

Identification of lysocin E using a silkworm model of bacterial infection

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Summary New antimicrobials with novel mechanisms need to be developed to combat antimicrobial-resistant pathogenic bacteria. The current authors recently reported discovery of a new antibiotic named "Lysocin E". Lysocin E was identified using a silkworm model of bacterial infection. The current review discusses the advantages of using a silkworm model of bacterial infection to identify and develop therapeutically efficacious antimicrobials. This review also discusses the discovery of lysocin E and its novel mechanism of action.

Keywords: Antimicrobial, lysocin E, silkworm, multidrug-resistant pathogens

1. The antimicrobial crisis caused by the spread of multidrug-resistant pathogens

Due to aging of the population, industrialized nations are now seeing an increase in patients dying due to infectious diseases. Pneumonia is one such an infectious disease, and pneumonia was the 3rd leading cause of death in Japan in 2011. In addition, the spread of multidrug-resistant strains is a serious clinical problem. Recently, the WHO issued a warning that the worldwide spread of multidrug-resistant strains will cause a decrease in clinically useful antibiotics (1). New antibiotics with novel mechanisms must be developed to overcome these problems, but far fewer antibacterials have emerged over the past few years than in previous decades. Due to the heightened sense of crisis, governments in the US and Europe have given incentives to pharmaceutical companies to develop new antimicrobials.

2. General problems encountered when developing antimicrobials

One bottleneck in the development of antimicrobials is the decrease in the hit rate of therapeutically efficacious

compounds. Thus far, secondary metabolites produced by bacteria in soil have been used to search for drugs, and many antimicrobials have been identified and used clinically in humans. Natural products are still an attractive source in comparison to chemically synthesized compounds because these secondary metabolites are diverse and easier to develop antibiotics from. Indeed, most antimicrobials in clinical use were derived from natural products. If one wishes to develop a new antibiotic, it should at least have a structure unlike that of existing antibiotics since it must be patented to recoup investment. In addition, researchers have been less prone to screen natural products for new compounds because of the vast numbers of attempts to screen those products. Moreover, most compounds that are identified by simply screening for antimicrobial activity usually display poor pharmacokinetics and toxicity to the host. Thus, new antibiotics need to be screened for therapeutic activity in the early stages of drug development. A silkworm model of bacterial infection can indeed facilitate drug development.

3. A silkworm model of bacterial infection for drug development

Kaito *et al.* found that silkworms were killed by injecting hemolymph (the blood of silkworms) with bacteria that are pathogenic to humans such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* (2). The current authors used that silkworm model to test the therapeutic efficacy of antibiotics in clinical use. Antibiotics were used to treat silkworms infected with *S. aureus* and their therapeutic efficacy was quantitatively

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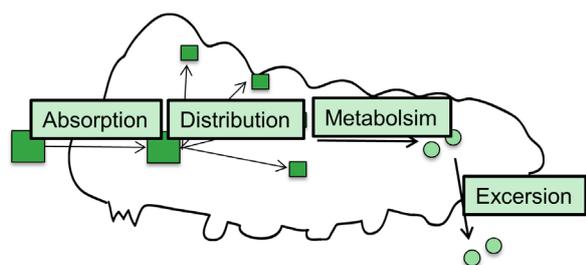


Figure 1. Pharmacokinetics in silkworms. Pharmacokinetics of drug absorption, distribution, metabolism, and excretion in silkworms are similar to pharmacokinetics in mammals.

Table 1. Therapeutic efficacy of antibiotics in silkworms infected with *S. aureus*

Drugs	ED ₅₀ (mg/kg·animal)	
	Silkworm	Mouse
Teicoplanin	0.3	0.1
Vancomycin	0.3	1
Minocycline	4	1
Flomoxef	0.2	0.3
Linezolid	9	4
Katanosin B	0.1	0.7

The ED₅₀ in the silkworm model and mouse model were similar. This suggests that the therapeutic efficacy of antibiotics can be evaluated using a silkworm model of bacterial infection.

evaluated by calculating the effective dose that produces an effect in half of the animal population taking the substance in question (ED₅₀). The ED₅₀ per body weight in the silkworm model was highly consistent with the ED₅₀ per body weight in a mammalian model (Table 1) (3). Moreover, this correspondence revealed pharmacokinetics in silkworms and mammals were similar. Pharmacokinetics consists of four factors: drug Absorption, Distribution, Metabolism, and Excretion (ADME) (Figure 1). These factors were present in the silkworm model as described below.

Absorption A previous study by the current authors suggested that absorption of small compounds by the mid-gut (the intestine of a silkworm) was affected by the molecular weight and hydrophobicity of compounds consistent to absorption in mammals (4). For example, vancomycin cannot be absorbed by the human intestine due to its high molecular weight and low hydrophobicity, and vancomycin similarly displayed no therapeutic efficacy when orally administered to silkworms.

Distribution and Excretion Pharmacokinetic parameters such as distribution and total clearance of several antibiotics in silkworms were similar to the same parameters in mammals (unpublished results).

Metabolism The current authors reported that silkworms have a cytochrome P450 reaction and a conjugation reaction (5). When 7-ethoxycoumarin was injected into hemolymph, this compound was metabolized into 7-hydroxycoumarin as a result of the

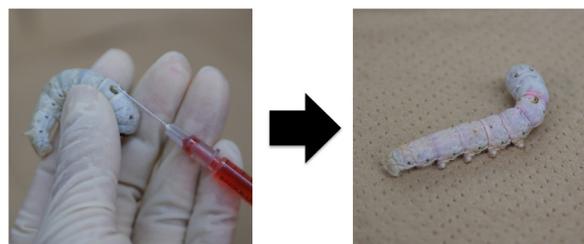


Figure 2. Injection of a sample into silkworm hemolymph. A silkworm is large enough to be handled by hand and the injection volume can be readily controlled because of their slow movement. When red dye was injected into hemolymph (left panel), the dye spread throughout the body (right panel).

cytochrome P450 reaction and was then transformed into a sugar conjugate form, as well as it is in mammals.

Besides these pharmacokinetic factors, the toxicity of a compound also affects the therapeutic efficacy of antibiotics. In a silkworm model, the lethal doses of cytotoxic compounds were highly correlated with that in rat model, suggesting that silkworms are a suitable model for evaluation of the acute phase toxicity of cytotoxic compounds (5,6). These results suggest that therapeutic effect of antibiotics on silkworm infection model is reflected by pharmacokinetics and cytotoxic effect, which mimics those in mammals (7). In other words, a silkworm model allows the evaluation of samples that may potentially become drugs.

4. Using a silkworm model of bacterial infection to screen new antibiotics for their therapeutic efficacy

Silkworms are inexpensive and present no ethical issues because they have been used in sericulture for over 4,000 years. In addition, silkworms are large enough to handle by hand and their slow movement allows quantitative injection (Figure 2). These features allow silkworms to be used to screen new antibiotics for their therapeutic efficacy. Choosing what to screen is important when screening for novel compounds. The current authors focused on natural products from bacteria supernatants. In general, re-isolation of same antibiotics from natural products by conventional methods, it tends to have been already saturated, however, we thought it would be possible to obtain novel therapeutically effective antibiotics if we screen by therapeutic effectiveness against silkworm model and used an own isolated library. Thus, a silkworm model of bacterial infection was used to screen the supernatant of bacteria in soil for therapeutic efficacy (Figure 3). In total, 14,651 strains of bacteria were collected from various regions of Japan and 2,794 supernatants displayed antibacterial activity against MRSA *in vitro*. These antibacterial supernatants were tested in a silkworm model of *S. aureus* infection, and ultimately 23 samples were found to display therapeutic efficacy. One of the soil bacteria produced "Lysocin E" (8).

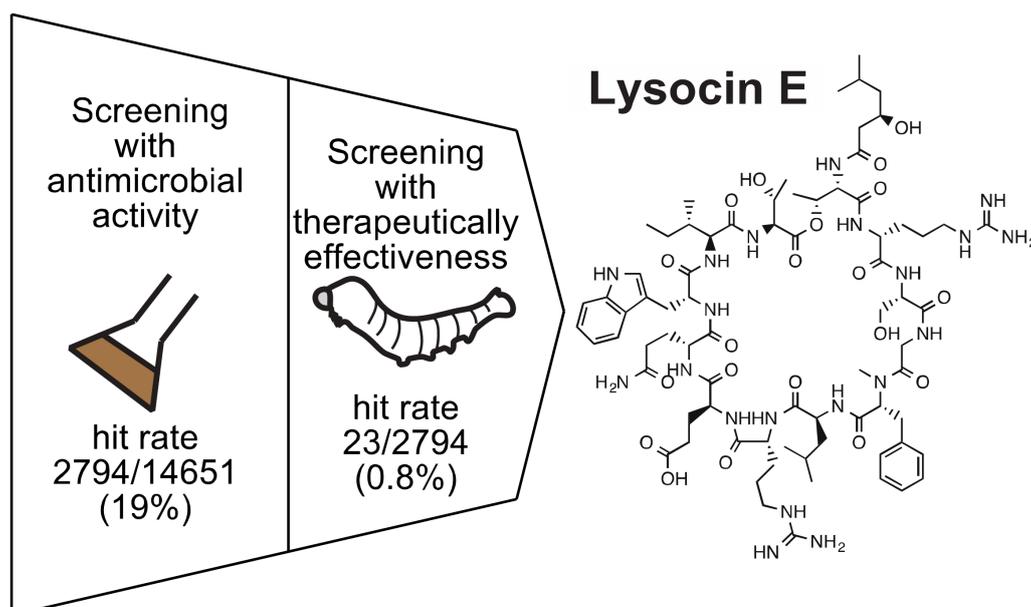


Figure 3. Strategy to screen for new antibiotics. First, samples that displayed antimicrobial activity against MRSA were screened for. Samples that were therapeutically efficacious were then screened again using a silkworm model of bacterial infection. A culture supernatant of bacteria in soil produced a new antibiotic named "Lysocin E".

5. Identification of lysocin E and determination of its structure

A supernatant of a *Lysobacter* sp. collected in Okinawa displayed potent therapeutic efficacy in a silkworm model of *S. aureus* infection despite its weak antimicrobial action. This therapeutically efficacious antibiotic was purified for evaluation in the silkworm model of bacterial infection. During purification, antimicrobial activity increased only 5-fold but therapeutic efficacy increased 300-fold (Table 2). This finding suggests that compounds that displayed only antimicrobial activity were efficiently removed during purification. It is usual case that amount of therapeutically effective compounds in culture supernatants is much smaller than the compounds that only inhibit microbial growth *in vitro*. Previous purification systems only discerned antimicrobial activity, so antimicrobial compounds with no therapeutic efficacy were identified. Now, however, therapeutically efficacious compounds can be efficiently identified using the silkworm model of bacterial infection. Indeed, nosokomycin, a new member of the moenomycin family of antibiotics, was identified using this model (9). New therapeutically efficacious compounds can be identified using the silkworm model of bacterial infection.

After purification, the structure of peak E was determined with amino acid analysis and mass spectrometry. The mass spectrum of peak E was distinct from the spectra for other natural products, so the purified compound was further analyzed with NMR and MS/MS. Results indicated that the antibiotic has a novel structure consisting of 12 d- and l-forms of amino acid residues with a short fatty acid chain (Figure 3). This

Table 2. Purification of lysocin E from culture supernatant

Items	ED ₅₀ (μg/g)	MIC (μg/mL)
Acetone extract	90	25
Butanol extract	4	0.6
Water precipitation	1.8	N.D.
ODS column chromatography	0.5	N.D.
Additional chromatography with an ODS column	0.3	5

During the purification of lysocin E, the reduction in the ED₅₀ was much higher than the reduction in the MIC. This suggests that the acetone extract contained antimicrobial compounds that were not therapeutically efficacious in the silkworm model of bacterial infection.

antibiotic was named "Lysocin E" in accordance with the standard system of antibiotic nomenclature. The most difficult aspect of determining the structure of lysocin E was ascertaining its absolute configuration. The chirality of each amino acid was determined with the exceptional 2D-HPLC system developed by Hamase *et al.* (10,11). The chirality for glutamine and glutamate could not be determined with heat treatment of lysocin E under acidic conditions because they were hydrolyzed into glutamate by the treatment, but both D and L forms of glutamate were detected. This problem was resolved by Urai *et al.*, who developed a new method to distinguish chirality using Hofmann rearrangement. Urai *et al.* are preparing a manuscript that describes how the structure of lysocin E was determined in detail.

6. Mechanistic analysis of lysocin E

Lysocin E displayed antimicrobial activity against

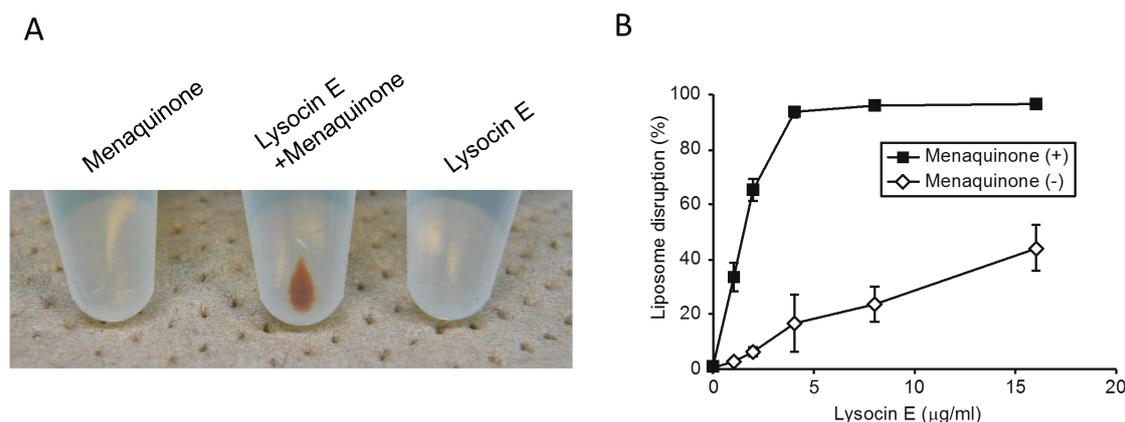


Figure 4. Mechanistic analysis of lysocin E. (A) Mixing lysocin E with menaquinone resulted in a red precipitate. (B) Lysocin E specifically disrupted menaquinone containing liposomes.

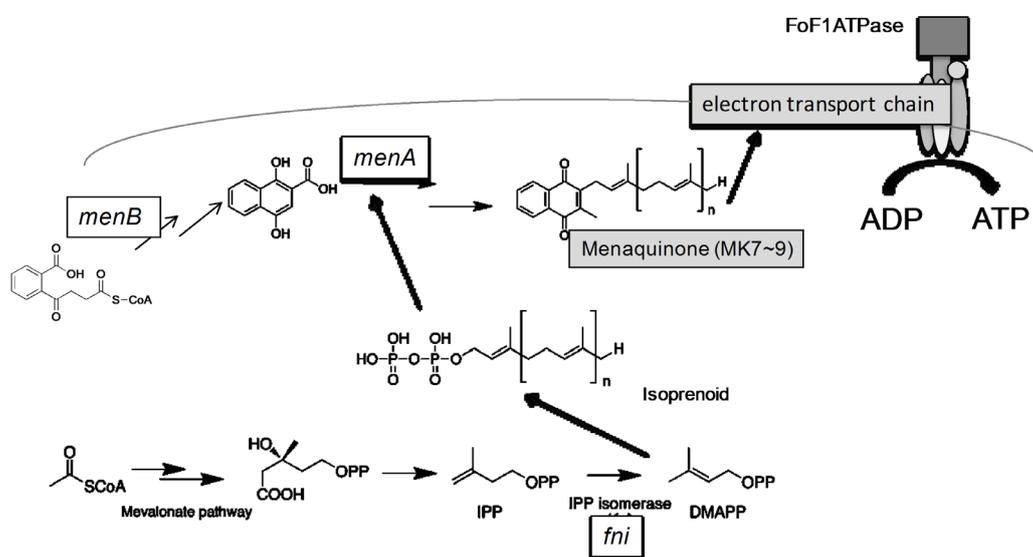


Figure 5. Diagram of the menaquinone synthesis pathway. Menaquinone is a cofactor in the respiratory chain of *S. aureus*, where it is involved in the synthesis of ADP into ATP by F_0F_1 ATPase. Mutants with a knocked out *menA* gene and *menB* gene completely lost the ability to synthesize menaquinone and were less sensitive to lysocin E.

some Gram-positive bacteria, including MRSA, and it demonstrated strong bactericidal activity against *S. aureus* in a brief period of time. This bactericidal activity was associated with membrane damage. In addition, lysocin E has a structure that differs considerably from that of other antibiotics with bactericidal activity. Thus, lysocin E was assumed to have a novel mechanism of action and different targets. Lysocin E-resistant and temperature sensitive-mutants were isolated to reveal the mechanism of action of lysocin E, and gene mutations were found in the menaquinone synthesis pathway. Furthermore, a precipitate was produced by mixing lysocin E with menaquinone (Figure 4A). In addition, the antimicrobial activity of lysocin E was inhibited by addition of menaquinone in an assay. These findings suggest that lysocin E binds to menaquinone directly rather than inhibiting biological processes performed

by proteins involved in menaquinone synthesis or the electron transport chain (Figure 5). This hypothesis was confirmed by microcalorimetry, which revealed that lysocin E interacted specifically with menaquinone, a bacterial co-factor in the electron transport chain, and not with ubiquinone, a mammalian cofactor. In addition, lysocin E was found specifically disrupt membranes containing menaquinone (Figure 4B) and to disrupt genes required for menaquinone synthesis. Mutants in which these genes were knocked out were highly resistant to lysocin E. The total synthesis of lysocin E was described by Murai *et al.* (12). They synthesized an enantiomer form of lysocin E and it displayed similar antimicrobial activity against *S. aureus*. Menaquinone is an achiral compound, so this phenomenon is theoretically reasonable and it is evidence indicating the substances that lysocin E targets. Therefore, lysocin E was concluded to target menaquinone on the

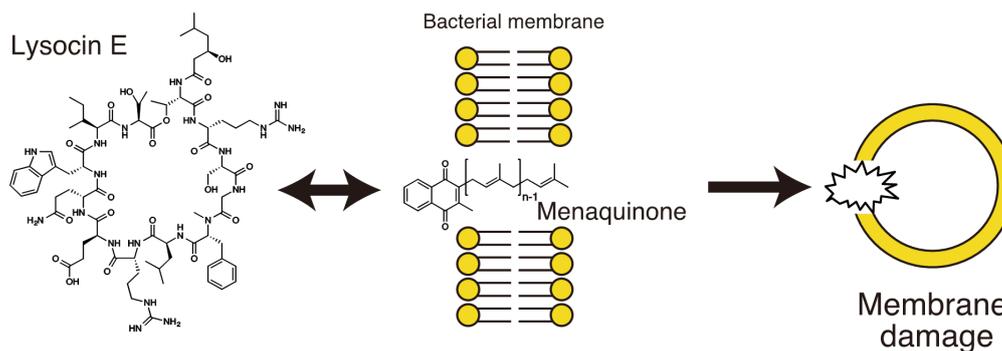


Figure 6. Diagram of the bactericidal action of lysocin E. Lysocin E binds to menaquinone on the surface of *S. aureus* and disrupts its membrane integrity.

bacterial membrane, and interaction between lysocin E and menaquinone would result in the rapid killing of bacteria (Figure 6).

7. Clinical usefulness of lysocin E

Lysocin E was screened for its therapeutic activity in a silkworm model of bacterial infection, so lysocin E would reasonably be expected to display therapeutic efficacy in a mouse model of systemic infection. Lysocin E displayed more potent therapeutic efficacy than vancomycin in a mouse model despite lysocin E having less antimicrobial activity than vancomycin. In addition, injection of lysocin E into the mouse abdomen at more than 500 times the ED_{50} did not kill mice. Furthermore, lysocin E did not cause any organ toxicity after it was repeatedly administered to mice. These features suggested that lysocin E could be useful in clinical treatment of humans, so pre-clinical tests are underway.

Another important factor is the emergence of resistant strains. Lysocin E targets the final product of menaquinone synthesis. Menaquinone is an essential cofactor in the respiratory chain of *S. aureus*. A respiratory chain is required for efficient synthesis of ADP into ATP by F_0F_1 ATPase, so a lack of or a reduction in menaquinone will cause slow growth and decrease the potential pathogenicity of bacteria. Thus, a lysocin E-resistant strain would presumably not be more drug-resistant, but further analyses are required.

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