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 meticillin-resistant S. aureus (MRSA).
Waldiomycin targets a common HK motif

**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, which form an ATP-binding surface, are indicated by ‘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; Gotot 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 suggests 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 angucycline 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 QuickChange Site-Directed Mutagenesis Kit (Strategene) using the primers listed in Table 2 and pETWalK366–611 (pBsGsubA;
Table 1. Bacterial strains and plasmids used in this study.

| Strain or Plasmid | Relevant genotype or Description | Source or Reference |
|-------------------|----------------------------------|---------------------|
| **Bacterial strains** |                                  |                     |
| *E. coli* DH5α     | F-Φ80lacZM15, Δ(kicZYA-argF) U169, deoR, recA1, endA1, hsdR17 (rK-, mK+), phoA, supE44, λ, thi-1, gyrA96, relA1 | Thermo             |
| *E. coli* BL21 (DE3) | F-,ompT, hsdS (rK- mK+), gal, dcm (DE3) | Laboratory stock    |
| *B. subtilis* 168 (Wild-type) | trpC2 | Laboratory stock    |
| *B. subtilis* TM0310 | tnpC2, aprE, pAPNC213-<chpA(Sp8)> | Morimoto et al. (2009) |
| *B. subtilis* SU1 | walK<sup>+</sup>-<sup>Cm</sup>6-yycH<sup>+</sup> | This study          |
| *B. subtilis* SU2 | walK<sup>+</sup>-<sup>Cm</sup>6-yycH<sup>+</sup>-<sup>lacZ</sup>-<sup>Sp</sup>5<sup>+</sup>-<sup>Cm</sup>6-yycH<sup>+</sup> | This study          |
| *B. subtilis* SU3 | walK(R377M/R378M) | This study          |
| *B. subtilis* SU4 | walK(R377M/R378M/S385A) | This study          |
| *B. subtilis* SU5 | walK(R377M/R378M/R389M) | This study          |
| **Plasmids**       |                                  |                     |
| pHT10              | Expression vector, <sup>Cm</sup><sup>+</sup>, Ap<sup>+</sup>, C-terminal c-Myc tag | MoBiTec            |
| pET-21a (+)        | Expression vector, Ap<sup>+</sup>, C-terminal His tag | Novagen            |
| pETWalK<sub>366-611</sub> (pBsGsubA) | WalK (Bs) (*B. subtilis* 168 aa 366 to 611) cloned into pET-21a (+) | Watanabe et al. (2012) |
| pETWalK<sub>366-611</sub> R377A | pET-21a (+), WalK<sub>366-611</sub> R377A | This study          |
| pETWalK<sub>366-611</sub> R377M | pET-21a (+), WalK<sub>366-611</sub> R377M | This study          |
| pETWalK<sub>366-611</sub> R378A | pET-21a (+), WalK<sub>366-611</sub> R378A | This study          |
| pETWalK<sub>366-611</sub> R378M | pET-21a (+), WalK<sub>366-611</sub> R378M | This study          |
| pETWalK<sub>366-611</sub> S385A | pET-21a (+), WalK<sub>366-611</sub> S385A | This study          |
| pETWalK<sub>366-611</sub> E387A | pET-21a (+), WalK<sub>366-611</sub> E387A | This study          |
| pETWalK<sub>366-611</sub> R389M | pET-21a (+), WalK<sub>366-611</sub> R389M | This study          |
| pETWalK<sub>366-611</sub> T390A | pET-21a (+), WalK<sub>366-611</sub> T390A | This study          |
| pETWalK<sub>366-611</sub> P391A | pET-21a (+), WalK<sub>366-611</sub> P391A | This study          |
| pETWalK<sub>366-611</sub> T393A | pET-21a (+), WalK<sub>366-611</sub> T393A | This study          |
| pETWalK<sub>366-611</sub> T394A | pET-21a (+), WalK<sub>366-611</sub> T394A | This study          |
| pETWalK<sub>366-611</sub> R377A/R378A | pET-21a (+), WalK<sub>366-611</sub> R377A/R378A | This study          |
| pETWalK<sub>366-611</sub> R377M/R378M | pET-21a (+), WalK<sub>366-611</sub> R377M/R378M | This study          |
| pETWalK<sub>366-611</sub> R377M/R378M/S385A | pET-21a (+), WalK<sub>366-611</sub> R377M/R378M/S385A | This study          |
| pETWalK<sub>366-611</sub> R377M/R378M/R389M | pET-21a (+), WalK<sub>366-611</sub> R377M/R378M/R389M | This study          |
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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 pETWalK 366–611 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 (CmR) 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 yycH gene, which was later used as a site for resolving the counter selectable marker, mazF, along with the lacI, spectinomycin resistance (SpR) and CmR 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 intermediate strain (Fig. 2D), three PCR fragments were generated (Fig. 2A). The first fragment, encompassing the walK and yycH 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 marker mazF and the lacI-SpR marker was amplified using primer 3 and primer 4 with B. subtilis TMO310 chromosomal DNA as the template. A CmR 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 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, yycH, mazF, lacI-SpR, and CmR 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 F/Bs WalK-R377MR378M R.

### 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 | GGATCGAGGAAGCAGGAAATTCGTGTCGCC |
|                   | Bs WalK-R377A R | GCAACAGAATTCGTTCCGATCC |
| R377M             | Bs WalK-R377M F | GGATCGAGGAAGCAGGAAATTCGTGTCGCC |
|                   | Bs WalK-R377M R | GCAACAGAATTCGTTCCGATCC |
| R378A             | Bs WalK-R378A F | GGATCGAGGAAGCAGGAAATTCGTGTCGCC |
|                   | Bs WalK-R378A R | GCAACAGAATTCGTTCCGATCC |
| R378M             | Bs WalK-R378M F | GGATCGAGGAAGCAGGAAATTCGTGTCGCC |
|                   | Bs WalK-R378M R | GCAACAGAATTCGTTCCGATCC |
| S385A             | Bs WalK-S385A F | GGCAATGTTGACCATGAGCTGAC |
|                   | Bs WalK-S385A R | CAGCTCATGCTGATCC |
| E387A             | Bs WalK-E387A F | GTTGTCAAGTTATCATCGAGTGGGAAATTCGTTGCG |
|                   | Bs WalK-E387A R | CGTAAGCGGGCGCAGCATGATACCATCATCGCAAC |
| R389M             | Bs WalK-R389M F | GTATCGACATGAGCTGATGACGCCGTTACGACA |
|                   | Bs WalK-R389M R | TGTCCGTAAGCGCGCTACGTCAGCTGATAAC |
| T390A             | Bs WalK-T390A F | CATGGACTGCGGCGGCGCCGCAACGCGAAGAT |
|                   | Bs WalK-T390A R | CATGGACTGCGGCGGCGCCGCAACGCGAAGAT |
| P391A             | Bs WalK-P391A F | CATGGACTGCGGCGGCGCCGCAACGCGAAGAT |
|                   | Bs WalK-P391A R | CATGGACTGCGGCGGCGCCGCAACGCGAAGAT |
| T393A             | Bs WalK-T393A F | CGTGGCGGCGCAGCATTGACCCGAGCTAT |
|                   | Bs WalK-T393A R | ATAGCTGCGCCAGTTGCGCAAGCAGCTATTA |
| T394A             | Bs WalK-T394A F | CGTGGCGGCGCAGCATTGACCCGAGCTAT |
|                   | Bs WalK-T394A R | ATAGCTGCGCCAGTTGCGCAAGCAGCTATTA |
| R377AR378A        | Bs WalK-R377AR378A F | GGATCGAGGAAGCAGGAAATTCGTGTCGCC |
|                   | Bs WalK-R377AR378A R | GCAACAGAATTCGTTCCGATCC |
| R377MR378M        | Bs WalK-R377MR378M F | AAAATGTGAGTAATGAGAATAATTCGTGTCGCC |
|                   | Bs WalK-R377MR378M R | ATTCGCAAGAAATTCGATCCTGGATCCTGATCCTTAT |

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 pETWalK 366–611 R377M/R378M as a template.
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   | GCCCAGCGGATTTGACCA        |
| rt-rpoB-R   | GCAATTCGGAGTATGAGAGGCTG   |
| rt-gyrA-F   | AGCTTACTGGGCTTGCAGT       |
| rt-gyrA-R   | AGCTTACTGGGCTTGCAGT       |
| rt-yoeH-2F  | ATGCTCCTTGGGCAAGGCGC      |
| rt-yoeH-2R  | GACCTGTTATCTTCTTGCGAAGAA |
| rt-yoeE-F   | GCGGAGACATCGACGATGTCA     |
| rt-yoeA-F   | CATTGCGGGCCAGCATG         |
| rt-yoeR-R   | CAAAGGCGGCTGAATATTTTCT    |
| rt-yoeA-2F  | CCGTAGAAACATTTTTTGGAAGAC |
| rt-yoeB-2R  | TGAATGGCCAATACTGCTTTTG    |
| primer 1    | AGATCTCTTATTTCCCGGAAAGC   |
| primer 2    | CGTACTGATTGGGTAGGATCCCCCG |
| primer 3    | TATCGACAGCGGAATTGACTCAAGC |
| primer 4    | CCGGGGATCTTACCCAATGCACTAG |
| primer 5    | GCTTTGACAAATCCGGATACG     |
| primer 6    | ATAATCGACAGCGGATACG       |
| primer 7    | AGAATATCGGATCGTAGATTTTTT |
| primer 8    | TTTATCGGACAGCGGAATTGACTCA |
| primer 9    | GAGAAGGAAGGTGGTAGGATCGGAG |
| primer 10   | GGCGTTTTATATTTTTGCGCTTT  |
| primer 11   | TCATGATTGGCGCCTTTGGAAGAA |
| primer 12   | TTATCCTACTCGCCGGGCACCCA  |
| primer 13   | CGCGAGTCGTCCCTAATGACGAGG |
| primer 14   | ACTGTATTCAAAAGACGTGTTAAG |

Table 4. IC_{50}s and MIC of waldiomycin against WalK mutants in vitro and in vivo, respectively.

| WalK allele | IC_{50} (µM) | MIC (µg/ml) | 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           |
| T990A       | 15.9 ± 2.5   | 5.7         | 4           |
| P991A       | 46.3 ± 16.9  | 5.7         | 4           |
| T993A       | 15.2 ± 0.4   | ND          | ND          |
| T994A       | 11.8 ± 0.7   | ND          | ND          |
| R377A/R378A | 14.8 ± 4.0   | ND          | ND          |
| R377M/R389M | 43.6 ± 3.1   | ND          | ND          |
| R377M/R378MS385A | 274.6 ± 55.2 | 5.7 | 4 |
| R377M/R378MR389M | 557.2 ± 10.2 | 5.7 | 4 |

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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 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<sup>R</sup> and Cm<sup>R</sup> (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<sup>R</sup> and Cm<sup>R</sup> (Figs. 2E and 2F). The SU5 strain, containing the triple substitution (R377M/R378M/S385A) 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<sup>R</sup> 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 pH10 (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<sup>R</sup> colonies were selected on an LB agar plate containing chloramphenicol. The walK gene was amplified 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 IC_{50} (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 pETWalK<sub>366–611</sub>. Using this cytoplasmic WalK domain containing the N-terminal 366 to C-terminal 611 amino acid residues, the IC_{50}s of waldiomycin against the WalK autokinase activity were measured as previously described (Fakhruzzaman et al., 2015). The data are pre-
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 \textit{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 (IC$_{50}$) 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 \textmu g/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 IC$_{50}$ 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 IC$_{50}$ value (557.2 \textmu g/ml) was observed in WalK with the triple substitutions, R377M/R378M/R389M. The IC$_{50}$ value of...
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The activities 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 yoeH, 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-

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

| HK   | IC₅₀ (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                                       |

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.
duced, 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 IC₅₀ 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 μg/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 IC₅₀. 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 IC₅₀ 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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