Targeting protein homodimerization: A novel drug discovery system

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Abstract To identify a novel class of antibiotics, we have developed a high-throughput genetic system for targeting the homodimerization (HD system) of histidine kinase (HK), which is essential for a bacterial signal transduction system (two-component system, TCS). By using the HD system, we screened a chemical library and identified a compound, I-8-15 (1-dodecyl-2-isopropylimidazole), that specifically inhibited the dimerization of HK encoded by the YycG gene of *Staphylococcus aureus* and induced concomitant bacterial cell death. I-8-15 also showed antibacterial activity against MRSA (methicillin-resistant *S. aureus*) and VRE (vancomycin-resistant *Enterococcus faecalis*) with MICs at 25 and 50 µg/ml, respectively.
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1. Introduction

The bacterial signal transduction system (two-component system, TCS) composed of histidine kinase (HK) and response regulators (RR) plays important roles in drug-resistance, pathogenesis and bacterial growth. HKs have recently emerged as compelling targets for antibacterial drug design for several reasons [1–4]. First, histidine phosphorylation in bacteria is distinct from serine/threonine and tyrosine phosphorylation in higher eukaryotes as a mode of signal transduction. Accordingly, a selective HK inhibitor is expected to have minimal toxicity in mammalian cells. Second, the high degree of structural homology in the catalytic domain of HKs suggests that multiple HKs could be inhibited within a single bacterium, thus, reducing the possibility of resistant strains emerging. Finally, as HKs are found throughout the genomes of Gram-positive and Gram-negative bacteria, a single HK inhibitor may exhibit a broad spectrum of activity provided that it can penetrate the cell envelopes of the target bacteria.

The YycG/YycF TCS is specific to low G+C Gram-positive bacteria, and it is highly conserved among this group of bacteria. The role of the YycG/YycF TCS system includes the proper regulation of bacterial cell wall or membrane composition [5,6], and it has been shown to be essential in *Bacillus subtilis*,

Streptococcus pneumoniae, *Enterococcus faecalis* and *Staphylococcus aureus* [7–10]. Notably, a temperature-sensitive mutant of this system has been shown to be lethal at a non-permissive temperature, indicating that this pathway provides an excellent target in designing novel antibiotics.

On the other hand, dimerization of HK is considered an essential step for autophosphorylation of this enzyme [11,12]. Cytoplasmic or truncated forms of HKs have previously been studied and shown to dimerize in vitro [13]. Solution state NMR [14] indeed revealed dimerization for the homodimeric core domain in *Escherichia coli* EnvZ HK, which belongs to the same Pho subfamily of HKs as YycG [15].

In order to develop a high-throughput genetic system for targeting the homodimerization (HD system) of HK that includes YycG, we used the pKWY2428 constructed by Novy [16], which is a homodimerization vector containing the N-terminal region (NλcI., 1–133 a.a.) of λcI. First of all, we constructed a chimeric repressor (NλcI-CYycG) in which NλcI was fused to the cytoplasmic region of YycG. NλcI-CYycG formed to repress the *tet* and *lacZ* genes expression in *E. coli*. However, the screening system with NλcI-CYycG was laborious and lacked reproducibility. Thus, we tried to construct a high-throughput homodimerization assay system based on the dimerization properties of the IclR repressor of *E. coli* using GFP as the reporter gene.

2. Materials and methods

2.1. Strains, plasmids and growth conditions

E. coli JM109 (*endA*1, *recA*1, *gyrA*96, *thi*, *hsdR*17(rK⁻,mK⁺), *relA*1, *supE*44, Δ(*lac-proAB*), (F′, *traD*36, *proAB*, *lacIqZ* ΔM15), and BL21(DE3) (F⁻, *ompT*, *hsdSB*, [rB⁻,mB⁻], *dcm*, *gal*, λ(DE3)) were cultured in Luria–Bertani broth (LB) to screen the chemical library and express N100, N100-zip or N100-CYycG, respectively. When necessary, LB was supplemented with ampicillin (100 µg/ml) or chloramphenicol (25 µg/ml).

2.2. Plasmids construction

pGMIiclR was constructed as follows: 99 bp *iclR* promoter (AE000475, 4226880-4226978) was amplified by PCR using a genomic DNA template from *E. coli* W3110 and the primers 5′-CCACGCAA-CATG<u>AGATCT</u>GTTCAAC-3′ and 5′-GAATGGGTGC<u>ATGCAT</u>-GACA<u>GTCTC</u>-3′, which contained *Bgl*II and *Eco*T22I sites (underlined) for cloning. The PCR product was digested with restriction enzymes and then ligated into *Eco*T22I and *Bgl*II sites of pGMI301 [17]. The *Bgl*II–*Bam*HI fragment (1260 bp) of pGMIiclR was cloned into the *Bgl*II–*Bam*HI sites of pTrc99A to obtain pF001 (Fig. 1). *iclR* (825 bp, AE000475, 4226882-4226058) was amplified by PCR using the primers 5′-CAGAAAAAGAGGAATTCATGGTCGCACCC-3′ and 5′-CTCTGCCCGCCAGGGTACCTCAGCGCATTC-3′ and

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genomic DNA from W3110. After this amplified DNA was digested with *Eco*RI and *Kpn*I, it was ligated into the corresponding sites of pF001 to obtain pFI001 (Fig. 1). To construct the truncated IclRs containing 117, 100 and 66 amino acid residues from the N-terminus (Fig. 2A), *Kpn*I sites were introduced into the corresponding sites of *iclR* gene on pFI001 by using the Quick Change site-directed mutagenesis kit (Stratagene) and the following primers (*Kpn*I site underlined): 5′-GCCATATTGACGGTACCGCCAGACTCTTCC-3′ and 5′-GGAAGAGTCTGGCGGTACCGTCAATATGGC-3′ for pFI004; 5′-GGGTGAACAATCGCGGTACCATTACGGCTC-3′ and 5′-GAGCCGTAATGGTACCGCGATTGTTCACCC-3′ for pFI003; and, 5′-GAAACCCTGCTGGGTACCCGTGGTTAGCAG-3′ and 5′-CTGCTAACCACGGGTACCCAGCAGGGTTTC-3′ for pFI002. These were digested with *Kpn*I and ligated by themselves to construct pFI004, pFI003 and pFI002 (Fig. 1 and Fig. 2A). The DNA fragments containing the DNA binding domain of λcI (NcI, 2–132 a.a. from N-terminal) [16], leucine zipper (zip, 33 a.a. sequence) of yeast GCN4 [18,19], dimerization domain of FadR of *E. coli* (CFad, 83–239 a.a. from N-terminal) [20], and the cytoplasmic domain of YycG of *S. aureus* (CYycG, 204–608 a.a. from N-terminal) [8] were prepared by PCR using the following primers and templates: 5′-GAAACAGGGTACCAGCACAAAAAGAAACC-3′ and 5′-CCAATTGAGGGTACCCGACACCCATCTCTC-3′ and pKWY2428 (for NcI); 5′-CCTATCCAGGGTACCAAACAGCTGGAAGAC-3′, 5′-CATAGGCCAGGTACCCCACGTTCACCCACC-3′, and pT18-zip (for zip); 5′-GAAATGGTACCGAAACACTGGCGCGACTGG-3′, 5′-CGGGGTACCTTATCGCCCCTGAATGGC-3′, and genomic DNA from W3110 (for CFad); 5′-GATTCGGTACCGCGCGAACGATTACCAAAC-3′, 5′-GTCGGTACCTTATTCATCCCAATCACCGTC-3′, and pYycGSa (for CYycG). After the DNA fragments containing zip, NcI, CFad and CYycG were digested with *Kpn*I, they were fused to the *Kpn*I site of pFI003 to construct pFI006, pFI007, pFI009 and pFI028, where the C-terminal part of IclR was replaced by zip, NcI, CFad and CYycG, respectively. The IclR, N100, N100-zip and N100-CYycG regions were amplified by PCR using following primers and templates: 5′-GACCATGGAACATATGGTCGCACCCATTCC-3′, 5′-CAGATCCCCGGGTCTCGAGGCGCATTCCAC-3′, and pFI001 (for IclR); 5′-GACCATGGAACATATGGTCGCACCCATTCC-3′, 5′-GTTGAACAGACTCGAGGGTACCATTACGC-3′, and pFI003 (for N100); 5′-GACCATGGAACATATGGTCGCACCCATTCC-3′, 5′-CAGATCCCCCTCGAGC-

CCACGTTCACCCAC-3′, and pFI006 (for N100-zip); 5′-GACCATGGAACATATGGTCGCACCCATTCC-3′, 5′-CCCCGGGTCTCGAGTTCATCCCAATCACCG-3′, and pFI028 (for N100-CYycG). After IclR, N100, N100-zip and N100-CYycG fragments were digested with *Nde*I and *Xho*I, they were ligated into the corresponding sites of pET21(a)⁺ (Novagen) to obtain pETIclR, pETN100, pETN100-zip and pETN100-CYycG, respectively. After that, N100-CYycG-Histag regions were amplified by PCR using the primers 5′-GGAGATATACATATGGTCGCACCCATTCCC-3′ and 5′-GTTAGCAGCGAATTCTCAGTGGTGGTGGTG-3′ along with pETN100-CYycG as template DNA. After this amplified DNA was digested with *Nde*I and *Eco*RI, it was ligated into the corresponding sites of pCold III (Takara) to obtain pCN100-CYycG.

2.3. Library screening

JM109 containing pFI001, PFI002, pFI003, pFI004, pFI006, pFI007, pFI009 or pFI028 was cultured at 37 °C in LB medium containing ampicillin (LB-amp, 100 µg/ml). After this, 1 µl of this culture was added to each 96-titer well containing LB-amp medium (199 µl) in the presence or absence of the drug from the chemical library. They were grown at 37 °C for 20 h, and the fluorescence levels at an excitation of 485 nm and at an emission of 535 nm, with optical density at 600 nm (OD$_{600}$), were measured with Wallac 1420 ARVOsx (Perkin–Elmer™ Life Science) and Model 3550 Microplate Reader (Bio-Rad) instruments, respectively. The fluorescent intensity of each sample was divided by its OD$_{600}$ value for normalization.

2.4. Purification of CYycG, IclR, N100, N100-zip and N100-CYycG

CYycG from *S. aureus* was purified as His6-tag as described previously [8]. IclR, N100 and N100-zip were also purified as His6-tag proteins using BL21 (DE3) containing pETIclR, pETN100 and pETN100-zip; however, it was difficult to purify N100-CYycG using pETN100-CYycG because it formed an inclusion body. To purify N100-CYycG, BL21 (DE3) containing both pCN100-CYycG and the chaperone plasmid pG-Tf2 (Takara) was cultured at 37 °C in an LB medium (ampicillin, 100 µg/ml; chloramphenicol, 25 µg/ml). After it was grown to an OD$_{600}$ of 0.5 at 37 °C, it was cultured at 15 °C for 24 h in the presence of IPTG (1 mM) and tetracycline (5 ng/ml) to purify N100-CYycG as a His6-tag protein.
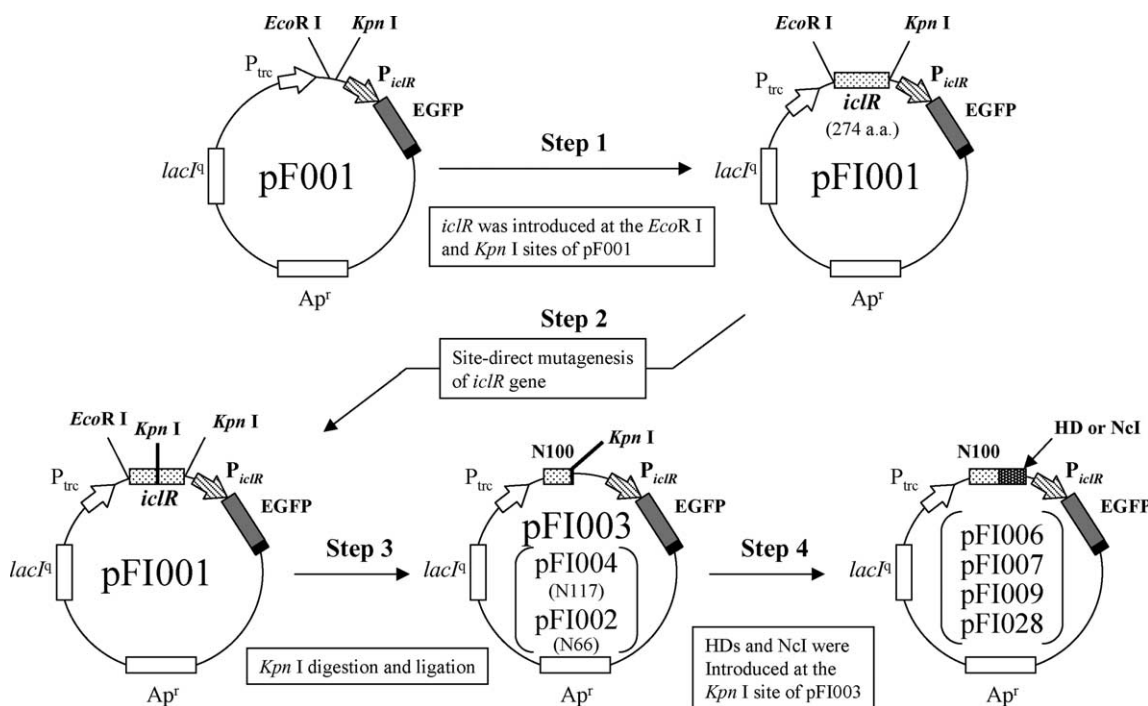

Fig. 1. Construction of plasmids for HD system. To construct the truncates IclRs containing 117, 100 and 66 amino acid residues from the N-terminal, *Kpn*I sites were introduced into the corresponding sites of *iclR* gene (step 2). HD, homodimerization domain; pFI006, zip; pFI007, CFad; pFI028, CYycG.
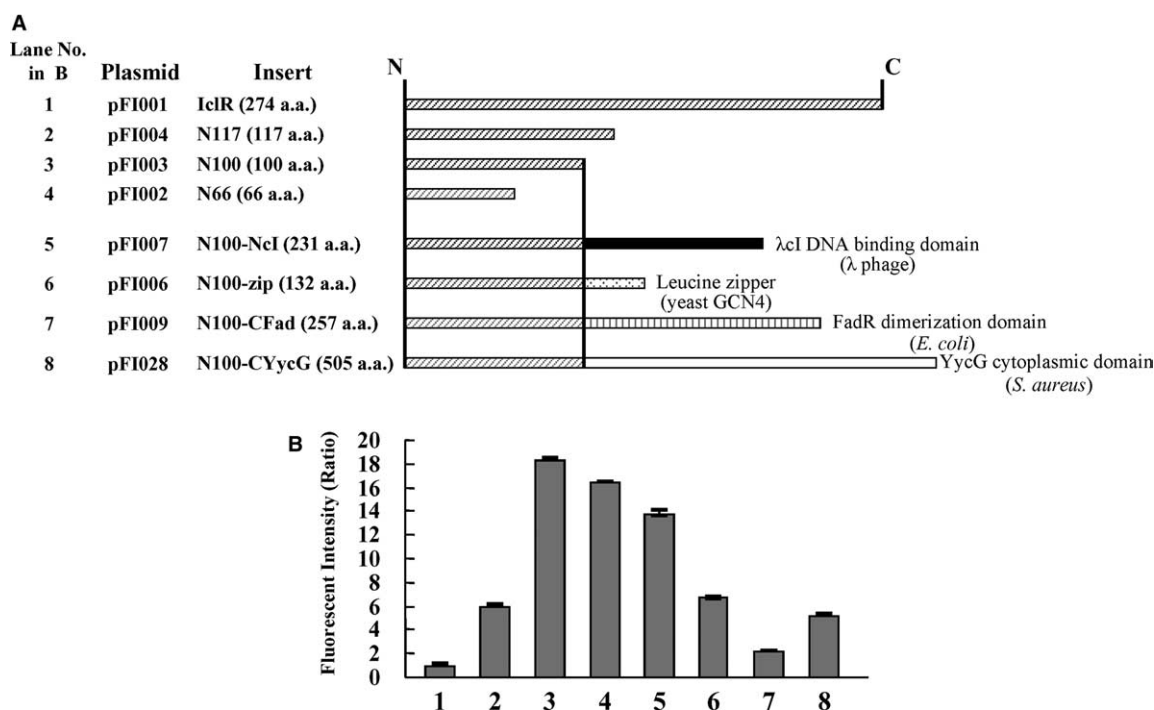
Fig. 2. Construction of IclR derivatives and their repressor activities. (A) Construction of *E. coli* IclR derivatives. Full length of IclR contains 274 amino acid residues. N66, N100 and N117 lack the C-terminal portions of IclR. N100 was fused to NcI, zip, CFad, or CYycG. (B) Fluorescent intensity of IclR and chimeric repressors. *E. coli* JM109 containing pFI001 (lane 1), pFI004 (lane 2), pFI003 (lane 3), pFI002 (lane 4), pFI007 (lane 5), pFI006 (lane 6), pFI009 (lane 7), or pFI028 (lane 8) was cultured and their fluorescent intensity was measured as described in Section 2. Each intensity was represented as relative value to that of JM109/pFI001.

2.5. Chemical cross-linking

IclR, N100, N100-zip or N100-CYycG (100 pmol) was incubated in 100 μl of HMKG buffer (50 mM HEPES [pH 7.5], 10 mM MgCl$_2$, 50 mM KCl, 20% glycerol) containing 300 μM imidazole for 30 min on ice. When necessary, dimethyl sulfoxide (DMSO) or I-8-15 was added and incubated for 15 min at room temperature. 0.3 mM disuccinimidyl suberate (DSS) was added to react with two side chains of protein targets to form a cross-linked complex [21]. After 2 h of incubation at room temperature, the reactions were stopped by the addition of 0.25 M glycine and trichloroacetic acid (TCA). The proteins were run on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The gels were electrotransferred onto polyvinylidene difluoride membranes (PVDF) that were then probed with polyclonal antibodies against IclR. The primary antibody was detected with a goat anti-rabbit IgG (H+L) alkaline phosphatase conjugated detection system (Bio-Rad).

2.6. Gel mobility shift assay

The ^{32}P-labeled probe was generated by PCR amplification using a primer pair for the *iclR* promoter (5′-CCACGCAACATGA-GATCTGTTCAAC-3′ and 5′-GAATGGGTGCATGCATGA-CAGTCTC-3′). The forward primer was labeled with 10 μCi of [γ-^{32}P]ATP (5000 Ci/mmol) by T4 polynucleotide kinase (Toyobo). *E. coli* W3110 genome DNA (100 ng) was used as the template for Ex *Taq* DNA polymerase (Takara). The PCR product with ^{32}P at its terminus was recovered from a polyacrylamide gel, and then a gel mobility shift assay was done. The ^{32}P-labeled probe (5000 cpm) was incubated with purified IclR, N100, N100-zip, N100-CYycG or bovine serum albumin (BSA) proteins for 20 min at 37 °C in 12.5 μl of binding buffer [10 mM Tris–HCl (pH 7.4), 3 mM MgCl$_2$, 5 mM CaCl$_2$, 1 mM EDTA, 50 mM KCl, 5% glycerol, 0.05% Nonidet P-40]. After adding a DNA dye solution (40% glycerol, 0.025% bromophenol, 0.025% xylene cyanol), the mixtures were directly subjected to 6% PAGE (pH 6.0). The radioactivity was measured by BAS1000 (Fuji Film) and the data were analyzed by Muti Gauge Ver. 2.0 (Fuji Film) for the gel mobility shift assay and the autophosphorylation assay.

2.7. Assay of HK inhibitor, I-8-15

Autophosphorylation of CYycG was performed as described previously [8]. Briefly, 5 pmol of CYycG was mixed with I-8-15 and then autophosphorylated with [γ-^{32}P]ATP for 5 min at room temperature in 10 μl of reaction buffer A [50 mM Tris–HCl (pH 7.5), 50 mM KCl, 50 mM MgCl$_2$] containing 2.5 μM ATP. To stop the reaction, a 2× sample buffer [120 mM Tris–HCl (pH 6.8), 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.1% BPB] was added. After SDS–PAGE, IC$_{50}$ was determined as described previously [8].

2.8. Antibacterial susceptibility

Antibacterial susceptibility testing was done by the broth microdilution method following the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS).

2.9. Synthesis of I-8-15 (1-dodecyl-2-isopropylimidazole)

2-Isopropylimidazole (1.44 g, 13.1 mmol) in 10 ml of chloroform was stirred at 70 °C and then dodecyl bromide (1.17 g, 4.69 mmol) was added dropwise to the solution and stirred for 1 day at the same temperature. The solution was evaporated to remove chloroform under pressure, and the residue was subjected to column chromatography on silica gel using the methanol and chloroform at a ratio of 1:15 (v/v) to afford 1-dodecyl-2-isopropylimidazole in 97.6% yield (1.30 g). The structure of the compound I-8-15 was identified as 1-dodecyl-2-isopropylimidazole using ^{1}H NMR and high-resolution mass spectroscopy.

3. Results and discussion

3.1. Construction of a vector for targeting protein homodimerization

From the results of the crystal structure analysis of the *Thermotoga maritime* IclR protein (TM IclR) [22], 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 (H4) [22]. 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 [22]. Database homology searches have identified many IclR-like proteins, known as the IclR family, which shows a conserved C-terminal region. *E. coli* IclR also forms a dimer and regulates both the *aceBAK* operon and its own expression [23–25]. To localize the essential domain for the IclR dimerization, we constructed four plasmids, pFI001, pFI002, pFI003 and pFI004, that contained a series of deletions of the *E. coli* IclR gene (Fig. 2). Each plasmid expresses a different length of IclR repressor and is, therefore, expected to differ in its ability to form a dimer [23,24] and in its binding activity to the *iclR* promoter sequence (Fig. 2). In order to quantify the dimerization ability of chimeric proteins as fluorescent intensity, the EGFP gene was fused directly downstream to the *iclR* promoter. In *E. coli* JM109 containing pFI001 (JM109/pFI001) expressing the full length (274 a.a.) of the *iclR* gene, EGFP expression (fluorescent intensity, 21 289 counts) was almost completely repressed in comparison with that of JM109/pF001 (733 941 counts). On the other hand, the fluorescent intensity of JM109/pFI003, JM109/pFI002 and JM109/pFI004 showed approximately 18-, 17- and 6-fold increases (Fig. 2B, lanes 2–4), respectively, compared to that of JM109/pFI001 (Fig. 2B, lane 1). These results suggest that a key region controlling the repressor activity of IclR resides between 274 and 100 a.a. residues from the N-terminal amino acid of IclR. It should be noted that the region between 117 and 100 a.a. corresponds to the α helix (H5) of TM IclR [22].

The results of Fig. 2B are consistent with the current model [7] in which the C-terminal domain of IclR modulates multimerization of IclR for DNA binding. To confirm the role of the C-terminal domain of IclR, a chimeric repressor plasmid (pFI006) (Fig. 2A, lane 6), in which the leucine zipper of yeast GCN4 [18,19] was fused to the N-terminal (100 a.a.) portion of IclR (N100-zip), was constructed and expressed in JM109. Our results from the reporter assay revealed that the chimeric molecule (N100-zip) effectively repressed the expression of EGFP (Fig. 2B, lane 6), while the repressor activity was significantly reduced in JM109/pFI007 (Fig. 2B, lane 5), where the DNA binding domain (NcI; residue 2-132) of the λcI repressor [16] was fused to N100. These results suggest that the above-identified domain (274–100 a.a.) of IclR (Fig. 2A, lanes 2 and 3) is essential for dimerization as well as DNA binding activity. To further confirm these results, we conducted cross-linking and gel shift analyses. Consequently, the dimer of IclR (full length) was observed in the presence of DSS (Fig. 3A, lanes 1 and 2). On the other hand, the N100 form of IclR did not react with polyclonal antibody against IclR (Fig. 3A, lanes 3 and 4), but the N100-zip formed a dimer and reacted with its antibody (Fig. 3A, lanes 5 and 6). To test whether IclR and N100-zip directly interact with the promoter region containing the iclR box, we carried out gel mobility shift assays (Fig. 3B). As a result, a marked shift was observed when IclR protein (0.1–0.4 pmol) was used (Fig. 3B, lanes 1–4). However, no shift bands appeared by the addition of the N100 fragment of IclR (0.1–0.4 pmol) (Fig. 3B, lanes 5–8) or BSA (150 pmol) (Fig. 3B, lane 13). In contrast, a significant gel shift was observed
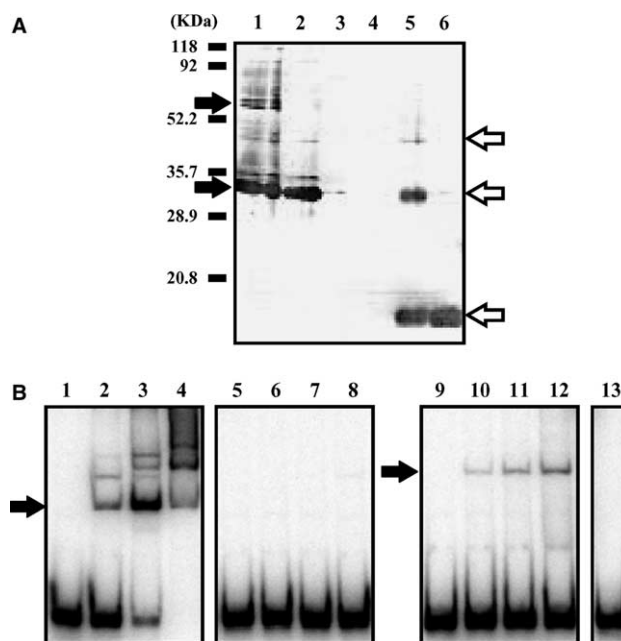

Fig. 3. Homodimerization of N100-zip. (A) Chemical cross-linking of N100-zip. IclR (lanes 1 and 2), N100 (lanes 3 and 4) or N100-zip (lanes 5 and 6) was incubated in the presence (lanes 1, 3 and 5) or absence (lanes 2, 4 and 6) of DSS. They were then analyzed by SDS–PAGE followed by Western blot with polyclonal antibody against IclR. Bottom and top of the black arrow show monomer and dimer of IclR. Bottom, middle and top of the white arrow show monomer, dimer and trimer of N100-zip, respectively. (B) Gel mobility shift assay. The labeled probe as described in Section 2 was incubated with the purified proteins, IclR (lanes 1–4), N100 (lanes 5–8), N100-zip (lanes 9–12) (lanes 1, 5 and 9: 0 pmol; lanes 2, 6 and 10: 0.1 pmol; lanes 3, 7 and 11: 0.2 pmol; lanes 4, 8 and 12: 0.4 pmol) and BSA (lane 13: 150 pmol) for 10 min at 37 °C in the binding buffer. The arrow shows the shift band of the DNA protein complex.

at a high concentration of N100-zip (0.1–0.4 pmol) (Fig. 3B, lanes 9–12). Therefore, the chimeric molecule consisting of the N100 of *E. coli* IclR and the leucine zipper of yeast GCN4 is indeed capable of forming a dimer and functionally repressing the *iclR* promoter.

To further validate our results, we constructed a chimeric gene using the homodimerized C-terminal domain (CFad; residues 83-240) of the FadR of *E. coli* [20], which contains a homodimerization domain (HD). We found that the chimeric repressor of N100 and CFadR is capable of suppressing a promoter such as N100-zip (Fig. 2B, lanes 6 and 7). These results confirm our notion that the region between 274 and 100 a.a. of IclR is involved in dimerization and that this region can be replaced with other functional homodimerization domains (Fig. 4).

3.2. A novel drug discovery system

To clarify the homodimerization domain of YycG, N100 was fused to YycG cytoplasmic domain (CYycG) to construct pFI028 (Fig. 2A). As shown in Fig. 2B lane 8, JM109/pFI028 showed a substantial decrease (up to 3-fold) of fluorescent activity compared to that of N100 (Fig. 2B, lane 3). This result strongly suggests that the cytoplasmic region of YycG is indeed involved in the homodimerization of the N100-CYycG chimera, which modulates the DNA binding activity for repressing EGFP gene expression (Fig. 4).
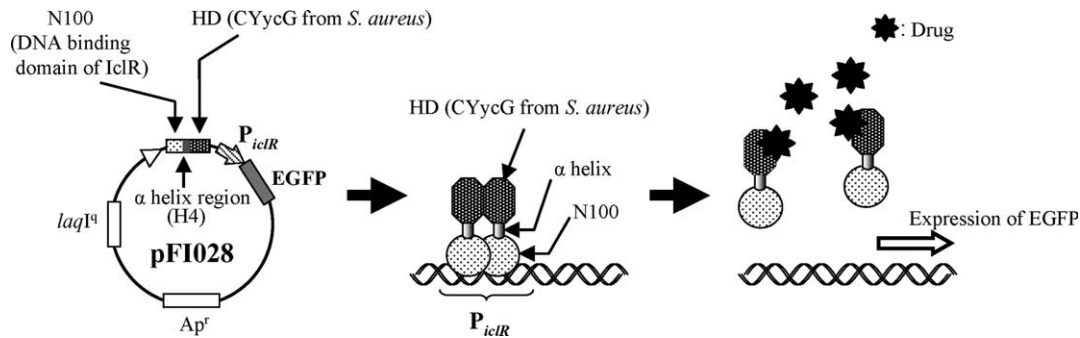
Fig. 4. Schematic diagram of homodimerization system.

Our assay system as described above effectively identifies and localizes the dimerization domain of virtually any protein that is capable of forming a homodimer. Therefore, the system is potentially useful for screening inhibitors of such dimerization. To test this possibility and to identify YycG inhibitors, we screened a chemical library against JM109/pFI028 and JM109/pFI001 in 96-titer wells (Fig. 5A). Through this screening process, we identified a compound named I-8-15, which significantly increased fluorescent intensity of JM109/pFI028, while no change in fluorescent intensity was observed in JM109/pFI001 in the presence or absence of I-8-15 (Fig. 5A). To confirm that I-8-15 indeed inhibits CYycG

dimerization, we performed a cross-linking experiment using N100-CYycG (Fig. 6, lanes 1 and 2). As a result, the dimer band of N100-CYycG was observed in the presence of DSS (Fig. 6, lane 2) but not in the absence of DSS (Fig. 6, lane 1). This result indicates that N100-CYycG in fact formed a dimer. Next, we investigated the effect of I-8-15 on the dimerization of N100-CYycG (Fig. 6, lanes 3 and 4). The dimer band of N100-CYycG remained in the presence of DSS and DMSO (Fig. 6, lane 3) but no band was observed in the presence of DSS and I-8-15 (Fig. 6, lane 4). On the other hand, the dimer band of N100-zip was observed even in the presence of DSS and I-8-15 (Fig. 6, lane 5). These results suggest that I-8-15 specifically inhibits the CYycG of S. aureus, thus, causing the conformational change and inhibiting the dimerization.

Next, we investigated the DNA binding ability of N100-CYycG for the IclR promoter in the presence of I-8-15 (50–200 μg/ml), but no inhibitory effect on the gel shift assay was observed (data not shown). Even though JM109/pFI028 (N100-CYycG) was incubated in the presence of I-8-15 (Fig. 5A), the fluorescent intensity increased only twice more than in the absence of I-8-15. Thus, we presumed that I-8-15 acts
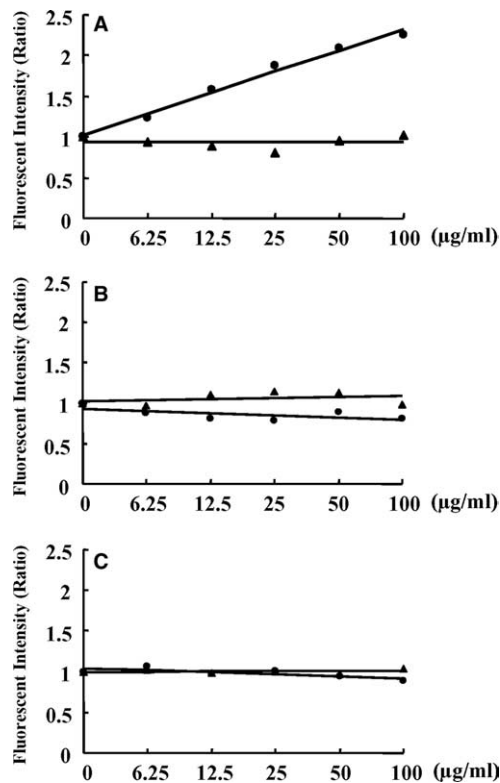

Fig. 5. Isolation of I-8-15. (A) Specific interference of I-8-15 against CYycG. E. coli JM109/pFI028 (●) or pFI001 (▲) was cultured in the presence of I-8-15, and fluorescence was measured as described in Section 2. (B, C) The effect of other HK inhibitors, Aranorosinol B and NH0891, against CYycG. E. coli JM109/pFI028 or pFI001 was cultured in the presence of Aranorosinol B or NH0891, and fluorescence was measured as described in Section 2.
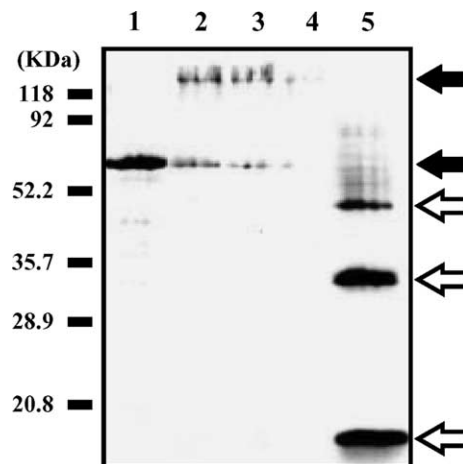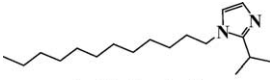

Fig. 6. Mode of action of I-8-15 against CYycG. After N100-CYycG (lanes 1–4) or N100-zip (lane 5) was incubated in the presence of DMSO (lane 3) or I-8-15 (lanes 4 and 5: 1000 μg/ml), DSS was added for chemical cross-linking reaction (lanes 2–5). These were then analyzed by SDS–PAGE followed by Western blot with polyclonal antibody against IclR. Bottom and top of the black arrow show monomer and dimer of N100-CYycG. Bottom, middle and top of the white arrow show monomer, dimer and trimer of N100-zip, respectively.

Table 1
Characterization of I-8-15

Mol. Wt.	278.48
IC_{50} (μM)	76.49
MIC (μg/ml)	
E. coli JM109	>200
B. sabtilis 168	25
MRSA[a]	25
VRE[b]	50

1 - Dodecyl - 2 - isopropylimidazole

Mol. Wt, molecular weight; IC_{50}, 50% inhibitory concentration of I-8-15 against in vitro CYycG autophosphorylation activity.
[a]Methicillin-resistant S. aureus 870307 isolated clinically.
[b]Vancomycine-resistant Enterococcus faecalis NCTC 12201 [27].

too weakly on CYycG to affect the gel shift assay. To show that I-8-15 indeed inhibits the activity of CYycG, in vitro autophosphorylation of CYycG was examined. I-8-15 inhibited autophosphorylation of CYycG with an IC50 of 76.5 μM (Table 1). Importantly, I-8-15 also significantly inhibited the growth of MRSA and VRE with MICs at 25 and 50 μg/ml, respectively (Table 1). We also tested two other HK inhibitors, Aranorosinol B [8] and NH0891 [26], that had previously been reported to inhibit CYycG at IC50 of 211 and 750 μM, respectively. However, when these compounds were added in the culture of JM109/pFI028 at a concentration of 100 μg/ml, no increase in fluorescent intensity was observed (Fig. 5B and C). These results suggest that I-8-15 acts on CYycG in a completely different manner from that of Aranorosinol B and NH0891 and exerts its bactericidal activity by inhibiting the autophosphorylation of CYycG.

References

[1] Walsh, C. (2003) Antibiotics: Actions, Origins, Resistance, American Society for Microbiology Publishing, Washington, DC.

[2] Matsushita, M. and Janda, D.K. (2002) Histidine kinase as targets for new antimicrobial agents. Bioorg. Med. Chem. 10, 855–867.

[3] Barrett, J.F. and Hoch, J.A. (1998) Two-component signal transduction as a target for microbial anti-infective therapy. Antimicrob. Agents Chemother. 42, 1529–1536.

[4] Macielag, M.J. and Goldschmidt, R. (2000) Inhibitors of bacterial two-component signaling systems. Exp. Opin. Invest. Drugs 9, 2351–2369.

[5] Howell, A., Dubrac, S., Andersen, K.K., Noone, D., Fert, J., Msadek, T. and Devine, K. (2003) Genes controlled by the essential YycG/YycF two-component system of Bacillus subtilis revealed through a novel hybrid regulator approach. Mol. Microbiol. 49, 1639–1655.

[6] Dubrac, S. and Msadek, T. (2004) Identification of genes controlled by the essential YycG/YycF two-component system of Staphylococcus aureus. J. Bacteriol. 186, 1175–1181.

[7] Martin, P.K., Li, T., Sun, D., Biek, D.P. and Schmid, M.B. (1999) Role in cell permeability of an essential two-component system in Staphylococcus aureus. J. Bacteriol. 181, 3666–3673.

[8] Watanabe, T., Hashimoto, Y., Yamamoto, K., Hirao, K., Ishihama, A., Hino, M. and Utsumi, R. (2003) Isolation and characterization of inhibitors of the essential histidine kinase, YycG in Bacillus subtilis and Staphylococcus aureus. J. Antibiot. 56, 1045–1052.

[9] Fabret, C. and Hoch, J.A. (1998) A two-component signal transduction system essential for growth of Bacillus subtilis: Implications for anti-infective therapy. J. Bacteriol. 180, 6375–6383.

[10] Hancock, L. and Perego, M. (2002) Two-component signal transduction in Enterococcus faecalis. J. Bacteriol. 184, 5819–5825.

[11] Surette, M.G., Levit, M., Liu, Y., Lukat, G., Ninfa, E.G., Ninfa, A. and Stock, J.B. (1996) Dimerization is required for the activity of the protein histidine kinase CheA that mediates signal transduction in bacterial chemotaxis. J. Biol. Chem. 271, 939–945.

[12] Tanaka, T., Saha, S.K., Tomomori, C., Ishima, R., Liu, D., Tong, K.I., Park, H., Dutta, R., Qin, L., Swindells, M.B., Yamazaki, T., Ono, A.M., Kainosho, M., Inouye, M. and Ikura, M. (1998) NMR structure of the histidine kinase domain of the E. coli osmosensor EnvZ. Nature 396, 88–92.

[13] Hidaka, Y., Park, H. and Inouye, M. (1997) Demonstration of dimer formation of the cytoplasmic domain of a transmembrane osmosensor protein, EnvZ, of Escherichia coli using Ni-histidine tag affinity chromatography. FEBS Lett. 400, 238–242.

[14] Tomomori, C., Tanaka, T., Dutta, R., Park, H., Saha, S.K., Zhu, Y., Ishima, R., Liu, D., Tong, K.I., Kurokawa, H., Qian, H., Inouye, M. and Ikura, M. (1999) Solution structure of the homodimeric core domain of Escherichia coli histidine kinase EnvZ. Nat. Struct. Biol. 6, 729–734.

[15] Throup, J.P., Koretke, K.K., Bryant, A.P., Ingraham, K.A., Chalker, A.F., Ge, Y., Marra, A., Wallis, N.G., Brown, J.R., Holmes, D.J., Rosenberg, M. and Burnham, M.K (2000) A genomic analysis of two-component signal transduction in Streptococcus pneumoniae. Mol. Microbiol. 35, 566–576.

[16] Park, S.H. and Raines, R.T. (2000) Genetic selection for dissociative inhibitors of designated protein–protein interactions. Nat. Biotechnol. 18, 847–851.

[17] Makinoshima, H., Nishimura, A. and Ishihama, A. (2002) Fractionation of Escherichia coli cell populations at different stages during growth transition to stationary phase. Mol. Microbiol. 43, 269–279.

[18] Karimova, G., Pidoux, J., Ullmann, A. and Ladant, D. (1998) A bacterial two-hybrid system based on a reconstituted signal transduction pathway. Proc. Natl. Acad. Sci. USA 95, 5752–5756.

[19] Blondel, A. and Bedouelle, H. (1991) Engineering the quaternary structure of an exported protein with a leucine zipper. Protein Eng. 4, 457–461.

[20] Raman, N., Black, P.N. and DiRusso, C.C. (1997) Characterization of the fatty acid-responsive transcription factor FadR. Biochemical and genetic analyses of the native conformation and functional domains. J. Biol. Chem. 272, 30645–30650.

[21] Martinez-Argudo, I., Martin-Nieto, J., Salinas, P., Maldonado, R., Drummond, M. and Contreras, A. (2001) Two-hybrid analysis of domain interactions involving NtrB and NtrC two-component regulators. Mol. Microbiol. 40, 169–178.

[22] Zhang, R.G., Kim, Y., Skarina, T., Beasley, S., Laskowski, R., Arrowsmith, C., Edwards, A., Joachimiak, A. and Savchenko, A. (2002) Crystal structure of Thermotoga maritima 0065, a member of the IclR transcriptional factor family. J. Biol. Chem. 277, 19183–19190.

[23] Donald, L.J., Hosfield, D.J., Curvelier, S.L., Ens, W., Standing, K.G. and Duckworth, H.W. (2001) Mass spectrometric study of the Escherichia coli repressor proteins, IclR and GclR, and their complexes with DNA. Protein Sci. 10, 1370–1380.

[24] Yamamoto, K. and Ishihama, A. (2003) Two different modes of transcription repression of the Escherichia coli acetate operon by IclR. Mol. Microbiol. 47, 183–194.

[25] Gui, L., Sunnarborg, A., Pan, B. and LaPorte, D.C. (1996) Autoregulation of iclR, the gene encoding the repressor of the glyoxylate bypass operon. J. Bacteriol. 178, 321–324.

[26] Yamamoto, K., Kitayama, T., Minagawa, S., Watanabe, T., Sawada, S., Okamoto, T. and Utsumi, R. (2001) Antibacterial agents that inhibit histidine protein kinase YycG of Bacillus subtilis. Biosci. Biotechnol. Biochem. 65, 2306–2310.

[27] Noble, W.C., Virani, Z. and Cree, R.G. (1992) Co-transfer of vancomycin and other resistance genes from Enterococcus faecalis NCTC 12201 to Staphylococcus aureus. FEMS Microbiol. Lett. 72, 195–198.