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Identification and Characterization of *hmr19* Gene Encoding a Multidrug Resistance Efflux Protein from *Streptomyces hygroscopicus* subsp. *yingchengensis* Strain 10-22

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Abstract The *hmr19* gene was cloned from *Streptomyces hygroscopicus* subsp. *yingchengensis* strain 10-22, a bacterium strain producing agricultural antibiotics. Sequence similarity comparison indicates that *hmr19* gene may encode a predicted protein with 14 putative transmembrane α-helical spanners, belonging to the drug:H^+ antiporter-2 family of the major facilitator superfamily. The expression of *hmr19* in the mycelium of strain 10-22 was detected by Western blotting analysis. Gene replacement technology was employed to construct an *hmr19* disruption mutant. The growth inhibition test against different antibiotics indicated that the mutant strain was 5–20 fold more susceptible to tetracycline, vancomycin and mitomycin C than the parental wild type strain. The mutant took up tetracycline much faster and accumulated more antibiotics than the wild type strain 10-22. While with the addition of an energy uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone, the characteristics of the accumulation of [^{3}H]tetracycline in these two strains were almost the same. It was thus concluded that *hmr19* encoded a multidrug resistance efflux protein.

Key words multidrug resistance (MDR); gene replacement (knockout); *Streptomyces*; *Streptomyces hygroscopicus* subsp. *yingchengensis* strain 10-22

The over-usage of antibiotics may result in the development of extensive antibiotic resistance in microorganisms [1]. Export of toxic compounds as means of resistance has been well documented in pathogenic bacteria as well as antibiotic-producing microorganisms [2,3]. Drug resistance efflux proteins comprise the primary efflux system, namely the ATP-binding cassette (ABC) family transporters energized by ATP, and the secondary active transporters, which constitute the major class of drug antiporters in microorganisms. The secondary active transporters are distributed in five superfamilies including the major facilitator superfamily (MFS), the small multidrug resistance (SMR) superfamily, the multidrug endosomal transporter (MET) superfamily, the resistance nodulation division (RND) superfamily and the multi-antimicrobial resistance (MAR) superfamily [4].

MFS is the most ubiquitous one among all these superfamilies, which contains 34 families known so far [5]. Members of this superfamily have been either shown or predicted to be integral membrane proteins and they catalyze the transportation *via* uniport, symport (solute:H^+ or solute:Na^+) and antiport (solute:H^+ or solute:solute) [6]. The six families of MFS known for drug export are DHA1 (TC 2.A.1.2), DHA2 (TC 2.A.1.3), DHA3 (TC 2.A.1.21), OCT (TC 2.A.1.19), SET (TC 2.A.1.20) and VNT (TC 2.A.1.22). Among them, the most widely identified and characterized are the DHA1 family with 12 putative transmembrane helical spanners and DHA2 family with 14 putative spanners [4,7].

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In this study, a coding segment CDS19 encoding an efflux protein (designated *h*ygroscopicus *m*ultidrug *r*esistance CDS*19* gene, *hmr19*) was identified *via* analyzing a 36.7 kb genomic DNA fragment clone (pHZ1392 [8]) of *Streptomyces hygroscopicus* subsp. *yingchengensis* strain 10-22, a producer of three anti-fungal antibiotics [9]. Sequence homology comparison of *hmr19* encoded putative protein against the GenBank protein database revealed that it was similar to many drug efflux pumps. Transmembrane domain analysis and motif search implied that the protein product of *hmr19* was a typical member of the DHA2 family. We further conducted functional studies upon the Hmr19 protein and characterized its multidrug resistance property.

Materials and Methods

Bacterial strains, culture condition and media

Bacterial strains and plasmids used in this study were listed in Table 1. *Escherichia coli* strains were grown at 37 °C in Luria-Bertani (LB) medium. Antibiotics kanamycin (50 mg/L) or chloramphenicol (170 mg/L) was

Table 1 Plasmids and strains used in this study

Plasmids and strains	Characteristics	Source or reference
E. coli strains		
DH5α	*supE ΔlacZU169 (Φ80 lacZ ΔM15) hasdR17 recA endA gyrA96 recA*	
BL21(DE3)	*HsdS gal (lacUV5-T7gene1 λcIts857 indI Sam7 nin5)*	
ET12567(pUZ8002)	*Dam13::Tn9 dcm6 hsdM hsdR recF143 zjj201::Tn10 galK2 galT22 ara14 lacY1 xyl5 leuB6 pUZ8002*	MacNeil [16]
S. hygroscopicus subsp. *yingchengensis* strains		
10-22	Wild type strain, producing three antibiotics 5102-I, 5102-II and 5102-III	From Deng ZX
EAK	*hmr19* gene knocked-out mutant strain. *hmr19*$^-$, Am^r, Thio^s	This study
Plasmids		
pET32a	Expression vector, T7 promoter. Ap^r	Novagen
p32E	*hmr19* franking with *Eco*RI and *Xho*I, cloned in pET32a	This study
p32ES	*Sal*I deletion/self-ligation of p32E, a 5′ terminal 396 bp *hmr19* DNA clone in pET32a	This study
pSP2	Used as a cloning vector. Ap^r, Cm^r	Pelzer *et al*. [17]
pSP3	Derivative plasmid from pSP2, without *Sph*I and *Pst*I sites	This study
pBluescriptKSII(+)	2.9 kb, used as a cloning vector. Ap^r	Fermentas
pBC-AM	Apramycin resistance, donor of *aac(3)IV*. Am^r	From Ding XM
B5K-pKSII	5.4 kb *Bam*HI fragment of pHZ1392 containing CDS19 and its flanking regions cloned into pBluescriptKSII(+)	This study
pSPB	Insertion DNA same as that in B5K-pKSII but cloned in pSP3	This study
pSP3eff	Recombinant clone containing cassette for *hmr19* gene disruption and probe for Southern blotting	This study
pHZ1358	Shuttle-vector of *E. coli* and *Streptomyces*, used for conjugation from *E. coli* to streptomycetes. Thio^r, Ap^r	From Deng ZX
pEA	Derivative plasmid from pHZ1358, containing the *hmr19* gene replacement cassette	This study

added when desired in the experiments. *E. coli* DH5α was used as the host for generating double-stranded plasmid DNA. *E. coli* BL21(DE3) was used as the host for protein expression. *E. coli* ET12567 was used for conjugation with streptomycetes. *S. hygroscopicus* subsp. *yingchengensis* strain 10-22 was grown on MS agar [10] for conjugation or TSB medium [10] for preparation of genomic DNA.

DNA preparation and amplification

S. hygroscopicus genomic DNA was isolated employing the Kirby mix method modified by Hopwood *et al*. [10]. General DNA manipulation, such as restriction enzyme digestion, was performed as described previously [8]. Oligonucleotides for PCR and sequencing were obtained from Sangon Ltd..

Cosmid sequencing and annotation

To clone an antibiotics biosynthetic gene cluster, cosmid pHZ1392 with a 36.7 kb genomic DNA fragment from *S. hygroscopicus* subsp. *yingchengensis* strain 10-22 was completely sequenced by limited shotgun and restriction fragment subcloning, and finished by primer walking [11]. The sequence was annotated with Frameplot 2.3.2 [12] to predict possible coding segments (CDSs). Multiple-sequence alignment was conducted using the NTI software [13]. The CDS19 in this genomic fragment was predicted to encoding a protein, which was probably a multidrug resistance efflux protein and designated *hmr19*. Multiple-sequence alignment analysis and HMMTOP program [14] were applied to predict the conserved motifs and transmembrane domains of Hmr19.

Expression of recombinant *hmr19* in *E. coli* and Western blotting analysis of the *S. hygroscopicus* 10-22 total protein

The *hmr19* gene was cloned into pET32a and expressed in *E. coli* BL21(DE3). Briefly, restriction sites *Eco*RI and *Xho*I were introduced into the flanking ends of the *hmr19* gene fragment *via* PCR primers for expression plasmid construction (5′-CATGAATTCGTAGTGCTCCCGG-3′ and 5′-TATCTCGAGGGTCGTCTCCTTGG-3′). The amplified 1.47 kb fragment was cloned into pET32a to construct p32E. Then p32E was digested with *Sal*I and self-ligated to generate p32ES containing the 5′ terminal 396 bp DNA sequence of the *hmr19* gene encoding the N-terminal 132 amino acid residues fused to the thioredoxin protein (TRX). The p32E and p32ES were individually transformed into BL21(DE3) to express corresponding recombinant proteins. The expressed proteins were analyzed by SDS-PAGE [18]. The Hmr19 protein purified from inclusion body was used to immunize rabbit to generate antiserum for Western blotting analysis. To verify the expression of *hmr19* in *S. hygroscopicus* 10-22, the spores of strain 10-22 were inoculated into TSB medium and cultured at 30 °C, 200 rpm for 24 h. Then total protein was extracted from mycelium for Western blotting analysis.

Construction of the *hmr19* gene knockout strain of *S. hygroscopicus*

A 5.4 kb *Bam*HI restriction fragment of pHZ1392 containing CDS19 and its flanking segments evenly on either side was cloned into pSP3 (derived from pSP2 [17] with *Sph*I and *Pst*I sites disrupted) to construct pSPB. The *aac(3)IV* gene cassette isolated from pBC-AM was inserted as the selective marker into pSPB to create pSP3eff, in which, the *hmr19* gene was replaced by *aac-(3)IV*. This gene disruption cassette was then cloned into pHZ1358 as a *Bam*HI fragment to create pEA as the gene knockout clone. The pEA was subsequently conjugated into *S. hygroscopicus* subsp. *yingchengensis* 10-22 *via* the donor *E. coli* strain ET12567(pUZ8002) as described by Flett *et al.* [19]. The original exconjugants were selected for apramycin and thiostrepton resistance (Am^r and $Thio^r$); while the donor *E. coli* was counterselected for nalidixic acid sensitive. After passed on MS agar without antibiotics for at least 3 cycles, the *hmr19* knockout clones were screened from the exconjugants for thiostrepton sensitivity ($Thio^s$) but retaining apramycin resistance (Am^r). Mutants showing double-crossover genotype were confirmed by Southern blotting [20] and Western blotting [21] analyses.

Phenotype scanning with antibiotics and dyes

Growth inhibition test (GIT) was performed by weighing the wet weight (*WW*) of mycelium cultured with antibiotics (*WWx*) or without antibiotics (*WWc*). The *WWx/WWc* ratio was used to indicate the level of growth inhibition. RIC_{50} was defined as the antibiotics concentration at which the ratio of *WWx/WWc* was about 0.5 and was used to indicate the differences in antibiotics susceptivity between the wild type strain 10-22 and the *hmr19* knockout strain EAK. GIT test was carried out as follows: the spores of the streptomycetes were inoculated into 25 ml TSB medium and cultured at 30 °C, 200 rpm for 24 h to grow up as mycelia at exponential-phase for the expression of CDS19. The culture was then diluted 5-fold with different concentration of antibiotics or toxic dye (Ethidium Bromide, EB) as desired and continued to

culture for 8 h. The cultured mycelium was centrifuged at 12,000 rpm for 5 min and the precipitates were collected for wet weight measurement. Antibiotics used were: lincomycin, erythromycin, spiromycin, kanamycin, hygromycin, tetracycline, spectinomycin, streptomycin, apramycin, vancomycin, ampicillin, and rifamycin. The RIC_{50} of different antibiotics were derived with Prism 3.0 software (GraphPad Software Company).

[^{3}H]tetracycline uptake assay of strain EAK and 10-22

[^{3}H]tetracycline uptake assay was modified as described [22,23]. Briefly, [^{3}H]tetracycline (PE Company) was diluted into 500 μl methanol to a concentration of 500 μM. About 5×10^8 spores of 10-22 or EAK were inoculated into 20 ml TSB and cultured at 30 °C, 200 rpm for 24 h. The mycelium at exponential-phase was collected by centrifugation at 3000 *g*, washed with 20 ml 50 mM PBS (pH 7.8) twice and resuspended to the same volume in new tubes. After 5 μM [^{3}H]tetracycline was added, 200 μl samples were retrieved at different time intervals of 1, 2, 3, 4, 5, 10, 15, 20, 30, 45, and 60 min. For each time point, samples were transferred into new Eppendorf tubes containing 600 μl 50 mM PBS, pH 7.8 and chilled on ice immediately to stop the uptake. Samples were further collected onto the filter of a plasmid extracting tube (V-gene Biotechnology Ltd.) by centrifugation at 10,000 rpm for 1 min. After washing 2–3 times with 800 μl 50 mM PBS containing 200 mM LiCl, pH 7.8, the filter was dried at 37 °C and the ^{3}H accumulated in mycelium was measured by Wallac1409 scintillation counter (Pharmacia). The total protein concentration of each sample was determined by BCA method [24]. To analyze the [^{3}H]tetracycline accumulation in the presence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, a protonophore that destroys proton motive force), 100 μM CCCP was added when the [^{3}H]tetracycline uptake test went on for 5 min.

Results

The *hmr19* gene encoded a protein belonging to the DHA2 protein family as indicated by sequence homology analysis

A genomic fragment of *S. hygroscopicus* subsp. *yingchengensis* probably related to antibiotics biosynthesis cloned in the shuttle cosmid (pHZ1392 [8]) was sequenced. Twenty-five predicted CDSs in this genomic DNA fragment were annotated (GenBank accession No. AY260760). Among them, CDS19 was predicted to encode a transmembrane protein and was designated *hmr19*. The *hmr19* starts with a GTG codon at nucleotide 26,954 and ends with a TGA stop codon at nucleotide 28,423, resulting in a 489 amino acid polypeptide with a predicted molecular mass of 48,687 Da (GenBank accession No. AY 260761).

Comparison of the deduced amino acid sequence of the *hmr19* encoded protein (Hmr19) with available protein sequences in GenBank employing BlastP revealed significant similarities, extending over the entire sequence, between Hmr19 and several efflux proteins [ALBF (AAL01877), PUR8 (P42670), EncT (AAF81738), AC22 (P46105) and CMCT (Q04733)] conferring drug resistance (Fig. 1). It was particularly interesting that Hmr19 was predicted to contain 14 putative transmembrane domains (Fig. 1), and all seven typical motifs of the DHA2 protein family [7] were identified. These characteristics suggested that *hmr19* likely encoded a probable drug resistance protein belonging to the DHA2 family [4,7].

Expression of *hmr19* in the mycelium of 10-22 was detected by Western blotting analysis

The complete *hmr19* gene cannot be expressed in BL21 (DE3) for unknown reasons (data not shown). As an alternative, the N-terminal 132 amino acid peptide of Hmr19 was expressed as a fusion protein with TRX protein in p32ES (Fig. 2). Using the rabbit antiserum against this fusion protein, a specific band can be detected in the total protein extract of strain 10-22 mycelia cultured without any antibiotics (Fig. 3). Thus, it was revealed that Hmr19 was expressed in the mycelium of 10-22 without any antibiotics induction. The SDS-PAGE determined molecular weight (MW) of Hmr19 expressed in 10-22 is about 35kD as indicated in Fig. 3, which is much lower than the predicted MW of 48 kD.

The *hmr19* gene knockout strain of *S. hygroscopicus* subsp. *yingchengensis* is more susceptible to three antibiotics

In order to study the *in vivo* function of Hmr19, gene knockout strain of *S. hygroscopicus* subsp. *yingchengensis*, EAK, was constructed as in "Materials and Methods". The double-cross gene replacement of *hmr19* gene disruption with *aac(3)IV* gene cassette insertion in the chromosome of strain EAK was verified by Southern analysis (Fig. 4). It was further confirmed by Western blotting analysis that a specific positive band detected in the total protein of the wild type strain 10-22 disappeared in the mutant strain

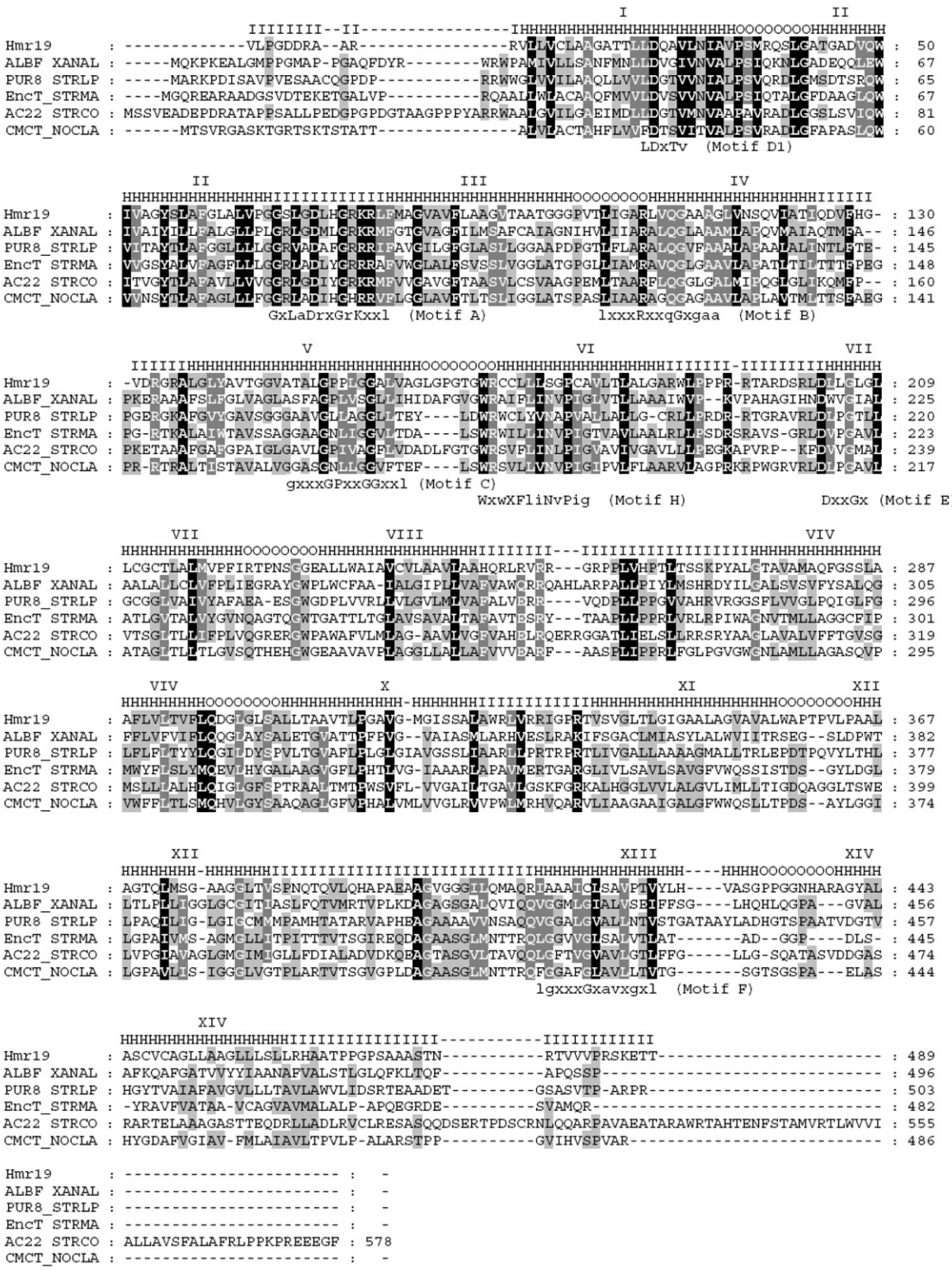

Fig. 1 Multiple-alignment, motif identification and transmembrane helices prediction for Hmr19 and related DHA2 family proteins

Sequences retrieved are described in Materials and Methods. Predicted spatial locations of peptide fragments of the 14 putative transmembrane helix (I–XIV) were indicated with letters above the sequences: "I", for cytosolic fragments; "H", for transmembrane fragments; "O", for extracellular fragments. All 7 motifs (Motif A–F) conserved in DHA2 family were indicated.

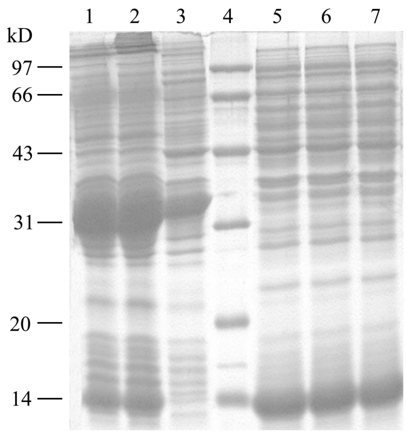

Fig. 2 SDS-PAGE analysis of the recombinant fusion protein containing the N-terminal peptide of Hmr19

Proteins were prepared as described in "Materials and Methods". 1–3, total proteins of *E. coli* transformed with p32ES and induced with IPTG; 5–7, total proteins of *E. coli* before induction; 4, molecular weight marker. The MW of the fusion protein in SDS-PAGE is shown to be about 35 kD.

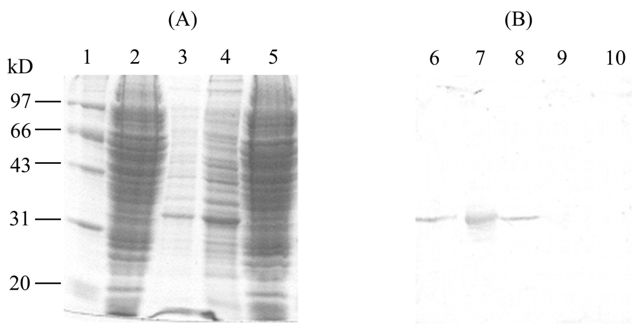

Fig. 3 Western blotting analysis of total proteins extracted from the *S. hygroscopicus* subsp. *yingchengensis* wild type strain 10-22, the *hmr19* gene knockout strain EAK, and the *E. coli* BL21(DE3) expressing the fusion protein of Hmr19 (p32ES)

Panel (A) and (B) were from a parallel SDS-PAGE experiment. (A) Coomassie Brilliant blue G-250 staining result. (B) The gel was transferred to a nylon membrane and detected by Western blotting. Lanes 1 and 10, MW markers; 2 and 9, total protein from the *hmr19* gene knockout strain EAK; 3 and 8, diluted total protein from *E. coli* BL21(DE3) expressing the Hmr19 fusion protein (p32ES); 4 and 7, the same samples as lanes 3 and 8 but with no dilution; 5 and 6, total protein from wild type strain 10-22.

EAK (Fig. 3).

In GIT assay, the *hmr19* gene knockout strain EAK was found to be much more susceptible to tetracycline, vancomycin and mitomycin C than the wild type strain 10-22. For tetracycline, the RIC_{50} decreased about 5-fold from about 5.25 mg/L of 10-22 to less than 1 mg/L of EAK [Fig. 5(A)]. For vancomycin, the RIC_{50} decreased about 20-fold from 4.27 mg/L to 0.2 mg/L [Fig. 5(B)]. For mitomycin C, the RIC_{50} decreased about 10-fold from about 47.8 mg/L to about 4.8 mg/L [Fig. 5(C)]. However, for the negative control streptomycin, the RIC_{50} of both strains were nearly the same at about 15 mg/L [Fig. 5(D)]. The three antibiotics had little similarities in molecular structure, except that they all had a free carboxamide group (data not shown), which was not found in other antibiotics that did not show significant susceptibility differences between the wild type strain 10-22 and the *hmr19* knockout mutant EAK.

[^{3}H]Tetracycline uptake test indicated that the multidrug resistant (MDR) effect of Hmr19 is energy dependent

The antibiotics susceptibility difference between the wild strain and the *hmr19* gene knockout mutant was further confirmed by the [^{3}H]tetracycline accumulation experiment. The time course of [^{3}H]tetracycline accumulation in the *S. hygroscopicus* subsp. *yingchengensis* wild type strain 10-22 and the *hmr19* gene knockout strain EAK were analyzed (Fig. 6). For both strains, tetracycline accumulation reached a nearly steady-state level after approximate 30 min co-incubation. However, the absolute level of steady-state accumulation differed significantly. After 30 min co-incubation, the [^{3}H]tetracycline accumulated in EAK was about 1500 cpm/mg, which was much higher than 630 cpm/mg for strain 10-22.

It has been known that all characterized drug exporters of the MFS superfamily probably function as H^+ antiporter [4,6]. In order to study the effect of membrane energization on the uptake of tetracycline mediated by Hmr19, the energy uncoupler CCCP was tested for its effect against the uptake of [^{3}H]tetracycline. Upon the addition of CCCP, [^{3}H]tetracycline accumulation in 10-22 increased rapidly and reached a higher level that was almost equal to the level observed in the *hmr19* gene knockout strain EAK, while the tetracycline accumulation in EAK was not affected.

Discussion

In this study, we characterized the *hmr19* gene from *S. hygroscopicus* subsp. *yingchengensis* 10-22 encoding a putative transmembrane protein of the DHA2 family. Although the *hmr19* gene was expressed in the bacterial mycelium without antibiotics induction, the phenotype of

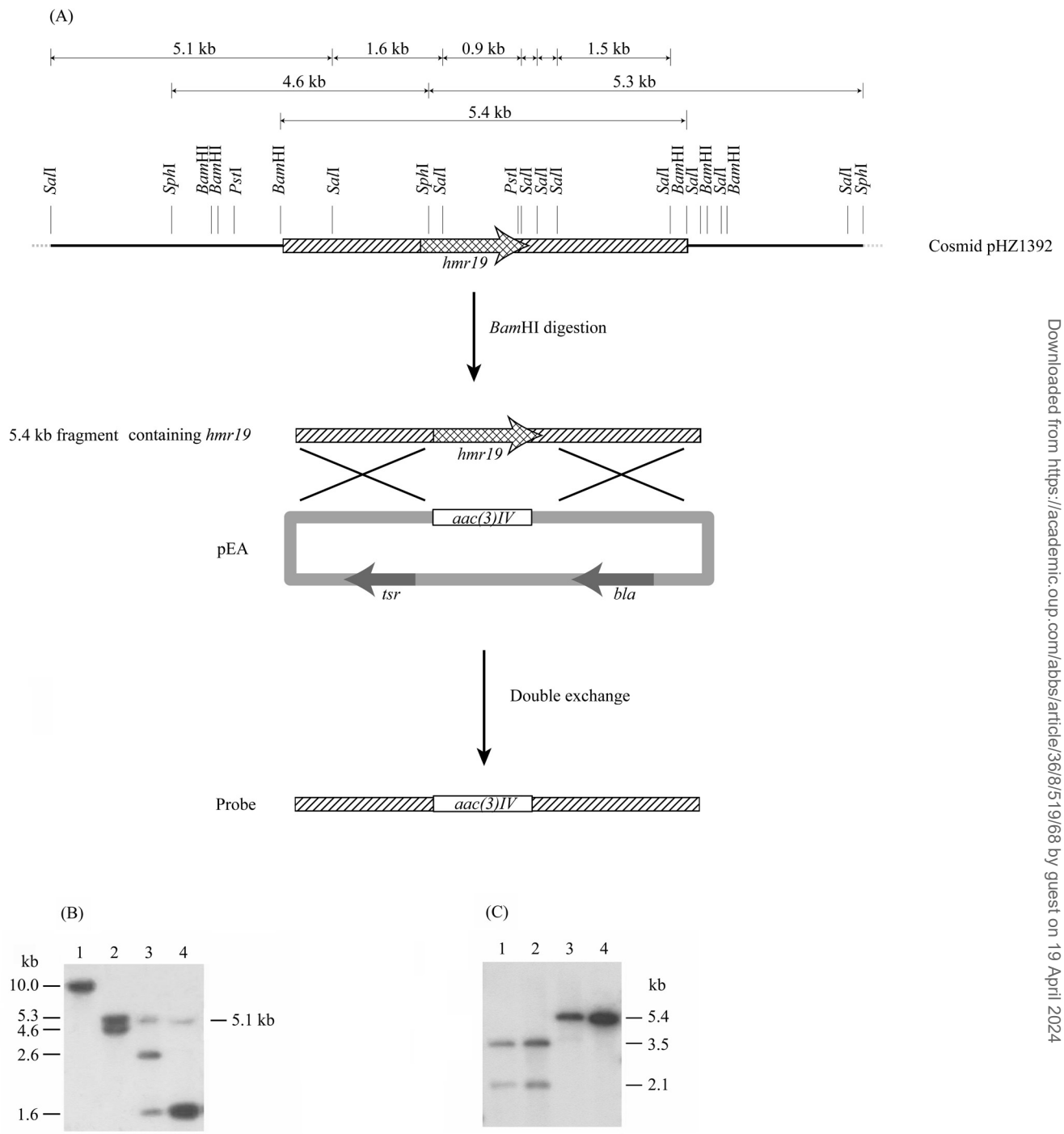

Fig. 4 Southern blotting analysis of the genomic DNA from the *S. hygroscopicus* subsp. *yingchengensis* wild type strain 10-22 and the *hmr19* gene knockout strain EAK

(A) The schematic illustration about the chromosomal *hmr19* gene replaced by an apramycin resistance cassette *aac(3)IV via* double-cross recombination. Change of specific restriction fragments for the *hmr19* gene disruption strain versus the strain 10-22 can be predicted. The 5.4 kb *Bam*HI fragment of the gene replacement construct in plasmid pSP3eff including the apramycin resistance cassette *aac(3)IV* flanked with part of *hmr19* segments was used as the probe for hybridization in (B,C). (B) Southern blotting analysis of EAK and 10-22 genomic DNA digested by *Sph*I and *Sal*I respectively. 1, genomic DNA from EAK digested with *Sph*I; 2, genomic DNA from 10-22 digested with *Sph*I; 3, genomic DNA from EAK digested with *Sal*I; 4, genomic DNA from 10-22 digested with *Sal*I. As expected, the two *Sph*I restriction fragments of 5.3 kb and 4.6 kb in 10-22 (lane 2) disappeared but a new fragment of 10 kb was shown in EAK (lane 1). One of the two 1.6 kb *Sal*I restriction fragments in 10-22 (lane 4) disappeared but a new fragment of 2.6 kb was shown in EAK (lane 3). (C) Southern blotting analysis of EAK and 10-22 genomic DNA digested with *Bam*HI and *Eco*RI. The 5.4 kb (*Bam*HI+*Eco*RI) fragment in 10-22 (lanes 3,4) disappeared but two new fragments of 3.5 kb and 2.1 kb were shown in EAK (lanes 1,2).

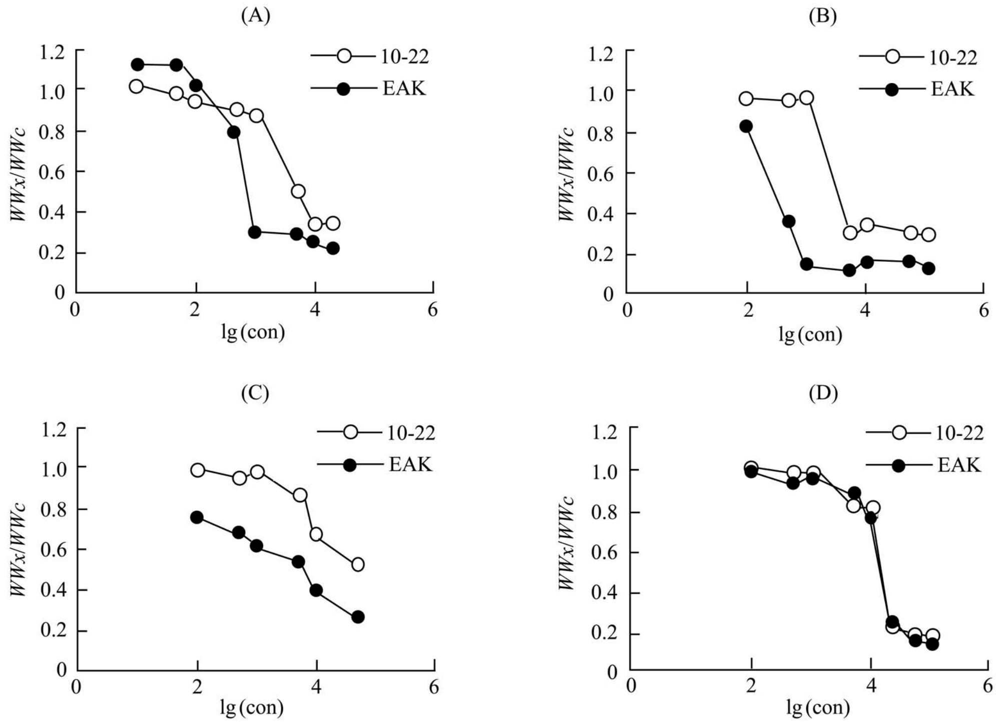

Fig. 5 Growth inhibition test (GIT) of the *S. hygroscopicus* subsp. *yingchengensis* wild type strain 10-22 and the *hmr19* gene knockout strain EAK

(A)–(D) The result of GIT with tetracycline, vancomycin, mitomycin C, and streptomycin (as a negative control), respectively. The concentration of antibiotics (μg/L) used was indicated in logarithmic scale lg (con). The value of *WWx/WWc* was defined in "Materials and Methods".

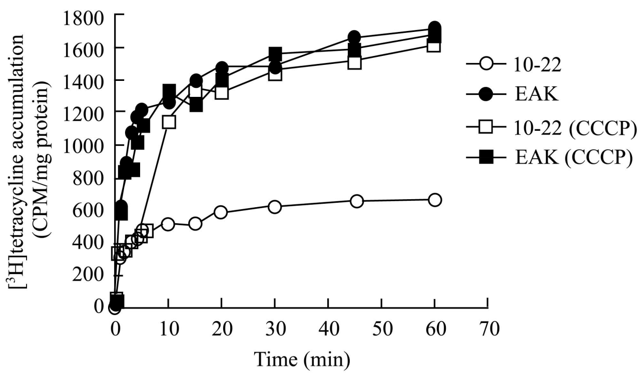

Fig. 6 [³H]Tetracycline uptake test for the *S. hygroscopicus* subsp. *yingchengensis* wild type strain 10-22 and the *hmr19* gene knockout strain EAK

When CCCP was added as an energy uncoupler 5 min after the uptake test started, [³H]tetracycline accumulation in the wild type strain 10-22 turned to be similar to that of the *hmr19* gene knockout strain EAK.

its knockout mutant indicated that the Hmr19 protein offered effective cellular protection against tetracycline, vancomycin and mitomycin C *via* the function of drug transporter, and this excretion of antibiotics from cytoplasm was an energy-dependent process. Thus, the Hmr19 is a member of MDR proteins.

Not all efflux proteins can be well expressed in *E. coli*, though successful examples were reported [25,26]. The most likely reason in failing to express *hmr19* in *E. coli* might be due to its multiple transmembrane structure. It also might lie in the codon usage bias of *E. coli* versus *Streptomyces*. It is significant that codons used commonly in *Streptomyces*, such as CGA for arginine, CGG for arginine, CCC for proline and TCG for serine are the rare codons for *E. coli* [27]. These four codons appeared 3, 12, 10 and 8 times respectively in *hmr19* gene. In addition, the possible toxicity of Hmr19 against *E. coli* might contribute to the failure as well.

The discrepancy between the molecular weight determined by Western blotting and the predicted one based on the amino acid sequence was quite significant (35 kD and 48 kD respectively). Similar phenomena were reported previously [25,26] and interpreted by the possible disproportion between gel mobility and molecular weight of membrane proteins. We did not further analyze the problem in this paper for it is more interesting to under-

stand the function of the protein.

Multidrug efflux pumps were known to be able to pump out a wide range of compounds, and it is often difficult to discern the critical structural feature of the substrates [2,7]. For Hmr19, a carboxamide group was found in the structure of all antibiotics substrates, *i.e.*, tetracycline, vancomycin and mitomycin C, but was not found in other antibiotics that apparently failed to be the substrates of the Hmr19 pump. It thus implied that this carboxamide group might be the common feature of the antibiotic substrates and might be critical in determining the specificity. Further experiments are required to elucidate whether this hypothesis is true or not.

The changes of antibiotic susceptibility were different for the three antibiotics. Resistance declined about 20-fold for vancomycin, 10-fold for mitomycin C while 5-fold for tetracycline in the *hmr19* gene knockout strain EAK. Among others, this might be explained by the fact that the generation of significant levels of resistance usually required synergistic cooperation between the slow influx of the drug crossing the outer membrane barrier and their direct efflux through efflux systems [28]. When *hmr19* gene was disrupted, the extrusion of the substrate antibiotics was stopped. Under this circumstance, the rate of accumulation of antibiotics in the cell solely depends on the rate of influx and thus the accumulation of these antibiotics in EAK will be a good model for testing the influx of these antibiotics through the membrane barrier.

Although Hmr19 was proved to be an MDR protein, there is no evidence correlating this ability to the resistance of *S. hygroscopicus* 10-22 against the three antibiotics (5102-I, -II and –III) produced by itself [8,11]. In fact, all the microorganisms with their complete genomic sequences available do evolve at least one predicted drug efflux system, while the soil or environmental bacteria actually bear the highest number of predicted drug efflux systems and thus, are regarded as the reservoir of drug resistance genes [29]. With this aspect, because streptomycetes are among the most important soil organisms for its production of various secondary metabolites broadly used in industry and for human health, it is especially important to understand their efflux systems.

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