ORIGINAL ARTICLE

Novel antibacterial compounds specifically targeting the essential WalR response regulator

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The WalK/WalR (YycG/YycF) two-component system, which is essential for cell viability, is highly conserved and specific to low-GC percentage of Gram-positive bacteria, making it an attractive target for novel antimicrobial compounds. Recent work has shown that WalK/WalR exerts an effect as a master regulatory system in controlling and coordinating cell wall metabolism with cell division in *Bacillus subtilis* and *Staphylococcus aureus*. In this paper, we develop a high-throughput screening system for WalR inhibitors and identify two novel inhibitors targeting the WalR response regulator (RR): walrycin A (4-methoxy-1-naphthol) and walrycin B (1,6-dimethyl-3-[4-(trifluoromethyl)phenyl]pyrimido[5,4-e][1,2,4]triazine-5,7-dione). Addition of these compounds simultaneously affects the expression of WalR regulon genes, leading to phenotypes consistent with those of cells starved for the WalK/WalR system and having a bactericidal effect. *B. subtilis* cells form extremely long aseptate filaments and *S. aureus* cells form large aggregates under these conditions. These results show that walrycins A and B are the first antibacterial agents targeting WalR in *B. subtilis* and *S. aureus*.

The Journal of Antibiotics (2010) 63, 127–134; doi:10.1038/ja.2010.4; published online 29 January 2010

Keywords: antibacterial agent; inhibitor; two-component signal transduction system; response regulator; WalR (YycF)

INTRODUCTION

In bacteria, signal transduction in response to a wide variety of environmental stimuli is mediated by pairs of proteins that communicate with each other through a two-component signal transduction system (TCS, Figure 1) involving protein phosphorylation. TCS consists of a histidine kinase (HK) and a response regulator (RR) that have global roles in bacterial growth as well as in drug resistance, virulence, biofilm formation and quorum sensing.¹ TCS inhibitors not only work as antibacterial agents but can also be developed as the inhibitors for drug resistance, biofilm formation, quorum sensing and virulence, as well as for plant-growth-regulating agents. In particular, TCSs are attractive as targets for antimicrobials for the following reasons. (1) Although many HK and RR genes are coded on the bacterial genome, few are found in lower eukaryotes. For example, only one HK and two RRs are present in the complete genome sequence of *Saccharomyces cerevisiae*, and none has been found from mammalian genomes. The HK/RR signal transduction system is distinct from serine/threonine and tyrosine phosphorylation in higher eukaryotes. (2) HKs and RRs possess a high degree of homology around their active sites. HKs are characterized by a conserved C-terminal catalytic domain containing a histidine residue, which is the site of autophosphorylation, and the N, G1, F and G2 boxes, which

presumably form a nucleotide-binding surface. RRs contain a conserved N-terminal domain that includes the aspartate residue that is phosphorylated, a pair of aspartate residues preceding this site and a lysine residue, all of which contribute to the acidic pocket for the phosphorylation site. Such a high degree of structural homology in the catalytic domain of HKs and in the receiver domain of RRs suggests that multiple TCSs within a single bacterium could be inhibited simultaneously by a single inhibitor, thereby lowering the frequency of the appearance of drug-resistant strains. (3) Some TCSs are essential for bacterial viability: YycG/YycF in *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Enterococcus faecalis*, *Streptococcus pyogenes*, *Streptococcus mutans* and *Streptococcus epidermidis*.^{2,3} The YycG/YycF TCS, recently renamed WalK/WalR to reflect its function,^{2,4} is highly conserved and specific to low-G+C Gram-positive bacteria, such as *B. subtilis* and *S. aureus*, in which it exerts an effect as a master regulatory system for cell wall metabolism and is essential for cell viability.^{4–8} As such, the WalK/WalR system constitutes an attractive target for the development of novel antimicrobial compounds, as inhibitors targeting WalK or WalR would be expected to have a bactericidal effect toward a broad range of major Gram-positive pathogens. Previously, a number of HK inhibitors have been identified,^{9–12} but these studies did not conclusively show a direct

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Received 14 December 2009; revised and accepted 4 January 2010; published online 29 January 2010

Figure 1 Two-component signal transduction system.

relationship between cell death and HK inhibition rather than some other uncharacterized nonspecific effect. However, structure-based virtual screening of compounds potentially targeting the kinase activity of *S. epidermidis* WalK found several to be active against not only *S. epidermidis* but also *S. aureus*, *S. aureus* and *S. mutans*.¹³

WalR is a typical RR of the OmpR/PhoB subfamily with a characteristic winged helix-turn-helix DNA-binding domain.^{14,15} These regulators control expression of their regulon genes by forming a head-to-head dimer of the receiver domains, using the conserved $\alpha 4$ - $\beta 5$ - $\alpha 5$ face for intermolecular interactions, paired with a head-to-tail dimer of the winged helix-turn-helix motifs that bind to the tandem DNA repeats of their binding site.¹⁶ Inhibiting the cognate sensor kinase may not be sufficient to disrupt signaling, as RRs can often be phosphorylated by non-cognate sensor kinases¹⁷ or small-molecule phosphate donors,¹⁸ making them the more attractive target for inhibition.^{16,19} Nevertheless, WalR-specific inhibitors have yet to be identified.

In this paper we describe a high-throughput screening system targeting the WalR RR, which uses a fusion between WalR and the DNA-binding domain of IclR to form a dimeric repressor and *egfP* as a reporter gene (Figure 2a). Using this system, we identified two novel inhibitors specifically targeting WalR in *B. subtilis* and *S. aureus* that were highly effective in killing these bacteria.

MATERIALS AND METHODS

Bacteria strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. Antibiotics were used at the following concentrations: ampicillin: 50 $\mu\text{g ml}^{-1}$ for *B. subtilis* and 100 $\mu\text{g ml}^{-1}$ for *E. coli*; chloramphenicol: 5 $\mu\text{g ml}^{-1}$ for *B. subtilis* and 10 $\mu\text{g ml}^{-1}$ for *S. aureus*; kanamycin: 5 $\mu\text{g ml}^{-1}$.

Plasmid construction

Primers used for plasmid construction are listed in Supplementary Table S1. To obtain pFI014, we PCR-amplified the DNA fragment containing the full length of *walR* of *B. subtilis* by using pBY33 as the template and YFIR4-399F and YFIR358-705R as the primers. The amplicons were digested with *KpnI* and ligated at the *KpnI* site of pFI003²⁰ (Figure 2a). For pArcA and pOmpR, the DNA fragments were prepared by PCR using the genomic DNA from *E. coli* W3110 as the template and the following primers: pFI003-arcA-F and pFI003-arcA-R for pArcA and pFI003-ompR-F and pFI003-ompR-R for pOmpR. The amplicons were digested with *KpnI* and ligated at the *KpnI* site of pFI003.

B. subtilis *walR* was also amplified by PCR with pBY33 as the template and primers pANBF-1 and BsYycF-R. The PCR product was digested with *KpnI* and

Figure 2 Screening of WalR inhibitors. (a) High-throughput screening system. N100, N-terminal 100-a.a. DNA-binding domain of IclR; HD, homodimerization domain; IclR Box, -21 to +14 on *iclR* promoter region; RRs, response regulators (WalR, ArcA and OmpR). (b) Repression of EGFP intensity by response regulators and IclR. JM109 containing pFI003 (column 1), pFI014 (column 2), pFI001 (column 3), pArcA (column 4) or pOmpR (column 5) was cultured and fluorescence intensity was measured as described in Materials and methods, indicated as a value relative to that of JM109 containing pFI003. (c) Structural formulas of walrycins A and B. (d) Induction of EGFP by walrycins A or B. JM109 containing pFI014 (open circles), pFI001 (squares), pArcA (triangles) or pOmpR (diamonds) was cultured in the presence or absence of walrycins A or B. Fluorescence intensity is presented here as a value relative to that in the absence of walrycins A or B.

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Source or reference
Strain		
<i>E. coli</i>		
JM109	Wild-type <i>E. coli</i>	Laboratory stock
BL21(DE3)	F [−] , <i>ompT</i> , <i>hsdS_B</i> (r _B [−] m _B [−]), <i>gal</i> , <i>dcm</i> (DE3)	Laboratory stock
<i>B. subtilis</i>		
168	<i>trpC2</i>	Laboratory stock
<i>S. aureus</i>		
N315	Methicillin-resistant <i>S. aureus</i>	Kuroda <i>et al.</i> ²³
Plasmid		
pFI001	rep _{MB1} , Ap ^r , P _{trc} - <i>iclR</i> (1–274 a.a.), P _{iclR} - <i>egfp</i> , <i>lacI</i> ^r , <i>rrnB</i> , <i>rrfB</i>	Furuta <i>et al.</i> ²⁰
pFI003	rep _{MB1} , Ap ^r , P _{trc} - <i>iclR</i> (1–100 a.a.), P _{iclR} - <i>egfp</i> , <i>lacI</i> ^r , <i>rrnB</i> , <i>rrfB</i>	Furuta <i>et al.</i> ²⁰
pFI014	pFI003, P _{trc} - <i>iclR</i> (1–100 a.a.)- <i>walR</i> (<i>B. subtilis</i>)	This work
pArcA	pFI003, P _{trc} - <i>iclR</i> (1–100 a.a.)- <i>arcA</i> (<i>E. coli</i>)	This work
pOmpR	pFI003, P _{trc} - <i>iclR</i> (1–100 a.a.)- <i>ompR</i> (<i>E. coli</i>)	This work
pKH55-21	pET21a(+), <i>Bam</i> HI— <i>Not</i> I fragment encoded ArcA	Yamamoto <i>et al.</i> ²⁷
pKH43-21	pET21a(+), <i>Bam</i> HI— <i>Not</i> I fragment encoded OmpR	Yamamoto <i>et al.</i> ²⁷
pBY33	pBluescript II SK+, <i>B. subtilis walRK</i>	Yamamoto <i>et al.</i> ¹⁰
pETBsFF	pET21a(+), <i>Nde</i> I— <i>Xho</i> I fragment encoded <i>B. subtilis</i> WalR	This work
pSCSawalRKH	pSC-B, <i>S. aureus walRK</i> <i>ycH</i>	This work
pETSa315VR	pET101/D-TOPO, <i>S. aureus walR</i> (<i>vicR</i>)	This work
pETBsG-tru	pET21a(+), <i>Bam</i> HI— <i>Not</i> I fragment encoded <i>B. subtilis</i> WalKtru	This work
pAN18	plasmid vector	Fukushima <i>et al.</i> ²¹
pABF	pAN18, <i>B. subtilis walR</i>	This work
pMK ₄ P _{prot}	plasmid vector	Archambaud <i>et al.</i> ²²
pSD1-7	pMK ₄ P _{prot} , <i>S. aureus walR</i>	This work

*Xba*I and then inserted into *Kpn*I and *Xba*I sites of pAN18²¹ to obtain pABF. A DNA fragment containing the *S. aureus walR* was amplified by PCR using primers OSA211 and OSA250 and *S. aureus* chromosomal DNA as a template. The PCR product was digested with *Bam*HI and *Pst*I and then cloned into pMK₄P_{prot}²² to obtain pSD1-7. For WalR protein purification, *B. subtilis walR* was amplified by PCR with pBY33 as the template and primers BsF-A-F and BsF-B-R. The PCR product was digested with *Nde*I and *Xho*I and then inserted into *Xho*I and *Nde*I sites of pET21a(+) (Novagen, Madison, WI, USA). The constructed plasmid was named pETBsFF. For WalKtru (C-terminal region, 204–611 a.a.) protein purification, *B. subtilis walK* was amplified by PCR with pBY33 as the template and primers YYCG(TRU)-F and YYCG(TRU)-R. The PCR product was digested with *Bam*HI and *Not*I and then inserted into *Bam*HI and *Not*I sites of pET21a(+). The constructed plasmid was named pETBsG-tru. A DNA fragment containing *walRKycH* of *S. aureus* was amplified by PCR with *S. aureus* N315²³ genomic DNA as the template and primers SAVicop-up and SAVicop-2dw. The PCR product was directly ligated to pSC-B (Stratagene, La Jolla, CA, USA) and was named pSCSawalRKH. *S. aureus walR* was subcloned from pSCSawalRKH to pET101/D-TOPO (Invitrogen, Carlsbad, CA, USA) by using primers TP-Sa315VR-F and TP-Sa315VR-3 and named pET-Sa315VR.

Chemical library

Two different kinds of chemical libraries were used for screening: one was produced by a new bioconversion system in recombinant *E. coli* cells;²⁴ the other contained 10 000 compounds from ChemBridge Corporation (San Diego, CA, USA).

Screening method

A chemical library was screened using the *E. coli*-based high-throughput enhanced green fluorescent protein (EGFP) screening system. JM109 cells containing plasmids pFI001, pFI003, pFI014, pArcA or pOmpR were grown at 37 °C in LB (Luria-Bertani) medium containing ampicillin. Then, 1 µl of this

culture was added to 96-titer wells containing 200 µl of LB–ampicillin medium and 1 µl of drugs, and the cultures were grown at 37 °C for 20 h. Fluorescence was measured using a Wallac 1420 ARVOsx (PerkinElmer Life Sciences, Boston, MA, USA) with excitation and emission wavelengths of 485 and 535 nm, respectively, and the optical density at 595 nm (OD₅₉₅) was measured using a Model 3550 Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA). For normalization the fluorescence intensity of each sample was divided by its OD₅₉₅ value.

Drug susceptibility assay

The MICs were examined by the serial agar dilution method using Mueller-Hinton agar (Difco Laboratories, Detroit, MI, USA).²⁵ The MIC was determined as the lowest concentration at which growth was significantly inhibited.

Trans-phosphorylation of WalR by phosphorylated WalKtru

To prepare phosphorylated (P-) WalKtru, WalKtru (0.5 µM) was incubated at room temperature for 15 min in kinase buffer (50 mM Tris-HCl pH 8.5, 200 mM KCl, 100 mM NH₄Cl, 25 mM MgCl₂, 12.5 mM ATP and 0.4 µCi [γ -³²P]ATP). After WalR (1 µM) was incubated at room temperature for 60 min in the presence of walrycins A or B, a P-WalKtru mixture was added and incubated for 15 min. The reaction was terminated by adding SDS sample buffer. The samples were analyzed using SDS-polyacrylamide gel electrophoresis. The gel was dried and exposed on an image plate and analyzed using FLA-7000 (Fuji Film, Tokyo, Japan).

RNA preparation and complementary DNA synthesis

For quantitative real-time PCRs (qRT-PCRs), the *B. subtilis* 168 and *S. aureus* N315 strains were grown with aeration to an OD 600 of 0.4, after which walrycins A or B was added and they were cultivated for another 5 min. The cells were harvested by centrifugation and immediately suspended in RNA-later (Ambion, Austin, TX, USA). RNA preparations were then performed with an SV total RNA Isolation System (Promega, Madison, WI, USA), followed by a

DNase I treatment with the TURBO DNA-free Kit (Ambion) to eliminate residual contamination by genomic DNA. Reverse transcription reactions were performed with the High Capacity complementary DNA Reverse Transcription Kit (ABI, Foster City, CA, USA).

qRT-PCRs

Primers (Supplementary Table S2) were selected with Primer Express Software Version 3.0 (ABI). qRT-PCRs were performed with complementary DNA (0.1 ng), 250 nM gene-specific primer pair and PowerSYBR Green Master Mix (ABI). PCR amplification, detection and analyses were performed using StepOne Real Time PCR system (ABI). PCR conditions included an initial denaturation step at 95 °C for 10 min, followed by a 40-cycle amplification (95 °C for 15 s and 60 °C for 60 s). The specificity of the amplified product and the absence of primer dimer formation were verified by generating a melting curve. Melting curve analysis was performed by an initial denaturation step of 95 °C for 15 s followed by 60 °C for 1 min and again 95 °C for 15 s. Fluorescence was measured continually during the melting curve cycle (a stepwise 0.3 °C temperature), 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 (C_T) was defined for each sample.

The expression levels were normalized using the 16S rRNA gene as an internal standard whose transcriptional level did not vary under our experimental conditions. Each assay was performed in triplicate and repeated using at least three independent RNA samples.

Preparation of 32 P-end-labeled DNA probes

Reverse primer (10 pmol, 5'-AAGACGTCATTGATAAAGACGCACTCCGGT-3') was phosphorylated with 10 μ Ci [γ - 32 P]ATP (5000 Ci mmol $^{-1}$, PerkinElmer) by using T4 polynucleotide kinase (Takara Bio, Otsu, Japan). The promoter fragment (P_{yochI}) was amplified by PCR using *B. subtilis* 168 genomic DNA as template, Ex Taq DNA polymerase (Takara Bio), and 32 P-labeled reverse primer and forward primer (5'-AATACAGGCTTATGCAAGATGACAGACTA-3'). The labeled fragments were purified and the radioactivity was measured with a liquid scintillation counter.

Gel mobility shift assay

Gel mobility shift assays were carried out as described in Yamamoto *et al.*²⁶ The 32 P-labeled probe was incubated at 30 °C for 10 min with purified His $_6$ -protein in binding buffer. After addition of a DNA dye solution (40% (v/v $^{-1}$) glycerol, 0.025% (w/v $^{-1}$) bromophenol blue and 0.025% (w/v $^{-1}$) xylene cyanol, the mixtures were subjected to 6% polyacrylamide gel electrophoresis.

Fluorescent dyes and fluorescence microscopy

Exponentially growing cells were mounted on 2% agarose pads containing growth medium for microscopy use and stained with Synapto Green (membrane) and SYBR green I (DNA). Images ($\times 1000$) were acquired on a Radiance 2100 confocal laser scanning microscope (Bio-Rad Laboratories).

Protein purification

E. coli BL21(DE3) cells containing the pETBsFF, pETSa315VR, pKH55-21 or pKH43-21 plasmids²⁷ were grown in 2 $\times$ YT medium at 30 °C. When the cell density reached an OD $_{600}$ of 0.6, IPTG was added at the final concentration of 1 mM, and the cell suspension was cultivated for another 3 h before the cells were harvested by centrifugation, washed with lysis buffer (50 mM Tris-HCl (pH 8.0), 100 mM NaCl) and then stored at -80 °C until use. Frozen cells were suspended in lysis buffer containing 1 mM phenylmethylsulfonyl fluoride and then lysed by sonication. The lysates were cleared by centrifugation and filtered through a 0.22- μ m filter. The filtrate was loaded onto a HiTrap HP column (ÄKTA prime system; GE Healthcare Bio-Science, Piscataway, NJ, USA) equilibrated with the lysis buffer, and His $_6$ -protein was eluted using an imidazole gradient from 0 to 500 mM. The fractions with the target protein were dialyzed against storage buffer (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 0.1 mM dithiothreitol and 10% (v/v $^{-1}$) glycerol), loaded onto a HiTrap Q FF (GE Healthcare Bio-Science) and eluted with a NaCl gradient of 0–500 mM in

storage buffer. Fractions containing the His $_6$ -tagged proteins were collected and loaded onto a HiTrap Desalting column (GE Healthcare Bio-Science).

Analytical size-exclusion chromatography

Size-exclusion chromatography analysis was performed on a fast-performance liquid chromatography ÄKTA system using a Superdex 200 10/300 GL or Superdex 75 10/300 GL (all three devices from GE Healthcare Bio-Science). Experiments were carried out at 4 °C and a flow rate of 0.25 ml min $^{-1}$ using 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1 mM EDTA and 0.1 mM dithiothreitol as the mobile phase while monitoring absorbance at 280 nm was determined. Samples (300 μ l) were prepared by incubating 2.5 nmol of protein in 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA and 0.1 mM dithiothreitol at 30 °C for 60 min in the presence or absence of 25 μ g ml $^{-1}$ of walrycin A. Before injection, samples were filtered through 0.22- μ m filters.

Limited proteolysis with trypsin

After WalR proteins were digested with trypsin (Promega) as previously described,²⁸ they were separated by SDS-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane (Immobilon-P transfer membranes; Millipore, Bedford, MA, USA), and detected using anti-WalR antibody and enhanced chemiluminescence plus western blotting system (GE Healthcare Bio-Science).

RESULTS AND DISCUSSION

High-throughput screening of inhibitors targeting WalR

From the results of the crystal structure analysis of the *Thermotoga maritima* IclR protein,²⁹ it was predicted that this protein is composed of two domains: the N-terminal DNA-binding domain containing the winged helix-turn-helix motif and a regulatory domain in the C-terminal of the protein, which is involved in its binding to a signal molecule. These two domains are linked by an α -helix.²⁹ The binding of a signal molecule to the C-terminal domain of IclR is presumed to repress the transcription of target genes by modulating either DNA-binding activity or multimerization of IclR.²⁹ Database homology searches have identified many IclR-like proteins, known as the IclR family, which show a conserved C-terminal region. *E. coli* IclR also forms a dimer and regulates both the *aceBAK* operon and its own expression.^{30–32} Furuta *et al.*²⁰ previously reported that the region between 100 and 274 a.a. of IclR is involved in dimerization and that this region can be replaced with other functional homodimerization domains (Figure 1). To design a high-throughput screening system targeting the WalR RR, we constructed a chimeric repressor gene consisting of sequences encoding the 100 amino-terminal amino acid residues of IclR that constitute the DNA-binding domain (N100) fused to the *walR*, *arcA* or *ompR* RR genes (Figure 2a). To quantify the dimerization capacity of the resulting chimerical repressor proteins in terms of fluorescence intensity, the *egfP* reporter gene was placed directly downstream from the *iclR* promoter (Figure 2a) as described previously.²⁰ In the presence of inhibitors targeting the RRs, the *egfP* gene is expressed and EGFP fluorescence intensity increases. WalR, ArcA and OmpR belong to the OmpR/PhoB subfamily, in which dimerization is essential for DNA-binding and transcriptional activation. Consequently, they are all capable of suppressing expression from the *iclR* promoter (Figure 2b). These results suggest that this high-throughput system could be used to screen inhibitors of RRs containing dimerization domains. To identify selective WalR inhibitors by measuring EGFP fluorescence intensity, we screened a chemical library against *E. coli* strains JM109/pFI014 (WalR) and JM109/pFI001 (IclR) in 96-titer wells. Through this screening process, we identified two compounds, compound A (4-methoxy-1-naphthol; CAS no. 84-85-5) and compound B (1,6-dimethyl-3-[4-(trifluoromethyl)phenyl]-pyrimido[5,4-e][1,2,4]triazine-5,7-dione; CAS no. 878419-78-4)

(Figure 2c) as WalR inhibitors. Both compounds strongly increased fluorescence intensity in a dose-dependent manner in strain JM109/pFI014 but did not significantly change fluorescence intensity of strain

JM109/pFI001 (Figure 2d). Furthermore, to clarify that they actually target WalR, we analyzed their effect on phosphotransfer from autophosphorylated WalKtru (cytoplasmic HK region) to WalR (Figure 3). As a result, phosphotransfer from P-WalKtru to WalR was inhibited with increased concentrations of compounds A and B. These results strongly suggest that both compounds target WalR to inactivate the IclR–WalR chimera repressor for inducing GFP expression. Therefore, we named them walrycin A and walrycin B.

Regulation of WalR regulon genes

To establish that these compounds exert an effect specifically on the WalK/WalR system, the effects of walrycins on *in vivo* expression of WalR regulon genes^{4,5,8,33,34} were analyzed using qRT-PCR). In *B. subtilis*, WalR activates expression of *ydjM* (cell wall-associated protein), *yocH* (cell wall hydrolase) and *yvcE* (*cwlO*, cell wall endopeptidase), whereas it negatively regulates expression of *yoeB* (modulator of autolysin activity) and *yjeA* (peptideglycan deacetylase). In *S. aureus*, WalR activates expression of several cell wall metabolism genes including *isaA* (cell wall transglycosylase) and *ssaA* (cell wall amidase). As shown in Figure 4, addition of walrycins A and B to *B. subtilis* led to lowered expression of *ydjM*, *yocH* and *yvcE* and increased expression of *yoeB* and *yjeA* within 5 min (Figures 4a and b). Similarly, transcription of *S. aureus* WalR regulon genes *isaA* and *ssaA* stopped within 5 min of adding walrycins A and B to the culture (Figures 4a and b). Furthermore, both walrycin compounds had antimicrobial

Figure 3 Inhibition of phosphotransfer from P-WalKtru to WalR. Phosphotransfer from P-WalKtru to WalR was performed in the presence or absence of walrycin A (a, lanes 1–5) or walrycin B (b, lanes 6–10): lane 1, 0 μM ; lane 2, 22.3 μM ; lane 3, 44.8 μM ; lane 4, 89.6 μM ; lane 5, 179.2 μM ; lane 6, 0 μM ; lane 7, 3.6 μM ; lane 8, 14.5 μM ; lane 9, 58.1 μM ; lane 10, 232.4 μM .

Figure 4 Transcriptional regulation of WalR regulon genes by walrycins A or B. Total RNAs were extracted from *B. subtilis* or *S. aureus* cells, after which walrycin A (a; 0, 25 and 50 $\mu\text{g ml}^{-1}$ for *B. subtilis* and 0, 50 and 100 $\mu\text{g ml}^{-1}$ for *S. aureus*) or walrycin B (b; 0, 0.78 and 1.56 $\mu\text{g ml}^{-1}$ for *B. subtilis* and 0, 3.13 and 6.25 $\mu\text{g ml}^{-1}$ for *S. aureus*) was added, and they were cultivated for 5 min. After reverse transcription, specific complementary (c)DNAs were quantified using qRT-PCR. The results are expressed as the means of relative quantification to that of 0 $\mu\text{g ml}^{-1}$ and s.d. of triplicate experiments using primers specific for WalR regulon genes, *gyrA* and 16S rRNA (normalizing gene).

Figure 5 Morphology changes induced by walrycins A or B. Confocal laser scanning microscopy of exponentially growing cells of *B. subtilis* 168 (a) and *S. aureus* N315 (b) in the presence or absence (Control) of walrycin A ($50\mu\text{g ml}^{-1}$ for *B. subtilis* and *S. aureus*) or walrycin B ($0.19\mu\text{g ml}^{-1}$ for *B. subtilis* and $0.78\mu\text{g ml}^{-1}$ for *S. aureus*).

activity, with MICs of walrycins A and B of 64 and $0.39\mu\text{g ml}^{-1}$ for *B. subtilis* 168 and 128 and $3.13\mu\text{g ml}^{-1}$ for *S. aureus* N315, respectively. These results indicate that walrycins A and B target WalR and lead to cell death in both *B. subtilis* and *S. aureus*.

The MICs of walrycin B against *B. subtilis* and *S. aureus* were shown to be stronger than those of walrycin A, at which concentration the WalR regulon gene expressions were specifically effected to cause cell death. These results suggest that walrycins A and B actually target WalR in the *B. subtilis* and *S. aureus*. On the other hand, walrycins A and B inhibited phosphotransfer from WalK to WalR at similar concentrations *in vitro*. However, walrycin A induced EGFP at lower concentrations than did walrycin B in a high-throughput genetic system using *E. coli* (Figure 2). Walrycin A might enter *E. coli* cells easily, but not *B. subtilis* and *S. aureus*, whereas the reverse is true for walrycin B.

To confirm that walrycins target the WalR RR, the effects of WalR overproduction were analyzed. When WalR was overexpressed from a multi-copy plasmid (*B. subtilis* strain 168/pABF and *S. aureus* strain N315/pSD1-7), the MICs for walrycin A were increased twice over those of the control strains (168/pAN18 and N315/pMK₄P_{prot}) to 128 and $256\mu\text{g ml}^{-1}$, respectively. Under the same conditions, MICs for walrycin B were increased fourfold for *B. subtilis* and twofold for *S. aureus*. These results indicate that the walrycins specifically target WalR to cause cell death in both *B. subtilis* and *S. aureus*.

In this study we have shown that walrycins inhibit WalR activity in *B. subtilis* and *S. aureus*. These activities are involved in many crucial cell functions and influence cell development. Because autolysins are involved in the separation of daughter cells, one would expect WalR-

depleted cells to be filamentous or aggregated. We used a confocal laser microscope to examine the effect of walrycin A on the morphology of *B. subtilis* and *S. aureus* cells. As shown in Figure 5, untreated *B. subtilis* and *S. aureus* cells had a typically short rod morphology or were well separated, whereas walrycins A and B-treated cells formed extremely long aseptate filaments or aggregates. Furthermore, after *B. subtilis* was incubated in the presence of walrycins A or B, cell lysis was observed (data not shown). These results are consistent with the previously reported phenotypes of WalR-depleted mutants.^{4,6,33}

A mode of action

B. subtilis and *S. aureus* WalRs, which were overproduced and purified from *E. coli*, revealed both monomeric and dimeric forms (Figure 6a-1, 2), whereas ArcA and OmpR formed the monomer as previously reported.^{35–37} After WalR was treated with walrycin A at 30°C for 60 min followed by size-exclusion chromatography, a decrease in the concentration of WalR monomer and a concomitant increase in the dimeric form (Figure 6a-1, 2) were observed. This increase in the proportion of dimeric protein was not observed when purified ArcA or OmpR was incubated with walrycin A (Figure 6a-3, 4). As these results suggest that walrycin A can exert an effect specifically on monomeric WalR to increase dimer formation, we performed a size-exclusion chromatography on fractionated WalR-monomers with or without walrycin A treatment. As shown in Figure 6a-5, almost half of the monomer had transitioned into the dimeric form. When dimeric WalR was treated with walrycin A under the same conditions, however, no change was observed (Figure 6a-6). These results clearly show that walrycin A exerts an effect specifically on the monomeric

Figure 6 A mode of action of walrycin A targeting WalR. (a) Walrycin A interacts with monomeric WalR. After each protein (1–6) was incubated at 30 °C for 60 min without (solid line) or with (dotted line) 25 $\mu\text{g ml}^{-1}$ of walrycin A, size-exclusion chromatography was performed as described in Materials and methods. WalRs (*B. subtilis*), ArcA and OmpR were separated using a Superdex 200 10/300 GL column, and *S. aureus* WalR was separated using a Superdex 75 10/300 GL column (GE Healthcare Bio-Science). 1, WalR (*B. subtilis*); 2, WalR (*S. aureus*); 3, ArcA (*E. coli*); 4, OmpR (*E. coli*); 5, the monomeric WalR (*B. subtilis*); 6, dimeric WalR (*B. subtilis*). M, monomer; D, dimer. (b) Conformational changes of WalR(D) and WalR(D'). WalR was digested with trypsin (protein to trypsin, 20:1). A total of 30 ng of WalR(D) (lanes 1–6) and WalR(D') (lanes 7–12) were digested with 1.5 ng of trypsin for 0 (lanes 1 and 7), 15 (lanes 2 and 8), 30 (lanes 3 and 9), 60 (lanes 4 and 10), 90 (lanes 5 and 11) and 120 min (lanes 6 and 12). (c) Gel mobility shift analysis with WalR(D) and WalR(D'). A radiolabeled DNA fragment carrying the promoter of *yocH* was prepared as described in Materials and methods. The mobility of fragments without WalR addition is shown in lanes 1 and 5. Lanes 2–4 (WalR(D)) and 6–8 (WalR(D')) show mobility after binding of 2.5 (lanes 2 and 6), 5 (lanes 3 and 7) or 10 pmol (lanes 4 and 8) of WalR.

form of WalR to increase the formation of WalR dimers. We designated the dimer that formed after the reaction of walrycin A and the WalR monomer as WalR(D') and evaluated its structural characteristics (Figure 6b). WalR(D) and WalR(D') were digested with trypsin, and the fragments were separated by SDS-polyacrylamide gel electrophoresis and analyzed using western blotting (Figure 6b). Results showed fragments arising from trypsin-digested WalR(D'), which were not detected in the WalR(D)-digested sample. These results indicate a possible altered conformation for WalR(D') generated by the reaction with walrycin A when compared with endogenous WalR(D). The ability of WalR(D') to bind to the promoter region of the *B. subtilis yocH* gene, which contains a WalR-binding site, was analyzed using gel mobility shift analysis (Figure 6c). Results showed that WalR(D) bound to the WalR box of P_{yocH} and formed a DNA-WalR(D) complex that migrated at a slower rate than the free DNA. However, no protein/DNA complex was formed when the *yocH* probe was incubated with WalR(D'), indicating that no binding took place. These results show that walrycin A reacts with the WalR monomer to form a WalR dimer (WalR(D')) with a different conformation from that of the endogenous dimeric WalR, and which lacks the ability to bind to WalR(D) operator sites (Figure 7).

Concerning the difference in the mode of action between walrycins A and B, we conducted an *in vitro* phosphotransfer experiment from

Figure 7 A model of walrycin action.

P-WalKtru to WalR in the presence or absence of walrycins (Figure 3). As a result, phosphotransfer was inhibited by both walrycins A and B. These results suggest that they likely inhibit WalR activity by a similar mechanism: by targeting WalR. Walrycin B is known as an analog of toxoflavin (a phytotoxin from *Burkholderia glumae*), which has been shown to have a strong MIC for *B. subtilis* and *S. aureus* but whose mode of action is not clear.³⁸ The compound could also interact with WalR to cause bactericidal effects. Recently, Kim *et al.*³⁹ reported that toxoflavin binds ToxR (an LysR-type transcriptional regulator) to activate synthetic gene expression of toxoflavin.

Walrycins are a new class of potent small-molecule compounds that kill bacterial cells by targeting the RR WalR and inhibiting this essential signal transduction pathway. They not only have therapeutic potential but will also prove to be useful reagents for the further study of the WalK/WalR TCS.

ACKNOWLEDGEMENTS

We thank Yamamoto K for plasmids pKH55-21 and pKH43-21 and Yamamoto K, Kobayashi K, Nagamatsu T and Nishiyama S for valuable discussions. We also thank Eguchi Y and Ho K for critically reviewing the manuscript and Kubo N, Shiraki S, Suzuki T, Otsuka A and Hirai Y for technical support. This work was supported by a Grant-in-Aid for Scientific Research (A, 20248012) of the Japan Society for the Promotion of Science (JSPS), the Research and Development Program for New Bio-Industry Initiatives (2006-2010) of the Bio-Oriented Technology Research Advancement Institution (BRAIN). Work in the group of Msadek T was supported by research funds from the European Commission (Grants BACELL Health LSHG-CT-2004-503468, StaphDynamics LHSM-CT-2006-019064 and BaSysBio LSHG-CT-2006-037469), the Centre National de la Recherche Scientifique (CNRS URA 2172), and the Institut Pasteur (GPH no. 9).

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (<http://www.nature.com/ja>)