FACULTY OF SCIENCES

# The role of the 92-kb plasmid, quorum sensing, and interspecies communication in the virulence of *Burkholderia cenocepacia* K56-2

Sofie Depluverez
Student number: 00802404

Supervisor(s): Prof. Dr. Bart Devreese

A dissertation submitted to Ghent University in partial fulfillment of the requirements for the degree of Doctor of Sciences: Biochemistry and Biotechnology

Academic year: 2017 - 2018

GHENT UNIVERSITY

L-ProBE
LABORATORY for PROTEIN BIOCHEMISTRY and BIOMOLECULAR ENGINEERING

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Sofie

# *NEDERLANDSE SAMENVATTING*

Mucoviscidose of taaislijmziekte is de vaakst voorkomende, dodelijke genetische ziekte in het blanke deel van de wereldbevolking. De dehydratie van de longvloeistof in de longen van deze patiënten bemoeilijkt het efficiënte mucociliaire transport van partikels en bacteriën. Dit leidt tot een verminderde weerstand tegen chronische en terugkerende longinfecties die vaak gepaard gaan met longontsteking en schade aan het longweefsel. De levensverwachting van mucopatiënten ligt dan ook een stuk lager, met een gemiddelde van ongeveer 40 jaar. De pathogene bacterie die in de meeste gevallen verantwoordelijk is voor deze longinfecties is *Pseudomonas aeruginosa*. Deze is echter vaak vergezeld door andere infecterende species, zoals *Stenotrophomonas maltophilia* en *Burkholderia cenocepacia*. Ondanks dat deze laatste slechts teruggevonden wordt bij 3 à 4% van de mucopatiënten, is de impact ervan niet te onderschatten. *B. cenocepacia* infectie kan immers leiden tot de ontwikkeling van het 'cepacia' syndroom, gekenmerkt door een vergevorderde necrotiserende longontsteking en sepsis, vaak met fatale afloop. Om dit te bewerkstelligen beschikt deze bacterie over een brede waaier aan virulentiefactoren, nodig voor invasie, kolonisatie en overleving in gastheercellen.

Het doel van het onderzoek, verricht in het kader van deze thesis, omvat het bestuderen van verschillende pathways betrokken in de regulatie, expressie en secretie van virulentie-geassocieerde factoren, waarbij gebruik gemaakt wordt van verscheidene proteomics-gebaseerde analysemethoden. Het eerste experimentele hoofdstuk beschrijft de rol van het 92-kb plasmide in de virulentie en biofilmvorming van *B. cenocepacia* K56-2. Dit plasmide bevat niet alleen de genen nodig voor het behoud van het plasmide, maar eveneens deze die coderen voor een functioneel type IV secretiesysteem, genaamd T4SS-1. Dit vaak voorkomend type secretiesysteem is noodzakelijk voor het overleven van de pathogeen binnenin de gastheercel en is verantwoordelijk voor het veroorzaken van het "*plant tissue watersoaking*" fenotype op ajuinweefsel. Tot nu toe zijn er in *B. cenocepacia* K56-2 nog geen effector eiwitten geïdentificeerd voor dit secretiesysteem, en de exacte rol ervan tijdens infectie dient nog onderzocht te worden. Het 92-kb plasmide bevat verder ook nog een operon (pBCA054-055) dat mogelijks betrokken is in *"quorum sensing"* (QS) signalisatie.
In deze studie werd het secretoom van een wildtype *B. cenocepacia* K56-2 stam vergeleken met dat van een stam waarbij de volledige T4SS-1 cluster, dewelke ook het mogelijke QS operon bevat, werd verwijderd (ΔpBCA017-059). Hierbij werd gebruik gemaakt van verschillende label-vrije en doelgerichte proteoomanalyse methoden.
De sterk verlaagde abundantie van twee T6SS eiwitten, Hcp1 en TecA, in het secretoom van de mutant kan ofwel verklaard worden door afwezigheid van het pBCA054-055 operon, ofwel door het feit dat de ΔpBCA017-059 mutant gecreëerd werd in de MHK achtergrond, waarbij de afwezigheid van een gentamicine resistentie effluxpomp de stam gevoeliger maakt voor secundaire mutaties die mogelijks de dysregulatie van het T6SS kunnen veroorzaken. Om hierover uitsluitsel te verkrijgen, werden twee nieuwe mutanten gemaakt, deze keer in de K56-2 achtergrond, in dewelke slechts een deel van de T4SS-1 structurele genen ontbreken (ΔpBCA017-033) of waarin het mogelijke QS operon werd verwijderd (ΔpBCA054-055). Geen van beide nieuwe mutanten vertoonde een afwijkende T6SS activiteit, wat er op wijst dat voorzichtigheid geboden is bij het gebruik van de MHK stam om de virulentie van *B. cenocepacia* te bestuderen. De laatste mutant vertoonde evenwel een verlaagde biofilm massa en een afwijkende biofilm morfologie ten opzichte van het wildtype. De ΔpBCA017-

033 mutant werd gekenmerkt door een verminderde secretie van verscheidene faageiwitten, wat mogelijks wijst op een defecte inductie van profagen, een proces dat normaal spontaan verloopt in *B. cenocepacia*. Verder onderzoek is echter vereist om dit fenomeen volledig uit te klaren.

Het tweede experimentele hoofdstuk van deze thesis focust op de rol van *"diffusible signal factors"* (DSFs) als moleculen voor intra- en interspecies communicatie. Eerst en vooral werd de endogene productie van BDSF door planktonisch gegroeide *B. cenocepacia* K56-2 culturen in kaart gebracht. Deze is maximaal in de mid-exponentiële fase, gevolgd door een scherpe daling in de vroeg-stationaire fase. Dit duidt mogelijks op het bestaan van een turnover mechanisme, gelijkaardig aan dat van *X. campestris* pv. *campestris*. Gebruik makende van *"Selected Reaction Monitoring"* (SRM) op een geselecteerd panel gesecreteerde virulentiefactoren, stelden we vast dat de exogene BDSF concentratie nodig voor het effectief veroorzaken van een respons in *B. cenocepacia* K56-2 minstens 75 µM bedraagt. Deze concentratie is veel hoger dan de in het vorige experiment bepaalde fysiologische concentratie en dan de BDSF concentratie gemeten in sputum stalen van mucopatiënten. Desalniettemin zijn er een aantal argumenten waarom we ons werk verder gezet hebben gebruik makende van dergelijke hoge concentraties. Ten eerste werden gelijkaardige hoge concentraties gebruikt in de meeste voorgaande *in vitro* studies, bijvoorbeeld voor transcriptoom analyse, om een betere vergelijking toe te laten. Ten tweede kunnen mogelijks de *in vivo* concentraties waaraan bepaalde cellen blootgesteld worden lokaal veel hoger liggen dan de globaal bepaalde fysiologische concentratie, bijvoorbeeld voor cellen in de directe omgeving van BDSF-producerende cellen. Toevoeging van BDSF aan exponentiële fase *B. cenocepacia* K56-2 culturen leidde tot een verhoogde secretie van het T2SS-afhankelijke metalloprotease ZmpA, het eiwit vereist voor toxiciteit in *Caenorhabditis elegans*, en van een lipase behorende tot de GDSL familie. Verder veroorzaakte deze stimulatie een verlaagde secretie van de T6SS eiwitten Hcp1 en TecA. Een mogelijke verklaring voor deze observaties is de BDSF-afhankelijke reductie van het intracellulaire cyclische-di-GMP niveau, wat leidt tot een omschakeling van een sessiele naar een planktonische, meer virulente levensstijl, gekenmerkt door verminderde biofilmvorming en T6SS activiteit. Deze hypothese wordt tevens versterkt door de observatie dat de aanwezigheid van DSFs een negatief effect heeft op de hoeveelheid biofilm die gevormd wordt.
Verder konden we aantonen dat *B. cenocepacia* K56-2 bovendien de mogelijkheid bezit om de aanwezigheid van *S. maltophilia* en *P. aeruginosa* DSFs op te merken en vervolgens daarop te reageren. Hoewel de effecten gelijkaardig zijn aan deze veroorzaakt door BDSF, is de omvang ervan beduidend kleiner.

Om beter inzicht te krijgen in de communicatie tussen verschillende CF pathogenen, richt het laatste experimentele hoofdstuk zich op de interacties die plaatsvinden tussen pathogenen die vaak samen voorkomen in de longen van mucopatiënten. Aangezien *B. cenocepacia* K56-2 en *S. maltophilia* 44/98 in vloeibare culturen steeds overgroeid worden door *P. aeruginosa* PAO1, werden de *B. cenocepacia* K56-2 culturen gegroeid in aanwezigheid van (geconcentreerd) cel-vrij supernatant afkomstig van *P. aeruginosa* PAO1 of *S. maltophilia* 44/98. Op deze manier konden zowel QS-afhankelijke als QS-onafhankelijke interacties tussen deze species geobserveerd worden bij min of meer fysiologische concentraties, zoals een effect op T6SS activiteit, flagellatie, conjugatie en biofilmvorming. Hoewel in deze studie enkel de effecten op het secretoom van *B. cenocepacia* K56-2 in kaart werden gebracht, zou het uitermate interessant zijn om na te gaan hoe de communicatie in de andere richtingen

verloopt, meer specifiek in de gebruikte *S. maltophilia* en *P. aeruginosa* stammen, alsook wat het effect is op gentranscriptie en de veranderingen in de expressie van intracellulaire eiwitten.
Verder verdiepend onderzoek omtrent deze topics kan uiteindelijk bijdragen aan de rationele ontwikkeling van nieuwe anti-pathogene therapieën om zo de intrinsieke resistentie van deze pathogeen tegen de meeste van de huidige antibiotica te omzeilen. Door universele secretiesystemen, hun effectoren of geconserveerde QS mechanismen te viseren, kan het toepassingsgebied van nieuwe behandelingen uitgebreid worden naar een brede waaier van Gramnegatieve pathogenen.

# *ENGLISH SUMMARY*

Cystic fibrosis (CF) is the most common lethal inherited disorder in Caucasians. In the lungs of patients with CF, the mucociliary clearance is impaired due to dehydration of the airway surface fluid. As a consequence, people with CF are highly susceptible to chronic or intermittent pulmonary infections, often causing extensive lung inflammation and damage, accompanied by a decreased life expectancy. The most commonly isolated pathogen, *Pseudomonas aeruginosa*, is often accompanied by other infecting species, such as *Stenotrophomonas maltophilia* and *Burkholderia cenocepacia*. Although the latter pathogen is encountered in only 3 to 4% of patients with CF, it is notorious because of its capacity to cause 'cepacia' syndrome, an often lethal necrotizing pneumonia. To accomplish this, it disposes of a whole array of virulence factors required for invasion, colonization and survival inside host cells.

The research performed in the context of this thesis aimed at characterizing different pathways affecting the regulation, expression or secretion of virulence-associated properties using various proteomic approaches. The first chapter concerns the role of the 92-kb plasmid in the virulence and biofilm formation of *B. cenocepacia* K56-2. This plasmid not only contains several genes required for plasmid maintenance, but also encodes a functional type IV secretion system (T4SS), termed T4SS-1. This widely encountered secretion system has been proven to be required for intracellular survival of this pathogen inside macrophages and is responsible for causing the plant tissue watersoaking phenotype on onion tissue. In *B. cenocepacia* K56-2, no effector proteins have been identified so far and its exact role has yet to be discovered. Furthermore, the plasmid also harbors an operon (pBCA054-055) that is potentially involved in quorum sensing (QS) signaling.
Using label-free and targeted proteomics, we compared the secretome of the wild type strain with that of a strain in which the entire T4SS-1 cluster, also encompassing the operon potentially linked to quorum sensing, was deleted (ΔpBCA017-059).
We observed an unexpected decrease in abundance of two T6SS proteins, Hcp1 and TecA, in the secretome of this mutant. This might be caused by the absence of the operon possibly linked to QS. Another possible explanation is the fact that the ΔpBCA017-059 mutant was created in the so-called MHK background, a strain lacking a gentamicin resistance pump which could be prone to other mutations causing dysregulation of the T6SS. In order to obtain a more decisive answer, two new mutants were created in the K56-2 background, in which only a subset of T4SS-1 structural genes was deleted (ΔpBCA017-033), or in which the regulatory operon possibly linked to quorum sensing was removed (ΔpBCA054-055). Neither of them did show aberrant T6SS activity, which indicates that care should be taken to the use of the MHK strain to study *B. cenocepacia* secretion systems and/or virulence. The latter mutant showed a significant reduction in the biofilm mass formed and an aberrant biofilm morphology. The ΔpBCA017-033 mutant was characterized by a reduced secretion of several phage proteins, and might therefore be deficient in prophage induction, a process which normally occurs spontaneously in *B. cenocepacia*. Further research into this phenomenon is required to generate a valid explanation for this observation.

The second chapter of this thesis focuses on the role of diffusible signal factors (DSFs) as intra- and interspecies communication molecules. First, we found that the production of BDSF by planktonically grown *B. cenocepacia* K56-2 cultures is maximal in mid-exponential phase, followed by a sharp

decline in early stationary phase. This suggests the existence of a turnover mechanism, similar to what was previously found in *X. campestris* pv. *campestris*. Using Selected Reaction Monitoring (SRM) on a panel of secreted virulence factors, we determined the working concentration of exogenously added BDSF to wild type *B. cenocepacia* K56-2 to be at least 75 µM, under the conditions tested. This is a much higher concentration than the physiological production levels determined in the previous experiment and significantly exceeds the concentration observed in sputum samples. However, there are some arguments why we continued our work with such concentrations. Similar concentrations where previously used in most *in vitro* studies, for example for transcript analysis, to allow better comparisons. More speculatively, *in vivo*, local concentrations might be higher and have an impact on single cells exposed to BDSF producing cells.
Addition of BDSF to exponential phase *B. cenocepacia* K56-2 cultures led to the increased secretion of the T2SS-dependent metalloprotease ZmpA, the nematocidal protein AidA, and of a GDSL family lipase. Furthermore, it reduced secretion of the T6SS proteins Hcp1 and TecA. These observations might be explained by the BDSF-dependent reduction in intracellular cyclic-di-GMP levels, causing a switch from a sessile lifestyle to a planktonic, more virulent status, accompanied by decreased biofilm formation and T6SS activity. This hypothesis is strengthened by our finding that biofilm formation is reduced in the presence of DSFs. *S. maltophilia* and *P. aeruginosa* DSFs can be effectively sensed by *B. cenocepacia* K56-2 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.

To gain further insight in the communication between different CF pathogens, the last chapter focuses on the interactions occurring between frequently co-isolated CF pathogens. Since *B. cenocepacia* K56-2 and *S. maltophilia* 44/98 are outcompeted in liquid co-cultures with *P. aeruginosa* PAO1, we monitored the effects on the secretome of *B. cenocepacia* K56-2 grown in the presence of (concentrated) cell-free supernatant (CFS) from *S. maltophilia* 44/98 or *P. aeruginosa* PAO1. This allowed us to observe both QS-dependent and QS-independent interactions between these species at more or less physiologically relevant concentrations, including effects on T6SS activity, flagellation, conjugation and biofilm formation. Here, we only monitored the effects on the secretome of *B. cenocepacia* K56-2, but it would be extremely interesting to also evaluate how the communication occurs the other way around, more specifically in the *S. maltophilia* and *P. aeruginosa* strains used here, as well as the effect on gene transcription and the intracellular proteome.

Further research into these topics may ultimately aid in the design of novel anti-pathogenic drugs in order to overcome the intrinsic resistance of this pathogen against the majority of the currently available antibiotics. By targeting secretion systems and/or their effectors and conserved QS mechanisms, the application range of novel therapeutics can be broadened to a whole range of Gram-negative pathogens.

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| | |
|---|---|
| 3-oxo-C12-HSL | N-(3-oxododecanoyl)-L-homoserine lactone |
| ACN | Acetonitrile |
| AHL | N-acylhomoserine lactone |
| Ara4N | 4-amino-4-deoxy-L-arabinose |
| AsialoGM1 | Asialoganglioside 1 |
| ATP | Adenosine triphosphate |
| AUC | Area under curve |
| *Bcc* | *Burkholderia cepacia* complex |
| BcCVs | Bacteria-containing vacuoles |
| BDSF | *Burkholderia* diffusible signal factor, cis-2-dodecenoic acid |
| BSA | Bovine Serum Albumin |
| C6-HSL | N-hexanoylhomoserine lactone |
| C8-HSL | N-octanoylhomoserine lactone |
| cAMP | Cyclic adenosine monophosphate |
| cCFS | Concentrated cell-free supernatant |
| CF | Cystic Fibrosis |
| CFS | Cell-free supernatant |
| CFTR | CF transmembrane conductance regulator |
| CGD | Chronic granulomatous disease |
| CK13 | Cytokeratin 13 |
| CV | Crystal violet |
| Cyclic-di-GMP | Cyclic diguanylate |
| DNA | Deoxyribonucleic acid |
| Dnase I | Deoxyribonuclease I |
| DSF | (*Stenotrophomonas*) Diffusible signal factor, cis-11-methyl-2-dodecenoic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| ET-12 | Edinburgh/Toronto-12 |
| FEV1 | Forced expiratory volume in 1 second |
| FWHM | Full width at half maximum |
| GI-T4SSs | Genomic island-associated T4SSs |
| GlcNAc | N-acetylglucosamine |
| Glu-Fib | [$Glu^1$]-Fibrinopeptide B |
| GTPase | Guanosine triphosphatase |
| HGT | Horizontal gene transfer |
| HMW | High molecular weight |
| ICEs | Integrative and conjugative elements |
| IL-1β | Interleuking 1 beta |
| IL-8 | Interleukin 8 |
| IM | Inner membrane |
| LB | Lysogeny Broth |
| LPS | Lipopolysaccharide |
| Lvh | *Legionella* virulence homolog |
| Mbp | Mega base pairs |
| MHC | Major histocompatibility complex |

| | |
|---|---|
| MIC | Minimal inhibitory concentration |
| mRNA | Messenger RNA |
| MVs | Membrane vesicles |
| MWCO | Molecular weight cutoff |
| NF-κB | Nuclear factor kappa B |
| OM | Outer membrane |
| PAS | Per-Arnt-Sim |
| PDSF | *Pseudomonas* diffusible signal factor, cis-2-decenonic acid |
| PE | Pulmonary exacerbation |
| PERT | Pancreatic enzyme replacement therapy |
| PQS | *Pseudomonas* quinolone signal |
| Ptw | Plant tissue watersoaking |
| QS | Quorum sensing |
| ROS | Reactive oxygen species |
| rRNA | Ribosomal RNA |
| SRM | Selected reaction monitoring |
| T1SS | Type I secretion system |
| T2SS | Type II secretion system |
| T3SS | Type III secretion system |
| T4CP | Type IV coupling protein |
| T4SS | Type IV secretion system |
| T5SS | Type V secretion system |
| T6SS | Type VI secretion system |
| T7SS | Type VII secretion system |
| T8SS | Type VIII secretion system |
| Tai | Type VI amidase immunity |
| TAP | Transporter associated with antigen processing |
| TCA | Trichloroacetic acid |
| TLR | Toll-like receptor |
| TNRF1 | Tumor necrosis factor receptor I |
| TPS | Two-partner secretion |
| vATPase | Vacuolar ATPase |
| *Xcc* | *Xanthomonas campestris* pv. *campestris* |
| *Xoo* | *Xanthomonas oryzae* pv. *oryzae* |

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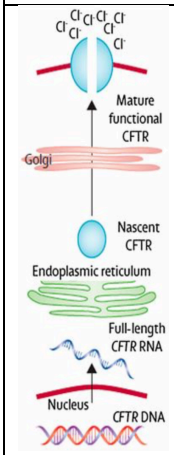
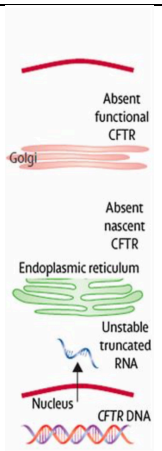
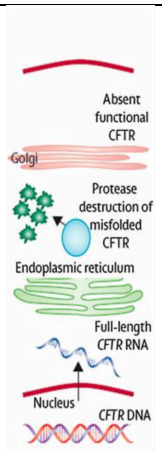
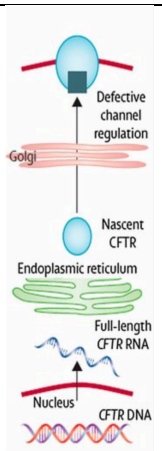
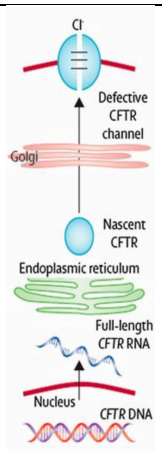
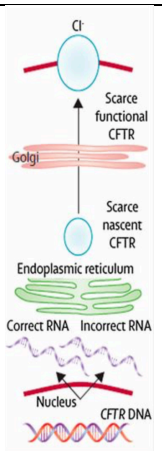
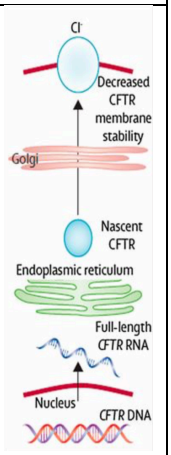
# *CHAPTER I: INTRODUCTION*

# 1 CYSTIC FIBROSIS

## 1.1 Cause

Cystic fibrosis (CF), the most common lethal inherited disorder in Caucasians, is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene, a cAMP-regulated epithelial chloride channel (Cohen and Prince, 2012). CFTR expression and function has been demonstrated in epithelial cells of the lower and upper respiratory tracts (Carvalho-Oliveira et al., 2004; Engelhardt et al., 1992; Kälin et al., 1999; Kreda et al., 2005; Penque et al., 2000; Trezise et al., 1993; Trezise and Buchwald, 1991), the gastrointestinal tract (Kälin et al., 1999; Mall et al., 2004; Manson et al., 1997; Tizzano et al., 1993; Trezise and Buchwald, 1991), salivary glands (Best and Quinton, 2005; Trezise and Buchwald, 1991) and male and female reproductive tracts (Tizzano et al., 1993; Trezise et al., 1993; Trezise and Buchwald, 1991). Today, over 1500 mutations in the *CFTR* gene have been reported to be potentially CF-causing, and these are grouped into distinct classes based on their effect on the synthesis and/or function of the CFTR protein (De Boeck et al., 2014; Dorfman, 2015). Class I mutations result in a truncated and mostly non-functional CFTR protein, while class II mutations lead to an aberrantly folded protein that is targeted for proteasomal degradation. A defective regulation of the ion channel, resulting in an impaired chloride ion flow through the channel, is caused by class III mutations. *CFTR* mutations leading to reduced conductance are grouped into class IV. Splice site mutations, class V mutations, which only partially disrupt correct splicing, lead to significantly decreased amounts of functional protein (Tsui, 1992; Welsh and Smith, 1993). Class VI missense mutations result in an unstable protein (Elborn and Vallieres, 2014).
The severity of the associated lung disease depends on the type of mutation present and whether the individual is homozygous or heterozygous for this particular mutation. Typically, patients carrying class IV or V mutations display a milder phenotype, since basal levels of functional CFTR are present on the cell membrane (Koch et al., 2001). By far the most common mutation is ΔF508, a class II mutation, carried by approximately 80% of CF patients (De Boeck et al., 2014). The ΔF508 mutation alters the secondary structure of the *CFTR* mRNA, leading to mRNA “misfolding” and a decreased translation rate, which contributes to ΔF508 CFTR protein misfolding (Bartoszewski et al., 2010).

**Table 1. Classes of *CFTR* mutations.** Adapted from Boyle and De Boeck (2013).

| Normal | I | II | III | IV | V | VI |
|---|---|---|---|---|---|---|
|  |  |  |  |  |  |  |
| **CFTR defect** | No functional CFTR protein | CFTR trafficking defect | Defective channel regulation | Decreased channel conductance | Reduced synthesis of CFTR | Decreased CFTR stability |
| **Type of mutations** | Nonsense; frameshift; canonical splice | Missense; amino acid deletion | Missense; amino acid change | Missense; amino acid change | Splicing defect; missense | Missense; amino acid change |
| **Specific mutation examples** | Gly542X<br>Trp1282X<br>Arg553X<br>621+1G ⟶ T | Phe508del<br>Asn1303Lys<br>Ile507del<br>Arg560Thr | Gly551Asp<br>Gly178Arg<br>Gly551Ser<br>Ser549Asn | Arg117His<br>Arg347Pro<br>Arg117Cys<br>Arg334Trp | 3849+10kbC ⟶ T<br>2789+5G ⟶ A<br>3120+1G ⟶ A<br>5T | 4326delTC<br>Gln1412X<br>4279insA |

## 1.2 Symptoms and clinical manifestations

Since the *CFTR* gene is normally expressed in different tissues throughout the human body, the pathophysiology associated with a defective protein manifests itself in multiple organs (Gregory et al., 1990; Trezise and Buchwald, 1991; Ward et al., 1991).

### 1.2.1 Respiratory system

Defects associated with aberrant CFTR function in the lungs include a dramatic change in the composition of the airway surface fluid, excessive oxidative stress in the airways and dysregulation of immune cell function. In healthy individuals, the majority of bacteria trapped in the mucus layer are removed and destroyed by mucociliary clearance and immune system function. However, in the lungs of people with CF, the mucociliary clearance is impaired due to the dehydrated nature of the airway surface layer. In addition, the immune response is compromised because of anomalies in immune activation caused by CFTR dysfunction in e.g. neutrophils, macrophages and T-cells. The latter leads to signaling abnormalities in essential pathways involved in the transcription of inflammatory mediators in *CFTR*-deficient cells. As a consequence, people with CF are highly susceptible to chronic or intermittent pulmonary infections, often causing extensive lung damage and inflammation, accompanied by a decrease in life expectancy (Cohen and Prince, 2012; Gibson et al., 2003; Govan and Deretic, 1996; Ratner and Mueller, 2012).

### 1.2.2 Gastrointestinal system

Despite the fact that CF is mostly known as a lung disease, CFTR protein levels are the highest in tissues of the gastrointestinal tract, according to ProteomicsDb, a human proteome database (www.proteomicsedb.org) based on organ specific LC-MS/MS data. In the digestive tract, the most common symptom is pancreatic insufficiency. The decreased sodium bicarbonate secretion by malfunctioning CFTR reduces the efficacy of pancreatic enzymes, and increases the precipitation of bile salts. The latter process is worsened by a lowering of the pH (hyperacidity) in the duodenum, which also negatively affects nutrient absorption and leads to premature activation of the digestive enzyme trypsin. On top of that, mucus plugs obstructing the pancreatic canaliculi prevent the efficient release of digestive enzymes in the duodenum, contributing to the poor digestion of proteins, lipids and carbohydrates (Haack and Garbi Novaes, 2013; Li and Somerset, 2014; Panagopoulou et al., 2014; Pencharz and Durie, 2000; Reis and Damaceno, 1998; Taylor and Aswani, 2002; Wat et al., 2008; Wilschanski et al., 2004). Extensive inflammation in the gastrointestinal tract is also a frequent hallmark of cystic fibrosis. However, it is still under debate whether this is due to the basic cellular defect of CF or whether it occurs secondary to bacterial infection (Balough et al., 1995; Munck, 2014; Smyth et al., 2000). Either way, because of the chronic bacterial infections, either in the lungs or in the gut, CF patients are forced to take antibiotics for prolonged and repeated time periods. This may disturb the otherwise beneficial host-microbiota relationship, aggravating the digestive problems (Davies and Bilton, 2009; Debyser et al., 2016; Dethlefsen and Relman, 2011).

### 1.2.3 Reproductive system

Over 98% of male CF patients have to deal with fertility problems, due to congenital absence of the vas deferens (Boyd et al., 2004). Infertility in women with CF is much less common and affects about 50% of the female CF population. The most common abnormality is a thick and dehydrated tenacious cervical mucus, which impairs cervical penetration by sperm during ovulation (Edenborough, 2001; Jarzabek et al., 2004; Sueblinvong and Whittaker, 2007).

## 1.3 Treatment

After more than 25 years of extensive research, there is still no cure for cystic fibrosis. However, the quest for the ultimate treatment has led to some significant breakthroughs (Bosch and De Boeck, 2016). When the CFTR gene was first discoverd, the prospects for the use of gene therapy as a cure were promising. However, after many clinical trials, it appeared that correcting the CFTR defect using gene therapy was not that evident (Griesenbach and Alton, 2013). Currently, most of the therapeutic strategies in use therefore aim at alleviating the symptoms and complications that arise from CFTR mutation. One strategy intends to increase the fluidity of airway secretions by inhalation of hypertonic salt solutions or osmotic agents, like mannitol (Elkins et al., 2006). A similar mucus-modulating strategy using human DNase I (more commonly known as dornase alfa) consists of reducing mucus viscosity by hydrolyzing DNA originating from bacteria and neutrophils (Griese et al., 1997; McCoy et al., 1996). Application of systemic corticoids to slow down the progression of lung

disease in people with CF is not widespread, since it is associated with severe side effects including glucose intolerance, diabetes and growth retardation (Cheng et al., 2015). As an alternative, nonsteroidal anti-inflammatory drugs like ibuprofen inhibit the migration, adherence, swelling and aggregation of neutrophils, thereby significantly reducing lung inflammation (Flynn et al., 1984; Kaplan et al., 1984; Konstan et al., 1995; Maderazo et al., 1984; Rinaldo and Pennock, 1986; Shimanuki et al., 1985; Sordelli et al., 1985; Venezio et al., 1985). Azithromycin, a macrolide antibiotic, is being used as a potential anti-inflammatory treatment for patients. Evidence from several studies demonstrates that azithromycin treatment improves respiratory function and reduces the frequency of pulmonary exacerbations (Clement, 2006; Equi et al., 2002; Wolter et al., 2002). Inhibitors of the cyclic-di-GMP specific type 5 phosphodiesterase are able to correct the transport of chloride ions across the nasal mucosa (Lubamba et al., 2008, 2011). Compounds such as sildenafil, vardenafil and tadalafil were already approved for the treatment of pulmonary arterial hypertension and erectile dysfunction and have shown promising anti-inflammatory properties useful for people with CF (Huaux et al., 2013; Lubamba et al., 2012). Correctors, compounds enhancing ΔF508-CFTR trafficking to the membrane, and potentiators, compounds able to increase the open probability of the aberrant CFTR channel, comprise a promising new group of pharmaceuticals. The combination of Ivacaftor (a potentiator) and Lumacaftor (a corrector) significantly increased chloride secretion by ΔF508-CFTR (Kuk and Taylor-Cousar, 2015).
In order for people with CF to maintain an appropriate nutrional status, the intake of several food supplements is recommended. Examples of these are fat-soluble vitamins and pancreatic enzymes (as part of the pancreatic enzyme replacement therapy, PERT) (Kalnins and Wilschanski, 2012). The supplied pancreatic enzymes are of porcine origin and contain proteases, lipases and amylases (Somaraju and Solis-Moya, 2014).
Another important aspect of the treatment of people with CF consists of specialized physiotherapy. The role of the physiotherapist not only includes airway clearance, but also inhalation therapy and advice on exercising, posture and mobility. Airway clearance is accomplished through postural drainage and percussion with the addition of forced expiration by using gravity to assist mobilization of secretions towards the central airways (Prasad, 1999).

## 1.4 Lung infections in people with CF

As stated in section 1.2, people with CF are highly susceptible to chronic lung infections because of an impaired mucociliary clearance and an aberrant immune function, accompanied by extensive lung inflammation. Starting in early childhood, the lungs of people with CF are already colonized with pathogens like *Staphylococcus aureus* and *Haemophilus influenzae*. The microbial population shifts over the years towards chronic *Pseudomonas aeruginosa* infection, often accompanied by other opportunistic pathogens like *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans* and *Burkholderia cenocepacia* (Filkins and O'Toole, 2015; Lyczak et al., 2002) (Figure 1). The above-mentioned infecting species colonize an environment characterized by the presence of an extremely diverse population of anaerobes, which represent the predominant microbial population in CF lungs (Sherrard et al., 2016). The resulting polymicrobial communities often complicate treatment by forming mixed biofilms and/or sharing antibiotic resistance mechanisms. Lung infections accompanied by extensive inflammatory responses pose the major clinical threat for people with CF

today, with as much as 80 to 95% of them ultimately dying from the consequences of respiratory failure brought on by chronic bacterial infection (Lyczak et al., 2002).

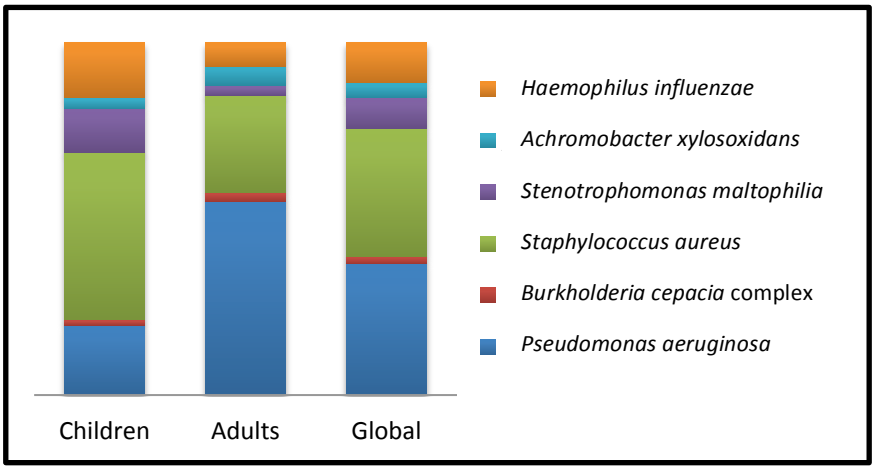



**Figure 1. Prevalence of major infecting bacterial species in children versus adults suffering from CF.** Data obtained from: "The changing microbial epidemiology in cystic fibrosis" (LiPuma, 2010).

# 2 BURKHOLDERIA CENOCEPACIA

## 2.1 Properties of *Burkholderia cenocepacia* and the *Burkholderia cepacia* complex

*Burkholderia cenocepacia* is a rod-shaped, motile, gram-negative ß-proteobacterium that has been isolated from numerous environmental and clinical sources (Vandamme et al., 1997, 2003). Its genome contains over 8 Mbp and consists of three circular chromosomes[1] and a plasmid. Over 9% of its genetic material is occupied by genomic islands, which may play a key role in the adaptation to survival in the different niches occupied by this pathogen (Holden et al., 2009). *B. cenocepacia* is a prominent member of the *Burkholderia cepacia* complex (*Bcc*), currently a group of 20 closely related, but yet distinct bacterial species (Martinucci et al., 2016; Spilker et al., 2015; Vandamme and Dawyndt, 2011). *Bcc* members exhibit a high degree of 16S rRNA (98-100%) and recA (94-95%) gene sequence similarity and moderate levels of DNA-DNA hybridization (30-50%) (Coenye et al., 2001). This group comprises *B. cepacia*, *B. cenocepacia*, *B. multivorans*, *B. pseudomultivorans*, *B. stabilis*, *B. vietnamiensis*, *B. dolosa*, *B. ambifaria*, *B. anthina*, *B. pyrrocinia*, *B. ubonensis*, *B. latens*, *B. diffusa*, *B. arboris*, *B. seminalis*, *B. metallica*, *B. contaminans*, *B. lata*, *B. gladioli*, *B. stagnalis* and *B. territorii* (Peeters et al., 2013a; Spilker et al., 2015). *Bcc* isolates can be encountered in soil, water, the rhizosphere of plants, the human body, different animal species and in the hospital environment. Some of them have been exploited for their beneficial properties, such as bioremediation of recalcitrant xenobiotics, biocontrol against plant pathogens and plant growth promotion. However, other members of the Bcc, like *B. cenocepacia* and *B. multivorans*, are opportunistic human pathogens in vulnerable patient groups, limiting their potential in biotechnological applications (Coenye and Vandamme, 2003). On top of that, most members of the *Bcc* are inherently multidrug resistant, thereby complicating effective treatment of infections caused by these pathogens (Nzula et al., 2002).

## 2.2 Epidemiology of *Burkholderia cenocepacia*

Amongst all members of the *Bcc*, *B. multivorans* and *B. cenocepacia* are the ones most frequently isolated from human sources, in particular from immunocompromised individuals, such as patients with CF or chronic granulomatous disease (CGD). These two opportunistic pathogens together account for approximately 85–97% of all *Bcc* infections in people with CF (Drevinek and Mahenthiralingam, 2010). The prevalence of *Bcc* bacteria is highest in adult patients, ranging between 3 and 4% (LiPuma, 2010). *B. cenocepacia* isolates can be subdivided into four distinct lineages (IIIA, IIIB, IIIC and IIID), based on recA sequencing and multilocus sequence typing (Vandamme et al., 2003). Almost all clinically relevant isolates belong to the IIIA or IIIB lineage (Mahenthiralingam et al., 2005). One of the most highly epidemic groups belonging to the IIIA lineage is known as ET-12 (Edinburgh/Toronto-12), comprising the well-characterized *B. cenocepacia* J2315 and K56-2 strains (Darling et al., 1998; LiPuma et al., 2001; Livesley et al., 1998). These strains often cause chronic infections in people with CF, resulting in approximately 20% of the cases in a

[1] *B. cenocepacia*'s third chromosome is currently also considered to be a megaplasmid, since it is not essential for growth and survival of the pathogen (Agnoli et al., 2014).

fatal 'cepacia syndrome', characterized by necrotizing pneumonia, bacteremia, sepsis and eventually death (Isles et al., 1984; LiPuma, 2010). The intrinsic resistance of these pathogens against many of the currently used antibiotics complicates effective treatment of these infections (Nzula et al., 2002). Inter-patient transmission of *B. cenocepacia* among people with CF or from their non-CF siblings has been reported (LiPuma et al., 1990). Infections with *B. cenocepacia* require pre- and post-operative procedures during lung transplantation to be adapted properly, and due to the potential postoperative persistence of these infections, they are associated with elevated mortality rates (LiPuma, 2001).

Isolates of *B. cenocepacia* are able to infect and survive in epithelial cells (Sajjan et al., 2006; Schwab et al., 2002), macrophages (Lamothe et al., 2007; Lamothe and Valvano, 2008), neutrophils (Bylund et al., 2005, 2006; Saldías et al., 2009), and dendritic cells (Macdonald and Speert, 2008). Internalized *B. cenocepacia* cells are able to delay the maturation of bacteria-containing vacuoles (BcCVs), which results in delayed acidification of the lumen of the vacuoles and inhibition of the assembly of the NADPH oxidase complex on the vacuolar membrane in macrophages (John et al., 1999; Keith et al., 2009; Lamothe et al., 2007; Lamothe and Valvano, 2008). The NADPH oxidase complex plays a crucial role in host defense by generating large quantities of superoxide anions, which serve as precursors of the more potent microbicidal reactive oxygen species (ROS) such as hydrogen peroxide and hydroxyl radicals (Fang, 2004; Segal, 2005; Takeya and Sumimoto, 2003). In epithelial cells, *B. cenocepacia* can alter the normal endocytic pathway and in dendritic cells, internalized bacteria can induce necrosis and alter cell function (Macdonald and Speert, 2008).

## 2.3 Virulence factors

In order to be able to invade the host and establish chronic infection, *B. cenocepacia* K56-2 possesses a wealth of virulence factors aiding in its colonization and replication, including toxins, extracellular enzymes, alternative sigma factors, iron acquisition systems, secretion systems, quorum sensing (QS) systems, adhesins, the ability to form biofilm, flagella, lipopolysaccharide (LPS) and multidrug resistance (Loutet and Valvano, 2010). These will be discussed in detail in the following sections and are summarized in Figure 2.

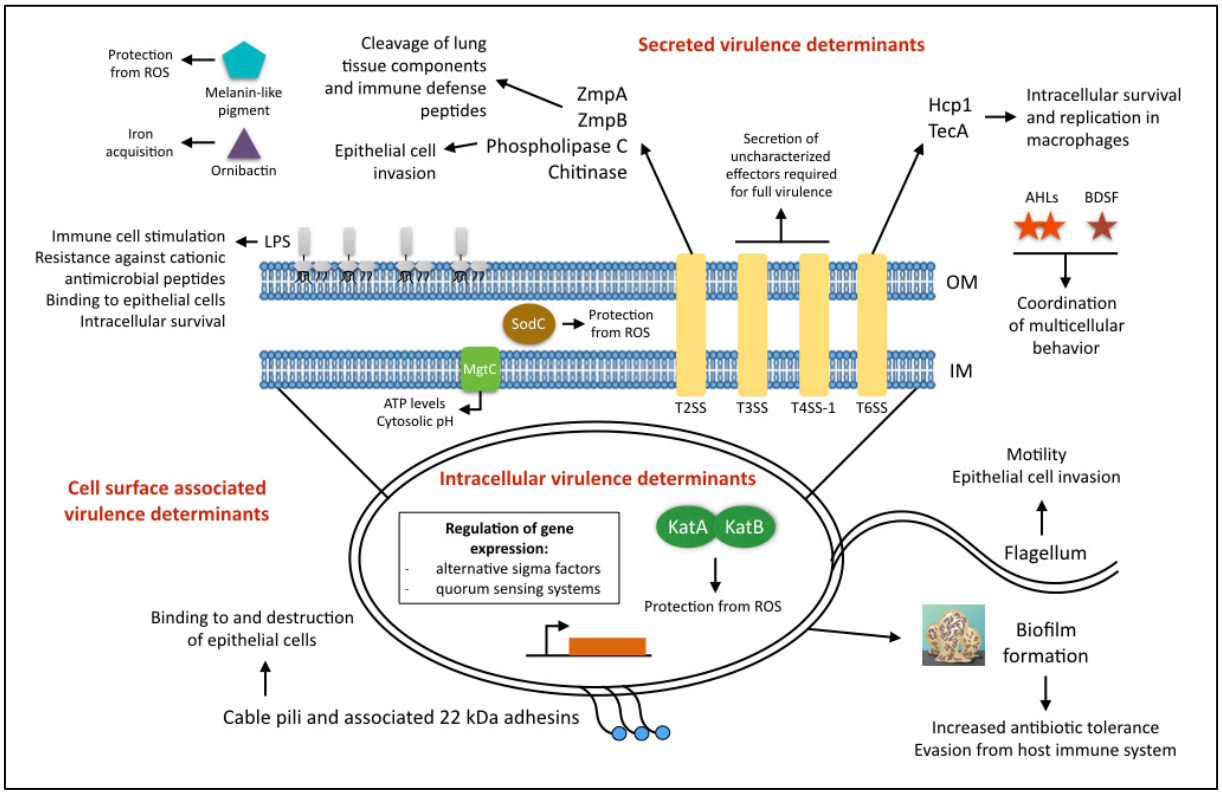



**Figure 2. Representation of the localization and known functions of *B. cenocepacia* virulence determinants.** The virulence determinants represented here are discussed in detail in section 2.3. IM = inner membrane, OM = outer membrane, BDSF = *Burkholderia* diffusible signal factor, AHLs = N-acylhomoserine lactones, LPS = lipopolysaccharide, ROS = reactive oxygen species. Picture adapted from Loutet and Valvano (2010).

### 2.3.1 The production of extracellular hydrolytic enzymes

Probably the most well-known virulence factors of *B. cenocepacia* comprise the two major zinc metalloproteases ZmpA and ZmpB. ZmpA and ZmpB belong to the M4 family of thermolysin-like proteases, which usually display specificity for hydrophobic amino acid residues and are inhibited by chelating agents, like EDTA (Corbett et al., 2003). They are expressed as pre-proenzymes, whose signal peptides dictate their secretion from the cytoplasm into the periplasm through the general secretory pathway (Pugsley, 1993). After autoproteolysis, the type II secretion system (T2SS) transports the mature 36 kDa ZmpA and 35 kDa ZmpB proteins across the outer membrane into the extracellular environment (Corbett et al., 2003; Kooi et al., 2005; Kooi and Sokol, 2009). ZmpA was found to cleave several components of lung tissue, including type IV collagen and fibronectin, and interferes with the immune defense, by hydrolyzing α-1 proteinase inhibitor, $\alpha_2$-macroglobulin and antimicrobial peptides such as LL-37, elafin and secretory leukocyte inhibitor (Kooi et al., 2005; Kooi and Sokol, 2009). Proteins digested by ZmpB also include α-1 proteinase inhibitor, $\alpha_2$-macroglobulin, type IV collagen and fibronectin, as well as transferrin, lactoferrin and several immunoglobulins. By interfering with the major protease inhibitors, ZmpA and ZmpB can disrupt the balance between human α-1 proteinase and its inhibitor, causing increased tissue damage and inflammation, while inactivation of $\alpha_2$-macroglobulin might result in increased dissemination of the pathogen leading to septicemia. Both ZmpA and ZmpB thus contribute to lung pathology, but only ZmpA plays a role in bacterial persistence (Kooi et al., 2006). Both metalloproteases are required for efficient IL-1β secretion in a type VI secretion system (T6SS)-dependent manner during intracellular infection of

macrophages, suggesting an interplay between T2SS and T6SS. They are also necessary for the maturation of BcCVs and intra-macrophage survival, independent of the T6SS (Rosales-Reyes et al., 2012a). They are therefore considered as the major virulence-associated proteins of *B. cenocepacia*. As outlined in section 2.3.7, expression of ZmpA and ZmpB is subject to QS-dependent regulation.

Another family of hydrolytic enzymes involved in the virulence of *B. cenocepacia* comprises the secreted lipases, which have been shown to play a role in lung epithelial cell invasion (Mullen et al., 2007). Phospholipase C is secreted by the T2SS of *B. cenocepacia* K56-2 (Goza, A., unpublished data) and is able to cleave the phosphodiester bond of phospholipids. In *Listeria monocytogenes*, another pathogen able to survive intracellularly, two different phospholipase C enzymes play a role in the escape from the phagosome membrane and in the invasion of adjacent cells (Dussurget et al., 2004; Smith et al., 1995).

Chitinases belong to the glycoside hydrolase family of enzymes that are capable of cleaving the glycosidic bonds of glycans (Frederiksen et al., 2013). Expression of a *P. aeruginosa* chitinase was shown to be increased during growth in artificial cystic fibrosis sputum, as well as in clinical isolates (Fung et al., 2010; Salunkhe et al., 2005). Analogous to phospholipase C, chitinase is thought to be exported via the T2SS in *B. cenocepacia* K56-2 (Goza, A., unpublished data). The target molecules in the host have not been identified so far, but the absence of endogenous host chitin suggests that other GlcNAc-containing glycans, for example in glycoproteins, might be the targets of this type of enzyme (Frederiksen et al., 2013).

### 2.3.2 Iron acquisition strategies

Since the amount of free iron available in the host is extremely limited, infecting pathogens have developed different strategies to gather iron in a very efficient way. Siderophores are relatively low molecular weight, high-affinity iron chelating compounds that can scavenge iron from the environment and make it available for the bacterial cell (Neilands, 1995). *B. cenocepacia* strains predominantly produce the siderophore ornibactin (Figure 3), sometimes accompanied by small amounts of pyochelin (Darling et al., 1998; Thomas, 2007). Production of ornibactin is required for the full virulence of this pathogen in a rat agar-bead model of lung infection, as well as in *Galleria mellonella* and *Caenorhabditis elegans* infection models (Sokol et al., 1999; Uehlinger et al., 2009; Visser et al., 2004). Other types of siderophores produced by members of the *Bcc* include salicylic acid and cepabactin (Darling et al., 1998).

Another important mechanism for *B. cenocepacia* to acquire iron during infection is through the production of the iron-binding protein ferritin. Bound iron can subsequently be released via proteolytic degradation of the ferritin protein (Whitby et al., 2006).

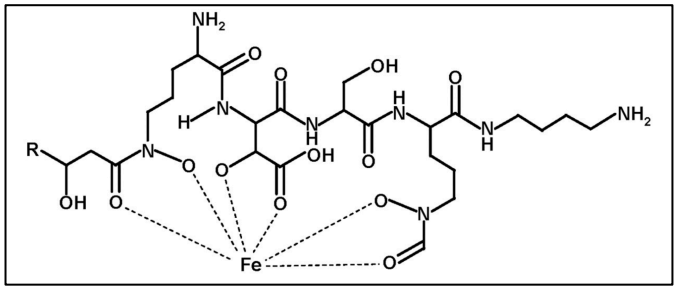



**Figure 3. Structure of ornibactin, the major siderophore produced by *B. cenocepacia*.**

### 2.3.3 Motility and adherence

Swimming motility of *B. cenocepacia* is assured through the presence a single, long, polar flagellum (Hales et al., 1998). Biogenesis of flagella proceeds via a highly complex, hierarchical pathway, involving over 40 structural and regulatory genes (Neidhardt and Curtiss, 1996). The main structural component of flagella is the flagellin protein FliC, accompanied by several hook and basal body proteins. The flagellar type III secretion system translocates structural proteins across the membrane, upon which they are added to the distal end of the growing organelle (Macnab, 1999). In *P. aeruginosa*, flagella are believed to be important for the dissemination of bacteria from local infection sites to other organs (Drake and Montie, 1988). Furthermore, flagellum-mediated motility was shown to facilitate penetration of host epithelial barriers and thereby contributes to host cell invasion by *B. cenocepacia* J2315 (Tomich et al., 2002). Flagella function as virulence determinants in a murine agar-bead model of *B. cenocepacia* K56-2 infection and are able to initiate signaling cascades in HEK293 cells by interacting with Toll-like receptor 5 (TLR5), causing activation of NF-κB (Urban et al., 2004). A recent study has shown that glycosylation of *B. cenocepacia* K56-2 flagellin with 4,6-dideoxy-4-(3-hydroxybutanoylamino)-D-glucose might reduce interaction with host TLR5 and thereby provides a means to escape the action of the immune system (Hanuszkiewicz et al., 2014). Loss of motility of *B. cenocepacia* CF isolates is suggested as a marker for the development of the life-threatening 'cepacia syndrome' (Kalferstova et al., 2015).

Host specificity and tissue tropism are very often determined by the specific adherence mechanisms present in a given pathogen (Krogfelt, 2015). Next to flagella that might function as adhesins, *B. cenocepacia* strains of the ET-12 lineage also possess cable pili, large appendages spread across the entire surface of the bacterium (Figure 4). A 22 kDa cable pili-associated adhesin protein was found to bind to human respiratory mucins (Sajjan et al., 1992; Sajjan and Forstner, 1993) and was recently shown to mediate adherence of cable-piliated *B. cenocepacia* BC7 to cytokeratin 13 (CK13) expressed on squamous airway epithelial cells (Urban et al., 2005). Both cable pili and the associated 22-kDa adhesin are required for the persistence and spread of *B. cenocepacia* BC7 infections. They are also needed for eliciting maximal inflammatory responses (Goldberg et al., 2011). The trimeric autotransporter adhesin BcaA (BCAM0224) forms stable trimers on the bacterial cell surface and is needed for early stages of biofilm formation, swarming motility, evasion of the host immune system, and adhesion to and invasion of human bronchial epithelial cells (Mil-Homens et al., 2010, 2014). Two other genes (BCAM0219 and BCAM0223) lying in close proximity of *bcaA*, presumably also encode trimeric autotransporters, of which BCAM0223 was shown to be involved in hemagglutination, serum resistance, epithelial cell adhesion and virulence (Mil-Homens and Fialho, 2012).

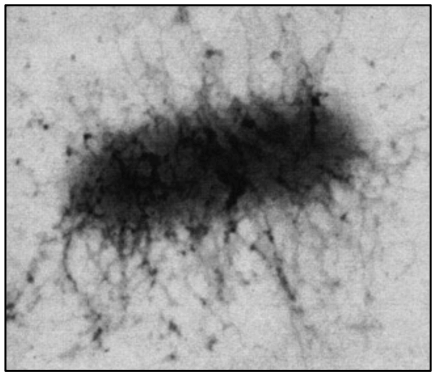


**Figure 4. Transmission electron micrograph of piliated *B. cenocepacia* strain BC7.** Picture reprinted from Tomich and Mohr (2004).

### 2.3.4 Lipopolysaccharides/surface polysaccharides

The major surface component of *B. cenocepacia* and the vast majority of Gram-negative bacteria is LPS. It is a complex glycolipid located in the outer leaflet of the outer membrane and is composed of lipid A, a core oligosaccharide and, in some bacteria, the O antigen (Ortega et al., 2009; Raetz and Whitfield, 2002). In *Burkholderia* spp., and thus also in *B. cenocepacia*, a unique feature of LPS is the constitutive presence of 4-amino-4-deoxy-L-arabinose (Ara4N) residues in the lipid A and inner core oligosaccharide. This modification reduces the net negative charge of LPS and promotes resistance against cationic antimicrobial peptides (Helander et al., 1994; Loutet et al., 2006). Functional expression of Ara4N was shown to be essential for *B. cenocepacia* viability and depletion of the proteins in the Ara4N biosynthesis pathway in conditional mutants increased susceptibility to antimicrobials and led to the accumulation of membranous material inside the cell (Ortega et al., 2007). LPS of clinical *B. cenocepacia* ET-12 strains is able to stimulate immune cells through TLR4-mediated NF-κB activation (Bamford et al., 2007) and LPS inner core oligosaccharide mutants show impaired virulence in *C. elegans*, *G. mellonella* and rat agar-bead models of infection and fail to survive in murine macrophages (Loutet et al., 2006; Ortega et al., 2009; Uehlinger et al., 2009).

### 2.3.5 Intracellular survival inside host cells

As mentioned before in section 2.1, *B. cenocepacia* isolates are able to infect and survive in a wide variety of human cells, including epithelial cells, macrophages, neutrophils and dendritic cells, where they can delay the maturation of BcCVs, alter the normal endocytic pathway and cell function, and induce necrosis.

In order to do that, *B. cenocepacia* possesses three types of secretion systems that are capable of injecting toxic proteins from the bacterial cytoplasm directly into host cells: type III secretion systems (T3SS), type IV secretion systems (T4SS) and T6SS (Depluverez et al., 2016). An elaborate discussion on the structure and function of those secretion systems can be found in section 3.3. Both the plasmid-encoded T4SS-1 and the T6SS have been shown to be required for intracellular survival and replication inside macrophages (Gavrilin et al., 2012; Sajjan et al., 2008a), although there is still some controversy about this subject (Valvano, 2015). A functional *B. cenocepacia* K56-2 T6SS can alter the activation of Rac1 and Cdc42 GTPases, leading to abnormal actin polymerization (Flannagan et al., 2012; Rosales-Reyes et al., 2012b). Although the T6SS is not involved in causing the phagosome maturation arrest, it does prevent the recruitment of the NADPH oxidase complex to the membrane of BcCVs (Rosales-Reyes et al., 2012b). Furthermore, the T6SS induces a proinflammatory response in macrophages, ultimately leading to cell death by pyroptosis (Rosales-Reyes et al., 2012a). It also disrupts the phagosomal membrane to allow the escape of the type II secreted metalloproteases ZmpA and ZmpB into the cytoplasm of the macrophages, which may reinforce the inflammatory response and results in the maturation arrest of BcCVs (Rosales-Reyes et al., 2012a). Recently, a novel T6SS effector, TecA, has been identified, which inactivates Rho GTPases by deamidation. This induces caspase-1 inflammasome activation and actin cytoskeletal defects (Aubert et al., 2015), and might therefore explain part of the effects attributed to the T6SS. A study from O'Grady and Sokol (2011), in which they compared gene expression in *in vitro* and *in vivo* environments, revealed the induction of the T4SS-1 *in vivo* in a rat agar-bead respiratory infection

model (O'Grady and Sokol, 2011). However, so far, no T4SS-1 effector proteins of *B. cenocepacia* have been identified.

Besides the aforementioned secretion systems, several other factors contribute or are even indispensable for the intracellular survival of *B. cenocepacia*. An *mgtC* mutant of *B. cenocepacia* K56-2 was unable to delay the phagosomal maturation and failed to survive in murine macrophages (Maloney and Valvano, 2006). The necessity for a functional MgtC, a protein responsible for ATP-dependent $Mg^{2+}$ transport, for intracellular survival was shown to be due to its ability to regulate bacterial physiological ATP levels and cytosolic pH (Lee et al., 2013). The secreted low molecular mass protein tyrosine phosphatase Dpm is presumably involved in impairing the recruitment of the 16 kDa subunit of the phagosomal vacuolar ATPase (vATPase), thereby delaying the acidification of the BcCVs (Andrade and Valvano, 2014; Rosales-Reyes et al., 2012b).

Since phagocytic cells generate reactive oxygen species to eliminate bacteria, factors involved in resistance against oxidative stress are priceless for the ability of *B. cenocepacia* to survive intracellularly. The presence of two catalases/peroxidases KatA and KatB protects the pathogen against hydrogen peroxide under iron-limiting conditions (Lefebre et al., 2005). SodC, a periplasmic superoxide dismutase, and a melanin-like pigment provide resistance against superoxide generated through the action of the NADPH oxidase complex (Keith et al., 2007; Keith and Valvano, 2007). Finally, two sigma factors, RpoN and RpoE, are also required for successful intracellular survival, although the exact mechanisms still have to be uncovered (Flannagan and Valvano, 2008; Saldías et al., 2008).

### 2.3.6 Biofilm formation

Biofilms are structured microbial communities encapsulated in extracellular matrix material consisting of proteins, polysaccharides and DNA (Pamp et al., 2007). Bacteria growing in biofilms display increased tolerance to antibiotic treatment and are less susceptible to the action of the host immune system (Burmølle et al., 2010; Hall-Stoodley and Stoodley, 2009). Generally, planktonically growing bacteria are associated with acute infections and, when diagnosed in an early stage and in an accurate way, can be treated quite successfully. However, once bacteria succeed to form biofilms, effective treatment becomes complicated and chronic infections often develop (Bjarnsholt, 2013). The transition between the planktonic and biofilm mode of growth is tightly regulated and encompasses many regulatory mechanisms. The intracellular second messenger cyclic-di-GMP plays a central role in this regulation and stimulates the production of several biofilm matrix components, like exopolysaccharides (Huber et al., 2002; Römling and Simm, 2009). Biofilm formation is also subject to QS-based regulation, as outlined in section 2.3.7. Other biofilm regulators include the small non-coding regulatory RNA *mtvR*, the alternative sigma factor RpoN, the LysR-type transcription factor ShvR and the hybrid sensor kinase AtsR (Aubert et al., 2008, 2013; Bernier et al., 2008; Ramos et al., 2013; Saldías et al., 2008; Subramoni et al., 2011). Although *B. cenocepacia* is able to form biofilms *in vitro* (Conway et al., 2002), there is still some debate regarding the relevance of *B. cenocepacia* biofilms in the lungs of people with CF (Schwab et al., 2014). *Bcc* bacteria in CF lungs were mainly found as single cells or small clusters within phagocytes, but not as biofilm-like structures (Schwab et al., 2014).

### 2.3.7 Quorum sensing

The coordination of the expression of all these virulence factors mainly occurs at the level of the QS systems. A concerted regulation of these energy-consuming virulence mechanisms is required for the optimal usage of nutrient sources, as well as for the unification of the different elements necessary for successful invasion and colonization of the host. *B. cenocepacia* employs two different QS systems responsible for the coordination of virulence factor expression and enabling the tuning of the behavior of different cells in the population (Loutet and Valvano, 2010). QS molecules are usually diffusible compounds that can alter gene expression when their concentration exceeds a certain treshold value (Fuqua et al., 1996).

#### *2.3.7.1 The CepIR QS system*

The first QS system, responsible for the production of N-octanoylhomoserine lactone (C8-HSL) and minor amounts of N-hexanoylhomoserine lactone (C6-HSL), is the CepIR system. In this system, CepI is the synthase, while CepR functions as the C8-HSL receptor. Upon binding of C8-HSL, CepR binds to specific DNA sequences leading to the induction or repression of target gene expression (Gotschlich et al., 2001; O'Grady et al., 2009a). The CepIR QS system is required for full virulence of *B. cenocepacia* towards nematode, wax moth, murine, alfalfa and onion infection models (Huber et al., 2001; Kooi et al., 2006; Sokol et al., 2003; Uehlinger et al., 2009). Traits positively influenced by the CepIR QS system include the production of the extracellular metalloproteases ZmpA and ZmpB and the nematocidal protein AidA, biofilm formation and swarming motility, while the production of the siderophore ornibactin is prone to negative regulation by CepIR (Aguilar et al., 2003; Huber et al., 2001, 2004; Kooi et al., 2006; Lewenza et al., 1999; Riedel et al., 2003; Sokol et al., 2003; Weingart et al., 2005). Evidence exists of the involvement of this QS system in the regulation of secretion, by influencing the expression of genes encoding type II, type III and type VI secretion systems (Aubert et al., 2008; Subsin et al., 2006).

#### *2.3.7.2 The CciIR QS system*

An exceptional property of the highly transmissible *B. cenocepacia* ET12 strains is the presence of a second acylhomoserine lactone (AHL)-based QS system, the CciIR system, located on a genomic island called *cci* (*cenocepacia* island). In analogy with the CepIR system, CciR is the receptor, while CciI is responsible for the synthesis of C6-HSL and minor amounts of C8-HSL. Much less is known about the genes regulated by this system, but it appears that both systems are intertwined, illustrated by the fact that CciR negatively regulates *cepI* and that CepR is required for the expression of the *cciIR* operon (Malott et al., 2005). The presence of a thirth LuxR type repressor CepR2, similar to CciR and CepR, further complicates the regulatory network. The *cepR2* gene was found to be repressed by CciR and is also involved in the regulation of virulence factor production (Malott et al., 2009). While CepR mainly functions as a positive regulator, CciR acts mainly as a negative regulator of gene expression (Suppiger et al., 2013).

### 2.3.7.3 Regulation of AHL-based QS systems

Since the AHL-based QS system controls many crucial virulence-associated traits in *B. cenocepacia*, a strict regulation of this system is essential to coordinate all these processes. It has been shown that the CepIR and the CciIR systems mutually interact, whereby CepR positively regulates the expression of the *cciIR* operon and CciR on its turn is responsible for the negative regulation of *cepI* expression (Figure 5) (Malott et al., 2005). A conserved hypothetical protein (BCAM1871) appears to induce AHL activity and positively regulates *cepIR* and *cciIR* expression (O'Grady et al., 2012). On top of that, BCAM1871 activates the expression of ShvR, a highly conserved LysR-type regulator involved in the negative control of *cepIR* and *cciIR* expression and the stimulation of biofilm formation (Bernier et al., 2008; O'Grady et al., 2009b, 2011a, 2012). The global virulence regulator AtsR, a hybrid sensor kinase, negatively controls the expression of the *cepI* and *cciI* genes, although it is also capable of regulating virulence factor production independent of the AHL-based QS system (Aubert et al., 2013).

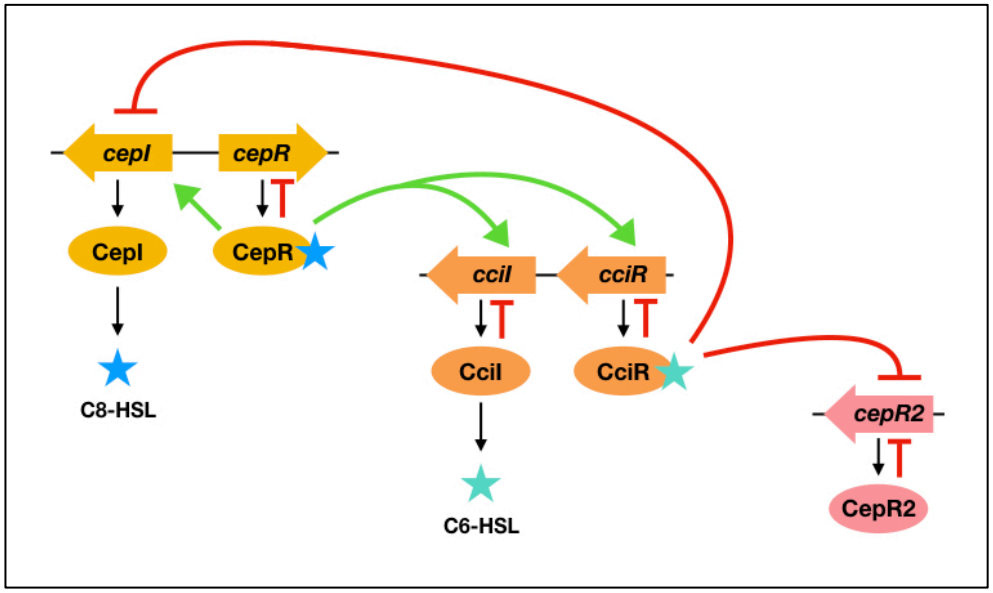



**Figure 5. Hierarchical organization of the CepIR, CciIR and CepR2 quorum sensing systems.** Figure adapted from O'Grady et al., 2009c.

### 2.3.7.4 The diffusible signal factor QS system

The second major QS system of *B. cenocepacia* uses fatty acids as signaling molecules, known as diffusible signal factors (DSFs). This system was first discovered in *Xanthomonas campestris* pv. *campestris*, a plant pathogen (Barber et al., 1997; Wang et al., 2004). The specific DSF synthesized by this bacterium is cis-11-methyl-2-dodecenoic acid (Wang et al., 2004). Production of DSF family molecules is also widespread among members of the *Bcc* (Suppiger et al., 2016). The structure of the major signalling fatty acid synthesized by *B. cenocepacia* was identified as cis-2-dodecenoic acid, also known as the *Burkholderia* diffusible signal factor (BDSF). The $rpfF_{Bc}$ gene (BCAM0581) encodes an enoyl-CoA hydratase with both dehydratase and thioesterase activity that enables the direct conversion of the acyl carrier protein thioester of 3-hydroxydodecanoic acid into cis-2-dodecenoic

acid (Bi et al., 2012; Boon et al., 2008; Deng et al., 2011). Intermediates of the fatty acid biosynthesis pathway presumably serve as the substrates for $RpfF_{Bc}$ *in vivo* (Ryan et al., 2015a). Adjacent to $rpfF_{Bc}$ lies the gene BCAM0580 encoding the BDSF receptor protein RpfR (Deng et al., 2012). This protein contains a PAS (Per-Arnt-Sim), GGDEF and EAL domain (Figure 6). The latter two domains are characteristic for respectively diguanylate cyclases and phosphodiesterases, which are commonly involved in cyclic-di-GMP turnover (Römling et al., 2005). Upon BDSF binding to the PAS domain of RpfR, its cyclic-di-GMP phosphodiesterase activity is switched on, thereby lowering the intracellular cyclic-di-GMP levels (Deng et al., 2012). Cyclic-di-GMP on its turn functions as a global regulator through binding to diverse receptors and effectors, including PilZ domain proteins, transcription factors and riboswitches (Ryan et al., 2012; Sondermann et al., 2012). It promotes biofilm formation and T6SS activity, and reduces motility and invasion (Romling et al., 2013). A second receptor protein, encoded by BCAM0227, appears to control only a subset of the whole range of BDSF-regulated genes (McCarthy et al., 2010). The latter protein 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 (Ryan et al., 2015a). Inactivation of *rpfR* or $rpfF_{Bc}$ lowers motility, adherence, biofilm formation and proteolytic activity (Deng et al., 2012; Ryan et al., 2009a), while inactivation of BCAM0227 reduced cytotoxicity towards Chinese hamster ovary cells and reduced virulence in an agar-bead mouse model of pulmonary infection, as well as towards larvae of the wax moth (McCarthy et al., 2010).

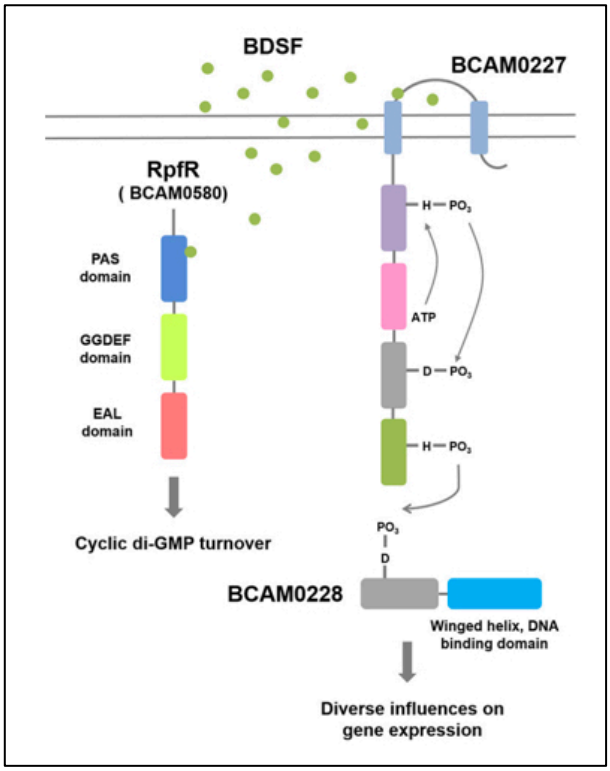



**Figure 6. Perception of BDSF signals by the RpfR and BCAM0227 receptor proteins.** Figure reprinted from Ryan et al. (2015a).

#### 2.3.7.5 Turnover of QS signals

Since QS is a highly energy-demanding process, it is now widely accepted that bacterial cells possess turnover systems responsible for the rapid degradation of QS signals in the post-quorum sensing phase (Grandclément et al., 2015; Zhou et al., 2017). Spontaneous lactonolysis, the opening of the lactone ring of AHLs by addition of a water molecule, is responsible for the short half life of AHLs under laboratory conditions (Byers et al., 2002; Delalande et al., 2005; Yates et al., 2002). But besides the natural turnover, bacteria very often contain specific quorum-quenching enzymes. The enzymatic degradation of AHLs by lactonases or acylases was first described in *Variovorax* and *Bacillus* genera (Dong et al., 2002; Leadbetter and Greenberg, 2000). Recently, the quorum-quenching AHL acylase PvdQ, produced by fluorescent pseudomonads, has been shown to degrade N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), a *P. aeruginosa* QS signal molecule (Sio et al., 2006).

Although several bacterial strains capable of rapidly degrading DSFs have been identified, including those belonging to the genera *Bacillus*, *Paenibacillus*, *Microbacterium*, *Staphylococcus*, and *Pseudomonas*, the mechanism by which DSF degradation occurs, remain obscure. A study by Newman et al. (2008) revealed that the *carAB* genes, encoding enzymes involved in carbamoylphosphate synthesis in *Pseudomonas* spp. strain G, were required for DSF inactivation (Newman et al., 2008). In *Xanthomonas campestris* pv. *campestris* (*Xcc*) and *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), RpfB has been identified as an enzyme potentially involved in DSF turnover, since RpfB transcript levels are low during mid-exponential growth, slightly increase in the late exponential growth phase, are maximal in early stationary phase, and subsequently decline, which matches the DSF production pattern during growth (Bi et al., 2014; Zhou et al., 2015, 2017).

Currently, no reports of DSF or AHL degrading enzymes in *B. cenocepacia* have been published yet, but it can be assumed that a similar system is present in this pathogen to strictly control DSF and AHL levels at any given time point during growth or infection.

#### 2.3.7.6 Crosstalk between the AHL and DSF QS systems in B. cenocepacia

Since both the AHL and the DSF type of QS systems regulate a similar set of virulence genes, there must be a certain degree of crosstalk between them. For example, Schmid et al. (2012) found that BDSF and C8-HSL co-regulate biofilm formation and the production of extracellular proteases and that both QS molecules are required for the expression of *bapA*, a beta-alanine specific aminopeptidase, and *bclACB*, an operon involved in lectin production in *B. cenocepacia* H111 (a CF sputum isolate that does not belong to the ET-12 lineage). Initially it was suggested that the two systems act in parallel. Later, it was shown that the BDSF-dependent QS system positively regulates AHL levels, which can be attributed to increased levels of *cepI* transcription (Deng et al., 2013b; Schmid et al., 2012; Udine et al., 2013). These data suggest a hierarchical arrangement of both QS systems, with the RpfFR system on top of the CepIR system (Schmid et al., 2012; Udine et al., 2013). In the J2315 strain, the expression levels of *cciI* and *cciR* rather than *cepI* were found to be reduced in a $rpfF_{Bc}$ mutant strain (Udine et al., 2013). However, it is important to stress that the specific regulation of QS systems might be highly strain-specific. Moreover, exact determination of AHL titers is not straightforward and is not well documented in older work in this area. Anyway, two possible working models were proposed. In the first one, it is hypothesized that an unknown cyclic-di-GMP

receptor protein stimulates the transcription of target genes, including *cciI*, *cciR* and *cepI*. The second model assumes that the two QS cascades converge and regulate a yet unknown common modulator, responsible for adjusting the expression of target genes (Schmid et al., 2012).

### 2.3.8 Other factors

The overview of the different virulence factors and regulators of *B. cenocepacia* discussed in the previous sections is probably just the tip of the iceberg. The whole arsenal of virulence-associated components is likely to be much more elaborate than what is currently known and/or understood. Several additional virulence traits, other than the ones reviewed above, include the phenylacetic acid catabolic pathway, the secretion of a nematocidal protein AidA and the serine proteases HtrA and MucD, the presence of genomic islands, the ability to switch to different colony morphologies and the production of exopolysaccharides like cepacian. These have been excellently reviewed by Drevinek and Mahenthiralingam (2010).

## 2.4 Antibiotic resistance

Efficient treatment of *Bcc* infections is often complicated by the high degree of resistance against many of the currently available antibiotics. Since the minimal inhibitory concentration (MIC) of antibiotics differs between different *Bcc* species and even among different strains of a single species, an accurate identification of the infecting pathogen is mandatory for the compilation of an effective treatment regime (Abbott et al., 2016). Several types of intrinsic or acquired resistance mechanisms have been identified in *Bcc* isolates, including reduced entrance of antibiotics due to properties of the cell envelope, active efflux into the extracellular environment, enzymatic inactivation of antibiotics, alteration of antibiotic targets by mutation or enzymatic modification, sequestration by specific drug-binding proteins, target overproduction and substitution of a susceptible target with a resistant target to create a metabolic bypass (Blair et al., 2011; Schweizer, 2012; Walsh and Wencewicz, 2016). A study by Zhou et al. revealed that more than 50% of the *Bcc* isolates from CF lungs were resistant to chloramphenicol, co-trimoxazole, ciprofloxacin, tetracycline, rifampin, avibactam, and co-amoxiclav (Zhou et al., 2007). Susceptibility testing on *B. cenocepacia* strains isolated from CF lungs showed high resistance (>90% of isolates) against amikacin, gentamicin, tobramycin, ciprofloxacin, levofloxacin, imipenem, ticarcillin/clavulanate, ampicillin/sulbactam and piperacillin and moderate resistance (60-90% of isolates) against aztreonam, ceftazidime, piperacillin/tazobactam, meropenem, doxycycline and minocycline. Up to 60% of the isolates were resistant to co-trimoxazole and temocillin (Abbott et al., 2016). Together, these studies clearly show that there is an urgent need for new treatment strategies in the battle against multidrug resistant *Bcc* bacteria, including *B. cenocepacia*. Novel, promising targets for the development of antimicrobial agents include a.o. QS systems, adhesins and secretion systems.

## 2.5 Role of *Burkholderia cenocepacia* in cystic fibrosis

As stated in section 1.2, people with CF are highly susceptible to chronic lung infections because of an impaired mucociliary clearance and an aberrant immune function accompanied by extensive lung inflammation. *Bcc* infections are encountered in only 3 - 4% of people suffering from CF worldwide (LiPuma, 1998), but the consequences are often life-threatening, since a significant proportion of the patients experience a rapid decline in lung function, accompanied by high fever, bacteremia, pneumonia and eventually death (Muhdi et al., 1996). The prevalence of another *Bcc* member, *B. multivorans*, is rising and, in various important CF regions, exceeds that of *B. cenocepacia* in people with CF. However, since it is less virulent and because of its lower associated mortality (Jones, 2004; Mahenthiralingam et al., 2001), much less research has focused on this pathogen, and it will not be discussed in this thesis either.

### 2.5.1 Localization

In people with CF, impaired mucociliary clearance and reduced activity of host defense products causes a situation in which bacteria bound to mucin can persist in the airway lumen and might gain access to airway epithelial cells (Boucher, 2004, 2007; Conner et al., 2007; Moskwa et al., 2007; Singh et al., 1998). Lung explants from patients with acute and chronic infection revealed the highest abundance of *B. cenocepacia* in the epithelia of the terminal and respiratory bronchioles, where they also exhibited intercellular migration, allowing penetration to the respiratory epithelium. High bacterial counts were also found in inflammatory cell infiltrates, in peribronchiolar and perivascular regions, in thickened alveolar septae and in luminal and parenchymal macrophages (Sajjan et al., 2001). Areas of necrotizing pneumonia were characterized by the presence of *B. cenocepacia*, reflected by the death of those patients from 'cepacia syndrome' (Sajjan et al., 2001). These findings are in contrast with the localization of *P. aeruginosa*, which is mainly encountered in the lumen of the airways, but not inside phagocytic cells (Baltimore et al., 1989).
Normal airway epithelial cells differentiated into a mucociliary phenotype are able to trap and kill bacteria in the apical mucus layer and can thereby prevent them from invading the cells. However, in CF epithelia, there is an inefficient killing of trapped bacteria, leading to persistent infections and eventually enabling the bacteria to invade host cells and survive intracellularly (Conner et al., 2007; Ganesan and Sajjan, 2012; Moskwa et al., 2007; Singh et al., 1998).
As for the mode of growth, no biofilm-like structures of *Bcc* bacteria were observed in the lungs of CF patients. They rather appeared to grow as single cells or small cell clusters, either in macrophages or within mucus or exfoliated epithelial cells (Schwab et al., 2014).

### 2.5.2 Interactions with the host

In order to invade host cells and establish chronic infection, *B. cenocepacia* isolates possess a whole arsenal of virulence factors, which were already briefly reviewed in section 2.3. Here, their relevance for establishing interactions and hijacking host cells will be discussed. The production of cable pili and the associated 22 kDa adhesin protein is essential for the binding of *B. cenocepacia* ET-12 strains to mucin and might even be necessary for the development of 'cepacia syndrome' (Sajjan et al.,

1992; Sajjan and Forstner, 1993). Adherence can also be mediated by the binding of flagella to the ganglioside asialoGM1, expressed on the apical surface of CF airway epithelial cells, thereby promoting invasion (Adamo et al., 2004; Feldman et al., 1998; Muir et al., 2004; Mullen et al., 2010). The resistance of this pathogen against antimicrobial peptides, such as β-defensin, through the action of extracellular proteases prevents its effective killing in the apical mucus layer (Baird et al., 1999; Kooi and Sokol, 2009). Together with the impaired mucociliary clearance in the lungs of CF patients, this leads to persistent infections and augments the probability of invasion of host cells.

The innate immune system in humans provides the first line of defense against any kind of threat from the outside, including bacterial pathogens. Interaction of bacterial products (LPS, flagella, pili,…) with receptors of the innate immune system, such as TLRs, activates pro-inflammatory cytokine responses required for the attraction of neutrophils. This process is usually beneficial for the host and assists in the efficient clean-up of infecting pathogens. However, in some cases, excessive chemokine responses caused by abberant stimulation of TLRs may lead to the exorbitant recruitment of neutrophils and concomitant lung tissue damage through the release of oxidants and proteases, like neutrophil elastase (Nauseef and Borregaard, 2014; Vandivier et al., 2006, 2009). The lipid A and O antigen components of LPS have been shown to be involved in the stimulation of pro-inflammatory cytokines like IL-1β through interaction with TLRs (Kotrange et al., 2011; De Soyza et al., 2004). Binding of flagella to asialoGM1, whose expression is increased in CF airway epithelial cells, enhances TLR5-based IL-8 production (Adamo et al., 2004; McNamara et al., 2006). Furthermore, *B. cenocepacia* has been shown to bind to the tumor necrosis factor receptor I (TNRF1), also leading to IL-8 expression. Cable pili and the associated 22 kDa adhesin have proven to be essential for maximal IL-8 production in squamous-differentiated bronchial epithelial cells, although mutants lacking them retained their capacity to bind to TNRF1 (Sajjan et al., 2008b). In *Staphylococcus aureus*, the binding ligand has been determined to be protein A, a major surface protein, but the ligand responsible for *B. cenocepacia* binding to TNRF1 has not been identified so far (Gómez et al., 2004; Sajjan et al., 2008b). Although TNRF1 normally plays an essential role in host defense, binding of pathogenic bacteria subverts this pathway towards an exaggerated immune response (Hehlgans and Pfeffer, 2005).

Next to the overwhelming immune response, *B. cenocepacia* has the ability to invade host cells and survive intracellularly in epithelial cells and several types of phagocytic cells (Bylund et al., 2005, 2006; Lamothe et al., 2007; Lamothe and Valvano, 2008; Macdonald and Speert, 2008; Sajjan et al., 2006; Saldías et al., 2009; Schwab et al., 2002). Once engulfed, *B. cenocepacia* resides in BcCVs, which progress normally to the early phagosomal stage. However, the accumulation of the late endosome or lysosome marker LAMP-1 is significantly delayed, causing a maturation arrest and a delayed acidification of the endosomal lumen. Recruitment of the 16 kDa subunit of the vATPase and assembly of the NADPH oxidase complex is likewise delayed, which interferes with the efficient removal of the engulfed pathogen (Keith et al., 2009; Rosales-Reyes et al., 2012b). Recent evidence suggests that *B. cenocepacia* is also able to escape from the BcCVs, triggering the selective autophagy pathway (Al-Khodor et al., 2014). The factors required for and/or contributing to intracellular survival were already outlined in section 2.3.5.

Several studies found a synergy between *B. cenocepacia* intracellular infection and the CFTR defect, since the maturation arrest is much more pronounced in CFTR-deficient macrophages than in normal macrophages (Lamothe and Valvano, 2008). Numerous explanations have been proposed, but currently, evidence indicates that defective CFTR leads to impaired autophagy and reduces the clearance of protein aggregates, leading to inflammation (Luciani et al., 2010, 2011, 2012). There is

thus a synergistic effect between *B. cenocepacia* infection and defective CFTR which downregulates autophagy, consistent with the delay in BcCV maturation (Valvano, 2015). In CFTR-deficient murine macrophages, *B. cenocepacia*-containing vacuoles rarely fuse with lysosomes and the infected cells produce higher IL-1β levels than normal macrophages (Abdulrahman et al., 2011).
Together, all these mechanisms contribute to the ability of *B. cenocepacia* to escape from the action of the innate immune system, resulting in either chronic colonization or an acute, often life-threatening infection.

### 2.5.3 Cooperation and crosstalk between CF pathogens

Pathogens infecting the lungs of CF patients are rarely confined to a single species, and it has been recognized that different species coexisting in the same environment have mutual interactions and can contribute to the pathogenesis or influence the clinical outcome of the disease (Amin et al., 2010; Bragonzi et al., 2012a; Chattoraj et al., 2010; Hibbing et al., 2010; Lopes et al., 2012; Rogers et al., 2010a; Twomey et al., 2012). Profiling the microbiome and understanding interspecies interactions occurring in CF lungs is of utmost importance to direct antibiotic therapy, since the presence of other species can modulate the antibiotic resistance profile of a given pathogen (Furiga et al., 2015; Pompilio et al., 2015). More and more studies are now focusing on the crosstalk between numerous CF pathogens, and on ways to interfere with this process to avoid its potentially fatal consequences. Mechanisms of inter-bacterial crosstalk often involve QS signaling and/or metabolic cooperation or competition (Elias and Banin, 2012).

Since *P. aeruginosa* remains the predominant pathogen in CF lung infections, the majority of the research aimed at the elucidation of microbial interactions, involves this pathogen. Pompilio et al. (2015) investigated the interactions between *P. aeruginosa* and the frequently co-isolated pathogen, *S. maltophilia*. They observed an inhibitory effect of *P. aeruginosa* on the growth of *S. maltophilia*, during both exponential and stationary growth phases for the planktonic and biofilm mode of growth. It was also shown that this effect required direct contact between both species, which suggest the potential involvement of a T6SS injecting specific proteins in *S. maltophilia* cells. The presence of *S. maltophilia* increased the expression of efflux pump genes in *P. aeruginosa*, as well as the production of exotoxin A, alkaline protease and alginate (Pompilio et al., 2015). In mixed biofilms, *S. maltophilia* significantly influences the architecture of *P. aeruginosa* biofilms, an effect dependent on the *S. maltophilia* QS signal cis-11-methyl-2-dodecenoic acid, a DSF family molecule. Addition of DSF to *P. aeruginosa* cultures increased the levels of bacterial stress tolerance proteins and the resistance against cationic antimicrobial peptides, like polymyxins (Ryan et al., 2008).
Under aerobic conditions, *P. aeruginosa* always outcompetes *B. cenocepacia* in mixed cultures. This effect was attributed to the production of pyocyanins by *P. aeruginosa* (Schwab et al., 2014; Tomlin et al., 2001). Secretion of toxic MVs is also used by *P. aeruginosa* to attack competing species. These MVs can contain hemolysins, proteases, elastases, phospholipases, alkaline phosphatases, Cif proteins and other antibacterial factors, such as murein hydrolases (Li et al., 1998; Tashiro et al., 2012, 2013). Several species belonging to the genus *Pseudomonas* and *Burkholderia* have been shown to produce rhamnolipids, glycolipidic biosurfactants with antimicrobial activity. They can permeate cells by intercalating into the membrane and they have been shown to be involved in the detachment of biofilms (Abdel-Mawgoud et al., 2010; Sotirova et al., 2008). Another factor

mediating competition between both species is the production of bacteriocins (Bakkal et al., 2010). These are potent, proteinaceous toxins with a relatively narrow killing range. A well-known example of a bacteriocin is pyocin, produced by *P. aeruginosa*. R and F type pyocins resemble bacteriophage tails and kill by depolarizing the cell membrane (Michel-Briand and Baysse, 2002). Much less is known about the cepaciacins, the bacteriocins produced by *Burkholderia*, except that they also appear to be phage-tail-like (Govan and Harris, 1985). Bacteriocins from both species are able to actively inhibit each other's growth (Bakkal et al., 2010).
In contrast to the competitive nature of *P. aeruginosa* in *in vitro* aerobic conditions, co-existence with other bacteria may be favored in more natural conditions. The mucoid form of *P. aeruginosa* is characterized by the elevated production of the acidic exoplysaccharide alginate (Govan and Deretic, 1996). Alginate facilitates persistence of *B. cenocepacia* in the lungs of CF mice by protecting bacteria from phagocytes and reducing the inflammatory response during the initial stages of infection (Chattoraj et al., 2010). *B. cenocepacia* positively influences biofilm formation of *P. aeruginosa*, while co-infection of both species in mice increased airway inflammation compared to infection with a single species (Bragonzi et al., 2012a). Addition of *P. aeruginosa* lyophilized cell-free supernatant (CFS) led to increased production of siderophores, lipases and proteases in *B. cenocepacia* cultures, an effect attributed to AHLs present in *P. aeruginosa* CFS (McKenney et al., 1995). This signaling using AHLs was found to be unidirectional, namely only from *P. aeruginosa* to *B. cenocepacia*, and not the other way around (Riedel et al., 2001). Recently, it has also been shown that *S. maltophilia* can respond to exogenous AHLs produced by *P. aeruginosa* and that this promotes swarming motility (Martinez et al., 2015).

Crosstalk between these bacteria seems to be much more mediated by DSFs. Cis-11-methyl-2-dodecenoic acid (DSF) produced by *S. maltophilia* was shown to restore biofilm formation in a BDSF-negative *B. cenocepacia* J2315 mutant (Deng et al., 2010). Likewise, BDSF is able to induce the secretion of MVs by *S. maltophilia*, although to a lesser extent than *S. maltophilia*'s own DSF (Devos et al., 2015). BDSF can be sensed by *P. aeruginosa* and influences expression of QS genes. This causes a decreased production of several QS signals, including 3-oxo-C12-HSL, *Pseudomonas* quinolone signal (PQS) and C4-HSL, resulting in a reduction of biofilm formation and virulence factor production. On top of that, BDSF is also able to inhibit the T3SS of *P. aeruginosa* and attenuates its virulence both *in vitro* and *in vivo* (Deng et al., 2013a). A schematic overview of the different interspecies interactions mediated by QS signals of the DSF family is given in Figure 7.

However, despite all these efforts and recent progress, it remains challenging to determine the true significance and consequences of interspecies interactions in CF infections.

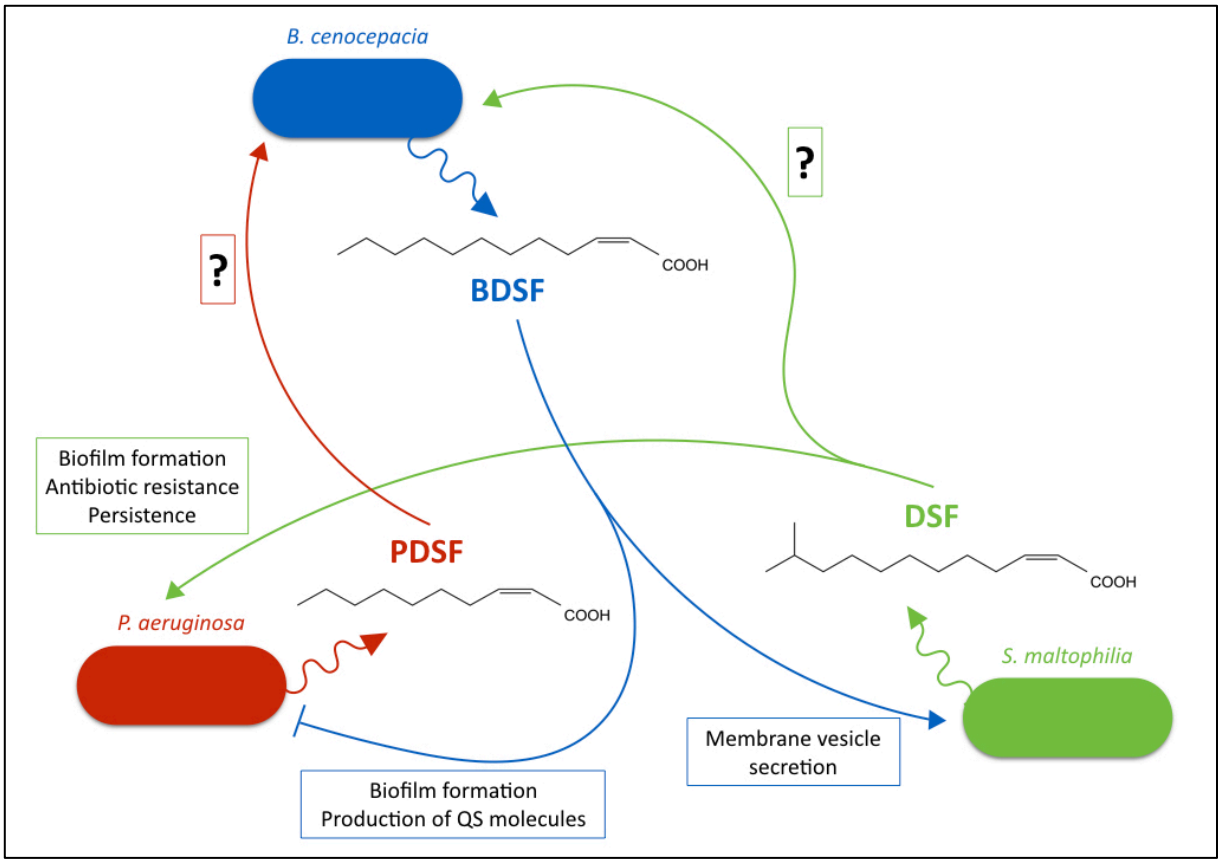



**Figure 7. Interspecies signaling via QS signals of the DSF family.** This overview summarizes the current knowledge regarding the role of DSF family QS molecules in interspecies communication. BDSF = *Burkholderia* diffusible signal factor, DSF = *Stenotrophomonas* diffusible signal factor, PDSF = *Pseudomonas* diffusible signal factor.

## 2.6 Consequences of *Bcc* infection in cystic fibrosis patients

Although *Bcc* bacteria, and most prominently *B. cenocepacia* and *B. multivorans*, have been associated with significant mortality in CF patients (Fauroux et al., 2004), the disease states linked to *Bcc* infection can vary considerably. This ranges from asymptomatic carriage to an accelerated decline in pulmonary function and eventually 'cepacia syndrome', an often fatal deterioration of lung function (Isles et al., 1984). Prolonged antibiotic therapy is only one of the implications for *Bcc*-infected people with CF, but these infections entail also other, far-reaching consequences.

### 2.6.1 Lung transplantation

A major issue associated with *Bcc* infection in CF patients is that there has been long-standing controversy regarding its potential contraindication for lung transplantation, depending on the infecting species (Chaparro et al., 2001; LiPuma, 2001; Murray et al., 2008; Olland et al., 2011; Snell et al., 1993; De Soyza et al., 2010; Webb and Egan, 1997). Lung transplantation is often the final therapeutic option for end-stage lung disease in people with CF. As for today, more and more reports indicate that a successful lung transplant can be achieved, even in patients with active *B.*

*cenocepacia* pneumonia, as long as appropriate post-operative treatments are applied (Chaparro et al., 2001; Salizzoni et al., 2014).

### 2.6.2 Transmission between infected CF patients

Patient-to-patient spread of *Bcc* isolates has been demonstrated in several independent studies (Goering, 1993; Govan et al., 1993; LiPuma et al., 1990; Mahenthiralingam et al., 1996; Pegues et al., 1994a). For example, in most CF centers, over 50% of the patients were long colonized by the same *Bcc* strain, which could not be recovered from environmental samples (LiPuma et al., 1990). Risk factors associated with the transmission of *Bcc* infections include kissing, hugging, dancing, intimate contact, frequent social contact and even sleeping in the same room (Govan et al., 1993; Pegues et al., 1994a). Acquisition of *Bcc* infection during hospitalization can occur through inadequate hand-washing, contamination of respiratory equipment, hand-shaking and cohosting of CF patients (Conly et al., 1986; Döring et al., 1996; Hamill et al., 1995; Pegues et al., 1994b).
All this evidence has led to a segregation policy, in which *Bcc*-positive and *Bcc*-negative CF patients cannot come into close contact with each other (Pegues et al., 1994a; Whiteford et al., 1995). For example, the Belgian Cystic Fibrosis Association prohibits the participation of *Bcc*-carriers in meetings and actions. These measures have dramatically reduced the rate of new colonization, but they also have a severe emotional and psychosocial impact on the lives of *Bcc*-infected patients. Not only do they need to live with the constant fear of infecting noncolonized patients, but they often get isolated from the rest of the CF society through implementation of the infection control measures (Fung et al., 1998). Therefore, a lot of controversy has arisen whether or not this segregation policy should be applied.

### 2.6.3 Influence of *Bcc* infection on the survival of CF patients

Besides the indirect consequences of *Bcc* infection on the quality of life of people with CF, it also directly affects the average lifespan of infected patients. The average life expectancy of patients who live past childhood is about 37 years according to The Cystic Fibrosis Foundation. A study by Jones et al. (2004) revealed a significantly shortened survival for patients with *B. cenocepacia* infection compared to matched patients with *P. aeruginosa* infection. The former group was also characterized by a greater number of hospital visits and required treatment more often (Jones, 2004). Patients infected with *B. cenocepacia* also display a significantly greater reduction in the forced expiratory volume in 1 second (FEV1) (Courtney et al., 2004; McCloskey et al., 1999). Results from these studies stress the important difference in clinical outcome between different *Bcc* species and even among strains from the same species.

# 3 THE ROLE OF BACTERIAL SECRETION SYSTEMS IN THE VIRULENCE OF GRAM-NEGATIVE AIRWAY PATHOGENS ASSOCIATED WITH CYSTIC FIBROSIS

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Running title: Protein secretion by CF pathogens

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Sofie Depluverez wrote the chapters on secretion systems. Simon Devos wrote the chapter on outer membrane vesicles. Bart Devreese edited the manuscript and is the supervisor of the two other authors.

**ABSTRACT**

Cystic fibrosis (CF) is the most common lethal inherited disorder in Caucasians. It is caused by mutation of the CF transmembrane conductance regulator (CFTR) gene. A defect in the CFTR ion channel causes a dramatic change in the composition of the airway surface fluid, leading to a highly viscous mucus layer. In healthy individuals, the majority of bacteria trapped in the mucus layer are removed and destroyed by mucociliary clearance. However, in the lungs of patients with CF, the mucociliary clearance is impaired due to dehydration of the airway surface fluid. As a consequence, patients with CF are highly susceptible to chronic or intermittent pulmonary infections, often causing extensive lung inflammation and damage, accompanied by a decreased life expectancy. This mini review will focus on the different secretion mechanisms used by the major bacterial CF pathogens to release virulence factors, their role in resistance and discusses the potential for therapeutically targeting secretion systems.

## 3.1 Bacterial infections involved in cystic fibrosis (CF) lung disease

The combination of a highly viscous, dehydrated mucus layer, defective mucociliary clearance and a number of yet unknown factors make patients with CF extremely susceptible to infections (LiPuma, 2010). *Pseudomonas aeruginosa* is the most prevalent Gram-negative species, infecting about 50% of all patients. It is detected in 25% of children, but approximately 70% of patients older than 25 years tested positive (Cystic Fibrosis Foundation, 2014). Members of the *Burkholderia cepacia* complex (*Bcc*) cause chronic infections in CF patients, which results in approximately 20% of the cases in fatal 'cepacia syndrome', characterized by necrotizing pneumonia, bacteremia, sepsis and eventually death (LiPuma, 2010). The prevalence of *Bcc* is highest in adults, affecting about 4% of the patients, with *B. cenocepacia* and *B. multivorans* accounting for 70% of the *Bcc* infections. Several reports indicate that the incidence of *Stenotrophomonas maltophilia* in CF patients has increased considerably in recent years (Denton and Kerr, 2002). This opportunistic nosocomial pathogen is mostly recovered from adolescent patients, with a prevalence of ± 15% (Cystic Fibrosis Foundation, 2014; Razvi et al., 2009). Prevalence of *Haemophilus influenzae* is maximal at an age of 2 - 5 years (32%) and decreases thereafter (Cystic Fibrosis Foundation, 2014). *Achromobacter xylosoxidans* is also an emerging CF pathogen with an overall prevalence around 6% (Razvi et al., 2009).
Common to all these species is their dramatic intrinsic or acquired resistance against most of the currently employed antibiotics, making these infections extremely difficult to eradicate. Efflux pumps, biofilm formation, decreased outer membrane permeability and inactivation of β-lactam antibiotics by chromosomally encoded β-lactamases are the main causes of resistance (Hoyle and Costerton, 1991; Waters, 2012).

## 3.2 Virulence factors

Each of the abovementioned species has its own repertoire of virulence factors, specifically adapted to its needs for invasion, colonization, replication and survival in the host (Table 2). Survival of *P. aeruginosa* is supported by the secretion of toxins and proteases, including pyocyanin, exotoxin A, elastase, alkaline phosphatase and phospholipase C (van 't Wout et al., 2015; Lee et al., 2005). Similar strategies are used by *B. cenocepacia* to invade and colonize host cells. Two zinc metalloproteases (ZmpA and ZmpB), phospholipase C, iron-chelating siderophores and cable pili participate in this process (Chung et al., 2003; Corbett et al., 2003; Darling et al., 1998; Sajjan et al., 1995; Uehlinger et al., 2009). Beside the production of a range of extracellular enzymes (lipase, fibrinolysin, hyaluronidase, protease, elastase, etc.), little is known about virulence factors contributing to the pathogenesis of *S. maltophilia* (Bottone et al., 1986). The extracellular capsule, adhesion proteins (HMW1 and HMW2, opacity-associated protein A), pili, haemocin and the IgA1 protease play a crucial role in the onset of the patient's inflammatory response by *H. influenzae* (Kostyanev and Sechanova, 2012; Rosadini, 2011).

## 3.3 The role of bacterial secretion systems in CF pathogenesis and virulence

Bacterial virulence factors are delivered either in the extracellular environment or directly into host cells. Most Gram-negative CF pathogens possess one or more specialized secretion systems to accomplish this task. Eight different secretion systems have been identified (Figure 8). Type I (T1SS), type III (T3SS), type IV (T4SS) and type VI (T6SS) secretion pathways use a single energy-coupled step to transport proteins across both the inner and outer membranes. The outer membrane-spanning type V secretion system (T5SS) and the double membrane-spanning type II secretion system (T2SS) translocate substrates that first have been transported into the periplasm by the Sec or Tat machinery (Costa et al., 2015). Type VII secretion system (T7SS) is restricted to Gram-positive bacteria and will not be discussed here. The type VIII secretion system (T8SS) refers to the curli biogenesis pathway (Chapman, 2002).

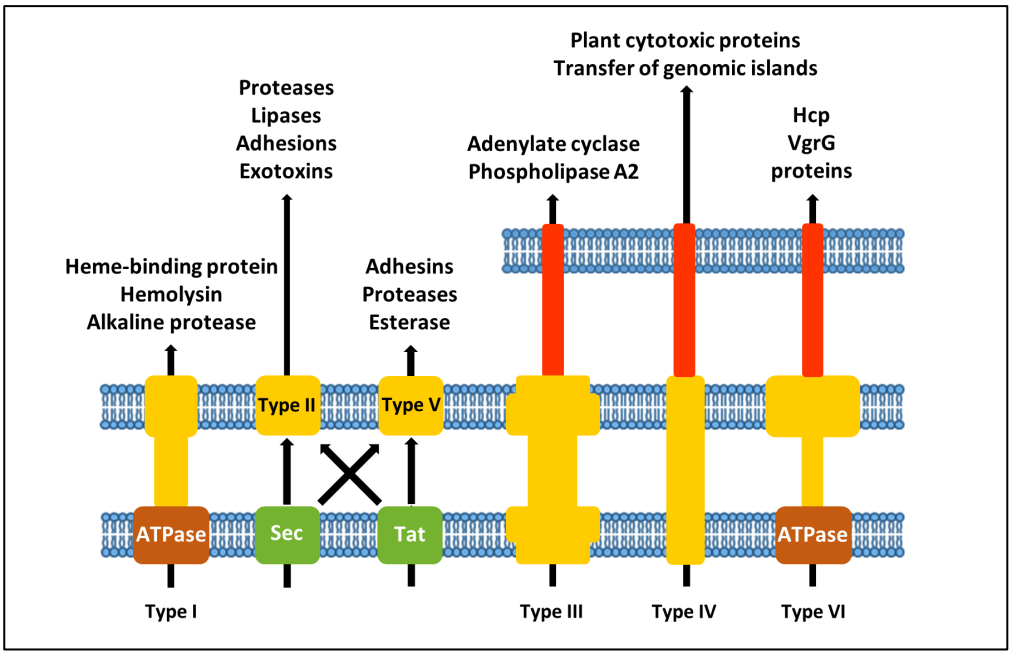


**Figure 8. Schematic overview of the different secretion systems of Gram-negative airway pathogens associated with CF.**

### 3.3.1 Type I secretion system (T1SS)

The type I secretion machinery is composed of an inner membrane associated ATP-binding cassette protein (which recognizes the secretion signal of the substrate), a membrane fusion adapter protein and a TolC-like outer membrane protein (Neidhardt and Curtiss, 1996). Substrate proteins are often very acidic and contain distinctive glycine-rich repeats that bind $Ca^{2+}$ ions (Baumann et al., 1993). Most of the transported proteins also contain repeats with a high degree of homology to adhesion molecules, suggesting a role for T1SS substrates in adherence (Hinsa et al., 2003).

The heme-binding protein HasAp from *P. aeruginosa*, important for iron acquisition, is an example of a protein secreted by T1SS (Létoffé et al., 1998). A second T1SS in *P. aeruginosa* is responsible for the secretion of the alkaline proteases AprA and AprX (Duong et al., 2001; Guzzo et al., 1991). In *B. pseudomallei*, the major haemolysin is exported through a T1SS (Harland et al., 2007). Three T1SS

clusters are present in the genome of *S. maltophilia* (Rocco, 2011), a potential substrate being the virulence-associated membrane protein Ax21 (Ferrer-Navarro et al., 2013).

### 3.3.2 Type II secretion system (T2SS)

The T2SS is important for the secretion of hydrolases. It consists of an outer membrane complex, a periplasmic pseudopilus, an inner membrane platform and a cytoplasmic ATPase. Substrates are transported into the periplasm as unfolded or folded proteins by the SecYEG translocon or the Tat transporter, respectively (Costa et al., 2015). Interaction of the T2SS with its substrates presumably occurs through recognition of a structural motif, rather than a linear secretion signal (Francetić and Pugsley, 2005; Lu and Lory, 1996; Sauvonnet and Pugsley, 1996).
In *P. aeruginosa*, the major extracellular protease LasB is secreted by the T2SS and is responsible for elastin degradation and cleavage of surfactant protein D, an important immune system protein (Alcorn and Wright, 2004; Olson and Ohman, 1992). Staphylolysin LasA, aminopeptidase PaAP and protease IV are other examples of type II secreted proteinolytic enzymes in *P. aeruginosa* (Cahan et al., 2001; Engel et al., 1998; Olson and Ohman, 1992). Another important family of T2SS substrates in this pathogen are lipases, like LipA, LipC, phospholipase C, PlcH, and PlcN, which are targeting the host membrane (Diaz-Laviada et al., 1990; Ostroff et al., 1990). CbpD, a T2SS-dependent chitin-binding protein, could serve as an adhesin, mediating colonization of eukaryotic cells (Folders et al., 2000). The type II secreted exotoxin A is responsible for ADP-ribosylation of elongation factor 2, resulting in protein synthesis inhibition and cell death (Allured et al., 1986). Also the *B. cenocepacia* zinc-dependent metalloproteases, ZmpA and ZmpB, are T2SS substrates (Nakazawa et al., 1996). They cleave antimicrobial peptides involved in innate immunity, like β-defensin-1, cathelicidin LL-37, elafin and secretory leukocyte inhibitor (Kooi and Sokol, 2009). *S. maltophilia* possesses two T2SS, Gsp and Xps (Karaba et al., 2013). The serine proteases StmPr1 and StmPr2 are substrates of the Xps T2SS and mediate degradation of extracellular matrix proteins (DuMont et al., 2015). *H. influenzae* does not contain the genes required to build a functional T2SS (Cianciotto, 2005).

### 3.3.3 Type III secretion system (T3SS)

Bacterial T3SS are nanomachines capable of injecting effector proteins into the cytoplasm or cell membrane of eukaryotic target cells, and are therefore also called injectisomes (Cornelis, 2006). The system consists of a double-membrane-spanning base composed of stacked rings and a needle-shaped filament that extends into the extracellular space (Marlovits et al., 2004). Different translocator proteins are first transported through the needle and inserted into the eukaryotic cell membrane to form a pore of about 2.8 - 3.0 nm (Dacheux et al., 2001; Schoehn et al., 2003). Effectors contain a non-cleavable N-terminal secretion signal and are targeted to the secretion machinery in an unfolded state (Cornelis, 2006).
Known T3SS effectors of *P. aeruginosa* include ExoS and ExoT, both containing a GTPase-activating function and an ADP-ribosyltransferase activity. By acting on the actin cytoskeleton, they are able to protect *P. aeruginosa* from phagocytosis (Barbieri and Sun, 2004). Accumulation of cyclic AMP in host cells is caused by the action of ExoY, an adenylate cyclase (Yahr et al., 1998). ExoU is responsible for acute cytotoxicity and lung tissue damage by its phospholipase A2 activity. Together

with ExoS, it prevents interleukin production by alveolar macrophages and modulates the early inflammatory response (Sato and Frank, 2004). A T3SS mutant of *B. cenocepacia* was attenuated in virulence in a murine model of infection, which indicates a role for the T3SS in evasion of the host immune system (Tomich et al., 2003). Currently, no effectors have been identified for this species. T3SS genes are not present in *S. maltophilia* (Crossman et al., 2008) or *H. influenzae* (Harrison et al., 2005).

### 3.3.4 Type IV secretion system (T4SS)

Like the T3SS, the T4SS is composed of a core complex spanning the inner and outer membrane and a pilus that protrudes into the extracellular environment (Christie et al., 2014). The secretion signals needed for translocation of effector proteins are generally localized at the C-terminus and consist of clusters of hydrophobic or positively charged residues (Alvarez-Martinez and Christie, 2009).
Two T4SSs with different functions are present in *B. cenocepacia*. The first is located on a 92 kb plasmid and is responsible for secretion of plant cytotoxic proteins. It also plays a role in the intracellular survival of *B. cenocepacia* in phagocytes. This plasmid is not present in all *B. cenocepacia* strains, but is found in ET-12 lineage strains including K56-2, BC7 and J2315. The second T4SS is chromosomally encoded and might be involved in plasmid mobilization, although the exact function is still unknown (Zhang et al., 2009). T4SS effectors of *Xanthomonas citri*, a close relative of *S. maltophilia*, have the capability of killing other bacterial species, thereby conferring a selective growth advantage in mixed bacterial communities (Souza et al., 2015). Whether the T4SS of *S. maltophilia* has a similar function, remains unknown. *H. influenzae* and *P. aeruginosa* do not contain a conventional T4SS.
A unique feature of the T4SS is that it can also transport nucleic acids. *P. aeruginosa* and *H. influenzae* possess one or more genomic island-associated T4SSs (GI-T4SSs) that play a crucial role in horizontal gene transfer (HGT) of integrative and conjugative elements (ICEs) (Juhas et al., 2007a). ICEs not only contain genes required for excision/integration and various accessory genes, but they often also harbor a T4SS, which completes the machinery for efficient transfer from donor to recipient cell (Guglielmini et al., 2011; Juhas et al., 2008; Wozniak and Waldor, 2010). A considerable part of the accessory genes are involved in antibiotic resistance or virulence. ICEHin1056 of *H. influenzae* carries ampicillin, tetracycline and chloramphenicol resistance genes (Juhas et al., 2007b), while PAPI-1 of *P. aeruginosa* encodes CupD type fimbriae essential for attachment and the PvrSR/RcsCB regulatory system involved in biofilm formation and antibiotic resistance (Mikkelsen et al., 2013). The chromosomally encoded T4SS of *B. cenocepacia* was also linked to plasmid mobilization (Zhang et al., 2009). Taken together, these mechanisms of HGT pose a major threat to our ability to combat infections occurring in CF patients by potentially transforming the lung microbiota into an antibiotic resistant community.

### 3.3.5 Type V secretion system (T5SS)

The T5SS is a single-membrane-spanning system that secretes virulence factors and mediates cell-to-cell adhesion and biofilm formation. The substrates are fused to their secretion pore to form a single polypeptide, also known as autotransporter. Unfolded autotransporters are delivered to the

periplasm via the SecYEG translocon. The exoproteins either remain associated with the outer membrane or are released in the extracellular environment after proteolytic cleavage (Leo et al., 2012). In a second type of T5SS, two-partner secretion (TPS), the substrate or passenger domain and the pore-forming domain are two separate proteins.
There is only one known autotransporter in *P. aeruginosa*, i.e. EstA. It can hydrolyze glycerol esters through its esterase activity and is involved in the production of rhamnolipids, cell motility and biofilm formation (Wilhelm et al., 2007). Three TPS systems have been characterized in *P. aeruginosa*: the LepA/LepB system, in which LepA is a protease activating NF-κB through digestion of PAR receptors (Kida et al., 2008), the CupB system, involved in the assembly of CupB fimbriae (Ruer et al., 2008) and the PdtA/PdtB system, where PdtA is related to High Molecular Weight (HMW) adhesins (Faure et al., 2014). The genome of *B. cenocepacia* J2315 contains four T5SS, two of them contain pertactin domains involved in adhesion, and the other two contain haemagglutinin repeats (Holden et al., 2009). Haemagglutinin autotransporters are also present in *S. maltophilia* (Ryan et al., 2009b). The HMW1 and HMW2 from *H. influenzae* are also TPS systems. The *H. influenzae* Hap, Hia and Hsf autotransporters mediate bacterial aggregation and microcolony formation and promote adherence to epithelial cells and extracellular matrix proteins (Fink et al., 2003; Spahich, 2011). Another T5SS substrate is the IgA protease, responsible for degradation of the major mucosal immunoglobulin (Fernaays, 2008).

### 3.3.6 Type VI secretion system (T6SS)

The type VI secretion machinery consists of a membrane complex and a tail complex, composed of structural elements that are equivalent to contractile phage tails (Basler et al., 2012). Although the T6SS plays a major role in the pathogenesis towards eukaryotic cells, it can also be used to target other bacteria in polymicrobial infections (Ho et al., 2014). Three T6SS are present in *P. aeruginosa*, but only two major substrates have been identified so far, Hcp and VgrGs. Hcp is believed to form nanotubes on the bacterial surface, which may allow transport of other T6SS effectors (Ballister et al., 2008). VgrGs could form trimeric complexes puncturing membranes allowing the passage of other proteins (Leiman et al., 2009). The *B. cenocepacia* T6SS modulates actin cytoskeleton dynamics and NADPH oxidase complex assembly, also through the action of Hcp and VgrGs (Pukatzki et al., 2007). *S. maltophilia* and *H. influenzae* do not contain T6SS genes.

**Table 2. Overview of the major virulence factors associated with the outer membrane or secreted by CF pathogens.**

| | ***Pseudomonas aeruginosa*** | ***Burkholderia cenocepacia*** | ***Stenotrophomonas maltophilia*** | ***Haemophilus influenza*** |
|---|---|---|---|---|
| Proteases | LasB[2], AprA[1], Staphylolysin LasA[2], aminopeptidase PaAP[2], protease IV[2], LepA[5], elastase[2] | ZmpA[2], ZmpB[2], MprA[2] | StmPr1[2], StmPr2[2], elastase[2] | IgA1 protease[5] |
| Lipases | LipA[2], LipC[2], phospholipase C[2], PlcH[2], PlcN[2], ExoU[3] | Phospholipase C | Lipase | / |
| Toxins | Pyocyanin, exotoxin A[2], Cif | Hemolysin | Zonula occludens toxin | / |
| Adhesion molecules | Chitin-binding protein CbpD[2], pili, ExoS[3], ExoT[3], alginate, fimbriae, flagellin | Cable pili, flagellin, fimbriae | Flagellin, fimbriae | HMW1[5], HMW2[5], pili, Hap[5], Hia[5], Hsf[5], opacity-associated protein A |
| Hydrolytic enzymes | Alkaline phosphatase, EstA[5] | Chitinase | Fibrinolysin, chitinase, hyaluronidase, DNase | Haemocin |

*Virulence factors of Achromobacter xylosoxidans have not been characterized yet. Indices indicate a known association with a certain secretion system (number corresponds to the type of secretion system).*

## 3.4 Membrane vesicles

Secretion of membrane vesicles (MVs) by both Gram-negative and Gram-positive bacteria is now considered as a true secretion system. The membranous nanoparticles are pinched off from the cell surface and carry membrane-associated and soluble proteins, nucleotides, and other molecules into the extracellular environment. MVs are involved in a series of biological functions, including nutrient acquisition, iron scavenging, antibiotic resistance and biofilm formation (Haurat et al., 2015).

MVs contribute to pathogenesis by delivering virulence factors and/or through modulation of the host immune system (Schwechheimer and Kuehn, 2015). *P. aeruginosa* MVs enable long-distance delivery of multiple virulence factors including alkaline phosphatase, hemolytic phospholipase C and Cif, a toxin that inhibits CFTR-mediated chloride secretion in the airways (Bomberger et al., 2009). Cif also enhances ubiquitination and subsequent degradation of the transporter associated with antigen processing (TAP), reducing MHC class I activation (Bomberger et al., 2014). Secretion of MV-associated hydrolases like (metallo)proteases, (phospho)lipases and peptidoglycan-degrading enzymes was also shown in *B. cenocepacia* (Allan et al., 2003). The *H. influenzae* MVs activate B-cells in a T-cell independent manner, possibly creating a diversion on the adaptive immune system and promoting survival within the host (Deknuydt et al., 2014).

Several studies highlighted the importance of MVs in antibiotic resistance. Exposure of *S. maltophilia* cells to β-lactam antibiotics led to a significant increase in MVs that are packed with β-lactamases (Devos et al., 2015). These MVs are capable of degrading β-lactams extracellularly, and even increase the β-lactam tolerance of the species *P. aeruginosa* and *B. cenocepacia* (Devos et al., 2016). Furthermore, β-lactamases were found in MVs of *P. aeruginosa* and *H. influenzae*, indicative for a general mechanism to respond to β-lactam stress (Ciofu et al., 2000; Schaar et al., 2011). MVs can also mediate export of antibiotics or extracellular capturing of antibiotics. When *P. aeruginosa* is

treated with the aminoglycoside gentamycin, it secretes gentamycin-containing MVs. These MVs also contain peptidoglycan hydrolase and were shown bactericidal against *B. cenocepacia* (Allan and Beveridge, 2003). Finally, MVs can aid in the inter- and intra-species spread of resistance genes (Schwechheimer and Kuehn, 2015).

## 3.5 Secretion systems as targets for anti-infective drugs

Development of novel therapies is crucial to manage the spread and impact of these pathogens on CF patients. Classical antibiotics mostly exert their function by inhibiting the growth of bacteria through interference with cell wall biogenesis, DNA replication, transcription and protein synthesis (Baron and Coombes, 2007). Unfortunately, the rate at which resistance against these traditional antibiotics emerges is alarming, partly due to the rise of mutations in the genes coding for antibiotic targets. Secretion system inhibitors are a novel class of anti-infectives that do not inhibit bacterial growth *per se* and are therefore likely to reduce, under certain circumstances, the rate at which resistance-causing mutations arise (Allen et al., 2014; Clatworthy et al., 2007; Rasko and Sperandio, 2010). Another advantage is the fairly high degree of conservation of these systems between a whole range of Gram-negative pathogens. Since secreted effectors often play a major role in immune evasion, targeting these important bacterial virulence mechanisms may restore pathogen clearance by the host's own immune system.
Kauppi et al. found that a family of acylated hydrazones of different salicylaldehydes can inhibit the T3SS at the level of substrate secretion/translocation (Kauppi et al., 2003). The related halogenated salicylaldehydes are capable of inhibiting the transcription of genes encoding T3SS components (Kenny et al., 1997). Thiazolidinones were found to target the formation or assembly of the T3SS needle apparatus. These compounds could also inhibit the T2SS in *Pseudomonas* and the type IV pili secretion system of *Francisella*, therefore it is hypothesized that they might act on the conserved outer membrane secretin (Felise et al., 2008; Kline et al., 2009). Other promising targets are the energy-generating ATPases of T2SS and T4SS (Sayer et al., 2014), the accessory lytic transglycosylases of T2SS, T3SS and T4SS (Koraimann, 2003) and the translocated effector proteins (Coburn et al., 2007; Figueira et al., 2013; Kidwai et al., 2013). By inhibiting T4SS-dependent secretion, horizontal transfer of antibiotic resistance genes could be reduced.

## 3.6 Concluding remarks

With as many as 90% of CF patients dying of fatal lung infections every year, it is crucial to find means to eradicate or at least control the growth and spread of these major CF pathogens. Secretion systems provide a useful target, since their effector proteins are responsible for a wealth of host cell compromising actions. Due to the fairly high degree of conservation in the composition of these secretion systems, an inhibitor has the potential to target a whole array of Gram-negative pathogens. Because the growth of the pathogens is unaffected by such compounds, the risk for resistance development is highly reduced. It is therefore essential to keep investing in the identification of novel effector proteins and structural elements of secretion systems, as well as in ways to block secretion of virulence factors and membrane vesicles.

# 4 TYPE IV SECRETION IN BURKHOLDERIA CENOCEPACIA K56-2

## 4.1 Versatile functions of T4SSs

Among the different secretion systems present in various bacteria, the T4SSs resemble bacterial conjugation systems. They are used for two main purposes during pathogenesis: the delivery of effector proteins to host cells and the exchange of genetic material with a phylogenetically diverse range of target cells, including bacterial, fungal, plant and human cells (Bates et al., 1998; Bundock et al., 1995; Cascales and Christie, 2003; Grohmann et al., 2003; Waters, 2001; Zhu et al., 2000). The transfer of effector proteins is quite similar to that of T3SSs or 'injectisomes' (Blocker et al., 2003) and is indispensable during the infection process of several plant and mammalian pathogens (Boschiroli et al., 2002; Burns, 2003; Censini et al., 1996; Schulein and Dehio, 2002; Seubert et al., 2003; Vogel, 1998; Zhu et al., 2000). T4SS activity usually requires direct contact with the eukaryotic host cell. Interactions between effectors and host molecules can suppress host defense mechanisms, facilitate intracellular growth and subvert nutrient synthesis towards the benefit of the infecting pathogen (Cascales and Christie, 2003). Genetic exchange is a crucial mechanism that enables pathogens to adapt to the constantly changing and challenging environments encountered during invasion of the host, since it contributes to genome plasticity. It is also very relevant in the clinical setting, where the transferred genetic material often contains antibiotic resistance genes, causing the emergence of multidrug resistance among clinical pathogens (De la Cruz and Davies, 2000). Based on their specific function, T4SSs are subdivided into four different classes: (i) effector translocation into host target cells, (ii) conjugation of chromosomal and plasmid DNA, (iii) DNA uptake and transformation, and (iv) DNA release into the extracellular milieu (Backert and Meyer, 2006).

## 4.2 Structural prototypes of T4SSs

Because of the sometimes overlapping functions of T4SSs, it is not always feasible to maintain a purely functional classification (Alvarez-Martinez and Christie, 2009). Therefore, a simpler grouping system was proposed, based on the structural properties of the different T4SSs. This way, most T4SSs can be divided into either a type IVa secretion system (T4SSa), exemplified by the *Agrobacterium tumefaciens* VirB/D4 T4SS, or a type IVb secretion system (T4SSb), with the *Legionella pneumophila* Dot/Icm T4SS as a prototype (Bhatty et al., 2013; Christie et al., 2005).

### 4.2.1 The VirB/D4 T4SS from *A. tumefaciens*

A schematic overview of the architecture of the T4SSa of *A. tumefaciens* is given in Figure 9. The fully functional T4SSa is composed of (i) the type IV coupling protein (T4CP) VirD4, (ii) the secretion channel consisting of energizing components (VirB4 and VirB11), transmembrane components (VirB3, VirB6, and VirB8 in the inner membrane (IM), and VirB7, VirB9, and VirB10 in the outer membrane (OM)) and the VirB1 hydrolase, and (iii) the pilus formed by VirB2 and VirB5 (Fronzes et al., 2009a, 2009b; Kerr and Christie, 2010; Low et al., 2014; Schröder and Lanka, 2005). The T4CP

VirD4 is an ATPase that targets the relaxosome-processed substrate to the T4SS machinery. This protein senses both intracellular (substrate docking) and extracellular (pilus-mediated host-pathogen interaction) signals and directs the gating of the T4SS translocation channel (Berry and Christie, 2011). Two additional ATPases, VirB4 and VirB11, are required to energize and coordinate substrate recruitment and movement through the translocation channel, as well as pilus biogenesis (Atmakuri et al., 2004; Ripoll-Rozada et al., 2013). VirB3 is an inner membrane protein whose function is still unknown. It is stabilized by VirB6, another essential integral membrane protein that plays an important role in the assembly of the T4SS (Hapfelmeier et al., 2000; Jakubowski et al., 2004). The essential VirB8 protein serves as the nucleating factor that recruits other T4SS components to the cellular poles for assembly (Kumar et al., 2000). VirB7 and VirB9 form a strong complex in the outer membrane, whereas VirB10 interacts with the VirB7/B9 complex, the IM T4SS components and the ATPases, and provides the central scaffold of the T4SS machinery (Baron et al., 1997; Beaupré et al., 1997; Das and Xie, 2000; Spudich et al., 1996). VirB9 is involved in the regulation of substrate selection and T-pilus biogenesis (Jakubowski et al., 2005). The lytic transglycosylase VirB1 displays muramidase activity that aids in the breakdown of structural restraints imposed by the peptidoglycan layer, which facilitates the T4SS assembly across the cell envelope (Fullner et al., 1996). VirB2 is the major component of the pilus, which is essential for substrate transport. The minor pilus component VirB5 is thought to play a role in pilus elongation, as it is required for the incorporation of VirB2 into the pilus (Lai and Kado, 1998; Schmidt-Eisenlohr et al., 1999). The latter two proteins might also function as specialized adhesins through binding to specific host cell receptors, thereby mediating host cell targeting (Backert et al., 2008).

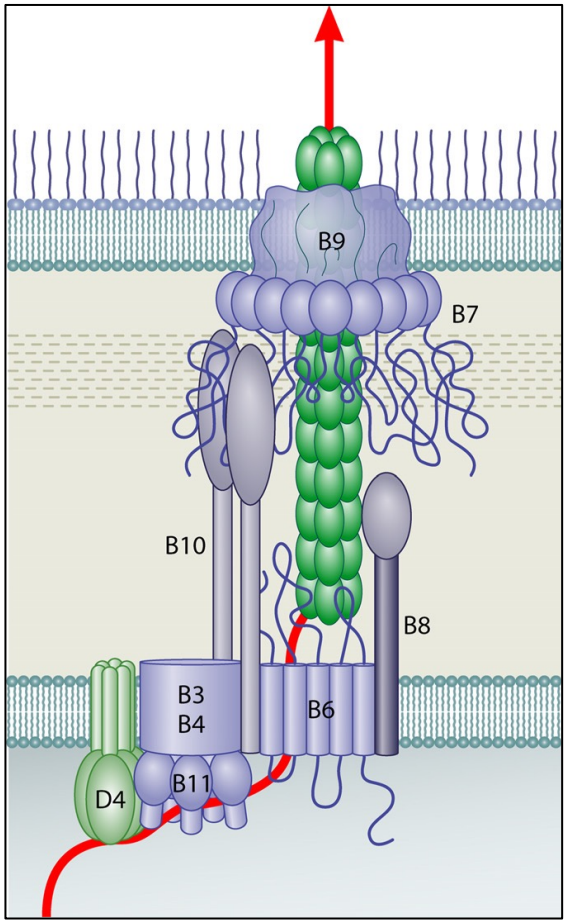


**Figure 9. Substrate translocation pathway and architecture of the VirB/D4 T4SS core complex of *A. tumefaciens*.** The substrate translocation pathway (red arrow) was developed for the *A. tumefaciens* VirB/VirD4 system on the basis of DNA-channel subunit contacts identified by formaldehyde cross-linking (Cascales, 2004). The DNA substrate cross-links with VirD4, VirB11, VirB6, VirB8, VirB2, and VirB9. These subunits are postulated to comprise the secretion channel, whereas other components, including VirB3, VirB4, and VirB10, promote channel assembly as protein scaffolds or through ATP-mediated conformational changes. Picture reprinted from Alvarez-Martinez and Christie (2009).

### 4.2.2 The Dot/Icm T4SS from *L. pneumophila*

Typically, T4SSb are more complex than T4SSa, where the Dot/Icm system consists of approximately 27 components, compared to 12 for the VirB/D4 system (Ghosal et al., 2017; Nagai and Kubori, 2011). Several components of the Dot/Icm T4SS are homologous to T4SSa proteins: the ATPases DotB, DotL and DotO were shown to have homology with VirB11, VirD4 and VirB4, respectively, and DotG demonstrates clear sequence similarity to VirB10 (Vogel, 1998; Yeo and Waksman, 2004). VirB9 and VirB7 also have homologs in the *L. pneumophila* T4SS, respectively DotH and DotC (Lawley et al., 2003). The prototypic structure of the Dot/Icm T4SS of *L. pneumophila* is shown in Figure 10. The T4SSb is composed of a T4CP subcomplex and a core transmembrane subcomplex (Vincent et al., 2006, 2012). The former consists of DotL, DotM, DotN, and the heterodimer pair IcmS/IcmW and has been shown to bind substrates in the cytoplasm and deliver them to the T4SS apparatus (Fronzes et al., 2009a; Vincent et al., 2012). DotL functions as the T4CP that recognizes substrates in the cytoplasm and interacts with IcmSW for the specific translocation of IcmSW-dependent substrates (Buscher et al., 2005; Sutherland et al., 2012). The transmembrane core complex of the Dot/Icm T4SS is composed of DotC, DotD, DotF, DotG and DotH and bridges both the inner and the outer membrane. It is functionally analogous to the *A. tumefaciens* VirB7-B10 subcomplex (Vincent et al., 2006). DotU and IcmF are accessory factors that stabilize the secretion machinery (Sexton et al., 2004a), while DotB is responsible for ATP hydrolysis, similar to its analog VirB11 (Sexton et al., 2004b). The DotO protein might be involved in the formation of a pore, potentially facilitating lysis of the infected macrophage (Kirby et al., 1998; Watarai et al., 2001). DotK is a putative lipoprotein, but has no effect on the intracellular growth in human macrophages (Yerushalmi et al., 2005). DotJ, DotV, DotE, DotA, DotI, DotP, IcmT and IcmV are located in the inner membrane, but the exact function of these proteins remains to be elucidated (Vincent et al., 2006). The cytoplasmic IcmQ protein is bound by its chaperone IcmR and is able to form pores in membranes (Duménil et al., 2004; Duménil and Isberg, 2001). IcmX is the only periplasmic Dot/Icm protein and is essential for the establishment of a replicative organelle in eukaryotic host cells (Matthews and Roy, 2000).

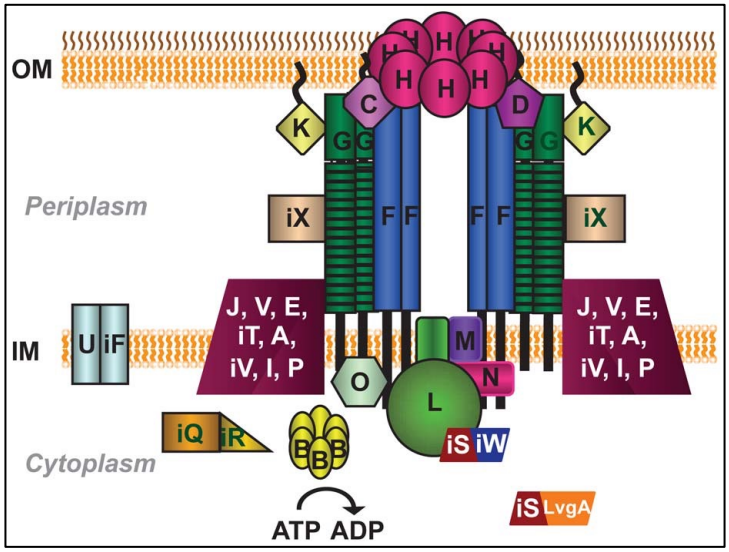


**Figure 10. Schematic overview of the *L. pneumophila* Dot/Icm T4SS.** The 27 proteins of the T4SS are shown at their predicted or experimentally determined subcellular locations in the outer membrane (OM), periplasm, inner membrane (IM) and cytoplasm. Dot proteins are labeled with the last letter of their name. Icm proteins are designated in the same manner, but are prefaced with an 'i'. Picture reprinted from Sutherland et al. (2013).

## 4.3 The T4SSs of *B. cenocepacia* K56-2

Two different type IVa T4SSs have been identified in *B. cenocepacia* K56-2. The first T4SS (T4SS-1) is located on the 92-kb plasmid and is required for causing the plant tissue watersoaking (ptw) phenotype on onion tissue (Engledow et al., 2004). The second, chromosomally encoded T4SS (T4SS-2) with complete homology to the VirB/D4 system of *A. tumefaciens*, is involved in plasmid mobilization (Zhang et al., 2009). Since both T4SSs have a lower G+C content compared to the rest of the genome, it is likely that they have been acquired through horizontal gene transfer (Engledow et al., 2004). Both T4SS gene clusters are also present in other *B. cenocepacia* strains (ST32 isolate 1232, HI2424, J2315 and BC7), although strains lacking the megaplasmid accordingly lack the T4SS-1 (e.g. *B. cenocepacia* strains H111, AU1054 and MC0-3). However, the distribution of plasmid- and genome-encoded T4SSs across other members of the *Bcc* is currently unknown.

### 4.3.1 The plasmid-encoded T4SS-1

The T4SS-1 located on the 92-kb plasmid was first identified during random transposon mutagenesis of *B. cenocepacia* followed by screening for the ptw phenotype using the plant tissue watersoaking assay (Engledow et al., 2004). The *ptw* gene cluster (Figure 11) displays homology to the *A. tumefaciens* VirB/D4 T4SS: PtwD4 (TraD), PtwB4 (TraB), PtwB5 (TraC), PtwB7 (TraN), PtwB8 (TraE), PtwB9 (TraO), PtwB10 (TraF) and PtwB11 (TraG) correspond to respectively VirD4, VirB4, VirB5, VirB7, VirB8, VirB9, VirB10 and VirB11. Since the Tra nomenclature is often used to designate the components of the *B. cenocepacia* T4SS-1, the corresponding names are given between brackets. They originate from the homology between the *A. tumefaciens* VirB/D4 system and the T4SS encoded by the *E. coli* conjugative plasmid pKM101 (Gordon and Christie, 2014). PtwC might function as a relaxosome component, based on its homology with TrwC of the R388 plasmid (Llosa et al., 1994). PtwN and PtwU have no VirB/D homologs, but they are related to respectively TraN, an adhesin, and TraU, a protein involved in mating pair stabilization (Lawley et al., 2003). The other Ptw proteins do not possess homology with Vir or Tra proteins. The factor responsible for the ptw phenotype was found to be a heat-stable protein, although the exact identity is not known yet (Engledow et al., 2004).

The T4SS-1 was also shown to contribute to the intracellular survival and replication of *B. cenocepacia* in both professional and non-professional phagocytes. Data suggest that it may allow intracellular bacteria to inhibit the fusion of bacterium-containing autophagosomes with lysosomes (Sajjan et al., 2008a). However, the effector proteins responsible for this inhibition still need to be discovered.

Recently, a study by Fernández-González et al. (2016) suggested that the putative relaxase PtwC can act *in cis* on the *oriT* of its own plasmid. This implies that the plasmid-encoded T4SS-1 might play a dual role in both intracellular survival and horizontal DNA transfer (Fernández-González et al., 2016).

Furthermore, T4SS-1 genes are heavily upregulated in cells isolated *in vivo* (rat lungs) compared to *in vitro* grown cells (O'Grady and Sokol, 2011), suggesting an important role for this system during host infection. This is further exemplified by the observation that T4SS-1 defective mutants fail to induce IL-1β in macrophages (Rosales-Reyes et al., 2012a).

### 4.3.2 The chromosomally-encoded T4SS-2

The second T4SS of *B. cenocepacia* resides on chromosome 2 and again shows a high degree of modular similarity to the *A. tumefaciens* VirB/D4 T4SS (Engledow et al., 2004) (Figure 11). This T4SS-2 is dispensable for ptw effector(s) secretion, but appears to be involved in plasmid mobilization, illustrated by the abolished mobilization of plasmid pML122Tc in a *bc-virD4* mutant of *B. cenocepacia* K56-2 (Zhang et al., 2009). Secretion of IL-1β is not affected by the T4SS-2, and expression of the chromosome 2-encoded T4SS genes is similar in the *in vitro* and *in vivo* conditions (O'Grady and Sokol, 2011; Rosales-Reyes et al., 2012a). Since inactivation of *ptwD4* did not completely abolish the ability of the mutant to persist intracellularly, it might be a possibility that components of the chromosomally encoded T4SS-2 may compensate for the deficiency of the plasmid-encoded T4SS-1 (Sajjan et al., 2008a). In *L. pneumophila*, components of the Dot/Icm and Lvh (*Legionella* virulence homolog) T4SSs can interact with each other, with *lvh* gene products capable of substituting some components of the Dot/Icm system for conjugation, but not for intracellular growth (Segal et al., 1999). However, it is currently unknown whether a similar compensation between the Ptw T4SS and the bc-VirB/D4 T4SS of *B. cenocepacia* can occur (Zhang et al., 2009).

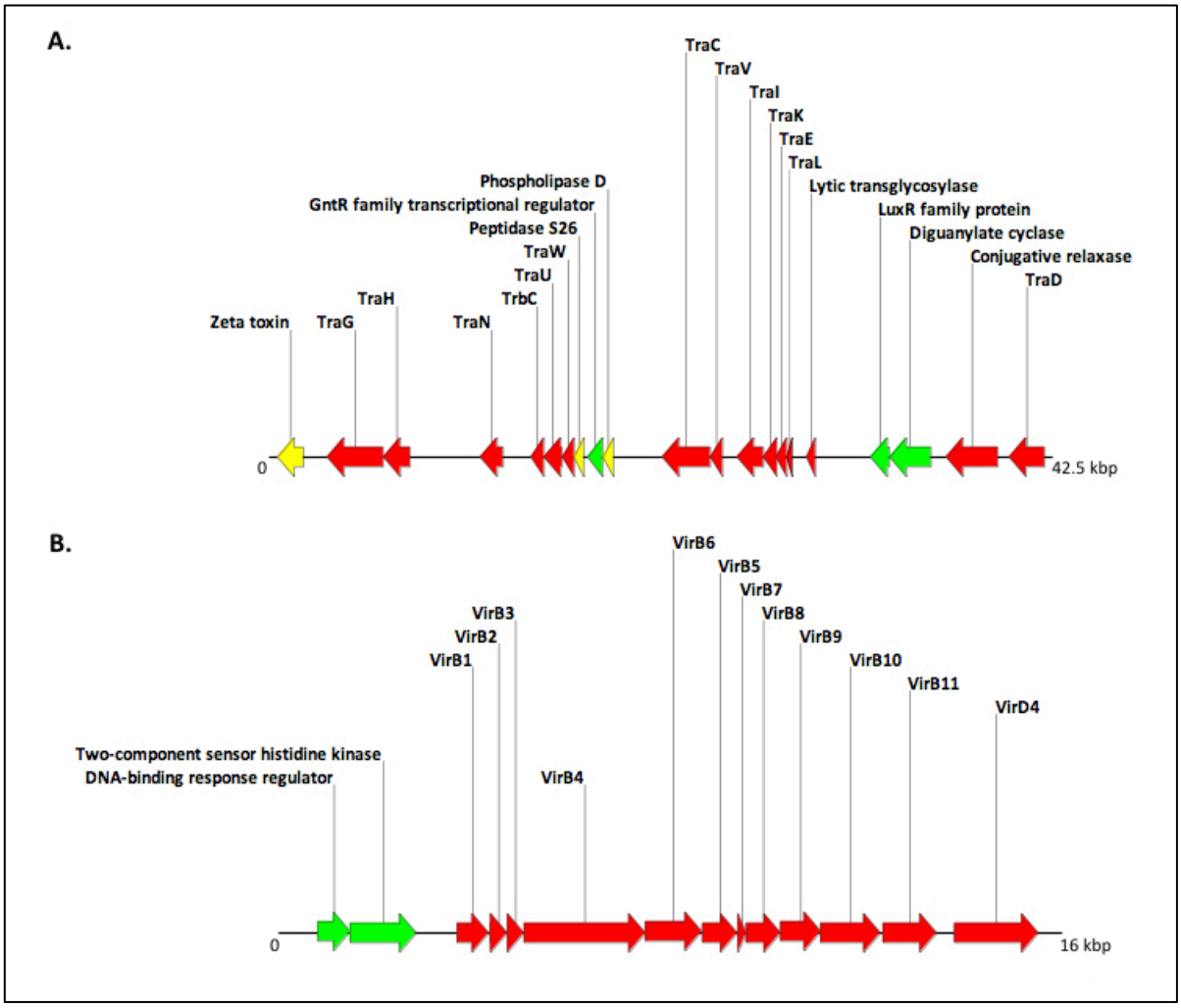


**Figure 11. Schematic representation of the two T4SS clusters of *B. cenocepacia* K56-2.** **A.** T4SS-1 cluster located on the 92-kb plasmid. The corresponding gene names in *B. cenocepacia* J2315 are pBCA017 (Zeta toxin), pBCA020 (TraG), pBCA021 (TraH), pBCA027 (TraN), pBCA030 (TrbC), pBCA031 (TraU), pBCA032 (TraW), pBCA033 (Peptidase S26), pBCA035 (GntR family transcriptional regulator), pBCA036 (Pospholipase D), pBCA041 (TraC), pBCA042 (TraV), pBCA044 (TraI), pBCA045 (TraK), pBCA046 (TraE), pBCA047 (TraL), pBCA049 (Lytic transglycosylase), pBCA054 (LuxR family protein), pBCA055 (Diguanylate cyclase), pBCA057 (Conjugative relaxase) and pBCA059 (TraD). **B.** T4SS-2 cluster located on chromosome II. The corresponding gene names in *B. cenocepacia* J2315 are BCAM0322 (DNA-binding response regulator), BCAM0323 (Two-component sensor histidine kinase), BCAM0324 (VirB1), BCAM0325 (VirB2), BCAM0326 (VirB3), BCAM0327 (VirB4), BCAM0328 (VirB6), BCAM0329 (VirB5), BCAM0330 (VirB7), BCAM0331 (VirB8), BCAM0332 (VirB9), BCAM0333 (VirB10) and BCAM0334 (VirB11). The VirD4 protein of *B. cenocepacia* K56-2 is homologous to the TraD protein of *B. cenocepacia* J2315, encoded by the pBCA059 gene. Protein names are listed above each gene. Structural genes are depicted in red, potential regulatory genes in green and accessory genes in yellow.

# *CHAPTER II: RESEARCH OBJECTIVES*

The overall original aim of this work was to characterize the role of the T3SS, the T4SS-1 and QS signaling in the virulence of the opportunistic Gram-negative pathogen *B. cenocepacia* K56-2 using different proteomic strategies. Both the T3SS and the T4SS-1 are capable of injecting effector proteins directly into host cells. A T3SS mutant shows an attenuated virulence in a murine model of infection, while the T4SS-1 is required for the intracellular survival and replication of this pathogen in macrophages. Therefore, identification of novel T3SS and T4SS-1 effector proteins will increase our understanding of pathogenesis and might eventually aid in the development of new antimicrobial agents directed against *B. cenocepacia* infection.

Currently, microbial proteomics is mainly employing untargeted approaches involving bottom up analyses of tryptic digests using either differential isotopic labelling or label-free mass spectrometric analysis. While these methods certainly provide insights into overall perturbations in the proteome, they sometimes lack sensitivity or accuracy to provide accurate data on the abundance of key proteins in the biological process under study. Therefore, we developed and applied targeted approaches using quantitative selected reaction monitoring (SRM) to quantify a selected panel of secreted virulence factors, focusing on the T3SS and T4SS-1 system.

A label-free proteomics-based secretome analysis of a T3SS mutant strain compared to the wild type was therefore conducted. However, under the conditions tested, no plausible T3SS effector proteins could be identified. Therefore, this part of the project was not further exploited.

In order to identify T4SS-1 effectors, the secretome of a *B. cenocepacia* K56-2 mutant strain with a deletion of the complete T4SS-1 gene cluster (ΔpBCA017-059) is compared to the wild type by label-free proteomics for both exponential and stationary phase cultures. Candidate effector proteins are validated using SRM. A targeted proteomics-based comparison of exponential and stationary phase cultures of wild type and mutants will potentially reveal growth phase-dependent differences. The unexpected absence of T6SS effectors in the secretome of the ΔpBCA017-059 mutant directed the research towards an investigation of the genes responsible for this effect. Therefore, two new mutant strains were obtained: one with a shorter deletion of the T4SS-1, ΔpBCA017-033, and one in which a potential regulatory operon linked to QS was deleted, ΔpBCA054-055. Again, label-free proteomics and targeted proteomics are used to characterize potential differences in the secretome between wild type and mutants. Given the potential link with QS, biofilm assays and biofilm microscopy are performed to quantify possible differences in biofilm mass and/or morphology.

The experiments regarding the role of the pBCA054-055 operon potentially involved in QS, directed the research towards an investigation of the sensitivity and responsiveness of *B. cenocepacia* towards different QS molecules. Since already much work had been done on the effects of acylhomoserine lactones on *B. cenocepacia* and addition of C8-HSL did not cause significant changes in the secretome, we mainly focused on the effect of the diffusible fatty acid signal molecules (DSFs).

The role of DSFs in the virulence of *B. cenocepacia* was already demonstrated by several enzymatic assays, but no comprehensive proteomic analysis was performed to obtain a detailed picture of the effect of DSFs on the secretome of this pathogen. Therefore, label-free and targeted proteomics are used to further elucidate the effect of stimulation of exponential-phase *B. cenocepacia* K56-2 cultures with various concentrations of BDSF over different periods of time. To determine the

physiological concentrations of BDSF produced during growth of *B. cenocepacia*, quantification of endogenous levels of BDSF using HPLC is also described. Furthermore, in the framework of interspecies crosstalk, this study also includes an investigation of the use of DSFs as a bacterial communication tool, by monitoring the influence of DSFs from frequently co-isolated CF pathogens on the secretome of *B. cenocepacia* via an optimized SRM method allowing the quantification of selected virulence factors. Again, the effect of the different QS molecules on the ability of *B. cenocepacia* to form biofilm is assessed.

Since crosstalk between CF pathogens probably not only occurs via QS, the effect of (concentrated) cell-free supernatant with and without quorum sensors on *B. cenocepacia* K56-2 is evaluated. Since the concentration procedure involves ultrafiltration using a 10 kDa molecular weight cut-off filter (MWCO), low molecular weight compounds, like QS molecules, are removed during this step and hence the observed effects are QS-independent. Some preliminary tests using *B. cenocepacia*, *S. maltophilia* and *P. aeruginosa* dual and triple species co-cultures are also performed during this research.

Globally, the aim of this thesis is to obtain a broader picture on the distinct virulence-associated properties of this opportunistic pathogen and the different and often complex ways in which they are regulated. Targeted proteomics hereby provides a powerful tool to monitor the effects of a whole array of different conditions on the expression/secretion of selected proteins, in our case the secretion of the main virulence factors of *B. cenocepacia*. It is an effective and reliable method to obtain quantitative info on any protein of interest. Protein secretion constitutes the first line of attack and defense of a pathogen during the infection process. Studying the secretome therefore provides a means for identifying and/or characterizing the prominent factors involved in this process and can assist in the development of novel therapeutics to combat the often life-treathening infections caused by this pathogen.

# *CHAPTER III: EXPERIMENTAL WORK*

# 5 PROTEOMICS ASSESSMENT OF THE ROLE OF THE 92-KB PLASMID FROM BURKHOLDERIA CENOCEPACIA K56-2

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**AUTHOR CONTRIBUTIONS**

Sofie Depluverez wrote the entire chapter and performed all the experiments, except for the analysis of biofilm morphology and viability, which were performed by Jasper Wille. Generation of mutants was performed at the laboratory of Prof. Dr. M. Valvano. Bart Devreese edited the chapter and is the supervisor of the author.

**ABSTRACT**

*Burkholderia cenocepacia* K56-2 possesses a 92-kb megaplasmid which encodes a.o. a type IV secretion system (T4SS-1) with homology to the *Agrobacterium tumefaciens* VirB/D4 system and the T4SS encoded by the *Escherichia coli* pKM101 conjugative plasmid. The T4SS-1 is required for effector secretion and intracellular survival inside macrophages, but currently no details are known regarding its mode of action or potential effector proteins. To identify proteins secreted through this system, we compared the secretome of wild type *B. cenocepacia* K56-2 with that of a ΔpBCA017-059 mutant, defective in its T4SS-1, using a label-free proteomics approach. In this study, two T6SS-associated proteins, Hcp1 and TecA, were found to be nearly absent in the secretome of the mutant. More in-depth targeted proteomics experiments, as well as the use of two other mutant strains (ΔpBCA054-055, with a deletion of an operon potentially linked to quorum sensing, and ΔpBCA017-033, a shorter T4SS-1 mutant) revealed that the previous observation might originate from a secondary mutation acquired by the original ΔpBCA017-059 mutant, since this mutant was created in the MHK background, a strain lacking a gentamicin resistance pump making it more prone to other mutations. The ΔpBCA054-055 and ΔpBCA017-033 mutants are able to secrete Hcp1 and TecA at wild type levels, but both mutants display an aberrant biofilm morphology and reduced formation. Furthermore, this work shows a potential link between T4SS-1 and prophage induction, exemplified by the reduced abundance of several phage proteins in the secretome of both the original (ΔpBCA017-059) and shorter (ΔpBCA017-033) T4SS-1 mutant. In conclusion, these results indicate that mutants created in the MHK background should be regularly screened for potential secondary mutations and that the integrity of the 92-kb plasmid is important for biofilm formation and prophage induction. However, additional research is required to provide valid explanations for these observations.

## 5.1 Introduction

The *Burkholderia cepacia* complex (*Bcc*) is currently a group of 20 genetically distinct, but phenotypically similar, Gram-negative bacteria that can colonize a wide variety of niches (Coenye et al., 2001; Peeters et al., 2013b; Spilker et al., 2015). Due to their extreme metabolic versatility, they have received considerable attention as agents for bioremediation and biological control. In contrast to their beneficial ecological roles, *Bcc* bacteria are important opportunistic pathogens that are able to infect multiple hosts, ranging from plants and animals to humans (Caballero-Mellado et al., 2007; Holmes et al., 1998). They often colonize the lower respiratory tract of immunocompromised individuals, particularly in people with cystic fibrosis (CF), where they can cause cepacia syndrome, a virtually lethal necrotizing pneumonia (Drevinek and Mahenthiralingam, 2010; Mahenthiralingam et al., 2002). Among the *Bcc*, *Burkholderia cenocepacia* is the major culprit of causing life-threatening infections in patients with CF. Because of its intrinsic resistance to the majority of clinically used antibiotics (Leitão et al., 2008) and its high rate of patient-to-patient transmission (Biddick et al., 2003), it is crucial to invest in the development of new, effective therapies that can limit the spread of this pathogen.

Since many proteins secreted by this organism aid in the colonization, invasion and infection of host cells, these so-called 'effector proteins' are highly ranked on the list of potential targets for novel antimicrobial drugs (Li and Xie, 2011). They can either be secreted in the extracellular environment, mainly by the T2SS, or directly injected into host cells by the T3SS, T4SS (T4SS-1 and T4SS-2) and T6SS (Bleves et al., 2010). Many T2SS and T6SS effector proteins are proven to be important virulence factors, like two zinc metalloproteases (ZmpA and ZmpB), phospholipase C, VgrG's, hemolysin-coregulated proteins (Hcp's), chitinase and multiple peptidases (Burtnick et al., 2014; Corbett et al., 2003; Kooi and Sokol, 2009; Pukatzki et al., 2007). Several T3SS effectors have been identified in other Gram-negative species, like *Burkholderia pseudomallei*, *Aeromonas salmonicida* subsp. *salmonicida*, *Salmonella enterica* serovar *typhimurium*, *Escherichia coli* and *Pseudomonas syringae* (Vanden Bergh et al., 2013; Vander Broek et al., 2015; Cardenal-Muñoz et al., 2014; Hockett et al., 2014; Pallett et al., 2014). T4SS effectors have been characterized in *Legionella pneumophila*, *Brucella* spp., *Coxiella burnetii* and *Salmonella enterica* serovar *thyphimurium* (Luo and Isberg, 2004; Myeni et al., 2013; Newton et al., 2013). However, so far, no T3SS or T4SS effector proteins have been identified in *B. cenocepacia*.

Type IV secretion systems can exert a multitude of functions and are classified into different subgroups, according to their main task: translocation of effectors into host cells, DNA conjugation, DNA uptake and transformation and release of DNA into the extracellular milieu (Zhang et al., 2009). Two different T4SSs are present in *B. cenocepacia*: T4SS-1 is located on the 92-kb plasmid, while a bc-VirB/D4 system (T4SS-2) is located on chromosome II. The latter is required for plasmid mobilization (Zhang et al., 2009). Only T4SS-1 is currently known to be responsible for effector secretion and is required for intracellular survival and replication inside macrophages (Sajjan et al., 2008a; Zhang et al., 2009). Moreover, there is compelling evidence that T4SS-1 is involved in the plant tissue watersoaking (ptw) phenotype, a plant disease trait (Engledow et al., 2004).

Surveying for effector proteins involved in these virulence traits, we started our work by searching for differences in the secretome of *B. cenocepacia* K56-2 wild type and a ΔpBCA017-059 mutant

strain (obtained form Prof. Miguel Valvano, Queen's University Belfast, UK) defective in its T4SS-1, by a label-free proteomics approach. An unexpected discovery was the fact that two known T6SS proteins, Hcp1 and TecA, showed a highly reduced abundance or were even completely absent from the secretome of the ΔpBCA017-059 mutant, in which the entire T4SS-1 operon is removed. On top of that, this mutant strain also displayed a dramatic change in abundance of proteins involved in amino acid metabolism and protein synthesis as well as a reduced ability to form biofilm compared to wild type *B. cenocepacia* K56-2. This observation raised questions. The original mutant potentially lacked a large number of genes that are not necessarily directly involved in T4SS-dependent secretion, such as the pBCA054-055 operon, potentially involved in QS signaling. In addition, this mutant was created in the so-called MHK background, a strain lacking a gentamicin resistance pump, which could be prone to other mutations (Prof. Dr. M. Valvano, pers. comm.). To sort this out, two new deletion mutants were created in the K56-2 environment (Figure 12), in which only a subset of T4SS-1 structural genes were deleted (ΔpBCA017-033), or in which the regulatory operon possibly linked to QS was removed (ΔpBCA054-055). The latter mutant showed a significant reduction in biofilm mass formation and an aberrant biofilm morphology, but did not show reduced secretion of Hcp1 or TecA, neither did the ΔpBCA017-033 mutant. This work indicates that care should be taken to the use of the MHK strain to study *B. cenocepacia* secretion systems. On top of that, we show that the integrity of the 92-kb plasmid is important for cell metabolism and biofilm formation and that there is a potential link between the T4SS-1 and the induction of genome-encoded prophages.

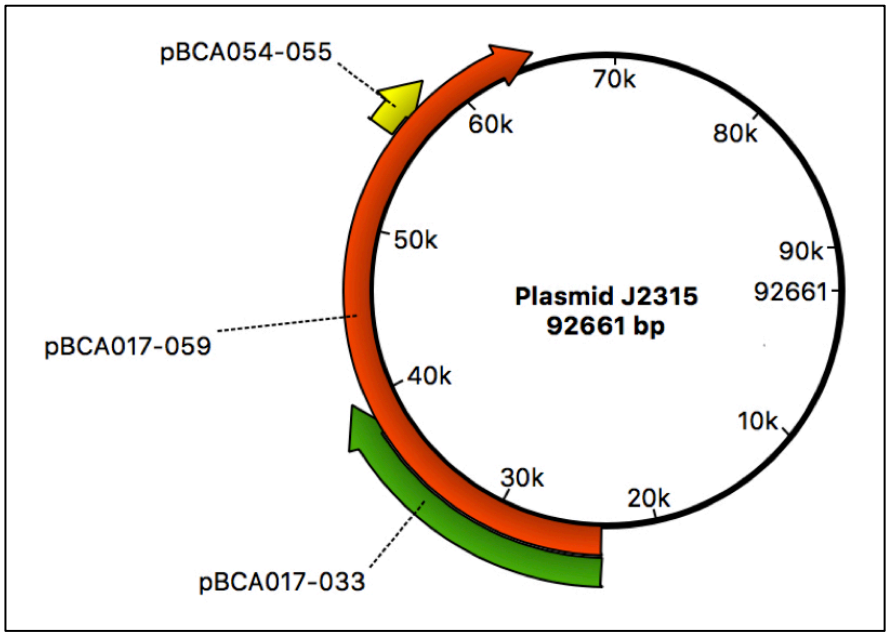


**Figure 12. Schematic representation of the plasmid regions deleted in the different mutant strains of *B. cenocepacia* K56-2 used in this study.**

## 5.2 Materials & Methods

### Chemicals and Solvents

All chemicals and reagents were obtained from Sigma-Aldrich, unless otherwise stated. Solvents for LC-MS were from Biosolve.

### Bacterial strains, growth and culture conditions

The different *B. cenocepacia* strains used in this work were prepared according to Flannagan and Valvano (2008) and were maintained in Microbank™ bacterial preservation systems (Fischer Scientific). In the ΔpBCA017-059 strain, the complete T4SS-1 located on the plasmid has been deleted. This mutant was created in the MHK background, which is a derivative of the K56-2 strain with a deletion in the genes encoding the gentamicin resistance pump (ΔBCAL1674-6), rendering the strain gentamicin sensitive ($Gm^S$) (Hamad et al., 2010). Since uncertainty raised about this strain, another mutant (ΔpBCA017-033) was created in the K56-2 background in which only structural T4SS-1 genes were deleted. The ΔpBCA054-055 mutant contains a deletion of a regulatory operon potentially linked to quorum sensing. The first gene, pBCA054, is a LuxR family regulatory protein, while pBCA055 is a diguanylate cyclase that might be involved in the regulation of cellular cyclic-di-GMP levels. In the ΔT6SS mutant (MHK background), the whole T6SS gene cluster located on chromosome 1 (ΔBCAL0333-52) has been deleted. Deletion of the global virulence regulator AtsR (BCAM0379) in the DFA35 mutant (ΔatsR, MHK background) causes overexpression of the T6SS. All cultures were grown on a shaker (200 rpm) at 37°C, unless otherwise stated.

### Isolation of extracellular proteins

Overnight *B. cenocepacia* cultures were diluted to an $OD_{600}$ of 0.1 in a total volume of 100 ml and were grown for 16h (stationary phase). The culture supernatant was filtered through a 0.22 μm 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 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 (4696xg, 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, except for the samples used for GELC-MS/MS analysis. Protein concentrations were estimated by Bradford's method (Coomassie Plus Protein Assay kit; Pierce).

### Isolation of intracellular proteins

Overnight cultures of wild type and ΔpBCA017-059 *B. cenocepacia* were diluted to an $OD_{600}$ = 0.1 and grown for 16h at 37°C, 200 rpm. Cells were collected by centrifugation (10 min, 6.000xg) and washed with phosphate-buffered saline (PBS). The cell pellet was dissolved in 2 ml lysis buffer (6 M urea, 2 M thiourea, Complete protease inhibitor cocktail in 50 mM ammonium bicarbonate). Cells were lysed by sonication (3 x 1 min on ice, 1s pulse on, 1s pulse off, 15% amplitude). Cell debris and unbroken cells were removed by centrifugation (15 min, 16.000xg) and intracellular proteins were precipitated with 4 volumes of acetone. After centrifugation (30 min, 16.000xg), the proteins were dissolved in 2 M urea in 50 mM ammonium bicarbonate. Protein concentrations were estimated by

Bradford's assay and proteins were reduced with dithiotreitol (DTT), alkylated with iodoacetamide (IAA) and digested overnight with sequencing-grade modified trypsin. Prior to reduction, alkylation and digestion, bovine serum albumin (BSA) was spiked in the samples to serve as an internal standard for normalization.

**Proteome analysis**

*GELC-MS/MS ANALYSIS*

From each sample, 10 μg of total protein was loaded onto a 1D SDS-PAGE gel. The proteins were separated and stained with Coomassie Brilliant Blue-G250. Each lane was cut into 10 slices. In-gel digestion was performed with sequencing-grade modified trypsin (Promega). Briefly, the gel pieces were destained by repeated washing with 50% acetonitrile/200 mM ammonium bicarbonate, dried and digested overnight with trypsin (37°C). The resulting peptides were extracted from the gel pieces with 60% acetonitrile/0.1% HCOOH, dried and dissolved in 2% acetonitrile/0.1% HCOOH.
Peptides obtained from in-gel digestion were separated by liquid chromatography and measured online with ESI mass spectrometry. LC-MS/MS analyses were performed using an Agilent 1200 HPLC system (Agilent Technologies) coupled to a LTQ FT Ultra mass spectrometer (Thermo Scientific, Xcalibur software). Peptides were loaded onto a trap column (Zorbax 300SB-C18, 5 μm, 5 mm x 0.3 mm, Agilent Technologies) and washed with 2% ACN/0.1% HCOOH for 10 min at a flow rate of 4 μl/min. From the trap column, the peptides were loaded onto an analytical column (Zorbax 300SB-C18, 3.5 μm, 150 mm x 75 μm, Agilent Technologies) and eluted by a binary gradient of solvent A (0.1% HCOOH in $H_20$) and solvent B (0.1% HCOOH in ACN) over a period of 30 min, with a flow rate of 0.3 μl/min. Eluting peptides were sprayed into the mass spectrometer source using D-chips (Advion) on which a 1.55 kV voltage was applied. The LTQ FT Ultra mass spectrometer was tuned and calibrated with caffeine, MRFA and UltraMark before measurement. A full survey scan in the FT (m/z 300 - 1400) with a resolution of 100 000 was followed by MS/MS analyses of the 5 most abundant ions in the LTQ via CID. Precursors were dynamically excluded for 90 s, and singly charged ions were rejected.

*DATABASE SEARCHING, CRITERIA FOR PROTEIN IDENTIFICATION AND PROTEIN QUANTIFICATION OF GELC-MS/MS DATA*

Tandem mass spectra were extracted by Extract MSn version 3.2. Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.5.0) and X!Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1)). Mascot was set up to search a custom *B. cenocepacia* K56-2 database (7672 entries, including common contaminants) assuming the digestion enzyme trypsin. X! Tandem was set up to search the *B. cenocepacia* K56-2 database, concatenated with the reversed sequences of this database, also assuming trypsin as the digestion enzyme. Two missed cleavages were allowed in both search engines. Mascot and X! Tandem searches were performed with a fragment ion mass tolerance of 0.30 Da and a parent ion tolerance of 10 ppm. Oxidation of methionine and propionamide modification of cysteine were specified in both Mascot and X! Tandem as variable modifications, whereas Glu -> pyro-Glu of the N-terminus, ammonia-loss of the N-terminus, Gln -> pyro-Glu of the N-terminus were only considered as variable modifications in X! Tandem.

Scaffold (version Scaffold_4.4.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm (Keller et al., 2002) with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters.
A Fisher's exact test was used to detect statistically significant differences and the Hochberg-Benjamini correction was applied to correct for multiple testing.
The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2016) partner repository with the dataset identifier PXD007645 and 10.6019/PXD007645.

*LC-MS/MS ANALYSIS*

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 µl/min and separated on a YMC Triart C18 column (3 µm, 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 µl/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.

*DATABASE SEARCHING, CRITERIA FOR PROTEIN IDENTIFICATION AND PROTEIN QUANTIFICATION OF LC-MS/MS DATA*

LC-MS/MS data were imported and analyzed in MaxQuant version 1.5.7.4. (Cox and Mann, 2008). Data from four biological replicates of each strain were searched against a custom database composed of *B. cenocepacia* J2315 sequences (downloaded from www.uniprot.org) using the Andromeda search engine (Cox et al., 2011). Carbamidomethylation of cysteine was set as a fixed modification. The variable modifications for the searches were oxidation of methionine, N-terminal acetylation, deamidation of asparagine and glutamine to pyroglutamate conversion, with a maximum of five modifications per peptide. Trypsin was set as the digestion enzyme, and maximum two missed cleavages were accepted. For the primary search, the precursor peptide tolerance was set to 10 ppm, and the MS/MS tolerance was 0.1 Da. The 'match between runs' feature of MaxQuant, which enables identification transfer between samples based on accurate mass and retention time, was applied with a match time window of one minute and an alignment time window of 20 minutes. A minimum of two razor and unique peptides was required for identification, and a minimum score of 40 was accepted for modified peptides. The FDR was set to 0.01 for peptide spectral matches, proteins and sites and calculated using a reverse decoy database. Minimal peptide length was seven amino acids.

Protein quantification was performed using the delayed normalization and maximal peptide ratio extraction, termed MaxLFQ, which determines the intensity ratio between proteins from different samples (Cox et al., 2014). Both unique and razor peptides were used for quantification and at least two ratio counts were required for a protein quantification to be valid. Statistical analyses were done using the Perseus software package (Tyanova et al., 2016). Prior to statistics, data were $\log_2$ transformed and only proteins that were detected in at least two samples were retained. Two-sample t-tests were performed to identify statistically significant differences, whereby a permutation-based FDR of 5% was used to correct for multiple testing. Only proteins with a fold change > 1.5 were considered significantly different.

*VALIDATION OF IDENTIFIED EFFECTOR PROTEINS BY SELECTED REACTION MONITORING*

Protein samples (identical to the ones used for the geLC-MS/MS analysis) were reduced with dithiothreitol, alkylated by iodoacetamide and digested with sequencing-grade modified trypsin. Prior to reduction and alkylation, bovine serum albumin (BSA) was spiked into the samples (10 ng BSA in 50 μg sample).

Six SRM methods were created, each cycling through a list of 33 transitions with a dwell time of 90 ms. The peptide mixtures were loaded on an Acclaim PepMap100 trap column (2 cm x 100 μm, C18, 5 μm, 100 Å) (Thermo Fisher Scientific, Waltham, MA, USA) using a flow rate of 5 μl/min for 3 min. After trapping, the peptides were analyzed on an Acclaim PepMap100 analytical column (L x ID 25 cm x 75 μm, C18, 3 μm, 100 Å) (Thermo) at a flow rate of 300 nl/min, on an Ultimate 3000-RSLC system (Thermo). As mobile phases, 0.1% formic acid in water and 0.1% formic acid in ACN were used to separate the peptides with a 30 min gradient, ranging from 2% to 40% ACN. The eluting peptides were sprayed directly into a 4000 QTRAP mass spectrometer (AB Sciex, Framingham, MA, USA) with the NanoSpray II ESI source equipped with a PicoTip Emitter (uncoated SilicaTip TM, 10 ± 1 μm). The system was operated in positive mode. The ionspray voltage was set at 3500 V, the capillary temperature at 60°C and the collision energy (CE) and declustering potential (DP) were calculated with the following equation: CE (V) = (0.5 x precursor ion m/z) + 5 and DP (V) = (0.0729 x precursor ion m/z) + 31.117.

Data were imported into Skyline (MacLean et al., 2010b) and for each peptide, the total area under curve (AUC) of the best peak was calculated. Values from individual peptides were normalized against BSA to get a quantitative measure of peptide abundance. A T-test (in case of equal variances) or a Fisher's exact test (in case of unequal variances) was performed to detect statistically significant differences between wild type and ΔpBCA017-059. If all peptides monitored for a given protein showed a significant difference, the protein was accepted to be significantly up- or downregulated.

*QUANTIFICATION OF T6SS EFFECTORS AND OTHER VIRULENCE FACTORS VIA SELECTED REACTION MONITORING*

Secreted proteins were isolated according to the previously described protocol. Protein samples were reduced, alkylated and digested with sequencing-grade modified trypsin. Prior to reduction, alkylation and digestion, BSA was spiked into the samples (132 ng in 10 μg sample) to serve as an internal standard.

Peptides were loaded on an ACQUITY UPLC M-Class Symmetry C18 trap column (100Å, 5 μm, 300 μm x 50 mm) at a flow rate of 15 μl/min for 5 min at 3% solvent B (0.1% formic acid in acetonitrile). After trapping, the peptides were loaded on a HSS T3 iKey (100 Å, 1.8 μm, 150 μm x 100 mm) at a

flow rate of 1 µl/min on an ACQUITY M-Class UPLC system (Waters). As mobile phases, solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in ACN) were used to separate the peptides with a 20 min gradient ranging from 3% to 50% solvent B. The UPLC effluent was introduced into a Xevo TQ-S triple quadrupole mass spectrometer (Waters), equipped with an electrospray ionization source. For each protein, nine transitions were monitored by SRM, each with a dwell time of 38 ms. The ESI-MS/MS parameters were set as follows: a capillary voltage of 3.6 kV, a cone voltage of 35 V, a source temperature of 120°C, a cone gas flow of 150 L/h, a nebulizer gas pressure of 6 bar and a nanoflow gas pressure of 0.2 bar. The collision voltage (CE) was optimized for each transition according to the following equation: CE (V) = (0.5 x precursor ion m/z) + 5. Nitrogen was introduced as the collision gas in the collision cell at a flow rate of 0.19 ml/min. Data acquisition was carried out by Masslynx 4.1 software and data were processed and analyzed using Skyline, as previously described.

**Biofilm formation and quantification**

Overnight *B. cenocepacia* wild type and mutant cultures were diluted to an $OD_{600}$ = 0.4 in LB. For each biofilm experiment, the wells of a round-bottomed polystyrene 96-well microtiterplate were inoculated with 100 µl of this dilution. Three biological replicates (each with a minimum of 12 technical replicates) were assayed per strain. After 24h of biofilm formation, the supernatant was removed and the wells were rinsed with 100 µl physiological saline.
For fixation of the biofilms, 100 µl 99% methanol was added (15 min), after which the supernatants were removed and the plates were air-dried. Then, 100 µl of a 0.1% crystal violet solution was added to all wells. After 20 min, the excess crystal violet was removed by washing the plates under running tap water. Finally, bound crystal violet was released by adding 150 µl of 33% acetic acid. The absorbance was measured at 595 nm and corrected against the background absorption. All steps were carried out at room temperature.

**Biofilm morphology and LIVE/DEAD staining**

Overnight *B. cenocepacia* K56-2 wild type and mutant cultures were diluted to an $OD_{600}$ = 0.2 in LB. For each biofilm experiment, the wells of a round-bottomed polystyrene 96-well microtiterplate were inoculated with 100 µl of this dilution. Three biological replicates were assayed per strain and the experiment was repeated three times. Cells were allowed to adhere to the plate for 4h, after which the culture medium was removed, wells were washed with physiological saline and fresh medium was added to all wells. After an additional 20h of biofilm formation, the supernatant was removed and the wells were rinsed with 100 µl physiological saline.
Biofilms were stained with the LIVE/DEAD™ *Bac*Light™ Bacterial Viability kit, according to the manufacturer's instructions (L7012, Thermo Fisher Scientific). Biofilm morphology was evaluated using an EVOS FL Auto Cell Imaging System (Invitrogen) with a 40x magnification. Both phase contrast and transmission microscopy images were recorded. For the fluorescence microscopy after LIVE/DEAD staining, the excitation wavelength was set to 470 nm, while the emission spectrum between 490 and 700 nm was recorded. Images were processed using the Image J processing software. The number of colony forming units (CFU) per biofilm was determined by plating entire biofilms on LB agar plates.

## 5.3 Results

### 5.3.1 Functional characterization of the plasmid region pBCA017-059, containing the plasmid-encoded T4SS-1

In order to gain more knowledge regarding the function of the plasmid-encoded T4SS-1, the secretome of T4SS-1 deficient *B. cenocepacia* K56-2 cultures (ΔpBCA017-059) was compared to that of wild type strains. Triplicate cultures of wild type and mutants were grown to stationary phase, at which time cells were removed from the culture medium by centrifugation. After filtration and addition of protease inhibitors, proteins present in the culture supernatant were collected by a sodium deoxycholate-assisted TCA precipitation. The secretomes were analyzed by a geLC-MS/MS approach. Three biological and two technical replicates were measured. In total, we identified 545 proteins with a minimum of 2 unique peptides among the secretomes of WT and ΔpBCA017-059 *B. cenocepacia* K56-2 (ProteomeXchange identifier PXD007645), originating from 59550 spectra. A comparison of the spectral counts was used to identify candidate effector proteins. A Fisher's exact test was applied to test for statistically significant differences. Only proteins identified with a probability higher than 99% and with at least two peptides, were retained. In the ΔpBCA017-059 mutant, 57 proteins showed a significantly lower abundance compared to the wild type strain, while 76 proteins were significantly more abundant in the secretome of the mutant strain. Results are summarized in Table 3 and Table 4.

Among the proteins downregulated in ΔpBCA017-059, several flagellar proteins could be identified, as well as one of the major zinc metalloproteases, ZmpA, an alkaline phosphatase, and multiple phage proteins, including a phage-like baseplate assembly protein (a Vgr family protein). Next to the decreased abundance of multiple metabolic and uncharacterized proteins, a remarkable observation is the absence of two T6SS effector proteins in ΔpBCA017-059. The latter proteins are a haemolysin-coregulated protein (Hcp1) and TecA, a Rho GTPase deamidase (Aubert et al., 2015). Proteins with significant higher abundance in the secretome of the mutant include numerous proteins involved in transport, nutrient uptake and amino acid metabolism, as well as several virulence factors, including the nematocidal protein AidA, a peptidase of the M28 family, the major cable pilus protein CblA and the zinc metalloprotease ZmpB.

**Table 3. Proteins significantly downregulated in stationary phase ΔpBCA017-059 cultures compared to wild type *B. cenocepacia* K56-2.**

| Accession | Gene in J2315 | Protein name | Total Spectrum Count WT | Total Spectrum Count ΔpBCA017-059 | Fold change[1] | P-Value |
|---|---|---|---|---|---|---|
| **Energy and amino acid metabolism** | | | | | | |
| EPZ86149.1 | BCAL0314 | Imidazoleglycerol-phosphate dehydratase | 3 | 1 | 3.00 | 0.0096 |
| EPZ87311.1 | BCAL2209 | Pyruvate dehydrogenase (acetyl-transferring), homodimeric type | 18 | 0 | Infinity | < 0.00010 |
| EPZ86162.1 | BCAL2736 | Isocitrate dehydrogenase, NADP-dependent | 19 | 0 | Infinity | < 0.00010 |
| EPZ91233.1 | BCAL2762 | Adenylate kinase | 8 | 2 | 4.00 | < 0.00010 |
| EPZ87406.1 | BCAL2284 | Acetyl-CoA synthetase | 3 | 1 | 3.00 | 0.0059 |
| EPZ85827.1 | BCAL2934 | Electron transfer flavoprotein domain protein | 17 | 7 | 2.43 | < 0.00010 |
| EPZ86508.1 | BCAL1516 | Dihydrolipoyllysine-residue succinyltransferase, E2 component of oxoglutarate dehydrogenase (succinyl-transferring) complex | 11 | 5 | 2.20 | 0.0014 |
| EPZ91662.1 | BCAL3246 | Mannose-1-phosphate guanylyltransferase/mannose-6-phosphate isomerase | 5 | 0 | Infinity | < 0.00010 |
| **Transcription and translation** | | | | | | |
| EPZ86786.1 | BCAL1506 | N utilization substance protein A | 2 | 0 | Infinity | 0.0082 |
| EPZ85919.1 | BCAL0260 | DNA-directed RNA polymerase, alpha subunit | 5 | 0 | Infinity | < 0.00010 |
| EPZ85430.1 | BCAL2950 | 30S ribosomal protein S1 | 24 | 7 | 3.43 | < 0.00010 |
| EPZ86159.1 | BCAL0227 | DNA-directed RNA polymerase, beta' subunit | 15 | 5 | 3.00 | < 0.00010 |
| EPZ86096.1 | BCAL0226 | DNA-directed RNA polymerase, beta subunit | 20 | 7 | 2.86 | < 0.00010 |
| EPZ85898.1 | BCAL0219/ BCAL0232 | Translation elongation factor Tu | 60 | 24 | 2.50 | < 0.00010 |
| **Stress response and chaperones** | | | | | | |
| EPZ89047.1 | BCAM1495 | Universal stress family protein | 3 | 0 | Infinity | 0.00024 |
| EPZ89510.1 | BCAM0050 | Universal stress family protein | 8 | 4 | 2.00 | 0.0096 |
| EPZ91923.1 | BCAL3299 | Catalase/peroxidase KatB | 33 | 21 | 1.57 | 0.002 |
| EPZ85575.1 | BCAL3192 | Putative oxidoreductase (redoxin) | 5 | 1 | 5.00 | 0.0021 |
| EPZ86165.1 | BCAL0389 | Thiol:disulfide interchange protein DsbC | 5 | 1 | 5.00 | 0.0021 |
| EPZ91694.1 | BCAL3270 | Chaperone protein DnaK | 37 | 9 | 4.11 | < 0.00010 |
| EPZ87278.1 | BCAL1919 | ATP-dependent chaperone protein ClpB | 12 | 4 | 3.00 | < 0.00010 |
| EPZ86026.1 | BCAL2442 | Chaperone protein HtpG | 5 | 2 | 2.50 | 0.0094 |
| EPZ91809.1 | BCAL3272 | Co-chaperone GrpE | 10 | 1 | 10.00 | < 0.00010 |
| EPZ87298.1 | BCAL1997 | Trigger factor | 29 | 18 | 1.61 | 0.0017 |
| **Motility** | | | | | | |
| EPZ91158.1 | BCAL0577 | Flagellar hook-associated protein 3 (HAP3) | 26 | 3 | 8.67 | < 0.00010 |
| EPZ91043.1 | BCAL0576 | Flagellar hook-associated protein 1 (HAP1) | 58 | 17 | 3.41 | < 0.00010 |
| EPZ90882.1 | BCAL0113 | Flagellar hook-associated protein 2 (HAP2) | 11 | 5 | 2.20 | 0.00087 |
| EPZ88369.1 | BCAM0987 | Flagellar hook-basal body protein | 28 | 16 | 1.75 | 0.00023 |
| EPZ90782.1 | BCAL0114 | Flagellin protein | 634 | 401 | 1.58 | < 0.00010 |
| **Virulence** | | | | | | |
| EPZ85968.1 | BCAL0343 | Type VI secretion system effector, Hcp1 family | 62 | 0 | Infinity | < 0.00010 |
| EPZ89291.1 | BCAM1857 | Rho GTPase deamidase TecA | 10 | 0 | Infinity | < 0.00010 |
| EPZ85371.1 | BCAL1083 | Putative exported alkaline phosphatase | 3 | 1 | 3.00 | 0.0036 |
| EPZ85518.1 | BCAL2958 | Outer membrane protein A | 10 | 4 | 2.50 | 0.0013 |
| EPZ85049.1 | BCAS0409 | Zinc metalloprotease ZmpA | 162 | 83 | 1.95 | < 0.00010 |
| EPZ86941.1 | BCAL1294 | Phage-like baseplate assembly protein | 4 | 0 | Infinity | < 0.00010 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Phenylacetic acid degradation** | | | | | | |
| EPZ90912.1 | BCAL0212 | Phenylacetate-CoA NADH oxidoreductase PaaE | 2 | 0 | Infinity | 0.0046 |
| EPZ90874.1 | BCAL0214 | Phenylacetic acid degradation protein PaaC | 21 | 8 | 2.63 | < 0.00010 |
| EPZ86116.1 | BCAL0408 | Phenylacetic acid degradation oxidoreductase PaaZ | 5 | 2 | 2.50 | 0.0029 |
| **Other function** | | | | | | |
| EPZ87335.1 | BCAL2063 | IMP dehydrogenase | 3 | 0 | Infinity | 0.0014 |
| EPZ91667.1 | BCAL3361 | Adenylosuccinate lyase | 10 | 2 | 5.00 | < 0.00010 |
| EPZ87416.1 | BCAL2206 | Phasin-like protein | 5 | 1 | 5.00 | < 0.00010 |
| EPZ85790.1 | BCAL3205 | Tol-pal system protein YbgF | 6 | 0 | Infinity | < 0.00010 |
| EPZ85542.1 | BCAL3203 | Tol-Pal system beta propeller repeat protein TolB | 6 | 2 | 3.00 | 0.009 |
| **Unknown function** | | | | | | |
| EPZ91139.1 | BCAL2769 | PF04461 family protein (nucleotide-binding protein) | 4 | 0 | Infinity | < 0.00010 |
| EPZ84687.1 | BCAS0206 | Methyltransferase domain protein | 4 | 0 | Infinity | < 0.00010 |
| EPZ90325.1 | BCAM1075 | Hypothetical phage protein | 3 | 0 | Infinity | 0.00043 |
| EPZ88827.1 | BCAM1035 | Hypothetical phage protein | 2 | 0 | Infinity | 0.0026 |
| EPZ89244.1 | BCAM1076 | Hypothetical phage protein | 5 | 1 | 5.00 | 0.00055 |
| EPZ85610.1 | BCAL3204 | Putative OmpA family lipoprotein, peptidoglycan-associated lipoprotein | 10 | 3 | 3.33 | < 0.00010 |
| EPZ89084.1 | BCAM1083 | Putative transmembrane phage protein | 7 | 3 | 2.33 | 0.0059 |
| EPZ87735.1 | BCAM1068 | Putative exported phage protein | 51 | 22 | 2.31 | < 0.00010 |
| EPZ88929.1 | BCAM1041 | Putative phage coiled coil domain protein | 21 | 10 | 2.10 | < 0.00010 |
| EPZ86836.1 | BCAL1571 | Phage tail sheath protein | 13 | 7 | 1.86 | 0.0029 |
| EPZ90408.1 | BCAM1069 | Hypothetical phage protein | 20 | 11 | 1.82 | 0.0011 |
| EPZ85589.1 | BCAL2970 | Hypothetical phage protein | 20 | 11 | 1.82 | 0,0011 |
| EPZ88604.1 | BCAM1067 | Hypothetical phage protein | 25 | 15 | 1.67 | 0,0013 |

[1] *Fold change of WT/ΔpBCA017-059. Only proteins with a fold change > 1.5 are listed.*

**Table 4. Proteins significantly upregulated in stationary phase ΔpBCA017-059 cultures compared to wild type *B. cenocepacia* K56-2.**

| Accession | Gene in J2315 | Identified Proteins | Total Spectrum Count WT | Total Spectrum Count ΔpBCA017-059 | Fold change[1] | P-value |
|---|---|---|---|---|---|---|
| **Amino acid metabolism/biosynthesis** | | | | | | |
| EPZ91933.1 | BCAL0010 | Phenylalanine 4-monooxygenase | 0 | 6 | Infinity | < 0.00010 |
| EPZ90836.1 | BCAL0074 | Glycine cleavage system H protein | 0 | 2 | Infinity | 0.0079 |
| EPZ89388.1 | BCAM0985 | 3-isopropylmalate dehydrogenase | 1 | 4 | 4.00 | 0.0012 |
| EPZ87789.1 | BCAM0986 | Aspartate-semialdehyde dehydrogenase | 4 | 13 | 3.25 | < 0.00010 |
| EPZ91209.1 | BCAL2790 | Kynurenine formamidase | 1 | 3 | 3.00 | 0.0041 |
| EPZ86245.1 | BCAL2354 | 2-isopropylmalate synthase | 2 | 4 | 2.00 | 0.0045 |
| EPZ85617.1 | BCAL2942 | Cysteine synthase B | 7 | 13 | 1.86 | 0.00026 |
| EPZ91790.1 | BCAL3452 | Arginine biosynthesis bifunctional protein ArgJ | 6 | 10 | 1.67 | 0.0033 |
| EPZ85766.1 | BCAL1059 | Succinylornithine transaminase/acetylornithine aminotransferase | 8 | 13 | 1.63 | 0.0031 |
| EPZ90856.1 | BCAL0201 | N-acetyl-gamma-glutamyl-phosphate reductase | 9 | 14 | 1.56 | 0.0057 |
| **Energy metabolism** | | | | | | |
| EPZ89515.1 | BCAM2703 | Methylisocitrate lyase | 0 | 3 | Infinity | 0.00015 |

| | | | | | | |
|---|---|---|---|---|---|---|
| EPZ90771.1 | BCAL0194 | NADP oxidoreductase coenzyme F420-dependent | 1 | 4 | 4.00 | 0.0019 |
| **Aromatic compound metabolism** | | | | | | |
| EPZ89072.1 | BCAM0804 | Catechol 1,2-dioxygenase | 1 | 3 | 3.00 | 0.0041 |
| EPZ90871.1 | BCAL0149 | Dienelactone hydrolase family protein | 3 | 7 | 2.33 | 0.0082 |
| **Purine and pyrimidine metabolism/biosynthesis** | | | | | | |
| EPZ85683.1 | BCAL2989 | Adenosine deaminase | 0 | 2 | Infinity | 0.0079 |
| EPZ86311.1 | BCAL2389 | Phosphoribosylamine--glycine ligase | 1 | 5 | 5.00 | < 0.00010 |
| EPZ86544.1 | BCAL1873 | Adenylosuccinate synthase | 9 | 14 | 1.56 | 0.0055 |
| EPZ91684.1 | BCAL3351 | Dihydroorotase | 1 | 5 | 5.00 | < 0.00010 |
| **General metabolism** | | | | | | |
| EPZ86949.1 | BCAL1850 | Zinc-binding alcohol dehydrogenase family protein | 0 | 2 | Infinity | 0.0079 |
| EPZ86318.1 | BCAL2446 | Aminotransferase, class I/II | 1 | 4 | 4.00 | 0.0031 |
| **Transport and nutrient uptake** | | | | | | |
| EPZ85892.1 | BCAL0282 | ABC transporter, solute-binding protein | 1 | 6 | 6.00 | < 0.00010 |
| EPZ86895.1 | BCAL1652 | Sulfate-binding protein | 1 | 5 | 5.00 | < 0.00010 |
| EPZ91427.1 | BCAL2813 | Periplasmic solute-binding family protein | 2 | 6 | 3.00 | 0.00078 |
| EPZ90993.1 | BCAL0765 | ABC transporter permease | 3 | 8 | 2.67 | < 0.00010 |
| EPZ85558.1 | BCAL2936 | Metal ABC transporter substrate-binding protein | 3 | 7 | 2.33 | 0.00065 |
| EPZ87653.1 | BCAM2556 | Purine nucleoside permease | 4 | 9 | 2.25 | 0.0004 |
| EPZ85428.1 | BCAL3041 | Maltose-binding protein | 5 | 11 | 2.20 | 0.00022 |
| EPZ91329.1 | BCAL0675 | ABC transporter, substrate-binding protein, family 5 | 8 | 17 | 2.13 | < 0.00010 |
| EPZ91350.1 | BCAL0544 | ABC transporter, substrate-binding protein, family 5 | 12 | 25 | 2.08 | < 0.00010 |
| EPZ87046.1 | BCAL1657 | Ribose transport system, substrate-binding protein | 53 | 108 | 2.04 | < 0.00010 |
| EPZ90924.1 | BCAL0151 | Branched chain amino acid ABC transporter, substrate-binding protein | 7 | 13 | 1.86 | 0.00033 |
| EPZ91980.1 | BCAL0043 | Branched chain amino acid ABC transporter, substrate-binding protein | 30 | 55 | 1.83 | < 0.00010 |
| EPZ90108.1 | BCAM2251 | Branched chain amino acid ABC transporter, substrate-binding protein | 6 | 11 | 1.83 | 0.0024 |
| EPZ86777.1 | BCAL1610 | Putative cystine-binding periplasmic protein | 34 | 62 | 1.82 | < 0.00010 |
| EPZ87892.1 | BCAM1293 | Glutathione ABC transporter, substrate-binding protein | 23 | 39 | 1.70 | < 0.00010 |
| EPZ85363.1 | BCAL1092 | Iron ABC transporter, solute-binding protein | 15 | 24 | 1.60 | 0.00019 |
| EPZ91922.1 | BCAL3358 | Glutamate-aspartate periplasmic-binding protein | 69 | 108 | 1.56 | < 0.00010 |
| EPZ85119.1 | BCAS0060 | Branched chain amino acid ABC transporter, substrate-binding protein | 26 | 40 | 1.54 | < 0.00010 |
| EPZ88573.1 | BCAM2618 | Lysine/arginine/ornithine ABC transporter, periplasmic lysine/arginine/ornithine-binding protein ArgT | 13 | 20 | 1.54 | 0.00085 |
| EPZ91036.1 | BCAL0763 | Metal ABC transporter substrate-binding protein | 28 | 42 | 1.50 | < 0.00010 |
| **Virulence and adhesion** | | | | | | |
| EPZ90396.1 | BCAM2308 | Leucyl aminopeptidase, M28 family | 6 | 18 | 3.00 | < 0.00010 |
| EPZ85253.1 | BCAS0293 | Nematocidal protein AidA | 6 | 16 | 2.67 | < 0.00010 |
| EPZ86304.1 | BCAL2466 | Ecotin | 3 | 7 | 2.33 | 0.00028 |
| EPZ88351.1 | BCAM2307 | Zinc metalloprotease ZmpB | 43 | 84 | 1.95 | < 0.00010 |
| EPZ88849.1 | BCAM2761 | Giant cable pilus CblA | 1 | 5 | 5.00 | < 0.00010 |
| **Translation** | | | | | | |
| EPZ87314.1 | BCAL2190 | Lysine--tRNA ligase | 4 | 8 | 2.00 | 0.0069 |
| EPZ91345.1 | BCAL0484 | Glutamyl-tRNA amidotransferase subunit A | 5 | 8 | 1.60 | 0.011 |
| **Other function** | | | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| EPZ85984.1 | BCAL2390 | Coproporphyrinogen III oxidase, aerobic | 0 | 3 | Infinity | < 0.00010 |
| EPZ89534.1 | BCAM0832 | Dyp-type peroxidase family protein | 0 | 2 | Infinity | 0.0041 |
| EPZ87505.1 | BCAL2198 | Cysteine desulfurase IscS | 0 | 2 | Infinity | 0.0011 |
| EPZ85989.1 | BCAL2733 | Multicopper oxidase | 6 | 11 | 1.83 | 0.0018 |
| EPZ91081.1 | BCAL2757 | Superoxide dismutase (Fe) SodB | 10 | 17 | 1.70 | 0.00032 |
| EPZ85090.1 | BCAS0205 | Taurine catabolism dioxygenase, TauD/TfdA family | 0 | 2 | Infinity | 0.0041 |
| **Unknown function** | | | | | | |
| EPZ89451.1 | BCAM1443 | Putative exported protein | 0 | 3 | Infinity | 0.0002 |
| EPZ86166.1 | BCAL2457 | Putative exported protein | 0 | 2 | Infinity | 0.0041 |
| EPZ89256.1 | BCAM2149 | Metallopeptidase, subfamily M20A | 1 | 4 | 4.00 | 0.0056 |
| EPZ86668.1 | BCAL1891 | 3'-5' exonuclease | 1 | 3 | 3.00 | 0.0013 |
| EPZ87472.1 | BCAL1962 | Deoxyribonuclease, TatD family | 1 | 3 | 3.00 | 0.0021 |
| EPZ86629.1 | BCAL1849 | Putative exported protein | 1 | 3 | 3.00 | 0.0062 |
| EPZ85208.1 | BCAS0292 | Inclusion body protein | 2 | 5 | 2.50 | 0.0029 |
| EPZ86033.1 | BCAL2418 | Putative exported protein | 8 | 17 | 2.13 | < 0.00010 |
| EPZ91750.1 | BCAL3281 | Metalloprotease TldD | 6 | 12 | 2.00 | 0.00097 |
| EPZ91732.1 | BCAL3310 | Putative exported protein | 13 | 22 | 1.69 | 0.00011 |

[1] *Fold change of ΔpBCA017-059/WT. Only proteins with a fold change > 1.5 are listed.*

From the candidate effector proteins identified in the label-free quantitative proteomics experiment, a number of proteins were selected for validation. These candidates were chosen based on their potential role in virulence or infection and/or because of their high degree of downregulation in the mutant strain. In order to obtain a confident quantification, BSA was spiked into the samples prior to digestion as an internal standard for normalization. Six biological replicates were analyzed on a Dionex 3000 Ultimate nanoLC system coupled to a QTRAP 4000 mass spectrometer (AB Sciex). For each peptide, the total area under curve (AUC) of the best peak was calculated. Values from individual peptides were normalized against BSA to get a quantitative measure of peptide abundance. A two-sample t-test was performed to detect statistically significant differences between wild type and ΔpBCA017-059.

Eight proteins showed a significant difference in abundance between wild type and ΔpBCA017-059. TecA was confirmed to be significantly downregulated in stationary phase cultures of ΔpBCA017-059 *B. cenocepacia*, as were Hcp1 and the phage-like baseplate assembly protein. On the other hand, the zinc metalloprotease ZmpB and a peptidase of the M28 family were confirmed to be significantly upregulated in the mutant strain. Results are summarized in Table 5.

In order to obtain firm evidence for the absence of T6SS effectors in the secretome of *B. cenocepacia* ΔpBCA017-059, a control experiment including wild type, ΔpBCA017-059 and ΔT6SS strains was performed. The secretome of three biological replicates from each strain grown to stationary phase was isolated and the relative abundance of Hcp1 and TecA in the different strains was determined by SRM on an Acquity M-class UPLC system connected to a Xevo TQ-S mass spectrometer (Waters) (Figure 13). These results confirm our previously obtained data and provide additional evidence that these proteins are also absent from ΔT6SS culture supernatants.

**Table 5. Fold changes and associated p-values of differentially expressed proteins between stationary phase WT and ΔpBCA017-059 *B. cenocepacia* K56-2 cultures, confirmed by SRM.**

| | Mean normalized intensity WT[1] | Mean normalized intensity ΔpBCA017-059[1] | Fold change[2] | P-value |
|---|---|---|---|---|
| **TecA** | | | | |
| ADALIAPDALLDTAGQSWR | 25.45 | 15.23 | 0.60 | 0.04380 |
| ASIPAGTAVGFAR | 35.44 | 4.14 | 0.12 | 7.4e-05 |
| FNFETGDQWDGR | 6.34 | 0.55 | 0.09 | 0.00024 |
| **Hcp1** | | | | |
| EEGLPLETFSLK | 494.84 | 16.30 | 0.03 | 5.1e-06 |
| IGGNQGGNTQGAWSLTK | 85.29 | 4.24 | 0.05 | 1.1e-05 |
| YAAVQWK | 85.13 | 2.31 | 0.03 | 0.00022 |
| **ZmpB** | | | | |
| ADGVAVTLYNPAYR | 35.31 | 87.23 | 2.47 | 1.9e-05 |
| VTNWNVGR | 13.65 | 37.77 | 2.77 | 0.0293 |
| YVNQTLGIK | 17.63 | 50.59 | 2.87 | 8.0e-05 |
| **Peptidase M28** | | | | |
| ETVHVVEIDDSR | 1.56 | 4.65 | 2.98 | 4.0e-05 |
| SPGADDDASGIASLTEALR | 4.79 | 11.09 | 2.32 | 2.6e-05 |
| **Phage-like baseplate assembly protein** | | | | |
| ETDLAFVER | 10.54 | 0.90 | 0.09 | 3.3e-07 |
| IFQEENTEAILAR | 2.31 | 1.02 | 0.44 | 0.0036 |
| QLAPDTVGLK | 3.91 | 1.04 | 0.27 | 0.00026 |

[1] *Mean normalized intensities calculated from three biological replicates.*
[2] *Fold change of ΔpBCA017-059/WT.*

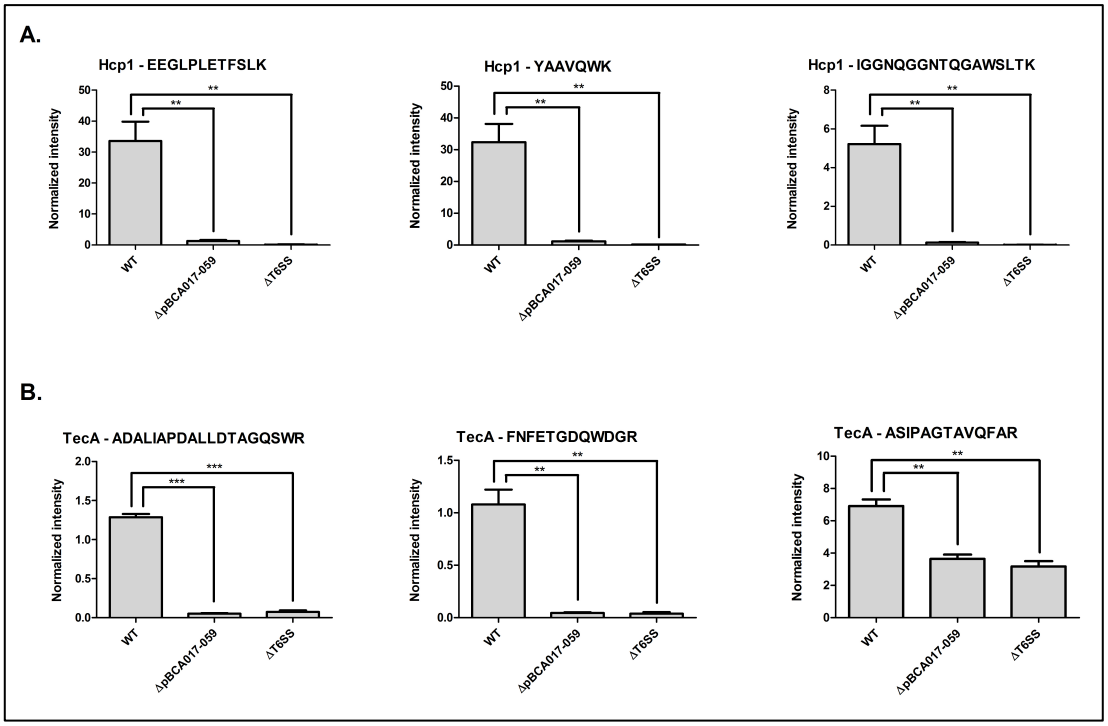


**Figure 13. Quantification of two T6SS proteins in the secretome of stationary phase cultures of wild type, ΔpBCA017-059 and ΔT6SS *B. cenocepacia* K56-2 by SRM.** Experiments were performed in triplicate. The statistical significance of the differences was assessed via two-sample t-tests. * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001 compared to the wild type control.

### 5.3.2 Impaired secretion of T6SS effectors in ΔpBCA017-059 is not due to decreased expression of T6SS genes

In order to gain insight in whether the deficiency of the T6SS to secrete its effector proteins was due to inhibition of its activity or assemblage or due to an impaired expression of T6SS genes, the intracellular proteome of wild type, ΔpBCA017-059 and ΔT6SS *B. cenocepacia* was collected and examined for the presence of Hcp1 and TecA. An absence of Hcp1 in ΔpBCA017-059 might point to a reduced expression of T6SS genes, whereas similar or increased abundance of these proteins suggests inhibition of T6SS activity.

Therefore, intracellular proteins of wild type, ΔpBCA017-059 and ΔT6SS were isolated from stationary phase cultures and the relative abundances of Hcp1 and TecA proteins in the different strains were determined by SRM. Quantitative data analysis shows no significant change in intracellular expression of TecA between wild type and both mutant strains (Figure 14). Hcp1 expression is not significantly decreased in ΔpBCA017-059, pointing to an inhibition of T6SS activity rather than suppression of T6SS gene expression. Since Hcp1 lies within the T6SS gene cluster, which is deleted in ΔT6SS, no Hcp1 protein could be detected in this mutant.

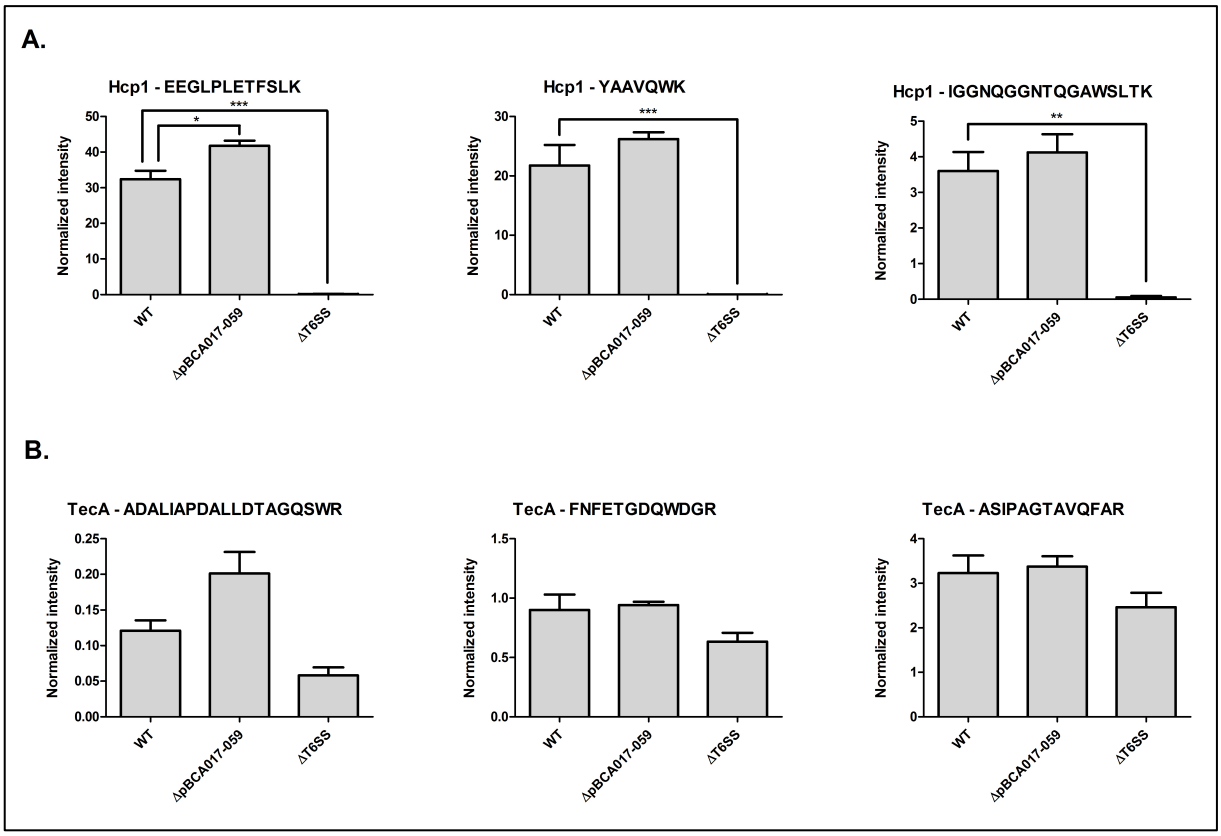


**Figure 14. Quantification of intracellular levels of two T6SS proteins in stationary phase cultures of wild type, ΔpBCA017-059 and ΔT6SS *B. cenocepacia* K56-2 by SRM.** Experiments were performed in triplicate. The statistical significance of the differences was assessed via two-sample t-tests. * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001 compared to the wild type control.

### 5.3.3 The ability of *B. cenocepacia* ΔpBCA017-059 to form biofilm is slightly reduced

Differences in biofilm formation between wild type and ΔpBCA017-059 were evaluated by a crystal violet assay. Cells were allowed to adhere for 4h, after which the culture medium was refreshed and biofilms were grown for an additional 20h. Three biological replicates, each with 24 technical replicates, were analyzed and a Welch two-sample t-test was used to detect statistically significant differences. Biofilm mass was slightly reduced in the ΔpBCA017-059 mutant strain (p-value = 8.009e-05). The mean normalized absorbance was 0.726 for wild type K56-2 and 0.441 for ΔpBCA017-059 (Figure 15).

### 5.3.4 Reduced biofilm formation of ΔpBCA017-059 is partly due to deletion of the regulatory operon pBCA054-055

In order to ascertain the complete knock out of the plasmid-encoded T4SS-1, a 41.61 kbp deletion was made (ΔpBCA017-059). However, this region also contains other genes, not encoding structural components of the T4SS-1. A bioinformatics analysis revealed the presence of several regulatory genes, such as a LuxR family regulatory protein (pBCA054) involved in quorum sensing regulation (Subsin et al., 2006) and a GGDEF domain containing membrane protein (pBCA055), a potential regulator of intracellular cyclic-di-GMP levels. If one or both proteins are involved in the regulation of T6SS activity, it might explain the observation that T6SS effectors are absent in ΔT4SS-1. In order to investigate this, additional deletion mutants were created. The first mutant strain contains a deletion of the operon comprising both candidate regulatory proteins (ΔpBCA054-055), while the second mutant has a non-functional T4SS-1 caused by deletion of an essential part of the T4SS-1 (ΔpBCA017-033), but leaving intact the candidate regulatory operon.
A crystal violet assay was performed in order to compare the biofilm formation potential of wild type, ΔpBCA054-055 and ΔpBCA017-033 *B. cenocepacia*. The ΔatsR strain was incorporated in this experiment as a positive control, since it is known to exhibit an enhanced biofilm formation potential (Aubert et al., 2008). Biofilms were grown for 24h at 37°C. The experiment was performed in triplicate. The mean normalized absorbance at 595 nm was found to be 0.989, 0.556 and 0.514 for wild type, ΔpBCA054-055 and ΔpBCA017-033, respectively (Figure 15). An unpaired two-sample t-test was used to detect statistically significant differences. Significantly less biofilm mass was formed by ΔpBCA054-055 and ΔpBCA017-033 compared to wild type *B. cenocepacia*, while ΔatsR formed significantly more biofilm, as was expected.
Crystal violet staining only reflects biofilm mass formation on a rather artificial substrate, regardless of the number of viable cells. Though significant, the reduction of biofilm in the mutants is quite small and therefore we investigated the biofilm formation further.

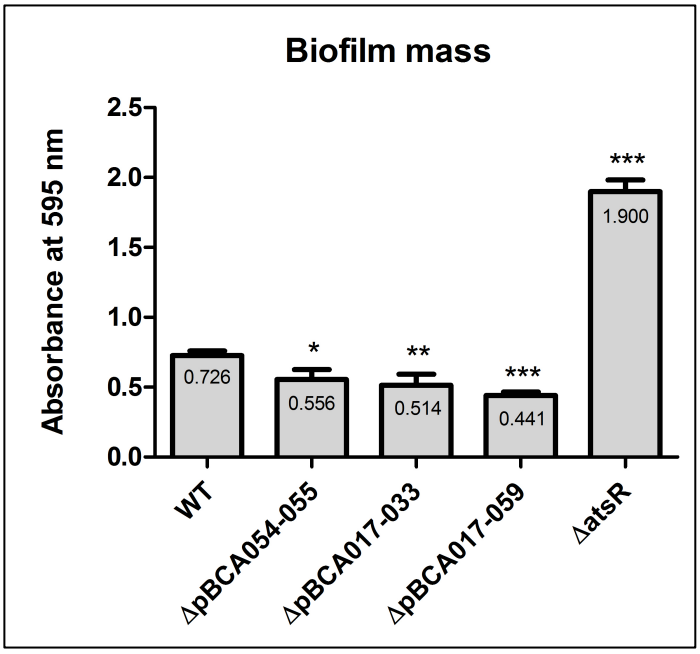


**Figure 15. Biofilm formation in wild type, ΔpBCA054-055, ΔpBCA017-033, ΔpBCA017-059 and ΔatsR *B. cenocepacia* K56-2.** Biofilms of wild type and mutants were grown for 24h and biofilm mass was quantified using the crystal violet assay. *** = p-value < 0.001, ** = p-value < 0.01, * = p-value < 0.05 compared to the wild type condition.

### 5.3.5 Both the ΔpBCA017-033 and ΔpBCA054-055 mutants show an aberrant biofilm morphology

Phase contrast microscopy analysis of 24h old biofilms of wild type and mutant *B. cenocepacia* K56-2 strains revealed a distinct biofilm morphology for both mutants compared to the wild type strain. Where multiple cell clusters, characteristic for a mature biofilm, are abundantly present in wild type biofilms, these are not observed in biofilms of either of the mutant strains. There were no significant differences in CFU when the biofilms were detached and cells were plated on LB agar plates. Also, LIVE/DEAD staining of the biofilms did not identify major differences in the number of dead cells present in wild type and mutant biofilms and indicated that the biofilms were viable (Figure 16).

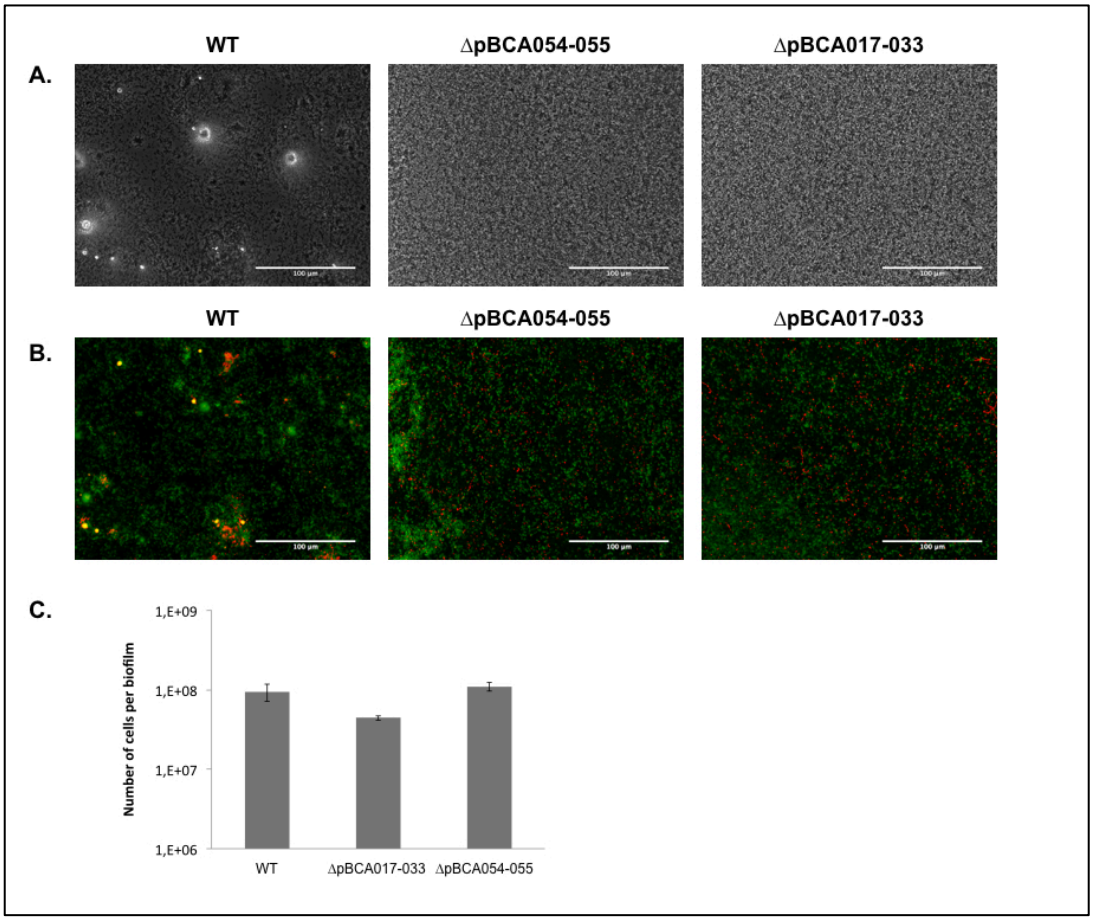


**Figure 16. Morphological comparison and viability analysis of biofilms formed by wild type, ΔpBCA054-055 and ΔpBCA017-033 *B. cenocepacia* K56-2. A.** Phase-contrast microscopy images of 24h biofilms recorded on an EVOS FL Auto Cell Imaging System (Invitrogen) with a 40x magnification. **B.** LIVE/DEAD staining of 24h biofilms. Excitation wavelength = 470 nm, emission spectrum recorded between 490 and 700 nm. **C.** Culturability of 24h biofilms evaluated by counting the number of CFU formed per biofilm.

### 5.3.6 Secretion of the T6SS effectors Hcp1 and TecA is not reduced in ΔpBCA054-055 and ΔpBCA017-033

To elucidate the possible role of the regulatory operon pBCA054-055 in the secretion of T6SS effectors, *B. cenocepacia* K56-2 wild type and mutant strains (ΔpBCA054-055 and ΔpBCA017-033) were grown to stationary phase and the secretion of virulence factors was monitored via SRM. The proteins followed were Hcp1, TecA, ZmpA, ZmpB, CblA and AidA, since these were observed to be differentially expressed between *B. cenocepacia* wild type and ΔpBCA017-059. Results are summarized in Figure 17. Secretion of Hcp1 and TecA is not abolished in these new mutants, on the contrary, TecA secretion is even slightly elevated in ΔpBCA054-055. No differences were observed between wild type and ΔpBCA017-033 for these two proteins. A significant upregulation of ZmpA and ZmpB was seen in ΔpBCA054-055 and ΔpBCA017-033. However, the highly increased secretion of the nematocidal protein AidA in the ΔpBCA017-059 mutant completely disappeared in the ΔpBCA054-055 and ΔpBCA017-033 mutants, while the slightly decreased abundance of CblA in the secretome of ΔpBCA017-059 was still observed in the secretome of ΔpBCA054-055, although not to a significant extent.

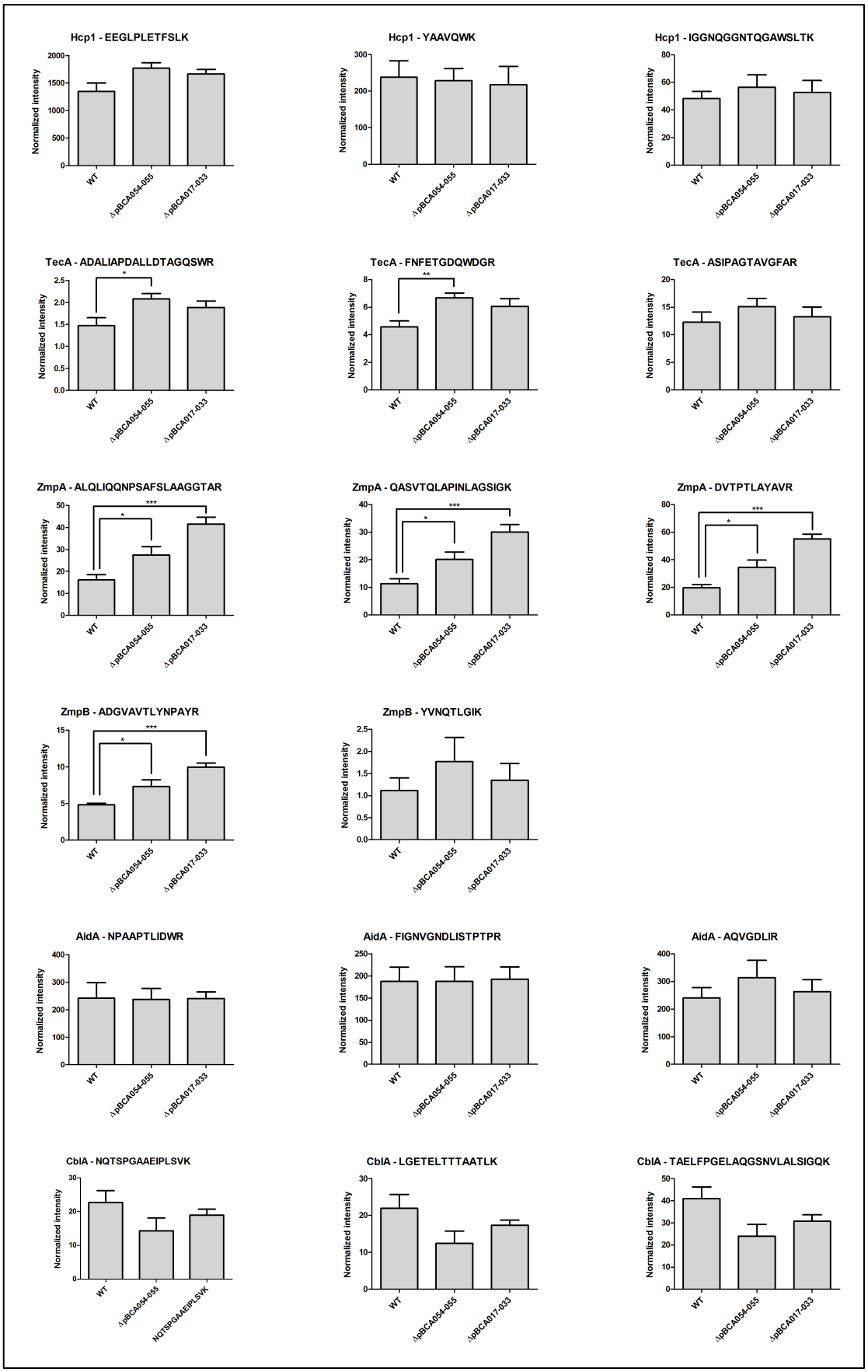


**Figure 17. Quantification of selected virulence factors in the secretome of stationary phase cultures of wild type, ΔpBCA054-055 and ΔpBCA017-033 *B. cenocepacia* K56-2 by SRM.** Experiments were performed in quadruplicate. The statistical significance of the differences was assessed via two-sample t-tests. * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001 compared to the wild type control.

In order to obtain a more complete view of the effect of deletion of the regulatory operon or part of the T4SS-1, a label-free proteomics study was conducted on the secretomes of the ΔpBCA054-055 and ΔpBCA017-033 mutants compared to wild type *B. cenocepacia* K56-2.

Only two proteins were significantly less abundant in the secretome of the ΔpBCA054-055 mutant compared to wild type *B. cenocepacia* K56-2: the giant cable pilus protein CblA and a putative exported protein BCAM0942 (Table 6). On the other hand, 23 proteins showed a significant increase in abundance in the ΔpBCA054-055 strain. Among these, some are involved in transcription and translation, comprising two ribosomal proteins, two elongation factors and a DNA-directed RNA polymerase. Others are typical energy metabolism-associated proteins, including ATP synthase subunits, adenylate kinase and transaldolase. Also multiple chaperone proteins and several proteins of unknown functions showed an increased abundance in this mutant (Table 7).

In the secretome of ΔpBCA017-033, the proteins showing a significant decrease in abundance compared to the wild type included four flagellar proteins, the major cable pilus protein CblA and three phage proteins belonging to a big phage cluster (BCAM1068, BCAM1069 and BCAM1075), next to several other proteins that are listed in Table 8. The flagellar and phage proteins are of particular interest since they were also decreased in the secretome of the ΔpBCA017-059 mutant. A total of 43 proteins displayed an increased abundance in the mutant strain (Table 9). Many of the proteins found to be increased in the secretome of the ΔpBCA054-055 mutant showed a similar trend in ΔpBCA017-033. Proteins uniquely upregulated in the ΔpBCA017-033 mutant include several uncharacterized exported proteins, the serine protease MucD1 and the major metalloprotease ZmpA. Surprisingly, the latter protein was found to be downregulated in the secretome of the ΔpBCA017-059 mutant, whereas it is upregulated in the ΔpBCA017-033 mutant. Secretion of the T6SS effectors Hcp1 and TecA was not significantly different between wild type, ΔpBCA054-055 and ΔpBCA017-033, as was seen in the targeted proteomics analysis.

**Table 6. Proteins significantly downregulated in stationary phase ΔpBCA054-055 cultures compared to wild type *B. cenocepacia* K56-2.**

| Accession | Gene in J2315 | Description | Average LFQ intensity[1] WT | Average LFQ intensity ΔpBCA054-055 | Fold change[2] | -Log10 P-value |
|---|---|---|---|---|---|---|
| **Adhesion** | | | | | | |
| B4ELG0 | BCAM2761 | Giant cable pilus CblA | 92292.44921 | 43294.61582 | 2.13 | 1.49458 |
| **Unknown function** | | | | | | |
| B4EF87 | BCAM0942 | Putative exported protein | 17819.26241 | 11265.31473 | 1.58 | 1.28518 |

[1] *Determined using the label-free quantitation algorithm of MaxQuant*
[2] *Fold change of WT/ΔpBCA054-055. Only proteins with a fold change > 1.5 are listed.*

**Table 7. Proteins significantly upregulated in stationary phase ΔpBCA054-055 cultures compared to wild type *B. cenocepacia* K56-2.**

| Accession | Gene in J2315 | Description | Average LFQ intensity[1] WT | Average LFQ intensity ΔpBCA054-055 | Fold change[2] | -Log10 P-value |
|---|---|---|---|---|---|---|
| **Energy metabolism** | | | | | | |
| B4EBY7 | BCAL0957 | Succinate--CoA ligase [ADP-forming] subunit alpha | 28334.39282 | 59437.05441 | 2.10 | 4.35914 |
| B4E6F9 | BCAL2433 | Transaldolase | 17883.81203 | 31557.056 | 1.76 | 2.49795 |
| B4E9G1 | BCAL2762 | Adenylate kinase (AK) | 68973.28216 | 120514.7328 | 1.75 | 4.69825 |
| B4EEY6 | BCAL0033 | ATP synthase F1 subunit delta | 42973.95686 | 71278.28477 | 1.66 | 1.88312 |
| B4EEY9 | BCAL0036 | ATP synthase F1 subunit beta | 31400.50193 | 50502.77353 | 1.61 | 2.78008 |
| **Transcription and translation** | | | | | | |
| B4E5D9 | BCAL0253 | 50S ribosomal protein L15 | 22453.19818 | 108138.8205 | 4.82 | 4.47861 |
| B4EBH0 | BCAL1942 | 50S ribosomal protein L9 | 89526.36563 | 223017.9025 | 2.49 | 2.57669 |
| B4ECN0 | BCAL2090 | Elongation factor Ts (EF-Ts) | 53748.98908 | 174558.3688 | 3.25 | 3.20753 |
| B4E5A5 | BCAL0219/BCAL0232 | Elongation factor Tu (EF-Tu) | 233235.0364 | 381870.7783 | 1.64 | 1.10943 |
| B4E5E6 | BCAL0260 | DNA-directed RNA polymerase subunit alpha | 11489.27386 | 24007.12464 | 2.09 | 1.57202 |
| **Stress response and chaperones** | | | | | | |
| B4ECY3 | BCAL3192 | Putative oxidoreductase | 35353.53034 | 143797.0945 | 4.07 | 3.83207 |
| B4EJI6 | BCAM1495 | Putative universal stress protein | 19917.16929 | 44718.99049 | 2.25 | 3.51308 |
| B4EG36 | BCAM0050 | Universal stress-related protein | 189035.8058 | 392076.324 | 2.07 | 3.24589 |
| B4EDZ2 | BCAL3270 | Chaperone protein DnaK (Hsp70) | 129966.0245 | 530456.5285 | 4.08 | 2.1439 |
| B4EBM4 | BCAL1997 | Trigger factor (TF) | 72605.94193 | 167479.5011 | 2.31 | 3.67074 |
| B4EDZ4 | BCAL3272 | Protein GrpE (Hsp70 cofactor) | 94001.75034 | 181084.0559 | 1.93 | 3.61545 |
| B4EB05 | BCAL1919 | Chaperone protein ClpB | 75429.08649 | 143406.4114 | 1.90 | 1.01031 |
| **Other function** | | | | | | |
| B4E7A4 | BCAL3530 | DNA-binding protein HU-alpha | 26241.57455 | 455834.3123 | 17.37 | 4.5122 |
| B4EDT5 | BCAL2206 | Phasin-like protein | 76104.80906 | 190363.8484 | 2.50 | 2.70726 |
| B4ELF4 | BCAM2755 | Photosynthetic reaction center subunit H | 35812.27791 | 85004.40592 | 2.37 | 4.48069 |
| **Unknown function** | | | | | | |
| B4E9G8 | BCAL2769 | UPF0234 protein BceJ2315_27070 | 20164.54641 | 37887.25507 | 1.88 | 2.86778 |
| B4EFP1 | BCAM1035 | Hypothetical phage protein | 8498.740481 | 13596.28605 | 1.60 | 1.15171 |
| B4EB46 | BCAL2956 | Putative exported protein | 504685.4608 | 769600.3875 | 1.52 | 2.55665 |

[1] *Determined using the label-free quantitation algorithm of MaxQuant*
[2] *Fold change of WT/ΔpBCA054-055. Only proteins with a fold change > 1.5 are listed.*

**Table 8. Proteins significantly downregulated in stationary phase ΔpBCA017-033 cultures compared to wild type *B. cenocepacia* K56-2.**

| Accession | Gene in J2315 | Description | Average LFQ intensity[1] WT | Average LFQ intensity ΔpBCA017-033 | Fold change[2] | -Log10 P-value |
|---|---|---|---|---|---|---|
| **Energy and amino acid metabolism** | | | | | | |
| B4EJR0 | BCAM2561 | Putative 4-aminobutyrate aminotransferase | 17732.80736 | 6529.242909 | 2.72 | 2.3767 |
| B4E9X7 | BCAL1796 | Putative saccharopine dehydrogenase | 59990.64822 | 32290.00402 | 1.86 | 3.10872 |
| B4E9J0 | BCAL2791 | Kynureninase | 10936.27142 | 7144.414159 | 1.53 | 1.99818 |
| B4E5W6 | BCAL3436 | Proline--tRNA ligase | 22045.93432 | 15018.7279 | 1.47 | 2.2332 |
| B4EAG1;B4EAU3 | BCAL1862/BCAL0832 | Acetyl-CoA acetyltransferase, putative poly-beta-hydroxy-butyrate storage protein | 141805.1104 | 97001.87408 | 1.46 | 2.92688 |
| **Adhesion and motility** | | | | | | |
| B4E8L9 | BCAL0577 | Flagellar hook-associated protein 3 (HAP3) | 101542.5592 | 55030.73587 | 1.85 | 1.48989 |
| B4E8L8 | BCAL0576 | Flagellar hook-associated protein 1 (HAP1) | 84572.44106 | 48318.063 | 1.75 | 1.31122 |
| B4EDI8 | BCAL0114 | Flagellin | 3685873.174 | 2314750.071 | 1.59 | 1.95211 |
| B4E8L0 | BCAL0567 | Flagellar hook protein FlgE | 205602.7449 | 139794.6865 | 1.47 | 2.38398 |
| B4ELG0 | BCAM2761 | Giant cable pilus CblA | 92292.44921 | 58994.79282 | 1.56 | 1.41285 |
| **Stress response** | | | | | | |
| B4E7Z2 | BCAL0501 | ATP-dependent protease subunit HslV | 14350.69844 | 7034.830755 | 2.04 | 2.37821 |
| B4E9H9 | BCAL2780 | Putative exported protein (thioredoxin) | 41694.6599 | 28802.37183 | 1.45 | 2.31585 |
| **Other function** | | | | | | |
| B4EPD9 | BCAS0751 | Putative gamma-glutamyltransferase | 157420.8017 | 90242.84935 | 1.74 | 1.7418 |
| B4EG92 | BCAM1112 | Biodegradative arginine decarboxylase | 34023.05337 | 20198.35247 | 1.68 | 1.65501 |
| B4EEU0 | BCAL3351 | Dihydroorotase | 16277.03157 | 10725.32194 | 1.52 | 1.29412 |
| **Unknown function** | | | | | | |
| B4EG55 | BCAM1075 | Hypothetical phage protein | 20684.56026 | 10163.66658 | 2.04 | 1.58273 |
| B4EG48 | BCAM1068 | Putative exported phage protein | 461077.583 | 236216.4645 | 1.95 | 2.55606 |
| B4EMR4 | BCAM1931 | Putative porin | 395008.7265 | 225290.1949 | 1.75 | 3.40394 |
| B4E873 | BCAL1577 | Hypothetical phage protein | 34621.96529 | 20028.96151 | 1.73 | 0.97602 |
| B4EG49 | BCAM1069 | Hypothetical phage protein | 124167.2348 | 80306.26935 | 1.55 | 1.2493 |

[1] *Determined using the label-free quantitation algorithm of MaxQuant*
[2] *Fold change of WT/ΔpBCA054-055. Only proteins with a fold change > 1.5 are listed.*

**Table 9. Proteins significantly upregulated in stationary phase ΔpBCA017-033 cultures compared to wild type *B. cenocepacia* K56-2.**

| Accession | Gene in J2315 | Description | Average LFQ intensity[1] WT | Average LFQ intensity ΔpBCA017-033 | Fold change[2] | -Log10 P-value |
|---|---|---|---|---|---|---|
| **Energy metabolism** | | | | | | |
| B4EBY7 | BCAL0957 | Succinate--CoA ligase [ADP-forming] subunit alpha | 28334.39282 | 50498.39799 | 1.78 | 3.71169 |
| B4E9G1 | BCAL2762 | Adenylate kinase (AK) | 68973.28216 | 111342.897 | 1.61 | 4.66515 |
| B4EEY6 | BCAL0033 | ATP synthase F1 subunit delta | 42973.95686 | 64430.22445 | 1.50 | 1.65096 |
| B4EEY9 | BCAL0036 | ATP synthase F1 subunit beta | 31400.50193 | 46926.00426 | 1.49 | 2.21742 |
| **Transcription and translation** | | | | | | |
| B4E5D9 | BCAL0253 | 50S ribosomal protein L15 | 22453.19818 | 99052.10402 | 4.41 | 4.46541 |

| | | | | | | |
|---|---|---|---|---|---|---|
| B4EBH0 | BCAL1942 | 50S ribosomal protein L9 | 89526.36563 | 197504.3633 | 2.21 | 2.278 |
| B4EAR6 | BCAL0804 | 50S ribosomal protein L16 | 23259.78273 | 34360.77605 | 1.48 | 2.17431 |
| B4ECN0 | BCAL2090 | Elongation factor Ts (EF-Ts) | 53748.98908 | 151764.5172 | 2.82 | 3.93431 |
| B4E5A5 | BCAL0219/BCAL0232 | Elongation factor Tu (EF-Tu) | 233235.0364 | 363066.6777 | 1.56 | 1.0886 |
| B4E5E6 | BCAL0260 | DNA-directed RNA polymerase subunit alpha | 11489.27386 | 22927.10642 | 2.00 | 1.31349 |
| **Stress response and chaperones** | | | | | | |
| B4ECY3 | BCAL3192 | Putative oxidoreductase | 35353.53034 | 116993.9985 | 3.31 | 3.46518 |
| B4EJI6 | BCAM1495 | Putative universal stress protein | 19917.16929 | 41461.22149 | 2.08 | 3.10225 |
| B4EG36 | BCAM0050 | Universal stress-related protein | 189035.8058 | 360421.363 | 1.91 | 4.77363 |
| B4EDZ2 | BCAL3270 | Chaperone protein DnaK (Hsp70) | 129966.0245 | 520206.5359 | 4.00 | 2.18737 |
| B4EBM4 | BCAL1997 | Trigger factor (TF) | 72605.94193 | 160359.4668 | 2.21 | 3.53857 |
| B4EDZ4 | BCAL3272 | Protein GrpE (Hsp-70 cofactor) | 94001.75034 | 177292.8709 | 1.89 | 3.66529 |
| B4EDV8 | BCAL2229 | Chaperone protein DnaK (Hsp70) | 20049.79687 | 29392.28703 | 1.47 | 2.36944 |
| **Transport** | | | | | | |
| B4EF00 | BCAL0051 | Periplasmic solute-binding protein | 93034.2943 | 203338.6586 | 2.19 | 3.10889 |
| B4EGW5 | BCAM2251 | Putative amino acid solute binding component of ABC transporter | 62935.0024 | 94481.87664 | 1.50 | 2.06681 |
| **Virulence** | | | | | | |
| B4ENF7 | BCAS0409 | Zinc metalloprotease ZmpA | 102940.4166 | 183287.6431 | 1.78 | 2.3038 |
| B4EAH7 | BCAL1001/BCAL2869 | Serine protease MucD1/MucD2 | 50507.14945 | 83465.9414 | 1.65 | 2.65086 |
| **Protein folding and turnover** | | | | | | |
| B4E6W2 | BCAL0389 | Thiol:disulfide interchange protein DsbC | 61436.14193 | 112691.955 | 1.83 | 2.39516 |
| B4EBL2 | BCAL1985 | Peptidylprolyl isomerase | 75341.12733 | 130410.4541 | 1.73 | 2.11885 |
| B4EA13 | BCAL2829 | Serine peptidase, subfamily S1B | 45325.96138 | 82061.90357 | 1.81 | 2.11926 |
| **Other function** | | | | | | |
| B4E7A4 | BCAL3530 | DNA-binding protein HU-alpha | 26241.57455 | 492392.752 | 18.76 | 4.71437 |
| B4EA12 | BCAL2828 | DNA-binding protein HU-alpha | 165954.1291 | 245740.3093 | 1.48 | 1.7976 |
| B4EDC2 | BCAL3205 | Cell division coordinator CpoB | 44788.7878 | 100167.1551 | 2.24 | 3.53113 |
| B4EDT5 | BCAL2206 | Phasin-like protein | 76104.80906 | 170871.2321 | 2.25 | 2.69543 |
| **Unknown function** | | | | | | |
| B4ELF4 | BCAM2755 | Putative exported protein | 35812.27791 | 126842.1754 | 3.54 | 4.04237 |
| B4E6X6 | BCAL1411 | Putative exported protein | 97192.00349 | 242726.988 | 2.50 | 4.37907 |
| B4E9X9 | BCAL1798 | Putative exported protein | 36642.36496 | 79364.15844 | 2.17 | 2.99041 |
| B4EB46 | BCAL2956 | Putative exported protein | 504685.4608 | 1036041.24 | 2.05 | 3.83338 |
| B4EFE1 | BCAM2073 | Putative exported protein | 240837.413 | 472244.6739 | 1.96 | 4.24485 |
| B4EFA8 | BCAM0963 | Putative exported protein | 99093.30719 | 194286.3474 | 1.96 | 4.27009 |
| B4E6C7 | BCAL2401 | Putative exported protein | 21070.32578 | 40890.17641 | 1.94 | 1.95511 |
| B4ECJ5 | BCAL1033 | Putative exported protein | 48651.61244 | 92585.58681 | 1.90 | 2.90575 |
| B4E6D9 | BCAL2413 | Putative exported protein | 20015.77699 | 37659.46802 | 1.88 | 4.20723 |
| B4E9G8 | BCAL2769 | UPF0234 protein BceJ2315_27070 | 20164.54641 | 37112.04808 | 1.84 | 3.45115 |
| B4EB48 | BCAL2958 | Putative ompA family protein | 145327.6812 | 240224.7047 | 1.65 | 3.46399 |
| B4EIZ8 | BCAM1443 | Putative exported protein | 181703.2879 | 285404.7856 | 1.57 | 1.56034 |
| B4EPD8 | BCAS0750 | Putative exported protein | 45189.50082 | 70864.47735 | 1.57 | 3.59414 |
| B4EP60 | BCAS0666 | Putative ankyrin-repeat exported protein | 22690.88094 | 34725.31113 | 1.53 | 2.11879 |
| B4EBI9 | BCAL1961 | Putative exported protein | 170380.4169 | 252946.8943 | 1.48 | 2.90581 |

[1] *Determined using the label-free quantitation algorithm of MaxQuant*
[2] *Fold change of WT/ΔpBCA054-055. Only proteins with a fold change > 1.5 are listed.*

### 5.3.7 Differences in T6SS effector secretion between wild type and ΔpBCA017-059 are growth phase-dependent

To gain more insight in the dysregulation of T6SS activity in the ΔpBCA017-059 mutant, the secretion of T6SS effectors was evaluated during different stages of bacterial growth. The secretome of *B. cenocepacia* wild type and mutant strains was isolated after both 6h (exponential phase) and 24h (stationary phase) of growth. The difference in T6SS effector/virulence factor secretion between exponential and stationary phase cultures of *B. cenocepacia* wild type and ΔpBCA017-059 was assessed via targeted proteomics.

A remarkable difference in Hcp1 secretion during exponential and stationary phase was seen for the ΔpBCA017-059 mutant. There is a significant reduction in secretion of Hcp1 during stationary phase in the ΔpBCA017-059 mutant (p-value < 0.05), while in wild type cultures its secretion tends to increase with longer growth times. During exponential growth, Hcp1 secretion is significantly lower in ΔpBCA017-059 (fold change of 3.17), but not completely abolished. In the stationary growth phase however, there is very little Hcp1 secretion in the mutant, on the limit of detection. Although the difference is big compared to wild type cultures (fold change of 27.75), it is not significant due to the very high degree of biological variation in T6SS activity among the stationary phase wild type replicates. This variation is not observed for other proteins targeted during the same analysis of this condition (e.g. CblA and ZmpB). The situation for the other T6SS effector protein TecA is quite comparable (fold change of 2.97 during exponential phase and 26.37 during stationary phase), except that its abundance in the ΔpBCA017-059 mutant is similar during exponential and stationary phase (Figure 18).

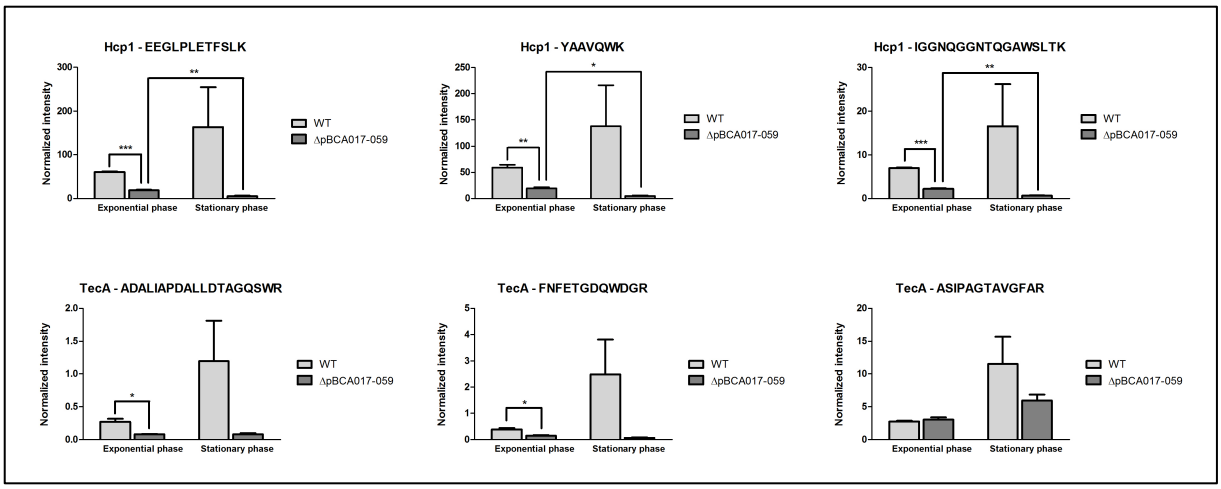


**Figure 18. Quantification of two T6SS proteins in the secretome of exponential and stationary phase cultures of wild type and ΔpBCA017-059 *B. cenocepacia* K56-2 by SRM.** Experiments were performed in triplicate. The statistical significance of the differences was assessed via two-sample t-tests. * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001.

In order to obtain a broader view regarding the virulence of this pathogen during the different phases of its growth, the secretion of other known virulence factors was also quantified for both wild type and ΔpBCA017-059 cultures. The two major metalloproteases, ZmpA and ZmpB, both show an elevated abundance in the secretome of exponential phase ΔpBCA017-059 cultures compared to wild type, with a fold change of 2.07 and 1.97, respectively (Figure 19). On top of that, the increased

abundance of ZmpA and ZmpB in the secretome of the ΔpBCA017-059 mutant is maintained during stationary phase (fold change of 1.53 and 1.78, respectively), although it is not significant for ZmpA, again due to the high degree of biological variation. The latter observation is in contrast with the observed downregulation of ZmpA in ΔpBCA017-059 in the label-free proteomics analysis.

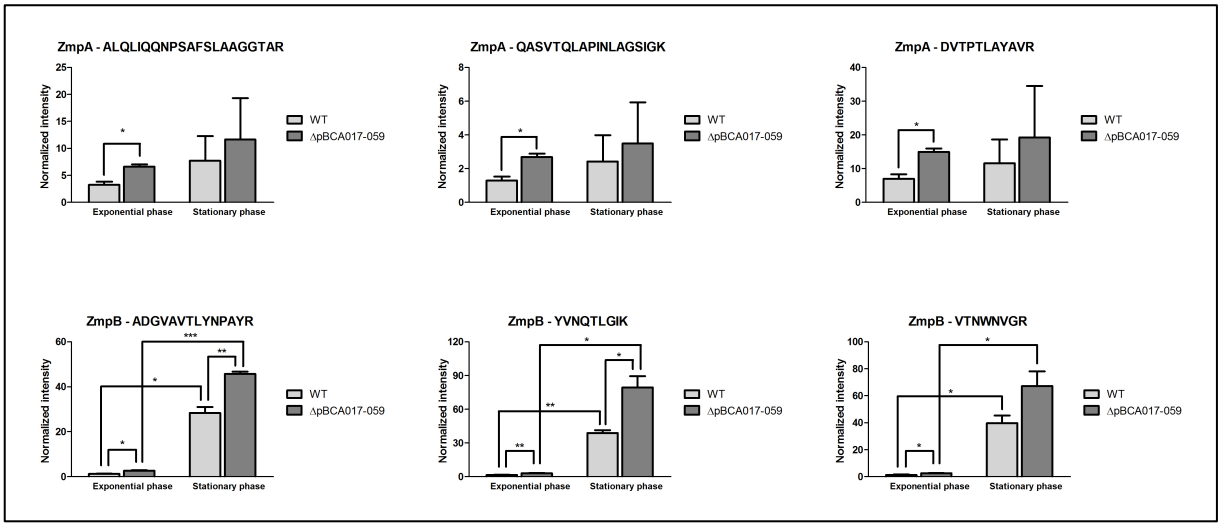


**Figure 19. Quantification of the metalloproteases ZmpA and ZmpB in the secretome of exponential and stationary phase cultures of wild type and ΔpBCA017-059 *B. cenocepacia* K56-2 by SRM.** Experiments were performed in triplicate. The statistical significance of the differences was assessed via two-sample t-tests. * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001.

Secretion of the nematocidal protein AidA is significantly higher in the ΔpBCA017-059 mutant, both in exponential and stationary phase, with a respective fold change of 4.71 and 2.93. The giant cable pilus protein has a slightly lower abundance in the ΔpBCA017-059 mutant, but this is not significant at the 5% confidence level and the fold change is small (1.20 in both exponential and stationary phase) (Figure 20).

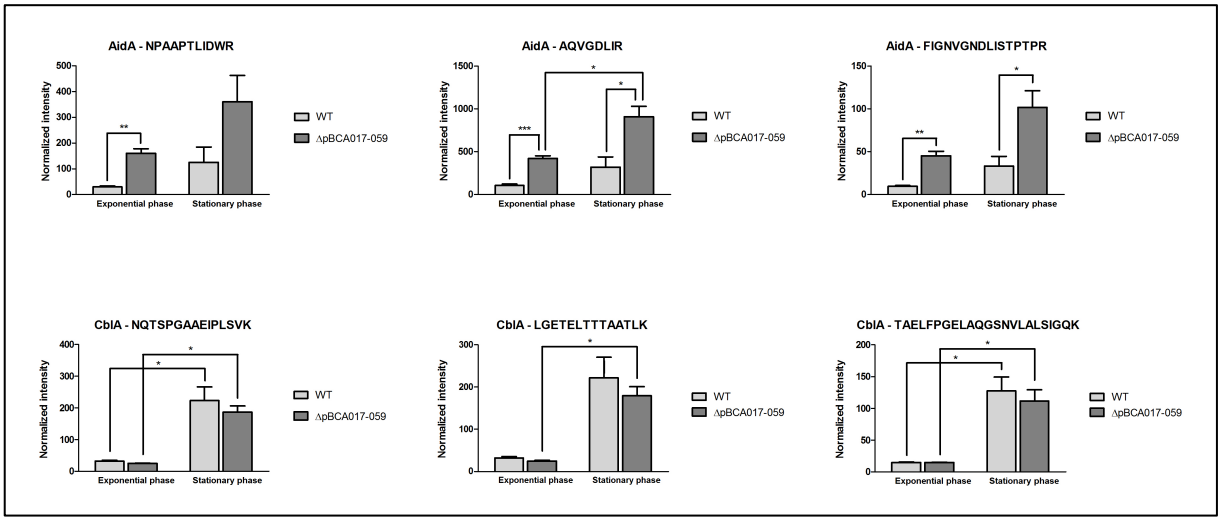


**Figure 20. Quantification of the nematocidal protein AidA and the giant cable pilus protein CblA in the secretome of exponential and stationary phase cultures of wild type and ΔpBCA017-059 *B. cenocepacia* K56-2 by SRM.** Experiments were performed in triplicate. The statistical significance of the differences was assessed via two-sample t-tests. * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001.

## 5.4 Discussion

*Burkholderia cenocepacia* is an opportunistic pathogen causing chronic lung infections in immunocompromised individuals, such as CF patients. It has the ability to damage the lung tissue and survive inside macrophages. This pathogen possesses a whole array of virulence factors aiding in invasion, survival and replication in the host. Since many of those act on the extracellular environment or directly inside host cells, it is crucial to gain more knowledge on the different secretion mechanisms used. The plasmid-encoded T4SS-1 is capable of injecting effector proteins directly into the host cell and has been proven to be a valuable weapon, since it contributes to intracellular survival of *B. cenocepacia* in host cells and has been linked to the plant tissue watersoaking phenotype caused during plant infection (Sajjan et al., 2008a; Zhang et al., 2009). However, no detailed study on secretome level has been performed to decipher the exact role of the plasmid-encoded T4SS-1 in protein secretion. On top of that, the reason why the T4SS-1 contributes to the *in vivo* virulence of *B. cenocepacia* K56-2 remains to be elucidated. Therefore, we tried to characterize the function of the T4SS-1 (and of the plasmid in general) in more detail.

In this study, the secretome of a *B. cenocepacia* K56-2 strain with a deletion of the complete T4SS-1 (ΔpBCA017-059), but also lacking the gentamicin resistance pump (MHK background), was compared to that of the wild type strain to gain deeper insight in the function of this plasmid region. The mutant strain surprisingly displayed an impaired secretion of two T6SS proteins, Hcp1 and TecA, in stationary phase cultures. Since the pBCA017-059 deleted region also contains genes that are not directly required for T4SS-1 structure or function, we postulated that this gene region might contain one or more genes involved in the regulation of T6SS activity. A closer look on this region revealed the deletion of an operon that might be linked to QS, pBCA054-055. The first gene of this operon, pBCA054, is a LuxR family regulatory protein, while pBCA055 is a diguanylate cyclase that might be involved in the regulation of cellular cyclic-di-GMP levels. Since it has been shown that T6SS activity is subject to QS-dependent regulation (Chambers et al., 2006; O'Grady et al., 2009c), it was worthwhile to further investigate whether this operon plays a role in the secretion of T6SS proteins. Therefore, a novel mutant was created in which the pBCA054-055 operon was deleted. Further, a "true" T4SS-1 deletion mutant (ΔpBCA017-033) was made, where only structural T4SS-1 genes were removed, leaving intact any potential regulatory genes, to confirm that the impaired secretion of Hcp1 and TecA was not due to the absence of a functional T4SS-1. However, both new mutants did not show any reduction in the secretion of these T6SS proteins, nor did they explain the significant upregulation of the nematocidal protein AidA, that was seen in the original ΔpBCA017-059 mutant. Our original observation that T4SS-1 deletion (ΔpBCA017-059) resulted in impaired T6SS activity could thus not be confirmed. There could be several explanations for this. Firstly, other genes that are not contained in the 'new' mutants could affect T6SS activity. However, we have checked the functions of these genes, and could not convincingly link those to the T6SS (Supplementary Table S1). A more plausible explanation could be that the MHK strain has drifted from the original K56-2 strain and gathered other mutations. We observed changes in both T6SS and T2SS secreted proteins, both of which are QS-dependent. It could have been that the MHK strain has collected partial QS-defective phenotypes (Prof. M. Valvano, pers. communication).

The label-free proteomics study comparing the secretome of wild type and ΔpBCA017-059 *B. cenocepacia* also revealed the significant decrease in the abundance of phage proteins in the

secretome of the ΔpBCA017-059 mutant. The majority of these phage proteins lies within a phage cluster in the genome of J2315, presumably ranging from BCAM1052 to BCAM1097. The proteins encoded in this phage region show significant homology to proteins from the *Burkholderia* phage Bups phi 1, a yet uncharacterized phage, and to a lesser extent to the *Acinetobacter* phage Ab105-1phi and the *Salmonella* phage SEN34. When comparing the secretome of wild type *B. cenocepacia* K56-2 with that of the shorter T4SS-1 mutant (ΔpBCA017-033), again three phage proteins lying within this cluster were found to be significantly less abundant in the secretome of the mutant. Also, proteins from another phage cluster present on chromosome I are downregulated in both mutant strains. This cluster represents the Mu-like phage KS10, ranging from bp 1,766,551 to bp 1,728,918 on chromosome I of *B. cenocepacia* J2315 (Goudie et al., 2008). It thus seems that the ΔpBCA017-059 and ΔpBCA017-033 mutants are defective in prophage induction, indicating that this observation is not biased by the original MHK host.
A link between type IV secretion and phage recognition has recently been described (Berry and Christie, 2011; Lang et al., 2010), although this does not explain the decreased production of phages in the mutant. The absence of a plasmid-encoded phage inducer or antirepressor might account for the observed decrease in phage production in the ΔpBCA017-059 and ΔpBCA017-033 mutants, but has not been identified so far.

The reduced biofilm formation potential of the ΔpBCA017-059 mutant might, at least in part, be due to deletion of the regulatory operon pBCA054-055, since deletion of these two genes, while leaving the T4SS-1 intact, resulted in a significantly lower degree of biofilm formation and an aberrant biofilm morphology. A plausible explanation for this observation is the fact that pBCA055 encodes a diguanylate cyclase. Diguanylate cyclases contain a GGDEF (Gly-Gly-Asp-Glu-Phe) domain and catalyze the conversion of guanosine triphosphate to the second messenger cyclic-di-GMP, which promotes biofilm formation (Boyd and O'Toole, 2012; Ryan, 2013). Deletion of the diguanylate cyclase causes a reduction in the intracellular cyclic-di-GMP level and consequently a lower amount of biofilm is formed. The aberrant biofilm morphology observed for the ΔpBCA017-033 might point to a biofilm maturation defect. Since prophages have previously been linked to biofilm formation (Carrolo et al., 2010; Nanda et al., 2015; Petrova et al., 2011; Rice et al., 2009; Webb et al., 2004), a possible explanation for the aberrant biofilm morphology in the ΔpBCA017-033 mutant might come from its deficiency to induce its genome-encoded phages. However, further research is needed to confirm these hypotheses.

**Supplementary Table S1. Annotation of genes encoded on the 92-kb plasmid of *B. cenocepacia* K56-2.**

| Gene in J2315 | Gene in K56-2 | Annotation in K56-2 (if not available, in J2315) | Conserved domains |
|---|---|---|---|
| x | BURCENK562V_RS34490 | Type I restriction protein of type I restriction-modification system | Type I site-specific restriction-modification system, R (restriction) subunit and related helicases |
| pBCA083 | BURCENK562V_RS34495 | Transposase | HTH superfamily |
| pBCA084 | BURCENK562V_RS34500 | Transposase | HTH21 superfamily, RVE superfamily: integrase core domain |
| x | BURCENK562V_RS34505 | Carbamoyltransferase domain protein | Carbamoyltransferase C-terminus |
| pBCA086 | BURCENK562V_RS34510 | UDP-N-acetylmuramate dehydrogenase | MurB_C superfamily |
| pBCA088 | BURCENK562V_RS34515 | Hypothetical protein | Metallo-dependent hydrolases superfamily |
| pBCA090 | BURCENK562V_RS34520 | Integrase | XerC superfamily |
| pBCA091 | BURCENK562V_RS34525 | Hypothetical protein | NO |
| pBCA092 | BURCENK562V_RS34530 | Hypothetical protein | NO |
| pBCA093 | BURCENK562V_RS34535 | Transcriptional regulator | PIN domain |
| pBCA094 | BURCENK562V_RS34540 | Twitching motility protein PilT | MazE_antitoxin domain |
| pBCA095 | BURCENK562V_RS34545 | DNA polymerase | LigD superfamily |
| pBCA001 | BURCENK562V_RS34550 | Chromosome partitioning protein | PRK13869 superfamily |
| pBCA002 | BURCENK562V_RS34555 | ParB-like protein | SpoOJ superfamily |
| pBCA003 | BURCENK562V_RS34560 | TrfA protein | Rep_3 superfamily |
| pBCA004 | BURCENK562V_RS34565 | DNA polymerase | DnaE domain |
| pBCA005 | BURCENK562V_RS34570 | DNA polymerase | PolY domain |
| pBCA006 | BURCENK562V_RS34575 | Cell division protein | SulA domain |
| pBCA007 | BURCENK562V_RS34580 | RNA-directed DNA polymerase | RVT_1 superfamily |
| pBCA008 | BURCENK562V_RS34585 | Hypothetical protein | SRAP domain |
| pBCA009 | BURCENK562V_RS34590 | Hypothetical protein | NO |
| pBCA010 | BURCENK562V_RS34595 | Hypothetical protein | NO |
| pBCA011 | BURCENK562V_RS34600 | Hemagglutinin | YadA domain |
| pBCA012 | BURCENK562V_RS34605 | Hypothetical protein | NO |
| pBCA013 | BURCENK562V_RS34610 | Hypothetical protein | NO |
| pBCA014 | BURCENK562V_RS34615 | Hypothetical protein | Pleckstrin-homology like domain |
| pBCA015 | BURCENK562V_C0072 | Hypothetical protein | NO |
| pBCA016 | BURCENK562V_RS34620 | Hypothetical protein | DUF1173 domain |
| pBCA017 | BURCENK562V_RS34625 | Toxin | Zeta toxin domain |
| pBCA018 | BURCENK562V_RS34630 | Hypothetical protein | NO |
| pBCA019 | BURCENK562V_RS34635 | Hypothetical protein | NO |
| pBCA020 | BURCENK562V_RS34640 | Conjugal transfer protein TraG | TraG domain |
| pBCA021 | BURCENK562V_RS34645 | Conjugal transfer protein TraH | TraH domain |
| pBCA022 | BURCENK562V_RS34650 | Hypothetical protein | DUF5131 domain |
| pBCA023 | BURCENK562V_RS34655 | HNH endonuclease | HNHc superfamily |
| pBCA024 | BURCENK562V_RS34660 | Hypothetical protein | NO |
| pBCA025 | BURCENK562V_RS34665 | Conjugal transfer protein TraF | TraF domain |
| pBCA026 | BURCENK562V_RS34670 | Hypothetical protein | NO |

| | | | |
|---|---|---|---|
| pBCA026a | BURCENK562V_RS34675 | Hypothetical protein | NO |
| pBCA027 | BURCENK562V_RS34680 | Conjugal transfer protein TraN | TraN domain |
| pBCA028 | BURCENK562V_RS34685 | Hypothetical protein | TraN domain |
| pBCA029 | BURCENK562V_RS34690 | Hypothetical protein | NO |
| pBCA030 | BURCENK562V_RS34695 | Conjugative transfer protein | TrbC_F-type superfamily |
| pBCA031 | BURCENK562V_RS34700 | Conjugal transfer protein TraU | TraU domain |
| pBCA032 | BURCENK562V_RS34705 | Conjugal transfer protein TraW | TraW domain |
| pBCA033 | BURCENK562V_RS34710 | Peptidase S26 | Peptidase S26 domain |
| pBCA034 | BURCENK562V_RS34715 | Putative lipoprotein | NO |
| pBCA035 | BURCENK562V_RS34720 | GntR family regulatory protein | MngR superfamily, FadR domain, HTH DNA-binding domain, C-terminal effector binding domain |
| pBCA036 | BURCENK562V_RS34725 | Endonuclease | PLDc domain, cardiolipin synthase |
| pBCA037 | BURCENK562V_RS34730 | Hypothetical protein | NO |
| pBCA037a | BURCENK562V_RS34735 | Hypothetical protein | NO |
| pBCA038 | BURCENK562V_RS34740 | Hypothetical protein | NO |
| pBCA039 | x | Hypothetical protein | NO |
| x | BURCENK562V_RS34745 | Hypothetical protein | NO |
| pBCA040 | x | Hypothetical protein | NO |
| x | BURCENK562V_RS34750 | Hypothetical protein | NO |
| pBCA041 | BURCENK562V_RS34755 | Conjugal transfer protein TraC | VirB4 superfamily |
| pBCA042 | BURCENK562V_RS34760 | Type IV conjugative transfer system lipoprotein TraV domain protein | TraV superfamily |
| pBCA043 | BURCENK562V_RS34765 | Disulfide isomerase | Thioredoxin-like superfamily, DsbA, DsbC, DsbG |
| pBCA044 | BURCENK562V_RS34770 | Conjugal transfer protein TraI | TraB, VirB10-like superfamily |
| pBCA045 | BURCENK562V_RS34775 | Hypothetical protein | TraK superfamily |
| pBCA046 | BURCENK562V_RS34780 | Putative type IV conjugative transfer system protein TraE | TraE superfamily |
| pBCA047 | BURCENK562V_RS34785 | Conjugal transfer protein TraL | TraL superfamily |
| pBCA048 | BURCENK562V_RS34790 | Hypothetical protein | NO |
| pBCA048a | BURCENK562V_C0101 | Hypothetical protein | NO |
| pBCA049 | BURCENK562V_RS34795 | Lytic transglycosylase | Lysozyme-like superfamily |
| pBCA050 | BURCENK562V_RS34800 | Hypothetical protein | NO |
| pBCA051 | BURCENK562V_RS34805 | LamB/YcsF family protein | LamB/YdjC superfamily |
| pBCA052 | x | Hypothetical protein | NO |
| pBCA053 | BURCENK562V_RS34810 | C4-dicarboxylate ABC transporter substrate-binding protein | Periplasmic-binding-protein type 2 superfamily |
| pBCA054 | BURCENK562V_RS34815 | Transcriptional regulator, LuxR family | HTH superfamily |
| pBCA055 | BURCENK562V_RS34820 | Diguanylate cyclase (GGDEF) domain protein | dCache1 domain, nucleotidyl_cyc_III superfamily |
| pBCA056 | BURCENK562V_RS34825 | Hypothetical protein | NO |
| pBCA057 | BURCENK562V_RS34830 | ATPase AAA | TraI superfamily |
| pBCA058 | x | x | x |
| x | BURCENK562V_RS34835 | Disulfide bond corrector protein DsbC | DsbC superfamily |
| pBCA059 | BURCENK562V_RS34840 | Type VI secretion protein | TrwD_AAD_bind superfamily |
| pBCA060 | x | Hypothetical protein | NO |

| | | | |
|---|---|---|---|
| pBCA061 | x | Hypothetical protein | NO |
| pBCA062 | BURCENK562V_RS34845 | Hypothetical protein | UFD1 superfamily |
| pBCA063 | BURCENK562V_C0110 | Hypothetical protein | NO |
| pBCA064 | BURCENK562V_RS34850 | Hypothetical protein | NO |
| pBCA065 | BURCENK562V_RS34855 | Hypothetical protein | DUF3717 domain |
| pBCA066 | BURCENK562V_RS34860 | Hypothetical protein | NO |
| pBCA067 | BURCENK562V_RS34865 | Hypothetical protein | NO |
| pBCA068 | BURCENK562V_RS34870 | Nuclease | ParB like domain, SpoOJ domain |
| pBCA069 | BURCENK562V_RS34875 | Hypothetical protein | NO |
| pBCA070 | BURCENK562V_C0117 | Hypothetical protein | NO |
| pBCA071 | BURCENK562V_RS34880 | Hypothetical protein | NO |
| pBCA072 | BURCENK562V_RS34885 | DNA-methyltransferase | AdoMet_MTases superfamily |
| pBCA073 | BURCENK562V_RS34890 | Hypothetical protein | NO |
| pBCA074 | BURCENK562V_RS34895 | Hypothetical protein | NO |
| pBCA075 | BURCENK562V_RS34900 | Hypothetical protein | NO |
| pBCA076 | BURCENK562V_RS34905 | Hypothetical protein | DUF945 domain |
| pBCA077 | x | Hypothetical protein | NO |
| pBCA078 | BURCENK562V_RS34910 | Hypothetical protein | NO |
| x | BURCENK562V_RS34915 | Hypothetical protein | NO |
| pBCA079 | x | Hypothetical protein | NO |
| pBCA080 | x | Hypothetical protein | NO |
| x | BURCENK562V_RS34920 | Hypothetical protein | NO |
| pBCA083 | BURCENK562V_RS34925 | Transposase | HTH superfamily |

# 6 *DIFFUSIBLE SIGNAL FACTORS ACT AS INTRA- AND INTERSPECIES COMMUNICATION MOLECULES IN* BURKHOLDERIA CENOCEPACIA *K56-2*

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**AUTHOR CONTRIBUTIONS**

Sofie Depluverez wrote the entire chapter and performed all the experiments. The quantification of endogenous BDSF production was performed in the framework of the master thesis of Jolien Schoovaerts. Bart Devreese edited the chapter and is the supervisor of the author.

**ABSTRACT**

Although the diffusible signal factor (DSF) family of quorum sensing (QS) molecules is widespread among Gram-negative bacteria, the current knowledge of the different processes controlled by this system is rather limited compared to the extensively characterized acylhomoserine lactone QS system. *Burkholderia*'s own DSF (cis-2-dodecenoic acid, BDSF) is known to be involved in both intra- and interspecies communication, with effects on cable pilus expression, biofilm formation and metalloprotease production. However, no proteome-wide characterization of the effects of BDSF on *Burkholderia* itself, nor the compilation of a comprehensive profile of the effects of DSFs from other species has been performed yet. First, we determine the profile and concentration of endogenous BDSF production by planktonically grown *B. cenocepacia* K56-2 using reversed-phase HPLC analysis. Production peaks in late-exponential phase with a maximum concentration of ± 1 µM. The observed profile also points to the existence of a BDSF turnover mechanism, similar to that observed in *Xanthomonas campestris* pv. *campestris*. In this study, we also investigate the working concentration of BDSF and the effect of different DSFs on the secretion of virulence factors in wild type *B. cenocepacia* K56-2 using label-free proteomics and selected reaction monitoring (SRM). Rather high DSF concentrations (75 - 100 µM) were required to observe differences in protein secretion under the growth conditions applied here. Effects include an increased secretion of the metalloprotease ZmpA, the nematocidal protein AidA and a GDSL family lipase, as well as a decrease of T6SS activity and biofilm formation. DSFs from *Stenotrophomonas* and *Pseudomonas* induce similar responses as BDSF in *B. cenocepacia* K56-2, although the magnitude of the observed effects is in general smaller. This further underscores the importance of DSFs in the regulation of virulence in *B. cenocepacia* K56-2. Moreover, the SRM assays developed during this work serve as valuable screening tools for the quantification of virulence factor production in a wide variety of conditions.

## 6.1 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 (Gibson et al., 2003). 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* (Cystic Fibrosis Foundation, 2014). 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 (Schwab et al., 2014; Stressmann et al., 2011). 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 (Bragonzi et al., 2012a). *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) (Ryan et al., 2008). DSFs are an important class of quorum sensing molecules that were first described in the plant pathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*) (Fouhy et al., 2007; Wang et al., 2004). 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 (Deng et al., 2010; Ryan et al., 2015a; Ryan and Dow, 2008, 2011). 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* (Twomey et al., 2012). 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 regarding interspecies communication through DSFs is presented in Figure 7 in section 2.5.3.

In *B. cenocepacia*, the $rpfF_{Bc}$ (BCAM0581) gene encodes an enoyl-CoA hydratase with both dehydratase and thioesterase activity, which enables the conversion of the acyl carrier protein thioester of 3-hydroxydodecanoic acid into cis-2-dodecenoic acid (Bi et al., 2012; Boon et al., 2008; Deng et al., 2011). Intermediates of the fatty acid biosynthesis pathway presumably serve as the substrates for $RpfF_{Bc}$ *in vivo* (Ryan et al., 2015a). The gene adjacent to $rpfF_{Bc}$ , BCAM0580, encodes the BDSF receptor protein RpfR (Deng et al., 2012). 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 (Römling et al., 2005). 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 (Deng et al., 2012). 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 (Ryan et al., 2012; Sondermann et al., 2012). Cyclic-di-GMP promotes biofilm formation and T6SS activity, and reduces motility and invasion (Romling et al., 2013). A second receptor protein, encoded by BCAM0227, appears to control only a subset of the BDSF-regulated genes (McCarthy et al., 2010). 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 (Deng et al., 2009, 2012; Ryan et al., 2009a), 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 (McCarthy et al., 2010).

The aim of this study was to investigate the effect of different diffusible signal factors on *B. cenocepacia* K56-2, a strain belonging to the ET12 lineage, the causative of cepacia syndrome in people with CF (Sajjan et al., 2008b). Since BDSF acts as an intraspecies signal that regulates cable pilus expression and metalloprotease production (Deng et al., 2009; Ryan et al., 2009), we investigated the effect of this quorum sensing molecule and its structural analogs DSF and PDSF, on the secretome of *B. cenocepacia* K56-2. First, we quantified the endogenous levels of BDSF produced by this strain, since no absolute values of extracellular BDSF concentrations are currently available. Quantification of BDSF in the culture supernatant of *B. cenocepacia* K56-2 was achieved through reversed-phase HPLC analysis. The concentration range in which exogenously added BDSF causes significant effects on known secreted virulence factors was determined using selected reaction monitoring (SRM). Label-free proteomic approaches were additionally performed to detect differences in the abundance of other 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 was assessed using SRM. Results show that under the growth conditions applied in this study, DSF affected the regulation of virulence-associated processes only at seemingly non-physiological concentrations. The SRM assays developed during this work, however, display large potential as screening tools to quantify virulence protein production in a wide variety of conditions. This method provides a rapid and easy way to quantify selected secreted proteins of interest more accurately.

## 6.2 Material and methods

**Chemicals and Solvents**

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

**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 $OD_{600}$ = 0.2 in fresh LB and grown for 5h. At this time point, cultures were stimulated with BDSF, PDSF or DSF (dissolved in methanol) at the appropriate concentration and were harvested after 30 min, 1h or 2h. Unstimulated cultures were supplemented with equal amounts of methanol and harvested immediately for time-dependent experiments or after two hours for all other experiments.

**Extraction and quantification of DSFs from culture supernatant**

DSFs were extracted according to Deng et al. (Deng et al., 2010). 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) was performed on an Ettan LC system (GE Healthcare Life Sciences) equipped with an Acclaim PepMap100 C18 column (3 µm, 250 mm x 75 µm) (Thermo Scientific) with as solvent A 0.1% TFA in $H_2O$ (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 concentration present in the different samples. Calibration solutions (0.1, 0.5, 1.0, 2.5, 5.0 and 10.0 ng/µl) were prepared by serial dilution of the individual stock solutions with water. Ten µL 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 µm 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).

**1D-LC-MS$^E$ analysis on a Synapt G1 mass spectrometer**

Proteins were reduced with dithiotreitol, alkylated with iodoacetamide and digested with sequencing-grade modified trypsin (Promega). Peptide mixtures (50 ng/µl in 0.1% HCOOH) were separated on a NanoAcquity UPLC® system (Waters Corporation, Milford, MA) operated in 1D mode. Solvent A and B were composed of 0.1% formic acid in water and 0.1% formic acid in acetonitrile (ACN), respectively. The sample (250 ng) was trapped on a Symmetry® C18 trapping column (5 µm, 180 µm x 20 mm; Waters) for 5 min at a flow rate of 5 µl/min. Peptides were separated on an Acquity UPLC® M-Class HSS T3 column (1,8 µm, 75 µm x 250 mm; Waters) at 40°C at 300 nl/min by increasing the acetonitrile concentration from 3% to 50% B over 45 min. The outlet of the column was directly connected to a PicoTip Emitter (uncoated SilicaTip™ 10 +/- 1 µm, New Objective) mounted on a Nanolockspray source of a SYNAPT™ G1 HDMS mass spectrometer (Waters). The time-of-flight (TOF) analyzer was externally calibrated with MS/MS fragments of human [Glu$^1$]-Fibrinopeptide B (Glu-Fib) from *m/z* 72 to 1285, and the data was corrected post-acquisition using the monoisotopic mass of the doubly charged precursor of Glu-Fib (*m/z* 785.8426) (lock mass correction). Accurate mass data were collected in a data independent positive mode of acquisition (MS$^E$) by alternating between low (5 V) and high (ramping from 15 to 35 V) energy scan functions. The selected *m/z* range was 125 to 2000 Da. The capillary voltage was set to 3.0 kV, the sampling cone voltage was 26 V and the extraction cone voltage 4 V. The source temperature was set on 80 °C.

**LC-MS$^E$ data analysis**

Identification of the proteins was performed with a strain-specific *B. cenocepacia* K56-2 database (7672 entries, downloaded from https://www.ncbi.nlm.nih.gov, including common contaminants) using the ProteinLynx Global Server version 4.1 software package. After an assessment query, the software automatically sets the peptide and fragment mass tolerances. The final mass errors were found to be ± 10 ppm for the precursor and ± 20 ppm for the product ions, respectively, which is achieved by lock mass correction using the monoisotopic value 785.8426 of the double charged precursor mass of [Glu$^1$]-Fibrinopeptide B and operating the instrument at a resolution of at least 10.000 FWHM. Peptide and protein identifications were performed by setting the minimal number of fragment ion matches per peptide to 5 and that per protein to 10. The minimal peptide match per protein was set to 2. Two missed trypsin cleavages were allowed and the tolerated modifications were oxidation of methionine and carbamidomethylation of cysteine. The protein false positive rate was set to 4%.

Protein quantification was performed using the Progenesis LC-MS software package (Nonlinear Dynamics), which uses multivariate statistics to assess differences in protein abundance via chromatographic peak patterns. The 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.

**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 µl/min and separated on a YMC Triart C18 column (3 µm, 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 µl/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 analysis**

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.
The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2016) partner repository with the dataset identifier PXD007057 and 10.6019/PXD007057.

**Selected Reaction Monitoring (SRM)**

Validation of differential protein analysis using LC-$MS^E$ was performed using a targeted proteomics approach implementing 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. 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 µg sample).

The peptide mixtures were analyzed on a M-class IonKey Xevo TQ-S system (Waters). Peptides were loaded on a M-class Trap Symmetry® C18 column (100 Å, 5 µm, 300 µm x 50 mm) (Waters) using a flow rate of 15 µl/min for 5 min. After trapping, the peptides were separated on an iKey HSS T3 analytical column (1.8 µm, 150 µm x 100 mm) (Waters) at a flow rate of 1 µl/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 x precursion ion m/z) + 5.

**SRM data analysis**

Data were imported into Skyline (MacLean et al., 2010a, 2010b) 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.

**Biofilm formation and quantification**

Overnight *B. cenocepacia* K56-2 cultures were diluted to an $OD_{600}$ = 0.4 in LB. For each biofilm experiment, the wells of a flat-bottomed polystyrene 96-well microtiter plate were inoculated with 100 μl of this dilution. Twelve wells were assayed per condition: either unstimulated or stimulated with 500 μM BDSF, 500 μM DSF or 500 μM PDSF. Three independent experiments were performed. After 24h of biofilm formation, the supernatant was removed and the wells were rinsed with 100 μl PBS. For fixation of the biofilms, 100 μl 99% MeOH was added (15 min), after which the supernatants were removed and the plates were air-dried. Then, 100 μl of a 0.1% crystal violet solution was added to all wells. After 20 min, the excess crystal violet was removed by washing the plates under running tap water. Finally, bound crystal violet was released by adding 150 μl of 33% acetic acid. The absorbance was measured at 595 nm. All steps were carried out at room temperature.

## 6.3 Results

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

To determine the concentration of BDSF released in the 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 (Figure 21). 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.

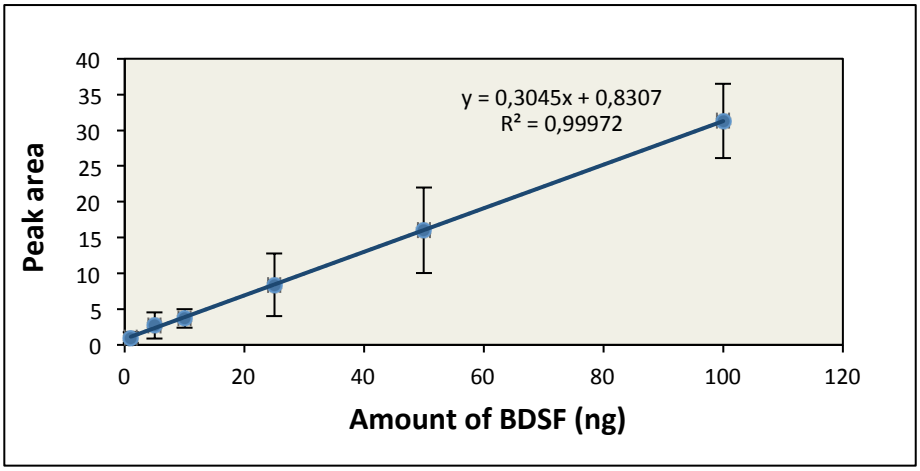



**Figure 21. BDSF standard curve.** Known amounts of synthetic BDSF were injected onto the HPLC system. The corresponding peak areas of three replicates were determined using the Unicorn software package. The equation and the corresponding $R^2$ value of the best fitting trendline is displayed on the chart.

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 $OD_{600}$ values at the time of extraction are plotted in function of the growth time (Figure 22). 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 four hours, but after 10 h total growth time, the concentration sharply declines to ± 600 nM and further to its basal level of ± 400 nM.

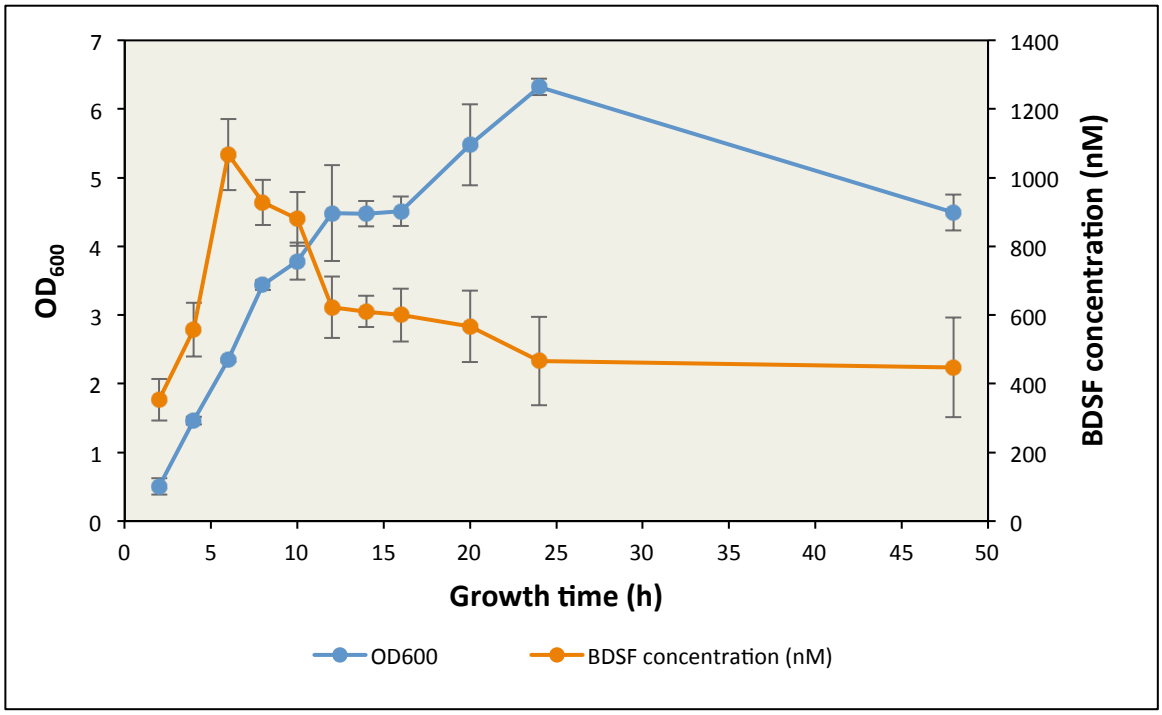



**Figure 22. BDSF concentrations in the culture supernatant of *B. cenocepacia* K56-2 cultures and corresponding $OD_{600}$ values in function of the growth time.** Values from three biological replicates with corresponding standard deviations are displayed on the graph.

### 6.3.2 Exogenously added BDSF only causes significant effects on the secretome at concentrations higher than 75 µM

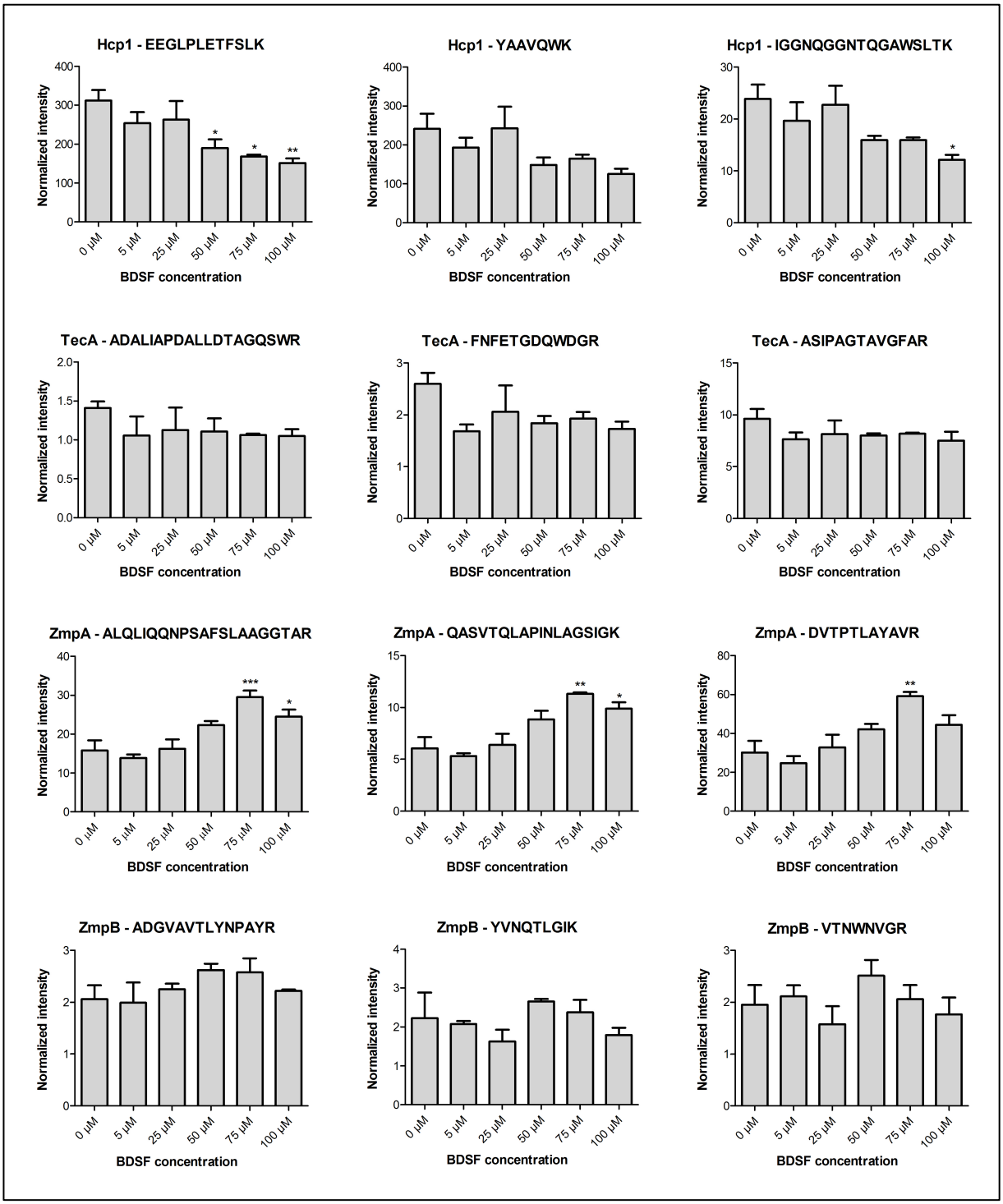
To obtain more insight in the effects of exogenous BDSF, different BDSF concentrations were added to exponential phase *B. cenocepacia* K56-2 cultures, starting from 5 µM BDSF up to 100 µM BDSF. These high concentrations were chosen to overcome the endogenous production of BDSF and to take into account the likelihood that local concentrations experienced by e.g. cells growing in a biofilm may exceed the production levels observed in the previous experiment. 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) for each BDSF concentration 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. 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 µM BDSF). Results are summarized in Figure 23.

A significant increase in abundance of ZmpA was detected upon stimulation with BDSF at a concentration of at least 75 µM. This resulted in a fold change of 1.90 compared to unstimulated cultures. Stimulation with 100 µM BDSF caused a 1.5-fold increase in ZmpA secretion. The abundance of the other type II secretion system (T2SS)-dependent metalloprotease, ZmpB, is not affected by addition of BDSF. The nematocidal protein AidA 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 µM BDSF (fold change of 1.75), comparable to the profile observed for ZmpA. Stimulation with 100 µM BDSF resulted in a fold change of 1.47 compared to unstimulated cultures.

Secretion of the T6SS protein Hcp1 was negatively influenced by BDSF, since addition of 100 µM 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 µM BDSF. TecA, a recently identified T6SS effector, did not show a significant decrease in secretion upon BDSF stimulation (fold change of 1.4 upon stimulation with 100 µM BDSF).

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


Hcp1 - EEGLPLETFSLK
Hcp1 - YAAVQWK
Hcp1 - IGGNQGGNTQGAWSLTK
TecA - ADALIAPDALLDTAGQSWR
TecA - FNFETGDQWDGR
TecA - ASIPAGTAVGFAR
ZmpA - ALQLIQQNPSAFSLAAGGTAR
ZmpA - QASVTQLAPINLAGSIGK
ZmpA - DVTPTLAYAVR
ZmpB - ADGVAVTLYNPAYR
ZmpB - YVNQTLGIK
ZmpB - VTNWNVGR
Normalized intensity
BDSF concentration
0 µM
5 µM
25 µM
50 µM
75 µM
100 µM

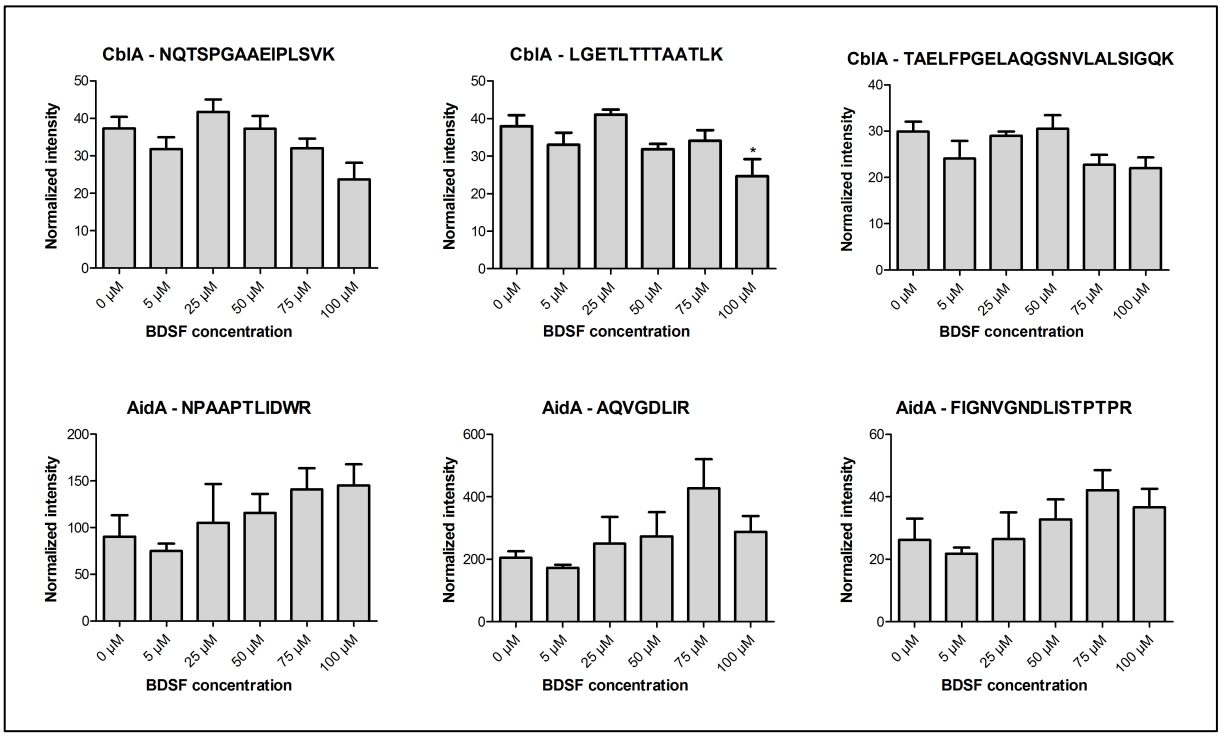



**Figure 23. Concentration-dependent effect of BDSF on the secretion of selected virulence factors in *B. cenocepacia* K56-2.** Exponential phase *B. cenocepacia* K56-2 cultures were stimulated with different concentrations of BDSF and grown for an additional 2 hours in the presence of the stimulus. The experiment was performed in triplicate. 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 compariosn tests. * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001 compared to the unstimulated control.

### 6.3.3 BDSF positively regulates protease production and motility and reduces T6SS activity

To further investigate the effect of BDSF on the secretome of *B. cenocepacia* K56-2, mid-exponential phase cultures were stimulated with 100 µM BDSF and the secretome was isolated after 30 min (T1), 1 hour (T1) or 2 hours (T2). Unstimulated cultures (T0) were used as a control. Secreted proteins from each time point were quantified by 1D-LC-$MS^E$ on a nanoAcquity UPLC system coupled to a Synapt G1 mass spectrometer. A total of 12 proteins showed a time-dependent upregulation upon BDSF stimulation, including the zinc metalloprotease ZmpA, flagellin, elongation factor Tu, chaperonin GroL, three ABC transporter substrate-binding proteins, a DNA-binding regulatory protein of the YebC/PmpR family, three metabolic enzymes and a hypothetical protein. Proteins displaying a time-dependent downregulation after stimulation include several chaperones and metabolic enzymes, as well as a porin and the T6SS protein Hcp1 (Figure 24).

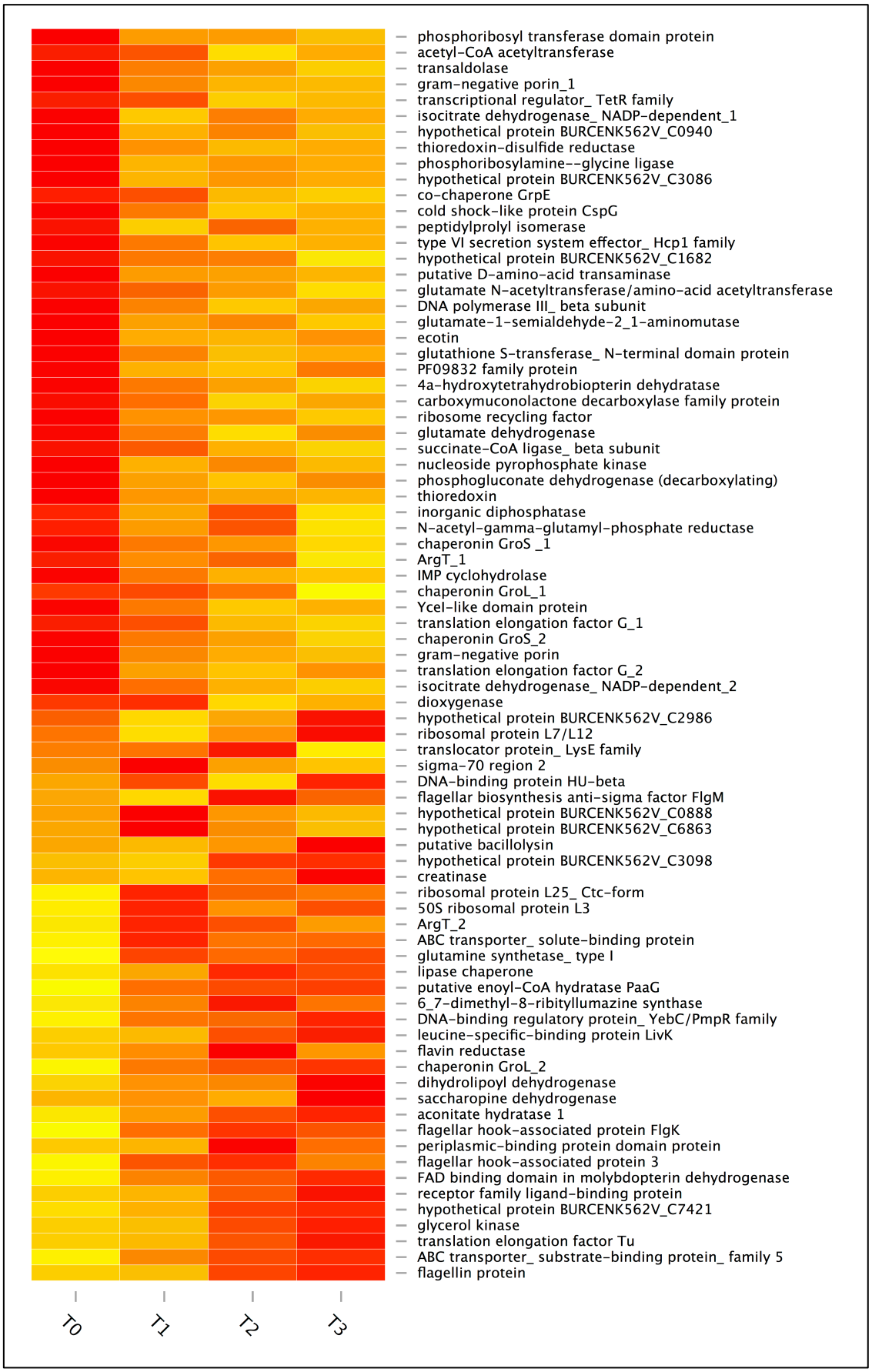



**Figure 24. Time-kinetic effect of BDSF stimulation on the secretome of *B. cenocepacia* K56-2 cultures.** Secreted proteins were quantified using LC-MS$^E$ on a Synapt G1 mass spectrometer. The secretome was analyzed immediately (T0) or 30 min (T1), 1h (T2) or 2h (T3) after stimulation. Yellow to red = low to high abundance.

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

To investigate the effect of BDSF on the secretome of *B. cenocepacia* K56-2 in more depth, mid-exponential phase cultures were stimulated with 100 μM 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 1D-LC-MS/MS using a TripleTOF 5600 mass spectrometer, operated in data-dependent mode. When cultures were stimulated with 100 μM BDSF, two T6SS proteins, Hcp1 and TecA, showed a 2-fold reduction in secretion (Table 10). Also, secretion of a putative exotoxin (BCAM1074) was significantly reduced in this condition. This protein contains a signal peptide (SignalP 4.1) and is predicted to be secreted (Phobius), 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 μM BDSF.

A remarkable observation is the significant increase in abundance of numerous ribosomal proteins upon BDSF stimulation (Table 11). 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.

**Table 10. Proteins significantly downregulated upon stimulation of *B. cenocepacia* K56-2 cultures with 100 μM BDSF.**

| Accession | Gene in J2315 | Protein name | Average normalized abundance Control | Average normalized abundance 100 μM BDSF | Fold change[1] | P-value |
|---|---|---|---|---|---|---|
| **Stress response and chaperones** | | | | | | |
| EPZ87343.1 | BCAL2013 | Peroxiredoxin, AhpC/TSA family protein | 658838.7605 | 420541.4191 | 1.57 | 0.016015818 |
| EPZ86665.1 | BCAL1233 | Hsp20/alpha crystallin family protein | 6439.435243 | 4151.038507 | 1.55 | 0.000997309 |
| **Motility** | | | | | | |
| EPZ91406.1 | BCAL0520 | Flagellar hook-length control protein FliK | 12691.85888 | 5341.255612 | 2.38 | 0.016296347 |
| **Transport and nutrient uptake** | | | | | | |
| EPZ89695.1 | BCAM1931 | Gram-negative porin | 149691.2977 | 49565.2581 | 3.02 | 0.00135363 |
| EPZ85519.1 | BCAL3008 | Gram-negative porin | 2010.911356 | 1191.111318 | 1.69 | 0.006229477 |
| **Virulence** | | | | | | |
| EPZ85968.1 | BCAL0343 | Type VI secretion system protein TssD/Hcp1 | 405128.4973 | 143721.9244 | 2.82 | 0.001607233 |
| EPZ89291.1 | BCAM1857 | Rho GTPase deamidase TecA | 5699.089629 | 2929.941101 | 1.95 | 0.002691733 |
| EPZ90584.1 | BCAM1074 | Exotoxin | 1214.73543 | 600.3221162 | 2.02 | 0.022897501 |
| **Unknown function** | | | | | | |
| EPZ86836.1 | BCAL1571 | Phage tail sheath protein | 3931.545638 | 1259.10665 | 3.12 | 0.005920957 |
| EPZ88604.1 | BCAM1067 | Hypothetical phage protein | 48862.30101 | 28350.9189 | 1.72 | 0.011225243 |
| EPZ90408.1 | BCAM1069 | Hypothetical phage protein | 43728.3551 | 28564.40184 | 1.53 | 0.005342623 |

[1] *Fold change is calculated for Control/100 μM BDSF. Only proteins with a fold change > 1.5 are listed.*

**Table 11. Proteins significantly upregulated upon stimulation of *B. cenocepacia* K56-2 cultures with 100 µM BDSF.**

| Accession | Gene in J2315 | Protein name | Average normalized abundance Control | Average normalized abundance 100 µM BDSF | Fold change[1] | P-value |
|---|---|---|---|---|---|---|
| **Energy and lipid metabolism** | | | | | | |
| EPZ85854.1 | BCAL2935 | Electron transfer flavoprotein domain protein | 1197.299718 | 2550.135836 | 2.13 | 0.001379081 |
| EPZ90956.1 | BCAL2783 | Cyclopropane-fatty-acyl-phospholipid synthase, SAM-dependent methyltransferase | 469.5467087 | 1946.028521 | 4.14 | 4.94E-05 |
| **Stress response and chaperones** | | | | | | |
| EPZ89510.1 | BCAM0050 | Universal stress family protein | 22796.71838 | 63912.71483 | 2.80 | 0.023067139 |
| EPZ87278.1 | BCAL1919 | ATP-dependent chaperone protein ClpB | 4417.238012 | 8261.143861 | 1.87 | 0.007633928 |
| **Transcription and translation** | | | | | | |
| EPZ86401.1 | BCAL1883 | Histidine--tRNA ligase | 565.694819 | 1210.519705 | 2.14 | 0.006532566 |
| EPZ86330.1 | BCAL0249 | 50S ribosomal protein L6 | 7619.615397 | 16204.8631 | 2.13 | 0.005486227 |
| EPZ87519.1 | BCAL1942 | 50S ribosomal protein L9 | 5063.497015 | 11901.15619 | 2.35 | 0.014330079 |
| EPZ87276.1 | BCAL1945 | 30S ribosomal protein S6 | 1861.343937 | 3089.093972 | 1.66 | 0.004483907 |
| EPZ91189.1 | BCAL0799 | 50S ribosomal protein L25, Ctc-form | 1436.552712 | 4411.145318 | 3.07 | 0.009915019 |
| EPZ85513.1 | BCAL2925 | 50S ribosomal protein L19 | 57.18479813 | 1902.760832 | 33.27 | 0.001000746 |
| EPZ86236.1 | BCAL0235a | 50S ribosomal protein L4 | 156.6355664 | 1091.892753 | 6.97 | 0.002405801 |
| EPZ86071.1 | BCAL0238 | 30S ribosomal protein S19 | 642.0645569 | 2226.242969 | 3.47 | 0.003319237 |
| EPZ86091.1 | BCAL0252 | 50S ribosomal protein L30 | 110.6453541 | 1855.913974 | 16.77 | 0.003490064 |
| EPZ86098.1 | BCAL0244 | 50S ribosomal protein L14 | 121.9994399 | 1068.975793 | 8.76 | 0.009618325 |
| EPZ85945.1 | BCAL0233 | 30S ribosomal protein S10 | 591.1146941 | 2180.661072 | 3.69 | 0.015024894 |
| EPZ86298.1 | BCAL0234 | 50S ribosomal protein L3 | 5011.547051 | 8915.629329 | 1.78 | 0.018449904 |
| **Transport and nutrient uptake** | | | | | | |
| EPZ86411.1 | BCAL1829 | OmpW family protein | 4072.005891 | 8099.005265 | 1.99 | 0.012228944 |
| EPZ85892.1 | BCAL0282 | Sn-glycerol-3-phosphate ABC transporter substrate-binding protein | 1165.940672 | 2074.029821 | 1.78 | 0.01448075 |
| **Virulence** | | | | | | |
| EPZ90945.1 | BCAL0217 | GDSL family lipase | 306.6300136 | 622.3485258 | 2.03 | 0.005556846 |
| **Other function** | | | | | | |
| EPZ85408.1 | BCAL2916 | Metalloprotease PmbA | 2433.859118 | 4018.637339 | 1.65 | 0.002792465 |
| EPZ91966.1 | BCAL3530 | DNA-binding protein HU-alpha | 560.9360851 | 4486.432951 | 8.00 | 0.00743517 |
| EPZ90830.1 | BCAL0216 | Phenylacetate-CoA oxygenase subunit PaaA | 1289.493201 | 2494.657891 | 1.93 | 0.013578114 |
| EPZ86322.1 | BCAL0377 | Metallo peptidase, subfamily M24B | 496.2269861 | 769.951094 | 1.55 | 0.018199821 |
| **Unknown function** | | | | | | |
| EPZ86731.1 | BCAL1411 | Hypothetical protein | 15621.16569 | 28554.13058 | 1.83 | 0.008948242 |
| EPZ87866.1 | BCAM0907 | Sulfurtransferase | 1744.004731 | 3967.445663 | 2.27 | 0.022421776 |
| EPZ85594.1 | BCAL3052 | PF09351 domain protein | 1885.275161 | 2965.087807 | 1.57 | 0.006677749 |
| EPZ85914.1 | BCAL2401 | PF11776 domain protein | 823.1198933 | 1452.015194 | 1.76 | 0.006960904 |

[1] *Fold change is calculated for Control/100 µM BDSF. Only proteins with a fold change > 1.5 are listed.*

These results were validated by quantifying a selected number of proteins by SRM on a M-class Acquity HPLC system coupled to a Xevo TQ-S mass spectrometer. Several proteins showing a significant up- or downregulation in the label-free proteomics approach or proteins with a potential role in virulence were selected for quantification to obtain a global view of the effect of BDSF on the regulation of virulence factor expression. The results (Figure 25) show a significant decrease in secretion of the T6SS protein Hcp1, as determined by SRM, although the difference was a bit smaller than detected in the label-free proteomics approach (fold change of 1.9 compared to 2.8). 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. ZmpA and AidA were significantly more secreted upon BDSF stimulation (fold change of 2.4 and 1.6, respectively), but this was not the case for ZmpB. BDSF also induced a significant decrease in the expression of the major cable pilus protein CblA, with an associated fold change of 1.5. 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. Two ribosomal proteins, L9 and L25, were confirmed to be significantly increased in the secretome of cultures stimulated with BDSF (fold change of 2.2 and 1.7, respectively). 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 two 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 (Figure 26).

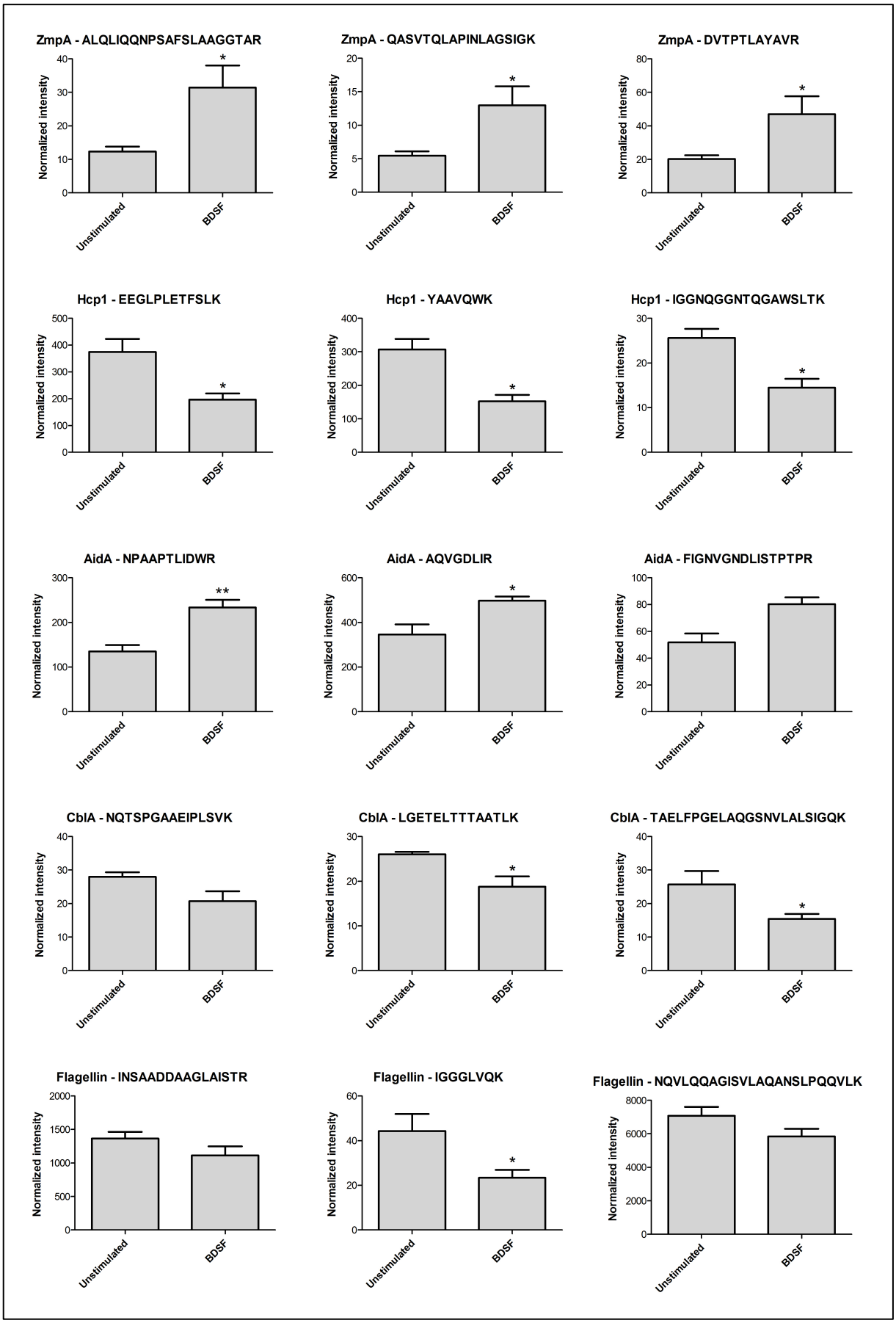



**Figure 25. Proteins confirmed to be differentially expressed upon stimulation with 100 µM BDSF.** Exponential phase *B. cenocepacia* K56-2 cultures were stimulated with 100 µM BDSF during 2 hours. Selected secreted proteins were quantified by SRM. * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001.

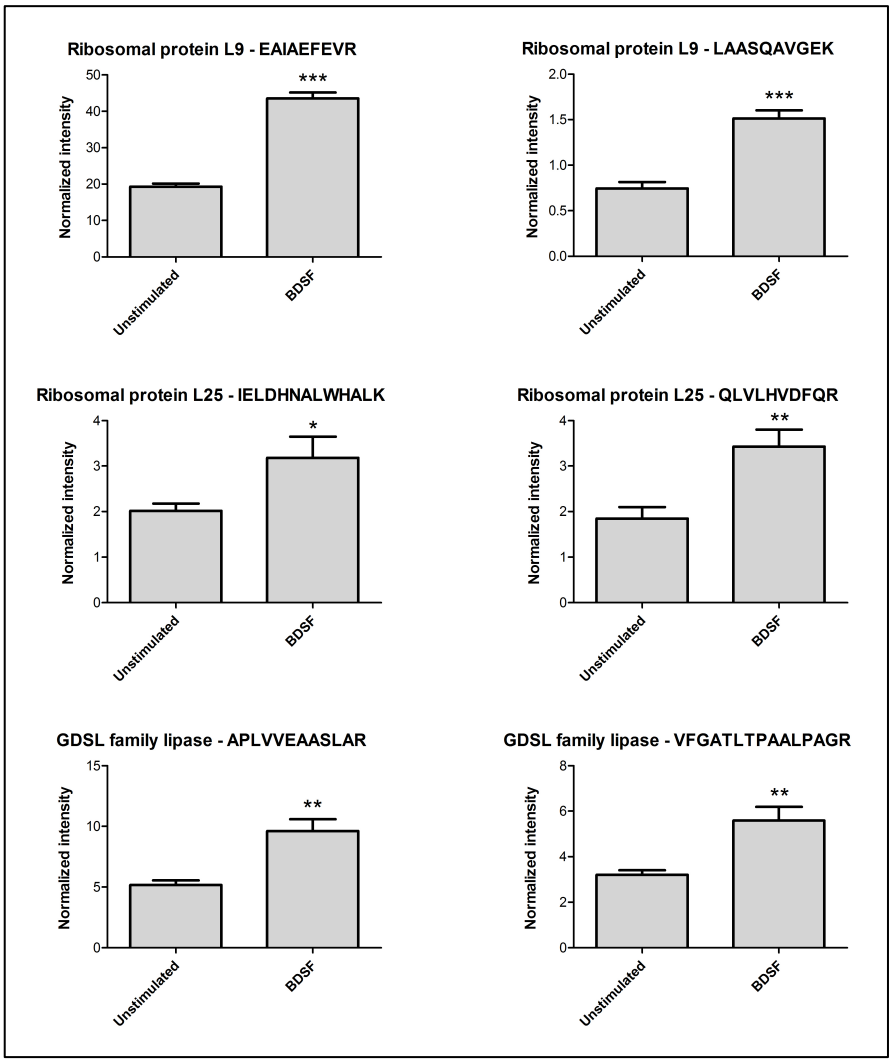



**Figure 26. Proteins confirmed to be differentially expressed upon stimulation with 100 µM BDSF.** Exponential phase *B. cenocepacia* K56-2 cultures were stimulated with 100 µM BDSF during 2 hours. Selected secreted proteins were quantified by SRM. * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001.

### 6.3.5 Diffusible signal factors from *P. aeruginosa* and *S. maltophilia* are recognized by *B. cenocepacia* K56-2 and have an effect on the secretion of virulence factors

To investigate the potential involvement of diffusible signal factors in interspecies communication, a panel of secreted, virulence-associated proteins from unstimulated cultures and cultures harvested 1 h and 2 h after BDSF, DSF and PDSF stimulation were quantified by SRM. These proteins were selected based on their response to BDSF, detected in the previous experiments, being ZmpA, ZmpB, Hcp1, the cable pilus protein CblA and the hypothetical protein BCAS0151.

Secretion of the hypothetical protein BCAS0151 is highly increased in a time-dependent manner upon BDSF, DSF and PDSF stimulation (Figure 27). This hypothetical protein does not contain any putative conserved domains and is conserved within the *Bcc*. Expression of the BCAS0151 gene was previously found to be significantly reduced in a *B. cenocepacia* H111 *rpfF* mutant (Schmid et al., 2012). Based on these results, it was expected that DSF and PDSF would have a similar effect on the secretion of ZmpA and ZmpB. However, the effect of DSF and PDSF stimulation on the secretion of

both metalloproteases was much smaller compared to the response generated by BDSF. For ZmpB, the increase upon DSF and PDSF stimulation was not significant at the 5% significance level, while for ZmpA, it was only significant for DSF for one of the two peptides measured (Figure 28).

A similar output was foreseen for the major cable pilus protein CblA (Figure 29). This protein however, was most strongly exported upon treatment with the *S. maltophilia* diffusible signal factor. PDSF generated a similar effect as BDSF, i.e. a small increase which is on the edge of significance.

A different pattern was observed for the secretion of the structural T6SS protein of the Hcp1 family upon BDSF stimulation. Secretion of this protein was slightly reduced in this case (Figure 30). However, when the cultures were stimulated with PDSF, its secretion significantly dropped. DSF generated a comparable response as PDSF, although to a slightly lower extent.

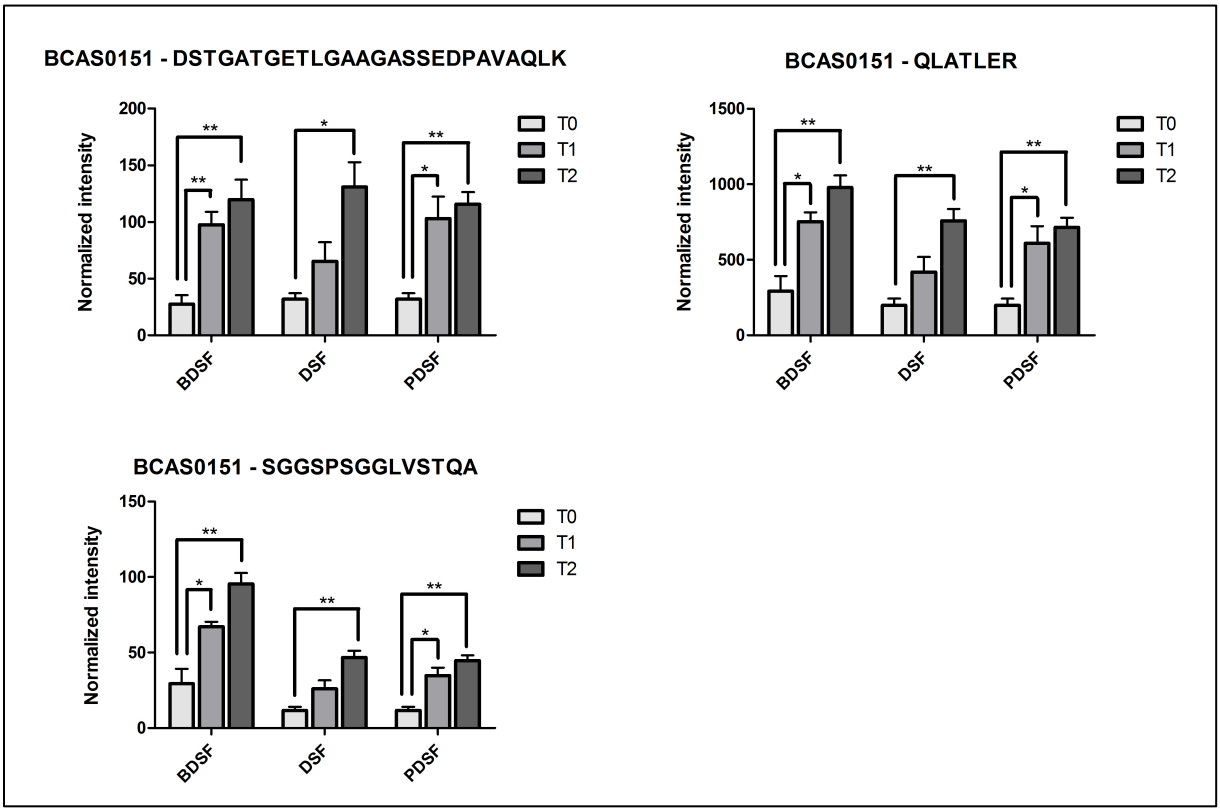



**Figure 27. Time-kinetic effect of diffusible signal factors from *Burkholderia* (BDSF), *Stenotrophomonas* (DSF) and *Pseudomonas* (PDSF) on the secretion of a hypothetical protein encoded by the BCAS0151 gene of *B. cenocepacia* K56-2.** Exponential phase *B. cenocepacia* K56-2 cultures were stimulated for 2h with 100 µM of the different DSFs. Protein abundances were quantified using SRM and two-sample T-tests were used to detect statistically significant differences. * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001.

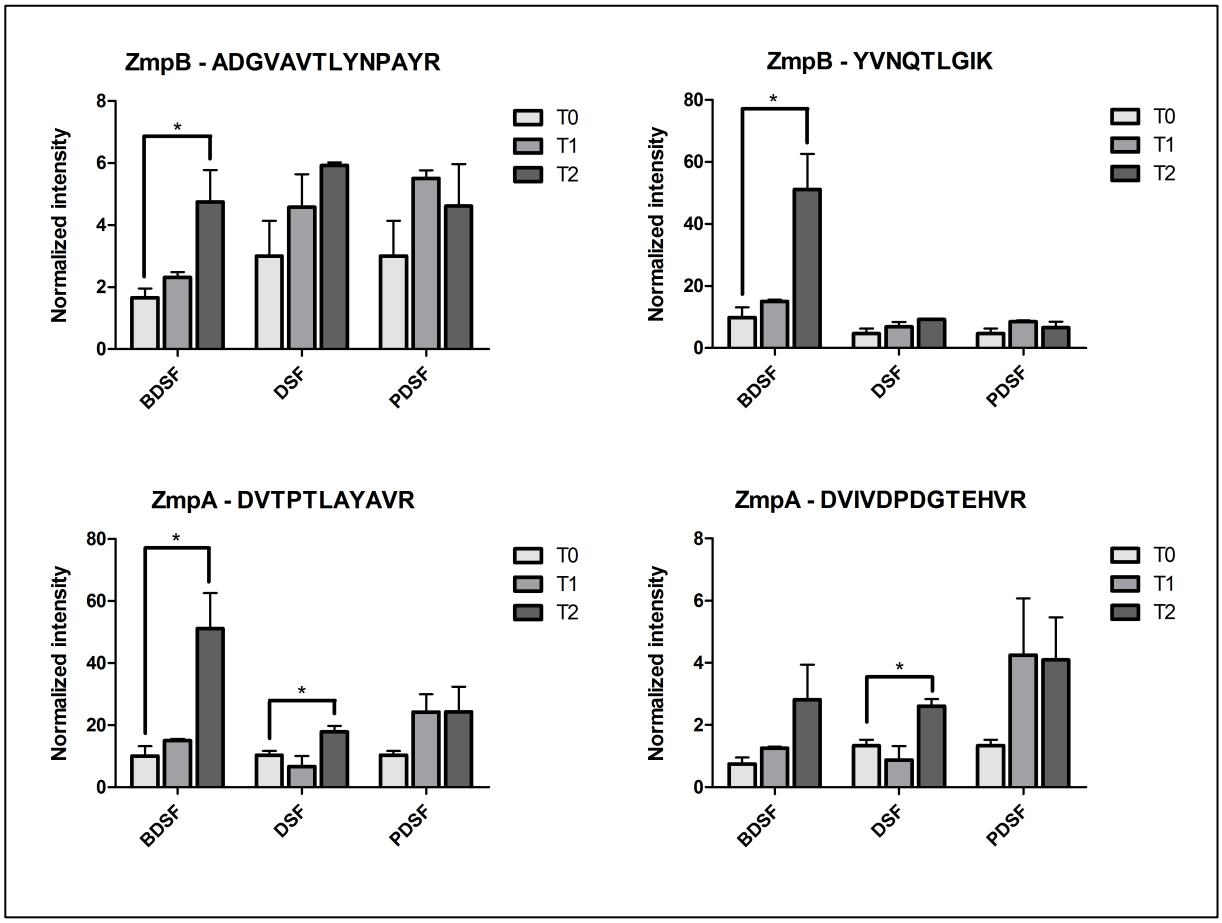



**Figure 28. Time-kinetic effect of diffusible signal factors from *Burkholderia* (BDSF), *Stenotrophomonas* (DSF) and *Pseudomonas* (PDSF) on the secretion of the zinc metalloproteases ZmpB and ZmpA of *B. cenocepacia* K56-2.** Exponential phase *B. cenocepacia* K56-2 cultures were stimulated for 2h with 100 µM of the different DSFs. Protein abundances were quantified using SRM and two-sample T-tests were used to detect statistically significant differences. * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001.

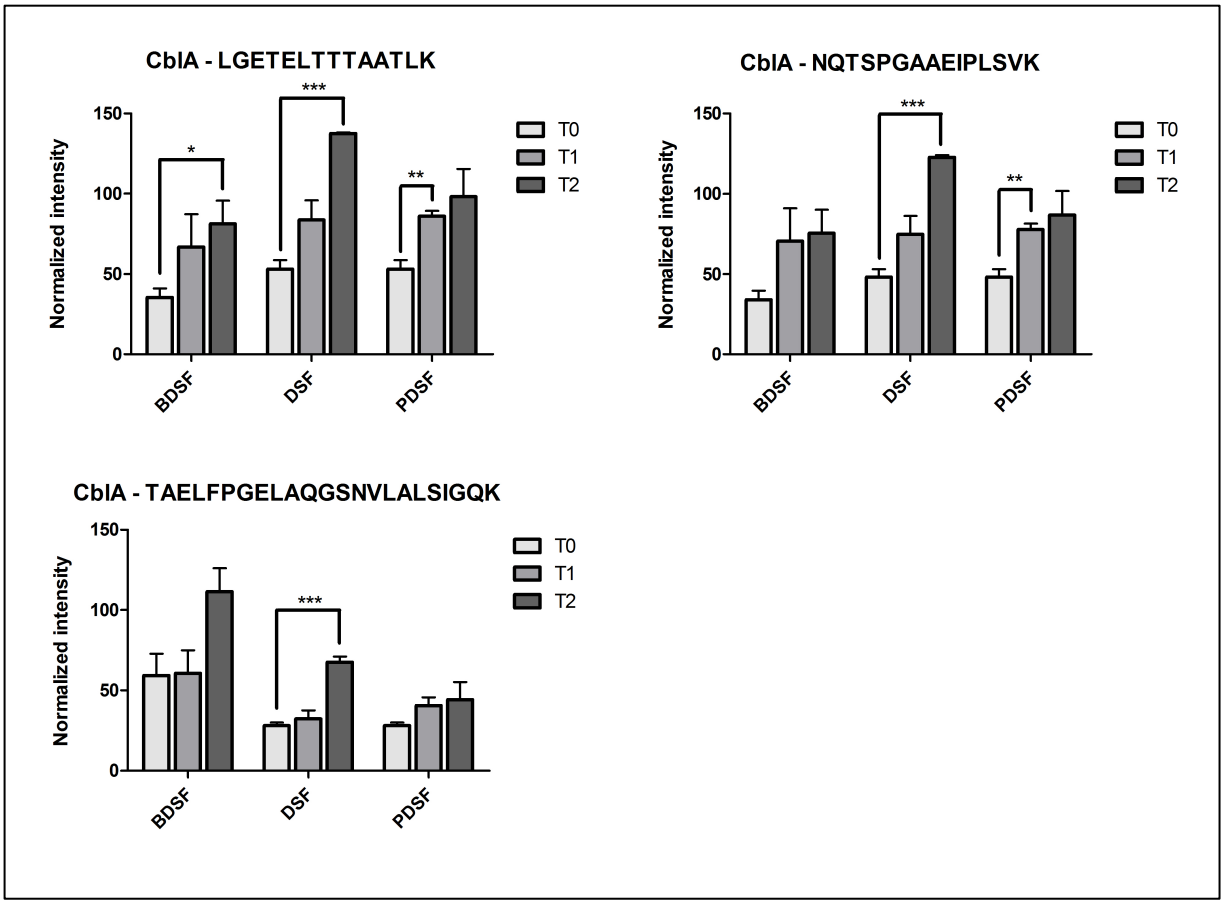



**Figure 29. Time-kinetic effect of diffusible signal factors from *Burkholderia* (BDSF), *Stenotrophomonas* (DSF) and *Pseudomonas* (PDSF) on the expression of the cable pilus protein CblA of *B. cenocepacia* K56-2.** Exponential phase *B. cenocepacia* K56-2 cultures were stimulated for 2h with 100 µM of the different DSFs. Protein abundances were quantified using SRM and two-sample T-tests were used to detect statistically significant differences. * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001.

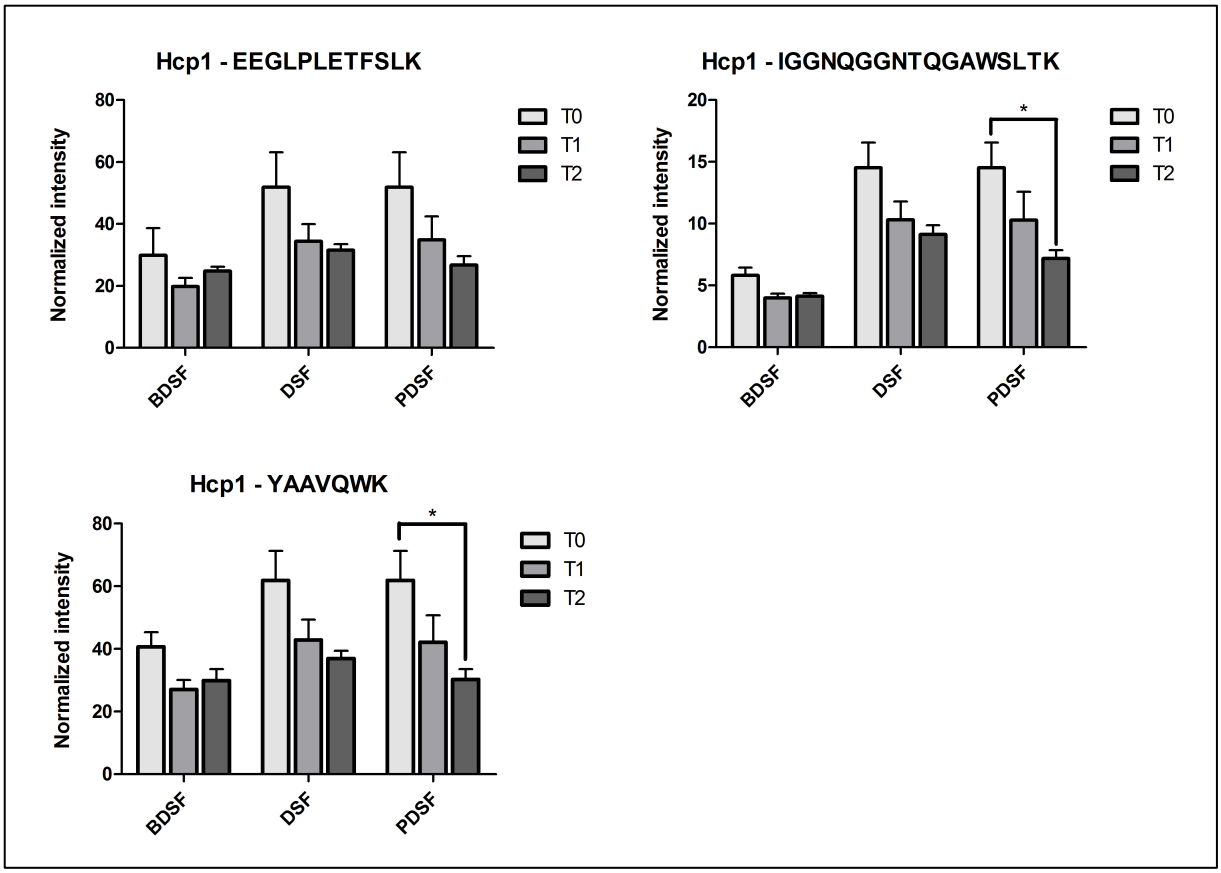



**Figure 30. Time-kinetic effect of diffusible signal factors from *Burkholderia* (BDSF), *Stenotrophomonas* (DSF) and *Pseudomonas* (PDSF) on the expression of the T6SS protein Hcp1 of *B. cenocepacia* K56-2.** Exponential phase *B. cenocepacia* K56-2 cultures were stimulated for 2h with 100 µM of the different DSFs. Protein abundances were quantified using SRM and two-sample T-tests were used to detect statistically significant differences. * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001.

### 6.3.6 DSF and PDSF alter the secretion of virulence factors in a similar way as BDSF, although the magnitude of the observed effects are much smaller

Since in the previous section, the time-dependent effect of BDSF, DSF and PDSF was evaluated in separate experiments and the unstimulated controls were harvested up to two hours earlier than the stimulated cultures, the difference in growth time could be a confounding factor. Therefore, a novel experiment was performed to compare the changes in the secretion of virulence factors brought about by 100 µM BDSF, DSF and PDSF after 2h of stimulation. Unstimulated cultures were used as a negative control and were isolated at the same timepoint as the stimulated cultures. Targeted proteomics was used to quantify the abundance of the T6SS proteins Hcp1 and TecA, the T2SS-dependent proteins ZmpA and ZmpB, the nematocidal protein AidA, and the major cable pilus protein CblA. Data were analyzed as described in section 6.3.2 and results are summarized in Figure 31.

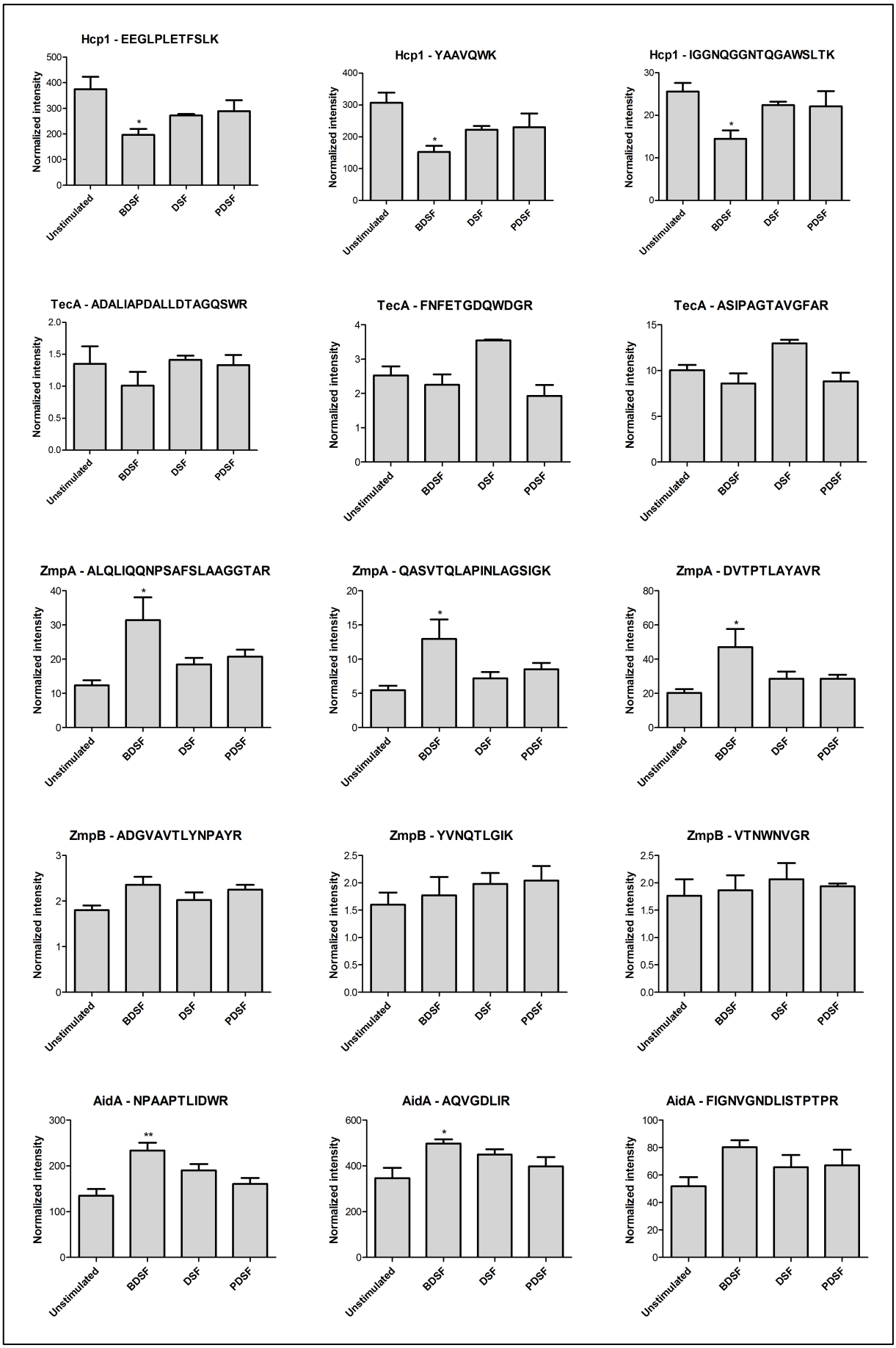
These data confirmed the significant decrease in Hcp1 secretion and CblA expression, as well as the increased secretion of ZmpA and AidA upon BDSF stimulation. Secretion of TecA was slightly reduced after 2 hours of growth in the presence of BDSF, although this difference was not significant at the 5% confidence level. Secretion of ZmpB is not affected by BDSF, as was already observed in previous

experiments (section 6.3.2). 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 12.

**Table 12. Effect of 100 µM BDSF, DSF and PDSF on the secretion of selected virulence factors.**

| | Fold change 100 µM BDSF | P-value[c] | Fold change 100 µM DSF | P-value[c] | Fold change 100 µM 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 |
| FNFETGDQWDGR | 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** | |
| ADGVAVTLYNPAYR | 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 |
| AQVGDLIR | 1.44 | < 0.05 | 1.30 | > 0.05 | 1.15 | > 0.05 |
| FIGNVGNDLISTPTPR | 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 |
| LGETELTTTAATLK | 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*


Hcp1 - EEGLPLETFSLK
Hcp1 - YAAVQWK
Hcp1 - IGGNQGGNTQGAWSLTK
TecA - ADALIAPDALLDTAGQSWR
TecA - FNFETGDQWDGR
TecA - ASIPAGTAVGFAR
ZmpA - ALQLIQQNPSAFSLAAGGTAR
ZmpA - QASVTQLAPINLAGSIGK
ZmpA - DVTPTLAYAVR
ZmpB - ADGVAVTLYNPAYR
ZmpB - YVNQTLGIK
ZmpB - VTNWNVGR
AidA - NPAAPTLIDWR
AidA - AQVGDLIR
AidA - FIGNVGNDLISTPTPR
Normalized intensity
Unstimulated
BDSF
DSF
PDSF

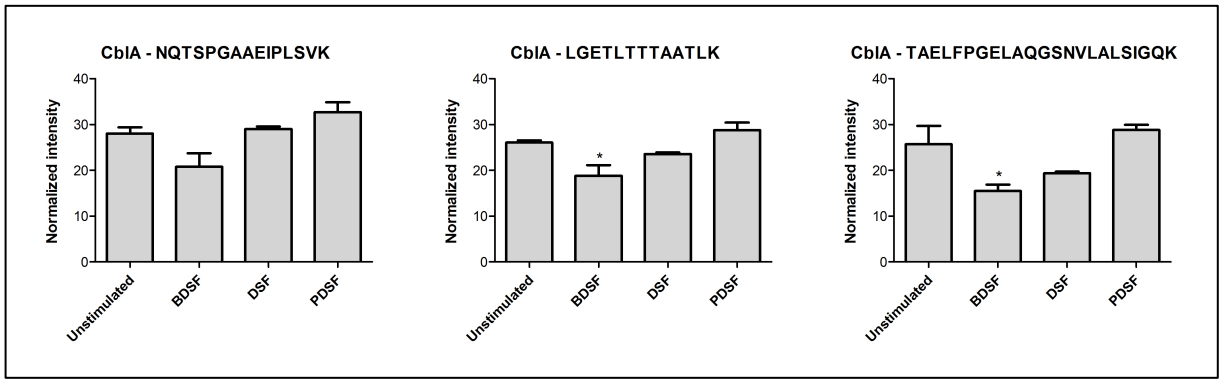



**Figure 31. Effect of 100 µM BDSF, DSF and PDSF on the secretion of selected virulence factors from *B. cenocepacia* K56-2.** Exponential phase *B. cenocepacia* K56-2 cultures were stimulated with 100 µM BDSF, DSF or PDSF and grown for an additional 2 hours in the presence of the stimulus. Virulence 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 compariosn tests. * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001 compared to the unstimulated control.

### 6.3.7 BDSF, DSF and PDSF negatively influence biofilm formation in *B. cenocepacia* K56-2

The effect of diffusible signal factors on the ability of *B. cenocepacia* K56-2 to form biofilms was evaluated by a crystal violet assay. Biofilms were grown for 24 h in the presence of 500 μM BDSF, DSF or PDSF. Methanol stimulation was used as a negative control. Biofilms grown in the presence of BDSF, DSF or PDSF had a significantly lower mass compared to the control biofilms. The mean absorbance values were 0.206, 0.214 and 0.149 for respectively BDSF, DSF and PDSF and 0.411 for the control condition (Figure 32).

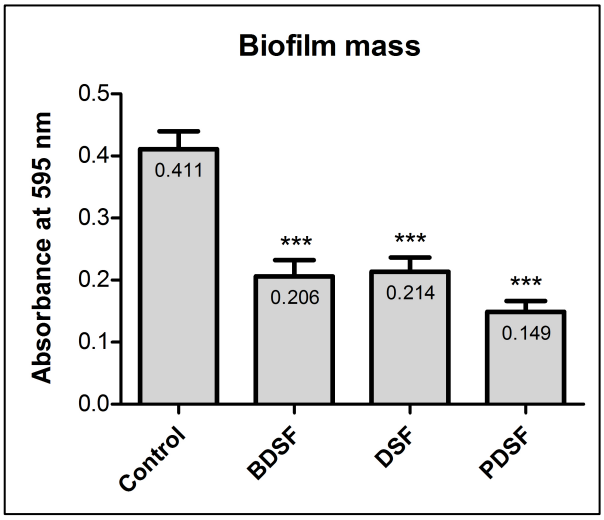



**Figure 32. Effect of diffusible signal factors of *Burkholderia* (BDSF), *Stenotrophomonas* (DSF) and *Pseudomonas* (PDSF) on biofilm formation of *B. cenocepacia* K56-2.** Biofilms were grown for 24h in the presence of 500 μM BDSF, DSF or PDSF and biofilm mass was quantified using the crystal violet assay. *** = p-value < 0.001 compared to the control condition.

## 6.4 Discussion

Since chronic lung infections pose a major problem for people with CF, an in-depth characterization of the infecting pathogens is of crucial importance to compile an efficient treatment plan. However, this is often complicated by the presence of polymicrobial communities displaying increased infection potential and antibiotic resistance. Understanding the interactions that take place between these different species is a fundamental step in the process of developing novel therapies and antimicrobials. Since quorum sensing plays a crucial role in interspecies communication, this research aimed at mapping the effects of quorum sensing molecules from the major CF pathogens *P. aeruginosa* and *S. maltophilia* on the expression/secretion of virulence factors in *B. cenocepacia*, the causative of cepacia syndrome in people with CF. In this study, we developed SRM based methods to measure the effects of the diffusible signal factor family of quorum sensors on the abundance of several secreted virulence factors. This has physiological importance since DSFs have been detected in the sputum of CF patients and it has already been demonstrated that they can have an influence on biofilm formation and virulence factor production (Deng et al., 2010; Ryan et al., 2008, 2009a; Twomey et al., 2012). However, no detailed study at the protein level has been performed so far. Moreover, perception of the *Pseudomonas* diffusible signal factor by *B. cenocepacia* has not been described yet.

*B. cenocepacia* produces the diffusible signal factor cis-2-dodecenoic acid, a 12-carbon fatty acid. However, up till now, the exact physiological production levels during *in vitro* growth have not been determined. Therefore, an HPLC-based method was developed for the absolute quantification of endogenously produced BDSF present in the culture supernatant of *B. cenocepacia* K56-2 at different points along its growth curve. The concentration of BDSF is maximal after 6 h of growth (1068 nM), corresponding to the mid-to-late exponential phase, and gradually decreases in the following four hours (± 900 nM after 10h). However, in early stationary phase, there is a sharp decline in BDSF concentration, with its value falling back to ± 600 nM after 12h of growth. This concentration is in the same range in sputum of patients with CF as was determined using a bioassay for DSF (250 nM, Twomey et al., 2012).

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 (He et al., 2010). The long-chain fatty-acid-CoA-ligase RpfB was found to counteract the activity of the DSF synthase RpfF and might therefore be involved in DSF turnover (Bi et al., 2014). This is supported by the observation that *rpfB* deletion boosted the production of DSF and BDSF during growth, while *rpfB* overexpression completely abrogated DSF signal production (Zhou et al., 2015). In *B. cenocepacia*, an RpfB homolog (BCAL1541) is present with an identity of 58% to that of *Xcc*. However, its regulation and role in BDSF signal turnover remain to be established (Zhou et al., 2017). The similarity between the BDSF profile of *B. cenocepacia* and the DSF profile of *Xcc* makes it reasonable that the *B. cenocepacia* homolog of RpfB is involved in BDSF turnover in *B. cenocepacia*, although the possibility exists that still other or additional types of quorum quenching are responsible for this phenomenon (Grandclément et al., 2015).

The working concentration of exogenously added BDSF was determined by stimulating exponential phase *B. cenocepacia* K56-2 cultures with different BDSF concentrations and determining the effect on the secretion of selected virulence factors via SRM. This analysis taught us that significant effects on the secretome, in the conditions tested, are only observed after stimulation with higher BDSF concentrations, starting from 75 µM. This is a much higher concentration than the one we determined in an extracellular extract from a standard liquid culture and what was observed in sputum samples by Twomey et al. There are some arguments why we continued our work with such concentrations. Similar concentrations were previously used in most *in vitro* studies, for example for transcript analysis (Mc Carthy et al. 2010), to allow better comparisons. More speculatively, *in vivo*, local concentrations might be higher and have impact on single cells exposed to BDSF producing cells. In the future, our HPLC method should allow to more accurately quantify DSF concentrations produced by cells grown under different conditions, for example those grown in synthetic cystic fibrosis media.

Under the conditions used, we observed a significant increase of a number of T2SS-dependent proteins upon BDSF stimulation, in the first place of the zinc metalloprotease ZmpA. Our results agree with previous findings showing that mutation of the BDSF synthase BCAM0581 resulted in decreased ZmpA promoter activity (Deng et al., 2009), suggesting that exogenous BSDF could enhance BCAM0581 transcription. 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 (O'Grady et al., 2011b). Secretion of the nematocidal protein AidA, a protein contributing to the killing of *C. elegans* by *B. cenocepacia* (Huber et al., 2004), 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 (Uehlinger et al., 2009), and was overexpressed in nonmucoid *B. cenocepacia* isolates compared to mucoid isolates (Zlosnik and Speert, 2010), its exact role during infection remains obscure.

Further, we showed a statistically significant upregulation of a GDSL family lipase upon stimulation of exponential phase cultures with 100 µM BDSF by both label-free and targeted proteomics. The function of this 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 (Flieger et al., 2002). Similar phospholipases belonging to class A have been implicated in the pathogenesis of *Campylobacter*, *Yersinia*, *Helicobacter* and *Salmonella* species (Dorrell et al., 1999; Grant et al., 1997; Ruiz-Albert et al., 2002; Schmiel et al., 1998).

Together, our data suggest that BDSF enhances the infectious potential of *B. cenocepacia*, by increasing secretion of virulence proteins that can modulate the host immune system. This is in contrast with the effect of BDSF on the T6SS, which is required for intracellular survival in macrophages (Flannagan et al., 2012; Rosales-Reyes et al., 2012b), but also has the ability to inhibit the growth of neighboring bacteria in mixed populations (Russell et al., 2011; Storz et al., 2016). Both label-free proteomics studies showed that secretion of the T6SS proteins Hcp1 and TecA is significantly reduced upon BDSF stimulation, but using targeted proteomics, only the reduced secretion of Hcp1 could be confirmed. This could be indicative for a decreased expression of the T6SS operon, since the gene encoding Hcp1 is part of it, while the *tecA* gene is not. To our knowledge, this is the first description of the direct effect on T6SS expression by diffusible signal factors in *B.*

*cenocepacia*. An explanation for this reduction might be that when the amount of BDSF exceeds a threshold, it activates the diguanylate phosphodiesterase domain of the BDSF receptor and thereby decreases intracellular cyclic-di-GMP levels. This causes a switch from a sessile lifestyle to a planktonic, more virulent status, accompanied by decreased biofilm formation and T6SS activity (Moscoso et al., 2011). Previously, it was shown that the regulatory protein AtsR is responsible for such a phenotype (Aubert et al., 2013). These authors showed that AtsR provoked responses that were both homoserine lactone QS-dependent and -independent. A direct link between AtsR activity and DSF signaling has not been demonstrated, but at least their responses seem to converge.

*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.
Results of the crystal violet assay clearly show a significant reduction in the amount of biofilm formed when the biofilms were grown in the presence of diffusible signal factors. This observation is in line with a general transition of this pathogen to a more virulent state upon stimulation with diffusible signal factors and is presumably caused by a DSF-dependent reduction of the intracellular cyclic-di-GMP concentration.
*B. cenocepacia* contains two diffusible signal factor receptor proteins: the major receptor RpfR (BCAM0580 in J2315) and a minor receptor encoded by the BCAM0227 gene in J2315 (Deng et al., 2012; McCarthy et al., 2010). BDSF binds to the PAS domain of RpfR, leading to a conformational change and activation of the phosphodiesterase (EAL) domain, leading to degradation of the second messenger cyclic-di-GMP. The latter is a universal regulator linking the perception of environmental or intracellular cues to specific alterations in cell function. How cyclic-di-GMP exerts its effects on virulence factor production and biofilm formation remains to be elucidated, but several intermediate signal transduction proteins must be involved (Ryan, 2013). BCAM0227 only controls a subset of the genes controlled by RpfR. It bears 36% identity to its homolog in *X. campestris* pv. *campestris*, RpfC, and the particular amino acids that were described to be essential for DSF binding to RpfC are not conserved or even absent in BCAM0227 (Cai et al., 2017). Therefore, it is crucial to further investigate the role of this receptor and the interplay with the RpfR signaling pathway.
Since several homologs of these receptors, containing analogues PAS, GGDEF and EAL domains, are present in the genome of *B. cenocepacia*, the question remains whether the diffusible signal factors from the different species are recognized through the same or through different receptor proteins. If the same receptor is to be used, the different output could be explained by a difference in the binding affinity, or by the efficiency of subsequent signal transduction.

Additional experiments investigating the effect of BDSF, DSF and PDSF stimulation on *B. cenocepacia* RpfR and BCAM0227 mutants are required to obtain better insights in the signal recognition/perception of these different quorum sensing molecules.

# 7 *INTERSPECIES INTERACTIONS BETWEEN* BURKHOLDERIA CENOCEPACIA *K56-2,* PSEUDOMONAS AERUGINOSA *PAO1 AND* STENOTROPHOMONAS MALTOPHILIA *44/98*

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**AUTHOR CONTRIBUTIONS**

Sofie Depluverez wrote the entire chapter and performed all the experiments. The biofilm assays were performed in the framework of the master thesis of Jolien Schoovaerts. Bart Devreese edited the chapter and is the supervisor of the author.

**ABSTRACT**

Treatment of lung infections in people with cystic fibrosis (CF) is often complicated by the fact that there is rarely a single infecting species with a defined antibiotic resistance profile. Instead, the culprits of pulmonary exacerbations are often polymicrobial communities, characterized by increased antibiotic resistance and infection potential. In order to develop more effecient treatment regimes, it is crucial to understand and characterize the interactions taking place among the members of such pathogenic consortia. The previous experimental chapter already demonstrated the importance of DSFs in interspecies crosstalk, and the role of acylhomoserine lactones has already been extensively described in the literature. However, yet other types of interactions can take place between species, such as secretion of toxic membrane vesicles containing different types of harmful enzymes or the production of bacteriocins. To study these protein-based interactions and potentially other, unknown types of crosstalk, we investigate the effects of (concentrated) cell-free supernatant (CFS) originating from stationary phase cultures of *Stenotrophomonas maltophilia* 44/98 or *Pseudomonas aeruginosa* PAO1 on the secretome and biofilm formation of *B. cenocepacia* K56-2 using label-free proteomics and biofilm assays. Growth of *B. cenocepacia* K56-2 in the presence of different types of CFS affects a.o. T6SS and T4SS-1 activity, as well as flagellation and cepacian biosynthesis. This work demonstrates the existence of QS-independent crosstalk between different CF pathogens, but requires an in-depth characterization of the composition of the different types of CFS to provide valid explanations for the observed results.

## 7.1 Introduction

Cystic fibrosis (CF) is a genetically inherited disease caused by mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, leading to the formation of a dehydrated mucus layer covering lung epithelial cells (Gibson et al., 2003). Patients suffering from CF are therefore highly susceptible to recurrent and often chronic lung infections, with approximately 90% of patients ultimately dying from the consequences of fatal lung infections. During CF airway disease relatively stable periods are frequently interrupted by acute pulmonary exacerbations (PEs). Due to the excessive immune responses evoked during these PEs, irreversible lung damage is inflicted on the patients (Amadori et al., 2009; Sanders et al., 2011) and frequently patients are unable to recover baseline pulmonary function after an acute PE.

One of the main culprits thought of causing PEs is *P. aeruginosa* (Goss and Burns, 2007; Sanders et al., 2010). However, several studies observed no increase in *P. aeruginosa* concentration immediately prior or at the time of PE (Stressmann et al., 2011), and recent epidemiological data highlights the potential role of co-infections in influencing the clinical outcome (Harrison, 2007; Rogers et al., 2010b; Ryan et al., 2008; Shank and Kolter, 2009; Sibley et al., 2008, 2009). Therefore, the pathophysiology of PEs might be related to changing microbial behavior or dynamics of interactions between the different species present in the CF lung (Pompilio et al., 2015). These polymicrobial communities are frequently characterized by extremely high levels of antibiotic resistance (Schwab et al., 2014; Stressmann et al., 2011). This phenomenon not only complicates treatment, but it may also considerably increase the disease progression and the mortality associated with these multispecies infections. It is therefore of crucial importance to gain more insight into the interactions occurring between bacterial CF community members.

Numerous studies have already been published in this context (for which I refer to section 2.5.3), but very few of them focused on the interactions influencing the behavior of *B. cenocepacia*. Mc Kenney et al (1995) studied the effect of concentrated cell-free supernatant of *P. aeruginosa* and showed by activity assays that the lipase and protease activity of *B. cepacia* increased. They also studied the effect on homoserine lactones, but at that time the role of DSFs was yet unknown (McKenney et al., 1995). In the previous chapter, we discovered that diffusible signal factors originating from the frequently co-isolated pathogens *S. maltophilia* and *P. aeruginosa* are able to modulate the secretion of virulence factors and the biofilm formation potential of *B. cenocepacia*. Furthermore, *P. aeruginosa* is able to secrete toxic MVs containing different types of noxious enzymes to attack competing species (Li et al., 1998; Tashiro et al., 2012, 2013). Bacteriocins are another type of proteinaceous toxins mediating competition between species (Bakkal et al., 2010). Together, these examples underscore the importance of protein-based interactions between different bacteria, next to the well-characterized QS-based communication.
In this study, we investigated the effect of (concentrated) cell-free supernatant (CFS) originating from stationary phase cultures of *S. maltophilia* and *P. aeruginosa* on the secretome and the biofilm formation of *B. cenocepacia*. We attempted to determine QS-dependent and/or QS-independent (e.g. protein-based) effects on *B. cenocepacia*, by using ultrafiltration to eliminate small molecular weight QS molecules from the CFS and focus on the role of larger biomolecules in interspecies crosstalk.

## 7.2 Materials and methods

**Bacterial strains and culture conditions**

*Burkholderia cenocepacia* K56-2, *Stenotrophomonas maltophilia* 44/98 and *Pseudomonas aeruginosa* PAO1 were routinely grown in Luria Broth (LB) on a shaker (200 rpm) at 37°C. Biofilms were grown for 24h in round-bottomed 96-well plates at 37°C without shaking.

**Collection of (concentrated) cell-free supernatant and addition to *B. cenocepacia* cultures**

Overnight cultures of the different strains were diluted to an $OD_{600}$ = 0.1 in fresh LB and grown for 18h, corresponding to stationary phase. Culture samples were centrifuged (6000xg, 15 min) and filtered through a 0.22 µm pore size filter (Sarstedt) to remove residual cells. The filtrate constitutes the cell-free supernatant (CFS). Optionally, this CFS was further concentrated via ultrafiltration through a 10 kDa MWCO filter (Amicon, Merck), yielding concentrated CFS. Protein concentrations of (un)concentrated CFS were estimated by Bradford's method (Coomassie Bradford Assay kit, Pierce).

Overnight *B. cenocepacia* cultures were diluted to an $OD_{600}$ = 0.1 in fresh LB and were supplemented with equal amounts of (concentrated) cell-free supernatant from the different species. Cultures were grown overnight at 37°C, 200 rpm.

**Isolation of extracellular proteins, 1D-LC-$MS^E$ and LC-$MS^E$ data analysis**

The methods used are described in section 6.2.

**Biofilm formation and quantification**

To investigate the effect of (un)concentrated CFS on biofilm formation, overnight *B. cenocepacia* K56-2 cultures were diluted to an $OD_{600}$ = 0.8. Fifty µl of this dilution was mixed with fifty µl (c)CFS containing a defined amount of protein. Three biological replicates were assayed for each condition. After 24h biofilm formation, the supernatant was removed and the wells were rinsed with 100 µl PBS.

For fixation of the biofilms, 100 µl 99% methanol was added (15 min), after which the supernatants were removed and the plates were air-dried. Then, 100 µl of a 0.1% crystal violet solution was added to all wells. After 20 min, the excess crystal violet was removed by washing the plates under running tap water. Finally, bound crystal violet was released by adding 150 µl of 33% acetic acid. The absorbance was measured at 595 nm. All steps were carried out at room temperature.

## 7.3 Results

### 7.3.1 Factors present in the cell-free supernatant of *S. maltophilia* 44/98 and *P. aeruginosa* PAO1 lead to significant changes in the secretome of *B. cenocepacia* K56-2

Since *B. cenocepacia*, *S. maltophilia* and *P. aeruginosa* are frequently co-isolated from the lungs of people with CF, and are able to communicate with each other through the action of DSFs (section 6.3.5 and 6.3.6), we evaluated the relevance of these and potentially other effects at physiological concentrations through the addition of CFS from different species to *B. cenocepacia* K56-2 cultures. Therefore, *B. cenocepacia* K56-2 cultures were grown overnight in the presence of CFS originating from stationary phase *S. maltophilia* 44/98 and *P. aeruginosa* PAO1 cultures. As a negative control, cultures were grown in the presence of *B. cenocepacia* K56-2 CFS. Differences in the secretome of three biological replicates of each condition were quantified by LC-$MS^E$ analysis on a NanoAcquity UPLC® system (Waters) coupled online to a Synapt G1 mass spectrometer (Waters). Only *Burkholderia* proteins with a fold change > 1.5, a p- and q-value < 0.05 and quantified with minimum 2 peptides were retained.

Growth of *B. cenocepacia* in the presence of *S. maltophilia* CFS leads to limited changes in the secretome. The most pertinent change is the increased abundance of a VgrG family protein (BCAL1294) (Table 13 and Table 14). This is a typical T6SS effector that can serve as a defense mechanism against competing species (Ho et al., 2014; Russell et al., 2011, 2014).

A much more pronounced effect is seen when *B. cenocepacia* K56-2 cultures are grown in the presence of *P. aeruginosa* CFS, with the concurrent downregulation of a whole series of flagellar proteins, pointing to a reduction in motility. Furthermore, both the T6SS effector protein Hcp1 and the previously found VgrG family protein are significantly downregulated in this condition compared to the control cultures (Table 15). This is in contrast with the situation for growth in the presence of *S. maltophilia* CFS, where the VgrG family T6SS effector is significantly upregulated. Several stress response and chaperone proteins, proteins involved in transcription or translation, and numerous uncharacterized proteins also showed a decrease in abundance when cultures were grown in the presence of *P. aeruginosa* CFS. One of the proteins found to have an increased abundance in the latter condition is the type VI amidase effector protein Tae4, a protein known to play a role in interspecies interactions occuring through the T6SS (Russell et al., 2012). Next to this protein, several proteins involved in transport and nutrient uptake are also increased (Table 16).

The effects observed in this experiment are the consequence of either QS-dependent or QS-independent processes.

**Table 13. *Burkholderia* proteins significantly downregulated upon growth of *B. cenocepacia* K56-2 in the presence of *S. maltophilia* 44/98 cell-free supernatant compared to growth in the presence of *B. cenocepacia* K56-2 cell-free supernatant.**

| Accession | Gene in J2315 | Description | Average normalized abundance Bc + Bc CFS[1] | Average normalized abundance Bc + Sm CFS[2] | Fold change[3] | P-value |
|---|---|---|---|---|---|---|
| **Energy and amino acid metabolism** | | | | | | |
| B4EBW5 | BCAL0935 | Putative periplasmic cytochrome c protein | 208.2585202 | 109.6370814 | 1.90 | 0.002564211 |
| B4E7I4 | BCAL1479 | Endoribonuclease L-PSP family protein | 150.1225777 | 97.22857547 | 1.54 | 0.029975752 |
| **Stress response and chaperones** | | | | | | |
| B4EP32 | BCAS0638 | 10 kDa chaperonin GroES | 2255.31371 | 1490.09679 | 1.51 | 0.006204925 |
| B4ED75 | BCAL2152 | Peptidyl-prolyl cis-trans isomerase PpiA | 2480.285266 | 1640.076485 | 1.51 | 0.022915109 |
| **Unknown function** | | | | | | |
| B4ECT3 | BCAL3142 | UPF0301 protein BceJ2315_30870 | 197.5557772 | 119.5459571 | 1.65 | 0.046363758 |

[1] *B. cenocepacia grown in the presence of B. cenocepacia cell-free supernatant*
[2] *B. cenocepacia grown in the presence of S. maltophilia cell-free supernatant*
[3] *Fold change is calculated for Bc + Bc CFS/Bc + Sm CFS*

**Table 14. *Burkholderia* proteins signficantly upregulated upon growth of *B. cenocepacia* K56-2 in the presence of *S. maltophilia* 44/98 cell-free supernatant compared to growth in the presence of *B. cenocepacia* K56-2 cell-free supernatant.**

| Accession | Gene in J2315 | Description | Average normalized abundance Bc + Bc CFS[1] | Average normalized abundance Bc + Sm CFS[2] | Fold change[3] | P-value |
|---|---|---|---|---|---|---|
| **Energy and amino acid metabolism** | | | | | | |
| B4E908 | BCAL2735 | Isocitrate dehydrogenase [NADP] | 4467.947488 | 8941.547722 | 2.00 | 0.005268982 |
| B4E9X7 | BCAL1796 | Putative saccharopine dehydrogenase | 242.6836894 | 1765.744935 | 7.28 | 0.000227065 |
| **Stress response and chaperones** | | | | | | |
| B4EDZ2 | BCAL3270 | Chaperone protein DnaK | 146.6654374 | 265.7694538 | 1.81 | 0.0371775 |
| **Transport** | | | | | | |
| B4E8D9 | BCAL2645 | Putative OmpA family membrane protein | 1139.379011 | 1882.431658 | 1.65 | 0.031044311 |
| B4ECV8 | BCAL3167 | C4-dicarboxylate ABC transporter substrate-binding protein | 266.0325477 | 1466.056891 | 5.51 | 0.000775028 |
| **Motility** | | | | | | |
| B4E8L9 | BCAL0577 | Flagellar hook-associated protein 3 (HAP3) | 2066.07073 | 5418.419106 | 2.62 | 0.044808399 |
| **Virulence** | | | | | | |
| B4E5H9 | BCAL1294 | VgrG family protein | 1280.280871 | 2802.557427 | 2.19 | 1.79E-05 |
| **Translation** | | | | | | |
| B4E5B7 | BCAL0231 | Elongation factor G | 6456.684196 | 9741.122526 | 1.51 | 0.001825074 |
| B4E5A5 | BCAL0219/ BCAL0232 | Elongation factor Tu | 1288.573938 | 2556.080657 | 1.98 | 0.002324356 |
| **Other function** | | | | | | |
| B4E6E4 | BCAL2418 | 3-carboxymuconate cyclase | 1329.280014 | 3573.308449 | 2.69 | 1.16E-05 |
| B4EDU2 | BCAL2213 | Oligopeptidase A | 1079.143364 | 1901.171946 | 1.76 | 0.002244967 |
| **Unknown function** | | | | | | |
| B4EMI2 | BCAM1919 | Hypothetical phage protein | 516.6668478 | 893.7458151 | 1.73 | 0.009056489 |

[1] *B. cenocepacia grown in the presence of B. cenocepacia cell-free supernatant*
[2] *B. cenocepacia grown in the presence of S. maltophilia cell-free supernatant*
[3] *Fold change is calculated for Bc + Sm CFS/Bc + Bc CFS*

**Table 15. *Burkholderia* proteins signficantly downregulated upon growth of *B. cenocepacia* K56-2 in the presence of *P. aeruginosa* PAO1 cell-free supernatant compared to growth in the presence of *B. cenocepacia* K56-2 cell-free supernatant.**

| Accession | Gene in J2315 | Description | Average normalized abundance Bc + Bc CFS[1] | Average normalized abundance Bc + Pa CFS[2] | Fold change[3] | P-value |
|---|---|---|---|---|---|---|
| **Energy and amino acid metabolism** | | | | | | |
| B4EAP2 | BCAL2934 | Electron transfer flavoprotein alpha-subunit | 2613.323018 | 459.9685504 | 5.68 | 6.01E-05 |
| B4EK69 | BCAM1606 | Electron transfer flavoprotein, alpha subunit | 433.9458064 | 169.8687881 | 2.55 | 0.00143063 |
| B4EEZ0 | BCAL0037 | ATP synthase epsilon chain | 234.3234127 | 74.10334286 | 3.16 | 0.000183678 |
| B4EBW5 | BCAL0935 | Putative periplasmic cytochrome c protein | 208.2585202 | 40.50842461 | 5.14 | 0.006012734 |
| B4EEC7 | BCAL0201 | N-acetyl-gamma-glutamyl-phosphate reductase ArgC | 1284.142855 | 346.276223 | 3.71 | 0.006253429 |
| **Stress response and chaperones** | | | | | | |
| B4E5V4 | BCAL3424 | Probable thiol peroxidase | 2074.435934 | 398.4326525 | 5.21 | 0.000138873 |

| | | | | | | |
|---|---|---|---|---|---|---|
| B4EG36 | BCAM0050 | Universal stress-related protein | 1919.48894 | 804.8138165 | 2.39 | 0.001545534 |
| B4ED76 | BCAL2153 | Peptidyl-prolyl cis-trans isomerase PpiB | 1331.135305 | 377.1693873 | 3.53 | 3.02E-05 |
| B4EDZ4 | BCAL3272 | Protein GrpE | 363.8067652 | 125.1577828 | 2.91 | 0.00241343 |
| B4ECT8 | BCAL3147 | 10 kDa chaperonin GroES | 6998.613886 | 1378.978145 | 5.08 | 6.71E-05 |
| B4EP32 | BCAS0638 | 10 kDa chaperonin GroES | 2256.852837 | 442.2245958 | 5.10 | 0.003451729 |
| B4EBE6 | BCAL0895 | Chaperone SurA | 140.5344826 | 63.33165601 | 2.22 | 0.001980235 |
| **Motility** | | | | | | |
| B4E8K5 | BCAL0562 | Negative regulator of flagellin synthesis (Anti-sigma-28 factor) FlgM | 5618.890454 | 502.7622584 | 11.18 | 4.44E-05 |
| B4E8L9 | BCAL0577 | Flagellar hook-associated protein 3 (HAP3) | 2085.130114 | 467.5879672 | 4.46 | 0.001143367 |
| B4EDI7 | BCAL0113 | Flagellar hook-associated protein 2 | 1500.781643 | 597.5733691 | 2.51 | 0.004574942 |
| B4E811 | BCAL0520 | Putative flagellar hook-length control protein FliK | 208.5751698 | 30.67983422 | 6.80 | 0.001242566 |
| B4E8L1 | BCAL0568 | Flagellar basal-body rod protein FlgF | 552.7770522 | 106.6829391 | 5.18 | 0.00963653 |
| B4E8K7 | BCAL0564 | Flagellar basal-body rod protein FlgB (Putative proximal rod protein) | 378.7646403 | 15.36892394 | 24.64 | 0.000545425 |
| **Transcription and translation** | | | | | | |
| B4E5F0 | BCAL1263 | Transcription elongation factor GreA | 626.636192 | 162.2910303 | 3.86 | 2.49E-05 |
| B4E7Z3 | BCAL0502 | RNA polymerase-binding transcription factor DksA | 316.2131446 | 116.4228201 | 2.72 | 0.000500248 |
| B4E5A5 | BCAL0219/ BCAL0232 | Elongation factor Tu | 1303.424348 | 714.2465812 | 1.82 | 0.004332254 |
| B4E912 | BCAL2739 | Elongation factor G | 697.856614 | 396.1605813 | 1.76 | 0.028025616 |
| B4E5D5 | BCAL0249 | 50S ribosomal protein L6 | 686.6204998 | 72.61685456 | 9.46 | 0.000128581 |
| B4EB40 | BCAL2950 | 30S ribosomal protein S1 | 200.6317089 | 102.5096562 | 1.96 | 0.00246819 |
| **Transport** | | | | | | |
| B4EDC0 | BCAL3203 | Protein TolB | 2489.805931 | 1559.454917 | 1.60 | 0.005873703 |
| B4EE76 | BCAL0151 | Extracellular ligand binding protein | 749.4767358 | 218.9219528 | 3.42 | 0.013332857 |
| **Virulence** | | | | | | |
| B4E6R5 | BCAL0343 | Putative type VI secretion system protein TssD/Hcp1 | 38147.8583 | 21010.01281 | 1.82 | 0.000705487 |
| B4E5H9 | BCAL1294 | VgrG family protein | 1280.280871 | 837.1482983 | 1.53 | 0.000368289 |
| B4E732 | BCAL2466 | Ecotin | 549.8455885 | 227.3407526 | 2.42 | 0.00033408 |
| **Other function** | | | | | | |
| B4E6I3 | BCAL2457 | Rod shape-determining protein RodA | 288.5502389 | 191.4331687 | 1.51 | 0.010070742 |
| **Unknown function** | | | | | | |
| B4EB46 | BCAL2956 | Putative exported protein | 4230.764002 | 1351.550287 | 3.13 | 0.000513627 |
| B4EHZ4 | BCAM2377 | Putative exported protein | 2060.032363 | 638.85965 | 3.22 | 0.000543646 |
| B4EFE1 | BCAM2073 | Putative exported protein | 2017.789527 | 500.0346723 | 4.04 | 2.79E-05 |
| B4EBI9 | BCAL1961 | Putative exported protein | 1127.922487 | 128.1003411 | 8.80 | 6.67E-06 |
| B4EG49 | BCAM1069 | Hypothetical phage protein | 926.5493137 | 267.227798 | 3.47 | 0.002155662 |
| B4EFV6 | BCAM2159 | Putative exported protein | 925.1247737 | 167.5552131 | 5.52 | 4.05E-05 |
| B4EIV3 | BCAM0365 | Putative short chain dehydrogenase | 1627.469957 | 401.9872223 | 4.05 | 1.45E-05 |
| B4EF87 | BCAM0942 | Putative exported protein | 247.1763232 | 41.27280373 | 5.99 | 7.13E-05 |

[1] *B. cenocepacia grown in the presence of B. cenocepacia cell-free supernatant*
[2] *B. cenocepacia grown in the presence of P. aeruginosa cell-free supernatant*
[3] *Fold change is calculated for Bc + Bc CFS/Bc + Pa CFS*

**Table 16. *Burkholderia* proteins signficantly upregulated upon growth of *B. cenocepacia* K56-2 in the presence of *P. aeruginosa* PAO1 cell-free supernatant compared to growth in the presence of *B. cenocepacia* K56-2 cell-free supernatant.**

| Accession | Gene in J2315 | Description | Average normalized abundance Bc + Bc CFS | Average normalized abundance Bc + Pa CFS | Fold change | P-value |
|---|---|---|---|---|---|---|
| **Stress response and chaperones** | | | | | | |
| B4EDZ2 | BCAL3270 | Chaperone protein DnaK | 146.6654374 | 678.4361319 | 6.26 | 0.000474843 |
| **Motility** | | | | | | |
| B4E8L8 | BCAL0576 | Flagellar hook-associated protein 1 (HAP1) | 1771.563841 | 7228.881542 | 5.37 | 0.000627643 |
| **Virulence** | | | | | | |
| B4EJ18 | BCAM1464 | T6SS amidase effector 4 Tae4 | 318.1878283 | 1699.630861 | 7.12 | 6.36E-06 |
| **Transport** | | | | | | |
| B4EAC3 | BCAL1824 | Putrescine-binding periplasmic protein | 2561.688659 | 5007.386801 | 2.24 | 0.003561385 |
| B4E628 | BCAL0305 | Toluene tolerance protein | 5488.014163 | 9450.242563 | 2.10 | 3.46E-05 |
| B4E8D9 | BCAL2645 | Putative OmpA family membrane protein | 1148.959879 | 1557.059731 | 1.56 | 0.029027412 |
| B4EBC5 | BCAL0874 | Transport protein | 141.9391684 | 223.6884755 | 1.99 | 0.024313048 |
| B4ECV8 | BCAL3167 | C4-dicarboxylate ABC transporter substrate-binding protein | 266.0325477 | 425.3111024 | 1.82 | 0.024985864 |
| B4EM61 | BCAM0759 | Periplasmic lysine-arginine-ornithine-binding protein ArgT | 199.0147289 | 309.2659413 | 1.88 | 0.030505406 |
| **Unknown function** | | | | | | |
| B4EMI2 | BCAM1919 | Hypothetical phage protein | 516.6668478 | 1019.995507 | 2.36 | 0.001288592 |
| B4EFA8 | BCAM0963 | Putative exported protein | 440.3197931 | 1008.176878 | 2.84 | 0.003755573 |

[1] *B. cenocepacia grown in the presence of B. cenocepacia cell-free supernatant*
[2] *B. cenocepacia grown in the presence of P. aeruginosa cell-free supernatant*
[3] *Fold change is calculated for Bc + Pa CFS/Bc + Bc CFS*

### 7.3.2 Significantly less biofilm mass is formed when *B. cenocepacia* K56-2 is grown in the presence of *P. aeruginosa* CFS

Because of the observed effect of CFS from *S. maltophilia* and *P. aeruginosa* on the motility and secretion of virulence factors of *B. cenocepacia*, we also assessed the potential effect on biofilm formation. Biofilms of *B. cenocepacia* K56-2 were grown in the presence of CFS originating from *S. maltophilla* 44/98 and *P. aeruginosa* PAO1. Again, cultures grown in the presence of *B. cenocepacia* K56-2 CFS were used as a negative control. Three biological replicates, each with twelve technical replicates, were analysed for every condition. The biofilm mass produced after 24h of growth was quantified using a crystal violet assay. Absorbance values were normalized against LB control wells and statistical analyses were performed using GraphPad Prism.

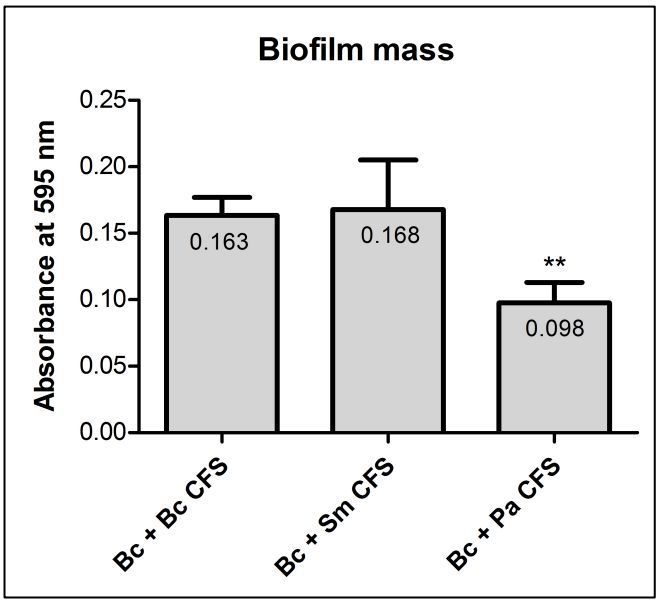


**Figure 33. Effect of cell-free supernatant from *S. maltophilia* 44/98 and *P. aeruginosa* PAO1 on biofilm formation of *B. cenocepacia* K56-2.** Biofilms were grown for 24h at 37°C in the presence of Sm or Pa CFS and compared to biofilms grown in the presence of *B. cenocepacia* K56-2 CFS. Biofilm mass was quantified using a crystal violet assay. Bc = *B. cenocepacia* K56-2, Sm = *S. maltophilia* 44/98, Pa = *P. aeruginosa* PAO1, CFS = cell-free supernatant. Mean normalized absorbance values are displayed in the graph. Error bars represent the standard error of the means.

The mean absorbance values for growth in the presence of *B. cenocepacia* CFS, *S. maltophilia* CFS and *P. aeruginosa* CFS were 0.163, 0.168 and 0.098 respectively (Figure 33). A significant difference in biofilm mass was observed for biofilms grown in the presence of *P. aeruginosa* CFS (p-value < 0.001), but not for biofilms grown in the presence of *S. maltophilia* CFS.

### 7.3.3 Factors present in the concentrated CFS of *S. maltophilia* 44/98 affect T6SS activity and motility of *B. cenocepacia* K56-2, but do not alter biofilm formation

The former experiments were performed on cell-free supernatants that contain both small molecular weight compounds and biopolymers like proteins. The measured effects could thus be partially due to the presence of signalling molecules like quorum sensors. In a second experiment, *B. cenocepacia* K56-2 cultures were grown overnight in the presence of concentrated CFS (cCFS) originating from stationary phase *S. maltophilia* 44/98 and *P. aeruginosa* PAO1 cultures. In this case, the CFS was concentrated by ultrafiltration through a 10 kDa MWCO membrane, and thus should be depleted of low-molecular weight compounds such as QS molecules. As a negative control, cultures were grown in the presence of concentrated *B. cenocepacia* K56-2 CFS. Differences in the secretome of three biological replicates of each condition were quantified by LC-MS$^{E}$ analysis on a NanoAcquity UPLC® system (Waters) coupled online to a Synapt G1 mass spectrometer (Waters). Only *Burkholderia* proteins with a fold change > 1.5, a p- and q-value < 0.05 and quantified with minimum 2 peptides were retained. Due to the highly distinct chromatographic profiles originating from the growth in the presence of *P. aeruginosa* cCFS, the alignment scores generated by Progenesis LC-MS were very low for this condition (± 50-55%, compared to 80% or higher for the other conditions). Despite our efforts to manually verify the peak matching, we conclude that a pairwise comparison of the LC-MS patterns with the control is not reliable. It seems that the samples still contain a relatively large amount of intact *Pseudomonas* proteins originating from the cCFS. This might indicate that the *Pseudomonas* extracts partially inhibit *B. cenocepacia* protein secretion and/or growth, although this has not been further verified.

When *B. cenocepacia* K56-2 cultures were grown in the presence of *S. maltophilia* cCFS, multiple proteins involved in transport and nutrient uptake showed a significantly increased abundance compared to cultures grown in the presence of concentrated *B. cenocepacia* CFS. This was also the case for several stress response and chaperone proteins, as well as for chitobiase, a putative virulence-associated protein, and the conjugative relaxase TrwC of the T4SS-1. There is also a remarkable increase in proteins involved in DNA replication and repair. The most interesting observation is the upregulation of the structural T6SS protein IcmF and the VgrG family T6SS effector protein that was also found upon growth in unconcentrated *S. maltophilia* CFS. Furthermore, growth in the presence of *S. maltophilia* cCFS leads to a significantly increased abundance of a diguanylate cyclase, and of an uncharacterized protein, encoded by the BCAM1006 gene, that lies within the cepacian biosynthesis cluster. Results are summarized in Table 17.

Among the proteins showing a reduced abundance in the secretome of cultures grown in the presence of *S. maltophilia* cCFS, there is a strong decrease in flagellar proteins, pointing to a reduced motility (Table 18). This was also seen for growth in the presence of unconcentrated CFS. Other proteins with a reduced abundance include the structural T4SS-1 protein TraC, a lectin and several exported and phage proteins of unknown function.

The changes in the secretome that are observed here, are caused by QS-independent processes, since the ultrafiltration step theoretically removes all compounds smaller than 10 kDa, thereby also eliminating QS molecules.

From this data, we can conclude that increased T6SS activity is observed when cultures are grown in both concencentrated and unconcentrated CFS, but that only concentrated CFS has an influence on motility and conjugation. It should be mentioned that the concentration step applied to generate the cCFS causes an increased abundance of biomolecules (> 10 kDa) compared to the unconcentrated CFS, which might also explain in part the different results for CFS and cCFS.

Biofilm mass obtained from *B. cenocepacia* K56-2 grown in the presence of concentrated CFS originating from *S. maltophilla* 44/98 and *P. aeruginosa* PAO1 did not differ significantly from biofilms grown in the presence of concentrated *B. cenocepacia* K56-2 CFS (data not shown).

**Table 17. *Burkholderia* proteins signficantly upregulated upon growth of *B. cenocepacia* K56-2 in the presence of *S. maltophilia* 44/98 concentrated cell-free supernatant compared to growth in the presence of concentrated *B. cenocepacia* K56-2 cell-free supernatant.**

| Accession | Gene in J2315 | Description | Average normalized abundance Bc + Bc cCFS[1] | Average normalized abundance Bc + Sm cCFS[2] | Fold change[3] | P-value |
|---|---|---|---|---|---|---|
| **Energy and amino acid metabolism** | | | | | | |
| B4EA25 | BCAL2841 | Phosphoglycerate kinase | 209.865311 | 578.7964443 | 2.24 | 0.013296018 |
| B4EFA6 | BCAM0961 | Aconitate hydratase | 488.2769777 | 1764.47305 | 2.46 | 0.00692511 |
| B4E972 | BCAL0650 | Putative pyruvate-flavodoxin oxidoreductase | 414.0250713 | 1427.367356 | 12.94 | 0.000210782 |
| B4E9X7 | BCAL1796 | Putative saccharopine dehydrogenase | 179.7364966 | 440.6819807 | 2.45 | 0.001876298 |
| **Stress response and chaperones** | | | | | | |
| B4EC19 | BCAL2012 | Putative oxidative stress related two-component regulatory system, sensor kinase protein | 416.9118287 | 4397.860394 | 7.04 | 0.000491162 |
| B4EH35 | BCAM0135 | Putative quinone oxidoreductase | 366.941583 | 355.6382437 | 3.27 | 0.002220641 |
| B4ED06 | BCAL1070 | Putative redoxin protein | 163.0253769 | 372.3388686 | 1.71 | 0.013053078 |

| | | | | | | |
|---|---|---|---|---|---|---|
| B4EBM4 | BCAL1997 | Trigger factor | 774.5835569 | 818.7378556 | 2.21 | 0.003205724 |
| B4EDZ2 | BCAL3270 | Chaperone protein DnaK | 208.8362174 | 444.2663985 | 9.48 | 0.000257651 |
| B4EF95 | BCAM0950 | Lipase chaperone | 446.590543 | 2654.914046 | 3.14 | 1.00E-04 |
| B4ED76 | BCAL2153 | Peptidyl-prolyl cis-trans isomerase PpiB | 420.5358809 | 1572.519554 | 2.70 | 0.000518067 |
| **DNA replication and repair** | | | | | | |
| B4EQ06 | BCAS0239 | UvrABC system protein A | 588.7538282 | 835.7740592 | 2.52 | 0.000197092 |
| B4EHU7 | BCAM1307 | Putative helicase | 211.2705599 | 2643.660016 | 28.94 | 0.000266653 |
| B4EDV9 | BCAL2230 | Putative ATP-dependent helicase | 126.1404121 | 1930.765252 | 12.00 | 0.000787735 |
| **Motility and adhesion** | | | | | | |
| B4E8L8 | BCAL0576 | Flagellar hook-associated protein 1 (HAP1) | 147.2073913 | 1491.162277 | 13.96 | 0.001574182 |
| **Transport and nutrient uptake** | | | | | | |
| B4EKA4 | BCAM2618 | Putative periplasmic lysine-arginine-ornithine-binding protein ArgT | 312.1359617 | 1344.203123 | 2.16 | 0.003637009 |
| B4EHT3 | BCAM1293 | ABC transporter_ substrate-binding protein | 444.9812291 | 486.6687828 | 1.81 | 0.000781118 |
| B4ENX8 | BCAS0582 | RND family efflux system transporter protein | 217.4514343 | 1109.324602 | 6.67 | 0.00191415 |
| B4EK02 | BCAM0499 | TonB-dependent receptor | 199.4569937 | 463.0623074 | 1.99 | 0.00366074 |
| B4EGW5 | BCAM2251 | Putative amino acid solute binding component of ABC transporter | 343.323407 | 792.9670667 | 1.75 | 0.003764868 |
| B4EJG9 | BCAM0446 | Outer membrane efflux protein | 303.5163533 | 416.4499017 | 2.72 | 0.012902206 |
| B4EDJ0 | BCAL0116 | Putative TonB-dependent siderophore receptor | 2192.062865 | 9565.121944 | 2.26 | 0.01479719 |
| **Virulence** | | | | | | |
| B4EAA7 | BCAL0782 | Putative chitobiase | 254.9550457 | 608.6927446 | 1.53 | 0.042655687 |
| B4E682 | BCAL1362 | Rhs element Vgr protein | 614.8587378 | 2231.249129 | 6.83 | 0.000122697 |
| **Other function** | | | | | | |
| B4ELB6 | BCAM1738 | Putative penicillin-binding protein | 693.6565871 | 3426.855072 | 2.91 | 0.005009304 |
| B4EJX1 | BCAM0469 | Putative aldehyde dehydrogenase | 81.16276975 | 503.0861685 | 9.67 | 7.36E-05 |
| B4EMK1 | BCAM0799 | 2_4-dienoyl-CoA reductase [NADPH] FadH | 119.8281043 | 291.0035239 | 1.55 | 0.000188896 |
| B4E5M6 | BCAL2348 | Polyribonucleotide nucleotidyltransferase | 182.7314989 | 2747.250051 | 10.62 | 0.000560201 |
| B4EQ54 | BCAS0288 | Putative phosphoribosylglycinamide synthetase protein | 395.5736678 | 1140.704496 | 5.46 | 0.001288033 |
| B4EQJ6 | pBCA057 | Putative conjugative transfer protein | 236.6914428 | 1434.614714 | 5.60 | 0.000450615 |
| B4E6S3 | BCAL0351 | Putative type VI secretion system protein TssM | 174.2686543 | 213.4821514 | 2.31 | 0.014888109 |
| B4ED05 | BCAL1069 | Diguanylate cyclase | 96.64633518 | 4185.738074 | 41.48 | 0.001769247 |
| B4E5S6 | BCAL3396 | Thiamine-monophosphate kinase | 129.4602797 | 1272.038011 | 458.37 | 0.001089541 |
| **Unknown function** | | | | | | |
| B4EHZ4 | BCAM2377 | Putative exported protein | 902.9858012 | 3529.691204 | 3.91 | 0.000525656 |
| B4EFL4 | BCAM1006 | Putative exported protein | 225.5561516 | 266.1601702 | 2.14 | 0.036293042 |
| B4EKM3 | BCAM0578 | Putative hydantoinase/oxoprolinase family protein | 118.6300361 | 180.1422237 | 1.60 | 0.003321711 |

[1] *B. cenocepacia grown in the presence of concentrated B. cenocepacia cell-free supernatant*
[2] *B. cenocepacia grown in the presence of concentrated S. maltophilia cell-free supernatant*
[3] *Fold change is calculated for Bc + Sm cCFS/Bc + Bc cCFS*

**Table 18. *Burkholderia* proteins signficantly downregulated upon growth of *B. cenocepacia* K56-2 in the presence of *S. maltophilia* 44/98 concentrated cell-free supernatant compared to growth in the presence of concentrated *B. cenocepacia* K56-2 cell-free supernatant.**

| Accession | Gene in J2315 | Description | Average normalized abundance Bc + Bc cCFS[1] | Average normalized abundance Bc + Sm cCFS[2] | Fold change[3] | P-value |
|---|---|---|---|---|---|---|
| **Energy metabolism** | | | | | | |
| B4EEY3 | BCAL3395 | NADP-dependent malic enzyme | 1484.757256 | 362.3083461 | 2.49 | 0.000519293 |
| B4EMS7 | BCAM1944 | Pyruvate dehydrogenase E1 component | 585.5686332 | 99.28732877 | 2.73 | 0.003275172 |
| **Stress response and chaperones** | | | | | | |
| B4E5V4 | BCAL3424 | Probable thiol peroxidase | 467.4674366 | 267.4043803 | 2.09 | 0.046337579 |
| B4ECT8 | BCAL3147 | 10 kDa chaperonin GroES | 3836.921689 | 1332.951903 | 5.49 | 0.000955247 |
| B4EP32 | BCAS0638 | 10 kDa chaperonin GroES | 4693.938895 | 369.3191509 | 5.60 | 0.001256071 |
| **Motility and adhesion** | | | | | | |
| B4E8K5 | BCAL0562 | Negative regulator of flagellin synthesis (Anti-sigma-28 factor) FlgM | 883.9452697 | 166.3197684 | 7.70 | 8.76E-05 |
| B4E8L1 | BCAL0568 | Flagellar basal-body rod protein FlgF | 812.4779558 | 92.91022983 | 3.71 | 0.00218809 |
| B4E811 | BCAL0520 | Putative flagellar hook-length control protein FliK | 234.9626816 | 18.71008471 | 6.70 | 0.002886872 |
| B4E8K7 | BCAL0564 | Flagellar basal-body rod protein FlgB | 218.0057724 | 190.4064588 | 1.63 | 0.008793849 |
| B4EFJ6 | BCAM0987 | Flagellar hook protein FlgE2 | 227.5604358 | 53.01991248 | 2.72 | 0.011640154 |
| B4E8D1 | BCAL2637 | Putative fimbriae usher protein | 1908.394104 | 2023.538767 | 1.58 | 0.027486072 |
| B4E8K8 | BCAL0565 | Flagellar basal-body rod protein FlgC | 1737.362874 | 4.526294328 | 60.44 | 0.000496807 |
| B4EH87 | BCAM0186 | Lectin | 355.1315463 | 148.3386909 | 2.39 | 0.000263498 |
| **Transport and nutrient uptake** | | | | | | |
| B4EMR4 | BCAM1931 | Putative porin | 4206.959582 | 3167.835465 | 2.48 | 0.000721211 |
| B4EJF8 | BCAM0435 | Cation efflux system protein | 3988.346116 | 70.5870269 | 1.72 | 0.005823262 |
| B4EMS9 | BCAM1946 | Putative quinoxaline efflux system transporter protein | 410.1812723 | 280.4282543 | 2.49 | 0.012556108 |
| B4EKB1 | BCAM2626 | Putative heme receptor protein | 545.2837444 | 164.7617309 | 2.38 | 0.043983776 |
| **Other function** | | | | | | |
| B4EAI5 | BCAL0993/ BCAL2877 | Malonyl CoA-acyl carrier protein transacylase FabD | 379.1641924 | 264.3965072 | 1.84 | 0.019292277 |
| B4EBL4 | BCAL1987 | Phosphoribosylformylglycinamidine synthase PurL | 424.242077 | 114.9853066 | 6.12 | 0.000101288 |
| B4EQF8 | BCAS0044 | Putative phosphoribosylglycinamide synthetase protein | 659.8470982 | 336.9115837 | 1.83 | 0.003095904 |
| B4EPD5 | BCAS0747 | Chromosome segregation protein SMC | 1219.207752 | 458.5490506 | 2.87 | 0.005381606 |
| B4EE27 | BCAL3306 | Protein translocase subunit SecD | 495.8563574 | 231.5196107 | 1.62 | 0.04707593 |
| B4EQ38 | BCAS0272 | Urea amidolyase, urea carboxylase subunit | 407.0436885 | 208.8390854 | 2.96 | 0.000136937 |
| B4EQH9 | pBCA041 | Putative TraC conjugative transfer protein | 7712.191624 | 6420.679723 | 1.77 | 0.003624451 |
| **Unknown function** | | | | | | |
| B4EB46 | BCAL2956 | Putative exported protein | 1153.016065 | 496.8557311 | 3.85 | 0.001298404 |
| B4EFE1 | BCAM2073 | Putative exported protein | 1519.411268 | 312.4645962 | 3.60 | 0.007855033 |
| B4E874 | BCAL1578 | Hypothetical phage protein | 975.2548901 | 87.56907504 | 7.27 | 0.006370109 |
| B4EPR7 | BCAS0151 | Uncharacterized protein | 1895.629872 | 1732.01361 | 1.82 | 0.014089075 |
| B4EBI9 | BCAL1961 | Putative exported protein | 1895.877021 | 195.4741369 | 3.26 | 0.001425721 |
| B4EG49 | BCAM1069 | Hypothetical phage protein | 406.8075253 | 8.887092704 | 60.77 | 5.05E-05 |
| B4EP13 | BCAS0619 | Two-component regulatory system, sensor kinase protein | 2303.959201 | 2208.514958 | 1.84 | 0.022116775 |

| | | | | | | |
|---|---|---|---|---|---|---|
| B4EN24 | BCAM0906 | Putative dienelactone hydrolase family protein | 481.0706475 | 176.8859867 | 1.84 | 0.010431298 |
| B4E8G2 | BCAL2669 | Putative exported protein | 260.5253488 | 114.4715326 | 1.71 | 0.020615083 |

[1] *B. cenocepacia grown in the presence of concentrated B. cenocepacia cell-free supernatant*
[2] *B. cenocepacia grown in the presence of concentrated S. maltophilia cell-free supernatant*
[3] *Fold change is calculated for Bc + Bc cCFS/Bc + Sm cCFS*

## 7.4 Discussion

Since polymicrobial communities present in the CF lung often complicate treatment through increased antibiotic resistance and infection potential, it is crucial to understand and characterize the interactions taking place between them in order to develop more efficient treatment plans. In the previous chapter, we already demonstrated the effects of synthetic DSFs from different co-isolated species on the secretome and biofilm formation of *B. cenocepacia*. Other studies that investigate interspecies communication between *P. aeruginosa* and *B. cenocepacia* mainly focused on the role of acylhomoserine lactones and on the impact of alginate (the major extracellular polysaccharide of *P. aeruginosa*) on *B. cenocepacia* virulence and immunotolerance (Chattoraj et al., 2010).

The aim of this investigation was the proteomic characterization of the effects of factors present at physiologically relevant levels in the (concentrated) CFS of two frequently co-isolated CF pathogens, *S. maltophilia* and *P. aeruginosa*. To differentiate the impact of protein factors from small molecular weight compounds like quorum sensors, we set up two different experiments. When using unconcentrated CFS, the observed effects can be caused by both QS-dependent or QS-independent processes. However, when concentrated CFS is used, only QS-independent effects are perceived, since filtration through a 10 kDa MWCO membrane eliminates the low molecular weight QS molecules (< 300 Da).

The effects caused by growth in the presence of *S. maltophilia* or *P. aeruginosa* CFS are different. In the latter condition, T6SS activity seems to be reduced, since Hcp1 is less abundant. This could be the result of the action of PDSF present in the *P. aeruginosa* CFS, as was discovered and described in the previous chapter. However, secretion of the type VI amidase effector, Tae4, is increased upon growth in the presence of *P. aeruginosa* CFS. Tae4 is a cell wall degrading enzyme that causes target cell lysis via its peptidoglycan amidase activity. Self-intoxication is overcome through the production of a specific cognate immunity protein, named Tai4 (type VI amidase immunity), with which it forms a toxin/antitoxin pair (Russell et al., 2012). The latter observation resembles the situation when cultures are grown in the presence of *S. maltophilia* CFS, since secretion of the VgrG-like T6SS effector was increased in this case. Another protein potentially implicated in interspecies competition is the putative chitobiase, a protein with beta-N-acetylhexosaminidase activity found to be upregulated in the presence of *S. maltopilia* cCFS. It acts by cleaving the terminal beta-1,4-linked N-acetylglucosamine from peptide-linked peptidoglycan fragments and is involved in cell wall remodeling.
This indicates that exposure to particular exogenous proteins is sufficient to induce the defense mechanism in *B. cenocepacia* against the presence of a competing species, since T6SS have been implicated in the killing of neighboring bacterial cells (Hood et al., 2010; Russell et al., 2011; Schwarz et al., 2010). The contradictory observations for growth in the presence of *P. aeruginosa* CFS might reflect the active competition between QS-dependent suppression of T6SS activity by *Pseudomonas* and activation of T6SS-dependent self-defense by *Burkholderia* in response to the presence of specific, yet unidentified *Pseudomonas* factors. The extensive downregulation of flagella-associated proteins in the presence of *P. aeruginosa* CFS might reflect a transition to a more sessile state.
The latter phenomenon is also observed for cultures grown in the presence of concentrated *S. maltophilia* CFS, with the significant downregulation of six flagellar proteins. Also, the expression of a lectin and of a fimbriae usher protein is significantly reduced in this condition, as well as that of the

conjugative transfer protein TraC, the T4SS-1 ATPase analogous to VirB4. Both the presence of unconcentrated *S. maltophilia* CFS and ultrafiltration-based concentrated *S. maltophilia* CFS stimulated the secretion of a T6SS Rhs element Vgr family protein and the production of the T6SS structural protein TssM, an IcmF/VasK-type protein with ATPase activity that is essential for bacterial killing in *Vibrio cholerae* (MacIntyre et al., 2010). Furthermore, the conjugative relaxase TraI of the T4SS-1, a protein involved in cepacian biosynthesis, and a diguanylate cyclase were found to be increased in the secretome of cultures grown in the presence of *S. maltophilia* cCFS. The latter enzyme is expected to be an intracellular enzyme. Increased abundance of such enzymes in extracellular extracts is often observed. Several hypotheses have been proposed to explain this, but most likely this is the result of increased abundance of these proteins inside the cells that are then released from dying cells or from extracellular vesicles. Unfortunately there was not sufficient time left to determine this, but speculatively this finding might on its turn indicate increased intracelllular cyclic-di-GMP levels. This global regulatory secondary messenger dictates the transition to a more sessile lifestyle, manifested by reduced motility and increased production of the exopolysaccharide cepacian, thereby promoting biofilm formation. Induction of T6SS activity by cyclic-di-GMP was already previously demonstrated for *P. aeruginosa* (Moscoso et al., 2011), and might explain the increased abundance of the Vgr family protein and TssM. The conjugative relaxase TraI catalyzes the unwinding of duplex DNA and acts as a sequence-specific DNA transesterase, providing the site- and strand-specific nick required to initiate DNA transfer. Together with the upregulation of two helicases, this might indicate the induction of horizontal gene transfer.

Regarding the effects on biofilm formation of *B. cenocepacia*, only the unconcentrated CFS of *P. aeruginosa* provoked a reduction in biofilm mass formation. Since this response was not observed for the concentrated CFS, it is likely to be caused by QS molecules present in the *P. aeruginosa* CFS. Therefore, it would be essential to determine the composition of both the unconcentrated and the concentrated CFS in future experiments. This will provide more insight in e.g. the concentration of QS molecules, the presence of secondary metabolites, protein content and other currently unknown factors. It might also help to explain differences in the response evoked by *S. maltophilia* or *P. aeruginosa* (c)CFS.

This preliminary study already revealed quite some information regarding the interactions that can occur between different species, but it is clear that such studies require more in-depth analyses of the intracellular and extracellular proteome, complemented with transcriptomic approaches. This will potentially unravel even more complex and highly regulated interaction networks. Moreover, we fully recognize that the experiments performed here were distinct from the real CF environment. There is definitely a need to develop good model systems to assess interbacterial interactions in such an environment, which probably requires further improvement of extraction methods and increased sensitivity to assess the bacterial secretomes. The targeted proteomics approach described in the previous experiments is certainly a promising tool for such investigations.

# *CHAPTER IV: GENERAL DISCUSSION*

The original overall goal of this thesis was the discovery of novel T3SS and T4SS-1 effector proteins. However, since a label-free proteomics-based comparison of the secretome of wild type and a ΔT3SS mutant strain under different conditions did not yield any plausible effector proteins, this research item was not further explored. The investigation of the secretome of a T4SS-1 deletion mutant (ΔpBCA017-059) did lead to some remarkable observations, including a possible link with T6SS activity. The search for a valid explanation for our findings led to the characterization of different plasmid regions and the assessment of the effects of the DSF family of quorum sensors. One of the main achievements of this project was the development of a quantitative SRM assay used to monitor the abundance of selected, secreted virulence factors of *B. cenocepacia* under different conditions. This tool allows the rapid and easy determination of the effects of various stimuli or mutations on the overall virulence potential of this pathogen, including T2SS and T6SS activity and piliation.

**Importance of the 92-kb plasmid of *B. cenocepacia K56-2***

The genome of *B. cenocepacia* strain K56-2 is highly similar to that of the completely sequenced J2315 strain and consists of three circular chromosomes (although one of them is recently been reconsidered as a giant plasmid) and a plasmid of approximately 92 kb (Varga et al., 2013). The latter has been implicated in the plant tissue watersoaking phenotype on onion tissue caused by *B. cenocepacia* infection. This phenotype was attributed to the presence of a T4SS, a system homologous to the VirB-VirD translocation system of *A. tumefaciens* and the transfer region of the IncN plasmid pKM101 (Christie, 2001; Engledow et al., 2004). The effector protein responsible for causing the ptw phenotype has not been identified so far. Moreover, there is compelling evidence that this T4SS-1 is required for intracellular survival and replication inside macrophages (Sajjan et al., 2008a; Zhang et al., 2009), but again the identity of the proteins responsible for this phenomenon remains to be uncovered. The plasmid is quite large, and contains accessory genes that are probably not involved in T4SS-1 function or assembly, but the function of these remains to be elucidated. This study aimed at discovering the role of the T4SS-1, which occupies a large part of the plasmid, in virulence factor secretion.

We started our work by searching for differences in the secretome of *B. cenocepacia* K56-2 wild type and a ΔpBCA017-059 mutant strain defective in its T4SS-1 by a label-free proteomics approach. An unexpected discovery was the fact that two known T6SS proteins, Hcp1 and TecA, showed a highly reduced abundance or were even completely absent from the secretome of the ΔpBCA017-059 mutant, in which the entire T4SS-1 operon is removed. On top of that, this mutant strain also displayed a dramatic change in abundance of proteins involved in amino acid metabolism and protein synthesis as well as a reduced ability to form biofilm compared to wild type *B. cenocepacia* K56-2. The large impact of this T4SS-1 KO mutation raised questions. We argued that the deletion also covered non T4SS-1 genes, for instance one or more regulatory genes that lie within the deleted T4SS-1 cluster. Therefore, two new deletion mutants were created by and obtained from Prof. Dr. M. Valvano. In the first mutant only a subset of T4SS-1 structural genes were deleted (ΔpBCA017-033), allowing to refine our analysis to *bona fide* T4SS-1 effectors. The second mutant lacked a regulatory operon possibly linked to quorum sensing (ΔpBCA054-055), mutation of which might explain also part of the T6SS responses. The latter mutant showed a significant reduction in formation and an aberrant biofilm morphology, but did not show reduced secretion of Hcp1 or TecA, neither did the ΔpBCA017-033 mutant. As a consequence, this ruled out a direct impact of T4SS-1 on T6SS activity. It

is not excluded that the original mutant has acquired a spontaneous mutation in e.g. a QS gene, thereby affecting the secretion of several virulence-associated proteins. We recall that the original mutant was made in a gentamicin resistant derivative strain, whereas all control experiments and the later mutants were in the original K56-2 background. In order to sort out this issue, either a complete resequencing of the genome of the ΔpBCA017-059 mutant is required to map potential undesired mutations, or a complementation experiment should be conducted. In the latter case, a plasmid bearing the pBCA017-059 genomic region is expressed *in trans* in the ΔpBCA017-059 mutant, and the secretion of virulence factors, such as Hcp1, TecA and AidA, is quantified by SRM. Only if secretion of these proteins remains affected, an undesired mutation in a regulatory gene possibly involved in QS has occurred. If secretion is restored to (near) wild type levels, the deleted pBCA017-059 region must contain yet another, unknown regulator of virulence factor secretion.

The aberrant biofilm formation of the ΔpBCA054-055 mutant could largely explain the reduced potential for biofilm formation of the original ΔpBCA017-059 mutant. This operon deserves further attention in future virulence studies. Firstly, pBCA055 encodes a diguanylate cyclase. Indeed, it carries the typical GGDEF (Gly-Gly-Asp-Glu-Phe) domain that is involved in the conversion of guanosine triphosphate to the second messenger cyclic-di-GMP, thereby promoting biofilm formation (Boyd and O'Toole, 2012; Ryan, 2013). Deletion of pBCA055 probably causes a reduction in the intracellular cyclic-di-GMP level and consequently a lower amount of biofilm is formed. A follow-up experiment in which intracellular cyclic-di-GMP levels are measured is needed to confirm this hypothesis. Extraction and quantification of intracellular cyclic-di-GMP has been extensively described, and can be achieved through ethanol extraction and subsequent reversed-phase HPLC-based quantification (Roy et al., 2013).

The aberrant biofilm morphology observed for the ΔpBCA017-033 mutant is more difficult to explain. Curiously, we observed a markedly reduced ability to induce genome-encoded phages for the ΔpBCA017-033 and ΔpBCA017-059 mutants, but not for the ΔpBCA054-055 mutant. Prophages have previously been linked to biofilm formation (Carrolo et al., 2010; Nanda et al., 2015; Petrova et al., 2011; Rice et al., 2009; Webb et al., 2004). Phage-induced cell lysis might be required for the release of crucial biofilm-promoting factors, such as eDNA (Gödeke et al., 2011). It has been shown that induction of the KS10 phage occurs spontaneously in *B. cenocepacia* K56-2 (Goudie et al., 2008), so this might also be the case for the poorly characterized Bups phi 1 phage. In order to prove this, extensive characterization of this phage is required, as well as the determination of susceptible host strains using plaque assays.

However, at this time, no valid explanation is available to account for the connection between prophage induction and the T4SS-1. The only described link between T4SS and phages consists of the observation that phages are able to bind T4SS components, leading to T4SS activation and phage penetration (Berry and Christie, 2011; Lang et al., 2010). Possibly, the absence of a plasmid-encoded phage inducer or antirepressor might account for the observed decrease in phage production in the ΔpBCA017-059 and ΔpBCA017-033 mutants, but has not been identified so far.

This study is the first to demonstrate the potential importance of the *B. cenocepacia* K56-2 plasmid in a multitude of functions: biofilm formation, phage induction and possibly also virulence factor secretion and amino acid metabolism in case no secondary mutation has occured in the MHK background. However, several additional experiments are still required to gather stronger evidence in order to support and/or validate our hypotheses, with the most important being the complementation of the different mutant strains with functional copies of the deleted plasmid regions and to determine whether the observed effect on virulence factor secretion is due to a

secondary mutation or to the loss of the concerned plasmid region. Due to time constraints, this could not be performed anymore within the context of this thesis. In case of the absence of any undesired spontaneous mutation, determination of the transcript levels of the different virulence factors would allow to distinguish between a regulatory effect on gene expression and a direct effect on secretion caused by deletion of the pBCA017-059 plasmid region.
Our original aim was to discover the true effector proteins secreted by the T4SS-1 responsible for the plant tissue watersoaking phenotype and the intracellular survival and replication inside macrophages. We have performed many proteomics experiments, not all are included in this thesis. Unfortunately, we could not come up with a clear list of such effector proteins. Future experiments should be directed towards identification of the trigger leading to activation of this secretion system. Experimental conditions mimicking the *in vivo* situation might contribute to this discovery process. This includes culturing the bacterial cells in the presence of human macrophages or onion tissue-derived cells and comparing the behavior of the wild type strain with that of the different mutants. The main obstacle using this approach is to find differentially secreted bacterial proteins amongst the huge amount of host cell proteins, comparable to finding a needle in a haystack. Another option might include the use of an attractive model organism, such as *Caenorhabditis elegans* or the larvae of the great wax moth *Galleria mellonella*, since their innate immune system shares a high degree of similarity with that of mammals (Hoffmann, 1995; Seed and Dennis, 2008). Further characterization of the plasmid-encoded proteins through sequential deletion of different plasmid regions is also required to obtain a complete view on the exact function of this remarkably large plasmid.

**Effect of diffusible signal factors on biofilm formation and virulence factor secretion**

Patients suffering from CF are generally much more susceptible to recurrent and chronic lung infections than healthy persons. About 80% of all adult people with CF are chronically infected with *P. aeruginosa* (Cystic Fibrosis Foundation, 2014). In most cases, several co-infecting bacteria are retrieved in the lungs, like *B. cenocepacia* and *S. maltophilia*. The relevance of polymicrobial communities in increasing antibiotic resistance and often accelerating disease progression has been demonstrated by multiple studies (Bragonzi et al., 2012b; Ryan et al., 2008; Schwab et al., 2014; Stressmann et al., 2011). This phenomenon severely complicates treatment and it is therefore essential to obtain deeper insights in the interactions taking place between the different infecting species. Several types of interactions can occur between bacterial cells, with the most well known and likely also the most important type being quorum sensing. Various classes of QS signals have been identified, such as AHLs, autoinducers, the *Pseudomonas* quinolone signal and DSFs. Already quite some work has been done on the latter class, but it is still far less extensively studied than the AHLs. Therefore, this study focused on determining the role of DSFs as an intraspecies signal in *B. cenocepacia*, as well as its function as a tool for interspecies communication.
*B. cenocepacia* produces the diffusible signal factor cis-2-dodecenoic acid, a 12-carbon fatty acid. Up till now, the exact physiological production levels during *in vitro* growth have not been determined. Therefore, we adapted an HPLC-based method for the absolute quantification of endogenously produced BDSF present in the culture supernatant of *B. cenocepacia* K56-2 at different points along its growth curve. A peak production of approximately 1 µM BDSF was achieved in the mid-to-late exponential phase, after which the concentration gradually decreased. In early stationary phase, a sharp decline in BDSF concentration was observed. The steep drop in extracellular BDSF

concentration in the stationary phase might be explained through the presence of a DSF turnover mechanism, in analogy with the situation in *Xcc*, where the long-chain fatty-acid-CoA-ligase RpfB was found to counteract the activity of the DSF synthase RpfF and might therefore be involved in DSF turnover (Bi et al., 2014). In *B. cenocepacia*, an RpfB homolog (BCAL1541) is present with an identity of 58% to that of *Xcc*. However, its regulation and role in BDSF signal turnover remains to be established (Zhou et al., 2017). The similarity between the BDSF production profile in function of the growth time of *B. cenocepacia* and the DSF production profile of *Xcc* makes it reasonable that the *B. cenocepacia* homolog of RpfB is involved in the BDSF turnover in *B. cenocepacia*, although the possibility exists that still other or additional types of quorum quenching are responsible for this phenomenon (Grandclément et al., 2015). The generation of an RpfB deletion mutant in *B. cenocepacia* K56-2 and subsequent analysis of the BDSF production pattern would be a valuable follow-up experiment to demonstrate the existence of an RpfB-based QS turnover mechanism.

The working concentration of exogenously added BDSF was determined by stimulating exponential phase *B. cenocepacia* K56-2 cultures with different BDSF concentrations and determining the effect on the secretion of selected virulence factors via SRM. This analysis taught us that significant effects on the secretome are only observed after stimulation with higher BDSF concentrations, starting from 75 µM. This concentration farly exceeds the endogenous production level. However, it is possible that *in vivo*, local concentrations, e.g. in biofilms, are higher than those determined in planktonic *in vitro* cultures and that this may increase the impact on a single cell around BDSF producing cells.

A label-free proteomics study, followed by SRM validation, was conducted to investigate the effect of addition of exogenous BDSF on the secretome of exponential phase *B. cenocepacia* K56-2 cultures to obtain a broader view on the relation between BDSF and virulence factor secretion. This analysis learned us that BDSF stimulation leads to increased secretion of the metalloprotease ZmpA, the nematocidal protein AidA, and a GDSL family lipase, while reducing secretion of the T6SS protein Hcp1. This can be explained by the BDSF-dependent activation of the diguanylate phosphodiesterase domain of the BDSF receptor, which decreases intracellular cyclic-di-GMP levels. Cyclic-di-GMP is a second messenger responsible for the regulation of several virulence properties. Low cyclic-di-GMP levels induce the bacterium to adopt a planktonic, more virulent lifestyle, accompanied by lower T6SS activity and decreased biofilm formation (Moscoso et al., 2011).

Since the abundance changes induced upon BDSF stimulation are rather small, a thorough transcript-level validation is required. Also, this study was limited to the evaluation of the changes in the secretome, but it would be highly interesting to monitor differences in intracellular protein expression too. Furthermore, evaluation of the effect of BDSF stimulation on a mutant defective in endogenous BDSF production is expected to result in more pronounced effects and will presumably allow the use of lower concentrations of exogenous BDSF. Today, it is still not completely understood how cyclic-di-GMP affects the expression of a pleithora of virulence- and biofilm-associated genes. Unravelling the signaling cascade provoked by cyclic-di-GMP would therefore be a very interesting topic for subsequent research. Since two different receptor proteins for BDSF have been identified, RpfR and BCAM0227, it would be useful to generate single and double mutants in order to evaluate the contribution of each receptor to the observed phenotypes.

Since quorum sensing plays a crucial role in interspecies communication, this research also aimed at mapping the effects of QS molecules from the major CF pathogens *P. aeruginosa* and *S. maltophilia* on the expression/secretion of virulence factors in *B. cenocepacia*. In this part of the study the effects of the DSF family of quorum sensors was investigated, since significant amounts have been detected in the sputum of CF patients and it has already been demonstrated that they can influence biofilm

formation and virulence factor production (Deng et al., 2010; Ryan et al., 2008, 2009a; Twomey et al., 2012). However, there is currently a lack of proteomics-based data regarding this subject. Both the time-dependent effects of BDSF, DSF and PDSF and the effects after 2h of stimulation on a selected panel of secreted virulence factors of *B. cenocepacia* K56-2 were assessed using SRM. This study learned us that *B. cenocepacia* can sense and respond to both *Stenotrophomonas* and *Pseudomonas*-derived DSFs. The generated effects on the secretome are typically smaller than the ones caused by *Burkholderia*'s own DSF. The different molecular "length" of BDSF, DSF and PDSF might explain the variation in the magnitude of the response. This observation allows us to hypothesize that the *Pseudomonas* and *Stenotrophomonas* DSFs are recognized by the same receptor as is the *Burkholderia* DSF. All three types of DSFs cause a significant reduction in biofilm formation, although high BDSF concentrations had to be used to observe these effects. These results are in line with a general transition of this pathogen to a more virulent state upon stimulation with DSFs and is presumably caused by a DSF-dependent reduction of the intracellular cyclic-di-GMP concentration. This further underscores the importance of DSFs in the regulation of virulence-associated processes and demonstrates their role as interspecies communication tools. Moreover, these results are the first to describe the perception of the *Pseudomonas* diffusible signal factor by *B. cenocepacia*.
Similar as for the intraspecies effects of BDSF, validation based on transcript levels is required to prove the biological significance of these results and an intracellular proteomics analysis could generate a broader view on the effects caused by the different classes of QS signals.
Because of the importance of QS as a general mechanism to regulate several virulence-associated properties (not only in *B. cenocepacia*, but in a whole range of Gram-negative pathogens), it is worthwile to keep searching for ways to modulate these signaling pathways. However, before efficient modulators or inhibitors can be developed, a thorough understanding of the complex regulatory networks underlying these pathways is required.

**Interspecies interactions between *B. cenocepacia K56*-2, *S. maltophilia* 44/98 and *P. aeruginosa* PAO1, three frequently co-isolated CF pathogens**

Since polymicrobial communities present in the CF lung often complicate treatment through increased antibiotic resistance and infection potential, it is crucial to understand and characterize the interactions taking place between them in order to be able to compose more efficient treatment plans. Whereas the previous section described only one aspect of communication, being the addition of DSFs originating from frequently co-isolated species, this study examined the effects of cell-free supernatant (CFS) from stationary phase cultures on the secretome of *B. cenocepacia* K56-2. This CFS not only contains QS molecules at their physiological concentration, but also preserves proteins and other (bio)molecules that are able to participate in interactions with bacterial cells from another species. The use of concentrated CFS (cCFS) on the other hand eliminates the effects of QS molecules, since they are filtered out during the concentration step and therefore allows us to evaluate QS-independent effects.
Growth in the presence of *S. maltophilia* or *P. aeruginosa* CFS causes the upregulation of respectively a VgrG-like T6SS effector and the type VI amidase effector Tae4. The latter protein is able to destroy the cell wall of target cells via its peptidoglycan amidase activity and is typically the toxin module of a toxin-antitoxin system. Self-intoxication is overcome through the production of Tai4, the specific cognate immunity protein (Russell et al., 2012). Activation of type VI effector secretion might

represent the induction of a defense mechanism in *B. cenocepacia* against the presence of a competing species, since T6SS have been implicated in the killing of neighboring bacterial cells (Hood et al., 2010; Russell et al., 2011; Schwarz et al., 2010). Although Tae4 shows a 7-fold increase in abundance upon growth in the presence of *P. aeruginosa* CFS, two other T6SS proteins, Hcp1 and a VgrG-like effector protein, are slightly downregulated in this condition (fold changes of 1.8 and 1.5, respectively). This contradictory observation might reflect the active competition between QS-dependent suppression of T6SS activity, possibly caused by QS molecules present in the *Pseudomonas* CFS and, on the other hand, activation of T6SS-dependent killing by *Burkholderia* in response to the presence of specific, yet unidentified *Pseudomonas* factors.

Similar to growth in the presence of unconcentrated *S. maltophilia* CFS, concentrated CFS stimulates the secretion of a T6SS Rhs element Vgr family protein and the expression of the T6SS structural protein TssM, an IcmF/VasK-type protein with ATPase activity that is essential for bacterial killing in *Vibrio cholerae* (MacIntyre et al., 2010). Furthermore, the conjugative relaxase TraI of the T4SS-1, a protein involved in cepacian biosynthesis, and a diguanylate cyclase were found to be increased in the secretome of cultures grown in the presence of *S. maltophilia* cCFS. These data led us to hypothesize that concentrated *S. maltophilia* CFS induces a diguanylate cyclase, which in turn increases intracelllular cyclic-di-GMP levels. This global regulatory secondary messenger dictates the transition to a more sessile lifestyle, manifested by reduced motility and increased production of the exopolysaccharide cepacian, thereby promoting biofilm formation. Induction of T6SS activity by cyclic-di-GMP was already previously demonstrated for *P. aeruginosa* (Moscoso et al., 2011), and might explain the increased abundance of the Vgr family protein and TssM. The conjugative relaxase TraI catalyzes the unwinding of duplex DNA and acts as a sequence-specific DNA transesterase, providing the site- and strand-specific nick required to initiate DNA transfer. Together with the upregulation of two helicases, this might indicate the induction of horizontal gene transfer. Factors present in the cCFS of *S. maltophilia* may cause *B. cenocepacia* to think that actual bacterial cells are in its neighbourhood, leading to the initiation of several otherwise contact-dependent reaction, like type VI secretion and horizontal gene transfer. Another protein potentially implicated in interspecies competition is the putative chitobiase, a protein with beta-N-acetylhexosaminidase activity found to be upregulated in the presence of *S. maltopilia* cCFS. It acts by cleaving the terminal beta-1,4-linked N-acetylglucosamine from peptide-linked peptidoglycan fragments and is involved in cell wall remodeling.

The effects of growth in the presence of *P. aeruginosa* cCFS could not be analyzed in a reliable way due to the high abundance of *Pseudomonas* proteins in the culture medium. This hampered the accurate alignment of chromatographic peak patterns, an essential part of LC-$MS^E$ data analysis. Without precise alignments, the quality of the subsequent quantitative analysis is very poor, due to many unmatched peaks.

Growth of *B. cenocepacia* biofilms in the presence of unconcentrated CFS of *P. aeruginosa* led to a significant reduction in biofilm mass formation. This is likely caused by the presence of QS molecules in the unfiltered CFS, since similar effects were not observed when the biofilms were grown in the presence of concentrated CFS. A decisive answer can be obtained through analysis of the composition of both the unconcentrated and concentrated CFS. This includes a.o. the determination of the concentration of various QS molecules (e.g. using our previously optimized HPLC-based approach), as well as metabolite and protein profiling. Moreover, a comparison of the CFS from *Stenotrophomonas* and *Pseudomonas* might provide an explanation for the different response generated by *B. cenocepacia*.

This study already revealed quite some information regarding the interactions that can occur between different species, but it likely represents only the tip of the iceberg. More in-depth analyses of the intracellular and extracellular proteome, complemented with transcriptomic approaches, will potentially unravel even more complex and highly regulated interaction networks. Here, we only monitored the effects on *Burkholderia*, but it would be extremely interesting to also evaluate how the communication occurs the other way around, more specifically in *Stenotrophomonas* and *Pseudomonas*.

The logical next step is to assess secretome and intracellular proteome changes during real co-cultures of *B. cenocepacia*, *S. maltophilia* and *P. aeruginosa*. However, there are still some challenges associated with these types of experiments. The first hurdle specific to this microbial community is the tendency of *P. aeruginosa* to quickly overgrow the other two species when cultured in LB medium, complicating quantitative proteomic analyses due to the vast excess of *Pseudomonas*-derived proteins. The use of alternative culture media and/or the physical separation of the different species using semi-permeable membranes could help to overcome this problem. The application of the latter culturing technique also offers the advantage that the intracellular proteins from the different species remain separated from each other, facilitating the monitoring of changes in intracellular proteomes. The major drawback of this set up is the preclusion of physical contact between the cells from different species, which does constitute an important type of interaction. A second obstacle is the difficulty to keep track of the population composition at any point in time. Plating on species-selective media is an excellent way to count the number of actually growing cells per species, but is time-consuming and labor-intensive. PCR-based cell counting methods using species-specific sequences is an alternative option, but requires extensive primer validation and the generation of standard curves for each species. The development of novel species quantification methods, either DNA- or protein-based, would certainly benefit the analysis of mixed cultures.

**Significance and concluding remarks**

The ever-growing problem of antibiotic resistance amongst human pathogens prompts us to design novel strategies to combat the often devastating infections they cause. In order to be able to develop novel types of antibacterial agents, a deeper understanding of the infection process and the virulence-associated properties of the different bacterial species is required. By interfering with essential steps in invasion, colonization or survival inside the host, the infection process can be slowed down or even completely stopped. This research aimed at elucidating a tiny piece of this complicated puzzle by investigating the role of the 92-kb plasmid containing the T4SS-1, which has previously been shown to play an important role in survival and replication in macrophages, and by deciphering part of the intricate network of QS-based regulation of virulence factor secretion. Secretion systems, such as the T4SS-1 investigated here, provide a useful target for antibacterial therapy, since their effector proteins are responsible for a wealth of host cell compromising actions. Due to the fairly high degree of conservation in the composition of these secretion systems, an inhibitor has the potential to target a whole array of Gram-negative pathogens. Because the growth of the pathogens is unaffected by such compounds, the risk for resistance development is highly reduced. However, since no T4SS-1 effector proteins of *B. cenocepacia* have been identified so far, it is of utmost importance to keep investing in research aimed at their discovery. Although our study

was unsuccessful regarding the discovery of novel effectors, we did found additional evidence for the importance of the plasmid-encoded T4SS-1 in e.g. biofilm formation and phage induction.

Since QS systems play a central role in the regulation of virulence in many Gram-negative pathogens and are sometimes involved in antibiotic tolerance, their inhibition represents an attractive path for the design of novel therapeutics. Like secretion system inhibitors, the use of QS inhibitors to attenuate bacterial pathogenicity rather than growth is alluring, given the reduced likelihood for the emergence of resistance against these types of drugs. They have already been proven to make biofilms more susceptible to antibiotic treatment and to improve the clearance of bacteria in animal models of infection (Rasmussen and Givskov, 2006). Understanding the role of the different QS systems will aid in the thoughtful design of appropriate inhibitors, abrogating as many of the deleterious effects as possible. In this context, our study aimed at broadening the existing knowledge on the DSF family of QS molecules, a family much less characterized than the well-known AHLs. We were able to show that *in vitro* grown *B. cenocepacia* K56-2 produces BDSF amounts in the nano- to micromolar range and that there likely exists a turnover mechanism similar to the one described in *Xcc*. Wild type strains only respond to fairly high concentrations of exogenously added BDSF and thereby undergo changes in virulence factor secretion and biofilm formation. On top of that, our research is the first to show the perception of the *Pseudomonas* DSF by *B. cenocepacia* K56-2, as well as it confirmed the response to *Stenotrophomonas* DSF, implicating that the DSF QS system is, next to the AHL system, participating in interspecies communication. Therefore, it should be considered to put more effort in the complete characterization of this system, as well as in the subsequent development and design of drugs targeting DSF QS signaling, next to the already established AHL QS inhibitors.
Next to QS-dependent communication between different CF pathogenic species, we were able to demonstrate the existence of QS-independent interactions. Extensive analysis of the concentrated cell-free supernatant used for these experiments will likely reveal more details on the nature of this type of interplay. More advanced studies using co-cultures of these pathogens are required to broaden our understanding of the various interactions occurring between the different species. This is relevant in the context of polymicrobial infections, and how these can be treated. Several studies have already shown that cooperating pathogens can increase the severity of infections, so further research into tools that can impede this type of cooperative crosstalk is essential to maintain our ability to combat these often devastating infections.

To conclude, this work has provided valuable knowledge regarding the role of the plasmid-encoded T4SS and the DSF-based QS system in the virulence of the cystic fibrosis pathogen *B. cenocepacia* K56-2. However, several follow-up experiments are needed to confirm the generated hypotheses and to fully understand and/or deploy the potential of these systems as targets for novel anti-pathogenic drugs.
Moreover, during this work we developed a widely applicable SRM method for the quantification of *B. cenocepacia* secreted virulence factors. This is a highly valuable tool for future research concerning the virulence potential of this pathogen. The whole process, from secretome isolation to mass spec analysis, only takes three days and provides a rapid and easy way to screen for effects of various stimuli on the virulence of *B. cenocepacia*. In general, this thesis underscores the use of targeted proteomics to accurately quantify selected proteins of interest. In label-free proteomic studies, several interesting proteins can be missed, especially when the instruments used are not the state-

of-the-art equipment. But even with highly sensitive mass specs, proteome profiling often fails to quantify all proteins belonging to a pathway of interest. Using SRM, one can specifically quantify the different proteins involved in a given biological process, on condition that the targets are already known. It also provides a means to quantifiy low abundant proteins that would otherwise be missed by traditional label-free proteomics methods, since it partially overcomes masking by highly abundant proteins.

Analysis of the secretome of *B. cenocepacia* provided an additional challenge during this research project, due to the low amount of protein secreted by this bacterium and the presence of several, uncharacterized compounds interfering with SDS-PAGE and HPLC separations. Both extensive concentration and clean-up of the culture medium was required to obtain high-quality samples for subsequent analysis. The protocol for secretome isolation developed during this thesis is widely applicable to prepare a broad range of impure samples with a low protein content for LC-MS analysis. To my opinion, the impact of impure samples on LC-MS performance is still frequently underestimated.

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# ADDENDUM

# *CHIP-BASED LC-MS MRM APPROACH FOR THE RELATIVE QUANTIFICATION OF* BURKHOLDERIA CENOCEPACIA *K56-2 SECRETED VIRULENCE FACTORS*

This addendum is an adapted version of the second experimental chapter (section 6). This paper has been redrafted as a technical paper that has been submitted to the journal Rapid Communications in Mass Spectrometry. This paper is currently under further revision.

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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. cenocepaci*a 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 chip based chromatography using IonKey™ technology 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 (Gibson et al., 2003). 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* (Cystic Fibrosis Foundation, 2014). 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 (Schwab et al., 2014; Stressmann et al., 2011). 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 (Bragonzi et al., 2012a). *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) (Ryan et al., 2008). DSFs are an important class of quorum sensing molecules that were first described in the plant pathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*) (Fouhy et al., 2007; Wang et al., 2004). 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 (Deng et al., 2010; Ryan et al., 2015b; Ryan and Dow, 2008, 2011). 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* (Twomey et al., 2012). 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 regarding interspecies communication through DSFs is presented in Figure 7.

In *B. cenocepacia*, the $rpfF_{Bc}$ (BCAM0581) gene encodes an enoyl-CoA hydratase with both dehydratase and thioesterase activity, which enables the conversion of the acyl carrier protein thioester of 3-hydroxydodecanoic acid into cis-2-dodecenoic acid (Bi et al., 2012; Boon et al., 2008; Deng et al., 2011). Intermediates of the fatty acid biosynthesis pathway presumably serve as the substrates for $RpfF_{Bc}$ *in vivo* (Ryan et al., 2015a). The gene adjacent to $rpfF_{Bc}$ , BCAM0580, encodes the BDSF receptor protein RpfR (Deng et al., 2012). 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 (Römling et al., 2005). 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 (Deng et al., 2012). 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 (Ryan et al., 2012; Sondermann et al., 2012). Cyclic-di-GMP promotes biofilm formation and T6SS activity, and reduces motility and invasion (Romling et al., 2013). A second DSF receptor protein, encoded by BCAM0227, appears to control only a subset of the BDSF-regulated genes (McCarthy et al., 2010). 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 (Deng et al., 2009, 2012; Ryan et al., 2009a), 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 (McCarthy et al., 2010).

*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 (Aubert et al., 2015; Corbett et al., 2003; Gingues et al., 2005; Kooi et al., 2006; Mahenthiralingam et al., 2005; Rosales-Reyes et al., 2012b). Today, there is no comprehensive quantitative information on the impact of DSFs on actual levels of these secreted virulence factors. The aim of this study was to develop and apply methodology to simultaneously quantify 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 (Sajjan et al., 2008b). 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 selected reaction monitoring (SRM) using a microfluidics based LCMS method (IonKey™ technology). This technology offers a plug and play integration of a high pressure microflow column with the ESI source. A Label-free proteomic approach was additionally performed to detect differences in the abundance of other 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 $OD_{600}$ = 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.

### Extraction and quantification of DSFs from culture supernatant

DSFs were extracted according to Deng et al. (Deng et al., 2010). 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) was performed on an Ettan LC system (GE Healthcare Life Sciences) equipped with an Acclaim PepMap100 C18 column (3 μm, 250 mm x 75 μm) (Thermo Scientific) with as solvent A 0.1% TFA in $H_2O$ (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/μl) were prepared by serial dilution of the individual stock solutions with water. Ten μL 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 μm 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 μl/min and separated on a YMC Triart C18 column (3 μm, 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 μl/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 (Mesuere et al., 2016). 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 μg 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 μm, 300 μm x 50 mm) (Waters) using a flow rate of 15 μl/min for 5 min. After trapping, the peptides were separated on an iKey HSS T3 analytical column (1.8 μm, 150 μm x 100 mm) (Waters) at a flow rate of 1 μl/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 x precursion ion m/z) + 5.

Data were imported into Skyline (MacLean et al., 2010a, 2010b) 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 the 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 (Figure 21). 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 $OD_{600}$ values at the time of extraction are plotted in function of the growth time (Figure 22). 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 ± 600 nM and further to its basal level of ± 400 nM. This concentration is in the same range as was determined for DSF in sputum of patients with CF (250 nM, Twomey et al., 2012). 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 (He et al., 2010).

### Exogenously added BDSF causes significant effects on the secretome only at concentrations higher than 75 µM 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, starting from 5 µM BDSF up to 100 µM BDSF. Cultures were grown for two more hours. The BDSF concentrations were chosen to differentiate from endogenously produced BDSF. 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 (Aubert et al., 2010; O'Grady and Sokol, 2011; Sajjan et al., 2000, 2002; Sajjan and Forstner, 1992; Sun et al., 1995; Tomich and Mohr, 2003)) 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 S2). 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 µM BDSF, collected at start of incubation with BDSF) (Figure 23).

Surprisingly, a significant increase in abundance of ZmpA was detected upon stimulation with BDSF only at a concentration of at least 75 µM. This resulted in a fold change of 1.90 compared to unstimulated cultures. Stimulation with 100 µM 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 µM BDSF (fold change of 1.75), comparable to the profile observed for ZmpA. Stimulation with 100 µM 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 (Deng et al., 2009), suggesting that exogenous BSDF could enhance BCAM0581 transcription. 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 (O'Grady et al., 2011b). Secretion of the T2SS dependent AidA, a protein contributing to the killing of *C. elegans* by *B. cenocepacia* (Huber et al., 2004), 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 (Uehlinger et al., 2009), and was overexpressed in nonmucoid *B. cenocepacia* isolates compared to mucoid isolates (Zlosnik and Speert, 2010), 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 µM 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 µM 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 µM BDSF).

Expression of the major cable pilus protein CblA was slightly reduced when cultures were stimulated with 100 µM 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 µM 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 10). 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 µM BDSF.

A remarkable observation is the significant increase in abundance of numerous ribosomal proteins upon BDSF stimulation (Table 11). 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 (Flieger et al., 2002). Similar phospholipases belonging to class A have been implicated in the pathogenesis of *Campylobacter*, *Yersinia*, *Helicobacter* and *Salmonella* species (Dorrell et al., 1999; Grant et al., 1997; Ruiz-Albert et al., 2002; Schmiel et al., 1998)

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 25). 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. Two ribosomal proteins, L9 and L25, were confirmed to be significantly increased in the secretome of cultures stimulated with BDSF (fold change of 2.2 and 1.7, respectively). Ribosomal proteins are frequently detected in secretome studies and could result from co-isolate membrane vesicles. 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.

### 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 31.

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 12.

## Conclusions

*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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**Supplementary Table S2. Transitions selected for SRM-based quantification.**

| 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+ |
| FIGNVGNDLISTPTPR | 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+ |
| | | E [y6] - 618.2842+ |
| | | E [y4] - 418.2045+ |
| ELEHASNVAAFEAAQAAAAK | 667.0008+++ | 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** | | |
| ALNYQYSVIQYSSVSPYAISSFLGR | 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+ |
| VSNQFNYVSGPGDHVNDPGAETFR | 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+ |

# *CURRICULUM VITAE*


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| 2011 - 2013 | Master of Science in Biochemistry & Biotechnology, Biochemistry & Structural Biology and Biomedical Biotechnology, Ghent University: passed with highest distinction<br>• Optimization of analytical methods for the study of antibiotic resistance in *Stenotrophomonas maltophilia* (L-ProBE) |
| 2008 - 2011 | Bachelor of Science in Biochemistry & Biotechnology, Ghent University: passed with highest distinction |
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## Professional skills

| | |
|---|---|
| Liquid chromatography | For protein/peptide separation and fatty acid analysis. Instruments: Ettan LC system (GE Healthcare), Agilent 1200 series nanoLC system, NanoAcquity UPLC system in 1D and 2D mode (Waters), M-class HPLC system (Waters), Ultimate 3000-RSLC system (Thermo Scientific) |
| Mass spectrometry | For targeted and label-free proteomics. Instruments: LTQ-FT Ultra MS (Thermo Scientific), Synapt G1 mass spectrometer (Waters), 4000 QTRAP mass spectrometer (ABSciex), Xevo TQ-S mass spectrometer (Waters), MALDI TOF/TOF 4800 Plus (ABSciex) |
| Data analysis software | Skyline, Progenesis LC-MS, Mascot Daemon and Distiller, Scaffold, MaxQuant and Perseus, ProteinLynx Global Server |
| Statistics | SPSS, R, GraphPad Prism |
| Other skills | PCR, agarose gel electrophoresis, bacterial cell culture, SDS-PAGE, enzymatic assays, protein extraction and purification |
| Soft skills | Strong analytic skills, ability to anticipate, flexibility, strong sense of responsibility, ability to work in a team and to communicate and discuss with colleagues, excellent planning skills, extensive language skills (both in English and French) |

## Training and conferences

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|---|---|
| 2016 | M-class IonKey Xevo TQ-S user training, Waters<br>Doctoral symposium of the Faculty of Sciences: **poster presentation**<br>Belgian Proteomics Association Conference: **poster presentation** |
| 2015 | Knowledge for Growth: **poster presentation** |
| 2014 | Quantitative Proteomics and Data Analysis, The Biochemical Society<br>ProteoMMX 3.0 meeting – strictly quantitative, Waters Corporation<br>Belgian Proteomics Association Conference: **poster presentation** |
| 2013 | Mass spectrometry data processing, Lennart Martens<br>Effective graphical displays, Doctoral Schools Course, organized by Principiae<br>Advanced academic slide design, Doctoral Schools Course |

## Guidance of students and practical courses

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|---|---|
| 2016 - 2017 | Guidance of a master thesis student<br>Integrated practical course Biochemistry & Microbiology: 2D-PAGE |
| 2015 - 2016 | Guidance of two master thesis students<br>Integrated practical course Biochemistry & Microbiology: 2D-PAGE |
| 2014 - 2015 | Guidance of a master 1 student<br>Integrated practical course Biochemistry & Microbiology: 2D-PAGE |

## Publications

Depluverez, S., Devos, S., and Devreese, B. (2016). The Role of Bacterial Secretion Systems in the Virulence of Gram-Negative Airway Pathogens Associated with Cystic Fibrosis. *Front. Microbiol.* 7, 1336. doi:10.3389/fmicb.2016.01336

## Extracurricular activities

Volleyball player: captain of a volleyball team. Excellent way to improve my leadership skills and to learn to work/play as one team, since organization is crucial during a volleyball game.

Trainer/coach of a junior volleyball team: transfer skills to young players and teach them how teamwork can improve their performance. Keep them motivated to give the best they can and to invest their time and energy in training.