Full Paper

Study on *in vivo* effects of bacterial histidine kinase inhibitor, Waldiomycin, in *Bacillus subtilis* and *Staphylococcus aureus*

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Two-component signal transduction systems (TCSs) represent one of the primary means by which bacteria sense and respond to changes in their environment, both intra- and extracellular. The highly conserved WalK (histidine kinase)/WalR (response regulator) TCS is essential for cell wall metabolism of low G+C Gram-positive bacteria and acts as a master regulatory system in controlling and coordinating cell wall metabolism with cell division. Waldiomycin, a WalK inhibitor, has been discovered by screening metabolites from actinomycetes and belongs to the family of angucycline antibiotics. In the present study, we have shown that waldiomycin inhibited autophosphorylation of WalK histidine kinases *in vitro* from *Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faecalis*, and *Streptococcus mutans* at half-maximal inhibitory concentrations of 10.2, 8.8, 9.2, and 25.8 μ M, respectively. Quantitative RT-PCR studies of WalR regulon genes have suggested that waldiomycin repressed the WalK/WalR system in *B. subtilis* and *S. aureus* cells. Morphology of waldiomycin-treated *S. aureus* cells displayed increased aggregation instead of proper cellular dissemination. Furthermore, autolysis profiles of *S. aureus* cells revealed that waldiomycin-treated cells were highly resistant to Triton X-100- and lyso-staphin-induced lysis. These phenotypes are consistent with those of cells starved for the WalK/WalR system, indicating that waldiomycin inhib-

ited the autophosphorylation activity of WalK in cells. We have also confirmed that waldiomycin inhibits WalK autophosphorylation *in vivo* by actually observing the phosphorylated WalK ratio in cells using Phos-tag SDS-PAGE. The results of our current study strongly suggest that waldiomycin targets WalK histidine kinases and inhibits the WalR regulon genes expression, thereby affecting both cell wall metabolism and cell division.

Key Words: angucycline antibiotics; *Bacillus subtilis*; *Enterococcus faecalis*; histidine kinase inhibitor; *Staphylococcus aureus*; *Streptococcus mutans*; two-component signal transduction systems; WalK/WalR

Introduction

Bacteria can adapt to physical, chemical, and biological stresses from the exterior and thereby survive in various environments. This adaptation process is mainly governed by the two-component signal transduction system (TCS), which consists of a transmembrane sensor histidine kinase (HK) and a cytosolic response regulator (RR) (Gao and Stock, 2009; Hoch and Silhavy, 1995; Utsumi, 2008). Depending on a particular environmental signal, the sensor HK autophosphorylates a conserved histidine residue in the dimerization domain, and then transfers the phosphoryl group to the conserved aspartic acid residue in the regulatory domain of its cognate RR. The phosphorylated

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Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant genotype or description	Source or reference
Bacterial strains		
<i>E. coli</i> BL21(DE3)	F ⁻ , ompT, hsdS _B (r _B ⁻ m _B ⁻), gal, dcm (DE3)	Laboratory stock
<i>B. subtilis</i> 168	trpC2	Laboratory stock
<i>S. aureus</i> N315	Methicillin-resistant <i>S. aureus</i>	Kuroda et al. (2001)
<i>E. faecalis</i> JCM5803	Wild type	MCRF
<i>S. mutans</i> UA159	Wild type	ATCC
Plasmids		
pHT10	Expression vector for <i>E. coli</i> and <i>B. subtilis</i> ; Ap ^r Cm ^r	MoBi Tec
pET-21a(+)	Expression vector, Ap ^r , C-terminal His tag	Novagen
pET-22b(+)	Expression vector, Ap ^r , C-terminal His tag	Novagen
pBsG-full	WalK (Bs) (<i>B. subtilis</i> 168 aa 1 to 611) cloned into pHT10	Watanabe et al. (2012)
pETYycG-tru	WalK (Bs) (<i>B. subtilis</i> 168 aa 207 to 611) cloned into pET-21a(+)	Okada et al. (2010)
pYycG Sa	WalK (Sa) (<i>S. aureus</i> N315 aa 204 to 608) cloned into pET-21a(+)	Watanabe et al. (2003)
pETefWalK	WalK (Efa) (<i>E. faecalis</i> JCM5803 aa 203 to 611) cloned into pET-21a(+)	Watanabe et al. (2012)
pETSMVicK31-450	WalK (Sm) (VicK of <i>S. mutans</i> UA159, aa 31 to 450) cloned into pET22b(+)	Eguchi et al. (2011)

MCRF, Microbial Chemistry Research Foundation; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance.

Fig. 1. Structure of waldiomycin.

RR then binds to the upstream promoter regions of target genes to regulate their expression. TCSs have global roles in bacterial functions such as growth, drug resistance, virulence, biofilm formation and quorum sensing (Utsumi, 2008). Therefore, inhibitors of TCS can be expected to work as antibacterial agents as well as inhibitors for drug resistance, biofilm formation, quorum sensing and virulence. Interrupting these critical signaling pathways may provide an alternative strategy for the development of novel classes of preventive and/or therapeutic agents (Barrett and Hoch, 1998).

The WalK/WalR TCS is highly conserved among Gram-positive bacteria having a low GC-content, including *Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Streptococcus mutans*, *Streptococcus pneumoniae* and *Streptococcus pyogenes* (Dubrac et al., 2008). This TCS has been reported to serve as a master regulatory system for cell wall metabolism and to be essential for cell viability (Bisicchia et al., 2007; Dubrac and Msadek, 2004; Dubrac et al., 2007; Fabret and Hoch, 1998; Martin et al., 1999). Thus, it has been suggested that WalK or WalR may serve as potential targets for the development of novel antibacterial agents (Fukushima et al., 2011; Szurmant et al., 2005; Türck and Bierbaum, 2012).

We previously described a sensitive differential growth

assay to screen for WalK inhibitors from natural sources (Okada et al., 2007). Using this screening method, we isolated three new inhibitors: walkmycin B (Okada et al., 2010), signermycin B (Watanabe et al., 2012) and waldiomycin (Igarashi et al., 2013). These inhibitors inhibit WalK autophosphorylation and thereby hinder WalK/WalR signal transduction. In another study, Qin et al. (2006) have found six WalK inhibitors using structure-based virtual screening (SBVS) of chemical molecules by targeting the ATP-binding domain. Inhibitors that target the WalR RR in *B. subtilis* and *S. aureus* have also been found (Gotoh et al., 2010).

In the current study, we have focused on the WalK inhibitor, waldiomycin (Fig. 1). This inhibitor belongs to the family of angucycline antibiotics and is structurally related to dioxamycin. It has antimicrobial activity against Gram-positive pathogens including methicillin-resistant *S. aureus* (MRSA) and *B. subtilis* (Igarashi et al., 2013). In an aim to further characterize this inhibitor, we have elucidated the *in vivo* effects of waldiomycin against *B. subtilis* and *S. aureus*.

Materials and Methods

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *B. subtilis* and *Escherichia coli* cells were grown under aerobic conditions at 37°C in a Luria-Bertani (LB) medium (1% polypeptone, 0.5% yeast extract, and 0.5% NaCl) with antibiotic added when required. *S. aureus* cells were grown in Trypticase Soy Broth (TSB) medium (1.7% Bacto tryptone, 0.3% Bacto soytone, 0.5% NaCl, 0.25% dipotassium phosphate, and 0.25% dextrose) at 37°C with aeration.

In vitro autophosphorylation activity. The cytoplasmic WalK domains (with C-terminal His tag) of Gram-positive *B. subtilis*, *S. aureus*, *E. faecalis*, and *S. mutans* were expressed and purified as described previously using *E. coli* BL21 (DE3) containing pETYycG-tru (Okada et al., 2010), pYycGSa (Watanabe et al., 2003), pETefWalK (Watanabe et al., 2012), and pETSMVicK31-450 (Eguchi

Table 2. Primers used in this study.

Primer name	Primer sequence (5' → 3')
<i>Sau</i> (qRT-PCR)	
rt-SArrn16S-2F	GTAAGATCAGCATGCTACGGTGAA
rt-SArrn16S-2R	TGTGACGGGCGGTGTGTA
rt-gyrA(Sa)-F	TTAATGATTGTTACTAATGCAGGTGTCA
tr-gyrA(Sa)-R	CTTGTGCTGCACGACCATTT
rt-isaA-F	CTGGTTCATCAGCAGCTCAAAAT
rt-isaA-R	GATGATTGCAGCCCATGTAGAA
rt-ssaA-F	GGTTACACAGTGAACAATACACCAAA
rt-ssaA-R	CGTGACCGTATGCACCTTGA
sceD-F	TCTAAAACAAATTGCTCAACGTGAA
sceD-R	CAGCTGCACCTGATGTTGGA
SA2353-F	GGGCTAACGCAGCTGCAT
SA2353-R	GCATGATAGCACCAACTTTTG
SA2097-F	GGCGGAGAAATCGGTTCTACT
SA2097-R	ACTGTGAATCCAGCACCTTGTG
SA0710-F	GCACATATTATGCCTTCGATCGT
SA0710-R	GCATTGCCCCATAAACTTCCT
SA0620-F	GCAGCTAATGATGGTTACCAAGTAAA
SA0620-R	CCAGGTGTGCTTTGCATAATTG
atIA-F	ACGTGTACCAGGTAAGTGACAGA
atIA-R	AATGCTGGATCTTGAGCTAAACG
rt_lytM-F	CCAAAAAATGCAAATGCCAAT
rt_lytM-R	TCTCTGCTGTTGCTGGATTCT
<i>Bsu</i> (qRT-PCR)	
rt_rrn16S-2F	CGTGAAGCTGGAATCGCTAGT
rt_rrn16S-2R	CAAGGCCCGGGAACGTAT
rt_gyrA-F	AGTCATTCCAGGGCCTGATT
rt_gyrA-R	ATTCGTATGCTTTCCGGATACC
rt_rpoB-F	GACCGGGCGGATTGACA
rt_rpoB-R	GCCATAGTGGGAGTAGTGAACGT
rt_yocH-2F	ATGTCCTTTGTGGCAGTTGCT
rt_yocH-2R	GCACCGTAATTTCTTTGCAGAA
rt_yvcE-F	GCGGACGCACTCAGTCTGA-3
rt_yvcE-R	TGCCAGCGTACGAATGAT
rt_yoeB-2F	CGCTAGAGAACATTTTGGAAACAC
rt_yoeB-2R	TGATGGGCAAACTGCTTTTTT
rt_yjeA-F	CATTGCCGCGGACGAT
rt_yjeA-R	CAAAGCGGCTGAAATTTTCTTT

Sau (qRT-PCR), *Staphylococcus aureus* quantitative RT-PCR; *Bsu* (qRT-PCR), *Bacillus subtilis* quantitative RT-PCR.

et al., 2011), respectively. The WalK cytoplasmic domains were preincubated in a kinase buffer {50 mM Tris-HCl (pH 8.5), 100 mM KCl, 100 mM NH₄Cl, 5 mM MgCl₂} at 30°C, as previously reported (Eguchi et al., 2011; Okada et al., 2007, 2010; Watanabe et al., 2003). Waldiomycin was then added. After a 5-min incubation, ATP solution (2.5 μM ATP and 16.7 nM [γ -³²P] ATP) was added, and the reaction mixture was incubated for an additional 10 min. The reaction was stopped by adding a 5×sodium dodecyl sulfate (SDS) sample buffer {300 mM Tris-HCl (pH 8.0), 10% SDS, 25% β-mercaptoethanol, 50% glycerol, 0.25% bromophenol blue}. The samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE). The gel was then dried, exposed to an imaging plate, and analyzed using FLA-7000 imaging analyzer (Fuji Film, Tokyo, Japan) and Multi Gauge version 3.0 software (Fuji Film, Tokyo, Japan). Prism 4.1 (GraphPad Software, CA) was used to determine the 50% inhibitory concentration (IC₅₀).

Extraction of total RNA. For quantitative reverse transcription PCRs (qRT-PCRs), the cells of *S. aureus* N315

and *B. subtilis* 168 were grown with aeration to an optical density at 660 nm (OD₆₆₀) of 0.3 at 37°C. After the addition of waldiomycin, the cultures were incubated for another 5 min. The cells were then harvested by centrifugation and immediately suspended in RNeasy lysis buffer (Ambion, Austin, TX). RNA was then purified with the SV Total RNA Isolation System (Promega, Madison, WI), followed by DNaseI treatment (Turbo DNA-free kit; Ambion) to eliminate residual contamination of genomic DNA. The purity and concentration of the RNA were determined by spectrophotometry and gel electrophoresis.

Complementary DNA (cDNA) synthesis. Reverse transcription reactions were carried out with the High-capacity cDNA Reverse Transcription Kit (ABI, Foster City, CA).

qRT-PCRs. PCR primers (Table 2) were designed with Primer Express Software Version 3.0 (ABI). Amplification of cDNA (1 ng) was carried out with 250 nM gene-specific primers and PowerSYBR Green master mix (ABI). PCR amplification, detection, and analyses were performed using the StepOne real-time PCR System (ABI). PCR conditions included an initial denaturation step at 95°C for 10 min followed by 40-cycle amplification (95°C for 15 s and 60°C for 60 s). The specificity of the amplified product and absence of primer dimer formation were verified by generating a melting curve with an initial denaturation step of 95°C for 15 s followed by 60°C for 1 min and 95°C for 15 s. Fluorescence was measured continually during the melting curve cycle (a stepwise 0.3°C) beginning at 60°C. The absence of contaminating genomic DNA was verified by testing each sample in control reactions without a previous reverse transcription step. The critical threshold cycle was defined for each sample, and expression levels were normalized using the 16S rRNA gene as an internal standard; 16S rRNA levels did not vary under our experimental conditions. Each assay was performed in triplicate.

Microscopy. For microscopic observations of bacterial morphology and organization, *S. aureus* N315 strains were grown to OD₆₆₀ of 0.3 at 37°C. Waldiomycin was added to exponentially growing cultures and the cells were incubated for an additional 1 h at the same temperature with shaking. Cells were stained with Gram's stain and observed under a light microscope at ×1,000 magnification.

Triton X-100- and lysostaphin-induced autolysis assays. Overnight cultures of strain *S. aureus* N315 were grown in TSB at 37°C to an OD₆₆₀ of 1.0 with aeration. After the addition of waldiomycin, the culture was incubated for an additional 10 min at the same temperature. The cells were then harvested (10 min; 5,400 × g) and resuspended in the same volume of phosphate buffered saline (PBS) containing either 0.1% Triton X-100 or 200 ng/ml lysostaphin (Wako Pure Chemical, Osaka, Japan). These suspensions were then incubated at 30°C (Triton lysis assay) or 37°C (lysostaphin assay) with aeration. Lysis was determined as the decrease in the OD₆₆₀ over time. The results were presented as percentage lysis, calculated by dividing the measured OD₆₆₀ by the initial OD₆₆₀.

Biofilm formation assays. The strain *S. aureus* N315 was grown overnight in TSB, diluted in TSB plus 0.25% glucose to an OD₆₆₀ of 0.1, and then distributed into sterile,

Fig. 2. Transcriptional regulation of the WalR regulon by waldiomycin.

Waldiomycin was added to exponentially growing cultures of *B. subtilis* (A) and *S. aureus* N315 (B). After 5 min incubation, the total RNA was isolated and quantified by qRT-PCR. Relative results were expressed as the mean and standard deviation from triplicate experiments using primers specific for WalR regulon genes, *gyrA* and 16S rRNA (normalizing gene). (C) Diagram of *S. aureus* cell wall peptidoglycan and cleavage sites for WalR-regulon encoded cell wall hydrolases. Peptidoglycan cleavage sites for WalR-regulon encoded cell wall hydrolases are indicated: a) Glycan strand glycosidic bond (glucosaminidase: *atlA*). b) Glycan strand glycosidic bond (lytic transglycosylases: *sceD* and *isaA*). c) N-Acetylmuramyl-L-alanyl amide bond (*atlA* amidase domain, *ssaA*, SA2353, SA2097, SA0710, SA0620), and d) Pentaglycine bridge glycylic-glycyl bond (*lytM* endopeptidase). NAM = N-acetylmuramic acid, NAG = N-acetylglucosamine.

96-well polystyrene plates (200 µL per well). Different concentrations of waldiomycin were added to the wells at 2 µL per well and mixed thoroughly. The plates were then incubated at 37°C for 24 h. The wells were washed gently twice with PBS and air dried. The adherent bacteria were stained with 0.1% crystal violet for 15 min at room temperature. The plates were then rinsed with PBS and air dried, and the stained biomass was resuspended in ethanol-acetone (80:20) for quantification. The absorbance of the solution was measured at 595 nm.

In vivo autophosphorylation assay. Overnight cultures of strain *B. subtilis* 168 transformed with pBsG-full were grown at 37°C in LB medium supplemented with chloramphenicol and 100 µM IPTG until the OD₆₆₀ reached 0.3. After the addition of waldiomycin, the cells were further grown for 1 h at the same temperature. Cells from 2 mL aliquot of culture were then collected by centrifugation (2,300 × g, 10 min, 4°C) and suspended into 200 µL of lysis buffer (100 mM NaCl, 50 mM Tris-HCl, pH 8.0). The cells were then disrupted by using Multi-beads shocker (Yasui Kikai, Osaka, Japan) at 2500 rpm, 60 sec ON, 60 sec OFF, 6 repeated cycles. Afterwards, the supernatant

sample was mixed with 5×SDS-sample buffer and were electrophoresed at 4°C with a constant current of 20 mA on a 50 µM Phos-tag SDS-polyacrylamide gel (6% acrylamide, 100 µM MnCl₂) for 150 min. After electrophoresis, the gel was soaked into 10 mM EDTA/Blotting buffer C (0.302% (w/v) Tris, 20% (v/v) methanol), thrice (10 min each), in order to remove Mn²⁺, and then soaked into Blotting buffer C for 10 min prior to Western Blot analysis.

Western blotting. The samples were separated by electrophoresis and then transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P membrane, Merck Millipore, Billerica, MA, USA). The PVDF membrane was blocked by 1% skim milk, probed with anti-WalK antibody as the primary antibody and enhanced chemiluminescence (ECL) peroxidase-labeled anti-rabbit antibody (GE Healthcare Bio-Science) as the secondary antibody. WalK was detected with LuminataTM Forte Western HRP (horseradish peroxidase) substrate (Merck Millipore), and signals were acquired with Image Quant Imager400 (GE Healthcare Bio-Science).

Fig. 3. Micrographs of *S. aureus* cells in the presence, or absence, of waldiomycin.

The *S. aureus* N315 strains were grown in TSB to an OD₆₆₀ of 0.3 at 37°C. Waldiomycin was added to the cultures and then incubated for an additional 1 h at the same temperature with shaking. Cells were stained with Gram's stain and observed under a light microscope at ×1,000 magnification. A: Waldiomycin treated cells. B: Waldiomycin untreated cells.

Results and Discussion

In vitro inhibitory effects of waldiomycin against WalK HKs

We examined the inhibitory activity of waldiomycin against WalK autophosphorylation *in vitro*. The half maximal inhibitory concentrations (IC₅₀s) against WalK HKs purified from *B. subtilis*, *S. aureus*, *E. faecalis*, and *S. mutans* were 10.2, 8.8, 9.2, and 25.8 μM, respectively. This shows that waldiomycin inhibits WalKs from different sources with a similar efficacy, indicating that waldiomycin is a general WalK inhibitor. Simocyclinones, a group of antibiotics structurally related to waldiomycin, are bifunctional antibiotics that inhibit bacterial DNA gyrase (Edwards et al., 2009). It has been shown that the aminocoumarin and angucycline moieties in simocyclinone are indispensable for potent inhibitory activity by interacting independently to the binding pocket of DNA gyrase. Similarly, the dioxolane carboxylic acid and angucycline moieties in waldiomycin are considered to be important for WalK HK inhibitory activity (Igarashi et al., 2013).

WalR regulon gene expression

Since we have shown that waldiomycin inhibited WalK *in vitro*, waldiomycin should exert an effect on the WalK/WalR system *in vivo*. Thus, the effect of this compound on *in vivo* expression of WalR regulon genes was analyzed using qRT-PCR. In *B. subtilis*, WalK/WalR activates expression of *yocH* (cell wall hydrolase), *yvcE* (*cwlO*, cell wall endopeptidase), *lytE* (autolysin) and *ydjM* (cell wall-associated protein), while negatively regulates the expression of *yoeB* (modulator of autolysin activity) and *yjeA* (peptidoglycan deacetylase) (Bisicchia et al., 2007; Howell et al., 2003; Salzberg and Helmann, 2007; Stapleton et al., 2007). In *S. aureus*, WalR positively regulates the expression of cell wall metabolism genes including *isaA* and *sceD* (cell wall transglycosylase/muramidase), *ssaA*, SA2353, SA2097, SA0620, SA0710, and *atlA* (cell wall

amidase), and *lytM* (cell wall glycyl-glycine endopeptidase) (Dubrac and Msadek, 2004; Dubrac et al., 2007, 2008). The cleavage sites for WalK/WalR regulon encoded cell wall hydrolases in *S. aureus* are indicated in Fig. 2C.

Addition of waldiomycin to the culture of *B. subtilis* led to lowered expressions of *yocH* (40%) and *yvcE* (13%) and increased expressions of *yoeB* (16-fold) and *yjeA* (7-fold) within 5 min (Fig. 2A). As for *S. aureus*, addition of waldiomycin led to lowered expressions of *isaA* (20%), *sceD* (30%), *ssaA* (20%), SA2353 (30%), SA2097 (40%) and SA0710 (60%) within 5 min (Fig. 2B). These results support the *in vivo* inhibition of the WalK/WalR system by waldiomycin. Although SA0620, *atlA*, and *lytM* are genes that are positively regulated by WalR, expression of SA0620 and *lytM* after waldiomycin treatment did not change, and that of *atlA* increased (Fig. 2B). We assumed that there may be some other regulatory factors besides WalR that may control the expression of these genes.

Effect of waldiomycin against cell morphology

Cell wall metabolism is involved in many cellular processes, and modifications of peptidoglycan degradation are linked to a wide range of phenotypes. A well-documented phenotype associated with a defect in autolysins is the propensity of planktonic cell suspensions to aggregate. As shown in our qRT-PCR results, waldiomycin inhibited the expression of several cell wall metabolism genes in *S. aureus*. We have tested the effects of this compound on *S. aureus* cell organization. As peptidoglycan hydrolases are involved in daughter cell separation, one would expect WalK/WalR inhibited cells to be aggregated in *S. aureus* instead of proper cellular dissemination. As shown in Fig. 3B, waldiomycin-untreated *S. aureus* cells exhibited typically well separated morphology, whereas waldiomycin-treated cells aggregated and formed clusters of aggregated cells (Fig. 3A). This cell aggregation phenotype is consistent with the previously reported phenotypes of WalK/WalR starved cells (Dubrac et al., 2007).

Fig. 4. Effect of waldiomycin on autolysis profiles of *S. aureus* cells mediated by Triton X-100 and lysostaphin.

The *S. aureus* N315 strains were grown in TSB to an OD₆₀₀ of 1. After the addition of waldiomycin at 6 μ g/ml (3/4MIC), the culture was incubated for an additional 10 min. Then the cells were pelleted and resuspended in PBS to test the effects of autolysis-inducing agents. Triton X-100 (A) and lysostaphin (B) mediated lysis of staphylococci was measured as the decline of OD₆₀₀ over time. Autolysis of waldiomycin-treated cells (open symbols) and untreated cells (closed symbols) were determined at 30°C in the presence of 0.1% Triton X-100 and at 37°C in the presence of 200 ng/ml lysostaphin.

Fig. 5. Effect of waldiomycin on biofilm formation of *S. aureus* N315. Biofilm formation assays of *S. aureus* were performed on microtiter plates in TSB plus 0.25% glucose with increasing concentrations of waldiomycin. Quantifications of biofilms were performed by measuring the OD₅₉₅ following crystal violet staining and resuspension in ethanol-acetone (80:20).

Effect of waldiomycin against Triton X-100- and lysostaphin-induced cell lysis

Different regulatory mechanisms are involved in the regulation of autolysis, such as modifying cell wall peptidoglycan (the substrate of murein hydrolases) and regulating the expression of genes associated with cell wall hydrolase activities (Groicher et al., 2000; Rice et al., 2003; Zheng et al., 2007). Since the WalK/WalR system effectively regulates bacterial autolysis, waldiomycin should have an effect on cell lysis. We tested the effect of waldiomycin on the autolysis of *S. aureus* induced by Triton X-100 or lysostaphin. As shown in Fig. 4A, waldiomycin-treated cells exhibited a high resistance to Triton X-100-induced autolysis; since only 30% cell lysis was observed after 3.5 h, while 96% cell lysis occurred in waldiomycin-untreated cells. This effect may be the consequence of waldiomycin repressed expression of WalR regulon genes. The effect of WalK/WalR inhibition on the lysostaphin sensitivity was also examined. Lysostaphin is a glycyl-glycine endopeptidase that specifically cleaves

Fig. 6. Effect of waldiomycin on *in vivo* autophosphorylation of WalK histidine kinase.

Overnight cultures of *B. subtilis* 168 were grown with aeration to OD₆₀₀ of 0.3 at 37°C. At this point, waldiomycin was added (Lane 1, 0 μ g/ml; 2, 1 μ g/ml; 3, 2 μ g/ml; 4, 4 μ g/ml; and lane 5, 8 μ g/ml (MIC)) and the cultures were incubated for an additional 1 h. Cells were harvested, disrupted, and analyzed by Phos-tag SDS-PAGE and Western blotting. P-WalK, phosphorylated WalK.

the pentaglycine cross-bridges of staphylococcal cell wall, leading to rapid lysis of the bacteria. As shown in Fig. 4B, only 39% cell lysis was seen for waldiomycin-treated cells after 30 min, whereas waldiomycin-untreated cells displayed a higher lysis rate, and 92% cells were lysed. These data showed that inhibition of WalK/WalR led to increased lysostaphin resistance. This effect could be due to a modification of cell wall peptidoglycan composition resulting in lowered accessibility of the pentaglycine bonds targeted by lysostaphin. These cell lysis phenotypes are also consistent with the previously reported phenotypes of WalK/WalR depleted cells (Dubrac et al., 2007), and support the fact that waldiomycin inhibits WalK *in vivo*.

Effect of waldiomycin on S. aureus biofilm formation

According to the previous report of Dubrac et al. (2007), depletion of the WalK/WalR system led to impaired biofilm formation in *S. aureus*. We checked the effect of

waldiomycin against *S. aureus* biofilm formation on a polystyrene surface. Cells produced multilayered weak biofilms in the DMSO treated (control) well. Contrary to our expectations, we found enhanced biofilm formation with increasing concentrations of waldiomycin. Sturdy biofilms were formed in the presence of a higher concentration of waldiomycin compared with the DMSO control. As shown in Fig. 5, the measured absorbance was 0.823 in the presence of 3/4MIC of waldiomycin, while it was 0.507 in the DMSO control well.

Autolytic activity of *atlA* gene has been linked to biofilm formation (Biswas et al., 2006; Heilmann et al., 1997). Since our qRT-PCR results revealed an increased expression of *atlA* gene due to the addition of waldiomycin to the *S. aureus* culture (Fig. 2B), this may be the reason why waldiomycin led to more robust biofilms in our biofilm formation assay.

In vivo inhibitory effect of waldiomycin against WalK HKs

The effect of waldiomycin on WalR regulon gene expression, cell morphology, and autolysis strongly suggests that waldiomycin inhibits WalK and thereby represses the WalK/WalR system in cells. In order to examine whether waldiomycin actually inhibited *in vivo* WalK autophosphorylation, we employed Phos-tag SDS-PAGE to analyze the level of WalK autophosphorylation in *B. subtilis*. Phos-tag SDS-PAGE is a technique that is capable of separating phosphorylated and unphosphorylated forms of protein (Kinoshita et al., 2006). This technique has been successfully applied to the detection of phosphorylated RRs *in vivo* (PhoB, WalR and PhoP) (Barbieri and Stock, 2008; Ishii et al., 2013; Wayne et al., 2012). As shown in Fig. 6, we were able to separate the phosphorylated WalK from the non-phosphorylated WalK (lane 1). When waldiomycin was added to exponential phase cells and incubated for 1 h before Phos-tag SDS-PAGE analysis, the phosphorylated WalK bands decreased with increasing concentrations of waldiomycin (lanes 2–5). This result clearly showed that waldiomycin applied from outside the cell inhibited WalK autophosphorylation inside the cell.

In this study, we have demonstrated that waldiomycin targets WalK histidine kinase and inhibits autophosphorylation activity of this essential histidine kinase both *in vitro* and *in vivo*, thereby inhibiting the WalK/WalR essential signal transduction pathway involved in critical biological processes during cell growth and division.

The continuing increase of hospital- and community-associated MRSA infections highlights an urgent need for the development of alternative potent antibacterial agents (Grundmann et al., 2006; Kazakova et al., 2005; Rice, 2006). By inhibiting the WalK/WalR TCS, waldiomycin has the potential to combat against different Gram-positive bacteria, including MRSA. However, the antimicrobial efficacy of this compound should be further improved by additional modification of the structure. Further pre-clinical studies of this compound will also be necessary before clinical application against infections caused by multidrug-resistant bacteria.

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