Lysocin E is a new antibiotic that targets menaquinone in the bacterial membrane

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To obtain therapeutically effective new antibiotics, we first searched for bacterial culture supernatants with antimicrobial activity in vitro and then performed a secondary screening using the silkworm infection model. Through further purification of the in vivo activity, we obtained a compound with a previously uncharacterized structure and named it ‘lysocin E’. Lysocin E interacted with menaquinone in the bacterial membrane to achieve its potent bactericidal activity, a mode of action distinct from that of any other known antibiotic, indicating that lysocin E comprises a new class of antibiotic. This is to our knowledge the first report of a direct interaction between a small chemical compound and menaquinone that leads to bacterial killing. Furthermore, lysocin E decreased the mortality of infected mice. To our knowledge, lysocin E is the first compound identified and purified by quantitative measurement of therapeutic effects in an invertebrate infection model that exhibits robust in vivo effects in mammals.

The discovery of new therapeutically effective compounds whose antimicrobial mechanisms are distinct from known antibiotics is urgently required to combat the worldwide development of multidrug-resistant bacteria, but progress in this area has been slow in recent years. The probability of obtaining compounds with therapeutic effects from high-throughput screening of natural products or chemical libraries by in vitro antimicrobial assay is very low. To overcome this problem, evaluation of the therapeutic effects of candidate compounds that act specifically against bacteria at an early stage of antibiotic development is greatly needed. The large number of mammals needed for screening, however, is problematic, owing to the high cost and concerns regarding animal welfare. We previously reported that silkworm infection models with pathogenic bacteria are useful for quantitative evaluation of the in vivo effects of antibiotics.

We applied the silkworm infection model to screen therapeutically effective antibiotics from culture supernatants of soil bacteria and discovered new antibiotics, including lysocin E. We demonstrate that lysocin E exerts antimicrobial activity through a distinct and new mechanism by specifically targeting menaquinone, an essential component of the electron transport system, in the bacterial membrane. Furthermore, we demonstrate that these antibiotics are therapeutically effective in a mouse model of systemic infection.

RESULTS

In vivo screening of new antibiotics

We previously demonstrated that the half-maximum effective dose (ED50, the dose that produces an effect in 50% of the subjects) values of known antibiotics determined in the silkworm infection model is consistent with those reported in mammalian models. In addition, we reported that the half-maximum lethal dose (LD50, the dose that kills 50% of the subjects) of cytotoxic compounds is comparable in both silkworms and mammals. Furthermore, the silkworm has a drug metabolism system similar to that of mammals. We previously reported that all of the tested antibiotics effective in mammals have in vivo effects in the silkworm model at comparable quantities. By using these reported advantages of the silkworm infection model, we began screening for compounds with therapeutic effects in vivo. We reported the identification of a new derivative of moenomycin A named nosokomycin A using the silkworm infection model. We performed further screening of effective samples from 14,651 culture supernatants of soil bacteria collected from various regions in Japan. Of 14,651 supernatants, we identified 2,794 (19%) that exhibited growth inhibitory activity in test tubes against MSSA1, a methicillin-sensitive strain of Staphylococcus aureus, as a primary screening. We further applied the culture supernatants that showed in vitro antimicrobial activities to the secondary screening using the silkworm infection model. We injected each sample into the silkworm hemolymph (blood) immediately after injection of the S. aureus suspension, and we selected samples exhibiting in vivo effects. Of the 2,794 crude samples, 23 had therapeutic effects on S. aureus–infected silkworms. The low hit rate (23 of 2,794; 0.8%) in the secondary screening with the silkworm model indicates that this method is highly effective for excluding antibiotics that are not therapeutically effective. In addition, we obtained several previously identified antibiotics with in vivo effects in mammals, such as katanosin B, in the present screening.
We further focused on a culture supernatant of the *Lysobacter* sp. RH2180-5 strain (Supplementary Results, Supplementary Fig. 1) among the crude samples exhibiting in vivo effects in the secondary screening using the silkworm model. We attempted to purify the active compounds by measuring therapeutic activities in various fractions using the silkworm infection model (Supplementary Table 1). We defined one unit of therapeutic activity as the amount that provides 50% survival of silkworms infected with the *S. aureus* MSSA1 strain. On the basis of the increase in specific activity, which is inversely proportional to the ED50 values, we purified these therapeutic substances. In the final step of purification, HPLC demonstrated that there were nine derivatives, peaks A–I, with common UV absorption patterns (Supplementary Fig. 2). We determined the structure of the most abundant compound, peak E, by NMR, MS, two-dimensional chiral HPLC and other methods, as described in Supplementary Figures 3–16 and Supplementary Tables 2–4. This structurally new antibiotic was a cyclic lipopeptide comprising 12 D- and L-forms of amino acid residues with a short fatty acid chain (Fig. 1a). We named this antibiotic lysocin E (1) after the source bacterial species, *Lysobacter*. Lysocin E was effective against some species of Gram-positive bacteria, including methicillin-resistant *S. aureus* (MRSA), whereas it was ineffective against tested Gram-negative bacteria and true fungi (Table 1). The chemical structures determined by MS analysis and antimicrobial activities of lysocin derivatives A–I (2–9) are shown in Supplementary Figure 17.

**Bactericidal action of lysocin E**

The structure of lysocin E is unique compared with that of other known cyclic peptides, and we thus expected that this antibiotic would have a new mechanism of action. A characteristic feature of lysocin E was its potent bactericidal activity. The addition of lysocin E to *S. aureus* culture medium rapidly decreased the number of viable cells (Fig. 1b). The killing capacity of lysocin E was much greater than that of other clinically used bactericidal antibiotics, such as Daptomycin, Gentamicin, and Vancomycin (Table 1).

**Figure 1 | Bactericidal effects of the new antibiotic lysocin E against *S. aureus*. (a) Chemical structure of lysocin E. (b) Bactericidal activity of lysocin E and other antibiotics. *S. aureus* Smith ATCC 13709 strain was incubated at 37 °C in Mueller-Hinton broth with each antibiotic (5 μg ml−1 vancomycin, 3.2 μg ml−1 daptomycin, 2.5 μg ml−1 gentamicin or 8 μg ml−1 lysocin E), and the number of viable cells was counted. Note that values for the lysocin E–treated group at 15 min, 30 min and 60 min were less than 104 colony-forming units (CFU) ml−1. (c) Induction of potassium leakage in *S. aureus* by lysocin E. *S. aureus* FDA 209P strain was incubated with the indicated concentrations of lysocin E at 37 °C for 30 min, and the amounts of potassium leaked and surviving number of cells were determined. As for the potassium release, we calculated the extracellular K+ concentrations relative to those of control groups treated with 10 μM melittin. (d) Effect of lysocin E on *S. aureus* membrane potential. Membrane potential was determined by measuring the intensity of fluorescence of DiSC3(5) (excitation 622 nm, emission 670 nm) in the presence of 4 μg ml−1 melittin. Error bars show ± s.d.

**Table 1 | Antimicrobial spectrum of lysocin E**

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>MIC (μg ml−1)</th>
</tr>
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<tbody>
<tr>
<td>Methicillin-susceptible <em>S. aureus</em> MSSA1 (clinical isolate)</td>
<td>4</td>
</tr>
<tr>
<td>Methicillin-resistant <em>S. aureus</em> MRSA4 (clinical isolate)</td>
<td>4</td>
</tr>
<tr>
<td><em>S. aureus</em> Smith ATCC13709</td>
<td>2</td>
</tr>
<tr>
<td>Staphylococcus simulans JCM2424</td>
<td>4</td>
</tr>
<tr>
<td>Staphylococcus haemolyticus JCM2416</td>
<td>1</td>
</tr>
<tr>
<td>Staphylococcus pseudintermedius JCM17571</td>
<td>4</td>
</tr>
<tr>
<td>Bacillus subtilis JCM2499</td>
<td>4</td>
</tr>
<tr>
<td>Bacillus cereus JCM20037</td>
<td>2</td>
</tr>
<tr>
<td>Listeria monocytogenes 104035</td>
<td>1</td>
</tr>
<tr>
<td>Streptococcus pneumonia (clinical isolate)</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Streptococcus sanguinis JCM5678</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Streptococcus agalactiae JCM5671</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Streptococcus pyogenes SS1-9</td>
<td>&gt;128</td>
</tr>
<tr>
<td><em>Serratia</em> marcescens (clinical isolate)</td>
<td>&gt;128</td>
</tr>
<tr>
<td><em>E. coli</em> W3110</td>
<td>&gt;128</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> PA01</td>
<td>&gt;128</td>
</tr>
<tr>
<td><em>Salmonella enterica</em> ATCC14028s</td>
<td>&gt;128</td>
</tr>
<tr>
<td><em>Candida albicans</em> ATCC10231</td>
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<tr>
<td><em>Candida tropicalis</em> pK233</td>
<td>&gt;128</td>
</tr>
<tr>
<td><em>Cryptococcus neoformans</em> H99</td>
<td>&gt;128</td>
</tr>
</tbody>
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Antimicrobial activities against various bacteria were determined by the microdilution method.
Genes involved in bacterial killing by lysocin E

To gain insight into the bacterial genes related to the antimicrobial action of lysocin E, we focused on a temperature-sensitive phenotype. A series of classical studies on antibiotic resistance revealed a relationship between drug resistance and the temperature-sensitive phenotype. Temperature-sensitive strains are obtained among bacterial populations resistant to several antibiotics and possessing mutations in genes related to the action of a respective drug (for example, gyrA for nalidixic acid\(^9\), pbp for penicillin\(^1\) and rpoB for rifampicin\(^2\)). Once the temperature-sensitive mutation is established as causative of antibiotic resistance, the gene responsible for antibiotic resistance can be determined using the gene responsible for temperature-sensitive growth. We previously established a method for determining the mutations responsible for the temperature-sensitive phenotypes in \(S. aureus\)^{1-4}. On the basis of these reports, we hypothesized that isolation and analysis of mutants that show both lysocin E resistance and temperature-sensitive phenotypes would allow us to identify the genes responsible for lysocin E resistance. We first selected temperature-sensitive mutants from \(S. aureus\) cultures treated with ethyl methanesulfonate (EMS; a mutagen that causes point mutations). We then screened genes from genome libraries of the wild-type \(S. aureus\) RN4220 strain, which suppresses the temperature-sensitive phenotype of the mutants, and obtained a plasmid with the genome region including the menA gene open reading frame. Sequence analysis of the mutant genome revealed a point mutation (G98A) in the menA gene, leading to an amino acid substitution (G33D). The product of the menA gene is involved in biosynthesis of menaquinone, a sole coenzyme used in the respiratory chain in \(S. aureus\); MenA conjugates the isoprenyl chain with the naphthoquinone to produce demethylmenaquinone\(^3\). The amount of menaquinone in the lipid fraction of the MenA\(^{G33D}\) point mutant was lower than 40% that of the wild-type strain, whereas complementation with the wild-type menA gene restored this menaquinone decrease. Consistently, menA gene complementation of the MenA\(^{G33D}\) mutant suppressed the lysocin E–resistant phenotypes observed in both the minimum inhibitory concentration (MIC, Table 2) and bacterial killing assays (Fig. 2a). Moreover, phage transduction experiments revealed that the resistance to lysocin E and the point mutation in the menA gene were cotransducible, indicating that the

<table>
<thead>
<tr>
<th>Table 2</th>
<th>MIC values of lysocin E against (S. aureus) menaquinone biosynthesis mutants</th>
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</thead>
<tbody>
<tr>
<td>Strain</td>
<td>MIC ((\mu)g ml(^{-1}))</td>
</tr>
<tr>
<td>WT</td>
<td>4</td>
</tr>
<tr>
<td>MenA(^{G33D})</td>
<td>8</td>
</tr>
<tr>
<td>WT/pSR</td>
<td>4</td>
</tr>
<tr>
<td>MenA(^{G33D}/pSR)</td>
<td>8</td>
</tr>
<tr>
<td>MenA(^{G33D}/pSRmenA)</td>
<td>4</td>
</tr>
<tr>
<td>ΔmenA</td>
<td>64</td>
</tr>
<tr>
<td>ΔmenB</td>
<td>64</td>
</tr>
</tbody>
</table>

MIC values of lysocin E against each strain were determined by the microdilution method in Mueller-Hinton broth after 18-48 h incubation. Regrowth of wild-type (WT) cells was not observed even after 48 h.

![Figure 2](https://example.com/figure2.png)

**Figure 2 | Antimicrobial effect of lysocin E on \(S. aureus\) mutants of menaquinone biosynthesis.** (a) Bactericidal effect of lysocin E on the MenA\(^{G33D}\) point mutant and the menA gene-complemented strain. WT, wild-type strain; pSR, empty vector; pSRmenA, vector containing the wild-type-derived menA gene. The CFU values in lysocin E–treated (16 \(\mu\)g ml\(^{-1}\)) samples were normalized with those in nontreated samples of each genotype. Statistical differences were analyzed by one-way analysis of variance (ANOVA) with Dunnett’s multiple comparison test (**P < 0.001**). (b) Bactericidal effect of lysocin E on the ΔmenA and ΔmenB mutants. Viable cell numbers in lysocin E–treated (16 \(\mu\)g ml\(^{-1}\)) samples relative to nontreated samples of each genotype were calculated. Statistical differences within each genotype were analyzed by Student’s t-test (**P < 0.0005**). (c) Potassium leakage in menaquinone biosynthesis mutants. Values in lysocin E–treated (16 \(\mu\)g ml\(^{-1}\)) samples were normalized with those in melittin-treated (10 \(\mu\)M) samples of each genotype. Statistical differences between the wild type (WT) and each mutant were analyzed by one-way ANOVA with Dunnett’s multiple comparison test (**P < 0.001**). (d) Killing effect of the ΔmenB mutant in the mouse infection model. The gene disruption mutant of the menB gene in Newman, a clinically virulent strain of \(S. aureus\), was used. Mice (n = 5) received intravenous injections with the indicated amounts of bacteria. Data represent mean ± s.d. of triplicates. Statistical differences were analyzed by one-way analysis of variance with Dunnett’s multiple comparison test (**P < 0.001**).
In contrast, melittin, a control agent that causes membrane damage, was not active at concentrations of 100 μg ml⁻¹ or 200 μg ml⁻¹. Consequently, the membrane damage exerted by lysocin E was markedly suppressed in the ΔmenB mutants. Moreover, we found that the potassium efflux resulting from the membrane-damaging effect of lysocin E was markedly suppressed in the ΔmenB mutants (Fig. 2c). In contrast, melittin, a control agent that causes membrane damage independent of menaquinone, induced the release of potassium in these mutants. These results suggest that lysocin E–dependent killing of S. aureus via membrane disruption requires menaquinone production. In addition to the above analyses using artificially constructed mutants (i.e., MenAΔ33Δ4 by EMS treatment or ΔmenA and ΔmenB by homologous recombination), we attempted to obtain naturally occurring mutants. We first determined the rates of spontaneous appearance of mutants resistant to lysocin E and other known antibiotics at concentrations fourfold higher than each MIC value. The rate of the spontaneous appearance of resistant colonies was 2 × 10⁻⁵ for lysocin E (16 μg ml⁻¹), whereas those for rifampicin (0.06 μg ml⁻¹) and arbekacin (4 μg ml⁻¹) were 8 × 10⁻⁶ and 2 × 10⁻⁶, respectively, after 2 d of incubation at 37 °C (values are the mean of 11 independent cultures). We then isolated the lysocin E–resistant strains and measured their individual MIC and menaquinone content. To avoid assessing sister colonies within the same bacterial culture, we isolated colonies from 11 lysocin E–containing plates, each spread with independently cultured bacterial suspensions. The 11 isolated strains all had MIC values of 64 μg ml⁻¹ lysocin E, and their menaquinone quantities were <3% of that of the wild type, consistent with a previous report [20]. We further constructed deletion mutants of the genes involved in the menaquinone biosynthesis pathway. Deletion of either menA or menB (encoding an enzyme required for naphthoquinone formation, which acts upstream of MenA⁵¹) led to a complete loss of menaquinone in S. aureus, consistent with a previous report [21]. We observed high MIC values of lysocin E (Table 2) and a marked resistance to bacterial killing by lysocin E (Fig. 2b) in the ΔmenA and ΔmenB mutants. Moreover, we found that the potassium efflux resulting from the membrane-damaging effect of lysocin E was markedly suppressed in the ΔmenA and ΔmenB mutants (Fig. 2c). In contrast, melittin, a control agent that causes membrane damage independent of menaquinone, induced the release of potassium in these mutants. These results suggest that lysocin E–dependent killing of S. aureus via membrane disruption requires menaquinone production. In addition to the above analyses using artificially constructed mutants (i.e., MenAΔ33Δ4 by EMS treatment or ΔmenA and ΔmenB by homologous recombination), we attempted to obtain naturally occurring mutants. We first determined the rates of spontaneous appearance of mutants resistant to lysocin E and other known antibiotics at concentrations fourfold higher than each MIC value. The rate of the spontaneous appearance of resistant colonies was 2 × 10⁻⁵ for lysocin E (16 μg ml⁻¹), whereas those for rifampicin (0.06 μg ml⁻¹) and arbekacin (4 μg ml⁻¹) were 8 × 10⁻⁶ and 2 × 10⁻⁶, respectively, after 2 d of incubation at 37 °C (values are the mean of 11 independent cultures). We then isolated the lysocin E–resistant strains and measured their individual MIC and menaquinone content. To avoid assessing sister colonies within the same bacterial culture, we isolated colonies from 11 lysocin E–containing plates, each spread with independently cultured bacterial suspensions. The 11 isolated strains all had MIC values of 64 μg ml⁻¹ lysocin E, and their menaquinone quantities were <3% of that of the wild type (Supplementary Table 5). On the basis of the strong relationship between resistance and menaquinone levels within these spontaneous mutants, we considered that menaquinone could be involved in the antimicrobial action of lysocin E.

Small colony variants of bacteria with attenuated growth abilities exhibit resistance against several antibiotics, such as lactoferrin B [31]. Because the above menaquinone synthesis mutants showed severe growth defects, we examined whether the slow-growth phenotype itself was responsible for the resistance to lysocin E. We evaluated the lysocin E susceptibilities of other mutants that had slow-growth phenotypes. Temperature-sensitive mutants of dnaA, dnaE, polC or ddlA, genes involved in bacterial DNA replication and peptidoglycan synthesis [31,32], had the same or lower MIC values...
Menaquinone-dependent antimicrobial activity of lysocin E

The above results led us to further examine whether a molecular interaction between lysocin E and menaquinone was involved in the antimicrobial action. When menaquinone (Fig. 3a) was added to the medium, the MIC value of lysocin E against S. aureus increased in a dose-dependent manner (Fig. 3b). In addition, water-insoluble precipitates formed when lysocin E and menaquinone were mixed in the medium (Fig. 3c), suggesting that interaction of lysocin E with exogenous free menaquinone molecules lowers its antimicrobial potential. In contrast, the addition of ubiquinone (Fig. 3d), a benzoquinone compound used as a coenzyme in the electron transport chain in mammalian mitochondria, neither affected the antimicrobial activity of lysocin E nor formed precipitates (Fig. 3e). This observation led us to assess the direct binding of lysocin E with menaquinone using microcalorimetry. An exothermic response was detected after the addition of menaquinone to lysocin E solution (Fig. 3f), indicating direct binding of both compounds. The calculated dissociation constant between menaquinone and lysocin E was 4.5 μM, and the stoichiometry of these compounds within the complex was 1:1. In contrast, the addition of ubiquinone to a lysocin E solution did not produce the exothermic reaction (Fig. 3g), suggesting that lysocin E specifically binds menaquinone. In addition, using the lysocin derivatives purified from the soil bacterial culture, we performed a dissociation experiment of the lysocin I–menaquinone complex with the same isothermal titration method as that used for lysocin E. Lysocin I, the second major compound (Supplementary Figs. 2 and 16) with the same MIC as lysocin E (Supplementary Fig. 17), also exhibited specific binding to menaquinone (Supplementary Fig. 20). To determine the mode of interaction between lysocin E and menaquinone, we performed gel filtration experiments to separate the lysocin E and menaquinone components from the complex in organic solvents. Both lysocin E and menaquinone were detected by gel filtration HPLC as separate peaks (Supplementary Fig. 21), suggesting that the mode of interaction is noncovalent.

We further tested the possibility that interaction of lysocin E with menaquinone present in the membrane might affect the membrane disruptive action of lysocin E using menaquinone-containing synthetic liposomes. To test this, we examined the membrane-disrupting effect of lysocin E by measuring the amount of calcein fluorescence in the presence or absence of menaquinone within the liposome membrane. We found that lysocin E selectively stimulated the disruption of membranes depending on the presence of menaquinone (Fig. 3g,h). This effect was specifically observed in lysocin E; the disruption of synthetic liposomes by daptomycin, another membrane-damaging cyclic lipopeptide, was not affected by the presence of menaquinone (Supplementary Fig. 22). Taken together, these findings suggest that lysocin E targets menaquinone within the cytoplasmic membrane of bacteria and promotes membrane disruption, which contributes to the acute bactericidal effect.

In vivo effects of the new antibiotics in mice

We next examined the therapeutic effects of lysocin E and nosokomycin A in a mouse systemic infection model with S. aureus. Both new antibiotics showed potent in vivo effects in mice infected with S. aureus (Fig. 4), and the ED₅₀ values were 0.5 mg per kg body weight and 12 mg per kg body weight, respectively (Supplementary Table 8). The ED₅₀ of lysocin E was smaller than that of vancomycin, which is widely used in human patients infected by MRSA, whereas the MIC value of lysocin E was larger than that of vancomycin. This finding suggested superior characteristics of lysocin E in the mouse body. Moreover, mice were not killed by intraperitoneal administration of lysocin E at a dose of 400 mg per kg body weight. Serum samples from mice 24 h after lysocin E injection (5 mg per kg body weight or 50 mg per kg body weight; doses were 10 or 100 times higher than the ED₅₀) showed no increase in the biochemical markers for tissue damage mainly to the liver and kidney compared with those from vehicle-injected mice (Supplementary Table 9), suggesting that lysocin E has low toxicity against mammals. Therefore, we concluded that the two new antibiotics, lysocin E and nosokomycin A, which were obtained by screening on the basis of the measurement of in vivo effects in the silkworm infection model, were also effective in a mouse model.

DISCUSSION

In this report, we used the silkworm infection model for two different purposes to identify new antibiotics. The first was a secondary screen of therapeutically effective culture supernatants of soil bacteria (23 hits) from various crude samples that were determined to be effective through a primary screen (2,794 hits) based on the inhibition of bacterial growth in vitro. The second was to purify therapeutically active compounds from these crude extracts. This step, using the silkworm system, seemed to be critical for excluding contaminants; we observed that the crude supernatant fractions contained other antibiotics that were not therapeutically effective (i.e., compounds that show in vitro antimicrobial activities but not in vivo therapeutic activities). We successfully used the silkworm infection model for what is to our knowledge the first time to identify new antibiotics, lysocin E and nosokomycin A, which have potent effects in a mouse systemic infection model.

Most notably, lysocin E exerts its antimicrobial activity through a new mechanism: a specific interaction with bacterial menaquinone. Lysocin E lyases bacterial cells and artificial liposomes depending...
on the presence of menaquinone; the membrane-damaging effect of lysoycin E against mutant cells with menaquinone deficiency and liposomes prepared without menaquinone supplementation is severely attenuated. Generally, the target of antibiotics should be specific to bacteria, and components of the outside membrane are thought to be good targets of antibiotics. For example, vancomycin and tripeptide recognize large molecular components required for peptidoglycan synthesis, and β-lactams target penicillin-binding proteins that exist within the cytoplasmic membranes. Other cyclic peptide antibiotics, including daptomycin, impair membrane potentials by making holes in bacterial membranes or directly disturbing the membrane, causing cell lysis. Besides these few examples of proteins and lipids, however, no other small molecule existing in the bacterial membrane has been demonstrated to be a specific target of antibiotics. Lysoycin E is to our knowledge the first antibiotic that kills bacteria via specific recognition of a low-molecular-weight compound within the bacterial membrane.

The bacterial species observed to be susceptible to lysoycin E, e.g., *Staphylococcus, Bacillus* and *Listeria*, all have menaquinone, consistent with the proposed antimicrobial mechanism of lysoycin E. Fungi lack menaquinone and are resistant to lysoycin E, further supporting that menaquinone is required for lysoycin E activity. In contrast, not all bacterial species with menaquinone are susceptible to lysoycin E; *E. coli*, for example, has menaquinone but is highly resistant. The resistance observed in several Gram-negative bacteria suggests that the presence of menaquinone is not sufficient for lysoycin E-dependent killing, and the reaction may be affected by surface properties in such species that differ from those in Gram-positive bacteria or some other factor.

In the course of analyzing the antimicrobial mechanism of lysoycin E, we demonstrated that mutations in the menA and menB genes that cause menaquinone deficiencies in *S. aureus* led to a marked resistance against lysoycin E. Because these mutants still exhibited susceptibility to lysoycin E at high concentrations, other factors may be involved in lysoycin E activity and should be investigated in future studies. Nevertheless, we found that spontaneous mutants with lysoycin E resistance produced very little menaquinone, supporting the strong relationship between the antimicrobial activity of lysoycin E and the presence of bacterial menaquinone as its potential target. Because the mouse-killing ability of the menaquinone-deficient *S. aureus* strain was lower than that of the parent strain, we assume that the spontaneous occurrence of lysoycin E-resistant mutants exhibiting menaquinone deficiency leading to slow growth and attenuated virulence phenotypes does not limit the clinical utility of lysoycin E.

We revealed *in vitro* that external supplementation with menaquinone, but not ubiquinone, lowered the antimicrobial potential of lysoycin E. Ubiquinone has a benzoquinone backbone with various lengths of isoprenyl chains. The differences in chemical structures and the inhibitory effects on the antimicrobial action of lysoycin E between menaquinone and ubiquinone led us to consider that the presence of the naphthoquinone backbone in menaquinone is important for exertion of the antimicrobial activity of lysoycin E. Further studies are needed to clarify the molecular mechanism of the membrane damage induced by lysoycin E after binding with menaquinone in the *S. aureus* cell membrane.

Lysoycin E and nosokomycin A were screened using the silkworm–*S. aureus* infection model. We previously reported the existence of drug metabolism pathways executed by cytochromes P450 and conjugation enzymes, which are common to mammals, in silkworms. Moreover, we reported that a silkworm bacterial infection model could be used to quantitatively evaluate the *in vivo* effects of antibiotics clinically used for human patients. The system was also useful for evaluating the toxicities of compounds in animal bodies. Indeed, the ED50 and LD50 values of compounds in the silkworm model are consistent with those in mammalian models. Although *in vivo* screening is limited in that low-titer antimicrobial leads with potential for future drug development may be missed, our discovery of lysoycin E and nosokomycin A suggests that new and therapeutically effective antibiotics can be identified by screening natural products using the silkworm bacterial infection model. In contrast to *S. aureus*, higher animals such as silkworm and mouse use ubiquinone as a coenzyme in the respiratory chain. Our results demonstrating that menaquinone, but not ubiquinone, bound to lysoycin E could explain the selective toxicity of lysoycin E against *S. aureus*. We consider that the silkworm model is applicable for evaluating the *in vivo* effectiveness of various compounds, simultaneously reflecting both drug toxicity and metabolism, thus allowing for the identification of new antibiotics with less severe side effects in host animals.

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### METHODS

Methods and any associated references are available in the online version of the paper.

### References


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**Author contributions**

H.H. performed screening and purification, chemical structure analysis, mechanism analysis, acute toxicity analysis and manuscript drafting. M.U. performed chemical structure analysis and mechanism analysis, and drafted the supplementary results. K.I. performed bacterial genetic analysis, mechanism analysis and manuscript drafting. J.Y. performed bacteriological analysis and mechanism analysis. A.P. performed screening and purification and mechanism analysis. M.M. critically discussed and confirmed the lysocin E chemical structure. K.H. performed chemical structure analysis. T. Katsu and J.S. performed mechanism analysis. T.A. performed bacterial genetic analysis. R.U. performed analysis of nosokomycin A. H.T. performed analysis of nosocomycin A and critically revised the article for important intellectual content. M.Y. and H.K. performed acute toxicity analysis. M.S. performed chemical structure analysis. K.S. critically revised the article for important intellectual content and final approval of the article.

**Competing financial interests**

The authors declare competing financial interests: details accompany the online version of the paper.

**Additional information**

Supplementary information and chemical compound information is available in the online version of the paper. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html. Correspondence and requests for materials should be addressed to K.S.
ONLINE METHODS
Preparation of soil bacteria culture supernatant. Soil samples were collected from various regions in Japan. Soil bacteria were isolated on GA plates or HV plates. Each isolated bacteria was inoculated in 5 ml YME medium and cultured at 30 °C for 5 d. The sample was added with an equal volume of acetone and centrifuged. The supernatant was evaporated and dissolved in 1 ml of 0.9% NaCl (saline).

Primary (in vitro) and secondary (in vitro) screening of antibiotics. In the primary screening, S. aureus suspensions containing 2 × 10⁶ colony forming units (CFU) per well supplied with test samples (100 μl well of fivefold concentrated culture supernatants) were incubated for 18–24 h at 37 °C to determine the in vitro antimicrobial activity.

Silkworm eggs (H. z. y. x. T. b. a. Ne) were purchased from Ehime Sanshu, and hatched larvae were fed artificial food, Silkmate 2S (Nosan) at 27 °C until the fourth molt stage. Culture supernatants (50 μl each) were injected into the hemolymph through the dorsal surface of 3 silkworms (fifth instar 2nd-day larvae fed with antibiotic-free food (Sysmes)). Immediately, each sample was subjected to GC/MS analysis (GCMS-QP2010 plus with RTx-5, 30 m × 0.25 mm × 0.25 μm (Restek, Shimadzu)).

Measurement of antimicrobial and bactericidal activity of lysocin E. The MIC assay was performed according to the Clinical and Laboratory Standards Institute protocols. The antimicrobial and antifungal activities of each compound were measured using the microdilution method and described previously. Measurement of the bactericidal activity was performed according to the National Committee for Clinical Laboratory Standards method.

Measurement of macromolecule synthesis in S. aureus. Measurement of [1H,N-acetylglucosamine incorporation into the S. aureus NCTC8325 strain was performed as previously described. Incorporations of radio-labeled thymidine, uridine and methionine into S. aureus RN4220 strain were measured as previously described.

Effect of lysocin E on the loss of membrane potential and potassium leakage. Membrane potential was measured using the fluorescence assay. S. aureus MSSA1 strain was grown to the mid-log phase in LB medium. Cells were washed with 5 mM HEPES buffer, pH 7.0, 50 mM glucose, 5 mM EDTA and resuspended in the same buffer to obtain an A₅₇₀ of 0.05. The fluorescence intensities (ex: 622 nm, em: 670 nm) were measured by a spectro fluorometer (Jasco FP-6200) in a chamber heated at 37 °C. Data were collected for 100 s after addition of the fluorescent dye 100 nM DisC(5) (3,3′-dipropylthiadicarbocyanine iodide) and each antibiotic.

NMR analysis. All NMR spectra were recorded at 500 MHz (1H) and 125 MHz (13C) with an ECA 500 instrument (Jeol Ltd.). Chemical shifts were given in ppm, with solvent signals (CDCl₃, δH 7.24 p.p.m., δC 77.0 p.p.m.; DMSO-d₆, δH 2.49 p.p.m., δC 39.5 p.p.m.; CD₃N, δH 8.71 p.p.m., δC 149.9 p.p.m.) used as an internal reference for samples. Signal assignments were made from double quantum filtered-correlation spectroscopy (DQF-COSY), heteronuclear multiple quantum coherence (HMQC), nuclear Overhauser effect spectroscopy (NOESY) and heteronuclear multiple bond coherence (HMBC) experiments. H NMR chemical shifts of overlapping signals were obtained from the center of the cross-peaks in the 2D spectra.

ESI-MS analysis. For high-resolution ESI-MS (HRESIMS) analysis, samples were dissolved in methanol containing 0.1% formic acid and analyzed by microTOF (Bruker Daltonics). For ESI/MS/MS analysis, samples were dissolved in 90% acetonitrile containing 0.1% formic acid and analyzed by micro TOFQ II (Bruker Daltonics).

Structural analysis of lysocin fatty acids. Lysocin E and I were hydrolyzed in 6 N HCl at 110 °C for 2 h and extracted with ether followed by analysis of 1H NMR in CDCl₃. The evaporated sample was also methyl-esterified using (trimethylsilyl)diazomethane and subjected to gas chromatography/mass spectroscopy (GC/MS) analysis (GCMS-QP2010 plus with RTx-5, 30 m × 0.25 mm × 0.25 μm (Restek, Shimadzu)).
S′-TGCTTCCGATACCCGGT-3′ and S′-AGTTTGGCACACCTTGCGTT-3′ for the menA gene and S′-TGAATTTAGCGAGGATGTC-3′ and S′-CACCATGCCAGCTTGCATCTT-3′ for the menB gene. PCR products were cloned into an S. aureus integration vector pSF151 (ref. 42), and plasmids were transferred into S. aureus RN4220 by electroporation. Integration within each mutant genome was confirmed by Southern blotting analysis.

Determination of menaquinone in S. aureus cells. Overnight cultured S. aureus wild-type cells (20 ml) and 200 ml of each mutant with adjusted density were suspended in 10 ml of ice-cold acetone containing 1 ml of glass beads. Extraction of menaquinone was performed according to a previous report 43. The extracted samples were dissolved in hexane, spotted on TLC LuxPlate silica gel 60 F254 (Millipore) and developed in a mixture of heptane and diethyl ether (85:15, v/v). The intensities of bands, detected under UV light at 254 nm, were analyzed by ImageJ software version 1.43t (NIH). The menaquinone quantities were also confirmed by HPLC analysis using a PEGASIL ODS SP100 (4.6 mm × 250 mm, 5 µm, Senshu Kagaku) and Cartridge Guard Column E (4.0 mm × 20 mm, 5 µm, GL Sciences) connected to Waters Delta 600 pump. A mixture of methanol and diisopropyl ether (4:1, v/v) was used as the mobile phase at flow rate of 1 ml min⁻¹. Menaquinone was detected by a Waters 2475 multi λ fluorescence detector (ex: 320 nm, em: 430 nm). Menaquinone-7 (Wako Pure Chemical Industries, Ltd.) was used as a standard.

Isothermal titration calorimetry. Menaquinone-4 dissolved in 5% DMSO was injected repeatedly 18 times every 300 s into a sample cell filled with lysocin E dissolved in 5% DMSO with stirring at 25 °C. The values of reaction stoichiometry and binding constant were computed using Origin software (GE Healthcare).

Liposome disruption assay. Calcein-encapsulated liposomes were prepared as previously described 44 with modification. Briefly, a lipid film prepared from PC/PG (1:1) containing 1.25 mol % menaquinone-4 or ubiquinone-10 (unless otherwise indicated) was rehydrated with 5 mM sodium HEPES (pH 7.5) containing 1 mM EDTA to remove free calcein. The thawed five times and filtered through a Sephadex G-50 column with 20 mM sodium HEPES (pH 7.5) containing 1 mM EDTA to remove free calcein. The liposome fraction was diluted fivefold with buffer, and the increase in fluorescence intensity (ex: 310 nm, em: 530 nm) was measured by a spectrofluorometer after the addition of antibiotics.

Mouse infection experiments. All mouse protocols followed the Regulations for Animal Care and Use of the University of Tokyo and were approved by the Animal Use Committee at the Graduate School of Pharmaceutical Science at the University of Tokyo (approval number 24-55). S. aureus cultured with brain-heart infusion broth (Difco) at 37 °C was injected intraperitoneally with 1% (w/v) mucin from swine stomach for lysocin E. One hour later, lysocin E or nosokomycin A dissolved in phosphate-buffered saline was injected subcutaneously or intravenously into ICR mice (18–20 g, 4 weeks old, CLEA Japan), respectively.

Statistical analysis. ED₅₀ values of antibiotics were calculated by multiple logistic regression analysis (Ekuseru-Toukei 2008, Social Survey Research Information). Other statistical analyses were performed using Prism 5 for Mac OS X, version 5.0d (GraphPad Software).


