Biological Characterization of White Line–Inducing Principle (WLIP) Produced by *Pseudomonas reactans* NCPPB1311

Pietro Lo Cantore,¹ Silvia Lazzaroni,² Manuela Coraiola,³ Mauro Dalla Serra,³ Claudia Cafarchia,⁴ Antonio Evidente,² and Nicola S. Iacobellis¹

¹Dipartimento di Biologia, Difesa e Biotecnologie Agro Forestali, Università degli Studi della Basilicata, Viale dell'Ateneo Lucano 10, 85100 Potenza, Italy; ²Dipartimento di Scienze del Suolo, della Pianta e dell'Ambiente, Università di Napoli "Federico II", Portici (Napoli), Italy; ³CNR-ITC Istituto di Biofisica-Sezione di Trento, Povo (Trento); ⁴Dipartimento di Sanità e Benessere Animale, Università di Bari, Italy

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The biological activities of the lipodepsipeptides (LDP) white line–inducing principle (WLIP), produced by *Pseudomonas reactans* NCPPB1311, and tolaasin I, produced by *P. tolaasii* NCPPB2192, were compared. Antimicrobial assays showed that both LDP inhibited the growth of fungi—including the cultivated mushrooms *Agaricus bisporus*, *Lentinus edodes*, and *Pleurotus* spp.—chromista, and gram-positive bacteria. Assays of the two LDP on blocks of *Agaricus bisporus* showed their capacity to alter the mushrooms' pseudo-tissues though WLIP was less active than that of tolaasin I. Contrary to previous studies, tolaasin I was found to inhibit the growth of gram-negative bacteria belonging to the genera *Escherichia*, *Erwinia*, *Agrobacterium*, *Pseudomonas*, and *Xanthomonas*. The only gram-negative bacterium affected by WLIP was *Erwinia carotovora* subsp. *carotovora*. Both WLIP and tolaasin I caused red blood cell lysis through a colloid-osmotic shock mediated by transmembrane pores; however, the haemolytic activity of WLIP was greater than that of tolaasin I. Transmembrane pores, at a concentration corresponding to $1.5 \times C_{50}$, showed a radius between 1.5 and 1.7 ± 0.1 nm for WLIP and 2.1 ± 0.1 nm for tolaasin I. The antifungal activity of WLIP together with the finding that avirulent morphological variants of *P. reactans* lack WLIP production suggests that WLIP may play an important role in the interaction of the producing bacterium *P. reactans* and cultivated mushrooms.

Additional keywords: *Pseudomonas tolaasii*.

Brown blotch of *Agaricus bisporus* and the yellowing of *Pleurotus ostreatus* are major diseases of these cultivated mushrooms caused by *Pseudomonas tolaasii* (Paine 1919). Besides *P. tolaasii*, other bacteria account for symptom development on the above hosts. *P. reactans*, known in literature for its use in the "white line" assay for the specific identification of *P. tolaasii* (Wong and Preece 1979), was demonstrated to be involved in disease development in the cultivated mushrooms

Agaricus bisporus and *Pleurotus* spp. (Iacobellis and Lo Cantore 1997, 1998b; Lo Cantore 2001; Lo Cantore and Iacobellis 2002; Iacobellis and Lo Cantore 2003) including *P. eryngii* (Iacobellis and Lo Cantore 1998a, 2003), a mushroom species cultivated in southern Italy.

The pathogenicity of some strains of *P. reactans* has been reported in independent studies (Goor et al. 1986; Kim et al. 1995; Wells et al. 1996), but the pathogenicity of this bacterium was neglected for a long time. Recently, additional bacteria, such as *P. costantinii* sp. nov. (Munsch et al. 2002) or *Pseudomonas* sp. strain NZ17 (Godfrey et al. 2001), a bacterium apparently closely related to *P. syringae*, have been reported to be the causal agents of brown blotch disease of *Agaricus bisporus*.

Strains of *P. reactans* produce an extracellular substance called the white line–inducing principle (WLIP), which has the ability to interact with tolaasin I, a toxic lipodepsipeptide (LDP) produced in vitro by virulent strains of *P. tolaasii*, and forms a white precipitate in the white line assay (Wong and Preece 1979). WLIP, biological features of which are substantially unknown, is a LDP with a molecular weight of 1,125 Da composed of an N-terminal β -hydroxydecanoic acid and a peptide moiety of nine amino acids, six of which are in the D-form. The molecule contains a lactone ring between D-allo-threonine (D-Thr³) and N-terminal L-isoleucine (Ileu⁹) (Mortischire-Smith et al. 1991). WLIP is structurally similar to another LDP called viscosin (Laycock et al. 1991; Neu et al. 1990), except for the chirality of leucine in position 5 that it is D- in WLIP and L- in viscosin. The latter substance is a potent biosurfactant that appears important in the biology of the pectolytic *P. fluorescens* SH10-3B strains responsible for broccoli head rot (Laycock et al. 1991). Interestingly, viscosin does not produce any white precipitate when coincubated with purified tolaasin I (Rainey et al. 1992).

The fact that avirulent morphological variants of *P. reactans* were shown to lack WLIP production (Iacobellis and Lo Cantore 2003; Lo Cantore 2001) suggests that WLIP may play an important role in the virulence and pathogenicity of the above bacterium. The lack of information, either on the biological features of WLIP or on its role in virulence and pathogenicity and the mechanisms involved, prompts us to characterize it also in comparison to tolaasin I which is considered as the main virulence factor of *P. tolaasii*, as being responsible for the symptom development on mushrooms (Rainey et al. 1991; 1992; Soler-Rivas et al. 1999). It was demonstrated to have antifungal activ-

Corresponding author: Nicola S. Iacobellis; E-mail: iacobellis@unibas.it

This paper is dedicated to the memory of Gianfranco Menestrina.

*The e-Xtra logo stands for "electronic extra" and indicates that Figure 1 appears in color online.

ity, and this trait is apparently due to the ability of this molecule to disrupt cell membrane functions by the transmembrane pore formation (Brodey et al. 1991; Hutchinson and Johnstone 1993; Lo Cantore et al. 2003b; Rainey et al. 1991).

Here, we report on the results regarding the toxicity of WLIP in comparison with tolaasin I, either on whole sporophores or pseudo-tissue blocks of *Agaricus bisporus*, as well as on the antimicrobial activity toward fungi, chromista, and bacteria. Furthermore, the haemolytic activity of the above substances was analyzed and compared. Some preliminary results have already been reported (Lo Cantore 2001; Lo Cantore et al. 2003a). The determination of antifungal activity of WLIP and its mechanism of action may lead to a better understanding of the role of the above metabolite in the virulence of *P. reactans*.

RESULTS

General characteristics

of *P. reactans* NCPPB1311 and LDP purification.

Strains NCPPB1311 and NCPPB2192 showed the LOPAT characteristics of the group V_a of *Pseudomonas fluorescens* (Lelliott and Stead 1987) and the features of *P. reactans* and *P. tolaasii*, respectively, to oxidate nutrients, as evaluated with the differential nutritional scheme of Goor and associates (1986). Furthermore, generated C-source utilization profiles of the above bacteria, evaluated with the computer-assisted system Biolog (Biolog Inc., Hayward, CA, U.S.A.), were different and clearly distinguished *P. reactans* from *P. tolaasii* (Lo Cantore 2001).

Table 1. Antimicrobial activity^a of white line-inducing principle (WLIP) and tolaasin I towards fungi and chromista

Fungi and chromista	No. strains	M.I.Q. (μg)	
		Tolaasin I	WLIP
<i>Fusarium solani</i>	3	0.32 ^b	5.12 ^b
<i>F. oxysporum</i>	1	0.32	2.56
<i>F. graminearum</i>	1	0.16	— ^c
<i>Sclerotinia sclerotiorum</i>	2	0.32 ^b	1.28 ^b
<i>S. minor</i>	1	0.16	0.64
<i>Sclerotium rolfsii</i>	1	0.32	— ^c
<i>Rhizoctonia solani</i>	1	0.08	0.64
<i>Trichoderma viride</i>	1	0.16	1.28
<i>Alternaria alternata</i>	1	0.64	ND ^d
<i>Verticillium dahliae</i>	1	0.16	1.28
<i>Phoma lingam</i>	1	0.16	2.56
<i>Septoria tritici</i>	1	0.64	ND
<i>Botrytis cinerea</i>	2	0.16 ^b	1.28 ^b
<i>Phytophthora nicotianae</i>	1	0.32	— ^c
<i>P. citrophthora</i>	1	0.32	— ^c
<i>Armillaria mellea</i>	2	0.32 ^b	2.56 ^b
<i>Heterobasidium annosum</i>	2	0.16 ^b	1.28 ^b
<i>Agaricus bisporus</i>	2	0.08 ^b	1.28 ^b
<i>Pleurotus eryngii</i>	3	0.32 ^b	2.56 ^b
<i>Pleurotus ostreatus</i>	2	0.16 ^b	1.28 ^b
<i>Pleurotus nebrodensis</i>	1	0.32	— ^c
<i>Lentinus edodes</i>	2	0.32 ^b	1.28 ^b
<i>Geotrichum candidum</i>	1	— ^c	— ^c
<i>Rhodotorula pilimanae</i>	1	0.64	— ^c
<i>Candida albicans</i>	1	5.12	— ^f
<i>C. parapsilosis</i>	1	— ^g	— ^f
<i>Malassezia pachydermatis</i>	1	— ^g	— ^f
<i>Cryptococcus neoformans</i>	1	2.56	— ^f

^a Measured as minimal inhibitory quantity (M.I.Q.). The experiments were repeated at least twice with three replicates.

^b Mean values obtained with the different strains.

^c No inhibitory activity was observed with 10.24 μg of WLIP.

^d ND = not determined.

^e No inhibitory activity was observed with 1.28 μg of tolaasin I.

^f No inhibitory activity was observed with 25 μg of WLIP.

^g No inhibitory activity was observed with 10 μg of tolaasin I.

P. reactans NCPPB1311 and *P. tolaasii* NCPPB2192 formed a white precipitate in King's medium B (KB) agar when grown one toward the other and caused brown lesions on tissue blocks of *Agaricus bisporus*. *P. reactans* NCPPB1311 was less virulent than *P. tolaasii* NCPPB2192 (results not shown).

Cultures (48 h old) of *P. reactans* NCPPB1311, assayed for antimicrobial activity on KB medium, showed an activity rate of 400 units/ml toward *Bacillus megaterium* but did not inhibit the growth of *Rhodotorula pilimanae*. On the contrary, cultures of *P. tolaasii* NCPPB2192 showed an activity of 3,200 and 800 units/ml toward *Bacillus megaterium* ITM100 strains and *R. pilimanae* ATTC26423, respectively.

The chemical nature of WLIP and tolaasin I was ascertained by comparing their ¹H nuclear magnetic resonance (NMR) and mass spectra (MS) data to those reported in literature (Mortishire-Smith et al. 1991; Nutkins et al. 1991). Furthermore, the absolute stereochemistry of WLIP was also confirmed by X-ray analysis, which gave WLIP crystallographic data in perfect agreement to those described for the LDP (Han et al. 1992).

Antimicrobial activity of WLIP produced by *P. reactans* NCPPB1311.

Crystalline WLIP and high-performance liquid chromatography (HPLC)-grade tolaasin I showed a different capability to inhibit target microorganisms (Tables 1 and 2). In particular, WLIP inhibited the growth of *Bacillus megaterium* ITM100 with a minimal inhibitory quantity (M.I.Q.) of 0.32 μg (Table 2). The M.I.Q. of tolaasin I toward *Rhodotorula pilimanae* ATCC26423 and *Escherichia coli* ITM103 was 0.64 μg, whereas against *Bacillus megaterium* ITM100, it was 0.04 μg (Tables 1 and 2).

Further antimicrobial assays showed that crystalline WLIP inhibited the growth of fungi, chromista, and gram-positive bacteria at M.I.Q. values higher than those of tolaasin I and, on the contrary, did not inhibit the growth of most of the tested gram-negative bacteria (Table 1 and 2). The only exception was the strain of *Erwinia carotovora* subsp. *carotovora* that

Table 2. Antimicrobial activity^a of white line-inducing principle (WLIP) and tolaasin I towards bacteria

Bacteria	No. strains	M.I.Q. (μg)	
		Tolaasin I	WLIP
Gram positive			
<i>Bacillus megaterium</i>	1	0.04	0.32
<i>Rhodococcus fascians</i>	2	0.16 ^b	1.28 ^b
<i>Curtobacterium flaccunfaciens</i>	1	0.16	0.64
<i>Clavibacter michiganensis</i> subsp. <i>sepedonicus</i>	1	0.32	0.64
<i>C. michiganensis</i> subsp. <i>michiganensis</i>	2	0.32 ^b	0.64 ^b
Gram negative			
<i>Escherichia coli</i>	1	0.64	— ^c
<i>Pseudomonas syringae</i> pv. <i>phaseolicola</i>	1	1.28	— ^c
<i>P. tolaasii</i>	1	— ^d	— ^c
<i>P. reactans</i>	1	1.28	— ^c
<i>P. corrugata</i>	1	0.64	— ^c
<i>Agrobacterium tumefaciens</i>	1	0.64	— ^c
<i>Erwinia herbicola</i>	1	0.64	— ^c
<i>E. carotovora</i> subsp. <i>carotovora</i>	1	0.32	5.12
<i>Xanthomonas campestris</i> pv. <i>phaseoli</i>	1	1.28	— ^c

^a Measured as minimal inhibitory quantity (M.I.Q.). The experiments were repeated at least twice with three replicates.

^b Mean values obtained with the different strains.

^c No inhibitory activity was observed with 10.24 μg of WLIP.

^d No inhibitory activity was observed with 1.28 μg of tolaasin I.

was inhibited at M.I.Q. = 5.12 µg (Table 2). No activity, either toward the yeast *R. pilimanae* or *Candida albicans*, *C. parapsilosis*, *Malassezia pachydermatis*, and *Cryptococcus neoformans*, yeast-like fungi responsible for systemic and cutaneous mycoses in animals and humans, was observed even when 10.24 or 25.00 µg of the substance was used.

Tolaasin I inhibited the growth of filamentous fungi and gram-positive bacteria and showed a small activity toward some gram-negative bacteria (Table 2). Of particular interest is the activity shown by tolaasin I toward some soil-inhabiting phytopathogenic fungi and chromista and the cultivated mushrooms *Agaricus bisporus*, *Pleurotus* spp., and *Lentinus edodes*. The M.I.Q. toward fungi and chromista ranged between 0.08 and 0.64 µg with strains of *Agaricus bisporus* and *Rhizoctonia solani*, the most sensitive among fungi used in this study. Besides *Bacillus megaterium*, other phytopathogenic gram-positive bacteria also were quite sensitive to the toxin, with a M.I.Q. ranging from 0.04 to 0.32 µg (Table 2). Among gram-negative bacteria, as expected, the strain of *P. tolaasii* was resistant to tolaasin I. In contrast to previous studies, tolaasin I was found to inhibit the growth of gram-negative bacteria belonging to genera *Erwinia*, *Escherichia*, *Agrobacterium*, *Pseudomonas*, and *Xanthomonas*. *E. carotovora* subsp. *carotovora*, *E. herbicola*, *Escherichia coli*, *A. tumefaciens*, *P. syringae* pv. *phaseolicola*, *P. corrugata*, *X. campestris* pv. *phaseoli*, and *P. reactans* strains showed a M.I.Q. ranging from 0.32 to 1.28 µg (Table 2). Furthermore, tolaasin I inhibited the growth of *C. albicans* and *C. neoformans*, but the sensitivity of the above fungi was lower when compared with that of phytopathogenic fungi. Under the applied assay conditions, no activity regarding the growth of *C. parapsilosis* or *Malassezia pachydermatis* was observed.

Effect of WLIP on tissue blocks and whole sporophores of *Agaricus bisporus*.

Assays of the two LDP WLIP and tolaasin I on tissue blocks of *Agaricus bisporus* showed that the deposition on their surface of a drop of solution containing 1.28 µg of WLIP caused brown sunken lesions (Fig. 1). Under the same assay condi-

tion, a similar effect was observed when drops of solution containing 0.08 µg of tolaasin I were used. Similar results were obtained when such LDP were deposited on the surface of whole sporophore caps (data not shown).

Haemolytic activity of WLIP and the effect of osmoprotectants.

Both WLIP and tolaasin I caused red blood cell (RBC) lysis. In both the agarose plate assay and the assay in solution, the haemolytic activity appeared to be higher for WLIP than for tolaasin I. The minimal haemolytic quantity (M.H.Q.) of WLIP and tolaasin I evaluated in the agarose plate assay were 0.64 and 2.56 µg, respectively (Table 3). Haemolytic activity of tolaasin I, as opposed to WLIP, appeared to be temperature dependent (Table 3). In the case of WLIP, incubation at 25 or 37°C did not influence the bovine RBC lysis, which in both treatments showed the same feature as estimated by the diameter of the clear zone. In contrast, tolaasin I was more active at 37°C. The temperature effect was ob-

Table 3. Minimal haemolytic quantity of white line-inducing principle (WLIP) and tolaasin I at different temperatures

Lipodepsipeptide quantity (µg)	Average diameter of the haemolysis area (mm) ^a			
	Tolaasin I		WLIP	
	25°C	37°C	25°C	37°C
10.24	8	16	24	24
5.12	7	11	22	22
2.56	7	7	18	18
1.28	0	0	11	11
0.64	0	0	9	9
0.32	0	0	0	0

^a Determined in agarose plate assay. The experiments were repeated at least twice with three replicates.

Fig. 1. Brown lesions on tissue blocks of *Agaricus bisporus* (lower three blocks in each treatment), caused by deposition of 5-µl solutions containing **A**, 5.12 µg of white line-inducing principle and **B**, 0.64 µg of tolaasin I, respectively. On upper blocks, 5 µl of sterile water was deposited.

Fig. 2. Dose dependence of the haemolytic activity of white line-inducing principle (WLIP) (squares) and tolaasin I (circles) after 4 h at room temperature. Dotted lines are best-fit experimental data points with Hill equation. Hill coefficients are 6.3 and 8.0 for tolaasin I and WLIP, respectively. Inset: Set of traces recorded on the microplate reader of kinetics of haemolysis of red blood cells (RBC) exposed to two-step sequential dilutions of tolaasin I (left) and WLIP (right), from top to bottom. Starting concentration (top panels) were 100 µg/ml tolaasin I and 6.25 µg/ml WLIP. The decrease of turbidity over time indicated the disappearance of intact RBC. All panels have the same linear axis, ranging from 0 to 4 h and from 0 to 110 mOD for x and y axes, respectively.

served at quantities of 10.24 or 5.12 μg but not at the M.H.Q. (2.56 μg) of the toxin.

In the solution assay, both tolaasin I and WLIP caused human RBC lysis (Fig. 2). WLIP was more effective than tolaasin I with regard to both C_{50} (i.e., the minimal concentration able to cause 50% haemolysis, which was close to 4.3 and 34.6 $\mu\text{g}/\text{ml}$ for WLIP and tolaasin I, respectively) and of the haemolysis rate (Fig. 2, inset). The Hill coefficient associated to the formation of the active unit appeared to be higher for WLIP (8 ± 2) than for tolaasin I (6 ± 1).

The haemolytic activity of both LDP can be prevented by adding osmoticants of adequate dimensions to the external medium. The results reported in Figure 3 suggest, at a concentration corresponding to $1.5 \times C_{50}$, a pore radius between 1.5 ± 0.1 and 1.7 ± 0.1 nm for WLIP and 2.1 ± 0.1 nm for tolaasin I.

The small molecular size of LDP suggests that such large pores could be formed by an aggregate of monomers, which is also consistent with the large Hill coefficients observed. The structure of the pore formed by WLIP appeared to be less stable with a more pronounced dependence on toxin concentration. In fact, at a higher toxin dose ($2 \times C_{50}$), a bigger osmoticant is necessary for full protection of RBC from lysis (Fig. 3, right bottom panel).

DISCUSSION

Growth in KB medium resulted in the production of high levels of the LDP WLIP and tolaasins by *P. reactans* NCPPB1311 and *P. tolaasii* NCPPB2192, respectively. However, the yield of WLIP (about 169 mg/l) was more than ten-fold higher than that of the tolaasin mixture (about 13 mg/l). Further purification of tolaasin by HPLC confirmed the previous indication (Shirata et al. 1995) that *P. tolaasii*, besides the already known tolaasins I and II (Nutkins et al. 1991), produced in vitro other related metabolites. Results on the chemical and biological characterization of tolaasins A to E, the new analogs of tolaasin I and II, have been recently reported (Bassarello et al. 2004; Lazzaroni et al. 2003).

The antimicrobial assays of crystalline WLIP and HPLC-grade tolaasin I showed also that WLIP is a LDP active in the growth inhibition of bacteria and fungi and confirmed this activity for tolaasin I, as previously reported (Rainey et al. 1991, 1992). Generally, the antimicrobial activity of tolaasin I was higher than that of WLIP. The gram-positive bacterium *Bacillus megaterium* proved to be the microorganism most sensitive to both tolaasin I and WLIP with a M.I.Q. of 0.04 and 0.32 μg , respectively. Also the other gram-positive bacteria used in this

Fig. 3. Haemolysis in the presence of osmotic protectants and Renkin fit. Upper panels: Representative traces of decrease in turbidity of a red blood cell suspension in the presence of different external polyethylene glycol (PEG) osmolites and constant lipodepsipeptide (LDP) concentrations (i.e., 100 and 6.25 $\mu\text{g}/\text{ml}$ for tolaasin I and white line-inducing principle (WLIP), respectively). PEG size is indicated next to each trace. Lower panels: Renkin representation of data collected in experiments similar to those reported in the upper panels. Curves through points are best fits according to Renkin equation (Schultz and Solomon 1961; Ginsburg and Stein 1987), which gives a pore radius for tolaasin I of 2.1 ± 0.1 nm. This value is not dependent on LDP concentration, at least at the concentrations used in this study (i.e., 50 to 100 $\mu\text{g}/\text{ml}$): the dotted lines represent the best fit of data collected at 76 $\mu\text{g}/\text{ml}$ of tolaasin I (triangles, $2 \times C_{50}$), solid lines refer to best fit of data collected at 50 (squares, $1.5 \times C_{50}$) and 100 $\mu\text{g}/\text{ml}$ (circles, $3 \times C_{50}$) of tolaasin I. On the other hand, WLIP shows different values of pore radii, ranging from 1.5 ± 0.1 to 1.7 ± 0.1 nm, depending on toxin concentration. Dotted lines are the best fit of data collected at 6.25 $\mu\text{g}/\text{ml}$ of WLIP (circles, $1.5 \times C_{50}$), and solid lines refer to best fit of data collected at 9 $\mu\text{g}/\text{ml}$ of WLIP (squares, $2 \times C_{50}$).

study were inhibited by both LDP (Table 2). The tested gram-negative bacteria were inhibited by tolaasin I but not by WLIP, except *E. carotovora* subsp. *carotovora* (Table 2).

Generally, fungi and chromista were inhibited by both LDP and, on average, tolaasin I was about eight- to tenfold more active than WLIP. Of interest is the fact that strains of *Agaricus bisporus*, *Pleurotus ostreatus*, *P. eryngii*, and *Lentinus edodes* were the most sensitive to both LDP. The assays of the two substances on tissue blocks and whole sporophores of *Agaricus bisporus* confirmed the antifungal activity of both WLIP and tolaasin I, which in fact, both caused significant brown and sunken lesions on the surface of the above *Agaricus* tissue blocks and on whole sporophores, although at different concentrations. Similar mushroom-tissue alterations were observed when WLIP solution was about 15 times more concentrated.

The results of this study clearly demonstrated that WLIP, like other LDP of bacterial origin (Bender et al. 1999), is toxic towards fungi, although the activity of this compound is generally lower than tolaasin I. The above findings suggest that WLIP, as already reported for tolaasin I (Brodey et al. 1991; Hutchison and Johnstone 1993; Rainey et al. 1991; 1992), may be important in the interaction with mushrooms. When considering the observed lower antifungal activity of WLIP, it is also necessary to remember that, at least in vitro, *P. reactans* NCPPB1311 produced a quantity of WLIP that is more than ten times higher than the quantity of tolaasin I produced by *P. tolaasii*-type strain NCPPB2192. It could be hypothesized that the lower antifungal activity of WLIP is compensated by the higher quantity produced in culture. So far *P. tolaasii* NCPPB1116, used by Rainey and associates (1991) and grown in conditions different from those adopted in this study, appears to produce higher levels of tolaasin. However, data on the possible generally different levels of LDP production in culture by strains of *P. reactans* and *P. tolaasii* are not available, and this feature needs to be confirmed with a representative number of strains.

Haemolysis assays showed that WLIP, as already reported for tolaasin I (Rainey et al. 1991), caused RBC lysis. This finding is in accord with the haemolytic activity of bacteria associated to cultivated mushrooms, including *P. reactans*, recently reported by Munsch and Alatossava (2002b). However, the assays clearly indicated the higher haemolytic activity of WLIP when compared with tolaasin I. Under assay conditions, the M.H.Q. of WLIP was more than four times higher than that of tolaasin I. WLIP and tolaasin I appear to cause RBC lysis through a colloid-osmotic shock, apparently due to transmembrane pore formation. In fact, in parallel studies, both LDP were also demonstrated to form channels in lipid vesicles (Coraiola et al., in press; Lo Cantore et al. 2003b).

The haemolytic activity of tolaasin I was, as previously reported (Rainey et al. 1991), temperature dependent. However, this effect was not observed in the case of WLIP. The higher temperatures might increase both the rate of tolaasin diffusion in solution and the membrane fluidity, facilitating the process of membrane destabilization by tolaasin I.

Haemolytic assays in aqueous solution showed that the M.H.Q. was about 3 and 12 µg/ml for WLIP and tolaasin I, respectively. In similar assays, Rainey and associates (1991) reported that a 1.5 µg/ml solution of tolaasin I determined a haemolytic effect. However, the latter experiments were performed on horse erythrocytes, at lower concentrations, and at 37°C.

A theoretical analysis of the protection experiments with the Renkin equation made it possible to assess the functional pore radius, which was between 1.5 ± 0.1 and 1.7 ± 0.1 nm for WLIP and 2.1 ± 0.1 nm for tolaasin I, respectively. These radius values are similar to those found for other LDP (Dalla Serra et al. 1999). The functional pore radius of tolaasin I, previously

estimated as being smaller (0.9 to 1.0 nm), according to reports by Rainey and associates (1991), could depend on the much lower tolaasin I concentration used in the above experiments. The small molecular size of LDP suggests that such large pores could be formed by an aggregate of monomers and, therefore, a pore diameter possibly dependent on the toxin concentration. For the smaller WLIP, this dependency is more marked and, at a higher toxin dose, a bigger osmoticant is necessary in order to fully protect RBC from lysis.

In conclusion, the findings reported in this paper indicate that WLIP is toxic to fungal as well as other cells. The activity of the above substances is possibly mediated by transmembrane pore formation, as demonstrated in the case of other LDP of bacterial origin (Bender et al. 1999; Menestrina et al. 2003). The antifungal activity of WLIP suggests that the substance—produced by *P. reactans* in such relatively high amounts, at least in vitro—may be important for the virulence and pathogenicity of the bacterium. The observation that phenotypic variants of *P. reactans* strains are avirulent and do not produce WLIP (Iacobellis and Lo Cantore 2003; Lo Cantore 2001) support the possibility that the latter compound may play an important role in the interaction of *P. reactans* with cultivated mushrooms. However, the high heterogeneity of the so-called *P. reactans* (i.e., bacterium associated with cultivated mushrooms and producing white precipitates when grown toward *P. tolaasii*) for several characteristics (Goor et al. 1986), including virulence (Lo Cantore 2001; Munsch and Alatossava 2002a), pyoverdine production (Munsch et al. 2000), and oxidative ability of potential nutrients evaluated with the computer-assisted system Biolog as well as repetitive extragenic palindromic-polymerase chain reaction fingerprinting (Lo Cantore 2001; N. S. Iacobellis et al. *unpublished data*), prompt further studies for a better characterization of the novel mushroom pathogen for an adequate taxonomic allocation.

MATERIALS AND METHODS

General characteristics of *P. reactans* NCPPB1311.

P. reactans NCPPB1311 and *P. tolaasii* NCPPB2192 were maintained under lyophilized conditions at 4°C. Subcultures were obtained by growing bacteria for 48 h on KB (King et al. 1954). Before use, the above bacteria were evaluated for the characteristics reported in the LOPAT (Lelliott and Stead 1987) and nutritional schemes (Goor et al. 1986), for the white line assay (Wong and Preece 1979), and for pathogenicity on tissue blocks of *Agaricus bisporus*, prepared following the procedure reported by Ercolani (1970). Furthermore, the bacteria were evaluated for their ability to oxidize carbon sources by utilizing computer-assisted Biolog (Biolog Inc., Hayward, CA, U.S.A.).

Purification of WLIP produced by *P. reactans* NCPPB1311.

P. reactans NCPPB1311 and *P. tolaasii* NCPPB2192 were grown at 25°C under shaking (180 rpm) in 500-ml Erlenmeyer flasks filled with 150 ml of liquid KB medium inoculated with 1.5 ml of a bacterial suspension containing about 10^8 CFU/ml. Every 24-h incubation, culture aliquots were centrifuged ($20,000 \times g$ for 15 min.), and subsequently, supernatants were filtered through Millipore membranes (0.22 µm). The resulting filtrates were evaluated for the presence of antimicrobial activity on *Bacillus megaterium* ITM100 and *Rhodotorula pilimanae* ATTC26423, according to a procedure reported by Lavermicocca and associates (1997). After 24 or 48 h, depending on the target organism, growth inhibition was evaluated, and the antimicrobial activity was expressed as units per milliliter. A unit is the quantity of the

metabolite dissolved in 10 µl that is capable of inhibiting the growth of the target microorganism in the area of deposition of the 10-µl drop (Sinden et al. 1971).

Tolaasins were isolated and purified from the cell-free culture filtrate, according to a modification of the method described by Peng (1986). The resulting tolaasin preparations, active in the inhibition of *Bacillus megaterium*, were further purified by reversed-phase HPLC on a Shimadzu instrument equipped

Table 4. Fungi, chromista, and bacteria used in the antimicrobial assays in this study

Target organisms	Strains	Source ^a
Fungi and chromista		
<i>Fusarium solani</i>	109, 114, 140	A. Zoina
<i>F. oxysporum</i>	219	A. Zoina
<i>F. graminearum</i>	ITM022016	A. Logrieco
<i>Sclerotinia sclerotiorum</i>	853, 902	A. Logrieco
<i>S. minor</i>	1185	A. Logrieco
<i>Sclerotium rolfsii</i>	1401	A. Logrieco
<i>Rhizoctonia solani</i>	1583	A. Logrieco
<i>Trichoderma viride</i>	ITM2685	A. Logrieco
<i>Alternaria alternata</i>	ITM2063	A. Logrieco
<i>Verticillium dahliae</i>	ITM1910	A. Logrieco
<i>Phoma lingam</i>	ITM2078	A. Logrieco
<i>Septoria tritici</i>	ITM2955	A. Logrieco
<i>Botrytis cinerea</i>	1636, ITM3715	A. Zoina, A. Logrieco
<i>Phytophthora nicotianae</i>	USB-F1	USB
<i>Phytophthora citrophthora</i>	USB-F2	USB
<i>Armillaria mellea</i>	196, 200	N. Luisi
<i>Heterobasidium annosum</i>	1, 2	N. Luisi
<i>Agaricus bisporus</i>	DS222, DS410	D. Sisto
<i>Pleurotus eryngii</i>	DS77, DS256, DS270	D. Sisto
<i>Pleurotus ostreatus</i>	DS226, DS245	D. Sisto
<i>Pleurotus nebrodensis</i>	DS260	D. Sisto
<i>Lentinus edodes</i>	DS46, DS93	D. Sisto
<i>Geotrichum candidum</i>	ITM104	ITM
<i>Rhodotorula pilimanae</i>	ATCC26423	ATCC
<i>Candida albicans</i>	Di.S.B.A.22	Di.S.B.A
<i>C. parapsilosis</i>	Di.S.B.A.12	Di.S.B.A
<i>Malassezia pachydermatis</i>	Di.S.B.A.10	Di.S.B.A
<i>Cryptococcus neoformans</i>	Di.S.B.A.32	Di.S.B.A
Gram-positive bacteria		
<i>Bacillus megaterium</i>	ITM100	ITM
<i>Rhodococcus fascians</i>	NCPPB2551, NCPPB3067	NCPPB
<i>Curtobacterium flaccumfaciens</i>	NCPPB559	NCPPB
<i>Clavibacter michiganensis</i> subsp. <i>sepedonicus</i>	NCPPB2137	NCPPB
<i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i>	DPP2, DPP3	DPP
Gram-negative bacteria		
<i>Escherichia coli</i>	ITM103	ITM
<i>Pseudomonas syringae</i> pv. <i>phaseolicola</i>	NCPPB2571	NCPPB
<i>P. tolaasii</i>	NCPPB2192	NCPPB
<i>P. reactans</i>	NCPPB1311	NCPPB
<i>P. corrugata</i>	NCPPB2445	NCPPB
<i>Agrobacterium tumefaciens</i>	NCPPB2437	NCPPB
<i>Erwinia herbicola</i>	ICMP9900	ICMP
<i>E.carotovora</i> subsp. <i>carotovora</i>	ICMP5702	ICMP
<i>Xanthomonas campestris</i> pv. <i>phaseoli</i>	ICMP3035	ICMP

^a A. Zoina of the Università di Napoli "Federico II", Italy; A. Logrieco of the Istituto di Scienze delle Produzioni Alimentari, CNR, Bari, Italy; USB = Università Degu Studi Della Basilicata; N. Luisi and D. Sisto of the Università di Bari, Italy; ITM = Istituto Tossine e Micotossine, Bari, Italy; ATCC = American Type Microorganism Collection, U.S.A.; Di.S.B.A. = Dipartimento di Sanità e Benessere Animale, Università di Bari, Italy; NCPPB = National Collection Plant Pathogenic Bacteria, U.K.; DPP = Dipartimento di Protezione delle piante, Università Tuscia, Viterbo, Italy; and ICMP = International Collection of Microorganism from Plants, Auckland, New Zealand.

with HPLC pump LC-10 ADVP and UV-VIS detector SPD-10AV. Analytical (25 cm × 4.6 mm) and semipreparative (25 cm × 10 mm) Brownlee Aquapore RP-300 (C8) columns were used for the analysis and purification of the above preparations, respectively. A solvent system of water (0.1% trifluoroacetic acid, vol/vol) and CH₃CN with flow rates of 1.0 and 4.7 ml/min for analytical and semipreparative columns, respectively, was used for elution. Preparation aliquots of 10 or 1 µg were used for preparative or analytical separation, respectively. All solvents were degassed before use. The fractions obtained from the semipreparative column were collected, partially dried under reduced pressure, and then, were lyophilized.

The isolation and purification of crude WLIP was performed according to the procedure reported by Mortishire-Smith and associates (1991). Crystalline WLIP was prepared by diffusing water vapor into a solution of crude WLIP in methanol.

The identity of both tolaasin I and WLIP was ascertained by NMR and MS. NMR spectra were recorded at 600 or 400 MHz in dimethyl sulfoxide (DMSO)-*d*₆ on Bruker (Karlsruhe, Germany) spectrometers. The same solvent was used as internal standard. Electrospray ionization MS were recorded on a Perkin-Elmer (Norwalk, CT, U.S.A.) API 100 LC-MS, with a probe voltage of 5,300 V and a declustering potential of 50 V. The absolute stereochemistry of WLIP was determined by X-ray analysis carried out on a colorless crystal obtained as above. Data acquired on an Enraf-Nonius CAD4 (Delft, Holland) single-crystal diffractometer were compared with those previously described for the LDP (Han et al. 1992).

Antimicrobial activity of WLIP produced by *P. reactans* NCPPB1311.

The antimicrobial activity of pure LDP has been evaluated on gram-positive and -negative bacteria, fungi, and chromista, according to the procedure reported by Lo Cantore (2001). The target organisms used in this study are reported in Table 4.

Crystalline WLIP was taken up in solution in DMSO (5 mg/ml), and then, twofold serial solutions in a buffer (20 mM Tris-HCl, 0.85% NaCl, pH 8) containing 1,024, 512, 256, 64, 32, and 16 µg/ml were prepared. For some assays, a solution containing 2,500 µg/ml was used.

HPLC-grade tolaasin I was taken up in solution in sterile distilled water (1 mg/ml), and then, twofold serial solutions in a buffer (20 mM Tris-HCl, 0.85% NaCl, pH 7) containing 128, 64, 32, 16, 8, 4, 2, and 1 µg/ml were prepared.

Drops (10 µl) of twofold serial dilutions of pure LDP solutions were deposited on the surface of Petri dishes containing 10 ml of potato dextrose agar (PDA). After the drop had dried, plates were overlaid with 3 ml of soft agar (0.7%) in which the target bacterium had been suspended to a final concentration of about 10⁷ CFU/ml.

Fungi and chromista were grown at 25°C on PDA plates, and after 3- or 5-days incubation, depending on the growth rate of the target microorganism, 10-µl drops of twofold serial dilutions of pure LDP solutions were deposited about 1 cm from the border of the colony.

After 24 or 48 h of incubation at 25°C (37°C in the case of *Escherichia coli*), the lowest tested concentration capable of inhibiting the growth of the target organism was recorded.

The quantity of the substances still causing the inhibition of the target organisms was referred to as M.I.Q. and was expressed in micrograms. The experiments were repeated at least twice with three replications.

Effect of WLIP on tissue blocks and whole sporophores of *Agaricus bisporus*.

Tissue blocks of *Agaricus bisporus*, prepared following the procedure reported by Ercolani (1970), were treated with 5-µl

drops of twofold serial dilutions of LDP solutions prepared as above, were incubated at 25°C, and were observed by eye after 16 and 40 h. Drops of sterile distilled water were used as control. The assays were performed at least twice with three replicates.

Fresh sporophores of *Agaricus bisporus* were rinsed twice with distilled sterile water and were placed in aluminum trays previously sterilized in an autoclave at 121°C for 21 min. Drops (5 µl) of twofold serial dilutions of LDP solutions were deposited on each cap of whole sporophores, which were then incubated at 25°C. For each toxin solution, three 5-µl drops on three sporophores were used. Drops of sterile distilled water were used as control. The assays were performed at least twice.

Haemolytic activity of WLIP and the effect of osmoprotectants.

Haemolytic activity of pure LDP was evaluated by an agarose plate assay and in solution on fresh heparinized RBC rinsed three times in a buffer (30 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7).

Haemolytic activity of tolaasin I and WLIP on bovine RBC was evaluated on the agarose plate assay. Plates containing 10 ml of sterile agarose (1% agarose in haemolysis buffer containing 20 mM Tris-HCl, 0.85% NaCl, pH 7) and seeded with RBC suspensions, adjusted with the above buffer to give 0.2 absorbance values at 590 nm, were used. Drops (10-µl) of twofold serial dilutions of LDP solutions were deposited on the surface of plates that were then incubated at 25 and 37°C for 16 h. The diameter of the clear zones was measured, and the haemolytic activity was expressed as M.H.Q. The experiment was repeated at least twice with three replicates.

Haemolytic activity of tolaasin I and WLIP on human RBC was determined by measuring the turbidity variation at 650 nm after the addition of LDP to RBC suspension. A 96-well microplate reader (UVmax, Molecular Devices, Sunnyvale, CA, U.S.A.) as described by Dalla Serra and associates (1999) was used. LDP solutions were serially diluted twofold from well to well with the above haemolysis buffer. The experiment started after supplementing each well with 100 µl of RBC suspended in the haemolysis buffer (final volume 200 µl per well). The apparent initial absorbance of intact RBC was about 100 mOD, which corresponded to 2.4×10^6 cells per well. The percentage of haemolysis was calculated as follows:

$$\% \text{ haemolysis} = 100 (A_i - A_f) / (A_i - A_w)$$

where A_i and A_f are the absorbance at the beginning and at the end of the experiment (4 h) and A_w the minimum value obtained after hypotonical lysis with pure water. All the experiments, performed at room temperature using flat bottom transparent microtiterplates, were repeated at least twice with three replicates.

Pore radius estimation was performed by using osmolyte protectants with different hydrodynamic radii as described by Tejuca and associates (2001). A 30-mM final concentration of the following osmolytes was added to the haemolysis buffer: sucrose (0.54), raffinose (0.66), stachiose (0.75) (from Acros Organics, Geel, Belgium), and polyethylene glycols 400 (0.56), 600 (0.69), 900 (0.85), 1,000 (0.92), 1,500 (1.2), 2,000 (1.4), 3,000 (1.7), and 4,000 (2.0) (from Fluka). The hydrodynamic radius of each osmolyte in nm, taken from Schultz and Solomon (1961), Kuga (1981), and Carneiro et al. (1997), is reported in brackets after the compound names. The optical density data were analyzed by a theoretical model based on the Renkin equation (Renkin 1954) as described by Ginsburg and Stein (1987).

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