY) or a plasmid encoding FLAG-tagged ykcC (pHY-ykcC-FLAG) were subjected to Western blot analysis using the anti-FLAG antibody. The membrane was stained with Coomassie Brilliant Blue and shown as a loading control (CBB).

B. Overnight bacterial cultures used in A were serially diluted 10-fold and spotted onto LB plates supplemented with or without vancomycin. The plates were incubated overnight at 37°C.

**Figure 7** Model of vancomycin resistance induced by the ykcB knockout

ΔykcB accumulates C55-P-glucose, which might change the cell surface structure and leads vancomycin resistance. In the ykcBstop/ykcCstop mutant, C55-P-glucose would not be accumulated because of the ykcC deficiency. The amount of diglucosyl diacylglycerol (Glc2-DAG) was not changed in the ykcBstop/ykcCstop mutant, suggesting that UDP-glucose is metabolized to other molecules in a ykcC-deficient background.
**Figure 1**

A. No drug vs Vancomycin (0.3 μg/ml)
- Parent
- ΔykcB

B. No drug vs Vancomycin (0.3 μg/ml)
- Parent/P\text{spark}
- ΔykcB/P\text{spark}
- ΔykcB/P\text{spark}-ykcB

C. No drug vs Ampicillin (0.1 μg/ml)
- Oxacillin (0.08 μg/ml)
- Cefazidime (1.8 μg/ml)
- Levofloxacin (0.06 μg/ml)
- Chloramphenicol (2.3 μg/ml)
- Tetracycline (3.8 μg/ml)

D. Survival of silkworms (%)
- Time after injection (h)
  - Parent
  - ΔykcB

E. CD\text{500}
- Time (h)
  - Parent
  - ΔykcB
Figure 2

A

Parent
\Delta ykB
\Delta mhqA
\Delta ykcC
delA
delB
delC
delD
delE
delF
delA / ykBstop
delA / ykBstop / ykcCstop

B

| No drug | Vancomycin (0.3 \mu g/ml) |
|---------|--------------------------|
| Parent  |                          |
| \Delta ykB |                       |
| \Delta mhqA |                     |
| \Delta ykcC |                     |

C

| No drug | Vancomycin (0.3 \mu g/ml) |
|---------|--------------------------|
| Parent  |                          |
| \Delta ykB |                       |
| delA   |                          |
| delB   |                          |
| delC   |                          |
| delD   |                          |
| delE   |                          |
| delF   |                          |
| delA / ykBstop |                |
| delA / ykBstop / ykcCstop |              |
Figure 4

A

| Parent | ΔykB | ΔykC | delA | delA ykcBstop | delA ykcCstop |
|--------|------|------|------|---------------|---------------|

α-LTA

B

* Relative amount of LTA

- Parent
- ΔykB
- ΔykC
- delA
- delA ykcBstop
- delA ykcCstop
- delA ykcBstop ykcCstop
Figure 7

Parent strain

| UD-Glc | YkcC | C55-P-Glc | YkcB | X |
|--------|------|----------|------|---|
|        |      |          |      |   |
| Glc₂-DAG |   |          |      |   |

Δykb

| UD-Glc | YkcC | C55-P-Glc | YkcB | X |
|--------|------|----------|------|---|
|        |      |          |      |   |
| Glc₂-DAG |   |          |      |   |

Vancomycin resistance

ykbstop / ykcCstop

| UD-Glc | YkcC | C55-P-Glc | Ykb | X |
|--------|------|----------|-----|---|
|        |      |          |     |   |
| Glc₂-DAG |   |          |     |   |

Other molecules