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Direct surfactin–gramicidin S antagonism supports detoxification in mixed producer cultures of *Bacillus subtilis* and *Aneurinibacillus migulanus*

Marina Rautenbach,[1] Hans André Eyéghé-Bickong,[1] Nicolas Maré Vlok,[1] Marietjie Stander[2] and Abré de Beer[1]

Correspondence
Marina Rautenbach
mra@sun.ac.za

[1]BIOPEP Peptide Group, Department of Biochemistry, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa

[2]Department of Biochemistry and LCMS-Central Analytical Facility, Science Faculty, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa

Antibiotic production as a defence mechanism is a characteristic of a wide variety of organisms. In natural evolutionary adaptation, cellular events such as sporulation, biofilm formation and resistance to antibiotics enable some micro-organisms to survive environmental and antibiotic stress conditions. The two antimicrobial cyclic peptides in this study, gramicidin S (GS) from *Aneurinibacillus migulanus* and the lipopeptide surfactin (Srf) from *Bacillus subtilis*, have been shown to affect both membrane and intercellular components of target organisms. Many functions, other than that of antimicrobial activity, have been assigned to Srf. We present evidence that an additional function may exist for Srf, namely that of a detoxifying agent that protects its producer from the lytic activity of GS. We observed that Srf producers were more resistant to GS and could be co-cultured with the GS producer. Furthermore, exogenous Srf antagonized the activity of GS against both Srf-producing and non-producing bacterial strains. A molecular interaction between the anionic Srf and the cationic GS was observed with circular dichroism and electrospray MS. Our results indicate that the formation of an inactive complex between GS and Srf supports resistance towards GS, with the anionic Srf forming a chemical barrier to protect its producer. This direct detoxification combined with the induction of protective stress responses in *B. subtilis* by Srf confers resistance toward GS from *A. migulanus* and allows survival in mixed cultures.

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INTRODUCTION

In diverse environmental microbial communities, various defence strategies have evolved to effectively compete for available resources. Peptide antibiotic production as a survival strategy is a characteristic of many micro-organisms, in particular bacteria from the genera *Lactobacillus* and *Bacillus* (Katz & Demain, 1977; Servin, 2004). Furthermore, survival via resistance to antibiotics from cohabiting micro-organisms is a natural evolutionary adaptation (D'Costa *et al.*, 2011) that occurs via two distinct strategies: constitutive (passive) resistance and adaptive (inducible) resistance.

Antimicrobial/antibiotic peptides are universal defence molecules, and micro-organisms produce numerous such peptides, either ribosomally or non-ribosomally (Sarika *et al.*, 2012; Walsh, 2004). Resistance, in particular total resistance, towards cationic antimicrobial peptides is limited (reviewed by Zasloff, 2002; Marr *et al.*, 2006), and therefore several antimicrobial peptides are still in consideration for the next generation of antibiotics (reviewed by Guaní-Guerra *et al.*, 2010; Baltzer & Brown, 2011). However, there is evidence for constitutive and adaptive resistance of bacteria towards antimicrobial peptides (reviewed by Ganz, 2001; Yeaman & Yount, 2003; Peschel & Sahl, 2006). Constitutive resistance is due to natural properties of a micro-organism that are present in the absence of peptide exposure. The molecular basis of many of the constitutive resistance observations towards antimicrobial peptides is unclear, although evidence points to differences in membrane structure, charge, shielding and energetics (reviewed by Yeaman & Yount, 2003). Adaptive resistance towards antimicrobial peptides depends on a triggered response that mainly involves several two-component regulatory mechanisms. This triggered response leads to bacterial reprogramming, which can entail a change in the cell membrane/cell wall/intracellular target or detoxification of the antimicrobial peptide by peptide-specific transport systems or by protease

Abbreviations: CD, circular dichroism; CMC, critical micelle concentration; ESMS, electrospray MS; GS, gramicidin S; Srf, surfactin; TFE, trifluoroethanol; UPLC-MS, ultraperformance liquid chromatography-linked MS.

activity (reviewed by Ganz, 2001; Yeaman & Yount, 2003; Peschel & Sahl, 2006). Furthermore, other cellular events such as sporulation (Schaeffer, 1969; Branda *et al.*, 2001) and biofilm formation enable some micro-organisms to survive environmental stress and resist antibiotics (Xu *et al.*, 2000; Mah & O'Toole, 2001; Davies, 2003).

Our study focuses on the sensitivity/resistance of several *Bacillus subtilis* strains to the lytic peptide gramicidin S (GS). *B. subtilis* has an intrinsic response that manifests itself in resistance to antimicrobial compounds (Butcher & Helmann, 2006; Mascher *et al.*, 2003). This takes place either by changes in the cell envelope (Cao & Helmann, 2004) or via detoxification by peptide-specific ABC transporter systems (Joseph *et al.*, 2002; Jordan *et al.*, 2008; Pietiäinen *et al.*, 2005) or proteases (Ellermeier & Losick, 2006). Furthermore, apart from the ability of *B. subtilis* to sporulate (Branda *et al.*, 2001), it has been shown to alter its cell surface properties and form biofilms (Kinsinger *et al.*, 2003), both resulting in a lower sensitivity to antimicrobial peptides (Cao & Helmann, 2004).

The anionic lipopeptide surfactin (Srf) has been implicated in both the sporulation (Cosby *et al.*, 1998; Nakano *et al.*, 1991) and the biofilm formation of its *B. subtilis* producer (Kinsinger *et al.*, 2003; Hofemeister *et al.*, 2004). Srf has other functions, such as acting as a signal molecule in the initiation of cannibalism and multicellularity/cell density increase (López *et al.*, 2009a, b). The cyclic Srf forms β-sheets and consists of a β-hydroxy fatty acid linked via a lactone bond to L-Glu-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu (Bonmatin *et al.*, 1994; Arima *et al.*, 1969). The competitor peptide in this study, GS, is produced by *Aneurinibacillus migulanus*. GS is a cyclic decapeptide with a β-sheet structure that is formed by its pentapeptide repeat, D-Phe-L-Pro-L-Val-L-Orn-L-Leu (Stern *et al.*, 1968; Laiken *et al.*, 1969). GS primarily acts as a defence molecule, but also retards the sporulation of its producer (Azuma & Demain, 1996; Nandi & Seddon, 1978). Both the anionic Srf and cationic GS have membranolytic activity (Maget-Dana & Ptak, 1995; Epand & Vogel, 1999) and are active against Gram-positive bacteria (Tsukagoshi *et al.*, 1970; Kondejewski *et al.*, 1996), as well as fungi (Midez *et al.*, 1989; Tsukagoshi *et al.*, 1970). However, Srf is generally less active than GS (this study), but possesses comparable haemolytic activity (Kracht *et al.*, 1999; Katsu *et al.*, 1988).

Srf and GS producer cultures have application in bio-control (Raaijmakers *et al.*, 2010; Bais *et al.*, 2004; Haggag, 2008) and may be important in the agricultural movement towards bio-control. The importance of bio-control and our search to find synergistic peptide combinations, such as iturin A and Srf (Thimon *et al.*, 1992; Maget-Dana *et al.*, 1992), motivated us to study the activity of GS and its producer *A. migulanus* on four *B. subtilis* strains, two Srf producers and two non-producers (Table 1). Srf is a multifunctional peptide, a characteristic that is shared with a number of other lipopeptides produced by bacteria (reviewed by Raaijmakers *et al.*, 2010). We will supply evidence for yet another function of Srf, namely that of a detoxifying agent that supports the resistance of *B. subtilis* towards GS from *A. migulanus*.

METHODS

Materials. Tryptone soy broth (TSB) was supplied by Merck and Difco Laboratories. Glucose and the components for Luria–Bertani (LB) broth and TGYM, tryptone, peptone, agar and yeast extract were supplied by Merck. Merck supplied the ethanol (>99.8 %), NaH_2PO_4 and Na_2HPO_4. Skimmed milk powder was supplied by Clover (Roodepoort, SA). Microtitre plates were from Nalge NUNC International. Culture dishes were from Quality Scientific Plastics USA. Srf, GS and 2,2,2-trifluoroethanol (TFE) were from Sigma. Acetonitrile (99.9 %, far-UV grade) and casein were from Romil and BDH, respectively. Analytical quality water was prepared by filtering water from a reverse osmosis plant through a Millipore MilliQ water purification system.

B. subtilis 168, OKB105 (*pheA1 sfp*), OKB120 (*pheA1 sfp srfA*::Tn*917*) and ATCC 21332 (*sfp*) were obtained from the Bacillus Genetic Stock Center (Ohio State University, OH, USA). *A. migulanus* ATCC 9999 and an additional culture of *B. subtilis*, ATCC 21332 (*sfp*), were obtained from the American Type Culture Collection (Manassas, VA, USA). Refer to Table 1 for more details.

Bacterial strain identity and verification of peptide production. Strain identities were confirmed using matrix assisted laser-desorption ionization time-of-flight (MALDI-TOF) MS analysis on a Bruker ultrafleXtreme (Bruker Daltonik) using a standardized methodology (Sogawa *et al.*, 2011). The ultrafleXtreme mass spectrometer was calibrated using the Bruker Daltonics bacterial test standard, as directed by the manufacturer's instructions. Bacterial cultures were grown overnight at 37 °C on tryptone soy (TS) agar (30 g TSB l^{-1}, 15 g agar powder l^{-1}). A single colony was deposited directly on an MTP BigAnchorChip 384 TF target plate (Bruker Daltonics). The bacterial sample was overlaid with 1 µl of a saturated solution of α-cyano-4-hydroxycinnamic acid in 30 % acetonitrile/0.10 % trifluoroacetic acid matrix. The samples were air-dried at room temperature to allow for co-crystallization with matrix. Samples were analysed using 5000 shots and a laser intensity of 45 %. Automatic data acquisition of mass spectra in the linear positive mode between 2 and 20 kDa was achieved using Flexcontrol software 3.0 (Bruker Daltonics). Spectrum peaks obtained were compared against the integrated spectral reference database using the MALDI Biotyper Software (Bruker Daltonics). Score values >2.0 were considered for classification.

The same protocol was followed to confirm the production of the peptide antibiotics by the strains *Bacillus* OKB105 and ATCC 21332 and *A. migulanus* ATCC 9999, with the only difference being the calibration standard. The ultrafleXtreme mass spectrometer was calibrated using PEG polymers to obtain a calibration range between 400 and 1400 Da. The bacterial samples were bombarded with 5000–20 000 shots and a laser intensity of 40 %. The mass peaks obtained were compared against MS data of Srf produced by *Bacillus* strains and GS produced by *A. migulanus* ATCC 9999 (Leenders *et al.*, 1999; Vater *et al.*, 2002, 2009).

Co-culture assays. Single colonies of *B. subtilis* 168, OKB120 and OKB105 were inoculated onto TS agar plates in close proximity (2–3 mm) to the GS-producing *A. migulanus* ATCC 9999. Due to the slow growth of *B. subtilis* ATCC 21332 on TS agar, this assay was performed on TGYM agar [0.5 % (w/v) peptone, 0.25 % (w/v) yeast extract, 0.1 % (w/v) glucose, 0.1 % (w/v) skimmed milk powder, 1.5 % (w/v) agar] for this bacterial strain. The plates were incubated

Table 1. Description of bacterial strains used in this study and their antimicrobial peptides, as detected by whole-cell MALDI-MS

NA, Not applicable.

Strain	Description	Antimicrobial peptide produced	Peptides detected with MALDI-MS (*m/z* of major molecular ions)
B. subtilis 168	Gram-positive; contains the Srf (Cosmina *et al.*, 1993) and fengycin synthetase operons (Tosato *et al.*, 1997); *srf* operon is not functional because it lacks a functional *sfp* gene (Kim *et al.*, 2000; Kunst *et al.*, 1997)	None reported	None detected
B. subtilis OKB105 (*pheA1 sfp*)	Gram-positive; strain 168 mutant with functional *sfp* gene and *srf* operon (Nakano *et al.*, 1988; Leenders *et al.*, 1999; Vater *et al.*, 2009); functional *sfp* gene from strain ATCC 21332 (Nakano *et al.*, 1988)	Srf	C_{13}-, C_{14}- and C_{15}-Srf with Na^+/K^+ adducts (1030.6, 1044,7 1046.6, 1058.6, 1060.7, 1074.7)
B. subtilis OKB120 (*pheA1 sfp srfA*::Tn*917*)	Gram-positive; strain OKB105 mutant with a mutation between modules 4 and 5 in the *srf* operon (Nakano *et al.*, 1988, 1991; Vollenbroich *et al.*, 1994; Vater *et al.*, 2009)	Tetrapeptide and shorter Srf fragments	C_n-β-OH-ELL and C_n-β-OH-EL with Na^+/K^+ adducts (494.5, 522.5, 602.6, 628.6)
B. subtilis ATCC 21332 (*sfp*)	Gram-positive; soil isolate that has functional *sfp* gene and *srf* operon (Arima *et al.*, 1968; Kluge *et al.*, 1988)	Srf	C_{13}-, C_{14}- and C_{15}-Srf with Na^+/K^+ adducts (1030.7, 1044.8, 1046.6, 1058.7, 1060.7, 1074.8, 1096.7)
A. migulanus ATCC 999	Gram-positive soil isolate; previously known as Nagano strain of *Bacillus brevis* (Stern *et al.*, 1968; Shida *et al.*, 1996)	GS	GS and its sodium adducts (1141.7, 1163.7, 1185.7)
M. luteus NCTC8340	Gram-positive	None reported	NA
E. coli HB101	Gram-negative	None reported	NA

for 24 h at 37 °C and then grown for an additional 72 h at room temperature. The interaction of the colonies was observed under a stereo microscope, and the images were recorded using a DCM-510 digital camera and ScopePhoto software (ScopeTek).

Dose–response assays. *B. subtilis* strains (168, OKB120, OKB105 and ATCC21332) were cultured from freezer or freeze-dried culture stocks on TS agar. *Escherichia coli* HB101 and *Micrococcus luteus* NCTC8340 were cultured from freezer stocks on LB agar or TS agar at 37 °C for 48 h before selected colonies were grown overnight in LB or TS broth. Target organisms were subcultured in TSB at 37 °C and grown to an OD_{620} of 0.6. Bacterial cultures were diluted to OD_{620} 0.2 for the dose–response assays in the 96-well plates (Lehrer *et al.*, 1991; du Toit & Rautenbach, 2000). A 90.0 µl volume of bacterial culture in each well was challenged with 10.0 µl GS over a dilution range from 50.0–0.20 µM. Alternatively, the bacterial culture was pre-incubated for 10 min with Srf (30 or 60 µM or a dilution range from 2 to 60 µM), before the addition of GS. Bacterial growth after 16 h was measured spectrophotometrically at 595 or 620 nm on a microtitre plate reader (note: contact with plastic was avoided as far as possible when handling Srf).

Curve fits and statistical analyses of data were done using GraphPad Prism 4 (GraphPad Software Inc.). Sigmoidal dose–response curves (variable slope with no weighing of data) were fitted to all assay results. Of the six or more determinations per concentration, only the mean was considered for fitting the curve. The IC_{50} was determined as described by du Toit & Rautenbach (2000).

Peptide interaction in the presence of target cells. As *M. luteus* does not produce any Srf, it was selected as a sensitive Gram-positive target for the investigation of peptide interaction in the presence of target cells. *M. luteus* was cultured as above and washed with 0.9 % NaCl by centrifugation and suspended in 0.9 % NaCl to OD_{620} 0.2. The cell suspension and controls (0.9 % NaCl) were then pipetted (190 µl) into a microtitre plate and incubated for 30 min at 37 °C with 30 µM Srf, 5 µM GS and a mixture of the two peptides (10.0 µl of treatment solution was used). The mixtures were then centrifuged for 8 min at 900 ***g***, and the supernatants (120 µl in triplicate) were analysed by ultraperformance liquid chromatography-linked MS (UPLC-MS). The remaining cells in the wells were then washed twice with 120 µl TSB, suspended in 120 µl TSB and incubated for 16 h at 37 °C before the survival was determined by OD620 measurement.

Electrospray MS (ESMS) and UPLC-MS of peptides and culture supernatants. Direct injection MS on peptides and UPLC-MS analyses of the peptide-treated *M. luteus* supernatants were done on a Waters Acquity ultraperformance liquid chromatograph connected to a Waters QTof Ultima mass spectrometer. Chromatographic separation was achieved on a Waters UPLC BEH C_{18} column (2.1 × 50 mm, 1.7 µm spherical particles, Waters), using a 0.1 % (v/v) trifluoroacetic acid (A) to acetonitrile (B) gradient [100 % A for 30 s, 0–30 % (v/v) B from 30 to 60 s, 30–60 % (v/v) B from 1 to 10 min, 60–80 % (v/v) B from 10 to 15 min at a flow rate 300 µl min^{-1}], followed by re-equilibration of the column to initial conditions. A capillary voltage of 3.5 kV was applied, with the source temperature set at 100 °C and cone voltage at 35 V. Data acquisition was in the positive mode, scanning through m/z=100 to 1999 (m/z is defined as the molecular mass to charge ratio). Representative scans were produced by combining the scans across the elution peak and subtracting the background.

Circular dichroism (CD). Analytical stock solutions (2.00 mM) of Srf and GS were prepared in ethanol/water (1 : 2, v/v) for CD studies. Peptides (10.0 µl) were then diluted to 10.0 µM in water (final volume was 2.00 ml, <0.2 % ethanol) before measurement. The

different ratios of peptide mixtures were prepared at least 30 min before analysis. CD spectra were obtained using a Chirascan spectropolarimeter (Applied Photophysics), and were collected at ambient temperature from 190 to 250 nm in water and 200 to 250 nm in TFE with a 1.00 cm path length. Data collection was set at over 0.05 s per step of 0.1 nm (5000 data points per nm), and three to five step scans were collected and averaged for each spectrum.

Mixed culture assays. To assess the survival in mixed cultures, cultures of *B. subtilis* OKB120 and *M. luteus* NCTC8340 were prepared from subcultured preparations and mixed in equal proportions according to c.f.u. counts. Cultures were then treated as described above under 'Methods, Peptide interaction in the presence of target cells', except that the treated cultures were diluted and spread on Luria agar plates. c.f.u. were counted after 48 h of growth at 37 °C.

RESULTS

Variable influence of the GS producer on *B. subtilis* strains

The *B. subtilis* strains showed typical *B. subtilis* colony morphology (Julkowska *et al.*, 2004), which was quite different from that of *A. migulanus* ATCC 9999 (Fig. 1). Although Srf production is linked to swarming (Kinsinger *et al.*, 2003; Kearns & Losick, 2003; Julkowska *et al.*, 2005), *B. subtilis* OKB120, producing lipopeptides with a partial Srf sequence (Vater *et al.*, 2009), and the non-producer *B. subtilis* 168 also showed some swarming behaviour (Fig. 1a–d). Only *B. subtilis* 168 showed visible sporulation (browning of colonies) over time under our growth conditions.

To assess the influence of the GS producer *A. migulanus* ATCC 9999 on the *B. subtilis* strains we co-cultured the different strains in close proximity (Fig. 1). The two non-producer strains, *B. subtilis* OKB120 and 168, grew away from the GS producer. *A. migulanus* ATCC 9999 outcompeted the non-producers, causing some decrease in colony growth with an inhibition zone at the interface between the colonies (Fig. 1a, b). The Srf producers *B. subtilis* 105 and *B. subtilis* ATCC 21332 grew well and survived in the presence of *A. migulanus* ATCC 9999, with the colonies of the GS producer actually being surrounded by the Srf producer with a total absence of an inhibition zone (Fig. 1c, d).

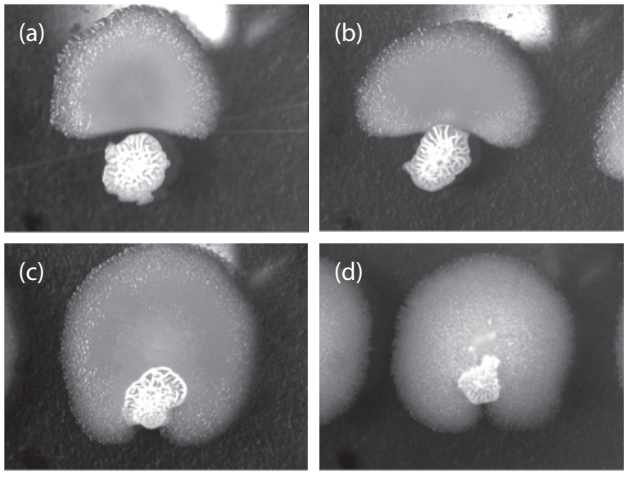

Fig. 1. Light microscope images of colonies of the GS producer *A. migulanus* ATCC 9999 (smaller white colony towards the bottom of each photograph) co-cultured on nutrient agar with *B. subtilis* strains (larger upper colony in each photograph). (a) *B. subtilis* 168, (b) *B. subtilis* OKB120, (c) *B. subtilis* OKB105 and (d) *B. subtilis* ATCC 12332.

Antagonism towards GS activity by exogenous Srf

In an effort to evaluate the observed results from our co-cultures assays, we challenged the different *B. subtilis* strains with purified GS in dose–response assays. The IC_{50} of GS towards the Srf producers, *B. subtilis* ATCC 21332 and OKB105 was higher, at 4.6 and 2.6 μM, respectively, than the 1.7 μM observed for the non-producers *B. subtilis* OKB120 and 168 (Table 2). This result correlated well with our co-culture observations. In order to simulate conditions with high Srf production, the *B. subtilis* strains were pre-incubated with 30 and 60 μM Srf. Pre-incubation with additional Srf protected its producers even more against GS, by almost doubling their respective IC_{50}s towards GS. Srf at 60 μM increased the GS IC_{50} towards *B. subtilis* OKB105 by almost 200 % to 7.4 μM. The GS IC_{50}s towards the non-producers *B. subtilis* OKB120 and *B. subtilis* 168 were also significantly increased (78 and 161 %, respectively) by 30 μM Srf to 3.2 and 4.8 μM (Table 2), similar to that of *B. subtilis* OKB105. The pre-incubation with a higher Srf concentration at 60 μM increased the GS IC_{50} towards *B. subtilis* OKB120 by >200 % to 5.2 μM. Antagonism towards GS activity was also observed with the Gram-negative bacterium *E. coli* as target organism, such that 40 μM Srf shifted the GS IC_{50} by almost 300 %, confirming the antagonism of Srf towards GS activity (Table 2).

Pre-incubation of a non-related Gram-positive bacterium, *M. luteus* NCTC8340, with Srf gave similar results to that found for the *B. subtilis* strains. The activity of GS was, as with other target cells, antagonized and the GS IC_{50} increased, respectively, by 77 and 273 % in the presence of 30 and 60 μM Srf (Table 2). At 100 μM Srf, almost all GS activity against *M. luteus* was suppressed, although at this concentration Srf on its own showed about 20 % *M. luteus* growth inhibition (results not shown). The short-term effect of Srf on GS activity towards *M. luteus* was assessed over 30 min. In the absence of Srf, we found that 5 μM GS killed about 50 % of the cells within 30 min. This latter result corroborated that lysis is the dominant mode of GS action (Epand & Vogel, 1999) and correlated well with the IC_{50} of 8 μM found with the 16 h dose–response assays (Table 2). Srf again improved cell survival to >70 % as assessed in subcultures in the presence of 5 μM GS. However, when challenged with GS at $2 \times IC_{50}$, 30 μM Srf failed to protect *M. luteus*, while 30 μM Srf protected *B. subtilis* OKB120, leading to 41 % survival.

Table 2. Influence of Srf on the antimicrobial activity of GS, as measured by changes in IC_{50} of GS towards the different bacterial target organisms in this study

n, Number of separate biological repeats, with each repeat consisting of three to six technical repeats. ND, Not determined.

Target organism	Activity parameter of peptides or combination					
	Srf	GS	30 μM Srf+GS		60 μM Srf+GS	
	IC_{50} ± SEM (μM)	IC_{50} ± SEM (μM) (*n*)	IC_{50} ± SEM (μM) (*n*)	Δ(IC_{50}) (%)	IC_{50} ± SEM (μM) (*n*)	Δ(IC_{50}) (%)
E. coli HB101	Inactive	18.5±0.5 (8)	71.6±3.1 (3)*†	287	ND	ND
M. luteus NCTC8340	>100	7.8±0.8 (9)	13.8±1.1(9)*	77	29±0.1(2)*	273
B. subtilis 168	Inactive	1.7±0.2 (6)	4.5±0.6 (6)*	161	ND	ND
B. subtilis OKB120	Inactive	1.7±0.2 (6)	3.2±0.3 (6)*	87	5.2±0.1 (2)*	206
B. subtilis OKB105	Inactive	2.6±0.1 (6)	4.6±0.6 (6)*	79	7.4±0.9 (2)*	187
B. subtilis ATCC 21332	Inactive	4.6±0.3 (4)	8.5±0.6 (4)*	86	ND	ND

*$P<0.001$ was determined with one-way ANOVA using a Newman–Keuls multiple comparison test for the GS IC_{50} determined in the presence of Srf compared with the IC_{50} of GS alone towards the target cell.
†Srf was added at 40 μM.

Dose-dependent antagonism of GS activity by Srf

The dose-dependent antagonism of GS activity by Srf was investigated over a broader concentration range against the two Gram-positive target strains in this study that do not produce Srf, *M. luteus* NCTC8340 and *B. subtilis* OKB120 (Fig. 2). A direct linear trend was observed between the percentage change in GS IC_{50} and the concentration of Srf for the two Gram-positive target cells (Fig. 2, insert). At a critical Srf concentration above 8 μM, antagonism towards GS activity increased considerably with *B. subtilis* OKB120 and *M. luteus* as targets (Fig. 2). However, for *M. luteus* as target organism, synergism between Srf and GS was observed below 8 μM Srf. *B. subtilis* OKB120 was protected over the whole Srf concentration range, from 0.9 to 60 μM Srf, against GS (Fig. 2). Srf at 0.9 to 15 μM increased the IC_{50} of GS towards *B. subtilis* OKB120 by 30–50 % (Fig. 2), leading to a substantial increase in surviving cells that would be an advantage in a competitive ecosystem.

Molecular interaction between Srf and GS

In order to assess the possibility of molecular interaction between the cationic GS and the anionic lipopeptide Srf, as well as the site of antagonism (aqueous or membrane phase), we analysed mixtures of the two peptides (in the presence and absence of bacteria/hydrophobic solvent) by ESMS and CD.

UPLC-MS analysis of supernatants of the *M. luteus* cultures, treated with either GS and/or Srf, revealed that the presence of cells and/or Srf had a significant influence on the recovery of GS (Fig. 3a). As expected, the presence of bacterial target cells lowered the recovery of free GS (as measured by the UPLC GS peak area) to 39±3 %. Srf in the absence of cells had a similar effect, leading to recovery of only 34±23 % free GS. The recovery of free GS was similar (24±19 %) when both Srf and cells were present. We also detected stable ESMS complexes of Srf and GS–Srf (Fig. 4), indicating that GS, in the absence and probably in the presence of cells, interacts with Srf. The survival of *M. luteus* collected in the bacterial culture pellet (Fig. 3b) correlated well with the recovery of GS as detected by

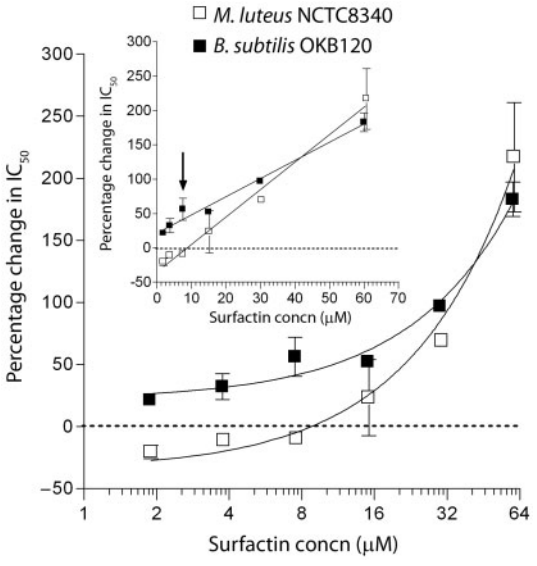

Fig. 2. Change of GS IC_{50} values as a response to Srf addition. Error bars show SEM of duplicate dose–response experiments, each with quadruplicate technical repeats. Linear regression lines were fitted with $R^2>0.98$; the $\log_2$ *x* axis scale is used for better illustration of the influence of Srf concentrations <8 μM. The graph with a linear-scale *x* axis is shown in the insert. The arrow indicates the critical concentration of 8 μM that is needed for a significant increase in GS IC_{50}.

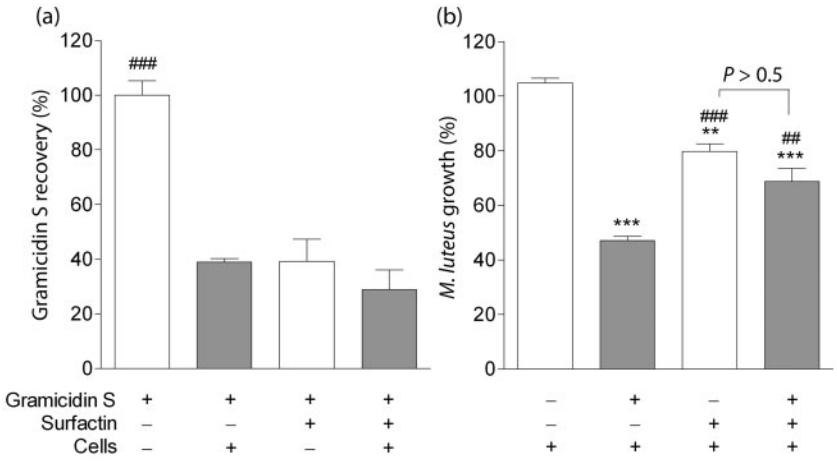

Fig. 3. (a) Recovery of GS from *M. luteus* culture supernatants (grey bars) and control samples without cells (white bars) as determined by UPLC-MS. #Recovery differed significantly ($P<0.001$) between the GS control sample (first bar) and other three samples. (b) Survival of *M. luteus* recovered in the culture cell pellet after treatment with GS or GS/Srf mixture (grey bars) and untreated or treated with Srf (white bars). The growth of the untreated *M. luteus* culture (first bar) differed significantly (***$P<0.001$; **$P<0.01$) from the three cell cultures treated with peptide. The GS-treated culture (second bar) differed significantly (###$P<0.001$; ##$P<0.01$) from the two cell cultures treated with either Srf or GS/Srf mixture. There was no significant difference ($P>0.5$) between the two cultures that were exposed to Srf. Mean and SEM are shown for 6–12 determinations each. Statistical analyses were done with one-way ANOVA using a Newman–Keuls multiple comparison test.

ESMS, with no significant difference ($P>0.5$) detected between the Srf-treated *M. luteus* and the culture treated with GS/Srf mixture.

To further assess the molecular interaction between Srf and GS, CD spectra of mixtures of the two peptides were determined in water to mimic the aqueous phase outside the target cell, and in TFE to mimic the hydrophobic phase in the membrane. The far-UV CD spectra of pure GS and Srf in water differed vastly, with Srf having a very weak spectrum and GS showing pronounced ellipticity minima at 206 and 216 nm (Fig. 5a), correlating with earlier CD studies on these peptides (Laiken *et al.*, 1969; Lee *et al.*, 2004; Jelokhani-Niaraki *et al.*, 2000; Volpon, 2001). Mixing GS with Srf below and above its critical micelle concentration (CMC) (2.5 and 10 μM) in water showed that both Srf concentrations induced a significant spectral change. Srf addition to GS induced a new maximum at 192 nm, a

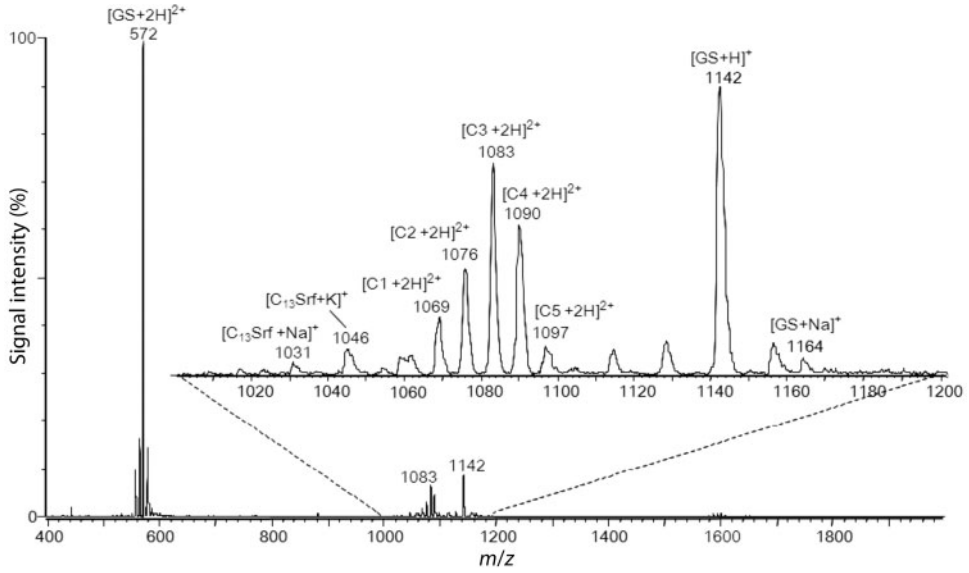

Fig. 4. ESMS spectrum of an Srf : GS (1 : 1) mixture, showing the ESMS-stable Srf–GS complexes. The doubly charged Srf–GS complexes are denoted as C1–C5. Singly and doubly charged species of GS and cationized species of C_{13}-Srf can also be seen.

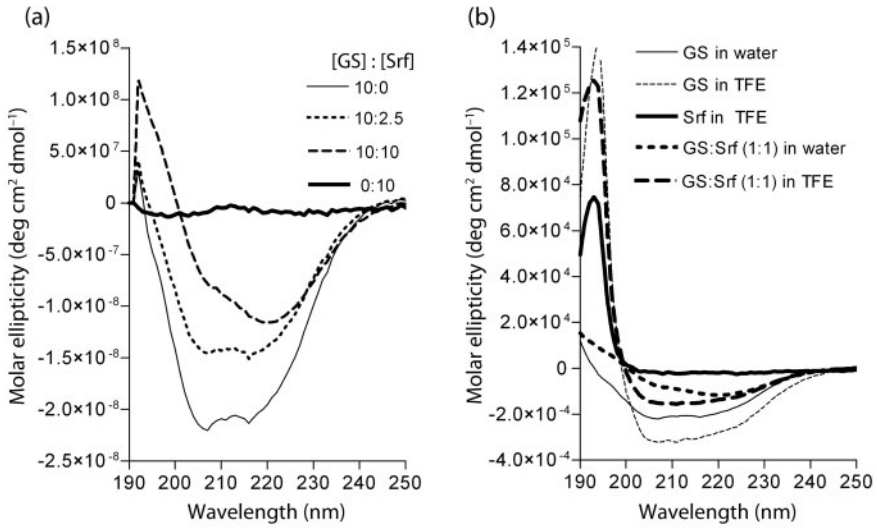

Fig. 5. CD spectra of 10 μM GS and/or Srf, or mixtures in water (a) or 50 % TFE (b). Srf : GS molar ratios are given in the legend. An average of three spectra were used to represent each CD spectrum by a point-to-point line. The molar ellipticity is given in terms of [GS + Srf] (total peptide concentration).

significant elipticity decrease at the minima, with 2 and 6 nm red shifts to 208 and 222 nm, respectively (Fig. 5a).

In the presence of the membrane-mimicking solvent TFE, the overall shape of the CD spectrum of GS correlated with type GS spectra observed in the presence of negatively charged phospholipids (Jelokhani-Niaraki *et al.*, 2000). The GS spectrum in TFE had a pronounced maximum at 196 nm, a broad minimum between 205 and 212 nm, and no 222 nm minimum (Fig. 5b). The spectrum of GS alone in TFE was significantly different from that of the Srf and GS mixture in water. An overall loss in the negative ellipticity in 50 % TFE was observed when Srf was mixed with GS, indicating interaction. However, spectrum shape and the ellipticity ratio $\theta_{206}/\theta_{222}$ remained similar to that of GS alone; thus, the bulk of the backbone structure(s) was unaffected (Fig. 5b). The detailed biophysical investigation of GS and Srf molecular interactions is beyond the scope of this study and will be reported elsewhere.

Srf contributes to its producer's survival in mixed cultures

The fact that all the target cells in our study were protected against GS by Srf may indicate that it does not offer a real benefit for its producer's survival. For example, when *B. subtilis* OKB120 and *M. luteus* were challenged with 5 μM GS, >90 % and ~50 %, respectively, of the two target strains were killed. However, when a 1 : 1 mixed culture of *M. luteus* and *B. subtilis* OKB120 was challenged with 5 μM GS in the presence of 30 μM Srf, survival of *B. subtilis* OKB120 increased dramatically to 80 %, but only marginally to nearly 60 % for *M. luteus*. Assisted by the Srf-induced swarming on agar, the *B. subtilis* OKB120 colonies also outcompeted nearby colonies of *M. luteus*, giving *B. subtilis* OK120 a competitive edge and a survival benefit.

DISCUSSION

The biological stress response in *B. subtilis* induced by antimicrobial peptides entails the K^+-induced signalling and expression of ABC transporter systems for detoxification, biofilm formation, cell density increase, swarming, sporulation and cannibalism. Srf is implicated as playing a role in all of these responses (Staroń *et al.*, 2011; López *et al.*, 2009a, b, Kinsinger *et al.*, 2003; Kearns & Losick, 2003; Julkowska *et al.*, 2005; Cosby *et al.*, 1998). However, we observed some swarming for all four *B. subtilis* strains in our study, but no sporulation by the Srf producers during our assay period (Fig. 1).

Three of the *B. subtilis* strains (168, OKB105 and OKB120) in this study are genetically identical, sharing the same stress response systems, except for the production of mature Srf (Table 1). *B. subtilis* 168 possesses ABC transport systems (Ohki *et al.*, 2003; Mascher *et al.*, 2003; Pietiäinen *et al.*, 2005), analogous to that of *B. licheniformis* (Podlesek *et al.*, 2000), which have been shown to detoxify a number of antimicrobial peptides. However, in spite of the ABC transport systems, only the Srf producers grew exceptionally well in a competitive co-culture with the GS producer (Fig. 1). Since the three *B. subtilis* strains 168, OKB105 and OKB120 share the same ABC transporter systems, it is possible that Srf enhanced and/or supported the detoxification role of these transporter systems in resistance to GS. We propose that another detoxification event may have taken place, namely Srf trapping of the

cationic GS, contributing to the resistance of the Srf producers to GS in the competitive co-cultures (Figs 1 and 6). This hypothesis was tested by evaluating the influence of exogenous Srf on the survival of *B. subtilis* and other target strains in the presence of GS. All the bacterial strains in this study, including the non-related bacteria *M. luteus* and *E. coli*, were significantly more resistant to GS in the presence of Srf, indicating that Srf plays a major role in this phenomenon (Table 2). Antagonism was most pronounced at concentrations above 7.8 μM, the CMC of Srf (Ishigami *et al.*, 1995), indicating the role of Srf micelles in antagonism towards GS activity (Figs 2 and 6).

As discussed elsewhere, the role of Srf in the *B. subtilis* strains may be that of a signalling molecule increasing the stress response in terms of detoxification, cell density increase and biofilm formation (López *et al.*, 2009a; Mascher *et al.*, 2003; Staroń *et al.*, 2011) (Fig. 6). *M. luteus* and *E. coli*, however, were also protected by Srf against the lytic action of GS, and our ESMS analyses (Fig. 4) and CD spectra of peptide mixtures (Fig. 5) indicated a direct solution-phase interaction between Srf and GS. CD spectra of Srf/GS mixtures showed major changes in organized hydrogen-bonded structures, possibly new β-sheet structures in the oligomeric peptide complexes. The CD spectral change of GS in the presence of micellar Srf is different from those reported for GS interacting with negatively charged phospholipid vesicles (Jelokhani-Niaraki *et al.*, 2000), indicative of differences in interacting structures. Changes in observed CD spectra of GS were also induced at 2.5 μM Srf, which is well below its CMC of 7.8 μM (Ishigami *et al.*, 1995), demonstrating that GS also interacts with non-micellar Srf in solution. We also observed ESMS-stable Srf–GS complexes with mixtures well below the CMC of Srf. The observed CD spectral changes and ESMS-stable complexes of the Srf/GS mixtures may therefore be the result of both ionic interactions and new hydrogen bond networks between the cationic GS and anionic Srf. These solution-phase complexes may lead to the loss of GS membrane interaction and activity.

Our UPLC-MS analyses of cell supernatants indicated that GS is trapped by both cells and Srf (Figs 3 and 4). Therefore, apart from the solution-phase interaction between the two peptides, the protection of the *B. subtilis* Srf producer may also be due to the absorption of Srf to their cell surfaces (Ahimou *et al.*, 2000), thereby shielding the cell wall and membrane from GS (Fig. 6). During interaction with the producer cell wall, Srf is orientated with the alkane chain exposed to the environment, thereby increasing cell hydrophobicity (Maget-Dana & Ptak, 1995; Ishigami *et al.*, 1995). At high Srf concentrations (above its CMC), some Srf micelles may also form outside the producer cell. Such assemblies would generate a high local Srf concentration outside the cells and at the cell–environment interface, resulting in the effective antagonism of the cationic GS both outside the cells and at the membrane/cell wall. The cell wall-absorbed Srf can interact or trap GS or shield the cell wall target from GS. CD spectra of the Srf/GS mixture in the membrane-mimicking solvent TFE, showed an intermediate loss in elipiticity (Fig. 5). This indicated that the higher-order structures of GS

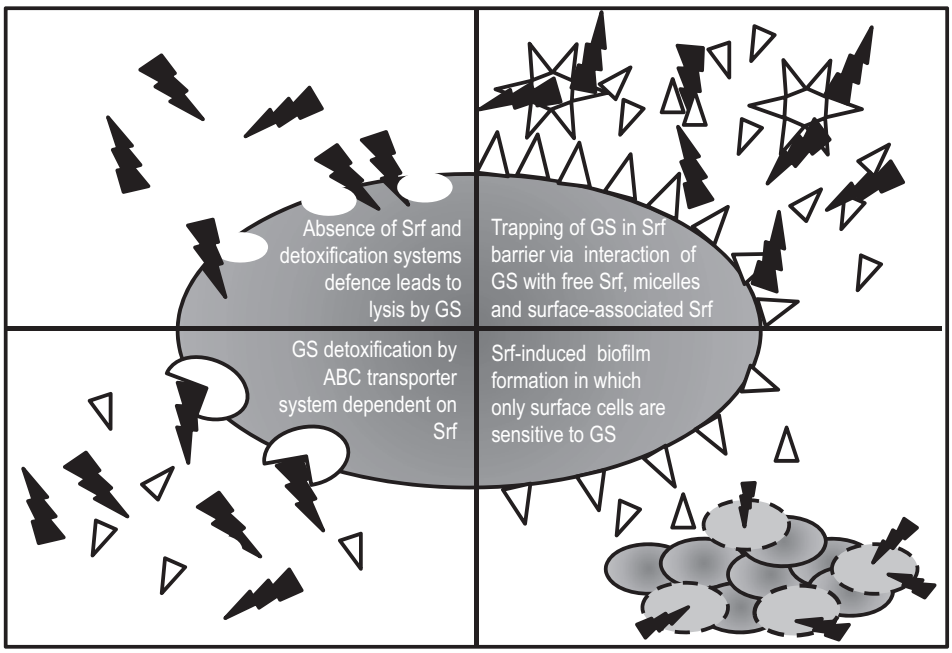

Fig. 6. Illustration summarizing the possible roles of Srf (white triangles, Srf micelles denoted by triangles in a star shape) in the protection of its producer *B. subtilis* (grey ellipses) against the lytic GS (black thunderbolts).

and Srf possibly interact with each other in a hydrophobic environment. If GS can interact with membrane-bound Srf, then GS would be trapped not only by complexing with solution-phase/micellar Srf, but also by Srf associated with the cell surface. Srf could then act as a detoxification agent by either blocking the GS interaction with the cell wall/membrane or trapping it outside the cell (Fig. 6).

Although all the target cells in our study were protected by Srf against GS, Srf does benefit its producer's survival above that of other organisms. Below its CMC, Srf was found to be synergistic with GS, probably because cell wall damage by GS assists Srf to gain entry to the cell membrane, where it disrupts the membrane via a postulated channel mechanism (Maget-Dana & Ptak, 1995; Ishigami *et al.*, 1995). The high Srf concentrations needed for more pronounced GS antagonism fall well within biological concentrations, as *B. subtilis* Srf producer strains have been shown to produce between 100 mg l^{-1} and 7 g l^{-1} in the medium (Peypoux *et al.*, 1999), indicating that a high Srf concentration could be reached in an competitive ecosystem. This was illustrated by Bais *et al.* (2004), who showed high Srf concentrations in extracts of *Arabidopsis* roots covered with a *B. subtilis* biofilm. In microbial ecosystems, high Srf concentrations will only be reached at a close proximity to the producer organism, as the hydrophobic Srf has low solubility in water, limiting diffusion and ensuring a high localized Srf concentration, thereby primarily protecting the *B. subtilis* Srf producer. Such a localized high Srf concentration will restrain and limit the access of GS to its membrane target by forming a chemical barrier around the Srf producer, as well as trapping GS in solution-phase complexes (Fig. 6). Our results indicated that Srf could have some similarity to the anionic capsular exopolysaccharide, alginic acid, from *Pseudomonas aeruginosa* in sequestering cationic antimicrobial peptides and increasing resistance (Friedrich *et al.*, 1999). The antagonism of GS by Srf also shares similarities with the inactivation of defensins by complexation with staphylokinase from *Staphylococcus aureus* (Jin *et al.*, 2004). Therefore, we tentatively classify the Srf-supported resistance to GS as constitutive.

Conclusions

Srf is produced by a vast number of *Bacillus* species from diverse geographical areas (Price *et al.*, 2007), and many functions have been assigned to this peptide, such as antimicrobial, signalling and surfactant functions. We propose yet another function for Srf, namely that of a detoxifying agent against cationic antimicrobial peptides, such as GS, from cohabiting bacteria.

This study leaves the question of whether the Srf–GS antagonism is a rare phenomenon or whether it is a more widespread resistance strategy utilized by cohabiting microorganisms in ecosystems. Such a resistance strategy in mixed producer populations with strains producing cationic peptides and anionic peptides/lipopeptides, or when strains/peptide products are mixed by design or coincidence, could have major consequences in bio-control, as certain combinations may lead to antagonistic mixtures and a consequent loss of antimicrobial activity.

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