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Sulfonamide-based DSF analogues interfere with quorum sensing in *S. maltophilia* and *B. cepacia*

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Running title

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ABSTRACT

Aim: *Stenotrophomonas maltophilia* (*Sm*) and *Burkholderia cepacia* complex (BCC) are gram-negative bacterial pathogens, which are typically multi-drug resistant and excellent biofilm producers. These phenotypes are controlled by quorum sensing (QS) systems from the DSF (Diffusible Signal Factor) family. We aim to interfere with this QS system as an alternative approach in combatting such difficult-to-treat infections. **Materials & methods:** A library of sulfonamide-based DSF bioisosteres was synthesised and tested against the major phenotypes regulated by QS. **Results and Conclusion:** Several analogues display significant antibiofilm activity while the majority increase the action of the last-resort antibiotic colistin against *Sm* and BCC. Most compounds inhibit DSF synthesis in the *Sm* K279a strain. Our results support the strategy of interfering with QS communications to combat multi-drug resistance.

Introduction

Members of the *Stenotrophomonas maltophilia* (*Sm*) and the *Burkholderia cepacia* complexes (BCC) are gram-negative bacterial species from different orders that share several common characteristics [1]. Although both bacterial complexes are mostly ubiquitous and frequently associated with plants [2–5], they are also recognised as important nosocomial and cystic fibrosis (CF) pathogens [6–9]. As human pathogens, these bacteria seem to have a preference for respiratory tract infections [10]. Other relevant major traits shared by *Sm* and BCC include their elevated ability to form biofilms on biotic and abiotic surfaces -including medical devices- and their high degree of antimicrobial resistance, isolates of which are typically multidrug resistant (MDR) [11].

In addition, both pathogens regulate bacterial behaviour such as virulence in response to their population density through similar quorum sensing (QS) systems mediated by the fatty acid signals of the DSF (diffusible signal factor) family [12–14].

Antimicrobial resistance (AMR) is acknowledged as the biggest challenge in modern medicine, since the rapid emergence of MDR isolates, including pan-resistant pathogens, significantly hampers the effective treatment of infected patients [15,16].

To overcome AMR, innovative approaches have been proposed. For example, novel antimicrobial adjuvants may rescue the activity of current antimicrobials and limit the onset of resistance [17]. Compounds targeting virulence represent another promising alternative [18–20]. For those pathogens which produce biofilms in a clinical context, antibiofilm agents are also being explored [21].

QS or bacterial cell-to-cell communication [22], is a major regulatory hub for virulence, biofilm formation and AMR [23,24]. Strategies targeting QS mechanisms have attracted considerable interest in recent years, as the blocking of key components of QS signal synthesis or perception can significantly attenuate microbial virulence [25].

Sm and BCC utilise similar QS signals based on the DSF family which are comprised of *cis*-unsaturated fatty acids [12–14]. The major QS signal in *Sm* is DSF or *cis*-11-methyl-2-dodecenoic acid [26,27]. BCC produces a closely related molecule, namely BDSF (*Burkholderia* diffusible signal factor), whose structure is *cis*-2-dodecenoic acid [28,29]. DSF and BDSF are almost identical, differing only by the presence of a methyl group on C11 in DSF (Figure 1).

Although general mechanisms of DSF regulation apply to all bacteria displaying DSF-like communication, there are considerable differences between species but also within a species at a subpopulation level, as exemplified by *Sm rpf*-1 and *rpf*-2 groups. A

schematic illustration of the key components governing DSF and BDSF regulation in *Sm* and BCC, respectively, is presented in Figure 1.

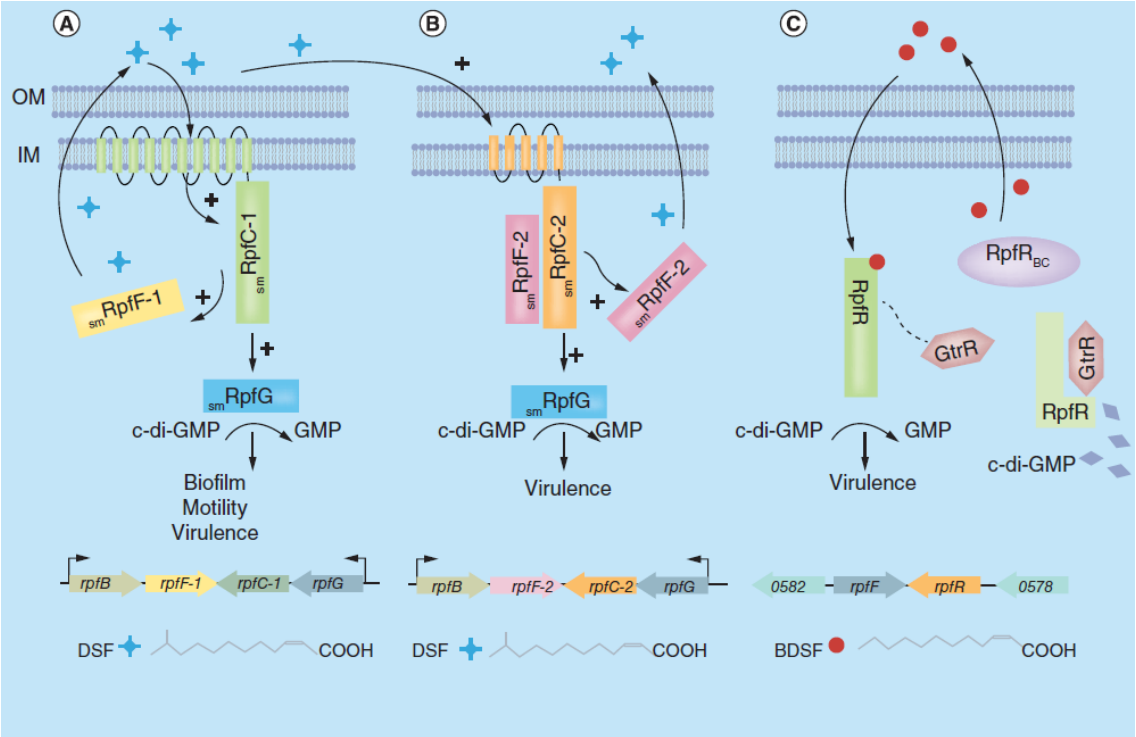

Figure 1. A) In the *Sm rpf*-1 system, RpfC-1 promotes RpfF-1 basal activity synthesizing DSF (*cis*-11-methyl-2-dodecenoic acid) that diffuses towards the extracellular environment. When the DSF concentration is high, RpfC-1 senses the signalling molecule and consequently phosphorylates the phosphodiesterase RpfG. RpFG then converts cyclic diguanylate monophosphate (c-di-GMP) to GMP thereby controlling the expression of genes which regulate biofilm formation, virulence and bacterial motility.

B) In the *Sm rpf*-2 system, RpfC-2 blocks RpfF-2, which in turns stops DSF synthesis. Exogenous DSF signals released by surrounding bacteria (e.g., *rpf*-1 strain) are detected by RpfC-2 liberating active RpfF-2 to produce DSF and thus stimulating bacterial virulence.

C) In BCC, BDSF (*cis*-2-dodecenoic acid) communication is governed by an unrelated cluster composed of the synthase RpfF and the receptor RpfR. When the concentration of BDSF is high, RpfR senses BDSF and promotes its c-di-GMP phosphodiesterases activity reducing intracellular levels of c-di-GMP and allowing the RpfR–GtrR complex to regulate the expression of genes involved in virulence.

Certain QS signals may also exert a collateral effect on surrounding microorganisms. For example, the *Pseudomonas* Quinolone Signal (PQS), and its precursor 4-hydroxy-2-heptylquinoline (HHQ), display antimicrobial activity against various bacteria and

yeasts [30,31]. Likewise, DSF and structurally similar fatty acids potentiate the activity of different antibiotics against a wide range of bacterial pathogens [32]. In *Xanthomonas campestris*, DSF is involved in biofilm dispersal [34]. The related fatty acid *cis*-2-decenoic acid (*cis*-DA) produced by *Pseudomonas aeruginosa* also promotes biofilm dispersion in several bacterial species [34]. Additionally, both BDSF and DSF inhibit hyphal transition of *Candida albicans* most probably by acting as antagonists of the DSF-related *C. albicans* signal farnesoic acid [29,35].

It has previously been reported that the *cis*-unsaturated double bond between C2 and C3 in DSF is a prerequisite for activity, since both the corresponding *trans*-unsaturated fatty acid and the fully saturated analogue produce significantly weaker biological responses [36]. Furthermore, the perceptive bacteria appear to be sensitive to shortening or elongation of the carbon backbone. These findings suggest that medicinal agents based on DSF or BDSF should avoid major changes to these structural features. For this reason, we wondered if replacing the carboxylic acid group with an appropriate sulfonamide might be worthy of investigation. Sulfonamides are considered bioisosteres of carboxylic acids and have a proven track record in medicinal chemistry [37]. Compounds modified in this fashion may display greater selectivity, less side effects, increased lipophilicity, decreased toxicity, improved pharmacokinetics or a reversal of agonistic/antagonist activity [38]. Sulfonamide derivatives of DSF or BDSF might be expected to disrupt cell-cell signalling and thereby constitute novel QS inhibitors [39].

Herein, we describe our work on the synthesis of a series of DSF and BDSF sulfonamide-based bioisosteres for testing against MDR isolates of the pathogens *Sm* and BCC, including strains resistant to the last-resort antibiotic colistin. We include our findings on the antibiofilm activity of these compounds as well as their ability to potentiate the effect of colistin both *in vitro* and *in vivo* using the *Galleria mellonella* infection model. We also investigate their potential anti-QS activity and lastly, we measure their toxicity on the human kidney cell line HK-2.

Experimental Protocols

General procedure for the preparation of acylsulfonamides 3a-3d:

A solution of dodec-2-ynoic acid (**2a** - 200 mg, 1.02 mmol, 1.0 eq) and the appropriate sulfonamide (1.1 mmol, 1.1 eq) in 10 mL dry dichloromethane was cooled to 0° C. DMAP (134 mg, 1.1 mmol, 1.1 eq) was then added at once. The mixture was stirred at 0° C for 15 min. EDCI (170 mg, 1.1 mmol, 1.1 eq) was added and gradually the temperature was raised to 25° C. Stirring was continued at this temperature for 16 h. After completion of the reaction, dichloromethane was added (20 mL), followed by 2M aqueous HCl solution (20 mL) and stirring continued for 30 sec (solution should reach pH 2-3). The organic layer was separated, dried over $MgSO_4$ and solvent was then removed by vacuum distillation. The crude mixture was purified by column chromatography on silica gel using CH_2Cl_2-MeOH (100:0-98:2).

***N*-(Methylsulfonyl)dodec-2-ynamide (3a)**

Yield: 36%

^{1}H NMR: δ (400 MHz, $CDCl_3$) 0.88 (t, 3H, *J* = 6.62 Hz), 1.23 – 1.43 (m, 12H), 1.52 – 1.62 (m, 2H), 2.36 (t, 2H; *J* = 7.07 Hz), 3.33 (s, 3H), 8.21 (bs, 1H).

^{13}C NMR: δ (100 MHz, $CDCl_3$): 4.11, 18.79, 22.66, 27.34, 28.86, 29.00, 29.24, 29.37, 31.83, 41.77, 73.54, 94.46, 150.53.

IR: ν (cm^{-1}): 3197, 2963, 2922, 2848, 2220, 1688, 1666, 1437, 1405, 1225, 1156, 1067, 974, 874, 619.

HRMS (ESI-TOF) m/z: [M – 1] Calcd for $C_{13}H_{22}NO_3S$ 272.1326; Found 272.1314.

***N*-(Phenylsulfonyl)dodec-2-ynamide (3b)**

Yield: 48%

^{1}H NMR: δ (400 MHz, $CDCl_3$) 0.80 (t, 3H, *J* = 6.64), 1.09 – 1.31 (m, 12H), 1.40 – 1.49 (m, 2H), 2.22 (t, 2H, *J* = 7.08 Hz), 7.49 (t, 2H, *J* = 7.73 Hz), 7.60 (t, 1H, *J* = 7.42 Hz), 7.97 – 8.02 (m, 2H).

^{13}C NMR: δ (100 MHz, $CDCl_3$): 14.11, 18.75, 22.65, 27.32, 28.83, 28.98, 29.23, 29.34, 31.82, 73.68, 94.02, 128.49, 129.05, 134.25, 138.19, 149.51.

IR: ν (cm^{-1}): 3215, 2924, 2854, 2226, 1670, 1449, 1431, 1350, 1217, 1160, 1088, 1056, 866, 813, 685.

HRMS (ESI-TOF) m/z: [M – 1] Calcd for $C_{18}H_{24}NO_3S$ 334.1482; Found 334.1477.

***N*-((2-Bromophenyl)sulfonyl)dodec-2-ynamide (3c)**

Yield: 67%

^{1}H NMR: δ (400 MHz, $CDCl_3$): 0.88 (t, 3H, *J* = 6.72 Hz), 1.19 – 1.40 (m, 12H), 1.50 – 1.58 (m, 2H), 2.31 (t, 2H; *J* = 7.11 Hz), 7.46 – 7.55 (m, 2H), 7.76 (dd, 1H, *J* = 1.71, 7.38 Hz), 8.30 (dd, 1H, *J* = 1.93, 7.74 Hz), 8.40 (bs, 1H).

^{13}C NMR: δ (100 MHz, $CDCl_3$): 14.12, 18.83, 22.66, 27.29, 28.84, 28.99, 29.24, 29.37, 31.84, 73.46, 94.70, 120.21, 127.80, 133.42, 135.05, 135.29, 137.34, 149.19.

IR: ν (cm^{-1}): 3203, 2916, 2848, 2226, 1694, 1574, 1425, 1350, 1260, 1223, 1162, 1125, 1050, 832, 762.

HRMS (ESI-TOF) m/z: [M – 1] Calcd for $C_{18}H_{23}NO_3SBr$ 412.0588; Found 412.0580.

_N_-(Cyclopropylsulfonyl)dodec-2-ynamide (3d)

Yield: 50%

^{1}H NMR: δ (400 MHz, $CDCl_3$): 0.88 (t, 3H, J = 7.09 Hz), 1.11 – 1.18 (m, 2H), 1.19 – 1.45 (m, 14H), 1.53 – 1.65 (m, 2H), 2.35 (t, 2H; *J* = 7.22 Hz), 2.94 (tt, 1H; J = 3.40, 4.84 Hz), 7.92 (bs, 1H).

^{13}C NMR: δ (100 MHz, $CDCl_3$): 6.46, 14.11, 18.80, 22.66, 27.38, 28.88, 29.01, 29.24, 29.37, 31.50, 31.84, 73.70, 77.23, 150.16.

IR: ν (cm^{-1}): 3389, 3193, 2918, 2230, 1698, 1456, 1435, 1343, 1315, 1294, 1221, 1188, 1060, 883, 705.

HRMS (ESI-TOF) m/z: [M + 1] Calcd for $C_{15}H_{26}NO_3S$ 300.1628; Found 300.1626.

General procedure for the preparation of acylsulfonamide 3e-3h:

A solution of 11-methyldodec-2-ynoic acid (**2b**) (350 mg, 1.664 mmol), DMAP (226 mg, 1.850 mmol, 1.05 eq), and EDCI (287 mg, 1.850 mmol, 1.05 eq) in DCM (15 mL) were stirred at 0°C for 15 mins under an atmosphere of N_2. The appropriate sulfonamide (1.769 mmol, 1.0 eq) was added and the mixture stirred for 20 h at room temperature. The reaction mixture was poured into 2M aqueous HCl (20 mL) and extracted with dichloromethane (3 x 60 mL). The organic extracts were then combined and washed with saturated brine solution, before drying over magnesium sulfate. Following filtration, the solvent was removed under vacuum. Finally, purification by column chromatography on silica gel using DCM-MeOH (100:0-98:2) afforded the target compounds.

11-Methyl-_N_-(methylsulfonyl)dodec-2-ynamide (3e)

Yield: 44%

^{1}H NMR: δ (400 MHz, $CDCl_3$) 0.86 (d, 6H, 6.69 Hz), 1.09 – 1.19 (m, 2 H), 1.20 – 1.44 (m, 8 H), 1.45 – 1.63 (m, 3 H), 2.36 (t, 2H, *J* = 7.20 Hz), 3.32 (s, 3H), 8.26 (bs, 1H).

^{13}C NMR: δ (100 MHz, $CDCl_3$) 18.79, 22.65, 27.32, 27.35, 27.95, 28.87, 29.03, 29.66, 38.96, 41.77, 73.55, 94.43, 150.55.

IR: ν (cm^{-1}): 3237, 2923, 2852, 2229, 1688, 1435, 1334, 1325, 1145, 976, 882.

HRMS (ESI-TOF) m/z: [M + 1] Calcd for $C_{14}H_{26}NO_3S$ 288.1628; Found 288.1619.

11-Methyl-*N*-(phenylsulfonyl)dodec-2-ynamide (3f)

Yield: 39%

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.85 (d, 6H, J=6.63 Hz), 1.08-1.19 (m, 2H), 1.19-1.29, (m, 6H), 1.29-1.39 (m, 2H), 1.45-1.57 (m, 3H), 2.29 (t, 2H, J=7.12 Hz), 7.52-7.61 (m, 2H), 7.67 (t, 1H, J=7.44 Hz), 8.08 (d, 2H, J=7.41 Hz).

^{13}C NMR (75 MHz) 18.75, 22.65, 27.37, 27.32, 27.94, 28.83, 29.01, 29.63, 38.95, 73.68, 93.94, 128.48, 129.05, 134.24, 138.21, 149.60.

IR (ATR) υ_{max} cm^{-1} 566, 587, 685, 737, 866, 1057, 1089, 1163, 1218, 1351, 1433, 1450, 1671, 2226, 2855, 2924, 3219.

HRMS (ESI-TOF) m/z: [M + 1] Calcd for $C_{19}H_{28}NO_3S$ 350.1784; Found, 350.1783.

***N*-((2-Bromophenyl)sulfonyl)-11-methyldodec-2-ynamide (3g)**

Yield: 51%

^{1}H NMR: δ (400 MHz, $CDCl_3$) 0.79 (d, 6H, J = 6.65 Hz), 1.02 – 1.11 (m, 2H), 1.13 – 1.33 (m, 8H), 1.38 – 1.52 (m, 3H), 2.23 (t, 2H, J = 7.19 Hz), 7.38 – 7.48 (m , 2H), 7.68 (dd, 1H, J = 1.68, 7.50 Hz), 8.22 (dd, 1H, J = 1.94, 7.76 Hz), 8.54 (bs, 1H).

^{13}C NMR: δ (100 MHz, $CDCl_3$) 18.82, 22.66, 27.30, 27.31, 27.94, 28.84, 29.02, 29.66, 38.97, 73.48, 94.69, 120.23, 127.78, 133.41, 135.04, 135.30, 137.35, 149.35.

IR: ν (cm^{-1}): 3219, 2952, 2917, 2850, 2228, 1697, 1427, 1348, 1163, 1052, 883.

HRMS (ESI-TOF) m/z: [M + 1] Calcd for $C_{19}H_{27}BrNO_3S$: 428.0890; Found 428.0878.

***N*-(Cyclopropylsulfonyl)-11-methyldodec-2-ynamide (3h)**

Yield: 22%

^{1}H NMR: δ (400 MHz, $CDCl_3$) 0.86 (d, 6H, J = 6.57 Hz), 1.11 – 1.19 (m, 4H), 1.20 – 1.45 (m, 10H), 1.46 – 1.66 (m, 3H), 2.36 (t, 2H, J = 7.19 Hz), 2.95 (tt, 1H, J = 3.34, 4.74 Hz), 8.02 (bs, 1H).

^{13}C NMR: δ (100 MHz, $CDCl_3$) 6.46, 14.11, 18.80, 22.66, 27.38, 28.88, 29.01, 29.24, 29.37, 31.50, 31.84, 73.70, 77.23, 150.16.

IR: ν (cm^{-1}): 3222, 2925, 2855, 2228, 1682, 1433, 1345, 1148, 880.

HRMS (ESI-TOF) m/z: [M + 1] Calcd for $C_{16}H_{28}NO_3S$ 314.1784; Found 314.1800.

General procedure for the preparation of BDSF analogues 4a-4d and DSF analogues 4e-4h:

Lindlar's catalyst (100 mg) and the appropriate acylsulfonamide (0.045 mmol, 1.0 eq) were added to dichloromethane (6 mL). This solution was shaken vigorously in a 60 PSI hydrogen atmosphere for 6 h using a Parr hydrogenator. The crude mixture was filtered and purified by careful column chromatography on silica gel using MeOH-DCM (0:100-1:99) to afford the target compounds.

(*Z*)-*N*-(Methylsulfonyl)dodec-2-enamide (4a)

Yield: 47%

^{1}H NMR: δ (400 MHz, $CDCl_3$) 0.80 (t, 3H, *J* = 6.62 Hz), 1.12 – 1.30 (m, 12H), 1.33 – 1.42 (m, 2H), 2.62 (q, 2H; *J* = 7.30 Hz), 3.27 (s, 3H), 5.63 (d, 1H; *J* = 11.28 Hz), 6.30 (dt, 1H; *J* = 7.53, 11.28 Hz), 8.27 (bs, 1H).

^{13}C NMR: δ (100 MHz, $CDCl_3$) 14.13, 22.68, 28.91, 29.29, 29.35, 29.40, 29.42, 29.51, 31.88, 41.73, 118.61, 154.81, 163.81.

IR: ν (cm^{-1}): 3268, 2918, 2848, 1696, 1629, 1435, 1398, 1323, 1260, 1174, 1109, 980, 929, 864, 823, 640.

HRMS (ESI-TOF) m/z: [M – 1] Calcd for $C_{13}H_{24}NO_3S$ 274.1482; Found 274.1472.

(*Z*)-*N*-(Phenylsulfonyl)dodec-2-enamide (4b)

Yield: 92%

^{1}H NMR: δ (400 MHz, $CDCl_3$) 0.80 (t, 3H, *J* = 6.8 Hz), 1.059 – 1.348 (m, 14 H), 2.516 (q, 2H, *J* = 7.2 Hz).

5.62 (d, 1H; *J* = 11.44 Hz), 6.18 (dt, 1H; *J* = 7.2, 11.44 Hz), 7.49 (t, 2H, J = 7.7 Hz), 7.58 (t, 1H; *J* = 7.40), 7.98 – 8.05 (m, 2H), 8.36 – 8.59 (bs, 1H).

^{13}C NMR: δ (100 MHz, $CDCl_3$): 22.67, 24.31, 28.88, 29.24, 29.27, 29.30, 29.39, 29.48, 31.87, 118.73, 128.30, 129.04, 133.94, 138.71, 154.10, 162.80.

IR: ν (cm^{-1}): 3287, 2956, 2916, 2848, 1729, 1702, 1625, 1582, 1449, 1427, 1335, 1260, 1174, 1082, 846.

HRMS (ESI-TOF) m/z: [M – 1] Calcd for $C_{18}H_{26}NO_3S$ 336.1639; Found 336.1624.

(*Z*)-*N*-((2-Bromophenyl)sulfonyl)dodec-2-enamide (4c)

Yield: 69%

^{1}H NMR: δ (400 MHz, $CDCl_3$): 0.80 (t, 3H, *J* = 6.72 Hz), 1.01 – 1.45 (m, 16H), 2.48 (dq, 2H, *J* = 1.29, 7.61 Hz), 5.69 (d, 1H, *J* = 10.86 Hz), 6.05 – 6.44 (m, 1H), 7.37 – 7.51 (m, 2H), 7.63 (d, 1H, *J* = 7.67 Hz), 8.28 (dd, 1H, *J* = 1.68, 7.94 Hz), 8.51 (bs, 1H).

^{13}C NMR: δ (100 MHz, $CDCl_3$): 14.13, 22.67, 28.85, 29.20, 29.27, 29.33, 29.37, 29.47, 31.87, 118.55, 120.09, 127.96, 133.27, 134.80, 135.22, 137.71, 154.52, 162.67.

IR: ν (cm^{-1}): 3224, 2918, 2848, 1704, 1637, 1576, 1431, 1341, 1280, 1252, 1184, 1139, 1095, 799, 701.

HRMS (ESI-TOF) m/z: [M – 1] Calcd for $C_{18}H_{25}BrNO_3S$ 414.0744; Found 414.0728.

(*Z*)-*N*-(Cyclopropylsulfonyl)dodec-2-enamide (4d)

Yield: 59%

^{1}H NMR: δ (300 MHz, $CDCl_3$): 0.87 (t, 3H, *J* = 6.62 Hz), 1.17 - 1.08 (m, 2H), 1.51 - 1.19 (m, 16 H), 2.69 (dq, *J* = 7.36, 1.74 Hz), 3.04 - 2.94 (m, 1H), 5.71 (dt, *J* = 11.51, 1.74 Hz), 6.34 (dt, *J* = 11.51, 7.54 Hz, 1H), 7.77 (bs, 1H).

^{13}C NMR: δ (75 MHz, $CDCl_3$): 6.30, 14.08, 22.65, 28.93, 29.26, 29.31, 29.34, 29.40, 29.49, 31.51, 31.86, 118.78, 153.98, 163.33.

IR: ν (cm^{-1}):3275, 2918, 2848, 1704, 1641, 1429, 1323, 1260, 1162, 1105, 1046, 950, 885, 864, 803, 709.

HRMS (ESI-TOF) m/z: [M + 1] Calcd for $C_{15}H_{28}NO_3S$ 302.1784; Found 302.1797.

(*Z*)-11-Methyl-*N*-(methylsulfonyl)dodec-2-enamide (4e)

Yield: 82%

^{1}H NMR: δ (400 MHz, $CDCl_3$) 0.86 (d, 6H, J = 6.62 Hz), 1.08 – 1.18 (m, 2H), 1.20 – 1.37 (m, 8H), 1.39 – 1.57 (m, 3 H), 2.69 (dq, 2 H, J = 1.69, 7.53 Hz), 3.34 (s, 3H), 5.70 (dt, 1H, J = 1.69, 11.33 Hz), 6.36 (dt, 1H, J = 7.53, 11.33 Hz), 8.22 (bs, 1H).

^{13}C NMR: δ (100 MHz, $CDCl_3$) 22.66, 27.36, 27.96, 28.91, 29.36, 29.40, 29.45, 29.81, 39.01, 41.74, 118.59, 154.82, 163.77.

IR: ν (cm^{-1}): 3268, 2954, 2921, 2851, 1698, 1633, 1442, 1399, 1323, 1175, 1108, 981, 867.

HRMS (ESI-TOF) m/z: [M + 1] Calcd for $C_{14}H_{28}NO_3S$ 290.1784; Found 290.1791.

(*Z*)-11-Methyl-*N*-(phenylsulfonyl)dodec-2-enamide (4f)

Yield: 64%

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.86 (d, 6H, J=6.62 Hz), 1.08-1.17 (m, 2H), 1.17-1.31 (m, 8H), 1.3-1.43 (m, 2H), 1.50 (h, 1H, J=6.57 Hz), 2.49-2.69 (m, 2H), 5.70 (d, 1H, J=11.39

Hz), 6.25 (dt, 1H, J=11.38 Hz, J=7.42 Hz), 7.52-7.61 (m, 2H), 7.65 (t, 1H, J=7.44 Hz), 8.09 (d, 2H, J=7.48 Hz).

^{13}C NMR (75 MHz) 22.66, 27.35, 27.95, 28.90, 29.25, 29.30, 29.43, 29.78, 39.01, 118.75, 128.30, 129.04, 133.93, 138.73, 154.07, 162.82.

IR (ATR) υ_{max} cm^{-1} 563, 595, 684, 718, 756, 847, 864, 1088, 1140, 1187, 1346, 1438, 1453, 1633, 1696, 2851, 2919, 3278.

HRMS (ESI-TOF) m/z: [M + 1] Calcd for $C_{19}H_{30}NO_3S$ 352.1941; Found, 352.1938.

(*Z*)-*N*-((2-Bromophenyl)sulfonyl)-11-methyldodec-2-enamide (4g)

Yield: 85%

^{1}H NMR: δ (400 MHz, $CDCl_3$) 0.85 (d, 6H, J = 6.58 Hz), 1.07 – 1.29 (m, 10 H), 1.30 – 1.40 (m, 2H), 1.49 (sep, 1H, J = 6.66 Hz), 2.55 (dq, 2H, J = 1.26, 7.37 Hz), 5.75 (dt, 1H, J = 1.26, 11.41 Hz), 6.28 (dt, 1H, J = 7.37, 11.41 Hz), 7.47 (dt, 1H, J = 1.57, 7.76 Hz), 7.54 (dt, 1H, J = 1.09, 7.78 Hz), 7.74 (dd, 1H, J = 1.09, 7.88 Hz), 8.35 (dd, 1H, J = 1.57, 7.82 Hz), 8.64 (bs, 1H).

^{13}C NMR: δ (100 MHz, $CDCl_3$) 22.66, 27.34, 27.95, 28.85, 29.21, 29.34, 29.41, 29.76, 39.00, 118.51, 120.04, 127.97, 133.26, 134.78, 135.20, 137.77, 154.51, 162.55.

IR: ν (cm^{-1}): 3227, 2952, 2917, 2848, 1706, 1642, 1434, 1341, 1186, 1097, 873.

HRMS (ESI-TOF) m/z: [M + 1] Calcd for $C_{19}H_{29}BrNO_3S$: 430.1046; Found 430.1041.

(*Z*)-*N*-(Cyclopropylsulfonyl)-11-methyldodec-2-enamide (4h)

Yield: 75%

^{1}H NMR: δ (400 MHz, $CDCl_3$) 0.85 (d, 6H, J = 6.45 Hz), 1.09 – 1.18 (m, 4 H), 1.20 – 1.36 (m, 8H), 1.36 – 1.56 (m, 5 H), 2.69 (dq, 2H, J = 1.53, 7.46 Hz), 2.94 – 3.04 (tt, 1H, J = 3.29, 4.78 Hz), 5.72 (dt, 1H, J = 1.53, 11.37 Hz), 6.34 (dt, 1H, J = 11.3, 7.46 Hz), 8.12 (bs, 1H).

^{13}C NMR: δ (100 MHz, $CDCl_3$) 6.32, 22.66, 27.36, 27.96, 28.95, 29.35, 29.46, 29.81, 31.47, 39.02, 118.80, 154.08, 163.52.

IR: ν (cm^{-1}): 3287, 2958, 2918, 2850, 1706, 1640, 1416, 1323, 1106, 861

HRMS (ESI-TOF) m/z: [M + 1] Calcd for $C_{16}H_{30}NO_3S$ 316.1941; Found 316.1940.

Bacterial strains

Bacteria used in this study include the species of the *Burkholderia cepacia* complex (BCC) *Burkholderia cepacia* (*Bc*) strain R6193, *Burkholderia cenocepacia* (*Bcc*) strain 289, *Burkholderia multivorans* (*Bm*) strain B10 and the representative *Stenotrophomonas maltophilia* (*Sm*) strains K279a (belonging to the *rpf*-1

subpopulation) and D457 (belonging to the *rpf*-2 subpopulation) [26]. To detect DSF production and inhibition, the reporter strain *Xanthomonas campestris* pv *campestris* (*Xc*) 8523 pL6engGUS [40] was used. More detailed information can be found in Supplementary Table 1.

Biofilm inhibition

The inhibitory effect of the compounds on biofilm formation in *Sm* and BCC organisms was investigated on a polystyrene surface using 96-well microtitter non-treated plates (BrandTech 781662). Briefly, 200 µl of bacterial cultures in LB medium adjusted to an optical density (OD_{620nm}) of 0.05 containing each compound at either 10 µM or 50 µM concentration were poured into wells and the plates were incubated for 24 h at 37 ºC. Control wells contained the same volume of the solvent DMSO. The next day, bacterial growth of biofilm plates was estimated by measuring the optical density of the wells at 620 nm. Biofilm plates were then rinsed with PBS, fixed at 60 °C for 1 h and stained for 15 min with 200 µl of crystal violet 0.1% (CV). The dye was removed and the plates were washed with distilled water and dried at 37 ºC for 30 min. CV (corresponding to the bacterial biomass adhered to the wells) was dissolved in 250 µl of 30% acetic acid for 15 min, and the optical density of the extracted dye was measured at 550 nm. Biofilm formation (OD_{550nm} of CV) was normalized by cell growth (OD_{620nm}) and reported as relative biofilm formation in percentage. Bacterial biofilm formation in the presence of the different compounds was compared to those containing the same volume of DMSO, which corresponded to 100% biofilm formation. Eight wells per compound per strain were used and the experiment was performed by triplicate. Statistical significance was analysed by the one-way ANOVA test.

Antimicrobial susceptibility testing

Minimal inhibitory concentration (MIC) of *Stenotrophomonas* and *Burkholderia* isolates to colistin in combination with the compound at a fixed dose of 10 µM or 50 µM were determined by the broth microdilution (BMD) method in cation-adjusted Muller Hinton Broth (CAMHB) in accordance with CLSI/EUCAST recommendations [41,42,43]. Breakpoint values were inferred by measuring the absorbance of the wells at 550 nm, and MICs were interpreted as those antibiotic concentrations that reduced ≥80% of bacterial growth compared to the positive control. All experiments were performed by triplicate in three different occasions.

Time-kill kinetics

Overnight cultures in CAMHB were diluted (1/100) in 10 mL of the same medium and incubated at 37ºC and 250 rpm to an optical density (OD_{620nm}) of 0.2. Kill kinetics were then initiated by the addition of the antibiotic colistin (concentration corresponding to the MIC in combination with the effective adjuvant) and the adjuvant at 50 µM concentration. Bacterial survival was monitored every 15 minutes during 2 h by plating serial dilutions on MH agar medium and expressed in percentage in relation to time point 0. Three replicates of each culture set were performed and the statistical analysis was calculated by the two-tailed unpaired t-test.

DSF and BDSF Bioassay

To evaluate the potential quorum sensing inhibitory effect of the compounds on DSF production in *S. maltophilia* K279a, the DSF bioassay using the reporter strain *Xanthomonas campestris* pv. *campestris* 8523 pL6engGUS [40] was used. The reporter strain was cultured overnight in 10 ml of NYG medium (2% glycerol, 0.5% peptone and 0.3% yeast extract) containing 10 µg/ml of tetracycline to an OD_{620nm} of 0.7. Then, cells were centrifuged and resuspended in 1 ml of fresh medium and added to 100 ml NYGA medium with 1% of Agar Noble (BD Difco) and 80 µg/ml of X-Glu (5-Bromo-4-chloro-3-indolyl ß-D-glucuronide sodium salt) (Sigma) and plated into petri plates. Then, an adjusted culture of the DSF-producer strain K279a (OD_{550nm} of 0.5) was used to seed a confluent culture onto the reporter plate by using a cotton stick. After drying the plates, 1 µl of each antagonist stocked at 5 mg/ml in DMSO was inoculated onto the double-cultured plate containing the DSF-reporter strain (*Xcc* 8523 pL6engGUS) into the agar and the DSF-producer strain (*Sm* K279a) onto the agar. Plates were incubated at 28ºC for 24 h and the presence of uncoloured halos indicated inhibition of DSF synthesis in *Sm* K279a.

1 µl of DSF and BDSF signals at the same stock concentration were spotted onto regular bioassay plates to validate their biological activity.

Sm and *Bc* strains used in this study were also tested on the regular bioassay by pin inoculation.

In vivo* efficacy using *Galleria mellonella

Larvae of *Galleria mellonella* were obtained from our own hatchery, which was established in collaboration with Professor Fernando García del Pino from the Zoology Department at the Universitat Autonoma de Barcelona.

To prepare bacterial inoculums, *Sm* and BCC isolates were grown overnight in 10 ml of BD Brain Heart Infusion (BHI) medium at 37 ºC in a rotary shaker. Then, cells were

centrifuged, washed in PBS and adjusted to contain ≈10^5 cells in a dose of 5 μl. The bacterial burden of the doses was confirmed by plating on BHI medium.

Thirty larvae per group were infected *via* left proleg with the aforementioned inoculum and incubated at 30 ºC for 1 h. Then, groups of infected larvae were treated by injecting *via* right proleg 5 μl of a PBS suspension containing either: i) DMSO (untreated group), ii) DMSO + colistin (colistin-treated group), or iii) compound + colistin (enhanced colistin-treated group).

To treat *Sm* K279a infections, single doses of 3.2 mg/kg of colistin and 21.52 mg/kg of **4g** (corresponding to the *in vitro* colistin MIC of 4 μg/ml in combination with 50 μM **4g**) was used. To treat larvae infected with *Sm* D457, single doses of 3.2 mg/kg of colistin and 20.82 mg/kg of **4c** (corresponding to the *in vitro* colistin MIC of 4 μg/ml in combination with 50 μM **4c**) were administered. Treatment of *Bc* R6193 infections was conducted by injecting single doses of 102.4 mg/kg of colistin and 21.52 mg/kg of **4g** (corresponding to the in vitro colistin MIC of 128 μg/ml in combination with 50 μM **4g**). An additional treatment with 102.4 mg/kg of colistin in combination with **4b** at 16.87 mg/kg was also applied to larvae infected with *Bc* R6193 (data not shown).

Experiments were performed by triplicate on different occasions using different batches of insects. Kaplan–Meier survival curves were plotted using GraphPad Prism 5.0a and survival analysis and statistical significance was determined using the log-rank test.

Toxicity by the MTT assay

The toxicity of the compounds was assessed *in vitro* on human proximal tubule cells (HK-2) by the EZ4U cell proliferation assay (Biomedica) following the manufacturer's instructions. In brief, HK-2 cells were cultured in DMEM/F12 with 10% FCS and 1% penicillin/streptomycin (GIBCO, Invitrogen) and seeded at a concentration of 4000 cells per well in 96-well tissue culture plates with clear bottoms (Falcon®), and plates were incubated overnight at 37 °C in a 5% CO_2 atmosphere. The next day, the medium was released and the DSF and BDSF derivatives were applied onto wells seeded with HK-2 cells at 50 μM concentration in 200 μl volume of DMEM/F12. Cell viability was determined by means of EZ4U assay after 24 and 48 h of exposure to the compounds, according to the manufacturer's instruction. Plates were read using a microplate reader (Victor 3, Wallac) at a wavelength of 450 nm and 620 nm, the latter used as a reference. The results were expressed as percentage of cell survival using untreated cells as control. Eight replicates per compound were performed and the experiment was conducted in two independent occasions. Statistical significance was measured by the one-way ANOVA test.

Results and discussion

Chemistry

Initially, a series of unbranched sulfonamide derivatives of BDSF was prepared. Starting from commercially available 1-undecyne (**1a**), dodec-2-ynoic acid (**2a**) was obtained by the lithiation of **1a** followed by addition of carbon dioxide gas (Figure 2). Early in our studies, we discovered that direct coupling of BDSF with a sulfonamide led to a mixture of *cis*- and *trans*-unsaturated products, which were often difficult to separate. For that reason, we adopted a strategy whereby sulfonamide coupling would preceed a stereoselective, partial hydrogenation to the target. Accordingly, **2a** was subjected to EDCI-mediated coupling with aliphatic and aromatic sulfonamides to afford acylsulfonamides **3a**-**3d**. Finally, partial hydrogenation of **3a**-**3d** in the presence of Lindlar's catalyst afforded BDSF analogues **4a**-**4d** exclusively as their *cis*-isomers.

The preparation of the corresponding DSF analogues incorporating an 11-methyl group was achieved in a similar manner, but starting from 10-methylundec-1-yne (**1b**). The synthesis of **1b** has been previously reported and relies on an alkyne zipper reaction to furnish the requisite terminal alkyne [44]. As before, lithiation of **1b** followed by addition of carbon dioxide furnished propargylic acid **2b**. EDCI coupling of **2b** with the appropriate sulfonamide furnished acylsulfonamides **3e**-**3h**. Semi-hydrogenation of **3e**-**3h** produced DSF analogues **4e**-**4h** with the required *Z*-configuration. For comparison purposes, pure samples of BDSF and DSF were prepared by the partial hydrogenation of **2a** and **2b** respectively.

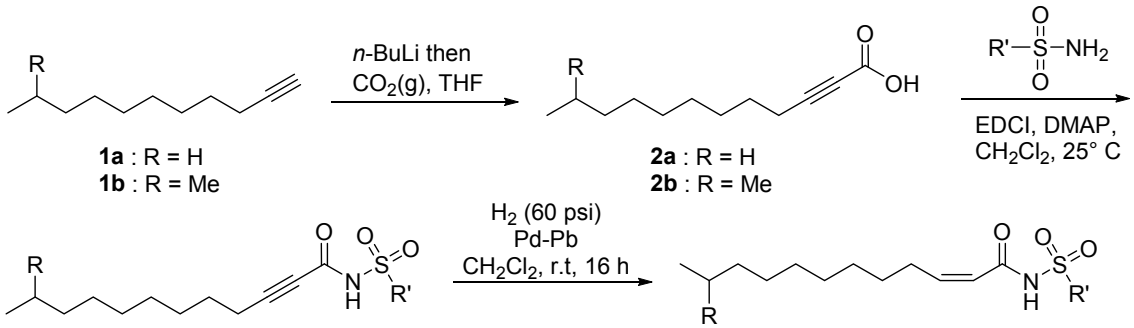

Figure 2. Synthesis of DSF and BDSF analogues.

Biological Evaluation

The effect of our library was tested against clinically relevant phenotypes regulated by QS in isolates of the two human pathogens which exploit DSF communication, namely *S. maltophilia* (*Sm*) and *B. cepacia* complex (BCC). To achieve representative results in terms of QS regulation in *Sm,* two clinical isolates belonging to the *rpf*-1 subpopulation (K279a) and the *rpf*-2 subpopulation (D457) [26] were investigated. For the BCC, three clinical isolates belonging to the species *B. cepacia* (*Bc* R6193), *B. cenocepacia* (*Bcc* 289) and *B*. *multivorans* (*Bm* B10) were selected (Supplementary Table 1).

Biofilm assays in the presence of our BDSF and DSF analogues revealed that **4g** was the most potent inhibitor, decreasing biofilm formation in all *Sm* and BCC specimens at 50 μM on a polystyrene surface (Figure 3). Similarly, DSF-derivative **4g**, as well as its BDSF analogue **4c**, displayed an inhibitory effect against *Bc. Bcc* and *Bm* proved even more sensitive with compounds **4b-h** inhibiting biofilm production in these species. Furthermore, a significant effect at 10 μM concentration was observed for **4c**, **4d**, **4f** and **4g** in *Bcc* and likewise for **4c**, **4f** and **4g** in *Bm*. In *Bc*, the presence of a brominated aromatic ring appears to be important for antibiofilm activity, since both **4c** and **4g** contain such a motif (Figure 2). This molecular feature is also important for biofilm inhibition in *Bcc* and *Bm* isolates, with these compounds displaying noticeably higher activity. In *Sm,* the presence of a methyl group on C11 appears to be a additional prerequisite for activity, with only **4g** displaying an inhibitory effect while its *des*-methyl analogue **4c** was inactive.

Sulfonamides **4b**, **4c**, **4f** and **4g** also moderately retarded growth of *Sm* isolates at 50 μM after 24 h incubation at 37 ºC (Supplementary Figure 1 A-B). Interestingly, phenyl-substituted **4f** displayed a small, but significant, inhibitory effect at the lower concentration of 10 μM. For BCC isolates, **4c, 4d**, **4f** and **4g** slightly reduced growth in *Bcc* 289 only (Supplementary Figure 1 D).

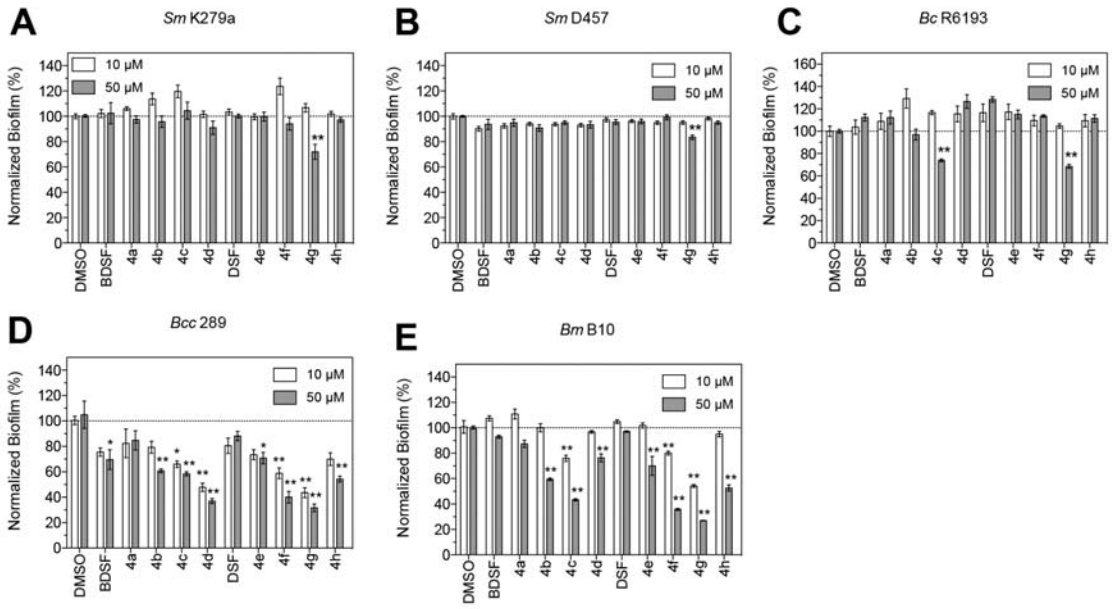

Figure 3. Inhibitory effect of **4a-4h** at 10 µM and 50 µM on the growth of *Sm* K279a (A), *Sm* D457 (B), *Bc* R6193 (C), *Bcc* 289 (D) and *Bm* B10 (E) in 96-well plate after 24 h incubation in LB at 37ºC. * *P<0.01; ** P<0.001.*

As pathogens, *Sm* and BCC compensate their limited pathogenicity with a strong ability to form biofilms, which notably contributes to their MDR capacity and may result in chronic infection. To date, few studies have been conducted with the aim of identifying or designing new antibiofilm compounds against BCC and *Sm*. Certain DSF-related fatty acids display intrinsic antibiofilm activity. Of these, *cis*-DA produced by *Pseudomonas aeruginosa* (*Pa*), has been shown to disperse mature biofilms of diverse gram-negative (GN) and gram-positive (GP) pathogens [34]. In *Sm rpf*-1 as in *Xc*, DSF appears to prevent biofilm formation. Our work confirms that DSF-based bioisosteric analogues can significantly inhibit biofilm formation in both *Sm* and BCC.

Given the moderate inhibitory effect on bacterial growth exhibited by certain compounds (e.g., **4b**, **4c**, **4d**, **4f** and **4g** against *Sm* and *Bcc* isolates), we wondered whether our molecules might possess intrinsic antimicrobial activity. However, this hypothesis was subsequently discounted as minimum inhibitory concentration (MIC) values above 500 µg/ml (corresponding to 1-3 mM) were recorded for all compounds including the natural signals DSF and BDSF against *Sm* and *Bc* R6193 isolates (Supplementary Figure 2). The observed effects were likely attributable to the antimicrobial influence of the solvent DMSO.

It has been reported that DSF induces resistance to various antibiotics, including polymyxin B, in *Pseudomonas aeruginosa* [45]. By contrast, DSF and related fatty acids enhance the activity of selected antibiotics against several other GN and GP pathogens [46]. Surprisingly, the antibiotic colistin has never been tested in combination with DSF or BDSF against *Sm* and BCC species.

Colistin is a last-resort antibiotic that is administered to patients suffering from nosocomial infections caused by GN pathogens when no other option exists. *Sm* and BCC are typical MDR pathogens, which considerably limits the therapeutic possibilities. Members of BCC are intrinsically resistant to colistin primarily because of its LPS composition which prevents colistin binding and activity [47]. These bacterial species display additional population mechanisms such as heteroresistance [48] and adaptive resistance [49]. Higher degrees of colistin susceptibility are observed in *Sm* isolates, although an increasing incidence of colistin-resistance has been recently observed [50,51]. Recently, heterogeneous colistin resistance phenotypes have also been identified in *Sm* isolates [52]. Importantly, it has been observed that colistin treatment induces biofilm formation in *Sm* [52]. Moreover, horizontal transference of plasmid-mediated colistin resistance genes among GN bacteria has also been reported, to the alarm of the scientific and medical communities [53].
Given that the *Sm* and BCC species are highly resistant to colistin monotherapy, we wondered whether the activity of colistin could be rescued by the addition of our DSF and BDSF derivatives.

The MIC to colistin of the isolates was assessed by the broth microdilution method (BMD) [41,42] in the presence of **4a**-**4h** at a fixed dose of 10 μM or 50 μM. As clinical breakpoints to colistin for *Sm* and BCC are not available (EUCAST), the breakpoint for *P. aeruginosa* (2 μg/ml) was instead used [43].
All six strains proved resistant to colistin with MICs of 16 and 64 in *Sm* K279a and *Sm* D457, respectively, and >256 μg/ml in the three BCC species (Table 1). None of our analogues increased resistance levels to colistin. In fact, most of the compounds, including the natural signalling molecules DSF and BDSF, reduced MIC values in comparison to the DMSO control for the majority of strains assayed. The observed enhancing effect was dose dependent and a generally greater MIC reduction was observed at 50 μM concentration. In *Sm* isolates, all of our molecules reduced MIC values 2- to 16-fold at 50 μM. The greatest reduction was observed in *Sm* D457 challenged with 50 μM of **4c**, which resulted in a MIC to colistin of 4 μg/ml. Aside from *Sm* D457 in the presence of **4b** or **4e**, co-administration of the remaining compounds at

50 μM reduced MICs of *Sm* resistant isolates below 8 μg/ml, a colistin concentration that can be readily reached with colistin inhalation therapy [54].

A 2- to 4-fold reduction of MIC values was also observed in *Bc* R6193 for 5 of the 8 sulfonamides at 50 μM, although antibiotic concentrations remained very high (≥128 μg/ml). In *Bcc* 289, all compounds resulted in decreased MICs, reaching a 16-fold reduction in the case of **4c**. By contrast, *Bm* B10 did not respond to any colistin-adjuvant combination with unaltered MICs recorded.

In order to discard an unspecific enhancing effect of saturated fatty acids, palmitic (C12), lauric (C14) and stearic (C16) fatty acids were also tested at 50 μM in combination with colistin, with unaltered MICs observed for *Sm* and *Bc* R6193 strains (data not shown).

The effect of **4a**-**4h** was also investigated in combination with ciprofloxacin and sulfametoxazole/trimetroprim (SXT), two antibiotics used in the treatment of *Sm* and BCC infections. Although certain antibiotic-adjuvant combinations showed a 2-fold reduction in MICs, no major effect was recorded for any isolate (Supplementary Table 2 and 3).

To further investigate the bactericidal effect of our library in combination with colistin, time-kill curves were performed for those compounds displaying an appreciable MIC reduction against *Sm* K279a, *Sm* D457 and *Bc* R6193. In cases where two or more analogues displayed similar colistin-enhancing activity, those compounds also exhibiting antibiofilm activity were selected (e.g., *Sm* K279a with **4g**). Colistin concentrations were selected based on the corresponding MIC values in combination with the appropriate compound. Following this criteria, *Sm* K279a was challenged with 50 μM of **4g** plus 4 μg/ml of colistin, *Sm* D457 was treated with 50 μM of **4c** plus 4 μg/ml of colistin, and *Bc* R6193 was challenged with 50 μM of **4g** plus 128 μg/ml of colistin. As shown in Figure 4 panels A-C, **4g** and **4c** in combination with 4 μg/ml of colistin significantly reduced the survival of *Sm* K279a and D457 respectively. By contrast, a combination of **4g** with 128 μg/ml of colistin did not decrease the survival of *Bc* R6193.

Our results indicate that for *Sm*, the addition of our compounds to colistin not only reduces MIC values, but also potentiates its bactericidal activity. In *Bc*, however, the colistin-compound combination solely potentiates its growth inhibitory effect. These findings are in line with those obtained by Deng and collaborators [46] who observed similar antibiotic-enhancing activity in experiments with DSF-related molecules.

Table 1. MICs to colistin of *S. maltophilia* and BCC isolates in the presence of the compounds at a fixed dose of 10 and 50 μM by the BMD method.

		colistin MIC (μg/ml)				
		S. maltophilia		*B. cepacia* complex		
Compound	Concentration (μM)	K279a	D457	*B. cepacia* R6193	*B. cenocepacia* 289	*B. multivorans* B10
w/o		16	64	>512	>256	>256
DMSO		16	64	>512	>256	>256
BDSF	10	**8**	**32**	**256**	>256	>256
	50	**4**	**8**	**256**	**64**	>256
4a	10	**16**	**32**	>512	>256	>256
	50	**8**	**16**	>512	**64**	>256
4b	10	**8**	**16**	**256**	128	>256
	50	**4**	**8**	**128**	**32**	>256
4c	10	**4**	**16**	>512	128	>256
	50	**4**	**4**	>512	**16**	>256
4d	10	**8**	**16**	>512	>256	>256
	50	**4**	**8**	**256**	**32**	>256
DSF	10	**8**	**32**	**256**	>256	>256
	50	**4**	**8**	**256**	**64**	>256
4e	10	**8**	**32**	**256**	>256	>256
	50	**8**	**16**	**128**	**32**	>256
4f	10	**8**	**16**	>512	>256	>256
	50	**4**	**8**	>512	**64**	>256
4g	10	**8**	**16**	**256**	128	>256
	50	**4**	**8**	**128**	**256**	>256
4h	10	**8**	**32**	**256**	>256	>256
	50	**4**	**8**	**256**	**32**	>256

ND: Not determined.
Numbers in bolt indicate ≥2-fold MIC reduction.

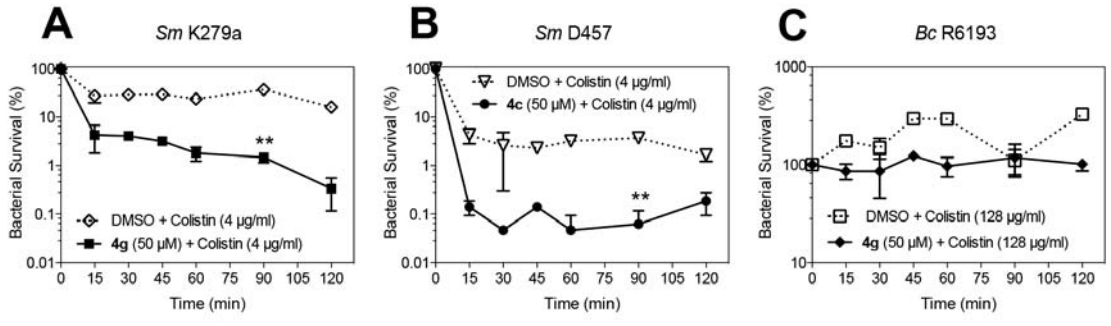

Figure 4. Time kill-curves of the *Sm* K279a (A), *Sm* D457 (B) and *Bc* R6193 (C) in the presence of the appropriate colistin-adjuvant combination (** *P<0.001*).

In an attempt to assess whether our compounds may interfere with QS communication, we designed a negative bioassay to test our library's inhibitory effect on DSF synthesis

611 in *Sm* strain K279a (see materials and methods for details). As shown in Figure 5, 7 of
612 the 8 sulfonamides produced a white halo indicating inhibition of DSF production in *Sm*
613 K279a. The DSF-inhibitory compounds included **4a**-**4e** and **4g**-**4h**, while **4f** produced a
614 blue halo indicating overactivation of the reporter strain. Such activation could be
615 attributable to either intrinsic activity of **4f** on the bioassay or an inducing effect on the
616 DSF synthesis of *Sm* K279a. Of the putative antagonists, **4a**, **4d**, **4e** and **4h** generated
617 the largest halos of inhibition. It is interesting to note that alkyl-subtituted, rather than
618 aryl-substituted, sulfonamides produced the larger halos of inhibition. Methyl-
619 substituted sulfonamides **4a** and **4e** and cyclopropyl-substituted sulfonamides **4d** and
620 **4h** appear to be the more effective inhibitors in this context. Of these, the BDSF
621 analogues **4a** and **4d** exhibit greater inhibition than the corresponding DSF derivatives
622 **4e** and **4h**.
623
624 As expected, DSF and BDSF effected activation of the reporter strain. To determine
625 whether or not the white halo corresponded to growth inhibition of the *Xc* reporter
626 strain, an equal volume of the sulfonamides was added to liquid cultures and the
627 optical density of the strains was read after incubation under the same conditions.
628 Although some compounds slightly reduced growth of the reporter strain, no correlation
629 was observed between the inhibitory halo in the bioassay and the growth inhibition in
630 the liquid culture (Supplementary Figure 2). These results, in combination with the MIC
631 experiments of the compounds alone (Supplementary Figure 1), support the
632 hypothesis that our molecules affect DSF synthesis independently of bacterial growth.
633
634 The same bioassay approach was adopted for *Sm* D457 and *Bc* R6193 strains to
635 measure inhibition of DSF synthesis. As previously reported, however, D457
636 (harbouring the cluster variant *rpf*-2) does not produce detectable levels of DSF under
637 these conditions [26,27] (Figure 5C). Although BDSF production was observed after
638 pin-inoculation of *Bc* in the regular bioassay (Figure 5C), the confluent growth of *Bc* on
639 the negative bioassay plate did not give a blue background corresponding to BDSF
640 activity and it was not possible to test the effect of the antagonists(data not shown).
641

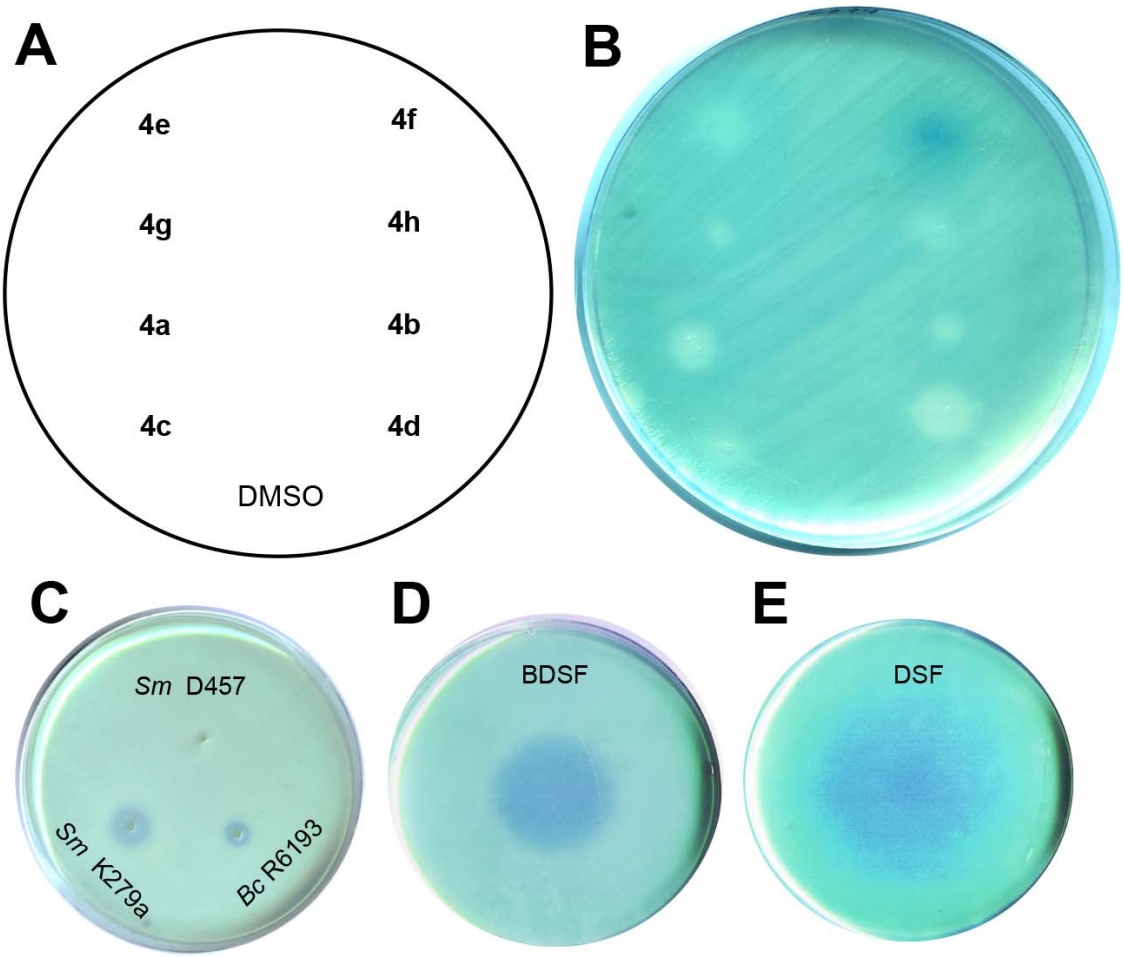

Figure 5. Determination of the inhibitory effect of the compounds on the DSF synthesis of *Sm* K279 using a bioassay (A and B). DSF production by *Sm* K279a, *Sm* D457 and *Bc* R6193 (C). Activity of synthetic DSF (D) and BDSF (E) on the bioassay.

To the best of our knowledge, this is the first time that interference with DSF-QS has been achieved in *Sm* [55]. Nonetheless, further research should be performed to validate DSF inhibition in larger liquid cultures and identify the exact mechanism by which these DSF and BDSF antagonists influence signal synthesis in *Sm* K279a.

Based on the encouraging results from the *in vitro* experiments, we next investigated our compounds' activity *in vivo* using the *Galleria mellonella* model of infection. To that end, we selected the strain-compound combination that exhibited greatest antibiofilm and colistin enhancing activity in *Sm* K279a, *Sm* D457 and *Bc* R6193 isolates. Accordingly, one group of 30 larvae was infected with 1-3 x 10^5 cfu of *Sm* K279a and treated with colistin alone (3.2 mg/kg) or in combination with **4g** (21.5 mg/kg). A second group was infected with the same inoculum of *Sm* D457 and treated with colistin alone (3.2 mg/kg) or in combination with **4c** (20.8 mg/kg). The final group was infected with an equal dose of *Bc* R6193 and challenged with colistin alone (102.4 mg/kg) or in

combination with **4g** (21.5 mg/kg). As with the time-kill curve experiments, treatment of *Bc* infections either with colistin alone or in combination did not result in a significant change in larvae survival. This result further confirms that colistin is not a suitable choice for treating *Bc* infections and that our analogues do not significantly increase colistin potency (data not shown). By contrast, **4g** increased the *in vivo* efficacy of colistin for *Sm* infections, being particularly effective against infections caused by the strain K279a (Figure 6A). Although **4c** was partially effective in the treatment of *Sm* D457 infections, the results were not significant (Figure 6B). These *in vivo* results are in line with those obtained in the MIC and time-kill curves experiments, with **4g** again proving to be the most effective agent against *Sm* K279a.

The increased efficacy observed for **4g** against *Sm* K279a infections may be attributable to a multifactorial effect. On the one hand, the more lipophilic nature of certain analogues may facilitate destabilization of the bacterial membranes, thereby potentiating colistin activity. Recently, it has been reported that addition of exogenous polyunsaturated fatty acids to *Klebsiella pneumoniae* decreased the MICs to polymyxin B and colistin, and inhibited biofilm formation due to interference with membrane phospholipids [56]. Likewise, deletion of *rpfF*-1 (the variant present in K279a) but not *rpfF*-2 (the variant of D457) leads to bacterial attenuation using the *Caenorhabditis elegans* and Zebrafish models, probably due to the inherent inactivity of RpfF-2 in the conditions tested [26].

Colistin was withdrawn from the clinical antibiotic pipeline because of its nephrotoxicity in the early 1980s, but has been recently reintroduced due to the emergence of MDR gram-negative bacteria [57]. Therefore, administration of colistin in combination with adjuvants that potentiate its activity at lower dosages is an interesting strategy. With this in mind, we measured the *in vitro* toxicity of our analogues using HK-2 human kidney cells [58]. The MTT assay revealed that none of the compounds display significant toxicity (Figure 6C).

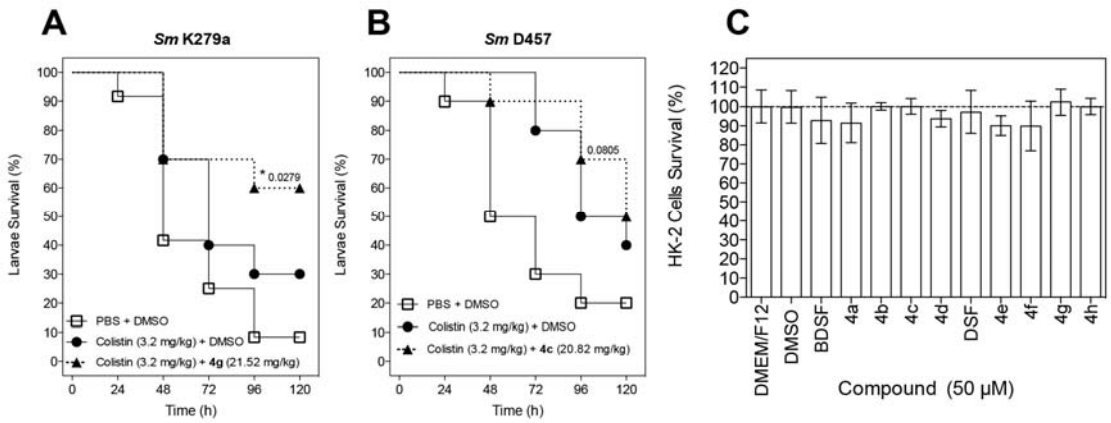

Figure 6. *In vivo* efficacy of **4g** and **4c** in combination with colistin against *Sm* K279a (A) and *Sm* D457 (B). MTT cytotoxic assay of **4a-4h** on HK-2 human kidney cells after 48 h of exposure (C).

Conclusion

The quorum sensing (QS) signals DSF and BDSF produced by *Stenotrophomonas maltophilia* (*Sm*) and species of the *Burkholderia cepacia* complex (BCC) participate in the regulation of clinically relevant phenotypes such as biofilm formation, antimicrobial resistance and bacterial virulence.

In this study, we have synthesized a series of DSF and BDSF derivatives containing bioisosteric sulfonamides in place of the original carboxylic acid groups. We have investigated their efficacy as biofilm inhibitors, antimicrobial adjuvants and QS antagonists against clinical isolates of *Sm* and BCC*,* which are multidrug resistant.

Biofilm assays for *Sm* identified **4g** as the most potent antibiofilm agent against the two representative strains K279a and D457. All of our compounds decreased MICs to colistin in *Sm* isolates. **4c** was observed to be particularly effective against *Sm* D457 causing a 16-fold MIC reduction (final MIC of 4 μg/ml). This was accompanied by an increase in bacterial mortality. In *Sm* K279a **4g**, the most potent biofilm inhibitor, also displayed a reduced MIC to colistin (4-fold; 4 μg/ml) and a significant increase in its bactericidal effect. Remarkably, a majority of our compounds reduced MICs to colistin below 8 μg/ml, a concentration that is reachable by inhalation therapy. Furthermore, treatment of *Galleria mellonella* larvae infected with either *Sm* D457 or K279a with the appropriate colistin-analogue combination resulted in increased larval survival, to a significant extent when K279a was treated with **4g**.

Although most of our compounds reduced MICs to colistin in *Bc* and *Bcc*, they failed to fully rescue the activity of this antibiotic. However, biofilm production in the BCC isolates *Bcc* 289 and *Bm* B10 proved highly sensitive to our sulfonamides, with **4c** and **4g** displaying a significant inhibitory effect at 10 μm concentration. The shared bromophenyl motif in **4c** and **4g** appears key to their activity.

Interestingly, all compounds except **4f** appear to block DSF production in *Sm* K279a, with a noticeably greater inhibitory effect observed in the BDSF derivatives over their corresponding DSF analogues. This is the first time that interference with DSF-QS has been achieved in *Sm*.

Overall, our results show that sulfonamide-containing bioisosteres of DSF and BDSF constitute a new family of bioactive agents with potential antibiofilm, antimicrobial and anti-QS effects. The novel analogues described in this study have been demonstrated to be effective against *Sm* MDR isolates. Future studies should be conducted to identify the precise mechanisms that underlie the variety of effects exhibited by these compounds in order to design more effective antimicrobial agents with a broader spectrum of action against other important MDR gram-negative bacterial pathogens.

Future Perspective

For the last seven decades, antibiotics have played a central role in medicine. Their discovery has rendered previously fatal infections easily treatable. To some extent, antibiotics have become victims of their own success, whereby widespread availability and inappropriate usage have promoted the growth of antimicrobial resistance. Indeed, such a scenario was predicted by Gerhard Domagk in his 1947 Nobel acceptance speech for discovering the first synthetic antibiotics. Currently, bacterial infections are responsible for 700,000 deaths around the globe each year. It is predicted that by 2050, more than 10 million individuals will die as a result of AMR. Given the decreasing number of effective antibiotics and the difficulties associated with the development of new classes of antibiotics, it is clear that alternative strategies are required. One possible approach relies on targeting quorum sensing and bacterial intercellular communication. Interference with quorum sensing can display multiple effects including disruption of resistance mechanisms. Additionally, such an approach does not produce the same evolutionary pressure which is associated with antibiotic usage. Agents which inhibit quorum sensing could offer a new lease of life to both existing antibiotics and to those antibiotics which have fallen out of use. Combination therapies, such as colistin/DSF bioiostere regimen outlined in this work, have significant potential in this regard. Furthermore, compounds which disrupt quorum sensing constitute useful probes for elucidating the underlying basis of bacterial resistance and ultimately designing new strategies for subverting AMR. Similarly novel approaches will be required if we are to successfully tackle AMR into the future.

SUMMARY POINTS

- Sulfonamide-based bioisosteres of DSF and BDSF possess potential antibiofilm and anti-quorum sensing activity against *Stenotrophomonas maltophilia* (*Sm*) and the *Burkholderia cepacia* complex (BCC).
- All of our compounds decrease MICs to colistin (2- to 16-fold) in *Sm* resistant isolates and a majority reduced MICs below 8 μg/ml, a concentration that is reachable by inhalation therapy.
- The 2-bromophenyl-substituted DSF analogue also displays significant antibiofilm activity against *Sm*.
-The majority of these novel compounds inhibit DSF production in *Sm.*
- Treatment of *Sm*-infected *Galleria mellonella* with a combination of colistin and the 2-bromophenyl-substituted DSF bioisostere increases larval survival to a significant extent.

-Most of our compounds reduce MICs to colistin in *B. cepacia* (*Bc*) and *B. cenocepacia* (*Bcc*), and the 2-bromophenyl-substituted DSF and BDSF analogues also exhibit significant antibiofilm activity against *Bc, Bcc and B. multivorans* (*Bm*) isolates.

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Author Contributions

VPK, CH, and TOS designed and synthesized the DSF and BDSF derivatives. PH, DY, XD, TOS and IG conceptually designed the experiments. PH performed most of microbiological experiments. PH and TOS authored the first draft. DY, XD, TOS and IG provided academic input and expertise, and critically reviewed the article. All authors have approved the final version.

802

Abbreviations

Sm - *Stenotrophomonas maltophilia*

BCC - *Burkholderia cepacia* complex

Bc - *Burkholderia cepacia*

Bcc - *Burkholderia cenocepacia*

Bm - *Burkholderia multivorans*

Xc - *Xanthomonas campestris*

Pa - *Pseudomonas aeruginosa*

rpf - Regulation of pathogenicity factors

QS - Quorum sensing

GN - Gram-negative

GP - Gram-positive

DSF - Diffusible signal factor

BDSF - *Burkholderia* diffusible signal factor

DA – Decenoic acid

DMSO - Dimethyl sulfoxide

BMD - Broth microdilution

MIC - Minimal inhibitory concentration

MDR - Multidrug resistance

CAMHB - Cation-adjusted Muller Hinton Broth

CV - Crystal violet

LPS - Lipopolysaccharide

EDCI - *N*-Ethyl-*N´*-(3-dimethylaminopropyl)carbodiimide

DMAP – Dimethylaminopyridine

DCM – Dichloromethane

PSI - Pounds per square inch

ESI-TOF - Electrospray ionisation time-of-flight mass spectrometry

References

Papers of special note have been highlighted as: * of interest; ** of considerable interest

1. Abbott IJ, Peleg AY. Stenotrophomonas, Achromobacter, and nonmelioid Burkholderia species: antimicrobial resistance and therapeutic strategies. *Semin Respir Crit Care Med*. 36(1), 99–110 (2015).

2. Eberl L, Vandamme P. Members of the genus Burkholderia: good and bad guys. *F1000Res* [Internet]. 5 (2016). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4882756/.

3. Hayward AC, Fegan N, Fegan M, Stirling GR. Stenotrophomonas and Lysobacter: ubiquitous plant-associated gamma-proteobacteria of developing significance in applied microbiology. *Journal of Applied Microbiology*. 108(3), 756–770.

4. Alavi P, Müller H, Cardinale M, *et al.* The DSF Quorum Sensing System Controls the Positive Influence of Stenotrophomonas maltophilia on Plants. *PLOS ONE*. 8(7), e67103 (2013).

5. Coenye T, Vandamme P. Diversity and significance of Burkholderia species occupying diverse ecological niches. *Environ. Microbiol.* 5(9), 719–729 (2003).

6. Brooke JS. Stenotrophomonas maltophilia: an Emerging Global Opportunistic Pathogen. *Clin Microbiol Rev*. 25(1), 2–41 (2012).

7. Drevinek P, Mahenthiralingam E. Burkholderia cenocepacia in cystic fibrosis: epidemiology and molecular mechanisms of virulence. *Clinical Microbiology and Infection*. 16(7), 821–830 (2010).

8. Adegoke AA, Stenström TA, Okoh AI. Stenotrophomonas maltophilia as an Emerging Ubiquitous Pathogen: Looking Beyond Contemporary Antibiotic Therapy. *Front Microbiol* [Internet]. 8 (2017). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5714879/.

9. Mahenthiralingam E, Urban TA, Goldberg JB. The multifarious, multireplicon *Burkholderia cepacia* complex. *Nature Reviews Microbiology*. 3(2), 144–156 (2005).

10. Spencer RC. The emergence of epidemic, multiple-antibiotic-resistant Stenotrophomonas (Xanthomonas) maltophilia and Burkholderia (Pseudomonas) cepacia. *Journal of Hospital Infection*. 30, 453–464 (1995).

11. Rajkumari N, Mathur P, Gupta AK, Sharma K, Misra MC. Epidemiology and outcomes of Stenotrophomonas maltophilia and Burkholderia cepacia infections among trauma patients of India: a five year experience. *J Infect Prev*. 16(3), 103–110 (2015).

12. Ryan RP, An S, Allan JH, McCarthy Y, Dow JM. The DSF Family of Cell–Cell Signals: An Expanding Class of Bacterial Virulence Regulators. *PLOS Pathogens*. 11(7), e1004986 (2015).

**Provides a comprehensive overview of cell-cell signalling.

13. Deng Y, Wu J, Tao F, Zhang L-H. Listening to a New Language: DSF-Based Quorum Sensing in Gram-Negative Bacteria. *Chem. Rev.* 111(1), 160–173 (2011).

*A review focussed on the DSF signal family.

14. Zhou L, Zhang L-H, Cámara M, He Y-W. The DSF Family of Quorum Sensing Signals: Diversity, Biosynthesis, and Turnover. *Trends Microbiol.* 25(4), 293–303 (2017).

15. Kåhrström CT. Entering a post-antibiotic era? *Nature Reviews Microbiology*. 11, 146 (2013).

16. Zaman SB, Hussain MA, Nye R, Mehta V, Mamun KT, Hossain N. A Review on Antibiotic Resistance: Alarm Bells are Ringing. *Cureus*. 9(6), e1403 (2017).

*A recent perspective on the growing threat of AMR.

17. González-Bello C. Antibiotic adjuvants – A strategy to unlock bacterial resistance to antibiotics. *Bioorganic & Medicinal Chemistry Letters*. 27(18), 4221–4228 (2017).

18. Rasko DA, Sperandio V. Anti-virulence strategies to combat bacteria-mediated disease. *Nature Reviews Drug Discovery*. 9(2), 117–128 (2010).

19. Dickey SW, Cheung GYC, Otto M. Different drugs for bad bugs: antivirulence strategies in the age of antibiotic resistance. *Nature Reviews Drug Discovery*. 16(7), 457–471 (2017).

20. Cegelski L, Marshall GR, Eldridge GR, Hultgren SJ. The biology and future prospects of antivirulence therapies. *Nature Reviews Microbiology*. 6(1), 17–27 (2008).

21. Ribeiro SM, Felício MR, Boas EV, *et al.* New frontiers for anti-biofilm drug development. *Pharmacology & Therapeutics*. 160, 133–144 (2016).

22. Papenfort K, Bassler BL. Quorum sensing signal–response systems in Gram-negative bacteria. *Nature Reviews Microbiology*. 14(9), 576–588 (2016).

23. Rutherford ST, Bassler BL. Bacterial Quorum Sensing: Its Role in Virulence and Possibilities for Its Control. *Cold Spring Harb Perspect Med*. 2(11), a012427 (2012).

24. Antunes LCM, Ferreira RBR, Buckner MMC, Finlay BB. Quorum sensing in bacterial virulence. *Microbiology (Reading, Engl.)*. 156(Pt 8), 2271–2282 (2010).

25. Defoirdt T. Quorum-Sensing Systems as Targets for Antivirulence Therapy. *Trends Microbiol.* 26(4), 313–328 (2018).

26. Huedo P, Yero D, Martínez-Servat S, *et al.* Two different rpf clusters distributed among a population of Stenotrophomonas maltophilia clinical strains display differential diffusible signal factor production and virulence regulation. *J. Bacteriol.* 196(13), 2431–2442 (2014).

* Characterization of DSF-QS in a clinical population of *S. maltophilia*

27. Huedo P, Yero D, Martinez-Servat S, *et al.* Decoding the genetic and functional diversity of the DSF quorum-sensing system in Stenotrophomonas maltophilia. *Front Microbiol*. 6, 761 (2015).

28. Deng Y, Wu J, Eberl L, Zhang L-H. Structural and functional characterization of diffusible signal factor family quorum-sensing signals produced by members of the Burkholderia cepacia complex. *Appl. Environ. Microbiol.* 76(14), 4675–4683 (2010).

29. Boon C, Deng Y, Wang L-H, *et al.* A novel DSF-like signal from Burkholderia cenocepacia interferes with Candida albicans morphological transition. *ISME J*. 2(1), 27–36 (2008).

30. Häussler S, Becker T. The Pseudomonas Quinolone Signal (PQS) Balances Life and Death in Pseudomonas aeruginosa Populations. *PLOS Pathogens*. 4(9), e1000166 (2008).

31. Reen FJ, Mooij MJ, Holcombe LJ, *et al.* The Pseudomonas quinolone signal (PQS), and its precursor HHQ, modulate interspecies and interkingdom behaviour. *FEMS Microbiol Ecol*. 77(2), 413–428 (2011).

32. Deng Y, Lim A, Lee J, *et al.* Diffusible signal factor (DSF) quorum sensing signal and structurally related molecules enhance the antimicrobial efficacy of antibiotics against some bacterial pathogens. *BMC Microbiol*. 14, 51 (2014).

33. Dow JM, Crossman L, Findlay K, He Y-Q, Feng J-X, Tang J-L. Biofilm dispersal in Xanthomonas campestris is controlled by cell–cell signaling and is required for full virulence to plants. *PNAS*. 100(19), 10995–11000 (2003).

34. Davies DG, Marques CNH. A fatty acid messenger is responsible for inducing dispersion in microbial biofilms. *J. Bacteriol.* 191(5), 1393–1403 (2009).

35. de Rossi BP, García C, Alcaraz E, Franco M. Stenotrophomonas maltophilia interferes via the DSF-mediated quorum sensing system with

945 Candida albicans filamentation and its planktonic and biofilm modes of
946 growth. *Rev. Argent. Microbiol.* 46(4), 288–297 (2014).

947 36. Wang L-H, He Y, Gao Y, *et al.* A bacterial cell-cell communication signal
948 with cross-kingdom structural analogues. *Mol. Microbiol.* 51(3), 903–912
949 (2004).

950 37. Meanwell NA. Synopsis of some recent tactical application of bioisosteres
951 in drug design. *J. Med. Chem.* 54(8), 2529–2591 (2011).

952 *A review of the successful application of bioisosteres in medicinal chemistry.

953

954 38. Olesen PH. The use of bioisosteric groups in lead optimization. *Curr Opin*
955 *Drug Discov Devel*. 4(4), 471–478 (2001).

956 39. Lowery CA, Salzameda NT, Sawada D, Kaufmann GF, Janda KD.
957 Medicinal chemistry as a conduit for the modulation of quorum sensing. *J.*
958 *Med. Chem.* 53(21), 7467–7489 (2010).

959 40. Slater H, Alvarez-Morales A, Barber CE, Daniels MJ, Dow JM. A two-
960 component system involving an HD-GYP domain protein links cell-cell
961 signalling to pathogenicity gene expression in Xanthomonas campestris.
962 *Mol. Microbiol.* 38(5), 986–1003 (2000).

963 41. Clinical and Laboratory Standards Institute. Methods for dilution
964 antimicrobial susceptibility tests for bacteria that grow aerobically.
965 Approved standard. 10th edition. M07 A10. Clinical and Laboratory
966 Standards Institute, Wayne, PA. (2015).

967 42. The European Committee on Antimicrobial Susceptibility Testing and
968 Clinical and Laboratory Standards Institute. Recommendations for MIC
969 determination of colistin (polymyxin E) as recommended by the joint CLSI-
970 EUCAST Polymyxin Breakpoints Working Group. (2016). Available from:
971 http://www.eucast.org/guidance_documents/.

972 43. European Committee on Antimicrobial Susceptibility Testing. Breakpoint
973 tables for interpretation of MICs and zone diameters. Version 8.0. (2018).
974 Available from: http://www.eucast.org/clinical_breakpoints/.

975 44. Kumar VP, Gupta MK, Horgan C, O'Sullivan TP. Synthesis of the quorum
976 sensing molecule Diffusible Signal Factor using the alkyne zipper reaction.
977 *Tetrahedron Letters*. 59(22), 2193–2195 (2018).

978 45. Ryan RP, Fouhy Y, Garcia BF, *et al.* Interspecies signalling via the
979 Stenotrophomonas maltophilia diffusible signal factor influences biofilm
980 formation and polymyxin tolerance in Pseudomonas aeruginosa. *Molecular*
981 *Microbiology*. 68(1), 75–86.

982 46. Deng Y, Lim A, Lee J, *et al.* Diffusible signal factor (DSF) quorum sensing
983 signal and structurally related molecules enhance the antimicrobial efficacy

of antibiotics against some bacterial pathogens. *BMC Microbiol*. 14, 51 (2014).

47. Ortega X, Silipo A, Saldías MS, Bates CC, Molinaro A, Valvano MA. Biosynthesis and Structure of the Burkholderia cenocepacia K56-2 Lipopolysaccharide Core Oligosaccharide. *J Biol Chem*. 284(32), 21738–21751 (2009).

48. El-Halfawy OM, Valvano MA. Antimicrobial Heteroresistance: an Emerging Field in Need of Clarity. *Clin. Microbiol. Rev.* 28(1), 191–207 (2015).

49. Olaitan AO, Morand S, Rolain J-M. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Front Microbiol* [Internet]. 5 (2014). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4244539/.

50. Juhász E, Iván M, Pintér E, Pongrácz J, Kristóf K. Colistin resistance among blood culture isolates at a tertiary care centre in Hungary. *J Glob Antimicrob Resist*. 11, 167–170 (2017).

51. Wei C, Ni W, Cai X, Zhao J, Cui J. Evaluation of Trimethoprim/Sulfamethoxazole (SXT), Minocycline, Tigecycline, Moxifloxacin, and Ceftazidime Alone and in Combinations for SXT-Susceptible and SXT-Resistant Stenotrophomonas maltophilia by In Vitro Time-Kill Experiments. *PLoS ONE*. 11(3), e0152132 (2016).

52. Martínez-Servat S, Yero D, Huedo P, *et al.* Heterogeneous colistin-resistance phenotypes coexisting in Stenotrophomonas maltophilia isolates influence colistin susceptibility testing. *Front. Microbiol.* [Internet]. 9 (2018). Available from: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02871/full.

53. Liu Y-Y, Wang Y, Walsh TR, *et al.* Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *The Lancet Infectious Diseases*. 16(2), 161–168 (2016).

54. Malott RJ, Wu C-H, Lee TD, *et al.* Fosmidomycin Decreases Membrane Hopanoids and Potentiates the Effects of Colistin on Burkholderia multivorans Clinical Isolates. *Antimicrob Agents Chemother*. 58(9), 5211–5219 (2014).

55. Huedo P, Coves X, Daura X, Gibert I, Yero D. Quorum Sensing Signaling and Quenching in the Multidrug-Resistant Pathogen Stenotrophomonas maltophilia. *Front Cell Infect Microbiol* [Internet]. 8 (2018). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5928129/.

56. Hobby CR, Herndon JL, Morrow CA, Peters RE, Symes SJK, Giles DK. Exogenous fatty acids alter phospholipid composition, membrane permeability, capacity for biofilm formation, and antimicrobial peptide susceptibility in Klebsiella pneumoniae. *MicrobiologyOpen*. 0(0), e00635.

57. Falagas ME, Kasiakou SK, Saravolatz LD. Colistin: The Revival of Polymyxins for the Management of Multidrug-Resistant Gram-Negative Bacterial Infections. *Clin Infect Dis*. 40(9), 1333–1341 (2005).

58. Huang JX, Kaeslin G, Ranall MV, *et al.* Evaluation of biomarkers for in vitro prediction of drug-induced nephrotoxicity: comparison of HK-2, immortalized human proximal tubule epithelial, and primary cultures of human proximal tubular cells. *Pharmacol Res Perspect* [Internet]. 3(3) (2015). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4492764/.