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ORIGINAL PAPER

Biologically Active Natural Products from Microorganisms and Plants

Anti-dormant mycobacterial activity and target molecule of melophlins, tetramic acid derivatives isolated from a marine sponge of *Melophlus* sp.

Masayoshi Arai[1] · Yoshi Yamano[1] · Kentaro Kamiya[1] · Andi Setiawan[2] · Motomasa Kobayashi[1]

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Abstract Tuberculosis (TB), caused by *Mycobacterium tuberculosis* infection, is a major world health problem that is responsible for the deaths of 1.5 million people each year. In addition, the requirement for long-term therapy to cure TB complicates treatment of the disease. One of the major reasons for the extended chemotherapeutic regimens and wide epidemicity of TB is that *M. tuberculosis* has the ability to persist in a dormant state. We therefore established a new screening system to search for substances with activity against dormant mycobacteria using *M. smegmatis* and *M. bovis* BCG cultivated in medium containing propionate as sole carbon source to induce dormancy. Subsequently, melophlins A (**1**), G (**2**), H (**3**), and I (**4**), tetramic acid derivatives, were re-discovered from the Indonesian marine sponge of *Melophlus* sp. as anti-dormant mycobacterial substances. Moreover, target analysis of melophlin A indicated that it targeted the BCG1083 protein of putative exopolyphosphatase and the BCG1321c protein of diadenosine 5′,5‴-P^1,P^4-tetraphosphate phosphorylase.

Keywords Melophlin · Marine sponge · Antibiotic · Tuberculosis · Carbon source · Dormant

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* infection, is one of the most common causes of morbidity and mortality in HIV-positive adults living in poverty [1]. A total of 8.7 million new TB cases and 1.5 million deaths from TB are estimated to occur each year [2]. In addition, *M. tuberculosis* has the ability to adapt to the environment of the infected region, called granuloma, and to shift into a dormant state. This unique attribute is recognized as a major reason for the requirement of extended (a minimum of 6 months) chemotherapy needed to treat TB. Therefore, new lead compounds having growth inhibitory activity against both active and dormant states of *M. tuberculosis* are urgently needed.

The environment of granuloma is known to be hypoxic, lower pH, and limited nutrient conditions, and these conditions are considered triggers of the dormancy response in *Mycobacterium* bacilli, such as isoniazid tolerance. Indeed, we succeeded in inducing dormancy in *M. smegmatis* and *M. bovis* BCG cultivated under hypoxic conditions, and isolated anti-dormant mycobacterial substances from marine organisms and marine-derived microorganisms [3–8].

On the other hand, recent studies suggest that fatty acids play crucial roles as major carbon and energy sources for *M. tuberculosis* within host tissues [9–11]. Therefore, fatty acids might be able to change mycobacterial metabolism, and induce dormancy in *Mycobacterium* bacilli. In this study, we validated the possibility that fatty acids can induce a dormancy response in *M. smegmatis* and *M. bovis* BCG, and found that propionate as sole carbon source

This article is dedicated to Professor Satoshi Ōmura in celebration of his 2015 Nobel Prize.

M. Arai and Y. Yamano contributed equally to this work.

✉ Masayoshi Arai
araim@phs.osaka-u.ac.jp

✉ Motomasa Kobayashi
kobayasi@phs.osaka-u.ac.jp

[1] Graduate School of Pharmaceutical Sciences, Osaka University, Yamada-oka 1-6, Suita, Osaka 565-0871, Japan

[2] Department of Chemistry, Faculty of Science, Lampung University, Jl. Prof. Dr. Sumantri Brodjonegoro No. 1, Bandar Lampung 35145, Indonesia

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induces dormancy in *M. smegmatis* and *M. bovis* BCG. We then established a new screening system to search for substances that were effective against dormant mycobacteria using propionate as sole carbon source. On the basis of bioassay-guided separation, melophlins A (**1**), G (**2**), H (**3**), and I (**4**) [12, 13], tetramic acid derivatives, were rediscovered as anti-dormant mycobacterial substances from the Indonesian marine sponge of *Melophlus* sp. To date, 19 melophlins have been isolated from the genus of *Melophlus*. Melophlins A and B were originally isolated by our group as substances that were able to reverse the cancer phenotype of *H-Ras*-transformed NIH3T3 fibroblast cells to the normal phenotype [12]. Thereafter, several group isolated other melophlins that were found to be cytotoxic, anti-bacterial, and anti-fungal substances [13, 14]. In 2009, Knoth et al. investigated the target molecule of melophlin A as an inhibitor of *Ras* signaling transduction, and showed that melophlin A bound to the dynamins of GTPase in HeLa cells [15]. However, the target molecules of melophlins **1**–**4** as anti-dormant mycobacterial substances remain unclear.

In order to identify the target molecule of anti-microbial substances, we generated a *M. smegmatis* library transformed with the genomic DNA library of *M. bovis* BCG, which randomly overexpressed the genes of *M. bovis* BCG, based on the idea that transformants overexpressing target proteins of anti-microbial compounds could confer resistance to the compound. In practice, we screened the transformants that exhibited resistance to the isolated anti-mycobacterial substances, and explored the genes that conferred resistance to anti-mycobacterial substances by analyzing the cosmids extracted from the resistant transformant of *M. smegmatis*. To date, we have succeeded in identifying the target molecules of halicyclamine A (target: DedA family protein) [16], trichoderin A (target: ATP synthase) [17], and agelasine D (target: putative dioxygenase) [8] by using this method. In this paper, we also present the target analysis of melophlin A as an anti-dormant mycobacterial substance using the transformant library of *M. smegmatis*.

Materials and methods

Materials

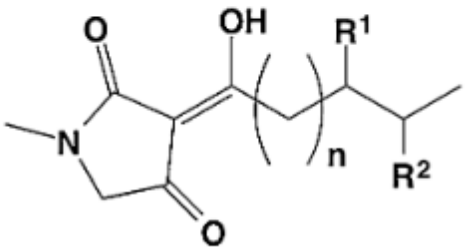
Middlebrook 7H9 broth, Middlebrook 7H10 agar, Middlebrook OADC Enrichment, and Luria–Bertani (LB) broth were obtained from BD (Franklin Lakes, NJ, USA). DNA restriction enzymes and T4 DNA ligase were obtained from New England BioLabs Inc. (Ipswich, MA, USA). The Expand High Fidelity PCR System (Roche Applied Science, Mannheim, Germany) was used for PCR. Other chemicals were purchased from Sigma (St. Louis, MO, USA) or Kishida Chemical Co., Ltd. (Osaka, Japan).

Isolation of melophlins 1–4

Melophlins **1**–**4** were isolated from the marine sponge of *Melophlus* sp. 10B15 collected at Biak, Indonesia (Fig. 1). Briefly, the MeOH extract (52 g) of the dry sponge was partitioned into a water–EtOAc mixture. Guided by bioassay, the active EtOAc-soluble portion (9.6 g; MICs against *M. smegmatis*: 25 μg/mL in the glucose medium, 3.0 μg/mL in the propionate medium) was subjected to silica gel column chromatography (eluted with $CHCl_3$–MeOH–H_2O, lower phase) and HPLC [COSMOSIL 5C18-MS-II (Nacalai tesque, Kyoto, Japan); eluted with MeOH–H_2O containing 0.1 % TFA] to afford melophlins A (**1**, 42 mg), G (**2**, 7 mg), and a mixture of melophlins H (**3**) and I (**4**) as active constituents. The mixture was further purified by HPLC [COSMOSIL Cholester (Nacalai tesque, Kyoto, Japan); eluted with MeCN–H_2O containing 0.1 % TFA] to isolate melophlins H (**3**, 23 mg) and I (**4**, 18 mg). Each melophlin was identified by ESI-TOF–MS and 2D-NMR analysis and compared with authentic spectral data [12, 13].

Bacterial culture

Mycobacterium strains were maintained and grown in Middlebrook 7H9 broth containing 10 % Middlebrook OADC enrichment, 0.2 % glycerol, and 0.05 % Tween 80, or on Middlebrook 7H10 agar containing 10 % Middlebrook OADC enrichment and 0.5 % glycerol. Three different carbon source media [basal medium (6.3 g/L $Na_2HPO_4{\cdot}12H_2O$, 1.0 g/L KH_2PO_4, 0.5 g/L $(NH_4)_2SO_4$, 0.1 g/L sodium citrate$\cdot 2H_2O$, 0.1 g/L $MgSO_4{\cdot}7H_2O$, 40 mg/L ferric ammonium citrate, 1.8 mg/L $ZnSO_4{\cdot}7H_2O$,



	R^1	R^2	n
Melophlin A (**1**)	H	H	14
Melophlin G (**2**)	H	H	10
Melophlin H (**3**)	H	CH_3	10
Melophlin I (**4**)	CH_3	H	10

Fig. 1 Chemical structures of melophlins **1**–**4**

1.0 mg/L pyridoxine, 1.0 mg/L $CuSO_4 \cdot 5H_2O$, 0.7 mg/L $CaCl_2 \cdot 2H_2O$, 0.5 mg/L biotin, 2.5 mL/L 20 % tyloxapol aq., 2.0 mL/L 20 % NaCl aq.) supplemented with 2.0 g/L glucose (glucose medium), 2.0 g/L propionate (propionate medium), or 0.1 g/L palmitate (palmitate medium), pH 6.6] were used for screening and bioassay using *M. smegmatis*. In the case of cultivation of *M. bovis* BCG for screening and bioassay, we added bovine serum albumin (fatty acid-free) (50 g/L) to each glucose, propionate, and palmitate medium.

Escherichia coli DH5α grown in LB broth was used for cloning and maintaining plasmids. *E. coli* HB101 was used to construct the genomic DNA library of *M. bovis* BCG and was grown in LB broth containing 0.3 % maltose and 1.2 g/L $MgSO_4$. The concentrations of antibiotics used were 100 μg/mL (carbenicillin), 150 μg/mL (hygromycin B), and 40 μg/mL (kanamycin) for *E. coli* strains, and 50 μg/mL (hygromycin B) and 20 μg/mL (kanamycin) for *Mycobacterium* strains.

Anti-microbial activity of melophlins 1–4 with different carbon sources

M. smegmatis and *M. bovis* BCG were pre-cultured in each glucose medium, propionate medium, and palmitate medium at 37 °C under shaking conditions until the optical density reached 0.8–1.0 at 600 nm. *M. smegmatis* (1×10^5 CFU/0.1 mL) or *M. bovis* BCG (1×10^5 CFU/0.1 mL) were inoculated in a 96-well plate, and then the serially diluted samples were added to the 96-well plate. The bacteria were incubated at 37 °C for 36 h in the glucose medium or 48 h in the propionate and palmitate media (for *M. smegmatis*), or for 7 days in the glucose medium or 14 days in the propionate and palmitate media (for *M. bovis* BCG). After incubation, 50 μL of MTT solution (0.5 mg/mL) was added to each well, and the plates were incubated at 37 °C for an additional 12 h. The optical density at 560 nm was measured to determine the MIC value.

Construction of the genomic DNA library and transformation of *M. smegmatis*

The methods for constructing the genomic DNA library from *M. bovis* BCG have been described previously [8]. Briefly, the Sau3AI restriction endonuclease-digested DNA fragments (approximately 30 kb) were ligated into an *E. coli–Mycobacterium* shuttle cosmid, pYUB415, using the double *cos* vector strategy [18]. The cell suspensions of *M. smegmatis* were mixed with the genomic DNA library and electroporated (2500 V, 25 μF, 1000 Ω) to transform *M. smegmatis*. The resulting suspensions were incubated at 37 °C for 4 h and then plated on Middlebrook 7H10 agar containing 50 μg/mL of hygromycin B.

Selection of melophlin A-resistant transformants and analysis of end sequences of cosmid

Over 4×10^3 clones of *M. smegmatis* transformed with the genomic DNA library, which were pre-cultured on propionate agar plate (propionate medium supplemented with 2 % agar), were used for screening melophlin A-resistant clones by cultivating on propionate agar plate containing 0.8 μg/mL of melophlin A (1×MIC). Subsequently, the clones resistant to melophlin A were grown in 7H9 broth containing hygromycin B, and cosmids for end sequencing were isolated using the standard alkaline lysis method. The cosmids extracted from the melophlin A-resistant transformants were subjected to end sequencing (Macrogen Japan, Tokyo, Japan). The sequence primers P1 (5′-GTACGCC ACCGCCTGGTTC-3′) and P2 (5′-GTGCCACCTGAC GTCTAAG-3′), designed based on the sequence of cosmid vector pYUB415, were used for analyzing the end sequences of the extracted cosmids. The sequences obtained were analyzed by BLAST search using the database of BCGList (http://genolist.pasteur.fr/BCGList/), TubercuList (http://genolist.pasteur.fr/TubercuList/), and Comprehensive Microbial Resource at the J. Craig Venter Institute (http://cmr.tigr.org/cgi-bin/CMR/CmrHomePage.cgi).

Preparation of *M. smegmatis* overexpressed candidate genes that might confer resistance to melophlin A (1)

The candidate gene regions or genes that conferred resistance to melophlin A were PCR amplified from the genomic DNA of *M. bovis* BCG using the primer pairs shown in Table 1. PCR was performed using a program of 30 cycles of 94 °C for 15 s, 57 °C for 30 s, and 68 °C for 1 min/kb. Following cloning into pCR2.1-TOPO (Life Technologies, Carlsbad, CA, USA) and sequencing, the cloned PCR fragment was excised using the primer-introduced restriction sites and then cloned into the mycobacterial expression vector pMV261, which had a *hsp60* promoter and kanamycin-resistance gene, or the promoter-less shuttle vector pMV206 containing the hygromycin B-resistance gene. *M. smegmatis* was transformed using the above-mentioned method. Descriptions of the plasmids and names of transformants are given in Table 1.

Results

Anti-mycobacterial activity of melophlins 1–4 in media with different carbon sources

It is known that the hypoxic, lower pH, and limited nutrient conditions in the granuloma of infected regions trigger the

Table 1 List of the transformants of *M. smegmatis* and description of the plasmids

Strains	Vectors	Description of plasmids	Sequence of primers (5′–3′) F: forward primer, R: reverse primer
ICHO1039	pMV206	Cloned S1-1 area[a] (1422912–1425666 bp of *M. bovis* BCG genome)[b]	F: TCTAGACGCGCTAGCTGGCATC, R: AAGCTTGGTCAGTCGGGCACAG
ICHO1040	pMV206	Cloned S1-2 area[a] (1425537–1429776 bp of *M. bovis* BCG genome)[b]	F: TCTAGACGATCGCCCGCAAGAC, R: AAGCTTTGGCGGTCAGCTGGTC
ICHO1041	pMV206	Cloned S1-3 area[a] (1429536–1434165 bp of *M. bovis* BCG genome)[b]	F: GGTACCAACCGCAACCGGTCAAC, R: TCTAGAGGCCGGTCTTCGGATG
ICHO1042	pMV206	Cloned S1-4 area[a] (1433943–1437194 bp of *M. bovis* BCG genome)[b]	F: TCTAGATCGCGCTGCAACACC, R: AAGCTTATCCGCGCCTGTGC
ICHO1043	pMV206	Cloned S1-5 area[a] (1436989–1440714 bp of *M. bovis* BCG genome)[b]	F: GATATCAACGGGCGGATGTCG, R: TCTAGATCGCCGCCAAGATCG
ICHO1044	pMV206	Cloned S1-6 area[a] (1440661–1444079 bp of *M. bovis* BCG genome)[b]	F: GATATCGCGTGCCGAGATCCTG, R: TCTAGAACCGCCGAATGCGTC
ICHO1045	pMV206	Cloned S1-7 area[a] (1444274–1449019 bp of *M. bovis* BCG genome)[b]	F: TCTAGAGGGCACCCACGAAACG, R: AAGCTTAGCGCGGTTCAGGTG
ICHO1046	pMV261	Cloned S2-1 area[a] (*mfd* gene) (1169441–1173295 bp of *M.bovis* BCG genome)[b]	F: AAGCTTATGGCCGCATCATGACC, R: ATCGATTGCGCCGAACGAGC
ICHO1047	pMV206	Cloned S2-2 area[a] (1173047–1176447 bp of *M. bovis* BCG genome)[b]	F: TCTAGATGCGGAAGGCTTGGTG, R: AAGCTTAACCGCGCTCGCTG
ICHO1048	pMV206	Cloned S2-3 area[a] (1174930–1178484 bp of *M. bovis* BCG genome)[b]	F: TCTAGAATGCGGCGGGTCATC, R: AAGCTTACGCGTGTGGCAGATG
ICHO1049	pMV206	Cloned S2-4 area[a] (1178885–1182306 bp of *M. bovis* BCG genome)[b]	F: TCTAGACCCGGCAAAGGCCATC, R: AAGCTTAGCGGAAACGCCTGTG
ICHO1050	pMV206	Cloned S2-5 area[a] (1182303–1184291 bp of *M. bovis* BCG genome)[b]	F: TCTAGACGGGCTTTGCCTGCTC, R: AAGCTTAGCGAGCGCTTGGC
ICHO1051	pMV261	Cloned *BCG1317* gene (1434036–1434985 bp of *M. bovis* BCG genome)[b]	F: GAATTCCCGCGGTTGCTGTTTCTC, R: ATCGATCACTGGCGCCGGAAAC
ICHO1052	pMV261	Cloned *BCG1318* gene (1434936–1435815 bp of *M. bovis* BCG genome)[b]	F: GAATTCTGGCCGGGATTGAGTGAC, R: ATCGATCAACGAGCGCTACCCTG
ICHO1053	pMV261	Cloned *BCG1319* gene (1435733–1436105 bp of *M. bovis* BCG genome)[b]	F: GAATTCGCAGCGCACCGGATTTC, R: ATCGATAGCGCCATCCTGGCTG
ICHO1054	pMV261	Cloned *BCG1320c* gene (1436174–1436665 bp of *M. bovis* BCG genome)[b]	F: GAATTCCTGCGCAAGACTGACCC, R: ATCGATCTCGACGCGCAGAAGC
ICHO1055	pMV261	Cloned *BCG1321c* gene (1436621–1437087 bp of *M. bovis* BCG genome)[b]	F: GAATTCATGCCCTGCGTGTTCTG, R: ATCGATGCGCTCAAGCCATCGG
ICHO1056	pMV261	Cloned *BCG1081* gene (1176342–1177063 bp of *M. bovis* BCG genome)[b]	F: GAATTCATGCCCGAAGCGAAACG, R: ATCGATGAGTTGCCGCGTGACC
ICHO1057	pMV261	Cloned *BCG1082* gene (1177046–1177516 bp of *M. bovis* BCG genome)[b]	F: GAATTCGTGGTCACGCGGCAAC, R: ATCGATGGGTTAGCGCCACTGC
ICHO1058	pMV261	Cloned *BCG1083* gene (1177501–1178486 bp of *M. bovis* BCG genome)[b]	F: GAATTCGCAGTGGCGCTAACCC, R: ATCGATACGCGTGTGGCAGATG

[a] Name of area corresponds to Fig. 3a, c

[b] Coordinate of *M. bovis* BCG genome is based on the database of BCG List (http://genolist.pasteur.fr/BCGList)

induction of dormancy in *Mycobacterium* bacilli [19–21]. In addition, *M. tuberculosis* in the granuloma presumably utilizes fatty acids as a major carbon source [10, 11]. Therefore, we speculated that dormancy would be induced in mycobacteria under culture conditions with fatty acids as carbon sources. Subsequently, we first evaluated the anti-microbial activity of isoniazid, which is a first-line anti-TB drug, against the *Mycobacterium* strains under culture conditions with propionate or palmitate as sole carbon source, because dormant mycobacteria show resistance to isoniazid [19, 22]. As shown in Table 2, isoniazid showed anti-microbial activity against both *M. smegmatis* and *M. bovis* BCG in the glucose medium with MIC values of 2.5 and 0.025 μg/mL, respectively. In the propionate medium, both strains were resistant to isoniazid, and the MIC value of isoniazid against these strains increased to 50 μg/mL. In contrast, *M. smegmatis* showed 80-fold greater resistance to isoniazid in the palmitate medium than in the glucose

medium, while the MIC of isoniazid against *M. bovis* BCG in the palmitate medium increased slightly compared with the MICs in the glucose medium. This result suggested that propionate is able to induce dormancy in *M. smegmatis* and *M. bovis* BCG, and that palmitate is not a suitable trigger for dormancy in *M. bovis* BCG. Therefore, we searched for substances that acted against dormant mycobacteria from the extract library of marine sponges using glucose medium and propionate medium.

On the basis of the bioassay-guided separation, melophlins A (**1**), G (**2**), H (**3**), and I (**4**) were isolated from the extract of the marine sponge of *Melophlus* sp. (Fig. 1). Melophlins **1**–**4** showed selective anti-microbial activities against both *M. smegmatis* and *M. bovis* BCG in the propionate medium relative to their activity in the glucose medium. Indeed, the MICs of melophlins **1**–**4** against *M. smegmatis* and *M. bovis* BCG in the propionate medium were 7–30 times and 4–8 times lower, respectively, than those in the glucose medium. Specifically, melophlin A (**1**) exhibited the highest selectivity among the isolated melophlins (Table 2). As a reference, MICs of melophlins **1**–**4** in the palmitate medium were similar to those in the glucose medium (Table 2). These results suggested that melophlins **1**–**4** would be effective against *Mycobacterium* spp., especially bacilli in a dormant state.

Isolation of the melophlin A-resistant strain from *M. smegmatis* strains transformed with a genomic DNA library

In general, the transformants that overexpress target proteins for an anti-microbial substance show resistance to the compound. Based on this idea, we generated 4,000 strains of *M. smegmatis* transformed with a genomic DNA library, which randomly overexpressed the genome fragments of *M. bovis* BCG. Melophlin A-resistant strains from the transformant library were then screened in dish cultures containing 0.8 μg/mL (1×MIC against *M. smegmatis*) of melophlin A. As a result, we were able to isolate 5 transformants, designated ICHO2016–20, as strains resistant to melophlin A (Fig. 2a). Of them, ICHO2016-2018 showed resistance to 3.2 μg/mL of melophlin A, while ICHO2019 and ICHO2020 showed resistance to 0.8 μg/mL of melophlin A (Fig. 2a).

Gene sequence analysis and identification of target molecule of melophlin A (1)

In order to analyze the gene sequence of *M. bovis* BCG in the cosmid, each resistant strain was cultured in Middlebrook 7H9 broth containing hygromycin B, and each cosmid (pYUB415_2016–2020) was extracted from the corresponding melophlin A-resistant strains. Sequencing showed three cosmids (pYUB415_2016–2018) containing the 1422.304–1448.713 kb (26.409 kb) region of the *M. bovis* BCG genome fragment as a common gene region (Common region 1), and two cosmids (pYUB415_2019 and 2020) containing the 1169.370–1183.760 kb (14.390 kb) region of the *M. bovis* BCG genome fragment as a common gene region (Common region 2) (Fig. 2b). This finding suggested that melophlin A targeted the molecules included in each of these common regions.

To narrow down candidate genes that confer resistance to melophlin A, we next divided the 26.409 kb of common region 1 into 7 sub-regions (S1-1 to S1-7) as shown in Fig. 3a, and generated seven transformants of *M. smegmatis* (ICHO1039–ICHO1045) overexpressing each sub-region. Similarly, common region 2 (14.390 kb) was divided into five sub-regions (S2-1 to S2-5) as shown in Fig. 3c, and established five transformants (ICHO1046–ICHO1050) overexpressing each sub-region. The resistance of each transformant to melophlin A was then evaluated on the propionate agar plate. As shown in Fig. 3b and d, the transformants ICHO1042 and ICHO1048, which overexpressed the S1-4 sub-region containing five genes (*BCG1317*, *BCG1318*, *BCG1319*, *BCG1320c*, and *BCG1321c*) and the S2-3 sub-region containing three genes (*BCG1081*, *BCG1082*, and *BCG1083*) encoding candidate targets, respectively, showed resistance to melophlin A, whereas the wild-type and other transformants of *M. smegmatis* did not.

To identify the genes that conferred resistance to melophlin A, eight transformants of *M. smegmatis* which overexpressed each open reading frame (ORF) in the S1-4

Table 2 MICs of melophlins **1**–**4** against *M. smegmatis* and *M. bovis* BCG cultured in the media prepared with different carbon sources

Compounds	MIC (μg/mL)					
	M. smegmatis			*M. bovis* BCG		
	Glucose	Propionate	Palmitate	Glucose	Propionate	Palmitate
1	25	0.8	12.5	25	3.0	25
2	3.0	0.4	3.0	12.5	3.0	12.5
3	3.0	0.4	6.0	12.5	3.0	12.5
4	6.0	0.8	3.0	25	6.0	25
Isoniazid	2.5	50	200	0.025	50	0.05

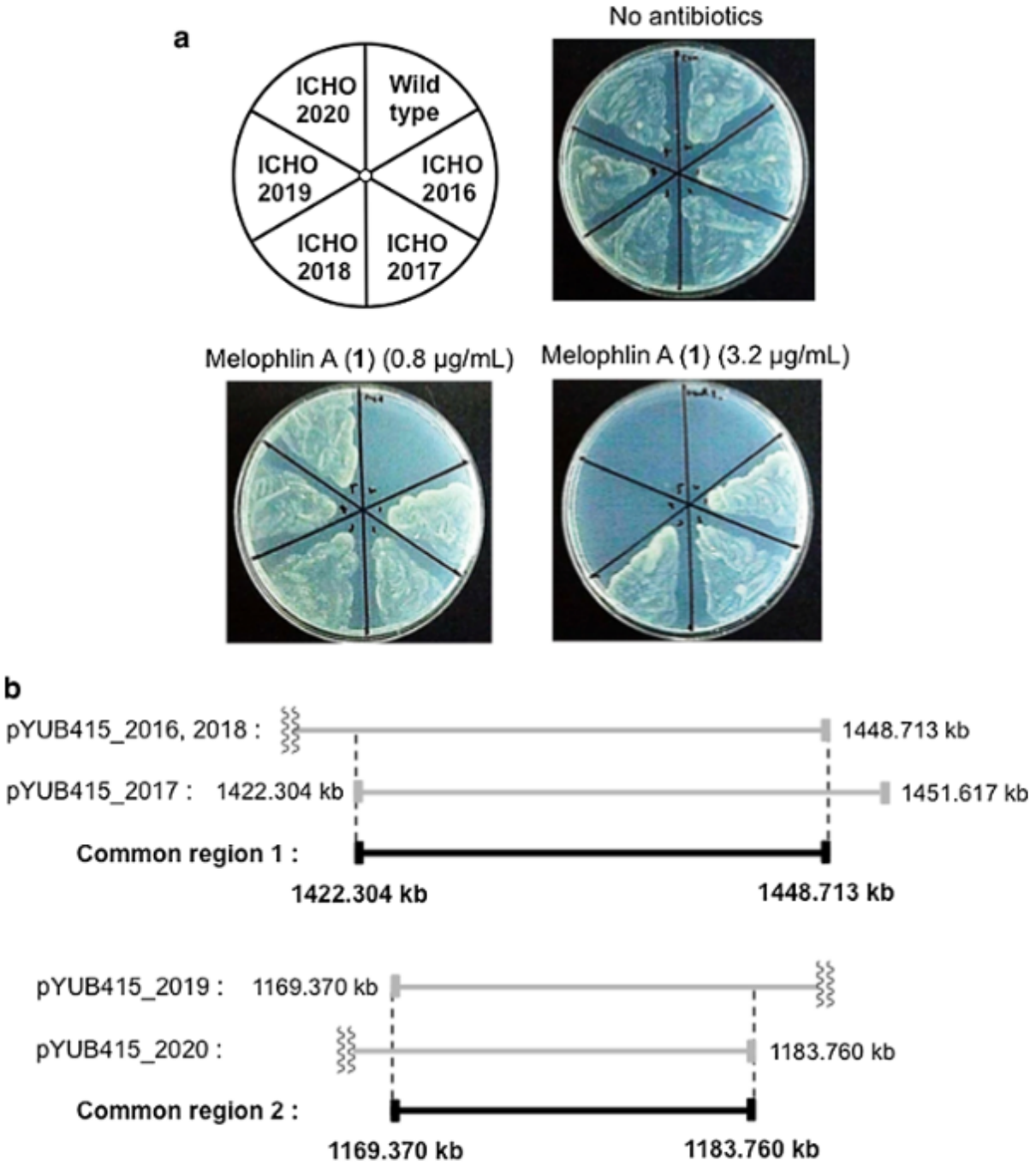


Fig. 2 **a** Growth of melophlin A-resistant transformants isolated from transformants of *M. smegmatis* with the genomic DNA library of *M. bovis* BCG. **b** Gene regions of *M. bovis* BCG in the cosmids from melophlin A-resistant transformants

and S2-3 sub-regions were generated, and then the growth of transformants on the propionate agar plate containing melophlin A was investigated. The results showed that the ICHO1055 strain, which overexpressed the *BCG1321c* gene, and the ICHO1058 strain, which overexpressed the *BCG1083* gene, conferred resistance to melophlin A (Fig. 4). In addition, the overexpressions of *BCG1321c* and *BCG1083* in *M. smegmatis* did not affect the MIC value of isoniazid in the propionate medium (50 μg/mL) (data not shown). Although further studies are necessary, these results suggested that BCG1083 and BCG1321c proteins might be target molecules of melophlin A as the anti-dormant mycobacterial substance.

Discussion

In our BLAST search of *BCG1083* and *BCG1321c* genes in the genome database of *M. tuberculosis* H37Rv, *BCG1083* and *BCG1321c* were more than 98 % identical to *Rv1026* and *Rv2613c* genes in the genome of *M. tuberculosis* H37Rv, respectively. In addition, the study of transposon mutagenesis revealed that *Rv1026* and *Rv2613c* might be essential genes in the *M. tuberculosis* H37Rv [23].

The *Rv1026* gene, called PPX2, encodes an exopolyphosphatase that has hydrolytic activity against long-chain polyphosphate [poly(P)] (Fig. 5a). Transient accumulation of poly(P) resulting from *Rv1026* deficiency has been reported to be associated with growth restriction, metabolic downshift, and isoniazid tolerance [24]. Therefore, poly(P) plays a significant role in various bacterial processes including protein synthesis, nucleotide synthesis, lipid metabolism, and energy use.

The amino acid sequence of Rv2613c includes a histidine triad (HIT) motif consisting of His-hydrophobic amino acid (Phi)-His-Phi-His-Phi-Phi, indicating that Rv2613c belongs to the HIT-like protein family. In general, this family protein has a diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap4A) hydrolase activity, which catalyzes Ap4A to adenosine 5′-triphosphate (ATP) and adenosine

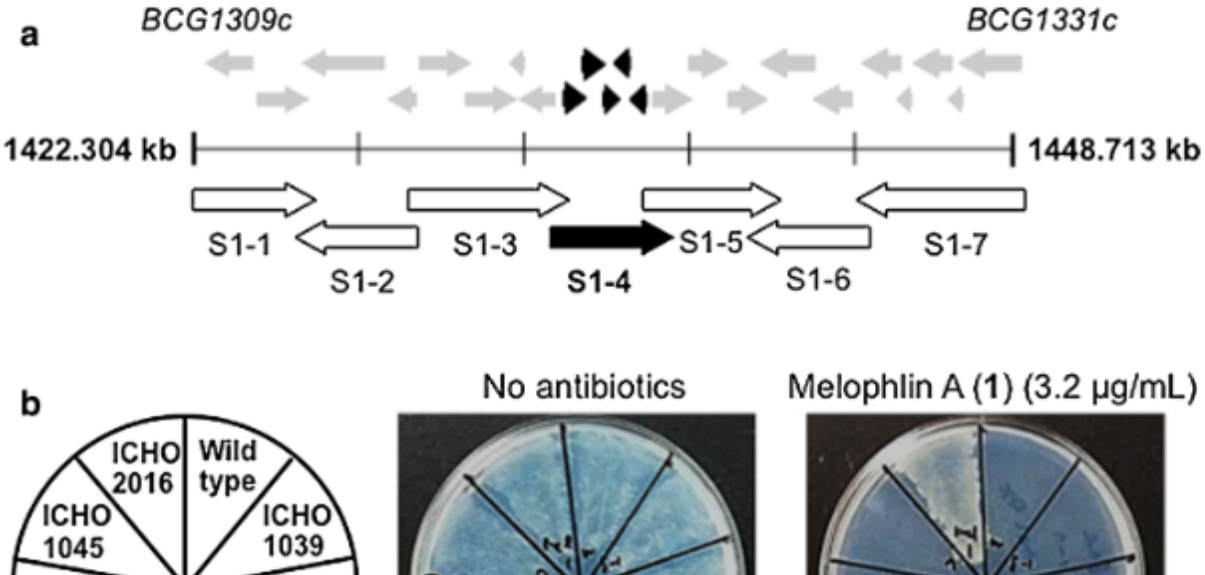


Fig. 3 **a**, **c** Cloning regions in common regions 1 and 2 of the *M. bovis* BCG genome. **b**, **d** The growth of each transformant on propionate agar plates containing melophlin A

5′-monophosphate (AMP) or 2 molecules of adenosine 5′-diphosphate (ADP) [25]. However, Mori et al. showed that the recombinant Rv2613c protein converted Ap4A into ATP and ADP in the presence of inorganic phosphate instead of exhibiting Ap4A hydrolase activity [26]. Thus, Rv2613c is now generally recognized as an Ap4A phosphorylase (Fig. 5b). Moreover, regulation of the Ap4A concentration is indispensable in some bacteria such as *E. coli* and *Salmonella enterica* [27, 28]. From these reports, control of the intracellular Ap4A concentration may be considered to play an important role in the survival and virulence of *M. tuberculosis*. Taken together, melophlin A (**1**) shows anti-dormant mycobacterial activity by inhibiting the functions of both exopolyphosphatase (BCG1083) and Ap4A phosphorylase (BCG1321c) proteins, while the mechanism underlying the selective anti-mycobacterial activity of melophlin A on the propionate medium is unclear.

In addition, melophlin A was reported to bind to dynamins of GTPase in mammalian cells, and to inhibit the receptor-mediated endocytosis, which requires normal dynamin function [15]. However, melophlin A does not inhibit the GTPase activity of dynamins [15]. Although the dynamins, BCG1083 (Rv1026) and BCG1321c (Rv2613c) have a phosphatase-like function in a wide sense, the mode of action of melophlin A against dormant mycobacteria might be different from the action of melophlin A in mammalian cells.

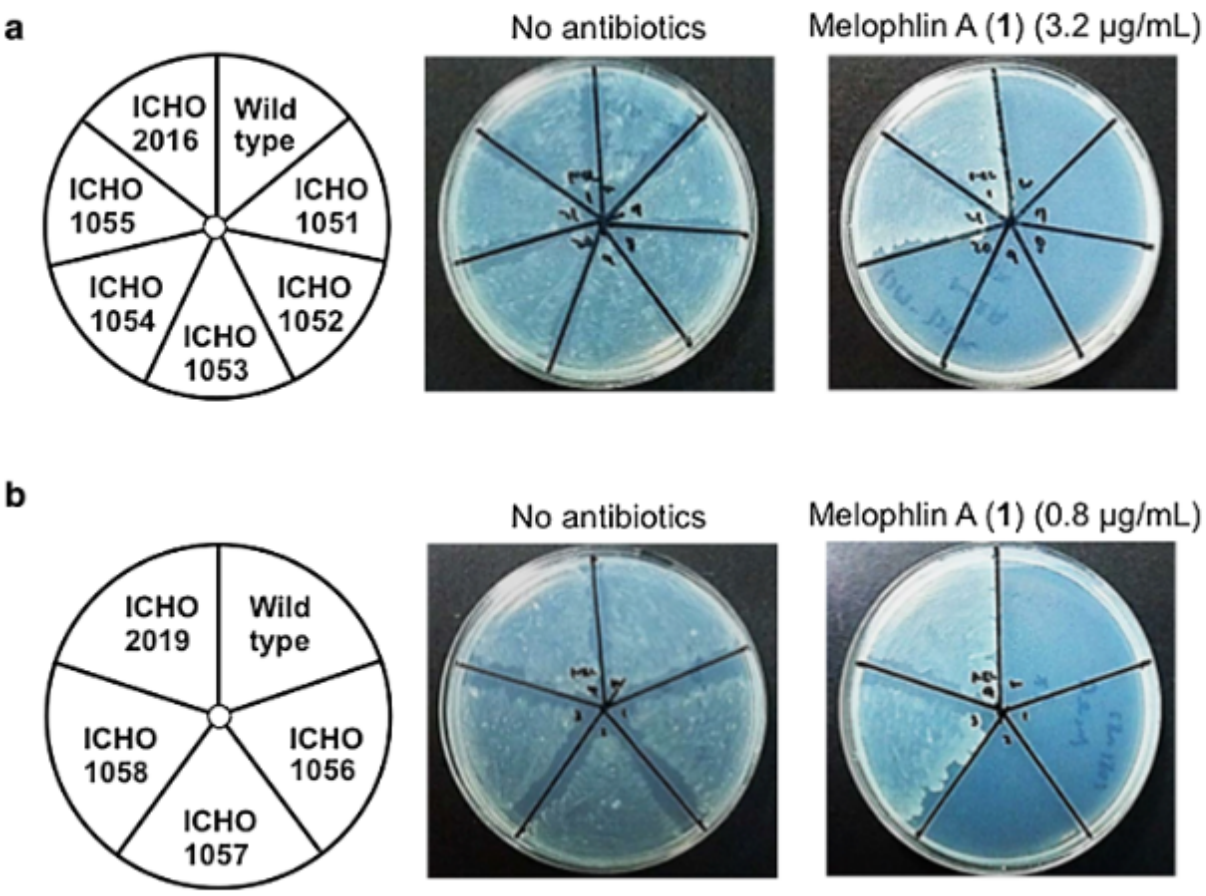


Fig. 4 The growth of each transformant which overexpressed the respective gene in the S1-4 sub-region (**a**) or S2-3 sub-region (**b**) on propionate agar plates containing melophlin A

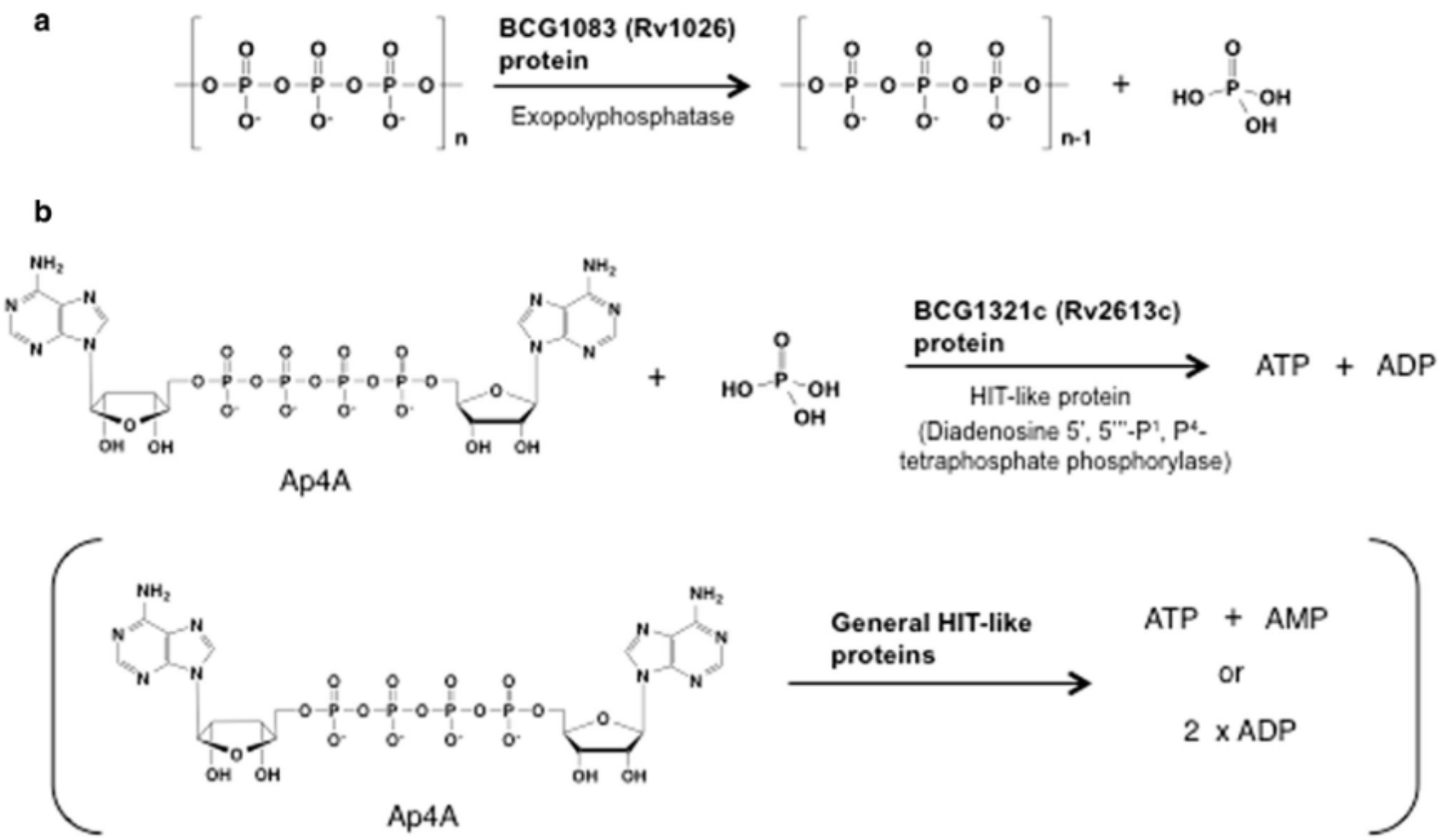


Fig. 5 Putative functions of BCG1083 (**a**) and BCG1321c (**b**) proteins as targets of melophlin A

Conclusion

In this study, we established a new screening system to search for anti-dormant mycobacterial substances using propionate as sole carbon source. Subsequently, melophlins **1**–**4** were discovered from the marine sponge of *Melophlus* sp. as anti-dormant mycobacterial substances. We then searched for the target molecules of melophlin A (**1**) using the *M. smegmatis* library transformed with the genomic DNA library of *M. bovis* BCG. This analysis revealed that the BCG1083 and

BCG1321c proteins are likely target molecules of anti-mycobacterial melophlin A. Although the binding affinities of melophlin A to these target proteins and the relationship between the functions of these target proteins and the anti-dormant mycobacterial activity of melophlin A in the propionate medium remain unclear, melophlins **1**–**4** would be expected to be a promising drug seed for TB chemotherapy.

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