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Identification of the Target Protein of Agelasine D, a Marine Sponge Diterpene Alkaloid, as an Anti-dormant Mycobacterial Substance

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One of the major reasons for the wide epidemicity of tuberculosis and for the necessity for extensive chemotherapeutic regimens is that the causative agent, *Mycobacterium tuberculosis*, has an ability to become dormant. Therefore, new lead compounds that are anti-bacterial against *M. tuberculosis* in both active and dormant states are urgently needed. Marine sponge diterpene alkaloids, agelasines B, C, and D, from an Indonesian marine sponge of the genus *Agelas* were rediscovered as anti-dormant-mycobacterial substances. Based on the concept that the transformants over-expressing targets of antimicrobial substances confer drug resistance, strains resistant to agelasine D were screened from *Mycobacterium smegmatis* transformed with a genomic DNA library of *Mycobacterium bovis* BCG. Sequence analysis of the cosmids isolated from resistant transformants revealed that the responsible gene was located in the genome region between 3475.051 and 3502.901 kb. Further analysis of the transformants over-expressing the individual gene contained in this region indicated that BCG3185c (possibly a dioxygenase) might be a target of the molecule. Moreover, agelasine D was found to bind directly to recombinant BCG3185c protein (K_D 2.42 μM), based on surface plasmon resonance (SPR). This evidence strongly suggests that the BCG3185c protein is the major target of agelasine D, and that the latter is the anti-mycobacterial substance against dormant bacilli.

Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* is one of the leading causes of mortality especially in areas where HIV infection is endemic.[1] The World Health Organization (WHO) estimated that 8.7 million were infected with TB and 1.4 million died from it in 2011.[2] It is known that *M. tuberculosis* has an ability to adapt to the environment of the infected region, called granuloma, and shifts into a dormant state. This unique attribute is recognized as a major reason for the necessity for extended (minimum 6 months) treatment. Therefore, new lead compounds, which have a growth inhibitory activity against both of the active growing and dormant states of *M. tuberculosis*, are urgently needed. Although the physiology of the latent *M. tuberculosis* infection remains unclear, it has been revealed that hypoxic conditions are able to induce dormancy in *Mycobacterium* species.[3–5] From this background, we have established a screening system for antibacterial substances that are effective against the dormant state of *Mycobacterium* species. So far, we have isolated several active substances, such as halicyclamines (macrocyclic alkaloids) from a *Haliclona* marine sponge,[6,7] trichoderins (new aminolipopeptides) from a culture of marine-sponge-derived *Trichoderma* fungus,[8] and neamphamide B (new cyclic depsipeptide) from a marine sponge of the genus *Neamphius*,[9] on the basis of bioassay-guided separation. In our continued screening of compounds from marine organisms, agelasines B, C, and D[10,11] from an Indonesian marine sponge of the genus *Agelas* were identified as substances against dormant mycobacteria. Agelasines with a diterpene alkaloid skeleton are known to exhibit several biological activities, such as anti-microbial,[12–17] anti-proliferative,[15–18] anti-protozoal,[19,20] anti-biofilm formation,[16] anti-fouling,[16] and inhibitory of Na^+/K^+-ATPase prepared from mammal tissues.[10,21] Agelasines D, E, and F have been reported to exhibit anti-microbial activity against *M. tuberculosis* H37Rv under active growing conditions;[13–15] however, details on the mode of action, activity against the dormant state of *Mycobacterium* bacilli, and target molecules of agelasines remain unclear.

Larsen et al. demonstrated that enoyl-Acp reductase (InhA) was the primary target of isoniazid, a front-line antibiotic in the treatment of TB: over-expression of *inhA* in *Mycobacterium smegmatis* conferred resistance to isoniazid.[22] In addition, a genomic DNA library from *M. tuberculosis* was applied in a complementation study with knockout mutants of *Mycobacterium* sp.[23] These studies inspired us to establish a *M. smegmatis* library over-expressing random genes of *Mycobacterium bovis* BCG, and we constructed a system to identify target molecules of anti-mycobacterial substances. We succeeded in identifying the genes that confer resistance to anti-mycobacterial substan-

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ces such as halicyclamine A (target: DedA protein)[24] and trichoderin A (target: ATP synthase)[25] by analyzing the cosmids extracted from the respective compound-resistant transformants of *M. smegmatis*. Here, we studied in detail the anti-mycobacterial activity of agelasines and identified the binding protein of agelasine D by analyzing the cosmids from resistant *M. smegmatis* transformed with the genomic DNA library of *M. bovis* BCG.

Results and Discussion

Anti-mycobacterial activity of agelasines under aerobic and dormancy-inducing hypoxic conditions

Hypoxia is known to be a major factor inducing non-replicating persistence of tubercule bacilli. Wayne et al. proved that oxygen depletion triggered the dormancy response, in which mycobacterial bacilli are resistant to isoniazid.[3,26] Based on these observations, we established a screening system to search for substances that exhibit anti-bacterial activity against dormant mycobacteria. Under aerobic conditions the minimum inhibitory concentration (MIC) values of isoniazid against *M. smegmatis* and *M. bovis* BCG were 2.5 and 0.03 μg mL^{-1}, respectively, whereas MIC values were more than 25 μg mL^{-1} under a nitrogen atmosphere containing 0.2% oxygen (Table 1). In contrast, agelasines B, C, and D exhibited anti-mycobacterial activities against *M. smegmatis* and *M. bovis* BCG under both aerobic and hypoxic conditions (MIC 0.8–12.5 μg mL^{-1}). These results indicate that agelasines exhibit anti-bacterial activities against *Mycobacterium* spp. in both actively growing and dormant states. Among these, agelasine D (with an exomethylene moiety in the diterpene skeleton) exhibited the most potent anti-mycobacterial activity against the tested strains. To examine the mode of action of agelasines, time-killing curves against *M. bovis* BCG was constructed for agelasine D in both aerobic and hypoxic conditions. As shown in Figure 1, the colony-forming unit (CFU) count of *M. bovis* BCG decreased in a time-dependent manner in the presence of agelasine D (4×MIC); no colony was detected after 12 days' incubation under aerobic condition, or after 14 days incubation under hypoxic condition. These data indicate that agelasine D exhibits a bactericidal effect against *M. bovis* BCG under both aerobic and hypoxic conditions.

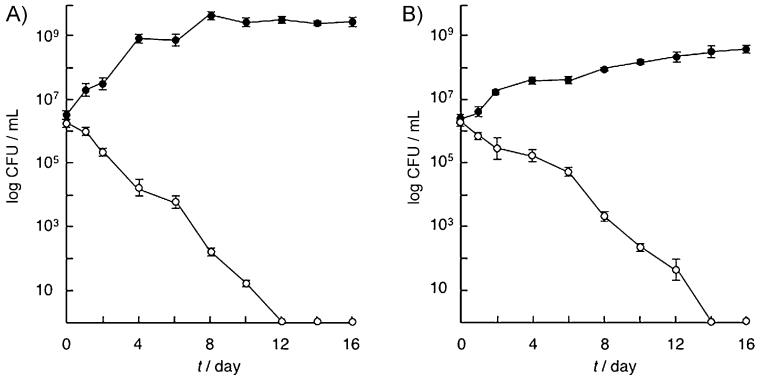
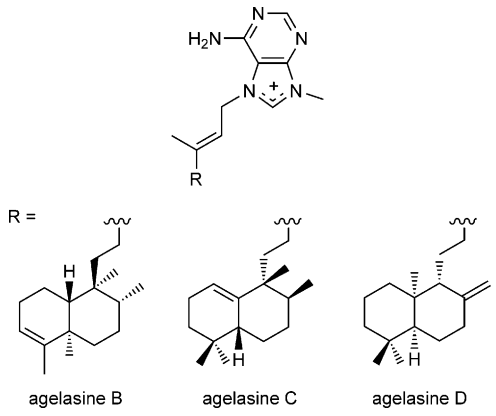


Figure 1. Time–kill curve of agelasine D against *M. bovis* BCG under aerobic and hypoxic conditions. The culture of *M. bovis* BCG (1×10^6 CFU mL^{-1}) was incubated in the presence (open circle) or absence (closed circle) of agelasine D under A) aerobic or B) hypoxic conditions. A 100 μL aliquot was collected at each time point, and serially diluted cultures were plated on Middlebrook 7H10 agar. The number of colonies was counted after four-week incubation.

Table 1. MICs of agelasines against *M. smegmatis* and *M. bovis* BCG under aerobic and hypoxic conditions.

	MIC [μg mL^{-1}]			
	M. smegmatis		*M. bovis* BCG	
Compounds	Aerobic	Hypoxic	Aerobic	Hypoxic
agelasine B	3.13	3.13	6.25	12.5
agelasine C	3.13	3.13	6.25	12.5
agelasine D	0.8	0.8	1.56	3.13
isoniazid	2.5	25	0.03	>100



Isolation of the agelasine-D-resistant clones from *M. smegmatis* transformed with genomic DNA library

In order to identify a gene that gives resistance to agelasine D when overexpressed, we generated transformants of *M. smegmatis*, which were transformed with the genomic DNA library of *M. bovis* BCG. The transformants of *M. smegmatis* that showed resistance to agelasine D were then screened from over 4000 transformants in dish cultures containing 1.6 μg mL^{-1} (2× MIC value against *M. smegmatis*) of agelasine D. We succeeded in isolating six transformants, designated ICHO2006–2011, as resistant to agelasine D (Figure 2 A). Each resistant strain was cultured in Middlebrook 7H9 broth supplemented with hygromycin B, and each cosmid (pYUB415_2006–2011) was extracted from the corresponding agelasine D-resistant strain. As a result of sequencing, four cosmids (pYUB415_2006, 2007, 2009, and 2010) were found to contain a 35.8 kb fragment (coordinates 3467.101–3502.901 kb). pYUB415_2008 and 2011 had inserts of 31.045 kb (3472.720–3503.765 kb) and 32.393 kb (3475.051–3507.444 kb), respectively. The gene that confers resistance to agelasine D was presumed to be con-

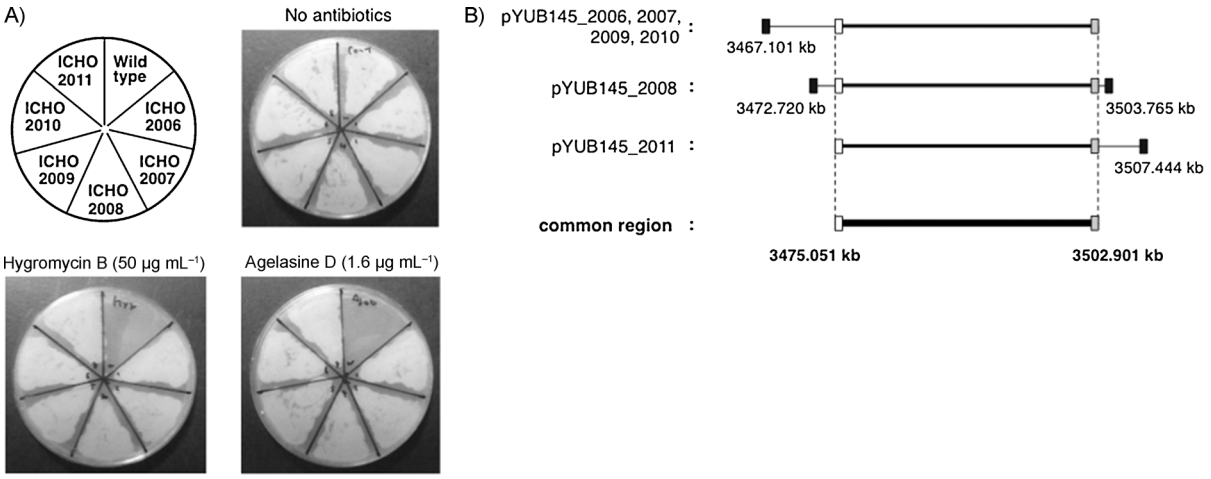


Figure 2. Growth of the agelasine-D-resistant *M. smegmatis* transformed with genomic DNA library of *M. bovis* BCG on the 7H10 agar plate containing agelasine D. A) Each strain (wild-type and clones ICHO2008–ICHO2011) was cultured in 7H9 broth, then cultures were adjusted to 1×10^5 CFU mL^{-1}. The 10 μL aliquots were spread on 7H10 agar with or without agelasine D (1.6 μg mL^{-1}). B) Gene region maps of the cosmids extracted from resistant transformants.

tained in the genome region of *M. bovis* BCG from 3475.051 to 3502.901 kb (27.85 kb; Figure 2B).

Second selection of *M. smegmatis* transformants expressing the candidate region for conferring resistance to agelasine D

We next divided the 27.85 kb of common region into ten subregions (S1–S10) as shown in Figure 3A. Then, the ten transformants (ICHO1020 ~ ICHO1029) of *M. smegmatis* were constructed to narrow down the candidate genes that confer resistance to agelasine D. Transformant ICHO1023 (S4 area overexpressed: genes *BCG3184c*, *BCG3185c*, *BCG3186c*, and *BCG3187c*) showed resistance against 1.6 μg mL^{-1} agelasine D, whereas wild-type and other transformants did not show resistance (Figure 3B).

Identification of the gene conferring resistance to agelasine D

To identify the gene conferring resistance to agelasine D, four *M. smegmatis* transformants were generated (ICHO1030–ICHO1033: over-expressing *BCG3184c*, *BCG3185c*, *BCG3186c*, and *BCG3187c*, respectively), and the growth of each was examined on 7H10 agar containing 1.6 μg mL^{-1} agelasine D. Only ICHO1031 (*BCG3185c*, a putative dioxygenase) showed resistance to agelasine D (Figure 3C). These results strongly suggest that BCG3185c protein is the target of agelasine D as an antidormant mycobacterial substance.

Binding affinity of agelasine D with the BCG3185c protein

A surface plasmon resonance (SPR)-based binding assay (Biacore T200 system) was used to determine whether agelasine D binds directly to the BCG3185c protein. Agelasine D interacted with BCG3185c, as demonstrated by the concentration-dependent responses and clear exponential curves during both the association and dissociation phases (Figure 4A). The K_D value of the interaction was 2.42 μM. As a negative control, we also evaluated K_D for the interaction between BCG3185c and streptomycin, which targets the 23S ribosome, and found a value of 1.47 mM (Figure 4B). These data indicate that agelasine D binds directly to the BCG3185c protein.

Effect of *BCG3185c* over-expression on the degradation rate of agelasine D

It is known that some microorganisms degrade exogenous chemical compounds with dioxygenases.[27–29] For example, the NidA3B3 protein in *Mycobacterium vanbaalenii* PYR-1 (ring-hydroxylating dioxygenase) degrades aromatic hydrocarbons such as naphthalene, anthracene, and pyrene to their corresponding *cis*-dihydrodiols.[29] Although the substrate and function of the BCG3185c protein in *M. bovis* BCG are unclear, BCG3185c is possibly a dioxygenase, because the conserved Rieske-type [2Fe–2S] cluster binding motif (–$CXHX_{17}CX_2H$–)[30] is found in the amino acid sequence (Cys95 to His118). Moreover, transformation of bacteria occasionally increases the degradation rate of chemical compounds by expression of transporter or metabolism-related genes. In order to exclude the possibility that agelasine D resistance was the result of the degradation of agelasine D in the bacilli, we compared the degradation rate of agelasine D in wild-type *M. smegmatis* with that in ICHO1031 (gene *BCG3185c*). There was no significant difference in the amount of agelasine D between ICHO1031 and wild-type *M. smegmatis* after 24 h incubation (Table 2, Figure S1 in the Supporting Information). This indicates that BCG3185c does not degrade agelasine D, and strongly suggests that agelasine D binds to BCG3185c directly and thus inhibits its function.

In our search for dioxygenase genes, we found six genes (*BCG0703*, *BCG0965c*, *BCG3185c*, *BCG3476*, *BCG3617*, and *BCG3633c*) in the *M. bovis* BCG genome database. These six genes supposedly correspond to genes *Rv0654*, *Rv0913c*,

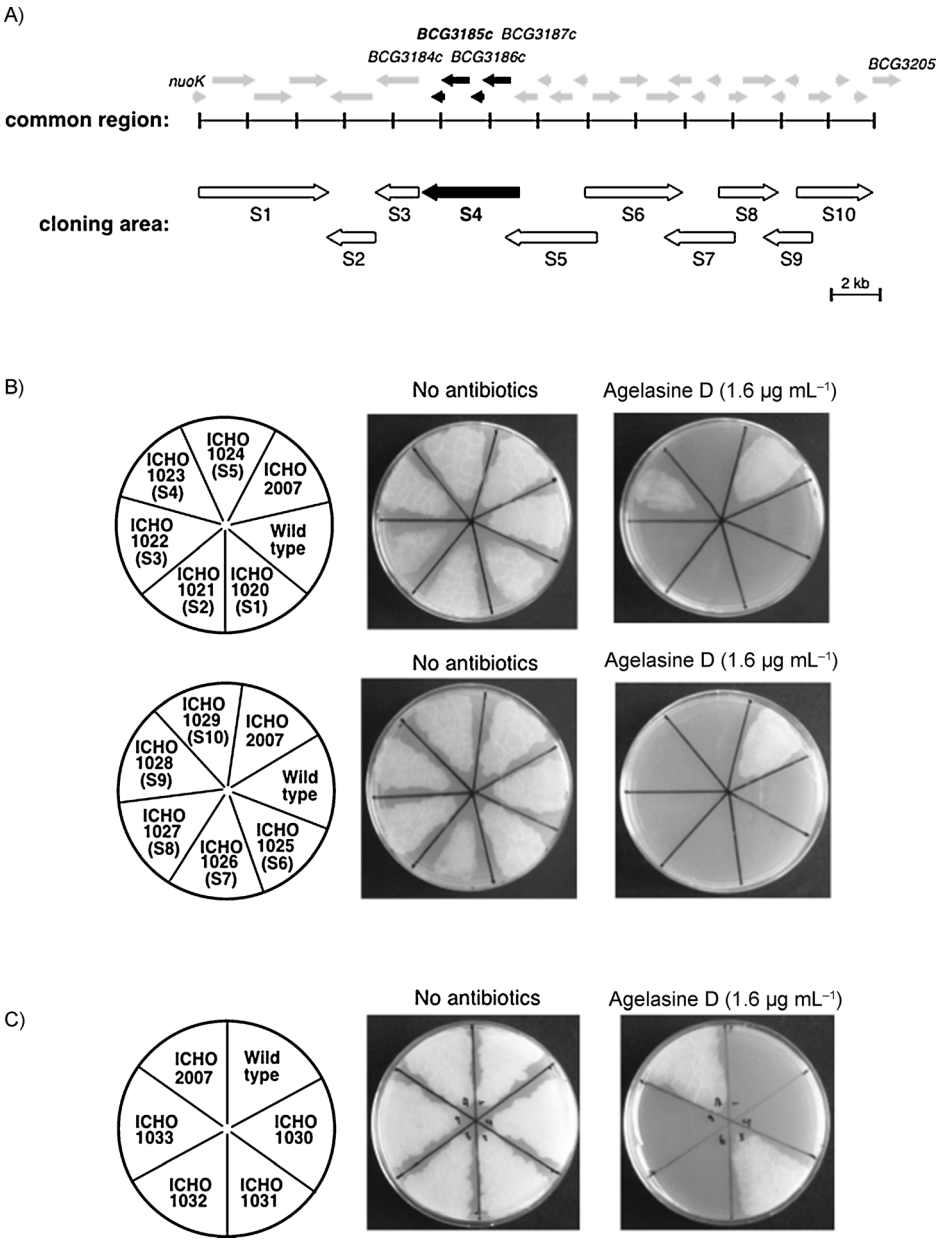


Figure 3. Growth of the transformants of *M. smegmatis* over-expressing candidate area or gene conferring resistance to agelasine D on 7H10 agars plate containing agelasine D. A) Gene map of the sub-cloning area to prepare transformants of *M. smegmatis*. B) Strains ICHO1020–ICHO1029 (over-expressing each S1–S10 area), and C) ICHO1030–ICHO1033 (over-expressing each *BCG3184c–BCG3187c gene*), wild-type *M. smegmatis,* and ICHO2007 were cultured in 7H9 broth, and then the cultures were adjusted to 1×10^5 CFU mL^{-1}. The 10 μL aliquots of each culture were spread on 7H10 agar plate with or without agelasine D (1.6 μg mL^{-1}).

Table 2. Effect of *BCG3185c* over-expression on the degradation rate of agelasine D.

Incubation time [h]	Ratio[a] Wild-type	ICHO1031
0	0.28	0.26
6	0.37	0.37
12	0.39	0.40
24	0.41	0.44

[a] Peak area of agelasin D/peak area of internal standard (rifampicin).

Rv3161c, *Rv3406*, *Rv3553*, and *Rv3568* (*hsaC*), respectively, in the genome of *M. tuberculosis* H37Rv. Although the detailed functions of most dioxygenase genes, including *BCG3185c* and *Rv3161c*, are still unknown, dioxygenases are involved in the maintenance of bacterial homeostasis and in pathogenicity in general. It has been reported that Rv0654 is a carotenoid cleavage oxygenase.[31] *M. tuberculosis* might utilize carotenoids such as β-carotene, lutein, and lycopene from its host to produce cleavage compounds for growth. Rv3568 (hsaC) is associated with cholesterol catabolism. In *M. tuberculosis*, cholesterol is converted to 3,4-dihydroxy-9,10-secoandrost-1,3,5(10)-triene-9,17-dione (3,4-DHSA) by multiple enzymatic steps. Rv3568 (hsaC) catalyzes extradiol ring cleavage of 3,4-DHSA to 4,5-9,10-diseco-3-hydroxy-5,9,17-trioxoandrosta-1(10),2-diene-4-oic acid (4,9-DSHA). A knockout mutant of *Rv3568* (*hsaC*) showed defective colony formation in guinea pig lungs.[32] These findings suggest that BCG3185c also plays an important role in the maintenance of bacterial homeostasis. To confirm the importance of BCG3185c as a drug target for the treatment of TB, testing of the *BCG3185c* gene is in progress.

Conclusions

We have identified agelasines B, C, and D as anti-mycobacterial substances effective against *Mycobacterium* sp. in the dormant state. Agelasine D showed bactericidal effect under both active growing and dormancy-inducing conditions. Based on the concept that a transformant over-expressing the target molecule of anti-microbial compound becomes resistant to that compound, we screened resistant *M. smegmatis* transformed with a genomic DNA library of *M. bovis* BCG. We found that the transformant over-expressing the *BCG3185c* gene exhibited resistance to agelasine D, without changing the degradation rate of agelasine D. In addition, we found strong evidence suggesting that the BCG3185c protein is a major target of agelasine D

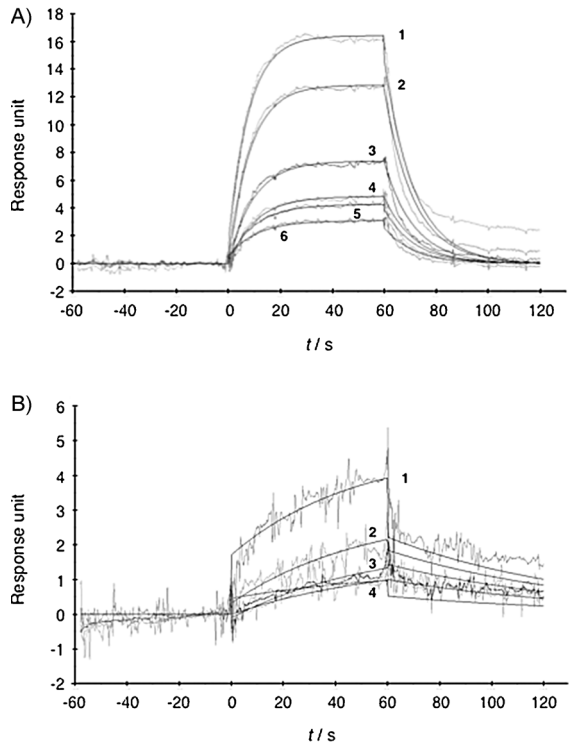


Figure 4. SPR binding analysis of A) agelasine D and B) streptomycin to immobilized BCG3185c protein. Various concentrations were injected through the BCG3185c protein–immobilized flow cells, and binding affinity was analyzed for 120 s at a flow rate of 30 μL min^{-1}. The experimental data were fitted by using the 1:1 Langmuir binding model to obtain affinity constant K_D. A) [agelasine D] 1: 0.63, 2: 0.50, 3: 0.25, 4: 0.16, 5: 0.13, 6: 0.08 μg mL^{-1}; B) [streptomycin] 1: 2.50, 2: 2.00, 3: 1.50, 4: 1.00 μg mL^{-1}.

as an anti-mycobacterial substance, by analyzing the binding affinity of agelasine D to the BCG3185c protein.

Experimental Section

Materials: Middlebrook 7H9 broth, Middlebrook 7H10 agar, Middlebrook OADC Enrichment, and lysogeny broth (LB) were purchased from BD (Franklin Lakes, NJ). Gene amplification was performed by Expand High Fidelity PCR System (Roche Applied Science). Restriction endonucleases and T4 DNA ligase were purchased from New England BioLabs. Antibiotics (carbenicillin, chloramphenicol, hygromycin B, isoniazid, kanamycin, and rifampicin) and other chemicals were purchased from Sigma. Agelasines B, C, and D were isolated from the marine sponge *Agelas* sp. 10E47 collected at Biak, Indonesia. Briefly, a methanol extract (5.4 g) of the dried sponge was partitioned in water/EtOAc (1:1). The EtOAc-soluble portion (2.7 g) was further partitioned in *n*-hexane/90% aqueous methanol (1:1). On the guidance of bioassay, the 90% methanol-soluble portion (2.0 g) was fractionated by silica gel column chromatography (solvent: $CHCl_3$/MeOH/H_2O 10:3:1 containing 1% NH_3 aq, lower phase) and HPLC [column: COSMOSIL 5C18-MS-II (Nacalai Tesque, Kyoto, Japan); solvent: MeOH/H_2O 85:15 containing 0.1% TFA] to afford a mixture of agelasines. The mixture of agelasines was further purified by HPLC [COSMOSIL 5C18-MS-II; MeCN/H_2O 60:40 containing 0.1% TFA] to isolate agelasines B (8.3 mg), C (6.6 mg) and D (52.2 mg). Each compound was identified with authentic sample by comparing ESI-TOF-MS and 2D NMR data.[10,11]

Bacterial strains and culture conditions: *M. smegmatis and M. bovis* BCG were maintained at 37 °C on Middlebrook 7H10 agar supplemented with 10% Middlebrook OADC and 0.5% glycerol, or in Middlebrook 7H9 broth containing 10% Middlebrook OADC, 0.2% glycerol, and 0.05% Tween 80. *Escherichia coli* DH5α was grown in LB and used for cloning and maintaining plasmid. *E. coli* HB101 was grown in LB supplemented with 0.3% maltose and $MgSO_4$ (10 mM) and used for construction of the genomic DNA library of *M. bovis* BCG. *E. coli* Lemo 21 (New England Biolabs; which expresses the *lysY* gene, a natural inhibitor of T7 RNA polymerase under L-rhamnose-dependent promoter) was used to generate the recombinant histidine-tagged BCG3185c protein and grown in super optimal broth (SOB). Antibiotics were added at the following concentrations to selected transformants: carbenicillin (100 μg mL^{-1}), hygromycin B (150 μg mL^{-1}), kanamycin (40 μg mL^{-1}), and chloramphenicol (30 μg mL^{-1}) for *E. coli* strains, and hygromycin B (50 μg mL^{-1}) and kanamycin (20 μg mL^{-1}) for *Mycobacterium* strains.

Anti-microbial activity of agelasines under aerobic and hypoxic conditions: MIC values against *M. smegmatis* and *M. bovis* BCG were determined by using the established MTT method.[33] Mid-log-phase bacilli (*M. smegmatis*: 1×10^4 CFU/0.1 mL; or *M. bovis* BCG: 1×10^5 CFU/0.1 mL) were inoculated in a 96-well plate, then serially diluted samples were added. For aerobic conditions, bacteria were incubated at 37 °C for 36 h (*M. smegmatis*) or 7 days (*M. bovis* BCG). For the hypoxic conditions, the protocol of Rustad et al. was used with minor modifications.[34] Mycobacterial bacilli were grown in Middlebrook 7H9 broth at 37 °C under nitrogen atmosphere containing oxygen (0.2%) until $OD_{600}=0.8$. Subsequently, bacilli were inoculated in a 96-well plate (same density) under aerobic conditions and incubated at 37 °C under nitrogen atmosphere containing oxygen (0.2%) for 96 h (*M. smegmatis*) or 14 days (*M. bovis* BCG). After incubation, MTT solution (50 μL, 0.5 mg mL^{-1}) was added to each well and incubated at 37 °C for an additional 12 h under aerobic or hypoxic conditions. OD_{560} was measured to determine MIC value.

Time-killing curve of agelasine D against *M. bovis* BCG under aerobic and hypoxic conditions: *M. bovis* BCG was grown in Middlebrook 7H9 broth at 37 °C under aerobic condition or nitrogen atmosphere containing oxygen (0.2%) until $OD_{600}=0.8$. *M. bovis* BCG culture in Middlebrook 7H9 broth was adjusted to 1×10^6 CFU mL^{-1}, then agelasine D (4×MIC) was added. An aliquot (100 μL) was collected at each time point, and serially diluted cultures were plated on Middlebrook 7H10 agar to measure CFU. The numbers of colonies were counted after four-week incubation.

Construction of genomic DNA library and preparation of *M. smegmatis* transformants: The genomic DNA of *M. bovis* BCG, which was isolated by use of the hexadecyltrimethylammonium bromide (CTAB) protocol,[35] was digested with restriction endonuclease Sau3AI to produce DNA fragments of approximately 30 kb. The double *cos* vector strategy with the *E. coli--Mycobacterium* shuttle cosmid pYUB415 was carried out to construct the genomic DNA library.[36] Briefly, pYUB415 was treated with restriction endonucleases of XbaI and BamHI to generate left- and right-arm fragments, and then the digested genome fragments were ligated into both left and right arms prepared from pYUB415. After in vitro packaging by using the MaxPlax Lambda Packaging system (Epicentre, Madison, WI), the resulting recombinant cosmids were transduced to *E. coli* HB101, and carbenicillin-resistant clones were

selected as positive transformants of *E. coli* HB101. Then, cosmids for the transformation of *M. smegmatis* were extracted from over 3×10^5 independent clones of *E. coli* HB101 by using a standard alkaline lysis method.[37] To generate the *M. smegmatis* transformed with the genomic DNA library, *M. smegmatis* was grown as described above until the OD_{600} reached 0.8–1.0. The cultures were centrifuged, and the resulting pellets were washed twice with 10% glycerol and re-suspended with $^1/_{10}$th of the initial culture volume of 10% glycerol solution. The bacterial suspensions were electroporated (2500 V, 25 μF, 1000 Ω) with the gnomic DNA library. The resulting suspensions were incubated at 37 °C for 4 h and then plated on Middlebrook 7H10 agar supplemented with hygromycin B (50 μg mL^{-1}).

Selection of agelasine D-resistant transformants and analysis of end sequence of cosmids: In order to isolate agelasine D-resistant clones, over 4×10^3 transformants of *M. smegmatis* with the genomic DNA library were cultured on 7H10 agar supplemented with agelasine D (1.6 μg mL^{-1}; 2×MIC). Subsequently, the agelasine D-resistant transformants were grown in the 7H9 broth supplemented with hygromycin B, and then cosmids were isolated for end sequencing by standard alkaline lysis.[37] The end sequencing of the isolated cosmids was performed by Macrogen Japan (Tokyo, Japan) with the primers P1 (5′-GTACGCCACCGCCTGGTTC-3′) and P2 (5′-GTGCCACCTGACGTCTAAG-3′), which were based on the sequence of cosmid vector pYUB415. The obtained sequences were analyzed by BLAST search with databases BCGList (http://genolist.pasteur.fr/BCGList/), TubercuList (http://genolist.pasteur.fr/TubercuList/), and Comprehensive Microbial Resource in J. Craig Venter Institute (http://cmr.tigr.org/cgi-bin/CMR/CmrHomePage.cgi). Description of plasmids and name of transformants are shown in Table S1.

Generation of *M. smegmatis* over-expressing candidate genes that confer resistance to agelasine D: The candidate gene regions or genes that confer resistance to agelasine D were PCR amplified from the cosmid pYUB415_2008 by using the primer pairs shown in Table S1). The amplification consisted of 30 cycles of 15 s at 94 °C, 30 s at 57 °C, and 1 min kb^{-1} at 68 °C. The PCR products were subjected to DNA sequencing after cloning into pCR2.1-TOPO (Life Technologies). Then, the cloned PCR fragment was excised by using primer-introduced restriction endonuclease sites and ligated into the mycobacterial expression vector pMV261 (*hsp60* promoter, kanamycin resistance gene) or promoterless shuttle vector pMV206 (hygromycin B resistance gene). The transformation of *M. smegmatis* was carried out according to the above-mentioned protocol. A description of plasmids and names of transformants are shown in Table S1.

Preparation of the recombinant BCG3185c protein: To generate histidine-tagged BCG3185c protein, the *BCG3185c* gene was PCR amplified from pYUB415_2008 with primer pairs His_BCG3185c_F (5′-GGTACCCGCGCTGTTGACAATCG-3′) and His_BCG3185c_R (5′-AAGCTTCCGGCAAGGCTAGCTG-3′), and ligated into the KpnI and HindIII restriction enzyme site of pREST B (Life Technologies). Following sequencing, *E. coli* Lemo21 was transformed with the pREST B vector containing *BCG3185c* and was cultured in SOB medium containing IPTG (400 μM) and L-rhamnose (750 μM) at 20 °C for 48 h. After centrifugation (8000 *g*, 20 min), the mycelium was sonicated in HBS buffer (GE Healthcare) containing imidazole (5 mM) and magnesium acetate (10 μM) and centrifuged (10000 *g*, 30 min). The supernatant was then passed through a TALON metal affinity resin column (1 mL; Takara Bio Inc., Shiga, Japan). Bound protein was washed with HBS buffer containing imidazole (5 mM) and subsequently eluted by using stepwise imidazole (50 and 150 mM) in HBS buffer. From SDS-PAGE analysis (Figure S2), the 150 mM imidazole eluate was selected for the analysis of binding kinetics after dialysis against HBS-P (GE Healthcare) supplemented with EDTA (50 μM).

Analysis of binding affinity of agelasine D with BCG3185c: The binding kinetics of agelasine D with BCG3185c was analyzed by using a Biacore T200 SPR system (GE healthcare). His-tagged BCG3185c (60 μg mL^{-1}) was immobilized on sensor chip NTA until the response units (RU) value reached 3000 with the NTA Reagent Kit (GE healthcare). Different concentrations of agelasine D or streptomycin were injected, and binding affinity was analyzed for 120 s at a flow rate of 30 μL min^{-1}. The experimental data were fitted by using the 1:1 Langmuir binding model (Biacore T200 Evaluation software version 1.0) to obtain affinity constant K_D (k_d/k_a).

Comparison of degradation rate of agelasine D between wild-type of *M. smegmatis* and the *BCG3185* over-expressing strain: Wild-type *M. smegmatis* and the ICHO1031 strain over-expressing *BCG3185c* (1×10^8 CFU mL^{-1}, 25 mL culture each) were incubated in the presence of agelasine D (0.4 μg mL^{-1}, 0.5×MIC). An aliquot (5 mL) was collected at each time point after confirmation that the two cultures showed equal OD_{600} values. Rifampicin (0.5 mg mL^{-1}) was added to the culture as a control. Subsequently, each bacillus culture was washed three times with PBS, and then agelasine D and rifampicin were extracted with MeOH and acetone. After the solvents had evaporated, the residues were dissolved in MeOH (200 μL) and analyzed by reversed-phase HPLC (COSMOSIL 5CN-MS; 10×250 mm; detection at 280 nm) with elution in MeOH/H_2O (6:4) containing TFA (0.1%). Agelasine D and rifampicin were eluted with retention times of 12.0 and 10.0 min, respectively (Figure S1).

Statistical analysis: All experiments were performed in triplicate from at least 3 independent experiments; data are mean ± SD. Differences between the data sets were determined by a Dunnett's test ($p < 0.01$ was regarded as significant).

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