



Helper bacteria halt and disarm mushroom pathogens by linearizing structurally diverse cyclolipopeptides

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The bacterial pathogen *Pseudomonas tolaasii* severely damages white button mushrooms by secretion of the pore-forming toxin tolaasin, the main virulence factor of brown blotch disease. Yet, fungus-associated helper bacteria of the genus *Mycetocola* (*Mycetocola tolaasinivorans* and *Mycetocola lacteus*) may protect their host by an unknown detoxification mechanism. By a combination of metabolic profiling, imaging mass spectrometry, structure elucidation, and bioassays, we found that the helper bacteria inactivate tolaasin by linearizing the lipocyclopeptide. Furthermore, we found that *Mycetocola* spp. impair the dissemination of the pathogen by cleavage of the lactone ring of pseudodesmin. The role of pseudodesmin as a major swarming factor was corroborated by identification and inactivation of the corresponding biosynthetic gene cluster. Activity-guided fractionation of the *Mycetocola* proteome, matrix-assisted laser desorption/ionization (MALDI) analyses, and heterologous enzyme production identified the lactonase responsible for toxin cleavage. We revealed an antivirulence strategy in the context of a tripartite interaction that has high ecological and agricultural relevance.

antivirulence | brown blotch disease | cyclic lipopeptides | *Mycetocola* | tolaasin

Edible mushrooms are an important food source. To maintain high-yield industrial-scale mushroom farming, it is vital to prevent infections. Numerous bacterial pathogens are known to cause detrimental mushroom diseases, such as cobweb, soft rot, cavity, and brown blotch disease (1). Therefore, many studies have been directed toward understanding the factors promoting virulence, which provides the basis for development of rational measures to prevent mushroom destruction. In general, bacterial pathogens require signal molecules and surfactants to colonize mushrooms (2). To weaken the host and to overcome defense mechanisms, they typically employ virulence factors including chitinolytic enzymes and antifungal agents (3, 4). A set of cyclolipopeptides (CLPs) including tolaasin I (1) has been identified as the major virulence factor of *P. tolaasii*, the causative agent of brown blotch disease (1, 5–7). The pore-forming toxin (8, 9) causes characteristic browning symptoms in the white button mushroom (*A. bisporus*), one of the most important cultivated mushrooms worldwide (10, 11). Furthermore, tolaasin-producing bacteria cause diseases of strawberries, cauliflower, and tobacco (12, 13), which calls for unifying strategies to control these pervasive pathogens even beyond mushroom farming.

In contrast to the highly unfavorable use of antibiotics in agriculture, the concept of employing helper bacteria to control pathogens is a potentially less disruptive alternative (Fig. 1). As counterparts to the bacterial pathogen, several antagonistic bacteria have been discovered that either show predatory behavior toward the toxin producer (14) or inactivate its main virulence factor (15–19). For example, the helper bacterium *Pseudomonas reactans* produces a CLP named white-line-inducing principle (WLIP) that precipitates together with tolaasin, thus, forming a characteristic white line when cocultivated with *P. tolaasii* on solid agar (18). However, WLIP is also toxic against the mushroom host

via membrane pore formation and shows hemolytic activities, which precludes its use as a biocontrol agent (20, 21). More promising is the use of helper bacteria that neutralize the effect of tolaasin. Examples of this tolaasin negation activity are provided by members of the genus *Mycetocola* (*M. tolaasinivorans* and *M. lacteus*), which were jointly isolated from the rotting fruiting bodies of mushroom *Pleurotus ostreatus* (16, 17). The saprophytic gram-positive bacteria, the first members of the novel genus *Mycetocola* in the family of *Microbacteriaceae*, are nonpathogenic with respect to the mushroom and were shown to detoxify tolaasin (16). Yet, the mechanism of tolaasin detoxification by these helper bacteria has remained elusive. Here, we report the molecular basis of the antivirulence strategy of two different *Mycetocola* species. We show that they not only efficiently inactivate the antifungal agent, but also cleave an important bacterial swarming mediator, thus, blocking both pathogenicity and motility of the pathogen.

Results and Discussion

To gain insight into the pathogen-helper bacteria interaction, we conducted coinoculation experiments of *P. tolaasii* with *M. tolaasinivorans* or *M. lacteus* on mushroom slices (*A. bisporus*). Whereas mushroom blocks inoculated with *P. tolaasii* alone showed brown sunken lesions, the presence of *M. tolaasinivorans* prevented infection symptoms. Nonetheless, *A. bisporus* was found to be a suboptimal infection model to monitor the inactivation of tolaasin since inoculation with *M. lacteus* alone led to slight browning of the mushroom tissue, likely owing to its saprophytic lifestyle. Therefore, we turned to potato tubers that

Significance

P. tolaasii is the causative agent of brown blotch disease in the common button mushroom *Agaricus bisporus* and responsible for tremendous losses in mushroom farms every year. The pathogen relies on two different cyclic lipopeptides during infection: the pore forming toxin tolaasin and the motility conferring biosurfactant pseudodesmin. In this study, we show how enzymes produced by helper bacteria of the genus *Mycetocola* provide protection to the mushroom via lipopeptide cleavage. Using bioactivity guided fractionation of the proteome, we could identify the key enzyme in toxin inactivation. Deciphering such protective interactions paves the way for the development of biocontrol agents for use in both agriculture and medicine.

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The authors declare no competing interest.

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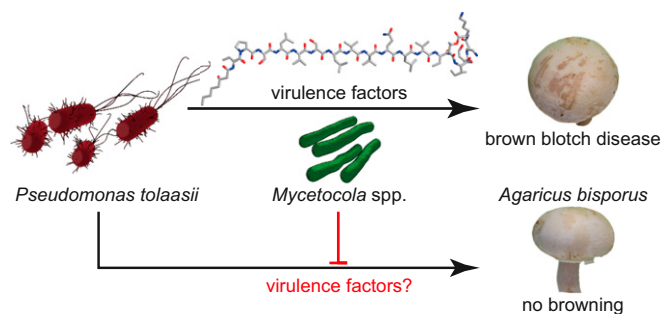


Fig. 1. Tripartite interaction of mushroom pathogen, helper bacteria, and fungal host. *P. tolaasii* produces virulence factors that cause bacterial brown blotch in *A. bisporus*. *Mycetocola* spp. can prevent decay of the mushroom cap.

reportedly develop black spots in the presence of the toxin (17). Blackening caused by *P. tolaasii* alone was completely inhibited in coinoculated potato slices (Fig. 2A), indicating effective protection against tolaasin.

To shed light on the chemical fate of the peptide toxin, we monitored the metabolic profiles of liquid cocultures by HPLC-MS. In the axenic *P. tolaasii* culture, we detected **1** as the main component of a mixture of known tolaasins (22). In contrast, we found that the titers of **1** were drastically reduced in cocultures with each of the helper bacteria (Fig. 2B). The intensity of a double-charged ion with a m/z difference of 9 was dominant. This mass difference of 18 Da in the uncharged molecule could result from the hydration of one of the dehydrobutyrine units or the hydrolysis of one of the amide bonds in the cyclic part of **1**. Tandem mass spectrometry (MS/MS) measurements ruled out these possibilities and unequivocally showed that the lactone is cleaved to yield the linearized congener known as tolaasin C (**2**, Fig. 2C) (22). A similar cleavage potential has been implicated for *Microbacterium* sp. K3–5 based on MS data (15). We confirmed the identity of **2** by comparison of its retention time with an authentic reference obtained by hydrolysis of **1** with lithium hydroxide (Fig. 2B).

To corroborate these findings on solid media and to dissect microbial interaction processes, we monitored bacterial populations by MALDI imaging. In the colony of the pathogen, **1** is detected as the main product. In addition, small amounts of **2** are colocalized with **1**, which was not unexpected since **2** occurs naturally in axenic *P. tolaasii* cultures (22). In the zone where *P. tolaasii* was mixed with *M. tolaasinivorans* or *M. lacteus*, no **1** was detected but high amounts of **2** were (Fig. 2D), clearly indicating that both types of helper bacteria are able to cleave the toxin.

When examining the phenotypes of the cocultures, we noted that the helper bacteria also impair the swarming ability of the pathogen. When grown axenically, *P. tolaasii* colonies spread uniformly in every direction, forming faint concentric circles that likely indicate different growth states. When coinoculated with *M. tolaasinivorans* or *M. lacteus*, however, the swarming diameter of *P. tolaasii* is reduced to approximately half the size of axenic colonies (Fig. 3A, see also *SI Appendix*, Fig. S3). Furthermore, marked phenotypic changes, such as zone formation and three-dimensional biofilmlike structures were observed (*SI Appendix*, Fig. S4).

Metabolic profiling and MALDI imaging of the cocultures yielded further insight into the chemical basis of this phenomenon. In liquid cocultures, we observed the emergence of a new compound with m/z 1142 $[M - H]^-$ that is not detectable in the requisite axenic cultures (Fig. 3B). MS/MS experiments revealed a fragmentation pattern typical of a peptide and an amino acid sequence that matches with viscosinamide A (**3**) and pseudodesmin A (**4**), two related CLPs that only differ in the absolute

configuration of the leucine residue at position 5 (*SI Appendix*, Fig. S5) (23, 24). A mass difference of 18 Da compared to the reported CLPs, and the MS/MS fragmentations (Fig. 3C) implied that the observed species corresponds to the linearized form of one of these molecules. To determine its structure, we isolated the CLP from an up-scaled *P. tolaasii* culture (2 L). By analysis of the NMR spectra and comparison of ^1H and ^{13}C chemical shifts with reference data (24, 25), we found that the isolated CLP is **4** (Fig. 3D, see also *SI Appendix*), a member of the viscosin family of biosurfactants involved in bacterial swarming and motility (24). We named the linearized congener pseudodesmin C (**5**) (Fig. 3E) and confirmed its identity further by comparison with an authentic reference generated by hydrolysis

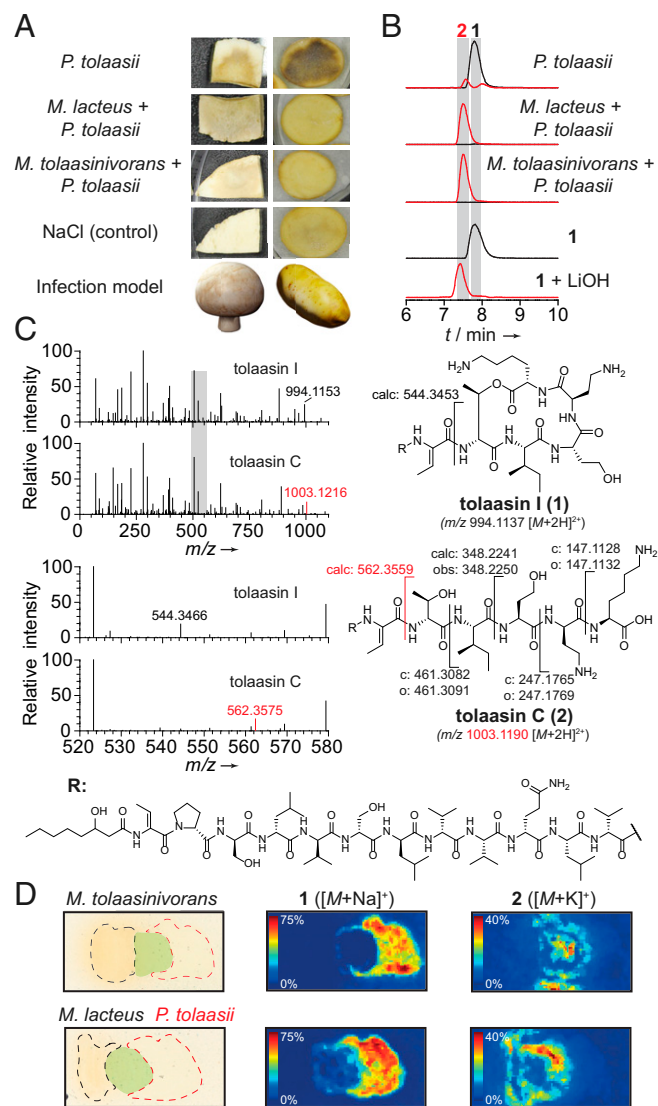


Fig. 2. Helper bacteria inactivate the toxin produced by the mushroom pathogen. (A) In vivo infection assays with mushroom and potato tuber slices. (B) Extracted-ion chromatogram (EIC) traces of **1** (black, 994.1137 $[M + 2H]^{2+}$) and **2** (red, 1003.1190 $[M + 2H]^{2+}$) obtained from high-performance liquid chromatography (HPLC) analysis of culture extracts, isolated **1**, and **1** hydrolyzed with 5 eq LiOH, 1 h, RT. (C) Comparison of MS/MS fragmentation patterns of **1** and **2** with highlighted key fragments. Gray bar indicates magnified area. c: calculated; o: observed masses. (D) MALDI imaging of solid cultures of *P. tolaasii* with *M. lacteus*, and *M. tolaasinivorans*, respectively, showing spatial distribution of **1** and **2**. Black: colony of *M. tolaasinivorans* and *M. lacteus*, respectively; red: colony of *P. tolaasii*; green: mixed colonies.

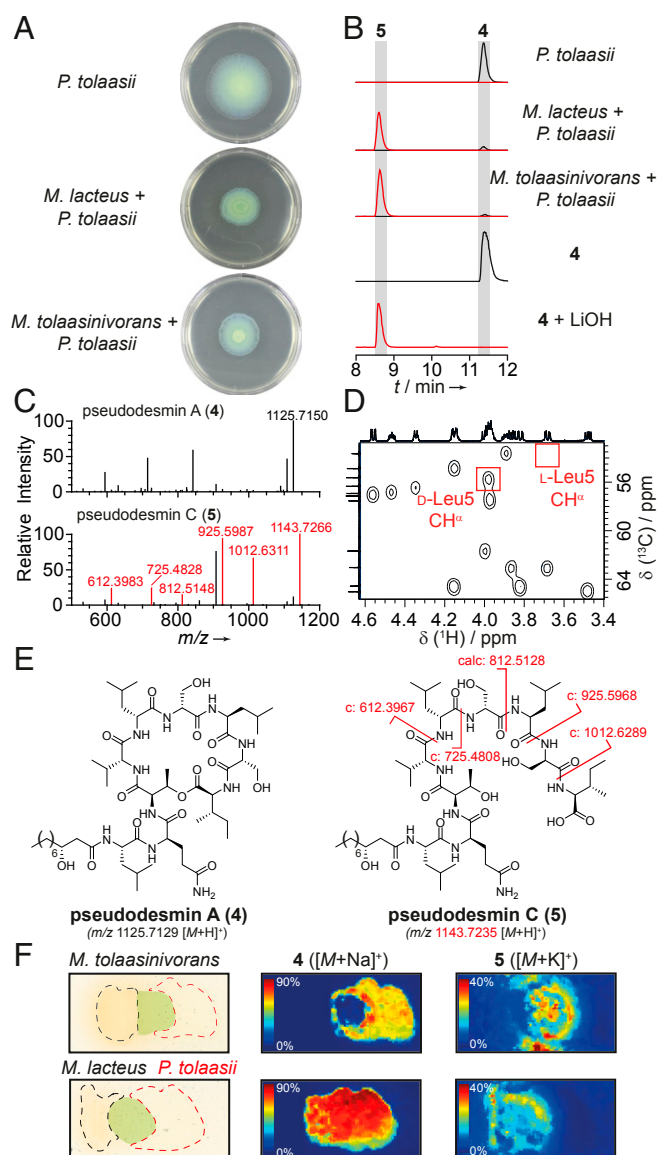


Fig. 3. Cleavage of **4** impairs dissemination of the pathogen. (A) Swarming assays of *P. tolaasii* and respective cocultures on soft Kings B agar. (B) EIC traces of **4** (black, 1125.7129 [M + H]⁺) and **5** (red, 1143.7235 [M + H]⁺) obtained from HPLC analysis of culture extracts, isolated **4**, and **4** hydrolyzed with 5 eq LiOH, 1 h, RT. (C) MS/MS analysis of **4** and **5** with key fragments highlighted in red. (D) Heteronuclear single quantum coherence correlations of the CH^α region of **4** with red boxes indicating expected signals for D-Leu CH^α and L-Leu CH^α in position 5 based on literature data. (E) Structures of **4** and **5** with key fragments highlighted in red. c: calculated masses of fragments. (F) MALDI imaging of cultures of *P. tolaasii* with *M. lacteus* or *M. tolaasinivorans*, showing spatial distribution of **4** and **5**. Black: colony of helper bacteria; red: colony of pathogen; green: mixed colonies.

of pure **4** with aqueous LiOH (Fig. 3B) as well as by evaluation of NMR data (SI Appendix).

To address whether the cleavage of **4** into **5** in coculture impairs the swarming of *P. tolaasii*, we scrutinized individual and coinoculated colonies with MALDI imaging (Fig. 3F). In stark contrast to the axenic *P. tolaasii* colony, intact **4** could not be detected in the zone where *P. tolaasii* and *M. tolaasinivorans* interacted. In lieu of the cyclopeptide, high levels of **5** accumulated in this area. In the coculture of *P. tolaasii* with *M. lacteus*, we still observed **4** as major congener. However, the linear **5** was

also formed in the mixed zone of the cultures. These results show that the presence of helper bacteria leads to the linearization of the surfactant **4** and, therefore, to a reduction of the swarming ability of the pathogen.

To unambiguously demonstrate that intact **4** is essential and sufficient for swarming, we generated a pseudodesmin-deficient *P. tolaasii* mutant. The putative pseudodesmin biosynthesis gene cluster (BGC) was identified by means of antiSMASH (26) analysis and predicted substrate specificities of the adenylation domains (SI Appendix, Fig. S14). To confirm the cluster identity and to create a nonproducing strain, we deleted a 1.7 kb fragment in the nonribosomal peptide synthetase (NRPS)-encoding gene *pseB* by homologous double crossover (Fig. 4A). Colonies of the verified null producer (Δ *pseB* mutant) were unable to swarm (Fig. 4B). To confirm the individual roles of the two CLPs, we also deleted a 1.7 kb segment within the NRPS gene (*tolA*) of the tolaasin biosynthetic locus that is homologous to the tolaasin BGC in *Pseudomonas costantinii* (SI Appendix, Fig. S15) (1). The resulting Δ *tolA* mutant did not cause browning or other visible disease symptoms in any of the tested model systems (Fig. 4B), once again corroborating the key role of **1** in pathogenesis. Whereas the swarming behavior of the tolaasin-deficient mutant was altered, motility was still observed (Fig. 4B). Thus, this surface-active lipopeptide can be considered as a swarming-enhancing factor. In contrast, **4** is essential and sufficient for bacterial motility on soft agar and might be beneficial in colonizing the hydrophobic mushroom caps. The hydrolytic agents produced and released by *M. tolaasinivorans* and *M. lacteus*, therefore, not only inactivate the pore-forming toxin secreted by *P. tolaasii*, but also impair bacterial movement by cleaving its main motility factor.

In order to identify the hydrolytic factor, we investigated the cell lysate of a mixed culture of *M. tolaasinivorans* and *M. lacteus* as these are the native bacterial flora of cultivated mushroom (16). Whereas tolaasins were cleaved by treatment with the crude cell lysate, no conversion was observed using heat-inactivated (HI) aliquots of the same lysate, indicating that an enzyme is responsible for the lactone cleavage. The obtained cytosolic proteome was then fractionated by ammonium sulfate precipitation, and active fractions were further subjected to fast protein liquid chromatography (FPLC)-based separations using different anion exchange columns and hydrophobic interaction chromatography as well as size exclusion chromatography (Fig. 4C).

Active fractions were analyzed using MALDI-time of flight and LC-MS in combination with the *in silico* proteome generated from genomic sequences of both strains. Then, we filtered the hit lists for basic local alignment search tool (BLAST)-annotated hydrolases/peptidases, and hits occurring in at least four out of nine analyses were chosen for heterologous production in *Escherichia coli*. The majority of hits derived from *M. lacteus* and all hits from *M. tolaasinivorans* were represented by homologs. Therefore, we focused on the production of five different annotated peptidases from *M. lacteus* (Fig. 4C). All candidates were produced in *E. coli* using a pET-28-based expression system, yet only one of them cleaves **1** (Fig. 4D, also see SI Appendix, Figs. S16 and S17) and was, thus, named tolaasin-degrading factor of *M. lacteus* (TdfL). A BLAST search revealed that the closest homologs of TdfL are also encoded in the genomes of *Mycetocola tolaasinivorans* and *Mycetocola saprophilus* (pairwise identity of 80.7% and 88.8%, respectively), both identified as tolaasin oligopeptidases. TdfL is annotated as a serine protease of the S9 prolyl oligopeptidase family, and we could identify the catalytic triad (Ser, Asp, His) in all three homologs (SI Appendix, Fig. S19).

To prove that these enzymes have the same activity, we heterologously produced the homolog encoded in the *M. tolaasinivorans* genome, named tolaasin-degrading factor of *M. tolaasinivorans* (TdfT) and tested the His-tagged protein in the *in vitro* assay. Like TdfL, TdfT effectively cleaves the lactone bond of **1**, whereas the

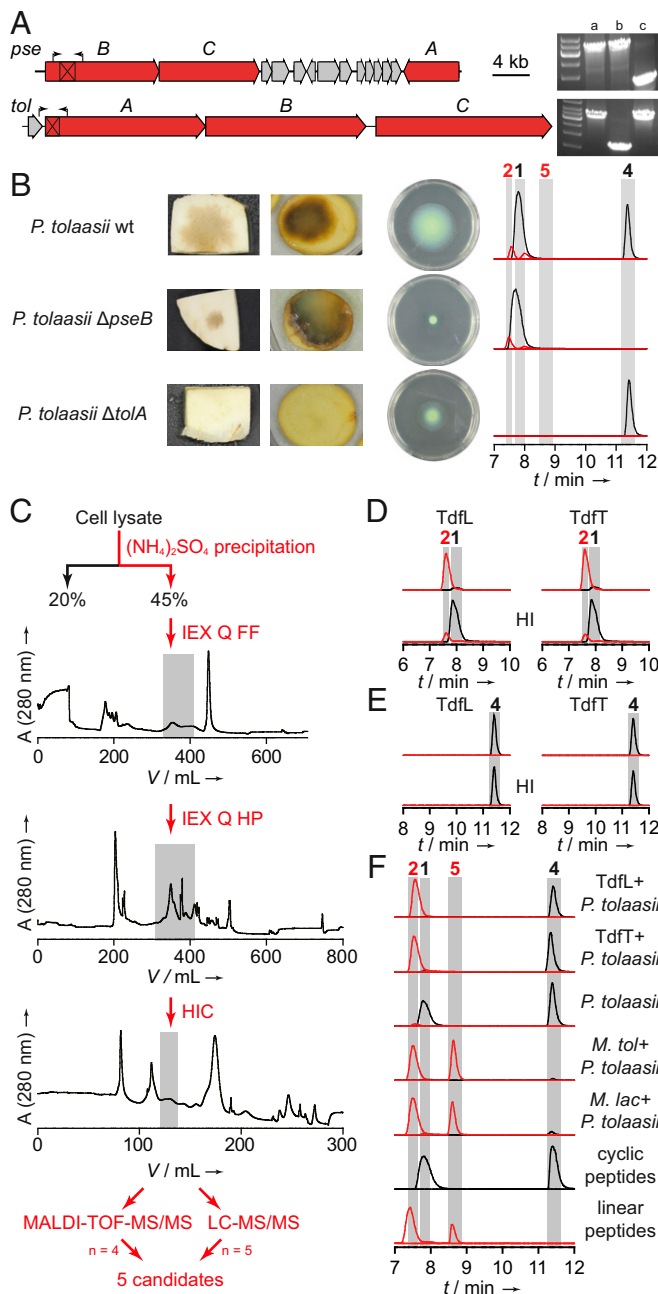


Fig. 4. Phenotypes of *P. tolaasii* knockout mutants, and identification of hydrolytic principle. (A) Gene clusters coding for pseudodesmin (*pse*) and tolaasin (*tol*) biosynthesis. Black boxes mark deleted areas, arrows indicate primer binding sites used for knockout verification. Agarose gel shows obtained amplicons from (a) *P. tolaasii* wild type; (b) *P. tolaasii* Δ *tolA*; (c) *P. tolaasii* Δ *pseB*. (B) Pathogenicity and swarming ability of *P. tolaasii* strains deficient in the production of tolaasin and pseudodesmin; EIC traces of 1, 2, 4, and 5. (C) Exemplary flow chart of bioactivity-guided fractionation approach. IEX: anion-exchange chromatography; Q: quarternary ammonium anion exchanger; FF: sepharose fast flow; HP: high performance; HIC: hydrophobic interaction chromatography. Gray boxes indicate active fractions that were further fractionated. (D) EIC traces of 1 (black, 994.1137 [M + 2H]²⁺) and 2 (red, 1003.1190 [M + 2H]²⁺) obtained from HPLC analyses of in vitro assays of TdfL and TdfT using 1 as a substrate. HI: heat-inactivated. (E) EIC traces of 4 (black, 1125.7129 [M + H]⁺) and 5 (red, 1143.7235 [M + H]⁺) obtained from HPLC analysis of in vitro assays of TdfL and TdfT using 4 as a substrate. (F) EIC traces of 1, 2, 4, and 5. In all LC traces: black: cyclic peptides 1 and 4; red: linearized peptides 2 and 5.

HI aliquot is inactive (negative control) (Fig. 4D). Since neither TdfL nor TdfT showed any hydrolytic activity toward 4 (Fig. 4E), we concluded that these hydrolases are substrate specific detoxifiers and that the helper bacteria harbor at least one other hydrolytic enzyme responsible for the cleavage of the swarming factor 4.

To exclude that 4 is hydrolyzed indirectly by a nonenzymatic reaction, we compared the activity of heat-inactivated cultures of both *Mycetocola* strains with that of nondeactivated cell suspensions toward hydrolysis of 4. For both strains, no formation of 5 could be observed using the heat-treated cultures, indicating that the cleavage of 4 is facilitated by an enzyme (*SI Appendix, Fig. S20*). We further supplemented growth medium with purified TdfL or TdfT, respectively, prior to inoculation with *P. tolaasii* to check whether the hydrolytic activity is maintained under cultivation conditions. LC-MS analyses of the culture extracts showed the same activity pattern as in the in vitro assays, i.e., linearization of 1 but not 4 (Fig. 4). These results illustrate that hydrolysis of 4 is performed by an independent enzyme and not indirectly, for example, via change in the pH in the medium (pH of cocultures 7.5–7.7).

Conclusion

In conclusion, we elucidated the role of the mushroom helper bacteria *M. tolaasinivorans* and *M. lacteus* that protect *A. bisporus* from brown blotch caused by *P. tolaasii*. For both helper strains, we identified homologous enzymes that cleave tolaasins, the major virulence factors of the plant and mushroom pathogen *P. tolaasii*, yielding the inactive linear forms. *M. tolaasinivorans* and *M. lacteus* not only disarm the causative agent of the mushroom disease, but also hamper the colonization of the hydrophobic mushroom cap by linearizing a second cyclic lipopeptide, 4 (Fig. 5). We assigned a biosynthetic gene cluster and a function to 4, which is essential for swarming and is, therefore, crucial for the dissemination of the pathogen. Our findings are an important addition to the currently known enzymatic strategies of bacteria to sabotage microbial virulence. The enzymatic cleavage of a lipopeptide has been reported as a mechanism that confers resistance in competing bacterial species (27), and lactonases are employed by bacteria to interfere with other species' quorum sensing signaling (28). Yet, the observation that helper bacteria halt and disarm pathogens attacking their host is unprecedented. In this ecological context, the enzymatic cleavage of cyclic lipopeptides is a remarkable antivirulence strategy, which is in line with rational approaches to block pathogenesis and chemotaxis (29–34). Understanding the mechanism by which helper bacteria protect their host not only sets the basis for the development

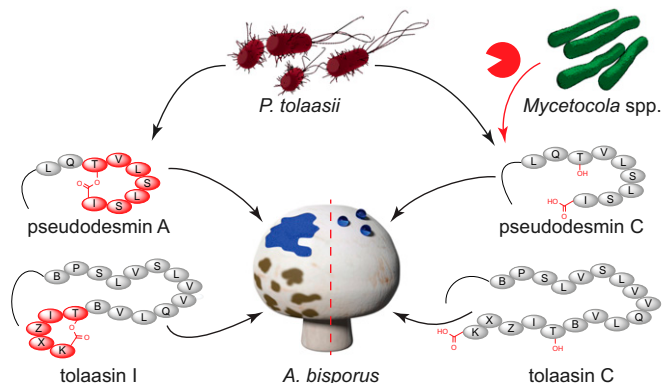


Fig. 5. *P. tolaasii* produces pseudodesmins as swarming agents and tolaasins as toxins against *A. bisporus*. In the presence of protective helper bacteria (*Mycetocola* spp.) the virulence factors are cleaved by enzymes, such as TdfL or TdfT. One letter code used to represent amino acids in the peptides. B: dehydrobutyryne; X: 2,4-diaminobutanoic acid; Z: homoserine.

of biocontrol strains used in agriculture, but also may inspire the design of protective symbionts for use in medicine.

Materials and Methods

Detailed materials and experimental methods can be found in the *SI Appendix* together with additional tables and figures. Genome sequences used in this study can be accessed via NCBI accession numbers NZ_RCUX00000000 (*M. tolaasinivorans*), NZ_RCUY00000000 (*M. lacteus*), NZ_PHHDD00000000, and NZ_CP020369 (both *P. tolaasii*).

Data Availability. All study data are included in the article and *SI Appendix*. Genome sequences used in this study can be accessed in the National Center

for Biotechnology Information (NCBI) GenBank, <https://www.ncbi.nlm.nih.gov/genbank/> [accession nos. NZ_RCUX00000000 (*M. tolaasinivorans*), NZ_RCUY00000000 (*M. lacteus*), NZ_PHHDD00000000, and NZ_CP020369 (both *P. tolaasii*)].

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