Small things matter:

Deciphering the role of small RNAs in

Burkholderia cenocepacia

Sanne Kiekens

Pharmacist

Thesis submitted to obtain the degree of Doctor in Pharmaceutical Sciences

Promotor: Prof. Dr. Tom Coenye

Co-promoter: Dr. Andrea Sass

Laboratory of Pharmaceutical Microbiology

2017

GHENT UNIVERSITY

FACULTY OF PHARMACEUTICAL SCIENCES

LPM Laboratory of Pharmaceutical Microbiology

Ghent, October 2017

Author

Apr. Sanne Kiekens

Promotors

Prof. Dr. Tom Coenye, Dr. Andrea Sass

Promotor

Prof. Dr. Tom Coenye

Laboratory of Pharmaceutical Microbiology, Ghent University, Belgium

Co-promotor

Dr. Andrea Sass

Laboratory of Pharmaceutical Microbiology, Ghent University, Belgium

Members of the examination and reading committee

Prof. Dr. Apr. Filip Van Nieuwerburgh (Chairman)

Laboratory of Pharmaceutical Biotechnology, Ghent University, Belgium

Prof. Dr. Apr. Koen Raemdonck (Secretary)

Laboratory for General Biochemistry and Physical Pharmacy, Ghent University, Belgium

Dr. Apr. Heleen Van Acker

Laboratory of Pharmaceutical Microbiology, Ghent University, Belgium

Prof. Dr. Bart Devreese

Laboratory for Protein Biochemistry and Biomolecular Engineering, Ghent University, Belgium

Prof. Dr. Ir. Jo Vandesompele

Department of Pediatrics and Medical Genetics, Ghent University, Belgium

Prof. Dr. Laurence Van Melderen

Departement of Bacterial Genetics and Physiology, Université Libre de Bruxelles, Belgium

DANKWOORD

Professor Coenye, ik wil je graag bedanken voor de kans om te starten bij LPM. Het zijn vier heel interessante jaren geweest waar ik veel heb bijgeleerd. Jij hebt me altijd moed ingesproken, was steeds positief en tussen al je drukke werk door had je nog tijd voor het verbeteren van mijn thesis. Ook ben ik je zeer dankbaar voor de congressen en opleidingen die ik heb mogen bijwonen gedurende mijn carrière bij LPM.

Professor Nelis, je passie voor het vak microbiologie was aanstekelijk, ik ging met plezier naar al je lessen en dat was ook één van de redenen waarom ik voor een doctoraat in microbiologie gekozen heb. Ik wil je bedanken voor de kans om te starten bij LPM en voor je enthousiasme en interesse tijdens de eerste jaren dat ik doctoreerde.

Andrea, mijn co-promotor, I don't know where to start ☺. We worked closely together for 4 years and had a lot of "it's *Burkholderia* "conversations. I want to thank you for all the information you gave me on this "bug". I enjoyed the conferences we went to, I known Mexico was way too hot for you, but I enjoyed. I admire you for your work and 'never give-up' attitude. Our last conference in Edingburgh, one guy admired us that we were working on *Burkholderia*… They started but then gave up. From the beginning you warned me that it wasn't going to be easy but if you go for it you will find what you need. So thank you Andrea, to never give up! And thank you for patience and help with writing my thesis.

Verder wil ik het volledige LPM-team (ook degene die het labo verlaten hebben, eenmaal een LPMer, altijd een LPMer…) bedanken voor alle leuke momenten. Ik zal deze 4 jaar niet snel vergeten door alle steun die jullie me gaven en door de leuke koffiepauzes, weekends en etentjes. Nog een extra bedankje aan iedereen die meegewerkt heeft aan het "LPM got talent" filmpje, als jullie nog foto's of gênante filmpjes nodig hebben, je weet me te vinden ☺.

Heleen, jij was één van de mede-moleculair werkers in LPM. Hoe vaak hebben we in de gang staan discussiëren welke kleine stap we nu konden aanpassen of optimaliseren. Dank je dat ik altijd bij je terecht kon. Zelfs als je het superdruk had, maakte je toch nog tijd voor mij en 1 blik was genoeg om tijd te maken voor koffiepauze ☺.

Lisa, over jou kan ik een boek schrijven, of nog beter over ons, maar hier zal ik het kort houden. 5 jaar hebben we blijkbaar samen gestudeerd om elkaar slechts af en toe te spotten. Maar dan kwamen we beide op het lumineuze idee om te starten bij LPM. Eerst hadden we samen microbiologie opleiding,

werden we lab maatjes en konden we uitplaat wedstrijdjes tegen elkaar houden. Niet veel later hebben we een volledige bokaal ingepalmd om onze bureau te maken. Duizend life-coach gesprekken en cocktails later kunnen we wel echt zeggen dat we buddy's door dik en dun zijn. **Annelien**, dat geldt ook voor jou, ik ben heel blij je te leren kennen. Eerst hebben we dans en bacterie info uitgewisseld, nu kunnen we overschakelen naar dans en bouw info en hopelijk dat bij een lekkere sushi schaal en een glaasje wijn. **Sarah**, jij was zowat de vaste waarde voor mij in het LPM labo. Jij kreeg alles voor elkaar, jij kon anderen helpen en dan tegelijk zelf nog honderden uitplatingen doen. Ik weet niet wat ze gaan doen zonder jou… Dank je om altijd te luisteren en te helpen. Ook ons Brno uitstapje/congres zal ik niet vergeten, allebei even snel KO maar een dessertje kon er wel nog bij! **Rosina**, je stond altijd direct klaar voor mij in de strijd tegen SAP. Ik heb het altijd heel leuk gevonden met je te babbelen en te discussiëren. Je was altijd heel enthousiast als ik iets vertelde. **Inne**, wat zou LPM zijn zonder jou. Jij zorgt voor een luchtige sfeer, houdt iedereen te vriend en niets is te bizar voor jou, zelfs koeknuffelen. Dank je om zo open te zijn en er gewoon te zijn. **Petra**, ik denk dat onze gesprekken zich vaak limiteerden tot 1 onderwerp, maar saai waren ze nooit. Reizen: en naar waar is de volgende… Ik neem zeker een voorbeeld aan jou, dat weten ze al op m'n volgend werk ☺. **Nele**, ook met jou heb ik veel leuke gesprekken gehad. Ik wens je heel veel succes in je verdere loopbaan, dat komt sowieso goed. **Lisa O**, je bent een heel enthousiaste en gemotiveerde werker. Het was heel leuk je in het labo te hebben. **Freija**, lopen we samen 15 km op de midzomernachtrun? Tuurlijk waarom niet. Je was altijd direct enthousiast als iemand iets voorstelde. Het was niet alleen fijn samen te lopen maar ook om samen te werken. **Gilles** en **Ilse**, jullie hebben me allebei opgeleid en daarna begeleid in de bizarre wereld van de *C. elegans* wormpjes, in de nog bizardere wereld van CLC workbench en alle andere zaken die ik waarschijnlijk nu al terug vergeten ben. Het was heel aangenaam met jullie samen te werken, ik heb veel van jullie geleerd. **Evelien**, ik weet nog in het begin als Lisa en ik wat verloren liepen jij direct klaar stond om te helpen. Dankjewel daarvoor. **Yunhui**, Cara, thank you to help me with *C. elegans* and for all the Chinese cookies. **QI**, you were always really helpful and kind, thank you. **Karl-Jan**, ook al zitten Lisa en ik niet meer in de bokaal om even op onze grond op therapie te komen, je bent altijd welkom bij mij (ik kan niet voor Lisa spreken…). Jij stond ook steeds klaar voor te helpen, na te denken of niets te doen ☺. **Eva**, het was altijd zalig met jou te tetteren in het labo (soms wel moeilijk te combineren met experimenten). Je enthousiasme voor musicals, Harry en bacteriën werkt aanstekelijk. Geniet nog van je laatste (o-ow) jaar bij LPM. **Charlotte**, ook met jou was het steeds lachen. Jij zal er voor moeten zorgen dat de mannen (duidelijk het zwakke geslacht) het blijven aankunnen. **Jasper**, bedankt om me te helpen bij flow en alle andere vragen die ik had. Je stond altijd direct klaar als ik hulp nodig had. **Aurélie**, ook bij jou kon ik steeds terecht met alle bedenkingen, vragen of wat dan ook. **Ian**, welkom in de wonderlijke onverstaanbare wereld van *Burkholderia*

mutanten. Ik ben blij dat we even samen hebben staan sukkelen en staan vloeken op de J2315. Blijven gaan zoals je bezig bent en er gaan zotte dingen ontdekt worden daar ben ik zeker van. **Eline**, ook jij was bijna een vaste waarde in de bokaal van Lisa en mij. Je bezoekjes waren altijd heel aangenaam en het werken met jou was minstens even aangenaam. Bedankt voor de prachtige microscopie foto's en je enthousiasme.

De zoektocht naar de functie van sRNAs heb ik niet alleen gedaan. Naast heel veel hulp van Andrea heb ik veel hulp gekregen van mijn 3 thesis studenten, **Hadiel**, **Severien** en **Charlotte H**. Zij hebben heel veel werk verricht en alhoewel het vaak niet goed uit kwam hebben ze me heel hard geholpen. Charlotte H, nog eens een extra bedankje om de laatste maanden zo leuk te maken in het labo, ondanks de vele frustraties. Binnenkort zullen we het er allemaal kunnen afdansen!

Tenslotte wil ik eigenlijk mijn hele entourage buiten LPM bedanken. Al mijn vrienden om te luisteren en zelfs geïnteresseerd te zijn in mijn bacterie onderzoek maar ook om mij te helpen ontspannen wanneer het echt nodig was. **Sanne**, **Ellen** en **Aline** (en jullie leuke aanhangsels) voor de etentjes, jacuzzi time, uitstapjes, Gentse feesten… **Gudrun** en **Inge**, voor alle micro en macro adventures. De **strekewijven**, vooral voor 5 jaar farmacie fun, vele goeie (verkleed) feestjes, golf initiaties…. Mijn mede **flamingo's** voor een onvergetelijke 2 jaar de Bouwmeestersstraat onveilig te maken en nu de andere kant van Gent. **Pierre** en **Lucia**, voor er gewoon altijd te zijn en voor pisco. **Brecht en klaartje**, voor Abu ☺ en voor geen watervrees te hebben. **Karen**, voor te kletsen en te gieren. Alle **spanjewijven** en de **KG bende** voor de gezellige avondjes en de zotte weekendjes. **Humdrum escapade** bende voor de vaak onverwachte zalige avonden. **QP**-ers om zo enthousiast te zijn, in alles. Ik zal jullie wel uitgebreid bedanken met een BBQ bij onze werf/housewarming.

Om mijn frustraties kwijt te geraken of gewoon ter ontspanning is dans ook een vaste waarde in mijn leven. Dank je **Fanny** voor je levendige lessen, ik kijk er naar uit om er weer volledig voor te gaan. Ook dank aan de "**Steam heat cats**" voor de leuke repetities en optredens. Ook wil ik mijn toekomstige werkgever, **Applied Maths,** bedanken om mij in het team op te nemen. Ik kijk er heel erg naar uit om nieuwe zaken te leren. Ja ik krijg niet genoeg van bacteriën.

Dan zeker niet te vergeten: mijn hele enthousiaste **familie** die elk feest wel vragen wanneer ze naar de receptie mogen komen met **Pit en Petere Jos** op kop, en mijn **schoonfamilie** moet daar zeker niet voor onderdoen. Bovenaan mijn bedankingslijstje staan toch wel **de moetsj**, **Hermpie** en de **Kiekens** gang. Bedankt om mij eigenlijk ALTIJD te steunen, welke richting ik ook uit ga. Jullie stimuleren me in alles,

niets is te zot voor woorden, eigenlijk hoe zotter hoe beter. Jullie “niet zeuren, gewoon doen” motto helpt me altijd overal doorheen en nu ook.

En nu komt de finale immense ‘dankuwel’ en ook wel ‘sorry’ voor mijn irritante momenten. **Ruben**, mijn roomie, huis- en Abu mede-eigenaar, reisbuddy, praatpaal en zoveel meer maar dus eigenlijk vooral het beste lief dat ik maar kan wensen. Als mijn familie me al zo hard steunt doe jij er nog wat druppels bovenop. Als het kan zou je die bacterie van een *Burkholderia cenocepacia* (opgeslagen in je gsm om goed te kunnen uitspreken) eens goed door elkaar schudden en zeggen naar mij te luisteren want anders…. Merci om altijd klaar te staan als ik het nodig heb, om mij te stimuleren in alles wat ik wil maar over twijfel, maar vooral gewoon om je enthousiaste zelve te zijn. Alleen met jou zou ik een huis kopen, het volledig in stof werken en er dan in gaan wonen met gewenste en ongewenste huisdieren ☺.

Table of Contents

List of Abbreviations

5'P	5'-monophosphate
5'PPP	5'-triphosphate
AHL	*N*-acyl homoserine lactone
asTSS	Internal antisense transcription start site
Bcc	*Burkholderia cepacia* complex
BCESM	*Burkholderia cepacia* epidemic strain marker
BDSF	*Burkholderia* diffusible signal factor
BSM	Basal salt medium
Cci	*Burkholderia cenocepacia* island
CDC	Centers for Disease Control and Prevention
cDNA	Complementary deoxyribonucleic acid
CDS	Coding sequence
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming units
CRISPR	Clustered regularly interspaced short palindromic repeats
Csr	Carbon storage regulator
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dRNA-seq	Differential RNA sequencing
dsRNA	Double-stranded ribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
EtOH	Ethanol
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ET-12	Electrophoretic type 12
HIV	Human immunodeficiency virus
H_2O_2	Hydrogen peroxide
isTSS	Internal sense transcription start site
LBA	Luria-Bertani agar
LBB	Luria-Bertani broth
LC-MS^E	Data-independent liquid chromatography/mass spectrometry
LMG	Laboratorium voor Microbiology, Faculty Sciences Ghent University
LPS	Lipopolysaccharide
MCS	Multiple cloning site
MFE	Minimum free energy
MIC	Minimum inhibitory concentration
miRNA	Micro ribonucleic acid
MQ	MilliQ water
mRNA	Messenger ribonucleic acid
NaCl	Sodium Chloride
NaOCl	Sodium hypochlorite

OD	Optical density
oTSS	Orphan transcription start site
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PMO	Phosphorothioate morpholino oligomer
PNA	Peptide nucleic acid
PNPase	Polynucleotide phosphorylase
PS	Physiological saline
pTSS	Primary transcription start site
qPCR	Quantitative polymerase chain reaction
QS	Quorum sensing
RBS	Ribosome binding site
RNA	Ribonucleic acid
RNase	Ribonuclease
RNAi	RNA interference
RNA-seq	RNA sequencing
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
SAH	S-adenosyl-L-homocysteine
SDS	Sodium dodecyl sulfate
sRNA	Small regulatory ribonucleic acid
TE	Tris/EDTA
TBE	Tris/borate/EDTA
TEX	Terminator exonuclease
tRNA	Transfer ribonucleic acid
TSS	Transcription start site
UTR	Untranslated region
WHO	World Health Organization

CHAPTER I. INTRODUCTION

1. Increasing drug resistance in bacteria

Infectious diseases are among the biggest threats to human health (1). According to a "Centers for Disease Control and Prevention" (CDC) report of 2013, at least 2 million people suffered from serious illness by bacterial infection, causing 23,000 deaths in the United States alone (similar results were described for Europe). In 2015, the World Health Organization (WHO) reported lower respiratory tract infections as the third deadliest disease, causing over 3 million deaths worldwide (2). Many of these deaths resulted from infection with multidrug-resistant bacteria (3-5).

Since the discovery of penicillin in 1928 by Alexander Fleming (6), antibiotics have been widely used to treat and prevent bacterial infections. Fleming not only persisted in finding the mechanism but he was also among the first scientists to warn about potential resistance. Since then, aided by Paul Ehrlich's systematic screening approach, a variety of antimicrobial drugs were developed. However, resistance increased steadily and nowadays it is a global threat (1). There is a need for a better surveillance in antibiotic treatment, as annually over 250 million doses of antibiotics are prescribed in the United States. Additionally the use of antibiotics as veterinary medicines, as biocides and as feed additives should be controlled and limited. It is partly because of this large amount of antibiotics in the environment that the occurrence of antibiotic resistant bacteria and antibiotic resistant genes increases (5).

Antibiotic resistance genes are typically genes involved in preventing entry of the antibiotic into the cell or in expulsion of the antibiotic from the cell before it reaches its target. Additionally, many bacteria harbour enzymes to deactivate or degrade antibiotics, such as β-lactamases found in Enterobacteriaceae, which enzymatically destroy β-lactam antibiotics (5, 7). Resistance in bacteria is either intrinsic or acquired through exchange of genetic information. Intrinsic resistance comprises the impermeability of bacterial cells, the activity of efflux pumps, the deactivation or degradation of antibiotics or the absence of the drug target. Horizontal gene transfer (uptake of free DNA and mobile genetic elements containing resistance genes) is the predominant mechanism to acquire resistance and *de novo* development of resistance occurs through spontaneous mutations (5, 8, 9). A less explored area is adaptive resistance. This phenomenon is probably caused by altered gene expression and emerges when bacterial cells are subjected to a gradual increase in concentration of an antimicrobial compound. It has a transient nature and often reverts upon removal of the antibiotic or stress factor (10, 11).

To conquer this resistance threat, the use of antibiotics needs to be monitored and the investigation of resistance mechanisms together with the development of new antimicrobial strategies is required (7). If no concerted effort is done, it is estimated that by 2050, antibiotic-resistant-infections could cause over 10,000,000 deaths per year, with a global economic cost of approx. $ 1 trillion (12). A major challenge in finding new strategies is the treatment of infections that do not elicit resistance, and therefore a profound knowledge of the bacterial adaptation process towards several stress factors is required.

2. *Burkholderia cenocepacia*

Burkholderia cenocepacia and *Burkholderia multivorans*, members of the *Burkholderia cepacia* complex (Bcc) are the predominant *Burkholderia* pathogens colonizing a cystic fibrosis (CF) lung. They can cause the "cepacia syndrome" in CF patients, an almost always fatal pneumonia with a rapid decline in lung function, often associated with bacteraemia (13).

2.1 *Burkholderia* and the *Burkholderia cepacia* complex

Burkholderia, a genus of Gram-negative bacteria first described in 1992, comprises over 90 diverse and versatile species and is still expanding. These bacteria are found in soil and fresh water, and the majority interacts with a variety of hosts (humans, animals, plants and fungi) in a harmful and/or beneficial manner. The genus is split into three deep-branching clades based on 16S rRNA gene data. The first clade consists of the Bcc, well-known but rare opportunistic pathogens, a group of plant pathogens (*Burkholderia gladioli*, *Burkholderia plantarii* and *Burkholderia glumae*) and the pathogens *Burkholderia mallei* and *Burkholderia pseudomallei*. Clade II and III comprises environmental- and plant-associated species, many of them beneficial to their host. The benefit of these bacteria lies in the production of various secondary metabolites, with proven antifungal, bactericidal or biopesticidal properties (14). For example, pyrrolnitrin, a chlorine-containing phenylpyrrole derivative synthesized out of tryptophan, shows antifungal activity and inhibits growth of *Candida albicans* and several Gram-positive bacteria (14, 15).

Of the entire *Burkholderia* genus, Bcc bacteria have been most intensively researched (16). This complex comprises at least 20 closely related species, among them the type species *Burkholderia cepacia*, able to cause persistent and often fatal infections (14, 17, 18). Originally described as a pathogen that cause soft onion rot, Bcc bacteria are nowadays known as human opportunistic pathogens (19). They cause lung infections in immunocompromised patients, but are harmless to healthy people. Not only do they adhere to epithelial cells, but they are also able to invade and survive intracellularly in epithelial cells, neutrophils and macrophages. Furthermore, Bcc bacteria possess several virulence factors (extracellular enzymes, biofilm formation, lipopolysaccharide production etc…) and they are resistant to a broad range of antimicrobial compounds which complicates treatment of Bcc infections (17, 20).

Besides their detrimental effects, Bcc bacteria have also shown beneficial effects for the environment; they produce antimicrobial compounds that protect and promote growth in plants. For these reasons,

the Environmental Protection Agency has registered Bcc strains for commercial use in the United States. However, this has raised concerns about the risk for vulnerable individuals (19).

2.2 Patients vulnerable for Bcc infections

For CF patients, Bcc infections often result in an increased morbidity and mortality. Cystic fibrosis is caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which codes for a chloride ion channel present in the epithelial cell membrane. A dysfunction of this chloride transporter causes impairment of several organs, but the effect is most pronounced in the respiratory tract. Highly viscous mucus is formed which causes an impaired mucociliary clearance in the lung (17, 21). The CF sputum becomes a perfect breeding ground for bacterial pathogens which colonize and persist in the lung. Besides members of the Bcc complex, many other bacteria colonize the CF lung, but Bcc bacteria and *Pseudomonas aeruginosa* cause the most problematic infections. They are characterized by a significant reduction of the patient's lung function while their response to antibiotic therapy is rather poor. *P. aeruginosa* is more widely distributed within the CF community (70-80%) compared to Bcc bacteria (3-30%) (21). However, due to frequent patient–to–patient transmission, the nearly impossible eradication and their often negative influence on health, Bcc bacteria are considered as clinically important pathogens. Furthermore, they are able to enter the bloodstream and cause septicaemia accompanied with acute respiratory failures, denoted as the cepacia syndrome. *B. cenocepacia* accounts for 50 - 90 % of all Bcc infections diagnosed in CF (7, 21).

Bcc bacteria are not carried as commensal organisms, but are transferred through social contact or are acquired form the environment. Disinfectants, intravenous solutions and contaminated medical devices have been reported as sources of Bcc bacteria (19). Due to the high risk for patient–to–patient transmission, clinical units of CF patients have set up a strict segregation policy since the 1990s. Treatment involves intravenous antibiotics and starts from the moment a CF patient is unwell and suffers from exacerbation of respiratory symptoms. Guidelines recommend a combination of at least two antibiotics for a period of 10 to 21 days. Typically, broad-spectrum antibiotics are chosen (e.g. β-lactams, macrolides, tetracyclines, aminoglycosides) (17, 21).

Not only CF patients are at high risk for invasive Bcc infections, there also have been reports of Bcc infections in immunocompromised patients (e.g. cancer and HIV-patients) (22). For patients suffering from chronic granulomatous disease (CGD), Bcc infections are the second most common cause of death (19, 23). CGD is a genetic immunodeficiency affecting phagocytes which results in an impaired production of reactive oxygen species. As a result, phagocytes fail in the oxidative killing of bacteria

and fungi and CGD patients suffer from recurrent infections associated with a dysregulated inflammatory response (19, 24, 25).

2.3 *Burkholderia cenocepacia*

2.3.1 ET-12 lineage

In the 1990s a highly transmissible lineage of *B. cenocepacia* emerged which made it one of the most clinically important members within the Bcc. The ET-12 lineage was first isolated in Canada and the UK, but rapidly reached Europe (26). Since then, several ET-12 strains have been isolated. Three strains (J2315, BC7 and K56-2) were isolated from patients with cepacia syndrome. Each strain harbours three chromosomes and one plasmid. Sequence data indicate a high similarity of their gene content, apart for several additional copies of insertion elements in the J2315 strain. Strain K56-2 is known as the less antibiotic-resistant strain (27). The ET-12 strains are the only Bcc strains encoding for the *Burkholderia cepacia* epidemic strain marker (BCESM) in combination with genes for cable pilus and the 22-kDa adhesin (19).

2.3.2 The genome of *B. cenocepacia* J2315

The first Bcc strain for which the whole genome sequence was completed was *B. cenocepacia* J2315 (LMG16656) (Figure 1) (13, 28). The *B. cenocepacia* J2315 genome contains 8.06 Mb and is, together with all Bcc species with genome sizes ranging from 6 Mb to 9 Mb, among the largest genomes found in Gram-negative bacteria (28, 29). *B. cenocepacia* J2315 has an average GC-content of 66.9 % and, compared to other *B. cenocepacia* strains, 21 % of its genome is unique which highlights its genomic plasticity. Most Bcc bacteria harbour three chromosomes and one or more plasmids. The two largest chromosome are stable and essential for survival as they contain ribosomal RNA operons and several essential genes (19), while the third is described as a nonessential megaplasmid (30). Most housekeeping functions such as cell division and central metabolism, are encoded on the first and largest chromosome and therefore this chromosome displays a higher level of conservation in Bcc strains. The two smaller chromosomes harbour a larger proportion of genes for functions such as protective responses and virulence factors (29). Due to these large genomes, a high number of accessory functions are present which allows Bcc strains to survive within a broad spectrum of environments (31).

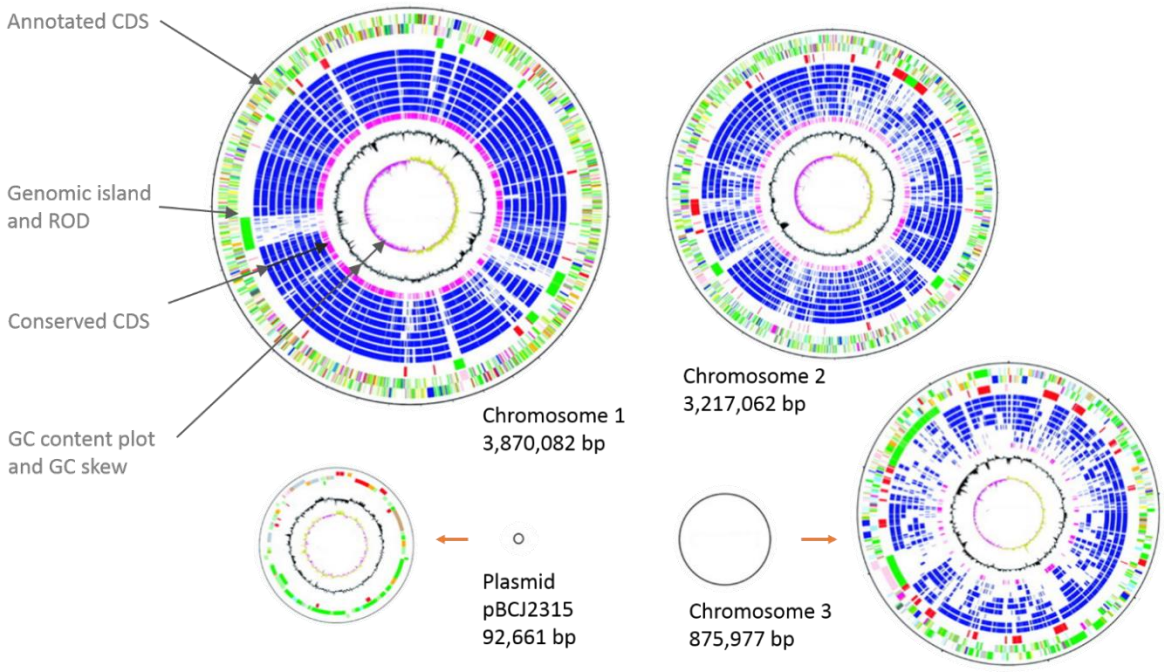


Figure 1. Genome of *B. cenocepacia* J2315. Chromosome 1 and 2 are drawn to scale. For chromosome 3 and the plasmid the empty circles represent the correct scale and the additional circles represent their content. From the outer side of the circle to the inner: two pair of coloured concentric circles represent annotated coding sequences on both coding strands. The subsequent circle represents coding sequences in genomic islands (green) and regions of difference compared to other Bcc strains (red). The blue circles represent homologues coding sequence in *Burkholderia cenocepacia* HI2424, *Burkholderia cenocepacia* AU1054, *Burkholderia contaminans* 383, *Burkholderia ambifaria* AMMD, *Burkholderia vietnamiensis* G4, *Burkholderia xenovorans* LB400, *Burkholderia pseudomallei* K96243 and *Burkholderia thailandensis* E264. The purple circle represents shared orthologues with *Ralstonia solanacearum* GMI1000. The black inner circle represents the GC-content and the middle circle represents the GC skew. If this is > 1 % it is indicated in khaki, < 1 % in purple (28).

Fourteen regions within the genome of *B. cenocepacia* J2315 are characterized by deviations in GC- content, by a high proportion of mobility genes (e.g. encoding transposases and integrases) and virulence genes and by the presence of more hypothetical proteins compared to the rest of the genome (28, 32). These regions are referred to as genomic islands and have likely been acquired through horizontal gene transfer. These fourteen regions together account for 9.3 % of the complete chromosome (13, 28). Genomic island 11 (BcenG11), originally designated as the *Burkholderia cenocepacia* island (cci), encodes for several genes linked to virulence and metabolism. Within this island the BCESM marker is encoded, a region formerly used as a marker for epidemic strains in the late 1990s (33). These genomic islands make up part of the unique regions of the *B. cenocepacia* J2315 genome. Additional regions of difference are mobile genetic elements, indel regions and allelic variants, which include the cable pilus locus and the 22-kDa adhesin, AdhA, important in the adhesion to epithelial CF cells (28).

Whole genome sequencing analysis revealed a multitude of new and interesting features of *B. cenocepacia* J2315 (28, 34), however about 15 % of the annotated genes have no known function or have only a general function prediction (35).

2.3.3 Virulence factors

To survive within a host, *B. cenocepacia* has several virulence factors, located on all three chromosomes and on the plasmid (19). Genomic islands in *B. cenocepacia* are known to encode for several virulence factors, illustrating the importance of lateral gene transfer. The *cci* pathogenicity island, unique to *B. cenocepacia* strains encodes for a fatty acid biosynthesis operon, several transcriptional regulators and a quorum sensing system (33). Next paragraphs briefly introduce the virulence factors of Bcc species.

2.3.3.1 Quorum sensing

Quorum sensing (QS) is the bacterial cell communication system. It operates through a synthase which produces signal molecules, and a regulator responsive to these molecules. *N*-acyl homoserine lactones (AHLs) are among the most used signal molecules in Gram-negative bacteria (14), usually produced by a synthase of the LuxI protein family and recognized by a LuxR protein family regulator (36). When a certain concentration threshold is reached, signal molecules bind to receptors resulting in increased or decreased expression of certain genes. All Bcc bacteria possess two QS systems, the CepIR system, a LuxIR-type QS system, and the RpfFR QS system (13, 37, 38). This second QS system uses a fatty acid molecule as signal molecule; cis-2-dodecenoic acid designated as the *Burkholderia* diffusible signal factor (BDSF) (39).

2.3.3.2 Biofilm formation

Like many other bacteria, *B. cenocepacia* is able to form biofilms, e.g. structured communities of bacterial cells embedded in a self-produced matrix. The oxygen and nutrient availability differs from the surface of the biofilm to the deeper layers of the aggregated structure. Therefore bacterial cells within the biofilm show differential growth rates and gene expression, depending on location relative to biofilms surfaces. This sessile lifestyle protects bacterial cells against the host immune defence and other environmental stresses and is often associated with chronic infections (40, 41).

2.3.3.3 Invasion of and Interaction with the host

To invade the host, surface structures such as lipopolysaccharide (LPS), flagella, pili and adhesins are important. Whereas flagella, pili and adhesins are important for motility and adherence, LPS is known to cause a strong immune response which contributes to host cell damage (39). This component consists of lipid A, core and O-antigen (13). The LPS structure of Bcc bacteria exhibits a lower anionic charge of the cell surface compared to other Gram-negative bacteria. Therefore cationic compounds, e.g. aminoglycosides and polymyxin B, are less able to bind and cause damage (42). Several pili have

been demonstrated for Bcc but for *B. cenocepacia* strains of the ET-12 lineage, the cable pilus is unique. It consists of large peritrichous individual fibres and is associated with the 22-kDa AdhA. However its specific role has not yet been elucidated (13, 28).

2.3.3.4 Iron acquisition

Iron is essential for all living organisms. Bacteria require iron for many cellular functions and metabolic pathways (43). To survive within the iron-limited environment of a CF lung, bacteria have evolved various mechanisms to capture free, non-bound iron from the host environment. Surprisingly, the total iron concentration in cystic fibrosis sputum is more than enough for bacterial growth, but it is only the soluble ferrous iron that can easily be used for bacterial growth without intervention of specific proteins. Over 50 % of total iron in the human body is stored in the erythrocytes, where it is bound to haemoglobin, and approx. 25 % is bound to ferritin. In order to survive within the CF lung, Bcc bacteria produce and excrete chelators, called siderophores, to scavenge the sequestered iron and transport it into the cell. Furthermore, they excrete proteases to degrade iron-containing proteins such as ferritin and they are able to take up iron-containing molecules, such as heme (21).

2.3.3.5 Secretion systems

Six secretion systems (Type I tot VI) are described in Bcc bacteria, and especially the Type III secretion system has been associated with pathogenesis (28). It is responsible for secretion of several exo-enzymes in order to modulate host cell interactions; metalloproteases and serine proteases are thought to affect host cells by proteolysis while phospholipases can induce an inflammatory response and play a role in membrane maintenance and cellular turnover (28, 39, 44).

2.3.3.6 Resistance against antimicrobial compounds

Treatment of Bcc infected patients is difficult due to the high innate resistance of these bacteria. They show resistance against polymyxins and aminoglycosides, due to their unique LPS structure, and against most β-lactams. Furthermore, Bcc bacteria can develop resistance to all other classes of antimicrobial agents. Resistance mechanisms are the expression of efflux pumps, selective degradation, drug target alteration and reduced permeability of the outer membrane by changes in porins (21, 28).

2.3.3.7 Resistance against oxidative stress

Within a host, bacteria encounter oxidative stress, caused by reactive oxygen species (ROS; e.g. hydrogen peroxide, hydroxyl radicals, superoxide and organic hydroperoxides). ROS can be present in

the sputum and can be induced by antibiotic treatment. A large amount of residual antibiotics may stimulate the production of hydroxyl radicals by the oxidation of free ferrous ions in the Fenton reaction. Additionally, bacteria also produce ROS intracellularly during respiration and metabolism (45). Bacteria, including Bcc bacteria, protect themselves against ROS by the production of oxidoreductases, cytochrome b, hydroperoxide resistance protein, catalases, alkyl hydroperoxide reductases and superoxide dismutases (21).

3. Gene expression and the complex regulation network

Gene expression is the process by which genetic instructions are used to synthesize specific gene products. Most genes encode for messenger-RNA (mRNA) which is further translated into proteins, however some of these gene products are functional RNA, transcribed from non-protein coding genes (46). In order to survive, bacteria need to sense their environment and respond to even subtle changes and this requires intricate regulatory networks to coordinate gene expression (47).

3.1 Gene expression: from genetic information to a functional product

3.1.1 Transcription

Transcription is the essential process by which genetic information, located on chromosomes or other genetic elements is transferred to ribonucleic acids (RNA). RNA polymerase and its associated transcription factors are the key players in this process. Due to their importance for cell viability, these proteins are highly conserved within bacteria and therefore an excellent target for broad spectrum antibiotics (48, 49). Bacterial RNA polymerase consist of six subunits labelled β, β', α, α ,ω and σ that together form the active enzyme. The sigma factor is able to dissociate from the core enzyme and is required to initiate binding to promotor regions on DNA. Promotor sequences vary within bacteria, but two regions are highly conserved and are recognised by the sigma factor; the Pribnow box, located 10 nucleotides upstream of the transcription start site (TSS) and a region 35 nucleotides upstream of the TSS. A comparison of many promotors revealed a consensus sequence TATAAT and TTGACA for the Pribnow box (-10 region) and for the -35 region, respectively.

Once a specific promotor sequence is recognized, the RNA polymerase core enzyme unwinds the double DNA helix and transcribes the non-template strand to its RNA complement, adding one nucleotide at a time with a rate of 30 to 100 nt/s *in vivo* (50). Transcription is terminated when a specific factor-dependent or independent terminator signal on the DNA template is reached (Figure 2). In the factor-dependent mechanism an RNA translocase called Rho binds the formed RNA molecule and moves down the chain towards the DNA-RNA-polymerase complex. Once a specific rho-dependent terminator site on the DNA template is reached, Rho induces a dissociation of both RNA and RNA polymerase from the DNA. In *Escherichia coli*, Rho is an essential protein (51). The second and most common mechanism is factor-independent. Transcription is ended by an intrinsic terminator consisting of a GC-rich symmetrical element containing an inverted repeat with a distal T-stretch. Once transcribed, this sequence forms a terminator hairpin which induces a dissociation of the RNA polymerase-DNA complex (46, 51, 52). Not all genes contain a promotor, a TSS and a terminator. Genetic information is organized in transcription units, consisting of one gene with a TSS and

terminator, or multiple genes with one TSS and terminator. Ribosomal RNA (rRNA) genes, for instance, are often grouped in one transcription unit. These genes are co-transcribed to one RNA molecule and further processed to several rRNAs (46).

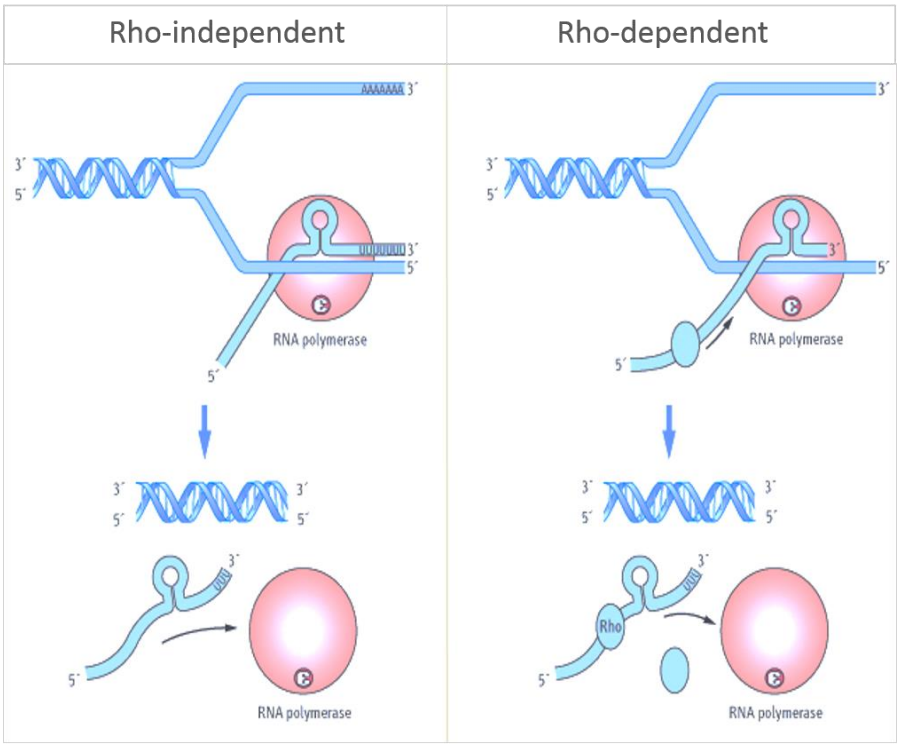


Figure 2. Transcription termination in bacteria. Transcription is terminated when a specific factor-dependent or independent terminator signal on the DNA template is reached. Left panel represents the factor-independent mechanism. Transcription is terminated by an intrinsic terminator sequence which forms a hairpin and induces a dissociation of the RNA polymerase-DNA complex. Right panel represents the rho-dependent mechanism. Rho moves along the newly transcribed chain until it reaches the RNA polymerase that pauses at a terminator site. Subsequently, Rho causes a dissociation of both RNA and RNA polymerase from the DNA (53).

Viruses are the only organisms with an RNA genome to store genetic information. All other organisms have evolved to a DNA genome, but RNA remains indispensable and accounts for approx. 20 % of the dry weight of prokaryotic cells (54). mRNA, the protein-coding RNA, is further converted into defined amino acids in the translation process. Many RNA molecules however do not code for proteins (non-coding RNA) but perform a major role in the process of protein synthesis (e.g. rRNA, the building block of the ribosomes, and transfer RNA (tRNA), the amino-acid carrier) or in the regulation of gene expression (e.g. small RNAs (sRNAs)) (46). The turnover of these molecules determines there lifetime and therefore their functionality. RNA is degraded internally by endoribonucleases or at the 5' or 3' -end by exoribonucleases. Enterobacteriaceae (e.g. *E. coli*) and related Gram-negative species possess several ribonucleases (Figure 3A). These include ribonuclease E (RNase E), which cleaves single-stranded RNA substrates, and RNase III, which cleaves double-stranded RNA (dsRNA) at specific locations (e.g. hairpin structures). An enzyme which aids in exoribonuclease activity is

pyrophosphohydrolase, RppH. RppH removes pyrophosphate from the 5'end of primary transcripts (54, 55). In *E. coli*, eight exonucleases are capable of RNA degradation in the 3'-5' direction. RNase II, a hydrolase, and polynucleotide phosphorylase (PNPase) are the primary agents in this 3'-5' exonucleolytic degradation of mRNA and are able to degrade poly(A) tails. In contrast to eukaryotic mRNA, where polyadenylation is a part of the transcription process and important for translation and stability of the mRNA, polyadenylation of prokaryotic mRNA affects fewer mRNA molecules and promotes RNA degradation (56, 57). Primary transcripts such as mRNA are protected from initial degradation by their 5'-triphosphate end. The pyrophosphate removal by RppH is known to be the initial and rate-limiting step and can be delayed by the presence of secondary structures in the 5'-untranslated region (UTR) of the mRNA (58). rRNA and tRNA molecules, are processed from larger primary transcripts to become functional and maintain their activity by the formation of strong secondary structures, which mask endonucleolytic cleavages sites (Figure 3B) (54).

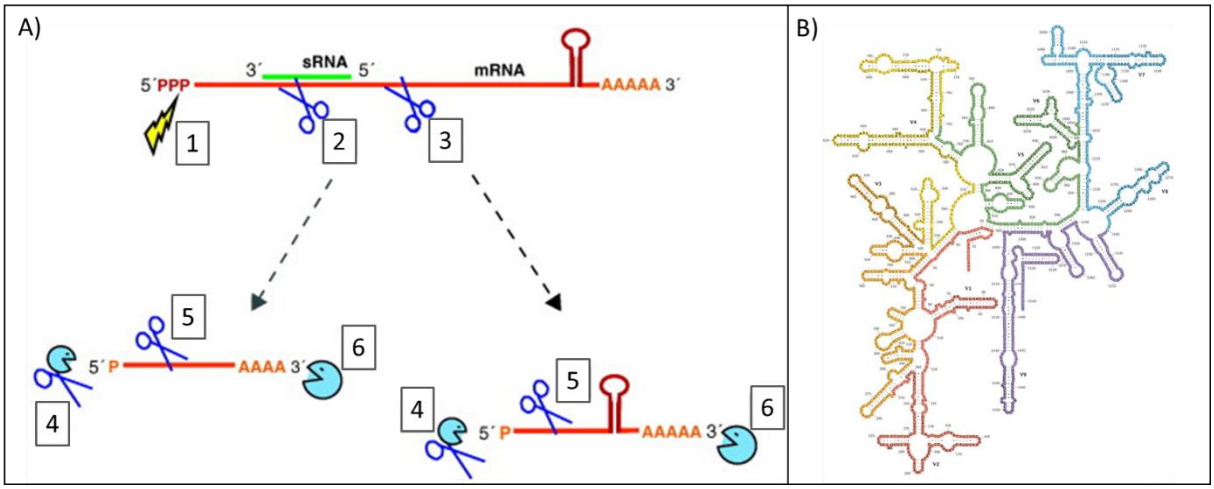


Figure 3. A) Processing and degradation of mRNA in *Escherichia coli*. A solid red line symbolizes mRNA. Endoribonucleases are visualised by scissors, exoribonucleases by 'packman', and pyrophosphohydrolase (RppH) by a flash. The top diagram depicts enzymatic attacks specific for primary transcripts, the ones at the bottom of the figure show degradation of processed or cleaved RNA. 1. Removal of a pyrophosphate by RppH. 2. Internal cleavage by RNase III at double stranded sites, created by a non-coding sRNA (shown on the figure) or by a secondary structure in the mRNA (not shown). 3. Internal cleavage at single stranded sites by RNase E. 4. Degradation in 5'-3' direction by exoribonucleases in some bacteria (not in *E. coli*). 5. Cleavage at single stranded sites in 5'-3' direction by RNase E. 6. 3'-5' degradation by exoribonucleases, e.g. RNase II and polynucleotide phosphorylase (PNPase) (54). B) Secondary structure of 16S rRNA of *E. coli* (59)

3.1.2 From coding RNA to functional proteins, the translation process

The initiation of translation begins with the association of mRNA to the small ribosomal subunit (30S). The 16S rRNA recognizes, by base-pairing, a specific sequence in the 5'UTR of the mRNA, i.e. the Shine-Dalgarno sequence (60, 61). In *E. coli* the Shine-Dalgarno sequence is located 4-9 nucleotides upstream of the start codon, although in other bacteria longer distances are found (61). Subsequently, the large (50S) ribosomal subunit connects to the smaller subunit to yield a 70S initiation complex. The start

codon, most often AUG, triggers the initiator fMet-tRNA and translation is initiated. Amino acids, carried by tRNA, are added to a growing polypeptide chain during elongation until the stop codon is reached and translation is terminated (46, 61). mRNA translation and particularly initiation of translation is a slow process and therefore an excellent target for post-transcriptional regulation. Besides that, it has an effect on mRNA stability (61, 62). Bacterial mRNA has an average half-life of 2 minutes (at 37°C) and is readily degraded when not protected by the ribosomes ("naked" mRNA) (58).

It is vital to have the correct amino acids inserted at the proper location of the polypeptide chain. Not only rRNA and the ribosomal proteins, but also initiation factors, elongation factors and release factors are responsible for this. Together they determine the speed and accuracy of the translation process (61). Once the polypeptide chain is released, it folds into a more stable structure, the final protein with biological activity and with an average half-life of 180 minutes in *E. coli*. For many proteins, co-translational folding occurs, in which the polypeptide chain folds spontaneously while still attached to the ribosomes (62). However, some proteins require assistance of molecular chaperones for correct folding. Additionally, proteins that function in the cytoplasmic membrane, in the periplasm (for Gram-negative cells) or outside the cell, harbour a signal sequence of 15-20 amino-acid residues at the beginning of their polypeptide chain which guides them to their location, where the signal sequence is subsequently removed by proteases (46).

3.2 Regulation of gene expression

All living organisms, including bacterial pathogens, often face stressful environmental conditions (temperature and pH changes, nutrient shortage, low oxygen levels, presence of competing organisms or cells, etc.). Both global regulatory networks and specific circuits regulate gene expression in response to nutritional cues or to specific stress factors (63). Regulation occurs on different levels, i.e. transcriptional, post-transcriptional and post-translational. Post-translational regulation appears to be the fastest response to environmental changes, however the regulation on transcriptional and post-transcriptional level has more benefits to the cells regarding energy and resources. The cell can focus on the production of proteins necessary for surviving in a specific environmental condition and prevents the production of unneeded proteins (46, 64).

3.2.1 Transcriptional regulation

Control at the initiation of transcription is thought to be the predominate regulation within a cell (60) and occurs by regulating the activity of RNA polymerases or by modulating promotor regions (65).

3.2.1.1 Enzyme-based regulation

Besides the housekeeping sigma factor, one or more alternative sigma factors with more stringent promotor sequence specificities are involved as well. Under most conditions the housekeeping sigma factor is the most abundant one, however, under specific circumstances the expression of certain alternative sigma factors increases. They compete with the housekeeping sigma factor and are able to guide RNA polymerase to specific promotors of a set of genes often stress-response related (65). RpoS, which is higher expressed when bacteria enter stationary phase, recognizes a subset of promotors for genes which are important for the transition of exponential to stationary phase (66). The activity of RNA polymerase relies not only on the abundance of certain sigma factors, but also on the proportion of functional non-sequestered enzymes. The non-coding sRNA 6S RNA, expressed in response to slow growth, mimics DNA promotors recognized by the housekeeping sigma factor subsequently changing the binding capacity of the RNA polymerase holoenzyme (65).

3.2.1.2 Promoter-based regulation

Regulation on the level of RNA polymerase activity is complemented by regulation at the promotor level through the interference of transcription factors or by nucleotide modifications. Transcription factors are repressors or activators which bind to an operator, a specific sequence of approx. 4-5 base pairs downstream of the promotor sequence. This way, an environmental cue which affects a transcription factor, often through a two-component regulatory system, can be coupled to the activity of certain promotors (65). A repressor binds an operator and inhibits RNA polymerase from binding to the promoter region. This occurs through steric hinder, when the operator region overlaps with the promoter region or it occurs by the induction of a loop which covers the promoter region (Figure 4A) (46, 65). Some repressors are only active in the presence of a small ligand, while others are active depending on their proportion in the cell and are inactivated by binding of small ligand which induces a dissociation of the repressor to its operator (46). An activator on the other hand supports the RNA polymerase to bind weak promotor sequences (i.e. sequences showing a poor match to the consensus sequence). They bind directly to RNA polymerase or they alter a site close to the promotor sequence to make it more available for RNA polymerase (Figure 4B) (46). Furthermore, nucleotide modification in the promoter or operator region can alter the affinity of a transcription factor or can directly affect the affinity of RNA polymerase. The most common modification is DNA methylation. For example, in *E. coli*, methylation of the *pap* operator decreases the affinity of a repressor eventually leading to transcription initiation (Figure 4C) (65).

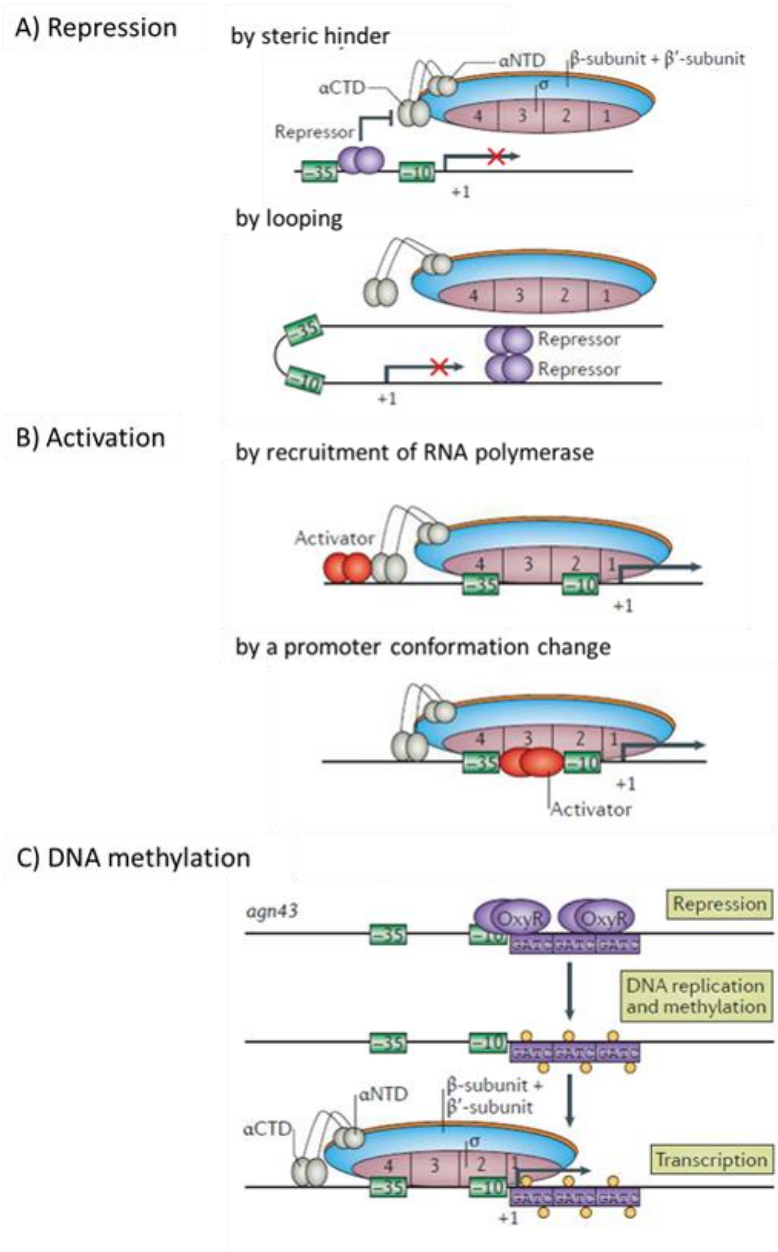


Figure 4. Promoter-based regulation. The principal mechanisms of regulation by transcription factors and chemical modification are shown for the *Escherichia coli* RNA polymerase holoenzyme with the housekeeping sigma factor. A) A repressor binds to an operator sequence and causes steric hindrance or induces a loop to block access of RNA polymerase to the promoter. B) An activator binds to the operator to recruit the RNA polymerase or to induce a change in the availability of the promoter for the RNA polymerase. C) OxyR causes transcriptional repression. After DNA replication, bases are methylated which prevents OxyR binding. RNA polymerase is able to bind and initiate transcription (65).

3.2.2 Post-transcriptional regulation

Eukaryotic post-transcriptional regulation has been widely investigated, however in bacteria, this phenomenon has been less studied. Due to a different cell structure and the lack of a nucleus, bacterial RNA is directly spread in the cytoplasm where it is considerably less stable compared to eukaryotic RNA (67). After transcription, the primary RNA molecule can be degraded into nucleotide monomers (RNA decay), or processed into shorter biological active fragments (RNA maturation). Both rRNA and

tRNA, quite stable RNAs due to their marked secondary structures, are the result of RNA maturation and are only degraded during stress or when defective (55, 68). Depending on different regulators such as RNA binding proteins, metabolites and small RNAs, an mRNA is guided through translation into a fully functional product or is degraded by ribonucleases (46, 55, 67). With an mRNA half-life of only a few minutes, regulation on the post-transcriptional level is ideal for bacterial cells to quickly adapt to environmental changes (68).

3.2.2.1 Translation control mechanisms

Ribosomes recognize the Shine-Dalgarno sequences of mRNA transcripts. Once the mRNA is bound to the ribosomes, it is partially protected from degradation by ribonucleases. Control at the level of translation occurs by a change in the stability of an mRNA transcript or by interfering with binding to the Shine-Dalgarno sequence (60).

3.2.2.1.1 Translational repressor proteins

Translational repressor proteins bind in the vicinity of the Shine-Dalgarno sequence and hinder the ribosomes to start translation. For example, translational repressor Crc in *Pseudomonas putida* binds to the *benR* and *alkS* mRNAs, which encode for transcriptional activators involved in benzoate and alkane degradation, to inhibit their translation (69). Some ribosomal proteins inhibit the translation of their own mRNA, when they are produced in excess, to maintain the correct balance of ribosomal components within the cell (60).

3.2.2.1.2 Riboswitches

Riboswitches are non-coding RNA regions on certain mRNAs, targeted by specific small molecule ligands, that regulate the expression of the mRNA of which they are a part of. They are usually located in the 5'UTR and undergo a structural rearrangement after binding of the ligand. Their region is composed of an aptamer part, the binding domain for the ligand, and an expression domain. The expression domain is located downstream of the aptamer and its secondary structure depends on the presence or absence of the ligand (67). Known ligands are amino acids, sugars and vitamins, and ligands are usually functionally related to the protein that the riboswitch-connected mRNA encodes. When the riboswitch folds into a stem-loop structure the ribosome binding site is sequestered and mRNA translation is inhibited. This effect on translation initiation is observed in Gram-negative bacteria, whereas in Gram-positive bacteria riboswitches preferably act on transcription initiation (68). One such ligand binding to a riboswitch is c-di-GMP. It recognizes riboswitches found on GEMM mRNAs (genes for the environment, for membranes and motility), first described in *V. cholerae* (46, 67).

3.2.2.1.3 Small RNAs

Small RNAs (sRNA) are important regulators of gene expression, affecting protein synthesis on the post-transcriptional level. sRNAs are small non-coding RNA molecules which base-pair with one or more mRNA targets to change their translation or degradation rate. Some sRNAs bind directly to proteins and alter their activity. When first described, all sRNAs were considered to represses gene expression, but in the meantime sRNAs that act as activators of gene expression were also discovered (68). Especially during stress, these regulators are of great importance for the bacterial cell in order to rapidly respond by changing the expression of certain proteins. The exact details of regulatory circuits involving sRNAs are not yet fully elucidated, but it is clear that regulation involving sRNAs is a complex interplay between environmental signals, regulatory proteins and other sRNAs and that these regulatory small molecules are crucial alongside their protein counterparts (67). The mechanisms of regulating with sRNAs are described in the next chapter.

3.2.2.2 Premature termination of transcription

A less common post-transcriptional regulation is premature termination of transcription. The nascent RNA strand adopts specific secondary structures, which causes RNA polymerases to abort transcription. Folding is the result of events at the ribosome or is regulated by proteins (46, 60).

3.2.3 Post-translational regulation

Post-translational regulation involves allosteric binding of small molecules to proteins, a well-known strategy for regulating protein activity in bacteria. However, recent findings have pointed to a widespread role for covalent post-translational protein modifications, previously regarded as a regulatory mechanism only occurring in eukaryotes (70). Through the improvement of mass spectrometry-based proteomics, it became clear that post-translational modifications are ubiquitous in bacteria. Especially phosphorylation and acetylation are prevalent, but also methylation and pupylation are observed. The latter is an equivalent of ubiquitination in eukaryotes; the prokaryotic ubiquitin-like (pup) protein is conjugated to amino acids of proteins to mark these proteins for degradation (70, 71). This type of regulation allows bacteria to rapidly adapt to external stimuli, faster than on the post-transcriptional level.

Allosteric binding of small molecules enables a fast and reversible modification of catalytic activity. In *E. coli*, the transcription factor Cra, a repressor of glycolytic enzymes and activator of gluconeogenesis enzymes, is inactivated by binding the intermediate fructose-1,6-bisphosphate, which accumulates during glycolytic growth (72). An example of a post-translation protein modification is the attachment

of phosphate onto functional groups of amino acid side chains by phosphorylation. For example the activity of S-adenosyl-l-homocysteine hydrolase in *Mycobacterium tuberculosis*, is decreased by phosphorylation at multiple sites by a Ser/Thr protein kinase (70, 71). Regarding host-pathogen interactions, both the chemotactic as well as the flagellar system undergo multiple instances of post-translational modifications (especially methylation, phosphorylation and acetylation) to translate an environmental signal into an altered phenotype (46, 71).

3.2.4 Regulation of gene expression as an antiviral defence system: CRISPR

RNA interference as an antiviral defence system was first discovered in eukaryotes. In bacteria, an equivalent system was found to protect them against virus attack. The CRISPR, or Clustered Regularly Interspaced Short Palindromic Repeats system, tackles both RNA and DNA viruses and other incoming genetic elements. Bacteria are able to build a memory of invading hostile virus sequences or foreign DNA or RNA, in a form of an adaptive or acquired immune system. Short sequences of foreign genetic material, i.e. spacers (26-72 bp), are incorporated into the host genome between short DNA repeats (21-48 bp), the CRISPRs (73, 74). The CRISPR region is transcribed as one RNA molecule and further processed by CRISPR associated proteins (CAS proteins), that cleave within repeated regions. When one of the CRISPR sequences base-pairs to a foreign nucleotide sequences, the complex will be destroyed by other CAS proteins. CRISPR RNAs are often referred as a class of regulatory RNAs (75). Over 70 % of bacterial sequenced genomes possess the CRISPR system (46), mostly found as one single CRISPR-CAS locus, however some bacteria harbour multiple CRISPR-CAS loci (74).

3.3 Key players of gene regulation found in *Burkholderia* species

Burkholderia species are found in a wide range of ecological niches where they have to conquer several environmental stresses (76). In the lung, a strong immune defence, aggressive antimicrobial substances, a heterogeneous distribution of nutrient and oxygen availability and the presence of other organisms represent such stress factors. Bcc bacteria persist in the host and adapt to it by a differential gene expression coordinated by several regulators (35, 77).

3.3.1 Sigma factors

21 genes for sigma factors are encoded on the *B. cenocepacia* J2315 genome (78). RpoD is the major house-keeping sigma factor. In stress response, alternative sigma factors have been found to play a crucial role. RpoE regulates the extracytoplasmic stress response and is required for the intracellular survival of *B. cenocepacia* K56-2 by delaying the phagolysosomal fusion in macrophages (79). Sigma factor RpoN is important in the response of *B. cenocepacia* H111 to nutrient-limiting environments,

for biofilm formation and motility but also for the virulence of *B. cenocepacia* H111 in the *Caenorhabditis elegans* model (80). In *B. cenocepacia* K56-2, this sigma factor has a function in the intracellular survival in macrophages (81).

3.3.2 Other regulatory proteins

Bioinformatic analysis of Bcc genome sequences revealed hundreds of transcriptional regulators belonging to several families (including AraC, ArsR, AsnC, DeoR, GntR, IcIR, LacI, LysR, MarR, MerR, TetR) and more than 28 putative two-component regulatory systems. However, only few regulators are functionally characterized. CepS, an AraC-type regulator, acts as an activator of several QS-regulated genes (82), while ShvR, a LysR-type regulator, is an important transcription factor for the coordination of colony morphology, biofilm formation and virulence and in the regulation of QS genes (83, 84). BceSR, a regulatory two-component system described in *B. cenocepacia* K56-2 and in other Bcc strains regulates protease production, QS, swimming, motility and virulence (84, 85). The EsaSR system, a two-component system in *B. cenocepacia* is known to be involved in resistance towards some antibiotics (i.e. chloramphenicol, ciprofloxacin, kanamycin, meropenem, novobiocin, and tetracycline) and in maintenance of membrane integrity (86). One of the major global regulators of the pathogenicity of *B. cenocepacia* is the AtsR/AtsT phosphorelay pathway. This mechanism is responsible for the negative regulation of genes involved in QS, biofilm formation, type VI secretion and protease secretion (87).

3.3.3 Quorum sensing, a regulatory system

All Bcc bacteria possess the CepIR system and the RpfFR QS system. The CepIR system is required for full virulence of Bcc in *C. elegans*. An active CepR binds to the cep box, a specific sequence located upstream the TSS of genes encoding toxins, lipases, proteases, iron-chelating siderophores and antifungal agents and changes their transcription rate (37, 38). In the RpfFR system, the signal molecule BDSF binds to the RpfR regulator and stimulates the c-di-GMP phosphodiesterase activity of this protein, resulting in a lower intracellular c-di-GMP level. Both QS systems regulate different target genes, however they have some target genes in common and both systems regulate biofilm formation, cell motility, proteolytic activity and virulence (88, 89).

B. cenocepacia harbours an additional QS regulator, CepR2, known to repress the transcription of the CepS activator and an additional QS system, the CciIR system, which interacts with the CepIR system. CciR negatively regulates the transcription of *cepI*, while CepR positively regulates *cciI* (90). Additional non-QS regulators are also able to affect the QS system within Bcc. For example, the previously mentioned ShvR regulator negatively controls *cepIR* and *cciIR* expression (82).

4. Small non-coding regulatory RNAs

4.1 What are small RNAs

In the early 1980s, a new class of gene expression regulators was found in bacteria and denoted as small non-coding regulatory RNA molecules or sRNAs (91). In eukaryotic cells, a homologous mechanism, RNA interference, has been widely investigated and it is assumed that over half of the total eukaryotic transcripts are non-coding RNAs (92, 93). The prokaryotic sRNA research knew a slower start, but in the beginning of the 21st century, aided by the optimization of genome-wide screening techniques, many bacterial sRNAs were discovered (75).

sRNAs are RNA molecules that are not translated into proteins. They form stable secondary structures and are located in untranslated regions of mRNA, in intergenic regions or antisense to coding regions (91). Their sizes typically range between 40 to 500 nucleotides, which is likely the main reason why early global screenings often failed to find these small molecules (94).

Together with proteins, these sRNAs regulate gene expression when a certain response to a changing environment is required. This regulation on multiple levels provides rapid response to changes (75). Compared to deploying protein regulators, the sRNA mode of regulation has several advantages for the cell. First, the synthesis of an sRNA requires less energy and cell machinery compared to the synthesis of a protein regulator (95). Secondly, the sRNA mode of regulation allows the cell to rapidly respond to a large change in input signals. A signal can induce synthesis of a large pool of sRNAs able to regulate protein expression much more reliably than protein regulators (96, 97).

At the moment over 200 sRNAs have been reported for Enterobacteriaceae with a genome size of about 4-5 MB which implies that approx. 5 % of all genes encode for sRNAs (75).

4.2 Mechanisms of action

sRNAs bind to specific targets. Some are known to interact with proteins, however the majority acts by post-transcriptional regulation through base-pairing with mRNA targets, often assisted by an RNA chaperone.

4.2.1 Proteins as sRNA targets

When sRNAs bind to a protein target, they change its conformation or they modulate its activity by competing with the normal DNA or RNA substrates of the protein (95). Two sRNAs families that act on proteins are known in bacteria.

The first group, with the *E. coli* 6S RNA as example, mimics the open complex of a DNA promotor and binds to the RNA polymerase holoenzyme containing house-keeping sigma factors, hereby changing the conformation and binding capacity of the enzyme. 6S RNA (180-200 nucleotides in size) accumulates in stationary phase and is responsible for down-regulating genes required for growth. The sequence itself is not conserved between species, but the secondary structure is (75).

An example of the second family is CsrB, which plays a role in the carbon storage regulator system. This sRNA regulates the activity of the CsrA translational regulatory protein by competing with mRNA targets. When this type of sRNA is present at high levels, the CsrA protein will preferably bind these sRNAs. The activity of the CsrA protein, a critical posttranscriptional regulator, with a role in switching life-styles or regulating metabolic pathways, is then repressed (46, 75).

4.2.2 Base-pairing sRNAs

The majority of sRNAs act by base-pairing with mRNA targets. This RNA:RNA interaction affects the gene-expression of the bound transcripts by either changing the translation rate or by altering mRNA stability. In some cases binding of an sRNA to an mRNA target can induce a premature transcription termination during the transcription process (46, 75).

Each sRNA binds to a specific region of the mRNA within the 5'UTR, the 3'UTR or the coding sequence of an mRNA. Occasionally, they base-pair to a specific intergenic region of a polycistronic mRNA and induce selective degradation (91). Base-pairing sRNAs are subdivided in two major groups, the cis-acting sRNAs and the trans-acting sRNAs, depending on their genomic location with respect to their target mRNA (Figure 5). Cis-acting sRNAs are encoded on the opposite strand of their target and share full complementarity with their target. Trans-acting sRNAs, the most abundant sRNAs, are encoded at distinct genomic locations or in the vicinity of their targets (91). Trans-acting sRNAs act through a mechanism analogous to that of eukaryotic microRNAs (miRNA); an intermolecular base-pairing interaction without full complementarity between target and sRNA sequence. However, they differ from miRNA in three ways: (i) bacterial sRNAs are generally approx. 100 nucleotides in length compared to 22 nucleotide long miRNAs; (ii) in bacteria, sRNAs are usually not processed (although some sRNAs are cleaved to a shorter functional form); and (iii) in contrast to eukaryotic miRNA, bacterial sRNAs more frequently base-pair 5'UTR, rather than the 3'UTR end (75, 98).

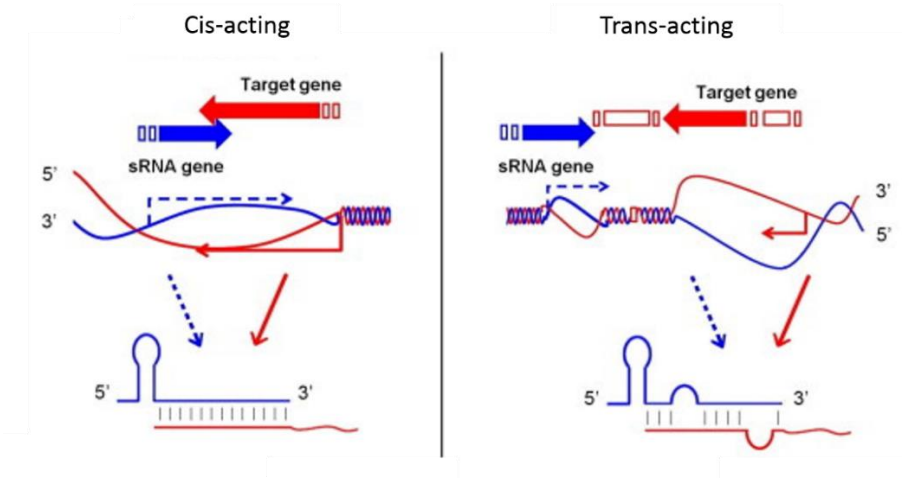


Figure 5. Genomic location of base pairing sRNAs. Cis-acting sRNAs are encoded on the opposite strand of their target and share full complementarity to their target. Trans-acting sRNAs, are encoded at distinct genomic locations or in the vicinity of their targets and base-pair to their target without full complementarity between target and sRNA sequence (99).

Interaction between an sRNA and mRNA mostly results in the degradation of both molecules (i.e. stoichiometric), instead of the recovery of the sRNA for further regulation (i.e. catalytic) (75, 100). Because of their imperfect complementarity, many trans-acting sRNAs require an RNA binding protein or chaperone that facilitates this base-pairing interaction (101). Despite this need for an RNA chaperone, partial complementarity is a major advantage and enables trans-acting sRNAs to regulate the expression of several genes or operons. For example, the sRNA RyhB in *E. coli* negatively regulates the expression of multiple operons, all of which encode iron-binding proteins (102-104).

Only a small part of the sRNA binds to its mRNA target. 6 to 8 nucleotides of this region, labelled as the seed region, are important for the interaction and are highly conserved. For example, both *Salmonella* and *E.coli* GcvB sRNAs have a conserved single-stranded G/U-rich region. This region binds specific to a C/A rich region of the 5'UTR of target mRNAs. Multiple targets encode this region, indicating one sRNA can simultaneously regulate multiple targets resulting in a rapid response to a particular stress (75). Additionally, one sRNA can regulate multiple targets due to different functional interaction domains or due to a different mechanism of action, which will depend on the input signal that stimulates expression of an sRNA. Vice versa, one single mRNA can be the target of multiple sRNAs (75, 101, 105, 106).

The outcome of both cis- and trans-regulation can be a change in translation initiation rate or an influence on the target mRNA stability. Most commonly, sRNA binding results in a lower translation rate of the target transcript. sRNAs base-pair to the Shine-Dalgarno sequence of a target mRNA and prevent the ribosomes to associate with the transcript (Figure 6). When ribosome binding is blocked, the mRNA transcript is unprotected and available for degradation by RNases. Not only specific binding

of the Shine-Dalgarno sequence can block ribosome entry, but also binding to the fifth codon in the open reading frame (e.g. *Salmonella* RybB RNA pairing with the *ompN* mRNA) or binding to regions 50 or more nucleotides upstream of the ribosome binding site. sRNA binding can also increase the degradation rate of the mRNA target. When an sRNA binds within the coding sequence downstream of the Shine-Dalgarno sequence, the interaction does not block ribosome binding, but the base-pairing accelerates RNase-dependent decay (Figure 6) (91). RNases degrade the sRNA-mRNA complex, which results in a lower translation rate for the target mRNA. An example of such base-pairing sRNA which interacts 23-26 codons downstream the *Salmonella ompD* mRNA is MicC (46, 75, 91).

In addition to the repression of translation, sRNA binding can also activate translation, although this occurs less frequently. Some mRNA transcripts are folded into secondary structures, e.g. hairpins, that prevent ribosomes from binding (Figure 6). sRNA binding can remodel this secondary structure and expose the ribosome binding site. This binding region is often located around the TSS, however, in some cases the interaction site is located more distinct. One sRNA can positively regulate a specific mRNA target and at the same time negatively regulate another target. It is important to keep in mind that the outcome of sRNA regulation does not only depend on the specific sRNA and its mechanism, but also on the availability of ribosomes, RNA chaperones, mRNA targets and RNases (75, 91).

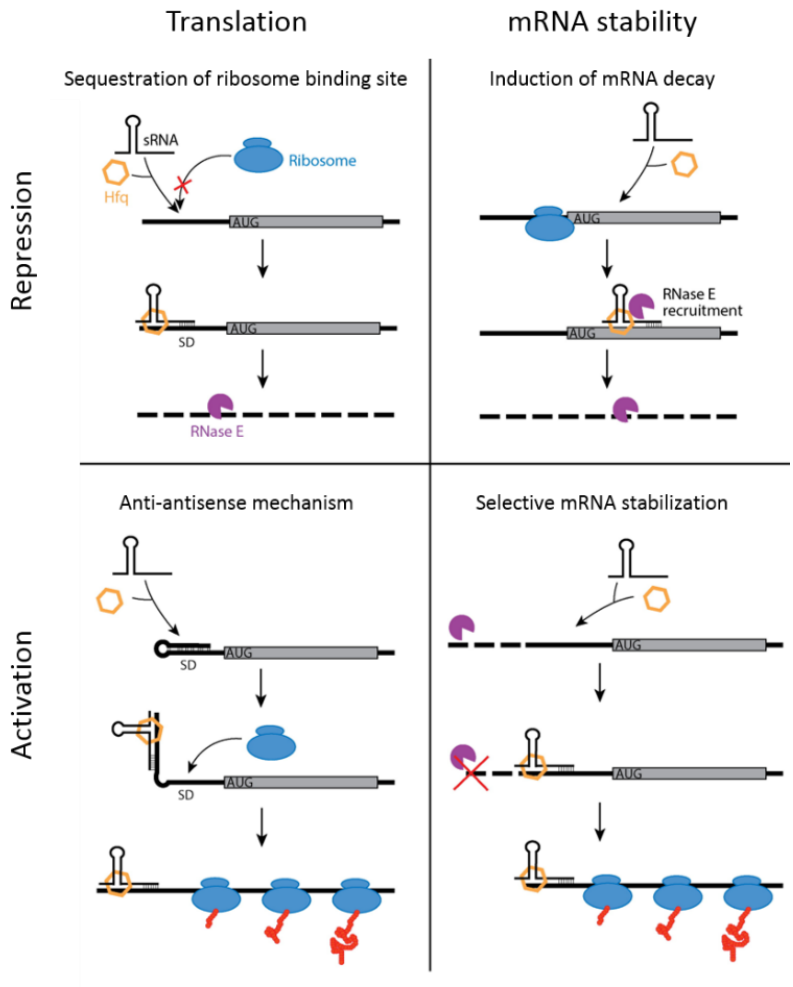


Figure 6. Possible mechanisms of action of base-pairing sRNAs. Left panel: repression and activation of translation. Right panel: negative (upper figure) or positive (lower figure) effect on transcript stability. SD: Shine-Dalgarno sequence. AUG: start codon. The red line depicts the formed polypeptide chain (101).

Interestingly, not all base-pairing sRNAs are non-coding. In some cases unusually long sRNAs represent both an sRNA and an mRNA. For example, in *E. coli* SgrS encodes an sRNA and a 43 amino acid protein SgrT. The sRNA base-pairs with the *ptsG* mRNA, encoding a sugar phosphate transporter, whereas SgrT inhibits the activity of the ptsG protein. All of the sRNA/mRNA pairs known so far function in the same regulatory network or metabolic circuit (75, 107).

Why one sRNA base-pairs to a specific mRNA target can be related to ΔG^{o} values of the interaction. This factor indicates the minimum free energy of a complex and is a measure of the stability of an sRNA-mRNA duplex. However, base-pairing is not only determined by these ΔG^{o} values. sRNAs are able to bind a specific target, often assisted by RNA chaperones, and form a stable duplex while the duplex' minimum free energy can be higher than an sRNA-mRNA duplex of a non-target mRNA (91).

4.2.2.1 Requirements for sRNA and mRNA in a base-pairing mechanism

Several other factors are important for regulation through sRNAs. First, base-pairing sRNAs have to be highly expressed in the cell in conditions in which they have their regulatory function. Secondly, their transcription is under a strict control of transcription activators and repressors and their sequence contains a strong Rho-independent terminator. The Rho-independent terminator consists of a stem loop followed by a U-stretch region and often encodes for an Hfq-binding site. A third important factor for base-pairing sRNAs is the seed region, the region of the sRNA responsible for base-pairing with the mRNA target. An sRNA can have one or more seed regions, mostly located in the single stranded regions of the sRNAs which are accessible for interaction. Because the seed region is the most crucial part for binding, it is more conserved than other regions of the sRNA. Finally, an sRNA is defined by multiple double-stranded regions which have a function to stabilize the RNA molecule and thereby allow base-pairing of the single-stranded regions to either their target mRNA or to an RNA chaperone. These structures are often conserved, however their sequences are not conserved but are enriched for nucleotides that interact intramolecularly (105).

Features important for the mRNA targets are an sRNA base-pairing sequence and in many cases, multiple Hfq-binding sites. The base-pairing site can be located in an unstructured region, when sRNAs repress the target, or the site is folded into a hairpin and the sRNA has a function in unfolding the structure (105).

4.2.2.2 RNA chaperones

Many base-pairing sRNAs require the help of an RNA chaperone to form the sRNA-mRNA duplex. Especially trans-encoded sRNAs with limited complementarity to their target mRNA often require the intervention of an RNA chaperone. RNA chaperones are RNA-binding proteins that facilitate the folding of RNA molecules. Unlike RNA helicases, RNA chaperones do not require ATP (108, 109). They bind both sRNA and target mRNA and facilitate their interaction. When a chaperone is present, it causes a local increase in both sRNA and target mRNA concentrations and it can unwind double stranded regions of both partners to optimize their interaction. The chaperone also offers protection against RNases, probably by masking RNase E cleavage sites (95), which increases the half-life of an sRNA (101). In Gram-negative bacteria, the first discovered chaperone was Hfq. Hfq is a close relative of the Sm/Lsm family of eukaryotic proteins and has a doughnut-shaped hexamer form (Figure 7A-B). It was initially identified as a host factor for replication of an RNA phage (91) and its role in RNA:RNA interactions was only discovered later. Several studies indicate that both the proximal and the distal

face of the hexamer bind RNA. The proximal side shows strong affinity for the U-rich end region of the sRNA while the distal face interacts with poly-A rich regions of mRNA (Figure 7C) (75, 91, 105).

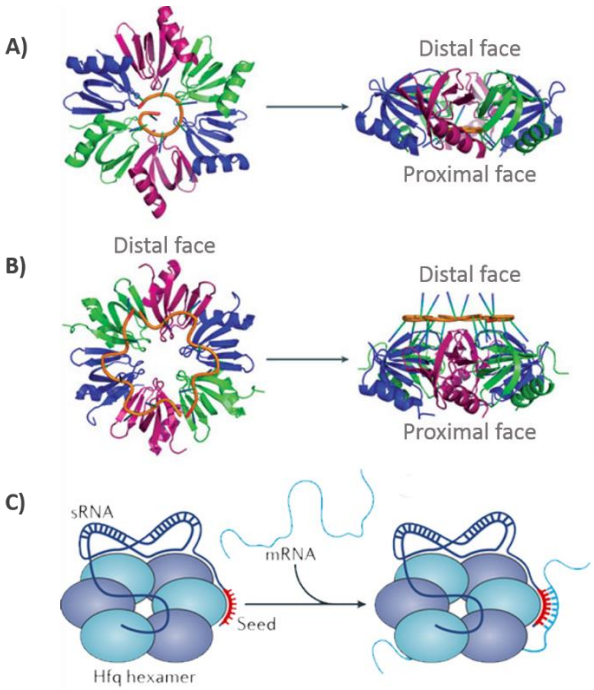


Figure 7. Hfq hexamer. A) Interaction with RNA (orange) at proximal face. B) Interaction with RNA (orange) at distal face (110). C) Hfq optimizes interaction between the sRNA and its mRNA target. The sRNA binds to the proximal face of Hfq while the target mRNA binds at the distal face of the hexamer (111).

Not all bacterial species harbour an Hfq protein, and not all trans-acting sRNAs require Hfq base-pairing. For example in *Staphylococcus aureus*, Hfq seems not required for RNA:RNA interactions (98). These findings suggest that more RNA chaperones exist (67). CsrA was found to be another sRNA binding protein and recent research found a FinO-like protein, ProQ, that has a role in stabilizing antisense sRNAs in *Legionella pneumophila, Salmonella enterica* and *E. coli* (108, 112, 113).

4.3 Evolution and conservation

4.3.1 (Functional) Conservation

Conservation analysis revealed a higher conservation for protein-binding regulatory sRNAs compared to base-pairing sRNAs (75). However, the secondary structure tends to be more conserved than the sRNA sequence. The sequence of base-pairing sRNAs in combination with their regulatory circuit is only conserved between closely related species, which can be expected as they bind a specific mRNA sequence (75). However in unrelated bacterial species, functional analogues without a conserved sequence can be found. The RyhB sRNA in *E. coli* and the PrrF sRNA of *P. aeruginosa* represent such

analogues. They are expressed under the same conditions and both bind the 5'UTR of similar targets, however their sequences or the sequences of their target are not similar (75, 114).

On the other hand, some sRNAs with similar sequences have a different function or expression pattern. The RyhB sRNA has a core function in *E. coli* and *Vibrio cholerae* with respect to iron homeostasis, while in *V. cholerae* it has an additional function related to respiration (105). The expression pattern of the GcvB sRNA is different in *S. enterica* and *V. cholera*. In *S. enterica* the level of this sRNA is high in exponential phase and decreases when stationary phase is reached, opposite to the high levels of this sRNA in *V. cholera* stationary phase cultures (105).

4.3.2 Origin and Evolution

To investigate the evolution and origin of sRNAs, it is helpful to consider their regulators. One sRNA regulator, the Fur repressor protein, regulates the expression of sRNAs located in the Fur regulon with a Fur-binding site in their promotor sequence and a core region for base-pairing specific targets. Both *Pseudomonas* and *E. coli* harbour a Fur protein and its corresponding sRNAs. The fur-binding site and core region sequences of the sRNAs are not conserved, which could indicate that these sRNAs have evolved independently within these bacteria (75).

sRNAs are acquired or could evolve through different mechanisms. First, sRNAs can enter the bacteria by horizontal transfer. Phages or transposons could contain sRNAs that further evolve to become complementary to host cell targets. Secondly, sRNA genes can duplicate. Multiple copies of a sRNA allow an increased regulatory effect or paralogs can differentiate. Thirdly, mutations within the genome can lead to new sRNAs (105). Finally, many sRNAs are derived from mRNAs. Especially the 3'UTR of mRNA is a good candidate sequence for sRNAs, because one feature, the rho-independent terminator stem loop, is already present. Also 5'UTRs are known to result in stable sRNAs; some sRNAs, encoded in the same orientation as their downstream gene, could have been the result of a previous mRNA promoter transformed into an independent transcript (75, 101).

4.4 Physiological role of sRNAs

Transcription of sRNAs is induced mostly under specific conditions, especially when cells are exposed to stress (101). During infection, bacterial cells have to cope with stresses coming from body fluids, phagocytosis and the immune response (73). sRNAs often play a subtle role in adaptation to low nutrient concentrations, unfavourable pH or temperature, lack of oxygen and oxidative-, osmotic-, or acid stress. Additionally they have shown to perform a regulatory role in cellular metabolism (carbon

metabolism, iron utilization), quorum sensing, envelope homeostasis, biofilm formation and virulence factor expression (Table 1) (73, 115).

Table 1. Role of sRNAs in specific regulatory networks (73).

Regulatory network	Species	sRNA	Main targets	Result[a]
Cellular metabolism				
Carbon metabolism	*E. coli*	Spot42	*galK*	-
		CsrB–CsrC	CsrA	-
		GlmY–GlmZ	*glmS*	+
	P. aeruginosa	RsmY–RsmZ	*rsmA*	-
Amino acid metabolism	*E. coli*	GcvB	*oppA, dppA, ssT*	-
		Sr1	*ahrC*	?
Iron regulation	*E. coli*	RyhB	mRNAs encoding Iron-binding proteins	-
	P. aeruginosa	PrrF1–2	*bfr, sdh, sodB*	-
QS and biofilm formation				
	V. cholerae	Qrr1–5	*hapR/aphA*	+
	S. aureus	RNAIII	mRNAs of QS	- / +
	E. coli	CsrB–CsrC	CsrA*, flhDC, pga*	- / +
		McaS	*flhDC*	+
		MicA	*ompA*	-
Stress response and adaptation to growth conditions				
Acid stress	*E. coli*	GadY	*gadXW*	+
		6S RNA	*pspF*	?
Oxidative stress	*E. coli*	OxyS	*rpoS, fhlA*	-
Osmotic stress	*E. coli*	FnrS	*sodB, maeA, gpmA, folEX*	-
		ArcZ	*arcB*	-
Outer membrane protein regulation in response to growth				
	E. coli	MicF	*ompF*	?
		OmrA-OmrB	Four porins	-
		RybB	*omp* mRNA	-
		RprA	*rpoS*	+
Pathogenesis				
	S. aureus	RNAIII	*hla*, *prot A*, coagulase, *rot*	- / +
	Streptococcus pyogenes	FasX	*fasBCA*	?
	Clostridium perfringens	VR-RNA	*colA/plc*	?
		Pel	*emm, sic, SpeD*	?
		RivX	*mga*	+

[a] - = repression of translation or induction of degradation, + = interaction promotes translation or stabilizes the mRNA, ? = mechanism not yet elucidated.

4.4.1 Protein sequestering sRNAs

6S RNA downregulates the transcription of many genes harbouring housekeeping sigma factor dependent promotors. It is suggested that 6S RNA is a "modest" global regulator as its mechanism probably does not result in the complete shut-off of genes (116). In *E. coli*, it functions to conserve resources and to optimize survival in nutrient-limiting conditions (116). Especially in late stationary phase, the majority of RNA polymerases linked to the housekeeping sigma factors is complexed with 6S RNA (117).

CsrB-like sRNAs bind regulatory proteins of the Csr system. This system controls the cell's central metabolism and nutrient utilization. The central component of this system is a repressor (CsrA in *E. coli* and RsmA in *Pseudomonas*) which regulates carbon starvation and glycogen biosynthesis but also mobility, QS and virulence. CsrB-like sRNAs sequester these repressors and antagonize their activity depending on the growth phase (73).

4.4.2 Base-pairing sRNAs

4.4.2.1 Frequent targets

Base-pairing sRNAs typically target genes coding for metabolic proteins, outer membrane proteins or transcription factors. Targets in metabolic pathways are succinate dehydrogenase and other nonessential iron-containing proteins (73, 105). The RyhB sRNA expression in *E. coli* increases in iron-limited conditions and represses the synthesis of over 18 nonessential iron-containing proteins. This decreases the cellular requirement for iron, and leaves more available for essential enzymes (73).

Almost half of the known targets of Hfq-binding sRNAs, are outer membrane proteins (95) and several studies in *E. coli* and *Salmonella* showed that all the major outer membrane porins can be down-regulated by one or more sRNAs. When the integrity of the outer membrane is compromised, sRNAs are induced (73). The cell requires a strict regulation of the outer membrane porins to maintain homeostasis. Down regulation of a specific membrane protein can minimize the entry of toxic molecules and phages (95). Outer membrane proteins are thought to be stable once inserted in the membrane, and mRNAs coding for these proteins have a long half-life. Their abundance is therefore mostly modulated by regulation of translation (73).

The last group of transcripts frequently targeted by base-pairing sRNAs are the transcription factors (105). One of them is the stationary sigma factor *rpoS* mRNA, one of the first targets characterized in *E. coli*. It is both positively as well as negatively regulated by sRNAs. sRNAs DsrA and RprA positively regulate this transcription factor in response to low-temperature and cell surface stress, respectively.

On the other hand, sRNAs OxyS and ArcZ induced by oxidative stress and anaerobic conditions, respectively, negatively regulate RpoS (73, 75).

4.4.2.2 Role in bacterial pathogenicity

Base-pairing sRNAs also play an important regulatory role in toxin production. They are able to repress genes encoding toxic proteins to keep the basal cellular toxin level low (75). sRNAs are also important factors in quorum sensing regulation in *V. cholerae*. At high cell densities the response regulator, LuxO, is phosphorylated and activates the transcription of a broad range of genes, among them five sRNAs. These sRNAs are involved in global regulation of the pathogenicity of *V. cholera*. They bind the *hapR* mRNA and inhibit its translation which stimulates the expression of virulence factors (e.g. toxins) and induces biofilm formation (73, 118). Examples of sRNAs that act both on cellular metabolism as well as on pathogenicity, are the CsrB-like sRNAs, RsmY and RsmZ in *P. aeruginosa*. On the one hand, these sRNAs regulate the global repressors RsmA and RsmE while on the other hand, they affect the expression of genes important for virulence and survival (73).

4.4.2.3 Role of sRNAs during stress

More genes controlled by sRNAs are the ones involved in the protection against acid-, oxidative-, osmotic- and anaerobic-stress. In *E. coli*, the sRNA GadY base-pairs and stabilizes the transcript of the major acid tolerance regulator GadX during acid stress (119) while during oxidative stress, the OxyS sRNA regulates adaptation to hydrogen peroxide and mechanisms to protect against oxidative damage (73). The first base-pairing sRNA discovered in *E. coli*, MicF, has a regulatory function in osmotic stress, by regulating one of the most abundant outer membrane proteins, OmpF (120). In anaerobic conditions, the sRNA FnrS is induced and inhibits expression of more than 32 proteins involved in oxygen-dependent energy metabolism (73).

4.5 Discovering sRNAs, their function and regulatory mechanism

The classical genetic screens often overlooked sRNA loci due to the lack of specific sequence characteristics present in protein coding genes. Similarly, their small size as well as the fact that they are only expressed under specific conditions has precluded their discovery in transcriptomic analyses (92). Additionally, deletion mutants often only show mild phenotypes (115). However, new transcriptomic techniques have improved the chances to discover novel transcripts in bacteria.

4.5.1 Discovering sRNAs

The first sRNAs were discovered by serendipity through investigation of protein coding sequences and their promoter regions, e.g. sRNA MicF was discovered by the investigation of the *ompC* promotor sequence. In the late 1990s, genome sequencing (especially of Enterobacteriaceae) enabled the first sRNA-screens using computational methods. These algorithms specifically looked for sequences with a high proportion of base-paired, structured regions (101), for DNA regions containing an orphan σ^{70} promotor followed in a short distance by a rho-independent terminator (121) or for conserved intergenic sequences (92, 122).

With the start of the whole-genome sequencing era in the beginning of the 21st century, techniques improved and the number of confirmed and suggested sRNAs increased drastically. In the best studied organisms, *E. coli* and *S. enterica,* almost 300 sRNAs have been described to date. sRNA research beyond these model bacteria confirmed the relative narrow distribution of conserved sRNAs across bacterial species (101).

Tiling-arrays were for some time the main global transcriptome analysis tool. However this method has many disadvantages; it has a limited resolution, there is the possibility of probe miss-hybridization or over-saturation and every organism requires its own specifically designed microarray often lacking acquired genes. Only since the development of high-throughput sequencing the rate of sRNA discovery has accelerated (101). Although, RNA extraction protocols specifically tailored to retaining short transcripts are required to allow sRNA discovery.

A more recently developed high-throughput RNA-sequencing technique that has provided a vast insight in the expression of sRNAs (and other transcripts) in a wide range of bacteria is differential RNA-sequencing (Figure 8) (123). This method takes advantage of the difference between primary bacterial transcripts, possessing a 5'-triphosphate (5'-PPP) end, and processed transcripts, that possess a 5'-monophosphate end (5'P). Most sRNAs and mRNAs are primary transcripts, while most tRNAs and rRNAs are processed transcripts. When an RNA sample is treated with a 5'-dependent terminator exonuclease (TEX), 5'-P transcripts are selectively degraded upon which the primary transcripts become relatively enriched (Figure 8A). When comparing a TEX-treated RNA-Sequencing library to an untreated RNA-Sequencing library of the same sample, transcription start sites of primary transcripts can be identified (Figure 8B). TSS located close to and in the same orientation of an annotated protein coding sequence are probable TSS for these protein coding genes. As very long 5'UTRs exist, even TSS located far from the protein coding sequence should be considered TSS for this gene. On the other hand, TSS without clear association with an annotated protein (e.g. they do not have a downstream

protein sequence in the same orientation) can be defined as new elements. The sequence downstream of such TSS can be an unannotated protein coding sequence or a non-coding RNA, when a short sequence is followed by a rho-independent terminator (101, 123).

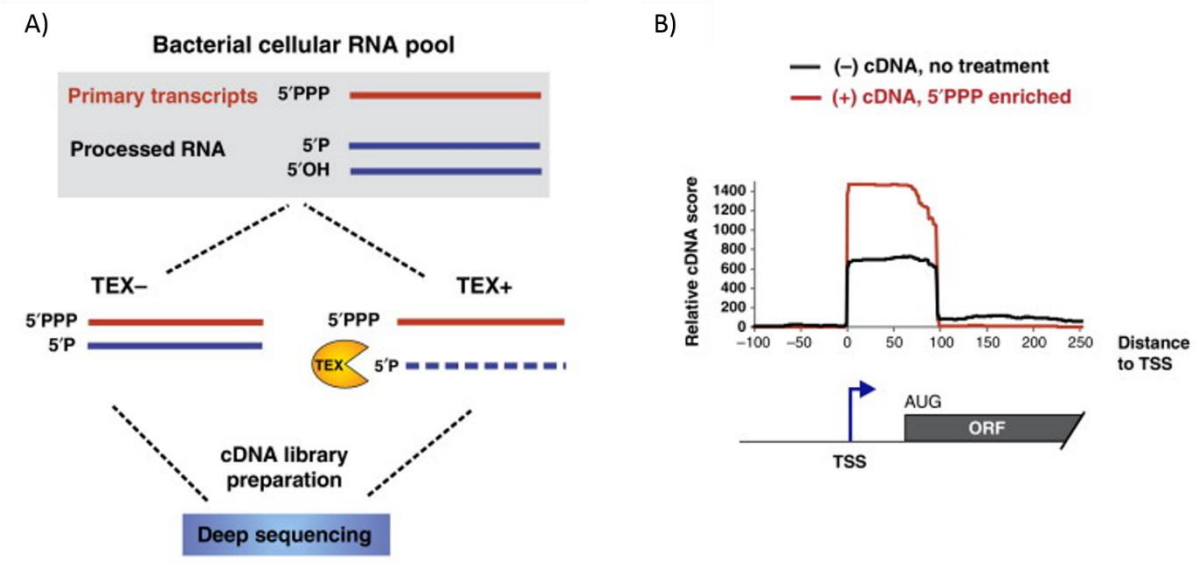


Figure 8. Differential RNA-sequencing. A) An RNA sample is split into two parts. One part is left untreated (TEX-), the other part is treated with a 5'-dependent terminator exonuclease (TEX) which degrades 5'-P transcripts (TEX+). Both samples are converted to complementary DNA (cDNA) and subjected to RNA-sequencing. B) The TEX-treated RNA-Sequencing library (Red line) is compared to the untreated RNA-Sequencing library (Black line). This enables the annotation of a transcription start sites (TSS, blue arrow) of a certain gene (grey bar) and could reveal TSS of new elements (unannotated protein coding genes or non-coding RNA genes) (123).

Not only global sequencing is used to discover sRNAs, other techniques that focus on chaperone-dependent sRNAs exist. One of them is RNA immunoprecipitation (RIP), which includes immunoprecipitation of an RNA-binding protein with further isolation and analysis of bound RNA (target mRNA and sRNA) by sequencing or microarray analysis. This directly identifies putative sRNAs which bind to the respective protein. A non-coding transcript that binds to an RNA chaperone necessary for regulation is likely to be a regulatory sRNA. A disadvantage of this method is that only stable protein chaperone:RNA interactions survive the procedure and only a subset of transcripts are identified. Refinements of this protocol to improve resolutions are being developed and are focussing on UV crosslinking of protein and bound RNA (101, 124).

When putative sRNA sequences are discovered by the above mentioned methods, further verification is still necessary. False-positive signals could be caused by cross-hybridization or spurious complementary DNA (cDNA) synthesis and some detected small transcripts could be transcriptional noise (105). On the other hand, sRNAs with important regulatory functions could be missed (false

negatives) due to their expression under specific conditions only, or because they are part of an mRNA, processed after transcription (101).

4.5.2 Investigation of RNA function

Identifying the function and regulatory network of newly discovered sRNAs is challenging. Phenotyping of a mutant strain with a particular sRNA overexpressed or deleted can reveal the sRNAs function, while computational predictions together with transcriptomics or proteomics of these sRNA mutants may aid in finding the exact regulatory mechanism (101).

4.5.2.1 Target-based investigation

Computational predictions are based on algorithms that calculate the possibility of interaction between an sRNA and an mRNA target. Some consider the sRNA conservation between closely related species (i.e. Copra RNA). Many of these algorithms are available and usually result in a large number of predictions, including many false positives. Therefore, additional information is necessary to further narrow down the list (99, 101).

The investigation of gene or protein expression changes of a sRNA mutant could reveal potential targets. However, it is important to take into account that these changes can be due to the direct interaction of an sRNA to a given target, or they occur as a downstream effect of this regulation. These downstream effects could be the result of the sRNA regulation, but it can also be caused by pleiotropic effects due to titrating RNA chaperones. Especially overexpression mutants show these downstream effects, together with the potential toxic effect of an overexpressed sRNA (101). In general, proteomics of sRNA mutants compared to a wild type would be the best method to evaluate sRNA regulation. It directly measures changes in protein abundance, and covers both the regulation of sRNA on the translation rate and degradation rate and it could reveal regulation on the protein level. However, proteomics is labour intensive and not all proteins of a cell are represented. Only the abundant proteins are detected and they have to be soluble in the extraction and digestion buffer. Therefore often transcriptomics is chosen but ideally both methods are combined (101, 125). Sievers et al. combined proteomics and transcriptomics to evaluate changes of an sRNA deletion mutant compared to the wild type of *Listeria monocytogenes*. 1587 proteins were identified but only 5 proteins were differentially expressed. Three of these proteins were also differentially expressed in the transcriptomic analysis (126). Whether gene or protein expression changes are directly or indirectly regulated cannot be determined by these methods, but this information does provide insights in the regulatory functions of sRNAs (127).

Actual RNA:RNA interaction can be validated by two major methods. An *in vitro* method is electrophoretic mobility shift assay (EMSA). This investigates binding of an sRNA to an mRNA by electrophoresis separated on a polyacrylamide gel. Conditions such as temperature, pH and buffer need to be optimized for every single interaction, and often Hfq has to be included for the interaction (125, 128). *In vivo*, a method that involves reporter genes expressed from plasmids is used. In this approach, the target is cloned as a translational fusion to a reporter gene (e.g. *lacZ*, *eGFP*, *mCherry*). The expression of the reporter is investigated in the presence or absence of a sRNA. If a change is observed when the sRNA is co-expressed or not, the interaction can be confirmed. For both methods, disruptive and compensatory mutations can further confirm specific base-pairing regions. Unfortunately, both methods and the additional mutations are labour intensive and species specific (101, 128)

4.5.2.2 Expression-based investigation

RNA-sequencing performed for specific growth conditions can link sRNAs to a certain regulatory network. For example, several studies investigated the role of sRNAs in establishing an intracellular infection. Mraheil et al. used *in vitro* tissue culture of murine macrophages infected with *L. monocytogenes*, isolated the pathogen cells and subsequently performed RNA-sequencing. This method revealed three sRNAs with a role in intracellular growth (129). In an *in vivo* infection model, *Salmonella* sRNA deletion mutants were injected into mice. The bacterial cell count of the spleen was monitored and showed differential counts for three sRNA deletion mutants (deleted sRNAs: IstR, OxyS, and SroA) (130). Other studies compared the transcriptome of bacteria grown *in vitro* to bacteria isolated from organs of infected animals. Six sRNAs were induced in *Yersina pestis* growing in lungs. Not only bacterial gene expression was investigated, but also the effect of intracellular growth of bacteria on the gene expression of the host cells. Interestingly, bacterial sRNAs can be found in host cells. They are transferred through active secretion or they are released after cell lysis (131). In this manner Liu et al. showed an effect of sRNAs OxyS and DrsA in *E. coli* on gene expression of *C. elegans* (132, 133).

4.6 sRNAs in *Burkholderia cenocepacia*

Several sRNAs in *B. cenocepacia* and in the Bcc complex have been discovered, but their biological function is not yet well understood (134). Remarkably, *B. cenocepacia* harbours two distinct Hfq-like proteins, Hfq and Hfq2. During exponential growth, *hfq* mRNA is highly expressed, while in stationary phase, the *hfq2* transcript is most expressed (134). Both these proteins are possibly required for survival in the *C. elegans* infection model (135).

The first attempt to discover sRNAs in *B. cenocepacia* was performed by computational methods (92). The QRNA software (136) was used to identify potential sRNA genes. Intergenic regions of *B. cenocepacia* J2315 and *Ralstonia solanacearum* GMI1000 were compared by BLAST. The intergenic regions conserved in both organisms were searched for regions with a more pronounced secondary structure. 213 non-coding RNA genes with a more pronounced secondary structure compared to the rest of the genome were selected. Two genes resulted in a hit in the noncoding RNA database (137) and one was found to be similar to the 6S RNA gene of *P. aeruginosa* (138). Additionally, four non-coding RNA genes were analysed by a microarray analysis which confirmed their expression. The authors concluded that the QRNA approach is useful as a first screen, but a second approach, such as the investigation of transcription start sites and potential terminators, should be included to reduce the number of false-positives (92).

Yoder-Himes et al. studied the expression of genes in two closely related *B. cenocepacia* strains. One strain, *B. cenocepacia* AU1054, was isolated from a cystic fibrosis patient and another, *B. cenocepacia* HI2424, was isolated from the soil in an agricultural field. RNA-sequencing was used to investigate the transcriptome of both strains grown in a synthetic cystic fibrosis sputum and in soil medium. 13 putative sRNAs, located in intergenic regions, were identified. Four of them, induced in soil medium, were conserved in four *B. cenocepacia* strains (AU1054, HI2424, MC0–3, J2315). However none of the 213 computationally-derived predicted non-coding RNAs (92) were confirmed in this study (139).

The response of *B. cenocepacia* J2315 cells to treatment with H_2O_2 and NaOCl was investigated by Peeters et al. by microarray analysis (140). After H_2O_2 treatment, 11 intergenic regions showed a different expression compared to their flanking genes, indicating they are potential sRNA genes. For seven of them, a differential expression pattern was observed when *B. cenocepacia* was grown in sputum compared to growth in a minimal medium (21). Two of them were also defined as sRNAs by Yoder-Himes et al. (139) and another two regions were identified as sRNA non-coding genes in the work of Coenye et al. (92). The same microarray was used for the detection of differential gene expression after treatment with NaOCl. 20 intergenic regions were found to have an expression pattern different than that of their flanking genes. Interestingly, one transcript showed a sequence and secondary structure similar to 6S RNA (140, 141).

When the response of *B. cenocepacia* J2315 sessile cells to chlorhexidine treatment was analysed by microarray analysis, 19 putative small RNAs were induced which suggests a role for them in chlorhexidine tolerance. However, none of them produced a hit when compared to the Rfam database (142).

Another research group investigated which sRNAs co-purified with the Hfq protein. This method revealed 24 new putative sRNAs (143). One of them a cis-acting sRNA later designated h2cR, was located on the opposite strand of the 5'UTR of *hfq2* and only conserved within the *Burkholderia* genus. This sRNA was expressed during exponential growth and negatively regulated the *hfq2* transcript by binding within the 5'UTR. Interestingly, a silencing mutant of this sRNA showed a reduced ability to colonize *C. elegans* which indicates a function for this sRNA in *B. cenocepacia* pathogenicity, probably by regulating Hfq2 expression (134, 144).

The most recent study on discovering sRNAs in *B. cenocepacia* was published by Sass et al. (34). Differential RNA-sequencing was performed on *B. cenocepacia* J2315 biofilm grown cells. Transcription start sites were mapped to the genome of *B. cenocepacia* J2315 and categorized based on their location in the genome (Figure 9). TSS in an intergenic region located less than 300 nt upstream of the start of an annotated gene, in sense with this gene and therefore linked as the TSS for this gene, were designated primary TSS (pTSS). Internal sense TSS (isTSS) or internal antisense TSS (asTSS) were located within an annotated gene. TSS found in an intergenic region not associated with any gene were denoted as orphan TSS (oTSS). All sequences that followed an oTSS, isTSS, asTSS and 5'UTRs longer than 72 nt (the average length of a 5'UTR) were compared to the Rfam database, nine sequences resulted in a hit. One of them was the previously detected 6S RNA (139, 140). Two hits were phage-related regulatory RNAs and another two were conserved regulatory motifs. One of these motifs was the SAH riboswitch of BCAL0145, an adenosylhomocysteinase, and the other was found as the *Burkholderiales*-specific sucA RNA, located in the 5'UTR of *sucA*, which probably functions as a riboswitch. For the remaining four sRNAs, the only information found was that they are toxic when expressed in *E. coli* (145). Sequences not resulting in a hit were compared to previously reported sRNAs. Six short transcripts found by this method were homologues to transcripts expressed in *B. cenocepacia* AU1054 and HI2424 found by Yoder-Himes et al. (139) and one transcript was also discovered in the Hfq-co-purification study of Ramos et al. (143).

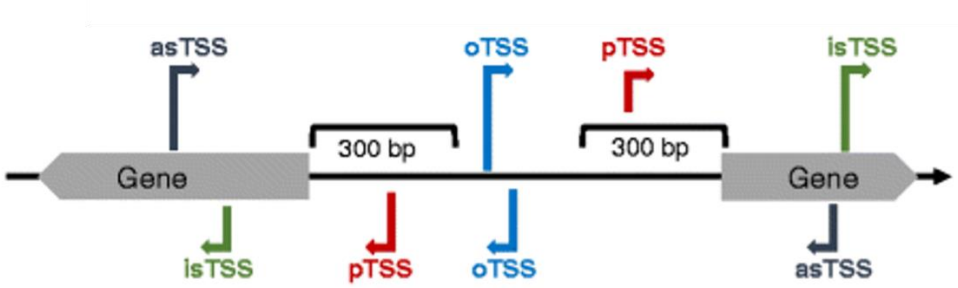


Figure 9. Categorisation of TSS. Primary TSS (pTSS) is a TSS in an intergenic region located less than 300 nt upstream of the start of an annotated gene, in sense with this gene. Internal sense TSS (isTSS) or internal antisense TSS (asTSS) are TSSs located within an annotated gene. Orphan TSS (oTSS) is a TSS found in an intergenic region not associated with any gene (34).

4.6.1 Concluding remarks

Optimization of sequencing techniques have led to the discovery of many putative sRNAs in *B. cenocepacia* and other Bcc species. Some of them were validated as expressed sRNAs and showed a match when compared to a non-coding RNA database. However may sRNAs still lack a functional characterization. At present, characterization is possible by evaluating phenotypes, transcriptomics and proteomics of an sRNA mutant.

CHAPTER II. OBJECTIVES

Burkholderia cenocepacia is one of the predominant *Burkholderia* species colonizing the CF lung and it has been associated with the often fatal "cepacia syndrome". Treatment of infections caused by *B. cenocepacia* and all other Bcc species is difficult due to their high innate resistance against a broad range of antimicrobial agents (13, 44). To completely understand and predict the response of bacteria to treatment it is important to understand all regulatory networks that play a role in resistance. The most recent discovered regulators are small RNAs which play important roles in fine-tuning gene expression in bacterial cells in a variety of environmental conditions, especially when cells encounter stress. sRNA-based regulation has been widely investigated in the model organisms *E. coli* and *Salmonella*. Methods for discovery and functional characterization of sRNAs were optimized for these organisms and several sRNAs were fully characterized (73, 75, 146). Different approaches have been used to discover putative sRNAs in *B. cenocepacia*, however, little is known about their role in the physiology of this bacterium (35) and elucidating this role is the main objective of this thesis.

In this dissertation I will focus on sRNAs, which had been identified by differential RNA-sequencing and which are highly expressed in *B. cenocepacia* biofilm cells (34). To obtain novel insights into the role of these sRNAs, their expression in different growth conditions will be investigated. In addition, the phenotype of sRNA deletion mutants will be studied. To this end, planktonic growth, biofilm formation, growth in the presence of antimicrobial compounds, membrane-, oxidative-, and osmotic stress, motility and virulence towards *C. elegans* will be tested (paper I). A phenotype can give clear indications on the role of the deleted sRNA, therefore we want to gain insight in the physiological role of a sRNA that showed phenotypes when deleted (ncS35) and into the regulatory pathways in which this sRNA plays a role in (paper II). For this we will determine the secondary structure and genomic location of this sRNA and expand our knowledge on the phenotype of the sRNA deletion mutant using various techniques, including microscopy and flow cytometry. In order to confirm that the observed phenotypes are solely due to the deletion of the sRNA, the sRNA will be overexpressed in the deletion mutant to determine whether this complements the observed phenotypes. RNA sequencing of wild type and deletion mutant together with a computational target prediction could further clarify the regulatory pathways in which this sRNA plays a role in. Finally, we will investigate the regulatory network of three sRNAs (ncS04, ncS16 and ncS27) that did not show a clear phenotype when deleted (paper III). To this end we will use a proteomics approach to investigate differential protein expression in sRNA deletion mutants. Potential targets will be predicted and qPCR will be used to investigate the expression of the computationally predicted targets. Subsequently, direct interactions of sRNAs with selected potential targets will be investigated using translational fusions of potential targets to the gene encoding a fluorescent protein. A plasmid containing this fusion will be expressed in wild type and in a sRNA deletionmutant and fluorescence will be measured.

CHAPTER III. EXPERIMENTAL WORK

Paper 1: Elucidation of the physiological roles of sRNAs in *Burkholderia cenocepacia* J2315

Sanne Kiekens, Andrea Sass and Tom Coenye

ABSTRACT

Bacterial sRNAs are non-coding regulatory RNA molecules that regulate gene expression on the post-transcriptional level. They can be important for membrane integrity, for metabolic remodelling upon changes in the environment and for a fast response to environmental stress. In this paper we selected nine sRNAs based on their high expression level in *Burkholderia cenocepacia* J2315 biofilm cells and investigated their expression in seven growth conditions. To shed light on their physiological role we constructed deletion mutants and investigated their phenotype. One sRNA deletion mutant showed differences in growth and motility compared to the wild type.

INTRODUCTION

Bacterial sRNAs are non-coding regulatory RNA molecules that interact with mRNA or proteins to interfere with their expression or to alter their activity. Their expression is often induced under specific conditions, mostly under stress conditions. For example, when the outer membrane integrity is disrupted, the synthesis of certain sRNAs is induced to down-regulate the expression of porins (75). sRNAs also play an important role in a metabolic remodelling upon changes in the environment, such as a reduction of iron or carbohydrate substrate availability, or changes in temperature and pH. For example, a low-iron environment stimulates the expression of the sRNA RyhB in *E. coli* while glucose starvation induces expression of the SpoT42 sRNA (147, 148). Furthermore, sRNAs are known to play a role in the response to osmotic stress, in virulence, in the transition from exponential to stationary phase, in motility and in biofilm formation (73, 75).

Many sRNAs have been discovered but their functional characterization is lagging behind. One approach used is the investigation of sRNA deletion mutants (127, 149). However, sRNA deletion mutants generally show mild to no phenotypes and it is therefore assumed that sRNAs mainly function by fine-tuning regulation of gene expression (149). Besides the investigation of sRNA deletion mutants, several other approaches have been used to elucidate the role of sRNAs. One study focussed on the influence of sRNAs on biofilm formation and motility of *Xanthomonas oryzae* by using plasmids containing sRNAs under the control of an inducible promotor. Of 99 mutants overexpressing sRNAs, 33 showed a significant effect on biofilm formation or on related phenotypes such as swimming motility (150). The investigation of phenotypes is often linked to a computational biology approach to predict interaction between sRNAs and mRNA targets (151).

OBJECTIVES

In *Burkholderia cenocepacia* J2315, several putative sRNAs were discovered by dRNA-seq (34). In the present study, the expression of nine sRNAs is confirmed. Four sRNAs were deleted and the phenotype of the resulting deletion mutants was investigated to shed light on the physiological role of the different sRNAs.

MATERIALS AND METHODS

Strains, plasmids and culture conditions

B. cenocepacia J2315 (LMG16656) was chosen as model pathogen and was used for the construction of four sRNA deletion mutants; ΔncS04, ΔncS16, ΔncS27 and ΔncS35. *E. coli* DH5α and *E. coli* DH5α λpir were used during the construction process for transformation and for the maintenance of several plasmids; the helper plasmid pRK2013 (ori_{colEI}, Km^r (152)), the suicide plasmid pGPI-SceI-XCm (ori_{R6K}, I-SceI restriction site, Tp^r and Cm^r (153)) and the broad host range replicative plasmid pDAI-SceI-SacB (ori_{pBBR1}, I-SceI nuclease, counter selectable marker SacB, Tet^r (153, 154)). *E. coli* OP50 was used as food source for *Caenorhabditis elegans* N2 (*glp-4*; *sek-1*). Bacterial strains were cultured on Luria-Bertani agar (LBA; Oxoid, Hampshire, UK) and Luria-Bertani broth (LBB; Oxoid) at 37°C. When appropriate, antibiotics were added for plasmid selection: chloramphenicol (Cm; Sigma-Aldrich, Brussels, Belgium), gentamicin (Gm; Sigma-Aldrich), kanamycin (Km; Sigma-Aldrich), trimethoprim (Tp; Ludeco, Brussels, Belgium) or tetracycline (Tet; Sigma-Aldrich). *C. elegans* was cultured on nematode growth medium (NGM) and fed with *E. coli* OP50. To synchronize nematode populations, purified populations were bleached with a 5 % sodium hypochlorite solution and eggs (resistant to bleach) were centrifuged, plated on NGM and incubated for three days at 25°C (155, 156). Additional media used were the basal salt medium (BSM, (157)) and the minimal medium M9 (158) supplemented with 0.4 % of a c-source (glucose or casamino-acids). 0.5 mM phenylalanine was added when no source of amino acids was present in the media. To investigate planktonic cultures, cells were grown with orbital agitation (250 rpm) at 37°C to optical density 0.5 (5×10^8 colony forming units (CFU) /mL) for exponential phase and to optical density 2.0 (2×10^9 CFU/mL) for stationary phase. Biofilms were grown in a round-bottomed 96-well microtiter plate (TPP, Trasadingen, Switzerland). Approx. 5×10^6 cells were added to one well. The plate was incubated for 24 h at 37°C, which included a washing step after 4 h with physiological saline (PS; 0.9 % NaCl in MQ). After 24 h the biofilm cells could be harvested. The supernatant was removed, the cells were washed with PS and the wells were refilled with PS. To detach the cells, the 96-well plate was sonicated at 40 kHz and shaked at 900 rpm for 5 min and this process was repeated. All cells were collected in one tube.

DNA extraction

Genomic DNA of *B. cenocepacia* J2315 was extracted using a modified bead-beater protocol (159). Briefly, a cell pellet (accounting for approx. 6 x 10^9 CFU) was resuspended in a TE buffer and subsequently mixed with a lysis buffer containing pronase (Roche, Vilvoorde, Belgium) and acid-washed glass beads. The suspension was vigorously vortexed for 10 seconds and the lysate was incubated at 37°C for 60 min. Both a saturated ammonium acetate solution and chloroform were added to the lysate and the solution was vortexed for 10 seconds. After centrifugation, the aqueous phase was transferred and purified by ethanol precipitation. Finally, the DNA pellet was dissolved in low-EDTA-TE buffer (0.1 mM EDTA) and RNase treated.

RNA extraction

Bacterial cells were pelleted at 4°C by centrifugation and stored at -80°C or immediately used for total RNA extraction. RNA was extracted using the Ambion RiboPure Bacteria kit (Life Technologies, Invitrogen, Merelbeke, Belgium) according to the manufacturer's instructions which included a 1 h DNase treatment at 37°C. One step within the final purification was optimized to retain sRNAs; the volume of ethanol was increased from 0.5 to 1.25 times the volume of the recovered aqueous phase. Purified RNA was DNase-treated for 1 h at 37°C.

Northern blot analysis

The expression of sRNAs in seven growth conditions was verified using northern blots. DIG-labeled DNA oligonucleotide probes specific for each sRNA candidate and for 5S which served as a loading control, were obtained from Sigma (Table 1). Three biological replicates were blotted. Total RNA (5 µg), a small RNA marker (Abnova, VWR, Amsterdam, The Netherlands) and an RNA ladder obtained from invitrogen were separated on a 10 % TBE-urea gel (Biorad, Temse, Belgium) in a 1 x TBE buffer (Biorad) by electrophoresis (1 h at 4°C and 80 V). The RNA was blotted (1 h at 4°C and 300 mA) and cross-linked (5 min exposure to UV-light) to a positively charged nylon membrane (Roche). The membrane was pre-hybridised for 30 min at 68°C in ULTRA-Hyb-oligo buffer (Ambion, Thermo Fisher, Aalst, Belgium) before 10 ng of one DIG-labelled probe was added, and hybridized overnight at 42°C. To visualize hybridization, the membrane was washed, treated with a blocking buffer (Roche) and incubated with an anti-DIG-Fab fragment (Roche) for 30 min followed by incubation with CSPD (Roche) for 10 min at 37°C. X-ray films (BioMax® Light X-ray films, Carestream, Rochester, NY, USA) were used to detect the signal.

Table 1. Primers and probes used in this study.

Primers or probes			Oligonucleotide sequence, 5'-3'
Construction of deletion mutant			
ΔncS04	Up	F:	ATAGAATTCTTCAGCATCGCGTATTCACC (EcorI)
		R:	AAAGCTAGCCCAACGATCTCGAAAATGACC (NheI)
	Down	F:	AAAGCTAGCGAATCCGCAGGTACCGTCA (NheI)
		R:	AAAAGATCTCTGCAACCTGAAGCACCATC (BgLII)
ΔncS16	Up	F:	AAAGGGCCCGCAGCAAGGCAACAATACGG (ApaI)
		R:	AAAGCTAGCGGCCTGCGACAGAAGTTCG (NheI)
	Down	F:	TATGCTAGCTTTGCCCGGAGAATTCGTG (NheI)
		R:	AAACCCGGGCGCATCTTGTTGGTCGCTTC (SmaI)
ΔncS27	Up	F:	ATTGAATTCTTGTCTGTGTGCGCTTCGAG (EcorI)
		R:	TATGCTAGCTTCGTCCCATGCAAACATCG (NheI)
	Down	F:	TATGCTAGCTCATGATGCGGGATTTCGAT (NheI)
		R:	TATAGATCTCAGGTAAGGGTGAAACGGT (BgLII)
ΔncS35	Up	F:	TATGAATTCTGATCGCCGACCCGAC (EcorI)
		R:	AAAGCTAGCCCGTGTGGATGTTCGC (NheI)
	Down	F:	TAAGCTAGCCGAAACGCATCTGTCAACC (NheI)
		R:	TATAGATCTCCAGTCGTCGATCTGCACC (BgLII)
Confirmation of deletion			
ncS04join		F:	GATGAAGCGGATGGAAGCTC
		R:	GACGCATGATCGAGTGGTTC
ncS16join		F:	GCATCAGTAAGCGCGACCAC
		R:	CACGGAAGTGAGGTCGGATG
ncS27join		F:	CCATGCCGAAGATCGTATCG
		R:	GTTTCAGGTCGCGTGTGAG
ncS35join		F:	CACATACATTCGCGGCAACT
		R:	CGAGCATCTTGTAGCGCATC
Northern blot probes			
ncS01-DIG			DIG-CGATTGTCTCCTCGTTCT
ncS04-DIG			DIG-CCTAATCGAAAAACGACAGG
ncS06-DIG			DIG-TGTTGTTCGACGACGATTGG
ncS11-DIG			DIG-ACCTGATTTCGCTCAGAA
ncS16-DIG			DIG-AACAACGACAAGAGGAGAA
ncS25-DIG			DIG-TCAACGAAAAAGTCTGCTTC
ncS27-DIG			DIG-CTGGTTCGGGCTTAATC
ncS35-DIG			DIG-TTGAGAGTCCCGGATTC
ncS37-DIG			DIG-AGTCACGGGATACATCG
5S RNA-DIG			DIG-AGAGTCGTTTCACGGTC

Restriction sites are underlined. F= forward primer, R= reverse primer, Up= primers for flanking sequences upstream, Down= primers for flanking sequences downstream, DIG = digoxigenin.

Construction of mutants

sRNA deletion mutants were constructed by allelic recombination (160). Briefly, sequences of approx. 1000 bp upstream and downstream of the sRNA sequence to be deleted were amplified using primer pairs listed in Table 1 and cloned into the suicide plasmid pGPI-SceI-Xcm. The plasmid was transformed into *E.coli* DH5α λpir by the $CaCl_2$ method (161), and into *B. cenocepacia* J2315 by triparental mating (152, 162). Ex-conjugants were sprayed with catechol and yellow colonies were selected for further propagation. Presence of concatenated DNA inserts was confirmed by PCR. pDAI-SceI-SacB, containing an endonuclease-encoding gene, was transformed into a *B. cenocepacia* pGPI-SceI-XCm mutant by triparental mating. Colonies remaining white after catechol treatment were selected and screened for sRNA deletion by PCR (Primer pairs listed in Table 1). Correct deletion was further confirmed by Sanger sequencing (GATC Biotech, Konstanz, Germany). The mutants were cured from the pDAI-SceI-SacB plasmid by successive transfers on LBA with 5 % sucrose.

Biofilm formation

Biofilm biomass was quantified using a crystal violet assay. Briefly, 100 µL 99 % methanol was added to the wells for fixating the cells. After 15 min the methanol was removed, the cells were treated for 20 min with 100 µL of a 0.1 % crystal violet solution, and washed. 100 µL of a 33 % acetic acid solution was added and absorbance was measured at 590 nm using a microtiter plate reader (Envision multilabel reader, PerkinElmer, Zaventem, Belgium).

Cell viability was determined by a resazurin-based assay. 20 µL of a CellTiter-Blue (Promega, Leiden, Belgium) solution was mixed with 100 µL PS and filled into the wells. Viable cells convert the resazurin to a fluorescent dye, measured after 1 h incubation at 37°C (excitation wavelength: 560 nm/emission wavelength: 590 nm).

The number of colony forming units per biofilm was determined by a plate count method. Cells were detached as described before, pooled and collected in one tube. The cells were centrifuged and resuspended in PS. A 10-fold dilution series was made and plated on LBA, plates were incubated for 48 h prior to counting.

For confocal laser scanning microscopy, biofilms were grown in a flat-black bottomed 96-well plate with glass bottom (Greiner Bio-one, Wemmel). Biofilms were visualised using a motorized Nikon TE2000-E inverted microscope (Nikon Benelux, Brussels, Belgium) with a Nikon C1 confocal laser scanning module. A Plan Apo VC60×1.4 NA oil immersion objective was used for focussing and suitable optical elements were used to obtain fluorescent signals. The cells were stained with a Live/Dead

solution (0.3 % SYTO 9 and propidium iodide in PS; Life Technologies) for 15 min. Excitation wavelength was 488 nm, the emission wavelengths were 500-530 nm and 660 nm for SYTO9 and for propidium iodide, respectively.

MIC determination

Susceptibility screening of *B. cenocepacia* J2315 and the deletion mutants towards various antimicrobial compounds and other stress agents was performed using broth microdilution according to EUCAST guidelines (163). A concentration range of an antibiotic or stress agent was added to a flat-bottom 96-well plate (TPP). Bacterial strains, grown in LBB, were added to the wells to obtain a final inoculum of approx. 5 x 10^5 CFU/mL. The optical density was observed after 24 h and 48 h at 590nm using an Envision multilabel plate reader (Perkin Elmer). The MIC was determined as the lowest concentration of an antimicrobial compound or stress factor that completely inhibits cell growth and where the optical density does not differ to blank wells. Used concentration range for antimicrobial compounds were for ceftazidime (TCI, Oxford, UK) 512 - 0.5 µg/mL, for chloramphenicol (Sigma) 120 - 0.12 µg/mL, for chlorhexidine (Fagron, Nazareth, Belgium) 120 - 0.12 µg/mL, for ciprofloxacin (Fluka, Sigma) 32 - 0.03 µg/mL, for meropenem (Hospira, Antwerp, Belgium) 128 - 0.125 µg/mL, for minocycline (Sigma) 64 - 0.06 µg/mL, for tobramycin (TCI, Zwijndrecht, Belgium) 1024 - 1 µg/mL, for trimethoprim (Ludeco) 512 - 0.5 µg/mL, for trimethoprim/sulfamethoxazol (Sigma) 512/9728 - 0.5/9.5 µg/mL, for temocillin (Eumedica SA, Brussels, Belgium) 4096 - 2 µg/mL, for cefsulodin (Sigma) 24500 - 11.96 µg/mL, for oxacillin (Sigma) 16384 - 8 µg/mL, and for aztreonam (TCI) 4096 – 2 µg/mL. For stress agents concentration ranges were for ethylenediaminetetraacetic acid (EDTA; Sigma) 5 - 0.005 %, ethanol (EtOH; Merck Millipore, Darmstadt, Germany) 50 - 0.048 %, for sodium chloride (NaCl; Panreac, Barcelona, Spain) 17 - 0.0166 %, for sodium hypochlorite (NaOCl; Sigma) 6.25 - 0.01 %, for sodium dodecyl sulphate (SDS; Merck) 1 - 0.001 % and for 2,2'-bipyridyl (Janssen, Beerse, Belgium) 1 - 0.001 mM. Additionally, the MIC set-up was used for determination of growth in a pH range. Bacterial strains, grown in LBB, were added to LBB adjusted with HCl or NaOH to a certain pH to obtain a final inoculum of approx. 5 x 10^5 CFU/mL.

Determination of growth curves

Growth curves were measured in LBB medium and LBB supplemented with different reagents: for osmotic stress, 1.5 % (w/v) NaCl was added to the medium, for membrane stress, 0.15 % EDTA (w/v), 0.015 % SDS (w/v) or 1.5 % ethanol (v/v), for oxidative stress, 0.045 % (w/v) NaOCl, and for an iron-depleted environment 0.25 mM 2,2'-bipyridyl. Approx. 2 x 10^5 CFU/well were added to a round-bottomed 96-well plate and the absorbance at 590 nm was measured for 60 h.

Motility assay

Swimming motility was investigated on soft agar plates. For wild type and each sRNA deletion mutant, a 1 µL aliquot of an liquid culture with optical density 0.1 was inoculated on a BSM 1.5 % agar for control and a BSM 0.3 % agar for swimming (150). The diameter of the swimming circle was evaluated after 20 h growth at 37°C.

C. elegans survival assay

Synchronized worms (L4 stage) were suspended in OGM, a medium containing 95 % M9 buffer (0.3 % KH_2PO_4, 0.6 % Na_2HPO_4, 0.5 % NaCl, and 0.012% $MgSO_4$ in water), 5 % Brain hart infusion broth (Oxoid) and 10 µg/mL cholesterol (Sigma). Nematodes were transferred to wells of a flat bottom 96-well plate to obtain approx. 30 nematodes per well. Liquid cultures of bacterial strains in OGM were added to the wells containing nematodes to obtain a final inoculum of 5×10^7 cells per well. Every well was filled to a final volume of 100 µL with OGM. Uninfected controls comprised wells containing 30 nematodes in OGM medium. Plates were incubated at 25°C. After 24 h and 48 h, nematodes were counted and observed using an EVOS microscope (Thermo Fisher Scientific, Waltham, MA, USA) to determine the ratio of living/dead nematodes. After 48 h incubation, the bacterial load in the nematodes was determined. Nematodes (both infected as well as uninfected nematodes) were collected in an Eppendorf tube (the content of three wells was pooled) and rinsed with M9 buffer supplemented with 1mM sodium azide to prevent vomiting. The nematodes were mechanically disrupted by 10 minutes vortexing with sterile silicon carbide beads. A dilution series of the supernatant was prepared on LBA 50 µg/mL Gm (selects against growth of *E. coli* OP50). The uninfected nematodes served as negative control. Plates were incubated for 48 h at 37°C prior to counting.

Statistics

Each experiment was performed at least in duplicate. For normally distributed values, an independent sample-t-test or ANOVA was performed, otherwise a Mann-Whitney test or a Kruskal-Wallis test was performed. Statistical tests were considered significant when $p < 0.05$.

RESULTS & DISCUSSION

Selected sRNAs

Nine putative sRNA genes previously detected by dRNA-seq (34) were investigated. Seven sRNAs are located on the large chromosome of *B. cenocepacia* J2315 and two are located on the second largest chromosome (Table 2). Four sRNAs were homologues to previously described non-coding RNAs in *B. cenocepacia*. A Hfq sRNA co-purification study described ncS04 as Bcj7 (143). The sRNAs ncS06 and

ncS11 are homologous to two intergenic regions of which the expression is induced by growth in soil medium (139). Finally, one sRNA, ncS27, resulted in a hit when compared to the Rfam database. ncS27 expression causes inhibition of *E. coli* growth and was therefore called 'toxic sRNA' (145). The function of the nine selected sRNAs in *B. cenocepacia* J2315 is currently not known.

Table 2. Nine sRNAs discovered by dRNA-seq in *B. cenocepacia* J2315

sRNA	**Genomic location**[a]	**Flanking genes**		**Other designation**
ncS01	L: 20581..20691	BCAL0019 Branched-chain amino acid ABC transporter permease	BCAL0020 Branched-chain amino acid ABC transporter periplasmic substrate binding protein	/
ncS04	L: 292949..293053	BCAL0264 Delta-aminolevulinic acid dehydratase	BCAL0265 GTP-binding cell division protein EngB	Bcj 7 (143)
ncS06	L: 603652..603828	BCAL0549 Hypothetical protein	BCAL0550 LamB/YcsF family protein	ncRNA7 (139)
ncS11	L: complement (2545296..2545503)	BCAL2293 Biopolymer transport protein	BCAL2294 LysR family regulatory protein	ncRNA13 (139)
ncS16	L: 2912201..2912281	BCAL2645 OmpA family membrane protein	BCAL2646 Methionyl-tRNA synthetase	/
ncS25	L: complement (3297991..3298074)	BCAL3007 Hypothetical protein	BCAL3008 Outer membrane porin protein	/
ncS27	L: complement (3666557..3666648)	BCAL3348a Hypothetical protein	BCAL3349 OsmC-like protein	Toxic RNA (145)
ncS35	M: complement (2304213..2304378)	BCAM2068 Hypothetical protein	BCAM2069 Hypothetical protein	/
ncS37	M: 2568766..2568918	BCAM2286 Putative short chain dehydrogenase	BCAM2287 Hypothetical protein	/

[a] L= large chromosome, M= medium chromosome.

Northern blotting

We confirmed the expression of the sRNAs in 7 growth conditions by northern blotting (Figure 1). Most sRNAs were highly expressed in biofilms, during growth in a minimal medium with glucose and during starvation conditions. Expression in planktonic cells (stationary and exponential phase) was typically lower. ncS01 and ncS04 additionally showed higher expression when exposed to H_2O_2 and SDS. The

ncS27-DIG probe hybridized to several spots on the membrane and showed a remarkably strong signal. Therefore, RNA extracted from a sRNA deletion mutant, ΔncS27, was used for hybridization. This mutant was confirmed by Sanger sequencing and PCR but the ncS27-DIG probe still hybridized to RNA of this mutant. We assume our probe is not exclusively hybridizing to sRNA ncS27 and for this reason the expression of this sRNA could not be confirmed beyond reasonable doubt (Figure 1).

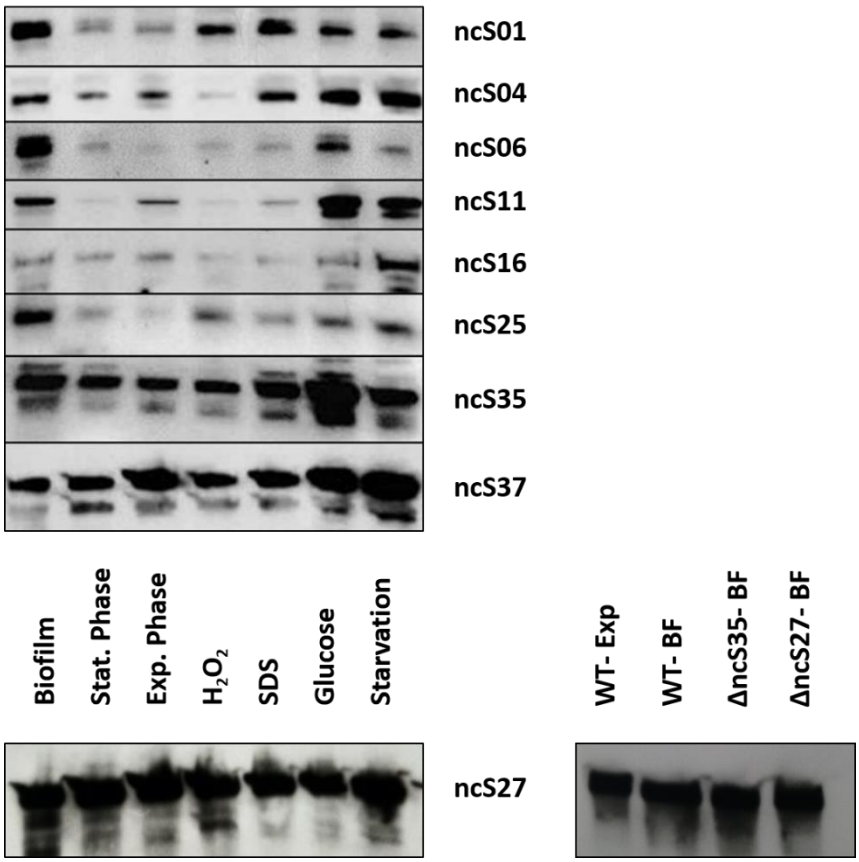


Figure 1. Validation of sRNA expression by Northern blotting. Expression was evaluated in biofilms, planktonic cells (both in stationary phase and in exponential phase) and in cells grown under stress conditions (LBB with 0.2 % H_2O_2 added 15 min prior to harvesting (H_2O_2), LBB supplemented with 0.015 % (w/v) SDS (SDS; membrane stress), in M9 supplemented with 0.1% (w/v) glucose or and in M9 supplemented with 0.2% (w/v) casamino acids (starvation). For ncS27, northern blotting of *B. cenocepacia* J2315 wild type (WT) and sRNA deletion mutants ΔncS35 and ΔncS27 confirms the non-specific hybridization of the used probe (BF = biofilm, Exp = exponential phase).

Evaluation of physiological role of sRNAs

Both deletion (164, 165) and overexpression (166) mutants are used for physiological characterization of sRNAs. Deletion mutants directly indicate whether a gene is essential and do not require addition of antibiotics for plasmid maintenance. Constructing overexpression mutants is easier than constructing deletion mutants, however there is a higher chance of pleiotropic effects due to the titration of an RNA chaperone (101). Because of the need for antibiotic selection during overexpression experiments, we decided to first pursue the construction of deletion mutants. *B. cenocepacia* and all other Bcc strains are difficult bacteria to genetically manipulate. Only a limited number of selective

markers can be used, as Bcc bacteria have an intrinsic resistance to almost all antibiotics used for plasmid selection (167). Secondly, plasmids can only be transformed into *B. cenocepacia* J2315 by conjugation. Additionally, the high GC-content (67%) (28) of *B. cenocepacia* J2315 makes successful primer design challenging. Despite these problems, we succeeded in constructing four sRNA deletion mutants (ΔncS04, ΔncS16, ΔncS27 and ΔncS35).

Biofilm phenotypes (biomass, metabolic activity and cell count) of sRNA deletion mutants showed no significant differences to wild type *B. cenocepacia* J2315 (Figure 2).

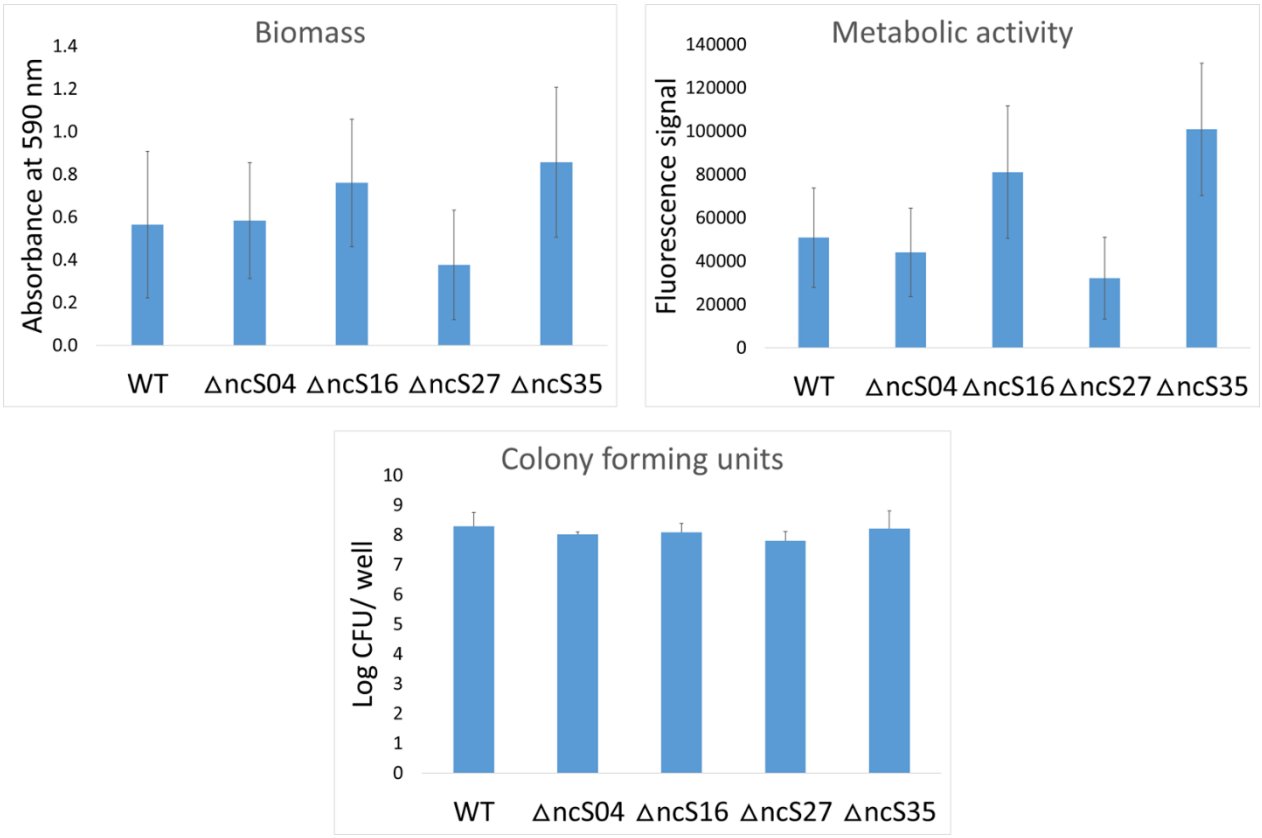


Figure 2. Biofilm formation of wild type (WT) and sRNA mutants. Biomass was evaluated using a crystal-violet assay, metabolic activity of biofilm cells by a resazurin based viability assay and colony forming units (CFU) within a biofilm were determined by plate counting. Error bars represent standard deviation. n=6, for the biomass and metabolic activity and n=3, for plate counts.

Despite the lack of statistical differences, ΔncS35 biofilms, formed in a 96-well plate, looked different from wild type biofilms (Figure 3A). To visualize biofilm formation, confocal imaging of biofilm cells was performed. All mutants showed a similar biofilm structure compared to the wild type, except ΔncS35 biofilm cells clustered to form larger aggregates than wild type biofilm cells (Figure 3B).

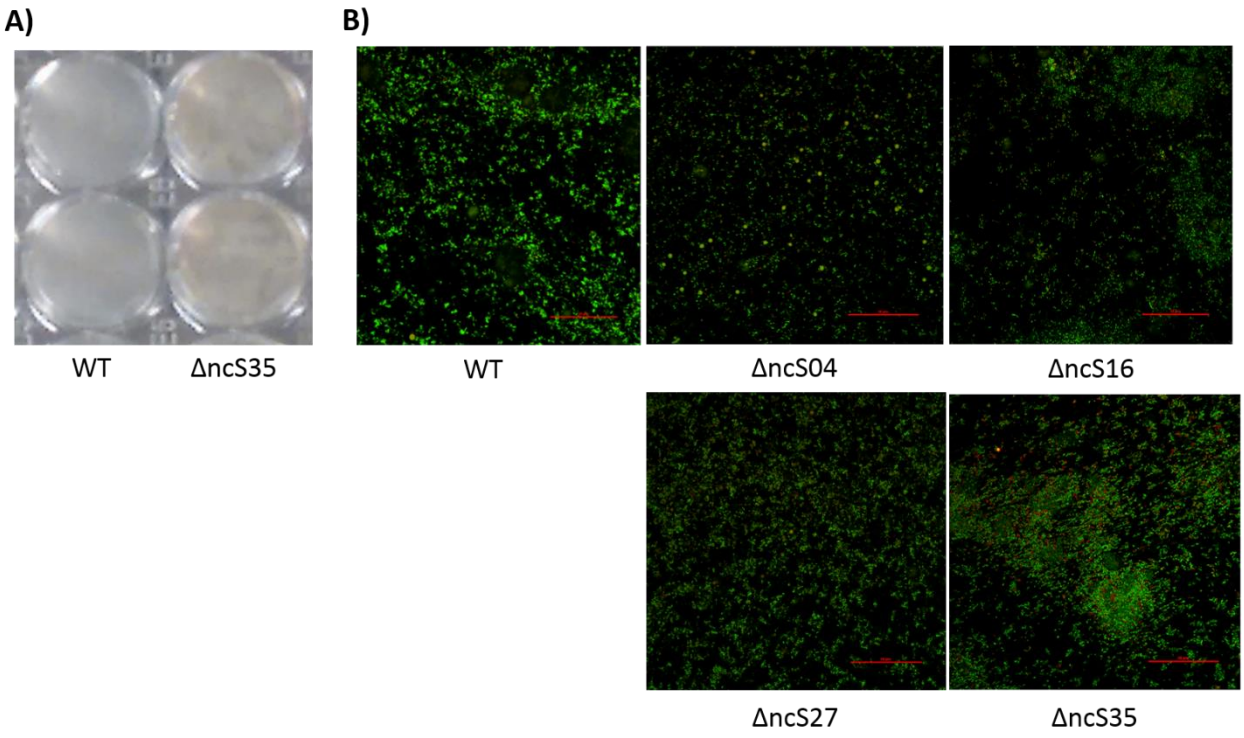


Figure 3. Biofilm formation. A) Biofilm cells of wild type (WT) and ΔncS35 after 24 h growth in a 96-well plate. B) Confocal laser scanning microscopy. Z-stack image of 24 h old biofilms of wild type (WT) and sRNA deletion mutants after Live/Dead staining. Scale bar indicates 50 µm.

MIC were determined for several antimicrobial compounds and stress factors (Table 3). The MIC was determined as the lowest concentration of a compound for which no growth was observed. For ΔncS04 and ΔncS27, no difference in MIC for any of the compounds was investigated compared to the wild type. For ΔncS16, only a slightly lower MIC for chlorhexidine was observed compared to the wild type. The MIC of ΔncS35 for two antimicrobial compounds, chlorhexidine and tobramycin the MIC was slightly lower compared to the wild type. Additionally the MIC for three beta-lactam antibiotics was decreased. For meropenem, a carbapenem, and ceftazidime, a third generation cephalosporin, the MIC was reduced 2-fold and for aztreonam, a monobactam, the MIC was reduced 4-fold. For three other beta-lactam antibiotics, temocillin (a carboxypenicillin), cefsulodin (a third generation cephalosporin), and oxacillin (a beta-lactamase resistant penicillin) no change in MIC was found. Due to the variability in this type of experiments, only a 4-fold difference in MIC is considered biologically relevant (168). Aztreonam is a monocyclic beta-lactam, selectively active against gram-negative bacteria. All beta-lactam antibiotics contain this four-membered lactam ring within their structure and they all act through irreversible acetylation of penicillin-binding proteins (PBPs), which are important for crosslinking the peptidoglycan layer of bacterial cell walls (169). Inhibition of PBPs will disrupt cell wall synthesis and ultimately lead to cell lysis (170). Beta-lactamases are a major determinant of

bacterial resistance towards beta-lactam antibiotics, however, efflux pumps or modifications of PBPs also contribute (171).

In *P. aeruginosa*, mutations in 19 genes were found to be associated with aztreonam resistance. Further investigations showed that mutations affecting the repressors of the *mexAB-oprM* efflux system and mutations within the target of aztreonam (*ftsI*), which were clustered around the aztreonam binding site, were most frequently observed. Additionally, genes involved in amino acid metabolism, energy metabolism and peptidoglycan metabolism were among mutated genes and resistance was associated with decreased growth rates (172). MexAB-OprM, extrudes almost all beta-lactams. The extrusion of aztreonam remains specific to the MexAB-OprM efflux system, whereas the extrusion of ceftazidime and cefsulodin is also mediated by other efflux pumps, e.g. MexCD-OprJ (173). Aztreonam-resistant *P. aeruginosa* were found to accumulate mutations in the transcriptional regulator NalC, causing an overexpression of MexAB-OprM (174). In *B. pseudomallei*, BpeAB-OprB is related to MexAB-OprM (175) and in *B. cenocepacia*, an RND homolog of MexAB-OprM, ORF2, was found (176), however whether they play a role in aztreonam resistance is not yet elucidated.

Table 3. Minimal Inhibitory Concentration (MIC) of antimicrobials and several stress compounds for *B. cenocepacia* J2315 (WT) and sRNA deletion mutants.

	WT	ΔncS04	ΔncS16	ΔncS27	ΔncS35
Antimicrobial compounds (MIC in µg/ml)					
Ceftazidime	16	16	16	16	8
Chloramphenicol	30	30	30	30	30
Chlorhexidine	15	15	7.5-15	15	7.5-15
Ciprofloxacin	8	8	8	8	8
Meropenem	16	16	16	16	8
Minocycline	8	8	8	8	8
Tobramycin	512	512	512	512	256-512
Trim/Sulf	16/304	16/304	16/304	16/304	16/304
Trimethoprim	512	512	512	512	512
Additional β-lactam antibiotics (MIC in µg/ml)					
Temocillin	64	-	-	-	64
Cefsulodin	25000	-	-	-	25000
Oxacillin	2048-4096	-	-	-	4096
Aztreonam	4096	-	-	-	512
Stress factors (MIC in % (w/v or v/v))					
EDTA	0.625	0.625	0.625	0.625	0.625
EtOH	1.56	1.56	1.56	1.56	1.56
NaCl	4.25	4.25	4.25	4.25	4.25
NaOCl	0.05	0.05	0.05	0.05	0.05
SDS	>1	>1	>1	>1	>1
Iron depletion (MIC in mM)					
Dipyridil	0.5-1	0.5-1	0.5-1	0.5-1	0.5-1

- = Not investigated. The average MIC is shown, n=3. Dark orange indicates a two-fold difference or higher in MIC compared to wild type. Pale orange indicates a slightly different MIC compared to the wild type. Trim/Sulf = trimethoprim/sulfamethoxazole.

Additionally, growth at range of different pH was investigated. All strains grow in the pH range of 5 to 8. In lower pH (≤4) and higher pH (≥9) no growth was observed. Remarkably, compared to the wild type, a higher optical density was observed for ΔncS35 under all conditions in which growth occurred (Figure 4).

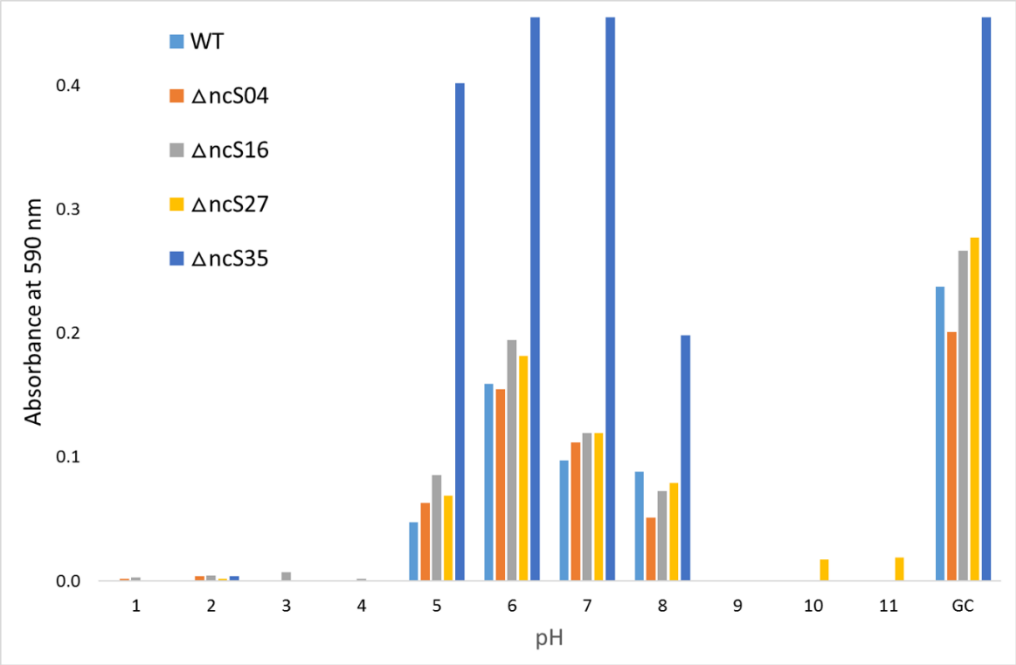


Figure 4. Growth at different pH for wild type (WT) and sRNA deletion mutants. GC= growth control in LBB without a change in pH (pH 7.5). n = 3, a representative figure is shown.

Growth curves for wild type and sRNA deletion mutants were determined at sub-MIC concentrations of various stress compounds and in pH 4.2 and 8.2 (Figure 5). In LBB, ΔncS35 showed a higher growth rate, with a doubling time of 360 minutes in the exponential phase, and a higher maximal optical density (approx. 1.0) than wild type with a doubling time of 960 minutes and a maximal optical density of approx. 0.8. This effect was less pronounced in medium supplemented with 0.045 % NaOCl, 0.15 % EDTA or 1.5 % NaCl. In LBB supplemented with 1.5 % ethanol, the growth curves and optical densities were similar to those obtained in LBB, indicating this amount of ethanol is probably not sufficient to cause stress and therefore no conclusions could be drawn of the effect of ethanol on sRNA deletion mutants. In pH 4.2 and 8.2 no differences for growth rate or final optical density for the sRNA deletion mutants compared to wild type was found.

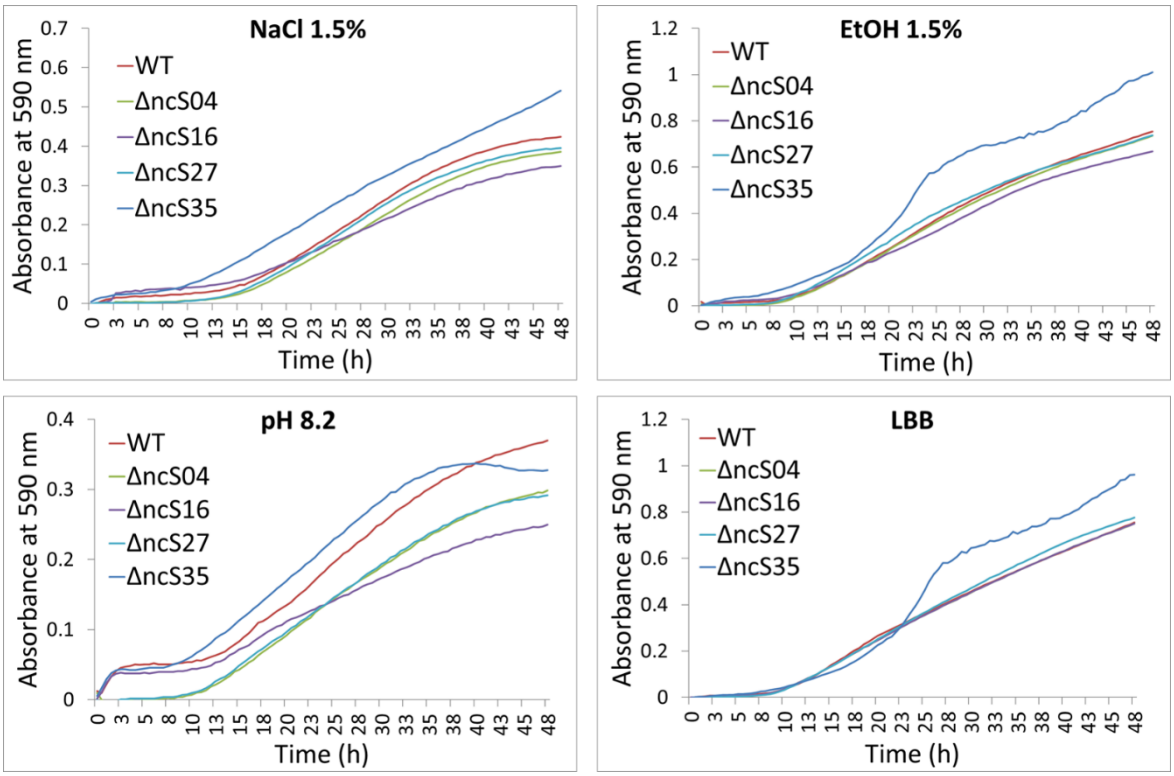
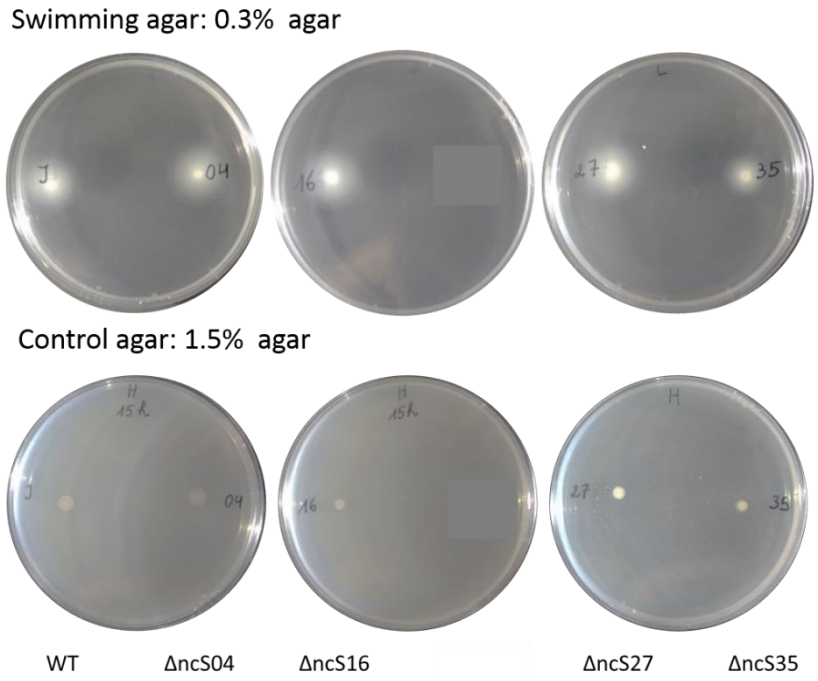


Figure 5. Growth curves of wild type (WT) and sRNA deletion mutants in different media (LBB supplemented with 1.5 % NaCl or 1.5 % Ethanol, LBB adjusted to pH 8.2 and in LBB).

In a motility assay (Figure 6), ΔncS35 demonstrated reduced swimming capacity, with a colony diameter less than half of the diameter of the wild type. Other sRNA deletion mutants did not show a difference in swimming motility.



Strain	Ø 1	Ø 2	Average
WT	1.10	1.10	1.10
ΔncS04	1.20	1.15	1.18
ΔncS16	1.10	1.20	1.15
ΔncS27	1.10	1.10	1.10
ΔncS35	0.70	0.80	0.75

Figure 6. Motility assay of wild type (WT) and sRNA deletion mutants. 1 µL of a cell suspension of OD 0.1 was spotted on a BSM soft agar plate (0.3 % agar) and on a control agar plate (1.5 % agar). After 24 h, the diameter was measured. n=2. A representative picture is shown. Ø = diameter.

The pathogenicity of wild type and sRNA deletion mutants was investigated using a *C. elegans* infection assay. *C. elegans* was infected with cell suspensions of OD 2.0. After 24 h and 48 h, the number of live and dead *C. elegans* was determined and after 48 h, the number of bacterial cells present in the worms was determined by plating (Figure 7). After 24 h, the variation in survival rates was quite high. After 48 h, less variation was observed but no significant differences were found between sRNA deletion mutants and the wild type. Also the amount of cultivable bacterial cells within *C. elegans* did not show significant differences.

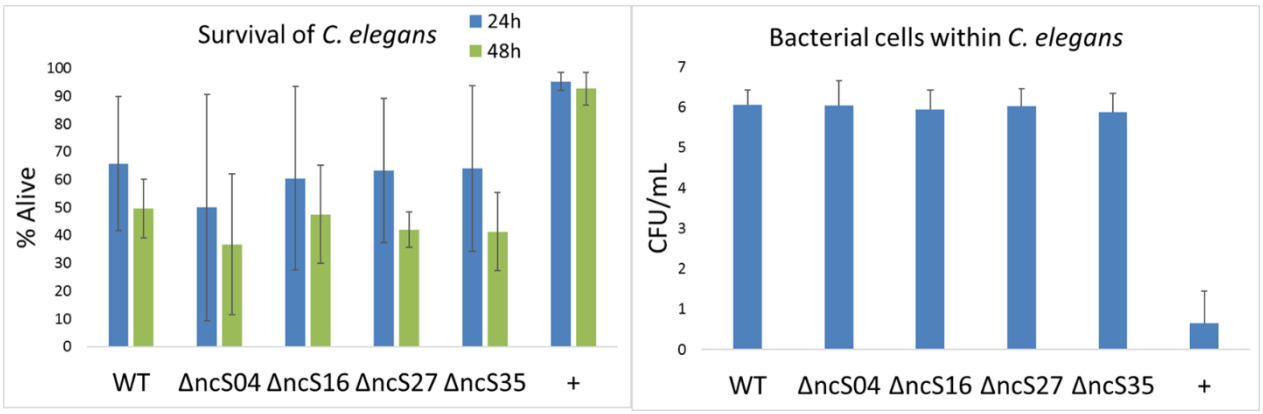


Figure 7. Infection assay using *C. elegans*. The survival of *C. elegans* was assessed after 24 h and 48 h. After 48 h, the number of cultivable bacterial cells within *C. elegans* were counted. + = uninfected control, *C. elegans* grown in medium without bacterial strains. Error bars represent standard deviation (n=3).

SUMMARY AND CONCLUSIONS

This study investigates nine *Burkholderia cenocepacia* J2315 sRNAs, discovered by dRNA-seq. Northern blotting confirmed expression of eight sRNAs in the different growth conditions, although levels of expression varied according to the condition. Of these nine sRNAs, four sRNAs were successfully deleted in *B. cenocepacia* J2315 and the phenotype of these sRNA mutants was evaluated in various growth conditions with or without the addition of stress factors to elucidate the physiological role of these sRNAs. For ΔncS35, several phenotypes were observed. This indicates a function for this sRNAs in the regulatory networks affecting motility, biofilm formation and growth. Therefore this mutant was chosen for further investigations and is discussed in a next chapter. For the three other sRNA deletion mutants (ΔncS04, ΔncS16 and ΔncS27), no phenotype was observed in the conditions tested. One explanation for the lack of a phenotype could be that the relevant stress condition was not investigated, as most sRNAs are only expressed under a certain stress condition. On the other hand, several studies on sRNA deletion mutants demonstrate that some sRNA deletion mutants only show a mild phenotype compared to the wild type, while most mutants show no phenotype at all. Probably these sRNAs have minor regulatory roles or they are functional redundant (127). Additional experiments are required to prove whether these sRNAs indeed do not function in the tested regulatory networks.

ACKNOWLEDGEMENTS

We thank Eline Teirlinck for support with confocal microscopy.

FUNDING

This work was supported by the Belgian Science Policy Office (Belspo) through the Interuniversity Attraction Pole Program.

Paper 2: The sRNA ncS35 regulates growth in *Burkholderia cenocepacia* J2315

Sanne Kiekens, Andrea Sass, Filip Van Nieuwerburgh, Dieter Deforce and Tom Coenye.

mSphere. Submitted.

2017

ABSTRACT

Burkholderia cenocepacia isolate J2315 is a member of the *Burkholderia cepacia* complex. It has a large genome with three replicons and one plasmid; 7261 genes code for annotated proteins, while 113 genes code for functional RNAs. Small regulatory RNAs of *B. cenocepacia* have not yet been functionally characterised. We investigated a small regulatory RNA (designated ncS35) that was discovered by differential RNA-sequencing. Its expression under various conditions was quantified and a deletion mutant, ΔncS35, was constructed. Compared to planktonic growth in a rich medium, expression of ncS35 was elevated when *B. cenocepacia* J2315 was grown in biofilms and in minimal medium. Cells of the deletion mutant showed increased aggregation, a higher metabolic activity and growth rate, and an increased susceptibility to tobramycin. A transcriptomic analysis revealed upregulation of the phenylacetic acid and tryptophan degradation pathways in ΔncS35. Computational target prediction indicated that ncS35 likely interacts with the first gene of the tryptophan degradation pathway. Overall, we demonstrated that sRNA ncS35 is a non-coding RNA with an attenuating effect on metabolic rate and growth. It is possible that slower growth protects *B. cenocepacia* J2315 against stressors acting on fast-dividing cells and enhance survival in unfavourable conditions.

IMPORTANCE

Small RNAs play an important role in the survival of bacteria in diverse environments. We explored the physiological role of ncS35, a small RNA expressed in *B. cenocepacia* J2315, an opportunistic pathogen in cystic fibrosis patients. In CF patients, infections can lead to “cepacia syndrome”, a rapidly progressing and often fatal pneumonia. Infections with *Burkholderia* spp. are difficult to threat with antibiotics, because of their high intrinsic resistance and ability to form biofilms. We show that ncS35 attenuates growth, reduces the metabolic rate in *B. cenocepacia* and influences biofilm structure. This demonstrates that yet uncharacterised small RNAs with regulatory function can influence physiological traits of *B. cenocepacia* that are relevant for infection.

INTRODUCTION

Burkholderia cenocepacia is a member of the *Burkholderia cepacia* complex (Bcc). This group of bacteria consists of 20 closely related bacterial species found in different environments. Bcc bacteria can be isolated from soil or are associated with plants, they can be used as biocontrol or bioremediation agent, or show plant growth promoting activity. On the other hand, Bcc bacteria can also act as opportunistic pathogens, causing severe infections in immunocompromised patients (14, 18). Especially individuals with cystic fibrosis (CF) or chronic granulomatous disease are sensitive to infections with Bcc bacteria. In CF patients, infections with *B. cenocepacia* can lead to “cepacia

syndrome", a rapidly progressing pneumonia, ultimately leading to death. Due to the high innate resistance to most antibiotics and to their ability to form biofilms, eradication of Bcc bacteria is difficult (35, 177). *B. cenocepacia* isolate J2315 is a member of the highly transmissible ET-12 lineage (178). It harbours two large replicons of 3.87 and 3.22 Mb, one smaller replicon of 0.88 Mb and one plasmid of 0.09 Mb, in total encoding for 7261 annotated proteins. Genes on the largest replicon mainly encode core cellular functions, whereas the two other chromosomes mainly harbour genes encoding accessory functions (28, 179). Besides protein coding genes, 113 RNA non-coding genes were annotated, including 74 tRNA and 10 riboswitches (28, 34). Little is currently known about regulatory small RNAs (sRNAs) in *B. cenocepacia*, their function and regulatory networks (34, 92).

sRNAs are small non-coding RNA molecules in bacteria that possess regulatory functions on the post-transcriptional level (73). They are typically relatively short in length (40-500 nucleotides) and act mostly by base pairing with mRNA. Cis-encoded sRNAs are located on the opposite strand of their mRNA target and are therefore fully complementary to their target. Trans-encoded sRNAs, the most abundant type of sRNAs, act by imperfect antisense base pairing on mRNA targets which are located elsewhere on the chromosome. Due to the limited complementarity to their target, many trans-encoded sRNAs require the RNA chaperone protein Hfq to fulfil their function (180). By base pairing, translation initiation frequency or stability of the mRNA target can be modulated. Binding of the sRNA within the 5'UTR can occlude the ribosome-binding site (RBS) and inhibit ribosome binding, leading to attenuation of translation. Subsequently, RNases, enzymes involved in RNA turnover, can degrade unprotected mRNA. Initiation of translation can also be increased as sRNAs can bind to hairpin structures of mRNA targets, open them and reveal the RBS. Finally, mRNA stability can directly be affected by sRNAs, as binding within the coding sequence (CDS) of the mRNA target can result in an increased degradation of the sRNA-mRNA duplex (180, 181).

sRNAs are only conserved between closely related species. They are involved in fast fine-tuning of gene expression, essential when bacteria have to survive stress, e.g. carbon or iron starvation, unfavourable pH or temperature, oxidative and membrane stress. They are known to play a role in pathogenicity, through regulating production of virulence factors, and in biofilm formation (146).

Genome-wide RNA-sequencing (RNA-seq) methods make it possible to obtain detailed information about transcriptome structure of an organism. An often-used technique is differential RNA-sequencing (dRNA-seq), which allows global mapping of transcription start sites (TSS). Briefly, for dRNA-seq an RNA sub-sample is treated with a 5'-monophosphate-dependent exonuclease (TEX). Bacterial primary transcripts carry a 5'-triphosphate end (5'PPP) and processed transcripts carry a 5'-monophosphate

(5'P). TEX specifically degrades 5'P-RNAs. Comparing abundance of sequenced 5'-ends of TEX-treated RNA with those of untreated RNA identifies TSS as loci with increased coverage in the TEX-treated sub-sample. TSS from annotated proteins, rRNAs and tRNAs can be distinguished from TSS of undiscovered proteins and sRNAs by their position relative to annotated genes (123).

In the present study, one transcript discovered by dRNA-seq (34), designated ncS35, not associated with a coding sequence and resulting in a short transcript, was characterized.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were maintained on Luria-Bertani agar at 37°C (LBA; Oxoid, Hampshire, UK) and liquid overnight (O/N) cultures were grown in Luria-Bertani broth (LBB; Oxoid) at 37°C with orbital agitation (150 rpm). Where appropriate, antibiotics were added for plasmid selection: ampicillin (Amp; Sigma-Aldrich), chloramphenicol (Cm; Sigma-Aldrich, Brussels, Belgium), gentamicin (Gm; Sigma-Aldrich), kanamycin (Km; Sigma-Aldrich), trimethoprim (Tp; Ludeco, Brussels, Belgium) or tetracycline (Tet; Sigma-Aldrich). Overexpression mutants were grown in LBB supplemented with 600 µg/mL Tp and 0.2 % (w/v) rhamnose. M9 medium was used as a minimal medium. *B. cenocepacia* J2315 is auxotrophic for phenylalanine, therefore in absence of a source of amino acids, 0.5 mM phenylalanine was added.

Planktonic cultures were grown at 37°C with orbital agitation (250 rpm). For exponential phase, cells were harvested at optical density (OD) of 0.5 (5 x 10^8 colony forming units (CFU) /mL), for late exponential phase at optical density 1.0 (1 x 10^9 CFU/mL) and for stationary phase at optical density 2.0 (2 x 10^9 CFU/mL). Biofilms were cultivated in microtiter plates (140). To harvest biofilm cells, 24 h grown biofilms were rinsed with physiological saline (PS). To detach the cells, 100 µL PS was added to each well and the plate was sonicated at 40 kHz and shaken at 900 rpm for 5 min. Cells from two cycles of shaking and sonicating were pooled and collected in one tube.

DNA extraction

Genomic DNA from *B. cenocepacia* J2315 was isolated after mechanical disruption with glass beads using a modified bead-beater protocol (159). A culture aliquot of approx. 5 x 10^9 CFUs was centrifuged and the pellet resuspended in Tris-EDTA (TE) buffer, mixed with a lysis buffer containing pronase (Roche, Vilvoorde, Belgium) and glass beads and vortexed for 10 seconds. The lysate was further incubated at 37°C for 60 min and mixed with a saturated ammonium acetate solution and chloroform.

After centrifugation, the clear aqueous phase was purified by ethanol precipitation, dissolved in low-EDTA-TE buffer and RNase treated.

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Reference or source
B. cenocepacia J2315 (LMG 16656)	CF sputum isolate	BCCM/LMG Collection
B. cenocepacia ΔncS35	sRNA ncS35 deletion mutant	This study
E. coli DH5α		Lab stock
E. coli DH5α λpir	Propagation of suicide plasmid	Biomedal, Seville, Spain
E. coli JM109	Cloning of PCR products	Promega, Leiden, Belgium
pGEM	Parental vector for cloning of PCR products, ori_{pUC}, ori_{F1}, Amp^r	Promega
pRK2013	Helper plasmid, ori_{colEI}, Km^r	(152)
pGPI-SceI-XCm	Suicide plasmid, ori_{R6K}, I-SceI restriction site, Tp^r and Cm^r	(153)
pDAI-SceI-SacB	Broad host range replicative plasmid, ori_{pBBR1}, I-SceI nuclease, counter selectable marker SacB, Tet^r	(153, 154)
pSCrhaB2	Expression vector containing a rhamnose-inducible promoter, ori_{pBBR1}, *rhaR* and $RhaS\text{-}P_{rhaB}$, Tp^r	(182)
pSCrhaM2	pSCrhaB2 lacking Shine-Dalgarno sequence and start codon	This study
pSCrhaM2 + ncS35	pSCrhaM2 overexpressing sRNA ncS35	This study

RNA extraction

Bacterial cells were collected and RNA was extracted using RiboPure Bacteria kit (Life Technologies, Merelbeke, Belgium). The manufacturer's instructions were followed aside from a change in the final RNA purification protocol: the volume of ethanol was increased to 1.25 times the volume of the recovered aqueous phase to retain sRNAs. Purified RNA was DNase-treated for 1 h at 37°C.

RACE

5'RACE was performed as described by Sass et al. (34), using the 5'RACE System from Invitrogen (Thermo Fisher, Aalst, Belgium). cDNA was generated from total RNA, extracted from biofilms, using gene specific primer ncS35-RC, and then poly(C)-tailed. cDNA was amplified using a nested gene specific primer (ncS35-RB), and the abridged anchor primer from the kit.

For 3'RACE, total RNA was first poly(A)-tailed (Poly(A) Polymerase Tailing Kit, Epicentre, Chicago, U.S.A.). cDNA was generated using the 3'RACE System from Invitrogen (Thermo Fisher), with its oligo d(T) adapter primer. cDNA was amplified using a gene specific primer (ncS35-FA) and the abridged universal amplification primer from the kit. PCR products were re-amplified with a nested gene specific primer (ncS35-FB).

PCR products were cloned using the pGEM-T vector system (Promega). Inserts were amplified and sequenced using standard primers M13F and M13R (Promega). Alignments of RACE sequences were performed with Clustal Omega [https://www.ebi.ac.uk/Tools/msa/clustalo/]. Primer sequences are listed in Table 2.

Computational analysis

Conservation of ncS35 was investigated using BLASTn (183). Input parameters were changed to word size 7, match/mismatch Scores 1/-1, gap existence cost -0, gap extension cost 2. Cut-off was 65 % for query coverage and 60 % for sequence similarity. Genome sequences included in the BLASTn search are listed in Suppl. Table S1. To search for homologues with known function, ncS35 sequence was compared to the Rfam database (184). The Mfold web server was used to predict secondary structure using standard parameters (temperature: 37°C, ionic strength: 1M NaCl) (185). z-scores for MFE were calculated using 500 randomly mononucleotide-shuffled sequences (obtained using CLC Genomics Workbench v. 8.5.1). The z-score is the number of standard deviations (σ) the MFE of the actual sequence (x) deviates from the mean MFE (μ) of shuffled sequences, i.e. $(x - \mu)/\sigma$. Putative targets of ncS35 were predicted with CopraRNA (Comparative Prediction Algorithm for sRNA Targets, (186)). Eight genome-sequenced Bcc strains were used as input (Suppl. Table S3). Output is a list of 100 targets with the smallest p-values, present in at least 50 % of input strains. Because p-values are corrected and weighted based on a phylogenetic tree of input genomes, this output can vary accordingly. Therefore the analysis was repeated five times and only hits occurring in all five results lists were retained.

Table 2. Primers and probes used in this study.

Primers or probes		Oligonucleotide sequence, 5'–3'
Construction of deletion mutant		
upstream	UM2068-F	TATGAATTCTGATCGCCGACCCGAC (EcorI)
flanking	UM2068-R	AAAGCTAGCCCGTGTGGATGTTCGC (NheI)
sequence		
downstream	DM2069-F	TAAGCTAGCCGAAACGCATCTGTCAACC (NheI)
flanking	DM2069-R	TATAGATCTCCAGTCGTCGATCTGCACC (BGLII)
sequence		
Confirmation of deletion		
ncS35join-F		CACATACATTCGCGGCAACT
ncS35join-R		CGAGCATCTTGTAGCGCATC
Construction of modified plasmid pSCrhaM2		
M2-F		TTACTAGTAAGGTACCCGGGGATCCTCTAGAGT
M2-R		ATTACGACCAGTCTAAAAAGCGCCTG
Construction of an overexpression mutant		
ncS35ov-F		ATACTAGTATGGCGCGACGACAAGTG (SpeI)
ncS35ov-R		TTTAAGCTTGCCGCGACATGACCTGT (HindIII)
Northern blot probes		
ncS35-DIG		DIG-TTGAGAGTCCCGGATTC
5S RNA-DIG		DIG-AGAGTCGTTTCACGGTC
RACE and qPCR		
ncS35-RB		CTGCGTGCCATGGACATATGT
ncS35-RC		TGGATGTTCGCTCAGGGCTC
ncS35-FA		GACAAGTGCGCGCAACGA
ncS35-FB		ACATATGTCCATGGCACGCAG

Restriction sites are underlined. F= forward primer, R= reverse primer, DIG = digoxigenin.

Quantitative RT-PCR analysis (qPCR)

RNA was extracted as described above. Additionally, a second DNase-treatment (NEB, Ipswich, MA, USA) was performed for 60 min and the RNA was purified with phenol-chloroform (Roti-Aqua-P/C/I, Carl Roth, Karlsruhe, Germany). Subsequently, an ethanol-sodium acetate mix (ethanol: 3 M Na-acetate 30:1, pH 6.5) was added to precipitate the RNA at −20°C over-night. After centrifugation and washing with 70 % ethanol, the RNA pellet was air dried and re-dissolved in water.

cDNA generation and qPCR were performed as described by Van Acker et al. (187), using three biological replicates. Primer sequences used for the analysis of ncS35 are ncS35-FB with ncS35-RC, respectively (Table 2, Suppl. Figure S1A). Data was normalised to eight control genes (primer sequences in suppl. Table S2). The final fold changes were calculated relative to a cDNA standard (mix of cDNA of all the samples of the different conditions).

Northern blot analysis

5 µg of total RNA was loaded onto a 10 % TBE-urea gel (Biorad, Temse, Belgium) and electrophoresed in 1 x TBE buffer (Biorad) at 4°C at 80 V for approx. 1 h. RNA was blotted on a positively charged nylon membrane (Roche) at 300 mA and 4°C for 1 h and cross-linked to the membrane by 5 min exposure to UV-light. Membranes were pre-hybridized at 68°C for 30 min in ULTRA-Hyb-oligo buffer (Thermo Fisher, Aalst, Belgium), then 10 ng of a DIG-labelled probe (Table 2, Sigma) was added and membranes were further hybridized overnight at 42°C. 5S RNA (Table 2) was probed as a loading control. Following hybridization, membranes were washed and treated with blocking buffer (Roche). Labelling with an anti-DIG-Fab fragment (Roche) was performed for 30 min followed by incubation with CSPD (Roche) for 10 min at 37°C. The signal was detected using X-ray films (BioMax Light X-ray films, Carestream, Rochester, NY, USA). Three biological replicates were blotted and representative images are shown. Full-size images of Northern blots are presented in Suppl. Figure S2.

Construction of mutants

PCR amplification was performed using Hot Star High-Fidelity (Qiagen, Venlo, Netherlands) or Phusion High-Fidelity (New England Biolabs, Bioké, Leiden, Netherlands) DNA polymerase. Correct insertions were confirmed by Sanger sequencing (GATC Biotech, Konstanz, Germany).

A deletion mutant, ΔncS35, was constructed by allelic recombination described by Aubert et al. (160). Primers for amplification of flanking regions are listed in Table 2. Deletion was confirmed by Sanger sequencing.

For complementation experiments, an RNA-expression plasmid, pSCrhaM2, was constructed from pSCrhaB2 (182), by removing its Shine-Dalgarno sequence and start codon using inverse PCR with primers M2 (Table 2). DNA was amplified using the LongRange PCR kit (Qiagen) with plasmid pSCrhaB2 as template. The PCR product was blunted using T4 DNA polymerase and self-ligated. Absence of Shine-Dalgarno and start codons as well as integrity of the multiple cloning site was confirmed by Sanger sequencing. sRNA ncS35 was cloned into pSCrhaM2 using primers ncS35ov (Table 2). Plasmids were transformed into ΔncS35 by triparental mating. Wild type and ΔncS35 strains transformed with

an empty pSCrhaM2 plasmid were used as control. All media used for mutant propagation and experiments contained 600 µg/mL Tp. Rhamnose was added when required to a final concentration of 0.2 % (w/v).

Biofilm analysis

Biomass was quantified using a crystal violet assay and cell viability was determined by a resazurin-based assay as described by Peeters et al. (188).

To determine the number of CFU per biofilm, cells were detached and collected as described before. Cells were centrifuged and resuspended in PS. A 10-fold dilution series was made and plated on LBA, plates were incubated for 48 h prior to counting.

For confocal laser scanning microscopy, biofilms were grown in a 96-well plate with glass bottom (Greiner Bio-one, Wemmel), stained with a Live/Dead solution (0.3 % SYTO 9 and propidium iodide in PS; Life Technologies) for 15 min and visualised using a motorized Nikon TE2000-E inverted microscope (Nikon Benelux, Brussels, Belgium, (189)).

Antimicrobial susceptibility testing

MICs were determined following EUCAST guidelines. A flat-bottomed 96-well plate, was used to test susceptibility to tobramycin (TCI Europe, Zwijndrecht, Belgium), meropenem (Hospira, Pfizer, Puurs, Belgium) and ceftazidime (Sigma-Aldrich). Growth was evaluated after 24 h and 48 h incubation at 37°C by measuring absorbance at 590 nm. The MIC was the lowest concentration for which no difference in absorbance was measured compared to uninoculated medium.

Growth and metabolic activity

Growth curves were measured in LBB medium. Approx. 2 x 10^5 CFU/well were added to a round-bottomed 96-well plate and the absorbance at 590 nm was measured for 60 h.

To assess colony formation, planktonic cultures were normalised to OD 1.0 (1 x 10^8 CFU/mL) and 10 µl drops of a dilution series were plated on LBA. Colony numbers and size were evaluated after 24 h, 48 h and 72 h.

To assess sedimentation rate, planktonic cultures normalized to OD 1.0 were left undisturbed and representative pictures were taken.

To measure metabolic activity, planktonic cultures normalized to OD 1.0 were centrifuged and resuspended in 100 µL PS with 20 µL CellTiter-Blue solution. After incubating 1 h at 37°C, fluorescence was determined (excitation wavelength: 560 nm, emission wavelength: 590 nm).

Flow cytometry

Cell size and granularity of planktonic cultures and biofilms were measured by an Attune Nxt flow cytometer and auto sampler from Invitrogen with the Attune Nxt software version 5.2 (Thermo Fisher). Planktonic cultures were investigated using suspension with OD 0.1 (approx. 1 x 10^7 CFU/mL). For biofilms, cells were grown and detached as described before. All media and solutions were filter-sterilized before use (Puradisk FP30; Whatman, Middlesex, UK). Cells were collected in a round-bottomed 96 well plate, and quantified with a blue excitation laser at 488 nm and appropriate filters.

Statistics

Each experiment included at least three biological replicates and representative images were taken. Results were checked for normality and when the data was normally distributed, an independent sample-t-test or ANOVA was performed, if not, a Mann-Whitney test or a Kruskal-Wallis test was performed. Statistical tests were considered significant when $p < 0.05$. To investigate whether a gene with altered expression level was also predicted as a potential target for the processed ncS35, a crosstabulation was performed including a Pearson Chi-Square test, which was considered significant when $p < 0.05$.

RNA-sequencing and data analysis

RNA was extracted as described before. Each condition included three biological replicates. RNA concentration was measured using the 'Quant-iT RiboGreen RNA assay' (Life Technologies, Grand Island, NY, USA) and RNA quality was assessed by capillary electrophoresis using the RNA 6000 Pico Chip (Agilent Technologies, Santa Clara, CA, USA). Subsequently, 2 µg of RNA was depleted for rRNA using the Ribo-Zero rRNA Removal Kit for Gram-negative Bacteria (Illumina, San Diego, CA, USA). Library preparation was performed using the 'Truseq stranded Total RNA library prep' protocol (Illumina, San Diego, CA, USA). Libraries were quantified by qPCR, according to Illumina's protocol 'Sequencing Library qPCR Quantification protocol guide', version February 2011. A DNA 1000 chip (Agilent Technologies, Santa Clara, CA, US) was used to control the library's size distribution and quality. Sequencing was performed on a high throughput Illumina NextSeq 500 flow cell generating 75 bp single reads.

Reads were mapped to the *B. cenocepacia* LMG 16656 reference genome (28) with a 95 % similarity cutoff using CLC genomics workbench version 8.5.1 (Qiagen, Venlo, Netherlands). Statistical analyses were performed using an EDGE (Estimated Degree of Gene Expression) test (190). Genes were reported as significantly differentially expressed in ΔncS35 compared to wild type when $p < 0.05$ and a fold change ≥ 1.5. RNA-seq raw data was submitted to ArrayExpress under accession number MTAB-5526.

DNA-sequencing

Both wild type and ΔncS35 were sequenced. An Illumina paired-end library was generated from 1 µg of genomic DNA. The DNA was fragmented to 200 bp using Covaris S2 sonication (Duty cycle: 10%, Intensity: 5, Cycles per burst: 200, Treatment time: 180s) and a sequence library was made for each sample using the Truseq DNA PCR free library preparation kit (Illumina, San Diego, CA, USA). The library was sequenced on an Illumina NextSeq 500, generating 75 bp single reads.

Sequencing reads from wild type and ΔncS35 were mapped to the *B. cenocepacia* J2315 reference sequence (28) using CLC workbench, with a 50 % length-fraction cut-off and a 80 % similarity cutoff. The Basic Variant Detection tool of CLC workbench was used to detect SNPs, and the InDels and Structural Variants tool to detect genome insertions and deletions. Variations to the reference sequence which were present in more than half of the reads in ΔncS35 but not in the wild type strain were reported.

RESULTS

Genome location and conservation of ncS35

We previously identified an orphan TSS in an intergenic region on the second largest chromosome of *B. cenocepacia* J2315 (34). This TSS is located on the opposite strand of its adjacent genes, BCAM2068 and BCAM2069 (Figure 1), suggesting that this orphan TSS produces an independently transcribed sRNA, which was designated ncS35. dRNA-Seq data further indicated that ncS35 is processed at a position 29 nt downstream of the TSS, revealed by a coverage peak depleted in the TEX-treated sub-sample (Figure 1A).

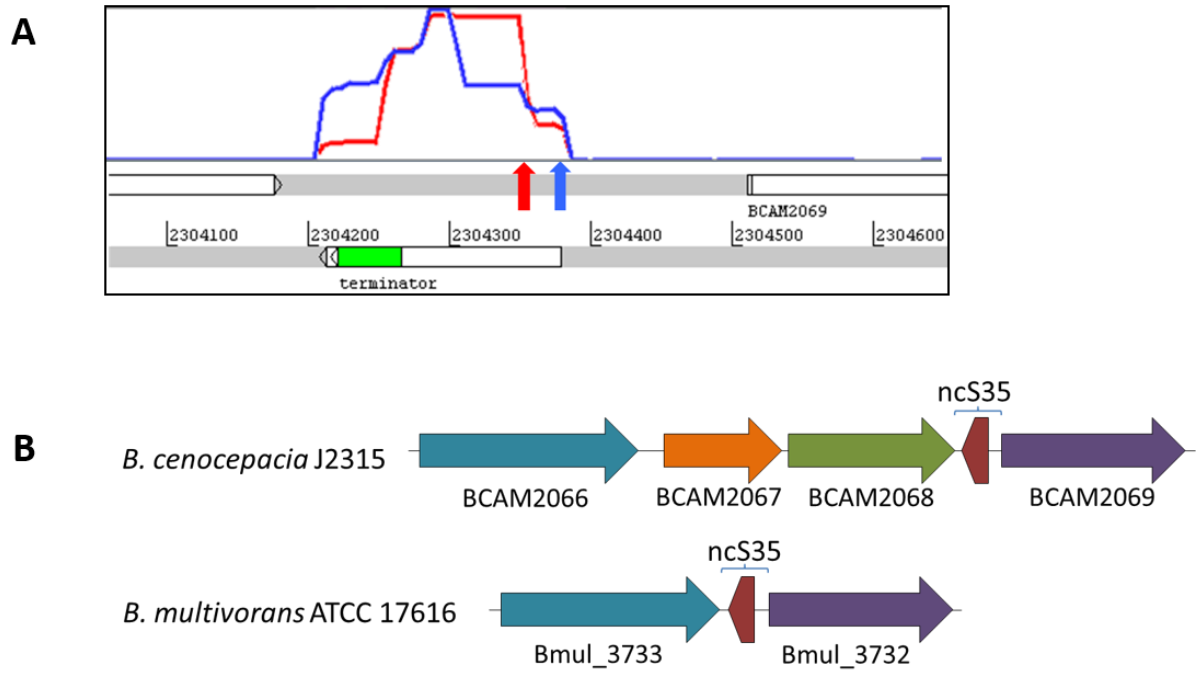


Figure 1. Genome location of ncS35. A) Coverage for ncS35 in dRNA-Seq data. Blue line: TEX-treated sub-sample, red line: untreated sub-sample. Blue arrow: location of TSS, red arrow: processing site. B) Synteny of adjacent genes. The red arrow represents ncS35, located on the opposite strand of its flanking genes, a terpene cyclase (BCAM2068, green) and a conserved hypothetical protein (BCAM2069, purple). BCAM2067 (orange, a putative undecaprenyl pyrophosphate synthetase, uppS), and BCAM2068 are adjacent to ncS35 in *B. cenocepacia* strains J2315, K56-2, H111 and in *B. dolosa* AU0158. In all other surveyed strains, the major facilitator protein (blue) is directly adjacent to ncS35 (*B. multivorans* ATCC 17616 is shown as a representative). Homologous genes have the same colour code.

5'RACE confirmed the TSS as the beginning of the transcript (position 2304378) as well as the processing site (position 2304350), indicating ncS35 can be cleaved to a shorter form. dRNA-Seq data also showed an abrupt decrease in coverage at a distinct location, and 3'RACE confirmed this transcript end (position 2304213). The full length ncS35 has a size of 166 nt and its computationally predicted secondary structure, with 4 hairpins (Suppl. Figure S1A), has a minimum free energy (MFE) of -77.80 kcal/mol and a negative z-score for MFE of 500 randomly mononucleotide-shuffled sequences (-3.97). The processed form has a size of 138 nt and its computationally predicted secondary structure, with a MFE of -67.10 kcal/mol and a z-score for MFE of -4.66, consists of 3 hairpins. The last hairpin, the rho-independent terminator, is followed by a stretch of 5 U's. The MFE for the full length and the processed ncS35 predicted secondary structure is lower than the MFE of 500 randomly mononucleotide-shuffled sequences (Suppl. Figure S1B).

BLASTn analysis showed that the sequence of full length ncS35 is conserved within the Bcc, while the sequence corresponding to the processed form is also present in the *Burkholderia pseudomallei* group. Other bacterial lineages do not harbour ncS35 and comparison to the Rfam database did not result in any hits.

In Bcc species, the relative orientation of ncS35 is conserved, but genes directly adjacent to ncS35 are not always homologous. In most strains investigated, ncS35 is flanked by a major facilitator superfamily protein and a conserved hypothetical protein (Figure 1B). In *B. cenocepacia* strains J2315, K56-2 and H111 and in *B. dolosa* AU0158 two genes (encoding *uppS* and a terpene cyclase) are inserted between ncS35 and the major facilitator superfamily protein (Figure 1B).

Expression of ncS35

qPCR and Northern blots showed that expression of ncS35 was significantly higher in biofilms than in planktonic cultures (Figure 2). Northern blots revealed one dominant fragment with a size of approximately 140 nt, and a less abundant fragment with a size of approx. 160 nt, indicating that processed ncS35 is the most abundant form. Compared to other growth conditions, expression is higher in biofilms, after growth in media with SDS and in minimal medium M9.

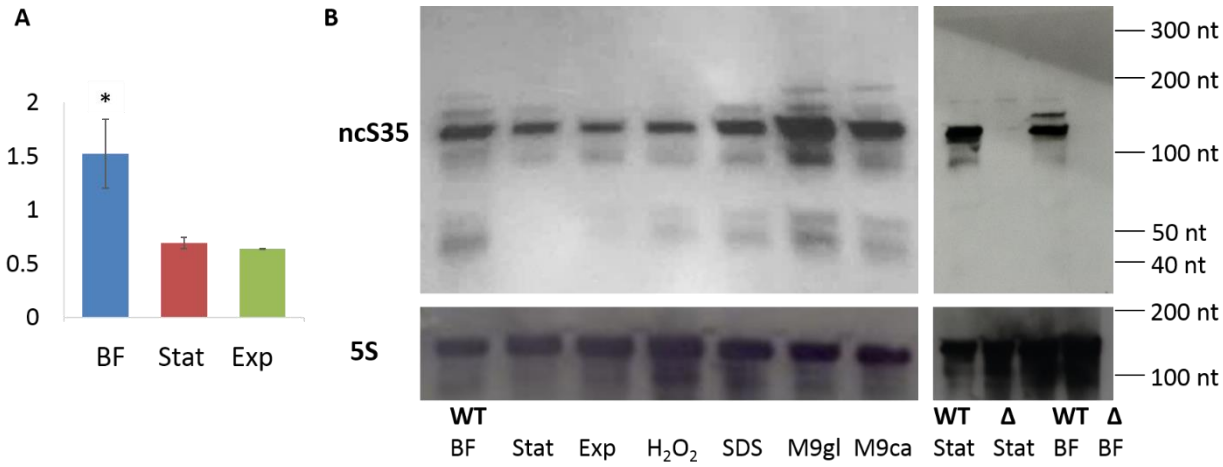


Figure 2. Expression of ncS35. A) qPCR. Expression of ncS35 in *B. cenocepacia* J2315 evaluated in exponential phase (Exp), stationary phase (Stat) and in biofilms (BF). Location of primer pair is visualised in Suppl. Figure S1A. Fold changes are calculated relative to a cDNA standard (mix of cDNA of all the samples of the different conditions). Error bars represent standard deviation. ncS35 expression is significantly higher in biofilms compared to all other conditions (asterisk, $p < 0.05$, $n = 3$). B) Northern blots. Left panels: Northern blot of ncS35 in *B. cenocepacia* J2315 (WT). Expression was evaluated in biofilms, stationary phase and in exponential phase. Response to stress was evaluated in various media: LBB with 0.2 % H_2O_2 added 15 min prior to harvesting, LBB supplemented with 0.015 % (w/v) SDS (membrane stress), and in M9 supplemented either with 0.1 % (w/v) glucose (M9gl) or with 0.2 % (w/v) casamino acids (M9ca; lower nutrient availability). 5S RNA was used as loading control. Right panels: Northern blots of WT and ΔncS35 (Δ) confirm deletion of ncS35 and specificity of probe hybridization.

Construction of ΔncS35

Due to the lack of hits in the Rfam database, a putative function could not be assigned to ncS35. To address this, a ncS35-deletion mutant, designated ΔncS35 was constructed. Northern blots and whole-genome sequencing confirmed the deletion from the genome (Figure 2B). DNA-sequencing revealed one 165 nt deletion between the wild type and ΔncS35 and 15 nucleotide variants (Suppl. Table S4).

The deletion was located on chromosome AM747721, region 2304238..2304402, spanning ncS35, showing the sRNA was successfully deleted. Of the 15 nucleotide variants, only three caused a change in amino acid sequence, and none were located in UTRs or promoter regions. In BCAL1675 (*amrB*) encoding a multidrug efflux system transporter protein, threonine is changed to alanine at amino acid position 708. In BCAL3010, *spoT*, encoding a guanosine polyphosphate pyrophosphohydrolase, leucine is changed to proline at position 37, a site not located in the catalytic domain. Finally, in BCAL3297, encoding a ferritin-like DNA-binding protein with oxidative damage protectant function, threonine is changed to isoleucine at amino acid position 40, within the catalytic domain.

Phenotype of ΔncS35

The phenotype of the deletion mutant was investigated using planktonic cultures and biofilm-grown cells. Complementation experiments were performed using wild type and ΔncS35 transformed with an empty plasmid pSCrhaM2 and ΔncS35 transformed with pSCrhaM2 overexpressing ncS35.

ΔncS35 showed the same MIC for tobramycin (256 µg/mL) as the wild type. However, a lower optical density was observed for ΔncS35 at concentrations near the MIC and partial complementation of this effect was possible (Suppl. figure S3). In ΔncS35, a two-fold decrease in MIC from 16 to 8 µg/mL was detected for meropenem and ceftazidime. This two-fold difference could not be complemented.

Confocal laser scanning microscopy revealed that ΔncS35 biofilm cells cluster to form larger aggregates than wild type biofilm cells (Figure 3A). However, no significant differences were observed for biomass (assessed by a crystal violet assay), for the number of metabolically active cells (assessed by a resazurin assay) and for the number of cultivable cells (determined by plating, data not shown). When grown planktonically, ΔncS35 cells precipitated faster compared to wild type cells (Suppl. Figure S4A). This phenotype was partially restored in the complemented mutant (Suppl. Figure S4B).

Flow cytometry was used to further assay aggregate formation. ΔncS35 formed larger cellular aggregates with a higher granularity in both planktonic cultures and in biofilm, as evidenced by higher forward and side scatter values (Suppl. Figure S4C). Aggregate formation of the complemented mutant was very similar to that of the wild type (Figure 3B).

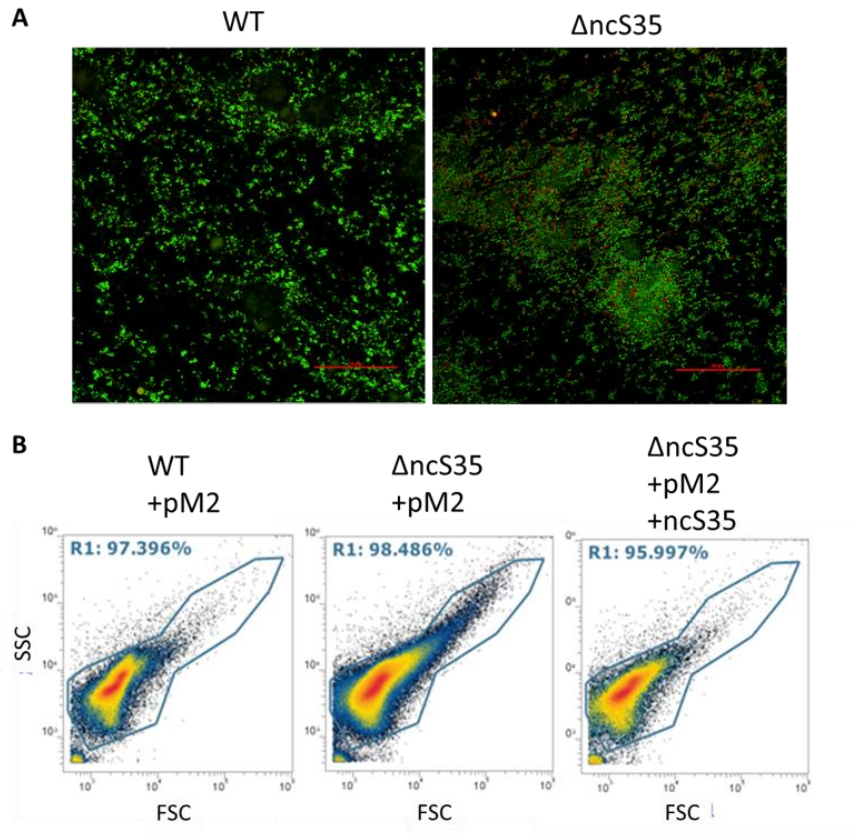


Figure 3. Cell aggregation in biofilms and planktonic culture. A) Confocal laser scanning microscopy. Z-stack image of 24 h old biofilms of wild type (WT) and ΔncS35 after Live/Dead staining. Scale bar indicates 50 µm (see figure 3, paper 1). B) Flow cytometry size/granularity plots of WT with empty plasmid (WT + pM2), ΔncS35 with empty plasmid (ΔncS35 + pM2), ΔncS35 with plasmid overexpressing ncS35 (ΔncS35 + pM2 + ncS35) biofilm cells grown in LBB 0.2 % rhamnose 600 Tp. X-axis represents forward scatter (FSC) and indicates cell size. Y-axis represents side scatter (SSC) and shows cell granularity. Gate R10 represents all the cells, dots outside this gate are background.

ΔncS35 grew to a higher optical density compared to the wild type in the rich medium LBB (Suppl. Figure S5A); complementation reduced optical density back to the levels of the wild type (Figure 4A). Between 5 h and 15 h, during exponential growth, the slopes of the growth curves indicate that ΔncS35 with the empty plasmid and ΔncS35 with the plasmid expressing ncS35 have a shorter doubling time (approx. 180 minutes) than wild type with the empty plasmid (approx. 270 minutes). After 24 h on LBA, ΔncS35 colonies were larger than colonies of the wild type or the complemented mutant (Suppl. Figure S5B).

Using a resazurin-based assay, a significant higher metabolic activity ($p < 0.05$) was observed for planktonically grown ΔncS35 cells compared to wild type cells or cells of the complemented mutant (Suppl. Figure S5C, Figure 4B).

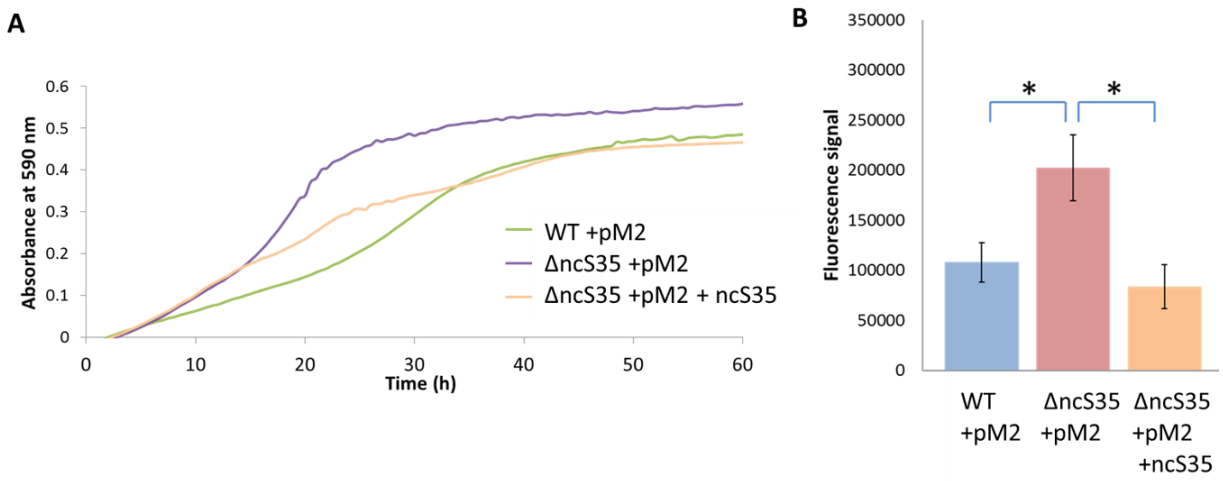


Figure 4. Complementation of growth and metabolic activity of planktonic cells. A) Growth curve in LBB with 600 Tp and 0.2 % rhamnose. Green line: WT with empty plasmid (WT + pM2), purple line: ΔncS35 with empty plasmid (ΔncS35 + pM2), orange line: ΔncS35 with plasmid overexpressing ncS35 (ΔncS35 + pM2 + ncS35). B) A cell suspension of WT with empty plasmid (WT + pM2), ΔncS35 with empty plasmid (ΔncS35 + pM2) and ΔncS35 with plasmid overexpressing ncS35 (ΔncS35 + pM2 + ncS35) was mixed with CellTiter-blue and fluorescence was measured after 1 h. Error bars represent standard deviation. Statistically significant differences are indicated by an asterisk ($p < 0.05$, $n = 3$).

Computationally predicted targets of ncS35.

To predict interactions between ncS35 and mRNAs, we used CopraRNA, an algorithm which takes accessibility of interaction sites and conservation of targets into account. Interactions were predicted for a stretch of 200 nt upstream to 100 nt downstream of the first nucleotide of annotated genes. Predicted interactions which fell outside of the annotated 5'UTR were discarded. 54 hits were retained for the full length, and 52 hits for the processed form; 20 hits were found in common for both (Suppl. Table S5). The predicted target with the lowest energy score and highest statistical significance both for full length and for processed ncS35 is a gene encoding an outer membrane protein (BCAM2255), with the predicted interaction site located inside the CDS, over a stretch of 40 nucleotides. Other predicted targets included genes encoding regulatory proteins, membrane transporters, lipoproteins and other membrane proteins, cytochrome c and other electron transport related proteins, flagellar proteins and ribosomal proteins.

Most predicted interaction sites were located inside the CDS of mRNA targets, approx. 50 nucleotides downstream of the start codon (Suppl. Figure S6). Interactions within the CDS can change the stability of the mRNA by increasing the degradation rate of the sRNA-mRNA duplex. To verify an increased mRNA degradation of predicted target genes in the mutant, mRNA abundance was quantified by RNA-seq.

Differential gene expression in ΔncS35

RNA-seq was performed on exponential and stationary phase planktonic cells, and on sessile cells of wild type and ΔncS35. In all three conditions, a total of 971 genes were upregulated and 1112 genes were down regulated in ΔncS35 compared to wild type. 14 genes were commonly up-regulated in ΔncS35 compared to wild type in all three conditions, and 4 genes were commonly down-regulated (Suppl. Figure S7, Suppl. Table S6).

Selected gene expression changes are listed in Table 3. In exponential phase, genes involved in metabolic activity are differentially regulated, including genes encoding proteins for substrate uptake and utilisation, respiration, and ornibactin and vitamin biosynthesis. Several genes for cable pilus biogenesis (BCAM2759, BCAM2762) are downregulated. In stationary phase, like in exponential phase, genes involved in metabolic activity are differentially regulated. The most upregulated genes in stationary phase belong to the phenylacetic acid degradation and tryptophan degradation pathways; these genes are also upregulated, but to a lesser extent, in exponential phase (Figure 5). Also upregulated in stationary phase are genes involved in flagellar assembly (several loci). Downregulated genes include the quorum-sensing-regulated zinc metalloprotease (BCAS0409), adhesin protein (BCAM2143) and lectin (BCAM0184-6).

Table 3. Selected gene expression changes in ΔncS35 compared to wild type.

	Exponential	Stationary	Biofilm	Annotation
Phenylacetic acid degradation pathway				
BCAL0212	3.8	19.9	-	Phenylacetic acid degradation NADH oxidoreductase PaaE
BCAL0213	5.3	15.8	-	Phenylacetic acid degradation protein PaaD
BCAL0214	6.0	29.0	-	Phenylacetic acid degradation protein PaaC
BCAL0215	5.0	28.7	-	Phenylacetic acid degradation protein PaaB
BCAL0216	4.5	16.3	-	Phenylacetic acid degradation protein PaaA
BCAL0406	4.5	2.7	-	Probable enoyl-CoA hydratase PaaG
BCAL0407	5.1	5.1	-	Beta-ketoadipyl CoA thiolase
BCAL0408	4.5	9.0	1.91	Phenylacetic acid degradation oxidoreductase paaZ
BCAM1711	5.4	12.5	-	Phenylacetate-coenzyme A ligase paaK
BCAM1712	6.4	6.08	-	3-hydroxybutyryl-CoA dehydrogenase
Tryptophan degradation pathway				
BCAL2790	1.99	14.02	-	Kynurenine formamidase
BCAL2791	1.93	10.64	-	Kynureninase
BCAL2792	-	12.26	-3.52	Tryptophan 2,3-dioxygenase
Respiration				
BCAL2141	-	13.38	3.51	Cytochrome o ubiquinol oxidase protein
BCAL2142	-	11.80	2.82	Cytochrome o ubiquinol oxidase subunit III
BCAL2143	-	13.19	2.51	Ubiquinol oxidase polypeptide I
BCAM2674	-	-	26.04	Cytochrome oxidase subunit I
BCAL3094	4.40	-	10.44	Oxygen-independent coproporphyrinogen III oxidase
BCAL2118	2.26	-	47.25	Isocitrate lyase
BCAM0166	20.57	-	58.31	NADH dehydrogenase
Efflux				
BCAL1674	2.30	-	-	Multidrug efflux system AmrA protein
BCAL1676	1.87	-5.47	-3.71	Multidrug efflux system outer membrane protein
BCAL1813	2.23	-	-	Multidrug efflux system outer membrane protein
BCAM2334	3.66	-	-	Efflux system transport protein
BCAM2335	3.90	-	-	Outer membrane efflux protein
BCAM2337	3.95	-	-	Multidrug resistance transporter protein
Motility				
BCAL3506	-	5.13	-	Flagellar motor switch protein FliM
BCAL0140	-	4.55	-	Flagellar biosynthetic protein FlhB
BCAL0524	-	3.31	-	Flagellar motor switch protein FliG
BCAL0561	-	2.18	-	Flagella synthesis protein FlgN
BCAL1677	-	3.39	-	Putative type-1 fimbrial protein
Surface protein				
BCAL3154	4.85	-	-	Glycine-rich surface protein

	Exponential	Stationary	Biofilm	Annotation
Pilus biogenesis				
BCAM2759	-2.03	-	-3.13	Putative minor pilin and initiator
BCAM2760	-	-	-6.51	Putative outer membrane usher
BCAM2761	-	-	-4.32	Giant cable pilus
BCAM2762	-2.84	-	-4.93	Giant cable pilus chaperone protein
BCAM2143	-	-2.98	-2.16	Cable pilus associated adhesion protein
BCAL1528	-	-2.95	-3.15	Flp type pilus assembly protein
BCAL1530	-	-3.08	-5.09	Flp type pilus assembly protein
BCAL1531	-	-	-5.29	Flp type pilus assembly protein
BCAL1532	-	-	-4.09	Flp type pilus assembly protein
Stress response				
BCAL1233	-	-	10.83	Heat shock Hsp20-related protein
BCAL1919	-	-	8.03	ClpB heat-shock protein
BCAL2119	-	-	9.79	Universal stress protein family protein
BCAL1234	-	2.55	12.01	Heat shock protein
Other genes, regulated in all three conditions				
BCAL0193	1.94	2.42	2.03	Exported protein
BCAL1055	2.62	2.10	1.65	Histidine transport system permease protein
BCAL2206	-2.00	-4.80	-6.19	Phasin-like protein
BCAM1775	2.34	2.00	2.50	Transglycosylase associated protein
BCAM2390	-1.86	-4.93	-5.21	Sarcosine oxidase delta subunit

RNA sequence data are represented as the mean fold changes of 3 biological replicates. Only values ≥ 1.5 fold change and $p < 0.05$ are shown. A hyphen indicates no significant changes ≥ 1.5-fold were found under this growth condition.

Gene expression in ΔncS35 biofilms is characterised by downregulation of genes related to cell division, transcription and translation as well as genes in cable pilus biogenesis (BCAM2759-62). Upregulated genes include an alternative NADH dehydrogenase (BCAM0166), isocitrate lyase (BCAL2118), certain chaperones and genes of the *lxa*-cluster (BCAM0275-0323).

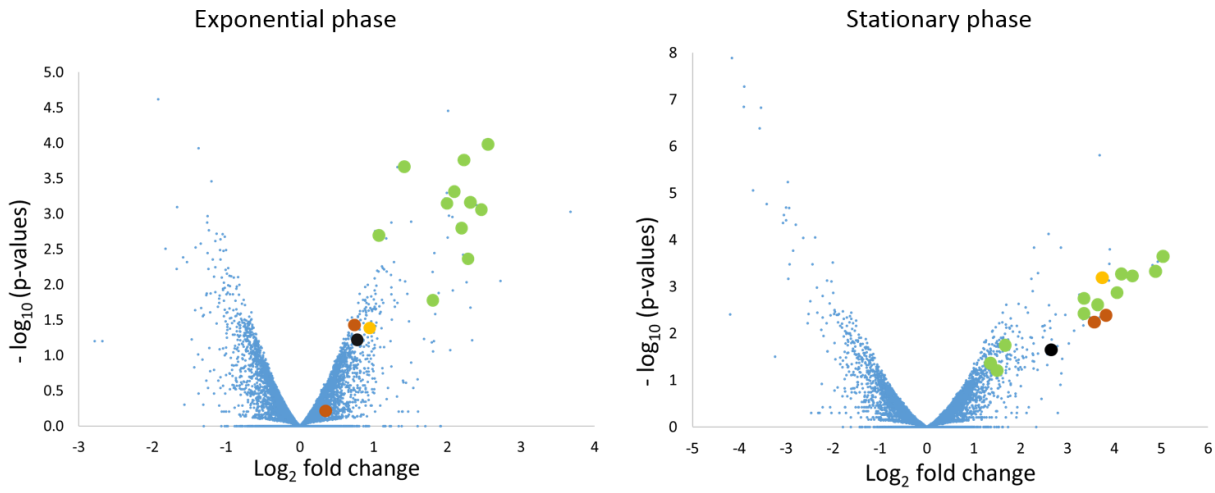


Figure 5. Volcano plot of gene expression data obtained by RNA-sequencing. X-axis: Log2 of the fold change of ΔncS35 compared to wild type in exponential and stationary phase. Y-axis: -log10 of the p-value. The green dots indicate genes involved in phenylacetic acid degradation and the orange dots indicate genes involved in tryptophan degradation. The black dot is BCAL0193 and the yellow dot is BCAL2790, two genes identified as putative targets of ncS35 by CopraRNA.

Two genes on the list of predicted targets change expression in ΔncS35 (Figure 5). They are predicted targets for the processed sRNA and are both upregulated. 0.3% of all annotated genes of *B. cenocepacia* J2315 are on the CopraRNA list of predicted targets for the processed ncS35 and on the list of genes that are differentially expressed in ΔncS35 when compared to the wild type. The Pearson Chi-square test resulted in a p-value of 0.143 meaning the presence of a gene on the CopraRNA list does not depend on the fact whether it changes expression between wild type and ΔncS35. Expression of BCAL0193, a putative exported protein, is upregulated in the mutant in all three conditions. Transcription for this gene starts 123 nt upstream of the start codon of the gene and the predicted interaction site is directly adjacent to the TSS (Figure 6). The second gene is BCAL2790, a putative arylformamidase, and the first gene in an operon containing genes for tryptophan degradation. It has a transcription start site 23 nt upstream of the start codon and is upregulated in the mutant in exponential phase and stationary phase. ncS35 is predicted to interact at the start codon of BCAL2790 (Figure 6). The predicted targets with the lowest energy score and highest statistical significance (see above) did not change expression significantly.

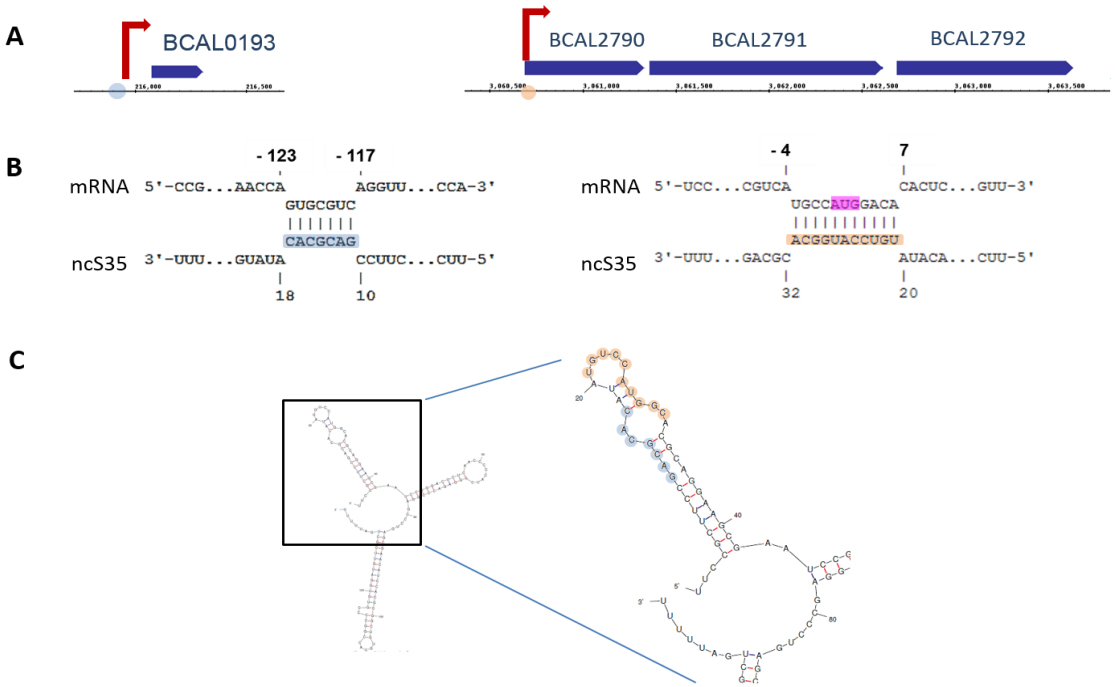


Figure 6. Location of interaction sites for computationally predicted targets upregulated in ΔncS35. In blue, interaction site for BCAL0193. In yellow, interaction site for BCAL2790. A) Location of genes and TSS. Interaction sites of ncS35 on these targets are depicted as circles. B) Interaction between ncS35 and mRNA. Pink refers to the start codon. C) Secondary structure of processed ncS35 with interaction sites highlighted.

DISCUSSION

In the present study, a novel sRNA of *B. cenocepacia* J2315, discovered by dRNA-seq and designated ncS35, is described. Secondary structure prediction revealed a thermodynamically favorable structure with stable stem-loops and a negative z-score, indicating its structural significance (191). Northern blots and RNA-seq coverage suggest that ncS35 is processed, and the processed form is more abundant. Combined with the pattern of ncS35 sequence conservation, this suggests the processed form is the active RNA species: the full sequence of ncS35 is only conserved among Bcc species while the processed form is also conserved in the *B. pseudomallei* group. Both groups are part of the first deep branching clade I of the *Burkholderia* genus, and are well-known human pathogens or opportunistic pathogens. Phytopathogenic *Burkholderia* spp. and members of *Burkholderia* clade II (14, 76) do not code for ncS35.

To investigate the function of ncS35, a deletion mutant was constructed. Mutant viability was not changed, showing that ncS35 is non-essential under laboratory conditions. ΔncS35 showed a higher growth rate in planktonic culture and entry into stationary phase was delayed compared to wild type. Overall metabolic activity was also increased. Growth-rate-related phenotypes could at least be partially complemented, confirming that ncS35 is involved in slowing down growth and attenuating metabolic activity of *B. cenocepacia* J2315.

The single nucleotide variants observed in the genome of ΔncS35 could have been selected for during mutant construction, where high concentrations of trimethoprim and tetracycline were used. The mutation in *spoT* could reduce its guanosin pentaphosphate (ppGpp) -hydrolyzing activity, resulting in higher levels in ppGpp, which is beneficial for survival in the presence of trimethoprim (192). On the other hand, a mutation in *spoT* could also represent a suppressor mutation, partially counteracting the growth-rate increasing effect of ncS35 deletion.

sRNAs that slow down growth have been described in *E. coli* (Spot 42, (193)) and in *Bacillus subtilis* (RnaC, (194)). Expression of Spot 42 is negatively regulated by cAMP and increases in media containing glucose. Spot 42 has a role in central carbon metabolism, regulating the expression of a pirin-like protein, which in turn regulates the activity of pyruvate dehydrogenase. Mutants overexpressing Spot 42 show a small-colony phenotype (195). RnaC is involved in regulating growth rate and entry into stationary phase in *B. subtilis* via repressing AbrB, a regulator necessary for fast growth. The impact of ncS35 on growth of *B. cenocepacia* could be similar to Spot 42 and RnaC, despite the lack of sequence homology, and despite that these sRNAs target different genes. The increased expression of ncS35 in minimal medium, particularly in the presence of glucose suggests a possible role of cAMP in its regulation.

The lower optical density observed in the mutant at near-MIC values of tobramycin could be an indirect effect of the higher growth-rate and higher metabolic activity of ΔncS35. Faster growing cells could be more susceptible to antibiotics, in particular to those inhibiting translation, such as tobramycin (196, 197). Moreover, it has been described that uptake of aminoglycosides is proton motive force-dependent (198) and could therefore be stimulated by a higher respiration rate.

Most computationally predicted interaction sites of ncS35 with its putative targets were located within the CDS of genes, and some in their 5'UTR. sRNAs binding to CDS can change mRNA-stability, while base-pairing in a 5'UTR can change the translation rate. In both cases, an increase in mRNA-degradation by RNases can be observed. The first base-pairing mechanism can directly lead to an increase in mRNA-degradation because sRNA-mRNA duplexes trigger cleaving by RNases (75). The second base-pairing mechanism can indirectly lead to an increase in mRNA-degradation via decreased ribosome binding. Ribosome binding provides protection against RNases and hence when binding is attenuated, mRNA degradation rate increases (75). In this case absence of ncS35 would lead to a reduced degradation rate for its targets, resulting in an upregulation of target gene expression in ΔncS35 compared to wild type.

Only two genes on the list of computationally predicted targets are upregulated in ΔncS35: BCAL0193, a putative exported protein and BCAL2790, the first gene of an operon containing genes encoding for tryptophan degradation. Computationally predicted targets not changing expression in our analysis are either false positives, or the conditions and selected method of analysis were not suitable to detect any interaction. The predicted interaction sites for BCAL0193 and BCAL2790 are located within the 5'UTR of the respective genes and near their TSS, as determined by dRNA-Seq (34). The interaction site for BCAL2790 overlaps with the start codon, while the interaction site for BCAL0193 overlaps with a potential alternative start codon GTG, which is in frame with the annotated gene. In both cases, it is possible that sRNA-binding affects translation rate; changes in mRNA abundance would therefore be caused indirectly by increased degradation of the un-translated mRNA.

The increased expression of tryptophan degradation could indirectly cause the upregulation of the phenylacetic acid degradation (PAA) pathway in exponential and stationary phase. These genes are among those with the highest fold-change upregulation in the mutant. An aromatic compound that is assumed to induce the PAA degradation pathway is hydroxyanthranilic acid, an intermediate in the degradation of tryptophan in *Burkholderia* spp. (199, 200). Upregulation of PAA degradation can also occur by a decrease in PaaR expression, a decrease in intracellular glucose concentration or an increase in the levels of aromatic precursors (200-202) but no indication was found that ncS35 directly regulates PaaR or glucose metabolism. Interestingly, in *E. coli* a confirmed target for Spot 42 is *paaK* (193), involved in phenylacetic acid degradation, but not directly targeted by ncS35. Pribytkova et al. found that deleting the phenylacetylCoA monooxygenase complex (ΔpaaABCDE) in *B. cenocepacia* K56-2, resulted in a decrease in pathogenicity against *C. elegans*. The deletion of this complex caused a higher PAA concentration in the cell, which negatively regulated *cepI* and *cepR* expression (203). However, in ΔncS35, no difference in *cepI* and *cepR* expression was observed. This could be due to the fact that in this mutant the phenylacetylCoA monooxygenase complex is still present, and as a consequence PAA levels are low. However, more research would be required to confirm this link.

The increased aggregation observed for ΔncS35 could be linked to several changes in gene expression: an upregulation of flagellar genes (several loci), of a fimbrial protein gene (BCAL1677) and/or of a glycine-rich surface protein (BCAL3154). Changes in expression of genes involved in cable pilus biogenesis (downregulated in exponential phase and in biofilms) could also be responsible for this increased aggregation as in a study on *B. cenocepacia* J2315 mutants, disrupted in cable pilus biogenesis, more auto-aggregation and a faster precipitation rate than for wild type cells was observed (204).

Gene expression in ΔncS35 biofilms shows an upregulation of the *lxa*-gene cluster (low-oxygen-activated) compared to wild type biofilms. This cluster encodes universal stress proteins and proteins involved in metabolism and is known to be induced under oxygen-limiting growth conditions (205). This suggests that ΔncS35 biofilm cells are more deprived of oxygen than those of wild type (205), possibly due to the larger cell aggregates found in mutant biofilms.

Little overlap in gene expression changes between the growth conditions was found, possibly caused by differential expression of ncS35 in those conditions. qPCR and northern blotting showed a higher expression in biofilms compared to planktonic cultures. Another possible explanation is that ncS35 affects gene expression differently depending on growth stages, due to changing targets and/or differential target expression. Finally, some sRNAs are known to sequester proteins and change their activity without affecting the mRNA abundance, which cannot be predicted from the sRNA sequence and would not become apparent in RNA-seq data (97).

SUMMARY AND CONCLUSIONS

In the present study we characterized a candidate sRNA (designated ncS35) in *B. cenocepacia* J2315. To investigate its function, a deletion mutant was constructed which showed various phenotypes. Cells of ΔncS35 showed an increased aggregation, a higher metabolic activity and higher growth rate and the susceptibility to tobramycin was increased. Gene expression analysis revealed upregulation of the PAA pathway in ΔncS35. This pathway could have been induced by increased tryptophan degradation in the mutant, a pathway possibly targeted by ncS35. Overall, it is shown that sRNA ncS35 is a non-coding RNA with an attenuating effect on metabolic rate and growth. Slower growth can protect bacteria against stressors acting on fast-dividing cells and/or enhance survival in unfavourable conditions.

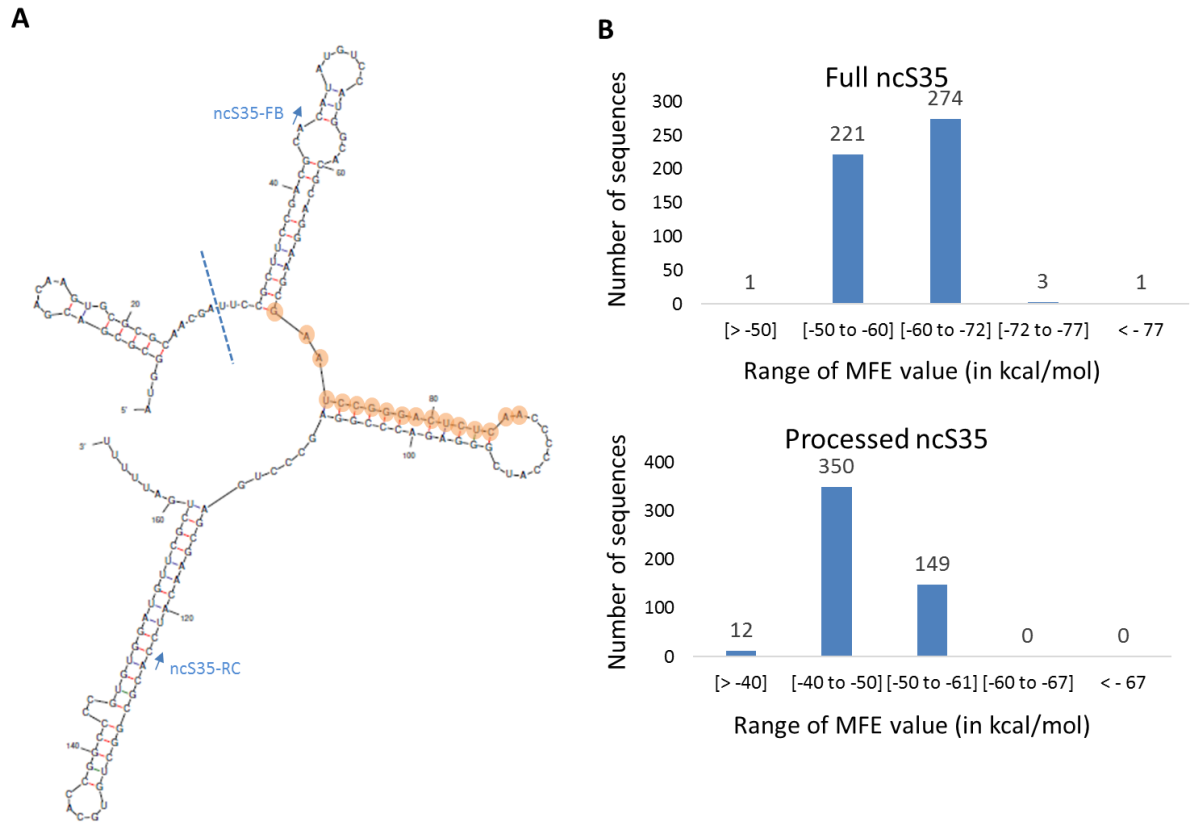
FUNDING

This work was supported by the Belgian Science Policy Office (Belspo) through the Interuniversity Attraction Pole Program.

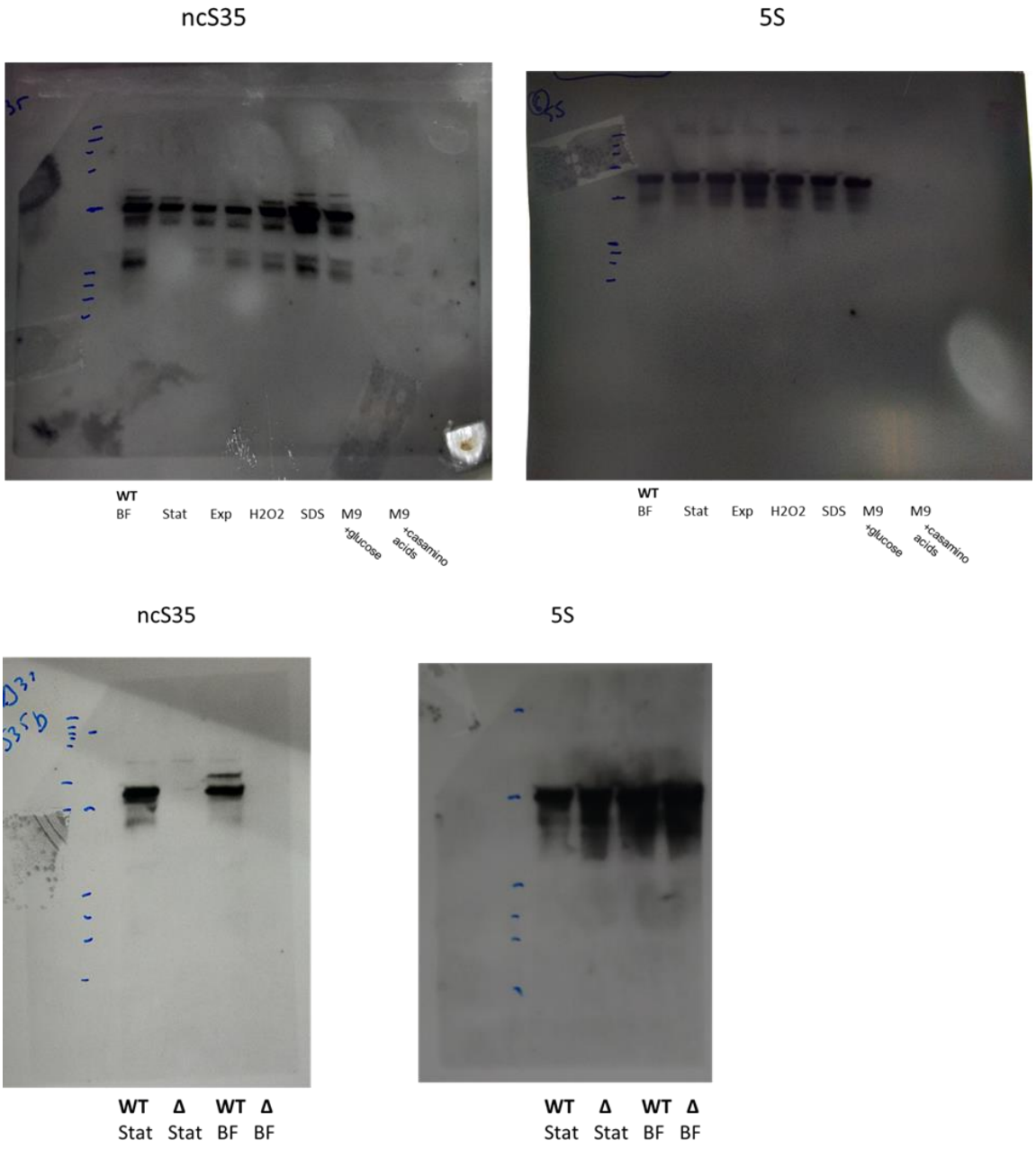
AUTHOR CONTRIBUTION STATEMENT

SK performed most experiments, wrote the draft manuscript and generated all figures. AS devised experiments, performed DNA-sequencing analysis, sRNA target analysis and RACE experiments, and contributed to the final manuscript. FVN and DD performed RNA-sequencing and initial RNA-seq data analysis and revised the manuscript. TC acquired funding, conceived the study and revised the manuscript.

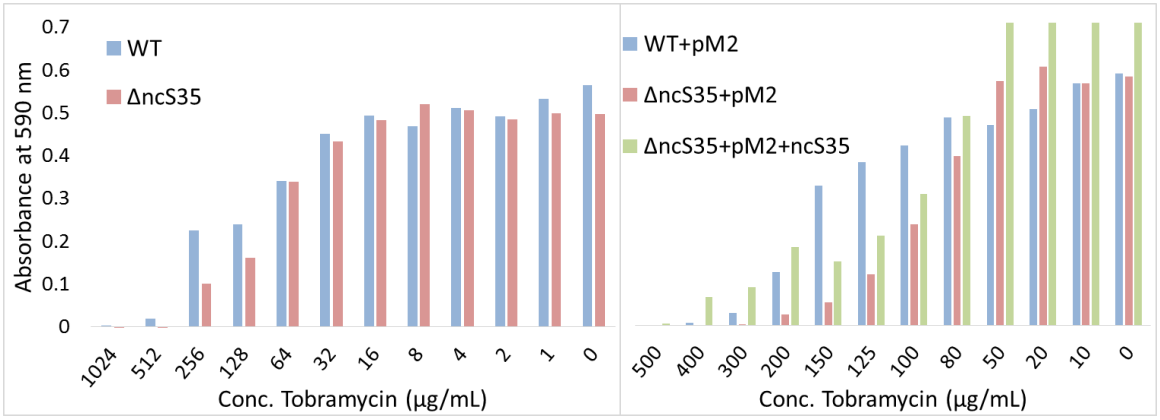
SUPPLEMENTARY DATA



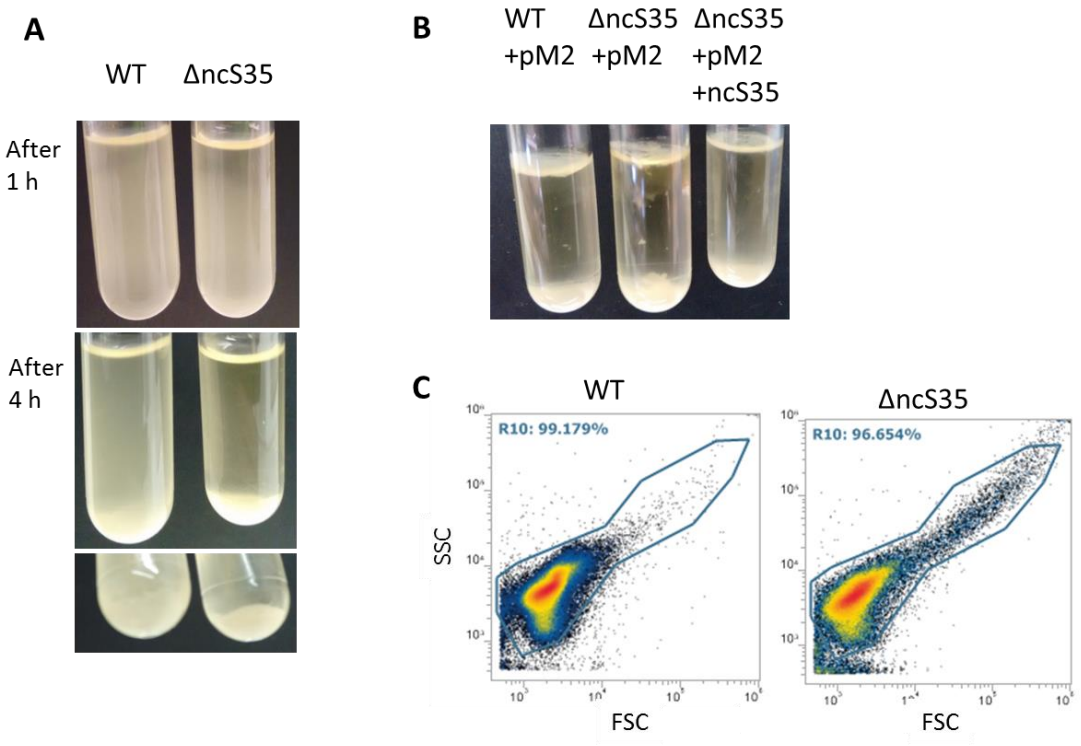
Supplementary Figure S1. Secondary structure for ncS35 A) Full length ncS35 predicted by Mfold. The dotted line indicates the processing site. Orange circles indicate the reverse complement of Northern blot ncS35-DIG probe. Blue arrows indicate the location of forward and reverse primer used for expression analysis by qPCR. B) MFE distribution of 500 randomly mononucleotide-shuffled sequences (CLC Genomics Workbench v. 8.5.1).



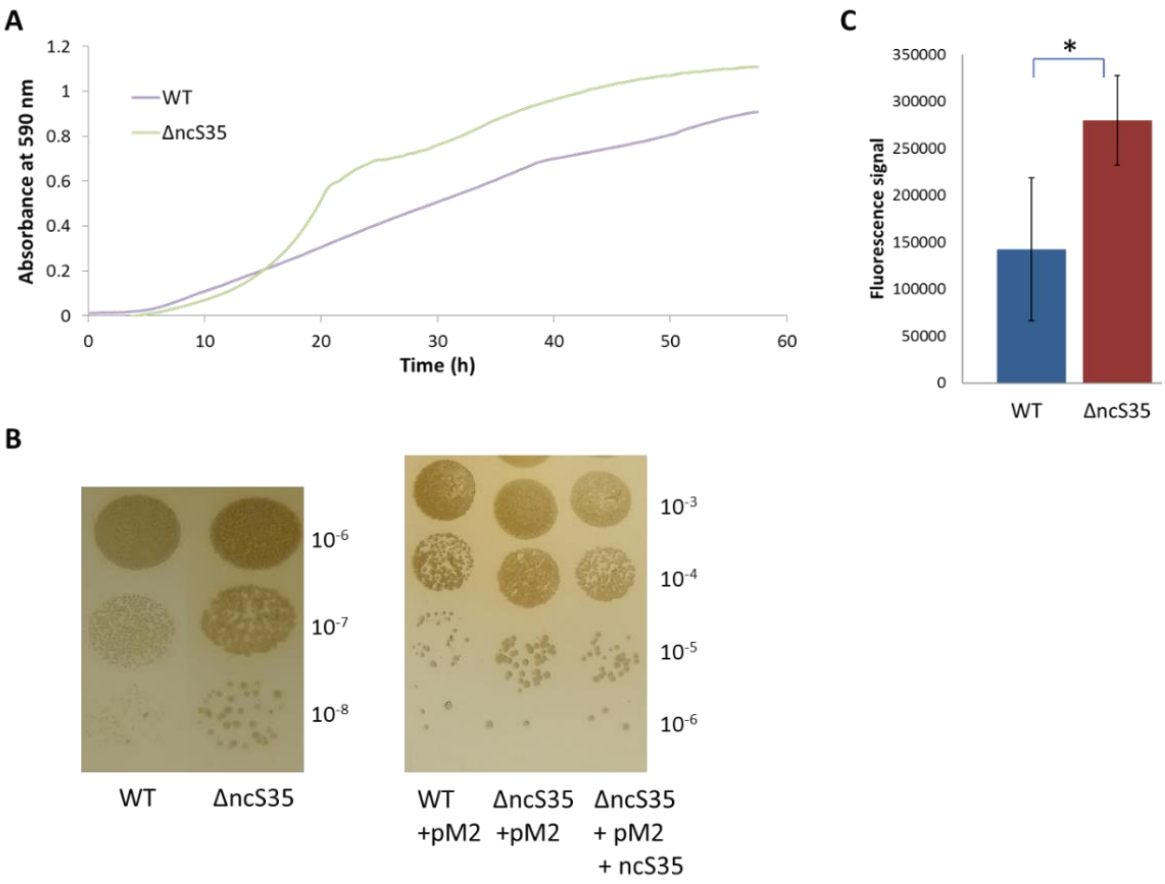
Supplementary Figure S2. Full-size images of northern blots represented in figure 2.



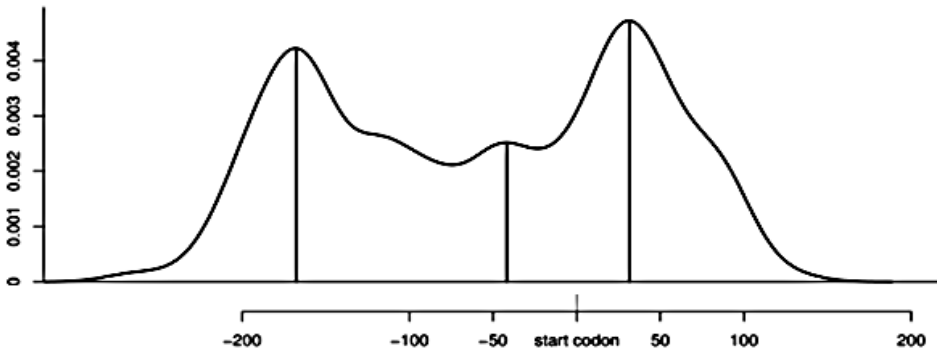
Supplementary Figure S3. Susceptibility of wild type and ΔncS35 to tobramycin. ΔncS35 grows to a lower optical density at near MIC concentration, while the MIC is unchanged (left panel). The effect could be partially complemented (right panel). WT: wild type, pM2 = empty vector control, pM2 + ncS35 = vector containing ncS35. For complementation experiments: strains were grown in LBB 600 Tp with 0.2% rhamnose. After 24 h the absorbance at 590 nm was measured. Figures show representative graphs of four biological replicates.



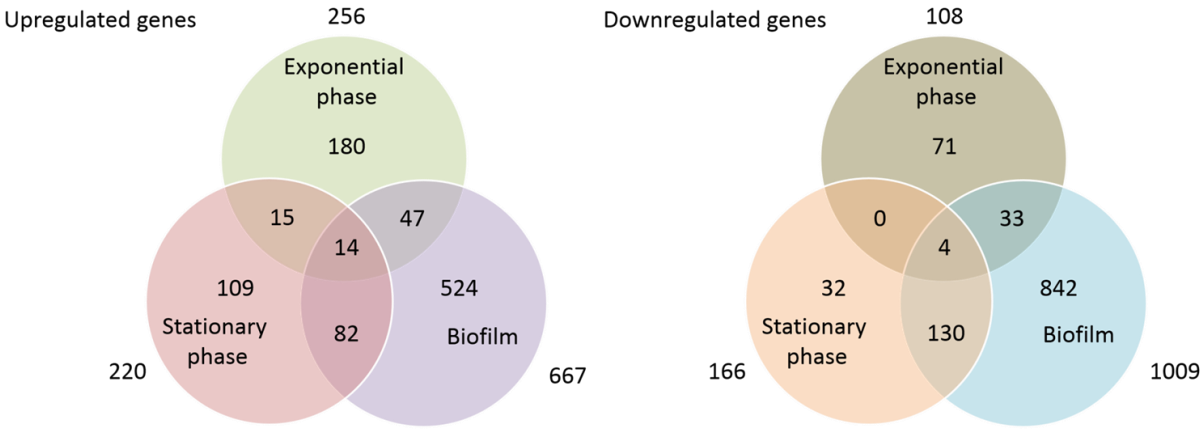
Supplementary Figure S4. Cell aggregation. A) Sedimentation of planktonic cultures. O/N cultures of wild type (WT) and ΔncS35 normalised to optical density 1.0 in polycarbonate tubes were photographed over time. B) Complementation of sedimentation. O/N cultures of WT with empty plasmid (WT + pM2), ΔncS35 with empty plasmid (ΔncS35, + pM2) and ΔncS35 with the plasmid overexpressing sRNA ncS35 (ΔncS35 +pM2 + ncS35) in LBB 600 TP 0.2 % rhamnose. C) Size/granularity plots of wild type (WT) and ΔncS35 biofilm cells by flow cytometry. X-axis represents forward scatter (FSC) and indicates cell size. Y-axis represents side scatter (SSC) and shows cell granularity. Gate R10 represents all the cells, dots outside this gate are background.



Supplementary Figure S5. Growth and metabolic activity of planktonic cells. A) Representative growth curve of wild type (WT) and ΔncS35 in LBB. Purple line is ΔncS35, green line is WT. B) Dilution series of wild type (WT) and ΔncS35 spotted on LBA and WT with empty plasmid (WT + pM2), ΔncS35 with empty plasmid (ΔncS35, + pM2) and ΔncS35 with the plasmid overexpressing sRNA ncS35 (ΔncS35 +pM2 + ncS35) spotted on LBB 600 TP 0.2 % rhamnose. Colonies were photographed after 24 h, representative pictures are shown. C) A cell suspension of wild type (WT) and ΔncS35 were mixed with CellTiter-blue and fluorescence was measured after 1 h. Error bars represent standard deviation. Statistically significant differences are indicated by an asterisk ($p < 0.05$, $n = 3$).



Supplementary Figure S6. Density plot of mRNA interaction sites. Putative mRNA targets were predicted by CopraRNA. The density plot is calculated from all predicted interactions with a p-value ≤ 0.01. y-axis: relative frequency of a mRNA nucleotide position in predicted interactions.



Supplementary Figure S7. Gene expression of wild type and ΔncS35 during three growth conditions. The Venn diagrams show the number of upregulated genes and downregulated genes in ΔncS35 compared to wild type in planktonic exponential phase, planktonic stationary phase and biofilm. Only genes with a significant differential gene expression of $p < 0.05$ and a fold change ≥ 1.5 were included.

Supplementary Table S1. Genome sequences included in the BLASTn search.

Bcc strains for analysing conservation of ncS35 in BCC	**non-BCC strains for analysing conservation**
B. cenocepacia J2315, K56-2, AU1054, MC0-3, H111 and HI2424 *B. lata* sp. 383 *B. vietnamiensis* G4 *B. multivorans* ATCC 17616 *B. ambifaria* AMMD *B. contaminans* MS14 *B. dolosa* AU0158 *B. cepacia* GG4 *B. pyrrocinia* DSM 10685	*B. pseudomallei* group: *B. thailandensis* E264 *B. pseudomallei* K96243 *B. mallei* ATCC 23344 *B. xenovorans* group: *B. xenovorans* LB400 *B. phytofirmans* PsJN *B. gladioli* BSR3 *B. phymatum* STM815 *B. glumae* BGR1 *B. rhizoxinia* HKI 454

Supplementary Table S2. Normalisation of qPCR data. Primers sequences of eight control genes.

Gene	**Primer sequence, 5'-3'**
BCAL2367	F: ACCATTTCCGCAACAAGGAC R: TGAAATCGGCCATGTACTGC
BCAL0026	F: TATGAAGTGCTGGTCGATGG R: TCAGCACGAAATCGTAGTCG
BCAL0813	F: AGCTCAATCCGGAAGTCGTG R: AGCTGCTGTTTCAGCGATCC
BCAL0972	F: TCTCGAAGGTCTGGCACGAG R: CGTGATGTCGTGCTTCATCG
BCAL1895	F: CAGCGGGTACGGGTTTCTTC R: GTTCTGGCCGTTGTTGATGC
BCAL2553	F: TGATCTGGGTGGTCAAGCTG R: TGCAGGTCAAAATCGTCGTC
BCAM0918	F: GAGATGAGCACCGATCACAC R: CCTTCGAGGAACGACTTCAG
BCAS0059	F: ATGCGGAATTCCAACAGGAG R: GCCCTTGCTCGAATAGTTGG

Supplementary Table S3. Strains used for CopraRNA analysis.

Bcc strains as input genomes for CopraRNA
B. cenocepacia J2315, AU1054, MC0-3 and HI2424 *B. lata* sp. 383 *B. vietnamiensis* G4 *B. multivorans* ATCC 17616 *B. ambifaria* AMMD

Supplementary Table S4. DNA-sequencing. Nucleotide variants (SNPs and indels) in ΔncS35 compared to wild type. * part of pseudogene BCAL2591 is missing.

Variants									
Chromosome	**Reference Position**	**Length**	**Reference**	**Allele**	**Frequency**	**Gene**	**Annotation**	**change?**	**Effect?**
AM747720	1832159	1	A	G	98.34	BCAL1675	amrB, multidrug efflux system transporter protein	ACC to GCC threonine to alanine	not in transmembrane helix
AM747720	2848513	1	T	G	100	BCAL2589	transposase	*	/
AM747720	2848515	1	-	A	84.52	BCAL2589	transposase	*	/
AM747720	2848517	1	T	C	100	BCAL2589	transposase	*	/
AM747720	2848525	3	ATG	GCC	97.37	BCAL2589	transposase	*	/
AM747720	2848530	2	CT	AG	100	BCAL2589	transposase	*	/
AM747720	2848537	1	C	T	100	BCAL2589	transposase	*	/
AM747720	2848543	1	C	T	100	BCAL2589	transposase	*	/
AM747720	2848545	1	C	G	100	BCAL2589	transposase	*	/
AM747720	3303206	1	A	G	93.16	BCAL3010	spoT, guanosine-3',5'-bis(diphosphate) 3'-pyrophosphohydrolase	CTG to CCG, leucine to proline	before catalytic region
AM747720	3612374	1	G	A	100	BCAL3297	ferritin DPS-family DNA binding = oxidative damage protectant	ACC to ATC, threonine to isoleucine	within binding domain
AM747720	3769393	1	A	G	100	upstream BCAL3441	rpmA, 50S ribosomal protein L27	IR within operon, no TSS affected	No effect
AM747721	1151529	1	G	A	97.77	BCAM1049	phage protein	CCC to CCT, stays proline	No effect
AM747721	2305140	1	G	A	98.1	BCAM2069	CHP	GTG to GTA, stays valine	No effect
AM747722	168206	1	G	A	98.65	upstream BCAS0151	HP	45 nt upstream TSS	No effect
InDels									
Chromosome	**Region**	**Type**	**Length**			**Gene**	**Annotation**	**change?**	**Effect?**
AM747721	2304238.. ..2304402	Complex	165			intergenic region	ncS35	deletion of ncS35	Phenotypes

Supplementary Table S5a. Complete list of predicted interactions by CopraRNA for processed ncS35. [a]Interaction position in mRNA relative to start codon (position 1-3). In grey: genes for which predicted interaction is located outside of the 5'UTR. In bold: genes upregulated in ΔncS35 (RNA-seq data). In italic: genes also predicted as a target for full length ncS35.

			Interaction position in mRNA[a]		Interaction position relative in sRNA		
FDR	p-value	Target in *B. cenocepacia* J2315	start	end	start	end	Annotation
0.957527	0.013215	BCAL0053	-168	-155	5	17	PadR family regulatory protein
0.957527	0.020495	BCAL0115	-59	-51	106	114	30S ribosomal protein S21
0.957527	0.00443	*BCAL0158*	-12	-5	106	113	metallo-beta-lactamase superfamily protein
0.95753	**0.01095**	**BCAL0193**	**-123**	**-117**	**11**	**17**	**putative exported protein**
0.957527	0.020654	*BCAL0230*	-119	-112	106	113	30S ribosomal protein S7
0.957527	0.013439	BCAL0267	-32	-26	59	65	putative cytochrome c biogenesis protein
0.957527	0.016024	BCAL0376A	-150	-141	104	113	putative membrane protein
0.957527	0.002792	*BCAL0896*	-155	-140	101	115	4-hydroxythreonine-4-phosphate dehydrogenase
0.957527	0.004039	*BCAL0953*	-11	-1	73	83	recombinase A
0.957527	0.018995	*BCAL1115*	-164	-156	104	112	putative ABC transporter membrane protein
0.957527	0.007387	*BCAL1446*	-126	-120	59	65	putative lipoprotein
0.957527	0.006474	BCAL1457	-142	-121	4	25	LysR family regulatory protein
0.957527	0.012203	BCAL1727	87	94	60	67	precorrin-6Y C5 15-methyltransferase (decarboxylating)
0.957527	0.012496	BCAL1822	29	37	106	114	putrescine transport system permease
0.957527	0.013417	BCAL1824	18	32	101	115	putrescine-binding periplasmic protein
0.957527	0.017881	*BCAL1908*	-67	-47	3	23	lipoyl synthase
0.957527	0.006047	*BCAL2000*	19	30	104	115	2-dehydropantoate 2-reductase
0.957527	0.012253	BCAL2069	-85	-70	100	115	conserved hypothetical protein
0.957527	0.01892	BCAL2097	-6	7	21	34	putative membrane protein
0.957527	0.013745	*BCAL2120*	-192	-175	1	19	LysR family regulatory protein

0.957527	0.018214	BCAL2194	-96	-84	3	15	chaperone protein HscA
0.957527	0.011199	BCAL2302	-179	-165	101	113	excinuclease ABC subunit B
0.957527	0.017375	BCAL2429	69	77	106	114	putative cytochrome C -related protein
0.957527	0.012382	BCAL2451	-124	-113	73	84	putative membrane protein
0.957527	0.009918	BCAL2680	28	38	104	115	uroporphyrin-III C-methyltransferase
0.957527	0.011332	BCAL2708	31	45	101	115	putative amino-acid transport permease
0.957527	0.022124	BCAL2732	38	68	59	83	cold shock-like protein
0.957527	0.012585	BCAL2750	-62	-51	103	115	putative methyltransferase
0.957527	0.022228	BCAL2785	30	42	2	15	putative peptide methionine sulfoxide reductase
0.95753	**0.01627**	**BCAL2790**	**-4**	**7**	**21**	**31**	**putative cyclase**
0.957527	0.01901	BCAL2801	-193	-183	105	115	major facilitator superfamily protein
0.957527	0.008993	BCAL2934	-25	-5	1	17	electron transfer flavoprotein alpha-subunit
0.957527	0.021246	BCAL3044	10	17	103	110	bifunctional glucokinase/RpiR family transcriptional regulator
0.957527	0.018722	*BCAL3107*	-187	-177	103	113	urease accessory protein
0.957527	0.008249	*BCAL3166*	-54	-48	58	64	putative lipoprotein
0.957527	0.005133	*BCAL3262*	-53	-47	28	34	DnaA regulatory inactivator Hda
0.957527	0.017847	*BCAL3344*	-115	-106	106	115	tyrosyl-tRNA synthetase
0.957527	0.015044	BCAL3396	-184	-158	2	27	thiamine monophosphate kinase
0.957527	0.016194	BCAM0580	-8	6	22	34	RNase II stability modulator
0.957527	0.023252	BCAM0687	-124	-112	101	115	putative threonine efflux pump
0.957527	0.013106	BCAM0700	29	45	101	116	TetR family regulatory protein
0.957527	0.01955	*BCAM0944*	-155	-146	107	116	putative lipoprotein
0.957527	0.005342	*BCAM1199*	30	42	104	115	two-component regulatory system response regulator protein
0.957527	0.010451	BCAM1357	-143	-132	101	112	gluconate 2-dehydrogenase flavoprotein subunit
0.957527	0.008883	BCAM1475	72	84	101	113	putative membrane protein
0.957527	0.018244	BCAM1477	-83	-69	101	114	aromatic amino acid transport protein
0.957527	0.021614	BCAM1538	60	75	101	114	putative dehydrogenase monooxygenase subunit
0.957527	0.012859	*BCAM1575*	-4	7	21	31	putative cell division-related metallo peptidase
0.957527	0.002276	*BCAM1755*	-199	-185	101	115	GntR family regulatory protein

0.957527	0.011325	BCAM1841	-167	-154	17	30	major facilitator superfamily protein
0.957527	0.015464	BCAM2120	-77	-66	22	34	metallo-beta-lactamase superfamily protein
0.026191	8.28E-06	*BCAM2255*	10	49	4	43	putative OmpA/MotB family outer membrane protein
0.103536	4.91E-05	*BCAM2290*	-123	-108	101	116	putative gamma-butyrobetaine dioxygenase
0.957527	0.006746	BCAM2292	-151	-139	101	115	conserved hypothetical protein
0.957527	0.002871	*BCAM2300*	-108	-97	101	112	putative cytochrome c
0.957527	0.002589	*BCAM2326*	51	63	101	113	serine hydroxymethyltransferase
0.957527	0.011267	BCAM2337	87	99	101	113	putative multidrug resistance transporter protein
0.957527	0.001232	*BCAM2439*	53	92	3	42	TonB-dependent receptor
0.957527	0.002457	*BCAM2533*	-64	-51	101	114	two-component regulatory system sensor kinase
0.957527	0.022141	BCAS0135	-2	7	21	29	AraC family regulatory protein
0.957527	0.001685	*BCAS0300*	-60	-51	105	114	flp type pilus subunit

Supplementary Table S5b. Complete list of predicted interactions by CopraRNA for full length ncS35. [a]Interaction position in mRNA relative to start codon (position 1-3). In grey: genes for which predicted interaction is located outside of the 5'UTR.

			Interaction position in mRNA[a]		Interaction position relative in sRNA		
FDR	p-value	Target in *B. cenocepacia* J2315	start	end	start	end	Annotation
0.954905	0.013542	BCAL0029	63	76	129	142	putative ATP synthase protein I AtpI
0.954905	0.011103	BCAL0158	-12	-5	134	141	metallo-beta-lactamase superfamily protein
0.954905	0.022906	BCAL0230	-119	-112	134	141	30S ribosomal protein S7
0.954905	0.009707	BCAL0289	-52	-16	4	36	glutamate synthase large subunit
0.954905	0.016214	BCAL0519	-185	-175	132	142	putative GMC oxidoreductase
0.954905	0.023929	BCAL0601	-88	-50	3	43	putative gamma-glutamyl-gamma-aminobutyrate hydrolase
0.954905	0.017144	BCAL0726	-16	5	20	38	putative lipoprotein
0.954905	0.022847	BCAL0746	-46	-36	51	62	conserved hypothetical protein
0.954905	0.017854	BCAL0748	-125	-105	1	19	putative membrane protein
0.954905	0.007989	BCAL0896	-155	-140	129	143	4-hydroxythreonine-4-phosphate dehydrogenase
0.954905	0.014007	BCAL0953	-11	0	101	111	putative recombinase A
0.954905	0.007078	BCAL0960	62	94	1	28	O-antigen polymerase family protein
0.954905	0.022559	BCAL1050	89	95	3	9	putative glycosyltransferase
0.954905	0.01779	BCAL1115	32	50	3	23	putative ABC transport system, membrane protein
0.954905	0.009047	BCAL1446	-173	-161	97	110	putative lipoprotein
0.954905	0.02188	BCAL1845	-167	-160	87	94	putative membrane protein
0.954905	0.015301	BCAL1908	-67	-47	31	51	lipoyl synthase
0.954905	0.016785	BCAL1998	14	41	3	27	putative kinase
0.954905	0.017015	BCAL2000	19	30	132	143	putative reductase
0.954905	0.024192	BCAL2120	-189	-164	22	43	LysR family regulatory protein

0.954905	0.017417	BCAL2145	-52	-43	3	12	NADH-ubiquinone oxidoreductase subunit
0.954905	0.001095	BCAL2183	29	71	3	44	metallo-beta-lactamase superfamily membrane protein
0.954905	0.014858	BCAL2239	-17	13	1	28	putative gamma-glutamyl-gamma-aminobutyrate hydrolase
0.954905	0.017672	BCAL2286	-200	-183	3	22	putative membrane protein
0.954905	0.02248	BCAL2318	-92	-82	129	139	conserved hypothetical protein
0.954905	0.008804	BCAL2330	70	91	1	20	putative membrane protein
0.954905	0.008305	BCAL2339	81	90	18	27	NADH dehydrogenase I chain F
0.954905	0.018147	BCAL2657	-34	-13	1	22	putative bifunctional cobalamin biosynthesis protein
0.954905	0.024206	BCAL2917	-148	-134	8	21	conserved hypothetical protein
0.954905	0.014144	BCAL2934	-25	-5	29	45	electron transfer flavoprotein alpha-subunit
0.189306	8.96E-05	BCAL2973	6	51	2	45	putative exported protein
0.954905	0.024129	BCAL3107	-187	-177	131	141	urease accessory protein
0.954905	0.021502	BCAL3166	-54	-48	86	92	putative lipoprotein
0.954905	0.009954	BCAL3262	-53	-47	56	62	DnaA-homolog protein Hda
0.954905	0.014899	BCAL3334	-95	-89	99	105	putative nitrogen regulation-related protein
0.954905	0.018429	BCAL3344	-115	-106	134	143	tyrosyl-tRNA synthetase
0.954905	0.009543	BCAL3372	-197	-183	129	142	putative lipoprotein
0.954905	0.008346	BCAL3469	41	60	8	26	cell division protein FtsL
0.954905	0.004018	BCAL3504	-14	-1	26	39	flagellar protein FliO
0.954905	0.012117	BCAM0025	-179	-169	131	142	putative membrane protein
0.954905	0.021493	BCAM0572	67	83	11	28	conserved hypothetical protein
0.954905	0.012541	BCAM0618	-177	-160	3	23	two-component regulatory system, response regulator protein
0.954905	0.016555	BCAM0739	-155	-140	129	142	AraC family regulatory protein
0.954905	0.023127	BCAM0790	-92	-65	3	28	outer membrane efflux protein
0.954905	0.021748	BCAM0944	-155	-146	135	144	putative lipoprotein
0.954905	0.014689	BCAM0948	-174	-167	16	23	TonB-dependent receptor
0.954905	0.013368	BCAM1199	30	42	132	143	two-component regulatory system, response regulator protein
0.954905	0.01981	BCAM1205	-83	-75	132	140	putative membrane protein
0.954905	0.021866	BCAM1382	2	18	8	24	two-component regulatory system, response regulator protein

0.954905	0.004443	BCAM1503	-11	21	3	31	putative methyl-accepting chemotaxis protein
0.954905	0.007183	BCAM1575	-4	7	49	59	putative cell division-related metallo peptidase
0.954905	0.002186	BCAM1755	-199	-185	129	143	GntR family regulatory protein
0.954905	0.013021	BCAM2013	49	64	26	43	putative short chain dehydrogenase
0.954905	0.018483	BCAM2166	-102	-88	29	43	LysR family regulatory protein PenR
0.012711	4.01E-06	BCAM2255	10	49	32	71	putative OmpA/MotB family outer membrane protein
0.481888	0.000304	BCAM2290	-123	-108	129	144	putative gamma-butyrobetaine dioxygenase
0.954905	0.005116	BCAM2300	-108	-97	129	140	putative cytochrome c
0.954905	0.007248	BCAM2326	51	63	129	141	putative serine hydroxymethyltransferase
0.954905	0.009102	BCAM2439	53	92	31	70	TonB-dependent receptor
0.954905	0.004092	BCAM2533	-64	-51	129	142	two-component regulatory system, sensor kinase protein
0.954905	0.002086	BCAM2653	88	98	3	13	putative exported protein
0.954905	0.00677	BCAS0300	-60	-51	133	142	flp type pilus subunit
0.954905	0.016342	BCAS0557	-27	-12	8	23	metallo-beta-lactamase superfamily protein
0.954905	0.002106	BCAS0705	-133	-121	129	141	putative pyridine nucleotide-disulphide oxidoreductase

Supplementary Table S6. RNA-sequencing. Complete list of significant gene expression changes in ΔncS35 compared to wild type ($p < 0.05$, fold change ≥ 1.5).

Fold changes in ΔncS35 compared to wild type				
Locus tag	**Exponential phase**	**Stationary phase**	**Biofilm**	**Annotation**
BCAL0003			-2.92	MarR family regulatory protein
BCAL0004	1.76		-2.33	cysteine peptidase, family C26
BCAL0006			-2.30	putative protein lysine methyltransferase
BCAL0007			-3.24	two-component regulatory system, sensor kinase protein
BCAL0008			-3.01	two-component regulatory system, response regulator protein
BCAL0008A			-3.19	conserved hypothetical protein
BCAL0009			-3.27	pterin-4-alpha-carbinolamine dehydratase
BCAL0010			-5.36	phenylalanine-4-hydroxylase
BCAL0011			-2.08	AnsC family regulatory protein
BCAL0014	2.40			putative GMC oxidoreductase
BCAL0021			-1.97	putative branched-chain amino acid ABC transporter permease
BCAL0024			-1.96	tRNA uridine 5-carboxymethylaminomethyl modification enzyme GidA
BCAL0036			-2.01	ATP synthase beta chain
BCAL0040			1.69	uroporphyrinogen decarboxylase
BCAL0043			-2.41	putative extracellular ligand-binding protein
BCAL0046			-3.84	putative fatty-acid CoA ligase
BCAL0051			-2.01	periplasmic solute-binding protein
BCAL0053			2.08	PadR family regulatory protein
BCAL0055			3.21	putative heavy metal resistance membrane ATPase
BCAL0057			-2.37	putative membrane protein
BCAL0065			-3.10	conserved hypothetical protein
BCAL0068	2.17			acetolactate synthase, large subunit
BCAL0069			2.39	conserved hypothetical protein, antitoxin
BCAL0070			1.56	plasmid stabilisation system protein, toxin
BCAL0073		-8.43		glycine dehydrogenase (decarboxylating)
BCAL0074		-3.18	-2.20	glycine cleavage system H protein
BCAL0075		-3.78		aminomethyltransferase
BCAL0081			1.63	prophage integrase
BCAL0086			2.36	hypothetical phage protein
BCAL0088	-1.76		-2.56	hypothetical phage protein
BCAL0093			3.87	putative phage tail protein
BCAL0094			4.24	phage major tail tube protein
BCAL0111		2.85		putative TPR repeat protein
BCAL0114			-2.67	flagellin (type II)
BCAL0121			-4.56	aquaporin Z
BCAL0122		2.19		histone-like nucleoid-structuring (H-NS) protein

BCAL0123			2.02	putative glycosyltransferase
BCAL0124			-2.40	flagellar regulon master regulator subunit FlhD
BCAL0127			1.82	chemotaxis protein MotB
BCAL0128			1.85	chemotaxis two-component response regulator CheY
BCAL0132			2.18	chemotaxis protein methyltransferase cheR
BCAL0140		4.55		flagellar biosynthetic protein FlhB
BCAL0155			1.69	putative cation efflux protein
BCAL0159			-3.21	putative RlpA-like lipoprotein
BCAL0161	2.28		2.37	conserved hypothetical protein
BCAL0165		1.80	2.13	putative plasmid replication-associated protein
BCAL0170		5.29	2.07	conserved hypothetical protein
BCAL0171			2.03	putative plasmid conjugal transfer protein
BCAL0172		2.01		putative plasmid conjugal transfer protein
BCAL0174		1.69		putative plasmid conjugal transfer protein
BCAL0178		2.01		putative DNA methyltransferase
BCAL0179			-3.12	hypothetical protein
BCAL0182			1.71	putative plasmid recombinase
BCAL0193	1.94	2.42	2.03	putative exported protein
BCAL0195	1.93			putative amino acid transport protein
BCAL0203			1.57	phosphatidylethanolamine-binding protein
BCAL0205			-2.03	NADP-dependent malic enzyme
BCAL0206A	2.51		3.14	putative outer membrane protein
BCAL0207		77.74		4-hydroxyphenylpyruvic acid dioxygenase
BCAL0209			-2.51	acetyltransferase (GNAT) family protein
BCAL0212	3.76	19.85		putative phenylacetic acid degradation NADH oxidoreductase PaaE
BCAL0213	5.26	15.76		phenylacetic acid degradation protein PaaD
BCAL0214	5.97	29.02		phenylacetic acid degradation protein PaaC
BCAL0215	5.01	28.71		phenylacetic acid degradation protein PaaB
BCAL0216	4.46	16.33		phenylacetic acid degradation protein PaaA
BCAL0225			-2.31	50S ribosomal protein L7/L12
BCAL0226			-2.17	DNA-directed RNA polymerase beta chain
BCAL0227			-2.21	DNA-directed RNA polymerase beta' chain
BCAL0231			-2.90	elongation factor G
BCAL0232			-2.79	elongation factor Tu (EF-Tu)
BCAL0233	-1.60		-5.75	30s ribosomal protein S10
BCAL0234			-3.39	50S ribosomal protein L3
BCAL0235			-3.88	50S ribosomal protein L4
BCAL0236			-4.30	50S ribosomal protein L23
BCAL0237			-4.20	50S ribosomal protein L2
BCAL0238			-3.28	30S ribosomal protein S19
BCAL0239			-3.02	50S ribosomal protein L22
BCAL0240			-4.13	30S ribosomal protein S3
BCAL0241			-3.22	50S ribosomal protein L16
BCAL0242			-4.91	50S ribosomal protein L29

BCAL0243			-4.05	30S ribosomal protein S17
BCAL0244			-2.91	50S ribosomal protein L14
BCAL0245			-3.60	50S ribosomal protein L24
BCAL0246			-4.06	50S ribosomal protein L5
BCAL0247			-5.01	30S ribosomal protein S14
BCAL0248			-4.62	30S ribosomal protein S8
BCAL0249			-4.88	50S ribosomal protein L6
BCAL0250			-3.87	50S ribosomal protein L18
BCAL0251			-6.03	30S ribosomal protein S5
BCAL0252			-2.38	50S ribosomal protein L30
BCAL0253			-3.51	50S ribosomal protein L15
BCAL0254			-4.60	preprotein translocase SecY subunit
BCAL0255	-1.68		-6.16	translation initiation factor IF-1
BCAL0259			-2.84	30S ribosomal protein S4
BCAL0260			-3.53	DNA-directed RNA polymerase alpha chain
BCAL0261			-2.94	50S ribosomal protein L17
BCAL0262			1.82	putative divalent-cation tolerance protein CutA
BCAL0263			1.63	thiol:disulfide interchange protein DsbD
BCAL0265			1.52	putative GTP-binding cell division protein EngB
BCAL0268			-3.13	putative cytochrome c biogenesis protein
BCAL0269			3.92	putative oxidoreductase
BCAL0270			2.83	ferric reductase-like transmembrane component
BCAL0281			-1.86	putative phosphohydrolase
BCAL0282		-4.42	-1.88	putative ABC transporter extracellular solute-binding protein
BCAL0283		-5.09		putative ABC transporter permease
BCAL0285		-4.16	-2.77	ABC transporter ATP-binding protein
BCAL0292	1.95			2',5' RNA ligase family protein
BCAL0299	1.69			thiazole biosynthesis protein ThiG
BCAL0303			-3.35	putative membrane protein
BCAL0304			-2.34	VacJ-like lipoprotein
BCAL0305			3.22	putative exported protein
BCAL0306			2.52	conserved hypothetical protein
BCAL0309			-2.59	BolA-like protein
BCAL0310			-3.07	UDP-N-acetylglucosamine 1-carboxyvinyltransferase
BCAL0311			-2.35	ATP phosphoribosyltransferase
BCAL0318			-1.94	imidazole glycerol phosphate synthase subunit HisF
BCAL0319			-2.96	phosphoribosyl-AMP cyclohydrolase
BCAL0320			-3.58	phosphoribosyl-ATP pyrophosphatase
BCAL0326			1.85	serine peptidase, subfamily S1B
BCAL0331			-1.95	putative stringent starvation protein A
BCAL0332			-1.98	putative stringent starvation protein B
BCAL0337			-2.21	putative type VI secretion system protein TssL
BCAL0338			-1.88	putative type VI secretion system protein TssK
BCAL0343			-2.01	putative type VI secretion system protein TssD

BCAL0344			-2.71	putative type VI secretion system protein TssE
BCAL0345			-2.57	putative type VI secretion system protein TssF
BCAL0347			-4.08	putative type VI secretion system protein TssH
BCAL0348			-3.87	putative type VI secretion system protein TssA
BCAL0349			-3.24	putative outer membrane protein
BCAL0350			-3.18	conserved hypothetical protein
BCAL0351			-2.28	putative type VI secretion system protein TssM
BCAL0360			-2.35	conserved hypothetical protein
BCAL0364			1.56	TetR family regulatory protein
BCAL0368		3.25		cold shock-like protein CspD
BCAL0375			2.53	glutamyl-tRNA reductase
BCAL0376a	1.71			putative membrane protein
BCAL0380			-1.93	ABC transporter ATP-binding subunit
BCAL0382	2.37			LysR family regulatory protein
BCAL0385			-7.34	putative amino acid permease
BCAL0391			7.41	acyl carrier protein phosphodiesterase
BCAL0394			2.28	uracil-DNA glycosylase
BCAL0396			-1.96	indole-3-glycerol phosphate synthase
BCAL0397			-2.01	anthranilate phosphoribosyltransferase
BCAL0398			-2.38	anthranilate synthase component II
BCAL0404	3.23			phenylacetate-coenzyme A ligase paaK
BCAL0405	2.81			phenylacetic acid degradation protein PaaI
BCAL0406	4.46	2.72		probable enoyl-CoA hydratase PaaG
BCAL0407	5.11	5.13		beta-ketoadipyl CoA thiolase
BCAL0408	4.54	9.01	1.91	putative phenylacetic acid degradation oxidoreductase paaZ
BCAL0409	2.11			putative phenylacetic acid degradation enoyl-CoA hydratase PaaF
BCAL0409a	-1.76	2.59		hypothetical protein
BCAL0412		2.56	1.73	conserved hypothetical protein (fragment)
BCAL0413		2.62		conserved hypothetical protein
BCAL0414		2.06		
BCAL0418		1.77		type I restriction enzyme specificity protein
BCAL0419		2.10		hypothetical protein
BCAL0423			-2.10	chromosomal replication initiator protein DnaA
BCAL0426			-1.88	putative membrane protein
BCAL0432	-1.62			putative membrane protein
BCAL0434			-2.49	putative exported protein
BCAL0435			-3.10	conserved hypothetical protein
BCAL0437	2.03		3.77	O6-methylguanine-DNA methyltransferase
BCAL0438			3.60	putative DNA-3-methyladenine glycosylase II
BCAL0440	2.77			putative exported protein
BCAL0441			-2.83	putative L-lactate permease
BCAL0442			-2.99	LysR family regulatory protein
BCAL0465			3.28	peptide deformylase

BCAL0466	1.73		3.46	methionyl-tRNA formyltransferase
BCAL0467			1.77	putative LysE type translocator protein
BCAL0468	2.24		4.72	metallo peptidase, subfamily M48B
BCAL0471			-2.05	two-component regulatory system, sensor kinase protein
BCAL0474			-1.95	conserved hypothetical protein
BCAL0480			1.67	putative rod shape-determining protein
BCAL0481			1.68	putative rod shape-determining protein
BCAL0482			-1.96	putative rod shape-determining protein
BCAL0483			1.70	glutamyl-tRNA amidotransferase subunit C
BCAL0486			-2.08	putative polyphosphate kinase
BCAL0487			-2.06	endonuclease/exonuclease/phosphatase family protein
BCAL0500			2.36	ATP-dependent protease ATP-binding subunit HslU
BCAL0501			2.83	ATP-dependent protease HslV
BCAL0510			1.78	conserved hypothetical protein
BCAL0511			1.69	putative deoxygenases
BCAL0524		3.31		flagellar motor switch protein FliG
BCAL0525		3.70		flagellar M-ring protein FliF
BCAL0531			2.49	putative membrane protein
BCAL0532			1.87	conserved hypothetical protein
BCAL0533			1.79	putative amino-acid transporter transmembrane protein
BCAL0535	2.01			sensor kinase protein
BCAL0537			2.36	endonuclease/exonuclease/phosphatase family protein
BCAL0538			2.05	putative exported protein
BCAL0540		-4.15	-2.63	putative ATPase
BCAL0541			-2.05	putative FAD dependent oxidoreductase
BCAL0543			-2.88	Major Facilitator Superfamily protein
BCAL0544			-3.49	putative periplasmic dipeptide transport protein
BCAL0545			-3.03	putative dipeptide transport system permease protein
BCAL0546			-3.81	putative dipeptide transport system permease protein
BCAL0547			-3.24	putative dipeptide ABC transporter ATP-binding protein
BCAL0548			-2.57	putative dipeptide ABC transporter ATP-binding protein
BCAL0551	2.03			allophanate hydrolase subunit 2
BCAL0552		-3.41		allophanate hydrolase subunit 1
BCAL0558	1.76			tRNA nucleotidyltransferase
BCAL0561		2.18		flagella synthesis protein FlgN
BCAL0564		7.16		flagellar basal-body rod protein FlgB (putative proximal rod protein)
BCAL0565		4.85		flagellar basal-body rod protein FlgC (putative proximal rod protein)
BCAL0566		4.56		basal-body rod modification protein FlgD
BCAL0567		3.92		flagellar hook protein 1 FlgE1
BCAL0568		3.45		flagellar basal-body rod protein FlgF (putative proximal rod protein)
BCAL0570		3.67		flagellar L-ring protein precursor (basal body L-ring protein)

BCAL0571		3.71		flagellar P-ring protein precursor (basal body P-ring protein)
BCAL0579			2.16	LysR family regulatory protein
BCAL0588			-5.51	putative lipoprotein
BCAL0602			-2.08	MerR family regulatory protein
BCAL0605			-2.26	putative exported protein
BCAL0610			1.53	conserved hypothetical protein, toxin
BCAL0612			1.61	bifunctional glmU protein (includes: UDP-N-acetylglucosamine pyrophosphorylase; glucosamine-1-phosphate N-acetyltransferase)
BCAL0615	2.11			conserved hypothetical protein (fragment)
BCAL0618	2.33	-7.40		PfkB family carbohydrate kinase
BCAL0619		-7.92	-3.98	putative N-acylglucosamine 2-epimerase
BCAL0623			3.06	putative exported protein
BCAL0624			1.92	putative outer membrane porin protein precursor
BCAL0625			-3.02	LysR family regulatory protein
BCAL0628	2.10			AsnC family regulatory protein
BCAL0632	1.84			putative dehydrogenase
BCAL0638			3.26	LysR family regulatory protein
BCAL0648			2.88	putative transferase (pseudogene)
BCAL0650	1.80			putative pyruvate-flavodoxin oxidoreductase
BCAL0655	2.21			CutC family protein
BCAL0656	1.94			putative aminotransferase/butyrosin synthesis protein
BCAL0665	2.00			dethiobiotin synthetase
BCAL0669			-2.92	conserved hypothetical protein
BCAL0671	1.70			putative carbonic anhydrase
BCAL0673			-2.72	metallo-beta-lactamase superfamily protein
BCAL0675			-2.01	extracellular solute-binding protein
BCAL0680			-2.15	conserved hypothetical protein
BCAL0682			1.56	putative 5-methyltetrahydrofolate--homocysteine methyltransferase
BCAL0683		3.79	8.12	conserved hypothetical protein
BCAL0685	2.27		1.61	IclR family regulatory protein
BCAL0686	1.84			fumarylacetoacetate (FAA) hydrolase family protein
BCAL0687	1.81			enoyl-CoA hydratase/isomerase family protein
BCAL0688	2.15			conserved hypothetical protein
BCAL0690			2.43	conserved hypothetical protein
BCAL0694	1.91			putative biotin ligase
BCAL0700	1.88			putative lipoprotein
BCAL0705			1.95	putative D-amino acid aminotransferase
BCAL0706			2.76	conserved hypothetical protein
BCAL0707			2.01	LysR family regulatory protein
BCAL0708			2.04	conserved hypothetical protein
BCAL0725			1.52	putative thiol-disulfide oxidoreductase
BCAL0731			1.60	glutamate--cysteine ligase
BCAL0749			-2.02	putative cytochrome c oxidase

BCAL0750			-2.18	cytochrome c oxidase polypeptide I
BCAL0752			-3.14	putative cytochrome c oxidase assembly protein
BCAL0754			-2.48	putative cytochrome c oxidase subunit III
BCAL0755			2.09	putative membrane protein
BCAL0760			-2.37	probable lipoprotein
BCAL0765			-2.41	putative exported protein
BCAL0766			-2.92	putative branched-chain amino acid transport system permease protein
BCAL0767			-3.36	ABC transporter ATP-binding protein
BCAL0778			-6.30	putative N-acetylglucosamine-6-phosphate deacetylase
BCAL0781		-3.40	-4.23	phosphotransferase system, IIbc component
BCAL0787	2.20		4.69	RNA polymerase sigma-32 factor
BCAL0790			1.50	hydroxymethylglutaryl-CoA lyase-like protein
BCAL0797			-1.89	putative histidinol-phosphate aminotransferase
BCAL0799			-1.96	ribosomal L25p family protein
BCAL0802		2.50		putative 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase
BCAL0812			-2.92	sigma-54 modulation protein
BCAL0813	-1.64		-2.04	putative RNA polymerase sigma-54 factor
BCAL0815			-1.96	OstA-like protein
BCAL0831	2.33			putative storage protein
BCAL0832		-3.41	-2.29	putative poly-beta-hydroxy-butyrate storage protein phaA
BCAL0833			-3.00	putative Acetoacetyl-CoA reductase phbB
BCAL0835			-2.59	putative exported protein
BCAL0853	7.16			conserved hypothetical protein
BCAL0857			-2.34	putative exported protein
BCAL0861		1.85		Major Facilitator Superfamily protein
BCAL0862			-2.22	MarR family regulatory protein
BCAL0866			-2.38	D-aminoacylase
BCAL0867			-3.28	YjgF family protein
BCAL0874	1.84		2.90	putative membrane protein
BCAL0875			1.63	conserved hypothetical protein
BCAL0878			1.57	conserved hypothetical protein
BCAL0883	2.10			TetR family regulatory protein
BCAL0885			-3.81	putative 3-hydroxyacyl-CoA dehydrogenase oxidoreductase
BCAL0886			-2.68	putative 3-ketoacyl-CoA thiolase
BCAL0894			-2.32	putative exported protein
BCAL0895			-2.79	putative peptidyl-prolyl cis-trans isomerase
BCAL0896			-2.83	4-hydroxythreonine-4-phosphate dehydrogenase
BCAL0899		2.67	2.20	lactoylglutathione lyase
BCAL0900			2.34	conserved hypothetical protein
BCAL0901			-2.18	putative acyltransferase
BCAL0916			-2.11	putative hydrolase protein
BCAL0918			-7.75	conserved hypothetical protein

BCAL0919			-3.32	conserved hypothetical protein
BCAL0920	2.28			LacI family regulatory protein
BCAL0921			-3.54	fatty acid desaturase
BCAL0922			-6.69	2Fe-2S ferredoxin
BCAL0924			-3.89	putative glycerol uptake facilitator protein
BCAL0928			-6.77	conserved hypothetical protein
BCAL0930	1.83			putative gamma-glutamyltransferase precursor
BCAL0933			-2.18	putative ATP-dependent RNA helicase 1
BCAL0934			-2.60	putative periplasmic cytochrome c containing protein
BCAL0936	2.04		2.11	putative transmembrane transporter protein
BCAL0938a	2.52			conserved hypothetical protein
BCAL0939			1.59	conserved hypothetical protein
BCAL0940			4.03	putative transglycosylase
BCAL0941			2.51	LysR family regulatory protein
BCAL0947a	-2.38		-2.10	putative membrane protein
BCAL0950			-4.26	Major Facilitator Superfamily protein
BCAL0954			-2.04	RecX family regulatory protein
BCAL0956			-1.95	succinyl-CoA synthetase beta chain
BCAL0957			-2.33	succinyl-CoA ligase alpha-chain
BCAL0958			1.64	putative membrane protein
BCAL0961			-6.83	putative lipoprotein
BCAL0963	1.91			metallo peptidase, subfamily M48B
BCAL0964	2.51		1.89	conserved hypothetical protein
BCAL0965			1.58	putative hydrolase
BCAL0966			2.20	conserved hypothetical protein
BCAL0969			-2.11	AraC family regulatory protein
BCAL0978	1.73			putative membrane protein
BCAL0979	2.37			molybdopterin biosynthesis protein MoeA 2
BCAL0980			1.68	molybdopterin-guanine dinucleotide biosynthesis protein A 2
BCAL0981			3.31	molybdenum cofactor biosynthesis protein A 2
BCAL0982			-2.68	putative ribonuclease E
BCAL0983			2.02	ribosomal large subunit pseudouridine synthase C 2
BCAL0984	1.72			haloacid dehalogenase-like hydrolase
BCAL0986			-2.23	serine peptidase, family S49
BCAL0992			1.99	3-oxoacyl-[acyl-carrier-protein] synthase III 2
BCAL0995		2.32		acyl carrier protein (ACP) 2
BCAL0998			1.85	RNA polymerase sigma-E factor (sigma-24) 2
BCAL0999			1.87	sigma-E factor negative regulatory protein 2
BCAL1000			1.66	sigma-E factor regulatory protein RseB precursor 2
BCAL1004			-2.83	signal peptidase I 2 (leader peptidase Lep 2)
BCAL1011			1.51	sigma-54 interacting response regulator protein
BCAL1030			13.42	Pirin-like protein
BCAL1033		4.18	2.28	putative exported protein
BCAL1034		2.90	2.07	SCO1/SenC family protein

BCAL1040			-1.88	glycosyl transferases group 1 protein
BCAL1041	2.00			putative membrane protein
BCAL1042	1.80			putative membrane protein
BCAL1051			-2.96	radical SAM superfamily protein
BCAL1055	2.62	2.10	1.65	histidine transport system permease protein
BCAL1057	2.29			histidine ABC transporter ATP-binding protein
BCAL1058	2.22		-2.29	AraC family regulatory protein
BCAL1059	2.30			succinylornithine transaminase
BCAL1060	2.64			putative arginine N-succinyltransferase, alpha chain
BCAL1062	2.25			succinylglutamic semialdehyde dehydrogenase
BCAL1063	2.06		-3.27	succinylarginine dihydrolase
BCAL1064	2.27			putative succinylglutamate desuccinylase
BCAL1065			1.95	periplasmic solute-binding protein
BCAL1071			2.00	NAD dependent epimerase/dehydratase family protein
BCAL1081			1.51	multidrug resistance protein MdtA precursor
BCAL1096	1.82			putative exodeoxyribonuclease V gamma chain
BCAL1099			-2.91	putative release factor
BCAL1102			2.85	putative lipoprotein
BCAL1103			5.18	putative OsmB-like lipoprotein
BCAL1118			1.83	putative integrase
BCAL1119			1.60	conserved hypothetical protein
BCAL1120		1.79		conserved hypothetical protein
BCAL1122		2.27		conserved hypothetical protein
BCAL1123			2.84	putative exported protein
BCAL1124		1.71	3.16	conserved hypothetical protein
BCAL1124a			3.43	
BCAL1127			2.23	conserved hypothetical protein, toxin
BCAL1128			2.49	putative DNA-binding protein, antitoxin
BCAL1138		2.24	1.57	conserved hypothetical protein
BCAL1140		2.26	1.57	AraC family regulatory protein (fragment)
BCAL1143		2.21		putative transposase (fragment)
BCAL1143a	-2.00			putative dehydrogenase (fragment)
BCAL1146			1.78	AraC family regulatory protein
BCAL1167			-1.83	putative exported protein
BCAL1168		2.15		conserved hypothetical protein
BCAL1169		3.11		
BCAL1172		1.86		conserved hypothetical protein
BCAL1176			-2.68	putative fusaric acid resistance transport protein
BCAL1177	-1.52		-2.46	putative fusaric acid resistance transporter protein
BCAL1179		3.75		LysR family regulatory protein
BCAL1182	-1.93			TetR family regulatory protein
BCAL1186			-1.94	putative oxidoreductase
BCAL1187			-1.81	conserved hypothetical protein
BCAL1188			-2.27	putative 3-dehydroshikimate dehydratase

BCAL1189			-3.60	putative exported protein
BCAL1190			-2.87	putative lipoprotein
BCAL1202A	-1.68			hypothetical protein
BCAL1204			-2.50	putative helicase
BCAL1211			-3.53	conserved hypothetical protein
BCAL1212			-3.37	2-oxoisovalerate dehydrogenase alpha subunit
BCAL1213			-3.85	2-oxoisovalerate dehydrogenase beta subunit
BCAL1214			-3.95	lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex
BCAL1215		-5.09	-3.86	dihydrolipoamide dehydrogenase
BCAL1216	2.16			conserved hypothetical protein
BCAL1222			1.60	LysR family regulatory protein
BCAL1230	2.06			LysR family regulatory protein
BCAL1231			2.12	putative membrane protein
BCAL1232			4.38	conserved hypothetical protein
BCAL1233			10.83	putative heat shock Hsp20-related protein
BCAL1234		2.55	12.01	putative heat shock protein
BCAL1235	1.85		1.78	putative 3-hydroxyacyl-CoA dehydrogenase
BCAL1236	2.11		2.03	putative exported protein
BCAL1247			3.77	putative exported protein
BCAL1248			2.08	putative phosphoribosyl transferase protein
BCAL1249			2.19	putative PHB depolymerase
BCAL1266			2.00	putative ribosomal RNA large subunit methyltransferase
BCAL1267			1.81	FtsH endopeptidase
BCAL1275			-2.09	phosphate regulon two-component regulatory system, response regulator protein
BCAL1279			2.58	putative exported protein
BCAL1280			1.96	putative hydrolase
BCAL1294			-2.45	conserved hypothetical protein
BCAL1295			-2.32	conserved hypothetical protein
BCAL1296			-3.27	conserved hypothetical protein
BCAL1297			-4.15	hypothetical protein
BCAL1298			-4.44	hypothetical protein
BCAL1300			-2.09	conserved hypothetical protein
BCAL1317		3.54	2.60	putative phage integrase
BCAL1330			-3.48	2Fe-2S ferredoxin
BCAL1344			2.74	conserved hypothetical protein
BCAL1357			-2.17	putative exported protein
BCAL1358			-2.53	conserved hypothetical protein
BCAL1359			-4.48	conserved hypothetical protein
BCAL1360			-4.76	hypothetical protein
BCAL1362			-4.88	conserved hypothetical protein
BCAL1363			-4.54	conserved hypothetical protein
BCAL1364			-5.48	putative membrane protein
BCAL1365			-7.15	putative lipoprotein

BCAL1366			-4.69	conserved hypothetical protein
BCAL1368			-2.79	putative porin
BCAL1369			5.39	putative RNA polymerase sigma factor FecI
BCAL1370			8.03	iron uptake regulatory protein FecR
BCAL1371			3.55	putative TonB-dependent siderophore receptor
BCAL1385			-3.38	putative CDP-alcohol phosphatidyltransferase
BCAL1390		-3.12		endoglucanase precursor
BCAL1395			-4.88	putative cellulose synthase catalytic subunit (UDP-forming)
BCAL1396			-5.07	putative membrane protein
BCAL1397		-3.30	-7.15	conserved hypothetical protein
BCAL1409		8.09		phosphoesterase family protein
BCAL1413A			8.10	putative lipoprotein
BCAL1414			1.62	putative hydrolase
BCAL1420			-3.14	putative thioesterase
BCAL1427			-3.99	myo-inositol catabolism protein
BCAL1428			-3.88	putative amine catabolism-related protein
BCAL1429			-3.95	putative TPP-binding acetolactate synthase
BCAL1431			-5.07	putative ribose ABC transport system, substrate-binding exported protein
BCAL1432			-3.94	putative sugar ABC transporter ATP-binding protein
BCAL1433			-4.65	putative sugar transport system permease protein
BCAL1434			-6.66	putative myo-inositol catabolism protein
BCAL1435			-6.06	inositol 2-dehydrogenase
BCAL1436			-5.94	putative dehydratase
BCAL1437			-7.79	putative oxidoreductase
BCAL1439		-3.59		methylmalonic acid semialdehyde dehydrogenase
BCAL1441		1.86		SirA-like protein
BCAL1442			-2.80	conserved hypothetical protein
BCAL1443			-1.88	two-component regulatory system, response regulator protein
BCAL1444			-2.72	putative exported protein
BCAL1445			-2.88	two-component regulatory system, sensor kinase protein
BCAL1448			1.63	putative valyl-tRNA synthetase
BCAL1458			2.58	putative exported protein
BCAL1459			1.51	calcineurin-like phosphoesterase
BCAL1461			1.57	putative membrane protein
BCAL1463			1.63	putative tRNA processing exoribonuclease
BCAL1465			2.08	conserved hypothetical protein
BCAL1467			1.87	chorismate synthase
BCAL1472			1.60	succinyl-CoA:3-ketoacid-coenzyme A transferase subunit A
BCAL1474			1.84	putative dehydrogenase
BCAL1477			1.70	LysR family regulatory protein
BCAL1483	-1.92		-2.45	50S ribosomal protein L35

BCAL1485			-2.93	phenylalanyl-tRNA synthetase alpha chain
BCAL1486			-3.23	phenylalanyl-tRNA synthetase beta chain
BCAL1487			1.59	integration host factor alpha-subunit
BCAL1494			-2.15	putative monooxygenase
BCAL1499			1.88	putative exported protein
BCAL1504			-2.33	RNA pseudouridylate synthase family protein
BCAL1505	-1.70		-2.08	conserved hypothetical protein
BCAL1507			-2.49	translation initiation factor IF-2
BCAL1508			-3.68	putative ribosome-binding factor
BCAL1511			1.56	putative multidrug resistance transport protein
BCAL1512			2.15	outer membrane efflux protein
BCAL1513		1.84	4.06	MarR family regulatory protein
BCAL1517			-2.88	dihydrolipoamide dehydrogenase
BCAL1518			-3.34	AFG1-like ATPase
BCAL1519			-4.19	putative transposase
BCAL1520			-7.34	putative lipoprotein
BCAL1525			-5.57	flp type pilus subunit
BCAL1525a			-3.75	putative flp type pilus leader peptidase
BCAL1526			-3.98	putative flp type pilus assembly protein
BCAL1528		-2.95	-3.15	flp type pilus assembly protein
BCAL1530		-3.08	-5.09	flp pilus type assembly protein
BCAL1531			-5.29	flp type pilus assembly protein
BCAL1532			-4.09	flp type pilus assembly protein
BCAL1533			-5.71	putative lipoprotein
BCAL1535			-3.36	putative membrane protein
BCAL1536		-3.29	-4.75	putative sigma-54 related transcriptional regulatory protein
BCAL1539			4.27	putative exported protein
BCAL1545			-2.88	conserved hypothetical protein
BCAL1548	2.23	-3.28	-2.23	putative sugar ABC transport system, lipoprotein
BCAL1549	2.38	-2.99		putative sugar ABC transport system, membrane protein
BCAL1550	2.25			putative sugar ABC transporter ATP-binding protein
BCAL1552	-1.58			putative extracellular substrate-binding protein
BCAL1553			-2.17	putative ribonuclease
BCAL1554			-2.54	putative tRNA/rRNA methyltransferase
BCAL1617	1.77			putative hydratase
BCAL1625			1.55	tRNA-dihydrouridine synthase A
BCAL1626	-1.86			conserved hypothetical protein
BCAL1630			-2.49	conserved hypothetical protein
BCAL1632			-1.93	putative lipoprotein
BCAL1645			-1.88	conserved hypothetical protein
BCAL1648			2.97	putative stress-related protein
BCAL1649			1.72	putative membrane protein
BCAL1652		-3.78	1.78	sulfate-binding protein precursor
BCAL1653		-11.38		sulfate transport system permease protein

BCAL1655		-5.94		sulfate ABC transporter ATP-binding protein
BCAL1656		-3.74		aliphatic sulfonate utilization regulatory protein SsuR
BCAL1661	2.01			putative ribokinase
BCAL1663			2.18	PrkA family serine protein kinase
BCAL1664			2.15	conserved hypothetical protein
BCAL1670	-2.47		-2.71	putative amino acid transport system, membrane protein
BCAL1671			-2.10	metallo peptidase, subfamily M23B
BCAL1674	2.30			multidrug efflux system AmrA protein
BCAL1675		-3.72	-3.93	multidrug efflux system transporter protein AmrB
BCAL1676	1.87	-5.47	-3.71	multidrug efflux system outer membrane protein
BCAL1677		3.39		putative type-1 fimbrial protein
BCAL1688			2.60	putative RNA polymerase sigma factor
BCAL1689			1.51	MbtH-like protein
BCAL1691	1.73			putative iron transport-related ATP-binding protein
BCAL1692	2.35			putative iron transport-related membrane protein
BCAL1693			-2.48	putative iron transport-related membrane protein
BCAL1694			-2.10	putative iron transport-related exported protein
BCAL1696	2.27			ornibactin biosynthesis non-ribosomal peptide synthase
BCAL1697	2.82			ornibactin biosynthesis non-ribosomal peptide synthase
BCAL1698	2.51			ornibactin biosynthesis protein
BCAL1699	1.70			putative L-ornithine 5-monooxygenase
BCAL1701	2.12			ornibactin synthetase F
BCAL1702	2.00			putative ornibactin biosynthesis protein
BCAL1704			1.58	conserved hypothetical protein
BCAL1705			1.69	cobyrinic acid A,C-diamide synthase
BCAL1718		6.09	11.58	putative CoA-transferase
BCAL1721		-3.44	-4.15	putative transcriptional regulator
BCAL1722		-4.75	-6.48	putative exported chitinase
BCAL1727	2.28			precorrin-6Y C5,15-methyltransferase (decarboxylating)
BCAL1728	1.76			putative cobalamin biosynthesis-related protein
BCAL1733			-2.25	putative glutathione S-transferase
BCAL1747A			3.72	putative exported protein
BCAL1752	2.03			conserved hypothetical protein
BCAL1753			-2.22	LysR family regulatory protein
BCAL1777a		6.07		putative membrane protein
BCAL1791			-2.63	conserved hypothetical protein
BCAL1794			-2.22	putative membrane protein
BCAL1795			-2.29	putative ATP-ase
BCAL1796	14.97		-11.61	putative saccharopine dehydrogenase
BCAL1804			-2.89	Major Facilitator Superfamily protein
BCAL1805			-2.59	putative sugar kinase
BCAL1806			-5.75	conserved hypothetical protein
BCAL1813	2.23			multidrug efflux system outer membrane protein
BCAL1817			2.30	putative transcriptional regulatory protein

BCAL1818		-4.08	-4.47	metallo-beta-lactamase superfamily protein
BCAL1819	1.86		-7.06	conserved hypothetical protein
BCAL1820	2.60			putative membrane protein
BCAL1821			-2.61	putrescine transport system permease protein
BCAL1822			-2.08	putrescine transport system permease protein
BCAL1829			2.67	putative outer membrane protein
BCAL1830			3.80	putative 2-nitropropane dioxygenase
BCAL1832			1.79	conserved hypothetical protein
BCAL1833			-2.53	putative exported protein
BCAL1841			-4.37	conserved hypothetical protein
BCAL1842			-2.65	conserved hypothetical protein
BCAL1844			-2.12	conserved hypothetical protein
BCAL1850		-3.83		putative dehydrogenase
BCAL1854			-2.42	putative exported protein
BCAL1857			1.77	putative membrane protein
BCAL1860			-2.09	putative polyhydroxyalkanoate (PHA) synthesis regulatory protein
BCAL1861			-2.37	acetoacetyl-CoA reductase
BCAL1862		-3.60	-2.54	acetyl-CoA acetyltransferase
BCAL1864	1.77			conserved hypothetical protein
BCAL1868	-1.52			conserved hypothetical protein
BCAL1869	-1.51			putative exported protein
BCAL1875			-2.15	putative membrane protein
BCAL1880			-2.13	GTP-binding protein EngA
BCAL1883			-2.65	histidyl-tRNA synthetase
BCAL1884			-2.25	putative 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase
BCAL1888			1.83	putative membrane protein
BCAL1899			2.04	DNA polymerase III subunit gamma
BCAL1900			3.38	thioredoxin
BCAL1900A			5.69	conserved hypothetical protein
BCAL1909			-12.50	dihydrolipoyllysine-residue acetyltransferase component of acetoin cleaving system
BCAL1910			-9.71	acetoin:2,6-dichlorophenolindophenol oxidoreductase beta subunit
BCAL1911			-13.02	acetoin:2,6-dichlorophenolindophenol oxidoreductase alpha subunit
BCAL1913			-13.53	putative acetoin catabolism protein
BCAL1915		-2.87		putative decarboxylase
BCAL1918			-2.09	conserved hypothetical protein
BCAL1919			8.03	ClpB heat-shock protein
BCAL1920		2.04		putative DNA-binding protein
BCAL1925			-5.58	threonine synthase
BCAL1926			-5.37	homoserine dehydrogenase
BCAL1927			-2.09	putative aminotransferase
BCAL1935			-2.48	polysaccharide deacetylase

BCAL1936			-2.43	AhpC/TSA family protein
BCAL1939			-1.92	putative integral membrane transport protein
BCAL1940			-2.02	conserved hypothetical protein
BCAL1941			-2.11	replicative DNA helicase
BCAL1944	-1.74		-2.12	putative primosomal replication protein
BCAL1947			1.62	putative sigma factor
BCAL1949			2.75	glyoxylate carboligase
BCAL1952			-4.44	conserved hypothetical protein
BCAL1955			-2.20	putative membrane protein
BCAL1960			1.70	putative exported protein
BCAL1961			2.36	putative exported protein
BCAL1966			1.68	tRNA-modifying protein YgfZ
BCAL1970			-2.44	thioesterase superfamily protein
BCAL1979	2.50			putative fatty acid degradation protein
BCAL1981			1.84	conserved hypothetical protein
BCAL1990			-1.96	glucose-6-phosphate isomerase
BCAL1991			1.76	ABC transporter ATP-binding protein
BCAL1992			3.50	putative acyl-CoA thioesterase precursor
BCAL1994			2.25	ATP-dependent protease La
BCAL2008			2.00	putative exported protein
BCAL2013			2.32	AhpC/TSA family protein
BCAL2024			-1.99	large-conductance mechanosensitive channel protein
BCAL2030			1.94	putative purine catabolism-related protein
BCAL2040			1.69	polysaccharide deacetylase
BCAL2044			1.92	muramoyltetrapeptide carboxypeptidase
BCAL2062	1.77		2.04	putative membrane protein
BCAL2063			1.60	inosine-5'-monophosphate dehydrogenase
BCAL2075			-2.29	putative ATP-binding protein
BCAL2081			-1.95	UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase
BCAL2082			-2.22	chaperone protein Skp precursor
BCAL2087			-2.07	undecaprenyl pyrophosphate synthetase
BCAL2088			-1.93	ribosome recycling factor
BCAL2094			-2.66	putative RNA pseudouridylate synthase
BCAL2098			-2.31	putative chromosome partition protein
BCAL2101			-2.03	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase
BCAL2102			-2.74	ArsC family protein
BCAL2103			-3.77	succinyl-diaminopimelate desuccinylase
BCAL2104			-4.28	putative DNA methylase
BCAL2115A	1.76			family M22 non-peptidase homologue
BCAL2117			-4.49	putative ATP-dependent RNA helicase
BCAL2118	2.26		47.25	isocitrate lyase
BCAL2119			9.79	universal stress protein family protein
BCAL2120			7.00	LysR family regulatory protein

BCAL2121			5.70	putative dehalogenase
BCAL2122			2.66	malate synthase A
BCAL2123	2.14		1.70	conserved hypothetical protein
BCAL2126			-2.33	glutamyl-tRNA synthetase
BCAL2130			-2.12	binding-protein-dependent transport system protein
BCAL2133			2.87	putative membrane protein
BCAL2136A		5.53		putative exported protein
BCAL2137		5.83		two-component regulatory system, response regulator protein
BCAL2138		5.99		two-component regulatory system, sensor kinase protein
BCAL2139		5.69		two-component regulatory system, response regulator protein
BCAL2140		9.99	2.07	putative exported protein
BCAL2141		13.38	3.51	cytochrome O ubiquinol oxidase protein
BCAL2142		11.80	2.82	cytochrome o ubiquinol oxidase subunit III
BCAL2143		13.19	2.51	ubiquinol oxidase polypeptide I
BCAL2144		7.73	2.74	ubiquinol oxidase polypeptide II precursor
BCAL2146			-2.03	aspartokinase
BCAL2148			-2.52	acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha
BCAL2149			-4.59	HhH-GPD superfamily base excision DNA repair protein
BCAL2150			-2.59	cysteinyl-tRNA synthetase
BCAL2153			-3.52	peptidyl-prolyl cis-trans isomerase B
BCAL2154			-2.39	putative UDP-2,3-diacylglucosamine hydrolase (lipid A biosynthesis related protein)
BCAL2161			1.71	putative exported protein
BCAL2162			2.02	putative FkbP-type peptidyl-prolyl cis-trans isomerase
BCAL2163	-1.56			Cupin superfamily protein
BCAL2165			-2.52	metallo-beta-lactamase superfamily protein
BCAL2170			1.90	metallo peptidase, subfamily M50B
BCAL2190			-1.95	lysyl-tRNA synthetase
BCAL2191	-1.68		2.78	putative membrane protein
BCAL2192			1.54	conserved hypothetical protein
BCAL2194			1.77	chaperone protein HscA homolog
BCAL2195			2.49	co-chaperone protein HscB homolog
BCAL2196			3.52	HesB family protein
BCAL2197			3.10	putative iron-sulfur cluster scaffold protein
BCAL2198			4.05	cysteine desulfurase
BCAL2199		3.46	6.31	putative transcriptional regulator protein
BCAL2200			3.69	low molecular weight phosphotyrosine protein phosphatase
BCAL2201			8.89	putative membrane protein
BCAL2204			1.80	IclR family regulatory protein
BCAL2205			1.54	D-alanyl-D-alanine endopeptidase (penicillin-binding protein precursor)
BCAL2206	-2.00	-4.80	-6.19	phasin-like protein

BCAL2207			-6.60	putative dihydrolipoamide dehydrogenase
BCAL2208			-7.09	dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex
BCAL2209			-4.95	pyruvate dehydrogenase E1 component 1
BCAL2210			-1.95	two-component regulatory system, sensor kinase protein
BCAL2211			-1.95	two-component regulatory system, response regulator protein
BCAL2212			1.51	FolD bifunctional protein
BCAL2213			3.41	oligopeptidase A
BCAL2214			2.38	putative DNA polymerase IV
BCAL2215			-2.25	putative racemase
BCAL2223			-2.89	putative nitrogen regulation protein NR(II)
BCAL2228			1.89	putative membrane protein
BCAL2229			1.52	putative exported protein
BCAL2232			-2.52	Fe(2+) trafficking protein
BCAL2237			-13.87	putative aminotransferase
BCAL2238			-8.81	putative glutamine synthetase
BCAL2239			-5.91	putative gamma-glutamyl-gamma-aminobutyrate hydrolase
BCAL2240			-2.98	HutG protein
BCAL2242	1.83			putative imidazolonepropionase
BCAL2243	2.05			conserved hypothetical protein
BCAL2244			-2.77	urocanate hydratase
BCAL2246			-2.06	histidine ammonia-lyase
BCAL2251	2.16			Major Facilitator Superfamily protein
BCAL2274		2.03		putative exported protein
BCAL2275			1.51	conserved hypothetical protein
BCAL2284	1.69		-1.94	acetyl-coenzyme A synthetase
BCAL2287			-2.74	putative fumarate hydratase
BCAL2289			-2.03	putative glutamate racemase
BCAL2290			2.13	putative bacterioferritin ferredoxin protein
BCAL2292		2.09		putative bipolymer transport protein
BCAL2295			11.20	putative chromosome condensation protein
BCAL2296			2.70	conserved hypothetical protein
BCAL2297		3.65		conserved hypothetical protein
BCAL2299			1.84	putative permease protein
BCAL2300			1.93	putative exported protein
BCAL2301			2.13	putative exported protein
BCAL2305			-2.20	putative potassium channel subunit
BCAL2306			-2.06	conserved hypothetical protein
BCAL2307			-2.25	conserved hypothetical protein
BCAL2312		3.66		hypothetical protein
BCAL2315			1.79	putative membrane protein
BCAL2317			-2.82	family M14 unassigned peptidase
BCAL2322	1.92			putative enoyl-CoA hydratase

BCAL2323			-2.38	putative glutathione S-transferase
BCAL2324			-3.28	conserved hypothetical protein
BCAL2325			-2.11	conserved hypothetical protein
BCAL2329			-3.14	NUDIX hydrolase
BCAL2330			-2.66	putative membrane protein
BCAL2331			-3.24	NADH dehydrogenase I chain N
BCAL2332			-3.11	NADH dehydrogenase I chain M
BCAL2333			-2.95	NADH-ubiquinone oxidoreductase I chain L
BCAL2334			-3.06	NADH-ubiquinone oxidoreductase I chain K
BCAL2335			-2.69	NADH dehydrogenase I chain J
BCAL2336			-2.54	putative NADH dehydrogenase I chain I
BCAL2337			-2.74	NADH dehydrogenase I chain H
BCAL2338			-2.38	putative NADH dehydrogenase I chain G
BCAL2339			-3.23	NADH dehydrogenase I chain F
BCAL2340			-2.29	putative NADH dehydrogenase I chain E
BCAL2341			-2.10	NADH dehydrogenase I chain D
BCAL2344	-1.76			NADH dehydrogenase I chain A
BCAL2345			-2.50	protein-export membrane protein SecG
BCAL2347			-3.32	putative oxidoreductase
BCAL2348			-3.23	polyribonucleotide nucleotidyltransferase
BCAL2351			-2.59	putative lipoprotein
BCAL2352		-12.69	-10.68	putative carbonic anhydrase
BCAL2353		-11.38	-9.52	putative sulfate transporter
BCAL2354			-2.95	2-isopropylmalate synthase
BCAL2355			-2.94	putative phosphatidyltransferase
BCAL2357			-4.00	ketol-acid reductoisomerase
BCAL2358	-1.68		-3.77	acetolactate synthase isozyme III small subunit
BCAL2359	-1.87		-3.35	acetolactate synthase isozyme III large subunit
BCAL2360			-1.84	putative sigma factor
BCAL2364	-2.10		2.43	putative exported protein
BCAL2373			-2.04	putative globin
BCAL2375	1.75		1.60	putative membrane protein
BCAL2381			-26.08	potassium-transporting ATPase C chain
BCAL2382			-38.61	potassium-transporting ATPase B chain
BCAL2383			-26.53	potassium-transporting ATPase A chain
BCAL2384			-2.04	quinone oxidoreductase
BCAL2391			-3.10	putative nicotinate-nucleotide adenylyltransferase
BCAL2392			-2.39	conserved hypothetical protein
BCAL2393			-2.47	conserved hypothetical protein
BCAL2396			-2.15	conserved hypothetical protein
BCAL2397			-8.37	putative lipoprotein
BCAL2407			-2.32	putative glycosyltransferase, wabS
BCAL2410			1.82	conserved hypothetical protein
BCAL2411			1.60	putative glutamyl tRNA synthetase

BCAL2412			-2.09	putative ATP-dependent RNA helicase 2
BCAL2413			-2.85	putative exported protein
BCAL2417			-2.33	putative DNA translocase
BCAL2419			1.57	glycosyl hydrolases family protein
BCAL2420			-3.80	putative depolymerase/histone-like protein
BCAL2428			-2.02	putative cytochrome C precursor-related protein
BCAL2431			-2.19	conserved hypothetical protein
BCAL2434			-5.66	glyoxalase/bleomycin resistance protein/dioxygenase superfamily protein
BCAL2436		2.06		putative membrane protein
BCAL2440	1.88		1.65	conserved hypothetical protein
BCAL2441	2.60		2.71	chorismate lyase
BCAL2442			1.96	chaperone protein HtpG
BCAL2443			1.95	GntR family regulatory protein
BCAL2444		-3.73		conserved hypothetical protein
BCAL2448			-2.14	putative phenazine biosynthesis-like protein
BCAL2449			-2.44	putative cyclic-di-GMP signaling protein
BCAL2453			1.90	putative membrane protein
BCAL2454			-3.07	topoisomerase IV subunit A, antitoxin
BCAL2455			-3.21	topoisomerase IV subunit B, toxin
BCAL2456			-3.35	ABC transporter ATP-binding protein
BCAL2457			-2.33	putative exported protein
BCAL2459	-1.85			putative O-methyltransferase
BCAL2467		1.91	4.44	putative lipoprotein
BCAL2468			3.24	putative membrane protein
BCAL2471		-5.54	-2.37	conserved hypothetical protein
BCAL2472		-3.82		alpha,alpha-trehalose-phosphate synthase (UDP-forming)
BCAL2475a		2.15		conserved hypothetical protein
BCAL2476			-2.94	hypothetical protein
BCAL2476c			-2.45	putative lysozyme
BCAL2480		1.92		ATP-dependent DNA helicase (pseudogene)
BCAL2485			-3.79	putative iron-sulphur cluster binding electron transport protein
BCAL2493			-2.04	putative membrane protein
BCAL2496			-2.26	
BCAL2512			-2.66	putative membrane protein
BCAL2514			1.78	conserved hypothetical protein
BCAL2516		1.98		hypothetical protein
BCAL2518		1.70		putative DNA-binding protein
BCAL2519			2.59	putative O-antigen acetylase
BCAL2523			2.28	LysR family regulatory protein
BCAL2523A			2.33	putative membrane protein
BCAL2524			4.42	AraC family regulatory protein
BCAL2551			3.11	putative membrane protein
BCAL2555			1.52	conserved hypothetical protein

BCAL2559		1.74	1.83	putative transcriptional regulator
BCAL2563		2.28	1.92	putative IstB-like insertion sequence protein
BCAL2577			-2.12	DJ-1/PfpI family protein
BCAL2581		1.76	1.78	putative transposase-related protein (pseudogene)
BCAL2583		2.34	2.24	transposase
BCAL2584		1.92	4.62	metallo-beta-lactamase superfamily protein
BCAL2585		2.35	2.11	putative nitrilase
BCAL2586		2.05	2.33	AraC family regulatory protein
BCAL2587		3.07		putative alcohol dehydrogenase
BCAL2588		1.74		putative transposase (fragment)
BCAL2591		2.61	1.58	
BCAL2597		2.06		hypothetical protein
BCAL2600			-2.09	putative phage integrase protein
BCAL2606	1.83		2.81	two-component regulatory system, response regulator protein
BCAL2607			7.55	putative exported protein
BCAL2608			18.64	Pirin-like protein
BCAL2615			2.60	putative exported outer membrane porin protein
BCAL2623	1.94			putative aldehyde dehydrogenase
BCAL2626			1.85	putative DNA-binding protein
BCAL2631			-3.74	phosphoenolpyruvate carboxylase
BCAL2636			1.72	putative fimbriae chaperone
BCAL2637			2.43	putative fimbriae usher protein
BCAL2644			2.67	putative ATP-binding protein
BCAL2645			-2.42	putative OmpA family membrane protein
BCAL2648			-2.30	putative outer membrane protein
BCAL2650			-2.07	putative chromosome condensation and segregation protein
BCAL2657	1.90			putative bifunctional cobalamin biosynthesis protein
BCAL2669			1.53	putative exported protein
BCAL2678			-2.47	putative permease protein
BCAL2680		-14.77	-3.95	putative uroporphyrin-III C-methyltransferase
BCAL2681		-17.90	-4.53	putative sulfate adenylyltransferase subunit 1
BCAL2682		-14.49	-3.70	putative sulfate adenylyltransferase subunit 2
BCAL2683		-22.65	-2.90	putative phosphoadenosine phosphosulfate reductase
BCAL2684		-33.12	-2.75	conserved hypothetical protein
BCAL2685		-12.73		putative sulfite reductase
BCAL2690		-5.64		short chain dehydrogenase
BCAL2694			-2.40	putative dehydrogenase
BCAL2705			-2.92	ABC transporter ATP-binding protein
BCAL2706			-2.79	ABC transporter ATP-binding protein
BCAL2707			-2.29	putative transport system permease protein
BCAL2708			-2.23	putative amino-acid transport permease protein
BCAL2711			1.56	putative FkpB-type peptidyl-prolyl cis-trans isomerase
BCAL2716			-2.48	putative L-aspartate oxidase

BCAL2717			-2.16	putative nicotinate-nucleotide pyrophosphorylase
BCAL2724			-2.61	isoleucyl-tRNA synthetase
BCAL2725			-2.45	signal peptidase II
BCAL2726			-2.61	putative DNA/pantothenate metabolism flavoprotein
BCAL2727			-2.78	deoxyuridine 5'-triphosphate nucleotidohydrolase
BCAL2732		1.98		cold shock-like protein
BCAL2733			2.23	putative multicopper oxidase
BCAL2734			40.00	conserved hypothetical protein
BCAL2741		1.70		putative exported protein
BCAL2749			1.57	putative diguanylate phosphodiesterase
BCAL2769			2.85	conserved hypothetical protein
BCAL2770			2.14	putative membrane protein
BCAL2771			1.63	conserved hypothetical protein
BCAL2772	2.04			putative AMP-binding enzyme
BCAL2773	1.75			putative integrase/recombinase
BCAL2775			1.54	4Fe-4S ferredoxin
BCAL2776			1.99	putative hydrolase
BCAL2777			1.92	putative N-acetylmuramoyl-L-alanine amidase
BCAL2780			8.15	putative thioredoxin protein
BCAL2781			4.26	ThiF family protein
BCAL2782			1.53	putative pyridoxamine 5'-phosphate oxidase
BCAL2783			1.53	putative cyclopropane-fatty-acyl-phospholipid synthase
BCAL2785			2.11	putative peptide methionine sulfoxide reductase
BCAL2786		-2.94		putative selenium-binding protein
BCAL2790	1.99	14.02		putative cyclase, Arylformamidase
BCAL2791	1.93	10.64		putative kynureninase
BCAL2792		12.26	-3.52	putative tryptophan 2,3-dioxygenase
BCAL2793			-3.25	Major Facilitator Superfamily protein
BCAL2794			-4.36	putative ketopantoate reductase
BCAL2795		-4.63	-5.63	aldehyde dehydrogenase family protein
BCAL2796		-3.81		putative benzoylformate decarboxylase
BCAL2799	2.38			putative carbohydrate kinase
BCAL2808			-2.31	putative periplasmic ABC transporter substrate-binding component
BCAL2816		-3.44	-4.55	S-formylglutathione hydrolase
BCAL2817			-3.75	S-(hydroxymethyl)glutathione dehydrogenase
BCAL2819			2.20	putative permease protein
BCAL2820			-3.75	efflux system outer membrane protein
BCAL2821			-2.90	RND family efflux system transporter protein
BCAL2822			-1.95	efflux system transport protein
BCAL2823			1.97	TetR family regulatory protein
BCAL2824			1.86	isochorismatase family protein
BCAL2828			1.70	putative exported protein
BCAL2830	1.74			two-component regulatory system, sensor kinase protein

BCAL2833			-2.71	putative membrane protein
BCAL2834			-3.06	putative acylhydrolase
BCAL2835	1.71			conserved hypothetical protein
BCAL2836	1.73			phosphoribosylaminoimidazole carboxylase ATPase subunit
BCAL2840			-2.48	putative pyruvate kinase II protein
BCAL2857	-1.77			conserved hypothetical protein
BCAL2862			-2.75	pyridoxal phosphate biosynthetic protein PdxJ 1
BCAL2863			-2.32	DNA repair protein RecO 1
BCAL2867			1.56	GTP-binding protein LepA 1
BCAL2869			1.72	serine protease MucD 1
BCAL2870			1.77	sigma-E factor regulatory protein RseB precursor 1
BCAL2871			1.88	sigma-E factor negative regulatory protein 1
BCAL2878			2.00	3-oxoacyl-[acyl-carrier-protein] synthase III 1
BCAL2880		2.49		50S ribosomal protein L32 1
BCAL2883			-2.56	putative tetrapyrrole methylase
BCAL2884			-2.57	serine peptidase, family S49
BCAL2889	2.12		2.63	molybdenum cofactor biosynthesis protein A 1
BCAL2890	1.92		1.83	putative molybdopterin-guanine dinucleotide biosynthesis protein A 1
BCAL2891	2.35			molybdopterin biosynthesis protein MoeA 1
BCAL2901			-2.20	AraC family regulatory protein
BCAL2904		2.33		conserved hypothetical protein
BCAL2905			2.99	hypothetical protein
BCAL2907			1.56	multidrug resistance protein NorM
BCAL2912			1.71	putative thymidylate synthase
BCAL2919			1.57	oligoribonuclease
BCAL2925			-2.46	50S ribosomal protein L19
BCAL2926			-2.27	tRNA(guanine-N(1)-)-methyltransferase
BCAL2932	2.19		-2.14	leucine-responsive regulatory protein
BCAL2933	3.97	-3.98	-2.37	D-amino acid dehydrogenase small subunit
BCAL2936			-2.54	putative exported protein
BCAL2937			-2.47	ABC transporter membrane permease
BCAL2943			-2.91	putative exported protein
BCAL2944			-3.45	ADP-l-glycero-D-manno-heptose-6-epimerase
BCAL2949			-1.96	integration host factor beta-subunit
BCAL2962b			-2.25	conserved hypothetical protein
BCAL2965			1.67	conserved hypothetical protein
BCAL2965a			1.90	conserved hypothetical protein
BCAL2965b	-1.68			conserved hypothetical protein
BCAL2965C			1.83	conserved hypothetical protein
BCAL2965D			1.60	hypothetical protein
BCAL2971b	-1.68			conserved hypothetical protein
BCAL2977	1.75			NAD-dependent formate dehydrogenase beta subunit
BCAL2978			-2.34	NAD-dependent formate dehydrogenase alpha subunit

BCAL2979	2.45			NAD-dependent formate dehydrogenase delta subunit
BCAL2980	2.14			putative oxygenase
BCAL2982			-2.01	citrate-proton symporter
BCAL2985			2.28	GntR family regulatory protein
BCAL2986	2.06			putative amidotransferase
BCAL2990		-3.02		guanine deaminase
BCAL2992			1.65	putative lipoprotein
BCAL2993			-2.01	aminopeptidase N
BCAL2995	-1.57			LuxR superfamily regulatory protein
BCAL2998			-2.32	transglycosylase associated protein
BCAL2999			-4.13	putative exported protein
BCAL3003	3.02		3.51	hypothetical protein
BCAL3006			-3.23	cold shock-like protein
BCAL3008		-4.70		putative outer membrane porin protein
BCAL3011			2.27	DNA-directed RNA polymerase omega chain
BCAL3012			-2.07	guanylate kinase
BCAL3032			1.90	putative ATPase protein
BCAL3033			4.04	probable outer-membrane lipoproteins carrier protein
BCAL3035			1.83	thioredoxin reductase
BCAL3038	1.96			ABC transporter ATP-binding component
BCAL3039	2.00		-1.86	ABC transporter, membrane permease
BCAL3040	2.01		-2.60	ABC transporter, membrane permease
BCAL3041	1.95			maltose-binding protein
BCAL3043			-2.31	6-phosphogluconolactonase
BCAL3044			-2.16	glucokinase
BCAL3049			2.38	glutamate-1-semialdehyde 2,1-aminomutase
BCAL3050	1.73			multifunctional riboflavin biosynthetic protein (includes: diaminohydroxyphosphoribosylaminopyrimidine deaminase; 5-amino-6-(5-phosphoribosylamino)uracil reductase)
BCAL3052			-1.99	conserved hypothetical protein
BCAL3054			-2.63	6,7-dimethyl-8-ribityllumazine synthase
BCAL3055			-2.28	probable N utilization substance protein B
BCAL3056			-3.36	putative aminotransferase
BCAL3059A			1.56	putative exported protein
BCAL3076A		1.81	1.56	putative DNA-binding protein
BCAL3077	-1.52	2.16	2.35	putative membrane protein
BCAL3078		1.96	1.81	putative lipoprotein
BCAL3079			1.78	hypothetical protein
BCAL3080			2.19	hypothetical protein
BCAL3081			2.28	hypothetical protein
BCAL3082			3.00	putative phage resolvase/recombinase for integration and excision
BCAL3083	-1.69			putative membrane protein
BCAL3091			1.96	putative undecaprenol kinase
BCAL3094	4.40		10.44	oxygen-independent coproporphyrinogen III oxidase

BCAL3095			1.78	conserved hypothetical protein
BCAL3112	1.75			heptosyltransferase I
BCAL3117			-2.01	UDP-glucose epimerase
BCAL3118			-2.18	UDP-N-acetylglucosamine-1-P transferase
BCAL3121			-1.98	putative nucleotide sugar epimerase/dehydratase
BCAL3124			-2.53	glycosyltransferase
BCAL3125			-3.25	
BCAL3128			-3.48	glycosyltransferase
BCAL3129			-3.06	nucleotide sugar aminotransferase
BCAL3130			-2.97	ABC transporter ATP-binding protein
BCAL3131			-2.39	putative ABC transporter, membrane permease
BCAL3132			-2.23	dTDP-4-keto-L-rhamnose reductase
BCAL3134			-1.96	glucose-1-phosphate thymidylyltransferase
BCAL3138			-2.76	dihydroorotase-like protein
BCAL3147			1.60	10 kDa chaperonin 1
BCAL3148			-2.77	Polyketide cyclase / dehydrase and lipid transport Family
BCAL3149			-2.45	Outer membrane lipoprotein carrier protein LolA
BCAL3150			-2.18	putative exported protein
BCAL3151			-2.15	putative transmembrane anti-sigma factor
BCAL3153			-2.40	putative lipoprotein
BCAL3154	4.85			putative glycine-rich surface protein
BCAL3161			2.69	putative membrane protein
BCAL3162			5.47	hypothetical protein
BCAL3163			6.24	putative nucleotidyltransferase
BCAL3180			1.59	putative oxidoreductase
BCAL3182			-2.92	Major Facilitator Superfamily protein
BCAL3183			-3.60	putative hydrolase
BCAL3184		-5.88	-3.86	homogentisate 1,2-dioxygenase
BCAL3185			-2.63	4-hydroxybenzoate transporter
BCAL3187			-2.17	putative oxidoreductase
BCAL3190		2.29	1.57	IclR family regulatory protein
BCAL3192		-3.44	-2.40	putative oxidoreductase
BCAL3193			2.10	conserved hypothetical protein
BCAL3196			-2.82	putative ATP-binding regulatory protein
BCAL3197			-2.08	serine hydroxymethyltransferase
BCAL3205C	-1.55			hypothetical protein
BCAL3208a			1.72	conserved hypothetical protein, antitoxin
BCAL3223		2.08		
BCAL3228		2.53		hypothetical protein
BCAL3229		2.64		conserved hypothetical protein
BCAL3229A		1.87		hypothetical protein
BCAL3232		1.90		hypothetical protein
BCAL3234		2.41		glycosyltransferase
BCAL3235		3.68	1.58	putative UDP-galactopyranose mutase

BCAL3246			-2.65	putative GDP-mannose pyrophosphorylase
BCAL3247			-2.05	mechanosensitive ion channel protein
BCAL3259	2.00			tetracycline resistance protein, class C (pseudogene)
BCAL3260	1.68			conserved hypothetical protein
BCAL3263			-2.72	conserved hypothetical protein
BCAL3264			-2.59	putative polynucleotide adenylyltransferase
BCAL3266			-1.84	putative deoxyguanosine kinase/deoxyadenosine kinase
BCAL3267			-2.49	putative 3-methyl-2-oxobutanoate hydroxymethyltransferase
BCAL3268			1.54	chorismate binding enzyme
BCAL3269			2.98	putative DnaJ chaperone protein
BCAL3270			3.87	putative DnaK chaperone protein
BCAL3271			5.51	thioredoxin
BCAL3272	1.70		6.66	putative heat shock protein
BCAL3275			1.61	putative heat-inducible transcription repressor
BCAL3278		-3.10	-2.63	putative glutamate-ammonia-ligase adenylyltransferase
BCAL3282			-4.23	putative phospho-2-dehydro-3-deoxyheptonate aldolase
BCAL3283			-5.49	conserved hypothetical protein, antitoxin
BCAL3284			-7.37	conserved hypothetical protein, toxin
BCAL3285			1.97	flavohemoprotein hmpA
BCAL3286	1.85			cobalamin adenosyltransferase protein
BCAL3288			-2.06	putative glycolate oxidase subunit GlcD
BCAL3289	1.77			putative glycolate oxidase subunit GlcE
BCAL3297		-3.33	-2.56	putative ferritin DPS-family DNA binding protein
BCAL3299		-3.34		peroxidase/catalase KatB
BCAL3304			-2.84	putative queuine tRNA-ribosyltransferase
BCAL3305	1.76		1.61	preprotein translocase subunit
BCAL3311			1.94	putative exported protein
BCAL3312			1.97	putative cytochrome b-561 membrane protein
BCAL3313	1.74			putative membrane protein
BCAL3315			-2.31	mammalian cell entry related membrane protein
BCAL3317			-4.05	putative membrane protein
BCAL3322			-3.80	putative DNA-binding protein
BCAL3326	2.05			NAD(P) transhydrogenase subunit alpha
BCAL3327	1.83			NAD(P) transhydrogenase subunit alpha
BCAL3330			-2.96	putative glutamate synthase
BCAL3339			1.51	holliday junction DNA helicase
BCAL3345	1.94		1.97	conserved hypothetical protein
BCAL3346			2.02	putative HesB-like protein
BCAL3348			-2.13	50S ribosomal protein L13
BCAL3353			2.75	putative outer membrane autotransporter
BCAL3357			1.53	glutamate/aspartate transport system permease protein
BCAL3358			1.52	periplasmic glutamate/aspartate-binding protein
BCAL3359			3.54	putative glutamate dehydrogenase
BCAL3362		2.37	2.30	putative oxidoreductase

BCAL3363			3.19	TetR family regulatory protein
BCAL3364			2.47	putative gluconokinase
BCAL3365		1.55		putative gluconate permease
BCAL3366			-3.92	KHG/KDPG aldolase
BCAL3367			-3.68	phosphogluconate dehydratase
BCAL3370			1.66	gamma-glutamyl phosphate reductase
BCAL3372			-2.05	putative lipoprotein
BCAL3374	1.71			putative transport-related membrane protein
BCAL3380		-4.06		putative allantoicase
BCAL3384			1.96	Major Facilitator Superfamily protein
BCAL3391	1.82			glyoxalase/bleomycin resistance protein/dioxygenase superfamily protein
BCAL3396			1.61	putative thiamine-monophosphate kinase
BCAL3405			-3.22	putative L-arabinose transport system, exported protein
BCAL3412			-2.87	putative peptidoglycan biosynthesis-related protein
BCAL3413	1.82			shikimate 5-dehydrogenase
BCAL3414			-2.75	putative exoribonuclease II
BCAL3418			-1.88	conserved hypothetical protein
BCAL3421			-1.97	biotin carboxylase
BCAL3424			-1.93	thiol peroxidase
BCAL3425			2.65	putative sugar kinase
BCAL3426			3.04	putative lipoprotein
BCAL3427			-3.12	histone H1-like protein
BCAL3431	2.36		2.30	putative exported protein
BCAL3432			1.64	cytochrome c assembly protein
BCAL3436			1.58	prolyl-tRNA synthetase
BCAL3443			-2.31	octaprenyl-diphosphate synthase
BCAL3444			-2.07	putative membrane protein
BCAL3451			-2.14	conserved hypothetical protein
BCAL3452			-2.37	arginine biosynthesis bifunctional protein ArgJ
BCAL3456			-2.33	putative thioredoxin reductase
BCAL3457			-2.82	cell division protein FtsZ
BCAL3458			-2.41	cell division protein FtsA
BCAL3459			-3.37	cell division protein FtsQ
BCAL3460			-2.56	D-alanine--D-alanine ligase B
BCAL3461			-2.31	UDP-N-acetylmuramate--alanine ligase
BCAL3464			-2.47	UDP-N-acetylmuramoylalanine--D-glutamate ligase
BCAL3473			1.74	putative outer membrane porin
BCAL3477	1.87			putative catalase
BCAL3482			-2.75	conserved hypothetical protein
BCAL3483			-2.89	hypothetical protein
BCAL3485			-2.62	putative membrane protein
BCAL3488			-2.42	conserved hypothetical protein
BCAL3489		-4.02	-4.05	conserved hypothetical protein
BCAL3491	2.32			putative exported protein

BCAL3505		2.40		flagellar motor switch protein FliN
BCAL3506		5.13		flagellar motor switch protein FliM
BCAL3509	2.36			LrgA family protein
BCAL3512			2.14	MarR family regulatory protein
BCAL3513	2.12		6.28	putative membrane protein
BCAL3514			2.17	outer membrane efflux protein
BCAL3523			-2.65	type II secretion system protein G
BCAL3524			-2.55	putative type II secretion system protein
BCAL3525			-3.98	type II secretion system protein F
BCAL3526			-5.86	type II secretion system protein E
BCAL3527			-5.21	type II secretion system protein D
BCAL3529			-2.15	putative cobalamin synthesis protein/P47K
BCAL3530			-2.79	DNA-binding protein HU-alpha
BCAL3531	1.93			FAD dependent oxidoreductase
BCALr00219		2.59	3.04	tRNA-Trp
BCALr0075B	-2.02			misc_RNA, glycine riboswitch
BCALr0080		2.93	2.18	Group II catalytic intron
BCALr0164		3.07	3.03	tRNA-Ala
BCALr0217c	2.28	3.04	4.18	tRNA-Ala
BCALr0218a			2.10	tRNA-Tyr
BCALr0218c	2.20		2.51	tRNA-Thr
BCALr0296		2.14		misc_RNA, TPP riboswitch
BCALr0409c	2.41	2.90	4.77	tRNA-Ala
BCALr0457		2.79		tRNA-Lys
BCALr0472	-2.87	2.43		tRNA-Phe
BCALr0800	-2.16			tRNA-Gln
BCALr0914		2.54		misc_RNA, FMN riboswitch
BCALr0949	-1.75	2.09		tRNA Met
BCALr0970a			2.47	tRNA-Asn
BCALr0970b			2.39	tRNA-Asn
BCALr1019b	-1.61			tRNA-Gly
BCALr1019d		3.38	2.97	tRNA-Cys
BCALr1104		8.39	3.98	misc_RNA, TPP riboswitch
BCALr1117	-1.59		-4.04	misc_RNA, Group I catalytic intron
BCALr1279		3.13	4.05	tRNA-Pro
BCALr1290	-2.21			tRNA-Arg
BCALr1480			2.05	tRNA-Val
BCALr1492		7.78	10.00	tRNA-Pro
BCALr1551a		2.87	2.83	tRNA-Leu
BCALr1551b		2.92	2.77	tRNA-Leu
BCALr1552		2.89	2.81	tRNA-Leu
BCALr1614		5.13		tRNA-Met
BCALr1625	-1.96			tRNA-His
BCALr1708		1.86		misc_RNA, cobalamin riboswitch

BCALr1709		2.42	1.62	misc_RNA, cobalamin riboswitch
BCALr1899	1.63	2.44	2.86	misc_RNA, bacterial signal recognition particle RNA
BCALr1993a	-4.55			tRNA-Asp
BCALr1993b		2.11		tRNA-Val
BCALr2006	2.18	3.69	8.43	tRNA-Leu
BCALr2125a	-4.70			tRNA-Asp
BCALr2125c			1.66	tRNA-Asp
BCALr2125e	-4.46			tRNA-Asp
BCALr2125g		3.44	3.20	tRNA-Ala
BCALr2145			2.40	tRNA-Ser
BCALr2219			2.55	tRNA-Met
BCALr2231c	2.36	3.09	4.61	tRNA-Ala
BCALr2236	-2.12			TRNA-Val
BCALr2281	-2.80			tRNA-Ser
BCALr2344		1.87	4.62	tRNA-Leu
BCALr2458	-1.83			tRNA-Arg
BCALr2462		5.07	2.62	tRNA-Met
BCALr2665	2.20	1.62		misc_RNA, cobalamin riboswitch
BCALr2852a		2.88	2.78	tRNA-Cys
BCALr2852b	-1.51			tRNA-Gly
BCALr2852d	-1.60			tRNA-Gly
BCALr2899a			2.35	tRNA-Asn
BCALr2899b			2.41	tRNA-Asn
BCALr2990	1.73	2.07	1.92	misc_RNA, yybP-ykoY element
BCALr2994	-1.58			tRNA-Thr
BCALr3016	-2.01	2.20		tRNA-Ser
BCALr3029		2.32	2.97	tRNA-Ser
BCALr3205		10.15		tRNA-Lys
BCALr3349			1.76	misc_RNA, bacterial RNase P class A
BCALr3443	2.49	3.13	4.01	tRNA-Pro
BCALr3484	2.66	5.19	2.56	tRNA-Val
BCAM0003			1.80	putative partition protein ParA
BCAM0005			1.66	putative replication protein
BCAM0005A		1.71		hypothetical protein
BCAM0007			2.52	putative phage integrase
BCAM0008			2.27	conserved hypothetical protein
BCAM0010		-8.05	-1.87	2-amino-3-ketobutyrate coenzyme A ligase
BCAM0011		-10.24	-2.60	threonine 3-dehydrogenase
BCAM0012			-7.08	putative membrane protein
BCAM0013			-12.18	putative acetyltransferase-GNAT family
BCAM0014			3.75	TetR family regulatory protein
BCAM0020		-3.22	-6.62	putative membrane protein
BCAM0021		-3.63	-7.38	putative patatin-like phospholipase
BCAM0022		-3.75	-6.35	putative D-beta-hydroxybutyrate dehydrogenase

BCAM0023		-3.54	-6.96	acetoacetate decarboxylase
BCAM0029		-3.20		putative protein kinase
BCAM0030		-4.13	-4.22	conserved hypothetical protein
BCAM0031		-4.45	-5.07	conserved hypothetical protein
BCAM0032		-3.50		conserved hypothetical protein
BCAM0043			2.15	conserved hypothetical protein
BCAM0046			-1.86	hypothetical protein
BCAM0048	2.30		8.40	LysR family regulatory protein
BCAM0049	4.17		18.89	CRP family regulatory protein
BCAM0050a			1.87	conserved hypothetical protein
BCAM0056			3.51	LysR family regulatory protein
BCAM0058			-1.90	3-oxoadipate CoA-transferase subunit A
BCAM0061			-2.33	putative 3-oxoadipate enol-lactonase I
BCAM0062			-2.83	4-carboxymuconolactone decarboxylase
BCAM0064	1.85			conserved hypothetical protein
BCAM0065A		2.53		insertion element hypothetical protein
BCAM0069			-4.65	conserved hypothetical protein
BCAM0070			-3.62	putative hydrolase
BCAM0071			-4.32	puatative mandelate racemase/muconate lactonizing enzyme
BCAM0072			-3.68	putative thiamine pyrophosphate enzyme
BCAM0073			-3.29	hypothetical protein
BCAM0074			-2.19	conserved hypothetical protein
BCAM0129			-2.73	TetR family regulatory protein
BCAM0131		-3.59	-3.74	chaperone protein HchA
BCAM0142		-3.40	-3.42	putative acyl-CoA dehydrogenase family protein
BCAM0149		2.14		putative phospholipase D protein
BCAM0152		2.76	2.03	putative lipoprotein
BCAM0153		2.92	2.78	2-keto-3-deoxygluconate permease
BCAM0154		2.55	4.71	4-deoxy-L-threo-5-hexosulose-uronate ketol-isomerase
BCAM0155			4.81	2-deoxy-D-gluconate 3-dehydrogenase
BCAM0160			1.95	putative exported protein
BCAM0163	-1.81			putative microcin immunity protein
BCAM0164			5.00	putative lipoprotein
BCAM0165	5.65		3.98	conserved hypothetical protein
BCAM0166	20.57		58.31	NADH dehydrogenase
BCAM0167			7.18	LysR family regulatory protein
BCAM0168			1.62	AraC family regulatory protein
BCAM0181	-1.70			putative catalase
BCAM0184		-6.42	-5.10	lectin
BCAM0185		-4.66	-3.61	lectin
BCAM0186		-6.13	-5.10	lectin
BCAM0188			-3.90	N-acylhomoserine lactone dependent regulatory protein
BCAM0189			-2.91	AraC family regulatory protein
BCAM0190		-7.39	-4.05	putative aminotransferase-class III

BCAM0191		-3.65	-2.25	putative non-ribosomal peptide synthetase
BCAM0216			-2.00	conserved hypothetical protein
BCAM0217			-2.80	conserved hypothetical protein
BCAM0233			3.33	ArsR family regulatory protein
BCAM0234			4.13	putative arsenate reductase
BCAM0245			-5.36	
BCAM0253			-2.97	putative 3-oxoacyl-[acyl-carrier-protein] synthase
BCAM0254	-1.98		-2.49	putative acyl carrier protein
BCAM0260		2.39		putative amino acid transporter
BCAM0261		2.08		putative branched-chain amino acid transporter
BCAM0264			-1.98	putative membrane protein
BCAM0267	-1.53			putative gram-negative porin
BCAM0271			2.82	conserved hypothetical protein, antitoxin
BCAM0272			2.88	putative acetyltransferase-GNAT family, toxin
BCAM0273		1.90	2.68	conserved hypothetical protein
BCAM0274a	-1.79	2.41	3.32	conserved hypothetical protein
BCAM0275			1.96	conserved hypothetical protein
BCAM0275a	-1.73		2.72	conserved hypothetical protein
BCAM0276			1.81	putative universal stress protein
BCAM0279			1.52	putative nitroreductase
BCAM0280			2.37	putative phospholipid-binding protein
BCAM0281			1.79	putative sulfate transporter family protein
BCAM0284			1.76	putative cytochrome c
BCAM0285			3.05	conserved hypothetical protein
BCAM0290			2.50	putative universal stress protein
BCAM0291			2.04	putative universal stress protein
BCAM0292			2.92	putative universal stress protein
BCAM0293			1.86	putative acetate kinase
BCAM0294			2.00	putative universal stress protein
BCAM0295			2.41	conserved hypothetical protein
BCAM0296			1.68	acetoacetyl-CoA reductase
BCAM0297			1.59	putative poly(3-hydroxyalkanoate) polymerase
BCAM0300			2.22	metallo-beta-lactamase superfamily protein
BCAM0307			2.84	conserved hypothetical protein
BCAM0308			1.83	conserved hypothetical protein
BCAM0309			1.57	putative cell division-related metallo peptidase
BCAM0310			2.56	putative ribonucleotide reductase
BCAM0311			3.00	putative 6-phosphofructokinase
BCAM0312			2.77	putative polysaccharide deacetylase
BCAM0313			3.17	putative exported protein
BCAM0314			2.09	conserved hypothetical protein
BCAM0315			3.78	putative exported protein
BCAM0316			2.75	conserved hypothetical protein
BCAM0317			2.92	putative membrane protein

BCAM0318			2.03	putative cation-transporting ATPase
BCAM0319			1.85	putative universal stress protein
BCAM0325	-2.04			putative type IV secretion system protein VirB2
BCAM0329	-1.68		-3.09	putative exported protein
BCAM0330	-1.90			putative lipoprotein
BCAM0332			-2.18	putative type IV secretion system protein VirB9
BCAM0344			-2.58	hypothetical protein
BCAM0354			-1.90	putative transcriptional regulator
BCAM0361			-6.23	putative amidohydrolase
BCAM0362			-2.35	conserved hypothetical protein
BCAM0367			3.65	putative branched-chain amino acid transport protein
BCAM0368	1.88		2.97	putative branched-chain amino acid transport protein
BCAM0374		1.61		conserved hypothetical protein
BCAM0376			4.93	conserved hypothetical protein
BCAM0377			2.07	putative thioredoxin
BCAM0378			1.51	hypothetical upf0047 protein sll1880
BCAM0382			2.55	putative lipoprotein
BCAM0384			2.04	putative lipoprotein
BCAM0387	1.81			pyruvate dehydrogenase (cytochrome)
BCAM0394			-5.29	conserved hypothetical protein
BCAM0395			-10.44	conserved hypothetical protein
BCAM0397		-11.82	-17.70	conserved hypothetical protein
BCAM0398	1.83	-18.51	-19.84	conserved hypothetical protein
BCAM0399		-6.92		LysR family regulatory protein
BCAM0404			2.20	LysR family regulatory protein
BCAM0412a			-2.66	LysR family regulatory protein
BCAM0432			-2.66	putative exported protein
BCAM0456			-16.83	putative ThiJ/PfpI family protein
BCAM0457			-10.11	hypothetical protein
BCAM0458	2.73		-11.61	conserved hypothetical protein (fragment)
BCAM0459		-20.96	-10.52	cysteine desulfurase
BCAM0464			2.88	LysR family regulatory protein
BCAM0476	-1.83			hypothetical protein
BCAM0476a			1.55	hypothetical protein
BCAM0478			1.93	glucosamine--fructose-6-phosphate aminotransferase (isomerizing)
BCAM0489			2.43	MerR family regulatory protein
BCAM0497			-2.40	putative lipoprotein
BCAM0498			-3.16	putative aldehyde dehydrogenase
BCAM0501			3.71	LysR family regulatory protein
BCAM0502		73.24		conserved hypothetical protein
BCAM0508		8.90		putative periplasmic binding protein
BCAM0509			-2.95	putative FAD dependent oxidoreductase
BCAM0510			-3.34	putative membrane protein
BCAM0512			-2.00	putative aminotransferase

BCAM0513			-2.28	conserved hypothetical protein
BCAM0519	-1.92			NADP-dependent malic enzyme
BCAM0525		9.69		putative aminotransferase protein
BCAM0525A		11.98		hypothetical protein
BCAM0526		3.30		pyridoxamine 5'-phosphate oxidase family protein
BCAM0528			-4.59	putative oxidoreductase/short-chain dehydrogenase
BCAM0529			-4.52	putative hydrolase
BCAM0543			-37.58	putative aminotransferase
BCAM0544			-67.34	putative acetylglutamate kinase
BCAM0545			-28.45	putative PTS transport system
BCAM0568			-3.98	putative short chain dehydrogenase
BCAM0575			1.78	LysR family regulatory protein
BCAM0576			-11.91	conserved hypothetical protein
BCAM0577		-13.70	-20.03	Major Facilitator Superfamily protein
BCAM0578		-27.33	-20.33	putative hydantoinase/oxoprolinase family protein
BCAM0588			4.28	MarR family regulatory protein
BCAM0590			-2.39	putative glutathione S-transferase
BCAM0599		1.88		putative transcriptional regulator
BCAM0600	1.86			conserved hypothetical protein
BCAM0601	1.71			putative HSP70 protein
BCAM0602	2.13			conserved hypothetical protein
BCAM0603			-3.10	conserved hypothetical protein
BCAM0604			1.69	ornithine cyclodeaminase
BCAM0616			-2.34	hypothetical protein
BCAM0621			1.94	conserved hypothetical protein
BCAM0626		-2.81		putative DNA-binding protein, antitoxin
BCAM0629			-4.10	putative hydrolase
BCAM0630			-5.58	putative dehydrogenase
BCAM0632		-4.58	-5.45	putative acetyl transferase-GNAT family
BCAM0633		-4.43	-5.10	conserved hypothetical protein
BCAM0666			-3.51	putative bacterial extracellular solute-binding protein
BCAM0679	1.86		1.79	conserved hypothetical protein
BCAM0692			2.41	putative NADPH-dependent FMN reductase
BCAM0693			10.50	conserved hypothetical protein
BCAM0695			2.48	putative lipoprotein
BCAM0699			2.07	putative exported dehydrogenase
BCAM0700			1.69	TetR family regulatory protein
BCAM0705		2.54	2.19	putative membrane protein
BCAM0710		1.95		metallo peptidase, family M35
BCAM0716			4.43	putative exported protein
BCAM0723			-2.87	putative exported protein
BCAM0725			-2.13	conserved hypothetical protein
BCAM0726			-2.24	conserved hypothetical protein
BCAM0727		2.40	-2.80	conserved hypothetical protein

BCAM0740			3.17	conserved hypothetical protein
BCAM0748			2.22	putative diguanylate cyclase
BCAM0752			1.94	putative hydrolase
BCAM0753			1.74	putative membrane protein
BCAM0757			-24.13	putative porin
BCAM0758			-9.00	conserved hypothetical protein
BCAM0766			1.55	D-ribose-binding periplasmic protein precursor
BCAM0770	-1.78			conserved hypothetical protein
BCAM0771			-2.11	putative transposase
BCAM0779	-2.13			putative methyl-accepting chemotaxis protein
BCAM0782		-4.20	-3.19	putative prolidase
BCAM0795		1.69		MarR family regulatory protein
BCAM0802		-3.03	-2.76	putative NAD(P)H dehydrogenase
BCAM0803		-9.58	-4.44	muconolactone delta-isomerase
BCAM0804		-10.38	-3.47	catechol 1,2-dioxygenase 1
BCAM0805			-2.82	muconate cycloisomerase I 1
BCAM0810	1.82		-21.18	putative aromatic oxygenase
BCAM0811			-19.71	putative aromatic oxygenase
BCAM0812	2.40		-16.24	putative aromatic hydrocarbons catabolism-related dioxygenase
BCAM0813			-11.01	putative aromatic hydrocarbons catabolism-related reductase
BCAM0817			-2.59	putative acetolactate synthase
BCAM0818		-3.77	-2.94	putative aldehyde dehydrogenase
BCAM0822	1.85			putative chemotaxis-related protein
BCAM0823	2.19			putative chemotaxis-related methyltranferase protein
BCAM0835		10.77	10.13	AraC family regulatory protein
BCAM0843		-3.60		putative lipoprotein
BCAM0847			-2.53	conserved hypothetical protein
BCAM0848		-3.48	-3.96	glyoxalase/bleomycin resistance protein/dioxygenase superfamily protein
BCAM0850			-6.53	putative rubyerythrin
BCAM0853			-2.19	conserved hypothetical protein
BCAM0868	1.95			AraC family regulatory protein
BCAM0875	2.01			conserved hypothetical protein
BCAM0876	1.81			conserved hypothetical protein
BCAM0887		2.66		putative tautomerase
BCAM0892			1.54	putative molybdate-binding periplasmic protein precursor
BCAM0895	-1.65		1.52	conserved hypothetical protein
BCAM0896			1.50	putative organic hydroperoxide resistance protein
BCAM0900			1.50	putative exported protein
BCAM0902			1.75	putative exported protein
BCAM0905			1.94	putative NADH dehydrogenase
BCAM0908			1.76	putative iron-sulfur protein
BCAM0918			-2.15	RNA polymerase sigma factor RpoD

BCAM0929	-1.56		-2.81	putative exported protein
BCAM0938			-3.14	acetyltransferase (GNAT) family protein
BCAM0941			-2.64	6-phosphogluconate dehydrogenase, decarboxylating
BCAM0944	1.80			putative lipoprotein
BCAM0946a		2.33		putative membrane protein
BCAM0948			-4.44	TonB-dependent receptor
BCAM0949			-5.60	exported lipase LipA
BCAM0952			-4.85	ABC transporter ATP-binding protein
BCAM0953			-8.05	extracellular solute-binding protein
BCAM0954			-7.62	binding-protein-dependent transport system protein
BCAM0955			-6.84	binding-protein-dependent transport system protein
BCAM0961			-5.31	aconitate hydratase
BCAM0962			-3.75	2-methylcitrate dehydratase
BCAM0963			-3.02	putative exported protein
BCAM0964			-2.43	putative lyase
BCAM0965			-2.22	malate dehydrogenase
BCAM0970			-1.99	succinate dehydrogenase iron-sulfur protein
BCAM0971			-2.22	conserved hypothetical protein
BCAM0972			-2.77	citrate synthase
BCAM0979			-2.32	putative glutathione-S-transferase
BCAM0983			1.76	3-isopropylmalate dehydratase large subunit 1
BCAM0983A			1.64	putative entericidin B-like bacteriolytic toxin
BCAM0990		1.89	1.77	N-(5'-phosphoribosyl)anthranilate isomerase
BCAM0992			-2.33	putative DNA methylase
BCAM0997			2.05	colicin V production protein
BCAM1003			2.51	putative epimerase
BCAM1014	1.93		-2.46	putative 3-demethylubiquinone-9 3-methyltransferase
BCAM1016			3.38	putative ribonuclease
BCAM1017			-2.35	formate dehydrogenase, major subunit (pseudogene)
BCAM1019			-4.30	formate dehydrogenase, iron-sulfur subunit
BCAM1020			-3.04	formate dehydrogenase, cytochrome b556 (FDN) subunit
BCAM1021			-3.19	protein FdhE homologue
BCAM1022			-4.15	L-seryl-tRNA(sec) selenium transferase
BCAM1024			-1.94	putative phage integrase
BCAM1026	-1.81		-3.78	putative phage DNA-binding protein
BCAM1027			-2.82	hypothetical phage protein
BCAM1028			-2.32	hypothetical phage protein
BCAM1029	-2.01		-2.53	hypothetical phage protein
BCAM1031	-1.66		-2.89	hypothetical phage protein
BCAM1032	-1.75			putative phage DNA-binding protein
BCAM1047			1.93	hypothetical phage protein
BCAM1049			2.35	hypothetical phage protein
BCAM1050			2.22	hypothetical phage protein
BCAM1054			3.37	putative phage DNA-binding protein

BCAM1062	-2.04			hypothetical phage protein
BCAM1080	-2.11		-2.90	hypothetical phage protein
BCAM1081	-1.68		-3.58	hypothetical phage protein
BCAM1082			-2.32	hypothetical phage protein
BCAM1082A	-1.98		-3.92	putative exported phage protein
BCAM1089			-2.58	putative exported phage protein
BCAM1097	-2.19			putative membrane protein
BCAM1102		2.23		Major Facilitator Superfamily protein (pseudogene)
BCAM1111			1.94	ornithine decarboxylase
BCAM1119		-3.15		conserved hypothetical protein
BCAM1139		5.72	-2.66	MarR family regulatory protein
BCAM1150			-3.14	3-hydroxyisobutyrate dehydrogenase
BCAM1151		-6.99	-8.81	methylmalonate-semialdehyde dehydrogenase
BCAM1152			-2.29	Major Facilitator Superfamily protein
BCAM1156			-2.24	putative isochorismatase
BCAM1167		-3.71		conserved hypothetical protein
BCAM1188	-1.73		-3.30	putative L-asparaginase II
BCAM1189	-1.95			LysR family regulatory protein
BCAM1190	-3.29			aspartate ammonia-lyase
BCAM1191	-2.77			L-asparagine permease
BCAM1203	2.00			putative enoyl-CoA hydratase/isomerase
BCAM1204	4.63	-3.16		alanine racemase, catabolic
BCAM1205	-1.58			putative membrane protein
BCAM1209			2.10	glutamine ABC transporter, permease protein
BCAM1210			2.18	glutamine ABC transporter ATP-binding protein
BCAM1214		1.82	-2.58	putative beta-hydroxylase
BCAM1239			1.69	conserved hypothetical protein
BCAM1242A		2.10		putative exported protein
BCAM1243			-3.69	putative aminotransferase
BCAM1244			-5.79	putative phosphonopyruvate decarboxylase
BCAM1245			-4.65	putative phosphoenolpyruvate phosphomutase/sugar nucleotidyltransferase
BCAM1246			-5.10	putative nucleotidyltransferase
BCAM1248			-2.67	putative oxygenase
BCAM1258			1.86	putative DNA-binding protein
BCAM1259			6.39	RNA polymerase sigma factor
BCAM1261	2.17			putative membrane protein
BCAM1276			6.45	ArsR family regulatory protein
BCAM1277			6.10	putative membrane protein
BCAM1278			3.47	putative membrane protein
BCAM1279			3.35	conserved hypothetical protein
BCAM1280			2.07	putative GntR-family transcriptional regulator/aminotransferase
BCAM1280a			1.63	conserved hypothetical protein
BCAM1285	1.89			putative membrane protein

BCAM1293			-5.72	ABC transporter, substrate-binding protein
BCAM1294			-5.27	ABC transporter, permease protein
BCAM1295			-7.04	ABC transporter, permease protein
BCAM1304			3.46	conserved hypothetical protein
BCAM1305			3.86	putative transcriptional regulator
BCAM1306			3.47	putative amino acid permease
BCAM1307			3.42	putative helicase
BCAM1316a			-1.94	conserved hypothetical protein
BCAM1321			1.70	putative polyphosphate kinase
BCAM1324	2.34			LysR family regulatory protein
BCAM1325		3.14		conserved hypothetical protein
BCAM1333	-1.76			putative exopolysaccharide acyltransferase
BCAM1334	-1.65		-1.79	conserved hypothetical protein
BCAM1335			-2.33	glycosyltransferase
BCAM1347			-8.29	putative acyl carrier protein
BCAM1348			-5.65	putative cyclic nucleotide-binding protein
BCAM1354		3.32	3.09	putative membrane protein
BCAM1355		3.11	2.38	putative phosphotransferase
BCAM1357			-2.96	gluconate 2-dehydrogenase flavoprotein subunit
BCAM1361			1.86	sugar-binding periplasmic protein precursor
BCAM1362			1.64	putative penicillin-binding protein
BCAM1396			1.92	putative glycerophosphoryl diester phosphodiesterase
BCAM1407		-4.56	1.65	DJ-1/PfpI family protein
BCAM1408		-6.15		xenobiotic reductase
BCAM1411			-2.04	putative short-chain dehydrogenase
BCAM1412		-3.81	-2.91	conserved hypothetical protein
BCAM1413a			-2.08	conserved hypothetical protein
BCAM1417			-2.32	two-component regulatory system, sensor kinase protein
BCAM1419	1.80			efflux system outer membrane protein
BCAM1427		-5.92	1.87	LysE family transporter
BCAM1437			1.98	MarR family regulatory protein
BCAM1438			2.31	putative membrane protein
BCAM1441			-14.00	omega-amino-acid-pyruvate aminotransferase
BCAM1442			-10.75	putative methylmalonate-semialdehyde dehydrogenase
BCAM1443		-4.51	-4.07	putative exported protein
BCAM1451		3.63		putative exported protein
BCAM1452		3.46		putative fusaric acid resistance transporter protein FusC
BCAM1457			-2.03	cytosine deaminase
BCAM1458	1.86			putative voltage-gated chloride channel
BCAM1461			-4.12	conserved hypothetical protein
BCAM1466			-2.07	IclR family regulatory protein
BCAM1472			1.75	putative glycosyltransferase
BCAM1483	2.47			cyclic nucleotide-binding transcriptional regulator

BCAM1484	3.12			two-component regulatory system, response regulator protein
BCAM1485	2.45			putative ornithine cyclodeaminase
BCAM1491			1.50	putative exported protein
BCAM1495			1.84	putative universal stress protein
BCAM1498			-3.26	amino acid permease (pseudogene)
BCAM1500	2.17			putative universal stress protein
BCAM1501			2.11	conserved hypothetical protein
BCAM1502			2.85	conserved hypothetical protein
BCAM1503			2.48	putative methyl-accepting chemotaxis protein
BCAM1504			2.65	putative sigma-54 interacting transcriptional regulator
BCAM1511		-3.12		conserved hypothetical protein
BCAM1518	-1.79		-2.07	conserved hypothetical protein
BCAM1536		2.06		TetR family regulatory protein
BCAM1537		-2.99	-2.04	putative dehydrogenase, zinc-binding subunit
BCAM1538			-2.18	putative dehydrogenase, monooxygenase subunit
BCAM1539		-3.15	-2.41	putative dehydrogenase, iron-sulfur cluster subunit
BCAM1540			-3.82	putative dehydrogenase, molybdopterin binding subunit
BCAM1541			-2.97	putative dehydrogenase, cytochrome C subunit
BCAM1542			-2.50	putative aldehyde dehydrogenase
BCAM1543B			1.90	conserved hypothetical protein
BCAM1543C			1.81	putative transposase (fragment)
BCAM1550			-2.13	putative peptidoglycan-associated lipoprotein
BCAM1551			-3.89	putative dehydrogenase, cytochrome C subunit
BCAM1552	-1.71		-4.56	putative dehydrogenase, iron-sulfur binding subunit
BCAM1553	-1.69			putative dehydrogenase, molybdenum-binding subunit
BCAM1569			2.14	putative BNR/Asp-box protein
BCAM1570			3.72	alcohol dehydrogenase
BCAM1571			3.16	TonB-dependent receptor
BCAM1573			-2.80	alpha,alpha-trehalose-phosphate synthase
BCAM1576		-4.07	-5.01	phosphoesterase family protein
BCAM1581			-2.65	phosphoenolpyruvate carboxykinase (GTP)
BCAM1588			11.48	putative lyase
BCAM1591A			2.02	putative exported protein
BCAM1594			2.64	conserved hypothetical protein
BCAM1595			1.89	putative esterase
BCAM1600			-4.55	putative exported protein
BCAM1602			-3.11	conserved hypothetical protein
BCAM1618			-3.86	translation initiation factor IF-1
BCAM1619	-1.77		-2.28	putative DNA-binding cold-shock protein
BCAM1638	1.75		1.73	putative plasmid stability protein
BCAM1661			2.28	RNA polymerase sigma factor
BCAM1665			1.84	LysE family transporter
BCAM1666			4.19	LysR family regulatory protein
BCAM1668			2.11	conserved hypothetical protein

BCAM1671a	1.84			GntR family regulatory protein
BCAM1672			-5.20	mannonate dehydratase
BCAM1680			-2.72	putative exported protein
BCAM1692			-2.22	putative fumarylpyruvate hydrolase
BCAM1697		2.85	1.90	putative membrane-associated amino terminal protease
BCAM1700			2.01	putative acetyltransferase
BCAM1701			2.23	conserved hypothetical protein
BCAM1702			7.73	Major Facilitator Superfamily protein
BCAM1704			-3.39	2,3-butanediol dehydrogenase
BCAM1706			1.51	putative membrane protein
BCAM1708			-2.52	putative aldo/keto reductase
BCAM1709	1.76	2.66		MarC family protein
BCAM1710	4.68	8.52		putative enoyl-CoA hydratase/isomerase
BCAM1711	5.43	12.55		phenylacetate-coenzyme A ligase paaK
BCAM1712	6.41	6.08		3-hydroxybutyryl-CoA dehydrogenase
BCAM1723	3.32			putative membrane protein
BCAM1724			-2.21	MarR family regulatory protein
BCAM1727			-2.44	conserved hypothetical protein
BCAM1732		-3.51	-4.56	putative porin
BCAM1733			-4.03	putative membrane protein
BCAM1734		-4.51	-3.58	putative cytochrome C
BCAM1735		-4.33	-3.94	putative oxidoreductase
BCAM1736			-3.20	conserved hypothetical protein
BCAM1739		1.88		putative membrane protein
BCAM1741			2.01	Major Facilitator Superfamily protein
BCAM1742	2.69			putative exported protein
BCAM1745		5.45		putative magnesium-transporting ATPase
BCAM1746			-4.34	putative DNA-binding protein
BCAM1748			-2.56	D-alanyl-D-alanine carboxypeptidase (penicillin-binding protein precursor)
BCAM1750	-1.53			MarR family regulatory protein
BCAM1760			-7.30	putative multidrug resistance transporter protein
BCAM1762			-2.23	putative solute-binding protein
BCAM1766			3.43	putative regulatory protein, toxin
BCAM1767			3.22	putative regulatory protein, antitoxin
BCAM1769		-3.37		putative D-alanyl-D-alanine dipeptidase
BCAM1770			-2.97	putative ABC transporter, substrate-binding protein
BCAM1775	2.34	2.00	2.50	putative transglycosylase associated protein
BCAM1777A	-1.72		-2.05	putative exported protein
BCAM1780			2.00	peptidoglycan-binding lysm:peptidase m23b precursor
BCAM1790			2.29	putative gluconate dehydrogenase
BCAM1810	-1.72			putative cold shock protein
BCAM1811	-2.09			conserved hypothetical protein
BCAM1812	-2.48			putative arginase
BCAM1824			1.86	conserved hypothetical protein

BCAM1829	2.78		-2.07	putative universal stress protein
BCAM1830			-4.28	putative exported protein
BCAM1831			-2.85	putative cyclase
BCAM1839			-2.68	putative endoribonuclease
BCAM1871			-2.61	conserved hypothetical protein
BCAM1928			1.70	putative transcription elongation factor
BCAM1941			3.54	putative glutathione S-transferase
BCAM1943			1.71	MarR family regulatory protein
BCAM1960			1.81	calcineurin-like phosphoesterase
BCAM1961			1.82	copper binding protein
BCAM1966		5.21	1.67	ArsR family regulatory protein
BCAM1995			2.09	putative endoribonuclease
BCAM2003	1.85			AraC family regulatory protein
BCAM2006			-2.42	putative aspartate carbomyltransferase
BCAM2006a			1.75	putative plasmid stability protein (pseudogene)
BCAM2006b			2.07	putative plasmid stability protein (pseudogene)
BCAM2007			3.14	TonB-dependent siderophore receptor
BCAM2010		2.84		conserved hypothetical protein
BCAM2023			-2.56	conserved hypothetical protein
BCAM2039		2.91	2.90	putative transcriptional regulator
BCAM2053		-3.41	-38.08	putative type III secretion system protein
BCAM2059		-3.26		LuxR superfamily regulatory protein
BCAM2064		-5.59	-4.15	putative periplasmic trehalase precursor
BCAM2067			-3.11	putative undecaprenyl pyrophosphate synthetase
BCAM2068		-4.81	-8.68	conserved hypothetical protein
BCAM2069		-3.21	-2.72	conserved hypothetical protein
BCAM2070			-2.17	conserved hypothetical protein
BCAM2071			-2.25	putative acetyltransferase
BCAM2073		-2.86	1.66	putative exported protein
BCAM2076			-9.01	putative diaminopimelate decarboxylase
BCAM2080			-3.08	putative bifunctional NMN adenylyltransferase/NUDIX hydrolase
BCAM2081			-5.28	conserved hypothetical protein
BCAM2082			-4.97	putative S-adenosylmethionine decarboxylase proenzyme
BCAM2094		3.44	-4.32	putative gamma-glutamylputrescine synthetase
BCAM2095			-3.81	putative DNA-binding protein
BCAM2096	2.60			putative gamma-glutamylputrescine oxidoreductase
BCAM2099			-2.11	putative Pfp1 family protein
BCAM2105			-2.27	MerR family regulatory protein
BCAM2106		-4.11	-5.99	non-heme chloroperoxidase
BCAM2113			-2.35	Major Facilitator Superfamily protein
BCAM2114			-2.32	putative hydroxylase
BCAM2117			-3.86	LysR family regulatory protein
BCAM2118			-3.69	LysR family regulatory protein

BCAM2120			-2.57	metallo-beta-lactamase superfamily protein
BCAM2122			-2.90	acetaldehyde dehydrogenase
BCAM2126			-2.11	putative outer membrane porin
BCAM2127			-3.31	putative dienelactone hydrolase family protein
BCAM2129			-3.21	2-amino-3-carboxymuconate 6-semialdehyde decarboxylase
BCAM2130			-3.50	3-hydroxyanthranilate 3,4-dioxygenase
BCAM2138			-3.74	conserved hypothetical protein
BCAM2141	2.38			ABC transporter ATP-binding membrane protein, type 1 secretion
BCAM2143		-2.98	-2.16	cable pilus associated adhesin protein
BCAM2145		5.05		putative GABA permease
BCAM2146		5.62		NnrU family protein
BCAM2147			-2.70	conserved hypothetical protein
BCAM2148			-2.87	putative L-lactate dehydrogenase
BCAM2149			-1.88	metallo peptidase, subfamily M20A
BCAM2158		2.14		putative DNA-binding protein
BCAM2159		4.64	2.70	putative exported protein
BCAM2180		-3.22		putative peroxiredoxin OsmC family protein
BCAM2181	1.77		-2.94	putative exported protein
BCAM2182			-2.16	putative membrane protein
BCAM2191			-3.39	enoyl-CoA hydratase/isomerase family
BCAM2192		-4.21	-3.85	enoyl-CoA hydratase/isomerase family protein
BCAM2193		-5.37	-4.44	putative 3-hydroxyisobutyrate dehydrogenase
BCAM2194			-3.64	methylmalonate-semialdehyde dehydrogenase
BCAM2195			-2.49	putative AMP-binding enzyme
BCAM2206			-3.29	conserved hypothetical protein
BCAM2207			-3.44	conserved hypothetical protein
BCAM2209			1.53	conserved hypothetical protein
BCAM2215		4.93		putative copper resistance protein C precursor
BCAM2216		2.47		putative exported protein
BCAM2224			3.49	putative pyochelin receptor protein FptA
BCAM2231			3.48	transcriptional regulator PchR
BCAM2247		-3.16	-6.39	putative amino acid ABC transporter ATP-binding protein
BCAM2248		-4.15	-8.35	putative amino acid ABC transporter ATP-binding protein
BCAM2249			-5.38	putative amino acid transport system permease component of ABC transporter protein
BCAM2250		-3.05	-5.64	putative amino acid transport system permease component of ABC transporter protein
BCAM2251			-4.83	putative amino acid solute binding component of ABC transporter
BCAM2251A			-2.12	conserved hypothetical protein
BCAM2252			-2.56	putative RHS family protein (fragment, pseudogene)
BCAM2253A			-2.06	conserved hypothetical protein
BCAM2275			1.66	putative DNA-binding protein
BCAM2276	1.78			putative FAD dependent oxidoreductase

BCAM2280			-2.14	putative solute-binding component of ABC transporter
BCAM2288			-2.34	putative short-chain dehydrogenase
BCAM2289			-2.06	conserved hypothetical protein
BCAM2307			-10.59	zinc metalloprotease ZmpB
BCAM2316			-4.64	putative amino acid permease
BCAM2317			-8.42	putative exported protein
BCAM2318			-5.05	putative ferredoxin oxidoreductase protein
BCAM2319		-4.17	-7.43	iron-sulphur Rieske protein
BCAM2324			-6.96	conserved hypothetical protein
BCAM2325			-7.43	putative dipeptidase
BCAM2326			-4.24	putative serine hydroxymethyltransferase
BCAM2334	3.66			efflux system transport protein
BCAM2335	3.90			outer membrane efflux protein
BCAM2336	3.90			putative sugar transferase
BCAM2337	3.95			putative multidrug resistance transporter protein
BCAM2338	5.68			putative glycosyltransferase
BCAM2339	4.56			putative methyltransferase
BCAM2350			-2.25	ABC transporter ATP-binding protein
BCAM2353	1.93			conserved hypothetical protein
BCAM2356			2.09	conserved hypothetical protein
BCAM2357	2.14		2.18	putative alpha/beta hydrolase
BCAM2365			-3.74	putative signal-transduction and transcriptional regulator Fis and NtrC family protein
BCAM2380			1.63	putative D-isomer specific 2-hydroxyacid dehydrogenase
BCAM2383		-3.23		ABC transporter ATP-binding protein
BCAM2385		-3.17	-6.27	rifampin ADP-ribosyl transferase
BCAM2386			-3.51	putative SLT transglycosylase
BCAM2389			-4.39	putative sarcosine oxidase alpha subunit
BCAM2390	-1.86	-4.93	-5.21	putative sarcosine oxidase delta subunit
BCAM2391			-3.92	putative sarcosine oxidase beta subunit
BCAM2399			-3.35	putative hydroxypyruvate isomerase
BCAM2400			-5.61	NAD dependent epimerase/dehydratase family protein
BCAM2400a			-4.20	putative exported protein
BCAM2400b			-3.98	putative exported protein
BCAM2402			-2.15	conserved hypothetical protein
BCAM2405			4.01	putative membrane protein
BCAM2406			2.86	putative membrane protein
BCAM2412			-7.80	putative membrane protein
BCAM2413			-10.59	putative GNAT family N-acetyltransferase
BCAM2414			-2.41	putative membrane protein
BCAM2415		-3.98		putative NADPH-dependent FMN reductase
BCAM2417			-2.30	conserved hypothetical protein
BCAM2418			-9.99	putative haemagglutinin-related autotransporter protein
BCAM2420			-3.98	conserved hypothetical protein
BCAM2421			-3.80	conserved hypothetical protein

BCAM2422			-3.85	putative exported protein
BCAM2423			-4.62	conserved hypothetical protein
BCAM2424			-3.52	conserved hypothetical protein
BCAM2427			-3.66	conserved hypothetical protein
BCAM2428			-4.25	conserved hypothetical protein
BCAM2430	2.56			putative biotin carboxylase
BCAM2431	2.53			putative enoyl coenzyme A hydratase-like protein
BCAM2432	3.10			putative biotin-dependent carboxyl transferase
BCAM2433	4.96		1.60	putative acyl-CoA dehydrogenase
BCAM2434	2.76		2.44	TetR family regulatory protein
BCAM2435			1.63	MarR family regulatory protein
BCAM2444			-2.06	putative exported protein
BCAM2445	1.78		2.42	conserved hypothetical protein
BCAM2446			2.05	putative gram-negative porin
BCAM2462		2.04		putative gram-negative porin
BCAM2463		2.08	1.71	putative membrane protein
BCAM2467			-2.27	putative transposase
BCAM2469		9.44		putative amino-acid permease
BCAM2474	2.11			conserved hypothetical protein
BCAM2477		-3.91		serine peptidase, family S10
BCAM2478			2.05	serine-carboxyl peptidase
BCAM2482		2.79	1.75	agmatinase
BCAM2483			3.92	Major Facilitator Superfamily protein
BCAM2490			5.48	conserved hypothetical protein
BCAM2491			1.94	Major Facilitator Superfamily protein
BCAM2504			1.63	conserved hypothetical protein
BCAM2515			2.41	putative glucarate transporter
BCAM2517			-2.72	putative membrane protein
BCAM2530			4.00	putative monooxygenase
BCAM2536	1.74	-12.99	-8.60	putative alpha-beta hydrolase
BCAM2537		-11.40	-6.99	putative membrane protein
BCAM2539			-4.37	AraC family regulatory protein
BCAM2548			-2.32	TetR family regulatory protein
BCAM2554			-2.06	LysR family regulatory protein
BCAM2558	1.90		2.02	putative selenide, water dikinase
BCAM2561			-16.14	putative 4-aminobutyrate aminotransferase
BCAM2562			-10.29	putative succinate-semialdehyde dehydrogenase (NADP+)
BCAM2568	1.82		1.64	putative beta-ketoadipyl CoA thiolase
BCAM2569			5.84	IclR family regulatory protein
BCAM2589			5.05	IclR family regulatory protein
BCAM2590			-2.33	Major Facilitator Superfamily protein
BCAM2591			4.27	putative cytochrome P450 iron-sulfur protein
BCAM2600			-2.17	putative glucose-6-phosphate 1-dehydrogenase
BCAM2611		1.61	2.34	TetR family regulatory protein

BCAM2623			1.57	conserved hypothetical protein
BCAM2626			4.12	putative heme receptor protein
BCAM2639	2.39			putative membrane protein
BCAM2642	2.19		1.58	conserved hypothetical protein
BCAM2671	1.66			putative low-specificity L-threonine aldolase
BCAM2673			4.07	putative lipoprotein
BCAM2674			26.04	putative cytochrome oxidase subunit I
BCAM2675		2.23	5.92	putative cytochrome oxidase subunit II
BCAM2676			2.55	putative membrane protein
BCAM2677			2.05	putative membrane protein
BCAM2682			2.25	putative HicB family protein
BCAM2683			4.34	putative cation-transporting ATPase membrane protein
BCAM2684			3.10	putative acetyltransferase
BCAM2685			2.80	conserved hypothetical protein
BCAM2686			1.50	putative membrane protein
BCAM2687			4.52	MgtC family membrane protein
BCAM2699			-4.77	putative hydrolase
BCAM2700			-8.98	putative membrane protein
BCAM2701			-7.55	aconitate hydratase 1
BCAM2702			-5.02	2-methylcitrate synthase
BCAM2703			-2.10	probable methylisocitrate lyase
BCAM2711			-2.72	H-NS histone family protein
BCAM2712			-2.33	conserved hypothetical protein
BCAM2717	2.12		5.27	putative exported protein
BCAM2718			4.50	putative lipoprotein
BCAM2729			-2.50	MerR family regulatory protein
BCAM2730			-4.56	putative tripeptide permease
BCAM2739			-1.92	MoaA/NifB/PqqE family protein
BCAM2739A			-2.85	hypothetical protein
BCAM2740			-3.65	conserved hypothetical protein
BCAM2745			-10.31	conserved hypothetical protein
BCAM2746			-4.85	carbon starvation protein A
BCAM2747	2.10			putative epimerase
BCAM2749			-3.12	carboxymuconolactone decarboxylase family protein
BCAM2755			-2.02	putative exported protein
BCAM2759	-2.03		-3.13	putative minor pilin and initiator
BCAM2760			-6.51	putative outer membrane usher
BCAM2761			-4.32	giant cable pilus
BCAM2762	-2.84		-4.93	giant cable pilus chaperone protein
BCAM2772			-3.65	putative class II aldolase
BCAM2781			2.59	putative exported protein
BCAM2811			-2.19	conserved hypothetical protein
BCAM2817	8.62			glycolate oxidase subunit
BCAM2829			-2.09	putative VacJ family lipoprotein

BCAM2830	1.74			conserved hypothetical protein
BCAM2831			-3.27	squalene--hopene cyclase
BCAM2834	1.83			conserved hypothetical protein
BCAM2835		-2.98	-2.37	putative esterase
BCAMr0437		3.08		misc_RNA, ybP-ykoY element
BCAMr1023	1.84		2.67	tRNA-Sec
BCAMr2491c	2.51	3.07	4.29	tRNA-Ala
BCAMr2491d	-1.83		2.44	tRNA-Ile
BCAS0002			2.29	putative chromosome partitioning protein
BCAS0003			3.40	ParA protein
BCAS0007			2.01	TetR family regulatory protein
BCAS0009			-1.93	conserved hypothetical protein
BCAS0010			-1.93	putative activator of osmoprotectant transporter
BCAS0056			2.06	LysR family regulatory protein
BCAS0070			-2.42	two-component regulatory system, response regulator protein
BCAS0081			1.85	ABC transporter ATP-binding membrane protein
BCAS0082		1.83	5.38	serine peptidase, family S33
BCAS0090			-2.56	flavoprotein
BCAS0093	2.71		1.63	putative Fic family protein
BCAS0099			4.82	LacI family regulatory protein
BCAS0104			-3.75	A-type flagellar hook-associated protein 2 (HAP2)
BCAS0105			-2.54	putative exported protein
BCAS0117	2.90	2.58		putative reverse transcriptase
BCAS0118			-3.58	putative H-NS family DNA-binding protein
BCAS0124			-3.31	putative putrescine-binding periplasmic protein precursor
BCAS0128			-3.47	ABC transporter ATP-binding protein
BCAS0129			-4.54	putative binding-protein-dependent transport system component
BCAS0147			-4.02	putative exported protein
BCAS0161			3.99	LysR family regulatory protein
BCAS0172			-3.35	putative dehydrogenase
BCAS0173	-1.54		-2.88	putative tautomerase
BCAS0187			2.71	putative amino acid efflux protein-LysE family
BCAS0188	1.96		2.47	LysR family regulatory protein
BCAS0188A			2.20	hypothetical protein
BCAS0189			-2.61	conserved hypothetical protein
BCAS0191			-3.20	putative endoribonuclease
BCAS0192			-3.07	AraC family regulatory protein
BCAS0193			-2.35	putative dehydrogenase
BCAS0198			-3.72	putative transporter protein
BCAS0199			-8.27	putative transporter protein-Dct family
BCAS0201	2.09	-4.87	-7.37	putative FAD dependent oxidoreductase

BCAS0202	2.06	-4.54	-6.34	putative membrane protein
BCAS0203		-3.89	-8.58	ABC transporter protein
BCAS0204		-4.20	-7.52	ABC transporter ATP-binding protein
BCAS0205		-4.41	-7.02	TauD/TfdA taurine catabolism dioxygenase family protein
BCAS0206		-6.30	-8.08	putative methyltransferase family protein
BCAS0207		-3.50	-5.99	conserved hypothetical protein
BCAS0208		-4.79	-6.55	putative acyl-CoA dehydrogenase
BCAS0209		-5.42	-6.52	conserved hypothetical protein
BCAS0210		-5.70	-7.59	putative AMP-binding enzyme
BCAS0211		-5.17	-7.14	putative pyridoxal-dependent decarboxylase
BCAS0212		-4.55	-7.44	conserved hypothetical protein
BCAS0213		-3.89	-7.18	conserved hypothetical protein
BCAS0214		-5.56	-8.32	conserved hypothetical protein
BCAS0215		-5.51	-6.34	putative exported protein
BCAS0216		-4.31	-7.43	putative acyl carrier protein
BCAS0217		-3.61	-5.53	conserved hypothetical protein
BCAS0218			-4.47	hypothetical protein
BCAS0219			-4.30	putative exported protein
BCAS0220			-2.96	putative permease
BCAS0221	1.84		-2.03	ABC transporter ATP-binding protein
BCAS0223			-2.22	putative fatty acid desaturase
BCAS0224			-2.78	conserved hypothetical protein
BCAS0226	1.88			putative hydrolase
BCAS0228			4.13	conserved hypothetical protein
BCAS0229			2.17	putative periplasmic sugar-binding protein
BCAS0235		1.77	2.03	two-component regulatory system, response regulator protein
BCAS0242			-3.04	conserved hypothetical protein
BCAS0243			-4.80	conserved hypothetical protein
BCAS0244			-4.40	hypothetical protein
BCAS0245			-3.67	30S ribosomal protein S21 3
BCAS0245A			-2.65	hypothetical protein
BCAS0246			-2.21	conserved hypothetical protein
BCAS0256			-2.97	putative porin protein
BCAS0257			1.99	putative acetyltransferase
BCAS0260			-2.52	hypothetical protein
BCAS0262			-2.60	putative acetyltransferase
BCAS0266			-2.55	two-component regulatory system, sensor kinase protein
BCAS0267a			-2.49	putative calcineurin-like phosphoesterase family protein
BCAS0276			-2.33	conserved hypothetical protein
BCAS0282			-4.55	hypothetical protein
BCAS0290		-8.09	-3.44	conserved hypothetical protein
BCAS0291		-7.75	-4.03	periplasmic solute-binding protein
BCAS0292		-8.36	-4.70	conserved hypothetical protein

BCAS0293		-8.62	-6.87	nematocidal protein AidA
BCAS0294		-3.28		putative GtrA-like family protein
BCAS0321a	2.35			putative exported protein
BCAS0375			-2.12	putative aspartate aminotransferase
BCAS0377			1.95	putative membrane protein
BCAS0380			1.54	TetR family regulatory protein
BCAS0388			-3.32	putative periplasmic solute-binding protein
BCAS0406			-3.10	putative exported protein
BCAS0407			-2.04	hypothetical protein
BCAS0409		-5.39	-8.98	zinc metalloprotease ZmpA
BCAS0410		-3.77	-4.63	conserved hypothetical protein
BCAS0411			-3.11	hypothetical protein
BCAS0412			-3.65	putative membrane protein
BCAS0434			1.91	HipA-like protein, toxin
BCAS0452			2.52	putative membrane protein
BCAS0453			2.30	putative membrane protein
BCAS0455			1.52	putative CopG family protein
BCAS0456			3.36	conserved hypothetical protein
BCAS0457			4.40	alkylphosphonate uptake protein
BCAS0504			-1.87	putative phage transmembrane acetyltransferase
BCAS0505a			-3.21	hypothetical phage protein
BCAS0506			-3.58	putative phage tail protein gpI
BCAS0516			-2.30	hypothetical phage protein
BCAS0517			-2.34	putative phage tail tube protein
BCAS0521		-5.08		hypothetical phage protein
BCAS0531	-2.26			putative phage membrane protein
BCAS0534			-2.91	holin
BCAS0535			-4.97	hypothetical phage protein
BCAS0536		-3.93	-4.20	putative phage membrane protein
BCAS0537	-1.72	-5.20	-6.32	hypothetical phage protein
BCAS0538		-3.66	-3.60	putative phage membrane protein
BCAS0539	-1.64	-4.03	-3.92	cro/cI repressor transcription regulator
BCAS0540			-2.41	hypothetical phage protein
BCAS0547			-2.35	putative DNA-binding phage protein
BCAS0549			-3.09	hypothetical phage protein
BCAS0551			-4.60	HU DNA-binding protein
BCAS0552			-4.20	single-stranded DNA binding protein
BCAS0553			-3.32	Mu gp16 gemA
BCAS0554			-3.05	Mu protein C/ Mor gp17 transcription regulator
BCAS0569			4.68	conserved hypothetical protein
BCAS0581			1.59	putative transcriptional regulatory protein, antitoxin
BCAS0597			-2.10	DeoR family regulatory protein
BCAS0607	1.76			3-hydroxybutyryl-CoA dehydrogenase
BCAS0608		-3.24	-3.48	beta-ketothiolase

BCAS0609			-3.15	putative electron transfer flavoprotein-ubiquinone oxidoreductase
BCAS0610			-2.12	putative acyl-CoA dehydrogenase
BCAS0613			1.63	AraC family regulatory protein
BCAS0627		5.17		putative lipoprotein
BCAS0628			2.60	TetR family regulatory protein
BCAS0636	2.31			conserved hypothetical protein
BCAS0637	1.99			60 kDa chaperonin 3
BCAS0638			1.96	10 kDa chaperonin 3
BCAS0640			1.79	conserved hypothetical protein
BCAS0649			-3.39	hypothetical protein
BCAS0651			-2.12	
BCAS0658			1.56	putative transposition-related protein
BCAS0660			1.91	putative transposase
BCAS0660A			3.68	putative H-NS family DNA-binding protein
BCAS0661C			-2.22	hypothetical protein
BCAS0662			-3.08	conserved hypothetical protein
BCAS0663			-3.38	RHS-family protein
BCAS0664			-2.48	conserved hypothetical protein
BCAS0666			-2.23	putative ankyrin-repeat exported protein
BCAS0669	-1.53		-4.03	hypothetical protein
BCAS0674			2.11	conserved hypothetical protein
BCAS0675			2.40	conserved hypothetical protein
BCAS0676			3.88	hypothetical protein
BCAS0678			-2.11	hypothetical protein
BCAS0681			-3.65	
BCAS0693			-2.61	putative monooxygenase
BCAS0697			-2.77	LysR family regulatory protein
BCAS0704			-5.24	putative short-chain dehydrogenase/oxidoreductase
BCAS0707			-5.64	two-component regulatory system, response regulator protein
BCAS0708			-4.63	two-component regulatory system, sensor kinase protein
BCAS0709			-3.24	two-component regulatory system, response regulator protein
BCAS0710			-2.06	LysR family regulatory protein
BCAS0712			-2.04	AnsC family regulatory protein
BCAS0728			-2.18	cytidine deaminase
BCAS0730			-3.45	putative Na+ dependent nucleoside transporter family protein
BCAS0731			-22.72	D-hydantoinase
BCAS0732			-15.72	putative cytosine/purines, uracil, thiamine, allantoin permease
BCAS0733			-19.13	putative dihydroorotate dehydrogenase family protein
BCAS0734			-15.08	putative pyridine nucleotide-disulphide oxidoreductase family protein
BCAS0735			-6.63	metallo peptidase, family M20 unassigned

BCAS0736			-2.56	TetR family regulatory protein
BCAS0737		-4.47	-2.63	putative acetyl-CoA acetyltransferase
BCAS0738		-4.21	-2.33	putative short-chain dehydrogenase family protein
BCAS0743	-1.58		-2.42	putative acetyltransferase-GNAT family
BCAS0746			-2.24	putative metal dependent phosphohydrolase
BCAS0757			1.57	conserved hypothetical protein
BCAS0758			1.87	conserved hypothetical protein
BCAS0762			2.57	putative exported protein
BCAS0763			-2.84	putative dienelactone hydrolase family protein
BCAS0771	4.30			putative adenylosuccinate synthetase
BCAS0773			-2.34	putative exported protein
BCAS0773A	-1.57			hypothetical protein
BCASr0743c	2.49	2.71	4.32	tRNA-Ala
BCASr0743d			2.06	tRNA-Ile
pBCA019			1.99	putative membrane protein
pBCA020			2.12	putative TraG conjugative transfer protein
pBCA026a			-3.18	hypothetical protein
pBCA028			-2.00	hypothetical protein
pBCA029			-2.68	putative membrane protein
pBCA030			-2.59	putative conjugative transfer protein
pBCA031	-1.53			putative TraU conjugative transfer protein precursor
pBCA047		3.26	-2.78	putative membrane protein
pBCA048		6.96		putative membrane protein
pBCA050	-1.75	1.95		hypothetical protein
pBCA051			-6.81	LamB/YcsF family protein
pBCA052			-7.66	putative exported protein
pBCA053			-4.10	putative extracellular solute-binding protein
pBCA054	-1.56		-2.82	LuxR family regulatory protein
pBCA055			-2.74	putative membrane protein
pBCA056			-2.49	hypothetical protein
pBCA057			-3.50	putative conjugative transfer protein
pBCA060			-2.58	hypothetical protein
pBCA070	-2.86			hypothetical protein
pBCA079			-1.83	hypothetical protein
pBCA090			2.70	putative integrase
pBCA092			1.86	hypothetical protein
pBCA093			2.35	PIN domain protein, toxin
pBCA094			2.99	conserved hypothetical protein, antitoxin
pBCA095			1.67	putative ligase

Paper 3: Investigating the targets of small RNAs in *Burkholderia cenocepacia* J2315

Sanne Kiekens, Andrea Sass, Sofie Depluverez, Bart Devreese and Tom Coenye

ABSTRACT

sRNAs are small non-coding RNA molecules that regulate gene expression at the post-transcriptional level. We investigated three sRNAs (ncS04, ncS16 and ncS27) in *Burkholderia cenocepacia* J2315, an opportunistic pathogen. None of them constructed deletion mutants showed a phenotype. Computational target prediction suggested a role of these sRNAs in regulation of outer membrane protein expression, therefore differential expression of outer membrane proteins was analysed using LC-MS^E and differential transcription of selected computationally predicted targets was investigated using qPCR. LC-MS^E revealed that BCAL3204, a putative OmpA family lipoprotein and a computationally predicted target for ncS16 was upregulated in ΔncS16 compared to wild type. Interaction between this sRNA and mRNA target was further investigated using translational fusions which confirmed that BCAL3204 is a target for ncS16. For the other two investigated sRNAs (ncS04 and ncS27) no clear differential expression was observed at the protein level. Preliminary data on sRNA ncS25 revealed that BCAL3473, a putative outer membrane porin, and the computationally predicted target with the lowest p-value for ncS25 is higher expressed in ΔncS25 compared to wild type investigated by qPCR. However, BCAL3473 could not be confirmed as a direct target for ncS25 using translational fusions.

IMPORTANCE

Burkholderia species are found in a wide range of environments, often associated with a host in a harmful or beneficial manner. *B. cenocepacia* is an opportunistic pathogen causing an increased morbidity and mortality in cystic fibrosis patients. Treatment of *B. cenocepacia* infections is difficult due to their high innate resistance against a broad range of antibiotics. To fully understand the resistance mechanisms of these bacteria and their ability to survive in a wide range of environments, a comprehensive understanding of their global regulatory networks is essential. We investigated the role of four small non-coding regulatory RNA molecules in the expression of outer membrane proteins, which protect cells against damage, expel antibiotics and are important for invasion of a host.

INTRODUCTION

sRNAs are small non-coding RNA molecules with an important function in post-transcriptional regulation of gene expression. The majority of sRNAs target mRNA through a base-pairing mechanism (75). These sRNAs are expressed mostly under specific conditions and base-pair with their mRNA either through full (cis-acting sRNAs) or limited complementarity (trans-acting sRNAs), often assisted by an RNA chaperone such as Hfq (180). A considerable number of sRNAs have been discovered in the model species *Escherichia coli* and *Salmonella* (97) and studies have shown that in these species genes coding for outer membrane proteins, such as porins, are typical targets for sRNA-based regulation (73, 95).

Burkholderia cenocepacia, a Gram-negative bacillus and member of the *Burkholderia cepacia* complex (Bcc), causes respiratory tract infections in cystic fibrosis patients, infections that can lead to ‘‘cepacia syndrome’’, an often fatal sepsis (13). Treatment of Bcc infections is difficult due to the high innate resistance of *B. cenocepacia* against a broad range of antimicrobial compounds and segregation of infected patients is needed as these bacteria are easily transmitted through social contact (17). To fully understand the antimicrobial resistance in these bacteria, a comprehensive understanding of their global regulatory networks is essential. sRNAs are one of the key players in the complex regulation of gene expression. In *B. cenocepacia* J2315, several techniques have so far been used for sRNA discovery, including microarray analysis (140, 142), co-immunoprecipitation with Hfq (143), computational methods (92) and transcriptomics (139). Several sRNAs were linked to a certain stressor (140, 142) and one was homologous to the 6S RNA (206). One sRNA, h2cr, was fully characterized and is involved in the regulation of expression of *hfq2*, one of the two genes encoding Hfq-like proteins in *B. cenocepacia* J2315 (134).

Differential RNA-seq (dRNA-seq) of biofilm-grown *B. cenocepacia* J2315 cells (34) revealed the presence of transcripts which had several features in common with typical regulatory sRNAs. dRNA-seq enables the identification of transcription start sites (TSS) by comparing two differentially treated cDNA libraries. One RNA sub-sample is treated with a Terminator 5’-Phosphate-Dependent Exonuclease (TEX), which degrades processed transcripts, thereby enriching un-processed or primary transcripts, while the other RNA sub-sample is not treated with TEX. Transcripts with a TSS in an intergenic region, not associated with a coding sequence, with a pronounced secondary structure and a rho-independent terminator followed by a U-rich 3’end and which were conserved among Bcc species, were considered as potential sRNAs. Four were chosen for further characterisation because they had a marked predicted secondary structure; ncS04, ncS16, ncS25 and ncS27. ncS04 was previously detected in a Hfq sRNA co-purification study (143) and the Rfam database identified ncS27 as “toxic small RNA”, named so because homologous sRNAs inhibited growth when expressed in *E. coli* (206). ncS16 and ncS25 were selected based on their genome context, as sRNAs are often encoded near proteins which are part of their regulatory circuit. Their flanking genes are outer membrane proteins, typical targets for sRNAs. Northern blotting and qPCR confirmed their expression in several growth conditions (planktonic and biofilm) and during growth in the presence of several stresses (oxidative stress, membrane stress and limited nutrient availability) (207).

For an initial characterization of the function and regulatory network of these sRNAs, we analysed the phenotype of deletion mutants, performed computational target analysis and attempted to confirm the predicted targets using a range of techniques.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

B. cenocepacia J2315 (LMG16656) was used for the construction of sRNA deletion mutants, ΔncS04, ΔncS16, ΔncS25 and ΔncS27. *E. coli* DH5α and *E. coli* DH5α λpir were used during the construction process for transformation and for the maintenance of the helper plasmid pRK2013 (ori_{colEI}, Km^r (152)), the suicide plasmid pGPI-SceI-XCm (ori_{R6K}, I-SceI restriction site, Tp^r and Cm^r (153)) and the broad host range replicative plasmid pDAI-SceI-SacB (ori_{pBBR1}, I-SceI nuclease, counter selectable marker SacB, Tet^r (153, 154)).

Bacteria were maintained on Luria-Bertani agar (LBA; Oxoid, Hampshire, UK) at 37°C. The liquid medium used for mutant construction was Luria-Bertani broth (LBB; Oxoid) and antibiotics for plasmid selection were chloramphenicol (Cm; Sigma-Aldrich, Brussels, Belgium), gentamicin (Gm; Sigma-Aldrich), kanamycin (Km; Sigma-Aldrich), trimethoprim (Tp; Ludeco, Brussels, Belgium) or tetracycline (Tet; Sigma-Aldrich).

To investigate planktonic cultures, cells were grown with orbital agitation (250 rpm) at 37°C to optical density at 590 nm of 0.5 (5 x 10^8 colony forming units (CFU) /mL) for exponential phase and 2.0 (2 x 10^9 CFU/mL) for stationary phase. Biofilms were grown in a round-bottomed 96-well microtiter plate (TPP, Trasadingen, Switzerland). Approx. 5 x 10^6 cells were added to one well. The plate was incubated for 24 h at 37°C, which included a washing step after 4 h with physiological saline (PS; 0.9 % NaCl in MQ). After 24 h the biofilm cells were harvested. The supernatant was removed, the cells were washed with PS and the wells were refilled with PS. To detach the cells, the 96-well plate was sonicated at 40 kHz and shaked at 900 rpm for 5 min and this process was repeated.

DNA extraction

Genomic DNA of *B. cenocepacia* J2315 was extracted using a modified bead-beater protocol (159). Briefly, a cell pellet (containing approx. 6 x 10^9 CFU) was resuspended in a TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8 in MQ) and subsequently mixed with a lysis buffer (50 mM Tris-HCl pH 8, 70 mM EDTA pH 8, 1 % SDS in MQ) containing pronase (Roche, Vilvoorde, Belgium) and acid-washed glass beads (Sigma). The suspension was vigorously vortexed for 10 seconds and the lysate was incubated at 37°C for 60 min. Both a saturated ammonium acetate solution (3 M) and chloroform were added to the lysate and the solution was vortexed for 10 seconds. After centrifugation, the aqueous phase was transferred and purified by ethanol precipitation. Finally, the DNA pellet was dissolved in low-EDTA-TE buffer (0.1 mM EDTA) and treated with RNase at 37°C for 60 min.

RNA extraction

Bacterial cells were pelleted at 4°C by centrifugation and stored at -80°C or immediately used for total RNA extraction. RNA was extracted using the Ambion RiboPure Bacteria kit (Life Technologies, Invitrogen, Merelbeke, Belgium) according to the manufacturer's instructions which included a 1 h DNase treatment at 37°C. To increase recovery of sRNAs, the volume of ethanol was increased from 0.5 to 1.25 times the volume of the recovered aqueous phase. Purified RNA was DNase-treated for 1 h at 37°C.

Quantitative RT-PCR analysis (qPCR)

RNA was extracted as described above and cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Primers (Table 1) were used in a concentration of 600 nM. For each sample a technical replicate was included on the plate and at least three biological replicates were included. qPCR experiments were performed on a Bio-Rad CFX96 Real-Time System C1000 Thermal Cycler with an initial denaturation at 95°C for 3 min followed by 49 amplification cycles, consisting of a denaturation step (15 sec at 95°C), an annealing step (30 sec at 64°C) and an extension step (15 sec at 72°C). A melting curve analysis was included at the end of each run. Data was normalised to two control genes (BCAM0918 and BCAL0026). Fold changes were calculated to a cDNA standard (mix of cDNA of all the samples of the different conditions).

Construction of mutants

Hot Star High-Fidelity (Qiagen, Venlo, Netherlands) or Phusion High-Fidelity (New England Biolabs, Bioké, Leiden, Netherlands) DNA polymerase were used for PCR amplification. Subsequently, PCR products were purified using NucleoSpin Gel and a PCR clean-up kit (Machery-Nagel, Düren, Germany). Plasmid extraction and isolation was carried out using the Wizard Plus SV Minipreps DNA purification system (Promega). Restriction digests and ligation reactions were performed according the manufacturer's instructions (Promega). Correct insertions were confirmed by PCR using GoTaq G2 DNA polymerase (Promega).

sRNA deletion mutants were constructed by allelic recombination (160). Briefly, sequences of approx. 1000 bp upstream and downstream of the sRNA sequence to be deleted were amplified using primer pairs listed in Table 1 and cloned into the suicide plasmid pGPI-SceI-Xcm. The plasmid was transformed into *E.coli* DH5α λpir by the $CaCl_2$ method (161), and into *B. cenocepacia* J2315 by triparental mating (152, 162). Ex-conjugants were sprayed with catechol and yellow colonies were selected for further propagation. Presence of concatenated DNA inserts was confirmed by PCR. pDAI-SceI-SacB, containing an endonuclease-encoding gene, was transformed into a *B. cenocepacia* pGPI-SceI-XCm mutant by

triparental mating. Colonies remaining white after catechol treatment were selected and screened for sRNA deletion by PCR. Correct deletion was further confirmed by Sanger sequencing. The mutants were cured from the pDAI-SceI-SacB plasmid by successive transfers on LBA with 5 % sucrose. Absence of the sRNA was confirmed by Sanger sequencing (GATC Biotech, Konstanz, Germany).

Table 1. Primers or probes used in this study.

Primers			Oligonucleotide sequence, 5'–3'
Construction of deletion mutant			
ΔncS04	Up	F:	ATAGAATTCTTCAGCATCGCGTATTCACC (EcorI)
		R:	AAAGCTAGCCCAACGATCTCGAAAATGACC (NheI)
	Down	F:	AAAGCTAGCGAATCCGCAGGTACCGTCA (NheI)
		R:	AAAAGATCTCTGCAACCTGAAGCACCATC (BgLII)
ΔncS16	Up	F:	AAAGGGCCCGCAGCAAGGCAACAATACGG (ApaI)
		R:	AAAGCTAGCGGCCTGCGACAGAAGTTCG (NheI)
	Down	F:	TATGCTAGCTTTGCCCGGAGAATTCGTG (NheI)
		R:	AAACCCGGGCGCATCTTGTTGGTCGCTTC (SmaI)
ΔncS25	Up	F:	AATAGATCTAATCTTCACCGCCATTGTCC (BgLII)
		R:	AAAGCTAGCCTTTGTCGGCGCGACTTC (NheI)
	Down	F:	TTTGCTAGCTCCATTTCATTTCGCTGCAC (NheI)
		R:	TTTGGGCCCCACGGTTGTTCGAGAACTGG (ApaI)
ΔncS27	Up	F:	ATTGAATTCTTGTCTGTGTGCGCTTCGAG (EcorI)
		R:	TATGCTAGCTTCGTCCCATGCAAACATCG (NheI)
	Down	F:	TATGCTAGCTCATGATGCGGGATTTCGAT (NheI)
		R:	TATAGATCTCAGGTAAGGGTGAAACGGT (BgLII)
Primers used for target expression analysis			
BCAL3204		F:	ACGCGCAGTACCTGAAGAGC
		R:	CGAGGTTGTACTCGCTCGTG
BCAL3473		F:	ACAACGCGAACACGTACCAG
		R:	TGAAGTTGATTTCGCCGTTG
Primers of control genes used for target expression analysis			
BCAM0918		F:	GAGATGAGCACCGATCACAC
		R:	CCTTCGAGGAACGACTTCAG
BCAL0026		F:	TATGAAGTGCTGGTCGATGG
		R:	TCAGCACGAAATCGTAGTCG
Primers used for translational fusions			
mCherry		F:	ATATTCTAGAGTGAGCAAGGGCGAGGAGG (XbaI)
		R:	AATGCATGCTTACTTGTACAGCTCGTCCATGCC (SphI)
BCAL3204 -fusion		F:	AAAGGATCCTCGCAACGTTCTGGCTGCAG (BamHI)
		R:	AAATCTAGAGAGCGCGCTGATCATCATCAC (XbaI)
BCAL3473 -fusion		F:	AAAGGTACCAAGTTGATGGGGCCCGTCAACAGAAC (KpnI)
		R:	AAATCTAGAGCCGTAAAGCGTCACGCTGCTTTG (XbaI)

Restriction sites are underlined.

Computational analysis

The Mfold web server was used to predict secondary structure using standard parameters (temperature: 37°C, ionic strength: 1M NaCl) (185). The CopraRNA (Comparative Prediction Algorithm for sRNA Targets, (186)) software was used to computationally predict targets. This algorithm combines structural information of the sRNA with a conservation analysis between at least three organisms. Therefore, sRNA homologues of the next genome-sequenced Bcc strains were used: *B. cenocepacia* J2315, HI2424, AU1054 and MC0-3, *B. lata* sp. 383, *B. vietnamiensis* G4, *B. multivorans* ATCC 17616, *B. ambifaria* AMMD. This calculation results in a list of 100 targets with the smallest p-values, conserved in at least 50 % of input strains. A density plot for mRNA interaction sites is calculated from all predicted interactions with a p-value ≤ 0.01.

SDS-PAGE and proteomics

Extraction of outer membrane proteins

The cell pellets were dissolved in 1 ml TSE buffer (200 mM Tris HCl pH 8, 500 mM sucrose, 1 mM EDTA, Complete protease inhibitor cocktail (Merck)). After 30 min incubation on ice, the cellular debris was removed by centrifugation (30 min, 16.000g, 4°C) and the proteins present in the supernatant were precipitated with 10 % trichloroacetic acid. The protein pellets obtained after centrifugation were washed with ice-cold acetone and dissolved in 2 M urea/50 mM ammonium bicarbonate. Protein concentrations were estimated by Bradford's method (Coomassie Plus Protein Assay; Pierce).

SDS-PAGE

10 µg of proteins were loaded on 12.5 % acrylamide/bis-acrylamide gels. Electrophoresis was performed 100V for 20 minutes and then at 150 V for one hour. Subsequently, gels were stained overnight with Coomassie Brilliant Blue G-250 (Sigma-Aldrich), and decolorized with 30 % methanol until protein bands were visible.

1D-LC-MS^E

Proteins were reduced, alkylated 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, 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 % over 45

min. The outlet of the column was directly connected to a PicoTip Emitter (uncoated SilicaTip 10 +/- 1 µm, New Objective, Woburn, MA, US) mounted on a Nanolockspray source of a SYNAPT^{TM} 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

Mass spectrometry data were processed using ProteinLynx Global SERVER (Waters). Identification of the proteins was performed using a strain-specific *B. cenocepacia* J2315 database (including common contaminants; Uniprot). 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. The p-values and q-values had to be smaller than or equal to 0.05 in order 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 > 2.0.

Translational fusions

Plasmid pSCrhaM2 was used for translational fusions. This plasmid is the rhamnose-inducible plasmid pSCrhaB2 lacking the start codon and Shine-Dalgarno sequence. It was constructed as described previously (208). Restriction and ligation were performed according manufacturer's instructions (Promega).

The 5'UTR and up to 40 nucleotides of the coding region of BCAL3204 and BCAL3473 were ligated in to the multiple cloning site (MCS) of pSCrhaM2 (primers used are listed in Table 1). Sequences encoding for the protein mCherry (209) lacking the start codon were ligated downstream with the target sequence. Plasmids were transformed into *E. coli* DH5α and colonies with the insert, confirmed by PCR, were conjugated into the wild type and into the sRNA mutants by triparental mating. Sanger sequencing was used to confirm the correct inserts.

All media used for further experiments contained 600 µg/mL Tp. Mutants were grown overnight in the basal salt medium (BSM, (157)) with 0.5 % (v/v) glycerol and 0.5 % (w/v) glucose. Cells were centrifuged and washed with BSM 0.5 % glycerol, to remove the glucose, which inhibits the rhamnose inducible promoter. Subsequently, cells were resuspended in LBB/BSM (50/50) to obtain an optical density of 1.0 (1 x 10^9 CFU/mL) and rhamnose was added to a final concentration of 0.2 %. 200 µL was added to the wells of a round-bottomed 96-well plate (TPP). The plate was incubated at 37°C and fluorescence (excitation wavelength: 555 nm/emission wavelength: 635 nm) was determined using a microtiter plate reader (Envision multilabel reader, PerkinElmer, Zaventem, Belgium).

Statistics

Each experiment was performed at least in duplicate. For normally distributed values, an independent sample-t-test or ANOVA was performed, otherwise a Mann-Whitney test or a Kruskal-Wallis test was performed. Differences were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

The four investigated sRNAs ncS04, ncS16, ncS25 and ncS27 are located on the large chromosome of *B. cenocepacia* J2315 (Figure 1A). dRNA-seq shows two peaks for ncS27, one is enriched by TEX-treatment and represents the TSS, and one is depleted by TEX-treatment, which indicates processing (Figure 1B). This processing site is located 29 nucleotides upstream of the TSS.

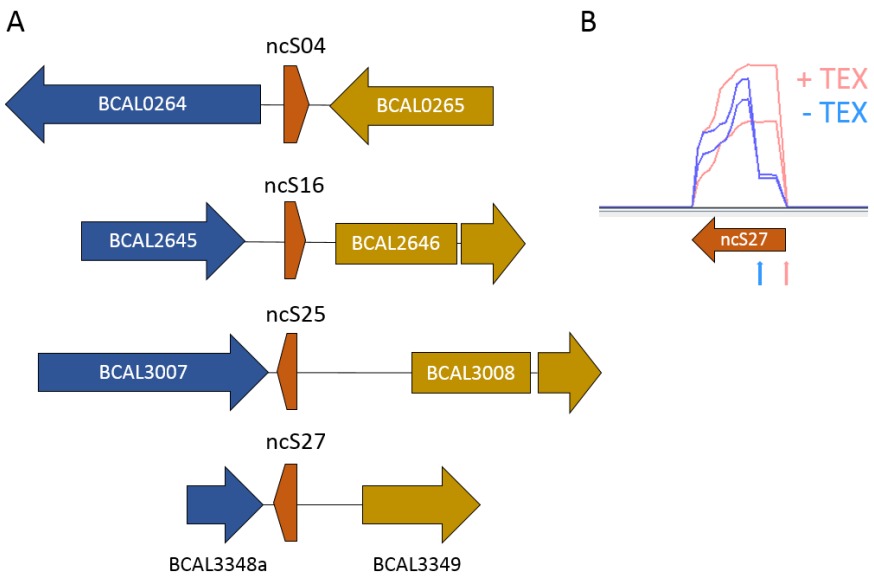


Figure 1. A) Genomic location of ncS04, ncS16, ncS25 and ncS27. BCAL0264, delta-aminolevulinic acid dehydratase; BCAL0265, GTP-binding cell division protein EngB; BCAL2645, OmpA family membrane protein; BCAL2646, methionyl-tRNA synthetase; BCAL3007, hypothetical protein; BCAL3008, outer membrane porin protein; BCAL3348a, hypothetical protein; BCAL3349, OsmC-like protein. Genes and their spacing are drawn to scale. Genes are represented as an arrow to indicate their orientation within the genome. B) Coverage for ncS27 in dRNA-Seq data. Red line: TEX-treated sub-sample, blue line: untreated sub-sample. Red arrow: location of TSS, blue arrow: processing site.

To determine the biological function of these sRNAs, sRNA deletion mutants were constructed and their phenotype was investigated. sRNAs ncS04, ncS16, ncS25 and ncS27 were successfully deleted from the genome of *B. cenocepacia* J2315 which indicates they are not essential, as found for most sRNAs (210). Mutant strains with either ncS04, ncS16 or ncS27 deleted (ΔncS04, ΔncS16, ΔncS27) did not differ from wild type with regards to various aspects of biofilm formation, susceptibility to a variety of antimicrobial agents and to membrane, oxidative and osmotic stress, growth in an iron-depleted environment, motility and virulence towards *C. elegans* (these data were obtained during this PhD and are shown in chapter III paper 1). Because sRNAs are responsible for the fine-tuning of gene regulation, many sRNA deletion mutants often show no or only very mild phenotypes, detectable in certain specific (stress) conditions only (180). Three explanations are possible for this lack of phenotype: (i) the deletion of the selected sRNAs does not influence the phenotype in our experimental set-up, (ii) these sRNAs have minor regulatory roles, and/or (iii) they are functionally redundant (127). To investigate the function and regulatory network of the sRNAs ncS04, ncS16 and ncS27, a combination of approaches was used. Preliminary data for ncS25 were also obtained: these are discussed in a separate section.

Computationally predicted targets

The CopraRNA software tool was used to predict potential mRNA targets for ncS04, ncS16 and ncS27 based on base-pairing properties and a conservation analysis. This analysis results in a ranked list of 100 potential mRNA targets (Supplementary Tables S1) and in a density plot for mRNA nucleotide positions calculated by the relative frequency of this position in all predicted interactions with a p-value ≤ 0.01. For the three sRNAs investigated, most interactions with potential mRNA targets are predicted within the region of 30 nucleotides upstream to 30 nucleotides downstream of the start codon (Figure 2).

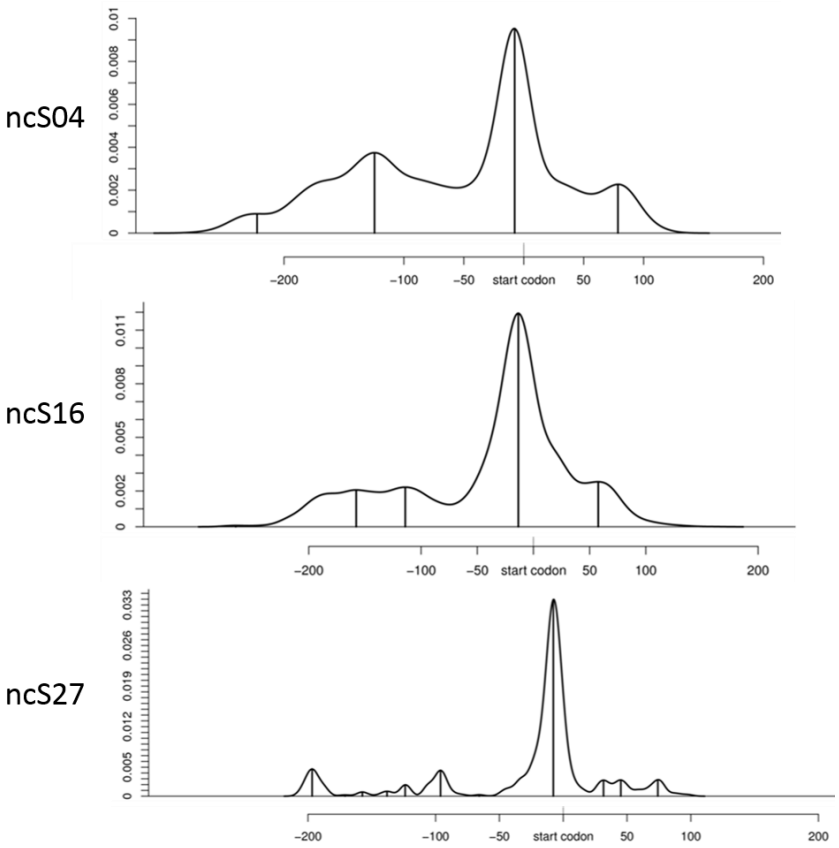


Figure 2. Density plots of mRNA interaction sites. Putative mRNA targets were predicted by CopraRNA for sRNA ncS04, ncS16 and ncS27. The density plot is calculated from all predicted interactions with a p-value ≤ 0.01. y-axis: relative frequency of a mRNA nucleotide position in predicted interactions.

This region includes the ribosome binding site and indicates a possible mechanism of action for these sRNAs, i.e. by translational repression or activation. sRNAs could mask the ribosome binding site and inhibit initiation of translation; alternatively they could open a secondary structure of the mRNA that inhibits ribosomes from binding and activate translation (75). Interaction with mRNA targets are predicted to mostly occur on the un-paired single stranded regions of the sRNA (Figure 3).

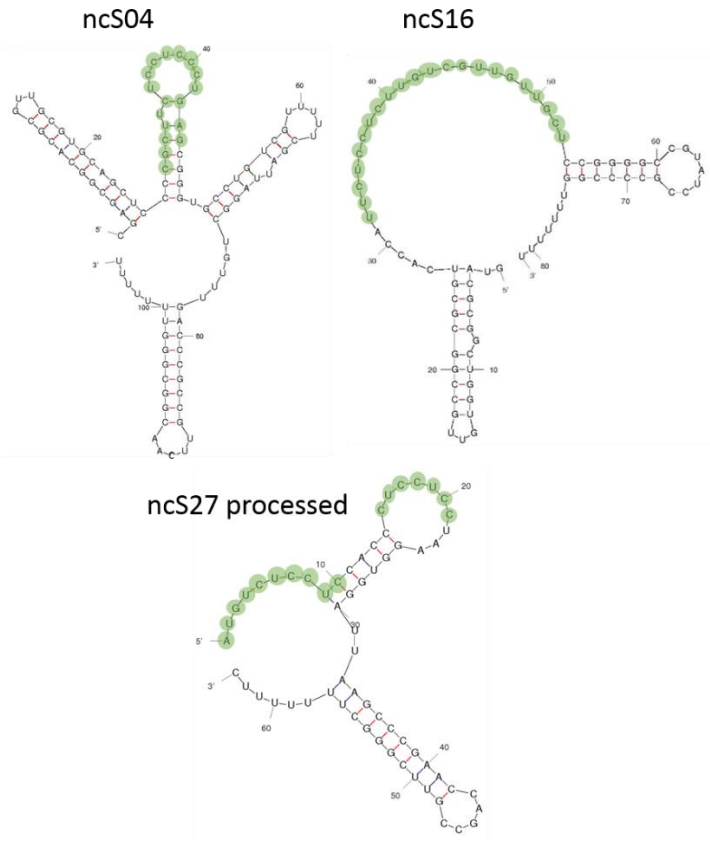


Figure 3. Secondary structures of ncS04, ncS16 and the processed form of ncS27. Structures were predicted by Mfold. Green circles indicate the predicted interaction sites.

For ncS04, the predicted target with the lowest p-value, is BCAL2958, a putative OmpA family protein. For ncS16, the predicted target with the lowest p-value is BCAL3055, the transcription antitermination protein NusB, and for ncS27, the predicted target with the lowest p-value is BCAL0206, an indolepyruvate ferredoxin oxidoreductase. Overall, the target lists contain many genes encoding cell envelope proteins such as outer membrane proteins (OMP) and porins. Almost half of the targets of known sRNAs are outer membrane proteins (73) and therefore further research was focussed on expression of these outer membrane proteins.

SDS-PAGE and proteomics

We used SDS-PAGE to investigate differences in protein composition in sRNA deletion mutants compared to wild type after growth in the presence of 0.005 % SDS in LBB (a situation mimicking membrane stress) (Figure 4).

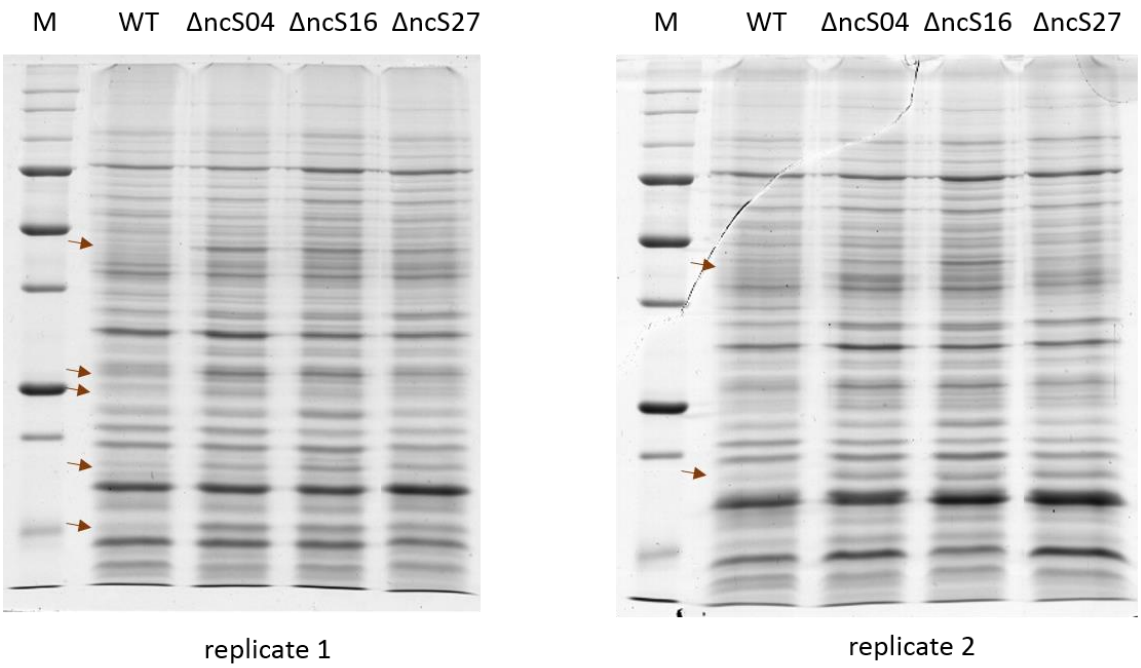


Figure 4. SDS-PAGE. A Coomassie Brilliant Blue stained gel after SDS-PAGE separation of proteins extracted from cells grown to stationary phase in LBB 0.005% SDS.

Differential protein expression was observed and LC-MS^E was used to identify differentially expressed proteins. Detected proteins with a fold change > 2.0, a q-value < 0.05 and at least two peptides available for quantification were considered relevant (Table 2, Supplementary Table S2). 367 proteins were detected, however only 208 proteins could be quantified. Of these 208 proteins approx. 30 were outer membrane proteins. For ΔncS04 only two proteins were significantly differentially expressed. A putative exported protein (BCAL1961) is more abundant with a fold change of 2.06 in ΔncS04 compared to wild type, while a putative lipoprotein (BCAL3153) is less abundant with a fold change of 3.54. In ΔncS16, eight proteins are significantly differentially expressed compared to wild type, all are upregulated in the mutant. Among them is a putative OmpA family lipoprotein, encoded by BCAL3204. It is upregulated with a fold change of 2.02 and is also predicted by Copra Analysis as a putative target. The protein with the highest fold change, 4.22, is a putative lipoprotein, encoded by BCAM0843. For ΔncS27, no significantly differentially expressed proteins could be identified.

Table 2. Outer membrane protein analysis of sRNA deletion mutants compared to wild type using LC-MS^E. Proteins with both a p-value as a q-value < 0.05, a fold change > 2.0 and at least two peptides available for quantification were considered relevant and are listed. **Bold** = predicted target by CopraRNA.

Accession no. Uniprot	Gene in *B. cenocepacia* J2315	Function	Fold change compared to WT	Corrected p-value
ΔncS04 compared to wild type				
B4EBI9	BCAL1961	Putative exported protein	2.06	2.33E-06
B4ECU4	BCAL3153	Putative lipoprotein	-3.54	1.12E-04
ΔncS16 compared to wild type				
B4EMP5	BCAM0843	Putative lipoprotein	4.22	7.59E-07
B4EG28	BCAM0042	Putative aldo/keto reductase	3.64	1.70E-05
B4E9M7	BCAL0677	Thiol:disulfide interchange protein	3.18	8.73E-06
B4EBR7	BCAL3033	Outer-membrane lipoprotein carrier protein lolA	2.82	4.35E-05
B4EKQ1	BCAM1652	Putative lipoprotein	2.30	1.68E-06
B4EDC0	BCAL3203	Translocation protein TolB	2.25	1.57E-04
B4EDC1	**BCAL3204**	**Putative OmpA family lipoprotein**	**2.04**	1.55E-04
B4EJQ5	BCAM2556	Putative purine nucleoside permease	2.00	3.65E-03

Very few of the computationally predicted targets could be detected and/or quantified by LC-MS^E. Of the predicted targets for which proteins were detected (7 for ncS04 and ncS16 and 4 for ncS27), only one was differentially expressed in a sRNA mutant; the above mentioned putative OmpA family lipoprotein which is upregulated in ΔncS16.

qPCR

The expression of BCAL3204 was further investigated on the level of transcription in ΔncS16 and wild type using qPCR. However in the four growth conditions tested (i.e. planktonic exponential phase, planktonic stationary phase, biofilm and planktonic stationary phase with added membrane stressor SDS) it was not significantly differentially expressed in ΔncS16 (Figure 5).

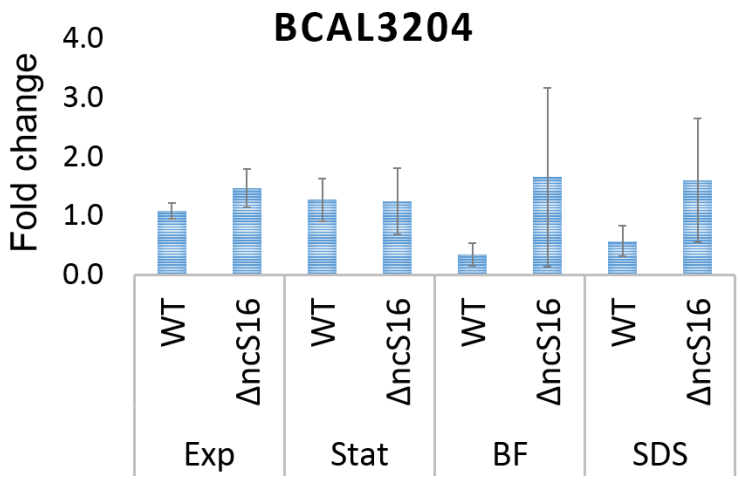


Figure 5. Expression of BCAL3204 in wild type (WT) and ΔncS16 evaluated in planktonic exponential phase (Exp), planktonic stationary phase (Stat), biofilm (BF) and planktonic stationary phase with added membrane stressor SDS (SDS) as determined with qPCR. Fold changes are calculated relative to a cDNA standard (mix of cDNA of different samples of different conditions). Error bars represent standard deviation (n=3).

Identification of interaction partners using translational fusions

The potential interaction between ncS16 and BCAL3204 was further investigated using a translational fusion. The interaction of ncS16 with BCAL3204 was predicted to be located within the 5'UTR, from 23 nucleotides upstream to 4 nucleotides upstream of the start of the annotated gene. Therefore a gene encoding a fluorescent protein, mCherry, was fused to the region of the mRNA containing approx 150 nucleotide upstream the start codon (5'UTR) and approx. 40 nucleotides of the coding sequence. The plasmid containing such a fusion was transformed into the wild type and into ΔncS16. Fluorescence was measured over a time range of 20 h (Figure 6A). After 20 h, the fluorescence signal was significantly higher in ΔncS16 compared to wild type, indicating the translation of BCAL3204 is upregulated in absence of ncS16 compared to the wild type (Figure 6B).

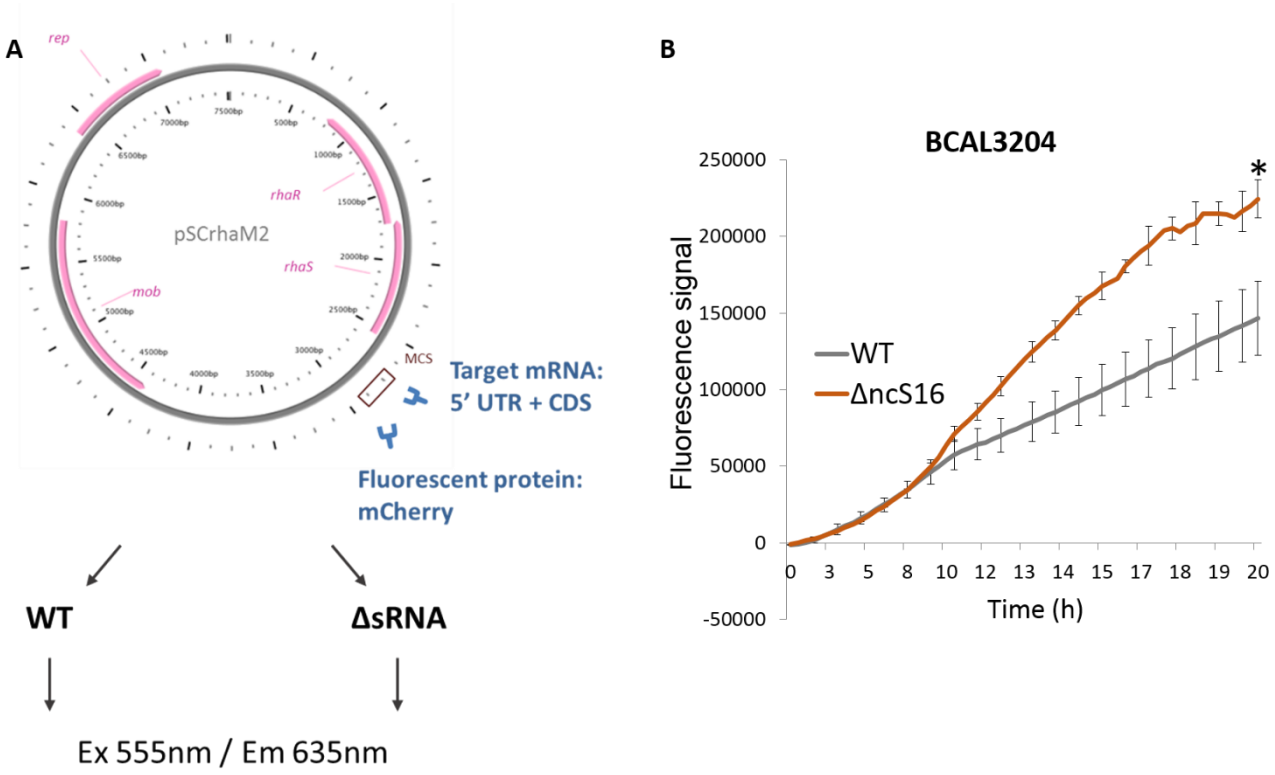


Figure 6. Translational fusions. A) The 5'UTR and approx. 40 nucleotides of the coding sequence (CDS) of a putative target is ligated to an mCherry coding sequence and cloned in the rhamnose inducible plasmid pSCrhaM2. The plasmid is transformed into the wild type (WT) and into a specific deletion mutant. *mob* = gene required for conjugal transfer of plasmid, *rep* = replication protein gene, *rhaR* and *rhaS* are transcriptional regulator genes of the rhamnose system and MCS = multiple cloning site. B) The fluorescence signal of mCherry measured during 20 h of wild type and ΔncS16 overexpressing BCAL3204 fused to mCherry grown in LBB/BSM (50/50) 0.2 % rhamnose 600 Tp. Error bars represent standard deviation. A statistical experiment was performed for the final fluorescence signal; a significant difference is indicated by an asterisk ($p < 0.05$, $n = 3$).

Together these results indicate that ncS16 binds to the BCAL3204 mRNA and possibly acts by inhibiting ribosome binding. This results in a lower protein expression, however not necessarily in a lower mRNA expression (211). BCAL3204, is annotated as OmpA/PAL, an outer membrane or peptidoglycan-associated lipoprotein (PAL). OmpA-like proteins are highly represented on the surface of Gram-negative bacteria and are linked to the adhesion, invasion and induction of cell death of host cells. Furthermore they have been found to play a role in antimicrobial resistance and immune invasion (212). PAL or the peptidoglycan-associated lipoprotein connects the outer membrane and the peptidoglycan (213). BCAL3204 probably encodes a PAL and, together with its adjacent genes BCAL3203 and BCAL3205, encodes for the Tol-PAL system membrane complex (214). The Tol-PAL system is well-known and conserved among Gram-negative bacteria and is involved in LPS O-antigen surface expression, outer-membrane stability, resistance to detergents and virulence (215). In *E. coli* it has been shown that PAL, can be released into the bloodstream where it contributes to provoke a septic shock (214). Furthermore, it has been shown that PAL is highly immunogenic in many bacteria (213); when it binds to host cells it can induce the release of cytokines such as TNF-α and various

interleukins (212). The Tol/PAL system BCAL3203-3205 was found upregulated in a *B. cenocepacia* strain related to J2315 in a rat agar bead infection model (21). BCAL3204 probably contributes to virulence in *B. cenocepacia* and could be important for the survival of this bacterium in the host.

Preliminary data on sRNA ncS25

CopraRNA analysis computationally predicted BCAL3473, a putative outer membrane porin, as the target with the lowest p-value for ncS25. The p-value for this interaction was remarkably lower compared to other predicted interactions and a relatively long interaction region of 29 nucleotides was predicted (Supplementary Table S1C). This protein was however not detected by LC-MS^{E}. The expression of BCAL3473 at the transcript level was evaluated by qPCR in exponential phase. BCAL3473 was significantly higher expressed with a fold change of 136 in ΔncS25 compared to a fold change of 6 in the wild type (Figure 7A). To investigate whether interaction occurred we performed an interaction analysis using a translational fusion to mCherry which included the computationally predicted interaction region of ncS25 with BCAL3473 (13 nucleotides upstream the first annotated nucleotide to 16 nucleotides within the coding sequence). However during a measurement of 20 h no significant difference in fluorescence is observed between ΔncS25 and the wild type (Figure 7B). Possibly, ncS25 does not target BCAL3473 directly and the differential expression of BCAL3473 in qPCR may be an indirect result of deleting this sRNA.

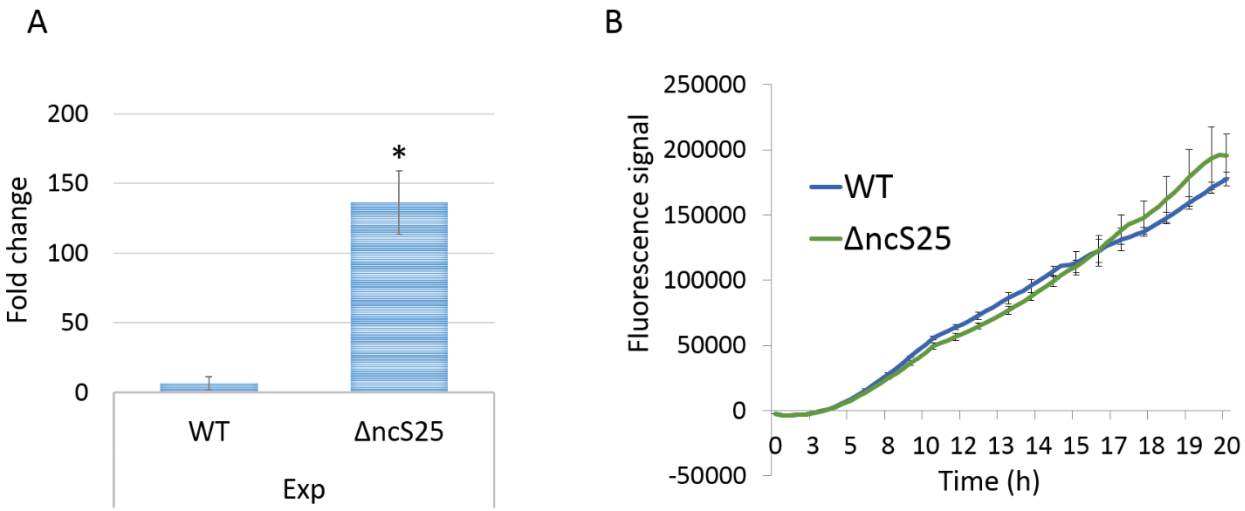


Figure 7. A) qPCR. Expression of BCAL3473 in wild type (WT) and in ΔncS25 evaluated in planktonic exponential phase (Exp). Fold changes are calculated relative to a cDNA standard (mix of cDNA of different samples in different conditions). B) The fluorescence signal of mCherry measured during 20 h of wild type and ΔncS25 overexpressing BCAL3473 fused to mCherry grown in LBB/BSM (50/50) 0.2 % rhamnose 600 Tp. Error bars represent standard deviation and statistically significant differences are indicated by an asterisk ($p > 0.05$, n=3).

In our study fluorescence is compared between wild type and a sRNA deletion mutant. It is possible that the sRNA expression in the wild type is not sufficient to cause detectable differences in fluorescence. One study in *E. coli* optimised a two-plasmid system to study translational fusions. This system comprises one low copy number plasmid (origin of replication pSC101, 3-4 plasmid copies/ cell) that expresses the mRNA fused to a reporter and one plasmid with a higher copy number (origin of replication ColE1, approx. 70 plasmid copies/cell) that expresses the sRNA to a higher level than the target (148). However, for *Burkholderia* species a two-plasmid system is not available due to the limited availability of cloning vectors that replicate in them, and due to their inherent resistance to a broad range of antimicrobials including the commonly used antibiotics employed for genetic selection. Additionally, most promotors used for gene expression analysis in *E. coli* do not function in *Burkholderia* species (167).

SUMMARY AND CONCLUSIONS

This study investigated potential targets of three sRNAs (ncS04, ncS16 and ncS27) which showed no phenotype when deleted and shows preliminary data for sRNA ncS25. Outer membrane protein analysis by LC-MS^E, showed that BCAL3204, a putative OmpA/PAL lipoprotein and a computationally predicted target for ncS16, was upregulated in ΔncS16. Interaction of sRNA ncS16 with BCAL3204 was confirmed using a translational fusion of this target to the gene encoding the fluorescent protein mCherry, however, no increased degradation of BCAL3204 was observed, as shown with by qPCR. For ncS25, the target with the lowest p-value was BCAL3473, a gene encoding a putative outer membrane porin. The mRNA level of this target was much higher in ΔncS25 compared to wild type, however it could not be confirmed as a direct target for ncS25 using translational fusion to mCherry. For the other two investigated sRNAs (ncS04 and ncS27) no targets could be confirmed.

FUNDING

This work was supported by the Belgian Science Policy Office (Belspo) through the Interuniversity Attraction Pole Program.

SUPPLEMENTARY DATA

Supplementary file 1: Tables S1. Computationally predicted targets by CopraRNA for ncS04 (Table S1A), for ncS16 (Table S1B), for ncS25 (Table S1C) and for ncS27 (Table S1D).

Supplementary file 2: Table S2. Proteomics data. Data of all detected proteins for wild type, ΔncS04, ΔncS16 and ΔncS27.

Supplementary Table S1A. Computationally predicted targets by CopraRNA for ncS04. Bold, proteins detected by proteomics; [a] Interaction position in mRNA relative to start codon (position 1-3).

			Interaction position in mRNA[a]		Interaction position relative in sRNA		
FDR	p-value	Target in *B. cenocepacia* J2315	start	end	start	end	Annotation
0.02814828	**9.70E-06**	**BCAL2958**	**-199**	**-192**	**35**	**42**	**putative ompA family protein**
0.057092923	2.95E-05	BCAL0749	-36	-29	35	42	putative cytochrome c oxidase
0.090124303	6.25E-05	BCAM1733	-138	-129	34	42	hypothetical protein
0.090124303	7.76E-05	BCAL2615	3	9	34	40	putative exported outer membrane porin protein
0.112753864	0.00013627	BCAL0029	-10	30	49	104	putative ATP synthase protein I AtpI
0.112753864	0.00016568	BCAL2114	-53	-47	35	41	uracil DNA glycosylase superfamily protein
0.112753864	0.000193989	BCAL3043	-8	-1	33	40	6-phosphogluconolactonase
0.112753864	0.000194254	BCAM1816	-17	-6	33	44	hypothetical protein
0.112753864	0.000216456	BCAL1663	-10	-4	36	42	PrkA family serine protein kinase
0.112753864	**0.000221178**	**BCAL3041**	**-13**	**-6**	**33**	**40**	**maltose-binding protein**
0.112753864	**0.000233083**	**BCAL2732**	**-88**	**-81**	**35**	**42**	**cold shock-like protein**
0.136716342	0.000306169	BCAL1965	-140	-124	23	38	putative lipoprotein
0.161172365	0.000388702	BCAL0890	-179	-172	27	34	thioesterase superfamily protein
0.163113559	0.000421482	BCAL1925	70	91	51	73	threonine synthase
0.165671989	0.000456633	BCAM1982	-15	-6	34	43	putative neutral zinc metallopeptidase
0.222221615	0.000651992	BCAL0824	-31	10	30	75	hypothetical protein
0.222221615	0.000689059	BCAM2161	-154	-117	35	77	two-component regulatory system sensor kinase
0.233186214	0.000767814	BCAL0287	-11	-4	33	40	putative outer membrane protein
0.233186214	0.000803398	BCAL3506	27	33	34	40	flagellar motor switch protein FliM
0.257208943	0.000930472	BCAL0206	-13	-7	34	40	indolepyruvate ferredoxin oxidoreductase
0.307419714	0.001314425	BCAM0619	-195	-171	27	48	hypothetical protein

0.307419714	0.00131925	BCAL2498	-128	-122	33	39	transposase
0.307419714	0.001353629	BCAL2826A	-51	34	1	76	hypothetical protein
0.307419714	0.001359181	BCAL2498	-128	-122	33	39	transposase
0.307419714	0.001421821	BCAL2830	-123	-113	33	42	two-component regulatory system sensor kinase protein
0.307419714	0.001517586	BCAL0016	17	23	35	41	putative branched-chain amino acid ABC transporter ATP-binding protein
0.307419714	0.001575104	BCAM0699	-13	-7	35	41	putative exported dehydrogenase
0.307419714	0.001581496	BCAL2230	-49	-43	35	41	putative ATP-dependent helicase
0.307419714	0.001592159	BCAL0199	-42	-36	37	43	putative lipoprotein
0.307419714	0.001715064	BCAM2652	-13	-6	37	44	TetR family regulatory protein
0.307419714	0.00172908	BCAM0751	-13	-7	35	41	LysR family regulatory protein
0.307419714	0.001747606	BCAL0344	-97	-80	55	73	putative type VI secretion system protein TssE
0.30788387	**0.001850992**	**BCAL1824**	**-13**	**0**	**27**	**41**	**putrescine-binding periplasmic protein**
0.30788387	0.001898467	BCAM2407	-16	-3	30	45	putative glycine betaine/L-proline ABC transporter substrate-binding protein
0.30788387	0.001949518	BCAL2031	-10	-4	34	40	hypothetical protein
0.30788387	0.001962395	BCAS0747	-14	-6	34	42	hypothetical protein
0.310669565	0.002033668	BCAL0194	18	42	50	74	putative oxidoreductase
0.345750633	0.002322872	BCAL3365	-12	-6	34	40	putative gluconate permease
0.346567202	0.00238806	BCAL2752	-173	-148	51	77	hypothetical protein
0.349193048	0.002466307	BCAL2824	-119	-107	55	67	isochorismatase family protein
0.350336702	0.002581791	BCAL1403A	-14	-8	32	38	hypothetical protein
0.350336702	0.002595087	BCAL3348	-83	-77	37	43	50S ribosomal protein L13
0.35826142	0.00272463	BCAL3396	-196	-190	34	40	thiamine monophosphate kinase
0.35826142	0.00277722	BCAL1414	-88	-62	37	65	putative hydrolase
0.384292311	0.00304521	BCAL2001	-24	-3	28	50	LuxR superfamily regulatory protein
0.393797573	0.003284887	BCAM0286	-12	-6	36	42	putative alcohol dehydrogenase
0.393797573	0.003310797	BCAL0323	-19	-9	33	41	twin arginine translocase protein A
0.393797573	0.003375581	BCAM1645	39	58	6	24	putative reductase component of oxidoreductase
0.393797573	0.003391883	BCAL1790	-19	5	27	50	putative iron-transport protein
0.414250132	0.003639407	BCAL2162	57	81	55	77	putative FkbP-type peptidyl-prolyl cis-trans isomerase

0.425888163	0.003815394	BCAS0306	-20	-6	37	50	hypothetical protein
0.425888163	0.003888385	BCAL1980	-16	-9	37	44	long-chain-fatty-acid--CoA ligase
0.429074734	0.004055751	BCAS0435	-7	14	50	73	oxidoreductase
0.429074734	0.004065308	BCAL1557	-11	-4	36	43	GntR family regulatory protein
0.434708139	0.004193567	BCAM2495	-10	-4	35	41	binding-protein-dependent transport system protein
0.439893037	0.004406171	BCAM1228	61	88	1	23	putative polysaccharide biosynthesis protein
0.439893037	**0.004446226**	**BCAL0409**	**-11**	**-5**	**33**	**39**	**enoyl-CoA hydratase**
0.439893037	0.00447092	BCAL1542	15	21	34	40	TetR family regulatory protein
0.43990158	0.004687869	BCAM0418	-73	-67	36	42	hypothetical protein
0.43990158	0.004698346	BCAL0795	-10	-4	34	40	phosphopantetheine adenylyltransferase
0.446437731	0.004845061	BCAM2336	-22	-3	28	46	putative sugar transferase
0.490819087	0.00541127	BCAL0184	-13	-7	34	40	putative glucarate transporter
0.490973059	0.005497545	BCAM1207	-137	-130	35	42	ABC transporter ATP-binding membrane protein
0.4957639	0.005664833	BCAM0841	-10	-3	35	42	hypothetical protein
0.4957639	0.005721995	BCAL2604	-11	-5	35	41	hypothetical protein
0.514102269	0.006022214	BCAM0575	72	85	11	24	LysR family regulatory protein
0.522743559	0.006213489	BCAM0673	-12	-6	33	39	IclR family regulatory protein
0.536631721	0.006502289	BCAM0107	-128	-122	33	39	LysR family regulatory protein
0.536631721	0.006656951	BCAM2659	-31	-24	36	43	putative tartrate transporter
0.536631721	0.006749431	BCAM1023	-30	-13	1	18	putative selenocysteine-specific elongation factor
0.536631721	0.006840783	BCAS0283	-52	-39	6	21	LysR family regulatory protein
0.542863035	0.007116092	BCAM0863	-13	-6	36	43	putative glycosyltransferase
0.542863035	0.007262806	BCAM0583	-89	-83	33	39	AraC family regulatory protein
0.542863035	0.007310585	BCAL1162A	-141	-135	37	43	putative transposase
0.542863035	0.007375527	BCAM0651	-20	-7	37	50	major facilitator superfamily protein
0.542863035	0.007563815	BCAL2789	54	60	34	40	AnsC family regulatory protein
0.542863035	**0.007576314**	**BCAM2821**	**-12**	**-6**	**35**	**41**	**malate synthase G**
0.542863035	0.007738704	BCAL0137	-8	-2	35	41	hypothetical protein
0.542863035	0.007770183	BCAM1423	32	57	2	21	acyl-CoA synthetase

0.542863035	0.007862043	BCAL1948	73	79	33	39	LysR family regulatory protein
0.542863035	0.007899037	BCAL0420	-15	-8	36	43	type I restriction component of type I restriction-modification system
0.542863035	0.007948899	BCAS0747	-14	-6	34	42	hypothetical protein
0.54359725	0.008092238	BCAL3474	-79	-54	30	50	Long-chain-fatty-acid--CoA ligase
0.54359725	0.008225219	BCAL0579	-124	-118	35	41	DNA-binding transcriptional activator GcvA
0.54359725	0.008240578	BCAM1818	-149	-134	27	41	hypothetical protein
0.548204757	0.008450829	BCAM0589	-11	-4	33	40	hypothetical protein
0.548204757	0.008532733	BCAL3276	-170	-153	32	44	NAD(+)/NADH kinase family protein
0.548204757	0.008611769	BCAM1541	-14	-7	35	42	putative dehydrogenase cytochrome C subunit
0.548204757	0.008695758	BCAM2255	72	96	2	29	putative OmpA/MotB family outer membrane protein
0.548204757	0.008877935	BCAL0115	75	94	58	77	30S ribosomal protein S21
0.548204757	0.008960663	BCAM1160	-31	-7	52	74	putative cyclic-di-GMP signaling protein
0.548204757	**0.009058782**	**BCAL0075**	**-122**	**-116**	**11**	**17**	**glycine cleavage system aminomethyltransferase T**
0.548204757	0.009065918	BCAL0359	30	50	3	24	putative lipoprotein
0.550226414	0.009194136	BCAL0638	-160	-127	34	65	LysR family regulatory protein
0.553897258	0.009462425	BCAL0549	-157	-144	11	24	hypothetical protein
0.553897258	0.009509687	BCAL3093	3	51	4	72	ABC transporter permease
0.553897258	0.009541727	BCAS0663	-143	-124	6	24	RHS-family protein

Supplementary Table S1B. Computationally predicted targets by CopraRNA for ncS16. Bold, proteins detected by proteomics; [a]Interaction position in mRNA relative to start codon (position 1-3).

			Interaction position in mRNA[a]		Interaction position relative in sRNA		
FDR	p-value	Target in *B. cenocepacia* J2315	start	end	start	end	Annotation
0.138894651	0.000165266	BCAL3055	-54	-28	29	53	transcription antitermination protein NusB
0.187641207	0.000255164	BCAL2604	-40	-2	29	65	hypothetical protein
0.207303475	0.000317139	BCAL1041	-7	36	7	54	hypothetical protein
0.228488795	0.000458428	BCAL2681	63	77	37	51	putative sulfate adenylyltransferase subunit 1
0.228488795	0.000494556	BCAS0357	-183	-166	32	51	xylose transporter ATP-binding subunit
0.228488795	0.000498657	BCAL0342	-39	-16	30	51	putative type VI secretion system protein TssC
0.228488795	0.000504905	BCAM0537	-26	9	17	51	family S33 serine peptidase
0.27095904	0.000709386	BCAL3044	-22	-9	35	48	bifunctional glucokinase/RpiR family transcriptional regulator
0.27095904	0.000728515	BCAL0749	-47	-20	29	53	putative cytochrome c oxidase
0.27095904	0.000736928	BCAM1733	-176	-130	32	78	hypothetical protein
0.286718627	0.000828526	BCAM1261	-122	-76	29	70	hypothetical protein
0.342640953	**0.001126752**	**BCAL2732**	**-33**	**-2**	**29**	**53**	**cold shock-like protein**
0.342640953	0.00115188	BCAS0747	-118	-96	27	52	hypothetical protein
0.342640953	0.001263522	BCAL0618	-111	-74	11	52	PfkB family carbohydrate kinase
0.342640953	0.001281881	BCAL2844	17	37	29	49	branched-chain amino acid aminotransferase
0.342640953	0.001289547	BCAM0055	-128	-104	29	52	GTP cyclohydrolase I
0.342640953	0.001387196	BCAS0747	-118	-96	27	52	hypothetical protein
0.342640953	0.001462353	BCAL1409	-30	-6	32	52	phosphoesterase family protein
0.342640953	**0.001521888**	**BCAL3204**	**-23**	**-4**	**31**	**52**	**putative OmpA family lipoprotein**
0.342640953	0.001605093	BCAL1864	-26	0	19	47	hypothetical protein
0.342640953	0.001605884	BCAL0241	-22	-4	31	47	50S ribosomal protein L16

0.342640953	0.001630792	BCAL0954	-13	14	19	44	recombination regulator RecX
0.454180635	0.002273531	BCAL2082	-21	-5	35	51	chaperone protein Skp
0.454180635	0.002316066	BCAL0795	-21	-4	34	52	phosphopantetheine adenylyltransferase
0.460003889	0.002500846	BCAM1645	-188	-158	8	48	putative reductase component of oxidoreductase
0.460003889	0.002502146	BCAL0781	-34	-8	29	52	PTS system transporter subunit IIBC
0.484931505	0.002746365	BCAL3269	50	70	36	52	chaperone protein DnaJ
0.484931505	0.002802596	BCAL0540	3	20	32	50	putative ATPase
0.496971013	**0.003201379**	**BCAL2958**	**-18**	**-2**	**32**	**48**	**putative ompA family protein**
0.496971013	0.003271208	BCAL2123	-29	7	20	52	hypothetical protein
0.496971013	0.003271339	BCAL3286	38	81	8	52	cobalamin adenosyltransferase protein
0.496971013	0.003301327	BCAL3165	76	99	29	52	putative exported hydrolase
0.496971013	**0.003323518**	**BCAL3297**	**37**	**52**	**38**	**53**	**putative ferritin DPS-family DNA binding protein**
0.496971013	0.003379031	BCAS0397	-145	-113	29	52	subfamily M20D metallo peptidase
0.510796256	0.003559858	BCAL0442	-29	-10	32	52	LysR family regulatory protein
0.609357916	0.004350337	BCAL1528	-18	58	5	53	flp type pilus assembly protein
0.614130975	0.004488804	BCAL3498	37	83	4	52	binding-protein-dependent transport systems inner membrane protein
0.621039698	**0.00482569**	**BCAM0972**	**-171**	**-151**	**35**	**54**	**type II citrate synthase**
0.621039698	0.005091724	BCAS0388	37	84	1	50	putative periplasmic solute-binding protein
0.621039698	0.005248262	BCAL1446	-16	35	14	44	putative lipoprotein
0.621039698	0.005300221	**BCAL3452**	-190	-176	38	52	bifunctional ornithine acetyltransferase/N-acetylglutamate synthase protein
0.621039698	0.005372389	BCAL0304	-30	-12	32	51	VacJ-like lipoprotein
0.621039698	**0.005455475**	**BCAL2820**	**-31**	**7**	**23**	**53**	**efflux system outer membrane protein**
0.621039698	0.00546494	BCAL1727	45	100	28	75	precorrin-6Y C5 15-methyltransferase (decarboxylating)
0.621039698	0.005532962	BCAL1895	16	27	42	53	stationary phase survival protein SurE
0.621039698	0.005556632	BCAL2718	-135	-93	29	78	quinolinate synthetase
0.621039698	0.005909409	BCAM2733	-195	-182	14	27	acylphosphatase
0.621039698	0.006072462	BCAM0970	-143	-134	38	47	succinate dehydrogenase iron-sulfur subunit
0.621039698	0.006088265	BCAL1965	-138	-84	27	81	putative lipoprotein
0.621039698	0.006148474	BCAM1207	24	65	5	53	ABC transporter ATP-binding membrane protein

0.621039698	0.006482595	BCAM1816	-177	-128	1	52	hypothetical protein
0.621039698	0.006485987	BCAL3460	-89	-33	15	67	D-alanine--D-alanine ligase
0.621039698	0.006629645	BCAL2334	-32	-10	32	53	NADH dehydrogenase subunit K
0.621039698	0.006683304	BCAL2393	-83	-63	32	50	rRNA large subunit methyltransferase
0.621039698	0.006717983	BCAL0594	-22	-5	31	52	hypothetical protein
0.621039698	0.006741392	BCAS0057	-180	-120	6	73	LysR family regulatory protein
0.621039698	0.007058915	BCAM2608	6	36	4	50	hypothetical protein
0.621039698	0.007067524	BCAM2459	-118	-98	29	52	putative transmembrane permease
0.621039698	0.007180043	BCAL0323	-31	-8	32	52	twin arginine translocase protein A
0.621039698	0.007317806	BCAL0645	-117	-84	5	46	sulfate-binding protein
0.621039698	**0.007337415**	**BCAL1824**	**57**	**74**	**35**	**53**	**putrescine-binding periplasmic protein**
0.621039698	0.007445983	BCAM2808	-25	-4	33	51	putative extracellular solute-binding protein
0.621039698	0.007447752	BCAL1326	-4	24	27	54	hypothetical protein
0.621039698	0.007495125	BCAL2016	-130	-85	21	73	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase
0.633160184	0.008060298	BCAL0540	3	20	32	50	putative ATPase
0.633160184	0.008255302	BCAM2243	-197	-163	19	52	hypothetical protein
0.633160184	0.008274386	BCAL0016	5	23	35	53	putative branched-chain amino acid ABC transporter ATP-binding protein
0.633160184	0.008287155	BCAL2114	-21	6	16	50	uracil DNA glycosylase superfamily protein
0.63450218	0.008412574	BCAL0023	-28	-12	30	44	putative branched-chain amino acid ABC transporter ATP-binding protein
0.640476573	0.008600654	BCAM0647	-52	21	11	78	putative ring-hydroxylating subunit beta
0.641575578	0.008724468	BCAM2411	-28	-7	32	51	hypothetical protein
0.642018224	0.008839619	BCAL3345	-21	19	5	52	anhydro-N-acetylmuramic acid kinase
0.664202383	0.009257963	BCAL1777	-18	-4	34	49	TonB-dependent receptor
0.667908632	0.009484859	BCAM1199	-24	-5	31	53	two-component regulatory system response regulator protein
0.667908632	0.009536686	BCAM0778	38	90	1	52	OmpA family protein
0.668584908	0.009773636	BCAL1269	-39	-5	29	52	phosphoglucosamine mutase
0.678983509	0.010041062	BCAM0364	-71	-55	35	52	putative lipoprotein
0.679282765	0.010160952	BCAL3289	-24	4	29	51	glycolate oxidase FAD binding subunit
0.696584827	0.010538169	BCAM1818	-153	-94	1	51	hypothetical protein

0.727162925	0.011168013	BCAM1659	-13	3	30	44	hypothetical protein
0.727162925	0.011342275	BCAL0035	62	83	25	51	F0F1 ATP synthase subunit gamma
0.727162925	0.011439136	BCAM2687	-185	-150	18	53	MgtC family membrane protein
0.727162925	0.011495181	BCAS0291	35	60	4	28	periplasmic solute-binding protein
0.732377787	0.01170211	BCAL0572	-160	-123	17	53	flagellar rod assembly protein/muramidase FlgJ
0.761192603	0.012291908	BCAL1948	35	79	33	72	LysR family regulatory protein
0.765751135	0.012625847	BCAL1413	57	95	3	51	glutaminyl-tRNA synthetase
0.778343645	0.013093511	BCAL2498	-139	-119	29	51	transposase
0.778343645	0.013098083	BCAL0606	-153	-118	17	54	putative transport related membrane protein
0.782855963	0.013307088	BCAM1982	-22	24	32	81	putative neutral zinc metallopeptidase
0.006402278	0.00000109	BCAL1872	14	36	29	52	putative nucleotide phosphoribosyltransferase
0.059822006	0.0000224	BCAS0750	-27	-3	30	53	hypothetical protein
0.059822006	0.0000305	BCAL0461	-21	4	29	52	LysR family regulatory protein
0.080145072	0.0000545	BCAL0730	-51	-21	21	50	ammonium transporter family protein
0.081723857	0.0000793	BCAS0058	-194	-167	33	54	putative oxidoreductase
0.081723857	0.0000833	BCAL3505	-58	-35	29	54	flagellar motor switch protein FliN

Supplementary Table S1C. Computationally predicted targets by CopraRNA for ncS25. Bold, proteins detected by proteomics; [a] Interaction position in mRNA relative to start codon (position 1-3).

			Interaction position in mRNA[a]		Interaction position relative in sRNA		
FDR	p-value	Target in *B. cenocepacia* J2315	start	end	start	end	Annotation
0.052029466	0.00000897	BCAL3473	-13	16	11	46	putative outer membrane porin
0.091437797	0.0000315	BCAL0035	61	77	39	54	F0F1 ATP synthase subunit gamma
0.189566268	0.000114168	BCAM2790	-16	24	20	55	putative 3-hydroxyphenylpropionic transporter MhpT
0.189566268	**0.000130668**	**BCAL2088**	**-17**	**-4**	**39**	**52**	**ribosome recycling factor**
0.336070957	0.000299727	BCAL2615	-7	7	40	55	putative exported outer membrane porin protein
0.336070957	0.000351143	BCAL0594	-11	9	19	44	hypothetical protein
0.336070957	0.000405393	BCAL0781	-19	-5	40	55	PTS system transporter subunit IIBC
0.358013016	0.000558273	BCAL3505	-50	-34	42	56	flagellar motor switch protein FliN
0.358013016	0.000562104	BCAL3038	-16	-9	48	55	ABC transporter ATP-binding protein
0.358013016	0.000616945	BCAL1506	-114	-102	42	56	transcription elongation factor NusA
0.464151002	0.000975789	BCAL0060	-20	-6	39	56	ethanolamine ammonia-lyase small subunit
0.464151002	0.001107171	BCAL2354	69	82	40	55	2-isopropylmalate synthase
0.464151002	0.001148143	BCAM2602	-136	-122	41	55	major facilitator superfamily protein
0.464151002	0.001148847	BCAS0397	-117	-96	38	55	subfamily M20D metallo peptidase
0.464151002	0.00119977	BCAL0749	-36	-25	44	55	putative cytochrome c oxidase
0.466325167	0.001322544	BCAL0442	-25	-9	40	55	LysR family regulatory protein
0.466325167	0.001366109	BCAL0624	-19	-2	38	56	putative outer membrane porin protein
0.510509821	0.001732741	BCAS0771	37	51	41	55	putative adenylosuccinate synthetase
0.510509821	0.001838768	BCAL0472	-15	-8	48	55	two-component regulatory system response regulator protein
0.510509821	0.001889515	BCAL0345	-16	8	20	51	putative type VI secretion system protein TssF
0.510509821	0.001892173	BCAM1787	-10	9	19	44	putative porin

0.510509821	0.001935415	BCAL1446	-16	-5	40	55	putative lipoprotein
0.51572898	0.002071105	BCAL3464	-29	-11	38	55	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase
0.51572898	0.002249854	BCAL0250	-53	-18	19	58	50S ribosomal protein L18
0.51572898	0.002256363	BCAL3055	-37	-7	19	55	transcription antitermination protein NusB
0.51572898	0.002495195	BCAL0328	7	19	40	55	ubiquinol-cytochrome c reductase iron-sulfur subunit
0.51572898	0.002566098	BCAL2718	-9	8	39	55	quinolinate synthetase
0.633173859	0.003382456	BCAL3427	-51	-44	48	55	histone H1-like protein
0.641833288	0.003621036	BCAM1423	-187	-150	40	74	acyl-CoA synthetase
0.641833288	0.003649922	BCAL0342	-14	2	39	56	putative type VI secretion system protein TssC
0.697147567	0.004173713	BCAL1071	5	14	47	56	NAD dependent epimerase/dehydratase family protein
0.697147567	0.004225343	BCAL0020	-16	-8	40	48	putative branched-chain amino acid ABC transporter periplasmic substrate binding protein
0.697147567	0.00434246	BCAL3008	-27	-7	40	56	putative outer membrane porin protein
0.697147567	0.004462079	BCAL0237	-151	-127	25	51	50S ribosomal protein L2
0.697147567	**0.004663422**	**BCAL1824**	**7**	**19**	**43**	**54**	**putrescine-binding periplasmic protein**
0.697147567	0.004685293	BCAL2931	2	24	23	45	radical SAM superfamily protein
0.710967265	0.005042378	BCAL1732	-13	5	42	58	MarR family regulatory protein
0.710967265	0.005308225	BCAL1777	-14	-2	44	56	TonB-dependent receptor
0.710967265	0.005397626	BCAM1477	-16	-4	40	55	aromatic amino acid transport protein
0.710967265	0.00566732	BCAL2498	-102	-72	28	56	transposase
0.710967265	**0.00594229**	**BCAL2732**	**-16**	**-1**	**42**	**55**	**cold shock-like protein**
0.710967265	0.006029045	BCAM1406	-118	-111	42	49	LacI family regulatory protein
0.710967265	0.00605624	BCAL2114	-15	-5	42	55	uracil DNA glycosylase superfamily protein
0.710967265	0.006169545	BCAM0528	37	49	43	55	putative oxidoreductase/short-chain dehydrogenase
0.710967265	0.006261328	BCAL1622	40	57	38	55	molybdenum-pterin binding protein II
0.710967265	0.006306444	BCAL2196	63	73	43	53	HesB family protein
0.710967265	0.00645531	BCAM2809	49	71	53	76	putative ABC transporter system permease
0.710967265	0.006540087	BCAL3276	-173	-154	40	56	NAD(+)/NADH kinase family protein
0.710967265	0.006798272	BCAM2301	-14	-8	40	46	putative methylamine dehydrogenase

0.710967265	0.007145132	BCAL1652	-22	-7	40	57	sulfate-binding protein
0.710967265	0.007494177	BCAL2730	-111	-91	37	55	putative ATP-dependent Clp protease ATP-binding subunit
0.710967265	0.007566362	BCAL0245	49	56	48	55	50S ribosomal protein L24
0.710967265	0.007567017	BCAM2410	35	48	42	54	putative glycine betaine/L-proline ABC transporter ATP-binding protein
0.710967265	0.007609662	BCAL3044	-19	-11	48	56	bifunctional glucokinase/RpiR family transcriptional regulator
0.710967265	0.007640205	BCAM2723	-121	-114	47	54	putative outer membrane porin protein
0.710967265	0.007703827	BCAL2647	3	10	48	55	hypothetical protein
0.710967265	0.007741083	BCAL1089	-17	-1	40	56	AnsC family regulatory protein
0.710967265	0.007910506	BCAL2752	-16	-7	46	55	hypothetical protein
0.710967265	0.007953117	BCAL1965	-143	-126	40	55	putative lipoprotein
0.710967265	0.00801716	BCAL0163	10	25	24	44	putative phospholipid-binding lipoprotein
0.710967265	0.008080172	BCAM0398	-22	-7	39	54	hypothetical protein
0.710967265	0.008205413	BCAM0119	-18	-5	39	54	hypothetical protein
0.710967265	0.008208652	BCAM1479	-14	-6	48	56	hypothetical protein
0.722987169	0.008608537	BCAL0753	-14	2	39	55	hypothetical protein
0.722987169	0.008738946	BCAM1604	-3	9	19	31	hypothetical protein
0.722987169	0.008845785	BCAL0884	-12	-5	40	47	putative acyl-CoA dehydrogenase oxidoreductase protein
0.731255694	0.009192187	BCAL0418	-87	-74	40	55	type I restriction enzyme specificity protein
0.731255694	0.009237908	BCAS0757	-17	-9	48	56	hypothetical protein
0.731255694	0.009324991	BCAL2393	-81	-64	40	56	rRNA large subunit methyltransferase
0.757014013	0.009846807	BCAL3260	-39	-5	42	76	hypothetical protein
0.757014013	0.009914366	BCAL2016	-101	-92	47	56	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase
0.764685406	0.010282467	BCAL1475	-12	-5	48	55	putative polysaccharide deacetylase
0.764685406	0.010755398	BCAM0841	-16	-4	40	55	hypothetical protein
0.764685406	0.010841326	BCAM1431	-13	-5	40	48	major facilitator superfamily protein
0.764685406	0.010847537	BCAM1007	4	11	48	55	putative polysaccharide biosynthesis protein
0.764685406	0.010893165	BCAM2152	-147	-136	45	56	hypothetical protein
0.764685406	**0.011147889**	**BCAL2609**	**55**	**67**	**42**	**54**	**periplasmic histidine-binding protein**
0.764685406	0.011154373	BCAL1321	51	63	44	55	glucosamine--fructose-6-phosphate aminotransferase

0.764685406	0.01117162	BCAS0052	-134	-128	52	58	sigma-54 dependent transcriptional regulator
0.764685406	0.011200803	BCAL3466	-17	-9	48	56	UDP-N-acetylmuramoylalanyl-D-glutamyl-2 6-diami nopimelate--D-alanyl-D-alanyl ligase
0.799872739	0.012156818	BCAL2656	-101	-89	43	55	cobyric acid synthase
0.799872739	0.012195912	BCAL1214	2	8	39	45	branched-chain alpha-keto acid dehydrogenase subunit E2
0.799872739	0.012499826	BCAL2498	-102	-72	28	56	transposase
0.799872739	0.012559122	BCAL3144	45	52	49	56	rubredoxin
0.799872739	0.012753632	BCAM2582	-140	-128	44	56	major facilitator superfamily protein
0.799872739	0.012826843	BCAS0495	-85	-78	48	55	benzoate 1 2-dioxygenase beta subunit
0.799872739	0.012976556	BCAL1357	-81	-69	44	56	hypothetical protein
0.799872739	0.013090125	BCAL2074	22	28	48	54	phosphoenolpyruvate synthase
0.799872739	0.013429043	BCAL2918	52	59	48	55	molybdenum cofactor biosynthesis protein MogA
0.799872739	0.013575653	BCAM2598	55	61	6	12	putative short-chain dehydrogenase
0.799872739	0.013586516	BCAL0756	-113	-98	40	55	hypothetical protein
0.799872739	0.013742541	BCAS0747	-17	-6	41	54	hypothetical protein
0.799872739	0.013834611	BCAL3053	67	73	49	55	bifunctional 3 4-dihydroxy-2-butanone 4-phosphate synthase/GTP cyclohydrolase II-like protein
0.799872739	0.014059749	BCAL2230	-37	-29	40	49	putative ATP-dependent helicase
0.799872739	0.014143553	BCAM1855	-22	-13	40	50	putative porin

Supplementary Table S1D. Computationally predicted targets by CopraRNA for ncS27. Bold, proteins detected by proteomics; [a] Interaction position in mRNA relative to start codon (position 1-3).

			Interaction position in mRNA[a]		Interaction position relative in sRNA		
FDR	p-value	Target in *B. cenocepacia* J2315	start	end	start	end	Annotation
0.771848754	0.000361311	BCAL0206	-17	-4	1	14	indolepyruvate ferredoxin oxidoreductase
0.805848319	0.000708233	BCAL2615	-18	12	1	30	putative exported outer membrane porin protein
0.805848319	0.00122514	BCAL0749	-37	-19	5	20	putative cytochrome c oxidase
0.805848319	0.001441905	BCAL0281	-66	-57	2	11	deoxyguanosinetriphosphate triphosphohydrolase-like protein
0.805848319	0.00162631	BCAM0663	-8	1	1	10	putative porin
0.805848319	0.001912368	BCAL0287	-11	-2	1	10	putative outer membrane protein
0.805848319	0.002009779	BCAL0023	-29	-10	2	19	putative branched-chain amino acid ABC transporter ATP-binding protein
0.805848319	0.002403025	BCAL0264	-121	-114	14	21	delta-aminolevulinic acid dehydratase
0.805848319	0.002477345	BCAM0744	-10	-1	1	10	major facilitator superfamily protein
0.805848319	**0.002652411**	**BCAL3041**	**-14**	**-4**	**1**	**11**	**maltose-binding protein**
0.805848319	0.003175842	BCAM1733	-136	-129	14	21	hypothetical protein
0.805848319	0.003324896	BCAL3043	-8	0	2	10	6-phosphogluconolactonase
0.805848319	0.003603356	BCAL0200	67	78	2	13	putative lipoprotein
0.805848319	0.003872319	BCAL2604	-11	9	5	21	hypothetical protein
0.805848319	0.004056643	BCAL3506	26	34	13	21	flagellar motor switch protein FliM
0.805848319	0.004185452	BCAL1948	73	80	12	19	LysR family regulatory protein
0.805848319	0.00450094	BCAL3396	-196	-177	1	20	thiamine monophosphate kinase
0.805848319	0.005571162	BCAL2284	-101	-87	1	14	acetyl-CoA synthetase
0.805848319	0.005717015	BCAM2056	-99	-93	15	21	type III secretion system protein
0.805848319	**0.00576993**	**BCAL2958**	**-198**	**-192**	**15**	**21**	**putative ompA family protein**

0.805848319	0.005987662	BCAM0583	-89	-82	2	9	AraC family regulatory protein
0.805848319	0.006658518	BCAM1506	-9	-3	2	8	putative phospholipid-binding lipoprotein
0.805848319	0.00676349	BCAM2433	-22	1	1	27	putative acyl-CoA dehydrogenase
0.805848319	0.006964514	BCAM2122	-184	-167	5	20	acetaldehyde dehydrogenase
0.805848319	0.007331773	BCAL3365	-14	-6	14	22	putative gluconate permease
0.805848319	0.008563505	BCAM1379	-10	1	1	11	ABC transporter substrate-binding protein
0.805848319	0.008987175	BCAL0719	-13	-3	1	11	CAIB/BAIF family protein
0.805848319	0.009012317	BCAS0770	42	50	12	20	LysR family regulatory protein
0.805848319	0.009358777	BCAM1441	-28	-19	12	21	beta alanine--pyruvate transaminase
0.805848319	0.009480999	BCAM2575	-47	-41	1	7	LysR family regulatory protein
0.805848319	0.009605116	BCAL0199	-10	-4	14	20	putative lipoprotein
0.805848319	0.009744469	BCAL1492	33	44	5	16	hypothetical protein
0.805848319	0.010006227	BCAL0850	-13	-3	1	11	glycolate permease
0.805848319	0.010367207	BCAL0795	-11	-4	14	21	phosphopantetheine adenylyltransferase
0.805848319	0.010391029	BCAL0184	-13	-5	2	10	putative glucarate transporter
0.805848319	0.010623247	BCAL1719	-12	-5	2	9	putative citrate transporter
0.805848319	0.01096113	BCAL2031	-10	-4	14	20	hypothetical protein
0.805848319	0.011754796	BCAS0747	-106	-98	13	21	hypothetical protein
0.805848319	0.011978454	BCAL0039	-10	-2	2	10	periplasmic cyclohexadienyl dehydratase
0.805848319	0.012235402	BCAM2594	-9	-3	15	21	putative alcohol dehydrogenase
0.805848319	0.012601883	BCAM2365	-9	0	1	10	putative signal-transduction and transcriptional regulator Fis and NtrC family protein
0.805848319	0.013005101	BCAL1387	84	90	41	47	putative phosphatase
0.805848319	0.013283906	BCAL0161	-60	-38	1	19	hypothetical protein
0.805848319	0.013377118	BCAL1342	-14	-7	1	8	putative hydroxyquinol 1 2-dioxygenase
0.805848319	0.013807739	BCAL2730	-9	0	2	11	putative ATP-dependent Clp protease ATP-binding subunit
0.805848319	0.013838247	BCAM1020	-12	-6	15	21	formate dehydrogenase cytochrome b556 (FDN) subunit
0.805848319	0.013957296	BCAL1423	-12	-6	15	21	ABC transporter ATP-binding protein
0.805848319	0.014172569	BCAM0679	78	84	41	47	hypothetical protein
0.805848319	0.014573495	BCAL2833	-14	-7	1	8	hypothetical protein

0.805848319	0.014676707	BCAM1205	-12	-4	2	10	hypothetical protein
0.805848319	0.015099941	BCAL3449	-64	-48	1	15	hypothetical protein
0.805848319	0.01535592	BCAM0363	-10	-4	1	7	putative lipoprotein
0.805848319	0.015543815	BCAL1732	-10	5	5	19	MarR family regulatory protein
0.805848319	0.015597367	BCAM2492	-18	23	2	28	hypothetical protein
0.805848319	0.015770607	BCAL3276	-159	-153	15	21	NAD(+)/NADH kinase family protein
0.805848319	0.015829594	BCAM0955	-16	-7	2	10	binding-protein-dependent transport system protein
0.805848319	0.015883792	BCAM1977	-91	-84	1	8	putative amino acid permease
0.805848319	0.016067223	BCAM1453	-182	-175	2	9	LysR family regulatory protein
0.805848319	0.01659285	BCAL3062	-41	-35	41	47	hypothetical protein
0.805848319	0.016735204	BCAM2443	-11	-4	1	8	putative cytochrome c
0.805848319	0.016872475	BCAL3029	-11	0	1	11	putative alkane monooxygenase
0.805848319	0.016875974	BCAS0609	-12	-5	1	8	putative electron transfer flavoprotein-ubiquinone oxidoreductase
0.805848319	0.01690671	BCAL1663	63	69	14	20	PrkA family serine protein kinase
0.805848319	0.017070883	BCAL2625	-9	-2	1	8	major facilitator superfamily protein
0.805848319	0.017503984	BCAL2149	-12	-2	1	10	HhH-GPD superfamily base excision DNA repair protein
0.805848319	0.017650373	BCAL0049	-12	-5	14	21	putative aminotransferase
0.805848319	**0.017753597**	**BCAL2732**	**-89**	**-82**	**12**	**19**	**cold shock-like protein**
0.805848319	0.017915186	BCAL1431	-32	-8	1	29	putative ribose ABC transporter substrate-binding exported protein
0.805848319	0.01814835	BCAL1864	-16	-9	14	21	hypothetical protein
0.805848319	0.018297841	BCAL0720	-8	-2	1	7	hypothetical protein
0.805848319	0.018599141	BCAS0471	-67	-46	2	27	outer membrane efflux protein
0.805848319	0.01914849	BCAL2789	53	61	13	21	AnsC family regulatory protein
0.805848319	0.019434275	BCAM1670	-73	-66	5	12	putative cyclic-di-GMP signaling protein
0.805848319	0.019822828	BCAL2676	-120	-97	2	24	leucyl aminopeptidase
0.805848319	**0.019904437**	**BCAL1868**	**-11**	**-3**	**1**	**9**	**hypothetical protein**
0.805848319	0.020265347	BCAM2052	-182	-175	14	21	hypothetical protein
0.805848319	0.020719489	BCAM2495	-10	-4	15	21	binding-protein-dependent transport system protein
0.805848319	0.020909848	BCAL1542	-12	-3	13	22	TetR family regulatory protein

0.805848319	0.020961357	BCAM2798	-10	-3	1	8	putative ABC transporter solute-binding protein
0.805848319	0.02107584	BCAS0137	-13	-6	14	21	hypothetical protein
0.805848319	0.021147366	BCAL2498	-128	-121	2	9	transposase
0.805848319	0.021799772	BCAM2566	-113	-107	41	47	putative GCN5-related N-acetyltransferase
0.805848319	0.022136251	BCAL3260	-12	-6	15	21	hypothetical protein
0.805848319	0.022163151	BCAL2752	33	39	2	8	hypothetical protein
0.805848319	0.022376311	BCAL2337	-15	-4	2	13	NADH dehydrogenase subunit H
0.805848319	0.022664488	BCAL1912	-12	-5	1	8	putative acetoin(diacetyl) reductase
0.805848319	0.022704368	BCAL2249	-9	-3	2	8	family S33 serine peptidase
0.805848319	0.02276183	BCAM2348	-10	-3	14	21	putative lipoprotein
0.805848319	0.022782343	BCAL2498	-128	-121	2	9	transposase
0.805848319	0.023042541	BCAL1911	35	42	1	8	acetoin:2 6-dichlorophenolindophenol oxidoreductase subunit alpha
0.805848319	0.02305028	BCAL2793	-158	-144	2	15	major facilitator superfamily protein
0.805848319	0.023458975	BCAL3385	-125	-119	1	7	putative L-idonate 5-dehydrogenase
0.805848319	0.023810551	BCAL0207	-11	-4	1	8	4-hydroxyphenylpyruvic acid dioxygenase
0.805848319	0.024031742	BCAL2114	-53	-47	15	21	uracil DNA glycosylase superfamily protein
0.805848319	0.024983479	BCAL2830	-123	-112	2	14	two-component regulatory system sensor kinase protein

Supplementary Table S2. Proteomics data. Average of all detected proteins for wild type, ΔncS04, ΔncS16 and ΔncS27 (n=6).

Accession	Gene in *B. cenocepacia* J2315	Peptide count	Peptides used for quantitation	WT	ΔncS04	ΔncS16	ΔncS27	Description
B4EE21	BCAL3299	43	34	22257.82	19844.54	26385.36	24498.75	Catalase-peroxidase 1
B4ECT7	BCAL3146	44	25	5313.519	5278.582	5582.365	5203.685	60 kDa chaperonin
B4EEU6	BCAL3358	32	25	34380.83	38081.71	27102.46	42778.26	Periplasmic glutamate/aspartate-binding protein
B4E5A5	BCAL0219	28	21	16939.82	16306.28	16225.44	16697.68	Elongation factor Tu
B4E5B7	BCAL0231	39	19	2388.59	2504.105	2461.727	2342.137	Elongation factor G
B4ED01	BCAL1065	27	18	14108.99	17163.27	13201.65	16738.96	Periplasmic solute-binding protein
B4EHD0	BCAM1250	23	18	2330.455	2969.802	2969.122	2461.197	Probable acetyl-CoA hydrolase/transferase
B4E5F7	BCAL1270	22	17	4420.07	4853.913	4560.441	5291.06	Ph phate-binding protein PstS
B4E8U8	BCAL1676	27	16	3394.17	3283.068	3324.926	3402.57	Multidrug efflux system outer membrane protein
B4EAC3	BCAL1824	23	16	4928.902	6707.485	4073.277	5640.36	Putrescine-binding periplasmic protein
B4ED28	BCAL1092	23	16	9462.174	9571.293	9149.099	10689.41	ABC transporter extracellular solute-binding protein
B4EPH7	BCAS0060	25	15	5799.441	6088.2	5006.978	8931.316	Extracellular amino acid-binding protein
B4EFA6	BCAM0961	24	15	1826.25	1972.331	2490.607	1833.828	Aconitate hydratase
B4EF00	BCAL0051	18	15	5662.692	4409.517	7616.657	6815.046	Periplasmic solute-binding protein
B4EFB0	BCAM0965	21	14	2732.374	3209.919	2863.632	2599.394	Malate dehydrogenase
B4E6Q6	BCAL0334	18	14	2375.797	2479.462	1786.557	2604.686	Periplasmic solute-binding protein
B4E7B9	BCAL0408	21	13	2387.278	2073.949	2750.193	2526.801	Putative phenylacetic acid degradation oxidoreductase
B4EF03	BCAL0043	21	13	4968.407	4642.877	3873.006	5230.105	Putative extracellular ligand-binding protein
B4EB46	BCAL2956	20	13	10473.7	14260.05	8221.35	10899.42	Putative exported protein
B4EEP2	BCAL2303	17	13	7076.97	4500.714	5690.559	7256.858	Aromatic amino acid aminotransferase
B4EMR4	BCAM1931	17	13	6890.481	6361.18	7613.881	5047.902	Putative porin
B4EA88	BCAL0763	16	13	10627.42	12328.97	11044.65	14428.07	Lipoprotein
B4E8I7	BCAL0544	16	11	6140.226	4993.143	5503.888	7358.351	Putative periplasmic dipeptide transport protein
B4EEY9	BCAL0036	15	11	1599.759	1894.443	1518.851	1598.57	ATP synthase subunit beta

B4EAC8	BCAL1829	14	11	7349.735	6780.437	6365.931	5961.757	Putative outer membrane protein
B4EDT5	BCAL2206	13	11	2318.35	1981.488	1926.54	1848.654	Phasin-like protein
B4EEU7	BCAL3359	13	11	1948.972	2340.504	2000.353	1992.435	Glutamate dehydrogenase
B4EE31	BCAL3310	13	11	9823.618	9798.917	11773.32	11799.07	Putative exported protein
B4EFB7	BCAM0972	15	10	1530.644	1653.387	1984.572	1759.433	Citrate synthase
B4EAW0	BCAL0849	13	10	4446.928	5077.249	3178.909	3534.473	Metallo peptidase_ subfamily M48B
B4EC20	BCAL2013	12	10	4403.416	4392.133	4026.539	4480.574	AhpC/TSA family protein
B4ED75	BCAL2152	11	10	4325.641	4531.318	3887.248	4936.653	Peptidyl-prolyl cis-trans isomerase
B4E628	BCAL0305	10	10	2789.657	2412.526	3185.133	3257.059	Putative exported protein
B4EGW5	BCAM2251	17	9	2229.087	2260.199	1611.046	2360.374	Putative amino acid solute binding component of ABC transporter
B4ECY3	BCAL3192	13	9	2440.267	1873.051	1927.259	2613.414	Putative oxidoreductase
B4E9M5	BCAL0675	13	9	1386.743	1452.323	1348.727	1677.886	Extracellular solute-binding protein
B4ELG0	BCAM2761	11	9	102006.9	55585.62	78089.91	138671.2	Giant cable pilus
B4E7J7	BCAL1493	16	8	929.8179	1000.167	1146.598	766.2186	Putative exported protein
B4E9G1	BCAL2762	15	8	1401.522	1423.879	1277.224	1426.608	Adenylate kinase
B4EE19	BCAL3297	14	8	18728.33	16601.75	17170.92	16966.28	Putative ferritin DPS-family DNA binding protein
B4ECX4	BCAL3183	14	8	752.8919	795.0367	703.1146	647.66	Putative hydrolase
B4EE32	BCAL3311	14	8	4082.298	3596.074	5234.131	4901.351	Putative exported protein
B4EN24	BCAM0906	13	8	1677.991	1994.564	1575.684	2116.829	Putative dienelactone hydrolase family protein
B4EDZ2	BCAL3270	13	8	1092.581	1356.344	1016.779	1286.672	Chaperone protein DnaK
B4EQ59	BCAS0293	12	8	14381.82	9620.417	8479.979	16062.48	Nematocidal protein AidA
B4EFJ5	BCAM0986	12	8	1585.572	1520.109	1433.36	1423.38	Aspartate-semialdehyde dehydrogenase
B4EB48	BCAL2958	10	8	7344.916	4335.6	6413.188	6725.548	Putative ompA family protein
B4EA12	BCAL2828	10	8	21866.29	22216.99	17015.29	26931.95	Putative exported protein
B4EAY5	BCAL1900	9	8	4159.93	4233.044	4138.086	4060.396	Thioredoxin
B4E9F6	BCAL2757	9	8	4604.625	4208.224	3541.019	4256.127	Superoxide dismutase
B4E5V4	BCAL3424	9	8	3002.409	2500.044	2724.922	3129.559	Probable thiol peroxidase
B4ECJ5	BCAL1033	8	8	6217.305	5619.348	5344.626	6990.236	Putative exported protein

B4E5A9	BCAL0223	8	8	915.0961	977.1037	950.2411	701.8936	50S ribosomal protein L1
B4E8T0	BCAL1657	16	7	973.5344	1503.631	1039.581	1185.618	Putative ribose transport system_ substrate-binding protein
B4E8A5	BCAL1610	16	7	1590.364	1772.197	1574.341	1808.772	Periplasmic cystine-binding protein
B4EDC0	BCAL3203	14	7	1520.36	1770.36	3427.409	1494.607	Protein TolB
B4EAU3;B4EAG1	BCAL0832, BCAL1862	14	7	2028.916	1795.309	2113.249	2332.862	Putative poly-beta-hydroxy-butyrate storage protein
B4E5F0	BCAL1263	11	7	1788.943	1939.734	1469.78	1991.612	Transcription elongation factor GreA
B4EBY7	BCAL0957	10	7	1025.684	1012.918	1042.771	895.8408	Succinyl-CoA ligase [ADP-forming] subunit alpha
B4EN10	BCAM0892	9	7	1301.844	1866.55	1158.482	1670.107	Putative molybdate-binding periplasmic protein
B4EMK6	BCAM0804	8	7	1021.398	1040.004	738.8685	920.2083	Catechol 1_2-dioxygenase 1
B4ECT8	BCAL3147	8	7	3246.428	2796.111	3088.704	3398.741	10 kDa chaperonin
B4ECZ5	BCAL1059	15	6	602.4485	791.7464	623.5734	615.4105	Acetylornithine aminotransferase
B4EBY6	BCAL0956	15	6	1235.867	1096.738	1273.601	1169.309	Succinyl-CoA ligase [ADP-forming] subunit beta
B4EAE8	BCAL1849	14	6	2256.142	1916.698	1438.845	2582.917	Putative exported protein
B4EA41	BCAL1012	12	6	1600.353	2494.115	1272.554	1693.021	Elongation factor P (EF-P) 1
B4EBI9	BCAL1961	11	6	1611.443	3313.797	1324.863	1816.733	Putative exported protein
B4EES4	BCAL3336	11	6	565.5518	453.4335	601.103	469.1595	Bifunctional purine biosynthesis protein PurH
B4ECN0	BCAL2090	11	6	621.8307	749.4482	721.4929	575.9545	Elongation factor Ts
B4EA90	BCAL0765	10	6	373.4657	632.7658	325.7238	398.0858	Putative exported protein
B4ED06	BCAL1070	10	6	1068.303	902.2861	955.6442	1151.562	Putative redoxin protein
B4EAP2	BCAL2934	10	6	1109.629	1043.308	993.4663	1041.293	Electron transfer flavoprotein alpha-subunit
B4EHT3	BCAM1293	9	6	896.0036	1066.444	833.0855	993.0497	ABC transporter_ substrate-binding protein
B4EC29	BCAL2022	8	6	701.6476	917.4433	756.0107	598.3758	PspA/IM30 family protein
B4EF87	BCAM0942	8	6	2753.929	2308.631	3416.493	2462.475	Putative exported protein
B4EAS3	BCAL0812	8	6	7328.411	5493.366	6021.222	7819.576	Sigma-54 modulation protein
B4EG36	BCAM0050	8	6	3738.671	2832.534	3315.837	3662.022	Universal stress-related protein
B4ECP1	BCAL2101	8	6	555.2078	565.364	657.2719	569.4221	2_3_4_5-tetrahydropyridine-2_6-dicarboxylate N-succinyltransferase
B4EAK7	BCAL0971	7	6	2822.845	2903.336	2548.562	2602.77	4Fe-4S ferredoxin

B4EA13	BCAL2829	6	6	480.9278	667.1284	552.6565	436.1171	Serine peptidase_ subfamily S1B
B4EDZ4	BCAL3272	6	6	1028.807	1116.309	885.414	960.8807	Protein GrpE
B4E8I5	BCAL0542	6	6	10960.8	9082.788	10403.07	11645.53	High-potential iron-sulfur protein
B4EM05	BCAM2827	13	5	3868.884	4628.063	3602.422	4554.847	Putative exported protein
B4E5Z0	BCAL0266	9	5	692.4332	741.9319	491.3524	606.0732	Putative cytochrome c4
B4ECY9	BCAL3197	8	5	268.1232	319.5179	336.122	237.5711	Serine hydroxymethyltransferase
B4EIZ8	BCAM1443	8	5	4117.345	2939.229	3235.494	4852.986	Putative exported protein
B4EK33	BCAM1570	8	5	939.474	806.4608	1135.127	990.5586	Alcohol dehydrogenase
B4E9I2	BCAL2783	8	5	2161.148	2366.462	1571.45	2571.73	Putative cyclopropane-fatty-acyl-phospholipid synthase
B4EPZ2	BCAS0226	8	5	808.3002	499.1259	644.9485	749.5465	Putative hydrolase
B4E6U0	BCAL0368	8	5	5407.616	4203.241	4944.432	5613.729	Cold shock-like protein CspD
B4E909	BCAL2736	8	5	568.4949	493.065	570.2155	538.8878	Isocitrate dehydrogenase [NADP]
B4E7M0	BCAL1516	7	5	2512.159	3418.099	2313.59	3097.058	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex
B4EDU2	BCAL2213	6	5	194.2117	231.0817	220.3532	178.3405	Oligopeptidase A
B4E6F9	BCAL2433	6	5	832.2445	730.216	789.6375	671.4036	Transaldolase
B4E912	BCAL2739	19	4	343.7163	395.9178	500.1596	358.4845	Elongation factor G
B4EKA4	BCAM2618	17	4	478.9086	612.9604	355.7914	541.0933	Putative periplasmic lysine-arginine-ornithine-binding protein
B4ELF8	BCAM2759	9	4	694.5133	486.1233	563.5267	864.6589	Putative minor pilin and initiator
B4EPX2	BCAS0206	9	4	920.7483	710.9216	828.243	940.9263	Putative methyltransferase family protein
B4EQ58	BCAS0292	8	4	2971.342	1738.655	2075.095	4053.904	Uncharacterized protein
B4EAI2	BCAL0996	8	4	538.2564	569.1799	470.3191	536.2578	3-oxoacyl-[acyl-carrier-protein] synthase 2
B4ECU4	BCAL3153	7	4	3334.3	941.2359	2713.481	2487.883	Putative lipoprotein
B4E9M7	BCAL0677	7	4	213.3144	391.5443	677.7232	256.1827	Thiol:disulfide interchange protein
B4EBS5	BCAL3041	7	4	371.3069	400.6002	344.3674	347.0335	Maltose-binding protein
B4EA23	BCAL2839	7	4	1929.563	1502.793	1958.352	2172.922	Fructose-bisphosphate aldolase
B4EEX6	BCAL3388	7	4	797.8682	1057.645	980.3941	932.0748	Glyceraldehyde-3-phosphate dehydrogenase

B4EEN9	BCAL2300	7	4	1664.236	1360.262	1424.316	1467.78	Putative exported protein
B4EN25	BCAM0907	7	4	478.74	533.6124	454.286	474.6922	Sulfurtransferase
B4EA25	BCAL2841	6	4	338.7541	364.2888	308.5226	317.7639	Phosphoglycerate kinase
B4EMR1	BCAM1928	6	4	540.9451	607.3985	357.6772	544.1045	Putative transcription elongation factor
B4EBL2	BCAL1985	6	4	477.694	411.794	405.3742	431.0806	Peptidylprolyl isomerase
B4E7U8	BCAL2609	6	4	673.9132	684.1183	610.1872	702.0648	Periplasmic histidine-binding protein
B4EDC1	BCAL3204	5	4	558.3483	580.8701	1139.807	535.7461	Putative OmpA family lipoprotein
B4E566	BCAL0009	5	4	773.4532	575.0127	635.0584	622.3332	Putative pterin-4-alpha-carbinolamine dehydratase
B4E6X6	BCAL1411	5	4	1403.906	1919.725	1506.532	1359.965	Putative exported protein
B4E9X7	BCAL1796	5	4	436.1789	622.9517	458.8415	474.4237	Putative saccharopine dehydrogenase
B4EP60	BCAS0666	5	4	528.2934	519.752	547.636	665.1202	Putative ankyrin-repeat exported protein
B4EJI2	BCAM1491	4	4	3320.257	2195.81	5321.684	3845.66	Putative exported protein
B4EKR8	BCAM1669	4	4	4454.401	2925.97	5081.521	5239.165	Putative exported protein
B4EB72	BCAL2973	4	4	2012.587	1434.531	2916.205	1354.44	Putative exported protein
B4EMR0	BCAM1927	4	4	1077.927	807.1305	1875.355	1470.782	Putative exported protein
B4EIQ7	BCAM0316	4	4	2409.759	2041.078	2464.632	2510.289	Uncharacterized protein
B4E6F5	BCAL2429	4	4	1140.54	1434.558	891.1053	1388.216	Putative cytochrome C-related protein
B4EF27	BCAL0074	4	4	1486.177	1171.805	1102.539	1500.662	Glycine cleavage system H protein
B4E8Z7	BCAL2724	4	4	514.3802	516.172	503.0813	641.7495	Isoleucine--tRNA ligase
B4EA04	BCAL2820	24	3	194.2153	229.8062	175.9651	144.2684	Efflux system outer membrane protein
Q4F6N6	BCAM2107	17	3	152.803	160.6168	159.7357	146.3647	Catalase-peroxidase 2
B4E640	BCAL0317	7	3	126.7804	127.6114	96.60235	95.33728	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide isomerase
B4E7M1	BCAL1517	7	3	324.9317	420.0285	411.4436	331.0049	Dihydrolipoyl dehydrogenase
B4E6Q3	BCAL0331	7	3	432.9368	502.0885	413.1457	452.4249	Putative stringent starvation protein A
B4EBL7	BCAL1990	6	3	226.5658	233.209	377.5018	226.9507	Glucose-6-phosphate isomerase
B4EAP3	BCAL2935	6	3	181.0494	157.7915	155.0197	155.9186	Electron transfer flavoprotein beta-subunit
B4E7Z3	BCAL0502	6	3	932.7564	917.1018	741.5478	1011.928	RNA polymerase-binding transcription factor DksA

B4EBI4	BCAL1956	6	3	1505.057	1524.877	1588.806	1316.199	Putative lipoprotein
B4EMP5	BCAM0843	5	3	734.1198	539.8668	3098.403	495.4561	Putative lipoprotein
B4EJQ5	BCAM2556	5	3	169.8035	237.925	339.855	172.0906	Putative purine nucle ide permease
B4EB66	BCAL2969a	5	3	456.7047	483.9668	393.353	537.7068	Uncharacterized protein
B4EMI2	BCAM1919	5	3	498.327	494.2816	470.1036	424.709	Hypothetical phage protein
B4ELZ9	BCAM2821	5	3	244.1467	250.7159	229.878	264.3375	Malate synthase G
B4EEX7	BCAL3389	5	3	422.1361	447.2152	450.8068	445.8009	Transketolase 1
B4EBR7	BCAL3033	4	3	125.9844	167.1047	354.9884	144.0167	Outer-membrane lipoprotein carrier protein
B4EIS8	BCAM0338	4	3	197.6791	285.4671	144.1343	220.0648	Uncharacterized protein
B4ECB7	BCAL3096	4	3	568.6334	800.739	504.2291	604.214	Protein-L-isoaspartate O-methyltransferase
B4EA66	BCAL0741	4	3	437.2215	361.4765	467.5065	501.284	Glutaredoxin 3
B4E605	BCAL0282	4	3	400.5798	447.4025	318.8959	478.3047	Putative ABC transporter extracellular solute-binding protein
B4EPY8	BCAS0222	4	3	787.9834	952.2904	668.2293	929.6293	Putative AMP-dependent synthetase
B4EPS9	BCAS0163	4	3	2282.917	2154.407	1901.33	2603.193	Putative pyridine nucleotide-disulphide oxidoreductase
B4E6W2	BCAL0389	4	3	260.2492	297.6022	305.0612	284.9242	Thiol:disulfide interchange protein DsbC
B4EKQ1	BCAM1652	3	3	548.7462	528.5579	1260.143	354.1191	Putative lipoprotein
B4ED89	BCAL2167	3	3	199.2271	266.6285	341.6703	162.6257	4-hydroxy-tetrahydrodipicolinate synthase
B4EIP0	BCAM2474	3	3	238.9002	357.4302	261.8196	206.181	Uncharacterized protein
B4E7K0	BCAL1496	3	3	35814.95	32726.86	30734.73	41558.83	Putative exported protein
B4EAX2	BCAL1887	3	3	1377.977	1388.622	1281.607	1327.584	Nucleoside diphosphate kinase
B4E707	BCAL1441	3	3	469.5706	322.2255	507.7104	358.3657	SirA-like protein
B4ED69	BCAL2146	3	3	414.6607	464.9172	375.4496	466.7272	Aspartokinase
B4EE76	BCAL0151	10	2	153.1114	238.3428	151.9694	268.9816	Extracellular ligand binding protein
B4EM61	BCAM0759	10	2	113.8481	127.0032	139.1172	131.0407	Periplasmic lysine-arginine-ornithine-binding protein
B4EAI5	BCAL0993	8	2	193.4355	247.4405	157.0698	196.4329	Malonyl CoA-acyl carrier protein transacylase
B4E7A4	BCAL3530	8	2	343.6529	366.646	307.9566	288.4918	DNA-binding protein HU-alpha
B4E905	BCAL2732	7	2	1087.915	713.9108	877.1592	1191.24	Cold shock-like protein

B4EEC7	BCAL0201	7	2	135.356	150.3493	149.0961	136.6425	N-acetyl-gamma-glutamyl-phosphate reductase
B4ECM8	BCAL2088	7	2	884.3988	876.6276	664.8396	888.8462	Ribosome-recycling factor
B4EG28	BCAM0042	6	2	119.3078	111.9226	434.8684	136.8307	Putative aldo/keto reductase
B4E8D9	BCAL2645	6	2	2152.442	1611.143	1938.717	2029.246	Putative OmpA family membrane protein
B4E5B0	BCAL0224	5	2	197.2369	345.0359	193.7662	155.5403	50S ribosomal protein L10
B4EBM4	BCAL1997	5	2	232.2239	238.5186	201.2168	234.861	Trigger factor
B4ECT1	BCAL3140	4	2	77.66306	120.6336	80.61725	122.6331	Bifunctional regulator/uracil phosphoribosyltransferase
B4EBP0	BCAL3006	4	2	1413.12	924.6383	981.7765	1314.945	Cold shock-like protein
B4EBI8	BCAL1960	4	2	994.7506	860.4868	1094.688	1119.601	Putative exported protein
B4E6T8	BCAL0366	4	2	287.0397	262.4527	222.1227	310.2556	Nitroreductase family protein
B4EMB9	BCAM1857	4	2	128.903	128.0106	146.2876	123.5809	Uncharacterized protein
B4E844	BCAL1548	3	2	48.84593	63.54528	85.89043	63.11204	Putative sugar ABC transport system_ lipoprotein
B4EDV4	BCAL2225	3	2	81.73728	94.44833	57.49472	65.3911	Uncharacterized protein
B4EFM0	BCAM1012	3	2	253.6155	295.486	283.8252	132.3109	Putative histone-like protein
B4E6D9	BCAL2413	3	2	294.5259	470.3964	265.0748	469.9708	Putative exported protein
B4EE74	BCAL0149	3	2	85.84713	93.54815	77.27289	72.1014	Putative dienelactone hydrolase
B4EML4	BCAM0812	3	2	254.0592	295.9709	153.1449	230.2895	Putative aromatic hydrocarbons catabolism-related dioxygenase
B4EBE6	BCAL0895	3	2	271.1299	248.5893	203.0967	277.6696	Chaperone SurA
B4EPX5	BCAS0209	3	2	151.9428	129.8707	188.2915	113.8114	Uncharacterized protein
B4EB43	BCAL2953	3	2	370.3952	240.2209	335.714	388.9103	Putative prephenate dehydrogenase
B4EDW1	BCAL2232	3	2	1978.321	1477.955	2198.377	2395.409	Probable Fe(2+)-trafficking protein
B4EK06	BCAM0504	3	2	482.9615	329.7396	538.3898	535.3812	CsbD-like protein
B4E6U4	BCAL0372	3	2	426.4851	397.7292	352.5314	264.7488	Glutaredoxin
B4EAI3	BCAL0995	3	2	2978.714	2361.494	2021.633	3250.789	Acyl carrier protein
B4EAG7	BCAL1868	3	2	567.9058	525.0399	389.2742	481.2039	Uncharacterized protein
B4EM48	BCAM0746	3	2	61.85813	61.00622	68.69739	70.14777	Argininosuccinate synthase
B4E9Z7	BCAL2813	3	2	232.9576	187.6013	184.2692	224.4559	Putative periplasmic solute-binding protein

B4EAX8	BCAL1893	3	2	195.9322	207.2828	191.2204	206.1635	Family M23 peptidase
B4E8U9	BCAL1677	2	2	38.58099	97.75334	62.37061	37.80468	Putative type-1 fimbrial protein
B4EMN1	BCAM0829	2	2	32.18316	65.38768	41.00347	26.76454	Putative riboflavin synthase alpha chain
B4E7M4	BCAL1520	2	2	151.2395	223.3531	83.61756	237.1882	Putative lipoprotein
B4EC25	BCAL2018	2	2	67.68738	96.45903	94.44448	59.67202	Acetylornithine deacetylase
B4EIB1	BCAM0299	2	2	96.19692	71.9143	81.50439	86.12744	Putative zinc-binding alcoholdehydrogenase
B4EJI6	BCAM1495	2	2	463.836	378.5286	362.0341	509.8656	Putative universal stress protein
B4EI96	BCAM0284	2	2	959.1032	719.2953	827.3102	1087.181	Putative cytochrome c
B4EH15	BCAM2299	2	2	143.8075	228.9726	172.765	147.4836	Putative cytochrome c
B4ELQ5	BCAM0704	2	2	724.7086	625.4182	1028.837	743.0171	Putative oxidoreductase
B4EF28	BCAL0075	2	2	366.1632	209.5416	323.5268	339.7939	Aminomethyltransferase
B4EBQ1	BCAL3017	2	2	283.9672	404.6803	235.1786	358.4635	Putative exported protein
B4EA89	BCAL0764	2	2	323.7714	259.0609	272.3106	415.1952	Putative exported protein
B4EH05	BCAM2289	2	2	344.2644	276.8685	408.1995	427.091	Uncharacterized protein
B4E5B1	BCAL0225	2	2	500.7343	536.1204	513.202	517.2409	50S ribosomal protein L7/L12
B4EA64	BCAL0739	2	2	180.5834	219.9951	195.271	187.4077	2_3-bisphosphoglycerate-dependent phosphoglycerate mutase
B4EDA1	BCAL2179	2	2	161.9429	191.2033	224.9889	174.2389	Enolase
B4EI88	BCAM0277	2	2	140.5054	109.4775	150.1787	124.4825	Uncharacterized protein
B4E5A7	BCAL0221	2	2	437.0288	392.0789	483.7191	431.5758	Transcription termination/antitermination protein NusG
B4EF29	BCAL0076	2	2	554.3243	584.5963	552.6185	628.173	Putative lipoprotein
B4E8W9	BCAL2696	2	2	671.8935	622.4345	737.4766	717.6865	Putative copper resistance protein
B4E6R5	BCAL0343	7	1	133.5204	107.0153	103.9613	153.6295	Putative type VI secretion system protein TssD
B4EKJ3	BCAM0548	6	1	35.95799	61.3092	32.15023	26.90787	60 kDa chaperonin
B4EF86	BCAM0941	6	1	126.4207	97.50877	169.2792	107.4701	6-phosphogluconate dehydrogenase_ decarboxylating
B4E6V5	BCAL0382	4	1	604.2643	184.6063	374.8789	372.9898	LysR family regulatory protein
B4EBY3	BCAL0953	4	1	145.4851	104.8162	148.8733	50.0732	Protein RecA
B4EMY8	BCAM0869	4	1	48.33906	39.56158	29.02632	44.31021	Peptidyl-prolyl cis-trans isomerase

B4EBA9	BCAL0858	4	1	1589.186	1328.411	1197.826	1916.683	Putative exported protein
B4EE04	BCAL3282	4	1	104.651	126.6923	92.67267	83.87928	Phospho-2-dehydro-3-deoxyheptonate aldolase
B4EKC7	BCAM2642	3	1	128.1826	129.9288	592.1421	105.0277	Uncharacterized protein
B4E9C1	BCAL1722	3	1	94.8232	134.5477	49.89389	115.2002	Putative exported chitinase
B4EBW5	BCAL0935	3	1	27.65643	85.36708	25.0018	23.70875	Putative periplasmic cytochrome c protein
B4ECX5	BCAL3184	3	1	27.44146	28.21344	26.38945	24.31575	Homogentisate 1_2-dioxygenase
B4EDS7	BCAL2198	3	1	380.4795	675.7829	284.2349	404.1713	Cysteine desulfurase IscS
B4EB22	BCAL1936	3	1	41.10123	35.30369	34.07555	43.7164	AhpC/TSA family protein
B4E9G8	BCAL2769	3	1	182.2265	243.5255	140.4116	198.9438	UPF0234 protein BceJ2315_27070
B4E732	BCAL2466	3	1	94.50893	102.057	82.04707	102.258	Ecotin
B4EB03	BCAL1917	3	1	157.593	138.0872	184.2934	117.9197	Putative exported protein
B4ELT9	BCAM1779	3	1	55.63562	61.12226	79.92834	56.36289	Beta-lactamase
B4E810	BCAL0519	3	1	42.44008	41.59073	53.15157	44.03687	Putative GMC oxidoreductase
B4EEW6	BCAL3378	3	1	195.3628	176.0689	190.634	187.6337	Ferric uptake regulator
B4EMQ9	BCAM1926	3	1	206.4548	162.4612	162.4646	214.2127	Uncharacterized protein
B4E6I3	BCAL2457	3	1	226.9791	183.3784	211.4271	220.47	Putative exported protein
B4E9Q6	BCAL0706	3	1	256.5908	214.2712	218.3643	262.1738	UPF0250 protein BCAL0706
B4EBT6	BCAL3052	3	1	173.3264	167.8139	182.8241	176.1835	Uncharacterized protein
B4ED76	BCAL2153	3	1	440.7365	433.6676	399.8719	438.2104	Peptidyl-prolyl cis-trans isomerase
B4E8L8	BCAL0576	2	1	10.41887	17.35155	94.2873	6.085734	Flagellar hook-associated protein 1 (HAP1)
B4EA67	BCAL0742	2	1	8.973161	72.41963	22.63	57.73216	Protein-export protein SecB
B4EEP6	BCAL2307	2	1	155.9057	64.42688	110.3735	218.3809	Uncharacterized protein
B4E5V6	BCAL3426	2	1	19.59285	18.6468	41.13225	13.21508	Putative lipoprotein
B4EEU0	BCAL3351	2	1	53.65738	32.91892	31.17094	32.45223	Dihydroorotase
B4EFB6	BCAM0971	2	1	11.27794	26.57431	16.7809	18.63173	Uncharacterized protein
B4EGQ4	BCAM1149	2	1	562.9484	722.0032	560.5237	428.8416	Putative lipoprotein
B4EDI8	BCAL0114	2	1	39.24486	32.01205	50.23967	50.35764	Flagellin
B4E829	BCAL1533	2	1	302.7386	280.2393	327.1952	376.9905	Putative lipoprotein
B4E7H0	BCAL1465	2	1	32.29912	39.0472	30.56972	24.2554	Uncharacterized protein

B4E8F9	BCAL2666	2	1	467.2336	446.759	397.0896	487.0431	Uncharacterized protein
B4E5Y2	BCAL3452	2	1	103.4401	119.6635	95.41302	108.3995	Arginine biosynthesis bifunctional protein ArgJ
B4EEZ0	BCAL0037	2	1	54.65757	73.66322	65.20063	56.91959	ATP synthase epsilon chain
B4E5R7	BCAL2389	2	1	81.36373	89.33759	79.76897	74.67818	Phosphoribosylamine--glycine ligase
B4E598	BCAL0212	2	1	204.9436	179.4783	194.3034	222.6772	Putative phenylacetic acid degradation NADH oxidoreductase PaaE
B4E9T3	BCAL1752	2	1	104.0504	121.1152	74.88547	85.14194	Uncharacterized protein
B4EEW4	BCAL3376	2	1	243.2029	267.1424	308.0876	235.0144	4-hydroxy-tetrahydrodipicolinate reductase
B4EJ50	BCAM2497	2	1	168.7964	137.9015	210.7125	134.0573	ABC transporter ATP-binding protein
B4E6H2	BCAL2446	2	1	17.80674	15.69611	24.64195	17.44527	Putative aminotransferase
B4E8B6	BCAL2622	2	1	88.27861	83.66621	80.73303	96.45894	Inorganic pyrophosphatase
B4E6H3	BCAL2447	2	1	31.97877	27.32952	34.36379	32.92159	Endoribonuclease L-PSP family protein
B4ECU7	BCAL3156	2	1	190.6911	133.0737	153.1071	194.417	Putative periplasmic lysine-arginine-ornithine-binding protein
B4EHS4	BCAM1284	2	1	65.85968	78.03135	61.32615	77.63159	Putative methyltransferase
B4EPC2	BCAS0734	2	1	93.07289	89.67082	92.12781	89.26358	Putative pyridine nucleotide-disulphide oxidoreductase family protein
B4EFW4	BCAM2167	2	1	202.8638	153.0481	207.4064	181.2123	Uncharacterized protein
B4EA22	BCAL2838	2	1	380.3943	380.2845	321.0452	429.9298	Phosphoribosylaminoimidazole-succinocarboxamide synthase
B4E5N9	BCAL2361	2	1	548.5083	463.4841	453.5518	582.911	Putative membrane protein
B4E5I4	BCAL1299	2	1	167.2092	142.8075	159.8001	181.9396	Uncharacterized protein
B4EIB9	BCAM0307	2	1	91.61142	78.46666	83.20067	95.85851	Uncharacterized protein
B4EJJ0	BCAM1500	1	1	16.5213	10.7226	18.57838	16.01749	Putative universal stress protein
B4EPW8	BCAS0202	1	1	976.0687	1080.608	1203.946	585.6753	Putative membrane protein
B4ECJ6	BCAL1034	1	1	14.22778	47.43861	19.8214	56.07641	SCO1/SenC family protein
B4E8V3	BCAL2680	1	1	5.248398	8.116808	12.87995	4.574417	Putative uroporphyrin-III C-methyltransferase
B4EF82	BCAM0937	1	1	254.0196	108.7642	214.0383	306.285	Putative monooxygenase_ luciferase-like
B4ENL1	BCAS0465	1	1	54.33948	65.84555	32.75564	47.21461	Putative tagatose-6-phosphate ketose/aldose isomerase

B4EE88	BCAL0163	1	1	96.36079	109.8493	95.8504	39.02804	Putative phospholipid-binding lipoprotein
B4ECZ9	BCAL1063	1	1	39.20232	77.50673	42.54932	30.04889	N-succinylarginine dihydrolase
B4E5M4	BCAL2346	1	1	106.5432	141.1715	98.03762	101.9593	Triosephosphate isomerase
B4EDE5	BCAL3228	1	1	41.7023	83.92451	45.96385	36.60006	Uncharacterized protein
B4EPR4	BCAS0147	1	1	1029.565	788.0132	974.7756	854.4104	Putative exported protein
B4EK20	BCAM0518	1	1	128.5045	219.1187	149.2274	96.73793	LysR family regulatory protein
B4E7I4	BCAL1479	1	1	34.54487	39.47196	27.77997	39.67481	Endoribonuclease L-PSP family protein
B4EEQ9	BCAL2321	1	1	31.05391	34.46147	26.90483	33.24116	Putative glutathione S-transferase
B4EI19	BCAM2400b	1	1	638.4281	730.8412	562.9979	724.0956	Putative exported protein
B4EP19	BCAS0625	1	1	60.58187	60.06932	75.72924	50.99556	Putative lipoprotein
B4EPK7	BCAS0090	1	1	1029.432	706.3759	973.4069	1096.808	NAD(P)H dehydrogenase (quinone)
B4EAD2	BCAL1833	1	1	495.3543	554.2727	352.571	629.7215	Putative exported protein
B4EBJ9	BCAL1971	1	1	218.5897	228.1578	156.0053	260.7128	Uncharacterized protein
B4ECX0	BCAL3179	1	1	160.4216	123.4927	160.1454	150.2713	Probable D-lactate dehydrogenase
B4E7C0	BCAL0409	1	1	58.16555	65.43464	56.09854	86.20869	Putative phenylacetic acid degradation enoyl-CoA hydratase PaaF
B4E9I4	BCAL2785	1	1	24.58814	23.98763	18.02984	17.70436	Peptide methionine sulfoxide reductase MsrA
B4EQ36	BCAS0270	1	1	55.08612	38.63617	47.2972	37.78362	Uncharacterized protein
B4EDU0	BCAL2211	1	1	156.48	186.7026	144.7382	161.1747	Two-component regulatory system_ response regulator protein
B4EK92	BCAM1629	1	1	627.1008	697.1716	611.4612	670.5971	Thiolase
B4EBG1	BCAL0910	1	1	54.19495	42.39394	54.96706	57.93708	Putative PhoH-family protein
B4EPY2	BCAS0216	1	1	4015.672	2962.83	3276.846	3477.69	Putative acyl carrier protein
B4EN28	BCAM0910	1	1	34.17301	31.09289	40.20434	31.70979	Putative geranyltranstransferase
B4EGV1	BCAM1196	1	1	37.29666	41.06636	35.70702	38.23369	Putative methyl-accepting chemotaxis protein
B4EPF2	BCAS0764	19	0					Multidrug efflux system outer membrane protein
B4EK69	BCAM1606	5	0					Electron transfer flavoprotein_ alpha subunit
B4E5Z6	BCAL0273	4	0					Protein CyaY
B4EH79	BCAM0178	4	0					Putative periplasmic solute-binding protein

B4ELU8	BCAM1787	4	0					Putative porin
B4EEY6	BCAL0033	3	0					ATP synthase subunit delta
B4EAC0	BCAL1821	3	0					Putrescine transport system permease protein
B4EBL5	BCAL1988	3	0					Putative D-amino acid dehydrogenase small subunit
B4EGJ5	BCAM0071	3	0					Puatative mandelate racemase/muconate lactonizing enzyme
B4EPS0	BCAS0154	3	0					Uncharacterized protein
B4EQA1	BCAS0335	3	0					Putative haemagglutinin-related autotransporter protein
B4E6R2	BCAL0340	2	0					Putative lipoprotein
B4E7B7	BCAL0406	2	0					Probable enoyl-CoA hydratase PaaG
B4E8N1	BCAL0589	2	0					Putative lipoprotein
B4EBE0	BCAL0889	2	0					Uncharacterized protein
B4EBG8	BCAL0917	2	0					Putative oxidoreductase
B4ECL5	BCAL1053	2	0					Putative membrane protein
B4EAC9	BCAL1830	2	0					Putative 2-nitropropane dioxygenase
B4EC82	BCAL2075	2	0					Putative ph phoenolpyruvate synthase regulatory protein
B4E5R3	BCAL2385	2	0					Methylglyoxal synthase
B4E7N5	BCAL2536A	2	0					Putative hydrolase
B4E8C4	BCAL2630	2	0					Porphobilinogen deaminase
B4E5W8	BCAL3438	2	0					Putative lipoprotein
B4EH52	BCAM0152	2	0					Putative lipoprotein
B4EIT7	BCAM0347	2	0					Ribonuclease VapC
B4EHX2	BCAM1331	2	0					Putative tyrosine-protein kinase
B4EIC4	BCAM1348	2	0					Putative cyclic nucleotide-binding protein
B4EPR0	BCAS0143	2	0					ABC transporter ATP-binding protein
B4EDK2	BCAL0128	1	0					Chemotaxis two-component response regulator CheY
B4EE68	BCAL0143	1	0					Putative flagellar biosynthesis protein
B4EE89	BCAL0164	1	0					Putative cytochrome c-551

B4E5A0	BCAL0214	1	0					Phenylacetic acid degradation protein PaaC
B4E634	BCAL0311	1	0					ATP phosphoribosyltransferase
B4E7B1	BCAL0400	1	0					Putative phosphoglycolate phosphatase
B4E802	BCAL0511	1	0					Putative deoxygenases
B4E8L3	BCAL0570	1	0					Flagellar L-ring protein
B4EBG6	BCAL0915	1	0					3_4-dihydroxy-2-butanone 4-phosphate synthase
B4EBW7	BCAL0937	1	0					Putative galactonate dehydratase protein
B4EAI9	BCAL0989	1	0					Uncharacterized protein
B4E697	BCAL1377	1	0					Citrate transporter
B4E713	BCAL1447	1	0					UTP--glucose-1-phosphate uridylyltransferase
B4E7M5	BCAL1522	1	0					Putative exported heme utilisation related protein
B4EBN4	BCAL2007	1	0					Squalene/phytoene synthase family protein
B4EC33	BCAL2026	1	0					Putative glyoxylate-realted oxidoreductase
B4ECR4	BCAL2123	1	0					Uncharacterized protein
B4EER0	BCAL2322	1	0					Putative enoyl-CoA hydratase
B4E8G2	BCAL2669	1	0					Putative exported protein
B4EA01	BCAL2817	1	0					S-(hydroxymethyl)glutathione dehydrogenase
B4ECY2	BCAL3191	1	0					Putative glutaryl-CoA dehydrogenase
B4EE37	BCAL3316	1	0					Putative lipoprotein
B4E5Y0	BCAL3450	1	0					NUDIX hydrolase
B4E5Y6	BCAL3456	1	0					Putative thioredoxin reductase
B4E6M9	BCAL3499	1	0					ABC transporter ATP-binding protein
B4E795	BCAL3521	1	0					Type II secretion system protein I
B4EFZ0	BCAM0005	1	0					Putative replication protein
B4EGP6	BCAM0122	1	0					BNR/Asp-box repeat protein
B4EHP4	BCAM0212	1	0					Uncharacterized protein
B4EI97	BCAM0285	1	0					Uncharacterized protein
B4EIV4	BCAM0366	1	0					5'-nucleotidase SurE
B4EL40	BCAM0619	1	0					Putative membrane protein

B4EF85	BCAM0940A	1	0					Putative methyltransferase
B4EJI3	BCAM1492	1	0					Putative exported protein
B4EMX1	BCAM1989	1	0					NAD dependent epimerase/dehydratase family protein
B4EN64	BCAM2019	1	0					AraC family regulatory protein
B4EFW2	BCAM2165	1	0					Beta-lactamase
B4EGE8	BCAM2216	1	0					Putative exported protein
B4EHY6	BCAM2368	1	0					Putative quinoprotein ethanol dehydrogenase
B4EIM0	BCAM2454	1	0					Putative FMN-binding protein
B4EJU4	BCAM2595	1	0					Alkyl hydroperoxide reductase AhpD
B4EJU6	BCAM2597	1	0					LysR family regulatory protein
B4EKV0	BCAM2681	1	0					Putative lipoprotein
B4ELE8	BCAM2749	1	0					Alkyl hydroperoxide reductase AhpD
B4ELZ7	BCAM2819	1	0					Glycolate oxidase iron-sulfur subunit
B4EPK8	BCAS0091	1	0					Putative pirin family protein
B4ENR9	BCAS0523	1	0					Hypothetical phage protein
B4EP67	BCAS0673	1	0					Uncharacterized protein
B4EPD8	BCAS0750	1	0					Putative exported protein

CHAPTER IV. BROADER INTERNATIONAL CONTEXT, RELEVANCE, AND FUTURE PERSPECTIVES

1. Increasing antimicrobial resistance and the high innate resistance of *B. cenocepacia*

Antimicrobial resistance of bacteria is becoming one of the biggest threats to human health. A better surveillance on the use of antibiotics is required and there is an urgent need for new antimicrobial compounds. *Burkholderia cenocepacia* J2315 is a multi-drug resistant bacterium isolated in the 1990s (178). It is an opportunistic pathogen, able to cause life-threating infections in immunocompromised patients such as cystic fibrosis patients and patients with chronic granulomatous disease. Strains from all Bcc species are able to cause infections in CF patients, however, *B. cenocepacia* and *B. multivorans* are the predominant strains and they are able to cause the deadly cepacia syndrome (216). The prevalence of Bcc infections in CF patients is rather low, approx. 3 % worldwide, but due to their variable and unpredictable clinical outcome they have become very important (39, 217). Their high intrinsic resistance to most clinically available antimicrobials impedes treatment (218, 219). Every single Bcc infection should be treated but treatment decisions should be made on a case-by-case basis (39, 217). *In vitro* susceptibility profiles of isolates are often used to determine the most appropriate antibiotic. However *in vitro* susceptibility is not always linked to an effective *in vivo* eradication of Bcc. Altered pharmacokinetics and difficulties in delivery of the drug to the lung cells in a sufficiently high concentration contribute to this ineffectiveness (39, 220). Currently, a combination of two or three antibiotics is used. Co-trimoxazole seems to be the drug of choice for CF patients that do not show allergic or hypersensitive reactions or intolerance. Alternative treatments are the administration of ceftazidime, meropenem and/or penicillins (217). The main concern for Bcc infections is the increasing resistance to co-trimoxazole due to an acquired outer membrane efflux pump or through increased production of dihydrofolate reductases (221, 222). Therefore new antimicrobial compounds are required.

Several virulence factors and resistance mechanisms of bacteria have been elucidated using modern technologies, including genomics and proteomics, which enable the development of new strategies to prevent or combat bacterial infections. Attempts have been made to develop protective vaccines against Bcc species. Metalloproteases and the O-antigen of the LPS have been studied as potential vaccine candidates. However, a vaccine effective against all Bcc species has not yet been found because these structures differ between species (39). Antibiotic resistance occurs through complex regulatory networks which are often not completely understood. Specific resistance genes or genes encoding virulence factors (e.g. quorums sensing) are of interest as targets for the development of

novel therapeutics (223). Furthermore, regulators involved in the expression of these virulence factors also represent promising targets (Figure 1) (39).

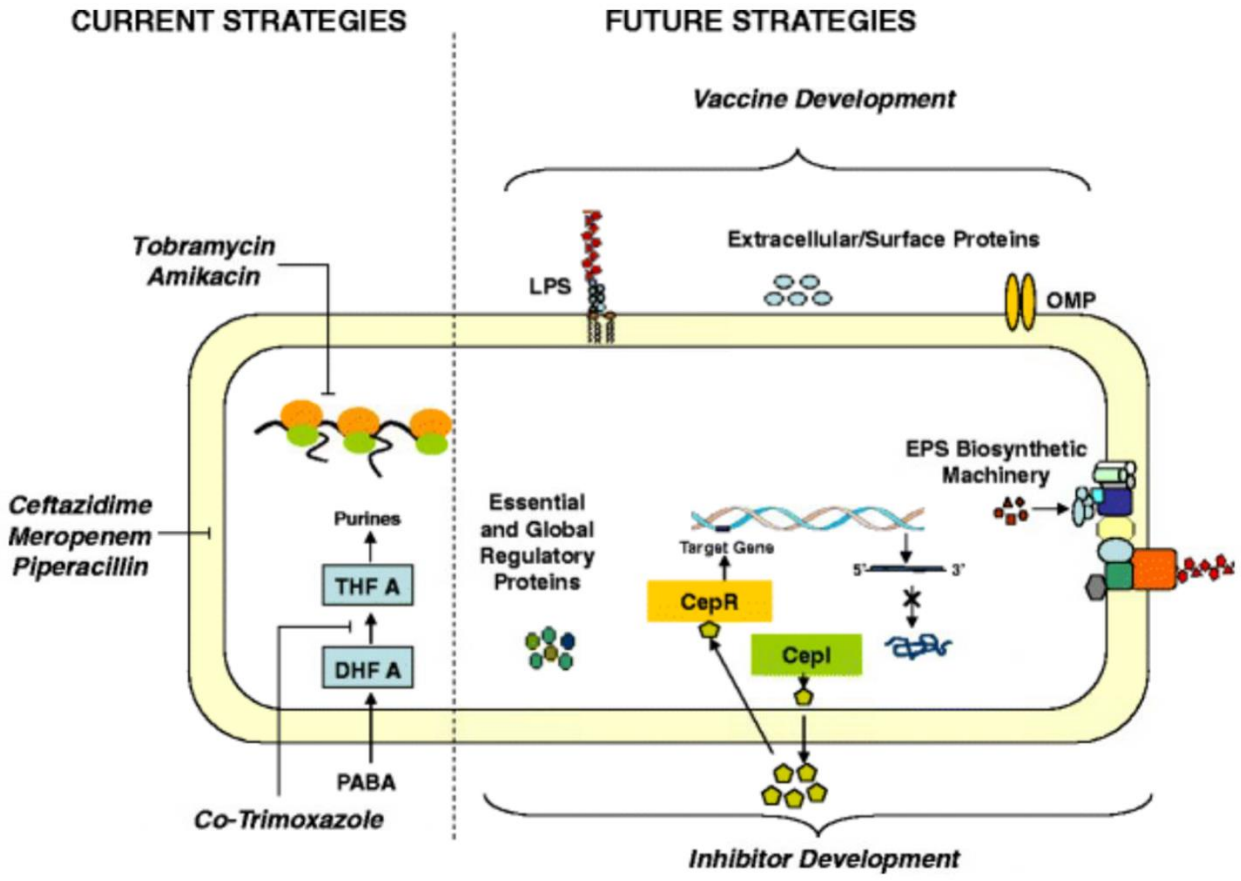


Figure 1. Current and future strategies to combat Bcc infections. Left side: Main currently used antibiotics. THF A = tetrahydrofolic acid, DHF A = dihydrofolic acid, PABA = p-aminobenzoic acid. Right panel: Future strategies involve vaccine development against lipopolysaccharide (LPS), extracellular proteins and outer membrane proteins (OMP), and the development of inhibitors targeting virulence factors (Quorum sensing, CepR/CepI and exopolysaccharides (EPS) biosynthesis) and essential regulators (39).

To predict and to prevent resistance, it is important to understand the complex regulatory networks of the bacterial response to antibiotics and other stress factors. In this dissertation, we focussed on recently discovered regulators, non-coding RNAs, and more specifically, the small RNAs.

2. The role of sRNAs in bacterial resistance

sRNAs are capable to minimize damage caused by antimicrobial compounds by activating antibiotic resistance pathways (224). When a bacterial cell encounters antimicrobial stress, the repertoire of expressed sRNAs changes. Every single antibiotic induces the expression of a unique set of sRNAs. Some antibiotics induce the expression of dozens of sRNAs, whereas others impact the expression of only a few (225).

sRNAs are known to play a role in the regulatory networks controlling antibiotic uptake, cell-wall modifications, efflux, expression of antibiotic degrading enzymes, and biofilm formation (Figure 2) (225, 226).

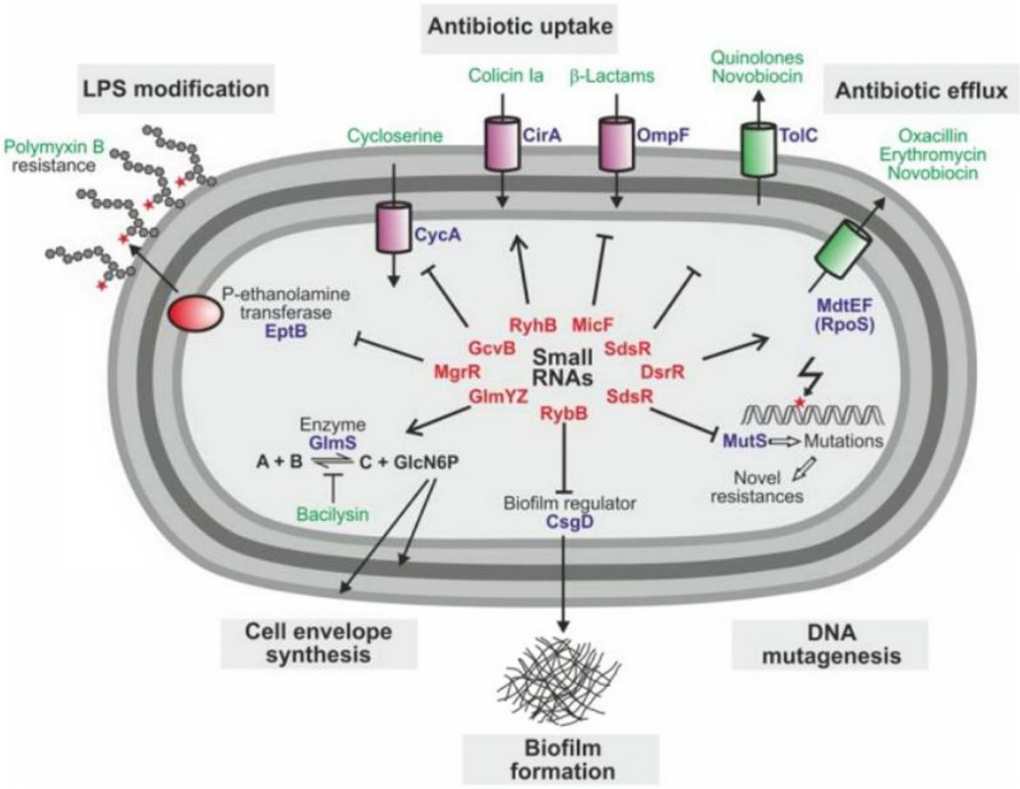


Figure 2. sRNAs with an impact on antimicrobial resistance or susceptibility in *Escherichia coli*. sRNAs are depicted in red, proteins they act on in blue and the antibiotic they affect in green (225).

sRNAs base-pair directly with resistance genes, or they indirectly cause a cascade of actions to coordinate a protective response to the antibiotic. Direct base-pairing is observed with the sRNAs MicF and GcvB in *Escherichia coli*, which repress translation of the OmpF porin and the serine transporter CycA, respectively. sRNA SroC causes an indirect antibiotic resistance effect by acting as a sponge for other sRNAs. It binds and sequesters the sRNA MgrR; lower levels of MgrR lead to higher expression of EptB, which modifies lipopolysaccharides with phosphoethanolamine. These modifications cause a reduction of the net anionic charge of LPS, preventing polymyxin B from binding (225, 227, 228). More examples of sRNAs with a role in antimicrobial resistance are shown in Table 1.

Table 1. Examples of sRNAs contributing to antimicrobial resistance or susceptibility in *Escherichia coli* and *Salmonella* (225).

sRNA	**Organism(s)**	**Compound**	**Mechanism**
DsrA	*E. coli*	Oxacillin, erythromycin, novobiocin	Upregulation of efflux pump MdtEF via RpoS
GcvB	*E. coli*	D-cycloserine	Represses *cycA*, which is required for drug uptake
GlmY, GlmZ	*E. coli, Salmonella*	GlmS inhibitors (e.g bacilysin)	Induces *glmS*
MicF	*E. coli, Salmonella*	Cephalosporins, norfloxacin	Represses *ompF*
MgrR	*E. coli*	Polymyxin B	Represses *eptB*, which modifies LPS
RybB	*E. coli*	Epigallocatechin gallate	Repression of the biofilm regulator CsgD leading to inhibition of biofilm formation
RyhB	*E. coli*	Colicin Ia	Activates synthesis of colicin Ia receptor CirA
SdsR (RyeB)	*E. coli*	Ampicillin	Represses *mutS*, which causes mutations that may confer resistance
SdsR (RyeB)	*E. coli, Salmonella*	Quinolones, novobiocin, crystal violet	Overexpression reduces resistance which is at least partially attributable to repression of *tolC* by SdsR

In the model organisms *E. coli* and *Salmonella* many sRNAs regulate antibiotic resistance genes. Recently, a sRNA expressed in *Staphylococcus aureus* was shown to play a role in the susceptibility of this bacterium to glycopeptide antibiotics. More sRNAs mediating antibiotic susceptibility are likely to be discovered in other pathogenic bacteria (225, 228, 229).

sRNAs not only have a direct or indirect effect on the expression of a resistance gene, but they are also able to increase mutation rates, promoting target modifications, and to stimulate horizontal transfer to spread resistance genes. For instance the master regulator of the general stress response in *E. coli*, RpoS, is both positively and negatively regulated by sRNAs, and activates the expression of several genes to counteract stress. Furthermore it induces the expression of the error-prone DNA polymerase IV and the sRNA SdsR. The error-prone DNA polymerase IV incorporates mismatched nucleotides, whereas sRNA SdsR causes a downregulation of the DNA mismatch-repair protein MutS, favouring the fixation of these spontaneous mutations (225, 230).

In *Burkholderia* species, several sRNAs have been discovered but little is known about their role in antibiotic resistance. Stubben et al. evaluated the response of *B. thailandensis* to a mixture of antibiotics using a microarray, which included probes for intergenic regions. The mixture of antibiotics consisted of carbenicillin, chloramphenicol, erythromycin and kanamycin, antibiotics to which *Burkholderia* species are naturally resistant. Nine intergenic regions were upregulated while one was downregulated (231). Another study in *B. cenocepacia* J2315 described the sRNA MtvR that acts as global regulator. When overexpressing this sRNA, the strain became more susceptible to the

aminoglycosides gentamicin and amikacin and to the β-lactams ceftazidime and imipenem (232). However, the latter paper was retracted, and it remains to be seen whether the reported role of MtvR can be confirmed in independent studies. Further research in this direction will likely reveal more sRNAs that affect antibiotic resistance genes or that play a role in the regulatory network of antibiotic resistance of *Burkholderia* species.

3. sRNA-like molecules in other organisms

Small non-coding regulatory RNAs have been discovered in all three domains of life. In eukaryotes, the sRNA mode of regulation is known as RNA interference (RNAi). Eukaryotic sRNAs, with a typical size of approx. 20 nucleotides, are much shorter than commonly observed for bacterial sRNAs. They regulate gene expression during development and in response to stress. Endogenous RNA molecules are processed in the nucleus and are subsequently transported to the cytoplasm where endoribonuclease Dicer cleaves them to functional products. The three main classes of regulatory RNA in eukaryotes are microRNAs (miRNA; endogenous RNA), small interfering RNAs (siRNA; exogenous RNA) and Piwi-interacting RNAs (piRNA). They preferably act on the 3'UTR of their target mRNA, aided by Argonaute proteins (149, 233, 234). Whereas bacterial sRNA deletion mutants only show mild phenotypes, eukaryotic sRNA deletion mutants show severe defects. miRNA mutations in *Caenorhabditis elegans* are correlated with defects in development of this organism and in humans, RNAi dysfunctions have been associated with mental retardation, several neurological diseases (e.g. Alzeimer' disease, Parkinson disease), cancers, and infertility (149, 234).

In Archaea, regulatory RNAs have been designated snoRNAs and are similar in size to bacterial RNAs. The first deletion mutants show growth defects, suggesting snoRNAs are of great importance in this domain of life (149).

4. RNA on the move

Numerous cases have been described in which RNA molecules are transferred between organisms. sRNA exchange is observed in organisms of the same species (e.g. breast-feeding of infants) but also between organisms of different species (Figure 3). This inter-play can be seen as an additional factor that changes gene expression in response to environmental inputs (235). The sRNAs OxyS and DrsA in *E. coli* were shown to downregulate genes of *C. elegans*. *E. coli* is used as food source for *C. elegans* and expresses these two sRNAs under stress conditions, possibly protecting the bacterium from overfeeding by *C. elegans*. OxyS binds to *che-2*, altering the chemosensory behavior of *C. elegans* while DrsA suppresses a lipase, leading to an altered lipid metabolism (132, 133).

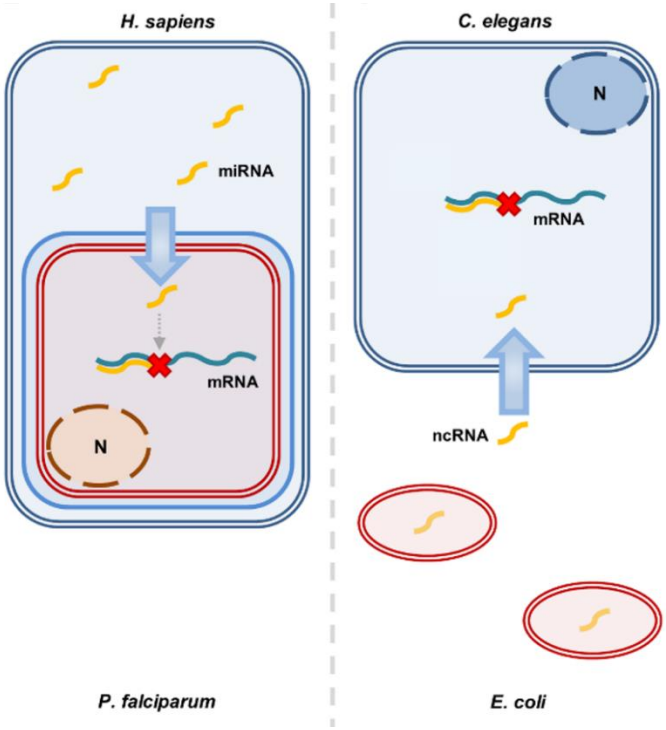


Figure 3. RNA transfer. Left panel: miRNA of *Homo sapiens* (Humans) can be translocated to *Plasmodium falciparum* where it interferes with the mRNA translation of this organism to counteract malaria. Right panel: *Escherichia coli* produces non-coding RNA (ncRNA), that are able to transfer to the nematode *Caenorhabditis elegans*, where they interfere with the translation of certain mRNA (235). N = nucleus

How RNA is transferred between organisms is still not completely understood and is likely to occur through different mechanisms. For DNA transfer from bacteria to eukaryotes, two mechanisms were found. Passive transfer of DNA occurs through the release of material after cell death or cell lysis. For example, two bacteria, *Listeria monocytogenes* and *Salmonella typhimurium*, were engineered to undergo lysis intracellularly. Subsequently, several proteins and DNA were found to be transferred to the eukaryotic cell (236, 237). On the other hand, some bacterial pathogens (e.g. *Bartonella* spp., *Rickettsia* spp., *Brucella* spp., *Helicobacter pylori*) were thought to actively transfer DNA to the eukaryotic cell by the use of a type IV secretion system (132, 238, 239). These two examples of DNA transfer from bacterial cells to eukaryotic cells make it seem likely that sRNA transfer can also occur.

B. cenocepacia is able to grow intracellularly (20), and as its genome encodes a type IV secretion system (240), it is possible that this bacterium transfers DNA or RNAs to host cells. Additionally, a study found an effect of expressing a sRNA of *B. cenocepacia* in *E. coli* and *Pseudomonas aeruginosa*. Expression of sRNA MtvR, only conserved among the *Burkholderia* genus, decreased the ability of *E. coli* and *P. aeruginosa* to form biofilms and increased the susceptibility of these bacteria to various antibiotics (tetracycline, chloramphenicol, ciprofloxacin, tobramycin and ampicillin). Furthermore, this sRNA binds to and promotes accelerated decay of the *Hfq* mRNA of *E. coli* (241). This indicates that sRNAs of a certain bacteria could affect other bacterial species and other organisms.

5. sRNA research: what does the future bring?

5.1 Discovering sRNAs

The first sRNAs were discovered by coincidence as they were thought to be a precursor of tRNA or as their DNA sequence was thought to be a promotor of a protein-coding gene. Early labour-intensive and low-throughput approaches found novel sRNA genes, but only since the use of high-throughput sequencing, many sRNAs have been discovered (242). The first sRNA research within *B. cenocepacia* J2315 was performed by a computational approach based on the knowledge of that time (92). Back then, identification of sRNAs was based on minimum free energy (MFE) of secondary structures and comparative genomics (242). We now know that sRNAs vary in length, are not well conserved even among related species, do not necessarily have a secondary structure with very low minimum free energy but do show a negative z-score. Negative z-scores indicate that the secondary structure of the native sequence is more stable than that of random shuffled sequences with the same length and composition (191, 242). Nowadays, sRNA discovery methods focus on what is actually expressed and not on assumptions of sRNA structure and composition (242). One method for identifying novel transcripts is differential RNA sequencing, which compares an RNA sample relatively enriched with primary transcripts by using a nucleotide monophosphate-specific exonuclease enzyme, to untreated RNA to map transcription start sites to a genome. In this dissertation we focussed on sRNAs discovered using this approach (34).

5.2 Validation of sRNA expression

The experimental set-up for discovering sRNAs is very important and many studies indicate sRNAs are often only expressed in a certain condition. In *B. cenocepacia*, 13 sRNAs were discovered in the strains AU1054 and HI2424 grown in synthetic cystic fibrosis sputum and in a soil medium (139). In *B. cenocepacia* J2315, 11 putative sRNAs were differentially expressed after H_2O_2 treatment, 20 after NaOCl treatment (140) and 19 after chlorhexidine treatment (142). Only two sRNAs were found in two studies, while all the others were found in a single study only.

Therefore before moving on to unravelling the physiological role of discovered putative sRNAs it is important to investigate their expression in different conditions by Northern blotting or by qPCR. Some sRNAs are expressed in various growth conditions, while some are only expressed when induced by a specific stress factor (242). In our study, expression of nine sRNAs discovered by dRNA-seq (34) was evaluated by Northern blotting in seven growth conditions. Each sRNA was detected in each condition, indicating they are expressed. Additionally, expression of some sRNAs varied between growth conditions which could already be an early indication of the role of a particular sRNA.

5.3 The physiological role of sRNAs and the search for their target

Identifying a target and elucidating the physiological role of bacterial sRNAs is challenging. Several algorithms have been described to computationally predict targets. Each method uses several parameters such as sRNA-mRNA duplex strength, conservation between closely related species, interaction site accessibility, number of potential Hfq binding sites etc. However, all methods result in many false-positive predictions. As our understanding of the mechanisms involved in sRNA-mRNA interactions is still increasing, algorithms could be improved and become more reliable in the future (242). In this research one algorithm was chosen for identification of potential targets, i.e. CopraRNA. Because of the strong possibility for false positive predictions, computationally predicted targets need to be verified by other methods.

For this dissertation we created sRNA deletion mutants to elucidate sRNA function. The deletion of genes remains the most powerful method to explore their function and determine whether they are essential for the organism or not. However for Bcc species this is not an easy task. There is a limited availability of cloning vectors due to the lack of suitable selectable and counter-selectable markers caused by the high intrinsic resistance of Bcc species. Furthermore introducing DNA into these bacteria can only be achieved by conjugational transfer (160, 167). Therefore many standard methods regarding genetic manipulations cannot be applied. We created markerless deletions in *B. cenocepacia* J2315 based on the I-SceI homing endonuclease system. Markerless deletions show less polar effects on adjacent genes and allow the deletion of multiple genes compared to gene interruptions marked with antibiotic resistance cassettes (243). The used method was first described in *B. cenocepacia* K56- 2. This strain is clonally related to J2315 but easier to manipulate genetically. However, for several studies genes have been successfully deleted from the J2315 strain using the I-SceI homing endonuclease system (142, 154, 187, 244).

In general, deleting or inactivating bacterial sRNAs only results in mild phenotypes. sRNAs are described as additional regulators that fine-tune gene expression. The lack of a clear phenotype when the sRNA is deleted is probably caused by the variety of pathways that provide a protective mechanism to bacteria in stress conditions (101). However, several sRNA deletion or knock-out mutants have shown interesting phenotypes; eight sRNA knock-out mutants of *Streptococcus pneumoniae* exhibited attenuation of sepsis in a murine infection model, indicating a role of these sRNAs in virulence (245) and the deletion of gcvB sRNA in *Yersinia pestis* resulted in a phenotype that suggests it affects growth rate and colony morphology (246). In several studies sRNAs were overexpressed. Overexpressing a sRNA often results in a more pronounced phenotype compared to its deletion. A study in *E. coli* found a different susceptibility to antibiotics for 17 out of the 26 investigated sRNA when overexpressed,

while only four of them generated a phenotype when they were deleted (227). The investigated sRNAs were Hfq-dependent and therefore it was assumed that the overexpression of a sRNA affects antibiotic resistance indirectly. Overexpression of one sRNA could sequester Hfq and outcompete other RNAs dependent on Hfq (225, 247). To specifically identify sRNA targets by proteomics or transcriptomics, pulsed ectopic expression could be useful, however, to characterize their physiological role, deletion of sRNAs is more promising (132). However, deletion is not possible for cis-acting sRNAs which are located complementary to their gene. For such sRNA (for example the cis-acting sRNA h2cR, which is located complementary to the *hfq2* gene in *B. cenocepacia* J2315) overexpression and silencing can be used (134).

In this dissertation we used transcriptomics and proteomics of deletion mutants to further elucidate the regulatory network of the investigated sRNAs. For ncS35, we performed transcriptomics because CopraRNA predicted interaction of this sRNA within the coding sequence. sRNAs that base-pair within the coding sequence of a mRNA probably affect the degradation rate of the mRNA target rather than the initiation of translation (146). This mechanism of action could either stabilize mRNA targets or induce the degradation of mRNA targets, detectable at the transcript level. As ΔncS35 showed a growth related phenotype, three growth conditions were tested (planktonic exponential phase, planktonic stationary phase and biofilm). This analysis resulted in 2055 differentially expressed genes for ΔncS35 compared to wild type in all three conditions, which represents approx. 28 % of all annotated genes. For the other sRNAs (ncS04, ncS16 and ncS27), CopraRNA predicted interactions with mRNA targets nearby the ribosome binding site which indicates that these sRNAs could interfere with the initiation of translation and not necessarily cause a difference at the transcript level. Furthermore, the predicted targets were mainly outer membrane proteins. Therefore we specifically grew cells in the presence of SDS to stress the membrane and quantified outer membrane protein abundance by LC-MS^E. In proteome analysis less abundant proteins are often not detected. In our study only 3 % of all annotated proteins in *B. cenocepacia* J2315 proteins was detected and only one protein that was predicted as a target by CopraRNA was detected and changed expression in a sRNA mutant (ΔncS16) compared to wild type. Transcriptomics on the other hand investigates the expression of all annotated genes. For both transcriptomics and proteomics the main challenge is to discriminate direct effects caused by a sRNA interaction from indirect effects. Deleting a sRNA often causes a change in the expression of several hundred genes. sRNAs are known to target multiple mRNAs, however the majority of the differential gene expression is caused by a regulatory cascade induced by the direct binding of a sRNA to its targets (247). Additionally, it is important to take into account that both methods can fail to detect a true direct effect and that the experimental set-up (growth conditions) is of great importance (126). To investigate the sRNA profile induced by a specific stress, the time of

exposure to this specific stress is important. Howden et al. investigated the sRNA profile induced by vancomycin and exposed strains to vancomycin for only 10 minutes to show transcriptional effects associated with the initial stress response and not the effects induced by bacterial killing and growth inhibition (248).

5.4 Validation of RNA:RNA interaction

Once a potential target is identified, base-pairing can be confirmed by electrophoretic mobility shift assays (EMSA, *in vitro*) or by translational fusions of the target to a reporter gene (*in vivo*). Both methods are time consuming and require optimizations specific to a particular sRNA (125, 128). For Hfq-dependent sRNAs, it is important to include the Hfq protein in order to obtain interaction. For EMSA, each interaction requires a specific optimization in terms of salt and magnesium concentration, annealing conditions and incubation time (249).

In this thesis, we optimised the translational fusion method in *B. cenocepacia* J2315 to confirm sRNA:mRNA interaction. Urban et al. described the construction of a two-plasmid system in *E. coli* (Figure 4). This requires two compatible (origins of replication from the same group should not be co-transformed) and complementary (the use of different antibiotic resistance genes as selective marker) plasmid vectors transformed into one strain (250, 251). In a two-plasmid system, one plasmid expresses the sRNA, while the other plasmid expresses the target fused to a reporter. The use of two plasmids facilitates the investigation of multiple targets for one sRNA. On the other hand, one mRNA can be the target of multiple sRNAs, so different plasmids each with a different sRNA can be transformed into *E. coli* containing the plasmid with a specific mRNA (148). However, we developed a one-plasmid method as only a single replicative plasmid is available for *Burkholderia* species. We used pSCrhaM2 with the target fused to a gene encoding a fluorescent protein (mCherry) and investigated fluorescence when this plasmid was transformed into wild type and sRNA deletion mutant. pSCrhaM2 is a 7519 bp plasmid based on pSCrhaB2, containing a rhamnose-inducible promotor, which has been successfully used to evaluate expression of genes (182, 252).

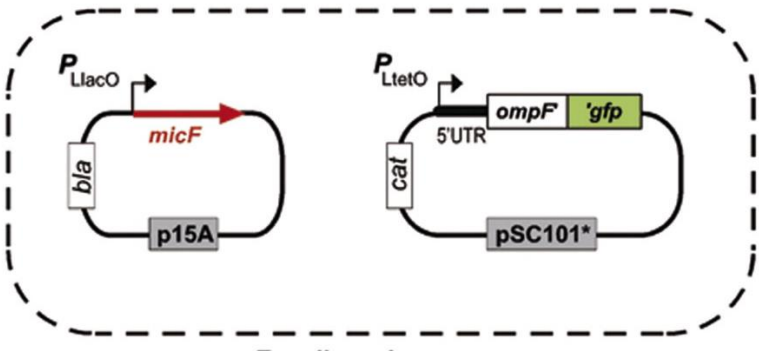


Figure 4. Two-plasmid fusion system in *Escherichia coli* to validate sRNA targets. micF is the sRNA and *ompF* is the putative target for this sRNA. The target is fused to *gfp*, which codes for a fluorescent protein. The fusion is cloned into a plasmid with pSC101* as origin of replication and is transcribed from a constitutive PLtetO promoter. The sRNA is cloned on a plasmid with p15A or ColE1 as origin of replication and is transcribed from the constitutive PLlacO promoter. Comparing the GFP fluorescence of *E. coli* transformed with only the plasmid containing the target fusion to a strain transformed with both plasmids could confirm the interaction between micF and *ompF* (125).

5.5 Future of sRNA research

At present, many sRNAs have been discovered but their functional characterization is lagging behind because methods are time-consuming and labour intensive while computational predictions are largely unreliable. Several promising strategies are being used to examine every aspect of sRNA regulation. Harris et al. developed SHAPE (selective 2'-hydroxyl acylation analysed by primer extension) to examine sRNA structure. This technique measures the reactivity of the 2' hydroxyl group of nucleotides. Single-stranded RNA will show a high activity, whereas RNA stem structures will show a lower activity (242). This could reveal unpaired regions of sRNAs to further optimize computational predictions.

Hfq co-immunoprecipitation is used and optimized to discover sRNA targets (242); high-throughput sequencing of RNA bound to Hfq after cross-linking the RNAs to the protein can reveal sRNAs and their targets. Once a specific target is known, it is easier to understand the role of a sRNA. Additionally, transposon insertion sequencing, a method used to monitor the fitness effects of gene disruptions, can give some insights in the role or importance of sRNAs (101).

6. sRNAs as future drug targets?

6.1 RNA as drug target

Using RNA as a drug target is nothing new. RNA serves as genetic material of viruses, as link between genetic information stored on DNA and the production of proteins and as structural scaffolding of the ribosomes (253). Targeting RNA is an emerging strategy for chemotherapy, and RNAs can be targeted at multiple accessible sites of their structure. Targeting proteins, which remains the mode of action for

most traditional drugs, is mostly restricted to their active sites. rRNA plays a critical role in protein synthesis. Due to marked differences between prokaryotes and eukaryotes, bacterial rRNA is an interesting target for drug action. Approximately two-third of a bacterial ribosome consists of rRNA. The 16S rRNA forms part of the smaller 30S ribosomal subunit and the 23S rRNA of the larger 50S subunit, while an eukaryotic ribosome consist of a small 40S subunit with 18S rRNA and a large 60S subunit containing 28S rRNA (254). Many antibiotics have been developed to target bacterial rRNA in order to inhibit protein translation, ultimately leading to inhibition of bacterial growth. The aminoglycosides streptomycin and spectinomycin, and tetracyclines bind to loops of the 16S rRNA while lincomycin, clindamycin and chloramphenicol target loops in the 23S rRNA (254, 255).

Currently there are no antibiotics that target tRNA. However the tRNA machinery has several promising targets, such as aminoacyl tRNA synthetases. At present, only mupirocin inhibits an aminoacyl tRNA synthetase, but scientist are focussing on the development of more compounds targeting these enzymes. Another antibiotic, puromycin, resembles the 3'end of the aminoacetylated tRNA and causes premature release of the polypeptide chain (254).

Compared to rRNA and tRNA, mRNA has a less complicated structure. In eukaryotes, small ligands targeting specific structures of mRNA (e.g. hairpins) have been developed. For example, an iron responsive element identified in Alzheimer's amyloid precursor and also present in several mRNAs has been of interest as a target for small molecules (254). Genes involved in diseases have been identified and complementary oligonucleotides have been constructed which within the cells can silence the mRNA to disrupt expression of its protein, a mechanism based on regulation by non-coding RNAs (256).

6.2 RNA silencing as an instrument against bacterial resistance

6.2.1 Riboswitch

With the discovery of riboswitches it emerged that many existing antimicrobial compounds (e.g. thiamine analog pyrithiamine, lysine analog DL-4-oxalysine) with a yet unknown mode of action in fact target riboswitches. Compounds mimicking the natural ligands of riboswitches (e.g. lysine, purine, flavin mononucleotide (FMN), cyclic-di-GMP) are able to silence essential genes of bacteria. Some riboswitches are widely distributed among bacteria and compounds targeting them are therefore broad-spectrum compounds, whereas other riboswitches are species-specific. A disadvantage of this approach is that many of the ligands are highly charged, which impedes their transport through the cell membrane. Additionally, resistance through mutations within the aptamer part of the riboswitch (ligand-binding part) has been reported (225).

An example of a compound targeting riboswitches is PC1, a pyrimidine compound, which targets the guanine riboswitch in *S. aureus* and *Clostridium difficile*. This riboswitch controls the expression of *guaA*, a GMP synthetase essential for virulence. PC1 was bactericidal only in bacteria where *guaA* expression is under the control of a guanosine riboswitch (257).

The genome of *B. cenocepacia* J2315 encodes 10 riboswitches, involved in major pathways such as vitamin synthesis (cobalamine, thiamine and glycine) (28, 34). Recently, two additional riboswitches were described; the S-adenosyl-L-homocysteine (SAH) riboswitch located in the 5'UTR of an adenosylhomocysteinase and the Burkholderiales-specific sucA RNA motif located upstream of *sucA*, encoding an enzyme of the citric acid cycle (34). However, compounds that interfere with these riboswitches have not yet been identified.

6.2.2 (s)RNA silencing

Some examples show that targeting sRNAs could be a promising approach. However interfering with sRNA regulation is still in their infancy and is more likely to provide a bacterial fitness reduction than to cause bacterial death (225). There are two general strategies for RNA silencing. Either the expression of an RNA is altered within the cell or a silencing agent is delivered to the cell (258). Ajoene is described as the major quorum sensing inhibitor present in garlic (259). At present it is the first reported compound that targets the quorum sensing system through modulation of sRNA expression in *P. aeruginosa* and *S. aureus*. It lowers the expression of sRNAs RsmY and RsmZ in *P. aeruginosa* and sRNA RNAIII in *S. aureus*, causing a lower expression of virulence factors (260).

RNA can be delivered to cells by using peptide nucleic acids (PNA) and phosphorothioate morpholino oligomers (PMO). In both models the sugar phosphate backbone of the RNA molecule is replaced, which impedes degradation by RNases and makes them far more stable than natural RNA molecules (256, 261). They can be used to either silence sRNAs or to directly silence mRNA. For sequence recognition and silencing these oligonucleotides need to have a sufficient length, on the other hand their high molecular weight and negative charge, as well as the presence of hydrogen bond donors and acceptors can hinder their uptake into the cell (256). This problem can be overcome by conjugation of these oligomers to cationic cell entry peptides (258, 262). The advantage of PNA and PMO oligomers is that, once delivered, they are retained longer compared to conventional antibiotics which are rapidly expelled by bacteria, and not removed by efflux pumps (258).

Several PMOs were developed to target essential genes. In *E. coli* and *S. typhimurium*, antisense PMO have been successfully tested which inhibit the expression of *acpP,* which encodes an acyl carrier

protein, thought to be essential for growth (263, 264). This gene is also conserved among Bcc species and Greenberg et al. succeeded to construct a PMO which targets *acpP* in Bcc species. It is active against Bcc infections *in vitro* and *in vivo* (265). Other interesting PMO targets are genes encoding quorums sensing systems and genes regulating the expression of virulence factors (39, 223). Interestingly, the *B. cenocepacia* sRNA MtvR when expressed in *E. coli* and *P. aeruginosa*, increased the susceptibility of these bacteria towards a variety of antibiotics. It could be interesting to use MtvR as candidate to develop sRNA-PMO's to act as an antimicrobial or as an adjuvant in antimicrobial therapy (241).

6.2.3 Hfq-dependent sRNAs

Many trans-acting sRNAs rely on RNA-chaperones to fulfill their function (e.g. Hfq, ProQ) (225, 227). Generally, inhibition of Hfq causes increased sensitivity to several stresses, an attenuation of virulence and an increase in susceptibility to antibiotics. The first Hfq inhibitor discovered is a peptide designated RI20. This peptide specifically binds at the proximal site of Hfq, the sRNA binding domain, and therefore inhibits all Hfq-sRNA interactions (225). Interestingly, a *Salmonella enterica Δhfq* strain was successfully used as a live attenuated vaccine in mice and was even found safe for pregnant mice (266). Within Gram-positive bacteria, Hfq function is not yet fully elucidated. Deletion of *hfq* has no major effects on the bacterial physiology in *S. aureus* and *B. subtilis*, while knockdown of Hfq expression in *C. difficile* shows multiple phenotypes relating to growth defects, and in *L. monocytogenes* this protein was found to play a role in stress tolerance and virulence (242, 267).

In *B. cenocepacia* J2315, a cis-encoded sRNA, h2cR, was found to negatively regulate the Hfq2 mRNA, which codes for one of the two Hfq-like proteins. Among bacterial species, only *Bacillus anthracis*, *Magnetospirillum magnetotacticum* and *Novosphingobium aromaticivorans*, and members of the *Burkholderia* genus harbour more than one copy of *hfq*-like genes (267, 268). To fully understand the effect of inactivation of *hfq* genes in *B. cenocepacia*, it is necessary to investigate the function and regulatory network of Hfq-dependent sRNAs.

7. sRNA research: why proceed?

Antimicrobial resistance is spreading and there is an urgent need for novel therapeutic approaches. The main strategy is finding new drugs targeting essential genes for bacteria, however, strategies to potentiate the activity of current antibiotics would also be useful. sRNAs are fascinating little molecules with a key role in gene expression. Investigating these molecules has provided us a better insight in the complex bacterial gene regulation, where they have shown to play a role in many cellular processes

including the response to antibiotics. Inhibition of one sRNA will probably not result in bacterial killing however sRNA-based therapeutic treatment of infectious diseases may become useful tools when combined with existing antibiotics (225, 226).

CHAPTER V. SUMMARY

Summary

Infectious diseases are a major threat to human health and the spread/increase of antimicrobial resistance is a major concern. *Burkholderia cenocepacia* J2315 is one of the predominant *Burkholderia* species colonizing the cystic fibrosis lung and is able to cause the "cepacia syndrome", a pneumonia with a rapid decline in lung function, often associated with bacteraemia. It is a member of the *Burkholderia cepacia* complex (Bcc), bacteria which are nowadays known as human opportunistic pathogens. Treatment of Bcc infections is difficult due to their high innate resistance against a broad range of antimicrobial compounds. Resistance in bacteria can be intrinsic, acquired through exchange of genetic information, or can be caused by altered gene expression (adaptive resistance). The focus of this dissertation is the investigation of the latest discovered group of gene expression regulators, the small RNAs, in *B. cenocepacia* J2315. sRNAs are small non-coding regulatory molecules that affect protein synthesis on the post-transcriptional level. While some sRNAs bind directly to proteins and alter their activity, most sRNAs act by base-pairing to mRNA targets to change their translation or degradation rate. Due to high-throughput RNA-sequencing, many sRNAs have been discovered in a wide range of bacteria. However, the identification of their function and regulatory network is lagging behind.

We investigated the expression of nine sRNAs in *B. cenocepacia* J2315, previously discovered by dRNA-seq. Northern blotting confirmed expression of eight sRNAs in seven different growth conditions, with levels of expression varying according to the condition. Of these nine sRNAs, five sRNAs (ncS04, ncS16, ncS25, ncS27 and ncS35) were successfully deleted in *B. cenocepacia* J2315. The phenotype of four sRNA deletion mutants (ΔncS04, ΔncS16, ΔncS27 and ΔncS35) was evaluated in various growth conditions with or without exposure to stress in order elucidate the physiological role of these sRNAs. Cell count, biomass and metabolic activity of biofilms, susceptibility to a variety of antimicrobial agents and to membrane, oxidative and osmotic stress, growth in an iron-depleted environment, motility and virulence towards *C. elegans* were determined. However, only a single mutant, ΔncS35, showed a phenotype in these experiments.

We subsequently further investigated ncS35 to elucidate its physiological role and regulatory network. ncS35 is processed to a shorter active form which is more expressed when *B. cenocepacia* J2315 was grown in biofilms and in minimal medium compared to planktonic growth. Cells of ΔncS35 showed an increased aggregation, a higher metabolic activity and higher growth rate, and the susceptibility to tobramycin was increased. Transcriptomics revealed upregulation of the phenylacetic acid and tryptophan degradation pathways in ΔncS35. The first gene of the tryptophan degradation pathway was computationally predicted to be a target of ncS35. Overall, it is shown that sRNA ncS35 is a non-

coding RNA with an attenuating effect on metabolic rate and growth. Slower growth can protect bacteria against stressors acting on fast-dividing cells and/or enhance survival in unfavourable conditions.

sRNA deletion mutants without a phenotype (ΔncS04, ΔncS16 and ΔncS27) were investigated through analysis of outer membrane proteins by LC-MS^E, because many targets computationally predicted for these sRNAs were OMPs. The expression of one predicted target of ncS16, a membrane lipoprotein which was more abundant in ΔncS16, was also evaluated on the transcriptional level using qPCR. Additionally, this mRNA was fused to a fluorescent protein to investigate actual interaction of the sRNA with its target. The interaction could be confirmed, as more fluorescence was produced in absence of ncS16. For the other two investigated sRNAs (ncS04 and ncS27) no targets could be confirmed. For ncS25, the most likely target based on computational predictions was an outer membrane porin which showed a significant higher mRNA expression in ΔncS25 compared to wild type. However, translational fusions could not confirm that this porin is a direct target of sRNA ncS25.

To conclude, methods for sRNA discovery in bacteria have improved, however, their functional characterization remains time-consuming and labour intensive. Additionally, computational target predictions are largely unreliable and contain many false-positive predictions. As understanding of mechanism of sRNA-mRNA interactions is still increasing, target predictions will become more reliable and sRNAs and their targets could become isolated together, which will make it easier to understand the physiological role of sRNAs. Of the functionally characterized sRNAs, many play a role in antimicrobial resistance and virulence. sRNAs are nowadays investigated as potential targets for the development of antibiotic chemotherapy, however strategies to use them are still in their infancy. In eukaryotes, sRNA-like molecules have been extensively studied where they are associated with mental retardation, neurological diseases, cancers, and infertility. Researchers have used RNA interference, a process in which complementary oligonucleotides can silence an mRNA linked to a disease to disrupt expression of its protein. Bacterial sRNAs are believed to fine-tune gene expression, therefore, anti-sRNA therapy is more likely to provide a bacterial fitness reduction rather than to cause bacterial death.

Samenvatting

Infectieziekten vormen een grote bedreiging voor de menselijke gezondheid en de verspreiding en vermeerdering van antimicrobiële resistentie is een grote zorg. *Burkholderia cenocepacia* is één van de overheersende soorten van het *Burkholderia* genus dat de long van mucoviscidose patiënten kan koloniseren en ernstige infecties kan veroorzaken. Verder is het ook in staat het ”cepacia syndroom”, een longontsteking met een snelle achteruitgang van de longfunctie, te veroorzaken. *Burkholderia cenocepacia* is lid van het *Burkholderia cepacia* complex (Bcc), bacteriën die bekend staan als opportunistische pathogenen. Behandeling van Bcc infecties is moeilijk vanwege hun hoge aangeboren resistentie tegen een breed scala aan antimicrobiële verbindingen. Bacteriële resistentie kan intrinsiek zijn, verkregen door uitwisseling van genetische informatie, of kan worden veroorzaakt door gewijzigde gen expressie (adaptieve weerstand). Dit proefschrift focust op de laatste ontdekte groep van genexpressie regulatoren, de small RNAs (sRNAs) in *B. cenocepacia* J2315. sRNAs zijn korte RNA moleculen die niet coderen voor proteïnen maar die de eiwitsynthese op het post-transcriptionele vlak regelen. Sommige sRNAs binden rechtstreeks aan eiwitten en veranderen hun activiteit, maar de meeste binden een mRNA doelwit en veranderen zo hun stabiliteit of de omzetting tot eiwitten. Door de ontwikkeling van snelle en betrouwbare RNA-sequencing methoden zijn reeds vele sRNA sequenties ontdekt, echter de identificatie van hun functie en hun regulerend effect is vaak niet geweten.

Wij hebben de expressie van negen sRNAs in *B. cenocepacia* J2315 onderzocht die eerder ontdekt waren door dRNA-seq. Northern blotting bevestigde de expressie van acht sRNAs in zeven verschillende groeicondities. Van deze negen sRNAs werden vijf (ncS04, ncS16, ncS25, ncS27 en ncS35) succesvol verwijderd in *B. cenocepacia* J2315. Het fenotype van vier sRNA deletiemutanten (ΔncS04, ΔncS16, ΔncS27 en ΔncS35) werd geëvalueerd in verschillende groei condities met of zonder blootstelling aan stress om de fysiologische rol van deze sRNAs te verduidelijken. Volgende karakteristieken werden bepaald: cel aantal, de biomassa en metabolische activiteit van biofilms, de gevoeligheid voor verschillende antimicrobiële middelen en voor membraan, oxidatieve en osmotische stress, de groei in een ijzer-arme omgeving, de motiliteit en de virulentie in het *C. elegans* infectie model. Echter, slechts één enkele mutant, ΔncS35, vertoonde een fenotype in deze experimenten.

Eerst hebben we ons gefocust op ncS35 om de werking van dit sRNA te verhelderen. Dit sRNA wordt omgezet tot een kortere actieve vorm die meer tot expressie komt wanneer *B. cenocepacia* J2315 werd gegroeid in biofilms en in minimaal medium ten opzichte van planktonische groei. ΔncS35 vertoonde een verhoogde aggregatie, een hogere metabolische activiteit en groeide aan een hoger tempo. Ook was de gevoeligheid voor tobramycine verhoogd. Het gen expressie profiel werd onderzocht en

onthulde een verhoogde expressie van het fenylazijnzuur- en tryptofaan katabolisme in ΔncS35. Ook werd het eerste gen in het tryptofaan katabolisme voorspeld als een mogelijk doelwit voor ncS35. sRNA ncS35 is dus een niet-coderend RNA met een limiterend effect op metabolisme en groei. Dit effect kan bacteriën beschermen tegen stress factoren die op snel delende cellen optreden en/of kan de overleving verbeteren in ongunstige omstandigheden.

Voor de sRNA deletie mutanten die geen fenotype vertoonden (ΔncS04, ΔncS16 en ΔncS27) werd een LC-MS^E analyse verricht voor de expressie van membraaneiwitten te onderzoeken, aangezien deze werden voorspeld als doelwit mRNAs. Een membraan lipoproteïne dat hoger tot expressie kwam in ΔncS16 en ook werd voorspeld als doelwit voor ncS16 werd verder geëvalueerd op het niveau van transcriptie door gebruik te maken van qPCR. Bovendien werd dit mRNA gefuseerd aan een fluorescerend eiwit om de werkelijke interactie van het sRNA met dit doelwit te onderzoeken. De interactie kon bevestigd worden, aangezien meer fluorescentie werd geproduceerd in afwezigheid van ncS16. Voor de overige twee onderzochte sRNAs (ncS04 en ncS27) konden geen doelwit mRNAs bevestigd worden. Voor het 5^e sRNA ncS25 was het meest waarschijnlijke doelwit mRNA een porine, dat eveneens een significante hogere mRNA-expressie in ΔncS25 vertoonde ten opzichte van het wild type. Een translationele fusie kon echter niet bevestigen dat dit mRNA een direct doelwit van sRNA ncS25 is.

Wat we kunnen concluderen is dat methoden naar het vinden van sRNA sequenties in bacteriën verbeterd zijn, maar dat hun functionele karakterisering tijdrovend en arbeidsintensief is. Bovendien zijn doelwit mRNA voorspellingen grotendeels onbetrouwbaar en zijn er veel vals positieven. Aangezien het inzicht naar het mechanisme van sRNA-mRNA interactie nog toeneemt, zullen deze voorspellingen betrouwbaarder worden. Ook worden verscheidene technieken geoptimaliseerd om sRNAs en hun doelwit mRNAs samen te isoleren, waardoor het gemakkelijker wordt om de fysiologische rol van deze sRNAs te begrijpen. Van de functioneel gekenmerkte sRNAs spelen velen een rol in antimicrobiële resistentie en virulentie. Daarom worden sRNAs onderzocht als potentiële doelwitten voor de ontwikkeling van antibiotica chemotherapie, hoewel strategieën om ze te gebruiken nog niet volledig ontwikkeld zijn. In eukaryoten zijn sRNA-achtige moleculen uitgebreid bestudeerd waar ze geassocieerd zijn met mentale achterstand, neurologische ziekten, kanker en onvruchtbaarheid. Onderzoekers hebben RNA interferentie, een proces waarin complementaire oligonucleotiden aan een mRNA binden, gebruikt om de expressie van een het eiwit te verstoren dat geassocieerd is aan een ziekte. Bacteriële sRNAs daarentegen worden geacht de gen expressie te verfijnen en daarom zal anti-sRNA therapie in bacteriën eerder een bacteriële fitness reductie geven in plaats van bacteriële dood te veroorzaken.

References

1. Aminov RI. 2010. A brief history of the antibiotic era: lessons learned and challenges for the future. Front Microbiol 1:134.
2. WHO G, Switzerland. 2017. The top 10 causes of death.
3. CDC A, GA. 2013. Antibiotic resistance threats in the United States. CDC, Atlanta, GA,
4. WHO G, Switzerland. 2014. Antimicrobial resistance: global report on surveillance 2014.
5. Sharma VK, Johnson N, Cizmas L, McDonald TJ, Kim H. 2016. A review of the influence of treatment strategies on antibiotic resistant bacteria and antibiotic resistance genes. Chemosphere 150:702-14.
6. Fleming A. 1929. On antibacterial action of culture of Penicillium, with special reference to their use in isolation of *B. influenzae*. Br J Exp Pathol 10:226–236.
7. Waclaw B. 2016. Evolution of Drug Resistance in Bacteria. Adv Exp Med Biol 915:49-67.
8. Gang Z, Jie F. 2016. The intrinsic resistance of bacteria. Yi Chuan 38:872-880.
9. Handel N, Schuurmans JM, Feng Y, Brul S, ter Kuile BH. 2014. Interaction between mutations and regulation of gene expression during development of de novo antibiotic resistance. Antimicrob Agents Chemother 58:4371-9.
10. Fernandez L, Hancock RE. 2012. Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. Clin Microbiol Rev 25:661-81.
11. Motta SS, Cluzel P, Aldana M. 2015. Adaptive resistance in bacteria requires epigenetic inheritance, genetic noise, and cost of efflux pumps. PLoS One 10:e0118464.
12. O'Neill J. 2014. Antimicrobial resistance: tackling a crisis for the health and wealth of nations. Welcome Trust and HM Government, London, United Kingdom.,
13. Drevinek P, Mahenthiralingam E. 2010. *Burkholderia cenocepacia* in cystic fibrosis: epidemiology and molecular mechanisms of virulence. Clin Microbiol Infect 16:821-30.
14. Depoorter E, Bull MJ, Peeters C, Coenye T, Vandamme P, Mahenthiralingam E. 2016. *Burkholderia*: an update on taxonomy and biotechnological potential as antibiotic producers. Appl Microbiol Biotechnol 100:5215-29.
15. el-Banna N, Winkelmann G. 1998. Pyrrolnitrin from *Burkholderia cepacia*: antibiotic activity against fungi and novel activities against *streptomycetes*. J Appl Microbiol 85:69-78.
16. Isles A, Maclusky I, Corey M, Gold R, Prober C, Fleming P, Levison H. 1984. *Pseudomonas cepacia* infection in cystic fibrosis: an emerging problem. J Pediatr 104:206-10.
17. Horsley A, Jones AM, Lord R. 2016. Antibiotic treatment for *Burkholderia cepacia* complex in people with cystic fibrosis experiencing a pulmonary exacerbation. Cochrane Database Syst Rev doi:10.1002/14651858.CD009529.pub3:CD009529.
18. De Smet B, Mayo M, Peeters C, Zlosnik JE, Spilker T, Hird TJ, LiPuma JJ, Kidd TJ, Kaestli M, Ginther JL, Wagner DM, Keim P, Bell SC, Jacobs JA, Currie BJ, Vandamme P. 2015. *Burkholderia stagnalis* sp. nov. and *Burkholderia territorii* sp. nov., two novel *Burkholderia cepacia* complex species from environmental and human sources. Int J Syst Evol Microbiol 65:2265-71.
19. Mahenthiralingam E, Urban TA, Goldberg JB. 2005. The multifarious, multireplicon *Burkholderia cepacia* complex. Nat Rev Microbiol 3:144-56.

20. Valvano MA. 2015. Intracellular survival of *Burkholderia cepacia* complex in phagocytic cells. Can J Microbiol 61:607-15.

21. Drevinek P, Holden MT, Ge Z, Jones AM, Ketchell I, Gill RT, Mahenthiralingam E. 2008. Gene expression changes linked to antimicrobial resistance, oxidative stress, iron depletion and retained motility are observed when *Burkholderia cenocepacia* grows in cystic fibrosis sputum. BMC Infect Dis 8:121.

22. Mann T, Ben-David D, Zlotkin A, Shachar D, Keller N, Toren A, Nagler A, Smollan G, Barzilai A, Rahav G. 2010. An outbreak of *Burkholderia cenocepacia* bacteremia in immunocompromised oncology patients. Infection 38:187-94.

23. Bylund J, Campsall PA, Ma RC, Conway BA, Speert DP. 2005. *Burkholderia cenocepacia* induces neutrophil necrosis in chronic granulomatous disease. J Immunol 174:3562-9.

24. Leiding JW, Holland SM. 2016. Chronic Granulomatous Disease.

25. Marciano BE, Spalding C, Fitzgerald A, Mann D, Brown T, Osgood S, Yockey L, Darnell DN, Barnhart L, Daub J, Boris L, Rump AP, Anderson VL, Haney C, Kuhns DB, Rosenzweig SD, Kelly C, Zelazny A, Mason T, DeRavin SS, Kang E, Gallin JI, Malech HL, Olivier KN, Uzel G, Freeman AF, Heller T, Zerbe CS, Holland SM. 2015. Common severe infections in chronic granulomatous disease. Clin Infect Dis 60:1176-83.

26. Speert DP, Henry D, Vandamme P, Corey M, Mahenthiralingam E. 2002. Epidemiology of *Burkholderia cepacia* complex in patients with cystic fibrosis, Canada. Emerg Infect Dis 8:181-7.

27. Varga JJ, Losada L, Zelazny AM, Kim M, McCorrison J, Brinkac L, Sampaio EP, Greenberg DE, Singh I, Heiner C, Ashby M, Nierman WC, Holland SM, Goldberg JB. 2013. Draft Genome Sequences of *Burkholderia cenocepacia* ET12 Lineage Strains K56-2 and BC7. Genome Announc 1.

28. Holden MT, Seth-Smith HM, Crossman LC, Sebaihia M, Bentley SD, Cerdeno-Tarraga AM, Thomson NR, Bason N, Quail MA, Sharp S, Cherevach I, Churcher C, Goodhead I, Hauser H, Holroyd N, Mungall K, Scott P, Walker D, White B, Rose H, Iversen P, Mil-Homens D, Rocha EP, Fialho AM, Baldwin A, Dowson C, Barrell BG, Govan JR, Vandamme P, Hart CA, Mahenthiralingam E, Parkhill J. 2009. The genome of *Burkholderia cenocepacia* J2315, an epidemic pathogen of cystic fibrosis patients. J Bacteriol 191:261-77.

29. Mahenthiralingam E, Drevinek P. 2007. Comparative genomics of *Burkholderia* species.

30. Agnoli K, Schwager S, Uehlinger S, Vergunst A, Viteri DF, Nguyen DT, Sokol PA, Carlier A, Eberl L. 2012. Exposing the third chromosome of *Burkholderia cepacia* complex strains as a virulence plasmid. Mol Microbiol 83:362-78.

31. Kim HS, Schell MA, Yu Y, Ulrich RL, Sarria SH, Nierman WC, DeShazer D. 2005. Bacterial genome adaptation to niches: divergence of the potential virulence genes in three *Burkholderia species* of different survival strategies. BMC Genomics 6:174.

32. Guo FB, Xiong L, Zhang KY, Dong C, Zhang FZ, Woo PC. 2017. Identification and analysis of genomic islands in *Burkholderia cenocepacia* AU 1054 with emphasis on pathogenicity islands. BMC Microbiol 17:73.

33. Baldwin A, Sokol PA, Parkhill J, Mahenthiralingam E. 2004. The Burkholderia cepacia epidemic strain marker is part of a novel genomic island encoding both virulence and metabolism-associated genes in *Burkholderia cenocepacia*. Infect Immun 72:1537-47.

34. Sass AM, Van Acker H, Forstner KU, Van Nieuwerburgh F, Deforce D, Vogel J, Coenye T. 2015. Genome-wide transcription start site profiling in biofilm-grown *Burkholderia cenocepacia* J2315. BMC Genomics 16:775.

35. Sousa SA, Feliciano JR, Pita T, Guerreiro SI, Leitao JH. 2017. *Burkholderia cepacia* Complex Regulation of Virulence Gene Expression: A Review. Genes (Basel) 8.

36. Whitehead NA, Barnard AM, Slater H, Simpson NJ, Salmond GP. 2001. Quorum-sensing in Gram-negative bacteria. FEMS Microbiol Rev 25:365-404.

37. Eberl L. 2006. Quorum sensing in the genus *Burkholderia*. Int J Med Microbiol 296:103-10.

38. Lutter E, Lewenza S, Dennis JJ, Visser MB, Sokol PA. 2001. Distribution of quorum-sensing genes in the *Burkholderia cepacia* complex. Infect Immun 69:4661-6.

39. Leitao JH, Sousa SA, Ferreira AS, Ramos CG, Silva IN, Moreira LM. 2010. Pathogenicity, virulence factors, and strategies to fight against *Burkholderia cepacia* complex pathogens and related species. Appl Microbiol Biotechnol 87:31-40.

40. Hoiby N, Bjarnsholt T, Moser C, Bassi GL, Coenye T, Donelli G, Hall-Stoodley L, Hola V, Imbert C, Kirketerp-Moller K, Lebeaux D, Oliver A, Ullmann AJ, Williams C. 2015. ESCMID guideline for the diagnosis and treatment of biofilm infections 2014. Clin Microbiol Infect 21 Suppl 1:S1-25.

41. Coenye T. 2010. Social interactions in the *Burkholderia cepacia* complex: biofilms and quorum sensing. Future Microbiol 5:1087-99.

42. Vinion-Dubiel AD, Goldberg JB. 2003. Lipopolysaccharide of *Burkholderia cepacia* complex. J Endotoxin Res 9:201-13.

43. Tyrrell J, Callaghan M. 2016. Iron acquisition in the cystic fibrosis lung and potential for novel therapeutic strategies. Microbiology 162:191-205.

44. Loutet SA, Valvano MA. 2010. A decade of *Burkholderia cenocepacia* virulence determinant research. Infect Immun 78:4088-100.

45. Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ. 2007. A common mechanism of cellular death induced by bactericidal antibiotics. Cell 130:797-810.

46. Snyder L, Champness W. 2003. Molecular Genetics of Bacteria.

47. Commichau FM, Stulke J. 2015. Trigger Enzymes: Coordination of Metabolism and Virulence Gene Expression. Microbiol Spectr 3.

48. Ma C, Yang X, Lewis PJ. 2016. Bacterial Transcription as a Target for Antibacterial Drug Development. Microbiol Mol Biol Rev 80:139-60.

49. Perez JC, Groisman EA. 2009. Evolution of transcriptional regulatory circuits in bacteria. Cell 138:233-44.

50. Mooney RA, Artsimovitch I, Landick R. 1998. Information processing by RNA polymerase: recognition of regulatory signals during RNA chain elongation. J Bacteriol 180:3265-75.

51. Grylak-Mielnicka A, Bidnenko V, Bardowski J, Bidnenko E. 2016. Transcription termination factor Rho: a hub linking diverse physiological processes in bacteria. Microbiology 162:433-47.

52. Lodish H, Berk A, Zipursky S. 2000. Molecular Cell Biology. 4th edition.

53. Baynes JW, Dominiczak MH. 2014. Medical biochemistry fourth edition ed. Edinburgh : Saunders Elsevier, Scotland.

54. Evguenieva-Hackenberg E, Klug G. 2011. New aspects of RNA processing in prokaryotes. Curr Opin Microbiol 14:587-92.

55. Rochat T, Bouloc P, Repoila F. 2013. Gene expression control by selective RNA processing and stabilization in bacteria. FEMS Microbiol Lett 344:104-13.

56. Régnier P, Marujo P. 2000-2013. Polyadenylation and Degradation of RNA in Prokaryotes. Landes Bioscience, Austin (TX).

57. Nicholson AW. 1999. Function, mechanism and regulation of bacterial ribonucleases. FEMS Microbiol Rev 23:371-90.

58. Pedersen M, Nissen S, Mitarai N, Lo Svenningsen S, Sneppen K, Pedersen S. 2011. The functional half-life of an mRNA depends on the ribosome spacing in an early coding region. J Mol Biol 407:35-44.

59. Yarza P, Yilmaz P, Pruesse E, Glockner FO, Ludwig W, Schleifer KH, Whitman WB, Euzeby J, Amann R, Rossello-Mora R. 2014. Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. Nat Rev Microbiol 12:635-45.

60. Alberts B, Johnson A, Lewis J. 2002. Molecular Biology of the Cell. 4th edition.

61. Gualerzi CO, Pon CL. 2015. Initiation of mRNA translation in bacteria: structural and dynamic aspects. Cell Mol Life Sci 72:4341-67.

62. Rodnina MV. 2016. The ribosome in action: Tuning of translational efficiency and protein folding. Protein Sci 25:1390-406.

63. Gerosa L, Kochanowski K, Heinemann M, Sauer U. 2013. Dissecting specific and global transcriptional regulation of bacterial gene expression. Mol Syst Biol 9:658.

64. Delihas N, Forst S. 2001. MicF: an antisense RNA gene involved in response of *Escherichia coli* to global stress factors. J Mol Biol 313:1-12.

65. Browning DF, Busby SJ. 2016. Local and global regulation of transcription initiation in bacteria. Nat Rev Microbiol 14:638-50.

66. Jishage M, Ishihama A. 1998. A stationary phase protein in *Escherichia coli* with binding activity to the major sigma subunit of RNA polymerase. Proc Natl Acad Sci U S A 95:4953-8.

67. Martinez LC, Vadyvaloo V. 2014. Mechanisms of post-transcriptional gene regulation in bacterial biofilms. Front Cell Infect Microbiol 4:38.

68. Picard F, Dressaire C, Girbal L, Cocaign-Bousquet M. 2009. Examination of post-transcriptional regulations in prokaryotes by integrative biology. C R Biol 332:958-73.

69. Hernandez-Arranz S, Moreno R, Rojo F. 2013. The translational repressor Crc controls the *Pseudomonas putida* benzoate and alkane catabolic pathways using a multi-tier regulation strategy. Environ Microbiol 15:227-41.

70. Pisithkul T, Patel NM, Amador-Noguez D. 2015. Post-translational modifications as key regulators of bacterial metabolic fluxes. Curr Opin Microbiol 24:29-37.

71. Cain JA, Solis N, Cordwell SJ. 2014. Beyond gene expression: the impact of protein post-translational modifications in bacteria. J Proteomics 97:265-86.

72. Chubukov V, Gerosa L, Kochanowski K, Sauer U. 2014. Coordination of microbial metabolism. Nat Rev Microbiol 12:327-40.

73. Michaux C, Verneuil N, Hartke A, Giard JC. 2014. Physiological roles of small RNA molecules. Microbiology 160:1007-19.

74. Bhaya D, Davison M, Barrangou R. 2011. CRISPR-Cas systems in bacteria and archaea: versatile small RNAs for adaptive defense and regulation. Annu Rev Genet 45:273-97.

75. Gottesman S, Storz G. 2011. Bacterial small RNA regulators: versatile roles and rapidly evolving variations. Cold Spring Harb Perspect Biol 3.

76. Sawana A, Adeolu M, Gupta RS. 2014. Molecular signatures and phylogenomic analysis of the genus *Burkholderia*: proposal for division of this genus into the emended genus *Burkholderia* containing pathogenic organisms and a new genus *Paraburkholderia* gen. nov. harboring environmental species. Front Genet 5:429.

77. Yang L, Jelsbak L, Molin S. 2011. Microbial ecology and adaptation in cystic fibrosis airways. Environ Microbiol 13:1682-9.

78. Menard A, de Los Santos PE, Graindorge A, Cournoyer B. 2007. Architecture of *Burkholderia cepacia* complex sigma70 gene family: evidence of alternative primary and clade-specific factors, and genomic instability. BMC Genomics 8:308.

79. Flannagan RS, Valvano MA. 2008. *Burkholderia cenocepacia* requires RpoE for growth under stress conditions and delay of phagolysosomal fusion in macrophages. Microbiology 154:643-53.

80. Lardi M, Aguilar C, Pedrioli A, Omasits U, Suppiger A, Carcamo-Oyarce G, Schmid N, Ahrens CH, Eberl L, Pessi G. 2015. sigma54-Dependent Response to Nitrogen Limitation and Virulence in *Burkholderia cenocepacia* Strain H111. Appl Environ Microbiol 81:4077-89.

81. Saldias MS, Lamothe J, Wu R, Valvano MA. 2008. *Burkholderia cenocepacia* requires the RpoN sigma factor for biofilm formation and intracellular trafficking within macrophages. Infect Immun 76:1059-67.

82. Ryan GT, Wei Y, Winans SC. 2013. A LuxR-type repressor of *Burkholderia cenocepacia* inhibits transcription via antiactivation and is inactivated by its cognate acylhomoserine lactone. Mol Microbiol 87:94-111.

83. Bernier SP, Nguyen DT, Sokol PA. 2008. A LysR-type transcriptional regulator in *Burkholderia cenocepacia* influences colony morphology and virulence. Infect Immun 76:38-47.

84. Winsor GL, Khaira B, Van Rossum T, Lo R, Whiteside MD, Brinkman FS. 2008. The *Burkholderia* Genome Database: facilitating flexible queries and comparative analyses. Bioinformatics 24:2803-4.

85. Merry CR, Perkins M, Mu L, Peterson BK, Knackstedt RW, Weingart CL. 2015. Characterization of a novel two-component system in *Burkholderia cenocepacia*. Curr Microbiol 70:556-61.

86. Gislason AS, Choy M, Bloodworth RA, Qu W, Stietz MS, Li X, Zhang C, Cardona ST. 2017. Competitive Growth Enhances Conditional Growth Mutant Sensitivity to Antibiotics and Exposes a Two-Component System as an Emerging Antibacterial Target in *Burkholderia cenocepacia*. Antimicrob Agents Chemother 61.

87. Khodai-Kalaki M, Aubert DF, Valvano MA. 2013. Characterization of the AtsR hybrid sensor kinase phosphorelay pathway and identification of its response regulator in *Burkholderia cenocepacia*. J Biol Chem 288:30473-84.

88. Boon C, Deng Y, Wang LH, He Y, Xu JL, Fan Y, Pan SQ, Zhang LH. 2008. A novel DSF-like signal from *Burkholderia cenocepacia* interferes with *Candida albicans* morphological transition. ISME J 2:27-36.

89. Schmid N, Pessi G, Deng Y, Aguilar C, Carlier AL, Grunau A, Omasits U, Zhang LH, Ahrens CH, Eberl L. 2012. The AHL- and BDSF-dependent quorum sensing systems control specific and overlapping sets of genes in *Burkholderia cenocepacia* H111. PLoS One 7:e49966.

90. Malott RJ, Baldwin A, Mahenthiralingam E, Sokol PA. 2005. Characterization of the cciIR quorum-sensing system in *Burkholderia cenocepacia*. Infect Immun 73:4982-92.

91. Wagner EG, Darfeuille F. 2005. Small Regulatory RNAs in bacteria. Nucleic Acids and Molecular Biology 17.

92. Coenye T, Drevinek P, Mahenthiralingam E, Shah SA, Gill RT, Vandamme P, Ussery DW. 2007. Identification of putative noncoding RNA genes in the *Burkholderia cenocepacia* J2315 genome. FEMS Microbiol Lett 276:83-92.

93. Mattick JS. 2001. Non-coding RNAs: the architects of eukaryotic complexity. EMBO Rep 2:986-91.

94. Grosshans H, Filipowicz W. 2008. Molecular biology: the expanding world of small RNAs. Nature 451:414-6.

95. Guillier M, Gottesman S, Storz G. 2006. Modulating the outer membrane with small RNAs. Genes Dev 20:2338-48.

96. Mehta P, Goyal S, Wingreen NS. 2008. A quantitative comparison of sRNA-based and protein-based gene regulation. Mol Syst Biol 4:221.

97. Waters LS, Storz G. 2009. Regulatory RNAs in bacteria. Cell 136:615-28.

98. Ternan N. 2013. Small regulatory RNA molecules in bacteria. OA Microbiology Dec 01:1-8.

99. Li W, Ying X, Lu Q, Chen L. 2012. Predicting sRNAs and their targets in bacteria. Genomics Proteomics Bioinformatics 10:276-84.

100. Masse E, Escorcia FE, Gottesman S. 2003. Coupled degradation of a small regulatory RNA and its mRNA targets in *Escherichia coli*. Genes Dev 17:2374-83.

101. Barquist L, Vogel J. 2015. Accelerating Discovery and Functional Analysis of Small RNAs with New Technologies. Annu Rev Genet 49:367-94.

102. Guillier M, Gottesman S. 2006. Remodelling of the *Escherichia coli* outer membrane by two small regulatory RNAs. Mol Microbiol 59:231-47.

103. Masse E, Gottesman S. 2002. A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. Proc Natl Acad Sci U S A 99:4620-5.

104. Masse E, Vanderpool CK, Gottesman S. 2005. Effect of RyhB small RNA on global iron use in *Escherichia coli*. J Bacteriol 187:6962-71.

105. Updegrove TB, Shabalina SA, Storz G. 2015. How do base-pairing small RNAs evolve? FEMS Microbiol Rev 39:379-91.

106. Shao Y, Feng L, Rutherford ST, Papenfort K, Bassler BL. 2013. Functional determinants of the quorum-sensing non-coding RNAs and their roles in target regulation. EMBO J 32:2158-71.

107. Neuhaus K, Landstorfer R, Simon S, Schober S, Wright PR, Smith C, Backofen R, Wecko R, Keim DA, Scherer S. 2017. Differentiation of ncRNAs from small mRNAs in *Escherichia coli* O157:H7 EDL933 (EHEC) by combined RNAseq and RIBOseq - ryhB encodes the regulatory RNA RyhB and a peptide, RyhP. BMC Genomics 18:216.

108. Attaiech L, Glover JN, Charpentier X. 2017. RNA Chaperones Step Out of Hfq's Shadow. Trends Microbiol doi:10.1016/j.tim.2017.01.006.

109. Semrad K. 2011. Proteins with RNA chaperone activity: a world of diverse proteins with a common task-impediment of RNA misfolding. Biochem Res Int 2011:532908.

110. Vogel J, Luisi BF. 2011. Hfq and its constellation of RNA. Nat Rev Microbiol 9:578-89.

111. Gorski SA, Vogel J, Doudna JA. 2017. RNA-based recognition and targeting: sowing the seeds of specificity. Nat Rev Mol Cell Biol 18:215-228.

112. Smirnov A, Forstner KU, Holmqvist E, Otto A, Gunster R, Becher D, Reinhardt R, Vogel J. 2016. Grad-seq guides the discovery of ProQ as a major small RNA-binding protein. Proc Natl Acad Sci U S A 113:11591-11596.

113. Gonzalez GM, Hardwick SW, Maslen SL, Skehel JM, Holmqvist E, Vogel J, Bateman A, Luisi BF, Broadhurst RW. 2017. Structure of the *Escherichia coli* ProQ RNA-binding protein. RNA 23:696-711.

114. Wilderman PJ, Sowa NA, FitzGerald DJ, FitzGerald PC, Gottesman S, Ochsner UA, Vasil ML. 2004. Identification of tandem duplicate regulatory small RNAs in *Pseudomonas aeruginosa* involved in iron homeostasis. Proc Natl Acad Sci U S A 101:9792-7.

115. Papenfort K, Vogel J. 2010. Regulatory RNA in bacterial pathogens. Cell Host Microbe 8:116-27.

116. Cavanagh AT, Wassarman KM. 2014. 6S RNA, a global regulator of transcription in *Escherichia coli*, *Bacillus subtilis*, and beyond. Annu Rev Microbiol 68:45-60.

117. Wassarman KM. 2007. 6S RNA: a small RNA regulator of transcription. Curr Opin Microbiol 10:164-8.

118. Bardill JP, Zhao X, Hammer BK. 2011. The Vibrio cholerae quorum sensing response is mediated by Hfq-dependent sRNA/mRNA base pairing interactions. Mol Microbiol 80:1381-94.

119. Opdyke JA, Kang JG, Storz G. 2004. GadY, a small-RNA regulator of acid response genes in *Escherichia coli*. J Bacteriol 186:6698-705.

120. Andersen J, Delihas N. 1990. micF RNA binds to the 5' end of ompF mRNA and to a protein from *Escherichia coli*. Biochemistry 29:9249-56.

121. Argaman L, Hershberg R, Vogel J, Bejerano G, Wagner EG, Margalit H, Altuvia S. 2001. Novel small RNA-encoding genes in the intergenic regions of *Escherichia coli*. Curr Biol 11:941-50.

122. Wassarman KM, Repoila F, Rosenow C, Storz G, Gottesman S. 2001. Identification of novel small RNAs using comparative genomics and microarrays. Genes Dev 15:1637-51.

123. Sharma CM, Vogel J. 2014. Differential RNA-seq: the approach behind and the biological insight gained. Curr Opin Microbiol 19:97-105.

124. Konig J, Zarnack K, Luscombe NM, Ule J. 2012. Protein-RNA interactions: new genomic technologies and perspectives. Nat Rev Genet 13:77-83.

125. Vogel J, Wagner EG. 2007. Target identification of small noncoding RNAs in bacteria. Curr Opin Microbiol 10:262-70.

126. Sievers S, Lund A, Menendez-Gil P, Nielsen A, Storm Mollerup M, Lambert Nielsen S, Buch Larsson P, Borch-Jensen J, Johansson J, Kallipolitis BH. 2015. The multicopy sRNA LhrC controls expression of the oligopeptide-binding protein OppA in *Listeria monocytogenes*. RNA Biol 12:985-97.

127. Liang H, Zhao YT, Zhang JQ, Wang XJ, Fang RX, Jia YT. 2011. Identification and functional characterization of small non-coding RNAs in *Xanthomonas oryzae* pathovar oryzae. BMC Genomics 12:87.

128. Mallick B, Ghosh Z. 2012. Regulatory RNAs: Basics, Methods and Applications. Springer Science & Business Media.

129. Mraheil MA, Billion A, Mohamed W, Mukherjee K, Kuenne C, Pischimarov J, Krawitz C, Retey J, Hartsch T, Chakraborty T, Hain T. 2011. The intracellular sRNA transcriptome of *Listeria monocytogenes* during growth in macrophages. Nucleic Acids Res 39:4235-48.

130. Santiviago CA, Reynolds MM, Porwollik S, Choi SH, Long F, Andrews-Polymenis HL, McClelland M. 2009. Analysis of pools of targeted *Salmonella* deletion mutants identifies novel genes affecting fitness during competitive infection in mice. PLoS Pathog 5:e1000477.

131. Yan Y, Su S, Meng X, Ji X, Qu Y, Liu Z, Wang X, Cui Y, Deng Z, Zhou D, Jiang W, Yang R, Han Y. 2013. Determination of sRNA expressions by RNA-seq in *Yersinia pestis* grown in vitro and during infection. PLoS One 8:e74495.

132. Ortega AD, Quereda JJ, Pucciarelli MG, Garcia-del Portillo F. 2014. Non-coding RNA regulation in pathogenic bacteria located inside eukaryotic cells. Front Cell Infect Microbiol 4:162.

133. Liu H, Wang X, Wang HD, Wu J, Ren J, Meng L, Wu Q, Dong H, Wu J, Kao TY, Ge Q, Wu ZX, Yuh CH, Shan G. 2012. *Escherichia coli* noncoding RNAs can affect gene expression and physiology of *Caenorhabditis elegans*. Nat Commun 3:1073.

134. Ramos CG, da Costa PJ, Doring G, Leitao JH. 2012. The novel cis-encoded small RNA h2cR is a negative regulator of hfq2 in *Burkholderia cenocepacia*. PLoS One 7:e47896.

135. Sousa SA, Ramos CG, Leitao JH. 2011. Burkholderia cepacia Complex: Emerging Multihost Pathogens Equipped with a Wide Range of Virulence Factors and Determinants. Int J Microbiol 2011.

136. Rivas E, Eddy SR. 2001. Noncoding RNA gene detection using comparative sequence analysis. BMC Bioinformatics 2:8.

137. Szymanski M, Erdmann VA, Barciszewski J. 2003. Noncoding regulatory RNAs database. Nucleic Acids Res 31:429-31.

138. Vogel DW, Hartmann RK, Struck JC, Ulbrich N, Erdmann VA. 1987. The sequence of the 6S RNA gene of *Pseudomonas aeruginosa*. Nucleic Acids Res 15:4583-91.

139. Yoder-Himes DR, Chain PS, Zhu Y, Wurtzel O, Rubin EM, Tiedje JM, Sorek R. 2009. Mapping the *Burkholderia cenocepacia* niche response via high-throughput sequencing. Proc Natl Acad Sci U S A 106:3976-81.

140. Peeters E, Sass A, Mahenthiralingam E, Nelis H, Coenye T. 2010. Transcriptional response of *Burkholderia cenocepacia* J2315 sessile cells to treatments with high doses of hydrogen peroxide and sodium hypochlorite. BMC Genomics 11:90.

141. Gardner PP, Daub J, Tate JG, Nawrocki EP, Kolbe DL, Lindgreen S, Wilkinson AC, Finn RD, Griffiths-Jones S, Eddy SR, Bateman A. 2009. Rfam: updates to the RNA families database. Nucleic Acids Res 37:D136-40.

142. Coenye T, Van Acker H, Peeters E, Sass A, Buroni S, Riccardi G, Mahenthiralingam E. 2011. Molecular mechanisms of chlorhexidine tolerance in *Burkholderia cenocepacia* biofilms. Antimicrob Agents Chemother 55:1912-9.

143. Ramos CG, Grilo AM, da Costa PJ, Leitao JH. 2013. Experimental identification of small non-coding regulatory RNAs in the opportunistic human pathogen *Burkholderia cenocepacia* J2315. Genomics 101:139-48.

144. Sousa SA, Ramos CG, Moreira LM, Leitao JH. 2010. The hfq gene is required for stress resistance and full virulence of *Burkholderia cepacia* to the nematode *Caenorhabditis elegans*. Microbiology 156:896-908.

145. Kimelman A, Levy A, Sberro H, Kidron S, Leavitt A, Amitai G, Yoder-Himes DR, Wurtzel O, Zhu Y, Rubin EM, Sorek R. 2012. A vast collection of microbial genes that are toxic to bacteria. Genome Res 22:802-9.

146. Beisel CL, Storz G. 2010. Base pairing small RNAs and their roles in global regulatory networks. FEMS Microbiol Rev 34:866-82.

147. McHugh JP, Rodriguez-Quinones F, Abdul-Tehrani H, Svistunenko DA, Poole RK, Cooper CE, Andrews SC. 2003. Global iron-dependent gene regulation in *Escherichia coli*. A new mechanism for iron homeostasis. J Biol Chem 278:29478-86.

148. Urban JH, Vogel J. 2007. Translational control and target recognition by *Escherichia coli* small RNAs in vivo. Nucleic Acids Res 35:1018-37.

149. Jaschinski K, Babski J, Lehr M, Burmester A, Benz J, Heyer R, Dorr M, Marchfelder A, Soppa J. 2014. Generation and phenotyping of a collection of sRNA gene deletion mutants of the *haloarchaeon Haloferax volcanii*. PLoS One 9:e90763.

150. Bak G, Lee J, Suk S, Kim D, Young Lee J, Kim KS, Choi BS, Lee Y. 2015. Identification of novel sRNAs involved in biofilm formation, motility, and fimbriae formation in *Escherichia coli*. Sci Rep 5:15287.

151. Modi SR, Camacho DM, Kohanski MA, Walker GC, Collins JJ. 2011. Functional characterization of bacterial sRNAs using a network biology approach. Proc Natl Acad Sci U S A 108:15522-7.

152. Figurski DH, Helinski DR. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Nati Acad Sc, USA Vol. 76:1648-1652.

153. Hamad MA, Skeldon AM, Valvano MA. 2010. Construction of aminoglycoside-sensitive *Burkholderia cenocepacia* strains for use in studies of intracellular bacteria with the gentamicin protection assay. Appl Environ Microbiol 76:3170-6.

154. Flannagan RS, Linn T, Valvano MA. 2008. A system for the construction of targeted unmarked gene deletions in the genus *Burkholderia*. Environ Microbiol 10:1652-60.

155. Cooper VS, Carlson WA, Lipuma JJ. 2009. Susceptibility of *Caenorhabditis elegans* to *Burkholderia* infection depends on prior diet and secreted bacterial attractants. PLoS One 4:e7961.

156. Stiernagle T. 2006. Maintenance of *C. elegans*. WormBook doi:10.1895/wormbook.1.101.1:1-11.

157. Hareland WA, Crawford RL, Chapman PJ, Dagley S. 1975. Metabolic function and properties of 4-hydroxyphenylacetic acid 1-hydroxylase from *Pseudomonas acidovorans*. J Bacteriol 121:272-85.

158. Sambrook J, Russell D. 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

159. Mahenthiralingam E, Campbell ME, Henry DA, Speert DP. 1996. Epidemiology of *Burkholderia cepacia* infection in patients with cystic fibrosis: analysis by randomly amplified polymorphic DNA fingerprinting. J Clin Microbiol 34:2914-20.

160. Aubert DF, Hamad MA, Valvano MA. 2014. A markerless deletion method for genetic manipulation of *Burkholderia cenocepacia* and other multidrug-resistant gram-negative bacteria. Methods Mol Biol 1197:311-27.

161. Cohen SN, Chang AC, Hsu L. 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc Natl Acad Sci U S A 69:2110-4.

162. Craig FF, Coote JG, Parton R, Freer JH, Gilmour NJ. 1989. A plasmid which can be transferred between *Escherichia coli* and *Pasteurella haemolytica* by electroporation and conjugation. J Gen Microbiol 135:2885-90.

163. Hasselmann CaM. 2003. Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. Clinical Microbiology and Infection 9.

164. Hobbs EC, Astarita JL, Storz G. 2010. Small RNAs and small proteins involved in resistance to cell envelope stress and acid shock in *Escherichia coli*: analysis of a bar-coded mutant collection. J Bacteriol 192:59-67.

165. Papenfort K, Pfeiffer V, Lucchini S, Sonawane A, Hinton JC, Vogel J. 2008. Systematic deletion of *Salmonella* small RNA genes identifies CyaR, a conserved CRP-dependent riboregulator of OmpX synthesis. Mol Microbiol 68:890-906.

166. Mandin P, Gottesman S. 2010. Integrating anaerobic/aerobic sensing and the general stress response through the ArcZ small RNA. EMBO J 29:3094-107.

167. Lefebre MD, Valvano MA. 2002. Construction and Evaluation of Plasmid Vectors Optimized for Constitutive and Regulated Gene Expression in *Burkholderia cepacia* Complex Isolates. Applied and Environmental Microbiology 68:5956-5964.

168. Wang F, Jain P, Gulten G, Liu Z, Feng Y, Ganesula K, Motiwala AS, Ioerger TR, Alland D, Vilcheze C, Jacobs WR, Jr., Sacchettini JC. 2010. Mycobacterium tuberculosis dihydrofolate reductase is not a target relevant to the antitubercular activity of isoniazid. Antimicrob Agents Chemother 54:3776-82.

169. Konaklieva MI. 2014. Molecular Targets of beta-Lactam-Based Antimicrobials: Beyond the Usual Suspects. Antibiotics (Basel) 3:128-42.

170. Drawz SM, Bonomo RA. 2010. Three decades of beta-lactamase inhibitors. Clin Microbiol Rev 23:160-201.

171. Poole K. 2004. Resistance to beta-lactam antibiotics. Cell Mol Life Sci 61:2200-23.

172. Jorth P, McLean K, Ratjen A, Secor PR, Bautista GE, Ravishankar S, Rezayat A, Garudathri J, Harrison JJ, Harwood RA, Penewit K, Waalkes A, Singh PK, Salipante SJ. 2017. Evolved Aztreonam Resistance Is Multifactorial and Can Produce Hypervirulence in *Pseudomonas aeruginosa*. MBio 8.

173. Masuda N, Sakagawa E, Ohya S, Gotoh N, Tsujimoto H, Nishino T. 2000. Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-oprM efflux pumps in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 44:3322-7.

174. Braz VS, Furlan JP, Fernandes AF, Stehling EG. 2016. Mutations in NalC induce MexAB-OprM overexpression resulting in high level of aztreonam resistance in environmental isolates of *Pseudomonas aeruginosa*. FEMS Microbiol Lett 363.

175. Podnecky NL, Rhodes KA, Schweizer HP. 2015. E ffl ux pump-mediated drug resistance in *Burkholderia*. Front Microbiol 6:305.

176. Guglierame P, Pasca MR, De Rossi E, Buroni S, Arrigo P, Manina G, Riccardi G. 2006. Efflux pump genes of the resistance-nodulation-division family in *Burkholderia cenocepacia* genome. BMC Microbiol 6:66.

177. LiPuma JJ. 1998. *Burkholderia cepacia* epidemiology and pathogenesis: implications for infection control. Curr Opin Pulm Med 4:337-41.

178. Govan JR, Brown PH, Maddison J, Doherty CJ, Nelson JW, Dodd M, Greening AP, Webb AK. 1993. Evidence for transmission of *Pseudomonas cepacia* by social contact in cystic fibrosis. Lancet 342:15-9.

179. Wong YC, Abd El Ghany M, Naeem R, Lee KW, Tan YC, Pain A, Nathan S. 2016. Candidate Essential Genes in *Burkholderia cenocepacia* J2315 Identified by Genome-Wide TraDIS. Front Microbiol 7:1288.

180. Storz G, Vogel J, Wassarman KM. 2011. Regulation by small RNAs in bacteria: expanding frontiers. Mol Cell 43:880-91.

181. Caldelari I, Chao Y, Romby P, Vogel J. 2013. RNA-mediated regulation in pathogenic bacteria. Cold Spring Harb Perspect Med 3:a010298.

182. Cardona ST, Valvano MA. 2005. An expression vector containing a rhamnose-inducible promoter provides tightly regulated gene expression in *Burkholderia cenocepacia*. Plasmid 54:219-28.

183. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol 215:403-10.

184. Daub J, Eberhardt RY, Tate JG, Burge SW. 2015. Rfam: annotating families of non-coding RNA sequences. Methods Mol Biol 1269:349-63.

185. Zuker M. 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 31:3406-15.

186. Wright PR, Richter AS, Papenfort K, Mann M, Vogel J, Hess WR, Backofen R, Georg J. 2013. Comparative genomics boosts target prediction for bacterial small RNAs. Proc Natl Acad Sci U S A 110:E3487-96.

187. Van Acker H, Sass A, Bazzini S, De Roy K, Udine C, Messiaen T, Riccardi G, Boon N, Nelis HJ, Mahenthiralingam E, Coenye T. 2013. Biofilm-grown *Burkholderia cepacia* complex cells survive antibiotic treatment by avoiding production of reactive oxygen species. PLoS One 8:e58943.

188. Peeters E, Nelis HJ, Coenye T. 2008. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. J Microbiol Methods 72:157-65.

189. Van Acker H, De Canck E, Van Nieuwerburgh F, Sass A, Deforce D, Nelis HJ, Coenye T. 2014. The BCESM genomic region contains a regulator involved in quorum sensing and persistence in *Burkholderia cenocepacia* J2315. Future Microbiol 9:845-60.

190. Magoc T, Wood D, Salzberg SL. 2013. EDGE-pro: Estimated Degree of Gene Expression in Prokaryotic Genomes. Evol Bioinform Online 9:127-36.

191. Clote P, Ferre F, Kranakis E, Krizanc D. 2005. Structural RNA has lower folding energy than random RNA of the same dinucleotide frequency. RNA 11:578-91.

192. Kwon YK, Higgins MB, Rabinowitz JD. 2010. Antifolate-induced depletion of intracellular glycine and purines inhibits thymineless death in *E. coli*. ACS Chem Biol 5:787-95.

193. Baekkedal C, Haugen P. 2015. The Spot 42 RNA: A regulatory small RNA with roles in the central metabolism. RNA Biol 12:1071-7.

194. Mars RA, Nicolas P, Ciccolini M, Reilman E, Reder A, Schaffer M, Mader U, Volker U, van Dijl JM, Denham EL. 2015. Small regulatory RNA-induced growth rate heterogeneity of *Bacillus subtilis*. PLoS Genet 11:e1005046.

195. Rice PW, Dahlberg JE. 1982. A gene between polA and glnA retards growth of *Escherichia coli* when present in multiple copies: physiological effects of the gene for spot 42 RNA. J Bacteriol 152:1196-210.

196. McKenney D, Allison DG. 1997. Influence of growth rate and nutrient limitation on susceptibility of *Burkholderia cepacia* to ciprofloxacin and tobramycin. J Antimicrob Chemother 40:415-7.

197. Evans DJ, Brown MR, Allison DG, Gilbert P. 1990. Susceptibility of bacterial biofilms to tobramycin: role of specific growth rate and phase in the division cycle. J Antimicrob Chemother 25:585-91.

198. Martins D, Nguyen D. 2017. Stimulating Central Carbon Metabolism to Re-sensitize *Pseudomonas aeruginosa* to Aminoglycosides. Cell Chem Biol 24:122-124.

199. Colabroy KL, Begley TP. 2005. Tryptophan catabolism: identification and characterization of a new degradative pathway. J Bacteriol 187:7866-9.

200. Luengo JM, Garcia JL, Olivera ER. 2001. The phenylacetyl-CoA catabolon: a complex catabolic unit with broad biotechnological applications. Mol Microbiol 39:1434-42.

201. Teufel R, Mascaraque V, Ismail W, Voss M, Perera J, Eisenreich W, Haehnel W, Fuchs G. 2010. Bacterial phenylalanine and phenylacetate catabolic pathway revealed. Proc Natl Acad Sci U S A 107:14390-5.

202. Hamlin JN, Bloodworth RA, Cardona ST. 2009. Regulation of phenylacetic acid degradation genes of *Burkholderia cenocepacia* K56-2. BMC Microbiol 9:222.

203. Pribytkova T, Lightly TJ, Kumar B, Bernier SP, Sorensen JL, Surette MG, Cardona ST. 2014. The attenuated virulence of a *Burkholderia cenocepacia* paaABCDE mutant is due to inhibition of quorum sensing by release of phenylacetic acid. Mol Microbiol 94:522-36.

204. Tomich M, Mohr CD. 2003. Adherence and autoaggregation phenotypes of a *Burkholderia cenocepacia* cable pilus mutant. FEMS Microbiol Lett 228:287-97.

205. Sass AM, Schmerk C, Agnoli K, Norville PJ, Eberl L, Valvano MA, Mahenthiralingam E. 2013. The unexpected discovery of a novel low-oxygen-activated locus for the anoxic persistence of *Burkholderia cenocepacia*. ISME J 7:1568-81.

206. Griffiths-Jones S, Bateman A, Marshall M, Khanna A, Eddy SR. 2003. Rfam: an RNA family database. Nucleic Acids Res 31:439-41.

207. Sass A, Kiekens S, Coenye T. 2017. Identification of small RNAs abundant in *Burkholderia cenocepacia* biofilms reveal putative regulators with a role in carbon and iron metabolism.

208. Kiekens S, Sass A., Van Nieuwerburgh F, Deforce D, Coenye T. 2017. The small RNA ncS35 regulates growth in *Burkholderia cenocepacia* J2315.

209. Henriques MX, Catalao MJ, Figueiredo J, Gomes JP, Filipe SR. 2013. Construction of improved tools for protein localization studies in *Streptococcus pneumoniae*. PLoS One 8:e55049.

210. Chen Z, Wang Y, Li Y, Li Y, Fu N, Ye J, Zhang H. 2012. Esre: a novel essential non-coding RNA in *Escherichia coli*. FEBS Lett 586:1195-200.

211. Moll I, Afonyushkin T, Vytvytska O, Kaberdin VR, Blasi U. 2003. Coincident Hfq binding and RNase E cleavage sites on mRNA and small regulatory RNAs. RNA 9:1308-14.

212. Sousa SA, Morad M, Feliciano JR, Pita T, Nady S, El-Hennamy RE, Abdel-Rahman M, Cavaco J, Pereira L, Barreto C, Leitao JH. 2016. The *Burkholderia cenocepacia* OmpA-like protein BCAL2958: identification, characterization, and detection of anti-BCAL2958 antibodies in serum from B. cepacia complex-infected Cystic Fibrosis patients. AMB Express 6:41.

213. Godlewska R, Wisniewska K, Pietras Z, Jagusztyn-Krynicka EK. 2009. Peptidoglycan-associated lipoprotein (Pal) of Gram-negative bacteria: function, structure, role in pathogenesis and potential application in immunoprophylaxis. FEMS Microbiol Lett 298:1-11.

214. O'Grady EP, Sokol PA. 2011. *Burkholderia cenocepacia* differential gene expression during host-pathogen interactions and adaptation to the host environment. Front Cell Infect Microbiol 1:15.

215. Paterson GK, Northen H, Cone DB, Willers C, Peters SE, Maskell DJ. 2009. Deletion of tolA in *Salmonella Typhimurium* generates an attenuated strain with vaccine potential. Microbiology 155:220-8.

216. Govan JR, Brown AR, Jones AM. 2007. Evolving epidemiology of Pseudomonas aeruginosa and the *Burkholderia cepacia* complex in cystic fibrosis lung infection. Future Microbiol 2:153-64.

217. Avgeri SG, Matthaiou DK, Dimopoulos G, Grammatikos AP, Falagas ME. 2009. Therapeutic options for *Burkholderia cepacia* infections beyond co-trimoxazole: a systematic review of the clinical evidence. Int J Antimicrob Agents 33:394-404.

218. Sarovich DS, Price EP, Limmathurotsakul D, Cook JM, Von Schulze AT, Wolken SR, Keim P, Peacock SJ, Pearson T. 2012. Development of ceftazidime resistance in an acute *Burkholderia pseudomallei* infection. Infect Drug Resist 5:129-32.

219. Speert DP. 2002. Advances in *Burkholderia cepacia* complex. Paediatr Respir Rev 3:230-5.

220. Horsley A, Webb K, Bright-Thomas R, Govan J, Jones A. 2011. Can early *Burkholderia cepacia* complex infection in cystic fibrosis be eradicated with antibiotic therapy? Front Cell Infect Microbiol 1:18.

221. Burns JL, Lien DM, Hedin LA. 1989. Isolation and characterization of dihydrofolate reductase from trimethoprim-susceptible and trimethoprim-resistant *Pseudomonas cepacia*. Antimicrob Agents Chemother 33:1247-51.

222. Zhou J, Chen Y, Tabibi S, Alba L, Garber E, Saiman L. 2007. Antimicrobial susceptibility and synergy studies of *Burkholderia cepacia* complex isolated from patients with cystic fibrosis. Antimicrob Agents Chemother 51:1085-8.

223. Sokol PA, Malott RJ, Riedel K, Eberl L. 2007. Communication systems in the genus *Burkholderia*: global regulators and targets for novel antipathogenic drugs. Future Microbiol 2:555-63.

224. Gould SE. 2014. Shooting the messenger: small RNA as a target for antibiotics.

225. Dersch P, Khan MA, Muhlen S, Gorke B. 2017. Roles of Regulatory RNAs for Antibiotic Resistance in Bacteria and Their Potential Value as Novel Drug Targets. Front Microbiol 8:803.

226. Lalaouna D, Eyraud A, Chabelskaya S, Felden B, Masse E. 2014. Regulatory RNAs involved in bacterial antibiotic resistance. PLoS Pathog 10:e1004299.

227. Kim T, Bak G, Lee J, Kim KS. 2015. Systematic analysis of the role of bacterial Hfq-interacting sRNAs in the response to antibiotics. J Antimicrob Chemother 70:1659-68.

228. Eyraud A, Tattevin P, Chabelskaya S, Felden B. 2014. A small RNA controls a protein regulator involved in antibiotic resistance in *Staphylococcus aureus*. Nucleic Acids Res 42:4892-905.

229. Sinel C, Augagneur Y, Sassi M, Bronsard J, Cacaci M, Guérin F, Sanguinetti M, Meignen P, Cattoir V, Felden B. 2017. Small RNAs in vancomycin-resistant *Enterococcus faecium* involved in daptomycin response and resistance. Scientific Reports 7:11067.

230. Gutierrez A, Laureti L, Crussard S, Abida H, Rodriguez-Rojas A, Blazquez J, Baharoglu Z, Mazel D, Darfeuille F, Vogel J, Matic I. 2013. beta-Lactam antibiotics promote bacterial mutagenesis via an RpoS-mediated reduction in replication fidelity. Nat Commun 4:1610.

231. Stubben CJ, Micheva-Viteva SN, Shou Y, Buddenborg SK, Dunbar JM, Hong-Geller E. 2014. Differential expression of small RNAs from *Burkholderia thailandensis* in response to varying environmental and stress conditions. BMC Genomics 15:385.

232. Ramos CG, Grilo AM, da Costa PJ, Feliciano JR, Leitao JH. 2013. MtvR is a global small noncoding regulatory RNA in *Burkholderia cenocepacia*. J Bacteriol 195:3514-23.

233. Wilson RC, Doudna JA. 2013. Molecular mechanisms of RNA interference. Annu Rev Biophys 42:217-39.

234. Ipsaro JJ, Joshua-Tor L. 2015. From guide to target: molecular insights into eukaryotic RNA-interference machinery. Nat Struct Mol Biol 22:20-8.

235. Knip M, Constantin ME, Thordal-Christensen H. 2014. Trans-kingdom cross-talk: small RNAs on the move. PLoS Genet 10:e1004602.

236. Kuo CY, Sinha S, Jazayeri JA, Pouton CW. 2009. A stably engineered, suicidal strain of *listeria monocytogenes* delivers protein and/or DNA to fully differentiated intestinal epithelial monolayers. Mol Pharm 6:1052-61.

237. Kong W, Brovold M, Koeneman BA, Clark-Curtiss J, Curtiss R, 3rd. 2012. Turning self-destructing *Salmonella* into a universal DNA vaccine delivery platform. Proc Natl Acad Sci U S A 109:19414-9.

238. Llosa M, Schroder G, Dehio C. 2012. New perspectives into bacterial DNA transfer to human cells. Trends Microbiol 20:355-9.

239. Fernandez-Gonzalez E, Backert S. 2014. DNA transfer in the gastric pathogen *Helicobacter pylori*. J Gastroenterol 49:594-604.

240. Zhang R, LiPuma JJ, Gonzalez CF. 2009. Two type IV secretion systems with different functions in *Burkholderia cenocepacia* K56-2. Microbiology 155:4005-13.

241. Ramos CG, Grilo AM, Sousa SA, Feliciano JR, da Costa PJ, Leitao JH. 2014. Regulation of Hfq mRNA and protein levels in *Escherichia coli* and *Pseudomonas aeruginosa* by the *Burkholderia cenocepacia* MtvR sRNA. PLoS One 9:e98813.

242. Harris JF, Micheva-Viteva S, Li N, Hong-Geller E. 2013. Small RNA-mediated regulation of host-pathogen interactions. Virulence 4:785-95.

243. Fazli M, Harrison JJ, Gambino M, Givskov M, Tolker-Nielsen T. 2015. In-Frame and Unmarked Gene Deletions in *Burkholderia cenocepacia* via an Allelic Exchange System Compatible with Gateway Technology. Appl Environ Microbiol 81:3623-30.

244. Udine C, Brackman G, Bazzini S, Buroni S, Van Acker H, Pasca MR, Riccardi G, Coenye T. 2013. Phenotypic and genotypic characterisation of *Burkholderia cenocepacia* J2315 mutants affected in homoserine lactone and diffusible signal factor-based quorum sensing systems suggests interplay between both types of systems. PLoS One 8:e55112.

245. Mann B, van Opijnen T, Wang J, Obert C, Wang YD, Carter R, McGoldrick DJ, Ridout G, Camilli A, Tuomanen EI, Rosch JW. 2012. Control of virulence by small RNAs in *Streptococcus pneumoniae*. PLoS Pathog 8:e1002788.

246. McArthur SD, Pulvermacher SC, Stauffer GV. 2006. The Yersinia pestis gcvB gene encodes two small regulatory RNA molecules. BMC Microbiol 6:52.

247. Papenfort K, Said N, Welsink T, Lucchini S, Hinton JC, Vogel J. 2009. Specific and pleiotropic patterns of mRNA regulation by ArcZ, a conserved, Hfq-dependent small RNA. Mol Microbiol 74:139-58.

248. Howden BP, Beaume M, Harrison PF, Hernandez D, Schrenzel J, Seemann T, Francois P, Stinear TP. 2013. Analysis of the small RNA transcriptional response in multidrug-resistant *Staphylococcus aureus* after antimicrobial exposure. Antimicrob Agents Chemother 57:3864-74.

249. Faner MA, Feig AL. 2013. Identifying and characterizing Hfq-RNA interactions. Methods 63:144-59.

250. Velappan N, Sblattero D, Chasteen L, Pavlik P, Bradbury AR. 2007. Plasmid incompatibility: more compatible than previously thought? Protein Eng Des Sel 20:309-13.

251. Brzoska AJ, Firth N. 2013. Two-plasmid vector system for independently controlled expression of green and red fluorescent fusion proteins in *Staphylococcus aureus*. Appl Environ Microbiol 79:3133-6.

252. Hanahan D. 1983. Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol 166:557-80.

253. Simons R, Grunberg-Manago M. 1998. RNA structure and function. New York.

254. Hong W, Zeng J, Xie J. 2014. Antibiotic drugs targeting bacterial RNAs. Acta Pharm Sin B 4:258-65.

255. Chukwudi CU. 2016. rRNA Binding Sites and the Molecular Mechanism of Action of the Tetracyclines. Antimicrob Agents Chemother 60:4433-41.

256. Matsui M, Corey DR. 2017. Non-coding RNAs as drug targets. Nat Rev Drug Discov 16:167-179.

257. Mulhbacher J, Brouillette E, Allard M, Fortier LC, Malouin F, Lafontaine DA. 2010. Novel riboswitch ligand analogs as selective inhibitors of guanine-related metabolic pathways. PLoS Pathog 6:e1000865.

258. Good L, Stach JE. 2011. Synthetic RNA silencing in bacteria - antimicrobial discovery and resistance breaking. Front Microbiol 2:185.

259. Jakobsen TH, van Gennip M, Phipps RK, Shanmugham MS, Christensen LD, Alhede M, Skindersoe ME, Rasmussen TB, Friedrich K, Uthe F, Jensen PO, Moser C, Nielsen KF, Eberl L, Larsen TO, Tanner D, Hoiby N, Bjarnsholt T, Givskov M. 2012. Ajoene, a sulfur-rich molecule from garlic, inhibits genes controlled by quorum sensing. Antimicrob Agents Chemother 56:2314-25.

260. Jakobsen TH, Warming AN, Vejborg RM, Moscoso JA, Stegger M, Lorenzen F, Rybtke M, Andersen JB, Petersen R, Andersen PS, Nielsen TE, Tolker-Nielsen T, Filloux A, Ingmer H, Givskov M. 2017. A broad range quorum sensing inhibitor working through sRNA inhibition. Scientific Reports 7:9857.

261. Geller BL. 2005. Antibacterial antisense. Curr Opin Mol Ther 7:109-13.

262. Mellbye BL, Puckett SE, Tilley LD, Iversen PL, Geller BL. 2009. Variations in amino acid composition of antisense peptide-phosphorodiamidate morpholino oligomer affect potency against *Escherichia coli* in vitro and in vivo. Antimicrob Agents Chemother 53:525-30.

263. Tilley LD, Mellbye BL, Puckett SE, Iversen PL, Geller BL. 2007. Antisense peptide-phosphorodiamidate morpholino oligomer conjugate: dose-response in mice infected with *Escherichia coli*. J Antimicrob Chemother 59:66-73.

264. Mitev GM, Mellbye BL, Iversen PL, Geller BL. 2009. Inhibition of intracellular growth of *Salmonella enterica* serovar Typhimurium in tissue culture by antisense peptide-phosphorodiamidate morpholino oligomer. Antimicrob Agents Chemother 53:3700-4.

265. Greenberg DE, Marshall-Batty KR, Brinster LR, Zarember KA, Shaw PA, Mellbye BL, Iversen PL, Holland SM, Geller BL. 2010. Antisense phosphorodiamidate morpholino oligomers targeted to an essential gene inhibit *Burkholderia cepacia* complex. J Infect Dis 201:1822-30.

266. Allam US, Krishna MG, Lahiri A, Joy O, Chakravortty D. 2011. *Salmonella enterica* serovar Typhimurium lacking hfq gene confers protective immunity against murine typhoid. PLoS One 6:e16667.

267. Keefer AB, Asare EK, Pomerantsev AP, Moayeri M, Martens C, Porcella SF, Gottesman S, Leppla SH, Vrentas CE. 2017. In vivo characterization of an Hfq protein encoded by the *Bacillus anthracis* virulence plasmid pXO1. BMC Microbiol 17:63.

268. Salgado-Garrido J, Bragado-Nilsson E, Kandels-Lewis S, Seraphin B. 1999. Sm and Sm-like proteins assemble in two related complexes of deep evolutionary origin. EMBO J 18:3451-62.

Curriculum Vitae

Sanne Kiekens

Overzet 12, 9000 Ghent, Belgium

+32 494 64 44 14

sanne.kiekens@gmail.com

https://www.linkedin.com/in/sanne-kiekens-984a02111/

22 September 1990, Beveren-Waas, Belgium

Education

2013 - 2017: PhD in Pharmaceutical Sciences
at Laboratory of Pharmaceutical Microbiology, Ghent University

2008 - 2013: Master in Pharmaceutical Sciences, Ghent University
Major in Drug development
Thesis "Ethyl glucuronide analysis in hair and urine with LC-MS/MS"
At Institute of Forensic Medicine, University of Bern, Switzerland

2002 - 2008: Sciences-Mathematics, Berkenboom Humaniora, Sint-Niklaas

Publications

Sass A., **Kiekens S.**, Coenye T. 2017. Identification of small RNAs abundant in *Burkholderia cenocepacia* biofilms reveal putative regulators with a role in carbon and iron metabolism. Nature Scientific Reports 7:15665.

Kiekens S., Sass A., Van Nieuwerburgh F., Deforce D., Coenye T. 2017. The sRNA ncS35 regulates growth in *Burkholderia cenocepacia* J2315. mSphere. Submitted.

Conferences

2017	IUAP meeting, Ghent, Belgium	Oral
2017	Microbiology Society Annual Conference, Edinburgh, UK	Oral
2016	Regulating with RNA in Bacteria and Archaea conference, Cancun, Mexico	Poster
2016	Knowledge for growth, Ghent, Belgium	Poster
2016	IUAP meeting, Louvain-La-Neuve, Belgium	Oral
2015	ESBG Eurobiofilm 2015, Brno, Czech Republic	Poster
2015	Knowledge for growth, Ghent, Belgium	Poster
2015	IUAP meeting, Namur, Belgium	Oral
2014	Knowledge for growth, Ghent, Belgium	Poster
2014	IUAP meeting, Leuven, Belgium	

Trainings

Doctoral schools UGent	Advanced Academic English, writing skills Grow your personal leadership – Grow your future career Effective image editing Communication skills Leadership training Summer School 'Let's Talk Science' Summer School ‘Molecular and physiological regulation of medical and environmental biofilms’