Full Paper

Characterization of H-box region mutants of WalK inert to the action of waldiomycin in *Bacillus subtilis*

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The WalK/WalR two-component system is essential for cell wall metabolism and thus for cell growth in *Bacillus subtilis*. Waldiomycin was previously isolated as an antibiotic that targeted WalK, the cognate histidine kinase (HK) of the response regulator, WalR, in *B. subtilis*. To gain further insights into the action of waldiomycin on WalK and narrow down its site of action, mutations were introduced in the H-box region, a well-conserved motif of the bacterial HKs of WalK. The half-maximal inhibitory concentrations (IC₅₀s) of waldiomycin against purified WalK protein with triple substitutions in the H-box region, R377M/R378M/S385A and R377M/R378M/R389M, were 26.4 and 55.1 times higher than that of the wild-type protein, respectively, indicating that these residues of WalK are crucial for the inhibitory effect of waldiomycin on its kinase activity. Surprisingly, this antibiotic severely affected cell growth in a minimum inhibitory concentration (MIC) assay, but not transcription of WalR-regulated genes or cell morphology in *B. subtilis* strains that harbored the H-box triple substitutions on the bacterial chromosome. We hypothesized that waldiomycin targets other HKs as well, which may, in turn, sensitize *B. subtilis* cells with the H-box triple mutant alleles of the *walK* gene to waldiomycin. Waldiomycin inhibited other

HKs such as PhoR and ResE, and, to a lesser extent, CitS, whose H-box region is less conserved. These results suggest that waldiomycin perturbs multiple cellular processes in *B. subtilis* by targeting the H-box region of WalK and other HKs.

Key Words: Antibiotics; *Bacillus subtilis* (*B. subtilis*); H-box region; Histidine kinase (HK); inhibitor; two-component system (TCS); Waldiomycin; WalK/WalR

Introduction

Bacteria use several sets of two-component systems (TCSs), each of which consist of a pair of proteins, a histidine kinase (HK) and a response regulator (RR), to respond to a wide variety of environmental stimuli. TCSs play essential roles in bacterial cell physiology, including drug-resistance, virulence, biofilm formation, quorum sensing, and cell growth (Inouye and Dutta, 2003; Utsumi, 2008). The WalK (HK)/WalR (RR) TCS is essential for cell wall metabolism and, thus, for cell growth in *Bacillus subtilis* (Fabret and Hoch, 1998), *Staphylococcus aureus* (Martin et al., 1999), and *Enterococcus faecalis* (Hancock and Perego, 2002), and has gained much attention as a novel antibacterial drug target against multidrug-resistant bacteria, such as methicillin-resistant *S. aureus* (MRSA)

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Fig. 1. (A) Model illustrating the domain structure of the WalK histidine kinase (HK).

A sensor domain is located between the two transmembrane (TM) helices. A HAMP domain, a PAS domain, and the DHp domain follow TM2. The catalytic and ATP-binding (CA) domain is at the C-terminus. The position of the histidine phosphorylation site is marked with an “H” together with a square indicating the H-box region. The positions of the N, G1, F and G2 boxes are marked with “N”, “G1”, “F” and “G2”, respectively. (B) Alignment of the H-box region amino acid sequences of HKs (WalK, ResE, PhoR, YcLK, YkoH, YvrG, BceS, and CitS) in *B. subtilis*. The position of the histidine phosphorylation site is marked in bold letters. Numbers on the H-box region amino acids of WalK indicate their sequence positions. Asterisks indicate amino acids that are conserved in all listed HKs. Twin dots and single dots indicate conservative and semiconservative substitutions, respectively.

and vancomycin-resistant *E. faecalis* (Bem et al., 2015; Gotoh et al., 2010; Schreiber et al., 2009) for the following reasons (Utsumi, 2008): (i) Although many HK and RR genes are coded on the bacterial genome, none have been identified in mammalian genomes. The HK/RR signal transduction system is distinct from serine/threonine/tyrosine phosphorylation signaling, which is more prevalent in higher eukaryotes than in prokaryotes. (ii) HKs possess a high degree of homology around their active sites. HKs are characterized by a conserved C-terminal domain containing a histidine residue (H-box; Fig. 1A), which is the site of autophosphorylation and the N, G1, F, and G2 boxes, which form an ATP-binding surface (Grebe and Stock, 1999). The high degree of structural homology in the C-terminal domains of HKs suggest that multiple

TCSs within a single bacterium can be inhibited simultaneously by a single inhibitor, thereby lowering the frequency of appearance of drug-resistant strains.

WalK is a membrane-linked HK that possesses two enzymatic domains at its cytoplasmic C-terminal region (Fig. 1A): a catalytic (kinase or ATP-binding) domain (CA), and a dimerization domain (DHp) containing an H-box in which phosphorylated histidine and several other amino acid residues are well conserved (Wang et al., 2013; Okajima et al., unpublished). While any specific signal(s) are unknown that alter autokinase and phosphatase activities of WalK from a sensor domain at periplasm and/or HAMP and PAS domains at cytosol, WalK inhibitors that target the CA or DHp domains have been reported (Qin et al., 2006; Watanabe et al., 2012). Some were found using structure-based virtual screening of chemical molecules by targeting the CA domain (Qin et al., 2006), although further derivation of these compounds was necessary to reduce their toxicity in experimental animals. We screened for WalK inhibitors from natural sources and isolated a new antibiotic, signermycin B (Watanabe et al., 2012), which inhibited autokinase activity by binding to the WalK DHp domain, thereby hindering WalK/WalR signal transduction involved in cell growth and division. These results suggested that both the CA and DHp domains of WalK may represent promising targets for the development of antibiotics that are active against drug-resistant bacteria.

Recently, we screened for WalK inhibitors from natural sources using a previously reported method (Watanabe et al., 2012) and isolated and characterized a new antibiotic, waldiomycin (Fakhruzzaman et al., 2015; Igarashi et al., 2013). Waldiomycin is an angucycline antibiotic composed of 1,3-dioxolane-2-carboxylic acid linked to an angucyclic polyketide via a tetraene linker and tetrahydropyran (Igarashi et al., 2013). It has antimicrobial activity against Gram-positive pathogens, including MRSA and *B. subtilis*. However, the detailed mechanism of the action by which waldiomycin inhibits WalK activity is still to be elucidated. In the present study, to determine how waldiomycin targets WalK in *B. subtilis*, mutants focused on the H-box region of WalK, some of which became almost completely resistant to the inhibitory action of waldiomycin, were constructed. Using both *in vitro* and *in vivo* methods, we narrowed down the site of action of waldiomycin by characterizing strains of *B. subtilis* with mutations in the H-box region of WalK, grown in the presence of this drug.

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 aerobically grown in lysogeny broth (LB) (1% polypeptone, 0.5% yeast extract, and 0.5% NaCl) at 37°C unless specified otherwise. Ampicillin and spectinomycin were used at 100 µg/ml and chloramphenicol at 50 µg/ml.

Construction of the mutants in the H-box region of WalK on its expression plasmid. Mutations in the H-box region of WalK were constructed with a QuikChange Site-Directed Mutagenesis Kit (Stratagene) using the primers listed in Table 2 and pETWalK_{366–611} (pBsGsubA;

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> DH5 α	F ⁻ , ϕ 80dZAM15, Δ (<i>lacZYA-argF</i>) U169, <i>deoR</i> , <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (rK ⁻ , mK ⁺), <i>phoA</i> , <i>supE44</i> , λ^- , <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i>	Thermo
<i>E. coli</i> BL21 (DE3)	F ⁻ , <i>ompT</i> , <i>hsdS</i> (τ_B^- m _B ⁻), <i>gal</i> , <i>dem</i> (DE3)	Laboratory stock
<i>B. subtilis</i> 168 (Wild-type)	<i>trpC2</i>	Laboratory stock
<i>B. subtilis</i> TMO310	<i>trpC2</i> , <i>aprE</i> ::pAPNC213- <i>chpA</i> (Sp ^R)	Morimoto et al. (2009)
<i>B. subtilis</i> SU1	<i>walk</i> ⁺ -Cm ^R - <i>yycH</i> ⁺	This study
<i>B. subtilis</i> SU2	<i>walk</i> ⁺ - <i>yycH</i> ⁺ - <i>mazF</i> ⁺ - <i>lacI</i> ⁺ -Sp ^R -Cm ^R - <i>yycH</i> ⁺	This study
<i>B. subtilis</i> SU3	<i>walk</i> K (R377M/R378M)	This study
<i>B. subtilis</i> SU4	<i>walk</i> K (R377M/R378M/S385A)	This study
<i>B. subtilis</i> SU5	<i>walk</i> K (R377M/R378M/R389M)	This study
Plasmids		
pHT10	Expression vector, Cm ^R , Ap ^r , C-terminal c-Myc tag	MoBiTec
pET-21a (+)	Expression vector, Ap ^r , C-terminal His tag	Novagen
pETWALK ₃₆₆₋₆₁₁ (pBsGsubA)	WALK (Bs) (<i>B. subtilis</i> 168 aa 366 to 611) cloned into pET-21a (+)	Watanabe et al. (2012)
pETWALK ₃₆₆₋₆₁₁ R377A	pET-21a (+), WALK ₃₆₆₋₆₁₁ R377A	This study
pETWALK ₃₆₆₋₆₁₁ R377M	pET-21a (+), WALK ₃₆₆₋₆₁₁ R377M	This study
pETWALK ₃₆₆₋₆₁₁ R378A	pET-21a (+), WALK ₃₆₆₋₆₁₁ R378A	This study
pETWALK ₃₆₆₋₆₁₁ R378M	pET-21a (+), WALK ₃₆₆₋₆₁₁ R378M	This study
pETWALK ₃₆₆₋₆₁₁ S385A	pET-21a (+), WALK ₃₆₆₋₆₁₁ S385A	This study
pETWALK ₃₆₆₋₆₁₁ E387A	pET-21a (+), WALK ₃₆₆₋₆₁₁ E387A	This study
pETWALK ₃₆₆₋₆₁₁ R389M	pET-21a (+), WALK ₃₆₆₋₆₁₁ R389M	This study
pETWALK ₃₆₆₋₆₁₁ T390A	pET-21a (+), WALK ₃₆₆₋₆₁₁ T390A	This study
pETWALK ₃₆₆₋₆₁₁ P391A	pET-21a (+), WALK ₃₆₆₋₆₁₁ P391A	This study
pETWALK ₃₆₆₋₆₁₁ T393A	pET-21a (+), WALK ₃₆₆₋₆₁₁ T393A	This study
pETWALK ₃₆₆₋₆₁₁ T394A	pET-21a (+), WALK ₃₆₆₋₆₁₁ T394A	This study
pETWALK ₃₆₆₋₆₁₁ R377A/R378A	pET-21a (+), WALK ₃₆₆₋₆₁₁ R377A/R378A	This study
pETWALK ₃₆₆₋₆₁₁ R377M/R378M	pET-21a (+), WALK ₃₆₆₋₆₁₁ R377M/R378M	This study
pETWALK ₃₆₆₋₆₁₁ R377M/R378M/S385A	pET-21a (+), WALK ₃₆₆₋₆₁₁ R377M/R378M/S385A	This study
pETWALK ₃₆₆₋₆₁₁ R377M/R378M/R389M	pET-21a (+), WALK ₃₆₆₋₆₁₁ R377M/R378M/R389M	This study

Table 2. Primers used in the site-directed mutagenesis of *walK* gene in this study.

WalK substitution	Primer name	Primer sequence (5' → 3')
R377A	Bs WalK-R377A F	GGATCAGGAAGCAAGAGAATTCGTTGCG
	Bs WalK-R377A R	CGCAACGAATTCTCTTCTTCCTGATCC
R377M	Bs WalK-R377M F	GGATCAGGAAATGAGAGAATTCGTTGCG
	Bs WalK-R377M R	GCAACGAATTCTCTCATTTCTGATCC
R378A	Bs WalK-R378A F	GGATCAGGAACGCGCAGAATTCGTTGCGAATG
	Bs WalK-R378A R	CATTCGCAACGAATTCTGCGCGTTCCTGATCC
R378M	Bs WalK-R378M F	ATGGATCAGGAACGCATGGAATTCGTTGCGAAT
	Bs WalK-R378M R	ATTCGCAACGAATTCCATGCGTTCCTGATCCAT
S385A	Bs WalK-S385A F	GCGAATGTAGCACATGAGCTG
	Bs WalK-S385A R	CAGCTCATGTGCTACATTCGC
E387A	Bs WalK-E387A F	GTTGCGAATGTATCACATGCGCTGCGGACGCCGTTACG
	Bs WalK-E387A R	CGTAAGCGGCGTCCGACGCGCATGTGATACATTCGCAAC
R389M	Bs WalK-R389M F	GTATCACATGAGCTGATGACGCCGCTTACGACA
	Bs WalK-R389M R	TGTCGTAAGCGGCGTCATCAGCTCATGTGATAC
T390A	Bs WalK-T390A F	CATGAGCTGCGGGCGCCGCTTACGACAATG
	Bs WalK-T390A R	CATTGTCGTAAGCGGCGCCCGCAGTCTCATG
P391A	Bs WalK-P391A F	CATGAGCTGCGGACGGCGCTTACGACAATG
	Bs WalK-P391A R	CATTGTCGTAAGCGCCGTCGCGAGTCTCATG
T393A	Bs WalK-T393A F	CTGCGGACGCCGCTTGCGACAATGCGCAGCTAT
	Bs WalK-T393A R	ATAGCTGCGCATTGTGCGAAGCGGCGTCCGCAG
T394A	Bs WalK-T394A F	CGGACGCCGCTTACGGCAATGCGCAGCTATTTA
	Bs WalK-T394A R	TAAATAGCTGCGCATTGCCGTAAGCGGCGTCCG
R377AR378A	Bs WalK-R377AR378A F	GGATCAGGAAGCAGCAGAATTCGTTGCGAATG
	Bs WalK-R377AR378A R	CATTCGCAACGAATTCTGCTGCTTCCTGATCC
R377MR378M	Bs WalK-R377MR378M F	AAAATGGATCAGGAAATGATGGAATTCGTTGCGAAT
	Bs WalK-R377MR378M R	ATTCGCAACGAATTCCATCATTTCTGATCCATTTT

Watanabe et al., 2012) as a template; except for the triple substitutions (R377M/R378M/S385A and R377M/R378M/R389M), where the constructs were amplified using pairs of primers (Bs WalK-S385A F/Bs WalK-S385A R and Bs WalK-R389M F/Bs WalK-R389M R) with pETWalK₃₆₆₋₆₁₁ R377M/R378M as a template. Nucleotide sequencing confirmed that the inserted DNA contained only the designed mutations.

Construction of WalK triple mutants on the chromosome of *B. subtilis*. The *B. subtilis* chromosomal mutant strains were constructed (Fig. 2) based on the marker-free deletion method (Morimoto et al., 2009). Note that the chloramphenicol resistant (Cm^R) marker (as well as the *walK* gene) was used as a site for homologous recombination (Fig. 2C) to facilitate the integration of another copy of the *ycyH* gene, which was later used as a site for resolving the counter selectable marker, *mazF*, along with the *lacI*, spectinomycin resistance (Sp^R) and Cm^R genes (Fig. 2E) from an intermediate strain (Fig. 2D).

To construct the SU2 strain (which was used for template DNA for PCR, as described below) (Fig. 2B), which represents gene orders in a prototypical intermediated strain (Fig. 2D), three PCR fragments were generated (Fig. 2A). The first fragment, encompassing the *walK* and *ycyH*

genes was amplified using primer 1 and primer 2 with wild-type *B. subtilis* chromosomal DNA as the template. The second fragment, including the counter selectable *mazF* marker and the *lacI*-Sp^R marker was amplified using primer 3 and primer 4 with *B. subtilis* TMO310 chromosomal DNA as the template. A Cm^R marker was amplified as the third fragment using primer 5 and primer 6 with pHT10 as the template. All PCR products were purified before the next step. The first and second PCR fragments were annealed together and then further amplified using primers 1 and 4. The resultant DNA fragment and the third PCR fragment were annealed together, further amplified using primers 1 and 6, and introduced into the SU1 strain (Fig. 2C), according to a standard method (Anagnostopoulos and Spizizen, 1961). The strain that integrated the combined DNA fragment A, encompassing the *walK*, *ycyH*, *mazF*, *lacI*-Sp^R, and Cm^R genes, on the chromosome by double crossover events (Fig. 2C) was selected on an LB agar plate containing spectinomycin (Fig. 2D).

To construct the SU3 strain harboring the double substitution (R377M/R378M) in the H-box region of the *walK* gene, a combined DNA fragment B (Fig. 2B) was amplified using primers 1, Bs WalK-R377MR378M R, Bs WalK-

Fig. 2. Schematic representation of the steps in the construction of the *walk* mutants on the *B. subtilis* chromosome.

(A) To construct the SU2 strain with the wild-type *walk* gene, three PCR fragments were generated using primers (i.e., 1–6), some of which were designed to harbor complementarity sequences (i.e., 2–3 and 4–5) at each 5' region, as described in detail in Materials and Methods. They were combined together as DNA fragment A. (B) To construct the *walk* mutant strains, SU3, SU4, and SU5, two PCR fragments were generated from the combined DNA fragment A, which had been replicated on the *B. subtilis* chromosome once, as described in detail in Materials and Methods. They were combined as DNA fragment B, containing a *walk* allele, which is indicated by a black box with a white star. A pair of primers, mutant forward (MF) and mutant reverse (MR), were complementary to each other, in this case. (C) The combined DNA fragment B (or A) was introduced into the SU1 strain that harbored the *Cm^R* gene between the *walk* and *yycH* genes. *Sp^R* colonies were selected on an LB agar plate containing Spectinomycin (Sp) to obtain an intermediate strain (D) harboring *walk* substitutions (or the wild-type sequence) and two copies of the *yycH* gene, which later became sites resolving the counter selectable *mazF* marker as well as the *lacI*-*Sp^R* and *Cm^R* markers (E). From several candidate colonies, one that had harbored only the desired substitutions (or wild-type sequence in the case of the SU2 strain) in the *walk* gene was screened by direct sequencing of colony PCR products. (F) A markerless strain with a *walk* allele was selected on an LB agar plate containing 1 mM IPTG and its *Sp*- and *Cm*-sensitive phenotypes were confirmed after its isolation.

Table 3. Primers used in qRT-PCR and the construction of WalK chromosomal mutants in this study.

Primer name	Primer sequence (5' → 3')
rt-rpoB-F	GACCGGGCGGATTGACA
rt-rpoB-R	GCCATAGTGGGAGTAGTGAACGT
rt-gyrA-F	AGTCATTCCAGGGCCTGATT
rt-gyrA-R	ATTCTGATGCTTTCCGGATCC
rt-yocH-2F	ATGTCCTTTGTGGCAGTTGCT
rt-yocH-2R	GCACCGTAATTTCTTTTGCAGAA
rt-yvcE-F	GCGGACGCACTCAGTCTGA
rt-yvcE-R	TGCCCAGCGTACGAATGAT
rt-yjeA-F	CATTGCCGCGGACGAT
rt-yjeA-R	CAAAGCGGCTGAAATTTTCTTT
rt-yoeB-2F	CGCTAGAGAACATTTTGGAAACAC
rt-yoeB-2R	TGATGGGCAAACTGCTTTTTT
primer 1	AGAGTTCTTATTTCCGCGAAGCCC
primer 2	CGTACTGATTGGGTAGGATCCCCGGTTATTCCACTCCACTGTTAGCCCCC
primer 3	TATCGACAGCGGAATTGACTCAAGC
primer 4	CCGGGGATCCTACCCAATCAGTACG
primer 5	GCTTGAGTCAATTCGCTGTCGATAGGCAATAGTTACCCTTATTATCAAG
primer 6	TTATAAAGCCAGTCATTAGGCCTATCTGACAA
primer 7	ATGAATAAGGTTGGTTTTTTTCGGTTCGATC
primer 8	CTTATCTTGATAATAAGGGTAAGTATGCTTCACGCTTCATCCCAATCATCCTCTTGTTTC
primer 9	GAACAAGAGGATGATTGGGATGAAGCGTGAGGCAATAGTTACCCTTATTATCAAGATAAG
primer 10	TCGTTTTTATATTTTCACGCTTCATTTATAAAGCCAGTCATTAGGCCTATCTGA
primer 11	TCAGATAGGCCAATGACTGGCTTTTATAAATGAAGCGTGAAAAATATAAAAAACGA
primer 12	TTATTCCACTCCACTGTTAGCCCCCTCCTT
primer 13	CGCGGATCCTTAATCAAATGAAGACGATCAACA
primer 14	ACTGTATTCAAACGACTGATTAGACCATTATAA

R377MR378M F, and 6 with SU2 chromosomal DNA as the template by PCR. The strain (an SU1 derivative) that integrated the combined PCR fragment by double crossover events (Fig. 2C) was selected on an LB agar plate containing spectinomycin (Fig. 2D). A clone harboring a designated *walK* allele was screened from several candidate colonies by the direct sequencing of colony PCR products. The selected intermediate strain was grown on an LB agar plate containing 1 mM IPTG to counter-select *mazF* along with the markers, *lacI*-Sp^R and Cm^R (Figs. 2E and 2F). Likewise, to construct the SU4 strain containing the triple substitution (R377M/R378M/S385A) in the H-box region of the *walK* gene, a combined DNA fragment B (Fig. 2B) was amplified using primers 1, Bs WalK-S385A R, Bs WalK-S385A F, and 6 with SU3 chromosomal DNA as the template by PCR. The selected intermediate strain was grown on an LB agar plate containing 1 mM IPTG to counter-select *mazF* along with the markers, *lacI*-Sp^R and Cm^R (Figs. 2E and 2F). The SU5 strain, containing the triple substitution (R377M/R378M/R389M) in the H-box region, was constructed in the same way as SU4, using primers Bs WalK-R389M R and Bs WalK-R389M F instead of Bs WalK-S385A R and Bs WalK-S385A F, respectively.

To construct the SU1 strain harboring the Cm^R cassette between the *walK* gene and the *yycH* gene, the parental strain of SU2, SU3, SU4, and SU5, first of all, three PCR fragments were generated using primers 7-12 with wild-type *B. subtilis* chromosomal DNA (for primer pairs 7/8 and 11/12) or pHT10 (for primers 9 and 10) as templates. The combined fragment was amplified using primer 13 and primer 14, and introduced into the *B. subtilis* 168 strain. Cm^R colonies were selected on an LB agar plate containing chloramphenicol. The *walK* gene was ampli-

Table 4. IC₅₀s and MIC of waldiomycin against WalK mutants *in vitro* and *in vivo*, respectively.

WalK allele	IC ₅₀ (μM)	MIC (μM)	MIC (μg/ml)
Wild-type	10.3 ± 1.0	5.7	4
R377A	20.4 ± 1.3	ND	ND
R377M	17.3 ± 3.6	ND	ND
R378A	20.2 ± 2.5	ND	ND
R378M	17.7 ± 0.4	ND	ND
S385A	44.6 ± 1.3	5.7	4
E387A	17.2 ± 0.4	ND	ND
R389M	23.5 ± 1.5	5.7	4
T390A	15.9 ± 2.5	5.7	4
P391A	46.3 ± 16.9	5.7	4
T393A	15.2 ± 0.4	ND	ND
T394A	11.8 ± 0.7	ND	ND
R377A/R378A	14.8 ± 4.0	ND	ND
R377M/R378M	43.6 ± 3.1	ND	ND
R377M/R378M/S385A	274.6 ± 55.2	5.7	4
R377M/R378M/R389M	557.2 ± 10.2	5.7	4

fied from the chromosome of all constructed strains and were confirmed to have only the designed substitutions or a wild-type sequence by direct nucleotide sequencing.

Measurement of IC₅₀s (50% inhibitory concentrations).

The cytoplasmic WalK domain (with a C-terminal His-tag) of *B. subtilis* was expressed and purified as previously described (Watanabe et al., 2012) using *E. coli* BL21 (DE3) containing pETWalK₃₆₆₋₆₁₁. Using this cytoplasmic domain containing the N-terminal 366 to C-terminal 611 amino acid residues, the IC₅₀s of waldiomycin against the WalK autokinase activity were measured as previously described (Fakhruzzaman et al., 2015). The data are pre-

Fig. 3. The effect of waldiomycin on the transcription of WalR-regulated genes.

Waldiomycin was added at 0.5 and 1 µg/ml to exponentially growing cultures of *B. subtilis*. After 5 min incubation, total RNA was isolated and quantified by qRT-PCR. The relative value in the transcription of each gene is presented as mean ± standard error from three independent experiments using primers specific for the WalR regulon gene, *gyrA*, and 16S rRNA. Data was first normalized with values of 16S rRNA and then respective target genes in the absence of waldiomycin (DMSO).

sented as the mean ± standard error of three independent experiments.

Antimicrobial activity. The minimum inhibitory concentrations (MICs) were determined by the broth microdilution method using LB. Bacterial suspensions were diluted to 10^5 – 10^6 cfu/ml and added to a 2-fold dilution series of waldiomycin in a 96-well microtiter plate. The plates were incubated at 37°C for 24 h. The MIC was determined as the lowest concentration at which growth was significantly inhibited.

Extraction of total RNA and qRT-PCR. The extraction of total RNA from *B. subtilis* 168 and its mutant strains (R377M/R378M/S385A and R377M/R378M/R389M), cultured in the presence or absence of waldiomycin and qRT-PCR, were performed as previously described (Fakhruzzaman et al., 2015). The primers used for qRT-PCR are listed in Table 3.

Microscopy. *B. subtilis* cells were grown to OD₆₆₀ of 0.3 in LB at 37°C with shaking. Waldiomycin was added to exponentially growing cultures to a final concentration of 4.0 µg/ml, and the cells were further shaken for 5 min at the same temperature. The cells were then observed under an Olympus CKX41 light microscope equipped with an LCAchN40XPh objective and WHB10X/20 eyepiece.

Results and Discussion

The amino acid residues of the H-box region of WalK are essential for the action of waldiomycin in vitro

Initially, we focused on characterizing the DHp domain of WalK (Fig. 1A) *in vitro* as the target of waldiomycin,

since another antibiotic, signermycin B, which was isolated by the same screening method as for waldiomycin, targeted the DHp domain of WalK (Watanabe et al., 2012). In addition, results from docking studies showed a potential interaction between waldiomycin and the H-box of the DHp domain of WalK (Okajima et al., unpublished). Based on the alignment of the H-box regions of HKs from *B. subtilis* (Fig. 1B), we selected and substituted the well-conserved amino acid residues, R377, R378, S385, E387, R389, T390, P391, T393, and T394 into alanine, which is generally employed for the construction of a series of substitution mutations because of its non-bulky and chemically inert (weak hydrophobic) properties, or methionine (only for R), which have a hydrophobic and similar side chain length to that of R, by site-directed mutagenesis. The WalK proteins with these single or combinations of substitutions were expressed and purified to examine the effect of waldiomycin. The half maximal inhibitory concentration (IC₅₀) values of waldiomycin against the WalK proteins with these single substitutions were elevated up to approximately 4-fold compared with that of the wild-type protein (Table 4). Among all the single substitutions in the H-box region, P391A exhibited the highest value of 46.3 µg/ml. We included residues immediately adjacent to the original H-box motif (amino acid positions 380 to 395 in WalK) as the candidate sites of action of waldiomycin because they are conserved within certain HKs at locations relatively close to the predicted site (Fig. 1B). The IC₅₀ values of WalK with a single substitution at R377 or R378 were approximately 2-fold compared with that of the wild-type protein (Table 4). The highest IC₅₀ value (557.2 µg/ml) was observed in WalK with the triple substitutions, R377M/R378M/R389M. The IC₅₀ value of

Fig. 4. Micrographs of *B. subtilis* cells grown in the presence or absence of waldiomycin.

The *B. subtilis* strains were grown in LB to an OD₆₆₀ of 0.3 at 37°C. Waldiomycin was added at 4 µg/ml to the cultures and then they were allowed to incubate for an additional 5 min at the same temperature with shaking. Cells were observed by light microscopy at 400× magnification. A, C, E: Untreated cells. B, D, F: Waldiomycin treated cells. Scale bars, 5 µm.

Table 5. IC₅₀s of waldiomycin against *B. subtilis* HKs.

HK	IC ₅₀ (µM)	Amino acid identity in the H-box region (%)
WalK	10.3 ± 1.0	100
PhoR	15.0 ± 5.1	68.4
ResE	16.0 ± 0.9	63.2
CitS	93.6 ± 29.9	21.1

WalK with the triple substitutions, R377M/R378M/S385A, was comparable (274.6 µg/ml). Here, we refer to amino acid positions 377 to 395 of WalK, which include three more residues in addition to the original H-box motif (Grebe and Stock, 1999) and overlap with the essential residues for the action of waldiomycin, as the “H-box region” for convenience. Consequently, the IC₅₀ values of the triple substitutions, R377M/R378M/S385A and R377M/R378M/R389M, in the H-box region of WalK were 26.4 ± 4.1 and 55.1 ± 4.5 times higher than that of the wild-type protein, respectively. Note that the circular dichroism spectra of the triple mutants were not altered from that of the wild-type protein and that the autokinase ac-

tivities of these mutants were not reduced in the absence of waldiomycin (data not shown). These results indicate that waldiomycin inhibits this HK at the H-box region and/or at a minimum, these residues are essential for its action but not for the kinase activity and maintaining the overall structure of WalK.

Triple substitutions in the H-box region of WalK on the *B. subtilis* chromosome suppress the effects of waldiomycin in vivo, but not on cell growth

To evaluate the effects of triple substitutions in the H-box region of WalK *in vivo*, the transcription of WalR-regulated genes was measured by qRT-PCR using RNA extracted from wild-type and mutant bacterial cells grown in the presence or absence of waldiomycin (Fig. 3). In *B. subtilis*, the WalK/WalR system activates the expression of the genes *yocH*, *yvcE*, and *lytE*, which all encode autolysins, and *ydjM* (Dubrac et al., 2008). In contrast, the WalK/WalR system negatively regulates the expression of *yoeB* and *yjeA*, which encode proteins that modulate autolysin activity. Moreover, the transcription of the *yocH* and *yvcE* genes in the wild-type cells were suppressed, while that of the *yoeB* and *yjeA* genes were in-

duced in the presence of waldiomycin (0.5 and 1 $\mu\text{g/ml}$). However, these effects were almost completely suppressed in *B. subtilis* strains with the triple substitutions, R377M/R378M/S385A and R377M/R378M/R389M (Fig. 3). These results were consistent with the effect of the triple mutations on the IC_{50} of waldiomycin.

B. subtilis strains depleted for WalK/WalR form filamentous cells (Fabret and Hoch, 1998; Fukushima et al., 2008), most likely because the *ftsQAZ* genes, which are important for cell division, are also part of the WalR regulon (Fukuchi et al., 2000). To further characterize the effect of triple substitutions in the H-box region of WalK *in vivo*, we observed cell morphology of the wild-type strain and the triple mutants in the presence of waldiomycin (5.7 μM or 4 $\mu\text{g/ml}$) by light microscopy. Consequently, waldiomycin induced filamentation of wild-type *B. subtilis* cells (Fig. 4B) but not of the strains with the triple substitutions (R377M/R378M/S385A and R377M/R378M/R389M) in WalK (Figs. 4D and 4F, respectively). These results support the observation that waldiomycin targets the H-box region of WalK and that the morphological changes of cells induced by waldiomycin are also mediated through the inhibition of WalK kinase activity in wild-type *B. subtilis* cells. Thus far, the *in vitro* and *in vivo* results are consistent with the notion that triple substitutions in the H-box region of WalK are inert to the action of waldiomycin.

Next, we tested the growth inhibitory effect of waldiomycin in an MIC assay. Unexpectedly, waldiomycin inhibited cell growth of the triple mutant strains to the same extent as wild-type *B. subtilis* (Table 4).

Waldiomycin inhibits multiple *B. subtilis* HKs *in vitro*

The data presented above (except for the MIC) demonstrate that triple substitutions in the H-box region resulted in resistance against the action of waldiomycin on WalK. Although some of the H-box regions of other HKs exhibit high degrees of homology with that of WalK (Fig. 1B and Table 5), it remains unclear whether, and if so how, waldiomycin distinguishes WalK from other HKs. Furthermore, it is unclear why it inhibited cell growth of the strains with triple mutations in the *walK* gene (Table 4). This may be a consequence of the well-conserved H-box region being the target of waldiomycin among all the HKs expressed in *B. subtilis* cells. Here, we hypothesized that waldiomycin inhibits both WalK and HKs that regulate other cellular processes in *B. subtilis*. We examined this hypothesis by incubating purified HK proteins (ResE, PhoR, and CitS) with waldiomycin and ATP. This resulted in the inhibition of PhoR and ResE (Table 5), which share the characteristic residues of the H-box region of WalK (Fig. 1B and Fig. S1), and, to a lesser extent, CitS, (Table 5), whose H-box region is less conserved (Fig. 1B and Fig. S1). To establish a relationship between the several HKs which represent potential targets of waldiomycin in *B. subtilis*, we carried out a phylogenetic analysis focused on the amino acid sequences of the H-box region. WalK, ResE, and PhoR are clustered together, whereas CitS is separate from this WalK cluster (Fig. S1). These results suggest that waldiomycin can target more HKs than those tested in this study, at their common structural motif, which

should in turn affect multiple cellular processes in *B. subtilis*.

In the present study, we examined the effects of substitutions focused on the H-box region of WalK on inhibiting the action of waldiomycin *in vitro* and *in vivo*. Additionally, we defined the H-box region, which includes three residues in addition to the original H-box sequence, as the target site of waldiomycin. This motif was further supported by the observation that waldiomycin inhibited other HKs such as ResE and PhoR, which share the characteristic residues in the H-box region with WalK, with comparable IC_{50} s. To a lesser extent, waldiomycin also inhibited CitS, which is segregated from the WalK cluster in the phylogenetic analysis of the H-box region sequences of HKs in *B. subtilis*. Previously, we reported the IC_{50} s of waldiomycin against the WalK HKs of *B. subtilis*, *S. aureus*, *E. faecalis*, and *Streptococcus mutans* (Fakhruzzaman et al., 2015; Igarashi et al., 2013) and showed that waldiomycin is a general WalK inhibitor. In this study, the target of waldiomycin was extended from WalK to other HKs in *B. subtilis*. Moreover, we demonstrated that it still inhibited the growth of *B. subtilis* cells with the triple mutations in WalK that had become inert to the action of waldiomycin. This was most likely because multiple cellular processes under the control of several HKs could be impaired at the same time, while in genetics the WalK/WalR system is the only essential TCS in *B. subtilis*. (Currently, we cannot rule out the possibility that waldiomycin may target another type of molecule other than HKs *in vivo* in *B. subtilis*.) Thus, the frequency of appearance of strains resistant to waldiomycin should not be that high. One can test if there is any alteration in such a frequency of the strain with the triple mutations in the *walK* gene from that of the wild-type strain. In addition, by studying other HK allele(s) whose H-box region harbors similar triple substitutions in the *walK* allele, it will be possible to identify other essential target(s) of waldiomycin *in vivo*, although this is a laborious task. In conclusion, waldiomycin targets several HKs, especially those with the WalK-type H-box region.

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Supplementary Materials

Supplementary figure is available in our J-STAGE site (<http://www.jstage.jst.go.jp/browse/jgam>).

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