Research

Insights into Detoxification of Tolaasins, the Toxins Behind Mushroom Bacterial Blotch, by *Microbacterium foliorum* NBRC 103072^T

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Abstract

Tolaasins are lipodepsipeptides secreted by *Pseudomonas tolaasii*, the causal agent of brown blotch disease of mushroom, and are the toxins that cause the brown spots. We previously reported that *Microbacterium foliorum* NBRC 103072^T is an effective tolaasin-detoxifying bacterium. In this study, we aimed to characterize the tolaasin-detoxification process of *M. foliorum* NBRC 103072^T. The tolaasin detoxification by *M. foliorum* NBRC 103072^T was carried out by hydrolyzation of tolaasins at two specific sites in the peptide moiety of tolaasins by its cells, and the resulting fragments were released from bacterial cells. The tolaasin-hydrolyzing activity can be extracted by a neutral detergent solution from *M. foliorum* NBRC 103072^T cells. Moreover, tolaasin adsorption to the bacterial cells occurred prior to hydrolyzation of tolaasins, which might contribute to the effective tolaasin detoxification by *M. foliorum* NBRC 103072^T. It is notable that the tolaasin-degradation process by *M. foliorum* NBRC 103072^T is carried out by hydrolyzation at specific sites in the peptide moiety of lipopeptide by bacterial cells as a novel biological degradation process of cyclic lipopeptides.

Keywords: biocontrol, lipodepsipeptide, *Microbacterium foliorum*, *Pseudomonas tolaasii*, tolaasin

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Brown blotch on mushroom, caused by *Pseudomonas tolaasii* is a problematic disease in the mushroom industry that can cause large economic losses (Goor et al. 1986; Han et al. 2012; Osdaghi et al. 2019; Paine 1919; Thorn and Tsuneda 1996; Tolaas 1915). Tolaasins, which are cyclic lipodepsipeptides secreted by *P. tolaasii*, have been identified as the toxins responsible for the disease's characteristic symptoms; unappealing brown or cream-colored lesions on pileus and stipe on the fruiting bodies of a wide range of edible mushroom species (Brodey et al. 1991; Hutchison and Johnstone 1993; Soler-Rivas et al. 1999). Seven homologs of tolaasins—tolaasin I, II, A, B, C, D, and E (Fig. 1A and B)—have been identified thus far (Bassarello et al. 2004; Nutkins et al. 1991). All of the tolaasin homologs consist of an octadecyl peptide and a β-hydroxyl fatty acid, and all except tolaasin C have a cyclic structure within the peptide

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moiety by linkage between the hydroxyl group of D -Thr14 and the C-terminal of L -Lys18. Tolaasins (except tolaasin C) show antimicrobial activities against a broad range of fungi and Gram-positive bacteria (Bassarello et al. 2004), and the cyclic structure of tolaasins might be important for their antimicrobial activities.

We previously reported that a bacterial isolate, *Microbacterium* sp. K3-5 (K3-5), shows tolaasin-detoxifying activity by hydrolyzation of tolaasins at the linkage between the hydroxyl group of D -Thr14 and the C-terminal of L -Lys18 (Tomita et al. 2018). We also demonstrated that tolaasins are directly hydrolyzed by bacterial cells of K3-5, whereas no hydrolyzing activity can be detected within K3-5 culture supernatants (Tomita et al. 2018). A few other reports have described inactivation of antimicrobial cyclic lipopeptides surfactin and daptomycin by *Streptomyces* strains (D'Costa et al. 2012; Hoefler et al. 2012). However, in those cases, the cyclic lipopeptide hydrolyzation was carried out by enzymes secreted by *Streptomyces* spp. into their culture supernatants.

We previously reported that all of the tested strains of *Microbacterium* spp. efficiently adsorb tolaasins, whereas no tolaasin-detoxifying activity can be detected in their culture supernatants. However, tolaasin adsorption onto *Microbacterium* cells is insufficient for tolaasin detoxification by most of the strains (Tomita et al. 2020).

Among the tested strains, we found that *Microbacterium foliorum* NBRC 103072^T (*M. foliorum* 103072) and K3-5 both show tolaasin-detoxifying activity. When K3-5 detoxifies tolaasins, hydrolyzed tolaasins are released from K3-5 cells into the supernatant, where they can be detected by liquid chromatography mass spectrometry (LC-MS) (Tomita et al. 2018). On the other hand, although *M. foliorum* 103072 detoxifies tolaasins as well as K3-5, no hydrolyzed tolaasins can be detected in the *M. foliorum* 103072 supernatants (Tomita et al. 2020), suggesting that the tolaasin-detoxification process by *M. foliorum* 103072 cells might differ from K3-5. Therefore, in this study, we aimed to characterize the tolaasin-detoxification process of *M. foliorum* 103072.

MATERIALS AND METHODS

Strains and medium

P. tolaasii 814, the causal agent of bacterial brown blotch on mushroom, was used for purification of tolaasins. *M. foliorum* NBRC 103072^T (Behrendt et al. 2001) was used as a tolaasin-detoxifying strain (Tomita et al. 2020). Strains were kept as glycerol stocks at -80°C until use. King's B medium (2.0% Proteose peptone, 0.15% K_2HPO_4 , 0.15% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and

A

Tolaasin I	$\text{R}_1=\text{COCH}_2\text{CH}(\text{OH})\text{CH}_2(\text{CH}_2)_3\text{CH}_3$, $\text{R}_2=\text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$, $\text{R}_3=\text{CH}_2\text{CH}_2\text{OH}$
Tolaasin II	$\text{R}_1=\text{COCH}_2\text{CH}(\text{OH})\text{CH}_2(\text{CH}_2)_3\text{CH}_3$, $\text{R}_2=\text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$, $\text{R}_3=\text{H}$
Tolaasin A	$\text{R}_1=\text{CO}(\text{CH}_2)_3\text{COOH}$, $\text{R}_2=\text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$, $\text{R}_3=\text{CH}_2\text{CH}_2\text{OH}$
Tolaasin B	$\text{R}_1=\text{COCH}_2\text{CH}(\text{OH})\text{CH}_2(\text{CH}_2)_3\text{CH}_3$, $\text{R}_2=\text{CH}(\text{CH}_3)_2$, $\text{R}_3=\text{CH}_2\text{CH}_2\text{OH}$
Tolaasin D	$\text{R}_1=\text{COCH}_2\text{CH}(\text{OH})\text{CH}_2(\text{CH}_2)_3\text{CH}_3$, $\text{R}_2=\text{CH}_2\text{CH}(\text{CH}_3)_2$, $\text{R}_3=\text{CH}_2\text{CH}_2\text{OH}$
Tolaasin E	$\text{R}_1=\text{COCH}_2\text{CH}(\text{OH})\text{CH}_2(\text{CH}_2)_3\text{CH}_3$, $\text{R}_2=\text{CH}_2\text{CH}(\text{CH}_3)_2$, $\text{R}_3=\text{H}$

B

FIGURE 1 Chemical structure of tolaasin. **A**, Tolaasin I, II, A, B, D, and E; **B**, Tolaasin C.

1.0% glycerol) was used for all bacterial cultivations (King et al. 1954).

Purification of tolaasins

Tolaasins were purified from bacterial cultures of *P. tolaasii* 814 as described previously (Tomita et al. 2020). Briefly, weakly acidic cation exchange resin (DIAION WK11; Mitsubishi Chemical, Tokyo, Japan) was added to the culture supernatants of *P. tolaasii* 814. After vigorous shaking, the resin was collected and washed by methanol (MeOH); then, tolaasins were eluted by MeOH containing 1.0% (vol/vol) formic acid. The elution was dried up in a rotary evaporator (N-1110; Tokyo Rikakikai, Tokyo, Japan). The resulting residue was dissolved in 50% (vol/vol) MeOH and applied onto an ODS column (Purif-pack ODS 100 μ m size: 60; Shoko Scientific, Yokohama, Japan). After washing the column with 50% (vol/vol) MeOH, tolaasins were eluted by MeOH containing 0.1% (vol/vol) formic acid.

LC-electrospray ionization tandem mass spectrometry analysis

For identification of tolaasin-degradation products, electrospray ionization tandem mass spectrometry (ESI-MS/MS) coupled with collision induced dissociation and the collision gas helium were used for further identification of the amino acid sequence of degraded tolaasin products. The selected precursor ions were acquired in auto LC-ESI-MS/MS modalities; then, the data were analyzed by using accurate-mass quadrupole-time-of-flight LC/MS with Agilent 6530 (Agilent Technologies, Santa Clara, CA, U.S.A.). Chromatographic separation was achieved using an Inert Sustain AQ-C18 (3 μ m, 1.0 by 150 mm; GL Sciences, Tokyo, Japan). The gradient had a constant flow rate of 0.07 ml/min, with solvent A = ultrapure water (UPW) containing 0.1% (vol/vol) formic acid and solvent B = acetonitrile containing 0.1% (vol/vol) formic acid. The linear gradient timetable consisted of 0 min, 70:30 (%A/%B); at 1 min, 70:30; at 13 min, 0:100; and at 15 min, 0:100. These data were analyzed using the Agilent Mass Hunter Qualitative Analysis Software (version B. 06.00).

LC-MS analysis

Quantification of tolaasins and tolaasin-degradation products was performed by an LC-MS system (Infinity II 1260 HPLC) coupled to a single quadrupole mass spectrometer (Agilent Infinity Lab LC-MSD) (Agilent Technologies, Tokyo, Japan) equipped with atmospheric pressure ionization electrospray operated in the positive mode. Chromatographic separation was achieved using an Inert Sustain C18 column (UP 2 μ m, 2.1 by 100 mm; GL Sciences, Tokyo, Japan). The gradient had a constant flow rate of 0.4 ml/min, with solvent A = UPW containing 0.1% (vol/vol) formic acid and solvent B = acetonitrile containing 0.1% (vol/vol) formic acid. The linear gradient timetable consisted of 0 min, 80:20 (%A/%B); at 4 min, 48:52; and at 12 min, 48:52.

Sample preparation for tolaasin I elimination and degradation assays

Cell suspension. *M. foliorum* 103072 was grown in King's B medium at 25°C for 48 h with shaking at 130 rpm. Bacterial cells were obtained by centrifugation and suspended in

phosphate-buffered saline (PBS) (10 mM phosphate-Na buffer [pH 6.8] and 140 mM NaCl) at an optical density at 600 nm of 1.0 (1.1×10^9 cells/ml).

Cell extract. Bacterial cells were obtained from 500 ml of shaking culture of *M. foliorum* 103072 grown as described above by centrifugation and washed by PBS twice. According to van der Woude et al. (2013), 10 ml of Triton X-100 in 10 mM sodium phosphate buffer (pH 7.2) was used for extraction of membrane proteins from bacterial cells (2.0×10^{11} cells) for 2 h at 4°C. After incubation, the supernatant was obtained by centrifugation at $10,000 \times g$ for 10 min and filtrated by cellulose acetate membrane (DISMIC-25CS, 0.20 μ m; Advantec, Tokyo, Japan).

Tolaasin I elimination and degradation assays

Elimination assay. Purified tolaasin (5 μ l, 2 mg/ml) was added to 120 μ l of the cell suspension and incubated for 4 h at 25°C. After incubation, 120 μ l of the supernatant was obtained by centrifugation at $10,000 \times g$ for 5 min and 80 μ l of MeOH was added to culture supernatants for LC-MS analysis, followed by filtration by cellulose acetate membrane (DISMIC-03CP 0.45 μ m; Advantec).

Degradation assay. Purified tolaasin (5 μ l, 2 mg/ml) was added to 120 μ l of crude enzyme extracts and incubated for 4 h at 25°C. After incubation, 80 μ l of MeOH was added to the crude enzyme extracts. To confirm the tolaasin degradation, LC-MS analysis was performed as described above.

Tolaasin elimination and degradation assay on the different pH condition

Cell suspension. Cell suspensions of *M. foliorum* 103072 were prepared as described above and resuspended by 120 μ l of 40 mM Britton-Robinson buffer (40 mM boric acid, 40 mM acetic acid, and 40 mM phosphoric acid; pH was adjusted by NaOH) (Britton and Robinson 1931). The pH of Britton-Robinson buffer solution ranged from 4 to 9. Purified tolaasin (5 μ l, 2 mg/ml) was added to 120 μ l of bacterial suspension with each pH buffer and incubated for 1 h at 25°C. Sample preparation and LC-MS analysis were performed as described above. Tolaasin I and tolaasin-degradation products (m/z 727 $[M+H]^+$) in the treatment with cell suspension and crude enzyme were quantified by LC-MS. The percentage of elimination of tolaasin I was calculated as follows:

$$\text{Tolaasin I elimination (\%)} = 100 - \frac{\{[\text{Peak area of tolaasin I (treatment of cell suspensions)}]\}}{[\text{Peak area of tolaasin I (control)}]} \times 100$$

Cell extracts. Bacterial cells were obtained by centrifugation from 1 liter of shaking culture of *M. foliorum* 103072 and washed by UPW twice. The cell pellets were resuspended in 10 ml of 0.5% TritonX-100 and incubated for 2 h at 4°C. After incubation, the crude enzyme extracts were obtained by centrifugation at $10,000 \times g$ for 10 min and filtrated by cellulose acetate membrane (DISMIC-25CS 0.20 μ m; Advantec). Crude enzyme extracts (60 μ l each) were twofold diluted with 80 mM Britton Robinson buffer at pH 4 to 9. Purified tolaasin (5 μ l, 2 mg/ml) was added to 120 μ l of the crude enzymes with each pH buffer and incubated for 5 h at 25°C. Sample preparation and LC-MS analysis were performed as described above. The percentage of elimination of tolaasin I was calculated as described above.

DNA extraction and amplification

The supernatant of cell lysate and the cell extracts described above were used as PCR template for detection of 16S ribosomal RNA (rRNA). Cell extracts were obtained as described above. The supernatant of cell lysate was obtained from the cell pellet collected by the extraction with 0.5% TritonX-100. After the cell pellet was resuspended with PBS, 0.2-mm-diameter Zirconia/Silica Beads (Bio Medical Science, Tokyo, Japan) were added to 200 μ l of the suspension and disrupted by a cell homogenizer (FastPrep 24 Instrument; Funakoshi, Tokyo, Japan) for 2 min. After the disruption of the cells, the supernatant was collected by centrifugation at $10,000 \times g$ for 10 min.

The cell extracts and supernatant of cell lysate were 1,000-fold diluted with UPW.

The 16S rRNA was amplified from the diluted samples by PCR using a set of universal bacterial primers for 16S rRNA gene: 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTACGACTT-3'). The PCR amplicons were electrophoresed on a 1% agarose gel with $1 \times$ Tris-acetate-EDTA buffer (50 mM Tris-base, 30 mM sodium acetate, and 3 mM EDTA in UPW; pH adjusted to 7.8 with 96% acetic acid). The gels were stained in ethidium bromide (1 μ g/ml) and visualized with the Bio-Rad ChemiDoc XRS+ system (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Semiquantification of PCR amplicons of 16S rRNA gene was performed by ImageJ image analysis software (<http://rsbweb.nih.gov/ij/index.html>).

Statistical analysis

The data were analyzed by one-way analysis of variance followed by Tukey's honestly significant difference post hoc test

or Student's *t* test with the freely available statistical analysis program tool js-STAR (version 9.1.8j; Tanaka and Nappa) and differences were considered to be significant at $P < 0.05$.

RESULTS

Hydrolyzation of tolaasins by *M. foliorum* 103072 cells

Four novel mass peaks of m/z 727, 640, 640, and 683 were detected by LC-MS in the supernatants derived from purified tolaasins added to *M. foliorum* 103072 cell suspension (Fig. 2B), whereas they were not detected in the purified tolaasins (Fig. 2A).

The MS² spectrum of the parent ion of protonated molecule $[M+H]^+$ at m/z 727.43 showed a set of daughter ions (Fig. 3A). Successive fragmentations from the two termini of the m/z 727.43 resulted in b-type ions at m/z 622.35, 523.30 ($-H_2O$, 505.27), 410.21, 323.17, and 226.14 along with corresponding y-type ions detected at m/z 502.27 ($-H_2O$, 484.25), 405.23, 318.20, and 205.12. These fragment ions were assigned to the sequence of β -hydroxyoctanoic acid chain- Δ but1-Pro2-Ser3-Leu4-Val5-Ser6, which corresponds to the N-terminal fragment derived from hydrolyzation of tolaasin I at the position of Ser6 and Leu7. The MS² spectrum of the parent ion of protonated molecule $[M+2H]^{2+}$ at m/z 639.91 were assigned to the fragment of Leu1-Val2-Val3-Gln4-Leu5-Val6- Δ but7-Thr8-Ile9-Hse10-Dab11-Lys12 cyclized via lactone formation between Thr8 and the C terminus, which corresponds to the C-terminal fragment derived from hydrolyzation of tolaasin I at the position of Ser7 and Leu8 (Fig. 3B).

FIGURE 2

Total ion chromatogram of tolaasins. **A**, Purified tolaasins treated in just phosphate-buffered saline. Masses (m/z) of peaks A, B, C, I, D, and II are 980, 987, 1,003, 994, 994, and 972, respectively. **B**, *Microbacterium foliorum* 103072-treated tolaasin. Masses (m/z) of novel peaks are 640, 727, and 683.

The other MS² spectrum of the parent ions of protonated ion [M+H]⁺ at *m/z* 640.39 and [M+2H]²⁺ at 683.43 was assigned to β -hydroxyoctanoic acid chain- Δ but1-Pro2-Ser3-Leu4-Val5 and Ser1-Leu2-Val3-Val4-Gln5-Leu6-Val7- Δ but8-Thr9-Ile10-Hse11-Dab12-Lys13 cyclized via lactone formation between Thr9 and the C terminus, respectively, and correspond to the N-terminal and C-terminal of tolaasin I when hydrolyzed at the position of Val5 and Ser6, respectively (Fig. 3C and D).

Hydrolyzation of tolaasins by bacterial cell extracts

Tolaasin hydrolyzation was observed from extracts of *M. foliorum* 103072 bacterial cells treated with Triton X-100 solution, whereas no tolaasin hydrolyzation was observed in the extract without Triton X-100 (Supplementary Fig. S1). Among the tested range of concentrations of Triton X-100 from 0 to 2%, tolaasin hydrolyzation was significantly higher from 0.05 to 0.5%. Therefore, we used 0.5% Triton X-100 to prepare the bacterial cell extracts for further analyses in this study. When tolaasins were added to these cell extracts, the distinctive peaks of *m/z* 727 [M+H]⁺, 640 [M+2H]²⁺, 640 [M+H]⁺, and 683 [M+2H]²⁺, which correspond to the hydrolyzation products of tolaasin I at the position of Ser6 and Leu7 or Val5 and Ser6, were detected in the extracts as well as the supernatants, respectively (Supplementary Fig. S2).

To estimate the damage to bacterial cells by the 0.5% Triton X-100 treatment, we used PCR to amplify the 16S rRNA gene in the supernatants of 0.5% Triton X-100 treatment and the cell lysate. The amount of 16S rRNA gene fragments detected by PCR was much lower in the supernatants of 0.5% Triton X-100 treatment compared with the supernatants of the cell lysate (Fig. 4).

Characterization of tolaasin detoxification by the bacterial cell extract compared with the bacterial cell suspension

In the cell suspension of *M. foliorum* 103072 (Fig. 5A), the amount of tolaasin I remaining in the supernatant rapidly decreased within 5 min (corresponding to approximately 60% of added tolaasin I). The ions of [M+H]⁺ of *m/z* 727 and [M+2H]²⁺ of *m/z* 640, which correspond to the N-terminal fragment of tolaasin I, II, A, B, and D and the C-terminal fragment of tolaasin I, A, and D when hydrolyzed at Ser6 and Leu7, increased linearly and reached a plateau at 45 min.

On the other hand, in bacterial cell extracts (Fig. 5B), added tolaasin I decreased linearly. The hydrolyzed products of [M+H]⁺ of *m/z* 727 and [M+2H]²⁺ of *m/z* 640 also increased linearly in the bacterial cell extract, and did not reach a plateau even after 5 h of incubation (Fig. 5B).

We also measured the levels of tolaasin I and its hydrolyzed products in the supernatants of cell suspensions and the bacterial cell extract at different pH values (Fig. 6), whereas we didn't observe any hydrolyzed products in the absence of the cell suspension or cell extract across the same pH range (data not shown).

Levels of tolaasin I decreased more rapidly at a higher pH in the supernatants of cell suspension and bacterial cell extracts (Fig. 6A and B). Tolaasin hydrolyzed products also accumulated more rapidly at higher pH in the cell suspension and bacterial cell extracts (Fig. 6C and D), whereas levels of tolaasin I decreased. These results showed that rapid hydrolyzation of tolaasins was observed at higher pH in both cell suspensions and bacterial cell extracts. On the other hand, it was notable that, although 36% of tolaasin I was eliminated in the cell suspensions at pH 4 (Fig. 6A), very little of the hydrolyzed products were observed (Fig. 6C).

FIGURE 3 Liquid chromatography tandem mass spectrometry spectrum of tolaasins hydrolyzed by *Microbacterium foliorum* 103072. **A**, *m/z* 727.43[M+H]⁺; **B**, *m/z* 639.91[M+2H]²⁺; **C**, *m/z* 640.39[M+H]⁺; and **D**, *m/z* 683.43[M+2H]²⁺.

DISCUSSION

We previously reported that the ability to adsorb tolaasins is widely distributed among *Microbacterium* spp. but that tolaasin adsorption alone is insufficient for tolaasin detoxification (Tomita et al. 2020). Among the tested strains, we found that a few strains, including the bacterial isolate K3-5, showed significant tolaasin detoxification activity. We previously revealed that the mode of tolaasin detoxification by K3-5 was through hydrolyzation of tolaasins at the lactone ring between the hydroxyl group of the D-Thr14 and the C-terminal L-Lys18 (Tomita et al. 2018). Our previous data suggested that *M. foliorum* 103072 might have a novel tolaasin-detoxification mechanism on its cells. Hence, in this study, we aimed to clarify the mechanisms of tolaasin detoxification by *M. foliorum* 103072.

LC-MS/MS analysis revealed that *M. foliorum* 103072 cells hydrolyzed the peptide moiety of tolaasins at two specific peptide bonds of Ser6-Leu7 and Val5-Ser6, and the resulting

fragments were released from the bacterial cells into supernatants (Fig. 3).

A few other articles have reported about inactivation of antimicrobial cyclic lipopeptides. The inactivation of the antimicrobial cyclic lipopeptides surfactin and daptomycin by *Streptomyces* strains is carried out by linearization of the cyclic structures of cLPs by secreted hydrolases (D'Costa et al. 2012; Hoefler et al. 2012). It is notable that the detoxification of tolaasins by K3-5 and *M. foliorum* 103072 is carried out by the cells themselves and not by secreted hydrolases, and then the resulting products are released from the cells (Tomita et al. 2020) (Figs. 2 and 3).

Recently, Hermenau et al. (2020) reported tolaasin detoxification by bacterial isolates of *Mycetocola* spp. The tolaasin detoxification by bacterial isolates of *Mycetocola* spp. was carried out by hydrolyzation at the lactone ring as well as K3-5, which was previously reported (Tomita et al. 2020). *Mycetocola* sp. is a member of the family *Microbacteriaceae* along with *Microbacterium* sp., suggesting that the tolaasin-detoxification process of K3-5 and *Mycetocola* spp. might be

FIGURE 4

Estimation of the damage to bacterial cells by the 0.5% Triton X-100 treatment. **A**, Agarose gel image of DNA products from PCR of 16S ribosomal RNA (rRNA) gene. **B**, Semiquantitative graph of DNA products from PCR of 16S rRNA gene. Mean $\pm$ standard error is represented ($n = 3$). An asterisk (*) denotes a significant difference with $P < 0.05$ by Student's t test.

FIGURE 5

Time-course analysis of tolaasin elimination and hydrolyzation. Black circles represent peak area of tolaasin I and white squares and triangles represent peak area of m/z 727 $[M+H]^+$ and m/z 640 $[M+2H]^{2+}$, respectively. **A**, Treatment by cell suspension and **B**, treatment by bacterial cell extract. Mean $\pm$ standard error is represented ($n = 3$).

distributed in *Microbacteriaceae*. In this study, we found a novel tolaasin-hydrolyzation process on *M. foliorum* 103072 which was carried out by cleavages at the two site-specific peptide bonds.

The site-specific tolaasin-hydrolyzing activities of *M. foliorum* 103072 could be observed in the extracts from *M. foliorum* 103072 cells treated with Triton X-100 (Supplementary Fig. S2). In these bacterial cell extracts, genomic DNA was detected at a much lower level compared with the cell lysate supernatant

(Fig. 4), suggesting that tolaasin-hydrolyzing enzymes are localized in the bacterial cell wall.

In the previous study, although only a few strains show tolaasin detoxification, we observed that effective tolaasin adsorption was widely distributed among *Microbacterium* spp. (Tomita et al. 2020), suggesting that the factors responsible for tolaasin adsorption might exist independently from tolaasin-detoxifying factors. Hence, in this study, we conducted a comparative study of tolaasin hydrolyzation and its

FIGURE 6

Tolaasin I elimination and hydrolyzation under the different pH conditions. Tolaasin I elimination: **A**, treatment by cell suspension and **B**, treatment by bacterial cell extract. Tolaasin-I hydrolyzation: **C**, treatment by cell suspension and **D**, treatment by bacterial cell extract. Mean + standard error is represented ($n = 3$). Values followed by different letters within the column are significantly different at $P < 0.05$ level of confidence according to Tukey's test.

FIGURE 7

Hypothesis of tolaasin detoxification by *Microbacterium foliorum* 103072.

elimination from bacterial cell suspensions and bacterial cell extracts (Fig. 5A and B). When added to the bacterial cell extracts, tolaasin I decreased linearly while the hydrolyzed tolaasin products (m/z 727[M+H]⁺ and 640[M+2H]²⁺) increased inversely with the decreasing tolaasin I (Fig. 5B), suggesting that tolaasin degradation does not follow first-order kinetics. On the other hand, approximately 60% of tolaasin I rapidly decreased within 5 min when added to the supernatants of bacterial cell suspension whereas hydrolyzed tolaasin products linearly increased and reached a plateau after 45 min (Fig. 5A). These data strongly suggest that the factors for tolaasin adsorption are located in the bacterial cell wall. Moreover, these adsorption factors might be important for the effective detoxification of tolaasins by *M. foliorum* 103072.

For further characterization of the bacterial cell suspensions and bacterial cell extracts, we evaluated tolaasin adsorption and hydrolyzation at a range of pH values (Fig. 6). Tolaasin I hydrolyzation was more active at pH values above 8 in both bacterial cell suspensions and bacterial cell extracts, whereas no hydrolyzation was observed at pH 4 (Fig. 6C and D). On the other hand, tolaasin I elimination was observed in the supernatants of bacterial cell suspensions (Fig. 6A), whereas no tolaasin I elimination was detected in the bacterial cell extracts at pH 4 (Fig. 6B). These data suggest that, although tolaasin-hydrolyzing enzymes lost the activity at pH 4, the factors for tolaasin adsorption exists on bacterial cells. Yang et al. (2017) reported that the adsorption of a cationic lipopeptide, brevivaccillin, to the cytoplasmic membrane of *Staphylococcus aureus* ATCC 6538 is inhibited by exogenous lipoteichoic acid (LTA), suggesting that an ionic interaction between brevivaccillin and LTA has a key role in inhibiting the adsorption of a cationic lipopeptide to Gram-positive bacterial cells. In our previous study, tolaasin I was extracted by 1 M NaCl from tolaasin-treated bacterial cells of *M. paraoxydans* NBRC 103076^T, which shows no tolaasin detoxification activity (Tomita et al. 2020). Based on these findings, we deduced that LTA in the cell wall of *Microbacterium* spp. might have an ionic interaction with cationic lipopeptides, which would explain tolaasin adsorption by *Microbacterium* spp. This hypothesis explains our observation that tolaasin I elimination and degradation activity decreased at lower pH, because lowering the pH causes the molecule to become more protonated and, hence, more positively (and less negatively) charged on the LTA (Fig. 7). Further study is needed for identification of receptors of tolaasins on bacterial cells.

Brodey et al. (1991) demonstrated that the whole structure of tolaasins is required to form an ion channel in a planar lipid bilayer by comparing tolaasin I with tolaasin-144, which lacks 3 amino acids in the peptide moiety. In this study, the resulting fragments from hydrolyzation of tolaasins at Ser6 and Leu7 were able to be detected in the *M. foliorum* 103072 cell-free extracts and the supernatants of *M. foliorum* 103072 cell suspension by LC-MS analysis (Fig. 3; Supplementary Fig. S2). Both of the hydrolyzed fragments increased in parallel up to 45 min in the supernatants of bacterial cell suspension (Fig. 5A) as well as in the cell-free extracts (Fig. 5B), suggesting that the resulting fragments were released from *M. foliorum* 103072 cells by hydrolyzation after adsorption of tolaasins to the bacterial cells. We previously reported that the cyclic structure of tolaasins is critical for its antimicrobial activity, especially for interaction with bacterial cells (Tomita et al. 2018). Our data suggested that the intact structure of tolaasins is important for their interaction with bacterial cells as well as for forming an ion channel in a lipid bilayer.

In conclusion, in this study, we showed that the detoxification of tolaasins by *M. foliorum* 103072 cells occurs though hydrolyzation of tolaasins at two specific sites in the peptide moiety, and it should be noted as a novel biodegradation process of cyclic lipopeptides. The hydrolases were able to be extracted from *M. foliorum* 103072 cells by a neutral detergent solution. By comparing the hydrolyzing activity of cell-free extracts and bacterial suspensions, we found evidence of tolaasin-adsorbing factors located on *M. foliorum* 103072 cell surfaces, which likely contribute to effective detoxification of tolaasins by *M. foliorum* 103072 cells.

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