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Abstract Current antibiofilm solutions based on planktonic bacterial physiology have limited efficacy in clinical and occasionally environmental settings. This has prompted a search for suitable alternatives to conventional therapies. This study compares the inhibitory properties of two biological surfactants (rhamnolipids and a plant-derived surfactant) against a selection of broad-spectrum antibiotics (ampicillin, chloramphenicol and kanamycin). Testing was carried out on a range of bacterial physiologies from planktonic and mixed bacterial biofilms. Rhamnolipids (Rhs) have been extensively characterised for their role in the development of biofilms and inhibition of planktonic bacteria. However, there are limited direct comparisons with antimicrobial substances on established biofilms comprising single or mixed bacterial strains. Baseline measurements of inhibitory activity using planktonic bacterial assays established that broad-spectrum antibiotics were 500 times more effective at inhibiting bacterial growth than either Rhs or plant surfactants. Conversely, Rhs and plant biosurfactants reduced biofilm biomass of established single bacterial biofilms by 74–88 and 74–98 %, respectively. Only kanamycin showed activity against biofilms of *Bacillus subtilis* and *Staphylococcus aureus*. Broad-spectrum antibiotics were also ineffective against a complex biofilm of marine bacteria; however, Rhs and plant biosurfactants reduced biofilm biomass by 69 and 42 %, respectively. These data suggest that Rhs and plant-derived surfactants may have an important role in the inhibition of complex biofilms.

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Introduction

Microbial biofilms have been implicated in recalcitrant healthcare-associated infections [24, 28, 47], the dissemination of community-acquired diseases [43] and hazardous concerns in the nutritional and environmental sectors [23]. Biofilms are sessile multicellular bacterial collectives with distinctly different physiologies from those of independent free-living bacteria [8]. Many biofilms are resistant to conventional antimicrobial technologies that were developed using a planktonic model [30]. In an effort to compensate for enhanced biofilm resistance, therapeutic doses of conventional antibiotics are often increased, accelerating harmful resistance patterns in bacteria. The detrimental consequences of this in the human population and wider environment have prompted a search for alternative solutions. One such safe and effective alternative to synthetic medicines and antimicrobial agents are biosurfactants [3, 18, 38].

Rhamnolipids (Rhs) are a group of biosurfactants produced by *Pseudomonas aeruginosa*. These have one (for mono-rhamnolipid) or two (for di-rhamnolipids) rhamnose sugar moieties (hydrophilic moiety) acylated to long-chain fatty acids or hydroxyl fatty acids (hydrophobic moiety)

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through one or two β-hydroxy fatty acid chains [32]. Their characteristic antimicrobial activity (mostly against planktonic bacteria) and biomedical applications have been extensively investigated [3, 4, 7, 13, 37]. However, there is surprisingly little information available on the extent to which Rhs and plant biosurfactants (PBs) inhibit mixed-species pre-existing bacterial biofilms. The inhibitory activity of several different Rh mixtures has been documented in relation to pre-existing single-species biofilms of *Salmonella typhimurium* [27], *Bordetella bronchiseptica* [16], microflora on vocal prosthesis [39] and *Bacillus pumilus* [10]. However, these studies have not examined this inhibitory action in direct comparison to existing antimicrobial solutions. Mixed bacterial biofilms are also important in inhibitory tests because many biofilms exist as complex polymicrobial colonisations [20, 41, 42, 46]. This complexity can add another dimension to their persistence [41]. In order to encompass a wide range of biofilm physiologies, this study compared the action of the biosurfactants with broad-spectrum antimicrobials on mixed bacterial biofilms.

Materials and Methods

Chemicals and Reagents

All solvents of analytical grade or other purities were supplied by VWR (HiPerSolv, Chromanorm Range, VWR international, Poole, Dorset, UK). Microbiological media and reagents were supplied by Oxoid Ltd (Basingstoke, Hampshire, UK) unless otherwise stated. Nutrient broth: Lab-Lemco Powder (1.0 g), yeast extract (2.0 g), peptone (5.0 g) and sodium chloride (5.0 g).

Broad-spectrum antibiotics: ampicillin (AMP) and kanamycin (KAN) were supplied by Gibco (Paisley, Scotland, UK) and chloramphenicol (CHL) was supplied by Acros (Geel, Belgium).

Rhs (at a 4 % w/v concentration, containing mono- and di-rhamnolipids mixtures) were produced as described previously [32, 35] and were diluted from a stock of 2 mg/ml solution.

The PB (SC1000) was supplied by Biobased Europe, Ayrshire, Scotland (http://www.biobasedeurope.com/). This is a non-ionic surfactant blend of plant oil extracts, fatty alcohols and tall oil. This colloid is water soluble and readily biodegradable and was formulated from a stock concentration of 2 mg/ml.

Bacterial Strains and Culture Conditions

Bacterial strains included *Escherichia coli* (ATTC 11775), *Citrobacter freundii* (ATTC 8090), *Klebsiella pneumoniae* (ATTC 13883), *Cronobacter sakazkii* (ATTC 29544), *Micrococcus luteus* (ATTC 4698), *Bacillus subtilis* (ATTC 6051) and *Staphylococcus aureus* (ATTC 12600). Bacteria for all experiments were removed from frozen stocks (−80 °C in glycerol) and thawed when necessary. Bacteria were cultured at 37 °C in nutrient broth (Oxoid Ltd) and enumerated by transferring an aliquot (10 µl) of growing bacteria to nutrient agar (NA) and incubating overnight at 37 °C.

Minimum Inhibitory Concentration

Minimum inhibitory concentrations (MICs) for planktonic bacteria were assessed using flat-bottomed 96-well high-bind plates (Costar ®, Corning Incorporated, Corning, NY, USA). To maintain consistent comparisons throughout biofilm analysis, all assays and dilutions were performed using nutrient broth. This meant that the analysis did not require media supplements for different species or adjustments for test substances such as broad-spectrum antibiotics. The highest concentration of the biosurfactant (test substance) was diluted through a series of twofold dilutions in 10 wells of a 96-well plate. To ensure that the MIC was within the range of observations, pilot experiments were carried out to determine the optimal range of concentrations for the test biosurfactant. The same determinations were also performed for the broad-spectrum antibiotics AMP, CHL and KAN. For MIC determinations, a 1-ml aliquot of bacteria that was subcultured (18 h at 37 °C) in sterile nutrient broth was incubated for a further 2–3 h in 50 ml of fresh media. The inoculum was adjusted to 1×10^4 cfu (determined from a growth curve for each bacterium in comparison to their OD at (600 nm)) and added to a 96-well plate. Appropriate controls of test substance only and media only were also added to wells in the same plate. Cultures along with test substances were shaken (130 rpm) overnight at 37 °C. The MIC was determined as the lowest concentration of test substance that inhibited visual growth of test bacteria after overnight incubation (16–20 h). Broth was removed from wells that showed visual inhibition and inoculated onto NA. The minimum concentration of test substance resulting in no bacterial growth was referred to as the minimum bactericidal concentration (MBC).

Radial Diffusion Assay

A modification of the ultrasensitive radial diffusion assay (RDA) was used to detect inhibitory activity of test substances on solid growth media [33, 21].

Preparation of Single-Species Biofilms

Single-species (homogenous) biofilms were prepared to assess the inhibition by biosurfactant in comparison to

broad-spectrum antibiotics [33]. Biofilm biomass was quantified by the crystal violet adhesion assay, commonly used in biofilm quantification [18, 25, 27, 33, 34]. The absorbance values were expressed as a percentage of a control biofilm for each organism, which contained an identical concentration of bovine serum albumin (BSA) (Sigma, St Louis, MO, USA). Most antibiotics and biosurfactants are also a potential carbon source for bacteria; therefore, BSA was used as the control substance due to its relatively benign nature in terms of bacterial inhibitory activity [6].

Preparation of Self-Assembling Marine Biofilm (SAMB)

A self-assembling marine biofilm (SAMB) was used to compare the ability of broad-spectrum antibiotics and biosurfactants to disperse a complex mixture of bacteria. This consisted of an assemblage of marine bacteria that formed a mixed marine bacterial biofilm (which was subsequently characterised in terms of species composition) on high-bind 99-well polystyrene plates [33]. Mixed biofilms were cultivated at a low temperature (10 °C) in a dilute nutrient medium of 50 % nutrient broth and 50 % seawater. Test substances were added to the four-day-old SAMB and subsequently cultivated for 4 days. The supernatant in the test well, referred to as planktonic bacteria, was removed from the wells. The biofilm biomass was quantified using the crystal violet adherence assay.

DNA-Based Characterisation of the SAMB: Cloning

After allowing the SAMB to form for 8 days, DNA was extracted from attached organisms constituting the biofilm. The biofilm was rinsed three times with sterile PBS to remove loosely associated bacteria prior to DNA extraction using a Power Biofilm kit (MO BIO Laboratories Inc., Carlsbad, California, USA). DNA was PCR amplified with each 50-µl reaction containing PCR buffer at 1×, 1.5 mM $MgCl_2$, 0.8 µM of universal primers U519F and U1068R [2, 45], 200 µM of each deoxynucleoside triphosphate, 1.25 U HotStar *Taq* polymerase (Qiagen, Hilden, Germany) and 1.5 µl of DNA. Reactions was run under the following conditions: 10 min at 95 °C, followed by 28 cycles of 45-s denaturing (95 °C), 45-s annealing (55 °C), 1-min elongation (72 °C) and an additional 10-min elongation at 72 °C. Four replicate PCRs were pooled, purified (MinElute Kit, Qiagen, Hilden, Germany) and 20 ng of PCR products were cloned using a TOPO-TA (Invitrogen) cloning kit. Recombinant clones were screened to ensure they were carrying inserts of the correct size prior to purification (QIAprep Miniprep kit, Qiagen, Hilden, Germany) and 53 plasmids were commercially sequenced (Beckman-Coulter Genomics, Essex, UK) using the primer U519F. Sequence reads were manually edited and aligned using Geneious Pro software version 5.5.6 (Geneious, Auckland, New Zealand). Sequences were analysed using the CLASSIFIER tool available through the Ribosomal Database Project [44]. Neighbouring sequences were used to reconstruct phylogenetic associations based on the neighbour-joining method with Tamura–Nei distances [40].

Data Analysis

Statistical analysis was performed using GraphPad Prism 4 software (Hearne Scientific Software Pty Ltd, Melbourne, Victoria, Australia). For MIC/MBC, data were given as mean ± standard error of the mean (SEM). For RDA test, the radius of inhibition (mm) was annotated as mean ± SEM for each test bacteria. For the calculations of the inhibition of single species of microbial biofilm, absorbance values (at 595 nm) from crystal violet adherence assay of control wells were subtracted from test wells to give a corrected value. This corrected value was expressed as a percentage of the control biofilm. The significance of corrected values relative to the control was calculated using a two-tailed students *t* test (unpaired) and annotated as $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. Outlying absorbance values that were less than the value of a standard blank well (i.e. a negative absorbance value in the crystal violet adherence assay) were adjusted using the value of a blank well, which was assigned as 0.01. Absorbance values from the test substances were expressed as a percentage of the control/control biofilm and used to create a bar graph depicting the mean ± SEM. This procedure was also followed for the mixed-species marine biofilm.

Results and Discussion

A comparison of solutions to bacterial colonisation cannot be adequately assessed through exclusively planktonic bacterial assays. In this manuscript, the efficacy of a Rh, a PB, AMP, KAN and CHL was compared over a range of bacterial physiologies. These included planktonic, agar (stranded planktonic), single-species biofilms and mixed-species bacterial biofilms [26].

Comparisons of the Minimum Inhibitory Concentrations of Antibiotics and Biosurfactants

Although bacteria exploit optimal growth conditions, there are very few environments in which they receive the optimum nutrition, agitation and aeration provided by laboratory-based planktonic culture. Rather, research suggests that many bacteria assume a more sessile physiology typified by a biofilm in

a more nutrient-poor environment [8]. However, in order to establish a baseline for inhibitory comparisons, the MIC and the MBC of antibiotics and biosurfactant were determined for mid-logarithmic cultures of common, medically relevant organisms: *E. coli*, *M. luteus* and *S. aureus*. The test substances used were AMP, CHL and KAN together with biosurfactants (Rh and a PB). Test substances were incubated with a predefined amount of bacteria as detailed in the methods section and incubated overnight at 37 °C at 130 rpm.

The resulting MIC/MBC data indicated that of the surface-active agents, only the PB had inhibitory activity against *E. coli*. However, wells containing Rh showed broad inhibitory activity against *M. luteus* and *S. aureus* (Table 1). The activity of these biosurfactants was weak in comparison to the broad-spectrum antibiotics AMP, KAN and CHL (Table 1). In some instances, the difference in MIC was 1:1,024 broad-spectrum antibiotics/biosurfactants, (based on individual replicate data). Additionally, the determination of the MIC for the Pb was slightly problematic given its opaque nature; however, it was assumed that the MIC should not be greater than the MBC and values were annotated accordingly. The antimicrobial effects of biosurfactants and in particular Rhs on planktonic bacteria have been noted as far back as 1971 [17]. However, different species of bacteria can vary widely in their sensitivity to inhibitory compounds even between strains of the same species [31], therefore standard laboratory strains were compared under controlled experimental conditions. The MIC for Rhs observed in Table 1 varied with different microorganisms and was generally higher for Gram-negative *E. coli* strain (>200 μg/ml) and lower for Gram-positive *M. luteus* (29 μg/ml) and *S. aureus* (50 μg/ml). This was supported by previous research on *S. aureus* (ATCC 6538) which had an observed MIC for Rhs at 32 μg/ml and the MIC for *E. coli* (ATTC 8739) and *M. luteus* (9631) was 64 μg/ml (mixture of 11 congeners) tested for 24 h (37 °C) [13]. More recent research has comparable MICs of >512 μg/ml for *E. coli* (ATTC 25922), 32–64 μg/ml for *M. luteus* and 128 μg/ml for *S. aureus* (ATTC 29213) [22]. The slightly higher MIC readings in these experiments may have been due to slower rotational incubation (130 rpm) of cultures necessitated by the depth of the microplate wells or a slightly difference in the composition of the Rhs components. Although there are few direct comparisons of the effects of Rhs with broad-spectrum antibiotics, independent measurements on the MIC of *E. coli* (ATTC 11775) support our observations with average MIC ranges recorded from 2 to 25 μg/ml [29, 36].

Radial Diffusion Assay

Another form of the planktonic physiology can be described as stranded planktonic growth and is routinely used when antibiotics are tested on agar plates [26]. This was also used to compare the inhibitory properties of the biosurfactants against the broad-spectrum antibiotics. Given the weak nature of the biosurfactant inhibition of growth in the previous planktonic tests, a modification of an ultra-sensitive inhibition assay was used [21]. Biosurfactants (20 μg) and broad-spectrum antibiotics (5 μg) were applied to wells in NA and the test substances absorbed into the agar for two hours. The different concentrations of test substances reflect the greater potency of the broad-spectrum antibiotics in this experiment. These ratios were maintained through the proceeding experiments in order that comparisons between inhibitory potentials could be made. Predefined inoculums of test bacteria (*B. subtilis*, *C. sakazakii*, *S. aureus*, *E. coli*, *M. luteus* and *K. pneumoniae*) were added to the agar plates and these were incubated overnight (37 °C). The inhibition data indicated that although both Rhs and the Pb possessed antimicrobial activity ~1/12 to 1/24 as effective as the broad-spectrum antibiotics AMP, CHL and KAN (when dilution factors were taken into account) (Fig. 1), the biosurfactant had some inhibitory activity against Gram-positive bacteria, but was ineffective against Gram-negative bacteria (Fig. 1). The data also show that on this occasion, the difference in the inhibition between the biosurfactant and the broad-spectrum antibiotics was less than the MIC determinations. These observations are supported by research using concentrations of 10–30 mg/ml Rh resulting in zones of inhibition of 15–20 mm for *S. aureus* (ATCC 25922) and no inhibition for *E. coli* [22].

Single-Species Biofilm Assay

It has been demonstrated that the pre-addition of biosurfactants can successfully inhibit the formation of biofilms in fungi [9], *S. typhimurium* [27], *B. bronchiasepta* [16], orally associated flora [39] and the marine bacteria *B. pumilus* [10]. However, a more realistic scenario is the application of biosurfactants to pre-existing biofilms [9, 10]. Therefore, biosurfactants and broad-spectrum antibiotics were incubated with biofilms of *B. subtilis*, *M. luteus* and *S. aureus* at similar concentrations to the previous RDA to enable a direct comparison between experiments. A 10-μl aliquot of biosurfactants (total concentration of 20 μg) and broad-spectrum antibiotics (total concentration of 5 μg) (ampicillin, chloramphenicol and kanamycin) was added to biofilms that had been cultivated for two days. The biofilm biomass was quantified by the crystal violet adherence assay. This assay was used for quantification because it allows for the measurement of both cell growth and biofilm biomass.

The quantification data revealed that the addition of the Rh and the Pb to pre-existing biofilms reduced *S. aureus* biofilm biomass by 85.6 ± 3.9 and 83.3 ± 6.1 %,

Table 1 Comparison of minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) in planktonic culture of biosurfactants and broad-spectrum antibiotics

	MIC (μg/ml)					MBC (μg/ml)				
	Rh	PB	AMP	CHL	KAN	Rh	PB	AMP	CHL	KAN
E. coli 11775	>200	133 ± 33.3	10.4 ± 2.1	3.1	0.5 ± 0.1	>200	133 ± 33.3	>25	>25	1.3 ± 0.3
M. luteus 4698	29.2 ± 11.0	116.7 ± 44.1	0.8 ± 0.4	2.1 ± 0.5	3.1 ± 1.6	150 ± 50	116.7 ± 44.1	1.3 ± 0.3	3.6 ± 1.4	4.7 ± 1.6
S. aureus 12600	50	133.3 ± 33.3	0.5 ± 0.1	3.1	3.1	>200	133.3 ± 33.3	0.6 ± 0.1	6.2	3.1

Rhamnolipids *Rh* and a plant biosurfactant *PB* were incubated with a defined inoculum of a: *E. coli*; b: *M. luteus*; and c: *S. aureus* in comparison to ampicillin *AMP*, chloramphenicol *CHL* and kanamycin *KAN*. Cultures were incubated overnight on a rotatory shaker at 37 °C/130 rpm. Values are expressed as mean ± standard error of the mean *SEM* in μg/ml for each test substance, $n = 3$

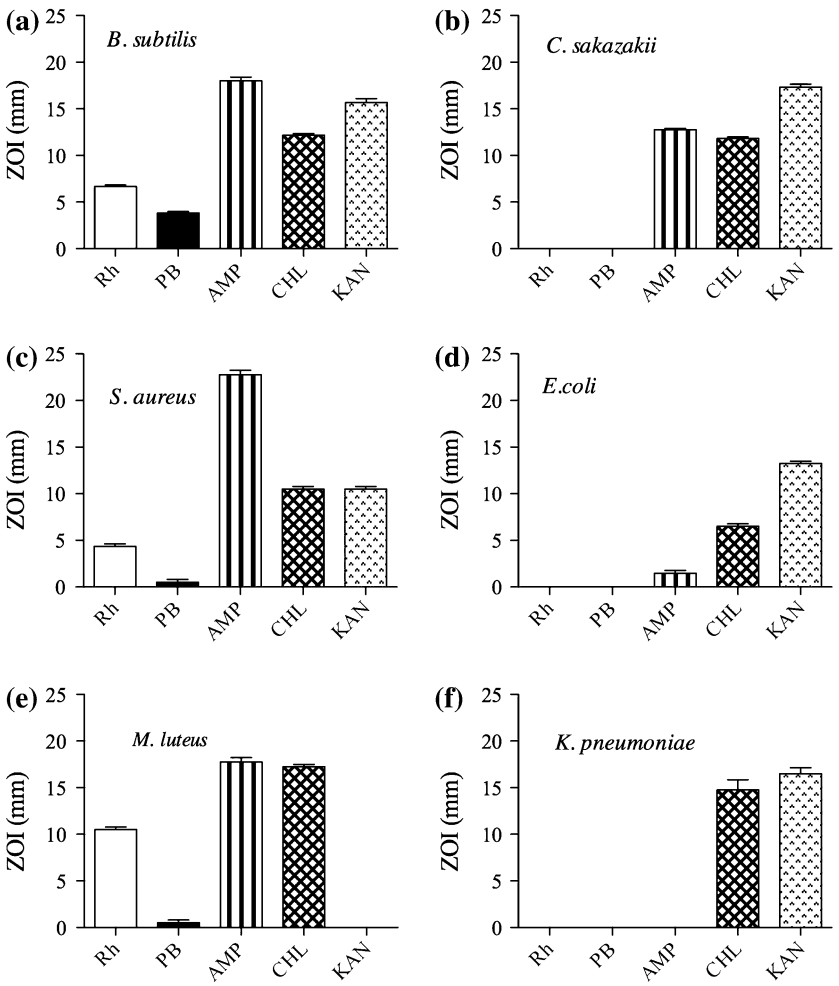

Fig. 1 Comparison of biosurfactants and broad-spectrum antibiotics in a radial diffusion assay. Aliquots of rhamnolipid (*Rh*) and a plant biosurfactant (*PB*) (20 μg/ml), ampicillin (*AMP*), chloramphenicol (*CHL*) and kanamycin (*KAN*) (5 μg/ml) were assayed in a radial diffusion assay against a lawn of **a** *B. subtilis*, **b** *C. sakazakii*, **c** *S. aureus*, **d** *E. coli*, **e** *M. luteus* and **f** *K. pneumoniae*. The inhibition of substances was determined by the measurement of the radius of the zone of inhibition *ZOI* after overnight incubation. The values are recorded as mean inhibition ±SEM (mm), $n = 4$

respectively ($P < 0.001$, $n = 12$). AMP and CHL did not significantly affect the biofilm biomass ($P > 0.05$, $n = 12$) and KAN had some inhibitory activity against *S. aureus* (34.7 ± 10.8 %) (Fig. 2a).

In the case of pre-existing biofilms of *B. subtilis*, the biofilm biomass was reduced by 88.4 ± 5.8 and 98.1 ± 1.7 % by Rhs and Pb, respectively ($P < 0.001$, $n = 12$) (Fig. 2b). The addition of AMP produced no significant inhibition, whilst CHL increased the biofilm biomass by 54.8 ± 20.3 % ($P < 0.05$) and KAN decreased it by 39.3 ± 9.5 % ($P < 0.01$, $n = 12$) (Fig. 2b). The addition of Rh to pre-existing biofilms of

M. luteus reduced biofilm biomass by 74.5 ± 6.6 % ($P < 0.001$, $n = 12$), whilst that of Pb produced no significant effect on the biofilm biomass ($P > 0.05$, $n = 12$) Fig. 2c). The addition of the broad-spectrum antibiotics, AMP, CHL and KAN, also produced no significant reduction in biofilm biomass (Fig. 2c). Biofilm growth after the addition of antibiotics is of concern and has been observed by other researchers, initially for aminoglycosides [15] and then for other antibiotics especially in sub-MIC doses [19]. This is not surprising given the microbial origins of the majority of antibiotics.

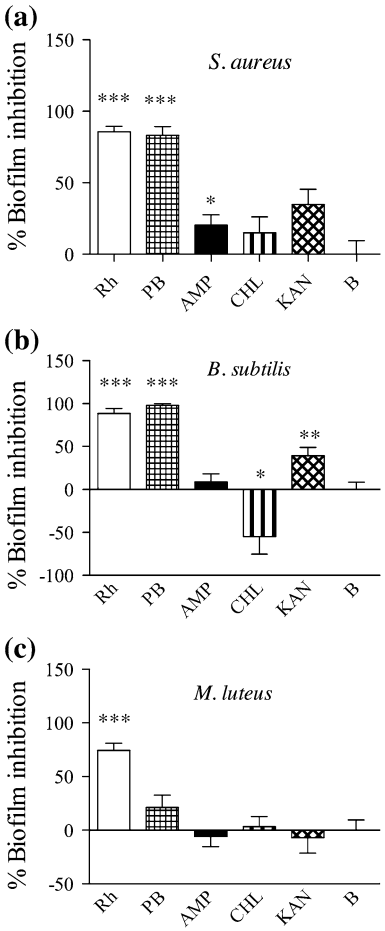

Fig. 2 Comparison of biosurfactants and broad-spectrum antibiotics on established single-species biofilms. Aliquots (10 µl) of rhamnolipids (*Rh*), a plant biosurfactant (*PB*) (total 20 µg), ampicillin (*AMP*), chloramphenicol (*CHL*) and kanamycin (*KAN*) (total 5 µg) were added apically to homogenous bacterial biofilms that had been cultivated for 2 days. The biofilms were **a** *B. subtilis*, **b** *M. luteus* and **c** *S. aureus*. After a further 2 days of incubation, the biofilms were quantified using the crystal violet adhesion assay. Quantification of biofilm biomass was in comparison to the control biofilm (*B*) which consisted of BSA + bacteria. Biofilm dispersal values were annotated as mean ± SEM, $n = 4$–12. A two-tailed students *t* test was used to determine if test biofilms significantly differed from the control biofilm of the same species (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

In contrast, Rh can successfully disperse pre-existing bacterial biofilms. This has previously been demonstrated with the marine bacteria, *B. pumilus* [10]. It has been reported that the most potent biosurfactant, surfactin, is capable of dispersing biofilms of *S. typhimurium* at concentrations of 5–50 µg [27]. Given this range, the data presented here illustrate that the dispersion efficiency of Rhs on preformed bacterial biofilms is relatively strong. Further research on *S. typhimurium* biofilms has revealed that concentrations of 100 µg of Rh were required (mixture of 11 congeners) to disperse pre-existing biofilms [27]. It was therefore noted that even with dilution factors taken into account, the efficacy of the broad-spectrum antibiotics declined in comparison to biosurfactants in biofilm assays. This decrease in efficiency has previously been noted by the group of Olsen for many antibiotics [7]. However, an increase in efficiency of biosurfactants from planktonic growth to biofilm has not been documented as often. Direct comparisons between planktonic bacterial and biofilm inhibition data are difficult because biofilm experiments are difficult to standardise. Each bacterial species, surface of attachment, temperature variation and nutrient status has the potential to create a different biofilm dynamic. Therefore, our experiments used similar reagents and surfaces for experiments to allow better comparisons.

DNA-Based Characterisation of the Self-Assembling Marine Biofilm (SAMB)

Although researchers have realized that the biofilm mode of growth is important in microbial colonisation, there has been less documentation on polymicrobial biofilms. This may be due to the multiplicity of potential bacterial permutations and the difficulties assessing the colonisation and succession patterns in such biofilms. However, it is know that the mixed biofilm dynamic can add an extra virulent dimension to some clinical biofilms [41]. Since there are many unknown factors in the assembly of complex biofilms, a large collection of marine bacteria was induced to form a mixed biofilm. This was referred to as a SAMB and subsequently characterised in terms of species composition (Fig. 3). The characterisation of the SAMB by cloning revealed a diverse assemblage of bacteria belonging to two phyla, Bacteroidetes and Proteobacteria, and covering as many as eight genera (Fig. 3 and Supplementary Table 1). Proteobacteria sequences were the most frequently observed accounting for 87 % of sequences recovered. *Pseudoalteromonas* was the most frequently observed genera accounting for 51 % of the total sequences, followed by Oceanisphaera (9 %), Colwellia (6 %) and Polaribacter (6 %). This illustrates an extremely wide diversity in the biofilm. There has been some research on the construction of test biofilms composed of two species

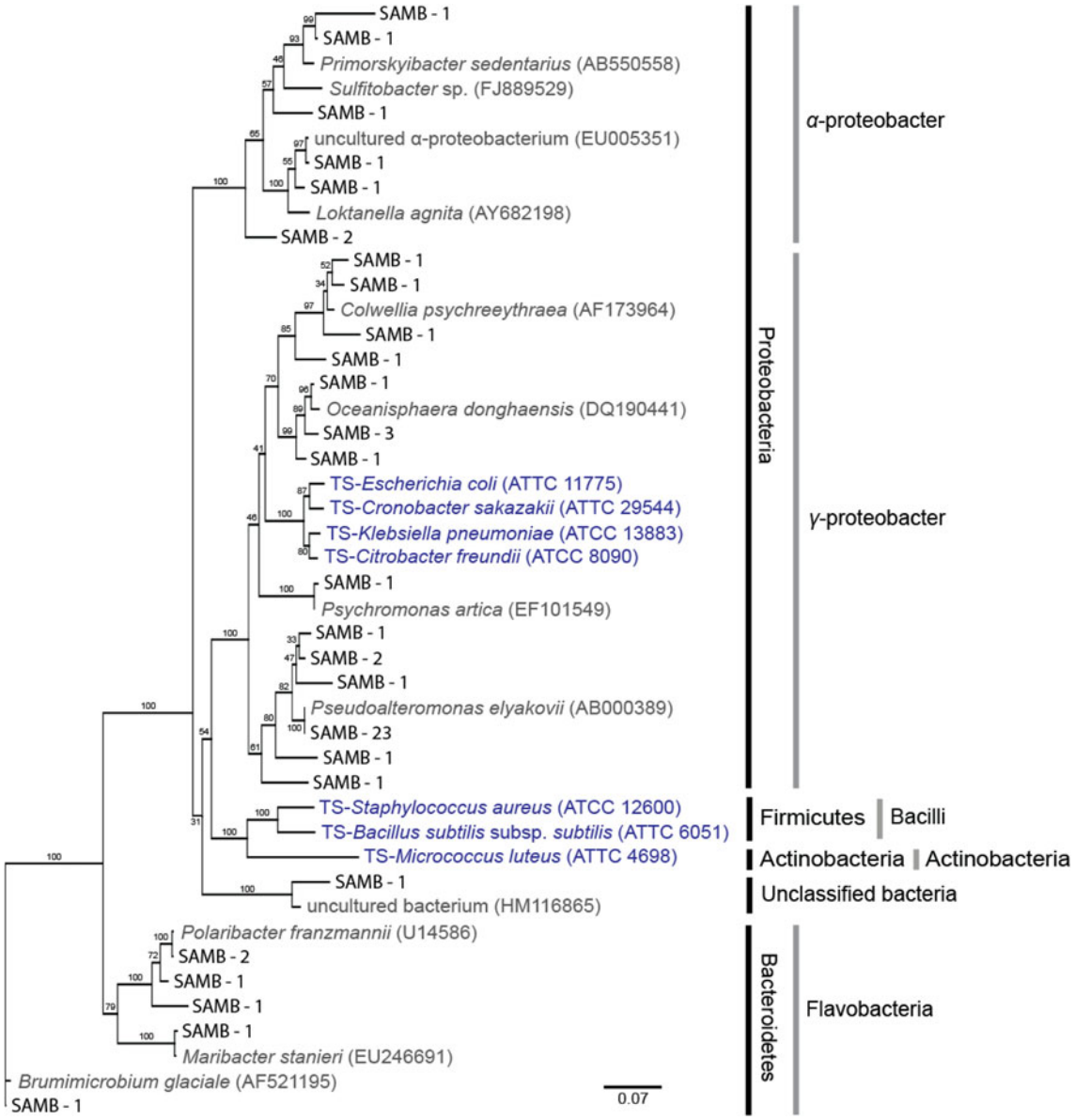

Fig. 3 Phylogenetic association based on partial 16S rRNA gene sequences of bacteria used in inhibition assays. Type strains (TS) used in the minimum inhibitory assay, radial diffusion assay, homogeneous biofilm (HB) inhibition tests and sequences cloned from the SAMB are depicted along with neighbouring sequences (followed by a GenBank accession no.). The SAMB entries are followed by a number indicating the total number of sequences recovered. *Vertical black* and *grey bars* indicate the represented Phyla and Classes, respectively. The Neighbour-joining method with Tamura–Nei distances was used for tree construction. *Scale bar* is equal to base pair substitutions per site

and also biofilms with up to 5 species [14, 41]. However, the complex biofilm systems such as SAMB [33] are not frequently documented and were originally designed to study the pathogenicity of cryptosporidium [48].

Self-Assembling Marine Biofilm Assay

The comparison of efficiency of antibiotics and biosurfactants against SAMB was addressed as follows. A 10 µl aliquot of biosurfactants (total added 20 µg) and broad-spectrum antibiotics (total added 5 µg) (ampicillin, chloramphenicol and kanamycin) was applied to a pre-existing biofilm of SAMB after 4 days of incubation at low temperatures as described in methods. The biofilms were incubated for a further 4 days before quantification of the biofilm biomass by crystal violet adherence assay.

The quantification indicated that the addition of Rhs to pre-existing SAMB reduced biofilm biomass by

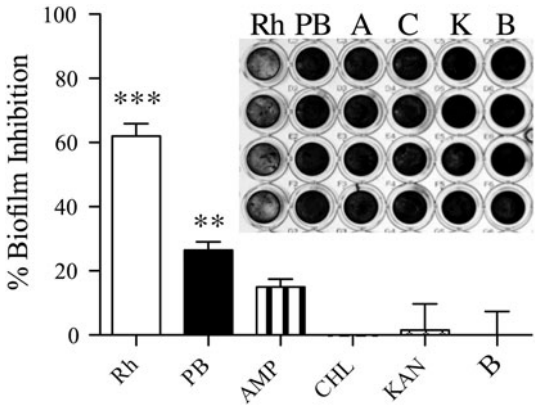

Fig. 4 Comparison of biosurfactants and broad-spectrum antibiotics on established mixed-species biofilms. Self-assembling marine biofilms (SAMB) were grown for 4 days before the addition of an aliquot (10 µl) of rhamnolipids (*Rh*), a plant biosurfactant (*PB*) (total 20 µg) and broad-spectrum antibiotics: AMP, CHL and KAN (total 5 µg). Biofilms were incubated for a further 4 days. Quantification of biofilm biomass (by crystal violet adherence assay) was in comparison to the control biofilm (*B*) which consisted of BSA + bacteria. Biofilm dispersal values were annotated as mean ± SEM, $n = 4$. The *inset* micrograph is a section of the 96-well high-bind test plate used in this experiment. A two-tailed Students *t* test was used to determine data significance (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

69.0 ± 3.4 % ($P < 0.001$, $n = 4$) relative to the control biofilm. The Pb reduced biofilm biomass by 26.5 ± 2.6 % relative to this control ($P < 0.05$, $n = 4$). The addition of AMP, CHL and KAN had no significant inhibitory effect on the biofilms relative to the growth of the control biofilm ($P > 0.05$, $n = 4$) (Fig. 4). The ratio of inhibition was ~5:1, biosurfactant/broad-spectrum antibiotic. Taking into account the fourfold dilution of the broad-spectrum antibiotics, this result shows a dramatic reversal of the effectiveness of the biosurfactants from the initial planktonic tests to the mixed biofilm assays. Similar experiments using a lipopeptide biosurfactant complex containing fusaricidins and polymyxins on a preformed biofilm of mixed marine organisms achieved a 72.4 % reduction of biofilm biomass [33]. Further observations on the marine bacteria *B. pumilus* showed that Rhs could disperse preformed biofilms by 93 % at a concentration of 100 mM [10]. Although the biofilm used here was of marine origin and the dispersal of its heterogeneous terrestrial counterparts has yet to be experimentally verified, the data demonstrate the great potential of Rhs to disperse mixed bacterial biofilms.

The mechanisms of biofilm inhibition by Rhs were not within the scope of this investigation; however, previous work suggests that biosurfactants are effective in biofilm dispersal because of their depletion of extracellular polymeric substances [10], their integral role in the biofilm cycle [5], promotion of cell surface hydrophobicity [1, 11] and their ability to overcome the theoretically low wettability of preformed biofilms [12]. Whether these mechanisms are exactly the same for mixed complexes of bacteria has yet to be experimentally verified.

Conclusions

After establishing a baseline for the comparison of biosurfactants and antimicrobial agents, the data have indicated that the inhibitory effect of biosurfactants increases drastically in comparison to broad-spectrum antibiotics in biofilm-orientated assays. Additionally, through the use of the SAMB, it was observed that the biosurfactants inhibited the formation of complex heterogeneous marine biofilms. This suggests that biosurfactants such as these may be able to play an important role in the development of solutions to single and mixed biofilms or even act as adjuvants to existing therapies aimed at tackling biofilm formation or eliminating established biofilms.

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Conflict of Interest The authors declare that they have no conflict of interest.

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