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Triggering of the Antibacterial Activity of *Bacillus subtilis* B38 Strain against Methicillin-Resistant *Staphylococcus aureus*

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Abstract When cultured in minimal growth medium, the B38 strain of *Bacillus subtilis* did not exhibit any antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) clinical isolate. Coculturing B38 strain with viable MRSA cells weakly increased antibacterial activity production (20 AU/ml). Addition of dead MRSA cells in a B38 culture, increased by 8-fold the *B. subtilis* strain antibacterial activity reaching 160 AU/ml against MRSA strain. This antibacterial activity recovered from cell-free supernatants was stimulated by an autoinducing compound which is sensitive to the action of proteinase K suggesting a proteinaceous nature. This compound was heat-stable till 80 °C and showed a molecular mass around 20 kDa as determined by SDS-PAGE. These results suggest that the production of antibacterial compounds by B38 strain is dependent on the amount of the autoinducing compound.

Keywords Antibacterial activity · Autoinducing compound · Induction · MRSA · *Bacillus subtilis*

Introduction

Microorganisms living in highly competitive environment generally produce secondary metabolites to inhibit the settlement of potential competitors. A new cultivation approach in

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which antagonistic microorganisms are challenged by exposure to other competitive species could be used for the enhancement of secondary metabolites production [1–5]. Several studies reported the induction of antimicrobial metabolite from lactic acid bacteria (LAB) especially when cocultured with living or dead Gram-positive bacteria cells [2, 4, 6, 7]. The production of some bacteriocins from LAB is a well-regulated process that could occur via a quorum-sensing mechanism which is mediated by small secreted peptides as pheromones [8–10]. These peptides are signal molecules that regulate bacteriocin production via two component signal transduction system including histidine protein kinase and a response regulator [11, 12]. This latter regulates the expression of various genes among which those encoding the peptide precursor of the signal molecule [12, 13]. Thus, pheromones known as autoinducing peptides generally induce their own synthesis [11, 14]. Unlike LAB microorganisms, only few reports dealt with the competition of living or heat-killed microorganisms for the production of antimicrobial activity from other producing microorganisms [1]. Multidrug resistant pathogenic microorganisms, especially methicillin-resistant *Staphylococcus aureus* strains (MRSA) are among the most problematic bacteria in public health. New antibiotics and therapy options are urgently needed to fight against MRSA infections. *Bacillus subtilis* species are known to produce antagonistic activities against many bacterial and fungal pathogens. In a previous study, we reported the isolation of a *B. subtilis* B38 strain that produced several antimicrobial compounds exhibiting a broad activity spectrum [15]. However, no antibacterial activity was found when the strain was grown in a minimal culture medium. Only a weak inhibitory activity (80 AU/ml) against a MRSA clinical isolate was found when the strain was cultured in TSB medium [15]. Optimization of the culture medium led to increase the antibacterial activity by 2-fold reaching 200 AU/ml [16]. In the present study, we investigated the putative induction of the antibacterial activity by coculturing the B38 strain with viable and dead (autoclaved) MRSA cells. Autoinduction of the antibacterial activity by consecutive cell-free supernatants was also undertaken.

Materials and Methods

Bacterial Strains and Media

B. subtilis B38 previously isolated from soil [15] was routinely maintained and cultured in TSB medium supplemented with 25% (*v*/*v*) glycerol at 30 °C. Under inducing conditions, this strain was grown in a minimal medium (MMYE) containing 0.7% $NH_4(SO_4)_2$, 0.1% NaCl, 0.1% K_2HPO_4, 0.01% $MgSO_4.7H_2O$, and 0.05% yeast extract. Bacterial strains used as inducers of the antibacterial activity were a MRSA clinical isolate provided by "Laboratoire de Microbiologie, Centre National de Greffe de Moelle Osseuse, Tunis, Tunisia" and *Pseudomonas aeruginosa* strain ATCC 27853. Indicator strains used for the detection of the antibacterial activity are listed in Table 1. Inducer and indicator strains were stored and grown in LB medium.

Antibacterial Activity Assay

Bacterial culture was centrifuged at 12,000×*g*, at 4 °C for 15 min, filtered through a 0.22 μm pore-size membrane, and cell-free supernatant was tested for antibacterial activity by using the disc-diffusion assay as previously described [15]. Antibacterial activity titer was expressed as activity units per milliliter ($AU\,mL^{-1}$) and corresponded to the reciprocal of the highest dilution showing a detectable inhibition zone of the indicator strain [17].

Table 1 Inhibitory spectrum of the antibacterial activity produced by *Bacillus subtilis* B38 cocultured with autoclaved MRSA or *Pseudomonas aeruginosa* cells

Indicator strains	Antibacterial activity (AU/ml)	
	Autoclaved MRSA	Autoclaved *P. aeruginosa*
Gram-positive bacteria		
Bacillus thuringiensis B15 LILM	300	280
Staphylococcus aureus ATCC 29213	160	140
Staphylococcus saprophiticus CP0002 LBCB	40	30
Staphylococcus aureus[a] LM	160	140
Staphylococcus epidermis[a] LM	80	60
Staphylococcus hominis[a] LM	40	30
Staphylococcus cohinii[a] LM	40	30
Listeria monocytogenes CIP 82110T	40	30
Enterococcus faecalis ATCC 29212	40	30
Gram-negative bacteria		
Escherichia coli ATCC 25922	40	30
Salmonella enteridis ATCC 13076	40	30
P. aeruginosa ATCC 27853	40	30

[a] Methicillin-resistant clinical isolates

ATCC American Type Culture Collection, *CIP* Collection de l'Institut Pasteur (Paris, France), *LILM* Laboratoire Interactions Légumineuses-Microorganismes (Centre de Biotechnologie de Borj-Cedria, Tunisia), *LM* Laboratoire de Microbiologie (Centre National de Greffe de Moelle Osseuse, Tunis, Tunisia)

Induction of the Antibacterial Activity by Viable MRSA Cells

Induction of the antibacterial activity of B38 strain was obtained by coculturing with MRSA inducer strain. Briefly, B38 cells (initial concentration: 10^7 CFU/ml) were cocultured with 10^6 CFU/ml MRSA cells in 50 ml of MMYE at 30 °C for 72 h. Aliquots were sampled every day to monitor the antibacterial activity against MRSA. After incubation for 24 h at 37 °C, bacteria were counted by plating out decimal dilutions of the coculture on LB medium or CHAPMAN stone agar (Pronadisa) for B38 and MRSA strain, respectively. Axenic cultures, cultures enriched with sterile medium and experiments with non antibiotic producer *E. coli* strain were used as negative controls.

Cell-free supernatant from culture of viable MRSA cells was also tested for its ability to induce antibacterial activity. Briefly overnight culture of MRSA cells was discarded by centrifugation (15,000×*g* at 4 °C during 20 min) and cell-free supernatant filtered through 0.22 μm membrane. 5% (*v*/*v*) was added to MMYE containing B38 strain. Incubation was performed as described above and aliquots were sampled every day to check for the antibacterial activity against MRSA. MMYE containing cell-free supernatant or B38 alone was used as negative control.

Induction of the Antibacterial Activity by Autoclaved Cells

Induction of the antibacterial activity of B38 cells was also performed using autoclaved MRSA or *P. aeruginosa* cells. 1 ml containing 10^6 CFU/ml of the inducer strain was

collected from overnight cultures and centrifuged at 12,000×*g* for 20 min at 4 °C. Bacteria were washed twice, re-suspended in sterile bidistilled water and autoclaved at 121 °C for 20 min. Bacterial pellets recovered by centrifugation at 12,000×*g* for 20 min at 4 °C were rinsed three times with sterile bidistilled water. Both supernatant containing cytoplasmic content [18] and cell pellet were added into 50 ml of MMYE inoculated with 10^7 CFU/ml of B38 cells and incubated at 30 °C for 72 h. Aliquots were sampled every day to check for antibacterial activity against MRSA strain. Bacterial count was performed as described above. MMYE containing either supernatant or autoclaved MRSA cells or B38 cells alone were used as negative controls.

To test the sensitivity of the inducer compound to proteolytic enzymes, autoclaved MRSA cell suspensions were treated with 1 U/ml proteinase K (invitrogen) [6]. The mixture was incubated overnight at 55 °C and then heated to 100 °C for 10 min to stop the reaction. Treated samples were tested for inducing antibacterial activity as described above. Untreated and heat-inactivated proteinase K samples were used as positive and negative control respectively.

Autoinduction of the Antibacterial Activity by Consecutive Cell-Free Supernatants

Cell-free supernatant $(CFS_0)_0$ resulting from B38 cell cultures enriched with autoclaved MRSA cells, was added (5% *v*/*v*) to 50 ml of MMYE medium inoculated with B38 cells (10^7 CFU/ml) and incubation conducted at 30 °C for 72 h. Resulting cell-free supernatant called CFS_1 was tested for its antibacterial activity and used for a second autoinduction. The supernatant of this second culture (CFS_2) was used for a third autoinduction. Cell-free supernatants (CFS_0, CFS_1, and CFS_2) added in MMYE medium without B38 cells were used as negative controls.

The amount of the autoinducing compound was determined in cell-free supernatants (CFS_0, CFS_1, and CFS_2) and expressed as autoinducing units per ml (AIU/ml). One autoinducing unit was defined as the amount of the autoinducer compound which promoted the production of the antibacterial activity by one activity unit. This was expressed as the reciprocal of the highest dilution exhibiting induction of the antibacterial compounds able to inhibit the indicator strain.

Purification of the Autoinducing Compound

One liter of MMYE containing autoclaved MRSA cells was inoculated with 10^7 CFU/ml B38 strain and incubation performed at 30 °C for 72 h. Cells were harvested by centrifugation at 12,000×*g* for 30 min at 4 °C and cell-free supernatant lyophilized. The resulting powder was re-suspended in bidistilled water and extensively dialyzed against bidistilled water at 8 °C for 24 h, using a 12 kDa cut-off dialysis membrane (Sigma, St. Louis, USA). Both retained and non retained fractions were further tested for their inducing activity. The active fraction was applied onto a Sep-Pak plus C18 cartridge (Waters, Division of Millipore Corp., Bedford, MA) and eluted using a discontinuous gradient of acetonitrile (0%, 20%, 40%, 60%, and 100%). It was then loaded onto DEAE-Sepharose column (Amersham Pharmacia Biotech) and elution performed using 10 mM ammonium acetate buffer at various pH i.e 7.5, 6, 5, 4, and 3. The resulting active fraction was further applied onto a C18 RP-HPLC column (250×4.6 mm) and elution performed using a linear gradient of acetonitrile/water containing 0.1% (*v*/*v*) trifluoroacetic acid over a 70 min period at a flow rate of 1 ml/min. All collected fractions were dried under vacuum, dissolved in bidistilled water and tested for their inducing activity.

Partial Characterization of the Autoinducing Compound

Sensitivity of the autoinducing compound towards proteinase K was tested on 2.5 ml of CFS_0 or CFS_1 as described above, except the reaction was stopped by heating at 80 °C for 30 min.

To analyze thermal stability, CFS_0 or CFS_1 were exposed to various temperatures ranging from 30 to 100 °C for 30 min or at 121 °C for 20 min and the induced antibacterial activity measured.

The purity of the autoinducing compound was tested on 16.5% Tris–tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tris–tricine SDS-PAGE) as described previously [19]. Appropriate SDS-PAGE standard markers (polypeptide, Bio-Rad) were used to estimate its molecular mass and bands were visualized using silver staining [20].

Thin Layer Chromatography and Bioautography Assays

Thin layer chromatography (TLC) analysis was performed with precoated silica gel 60 F_{254} plates (20×20 cm; layer thickness, 0.20 mm; Merck) on aluminum supports. TLC was conducted using 10-fold concentrated cell-free supernatants and *n*-butanol-methanol-H_2O (39:10:20, *v*/*v*/*v*) as mobile phase. The active compounds were revealed by bioautography assay as previously described [15]. The developed TLC plates were placed into petri disc, covered with the inoculated soft LB agar with MRSA strain and incubated at 28 °C for 24 h. Active spots were detected after spraying an aqueous solution of methylthiazolyltetrazolium bromide (5 mg/ml).

Results and Discussion

Induction of an Antibacterial Activity by Coculture with Living Cells

B38 strain didn't produce any antibacterial activity against MRSA pathogenic strain when grown in minimal culture medium. Coculturing B38 strain with living MRSA cells weakly stimulated the production of antibacterial activity against MRSA (20 AU/ml) at 48 h and 72h of incubation without affecting their growth (Fig. 1a). Coculture reduced the viability of the MRSA strain suggesting a bacteriostatic effect of the antibacterial compound (Fig. 1a). TLC-bioautography analysis pointed out the presence of one active spot with an R_f value of 0.47 (Fig. 1b) and two other spots with R_f values 0.70 and 0.82 that were detected only under UV illumination at 254 nm because of their low amount (Fig. 1c). When B38 strain was cultured with MRSA cell-free supernatant, no antibacterial activity was detected which suggested that induction was not mediated by a secreted metabolite of the inducer strain. The presence of competing microorganisms is a well known environmental factor affecting the production of antibacterial compounds [12, 21, 22]. Interestingly bacteriocin production from LAB such as *Lactobacillus plantarum* NC8 or J23 was obtained by coculturing with other species and was strictly dependent on bacterial cell-wall associated proteins [2, 4]. Conversely, production of an antimicrobial activity from marine bacteria was enhanced by signal molecules secreted by terrestrial inducer strains [1].

Induction of an Antibacterial Activity by Autoclaved MRSA Cells and Activity Spectrum

Coculturing B38 strain with autoclaved MRSA cells highly increased the antibacterial activity till 160 AU/ml (Fig. 2a), which is similar to that previously obtained in optimized

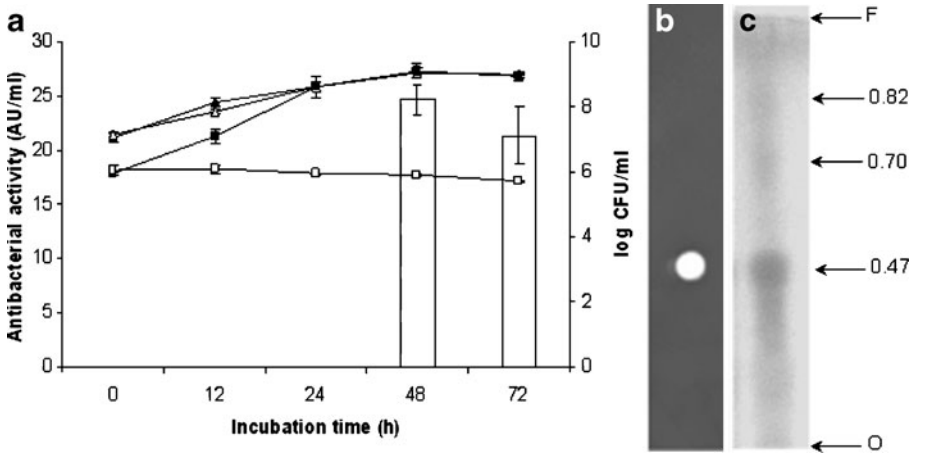

Fig. 1 Induction of an antibacterial activity by coculturing *Bacillus subtilis* B38 with viable MRSA cells (**a**); TLC-bioautography analysis of the antibacterial activity (**b**); detection of the antibacterial compounds under UV light at 254 nm **(c)**. 2 µl of 10-fold concentrated extract of *B. subtilis* B38 were deposited on TLC plate. The *arrow* indicated the R_f value of the growth inhibition zone of the indicator MRSA strain. *O* origin, *F* front of migration. *Columns* represented the antibacterial activity against MRSA (AU/ml) and the *curves* the growth (log CFU/ml) of *B. subtilis* B38 alone (*filled triangles*) and coculture (*open triangles*) and of MRSA alone (*filled squares*) and coculture (*open squares*). The standard error based on three repetition values was calculated and expressed as the *error bar*

medium [16]. No effect on B38 growth was observed which is in accordance with previous reports [2, 23].

Treatment of autoclaved MRSA cells with proteinase K completely abolished its ability to induce antibacterial activity suggesting its proteinaceous nature. TLC analysis confirmed the presence at higher amount (Fig. 2b) of three antibacterial compounds previously described [15].

Disruption of cell membrane might be a necessary step for the availability of the inducing compounds. Such compounds are not secreted into the culture medium since cell-free supernatant from inducer bacteria alone was inactive. Moreover, the induction was not due to a cytoplasmic compound since a culture enriched with supernatant from autoclaved cells did not exhibit any antibacterial activity. However, this activity increased after addition of cell debris to coculture and decreased after treatment with proteinase K. This suggests that the inducer might be cell-membrane associated proteins.

It has been previously reported that autoclaved *Lactococcus lactis* cells were able to induce the production of plantaricin NC8 by *L. plantarum* [6, 7]. Furthermore, the production of secondary metabolites by some marine bacteria also increased after coculturing them with heat-killed *S. aureus* cells [1]. Likewise, heat-killed fungi (120 °C for 20 min) increased the production of antifungal compounds by *Streptomyces* US80 [24].

In our case, the antibacterial activity induced by autoclaved MRSA or *P. aeruginosa* cells exhibited a wide spectrum as it was highly active against *Bacillus thuringiensis* B15 (280–300 AU/ml) and MRSA species (30–160 AU/ml) and to a lesser extent against *Enterococcus faecalis*, *Listeria monocytogenes*, or Gram-negative bacteria as *Escherichia coli*, *Salmonella enteridis*, and *P. aeruginosa* (30–40 AU/ml) (Table 1). As the inhibitory spectra were similar whatever the inducer strain used, one can speculate that the same antibacterial compound is produced, which confirmed previous data obtained for plantaricin NC8 [2]. In addition the inhibitory profile was the same than that previously described in optimized conditions [16].

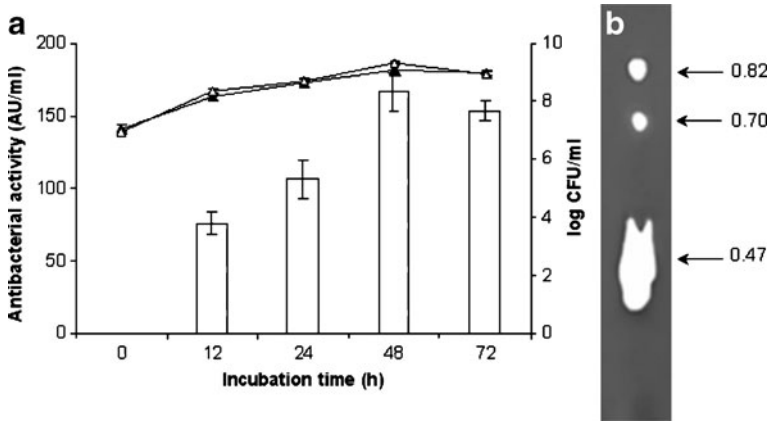

Fig. 2 Induction of the antibacterial activity by coculture of *Bacillus subtilis* B38 with autoclaved MRSA cells **(a)**; TLC-bioautography analysis of induced compounds **(b)**. *Columns* represent the induced antibacterial activity by coculture of *B. subtilis* B38 with autoclaved MRSA cells. *Curves* represent bacterial growth (log CFU/ml) of *B. subtilis* B38 alone (*filled triangles*) and cocultured with autoclaved MRSA cells (*open triangles*). The antibacterial activity was tested against the indicator MRSA strain. The standard error based on three repetition values was calculated and expressed as the *error bar*

Autoinduction of the Antibacterial Activity by Consecutive Cell-Free Supernatants

Cell-free supernatant from B38 strain cultured with autoclaved MRSA cells called CFS_0 was tested for its autoinducing activity (Table. 2). The addition of 5% CFS_0 induced antibacterial activity till 128.7 AU/ml after 72 h of incubation (Fig. 3a). It has already been reported that cell-free supernatant from *L. plantarum* cocultured with several Gram-positive bacteria induced NC8 bacteriocin, suggesting the presence of an autoinducing compound [2]. The second CFS_1 was also able to induce antibacterial activity (58.66 AU/ml) although to a lesser extent (2-fold lower) than CFS_0. The decrease in antibacterial activity is likely linked to the lower amount of autoinducing compound in CFS_1 (5 AIU/ml) when compared with CSF_0 (29 AIU/ml). CFS_2 supernatant having no autoinducing compound was no more effective (Table 2). The antibacterial activity induced with consecutive CFS_0 and CFS_1 supernatants was lower than that obtained with autoclaved cells (166.7 AU/ml). Thus, no significant effect on B38 growth was observed (Fig. 3a, b). TLC-bioautography analysis showed that the production of the compound with an R_f value of 0.47 decreased in consecutive supernatants (CFS_1 and CFS_2)

Table 2 Antibacterial and autoinducing activities in consecutive supernatants of B38 strain

Cell-free supernatants	Autoinducing activity[a] (AIU/ml)	Antibacterial activity[a, b] (AU/ml)
CFS_0	29.33±2.61	166.66±13.06
CFS_1	5.33±2.61	128.66±5.69
CFS_2	0	58.66±2.61

CFS_0 cell-free supernatant from coculture of *Bacillus subtilis* B38 and autoclaved cells of MRSA, *CFS_1* cell-free supernatant from *B. subtilis* B38 culture induced with CFS_0, *CFS_2* cell-free supernatant from *B. subtilis* B38 culture induced with CFS_1

[a] The antibacterial and autoinducing activities are the means of three repetition experiments

[b] The antibacterial activity was tested against the indicator MRSA strain

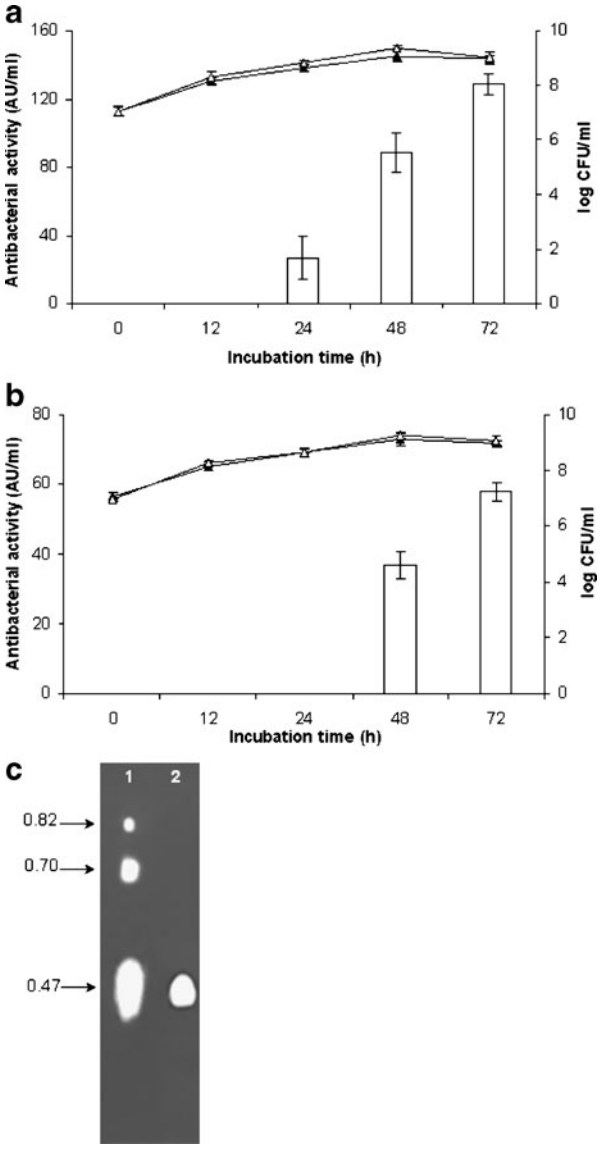

Fig. 3 Autoinduction of the antibacterial activity from *Bacillus subtilis* B38 by CFS_0 **(a)**, CFS_1 (**b**), and TLC-bioautography analysis **(c)**. *CFS_0* cell-free supernatant from *B. subtilis* B38 cocultured with autoclaved MRSA cells (*1*), *CFS_1* cell-free supernatant from *B. subtilis* B38 culture induced with CFS_0 (*2*). *Columns* represented the antibacterial activity (AU/ml) against MRSA. *Curves* represented the bacterial growth (log CFU/ml) of *B. subtilis* B38 alone (*filled triangles*), with cell CFS_0 or CFS_1 (*open triangles*). The standard error based on three repetition values was calculated and expressed as the *error bar*

whereas those with R_f values of 0.70 and 0.82 were undetectable in CFS_2 (Fig. 3c). The decrease in the production of antibacterial compounds by B38 in consecutive CFSs is inherent to the decrease in the amount of autoinducing components (Table 2). Accordingly, it has been shown that the production of plantaricin NC8 was also affected after culturing *L. plantarum* NC8 with consecutive CFSs, due to the reduction in autoinducer amount [2].

Table 3 Purification steps of the autoinducing compound

Purification steps	Total activity (AIU)	Specific activity (AIU/ml)	Purification fold	Recovery (%)
Growth medium	32,000	32	1	100
Dialysis	24,000	48	1.5	75
Sep-Pak C18	12,500	500	15.62	39
DEAE-Sepharose	9,000	900	28.12	28.12
C18 RP-HPLC	3,000	1,500	46.87	9.37

Purification and Partial Characterization of the Autoinducing Compound

The autoinducing compound was purified from CFS_0 and data are summarized in Table 3. Only the retained fraction from a 12 kDa cut-off dialysis membrane exhibited a specific activity of 48 AIU/ml. The resulting fraction was applied onto Sep-Pak C18 cartridge and active compound eluted at 60% acetonitrile (F60) exhibited a specific activity of 500 AIU/ml. F60 was further fractionated using DEAE-Sepharose and active compound eluted with 10 mM ammonium acetate pH 4 reaching a specific activity of 900 AIU/ml. Purification to near homogeneity was achieved after C18 RP-HPLC step (Fig. 4) reaching a specific activity of 1 500 AIU/ml corresponding to a 47-purification fold (Table 3).

Purified compound was heat-stable till 80 °C, inactive beyond 100 °C and sensitive towards proteinase K. Its molecular mass was around 20 kDa as determined by SDS-PAGE (Fig. 4 inset). This autoinducer appeared as different from the antibacterial compounds previously described [15], these latter being unaffected by autoclaving and proteinase K treatment. Altogether data suggest that this newly described autoinducer could act via different molecular mechanisms than previously reported for several Gram-positive bacteria including *Lactobacillus* [2, 9, 10, 25], *Carnobacterium* [23, 26], and *Enterococcus faecium* [27, 28]. Most of them are small stable cationic peptides containing 19 to 26 amino acid residues that are post-translationally processed and are sometimes modified [14, 21]. Generally these molecules have no other function than being implicated in interspecies communication [12], but exceptions include the lantibiotics nisin and subtilin which have not only signaling activity but also show antimicrobial activity [21, 29]. In addition to the regulation process of antimicrobial metabolite production, quorum sensing in Gram-positive bacteria could include genetic competence in *B. subtilis* [30] and *Streptococcus*

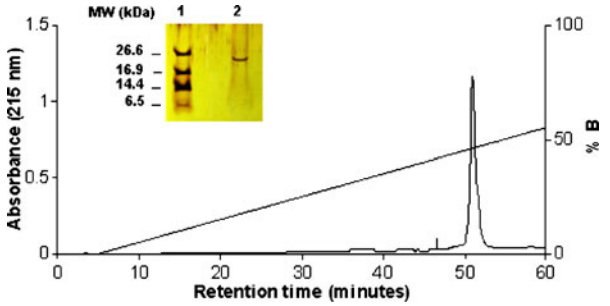

Fig. 4 Chromatogram of the autoinducing compound after C18 RP-HPLC. *Inset*, 16.5% Tris–tricine gel electrophoresis of the purified autoinducing compound; *lane 1*, low molecular weight markers (Bio-Rad); *lane 2*, autoinducing compound

pneumoniae [31] or virulence in *S. aureus* [32]. Quorum-sensing system of *Bacillus* species is composed of two regulatory proteins, Com P (histidine kinase) and Com A (response regulator) and an extracellular Com X pheromone composed by five to ten amino acid residues [33, 34]. Interestingly the same regulatory process is involved in the synthesis of the antimicrobial surfactin by *B. subtilis* [35].

Conclusions

B. subtilis B38 strain produced high level of antibacterial activity when stimulated by autoclaved MRSA or *P. aeruginosa* cells. An autoinducer compound secreted into cell culture medium has been purified to near homogeneity and partially characterized. Further work aiming to investigate the chemical structure as well as the molecular mechanism of this autoinducing compound is currently in progress. It will be interesting to test the effect of this autoinducer on interspecies communication and on antimicrobial production of related bacteria especially those used as biopreservative against spoiling microorganisms.

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