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Isolation and Characterization of Inhibitors of the Essential Histidine Kinase, YycG in *Bacillus subtilis* and *Staphylococcus aureus*

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The set of sensor kinase YycG and response regulator YycF is the only essential two-component system (TCS) in *Bacillus subtilis* and *Staphylococcus aureus*. We have developed a screening method for antibacterial agents that inhibit YycG, the essential histidine kinase (HK). To increase screening sensitivity, a temperature-sensitive *yycF* mutant (CNM2000) of *B. subtilis* with super-sensitivity to HK inhibitors was constructed, which was used for the screening of acetone extracts from 4000 microbes. A total of 11 samples showed higher sensitivity to CNM2000 than to wild-type parent 168, and seven of those were characterized to be potent inhibitors against autophosphorylation of YycG. One sample compound was purified and identified as aranorosinol B, a known antibacterial agent against Gram-positive bacteria including *B. subtilis* and *S. aureus*. Aranorosinol B inhibited YycG from both *B. subtilis* and *S. aureus* with a half-maximum inhibitory concentration (IC_{50}) of 223 and 211 μM, respectively.

The rapid emergence of antibiotic resistance in pathogenic bacteria has underscored the need for an accelerated approach to the discovery of new antibacterial agents. Bacterial genomics, bioinformatics and gene manipulation studies have led to the discovery of novel protein targets for antibacterial agents[1]. For instance, the two-component regulatory systems (TCSs) of bacteria, which consist of two proteins, histidine kinases (HKs) and response regulators (RRs), have received increasing attention for their potential as novel antibacterial drug targets for the following reasons[2~4]. First, TCSs are essential for coordinated expression of stress-response genes including those for virulence factors. Second, some TCSs regulate the expression of antibiotic resistance determinants including drug-efflux pumps[5~7]. Third, protein-histidine phosphorylation in the signal transduction pathway in bacteria is distinct from serine/threonine and tyrosine phosphorylation in higher eukaryotes. Finally, the 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, potentially leading to a lower frequency of drug-resistant bacteria. Recently, a number of TCS autophosphorylation inhibitors with inhibitory activity against multi-drug resistant bacteria have been developed[2~4,8,9]. However, the mode of actions remains mostly unsolved, and bactericidal properties may be attributable to multiple mechanisms. In some cases, bacterial growth inhibition is independent of TCS inhibition[2~4].

TCS is a fundamental system of bacterial response to environmental stresses in both Gram-negative and Gram-positive bacteria, but is not needed for steady-state growth without the stress. Thus, the genes for TCS are usually non-essential for cell growth. However, a small number of TCS-encoding genes are essential in both Gram-negative and Gram-positive bacteria[10~15]. In *Bacillus subtilis*, there are 36 HKs and 34 RRs[16], of which only one TCS, YycG

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(HK)/YycF (RR), is essential[11,17]. In fact, FABRET *et al.*[11] isolated *yycF* temperature-sensitive (ts) strain (JM17041) with A-to-C mutation at position 35103 (DNA Data-Bank accession no. D78193), which leads to histidine-to-proline (H215P) substitution. Such an essential TCS gene could be useful for the screening of antibacterial agents against TCS.

Homologues of the *B. subtilis* YycG/YycF pair have also been identified in *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Enterococcus faecalis* and *Streptococcus pyogenes*[11~14]. In *S. aureus*, a *yycF*-ts mutant has been isolated, which carries a Glu-to-Lys point mutation at position 63 in the receiver domain of the YycF response regulator[13]. Since this point mutation in *yycF* leads to severe defects in bacterial growth at non-permissive temperatures, the YycG/YycF system may be an excellent target of novel antibiotics[13]. Based on these considerations, we have developed in this study a screening method for the inhibitors of the essential HK, YycG. Aranorosinol B from *Gymnascella dankaliensis* was identified as a result of this screen.

Materials and Methods

Strains and Media

B. subtilis 168 (*trpC2*) and its ts mutant CNM2000 (*trpC2*, *yycF* [H215P], Cm^r), *E. coli* M15 [pREP4] (*lac*, *ara*, *gal*, *mtl*, *F*, *lacI*, Km^r) and BL21 (DE3) (F^-, *dcm*, *ompT*, *hsdS* [r_B^- m_B^-], *gal*, λ[DE3]) were cultured in Luria-Bertani broth (LB). *E. coli* M15 [pREP4] was used for expressing YycF and YycG from *B. subtilis*. *E. coli* BL21 (DE3) was used for the expression of *S. aureus* YycG and *E. coli* HKs. When necessary, LB was supplemented with ampicillin, kanamycin, chloramphenicol, and neomycin at concentrations of 100, 25, 5 and 3 μg/ml, respectively.

Plasmids

The plasmids used in this study are listed in Table 1. To obtain the cytoplasmic domain (Arg_{204} to Gln_{608}) of YycG from *S. aureus* 209P[13], a 1.2-kbp fragment containing *yycG* was amplified by PCR using genomic DNA as the template, SAGF-2 (5′-AGGATTCTTTGGATCCCGAACGATTACC-AA-3′) and SAGR-2 (5′-TTTAATATGCTGCGGCCGC-TTCATCCCAAT-3′) as primers and Ex *Taq* DNA polymerase (Takara). This fragment was digested with *Bam*HI and *Not*I, and ligated into pET-21a (+) between *Bam*HI and *Not*I sites to obtain pYycGSa.

To construct pHKs (Table 1), DNA fragments containing the cytoplasmic region of HKs were prepared by PCR using genomic DNA (*E. coli* W3110) as the template and a pair of primers (supplemental data shown in http://www.nara.kindai.ac.jp/nogei/seiken/primers.html). The PCR-amplified fragments were inserted into pET21a (+) to generate pHKs (Table 1).

Construction of *B. subtilis* CNM2000

pBY33 containing *B. subtilis yycFG*[18] was digested with *Bsg*I and then blunted with T4 DNA polymerase (Toyobo) (Fig. 1). A fragment Cm^r cassette digested with *Sma*I from pBEST4C was ligated into the blunted site of pBY33 to construct pBYC1. In addition, a fragment Nm^r cassette digested with *Xba*I from pBEST501 was ligated into the *Spe*I site of pBYC1 to make pBYCN1. The H215P mutation[11] of *yycF* was generated by PCR using QuikChange™ Site-directed Mutagenesis Kit (Stratagene), pBYCN1 and the primers YM1F (5′-GACAACCCGAGC-CCTCCAAATTGGATCGTC-3′) and YM1R (5′-GACG-ATCCAATTTGGAGGGCTCGGGTTGTC-3′) to obtain pBYCNM1. After pBYCNM1 digested with *Bam*HI was transformed into *B. subtilis* 168, a ts strain (CNM2000), which did not grow at 47°C on an LB plate, was obtained as a chloramphenicol-resistant and neomycin-sensitive strain.

Purification of YycF, YycG and HKs

B. subtilis YycF and YycG, *S. aureus* YycG and *E. coli* HKs were purified as His6-tag proteins as described previously[18].

Bioassay

In order to search for HK inhibitors, the acetone extracts of 4000 microbes were spotted on Trypticase Soy (0.75%) agar (1.5%) plates that were overlaid with 3 ml of top-agar (0.5%) containing 30 μl of overnight culture of *B. subtilis* 168 or CNM2000. After 24 hours of incubation at 37°C, we selected the samples that inhibited growth of CNM2000 more strongly than that of 168.

Autophosphorylation of HKs and Phosphotransfer to RRs

Autophosphorylation of HKs and their phosphotransfer to RRs were performed as described previously[9,18].

MAP Kinase Assay

0.5 U of p42 MAP kinase (New England Biolabs, Inc.) and 2 μM of myelin basic protein (MBP, Gibco BRL) were contained in a MAPK buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 2 mM DTT, 1 mM EDTA, 0.01% Brij 35). The phosphorylation of MBP was started by addition of

Table 1. Plasmids.

Plasmids	Characteristics	Sources
pBluescript II SK +	cloning vector, Ap^r	Stratagene
pQE30	expression vector, Ap^r	Qiagen
pET-21a (+)	expression vector, Ap^r	Novagen
pBEST4C	Ap^r, Cm^r	M. Itaya
pBEST501	Ap^r, Nm^r	M. Itaya
pBY33	pBluescript II SK$^+$ containing *yycFG* (3,916 bp)	K. Yamamoto[18]
pBYCN1	pBY33, Cm^r, Nm^r	This study
pBYCNM1	pBYCN1, *yycF* (H215P)	This study
pYycF*1	*Bam*H I—*Pst* I fragment (703 bp) encoding YycF of *B. subtilis* (2 to 235*3; SWISS PROT P37478)	K. Yamamoto[18]
pYycG*1	*Bam*H I—*Pst* I fragment (1,254 bp) encoding YycG of *B. subtilis* (204 to 611*3; SWISS PROT Q45614)	K. Yamamoto[18]
pYycGSa*2	*Bam*H I—*Not* I fragment (1,214 bp) encoding YycG of *S. aureus* (204 to 608*3; TrEMBL Q9XCM6)	This study
pHKs*2		This study
pKH41-14	*Bam*H I – *Not* I fragment (2,100 bp) encoding ArcB (79 to 778*3; SWISS PROT P22763)	
pKH21-11	*Bam*H I – *Not* I fragment (843 bp) encoding BaeS (187 to 467; SWISS PROT P30847)	
pKH37-3	*Bam*H I – *Not* I fragment (2,166 bp) encoding BarA (197 to 918; SWISS PROT P26607)	
pBasS	*Nde* I – *Not* I fragment (825 bp) encoding BasS (89 to 363; SWISS PROT P30844)	
pKH17-2	*Bam*H I – *Not* I fragment (1,962 bp) encoding CheA (1 to 654; SWISS PROT P07363)	
pKH49-2	*Bam*H I – *Not* I fragment (804 bp) encoding CpxA (190 to 457; SWISS PROT P08336)	
pKH54-11	*Bam*H I – *Not* I fragment (810 bp) encoding CreC (205 to 474; SWISS PROT P08401)	
pCusS	*Bam*H I – *Not* I fragment (819 bp) encoding CusS (208 to 480; SWISS PROT P77485)	
pKH52-4	*Bam*H I – *Not* I fragment (1,023 bp) encoding DcuS (203 to 543; SWISS PROT P39272)	
pKH42-4	*Bam*H I – *Not* I fragment (804 bp) encoding EnvZ (183 to 450; SWISS PROT P02933)	
pKH56-2	*Bam*H I – *Not* I fragment (1917 bp) encoding EvgS (559 to 1,197; SWISS PROT P30855)	
pHydH	*Nde* I – *Not* I fragment (729 bp) encoding HydH (223 to 465; SWISS PROT P14377)	
pPhoQ	*Nde* I – *Not* I fragment (810 bp) encoding PhoQ (217 to 486; SWISS PROT P23837)	
pKH2-2	*Bam*H I – *Not* I fragment (1,140 bp) encoding PhoR (52 to 431; SWISS PROT P08400)	
pKH14-4	*Bam*H I – *Not* I fragment (831 bp) encoding RstB (157 to 433; SWISS PROT P18392)	
pKH8-1	*Bam*H I – *Not* I fragment (1,683 bp) encoding TorS (354 to 914; SWISS PROT P39453)	
pKH45-3	*Bam*H I – *Not* I fragment (681 bp) encoding UhpB (274 to 500; SWISS PROT P09835)	
pKH19-3	*Bam*H I – *Not* I fragment (819 bp) encoding YedV (108 to 452; SWISS PROT P76339)	
pKH36-1	*Bam*H I – *Not* I fragment (843 bp)encoding YfhK (216 to 496; SWISS PROT P52101)	

*1, all fragments were cloned into pQE30; *2, all fragments were cloned into pET-21a (+); *3, position number of amino acid residues from N –terminal of each protein. Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Nm^r, neomycin resistance.

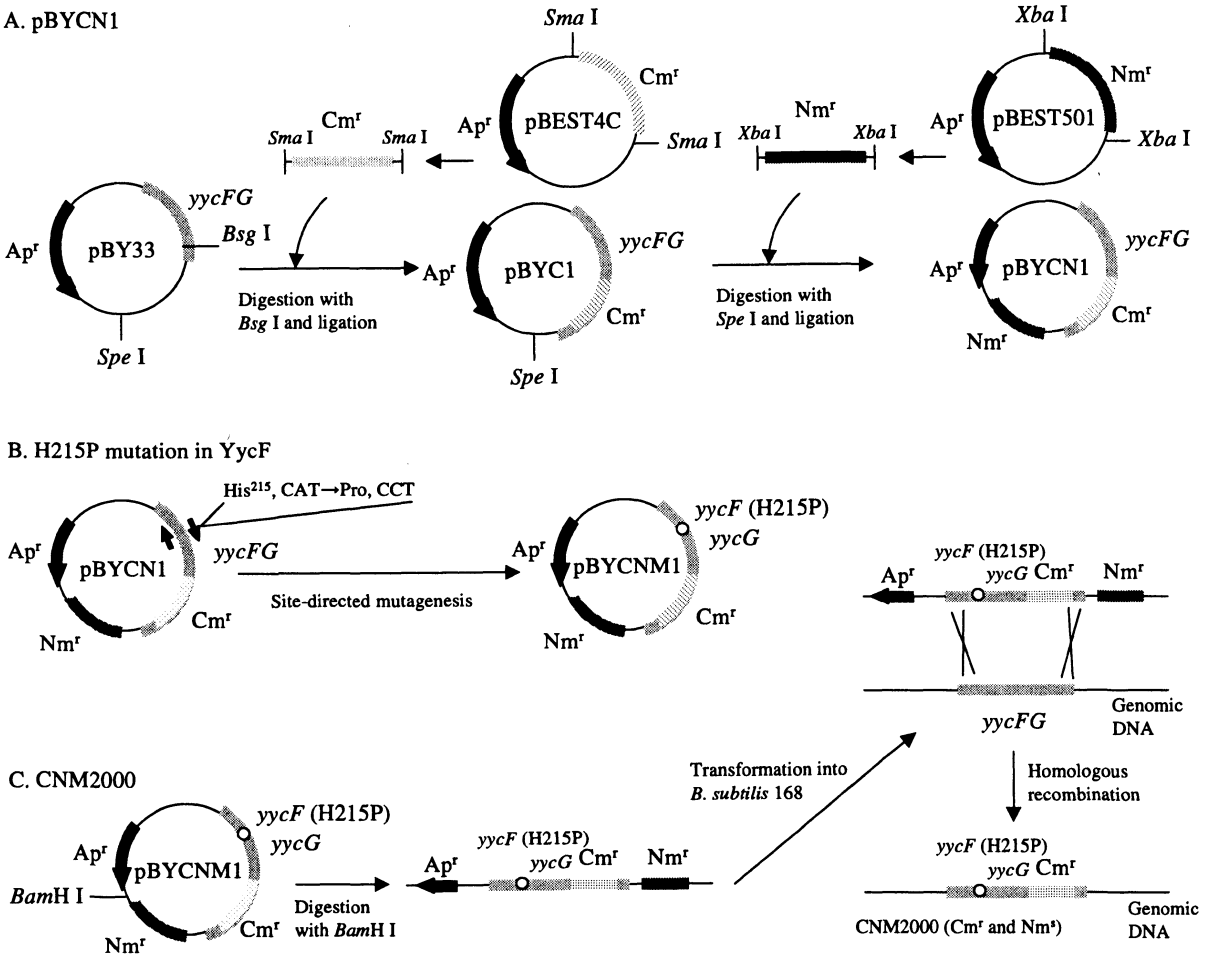
1 μCi of [γ-^{32}P]ATP diluted with unlabeled ATP to a final concentration of 2.5 μM. The reaction mixture (10 μl) was incubated for 10 minutes at 30°C before adding 10 μl of 2×sample buffer (120 mM Tris-HCl [pH 6.8], 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.1% BPB).

To assay the inhibitors, p42 MAP kinase and MBP were incubated for 5 minutes in the MAPK buffer containing the inhibitors and then [γ-^{32}P]ATP was added and reacted for 5 minutes.

Determination of IC_{50}

All the phosphorylated products were separated by using

Fig. 1. Construction of CNM2000.



SDS-polyacrylamide gel electrophoresis and quantitated with a phosphoimager (Fuji Bas 1000 Mac). IC_{50} was calculated from the intensity of bands of each phosphorylated product after subtraction of the background.

Isolation of Aranorosinol B from *Gymnascella dankaliensis*

An antibacterial substance was extracted with acetone from the culture broth of *G. dankaliensis* grown for 4 days at 25°C in two liters of medium (6% modified starch, 1% glucose, 6% corn steep liquor, 1% cotton seed meal, 1% soy bean powder, 1.6% KH_2PO_4, 1.2% Na_2HPO_4) after being precultured for 4 days at 25°C in 60 ml of medium (2% corn starch, 1% glycerol, 1% sucrose, 1% cotton seed meal, 1% gluten meal, 0.2% Tween 80). The acetone extract was passed through a column (100 ml) of DIAION HP20 (Mitsubishi Chemical Co., Ltd.). The column was washed with 50% aqueous methanol (300 ml) and eluted with methanol (500 ml). The eluate was diluted with an equal volume of water and passed through a column (350 ml) of YMC-GEL (ODS-AM120-S50, YMC Co., Ltd.). The column was eluted with 60% aqueous acetonitrile. The eluates were assayed for antibacterial activity and monitored by analytical HPLC. The active fractions containing antimicrobial substances were combined and diluted with an equal volume of water and passed through a column (350 ml) of YMC-GEL. The column was eluted with 55% aqueous acetonitrile. The active fractions were concentrated *in vacuo* to dryness and dissolved in a small amount of chloroform. *n*Hexane was added to the solution and the mixture was allowed to stand at room temperature to obtain the precipitate of the active substance. The active substance was obtained as a white

powder (110 mg). It was identified as aranorosinol B[19] (Fig. 5A) using ^{13}C NMR (Bruker DRX 500) and mass spectrometric (MS) analysis (Agilent 1100, LC-MS).

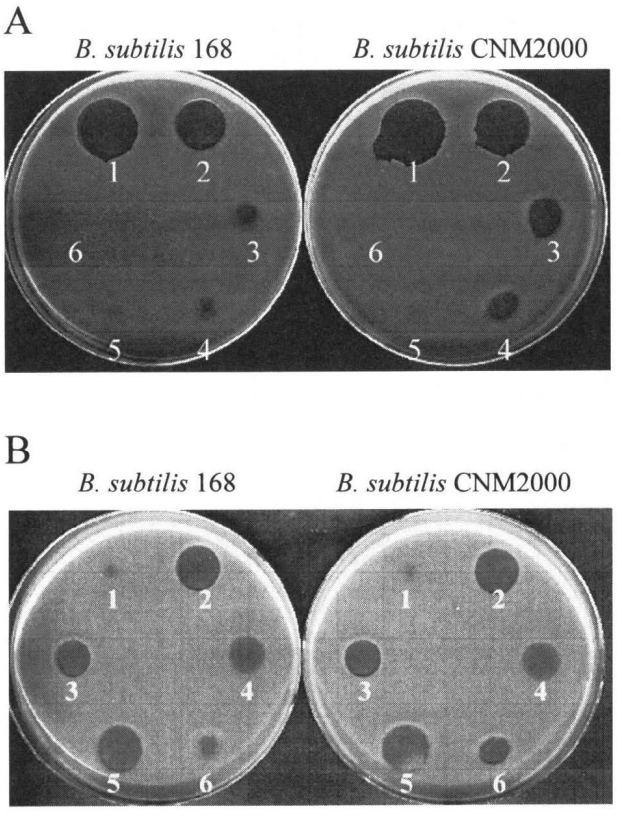
Results and Discussion

Isolation and Characterization of *B. subtilis* YycF Mutant

B. subtilis strain CNM2000, which carries the H215P mutation in YycF (the response regulator of YycG/YycF TCS), was constructed by site-directed mutagenesis in this study. As previously reported[11], this mutant has a ts phenotype in growth. The H215P mutation is located on the loop connecting the α3 helix to the β6 and β7 *C*-terminal strands of YycF. Substitution of a proline for a histidine at the site may perturb the DNA-binding properties at an elevated temperature[11]. Upon a shift to 47°C, the CNM2000 strain stopped growing after approximately 30 minutes and afterward the cell turbidity at OD_{600} decreased.

We examined the sensitivity of the *B. subtilis* CNM2000 mutant to cefazolin, amikacin, vancomycin, erythromycin, ofloxacin, and NH127 (3-benzyl-1-lauryl-2-methylimidazolium iodide)[9,18]. The growth of CNM2000 was found to be more sensitive than that of the *B. subtilis* wild-type 168 to only the antibacterial agent NH127, an HK inhibitor (Fig. 2). *B. subtilis* YycG was inhibited by NH127, but not by cefazolin, amikacin, vancomycin, erythromycin, and ofloxacin (Fig. 3). Furthermore NH127 inhibited autophosphorylation of YycG from both *B. subtilis* and *S. aureus* with the IC_{50} of 22.4 and 48.8 μM, respectively (Fig. 4A and 4B) although MAP kinase was not affected under the same conditions at all (Fig. 4D). Phosphotransfer from phosphorylated YycG to YycF of *B. subtilis* was also inhibited by NH127 with the IC_{50} of 25.6 μM (Fig. 4C).

Fig. 2. Sensitivity of *B. subtilis* 168 and CNM2000 to the antibacterial agents.



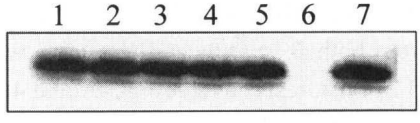
(A) NH127 was bioassayed using 168 (left) and CNM2000 (right) as described in the text. One microliter of NH127 at six concentrations (μg/ml) was spotted on Trypticase Soy agar plates containing 168 or CNM2000: 5000 (1), 500 (2), 50 (3), 5.0 (4), 0.5 (5), 0.05 (6).

(B) Cefazolin (1), amikacin (2), vancomycin (3), erythromycin (4), ofloxacin (5), and NH127 (6) were bioassayed using 168 (left) and CNM2000 (right) as described in the text. One microliter of each antibacterial agent (50 μg/ml) in DMSO was spotted on Trypticase Soy agar plate containing 168 or CNM2000.

Isolation and Characterization of Natural Antibacterial Agents That Inhibit YycG Histidine Kinase

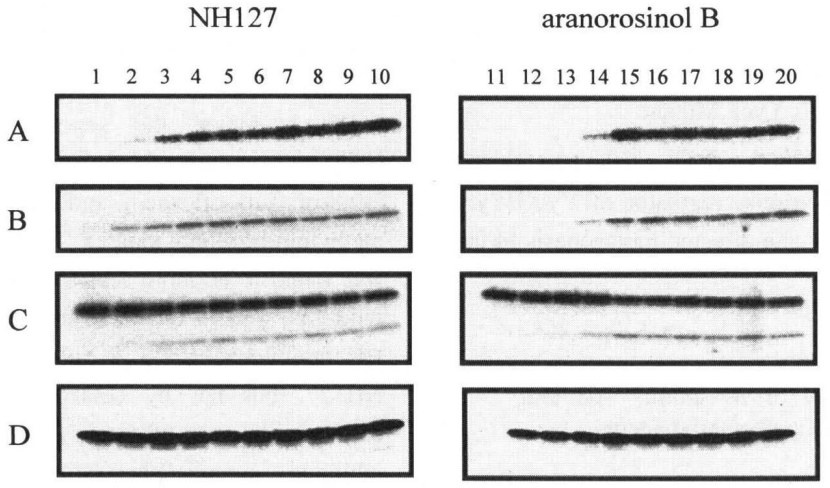
Since the *B. subtilis* mutant CNM2000 with YycF (H215P) mutation is highly sensitive to the HK inhibitor NH127 (Fig. 2), we used this strain for the screening of YycG inhibitors. For this purpose, acetone extracts from 4000 microbes were used in a bioassay of CNM2000 growth. A total of 11 samples showed greater activity against CNM2000 than strain 168. Seven of those samples significantly inhibited the autophosphorylation activity of

Fig. 3. Effect of the antibacterial agents on YycG autophosphorylation.



Autophosphorylation of YycG purified from *B. subtilis* was performed in the presence or absence of the antibacterial agents (50 μg/ml); lanes 1~7 contain cefazolin, amikacin, vancomycin, erythromycin, ofloxacin, NH127, and DMSO (control), respectively.

Fig. 4. Autophosphorylation, phosphotransfer, and p42 MAP kinase assays.



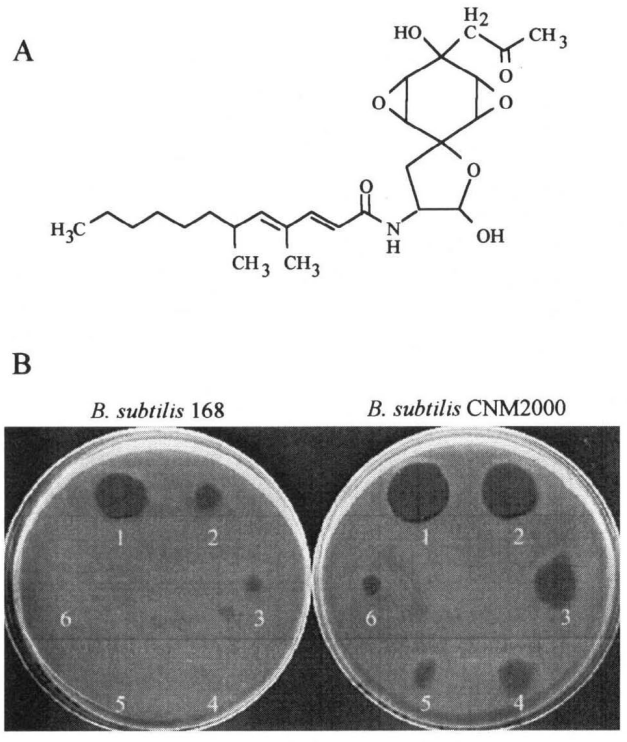
Autophosphorylation of YycG purified from *B. subtilis* (A) and *S. aureus* (B) and phosphotransfer from phosphorylated YycG (*B. subtilis*) to YycF (*B. subtilis*) (C) and p42 MAP kinase assay (D) were performed in the presence or absence of NH127 (lanes 1~10) or aranorosinol B (lanes 11~20) at nine concentrations (μg/ml): lane 1, 50.0; lane 2, 25.0; lane 3, 12.5; lane 4, 6.25; lane 5, 3.13; lane 6, 1.56; lane 7, 0.78; lane 8, 0.390; lane 9, 0.190; lane 10, vehicle control (DMSO); lane 11, 1000; lane 12, 500; lane 13, 250; lane 14, 125; lane 15, 62.5; lane 16, 31.3; lane 17, 15.6; lane 18, 7.80; lane 19, 3.90; lane 20, vehicle control (DMSO). In (C), after phosphorylation of YycG, drugs were added and then reacted with YycF as described previously[9,18].

YycG HK; the other four samples were only weakly active (data not shown). Starting with the most potent extract from the microbe WF140196, identified as *Gymnascella dankaliensis* (former name: *Pseudoarachniotus roseus*), a compound was purified, that was more potent against CNM2000 versus strain 168 [see Materials and Methods for details of the purification process]. The identification of WF140196 as aranorosinol B[19] was done by the comparison of ^{13}C NMR data. The ^{13}C NMR spectrum of WF140196 was measured in the same D-solvent system ($CDCl_3$-C_6D_6 (3 : 1)) as used by Roy *et al.* and the data were listed in Table 2 together with those of aranorosinol B. The data observed were in good agreement with the reported values within the range of experimental errors, identifying WF140196 as aranorosinol B (Fig. 5A). The purified aranorosinol B inhibited the autophosphorylation of YycG from both *B. subtilis* and *S. aureus* with IC_{50} of 223 μM and 211 μM, respectively (Fig. 4A and 4B), and the MAP kinase reaction with IC_{50} of 1477 μM. Aranorosinol B also inhibited phosphotransfer from phosphorylated YycG (*B. subtilis*) to YycF (*B. subtilis*) with IC_{50} of 223 μM (Fig. 4C). As expected, the mutant CNM2000 was more sensitive to the purified aranorosinol B than the wild-type strain 168

Table 2. Comparison of ^{13}C NMR spectrum.

position	aranorosinol B	WF140196	difference
2	96.7	96.4	0.3
3	52.4	52.1	0.3
4	37.1	37.3	-0.2
5	79.0	79.1	-0.1
6	59.6	59.3	0.3
7	58.2	58.0	0.2
8	66.4	66.3	0.1
9	57.5	57.3	0.2
10	58.7	58.5	0.2
1'	166.9	166.5	0.4
2'	117.9	117.4	0.5
3'	146.9	147.0	-0.1
4'	131.1	130.9	0.2
5'	147.7	148.0	-0.3
6'	33.3	33.2	0.1
7'	37.4	36.9	0.5
8'	27.6	27.6	0.0
9'	29.6	29.5	0.1
10'	32.0	31.9	0.1
11'	22.8	22.7	0.1
4'-CH_3	12.6	12.5	0.1
6'-CH_3	20.5	20.6	-0.1
11'-CH_3	14.1	14.1	0.0
1"	47.5	47.0	0.5
2"	210.4	210.4	0.0
3"	31.5	31.4	0.1

Fig. 5. Bioassay of aranorosinol B.



(A) Chemical structure of aranorosinol B.
(B) Purified aranorosinol B was bioassayed using 168 (left) and CNM2000 (right) as described in the text. One microliter of aranorosinol B at six concentrations ($\mu g/\mu l$) was spotted on Trypticase Soy agar plates containing 168 or CNM2000: 10.0 (1), 5.00 (2), 2.50 (3), 1.25 (4), 0.625 (5), 0.313 (6).

(Fig. 5B). Aranorosinol B, which had been isolated from *Pseudoarachniotus roseus*[19], did not show antibacterial activity against Gram-negative bacteria including *E. coli* and *Pseudomonas aeruginosa*, but MICs against *S. aureus* and *B. subtilis* were 31.25 and 15.62 μg/ml, respectively, which were also obtained in this study. The ts growth assay described here is presently being used in our laboratory for high-throughput screening for the identification of HK inhibitors and is of value in the subsequent stages of lead identification involving iterative rounds of chemical refinement.

Although aranorosinol B did not show antibacterial activity against Gram-negative bacteria, NH127 possessed antibacterial activity against both Gram-positive and Gram-negative bacteria[9,18]. To investigate the potential difference in the mode of action of aranorosinol B and NH127, we

Table 3. Effects of aranorosinol B and NH127 on *E. coli* HKs.

HKs	IC_{50} (μM)		Ratio (a/b)
	aranorosinol B[a]	NH127[b]	
ArcB	469	125	3.75
BaeS	274	109	2.51
BarA	706	577	1.22
BasS	224	158	1.42
CheA	1,407	88	15.99
CpxA	11,186<	173	64.66
CreC	213	149	1.43
CusS	761	160	4.76
DcuS	2,868	136	21.09
EnvZ	515	185	2.78
EvgS	189	387	0.49
HydH	1,084	183	5.92
PhoQ	11,186<	101	110.75
PhoR	338	297	1.14
RstB	190	183	1.04
TorS	11,186<	213	52.52
UhpB	101	170	0.59
YedV	78	122	0.64
YfhK	172	167	1.03

investigated the activity of these two compounds as inhibitors of HK autophosphorylation across a total of 19 sensor kinases from *E. coli*. The results are summarized in Table 3. A group of sensor kinases including CheA, CpxA, DcuS, PhoQ, and TorS were more sensitive to NH127 than to aranorosinol B, while other groups such as EvgS, UhpB and YedV were more sensitive to aranorosinol B than to NH127. These results suggested that the relative activities of these two agents against the different groups of sensor kinases could be a basis for their different antibacterial

properties.

Acknowledgments

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