

Bacillus subtilis from Soybean Food Shows Antimicrobial Activity for Multidrug-Resistant *Acinetobacter baumannii* by Affecting the *adeS* Gene^S

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Received: July 4, 2016
Revised: August 16, 2016
Accepted: August 24, 2016

First published online
September 2, 2016

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Supplementary data for this paper are available on-line only at <http://jmb.or.kr>.

pISSN 1017-7825, eISSN 1738-8872

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Exploring novel antibiotics is necessary for multidrug-resistant pathogenic bacteria. Because the probiotics in soybean food have antimicrobial activities, we investigated their effects on multidrug-resistant *Acinetobacter baumannii*. Nineteen multidrug-resistant *A. baumannii* strains were clinically isolated as an experimental group and 11 multidrug-sensitive strains as controls. The growth rates of all bacteria were determined by using the analysis for xCELLigence Real-Time Cell. The combination of antibiotics showed synergistic effects on the strains in the control group but no effect on the strains in the experimental group. Efflux pump gene *adeS* was absent in all the strains from the control group, whereas it exists in all the strains from the experimental group. Furthermore, all the strains lost multidrug resistance when an *adeS* inhibitor was used. One strain of probiotics isolated from soybean food showed high antimicrobial activity for multidrug-resistant *A. baumannii*. The isolated strain belongs to *Bacillus subtilis* according to 16S RNA analysis. Furthermore, *E. coli* showed multidrug resistance when it was transformed with the *adeS* gene from *A. baumannii* whereas the resistant bacteria could be inhibited completely by isolated *Bacillus subtilis*. Thus, probiotics from soybean food provide potential antibiotics against multidrug-resistant pathogenic bacteria.

Keywords: *Acinetobacter baumannii*, *adeS* efflux pump, probiotics, soy curd, antibiotics, multidrug resistance

Introduction

Acinetobacter baumannii is an opportunistic bacterial pathogen primarily associated with hospital-acquired infections [16], and highly antibiotic-resistant clinical *A. baumannii* has become increasingly prevalent [26, 30]. Furthermore, clinical isolates of *Acinetobacter* genus have been found to have multidrug-resistant (MDR) properties [9]. *A. baumannii* often causes various infectious diseases, such as bacteremia [13], urinary tract infection [35], and surgical wound infection [8]. The patients infected with MDR *A. baumannii* will have a higher rate of mortality and a longer hospital stay [33]. However, in recent years, a limited number of antibiotics is available for treating the infections caused by MDR strains. Therefore, it is necessary to explore novel

antimicrobial agents or treatment strategies for treating *A. baumannii* infection.

For antibiotic-resistant *A. baumannii*, a combination therapy is often considered [7]. For example, minocycline-based combination therapy has been suggested to be a possible choice for the treatment of infections caused by *A. baumannii*. However, the usage of combination therapy for infections caused by *A. baumannii* has not been well established yet [39]. Furthermore, a combination therapy is often used for drug-resistant bacteria but may produce contradictory effects on different strains. Thus, it is critical to understand the mechanism behind the antibiotic-resistant properties of *A. baumannii*. The medicine used against *A. baumannii* often leads to widespread antibiotic resistance, including imipenem [6], gentamicin [32], ciprofloxacin [2],

ampicillin/sulbactam [5], levofloxacin [36], and tobramycin [31]. To explore the molecular mechanisms of the multidrug resistance in *A. baumannii*, we investigated the resistance-related genes in the bacterium: β -lactamase gene (*bla*_{IMP}, *bla*_{VIM}, *bla*_{GIM}, *bla*_{SHV}, *bla*_{SIM}, *bla*_{CTX-M}, *oxa-23*) [4], fluoroquinolone-resistant genes (*qnrA*, *gyrA*, *parC*) [34], aminoglycoside modifying enzyme gene (*aphA6*) [37], insertion sequence (*ISAbal*) [21], integron (*Intl 15'*, *Intl 13'*) [15], and efflux pump genes (*adeB*, *adeR*, *adeS*) [25]. Expression of different types of metallo-beta-lactamases has been associated with carbapenem resistance [30]. Previous study showed that triple mutations in clinical isolates of *A. baumannii* contribute to fluoroquinolone resistance. The mutations in *parC* S80L or E84K (groups II and VII) have been supposed to contribute to alterations in efflux pump activity in the strains [34]. Horizontal gene transfer is an important reason for disseminating aminoglycoside resistance in *A. baumannii* [3]. The expression of *bla*_{OXA-95} was demonstrated to cause resistance to imipenem. The transposition of *ISAbal* upstream of the *bla*_{OXA-95} gene increases the high-level expression of *bla*_{OXA-95}, resulting in the resistance to imipenem in the strain [21]. Integrons are also linked with multiple-drug resistance of *A. baumannii*, and class I integron isolates bearing *AadA2-HP-dfrA* have been found to be prevalent in China hospitals [17]. Multidrug efflux transporters play an important role in the bacterial resistance [28].

Based on the molecular mechanisms, a new strategy is also needed to eliminate the antibiotic-resistant activity of *A. baumannii* [12]. It is necessary to explore novel safe antibiotics for MDR pathogenic bacteria. Some clinical studies showed that probiotics prevented hospital-acquired infections in patients. The protective effect of probiotics may be caused by preventing MDR-pathogen-induced immunosuppression [29]. Probiotics are safe and widely existed in soybean food [10]. Thus, the antibiotics of the probiotics in soybean food may be safe and effective to control MDR pathogenic bacteria.

Materials and Methods

Strains

A total of 30 strains of *A. baumannii* were isolated from patients with sepsis or osteomyelitis at the Clinical Laboratory Center of Beijing Friendship Hospital, Capital Medical University, from May 2011 to June 2012. The minimum inhibitory concentration (MIC) of all antibiotics was determined by broth microdilution. A total of 19 MDR strains of *A. baumannii* were isolated as the experimental group and 11 multidrug-sensitive strains of *A. baumannii* were isolated as the control group.

Preparation of Bacterial Inoculum

All the bacteria were cultured in nutrient agar at 37°C for 18 h. Isolated colonies were further subcultured on an agar slant at 37°C for 24 h. The bacteria suspension was adjusted to an OD value of 0.1 at 600 nm, which is equivalent to a concentration of 10⁶ CFU/ml.

Isolation of Probiotics from Soy Curd

Soy curd was purchased from a local supermarket and ground into pieces, and the mixture supernatants with probiotics were obtained by centrifugation at 70 ×g for 10 min. The supernatants were further diluted by 1,000-fold with sterile water. A 50 μl diluted mixture was plated on an agar plate with LB medium and cultured at 37°C overnight. A single colony was picked up and cultured with LB liquid medium at the same condition for 10 h. The supernatants were collected by centrifugation at 15,000 ×g for 10 min. The effects of probiotics on MDR pathogenic bacteria were measured by using the following steps. For the probiotics with high inhibitory activity against pathogenic bacteria, the species was identified with 16S RNA analysis.

Determination of Minimal Inhibitory Concentration

The antibiotic tests were conducted in accordance with the Guidelines of the Clinical & Laboratory Standards Institute. The MIC values of each antibiotic against *A. baumannii* were determined by using a two-fold serial dilution method. The tested antibiotics were pipetted in a sterile 96-well plate. The bacteria inoculum in MH broth was used as a positive control whereas the antibiotic in MH broth was used as a negative control. All samples were cultured in 96-well plates at 37°C for 24 h. The MIC was defined as the lowest concentration of an antibiotic if OD values were no more than 0.1 after 24 h culture. Each test was carried out in triplicates.

Determination of Fractional Inhibitory Concentration (FIC)

The concentrations of the two antibiotics were designed in accordance with a checkerboard method. Imipenem is an antibiotic that is widely used for treating many bacterial infections [23, 27], but imipenem-resistant pathogens have been reported by a number of literature [1, 14, 19, 38]. *A. baumannii* is difficult to eradicate and the combination therapy of imipenem and other antibiotics shows therapeutic results for controlling the pathogen [18]. Thus, we used combinations that are based on imipenem. Five antibiotic combinations were tested: imipenem + gentamicin, imipenem + ciprofloxacin, imipenem + ampicillin/sulbactam, imipenem + levofloxacin, and imipenem + tobramycin. A checkerboard broth dilution method was used to determine the MIC values, and the FIC index was calculated. The combined effects of imipenem with antibiotics on all *A. baumannii* strains were evaluated based on the FIC index values for each combination. The combination of imipenem and other antibiotics significantly inhibited bacterial growth. The FICI value was

calculated as FIC index = FIC of imipenem + FIC of other antibiotics, where FIC of imipenem = MIC of combination antibiotics with imipenem/MIC of imipenem alone; FIC of other antibiotics = MIC of combination antibiotics/each antibiotic alone. $FIC \leq 0.5$ indicates a synergistic effect, $0.5 < FIC \leq 1$ indicates an additive effect, $1 < FIC \leq 2$ means an independent effect, and $FIC > 2$ indicates an antagonistic effect.

AdeS Efflux Pump Inhibitor

Antibiotic susceptibility was analyzed with 1 mM MgSO₄ containing 0, 10, 25, or 50 µg/ml phenylalanine-arginine beta-naphthylamide (PaβN, an AdeS efflux pump inhibitor) and all bacteria were grown to logarithmic phase at 37°C for 24 h. All MICs were repeatedly measured three times.

Time-Kill Study

If combination antibiotics showed synergistic or antagonistic results, a time-kill curve was plotted to verify the results, and then the resistant factors were determined in corresponding strains by using a gene inhibitor. The growth rates of all bacteria were determined by using an xCELLigence-system (Roche Applied Science, USA) in a 96-cell plate.

Detection of Resistance Genes

The following MDR-related genes were amplified by PCR: β-lactamase gene (*bla*_{IMP}, *bla*_{VIM}, *bla*_{GIM}, *bla*_{SHV}, *bla*_{SIM}, *bla*_{CTX-M}, *oxa*-23), fluoroquinolone-resistant genes (*qnrA*, *gyrA*, *parC*), aminoglycoside modifying enzyme gene (*aphA6*), insertion sequence (*ISAbal*), integron (*IntI 15'*, *IntI 13'*), and efflux pump genes (*adeB*, *adeR*, *adeS*). Primers used are shown in Table S3.

Expression of *adeS* of *A. baumannii* in *E. coli*

adeS from *A. baumannii* was amplified from its genomic DNA as a 1,083-bp product and cloned into the NdeI and XhoI sites with a C-terminal 6×His tag on the vector. The constructed vectors were transformed into BL21 (DE3) *E. coli*. The bacteria were cultured overnight in LB medium with 100 µg/ml ampicillin at 37°C with 220 rpm shaking. Then, 50 µl start cultures were added to 5 ml of LB and grown at 37°C until OD_{600nm} reached at OD_{600nm} 0.6 and 0.1 mM IPTG was added and incubated further for 5 h. The protein was purified with a Ni-NTA purification system and verified by SDS-PAGE.

Determination of Polymyxin B Minimum Inhibitory Concentration

Overnight cultures were diluted 100-fold in fresh LB medium and grown until OD_{600nm} reached 0.5. Table S1 indicates the MIC ranges of three biological repetitions.

Antibiotics Growth Assay

Antibiotics growth assay was used to evaluate the function of AdeS. A single colony was added to 2 ml of LB with 100 µg/ml ampicillin and cultured overnight at 37°C in the presence or absence of different antibiotics and/or with different recombinations.

The cell density was measured at 600 nm by using a microplate reader (Thermo Scientific, USA).

Statistical Analysis

The data between the experimental group and the control group were analyzed by Student's *t* test (*t*) via IBM SPSS statistics base 20 (SPSS Inc, USA). There were significantly statistical differences if $p < 0.05$.

Results

MIC Determination

The MIC₅₀ and MIC₉₀ of the antimicrobial agents against the stains are shown in Table S1. In the experimental group, the strains were insensitive to most of the antibiotics with low values of MIC₅₀ and MIC₉₀, except for gentamicin and tobramycin with high values of MIC₉₀. In contrast, most strains were sensitive to those antibiotics in the control group.

FIC Determination

The FIC distribution of combination antibiotics against the tested stains was detected by a checkerboard broth microdilution method and results are shown in Table S2. The combination among minocycline, polymyxin, rifampicin, and moxifloxacin showed synergistic effects on most strains from the control and experimental groups with FIC values less than 0.5. On the other hand, most combinations of antibiotics showed synergistic effects on these strains in the control group.

Resistance Gene Identification

Using the primers in Table S3, MDR genes were identified by comparing the resistance of three strains A, B, and C from the control group and three strains A', B', and C' from the MDR group (Table 1). A total of 17 potential genes were examined, and most genes existed or were absent in both. The *adeS* gene specifically existed in all the strains from the MDR group and was absent in all the strains from the control group. The results suggest *adeS* may be an important gene for the multidrug resistance in *A. baumannii*.

Time-Kill Analysis

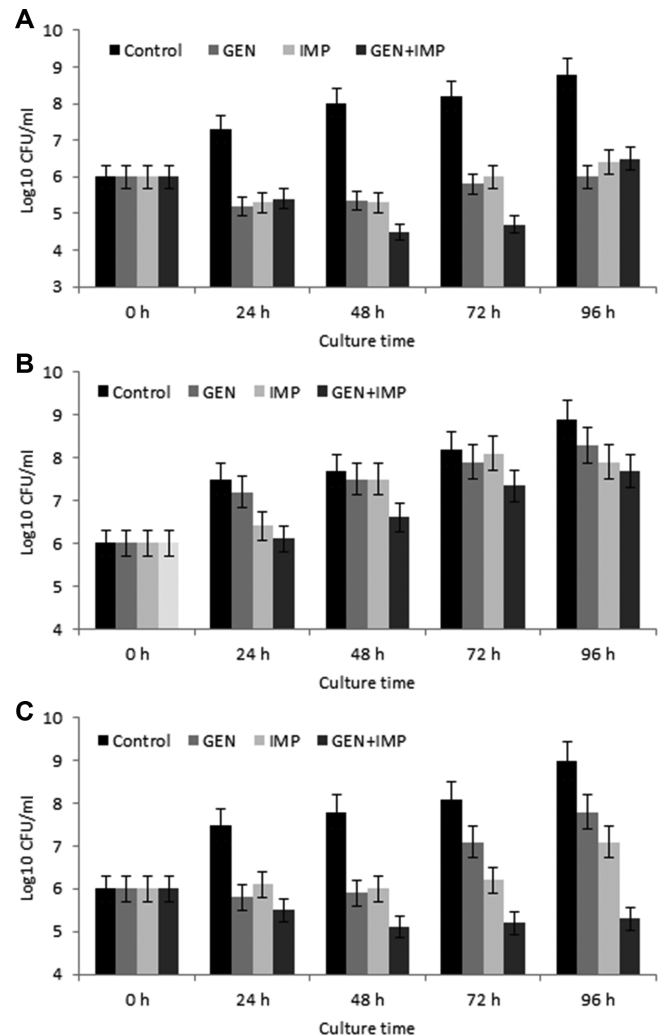
The time-kill curves are shown in Fig. 1. The combination antibiotics that showed synergistic effects were subjected to a time-kill assay. The combination of imipenem and gentamicin showed synergistic effects. Fig. 1A shows the combination of imipenem and gentamicin against the *A. baumannii* strains from the control group. Imipenem, gentamicin, and the combination of imipenem and

Table 1. Resistant genes identified by PCR.

		MSG			MRG		
		A	B	C	A'	B'	C'
β-Lactamase gene	<i>bla_{IMP}</i>	-	-	-	+	+	-
	<i>bla_{VIM}</i>	-	-	-	-	+	+
	<i>bla_{GIM}</i>	-	-	-	-	+	-
	<i>bla_{SHV}</i>	-	-	-	+	-	+
	<i>bla_{SIM}</i>	+	-	+	+	-	-
	<i>bla_{CTX-M}</i>	-	-	-	-	+	+
	<i>oxa-23</i>	-	-	-	+	+	-
Integron	<i>Int1 15'</i>	+	+	+	+	+	+
	<i>Int1 13'</i>	-	-	-	-	-	-
Efflux pump genes	<i>adeB</i>	-	-	-	-	-	-
	<i>adeR</i>	+	+	+	+	+	+
	<i>adeS</i>	-	-	-	+	+	+
Fluoroquinolone-resistant genes	<i>gyrA</i>	+	+	+	+	+	+
	<i>parC</i>	+	+	+	+	+	+
	<i>qnrA1-A6</i>	-	-	-	-	-	-
Aminoglycoside-resistant genes	<i>aphA6</i>	-	-	-	-	-	-
Insertion sequence	<i>ISAb1</i>	+	+	+	+	+	+

There are three strains from the multidrug-sensitive group (MSG) and three strains from the multidrug-resistant group (MRG), which are marked as A, B, C, and A', B', and C', respectively.

gentamicin all exhibited the reduction of 1 log₁₀ CFU/ml at 3 h, which was then followed by the reduction of 0 and 2 log₁₀ CFU/ml at 6 h. Fig. 1B shows that bacteriostatic action was observed with imipenem, gentamicin, and combination imipenem and gentamicin against *A. baumannii* strains from the experimental group. Only gentamicin showed the reduction of 0.1 log₁₀ CFU/ml at 3 h, which was then followed by the increase of 1 log₁₀ CFU/ml at 6 h. At 24 h, there was an increase of 1.5 log₁₀ CFU/ml when compared with the value at 0 h. There was no significantly statistical difference for the values between imipenem, or combination and controls without antibiotics ($p > 0.05$). There were significantly statistical differences for gentamicin, imipenem, and combination antibiotics from 3 to 24 h ($p < 0.05$). From Fig. 1C, bacteriostatic action was observed for imipenem, gentamicin, and combination imipenem and gentamicin against the *A. baumannii* strains in the experimental group treated with PaβN, an AdeS efflux pump inhibitor. Imipenem, gentamicin, and combination imipenem and gentamicin showed the reduction of 0.3, 0.5, and 1 log₁₀ CFU/ml at 3 h, which was then followed by the reduction of 0.5, 0.4, and 0.6 log₁₀ CFU/ml at 6 h. The results suggest an AdeS efflux

**Fig. 1.** Time-kill curve of imipenem + gentamicin against strains from control and experimental groups.

(A) The strains from the control group without multidrug resistance. (B) The strains from the experimental group with multidrug resistance. (C) The strains from the experimental group with multidrug resistance and treated with AdeS efflux pump inhibitor phenylalanine-arginine beta-naphthylamide. The error bars denote standard deviation.

pump correlates with multidrug resistance in *A. baumannii*, which can be reversed by an AdeS efflux pump inhibitor, PaβN.

Bacillus subtilis sp. m3 in Soy Curd Showed High Antibacterial Activity against *A. baumannii*

After a series of selection, 88 kinds of bacteria were isolated from soy curd by series dilution. Among the 88 samples, one strain showed high antibacterial activity for MDR *A. baumannii* according to MIC measurement. The strain has a 98% identical 16S RNA sequence with the

species *Bacillus subtilis* (GenBank No. LT546428.1) and was named as *Bacillus subtilis* sp. m3. The OD_{600nm} of *A. baumannii*

was still less than 0.1 after culture at 37°C for 24 h when 0.1 µl of supernatant of the *Bacillus* culture (OD_{600nm} = 2.6) was added to 1 ml LB medium with MDR *A. baumannii*.

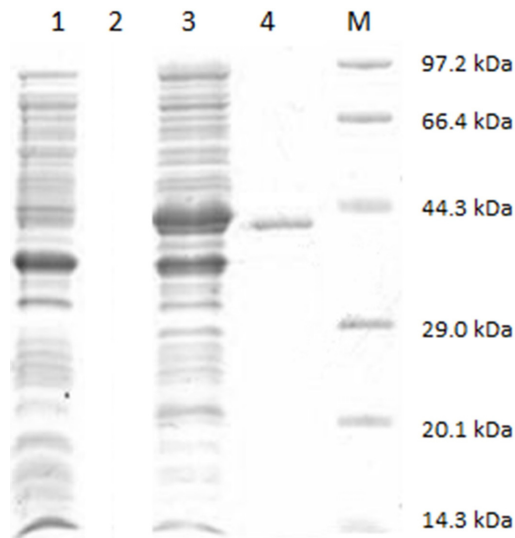


Fig. 2. SDS-PAGE analysis of *adeS* expression in *E. coli*. Lanes 1 and 2, the proteins were expressed in *E. coli* with empty vectors (pET21) and purified. No target protein can be found after Ni-NTA purification. Lanes 3 and 4, the proteins were expressed in *E. coli* with reconstructed vector pET21-*adeS* and purified. Target protein can be found after Ni-NTA purification.

Expression of *adeS* of *A. baumannii* in *E. coli*

The *adeS* gene of *A. baumannii* was successfully expressed in *E. coli* when it was transformed with the vector pET21a-*adeS* (Fig. 2). The protein was easily purified with the Ni-NTA system.

Effects of Recombined *adeS* on the Growth of *E. coli* Treated with Different Antibiotics

The engineered *E. coli* transformed with pET21a-*adeS* showed different resistance for different antibiotics and or combinations (Fig. 3). All the antibiotics could inhibit the growth of wild *E. coli* (Fig. 3A) whereas the *E. coli* transformed with empty vector pET21a only showed resistance for ampicillin/sulbactam (Fig. 3B). The engineered *E. coli* transformed with pET21a-*adeS* showed strong resistance to most antibiotics (Fig. 3C).

Bacillus subtilis* sp. m3 Showed High Antimicrobial Activity for the *E. coli* Transformed with *adeS

Bacillus subtilis sp. m3 showed high antibacterial activity

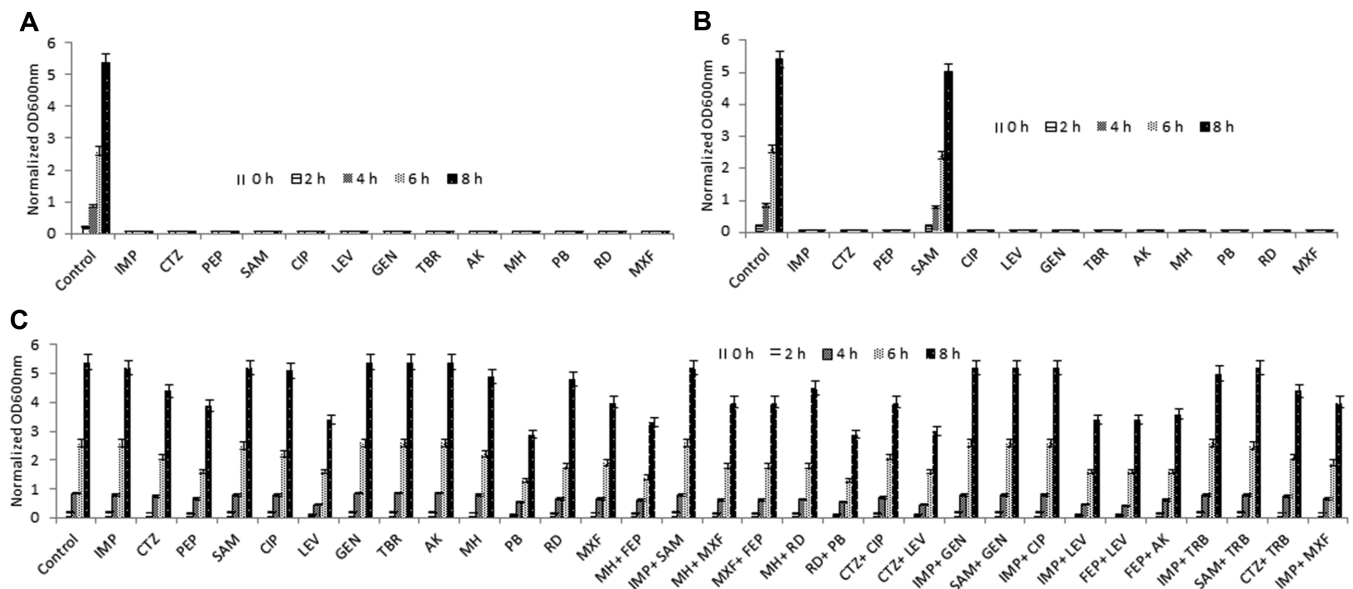


Fig. 3. The effects of *adeS* on *E. coli* for antibiotics resistance. (A) The effects of different antibiotics on *E. coli*. (B) The effects of different antibiotics on *E. coli* transformed with empty vector pET21a. (C) The effects of different antibiotics and/or with different combinations on the growth of *E. coli* transformed with reconstructed vector pET21a-*adeS*. IMP = imipenem; CTZ = ceftazidime; FEP = cefepime; SAM = ampicillin/sulbactam; CIP = ciprofloxacin; LEV = levofloxacin; GEN = gentamicin; TBR = tobramycin; AK = amikacin; MH = minocycline; PB = polymyxin B; RD = rifampicin; MXF = moxifloxacin. FIC ≤ 0.5 indicates a synergistic effect, 0.5 < FIC ≤ 1 indicates an additive effect, 1 < FIC ≤ 2 means an independent effect, and FIC > 2 indicates an antagonistic effect. All assays were performed in triplicates for each antibiotic or recombination. The data were presented as mean values ± SD.

for MDR *A. baumannii*. The OD_{600nm} of *A. baumannii* was still less than 0.1 after cultured at 37°C for 24 h when 0.1 μ l or 0.5 μ l of supernatant of the *Bacillus* culture ($OD_{600nm} = 2.6$) was added to 1 ml of LB medium with *A. baumannii*.

No drug-sensitive strain had the efflux pump gene *adeS* whereas all drug-resistant strains had *adeS*. To identify the role of AdeS in multidrug resistance of *A. baumannii*, the AdeS inhibitor, PA β N, was used, which can permeabilize the outer membrane of gram-negative bacteria [22]. Just as we expected, when the inhibitor was used, the multidrug resistance was lost in all the bacteria tested. To further confirm the results, *E. coli* was transformed with *adeS*. The engineered *E. coli* showed resistance for most antibiotics (Fig. 3). All the results suggested that AdeS plays an important role in multidrug resistance. Our results indicated that certain types of antibiotics may activate *adeS* in MDR *A. baumannii*, which in turn causes the multiple-composition efflux pump genes to overexpress the proteins (Fig. 4). Although other mechanisms still existed (Fig. 4), the efflux pump gene *adeS* may play an important role in the antibiotic resistance.

Discussion

Soy is a rich source of microorganisms, which can produce many antibiotics [24]. The *Bacillus* species are the

main microorganisms in soy curd and produce various antibiotic compounds, including polymyxin [20] and circlin [11]. On the other hand, with the increase of MDR pathogenic bacteria, it is necessary to explore novel antimicrobial agents with few side effects and high activity. Therefore, the antimicrobial probiotics in food soy curd for pathogenic bacteria were investigated here. Among all 88 samples isolated from soy curd, one strain showed high inhibitory activity against *A. baumannii*. After 16S RNA analysis, the strain was found to belong to *Bacillus* species. The isolated *Bacillus* species showed similar inhibitory functions for MDR *E. coli* transformed with the *adeS* gene of *A. baumannii*. These results further confirmed the inhibitory functions of *Bacillus* species from soybean for pathogenic bacteria.

Temperature is an important factor affecting the growth and secondary metabolites of *Bacillus* species. The target strain still showed good thermal stability and still had more than 80% total inhibitory activity at 40°C after 2-day culture. The results suggested that the isolated strain may be a thermophilic bacterium. pH is another factor affecting the growth of metabolites of *Bacillus* species. The results indicated that the strain had more than 90% total activity at pH ranging from 4 to 9 after 2 day culture. All these results will be beneficial to keep the long-time half-life for the antibiotics of probiotics in soy curd.

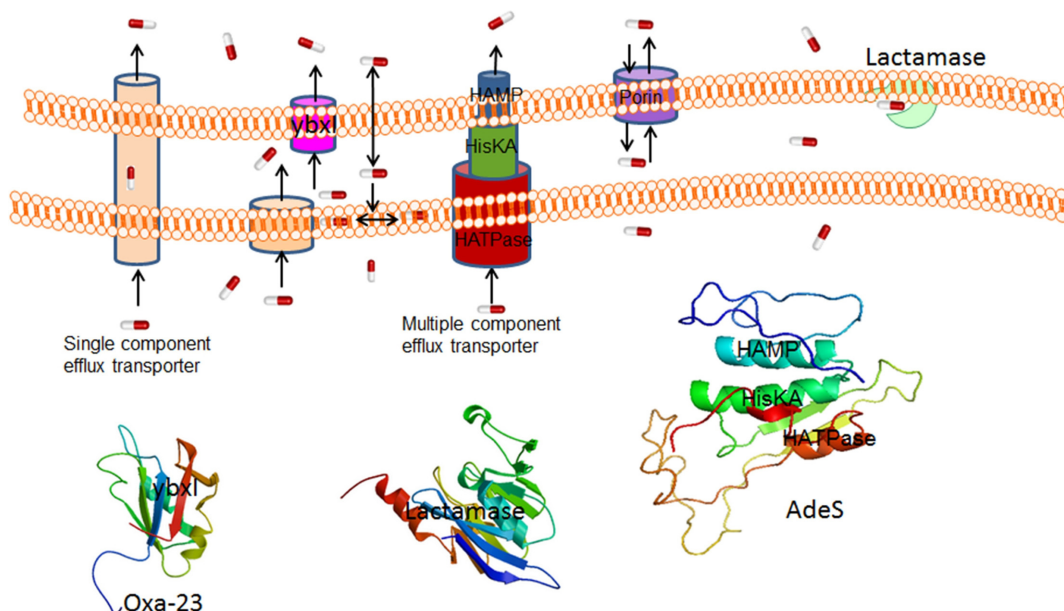


Fig. 4. The strategies for *A. baumannii* developing resistance for antibiotics.

Bacterial resistance to different antibiotics can be achieved by many different ways: producing lactamases, preventing antibiotics from binding cell wall; reducing transport of antibiotics into the periplasmic space via the changes in porins; using the mechanisms for exclusion of antibiotics via a single-composition efflux pump; and using the mechanisms for exclusion of antibiotics via a multiple-composition efflux pump.

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