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Antibiotics Production by *Bacillus subtilis* BG2571A Modulate Antimicrobial Activity in the Healthcare Sector

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ABSTRACT

Antibiotics are considered as weapons against some diseases. The utilization of antibiotics over the last decades have generated a strong selective pressure for the emergence of multi-resistant strains and nosocomial infections. Biofilm phenotype was demonstrated as a key parameter in spreading infections, especially in hospitals and healthcare units. Biofilm phenotype was associated with a high tolerance to exogenous stress, and treatments with conventional antibiotics were usually ineffective at eradicating them. Thus, the development of novel anti-biofilm drugs is actually of the upmost importance. Here, the antimicrobial and anti-biofilm activities towards pathogenic microorganisms of a set of non-ribosomaly synthetized peptides and polyketides isolated from *B. subtilis* BG2571A culture supernatant were presented.

Keywords: *Bacillus, antibiotics, anti-biofilm activity*

1. INTRODUCTION

Despite continuing efforts, the increasing prevalence of resistance among pathogenic bacteria to common antibiotics has become one of the most significant concerns in modern medicine [**1**]. Incidences of hospital-acquired and community-acquired antibiotic resistant *Staphylococcus aureus* infections have risen dramatically in recent years, with almost 50 % of hospital acquired *Staphylococcus aureus* infections. In 2010, the Infectious Diseases Society of America launched its 10 × 20 initiative, calling for a global commitment to new antibacterial drug development, with the goal of ten new antibiotic agents by the year 2020 [2].

Therefore, surveillance programs have been developed at different levels to control pathogens spreading in hospitals and, at the same time, to collect useful data to develop innovative thwarting strategies to prevent healthcare diseases [3]. However, occurrence of infections in hospital care units increases continuously and represents for the future insolvable challenges in treating infection with the conventional drug arsenal [4]. These healthcare associated diseases are most of the time caused by the contamination of hospital devices, surfaces, air and water by pathogenic bacteria [5]. The abusive use of antibiotics over the last decades have generated a strong selective pressure for the emergence of multi-resistant strains, exemplified by methicillin resistant *Staphylococcus aureus* (MRSA) that is insensitive to beta-lactame antibiotics, including penicillins and cephalosporins, or vancomycin-resistant *enterococci* (VRE). Moreover, recent studies shed light on the role of microbial biofilms in the diffusion and the persistence of microorganisms in environment, notably in hospitals, surgery rooms and intensive healthcare units [6]. Biofilm consists of densely packed microbial cells embedded in a self-synthesized extracellular polymeric matrix, composed mainly of polysaccharides, that is attached to a tissue or a surface [7]. It is the predominant life style of microorganisms in most natural, industrial and clinical environments. Biofilm phenotype is associated with a high tolerance to exogenous stress, and treatments with conventional antibiotics are usually ineffective at eradicating them [8].

All of these point out the urgent needs for antibiotics with alternative mode of action [9]. The recent advances in genome sequencing have highlighted the genus *Bacillus* as an unexpected source of antibiotic-like compounds. For some of them, such as *Bacillus subtilis*, more than 5 % of the genome has been found potentially devoted to the synthesis of polyketides (PKs), nonribosomal peptide (NRPs), bacteriocins and other unusual antibiotics [10, 11]. NRPs and PKs are synthetized by multimodular enzymatic complexes by elongation of activated monomers of amino and hydroxyl acid building blocks, respectively [12, 13, 14]. Difficidins, macrolactones, bacillaenes surfactins,

fengycins and iturins represent some important products of NRPS and PKS complexes [10]. Their mechanisms of action are related with the inhibition of peptide synthesis or the disruption of the membrane integrity [11]. Actually, the efficacy of some NRPs and PKs against pathogenic fungi, multi-resistant *Staphylococci*, including MRSA was ascertained [15]. Thereon NRPs and PKs could be regarded as an interesting alternative to conventional antibiotics to combat multidrug resistant bacteria. However, there is still an important lack of knowledge on their antimicrobial activity, especially regarding the biofilm phenotype. The objective of this research was to evaluate the antimicrobial activity of metabolites from *Bacillus subtilis* BG2571A against pathogenic microorganisms, with special emphasize on their anti-biofilm activity. This first characterization of strain BG2571A highlights a possible novel practical application of some antibiotics, in the prevention of microbial contamination of the hospital environments.

2. MATERIALS AND METHODS

2.1. Strains and media

Strain BG2571A was isolated from a surgery room at the Prince Régent Charles Clinic (Bujumbura, Burundi) using surface contact plates (RODAC plate, Oxoid, Rodano, Italy) filled with Plate Count Agar medium (PCA, see below). Different strains used for antimicrobial susceptibility and biofilm inhibition tests were *Bacillus cereus* ATCC17778, *Pseudomonas aeruginosa* ATCC15442, *Aeromonas hydrophila* ATCC7966 and *Aspergillus niger* ATCC9642. The different culture media were: Tryptone Soy Broth (TSB), Plate Count Agar (PCA), *Aeromonas* Selective Medium Base (ASMB), Mannitol Egg Yolk Polymyxin Agar (MEYPA), *Pseudomonas* Agar Base (PAB), Sabouraurd Dextrose Agar (SDA). They were all purchased from Oxoid. Landy medium was as described elsewhere [16]. Phosphate buffered solution (PBS, pH 7.3) contained NaCl 8 g/l, KCl 0.2 g/l, Na_2HPO_4 1.15 g/l, KH_2PO_4 0.2 g/l.

2.2. Strain identification

Strain BG2571A was identified by recA and recN sequence analysis [17]. Total DNA was prepared according to the protocol [18]. RecA and recN were amplified from genomic DNA using primer pair recAf/recAr (TGAGTGATCGTCAGGCAGCCT / TTCTTCATAAGAATACCACGAACCGC) and recNf/recNr (CTTTTGCGATCAGAAGGTGCGTATCCG/GCCAT TATAGAGGAACTGACGATTTC). Sequences were processed with Vector NTI software (Invitrogen) and similarity search was performed using BLAST algorithm against the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3. Influence of media on antibiotics activities

Antimicrobial activity was estimated by a modified agar diffusion assay [19]. Briefly, PCA plates were first homogeneously surface inoculated with 100 µl (0.5 McFarland unit) of the different sensitive cell suspensions. An isolated BG2571A colony grown for 24 hours was then spotted with a 1 µl microstreaker at the center of each plate. Antimicrobial activities were estimated by measuring the diameter (in mm) of the growth inhibition zone after 48 h of incubation at 30 °C. Experiments were performed in duplicate.

2.4. Culture in microtiter plate

Liquid cultures were performed in microtiter plates (flat-bottom 96 wells, Becton-Dickinson) in TSB medium as described [20] unless stated otherwise. Plate inoculations were performed from standardized cell suspensions obtained by diluting a 14 h preculture to a turbidity at 600 nm equivalent to one McFarland unit. For co-cultures experiments, 230 µl of culture medium were inoculated with 20 µl of both BG2571A and the sensitive cell suspensions. For monocultures, 250 µl of culture medium were inoculated with 20 µl of sensitive cell suspensions. Microtiter plates were incubated for 48 h at 30 °C [21].

2.5. Biofilm assessment

Biofilm quantification was performed by staining cells with crystal violet as described elsewhere [22, 14]. Briefly, after discarding the culture broth, attached cells were rinsed three times with PBS before being fixed with 250 µl of ethanol 96 % for 15 minutes. After ethanol removal and plate drying, 250 µl of crystal violet solution (2 %, w/v) were added to each well. Crystal violet was removed after a 5 min staining period and the wells were rinsed three times with distilled water and dried. The residual crystal violet was dissolved in 250 µl of a 33 % acetic acid solution [23] and the absorbance of this solution was measured at 570 nm using a microplate reader (ELX 800, Biotek).

2.6. Biomass quantification

For cell quantification, viable cell counts were performed as described [24]. Briefly, cultures were conducted on selective solid media as follow: ASMB for *A. hydrophyla*, PAB for *P. aeruginosa*, MEYPA for *B. cereus* and SDA for *A. niger*. For planktonic bacteria, 100 µl of culture broth from a 48 h culture were plated on selective medium after adequate dilutions. For viable cell enumeration in the biofilm, culture broth was first eliminated and the biofilm was rinsed twice with PBS. Attached cells were then recovered by swabbing with a sterile cotton-tipped swab (VWR-PBI International) rinsed with 2 mL of PBS and released from the biofilm by vigorous vortex mixing for 5 min at full speed as described elsewhere [25]. The resulting cell suspension was then plated on adequate selective medium after appropriate dilutions. Cell counts were performed after 48 h of incubation at 37 °C for bacteria and 25 °C for *A. niger*. Results were

expressed in CFU/ml for the planktonic phase and in CFU/cm^2 for the biofilm. A reduction of 1000-fold (log reduction magnitude of 3) or more in viable cell counts was considered as a significant antimicrobial effect [26]. In order to assess the effective correlation between plate counts and crystal violet staining, a Pearson correlation index was calculated for both mono- and co-cultures using standard methods.

2.7. Strain biological activities assessment

To assess the biological activity of BG2571A culture supernatant, a 24 h *A. hydrophila* biofilm was washed three times with PBS. After what 250 µl of supernatant from a standardized BG2571A cell suspension were added per well. For the negative control, 250 µl of fresh TSB was used. Biomass quantifications in the planktonic phase and in the biofilm were determined as described above after 4 h and 24 h of incubation at 30 °C. The supernatant from BG2571A culture was sterilized by filtration (Millipore Durapore, 0.22 µm pore size) before use. Experiments were performed in four replicates.

2.8. Identification of biologically active compounds

To produce biologically active compounds, strain BG2571A was grown in Landy medium at 37 °C for 72 h. Samples were extracted from the culture supernatant by solid phase extraction using C18 cartridges (900 mg, Alltech) . After binding and subsequent washing steps with MilliQ water (5 bed volume), metabolites were eluted with methanol (2 bed volume), dried under vacuum and resuspended in 100 µl of methanol. Resulting samples were analysed by reverse-phase high-pressure liquid chromatography (Waters Alliance 2695/diode array detector) coupled with single quad mass spectrometer (Waters SQD mass analyser) on an X-Terra MS 150*2.1 mm, 3.5µm column (Waters). Lipopeptides were eluted as described [27] whereas polyketides were eluted as described elsewhere [28]. The identity of each metabolite was obtained on the basis of the mass of molecular ions detected in the SQD by setting electrospray ionisation (both positive and negative mode) conditions in the MS as source temperature, 150 °C; desolvatation temperature, 325 °C; nitrogen flow, 550 l/min; cone voltage 80 V.

3. RESULTS

3.1. Strain identification

BG2571A strain identification was performed by recA and recN sequence analysis as previously described [28]. BLAST analysis showed that recN and recA sequences from strain BG2571A had 99 % and 100 % identity, respectively, with the sequence of *B. subtilis* DSM7, while scores of 88 % and 99 % were obtained for *Bacillus subtilis* genus (data not shown). Therefore, these results suggest that strain BG2571A belongs to *Bacillus subtilis*.

3.2. Evaluation of antimicrobial activity

Plate tests for antimicrobial susceptibility have clearly demonstrated that *B. subtilis* BG2571A was able to inhibit the growth of both some Gram positive and Gram negative bacteria, as well as pathogenic fungus. As shown in Table 1, *A. niger* and *A. hydrophila* were found the most sensitive microorganisms tested. Beside this, *P. aeruginosa* and *B. cereus* were found also sensitive, but in a lesser extent.

Table 1 Activity spectrum of *B. subtilis* BG2571A

Strain	Zone of inhibition (diameter in mm)
B. cereus ATCC 17778	8
P. aeruginosa ATCC 15442	6
A. hydrophila ATCC 7966	19
A. niger ATCC9642	33

ATCC: American Type Culture Collection (http://www.lgcstandards-atcc.org/)

3.3. Biofilm quantification and viable cell count

Preliminary experiments demonstrated, by mean of crystal violet staining, that all the sensitive strains tested are able to form biofilm *in vitro* on microtiter plates (data not shown). To further characterize the biological activity of *B. subtilis* BG2571A, cell counts of the susceptible strains were performed both in the planktonic phase and in the biofilm during mono and co-culture (i.e. in the presence of BG2571A) by mean colony numbering on selective medium. For viable cell counts from biofilm, we first assessed that our experimental protocol was accurate compared to crystal violet staining. Cell counts on selective medium, expressed in CFU/cm^2, were found significantly correlated to the value obtained by the staining method. Indeed, Pearson correlation indexes of 0.99 and 0.74 were obtained for mono- and co-culture, respectively (data not shown).

As shown in Figure 1, cell growth of *A. hydrophila* was significantly reduced when co-cultured with *B. subtilis* BG2571A. The reductions in specific cell counts between the mono and co-cultures were equal to 6 log of magnitude in average. By contrast, for *P. aeruginasa*, *A. niger* and *B. cereus*, a weaker or non-significant effect was observed (log reduction of 2, 2.1 and 2, respectively).

Co-culture with *B. subtilis* BG2571A also affected the biofilm formation for the sensitive strains. Specific cell counts of *A. hydrophila* and *A. niger* in the biofilm were significantly reduced in those conditions. Indeed, reductions in cell counts were by 4.6 and 3.2 log of magnitude for the two strains, respectively (Figure 2). By contrast, co-culture with BG2571A had little or no effect on biofilm formation for *P. aeruginosa* and *B. cereus*.

3.4. Effect of BG2571A supernatant in mixture culture

Even though planktonic cell growth and biofilm formation were affected in different manner to the sensitive strains when co-cultured with *B. subtilis* BG2571A, there is no experimental evidence for this effect being related to the production of antimicrobial compounds by BG2571A. Thereon, we further characterize this effect by culturing *A. hydrophila*, the most sensitive strain, in the presence of BG2571A culture supernatant. A significant reduction in cell counts could be observed in both the planktonic phase and in the biofilm upon treatment with BG2571A supernatant as shown in Figure 3. Cell counts from the biofilm were reduced by 5.3 and 4.8 log of magnitude in average after 4 h and 24 h of treatment, respectively. For the planktonic phase, which corresponds here to cells escaped from the biofilm, the observed effect was stronger with a reduction in cell viability of 7.8 and 7.6 log of magnitude, respectively.

3.5. Analysis of antimicrobial compounds produced by B. subtilis BG2571A

BG2571A culture supernatant collected after 72 h of growth in Landy medium was concentrated by solid phase extraction and analyzed by HPLC-ESI-MS. As shown in Table 2, six groups of mass peaks were detected. On the basis of data obtained from previous experiments [28], three of them were found to correspond to cyclic lipopeptides. Signals at *m/z* from 1031 to 1059, from 1472 to 1528 and from 1066 to 1080 were identified as surfactins, fengycins and iturins A homologues, respectively. Mass peaks at *m/z* of 1486 and 1514 and at 1500 and 1528 traduced the Ala/Val dimorphy of fengycin A and B. By contrast, the characteristic mass peaks corresponding to the calculated mass of the different homologues of mycosubtilin and bacillomycin D, the two other members of the iturin group, could not be detected. The three remaining groups of mass peaks were found to correspond to polyketides. Signals, at *m/z* 425, 511 and 525 were assigned to the molecular ions of macrolactin A, 7-o-malonyl macrolactin A and 7-o-succinyl macrolactin A, respectively. Signals at *m/z* 559, 583 and 605 were assigned to oxydifficidin, bacillaene A and bacillaene B based on data from the literature [29]. The characteristic peaks of the other secondary metabolites usually co-produced by *Bacillus* spp; i.e. bacilysin, its chlorinated derivative chlorotetain, the siderophore bacillibactin and the antimicrobial zwittermicin were not detected.

Table 2 Metabolite production of *B. subtilis* BG2571A detected by HPLC-ESI mass spectrometry

Metabolite	Observed mass peak	Assignment
Surfactin	1031 [M+Na]+	C13-surfactin
	1045 [M+Na]+	C14-surfactin
	1059 [M+Na]+	C15-surfactin
Fengycin	1472 [M+Na]+	Ala-6 C15-fengycin
	1486 [M+Na]+	Ala-6 C16-fengycin
	1500 [M+Na]+	Ala-6 C17-fengycin
	1514 [M+Na]+	Val-6 C16-fengycin
	1528 [M+Na]+	Val-6 C17-fengycin
Iturin A	1066 [M+Na]+	C14-iturin A
	1080 [M+Na]+	C15-iturin A
Macrolactin	425 [M+Na]+	Macrolactin A
	511 [M+Na]+	7-o-malonyl macrolactin A
	525 [M+Na]+	7-o-succinyl macrolactin A
Difficidin	559 [M-H]-	Oxydifficidin
Bacillaene	58 [M+H]+	Bacillaene A
	605 [M+Na]+	Bacillaene B

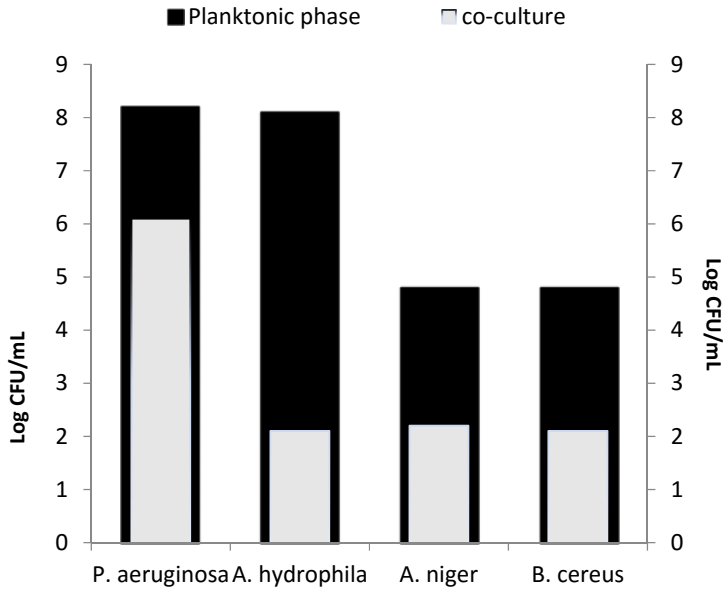

Figure 1: Cell concentration of the different sensitive strains, expressed in Log CFU ml^{-1}, in the planktonic phase during mono-culture (black) or co-culture with *B. subtilis* BG2571A (grey). Values are mean of three independent experiments.

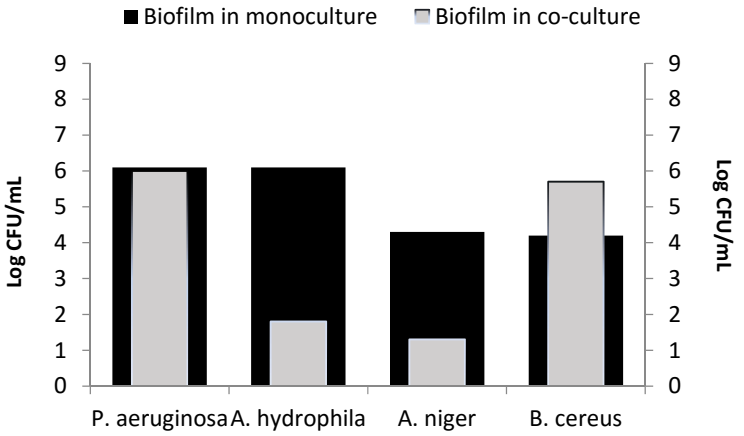

Figure 2: Cell concentration of the different sensitive strains, expressed in Log CFU ml^{-1}, in the biofilm during mono-culture (black bars) or co-culture with *B. subtilis* BG2571A (grey). Values are mean of three independent experiments.

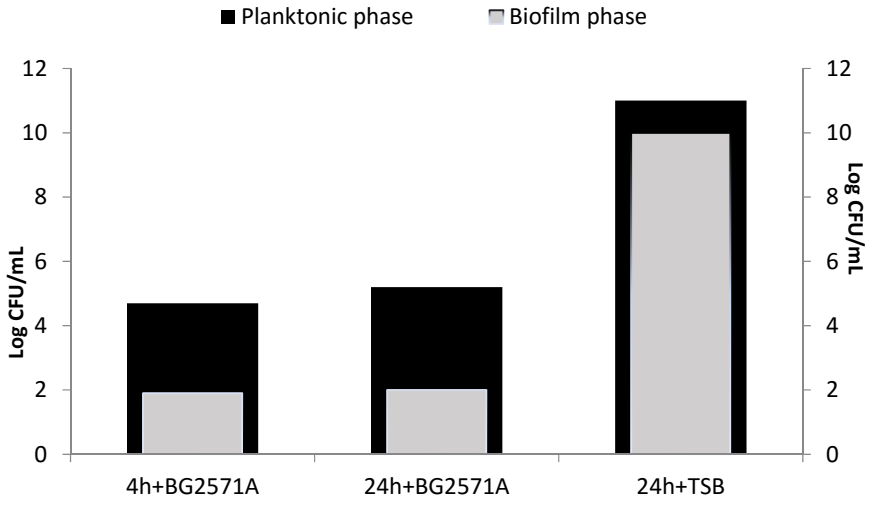

Figure 3: Cell concentration of *A. hydrophila*, expressed in Log CFU ml^{-1}, in the planktonic phase (black) and in the biofilm (grey) after 4 h and 24 h of incubation with *B. subtilis* BG2571A supernatant. TSB medium was used instead of BG2571A supernatant as negative control. Values are mean of four independent experiments.

4. DISCUSSION

The biofilm phenotype has been recognized as a key parameter in nosocomial infections. The formation of a highly hydrated extracellular polymeric phenotype or biofilm contributes to antimicrobial resistance by blocking the transport of antimicrobials through the biofilm matrix. Possible mechanisms for this to occur are by binding of the biofilm to them directly, as in the case of positively charged antibiotics, restricting their permeation and by restricting diffusion of larger antimicrobials [30]. Medical device-related infections are one of the most striking examples of biofilm-dependent infections. Any inserted medical devices could be colonized but intravenous catheters, due to their widespread use, are the most commonly device to be infected [31]. For instance, in European hospital, 56 % of bloodstream infections are catheter-associated [32]. Biofilm exhibits tolerance to biocides, chemotherapeutic agents and host-immune defenses. In consequences, biofilm-associated infections are extremely difficult to treat, rendering infections and chronic or recurrent diseases [31]. Development of preventing strategies, including the development of novel anti-biofilm drugs is of the upmost importance actually. The multiple modes of action utilized by antimicrobial peptides reduces the ability of microorganisms to develop resistance, with cidal activity also shown against bacteria resistant to standard antibiotics [33].

Among antibiotics, the cyclic lipopeptide daptomycin is the prototype molecule that has been approved by Food and Drug Administration in 2003 for the treatment of skin structure infection caused by pathogen Gram-positive, including methicillin-resistant *Staphylococcus aureus*, and in 2006 for the treatment of bacteremia [34]. However, the development of daptomycin resistance in *Enterococcus faecium* and *S. aureus* underlines the demand for daptomycin derivatives or novel related drugs. Among lipopeptide antibiotics, *B. subtilis* BG2571A was found here able to produce surfactins, iturins A, fengycins A and fengycins B. These cyclic lipopeptides are composed of seven (surfactin and iturin A) or 10 α-amino acids (fengycins) linked to a β-amino (iturins) or a β-hydroxy (surfactins and fengycins) fatty acid which may vary from C-13 to C-16 for surfactins, from C-14 to C-17 for iturins and from C-14 to C-18 for fengycins. These compounds have well recognized applications due notably to their antibacterial, antifungal, antiviral, antitumor and antimycoplasma activities[35,36,11,37].

In addition to these peptides, polyketides are the other dominant family of secondary metabolites having relevant bioactivities [11]. Macrolactin, difficidin and bacillaene were detected by LC-MS analysis in the culture supernatant of *B. subtilis* BG2571A. Macrolactins are macrolides containing three separate diene structure elements in a 24-membered lactone ring [38]. They are considered as potent antiviral, cytotoxic agents with antibacterial activity. Macrolactin A is able to inhibit murine melanoma cancer cells *in vitro* as well as the replication of herpes simplex viruses, and squalene synthase activity with, thus, possible applications in the prevention of cardiovascular disease by lowering cholesterol levels [39]. It also protects T lymphoblast cells against human immunodeficiency virus replication [38]. The macrolactin derivative 7-o-molonyl macrolactin had the ability to interfere with one or more stages of cell division of a number of multidrug-resistant Gram-positive bacteria such as

MRSA and VRE [40]. The hydroxyl group at C-15 was suggested to play an important role in the antibacterial activity of this compound [41]. Difficidin is an unsaturated 22-membered macrocyclic polyene lactone phosphate ester [42 with broad-spectrum antibacterial activity against aerobic and anaerobic bacteria. It was found effective to treat lethal bacteremia caused by *Klebsiella pneumoniae* in mice [43]. By using 4 radiolabelled amino acid, [45] demonstrated that difficidin was able to abolish protein synthesis *in vivo* and *in vitro*. Similarly to difficidin, bacillaene interfere with prokaryotic protein synthesis, probably by inhibiting the initiation step [45]. This compound is constituted by an open-chain enamine acid with an extended polyene system. It was found active on human pathogens such as *Serratia marcescens*, which is often associated with catheter-associated bacteremia; *Klebsiella pneumonia* or *Proteus vulgaris* as well as Gram-positive bacteria such as *S. aureus* [45].

While the antimicrobial activities of the above-mentioned compounds on planktonic bacteria is well documented in the literature, information on their activity on biofilm are scarce. As biocides antimicrobial peptides have the potential to eradicate the most resistant forms of clinically relevant biofilm forming pathogens. The formation of a highly hydrated extracellular polymeric phenotype or biofilm contributes to antimicrobial resistance by blocking the transport of antimicrobials through the biofilm matrix. Possible mechanisms for this to occur are by binding of the biofilm to them directly, as in the case of positively charged aminoglycoside antibiotics, restricting their permeation and by restricting diffusion of larger antimicrobials [46]. [47] demonstrated that surfactin is able to inhibit *Salmonella enterica* biofilm adhesion on pre-coated urinary catheters. They also report a similar effect for *Escherichia coli*, while this pretreatment was completely ineffective against *P. aeruginosa*. This anti-adhesion behavior of surfactin was also reported for *S. aureus* for pre-coated plastic device [48]. These authors also reported that fengycin was also able to inhibit bacterial biofilm formation, probably by interfering with cell membrane structure. In the light of this lack of information, we aimed to investigate more deeply this anti-biofilm activity. As a first step, we clearly demonstrated here that the different secondary metabolites produced by *B. subtilis* BG2571A are able to inhibit the growth of human pathogenic microorganisms.

5. CONCLUSION

The antimicrobial cocktail produced was found active against Gram negative, Gram positive and fungus such as *A. niger*. Moreover, this anti-biofilm activity was observed at the level of the biofilm formation, probably by inhibiting cell-adhesion, but also in the ability of this antimicrobial cocktail to inhibit the development of an existing biofilm as examplified with the BG2571A culture supernatant experiments. Lipopeptides such as polymyxin B and daptomycin are already utilized in topical formulations [49], therefore the potential might thus exist for the surfactins, fengycins and iturins containing peptides to be exploited similarly. Issues still remain with regard to the stability of peptide based formulations *in vivo* and the large scale production costs of these peptides. While the spectrum of the antimicrobial activities could be ascribed to the production of distinct antimicrobial compounds, how they act specifically on each on biofilm formation in combination remains to be characterized in more details. Experiments are still in progress to further characterize this synergism. However, this first report highlighted *B. subtilis* BG2571A as a candidate bacterium for control of pathogenic biofilm-former in the hospital healthcare units.

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