Microfluidics-based LC-MS MRM approach for the relative quantification of *Burkholderia cenocepacia* secreted virulence factors

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Abstract

Burkholderia cenocepacia is an opportunistic pathogen that is commonly isolated from patients with cystic fibrosis (CF). Several virulence factors have been identified, including extracellular enzymes that are secreted by type II and type VI secretion systems. The activity of these secretion systems is modulated by quorum sensing. Apart from the classical acylhomoserine lactone quorum sensing, *B. cenocepacia* also uses the diffusible signal factor system (DSF) i.e. 2-undecenoic acid derivatives that are recognized by specific receptors resulting in changes in biofilm formation, motility and virulence. However, quantitative information on alterations in the actual production and release of virulence factors upon exposure to DSF is lacking. We here describe an approach implementing microfluidics based chromatography combined with single reaction monitoring to quantify protein virulence factors in the secretome of *B. cenocepacia*.

Introduction

Cystic fibrosis (CF) is a genetically inherited disease caused by mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which leads to the formation of a dehydrated mucus layer covering lung epithelial cells ^[1]. Patients suffering from CF are highly susceptible to recurrent and chronic lung infections. About 80% of all adult people with CF are chronically infected with *Pseudomonas aeruginosa* ^[2]. In most cases this pathogen is accompanied by other infecting bacteria, like *Burkholderia cenocepacia* and *Stenotrophomonas maltophilia*, leading to the formation of polymicrobial communities, frequently characterized by extremely high levels of antibiotic resistance ^[3,4]. This phenomenon complicates treatment and aggravates disease progression. Bragonzi et al. (2012) demonstrated that *B. cenocepacia* positively influences biofilm formation by *P. aeruginosa* and co-infection increases the host inflammatory response ^[5]. *S. maltophilia* may have a considerable effect on the *P. aeruginosa* biofilm architecture and polymyxin tolerance, which depends on a diffusible unsaturated fatty acid, cis-11-methyl-2-dodecenoic acid (DSF) ^[6]. DSFs are an important class of quorum sensing molecules that were first described in the plant pathogen *Xanthomonas campestris* pv. *campestris* (Xcc) ^[7,8]. Currently, various DSFs have been identified in different species. For example, *B. cenocepacia* and *P. aeruginosa* synthesize cis-2-dodecenoic acid (BDSF) and cis-2-decenoic acid (PDSF), respectively ^[9–12]. Furthermore, nanomolar quantities of BDSF and DSF were detected in the sputum of CF patients and directly correlated with the presence of *B. cenocepacia* and *S. maltophilia* ^[13]. The authors also showed that *P. aeruginosa* isolates from people with CF retain the ability to respond to DSF, as shown by their increased tolerance to polymyxin B and E upon addition of synthetic DSF. A summary of the current knowledge about the impact of BDSF signaling on *B. cenocepacia* and potential interplay with other DSFs is presented in FIGURE 1.

In *B. cenocepacia*, the *rpff_{Bc}* (BCAM0581) gene encodes an enoyl-CoA hydratase with both hydratase and thioesterase activity, which enables the conversion of the acyl carrier protein thioester of 3-hydroxydodecanoic acid into cis-2-dodecenoic acid ^[14–16]. Intermediates of the fatty acid biosynthesis pathway presumably serve as the substrates for RpfF_{Bc} *in vivo* ^[17]. The gene adjacent to *rpff_{Bc}*, BCAM0580, encodes the BDSF receptor protein RpfR ^[18]. This protein contains PAS (Per-Arnt-Sim), GGDEF and EAL domains. The latter two domains are characteristic for diguanylate cyclases and phosphodiesterases, respectively, which are commonly involved in cyclic-di-GMP turnover ^[19]. Upon BDSF binding to the PAS domain of RpfR, its cyclic-di-GMP phosphodiesterase activity is switched on, thereby lowering intracellular cyclic-di-GMP levels ^[18]. Cyclic-di-GMP in turn functions as a global regulator through binding to diverse receptors and effectors, including PilZ domain proteins, transcription factors and riboswitches ^[20,21]. Cyclic-di-GMP promotes biofilm formation and T6SS activity, and reduces motility and invasion ^[22]. A second DSF receptor protein, encoded by BCAM0227, appears to control only a subset of the BDSF-regulated genes ^[23]. This receptor does not contain GGDEF or EAL domains, but possesses a sensor histidine kinase domain and a histidine-containing phosphotransfer domain, suggesting it is part of a two-component system. Its cognate response regulator protein might be BCAM0228, which contains a DNA-binding helix-turn-helix domain. Inactivation of *rpfR* or *rpff_{Bc}* lowers motility, adherence, biofilm formation and proteolytic activity ^[18,24,25], while inactivation of BCAM0227 reduces cytotoxicity towards Chinese hamster ovary cells and virulence in an agar-bead mouse model of pulmonary infection, and in the *Galleria mellonella* infection model ^[23].

B. cenocepacia Infection is associated with the secretion of virulence factors involving both type II (T2SS) and type VI (T6SS) secretion systems, including Zn metalloproteases ZmpA and ZmpB, and the

Rho GTPase TecA ^[26–31]. Today, there is no comprehensive quantitative information on the impact of DSFs on actual levels of these secreted virulence factors. In fact, assaying these virulence factors is typically based on very general and non-specific methods like assessing proteolytic activity on azocasein. The aim of this study was thus to develop and apply methodology to simultaneously quantify in a specific manner a panel of known virulence factors of *B. cenocepacia* K56-2, a strain belonging to the ET12 lineage, the causative of cepacia syndrome in people with CF ^[32]. First, we quantified the endogenous levels of BDSF produced by this strain using a reverse phase HPLC method. The concentration range in which exogenously added BDSF causes significant effects on a subset of known secreted virulence factors was determined using targeted proteomics. Lange et al. (2008) were the first to use multiple reaction monitoring for targeted quantitation of secreted virulence factors of *Streptococcus pyogenes* ^[33]. While MRM- based targeted proteomics has been largely recognized as an excellent tool for protein quantitation, it has not been applied to study the impact of quorum sensing on extracellular secretion of (opportunistic) pathogens associated with CF disease progression. Here, we introduce a microfluidics based LC-MS method (implementing IonKey™ technology) to fill this gap. In this system, the microcolumn is integrated with the electrospray probe, displaying high robustness and reduced post-column dead volumes compared to classical LC-MS set-ups.

A Label-free proteomic approach was additionally performed to detect differences in the abundance of secreted proteins upon BDSF stimulation and results were validated with SRM. Both the time-dependent effects of BDSF, DSF and PDSF and the effects after 2 h of stimulation on a selected panel of secreted virulence factors were assessed using SRM.

Material and methods

Chemicals and Solvents

Chemicals, including DSFs, and reagents were from Sigma-Aldrich, unless otherwise stated. Solvents for HPLC were from Biosolve.

Bacterial strains and culture conditions

The *B. cenocepacia* K56-2 wild type strain used in this work was received from Prof. Dr. Miguel Valvano (Queen's University, Belfast, UK) and was maintained in Microbank™ bacterial preservation systems (Fischer Scientific). All cultures were grown in Miller's Luria Broth (LB, Difco™) on a shaker (200 rpm) at 37°C, unless otherwise stated. Overnight cultures were diluted to an OD₆₀₀ = 0.2 in fresh LB and grown for 5 h. At this time point, cultures were stimulated with BDSF, PDSF or DSF (dissolved in methanol) at different concentrations (for BDSF) or at a final concentration of 100 µM (PDSF and DSF) and were harvested after 30 min (T1), 1 h (T2) or 2 h (T3). Unstimulated cultures were supplemented with equal amounts of methanol and harvested immediately (T0) for time-dependent experiments or after two hours for all other experiments. We also performed experiments where BDSF was added earlier, after 3h (collection of samples 2h after BDSF addition) and another where we collected samples in the stationary phase (+24h after BDSF addition)

Extraction and quantification of DSFs from culture supernatant

DSFs were extracted according to Deng et al. ^[9]. Briefly, the supernatant of *B. cenocepacia* K56-2 cultures was collected by centrifugation (10 min, 6000 xg) and was filtered twice through a 0.22 µm

filter. DSFs were extracted with an equal amount of ethyl acetate. Samples were mixed for 30 min, followed by centrifugation at 4696 xg for 15 min. The upper organic phase was collected and dried by vacuum centrifugation. The dried pellets were dissolved in 50% methanol/0.1% trifluoroacetic acid (TFA).

A reversed-phase HPLC analysis (adapted from Ionescu et al., 2016^[34]) was performed on an Ettan LC system (GE Healthcare Life Sciences) equipped with an Acclaim PepMap100 C18 column (3 μ m, 250 mm x 75 μ m) (Thermo Scientific) with as solvent A 0.1% TFA in H₂O (Milli-Q; MQ) and solvent B 0.1% TFA in methanol. A 15-min gradient starting from 25% solvent B and increasing to 100% solvent B was applied for the separation. The absorbance at 210, 220 and 254 nm recorded by the UV detector was used to monitor BDSF. Quantification was based on the 210-nm signal. BDSF was identified using reference data from the combination of the retention time and the UV spectral patterns of pure standard compounds. A standard curve of synthetic BDSF was used to derive the absolute BDSF concentrations present in the different samples. Calibration solutions (0.1, 0.5, 1.0, 2.5, 5.0 and 10.0 ng/ μ l) were prepared by serial dilution of the individual stock solutions with water. Ten μ l of each solution were injected into the HPLC system, all in duplicate, and a calibration curve was generated. Peak areas were integrated automatically using the Unicorn version 5.11 software package. To calculate the loss during ethyl acetate extraction, a blank LB sample containing 50 ng BDSF was extracted using the same protocol as the samples and the amount of BDSF recovered was quantified from the standard curve.

Isolation of extracellular proteins

Culture samples were centrifuged (6000 xg, 15 min) and filtered through a 0.22 μ m pore size filter (Sarstedt) to remove residual cells. Complete protease inhibitor cocktail (EDTA-free) (Roche) was added to the filtrate (1 tablet per 50 ml). After the addition of 0.02% sodium deoxycholate (DOC), the mixture was incubated on ice for 30 min. Proteins were precipitated overnight with 10% trichloroacetic acid (TCA) and collected by centrifugation (4696 xg, 1h, 4°C). The pellet was dissolved in 0.1 M Tris-HCl pH 8.0 and washed by ultrafiltration (Amicon Ultra-15, 10 kDa cutoff, Merck Millipore). A chloroform methanol extraction was performed to remove lipids and DNA. Protein concentrations were estimated by Bradford's method (Coomassie Bradford Assay kit, Pierce).

Shotgun proteomics by LC-MS/MS analysis on a TripleTOF 5600 mass spectrometer

After reduction, alkylation and digestion of proteins with trypsin, peptide samples were loaded onto an Eksigent Expert nanoLC 425 system, coupled on-line to a TripleTOF 5600 mass spectrometer (ABSciex). Peptides were trapped for 5 min at a flow rate of 8 μ l/min and separated on a YMC Triart C18 column (3 μ m, 0.3 x 150 mm) using a 90-min gradient ranging from 3% to 40% solvent B (0.1% formic acid in ACN) at a flow rate of 5 μ l/min. The outlet of the LC column was coupled directly to the inlet of a DuoSpray ESI source. The capillary voltage was set to 5500 and MS scans were recorded from 300 to 1250 m/z. The MS/MS scan range was set from 50 to 1800 m/z. The instrument was operated in data-dependent acquisition mode. Precursors were selected for fragmentation at a threshold of 500 cps, with a charge of +2 to +5 and a dynamic exclusion window of 10s.

LC-MS/MS data were imported in Progenesis LC-MS (Nonlinear Dynamics) for quantitative analysis. Identification of the corresponding peptides and proteins was performed by searching the spectra against the *B. cenocepacia* K56-2 database using Mascot (Matrix Science, London, UK; version 2.5.0). Trypsin was set as the digestion enzyme; the peptide and fragment tolerances were set to 10 ppm and 0.1 Da respectively and a maximum of two missed cleavages was allowed. Carbamidomethylation of

cysteine was set as a fixed modification, and oxidation of methionine and deamidation of asparagine to glutamine were set as variable modifications. Search results were imported back into Progenesis LC-MS. The protein p-values and q-values had to be smaller than or equal to 0.05 to accept a difference as significant. A minimum of 2 peptides per protein used for quantification was required, as well as a maximal fold change ≥ 1.5 .

Selected Reaction Monitoring (SRM)

The targeted proteomics approach implemented selected reaction monitoring. For each protein, a minimum of 6 transitions was selected for monitoring by SRM. Selection was based on the results of preliminary test experiments in which possible transitions were monitored for their ionization and detection efficiency. The specificity of the peptide sequence for a particular protein was verified using the Unipept web server ^[35]. Protein samples were reduced with dithiothreitol, alkylated with iodoacetamide and digested with sequencing-grade modified trypsin. Prior to reduction and alkylation, bovine serum albumin (BSA) was spiked into the samples at a ratio of 1:75 (132 ng BSA in 10 μ g sample). The peptide mixtures were analyzed on a M-class Acquity system coupled to a Xevo TQ-S system (Waters). Peptides were loaded on a M-class Trap Symmetry[®] C18 column (100 Å, 5 μ m, 300 μ m x 50 mm) (Waters) using a flow rate of 15 μ l/min for 5 min. After trapping, the peptides were separated on an iKey HSS T3 analytical column (1.8 μ m, 150 μ m x 100 mm) (Waters) at a flow rate of 1 μ l/min. As mobile phases, 0.1% HCOOH in MQ and 0.1% HCOOH in ACN were used to separate the peptides with a 20-min gradient, ranging from 3% to 50% ACN. The eluting peptides were sprayed directly into the Xevo TQ-S mass spectrometer (Waters) using the IonKey+ ESI source. The system was operated in positive mode. The source temperature was 120°C, the capillary voltage 3.6 kV and the cone voltage 35 V. The cone gas flow was set at 150 L/hr, the collision gas flow at 0.19 ml/min and the nebulizer and nanoflow gas pressures at 6 bar and 0.2 bar respectively. The collision energy (CE) was calculated for each peptide with the following equation: $CE (V) = (0.5 \times \text{precursor ion } m/z) + 5$.

Data were imported into Skyline ^[36,37] and for each peptide, the total area under curve (AUC) was calculated. Values from individual peptides were normalized against the intensity of a BSA peptide (QTALVELLK). A one-way Anova (in case of equal variances) or analogous non-parametric test (in case of unequal variances), followed by appropriate post-hoc tests, was performed to detect statistically significant differences between the different conditions.

Results and discussion

Endogenous levels of BDSF in *B. cenocepacia* K56-2 are maximal in late-exponential growth phase

To determine the concentration of BDSF released in LB medium by *B. cenocepacia* K56-2 cultures, fatty acids were extracted using ethyl acetate and quantified by reversed-phase HPLC analysis. The production of BDSF was assessed along different points in the growth curve, using a standard curve, which was established by injecting different amounts of BDSF standards (supplementary figure S1). To estimate the amount of BDSF lost during ethyl acetate purification and separation, a control sample with a known amount of BDSF (50 ng) spiked in LB was extracted using the same protocol.

The average amount of BDSF recovered from the 50-ng control sample was 8.72 ng (two replicate measurements), representing a 5.73-fold loss during extraction and/or separation. This loss is incorporated in the calculations of the BDSF concentrations present in the culture supernatants of *B.*

cenocepacia K56-2. The BDSF concentrations and corresponding OD₆₀₀ values at the time of extraction are plotted in function of the growth time (FIGURE 2). Production of BDSF reaches its maximum after 6 h of growth, during the late-exponential growth phase, with a concentration of 1068 nM after correction. It slightly decreases the next 4 h, but after 10 h, the concentration sharply declines to $\pm$ 600 nM and further to its basal level of $\pm$ 400 nM. This concentration is in the same range as was determined for DSF in sputum of patients with CF (250 nM)^[13]. The steep drop in extracellular BDSF concentration in the stationary phase might be explained through the potential presence of a DSF turnover mechanism. The existence of this mechanism was first demonstrated in the plant pathogen *X. campestris* pv. *campestris* (Xcc), where DSF molecules accumulated during early stationary phase and subsequently declined sharply^[38].

Exogenously added BDSF causes significant effects on the secretome only at concentrations higher than 75 μ M LB medium- grown *B. cenocepacia*

To obtain more insight in the effects of exogenously added BDSF, different BDSF concentrations were added to exponential phase *B. cenocepacia* K56-2 cultures grown for 4h in LB medium, starting from 5 μ M BDSF up to 100 μ M BDSF. This time point was chosen as at this time point endogenous production is still limited and to see what is the effect on cells that have thus not yet been exposed to BDSF. Cultures were grown for two more hours and extracellular proteins were isolated. Cultures were grown for an additional two hours in the presence of the stimulus. The secretion of known virulence factors (ZmpA, ZmpB, Hcp1, TecA, AidA and CblA^[39–45]) in each culture was quantified via SRM. Transitions were selected based on the results of preliminary test experiments in which possible transitions were monitored for their ionization and detection efficiency (Supplementary Table S1). Peak areas were calculated in Skyline and normalized against the internal standard (BSA - QTALVELLK). A One-way Anova and Dunnett's multiple comparison test were used to detect statistically significant differences by comparing each condition to the unstimulated control (0 μ M BDSF, collected at start of incubation with BDSF) (supplementary figure S2).

Surprisingly, a significant increase in abundance of ZmpA was detected upon stimulation with BDSF only at a concentration of at least 75 μ M. This resulted in a fold change of 1.90 compared to unstimulated cultures. Stimulation with 100 μ M BDSF caused a 1.5-fold increase in ZmpA secretion. The production of the other type II secretion system (T2SS)-dependent metalloprotease, ZmpB, is not affected by addition of BDSF. The nematocidal protein AidA, also T2SS dependent, showed a trend towards an increased secretion upon BDSF stimulation, although this increase was not significant at the 5% confidence level. The highest increment was observed after addition of 75 μ M BDSF (fold change of 1.75), comparable to the profile observed for ZmpA. Stimulation with 100 μ M BDSF resulted in a fold change of 1.47 compared to unstimulated cultures.

The poor response to BDSF at lower concentrations could be due to active turn-over mechanisms under the conditions used, which requires further investigations. However, the trends we observe agree with previous findings showing that mutation of the BDSF synthase BCAM0581 resulted in decreased ZmpA promoter activity^[25], suggesting that exogenous BDSF could enhance *zmpA* transcription. It is known that ZmpA production is under control of the acylhomoserine lactone quorum sensing, which is on its turn responsive to BDSF. Studies in the *B.cenocepacia* H111 strain showed increased protease activity upon exposure to BDSF, as a result of this interplay between the two quorum sensing systems^[46]. However, care should be taken to translate results from H111 to the K56-

2 strain as they differ in virulence towards nematode and insect models. The apparent unresponsiveness of ZmpB towards BDSF suggests a different regulatory mechanism for the two metalloproteases, and agrees with a previous study on the influence of the transcriptional regulator ShvR on type II secretion. Mutation of *shvR* led to decreased transcription of *zmpA*, while transcription of *zmpB* increased in the Δ *shvR* mutant ^[47]. Secretion of the T2SS dependent AidA, a protein contributing to the killing of *C. elegans* by *B. cenocepacia* ^[48], was also induced upon BDSF stimulation of *B. cenocepacia* K56-2 cultures. Since *aidA* mutation had no effect on the virulence of *B. cenocepacia* in the rat chronic respiratory infection model ^[49], and was overexpressed in nonmucoid *B. cenocepacia* isolates compared to mucoid isolates ^[50], its exact role during infection remains obscure.

Secretion of the T6SS protein Hcp1 was negatively influenced at higher concentrations of BDSF, since addition of 100 μ M BDSF caused a 2-fold reduction in its abundance in the secretome of exponential phase cultures. This decrease is less pronounced when lower concentrations of BDSF are added, and is only observed starting from stimulation with 50 μ M BDSF. TecA, a recently identified T6SS effector included in this analysis, did not show a significant decrease in secretion upon BDSF stimulation (fold change of 1.4 upon stimulation with 100 μ M BDSF).

Expression of the major cable pilus protein CblA was slightly reduced when cultures were stimulated with 100 μ M BDSF (fold change of 1.49), although this was significant for only 1 of the peptides targeted for this protein.

BDSF negatively regulates T6SS activity, while stimulating lipase production and expression of ribosomal proteins

To investigate whether BDSF has other effects on the secretome of *B. cenocepacia* K56-2, mid-exponential phase cultures were stimulated with 100 μ M BDSF and the secretome was isolated after 2 hours of growth in the presence of the stimulus. Unstimulated cultures collected at the same time point were used as a control. Secreted proteins from each condition (with three biological replicates) were quantified by label free proteomics using LC-MS/MS on a TripleTOF 5600 mass spectrometer, operated in data-dependent mode. This experiment confirms the reduced abundance of proteins depending on T6SS activity, i.e. Hcp1, the T6SS inner tube protein and the T6SS-effector TecA, showed a 2-fold reduction in secretion (Table 1). Also, secretion of a putative exotoxin (BCAM1074) was significantly reduced in this condition. This protein contains a signal peptide and is predicted to be secreted, although the exact function is unknown. It lies within a large cluster encoding phage proteins presumably spanning from BCAM1024 to BCAM1097. Three phage proteins, of which two (BCAM1067 and BCAM1069) belong to the same phage cluster, were decreased in the secretome of cultures stimulated with 100 μ M BDSF.

A remarkable observation is the significant increase in abundance of numerous ribosomal proteins upon BDSF stimulation (Table 2). A lipase belonging to the GDSL family and its adjacent gene encoding the phenylacetate-CoA oxygenase subunit PaaA, involved in phenylacetic acid degradation, also show a significantly increased secretion in this condition. The function of the lipase in *B. cenocepacia* is currently unknown. However, the lipase domain is similar to the PlaA lipase from *Legionella pneumophila*. This is a type II secreted lysophospholipase A with a role in the bacterial detoxification of lysophospholipids ^[51]. Similar phospholipases belonging to class A have been implicated in the pathogenesis of *Campylobacter*, *Yersinia*, *Helicobacter* and *Salmonella* species ^[52–55]

These results were validated by quantifying a selected number of proteins by SRM using the IonKey microfluidics separation technology coupled to the Xevo TQ-S mass spectrometer. The results confirm a significant decrease in secretion of the T6SS protein Hcp1. In contrast, the T6SS effector TecA, which is not encoded in the T6SS gene cluster, does not show a significantly reduced secretion in the SRM analysis (FIGURE 3). The expression of flagellin, the main building block of flagella, is somewhat reduced upon BDSF stimulation, although this difference was only significant at the 5% confidence level for one of the three targeted peptides. The significant increase in secretion of the GDSL family lipase found in the label-free proteomics study, was also confirmed here, with a fold change of 1.8 induced upon BDSF stimulation. Secretion of three phage proteins, encoded by BCAM1067 and BCAM1069, and of the exotoxin (BCAM1074) was reduced upon BDSF stimulation in the label-free proteomics study, although this could not be confirmed at the 5% confidence level in the targeted proteomics analysis.

Abundance of ZmpA and Aida were not found to be significant changed in the label free proteomics experiment, but we repeated the SRM experiment anyway. They were both significantly more secreted upon BDSF stimulation (fold change of 2.4 and 1.6, respectively), but this was not the case for ZmpB, as reported above. We confirmed also a significant decrease in the expression of the major cable pilus protein CblA upon BDSF treatment, with an associated fold change of 1.5. This confirms the sensitivity of the SRM method compared to a discovery type of proteomics experiment.

We also repeated the experiment on cells that were grown for only 3h before triggering with BDSF, to verify that the effects are indeed true to the exogenously added BDSF, as at that point endogenous production is low. The trends for ZmpA and Hcp1 were similar (Supplementary Figure S3). At that time point, changes in CblA were not yet observed. For cells grown to the stationary phase, we could not see significant differences between BDSF treated and untreated cells (data not shown).

Diffusible signal factors from *Pseudomonas* and *Stenotrophomonas* and have an effect on the secretion of virulence factors similar to BDSF

The SRM analysis of the T6SS proteins Hcp1 and TecA, the T2SS-dependent proteins ZmpA, ZmpB, and AidA, and the major cable pilus protein CblA was also used to assess the impact of DSF and PDSF after 2 h of stimulation. Unstimulated cultures were used as a negative control and were isolated at the same time point as the stimulated cultures. Results are summarized in FIGURE 4.

These data confirmed the significant decrease in Hcp1 secretion and CblA expression, as well as the increased secretion of ZmpA and AidA upon BDSF stimulation. Secretion of TecA was slightly reduced after 2 h of growth in the presence of BDSF, although this difference was not significant at the 5% confidence level. Secretion of ZmpB is not affected by BDSF, as was already observed in previous experiments. When comparing these results to the effects caused by DSF and PDSF, a similar influence on the secretion of these virulence factors can be observed. However, the magnitude of the observed differences induced by DSF and PDSF is much smaller than the effects caused by BDSF and is not significant at the 5% confidence level. The associated fold changes for each peptide and the average fold changes per protein are presented in Table 3.

Conclusions

We here presented a targeted proteomics approach to quantify secreted virulence factors based on microfluidics LC-MRM mass spectrometry. We applied this methodology to monitor the impact of intra- and interspecies generated diffusible signal factors on the virulence of *B. cenocepacia* K-56-2. *Stenotrophomonas* and *Pseudomonas* DSFs can be effectively sensed by *B. cenocepacia* and have a similar influence as BDSF on the secretion of virulence factors. However, the magnitude of the effects caused by DSF and PDSF stimulation is typically smaller than that resulting from BDSF stimulation. Differences in the secretion of virulence factors induced by DSF are somewhat larger than the ones caused by PDSF. This might be explained by the molecular “length” of the quorum sensors: DSF and BDSF are both 12-carbon fatty acids, while PDSF is only a 10-carbon fatty acid and is thus not expected to be recognized as efficiently as BDSF or DSF.

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Figure Legends

Figure 1. Interspecies signaling via QS signals of the DSF family. This overview summarizes the current knowledge regarding the role of DSF family QS molecules in interspecies communication. BDSF = *Burkholderia* diffusible signal factor, DSF = *Stenotrophomonas* diffusible signal factor, PDSF = *Pseudomonas* diffusible signal factor.

Figure 2. BDSF concentrations in the culture supernatant of *B. cenocepacia* K56-2 cultures and corresponding OD₆₀₀ values in function of the growth time. Values from three biological replicates with corresponding standard deviations are displayed on the graph.

Figure 3. Proteins confirmed to be differentially expressed upon stimulation with 100 µM BDSF. Exponential phase *B. cenocepacia* K56-2 cultures were stimulated with 100 µM BDSF during 2 hours. Selected secreted proteins were quantified by SRM. * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001.

Figure 4. Effect of 100 µM BDSF, DSF and PDSF on the secretion of selected virulence factors from *B. cenocepacia* K56-2. Exponential phase *B. cenocepacia* K56-2 cultures were stimulated with 100 µM BDSF, DSF or PDSF and grown for an additional 2 hours in the presence of the stimulus. Virulence factors in the secretome were quantified by SRM. The statistical significance of the differences was assessed via a one-way ANOVA, followed by Dunnett's multiple comparison tests. * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001 compared to the unstimulated control.

Table 1. Proteins significantly downregulated upon stimulation with 100 μ M BDSF (TripleTOF - Progenesis).

Accession	Gene in J2315	Protein name	Average normalized abundance Control	Average normalized abundance 100 μ M BDSF	Fold change ¹	P-value
Stress response and chaperones						
EPZ87343.1	BCAL2013	Peroxiredoxin, AhpC/TSA family protein	658838,76	420541,42	1,57	0,01602
EPZ86665.1	BCAL1233	Hsp20/alpha crystallin family protein	6439,44	4151,04	1,55	0,00100
Motility						
EPZ91406.1	BCAL0520	Flagellar hook-length control protein FliK	12691,86	5341,26	2,38	0,01630
Transport and nutrient uptake						
EPZ89695.1	BCAM1931	Gram-negative porin	149691,30	49565,26	3,02	0,00135
EPZ85519.1	BCAL3008	Gram-negative porin	2010,91	1191,11	1,69	0,00623
Virulence						
EPZ85968.1	BCAL0343	Type VI secretion system protein TssD/Hcp1	405128,50	143721,92	2,82	0,00161
EPZ89291.1	BCAM1857	Rho GTPase deamidase TecA	5699,09	2929,94	1,95	0,00269
Phage proteins						
EPZ90584.1	BCAM1074	Exotoxin	1214,74	600,32	2,02	0,02290
EPZ86836.1	BCAL1571	Phage tail sheath protein	3931,55	1259,11	3,12	0,00592
EPZ88604.1	BCAM1067	Hypothetical phage protein	48862,30	28350,92	1,72	0,01123
EPZ90408.1	BCAM1069	Hypothetical phage protein	43728,36	28564,40	1,53	0,00534

¹ Fold change is calculated for Control/100 μ M BDSF. Only proteins with a fold change > 1.5 are listed.

Table 2. Proteins significantly upregulated upon stimulation with 100 μ M BDSF (TripleTOF - Progenesis).

Accession	Gene in J2315	Protein name	Average normalized abundance Control	Average normalized abundance 100 μ M BDSF	Fold change ¹	P-value
Energy and lipid metabolism						
EPZ85854.1	BCAL2935	Electron transfer flavoprotein domain protein	1197,30	2550,14	2,13	0,00138
EPZ90956.1	BCAL2783	Cyclopropane-fatty-acyl-phospholipid synthase, SAM-dependent methyltransferase	469,55	1946,03	4,14	4,94E-05
Stress response and chaperones						
EPZ89510.1	BCAM0050	Universal stress family protein	22796,72	63912,71	2,80	0,02307
EPZ87278.1	BCAL1919	ATP-dependent chaperone protein ClpB	4417,24	8261,14	1,87	0,00763
Transcription and translation						
EPZ86401.1	BCAL1883	Histidine--tRNA ligase	565,69	1210,52	2,14	0,00653
EPZ86330.1	BCAL0249	50S ribosomal protein L6	7619,62	16204,86	2,13	0,00549
EPZ87519.1	BCAL1942	50S ribosomal protein L9	5063,50	11901,16	2,35	0,01433
EPZ87276.1	BCAL1945	30S ribosomal protein S6	1861,34	3089,09	1,66	0,00448
EPZ91189.1	BCAL0799	50S ribosomal protein L25, Ctc-form	1436,55	4411,15	3,07	0,00992
EPZ85513.1	BCAL2925	50S ribosomal protein L19	57,18	1902,76	33,27	0,00100
EPZ86236.1	BCAL0235a	50S ribosomal protein L4	156,64	1091,89	6,97	0,00241
EPZ86071.1	BCAL0238	30S ribosomal protein S19	642,06	2226,24	3,47	0,00332
EPZ86091.1	BCAL0252	50S ribosomal protein L30	110,65	1855,91	16,77	0,00349
EPZ86098.1	BCAL0244	50S ribosomal protein L14	122,00	1068,98	8,76	0,00962
EPZ85945.1	BCAL0233	30S ribosomal protein S10	591,11	2180,66	3,69	0,01502
EPZ86298.1	BCAL0234	50S ribosomal protein L3	5011,55	8915,63	1,78	0,01845
Transport and nutrient uptake						
EPZ86411.1	BCAL1829	OmpW family protein	4072,01	8099,01	1,99	0,01223
EPZ85892.1	BCAL0282	Sn-glycerol-3-phosphate ABC transporter substrate-binding protein	1165,94	2074,03	1,78	0,01448
Virulence						
EPZ90945.1	BCAL0217	GDSL family lipase	306,63	622,35	2,03	0,00556
Other function						
EPZ85408.1	BCAL2916	Metalloprotease PmbA	2433,86	4018,64	1,65	0,00279
EPZ91966.1	BCAL3530	DNA-binding protein HU-alpha	560,94	4486,43	8,00	0,00744
EPZ90830.1	BCAL0216	Phenylacetate-CoA oxygenase subunit PaaA	1289,49	2494,66	1,93	0,01358
EPZ86322.1	BCAL0377	Metallo peptidase, subfamily M24B	496,23	769,95	1,55	0,018200
Unknown function						
EPZ86731.1	BCAL1411	Hypothetical protein	15621,17	28554,13	1,83	0,00895
EPZ87866.1	BCAM0907	Sulfurtransferase	1744,00	3967,45	2,27	0,02242
EPZ85594.1	BCAL3052	PF09351 domain protein	1885,28	2965,09	1,57	0,00668
EPZ85914.1	BCAL2401	PF11776 domain protein	823,12	1452,02	1,76	0,00696

¹ Fold change is calculated for Control/100 μ M BDSF. Only proteins with a fold change > 1.5 are listed.

Table 3. Effect of 100 μ M BDSF, DSF and PDSF on the secretion of selected virulence factors.

	Fold change 100 μ M BDSF	P-value ^c	Fold change 100 μ M DSF	P-value ^c	Fold change 100 μ M PDSF	P-value ^c
Hcp1^a	1,89		1,30		1,26	
EEGLPLETFSLK	1,90	< 0.05	1,38	> 0.05	1,30	> 0.05
YAAVQWK	2,01	< 0.05	1,39	> 0.05	1,34	> 0.05
IGGNQGGNTQGAWSLTK	1,76	< 0.05	1,14	> 0.05	1,16	> 0.05
TecA^a	1,21		0,81		1,15	
ADALIAPDALLDTAGQSWR	1,34	> 0.05	0,96	> 0.05	1,02	> 0.05
FNFTGDQWDGR	1,12	> 0.05	0,71	> 0.05	1,31	> 0.05
ASIPAGTAVGFAR	1,17	> 0.05	0,77	> 0.05	1,14	> 0.05
ZmpA^b	2,41		1,41		1,55	
ALQLIQQNPSAFSLAAGGTAR	2,54	< 0.05	1,49	> 0.05	1,68	> 0.05
QASVTQLAPINLAGSIGK	2,37	< 0.05	1,31	> 0.05	1,56	> 0.05
DVTPTLAYAVR	2,33	< 0.05	1,41	> 0.05	1,41	> 0.05
ZmpB^b	1,16		1,18		1,21	
ADGVAVTLNYPAYR	1,30	> 0.05	1,12	> 0.05	1,25	> 0.05
YVNQTLGIK	1,11	> 0.05	1,24	> 0.05	1,27	> 0.05
VTNWNVGR	1,06	> 0.05	1,17	> 0.05	1,10	> 0.05
AidA^b	1,57		1,33		1,21	
NPAAPTLIDWR	1,73	< 0.01	1,41	> 0.05	1,19	> 0.05
AQVGD LIR	1,44	< 0.05	1,30	> 0.05	1,15	> 0.05
FIGNVGNDLISTPTR	1,55	> 0.05	1,27	> 0.05	1,30	> 0.05
CblA^a	1,47		1,13		1,13	
NQTSPGAAEIPLSVK	1,35	> 0.05	0,97	> 0.05	1,17	> 0.05
LGETELTTAATLK	1,39	< 0.05	1,11	> 0.05	1,11	> 0.05
TAELFPGELAQGSNVLALSIGQK	1,67	< 0.05	1,33	> 0.05	1,12	> 0.05

^a Fold changes are calculated by dividing the mean normalized intensity of the unstimulated control by the mean normalized intensity of the appropriate stimulated culture.

^b Fold changes are calculated by dividing the mean normalized intensity of the appropriate stimulated culture by the mean normalized intensity of the unstimulated control.

^c Associated p-values were calculated from a one-way ANOVA followed by a Dunnetts's multiple comparison test

Supplementary Table S1. List of peptides used for targeted proteomics and the transitions used to detect and quantify the peptides using SRM.

Peptide	Precursor ion mass	Fragment ion mass
BSA		
QTALVELLK	461.7477++	A [y7] - 785.5131+ L [y6] - 714.4760+ V [y5] - 601.3919+
ZmpA		
ALQLIQQNPSAFSLAAGGTAR	705.3833+++	F [y10] - 950.5054+ S [y9] - 803.4370+ L [y8] - 716.4050+ A [y7] - 603.3209+
QASVTQLAPINLAGSIGK	590.0036+++	A [y11] - 1040.6099+ P [y10] - 969.5728+ I [y9] - 872.5200+ N [y8] - 759.4359+ L [y7] - 645.3930+
DVTPTLAYAVR	603.3299++	T [y9] - 991.5571+ P [y8] - 890.5094+ L [y6] - 692.4090+ A [y5] - 579.3249+
ZmpB		
ADGVAVTLYNPAYR	755.3884++	T [y8] - 997.5102+ Y [y6] - 783.3784+ N [y5] - 620.3151+ P [y4] - 506.2722+
YVNQTLGIK	518.2953++	V [y8] - 872.5200+ N [y7] - 773.4516+ Q [y6] - 659.4087+ T [y5] - 531.3501+
VTNWNVGR	473.2487++	T [y7] - 846.4217+ N [y6] - 745.3740+ W [y5] - 631.3311+ N [y4] - 445.2518+
Hcp1		
EEGLPLETFSLK	681.8612++	P [y8] - 934.5244+ E [y6] - 724.3876+ T [y5] - 595.3450+
YAAVQWK	433.2320++	A [y6] - 702.3933+ A [y5] - 631.3562+ V [y4] - 560.3191+
IGGNQGGNTQGAWSLTK	844.9212++	T [y9] - 991.5207+ G [y7] - 762.4145+ W [y5] - 634.3559+

TecA		
ADALIAPDALLDTAGQSWR	992.5104++	D [y8] - 920.4221+ G [y5] - 633.3103+ S [y3] - 448.2303+
FNFETGDQWDGR	736.3155++	E [y9] - 1063.4439+ T [y8] - 934.4013+ G [y7] - 833.3537+
ASIPAGTAVGFAR	609.3355++	P [y10] - 946.5105+ G [y8] - 778.4206+ G [y4] - 450.2459+
AidA		
NPAAPTLIDWR	627.3355++	A [y9] - 1042.5680+ A [y8] - 971.5309+ P [y7] - 900.4938+
AQVGDLIR	436.2534++	V [y6] - 672.4039+ G [y5] - 573.3355+ L [y3] - 401.2871+
FIGNVGNDLISTPTR	567.6371+++	I [y7] - 771.4359+ S [y6] - 658.3519+ T [y5] - 571.3198+ P [y4] - 470.2722+
CblA		
NQTSPGAAEIPLSVK	756.4068++	P [y11] - 1081.6252+ A [y8] - 856.5138+ I [y6] - 656.4341+ P [y5] - 543.3501+
LGETELTTTAATLK	724.8958++	E [y10] - 1048.5885+ L [y9] - 919.5459+ T [y8] - 806.4618+ T [y7] - 705.4141+
TAELFPGELAQGSNVLALSIGQK	781.7567+++	L [y8] - 829.5142+ A [y7] - 716.4301+ L [y6] - 645.3930+ S [y5] - 532.3089+
BCAM1067		
QSQATVETGFFDPSNAFSAYGLFGK	890.4221+++	N [y11] - 1174.5891+ A [y10] - 1060.5462+ F [y9] - 989.5091+ S [y8] - 842.4407+ A [y7] - 755.4087+
GPADILR	371.2163++	A [y5] - 587.3511+ D [y4] - 516.3140+ I [y3] - 401.2871+ L [y2] - 288.2030+
BCAM1069		
AELEAEGGR	466.2276++	L [y7] - 731.3682+

ELEHASNVAAFEAAQAAAK	667.0008+++	E [y6] - 618.2842+ E [y4] - 418.2045+ A [y12] - 1119.5793+ A [y11] - 1048.5422+ F [y10] - 977.5051+ E [y9] - 830.4367+ A [y8] - 701.3941+
50S ribosomal protein L6		
LTLVGVGYR	489.2926++	T [y8] - 864.4938+ L [y7] - 763.4461+ V [y6] - 650.3620+ G [y5] - 551.2936+ V [y4] - 494.2722+
QQVGQVAAEVR	592.8227++	V [y9] - 928.5211+ G [y8] - 829.4526+ Q [y7] - 772.4312+ A [y5] - 545.3042+
YADEVVILK	525.2975++	A [y8] - 886.5244+ D [y7] - 815.4873+ E [y6] - 700.4604+ V [y5] - 571.4178+ V [y4] - 472.3493+
50S ribosomal protein L9		
EAIAEFEVR	532.2746++	I [y7] - 863.4621+ A [y6] - 750.3781+ E [y5] - 679.3410+ F [y4] - 550.2984+ E [y3] - 403.2300+
LAASQAVGEK	487.2693++	A [y8] - 789.4101+ S [y7] - 718.3730+ Q [y6] - 631.3410+ V [y4] - 432.2453+
LFGSVTNGDVAELLK	781.9249++	N [y9] - 958.5204+ G [y8] - 844.4775+ D [y7] - 787.4560+ V [y6] - 672.4291+ A [y5] - 573.3606+
50S ribosomal protein L25		
IELDHNALWHALK	780.4201++	H [y9] - 1089.5952+ N [y8] - 952.5363+ A [y7] - 838.4934+ L [y6] - 767.4563+ W [y5] - 654.3722+
QLVLHVDFQR	627.8513++	L [y7] - 914.4843+ V [y5] - 664.3413+ F [y3] - 450.2459+

VPLHFLNAEVSPAVK	810.9590++	L [y10] - 1027.5782+ N [y9] - 914.4942+ A [y8] - 800.4512+ E [y7] - 729.4141+ V [y6] - 600.3715+
Exotoxin		
ALNYQYSVIQYSSVSPYAISFLGR	938.4763+++	P [y10] - 1110.5942+ Y [y9] - 1013.5415+ A [y8] - 850.4781+ I [y7] - 779.4410+ S [y6] - 666.3570+
QEPGIVAEALSTSQANALQAK	709.3744+++	S [y9] - 930.5003+ Q [y8] - 843.4683+ A [y7] - 715.4097+ N [y6] - 644.3726+ A [y5] - 530.3297+
SVSFQVAAK	468.7611++	S [y7] - 750.4145+ F [y6] - 663.3824+ Q [y5] - 516.3140+ V [y4] - 388.2554+
GDSL family lipase		
APLVVEAASLAR	598.8535++	V [y9] - 915.5258+ V [y8] - 816.4574+ E [y7] - 717.3890+ A [y6] - 588.3464+ A [y5] - 517.3093+
VSNQFNYVSGPGDHSVNDPGAETFR	869.7335+++	V [y10] - 1105.5273+ N [y9] - 1006.4588+ D [y8] - 892.4159+ P [y7] - 777.3890+ G [y6] - 680.3362+
VFGATLTPAALPAGR	721.4117++	L [y10] - 966.5731+ T [y9] - 853.4890+ P [y8] - 752.4413+
Flagellin		
INSAADDAAGLAISTR	773.3970++	D [y10] - 974.5265+ A [y9] - 859.4996+ A [y8] - 788.4625+ G [y7] - 717.4254+ L [y6] - 660.4039+
IGGGLVQK	386.2398++	G [y7] - 658.3883+ G [y6] - 601.3668+ G [y5] - 544.3453+ L [y4] - 487.3239+
NQVLQQAGISVLAQANSLPQQVLK	849.8133+++	A [y10] - 1097.6313+ N [y9] - 1026.5942+

		S [y8] - 912.5513+
		L [y7] - 825.5193+
		P [y6] - 712.4352+
BCAS0151		
DSTGATGETLGAAGASSEDPAVAQLK	802.0540+++	E [y9] - 970.5204+
		D [y8] - 841.4778+
		P [y7] - 726.4509+
		V [y5] - 558.3610+
QLATLER	415.7402++	A [y5] - 589.3304+
		T [y4] - 518.2933+
		L [y3] - 417.2456+
SGGSPSGGLVSTQA	602.7938++	P [y10] - 916.4734+
		V [y5] - 505.2617+
		S [y4] - 406.1932+