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Full Paper

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Vetiver and cell death of *B. subtilis*

Full Paper

Analysis of cell death in *Bacillus subtilis* caused by sesquiterpenes from *Chrysopogon zizanioides* (L.) Roberty

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Abstract: Recently, the antibacterial effects of essential oils have been investigated in addition to their therapeutic purposes. Owing to their hydrophobic nature, they are thought to perturb the integrity of the bacterial cell membrane, leading to cell death. Against such antibiotic challenges, bacteria develop mechanisms for cell envelope stress responses (CESR). In *Bacillus subtilis*, a gram-positive sporulating soil bacterium, the extracytoplasmic function (ECF) sigma factor-mediated response system plays a pivotal role in CESR. Among them, σ^M is strongly involved in response to cell envelope stress, including a shortage of available bactoprenol. Vetiver essential oil, a product of *Chrysopogon zizanioides* (L.) Roberty root, is also known to possess bactericidal activity. σ^M was exclusively and strongly induced when the cells were exposed to Vetiver extract, and depletion of multi-ECF sigma factors (Δ*sigM*, Δ*sigW*, Δ*sigX*, and Δ*sigV*) enhanced sensitivity to it. From this quadruple mutant strain, the suppressor strains, which restored resistance to the bactericidal activity of Vetiver extract, emerged, although attempts to obtain resistant strains from the wild type did not succeed. Whole-genome resequencing of the suppressor strains and genetic analysis revealed inactivation of *xseB* or *pnpA*, which code for exodeoxyribonuclease or polynucleotide phosphorylase, respectively. This allowed the quadruple mutant strain to escape from cell death caused by Vetiver extract. Composition analysis suggested that the sesquiterpene, khusimol, might contribute to the bactericidal activity of the Vetiver extract.

Key Words: *Bacillus subtilis*; cell death; essential oils; sesquiterpenes; undecaprenol

Abbreviations: C55, undecaprenol; C55-P, undecaprenyl phosphate; C55-PP, undecaprenyl pyrophosphate; CESR, cell envelope stress responses; ECF, extracytoplasmic function; EO, essential oil.

Introduction

Essential oils (EOs) are volatile liquids extracted from various parts of aromatic plants and are widely used in cosmetics and perfumes, mainly for therapeutic purposes (Dhifi et al., 2016). Although EOs are complex molecular substances, they contain, in many cases, terpene family compounds, including monoterpenes and sesquiterpenes. Recently, much attention has been paid to antimicrobial activity exerted by EOs and their constituents, especially against clinically isolated and drug-resistant bacteria, partly because of their broad range of structural variation and generally recognized as safe (GRAS) status (Ebani et al., 2020; Klūga et al., 2021; Kot et al., 2019; Pandey et al., 2017; Sakkas et al., 2018; Sharma et al., 2021). Owing to their hydrophobic nature, EOs are thought to penetrate easily into the cell membrane of bacteria, alter their permeability and fluidity, and disturb the integrity of the envelope and cytoplasm, resulting in cell death (Dhifi et al., 2016). However, it is still not fully elucidated whether EOs attack the cell membrane blindly or whether specific proteins or components, the function of which is inhibited by EOs, exist on the cell surface or in the cytoplasm.

As the envelope is an essential structure of any bacterial cell, it is reasonable that the cell envelope integrity and its biosynthesis are used as a primary target of antibiotic action. Fosfomycin inhibits the first step of peptidoglycan synthesis by inactivating the enzyme UDP-N-acetylglucosamine-3-enolpyruvyltransferase (Kahan et al., 1974). Lipid II is a membrane-bound peptidoglycan precursor, in which a disaccharide (N-acetyl-glucosamine/N-acetyl-muramic acid) and a pentapeptide are covalently linked to the lipid carrier undecaprenol via a pyrophosphate ester bridge (Delcour et al., 1999). Vancomycin, a glycopeptide antibiotic, is a well-known inhibitor of cell wall synthesis through complexation with lipid II of peptidyl-d-alanyl-d-alanine moieties in peptidoglycan biosynthesis (Cegelski et al., 2002). Bacitracin forms a complex tightly with undecaprenyl pyrophosphate (C55-PP), thereby preventing the recycling of the lipid carrier (Stone and Strominger, 1971). Recently, it was reported that the cyclic lipopeptide antibiotics, friulimicin B and laspartomycin C, form a complex with undecaprenyl phosphate (C55-P) (Kleijn et al., 2016; Schneider et al., 2009). However, teixobactin binds to a highly conserved motif of lipid II in different manner from that of glycopeptide antibiotics (Ling et al., 2015). These newly discovered cell wall synthesis inhibitors are drawing attention because of the rare occurrence of drug-resistant strains.

It has been proposed that to respond to an antimicrobial attack and thereby, protect the integrity of the cell envelope, cells have evolved countermeasures referred to as cell envelope stress responses (CESR) (Jordan et al., 2008). This mechanism has been best studied in *Bacillus subtilis*, a gram-positive soil bacterium, and is considered a model microorganism (Radeck et al., 2017a). The CESR network includes several signal transduction systems, which transmit external stress signals via membrane-embedded sensor proteins inside a cell to adapt transcription profiles to an unfavorable environment. Bacterial sigma factors regulate transcription initiation with the RNA polymerase core enzyme, forming holoenzyme (Feklístov et al., 2014). Among signal transduction systems in CESR, extracytoplasmic function (ECF) sigma factors, a class of alternative sigma factors, mediates one of

the response systems, which plays a pivotal role in the CESR network, especially in *B. subtilis*. The function of ECF sigma factor is negatively regulated, in most cases, with cognate anti-sigma factor, which is a membrane-embedded protein, via direct protein-protein interaction, and is exerted when the anti-sigma factor senses an environmental signal and releases the sigma factor, resulting in the formation of RNA polymerase holoenzyme. (Asai, 2018; Helmann, 2016).

Among the seven ECF sigma factors of *B. subtilis*, σ^M, σ^W, and σ^X were proposed to be involved in CESR mechanisms (Radeck et al., 2017a). The promoter sequences recognized by ECF sigma factors are similar, and the signals that stimulate these ECF sigma factors overlap, resulting in control of their regulon genes with multiple sigma factors (Mascher et al., 2007). Although such a functional redundancy is seen, σ^M, σ^W, and σ^X are classified based on their response to various antibiotics and are suggested to be closely associated with cell envelope homeostasis, cell membrane integrity, and the homeostasis of the cytoplasmic membrane, respectively (Radeck et al., 2017a). Moreover, the C55-P inhibitor, friulimicin B, and laspartomycin C activate the ECF sigma factor-controlled stress response system, especially σ^M, in *B. subtilis* (Diehl et al., 2020; Wecke et al., 2009). It has also been suggested that one of the proposed signals of σ^M activation is the shortage of available C55-P within a cell (Inoue et al., 2013). In addition to the genes involved in the core biosynthesis pathways for cell envelope assembly, σ^M activates the expression of auxiliary genes for lipid II maintenance in response to damaging agents. Lipid II is assembled in the cytosol and then shuttled across the membrane to the outside of the cells by the flippases Amj and MurJ, both functionally redundant in *B. subtilis*, and the *amj* gene is transcribed with RNA polymerase containing σ^M (Meeske et al., 2015). After a disaccharide/pentapeptide building block is loaded on the cell wall, the pyrophosphate form of undecaprenol (C55-PP) is released from lipid II. C55-PP requires dephosphorylation by specialized UPP phosphatases for recycling (Manat et al., 2014). In *B. subtilis*, both BcrC and UppP are UPP phosphatases showing synthetic lethality, and σ^M (together with σ^I, σ^X, σ^V, and potentially, also σ^W) can activate the expression of *bcrC* (Cao and Helmann, 2002; Radecket al., 2017b). This suggests that σ^M plays a significant role, especially in recycling C55-P, which has received attention as a target for antibiotic action.

Chrysopogon zizanioides (L.) Roberty (formerly known as *Vetiveria zizanioides* (Linn.) Nash) is a perennial grass that originates in India and belongs to the family Poaceae. Extracts from the root of this plant, which are collectively known as Vetiver EO, have been traditionally used owing to its antibacterial, antifungal, insecticidal, anti-inflammatory, anticancer, and antioxidant activities (Bhushan et al., 2013). In particular, Vetiver EO and its constituents are effective against multidrug-resistant strains and biofilm-forming bacteria (Dwivedi et al., 2013; Hammer et al., 1999; Kačániová et al., 2020; Kannappan et al., 2017; Morris, 2021), but their precise mechanisms of action in vivo remain unclear. In this study, we investigated the involvement of Vetiver EO and its constituents in antibacterial action, based on the ECF sigma factor-mediated response system in *B. subtilis*.

Material and Methods

Bacterial strains, media, growth, and conditions. The strains used in this study are listed in Table S1. Bacterial cultures were grown in Luria–Bertani (LB) medium (10 g/L tryptone [Difco], 5 g/L yeast extract [Difco], and 5 g/L NaCl). The following antibiotics were added if necessary: for *B. subtilis*, spectinomycin, 5 μg/mL; chloramphenicol, 5 μg/mL; erythromycin, 10 μg/mL, and for *Escherichia coli*, ampicillin, 50 μg/mL. The Vetiver extract was then dissolved in ethanol. Transformation of *B. subtilis* and *E. coli* was performed as described previously (Anagnostopoulos and Spizizen, 1961; Asai et al., 2007). Bacterial cells were grown at 37°C.

Insertion of exogenous DNA into chromosomes. Insertion of exogenous plasmid DNA, pMutinT3, into the *B. subtilis* chromosome was carried out as described elsewhere (Moriya et al., 1998; Vagner et al., 1998). For gene disruption, the internal region of each gene was amplified. In addition, a proximal intergenic region was amplified to introduce the erythromycin resistance gene in the flanking region of the target gene. Finally, DNA amplification was performed by PCR using the primer pairs listed in Table S2.

β-Galactosidase assay. *B. subtilis* cells harboring promoter-*lacZ* fusion were grown at 37°C with shaking, as described above. Samples of the culture were withdrawn for β-galactosidase activity determination, as previously described (Asai et al., 2007). One unit of enzymatic activity was defined as 1000 $\times ABS_{420}$ / OD_{600} / mL/min.

Plant material. *C. zizanioides* root chips were purchased from a local market in India. One of the authors (Hideo Yamada, Yamada-Matsu Co., Ltd.) imported the material that did not contain significant moisture.

Fractional distillation of n-hexane extract from Vetiver EO. Fractional distillation of the hexane extract afforded Group A (oven temperature, 52–83°C/0.30 Torr), Group B (oven temperature 88–103°C/0.30 Torr), Group C (oven temperature >103°C/0.30 Torr), and residue as described elsewhere (Hasegawa, 2014).

Steam distillation of C. zizanioides root. Steam distillation of the plant roots (100 g) yielded a mixture of essential oil and water. The mixture was extracted with n-hexane and dried over anhydrous magnesium sulfate. The hexane solution was concentrated under reduced pressure by rotary evaporation approximately at 25°C to afford pale-yellow oil (212 mg; extractability, 0.21%).

n-Hexane extraction of C. zizanioides root. Odor compounds from the plant roots (100 g) were extracted with n-hexane (1 L) approximately at 25°C. Removing the solvent under reduced pressure by rotary evaporation approximately at 25°C afforded a brown solid (364.1 mg; extractability, 0.36%).

Silica gel chromatography. The hexane extract (364.1 mg) was chromatographed over a silica gel (60 mesh, Merck, Darmstadt, Germany) column. Gradient elution was carried out with chloroform: ethyl acetate in the ratios of 9:1 and 7:3 and ethyl acetate (100%) and was collected into 10 mL per fraction. The components of the fraction were monitored by thin-layer chromatography (silica gel 60 GF254) in $CHCl_3$. Removal of the solvent from the fraction, abundant in khusenic acid, afforded a colorless viscous liquid (136.1 mg).

Reduction of khusenic acid. $LiAlH_4$ (35.6 mg in 4 mL diethyl ether, anhydrous) was added to a flask that had been purged with nitrogen. The fraction of khusenic acid (136.1 mg) dissolved in 2 mL diethyl ether was added slowly to the flask, and the solution was stirred for 2 h approximately at 25°C. To the reaction mixture obtained, 0.05 mL of ice-cold water, 0.05 mL of 15 % NaOH, and 0.15 mL of ice-cooled water were added in this order. The organic layer was washed with saturated ammonium chloride and NaCl solutions. The organic solution was dried over anhydrous magnesium sulfate. The solvent was removed under reduced pressure by rotary evaporation at room temperature to afford a pale-yellow solid (40.3 mg: extractability, 0.30%). The mixture was purified by silica gel chromatography, and khusimol was obtained as a colorless viscous liquid, (19.2 mg).

Bacterial susceptibility testing Bacterial susceptibility was tested using the disc diffusion assay, monitoring of the growth curve, and colony-forming ability. Each plant extract was dissolved in ethanol. A rapidly growing culture of bacterial cell suspensions, inoculated into liquid soft agar medium (0.8% agar), was overlaid on a rigid agar plate. Sterile filter paper discs (Advantec, diameter 8 mm) were placed and soaked in 10 μL of each plant extract. Bacterial susceptibility was observed by monitoring the growth curve of the cells after the addition of compounds. Turbidity was automatically monitored as OD660 of the culture in a test tube at defined intervals by a compact rocking incubator (Advantec TVS062CA). Bacterial susceptibility was evaluated using a spot test. The OD600 of the culture was adjusted to 0.1 by diluting with fresh medium, and the serially diluted spots were loaded on a solid agar medium containing the compounds.

Suppressor Selection and Identification To isolate suppressor mutants, ECF sigma factor-depleted strains were inoculated on a solid agar plate containing the Group C extract of the bulb-to-bulb distillation of Vetiver EO. After repeated inoculation on the same agar plate, four suppressor strains were selected, and to identify suppressor mutations, whole-genome sequencing was performed on a Genome Analyzer II (Illumina Inc., San Diego, CA, USA) according to the manufacturer's protocols, as described previously (Shiwa et al., 2013; Yee et al., 2011). Analysis of sequences. Sequence reads from each sample were mapped onto the *B. subtilis* 168 reference genome (accession number NC _000964.3) by using BWA software (ver. 0.5.1) (Li and Durbin, 2009) using default parameters, as previously described (Shiwa et al., 2013).

Results and Discussion

We re-examined the antibacterial activity of Vetiver EO against representative gram-positive bacteria, *B. subtilis*, and gram-negative bacteria, *E. coli*, using a disc diffusion assay. Inhibitory zones provoked by the hexane extract of Vetiver EO were observed in *B. subtilis* but not in *E. coli* at the concentration tested (Fig. 1). This tendency was also observed in a previous report (Hammer et al., 1999). To investigate the compounds responsible for the antibacterial activity, we used fractional distillation to divide the commercial Vetiver EO into groups A to C with different characteristic odors (Hasegawa, 2014). Then, each group was used for the disc diffusion assay, and only the Group C extract showed significant antibacterial activity against *B. subtilis* (Fig. 1). We employed this Group C extract for further analyses. Susceptibility against Group C extract was determined by observing growth inhibition in liquid medium in the presence of the fraction ranging from a final concentration of 20 to 80 μg/mL (Fig. S1A). The turbidity was seriously retarded when the *B. subtilis* cells were exposed to more than 30 μg/mL of the Group C extract. Similar to the disc diffusion assay, the growth of *E. coli* cells was not seriously retarded even in the presence of Group C extract of more than 80 μg/mL, in which *B. subtilis* cells were drastically lysed (Fig. S1B).

Cellular biosensors composed of the reporter and the selected promoter, which are specifically induced with bioactive compounds, are used to predict which biosynthetic pathway of bacteria is interfered with (Urban et al., 2007). In that report, the promoters of the genes of *ypuA*, *yorB*, *yheI yvgS*, and *fabHB* were induced when the cellular biosynthetic pathways of the cell wall, DNA, proteins, RNA, and fatty acids, respectively, were disrupted. To determine the effect of Group C extract on cells, we introduced the gene fusion of these promoters and the *lacZ* gene in *B. subtilis*, and the β-galactosidase activity of the cells in the presence of 20 μg/mL Group C extract was measured. The promoter activity of *ypuN* was activated in response to the Group C extract, suggesting that cell wall synthesis would be impaired. Although this test was performed only once, the result inspired us to confirm the effect of Group C extract on the cell wall biosynthetic pathway via analysis of ECF sigma factors, σ^M, σ^W, and σ^X involved in CESR, and transcription of *ypuN*, which is a gene of unknown function, is known to depend on one of the ECF sigma factors of *B. subtilis*, σ^M (Jervis et al., 2007).

In most cases, the transcription of the ECF sigma factor genes is upregulated when the cells face external stressors, which threaten individual bioprocesses. Therefore, the type of stress signal can be predicted by measuring the strength of its transcription. In the next experiment, we prepared Group C extract again from Vetiver essential oil by using bulb-to-bulb diffraction because the extract was exhausted. Susceptibility against this Group C extract was also determined by observing growth inhibition in a liquid medium (Fig. 2A). As shown in Fig. 3, σ^M was activated by treating *B. subtilis* with sublethal amounts of Group C extract and vancomycin, whereas σ^W and σ^X were not, indicating that Group C extract affected cell wall biosynthesis. Antibiotics, which directly inhibit essential biosynthetic pathways, tend to show less biocidal effects on cells in the stationary phase than on

growing cells. Ampicillin, fosfomycin, and vancomycin were less effective against cells in the stationary phase at the same concentration at which the turbidity of the exponentially growing cells was decreased (Fig. S2), whereas lysozyme, which catalyzes the hydrolysis of glycoside bonds between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan caused cell lysis in both the exponential and stationary phases. However, cell lysis was induced immediately after the addition of Group C extract to the *B. subtilis* cells entering the stationary growth phase, and the turbidity (OD600) reached nearly to 0.1 within 10 hours (Fig. 2B). Teixobactin has remarkable bactericidal activity against *Staphylococcus aureus* at the late exponential and exponential growth phases (Ling et al., 2015). These observations suggest that the compounds included in Group C extract are associated with C55-P and its related compounds, resulting in inhibition of lipid II formation and cell wall biosynthesis.

Mutants of *S. aureus* or *Mycobacterium tuberculosis* resistant to teixobactin cannot be obtained (Ling et al., 2015). Therefore, to obtain mutants of *B. subtilis* resistant to Group C extract, cells were plated on the media with a lethal dose of the extract, aiming for the appearance of a surviving colony or were continued to grow in liquid media by serial dilution into fresh media with a lethal amount of the extract when the growth of culture was recovered, but these trials did not succeed (data not shown). This also supports the idea that the Group C extract of Vetiver EO has the same characteristic features as teixobactin. Group C treatment activated defense mechanisms, which are driven by ECF sigma factors. To elucidate whether *B. subtilis* cells would be sensitive to the extract without ECF sigma factors, the mutants of ECF sigma factors previously constructed (Asai et al., 2008) were tested. Among the *B. subtilis* strains possessing a single mutation of ECF sigma factor genes, *the sigM*-deleted mutant was the most sensitive (Fig.4 A-D). Among mutants with double ECF sigma factor deletion, a *sigM-containing* combination of deletion mutants, viz. ASK4710 (Δ*sigM*, Δ*sigX*), and ASK4709 (Δ*sigM*, Δ*sigW*), were more sensitive in ASK4710 and ASK4709 to the lower amount of the extract than the single *sigM* deletion mutant, ASK4706. Additionally, this sensitivity was increased by introducing additional deletions of *sigW* and *sigV*, which are associated with lysozyme resistance (Ho et al., 2011), into *sigM* and *sigX* deletion mutants (ASK4744). This mutant of quadruple ECF sigma factor deletions (and also *sigM* and *sigX* deletion mutant, ASK4710) did not form colonies on the solid plate containing 45 μg/mL Group C extract (Fig. 4D), but after leaving it for10 days approximately at 25°C, several colonies appeared (Fig. 4E). These colonies were picked and inoculated on fresh agar plates containing 45 μg/mL Group C extract for single colony isolation. This process was repeated until the strains showed distinctive colony formation even in the presence of a lethal dose of Group C extract and several strains regained resistance, which was lost with simultaneous inactivation of two or four ECF sigma factors (Fig. 4F and G). We considered four recovery mutants, which emerged from the quadruple deletion mutant, as suppressor strains, as shown in Fig. 4F, indicated in bold letters. We performed a whole-genome sequencing analysis of these strains to identify causal genes, and the results are summarized in Table 1.

Commercial EO is generally produced from a plant by steam distillation, whereas in laboratory

experiments extraction using a chemical solvent like hexane is also efficient way. We extracted a sample directly from the *C. zizanioides* root by steam distillation and hexane extraction, aiming to determine an antibacterial compound in the Vetiver extract. As shown in Fig. 2C and D, the specific activity of the steam distillation sample was higher than that of the hexane extraction sample by monitoring the turbidity profiles of the culture after the addition of samples. It was reported that tricyclic sesquiterpenes, khusimol (Fig. 5A), are the main constituents of the Vetiver EO (Champagnat et al., 2006; Mallavarapu et al., 2012), and khusimol and khusenic acid (Fig. 5) show antibiotic activity against mycobacteria (Dwivedi et al., 2013). In fact, it was shown that khusimol is fractionated into Group C by fractional distillation (Hasegawa, 2014). From our composition analysis, the steam distillation sample contained khusimol and khusenic acid (Fig. S3A), whereas the hexane extraction sample was rich in khusenic acid (Fig. S3B). It was supposed that khusimol would be an antibacterial compound against *B. subtilis*, although it was suggested that the antimycobacterial activity of khusenic acid is higher than that of khusimol (Dwivedi et al., 2013). We did not purify khusimol using a steam distillation sample because of its complex composition. (Fig. S3A). Alternatively, we obtained khusimol by reduction of crude khusenic acid contained in the hexane extraction sample (Fig. S3C).

Among the four suppressor mutants shown in Table 1, *xseB* and *pnpA*, all of which caused amino acid substitutions, were commonly found. These mutations were backcrossed with DNA sequences of the wild type, which was linked with the erythromycin resistance gene on pMutinT3 inserted at the proximal site by using DNA transformation, and the antibacterial activity of the steam distillation sample against the resultant strains was tested (Fig. 6A). The steam distillation sample showed characteristic features similar to those of the Group C extract. All the strains were sensitive to the sample, and their phenotype was similar to that of the quadruple deletion mutant, ASK4744, when the *xseB* or *pnpA* genes were authentic, suggesting that inactivation of these genes caused a suppression effect. To further confirm which genes are responsible for the suppressor phenotype, defined insertion mutations of the genes *pnpA*, *xseA*, *xseB*, *cshB*, *tagT*, and *glyA*, in which *xseB* constitutes an operon with *xseA*, were introduced individually into the quadruple deletion mutant, ASK4744, and the resultant strains were used to test resistance against steam distillation and khusimol sample by spot test (Fig. 6B). Insertional mutations of *pnpA* and *xseA* recovered colony formation ability of the quadruple deletion mutant, ASK4744, in the presence of steam distillation sample, but that of *xseB* did not. This was also observed in the presence of kushimol, although the suppressive effect of *pnpA* seemed to be less apparent than that of *xseA*. As insertion mutation was performed by a single crossing-over event of integrative plasmid, *xseA* insertion caused a polar effect on downstream *xseB*, resulting in the inactivation of both *xseA* and *xseB*. The gene *xseB* is 255 bp in length, and the plasmid was inserted at the 183rd nucleotide in *xseB*. Therefore, it is plausible that approximately three-quarters of XseB would be expressed, and the XseB function would not be completely lost. We constructed deletion mutants of *xseA*, *xseB*, and both *xseA* and *xseB* by insertion of a promoter- and terminator-less spectinomycin-resistant gene to avoid polar effects and confirmed that inactivation of *xseB* alone was responsible for the suppressive effect on growth inhibition of the quadruple deletion mutant,

ASK4744, in the presence of kushimol (Fig. S4).

The *xseA* and *xseB* genes encode large and small subunits of exonuclease VII, respectively, which hydrolyze single-stranded DNA and are involved in DNA repair and recombination (Lovett, 2011; Poleszak et al., 2012). In *E. coli*, overexpression of XseA without XseB causes cell death independent of intrinsic exonuclease VII activity (Jung et al., 2015). They hypothesized that XseA with XseB protects cells from DNA damage as an exonuclease VII enzyme complex in calm conditions, whereas when the cells are exposed to extremely unfavorable conditions, the N-terminal portion of XseA is cleaved proteolytically and causes cell death. As the accumulation of severe DNA damage induces cell lysis (Asakura and Kobayashi, 2009; Erental et al., 2014), such a critical condition could trigger XseA proteolysis. However, in our studies on *B. subtilis*, it seems that kushimol stimulates XseB-mediated cell lysis independent of exonuclease VII activity. In in silico and in vitro studies, it was proposed that khusenic acid and kushimol bind the bacterial DNA gyrase and inhibit its activity (Dwivedi et al., 2013); therefore, these compounds might induce severe DNA damage directly when incorporated into a cell.

The *pnpA* gene encodes polynucleotide phosphorylase (PnpA), a non-essential multifunctional protein involved in competence development, RNA degradation, and DNA repair in *B. subtilis* (Cardenas et al., 2009; Condon, 2003; Luttinger et al., 1996). It has been reported that in the type I toxin-antitoxin system, *bsrE*/SR5 from the *B. subtilis* chromosome, overexpression of toxin BsrE, which contains a transmembrane domain, causes cell lysis on agar plates, and SR5 is an antitoxin antagonist. In the pnpA-deleted strain, extended SR5 mRNA appeared and formed a duplex with *bsrE* mRNA, resulting in a reduction in the amount of *bsrE* RNA, probably because of digestion with double strand-dependent RNase (Müller et al., 2016). It is possible that kushimol and other components of Vetiver EO activate such a membrane-associated toxin-antitoxin system and cause cell lysis.

Sesquiterpenes such as kushimol possess many useful antibacterial properties. These are natural compounds that have been used as aroma oils for a long time, recognized as safe for humans (GRAS), and odor compounds, which exert effects on their objects (e.g., bacteria, fungi, insects) in a non-contact manner (Dhifi et al., 2016). In particular, resistant strains against them did not occur, at least during the period of our analysis. Inactivation of *pnpA* or *xseB* in *B. subtilis* strain harboring intact ECF sigma factors did not show a more resistant phenotype against Vetiver extract than the wild-type strain (data not shown), suggesting that ECF sigma factor-mediated protective mechanisms would be robust against disturbances in cell wall biosynthesis. In the present study, α-santalol (Hasegawa et al., 2013), a sesquiterpene alcohol, which is a major constituent of sandalwood (*Santalum album* L.)-derived EO, had similar biocidal activity against *B. subtilis* as kushimol (Fig. S5). Namely, α-santalol stimulated σ^M-mediated response, and moreover, inactivation of *pnpA* or *xseB* also made the quadruple deletion mutant, ASK4744, tolerable to a lethal dose of α-santalol. Our analysis using ECF sigma factor-depleted *B. subtilis* strains revealed an unexpected link between sesquiterpenes and a few different kinds of cell death mechanisms. Elucidation of the expression profiles of ECF sigma factors in *B. subtilis* is useful for screening valuable sesquiterpenes. It is difficult to investigate the bioactivity of

compounds such as sesquiterpenes in detail because of the paucity of the samples obtained by extraction and purification from plant materials. Research on efficient production systems for sesquiterpene compounds by using microorganisms has been actively conducted in recent years (Aguilar et al., 2019; Celedon et al., 2016; Zha et al., 2020; Zhou et al., 2021) and will provide a sufficient sample for research.

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Supplementary Materials

Supplementary materials are available on our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

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Figure Legends

Fig. 1. Inhibitory zones of crude extracts of commercial Vetiver essential oil. Susceptibility of the bacterial strains, *B. subtilis* 168 (A) and *E. coli* C600 (B), against hexane extract of Vetiver EO and Group A to C extracts of bulb-to-bulb distillation, was tested. The indicated amount of extracts were dissolved in ethanol, and 10 mL of each was soaked onto the filter disks. Experiments were performed in multiple replicates, and representative data are shown.

Fig. 2. Growth of the *B. subtilis* cells after antibiotic challenge with the extracts of Vetiver EO and *C. zizanioides* root. Group C extract of Vetiver EO was added at the logarithmic growth phase (A) or stationary phase (B). Hexane extract (C) and Steam distillation sample (D) of *C. zizanioides* root were added at the logarithmic growth phase. Samples were added at the time indicated with the arrows. Experiments were performed in multiple replicates, and representative data are shown.

Fig. 3. Activity of σ^M, σ^W, and σ^X after addition of Vetiver EO extract and the cell wall-attacking antibiotic. The cells of the BSU41 (*amyE*::P*sigM-lacZ*) (A), BSU42 (*amyE*::P*sigW-lacZ*) (B), and BSU43 (*amyE*::P*sigX-lacZ*) (C) strains were exposed to 0.2% ethanol (open circle), 15 μg/mL Group C extract of Vetiver EO (closed circle), and 0.2 μg/mL vancomycin (closed rectangle) from time zero. β-galactosidase activity of the cells was measured in multiple replicates at times indicated and shown with the error bars.

Fig. 4. Growth of the mutants deleted for ECF sigma factor genes on Group C extract of Vetiver EO. As shown in panel A, *B. subtilis* 168, ASK4705 (Δ*sig*X), ASK4706 (Δ*sig*M), ASK4707 (Δ*sig*W), ASK4709 (Δ*sig*M Δ*sig*W), ASK4710 (Δ*sig*M Δ*sig*X), ASK4737(Δ*sig*W Δ*sig*X), and ASK4744 (Δ*sig*M Δ*sig*W Δ*sig*X Δ*sig*V) were cultivated on the solid agar plates without (B) or with 30 μg/mL (C) and 45 μg/mL (D) of Group C extract for two days, and with 45 μg/mL of Group C extract for ten days (E). Panel F showed that suppressor mutants derived from ASK4709 and ASK4744 were inoculated on the solid agar plate with 45 μg/mL of Group C extract (G). The number after "R," 2 or 4, indicates a mutant emerged from the strain ASK4709 or ASK4744, respectively. The suppressor strains used for whole-genome-sequencing analysis are indicated in a bold letter.

Fig. 5. Chemical structures of khusimol (A) and khusenic acid (B).

Fig. 6. Certification and identification of a responsible mutation existed in the mutant suppressor strains. The colony-forming efficiency of *B. subtilis* wild-type cells (168) and the mutant strains on the medium supplemented with extracts of *C. zizanioides* root are shown. A. The mutant suppressor strains, in which a mutation resided in the indicated gene was restored to that of the wild-type strain by backcross, were tested. B. The strain ASK4744 (Δ*sigM* Δ*sigW* Δ*sigX* Δ*sigV*), in which the plasmid

pMutinT3 (indicated as pMut) was inserted into an indicated gene, was tested.

Table 1. The locations and identities of the suppressor mutation of the quadruple deletion mutants of ECF sigma factors.

Strain Name	Suppressor Number	Gene	Position of mutation*	Amino acid substitution or description of the mutation	Function
ASK4800	R42	***xseB***	20A → ins A	frameshift	exodeoxyribonuclease
		mgtE	-341C → T	intergenic	magnesium transporter
ASK4801	R44	***pnpA***	1315A → C	T439P	polynucleotide phosphorylase
		cshB	27T → A	Y9stop	RNA helicase
		clpX	278A → G	Y93C	protein degradation
		tagT	161T → C	L54P	cell wall modification
ASK4802	R45	***pnpA***	1324G → del 12 bp	deletion	-
		glyA	220G → A	A74T	serine hydroxy methyltransferase
ASK4803	R47	***xseB***	20A → del A	frameshift	-

All the strains listed are derivatives of strain ASK4744 (*trpC2* Δ*sigM* Δ*sigW* Δ*sigX* Δ*sigV*).

* Number of start codons (ATG) from the open reading frame.

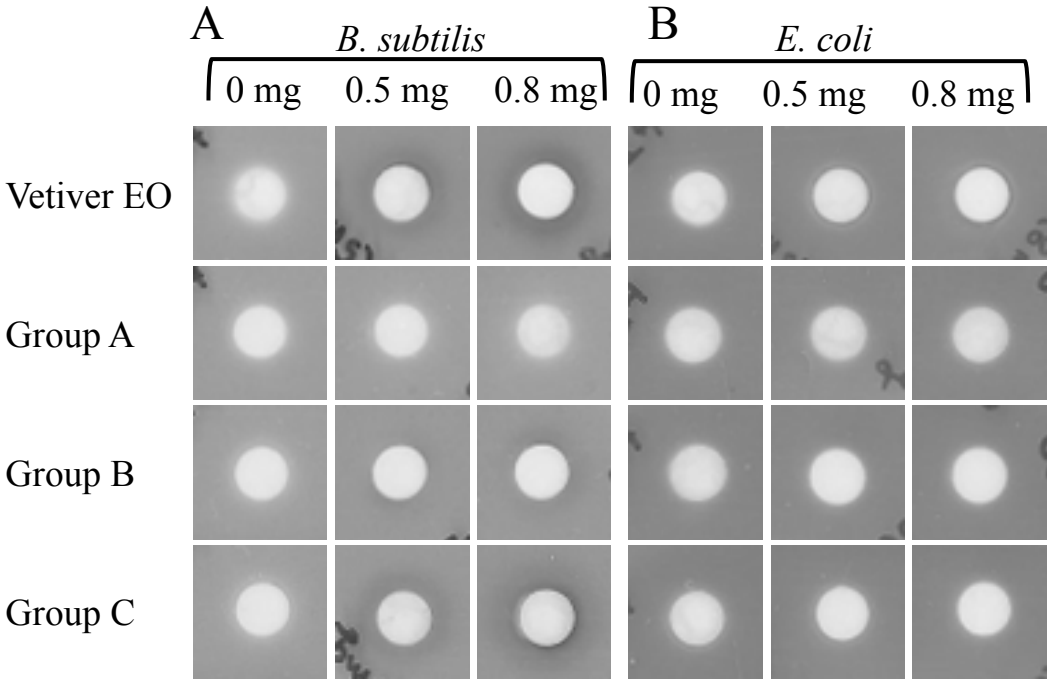
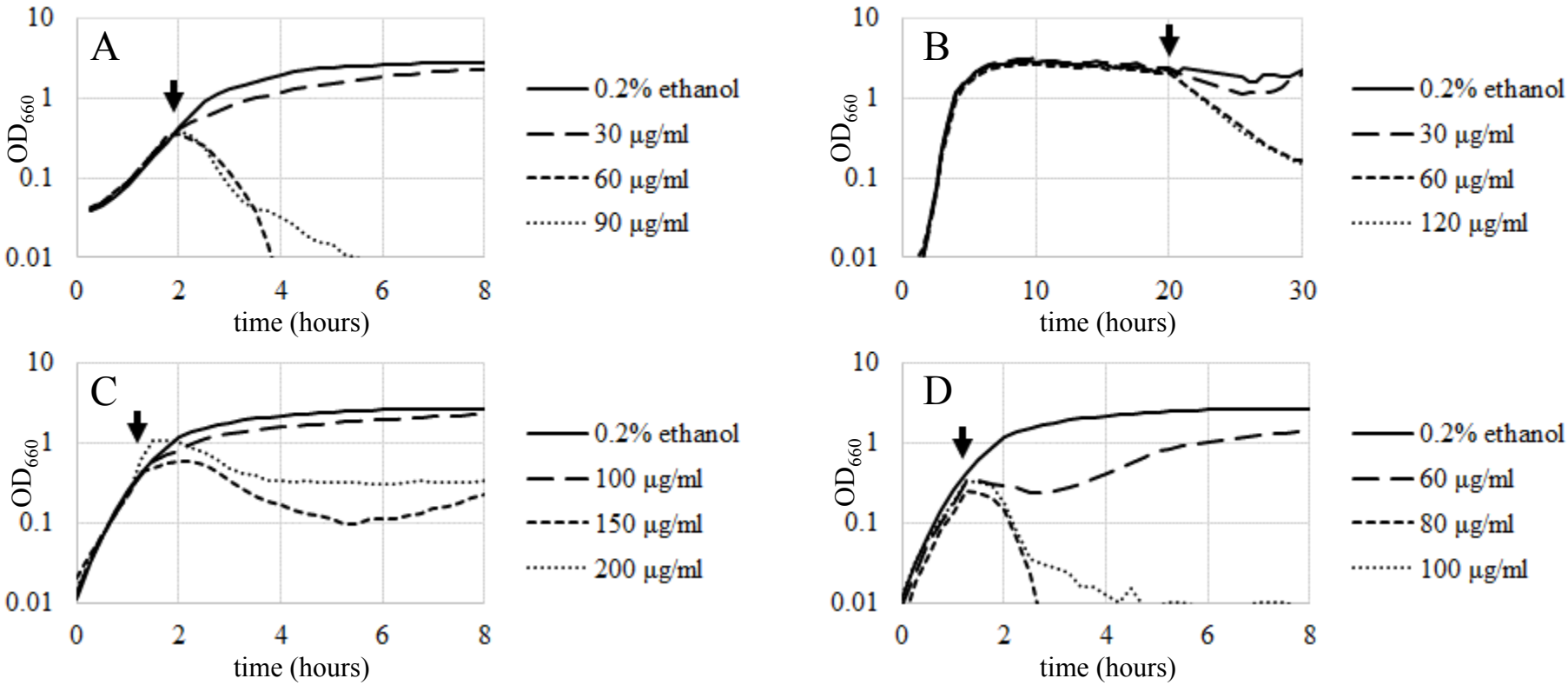


Fig. 1. Shinjyo et al.



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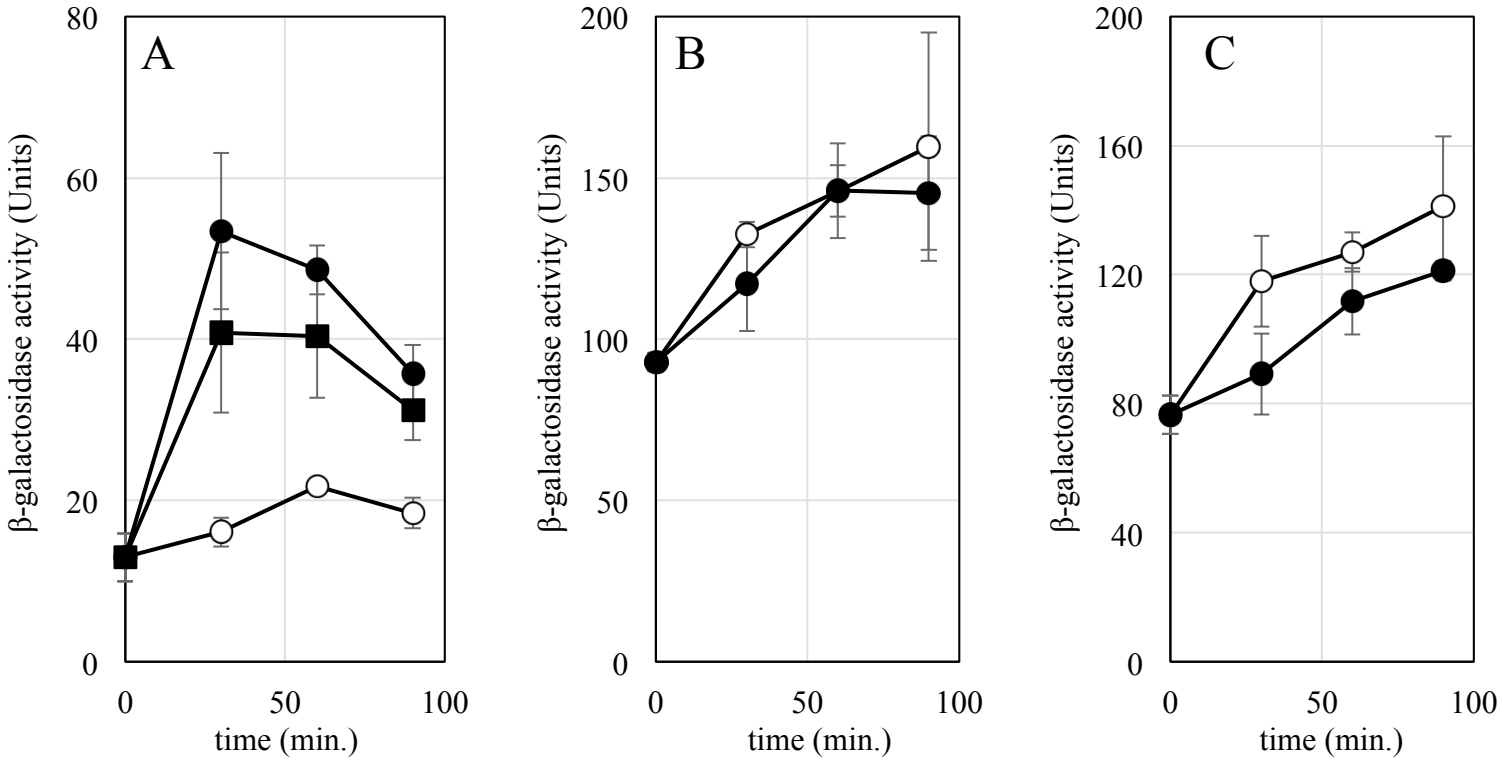

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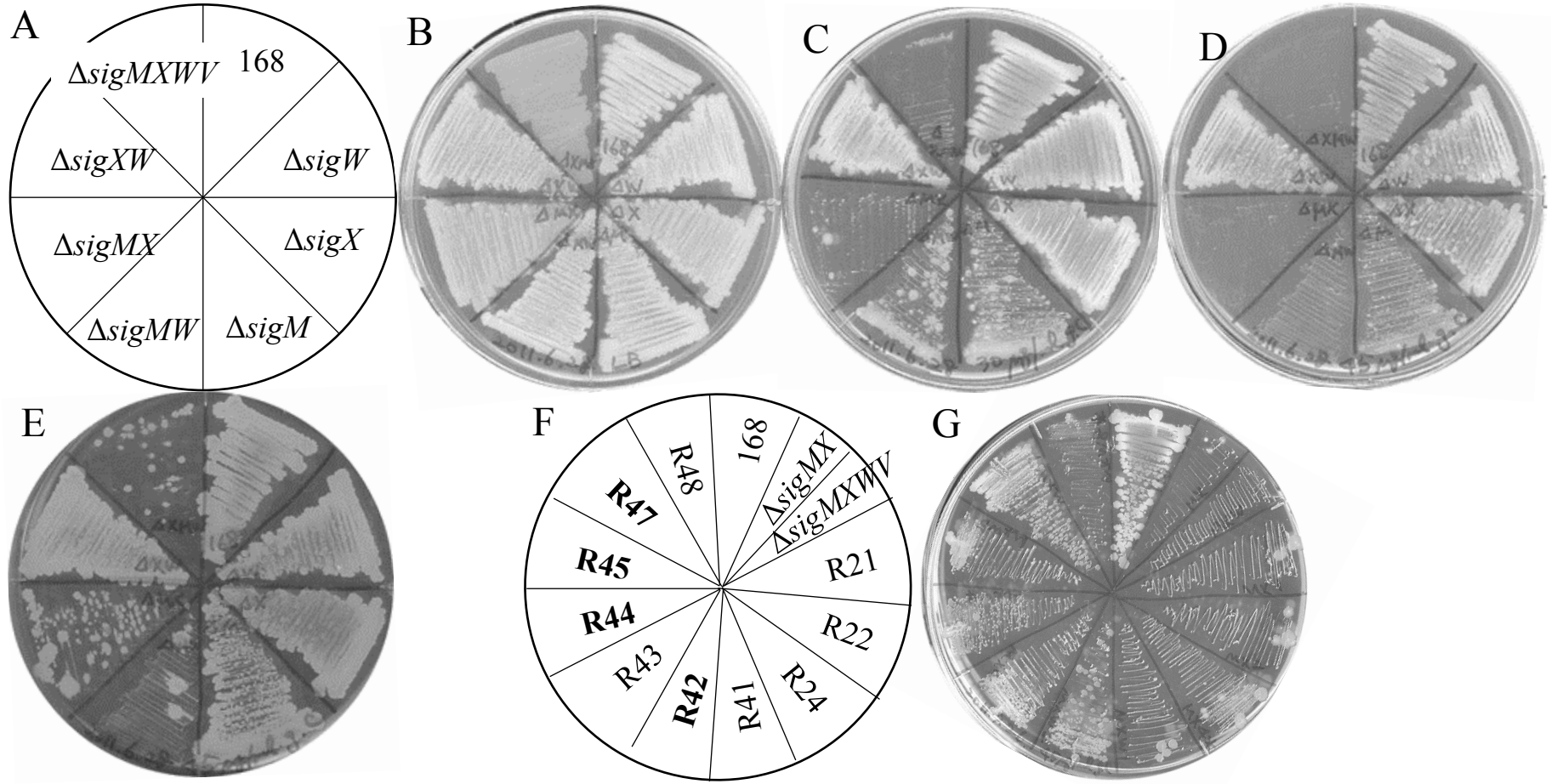

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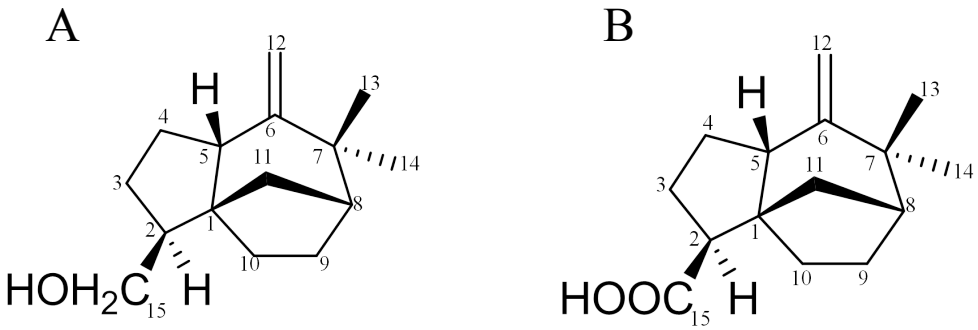

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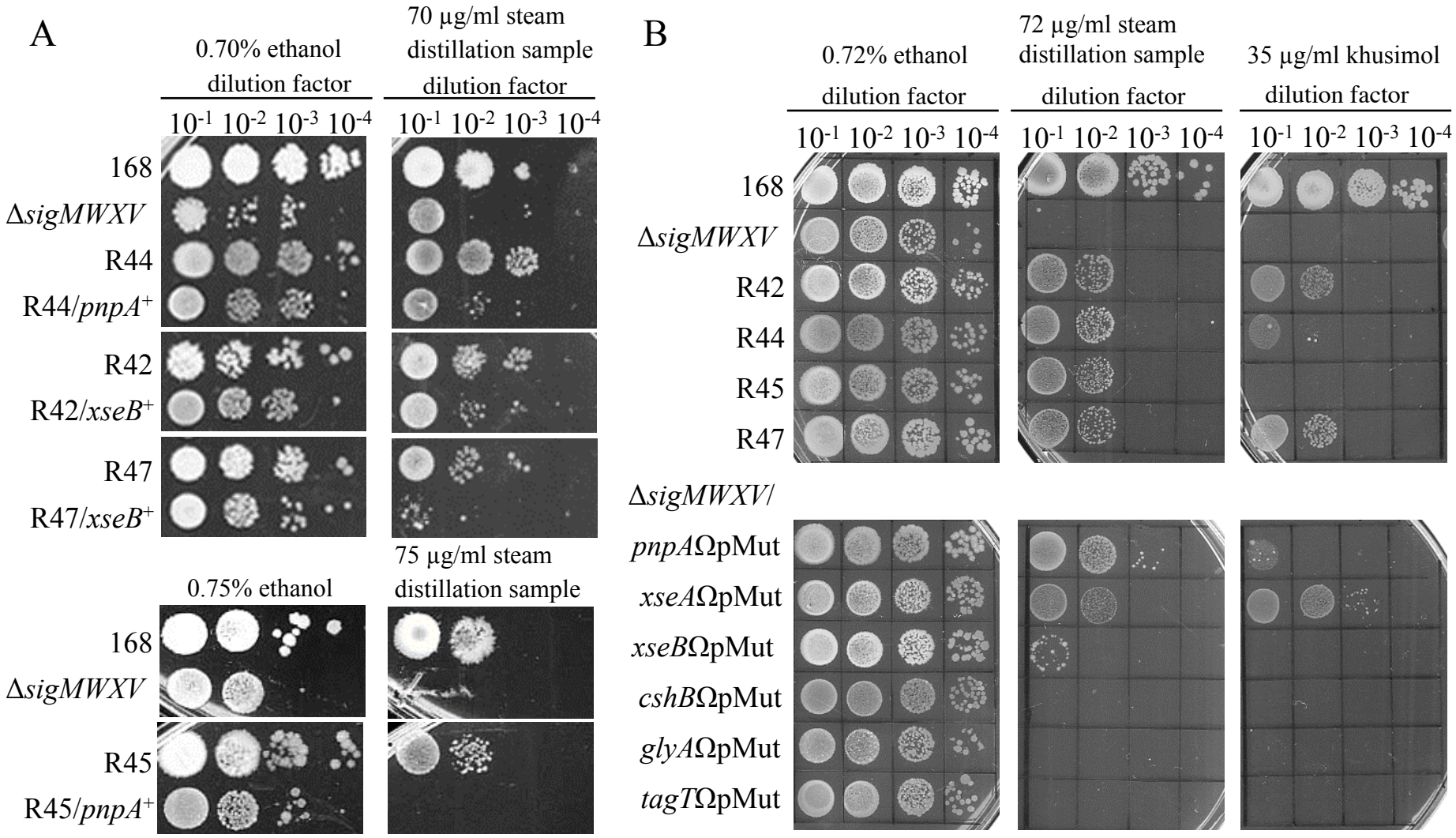

Fig. 6. Shinjyo et al.