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The intracellular behaviour of Burkholderia cenocepacia in murine macrophages

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Microbiology and Immunology

THE INTRACELLULAR BEHAVIOUR OF *BURKHOLDERIA CENOCEPACIA* IN MURINE MACROPHAGES

(Spine title: Intramacrophage behaviour of *B. cenocepacia*)

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by

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Abstract

Burkholderia cenocepacia is an opportunistic pathogen causing life-threatening infections in cystic fibrosis and other immunocompromised patients. The bacterium survives within macrophages by interfering with typical endocytic trafficking, resulting in delayed maturation of a *B. cenocepacia*-containing phagosome. We hypothesize that *B. cenocepacia* alters gene expression after internalization by macrophages, inducing genes involved in intracellular survival and host adaptation. Furthermore, we hypothesize that specialized bacterial secretion systems are involved in the interactions between intracellular bacteria and macrophages. In this work, we characterize later-stage infection of macrophages by *B. cenocepacia*, showing replication within an acidified endosomal compartment suggestive of a phagolysosome. We examine differential gene expression by intracellular *B. cenocepacia* using selective capture of transcribed sequences (SCOTS) with both competitive enrichment and microarray analysis. We identified 766 genes differentially regulated in intracellular bacteria, of which 329 were induced and 437 repressed. Affected genes are involved in all aspects of cellular life, including information storage and processing, cellular processes and signalling, and metabolism; in general, intracellular gene expression demonstrates a pattern of environmental sensing, bacterial response, and metabolic adaptation to the phagosomal environment. Deletion of various SCOTS-identified genes affects *B. cenocepacia* entry into macrophages and intracellular replication, as well as host-directed cytotoxicity and spread to neighbouring cells. Expression of secretion system genes is

differentially-regulated by intracellular *B. cenocepacia*. Although none of the five major secretion systems are essential for growth in culture, we show that bacterial secretion systems are involved in macrophage entry, intracellular replication, and host-directed cytotoxicity. Type IV secretion systems play a role in early interactions with macrophages, while type II and VI secretion systems contribute to post-internalization intracellular replication and host-directed cytotoxicity. As a whole, secretion systems appear to increase pathogenicity in macrophages while limiting the spread of *B. cenocepacia* infection.

Together, these studies advance our understanding of the intracellular behaviour of *B. cenocepacia* in macrophages. Further investigation into the remaining SCOTS-identified genes, as well as putative secreted effectors, will provide a better understanding of the adaptive responses of intracellular *B. cenocepacia*, leading to life in a phagosomal niche and host cell cytotoxicity.

Keywords

Burkholderia cenocepacia, macrophage, intracellular gene expression, microarray, SCOTS, secretion systems

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List of Abbreviations

2,4,5-T	2,4,5-trichlorophenoxyacetic acid
Ab	antibody
ABC	ATP-binding cassette
ABM	actin-based motility
ADP	adenosine diphosphate
AHL	*N*-acyl-homoserine-lactone
Ap	ampicillin
Ara4N	4-amino-4-*deoxy*-L-arabinose
AT	autotransporter
ATCC	American Type Culture Collection
Bcc	*Burkholderia cepacia* complex
BcCV	*Burkholderia cenocepacia*-containing vacuole
BCESM	*B. cepacia* epidemic strain marker
BCRRC	*B. cepacia* complex Research and Referral Repository for Canadian Clinics
BCV	bacteria-containing vacuole
BDSF	*Burkholderia* diffusible signal factor cis-2-dodecenoic acid
BLAST	Basic Local Alignment Search Tool
bp	base pair
C	carboxy
cAMP	cyclic adenosine 5'-monophosphate
cbl	cable
CC	coincidence cloning
cci	*B. cenocepacia* island
CE	competitive enrichment
CF	cystic fibrosis
CFU	colony forming unit
CFTR	cystic fibrosis transmembrane conductance regulator
CGD	chronic granulomatous disease
CK13	cytokeratin 13
COG	clusters of orthologous genes
DAPI	4',6-diamidino-2-phenylindole
DECAL	differential expression using customized amplification libraries
DIC	differential interference contrast
DIG	digoxigenin
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacteic acid
EEA1	early endosome antigen 1
eGFP	enhanced green fluorescent protein
EPS	exopolysaccharide
ER	endoplasmic reticulum

FBS	fetal bovine serum
FU	fluorescence units
GAP	GTPase-activating protein
GDI	guanine dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine exchange factor
GFP	green fluorescent protein
Gm	gentamicin
GTP	guanosine triphosphate
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
HHQ	2-heptyl-4(1*H*)-quinolone
I	intracellular
IAA	indole-3-acetic acid
ICU	intensive care unit
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IM	inner membrane
Kbp	kilobase pairs
kDa	kilodalton
Kan	kanamycin
kbp	kilo base pair
LAMP	lysosomal-associated membrane protein
LB	Luria-Bertani
LBPA	lysobisphosphatidic acid
LCV	*Legionella*-containing vacuole
LPG	lipophosphoglycan
LPS	lipopolysaccharide
Lys-PG	lysylphosphatidylglycerol
M6PR	mannose-6-phosphate receptor
Mbp	mega base pair
mg/mL	milligram per milliliter
MLST	multilocus sequence typing
MMST	MOPS – minimal salts – tryptose medium
MOI	multiplicity of infection
MOPS	3-(*N*-morpholino) propanesulfonic acid
mRFP1	monomeric red fluorescent protein 1
N	amino
NADPH	nicotinamide adenine dinucleotide phosphate
NB	nutrient broth
NBD	nucleotide binding domain
NET	neutrophil extracellular trap
NLR	NOD-like receptor
NME	non-macrophage-exposed
NOD	nucleotide oligomerization domain
nt	nucleotide
OD	optical density

OM	outer membrane
OMV	outer membrane vesicles
ori	origin of replication
PA	phenylacetic acid
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PES	polyethersulfone
PG	phosphatidylglycerol
p.i.	post-infection
PI(3)P	phosphatidylinositol-3-phosphate
PLA2	phospholipase A2
PLC	phospholipase C
PRR	pattern recognition receptor
ptw	plant tissue water soaking
q	quantitative
QS	quorum sensing
QSI	quorum-sensing inhibitors
RILP	Rab-interacting lysosomal protein
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SA	salicylic acid
SCOTS	selective capture of transcribed sequences
scs	selectively captured sequences
SCV	*Salmonella*-containing vacuole
SDS	sodium dodecyl (lauryl) sulfate
Sec	general secretory
SP	signal peptide
ST	sequence type
STM	signature-tagged mutagenesis
T1SS	type I secretion system
T2SS	type II secretion system
T3SS	type III secretion system
T4CP	type IV coupling protein
T4SS	type IV secretion system
T5SS	type V secretion system
T6SS	type VI secretion system
TAE	Tris-acetate-EDTA
Tat	twin-arginine translocation
TCS	two-component regulatory system
Tet	tetracycline
TEM	transmission electron microscopy
TLR	Toll-like receptor
TMR	tetramethylrhodamine

Tp	trimethoprim
TPS	two-partner secretion
TSB	trypticase soy broth
μg/mL	microgram per milliliter
v/v	volume per volume
w/v	weight per volume

Chapter 1 – Introduction

1.1 Burkholderia cenocepacia and the Burkholderia cepacia complex

1.1.1 The *Burkholderia cepacia* complex

The *Burkholderia cepacia* complex, or Bcc, is a group of at least 17 closely related organisms which are phenotypically similar but genetically distinct (493, 495). Originally identified in the 1940's as the causative agent of soft onion rot, or "sour skin", discoverer Walter Burkholder designated the organism *Pseudomonas cepacia*, from the Latin *cepia* for onion (63). Later studies showed *P. multivorans*, a metabolically diverse environmental isolate, and *P. kingii*, isolated from both clinical specimens and disinfectants, to be synonymous strains (442). In 1992, the genus *Burkholderia* was created, and seven *Pseudomonas* spp., including *P. cepacia* were transferred into the new genus (529). Two of the original seven species were later reclassified as *Ralstonia* spp. (530), and over sixty new species have since been described in the *Burkholderia* genus (http://www.bacterio.cict.fr). Marked heterogeneity among species designated as *B. cepacia* led to division into genomovars, genetically distinct organisms awaiting official species designation based on phenotypic distinction (493). Eventually, as the sensitivity and specificity of tests improved, each genomovar received a binomial species designation. Biochemical differentiation among members of the Bcc is difficult, thus genetic techniques are most often used for species determination (287). Sequence polymorphisms in the *recA* gene, encoding a protein essential for DNA recombination and repair, are discriminatory for most species (288), but members of the Bcc are best distinguished based on multilocus sequence typing (MLST). This strategy utilizes sequence polymorphisms in seven housekeeping genes located on the first two

chromosomes (*atpD, gltB, gyrB, recA, lepA, phaC,* and *trpB*); amplification of 300-400 base pair (bp) fragments of each gene creates an allelic profile which can be used to assign a clonal sequence type (ST) (31). Currently, the complex consists of *B. cepacia*, *B. multivorans, B. cenocepacia, B. stabilis, B. vietnamiensis, B. dolosa, B. ambifaria, B. anthina, B. pyrrocinia, B. ubonensis, B. latens, B. diffusa, B. arboris, B. seminalis, B. metallica, B. contaminans,* and *B. lata* (495). Isolates of all 17 species have been found in a clinical setting, with all but *B. ubonensis* isolated from cystic fibrosis patients; additionally, with the exception of *B. latens* and *B. metallica*, all species have also been isolated from the natural environment (505). Pre-classification literature from 1950 through mid-1990 refers to only *P. cepacia* or *B. cepacia*; current re-analysis has demonstrated that Burkholder's original onion pathogens were true *B. cepacia* species, *P. kingii* clinical isolates were *B. cenocepacia,* and *P. multivorans* environmental isolates were mainly *B. lata* and *B. cepacia* (491). Due to the complexity of taxonomic classification within the *B. cepacia* complex, many studies, particularly environmental surveys, in which Bcc bacteria are a serendipitous identification choose to identify the bacteria only as *B. cepacia*-like. Thus, current species designations will be utilized where possible, but when referring to literature where members of the Bcc were not distinguished, designations "*P. cepacia*", or "*B. cepacia*" will be used.

1.1.2 *Burkholderia cenocepacia*

Burkholderia cenocepacia, literally meaning "new *cepacia*", is an aerobic, Gram negative, non-spore-forming, motile, bacillus-shaped, β-proteobacterium (178, 492). The bacterium ranges in size from 1.0 to 2.0 μm long and from 0.6 to 0.9 μm wide, and grows at 37^{o}C and room temperature but not at 5^{o}C; growth at 42^{o}C is strain-dependent (492). *B. cenocepacia* is metabolically diverse; many functions are strain-specific (492), as are the production of pigment (231, 492), and lipopolysaccharide O antigen (356). The species *B. cenocepacia* may be further subdivided into four phylogenetic clusters based on *recA* gene sequence,

IIIA to IIID, with most clinically relevant isolates represented in the first two groups (134). *B. cenocepacia* is an opportunistic pathogen of cystic fibrosis (CF) patients (380) - the type strain for the species, LMG 16656, or J2315, was isolated from the sputum of a cystic fibrosis patient in Edinburgh in 1989 (492) - but *B. cenocepacia* infections are also found in non-CF patients (380). The bacterium is also frequently isolated from environmental reservoirs; in some instances, the same strain can be found in both clinical and environmental niches (30).

1.1.3 *B. cenocepacia* in the environment

B. cenocepacia and other members of the Bcc have been isolated from many different environmental sources, ranging from urban (327), woodland (495), and agricultural (268) soils, to European rivers (503), Chinese lakes (146), and the sediments of American streams (353), to Mexican radishes (30), Indian sugar cane (496), Brazilian flowers (495), and raw milk (346). *B. cenocepacia* engages in both pathogenic and beneficial interactions with plants, and can act as an endosymbiont for both fungi and insects. Though non-Bcc *Burkholderia* spp. are able to cause serious disease in higher mammals - most notably equine glanders resulting from *B. mallei* infection – there is only a single example of natural Bcc infection in animals, where *B. cenocepacia* caused an outbreak of subclinical mastitis in sheep (49).

1.1.3.1 Phytopathogens

"*B. cepacia*" was first discovered as a phytopathogen in onion crops (63). Though recent classification of Burkholder's original strains has identified true *B. cepacia* species, *B. cenocepacia* has also been isolated directly from the lesions of field-grown onion bulbs which naturally developed bacterial rot (452). In a field study in Michigan, *B. cenocepacia* was isolated from both the bulk soil and

the onion rhizosphere, with presence in the rhizosphere increasing with onion root development; 90% of the 480 *B. cenocepacia* isolates were highly virulent when assayed for pathogenicity on detached onion bulb scales (213).

In a natural setting, bacteria present in the surrounding soil and rhizosphere can be transferred to plant leaves and bulbs by splashing water from irrigation or rain (213). Pathogenicity in onion tissue requires an existent tissue wound for bacterial ingress, and moisture for effective spreading (228). Plant damage occurs by two separate mechanisms. Plant tissue watersoaking (ptw) is an accumulation of fluids in the intracellular spaces of plant tissues caused by a loss of cell membrane integrity (141); accumulation of fluids contributes to the spread of the bacteria through the tissues. Early studies in "*P. cepacia*" showed production of a pectic enzyme called polygalacturonase (486). Polygalacturonase is a secreted enzyme that hydrolyzes pectin, one of the principal macromolecular components in primary plant cell walls (313, 314). Bacterial infection lowered the pH of onion tissue from 5.5 to 4.0, conducive to polygalacturonase activity, and resulted in tissue maceration (486). "*B. cepacia*" has also been shown to cause disease in tomatoes, mushrooms, and orchids (399); *B. cenocepacia,* specifically, is the causal agent of banana finger-tip rot (262).

1.1.3.2 Beneficial interactions with plants

Although *B. cenocepacia* is pathogenic in several types of plant, many bacteria-plant interactions exist which are neutral or mutually beneficial. Early research in "*B. cepacia*" showed antagonism and repression of soil-borne plant pathogens, including the fungi *Alternaria* which affects canola and ginseng (200), *Aphanomyces* in peas, alfalfa, and snap beans (57), *Sclerotinia* in sunflowers (323), and *Pythium* in cucumbers and peas (57, 200). "*B. cepacia*" can also suppress damaging fungal growth in nursery seedlings (200). Field studies showed presence of "*P. cepacia*" in the rhizosphere – soil on root surfaces – of

maize; through production of pyrrolnitrin and a second unidentified antifungal agent, "*P. cepacia*" was antagonistic to five major fungal pathogens of maize (249). Paradoxically, endosymbiotic bacteria and fungi within the rhizosphere are able to promote plant resistance to stress and disease; "*B. cepacia*" is an endosymbiont of several species of the fungus *Glomus*, which establishes an endophytic relationship with sorghum (20). Plant-associated Bcc bacteria also promote growth by the fixation of N_2. *B. vietnamiensis*, shown to promote growth of sugar cane, is the only currently identified diazotroph in the complex (179), though an unclassified member of the Bcc is able to induce nitrogen-fixing root nodules in the wild rose wood tree legume *Dalbergia* spp. (375).

B. cenocepacia has been identified in the rhizosphere of onions (213) and maize (113). From the rhizosphere, bacteria may enter the root tissue and establish an endophytic relationship with a plant. *B. cenocepacia* has been isolated from the stem and roots of sugar cane (324), as well as wheat and lupine shoots (29). In sugarcane, endophytic *B. cenocepacia* produces the plant growth hormone IAA, and strongly inhibits hyphal growth of *Fusarium moniliforme*, causative agent of Pokkah boeng disease (324).

1.1.3.3 Endosymbiosis with insects

Unidentified *Burkholderia* spp. have been found as endosymbiotic gut bacteria in plant-associated insects, including Japanese broad-headed bugs *Leptocorisa chinensis* and *Riptortus clavatus* (235), and the ant *Tetraponera binghami* (488); no purpose for this relationship has been experimentally established. *B. cenocepacia* participates in a tri-symbiotic relationship with the leaf-cutting ant *Atta sexdens rubropilosa* and the fungus *Leucoagaricus gongylophorus* (96). Leaf-cutting ants maintain a fungus garden, protecting and providing leaf substrates to the obligate mutualistic fungus, which in turn supplies the ant with nutrients (416). Bcc bacteria isolated from ant nests inhibit several species of entomopathogenic and general saprophytic fungi, as well as the specialized

garden parasite *Escovopsis weberi*, but not the mutualist *L. gongylophorus* (416); thus, in such a complex relationship, Bcc bacteria maintain the integrity of the fungus garden while protecting the ant from fungal infection.

1.1.3.4 Bioremediation

Bcc bacteria possess extraordinary metabolic diversity, to the extent that the bacteria can survive in simple tap water (279) and can utilize antibiotics such as penicillin as a sole carbon source (41). As a consequence of this metabolic diversity, Bcc bacteria have shown promise in bioremedial applications. 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) is a herbicide to defoliate broad-leaf plants which is persistent in the environment, poorly biodegradable, and a major component of Agent Orange; 2,4,5-T can be broken down by "*P. cepacia*" (74). Insecticide and anti-parasitic abamectin, an environmental pollutant affecting non-target soil vertebrates and aquatic systems, can be used as a carbon source by "*B. cepacia*" (12). Bcc bacteria are also able to degrade the fungicide carbendazim (223), insect repellent and plasticizer dimethyl phthalate (511), solvent trichloroethylene (158), and pesticide endosulfan (208), among others. Endophytic and rhizospheric strains of Bcc bacteria have shown degradation of phenol (510). In a biofilm with cyanobacteria, Bcc bacteria were able to degrade diesel in hydrocarbon-rich industrial wastewater (75); indeed, a strain of *B. cenocepacia* isolated from oil-contaminated soil produces a biosurfactant that enhances the solubilization of multiple pesticides (514).

1.1.3.5 Commercial potential

Bcc bacteria have the ability to degrade serious environmental pollutants, antagonize soil-borne pathogens, and promote plant growth, and as such, could be of great benefit to agriculture and industry. As a biocontrol agent, Bcc bacteria have the potential to replace, at least in part, such common chemical

biocides as captan, thiram, benomyl, and chloropicrin, all of which have toxicity or carcinogenic issues (360). Several strains of *B. ambifaria* and a strain of *B. cenocepacia* were registered as biopesticides in the mid-1990s and used commercially until withdrawn from use a decade later (79). Field trials with *B. vietnamiensis* efficiently removed trichloroethylene, one of the most abundant organic aquifer contaminants, from groundwater (244), and a US patent was obtained (79). Industrial applications continue to be explored, with development of a Bcc-containing microalgae-bacteria consortium to degrade crude oil (474), and a bioreactor utilizing Bcc to remove petroleum hydrocarbons from refinery wastewater (75). An engineered endophytic strain of *B. cepacia* was used to improve lupine phytoremediation of soil co-contaminated with the organic pollutant trichloroethylene and toxic nickel (520). However, commercial applications must be weighed against capacity for opportunistic infection, as identical strains have been isolated from both clinical and environmental sources (30); until Bcc infections can be successfully addressed, the biotechnological potential present among members of the complex cannot ethically be developed.

1.1.4 *B. cenocepacia* as an industrial contaminant

Outside the natural environmental, Bcc bacteria are also able to survive in a wide variety of man-made products. "*P. cepacia*" contamination was found in serum albumin (461), the anaesthetic fentanyl (53, 439), and commercial iodine and benzalkonium chloride antiseptics (18, 161); in one study, *"P. cepacia"* survived for 14 years in an inorganic salt solution containing benzalkonium chloride as an antimicrobial (173). "*B. cepacia*" contamination of oxymetazoline nasal spray resulted in an infection outbreak in the United States and recall of the product (132). Bcc bacterial contamination has also been identified in prefabricated moist washcloths (310), ultrasound echocardiographic gel (304), multiple catheter disinfectants (183, 190), the antiemetic bromopride (312), trypan blue ophthalmic solution (470), heparin (531), alcohol-free mouthwash (245), Albuterol

nebulization solution (34), and moisturizing body milk (14), among others. Product contamination can be compounded, as "*B. cepacia*" contamination of trypan blue, a dye used to examine tissues, led to contamination of a cornea tissue bank, though no post-graft infection was reported for any of the 169 contaminated grafts (331).

In an 8 year study of 327 US Food and Drug Administration (FDA) non-sterile and sterile product recalls, "*B. cepacia*" was the most frequently isolated microbe in 22% and 2.5% of the recalls, respectively (216). Among Bcc bacteria, *B. cenocepacia* is one of the most common industrial contaminants (287). However, Bcc contamination may arise from other sources, including water. An outbreak in a haemodialysis unit in Brazil was linked to "*B. cepacia*" contamination of reverse osmosis tubing in the water reservoir (454). Hospital tap water has also been linked to multiple infection outbreaks (279, 391). Bcc-contaminated water is not unique to a hospital environment, as "*B. cepacia*" is also the primary contaminant of the Space Shuttle water system both pre- and post-flight (237). The presence of *B. cenocepacia* within a hospital setting may contribute to opportunistic infection. While recall of contaminated products is fairly straightforward, eradication of *B. cenocepacia* from an infected hospital unit water source is more complex (243).

1.1.5 *B. cenocepacia* as an opportunistic human pathogen

The first clinical isolate of "*P. cepacia"* was found in urine in 1951; over the next 14 years, isolates were identified in a vast number of clinical sources, including blood, eye, stool, throat, myocardial abscess, spinal fluid, sputum, knee joint, and many others (220). Contaminated medical products led to frequent nosocomial infection outbreaks, including endocarditis, respiratory infection, and septicemia (39, 325, 420, 459). Infections were seen in patients with conditions including cancer, congenital heart disease, fistulae, quadriplegia, diabetes, and surgically-repaired major trauma (138). Today, Bcc bacteria are generally considered

opportunistic pathogens, or bacteria which do not typically cause disease in a healthy host, but exploit a niche created in the absence of a functional immune system. Bcc infection is most commonly seen in individuals with cystic fibrosis, although infections are also seen in immunocompromised patients, including those with chronic granulomatous disease, and, more rarely, in immunocompetent individuals (described in detail below). With the exception of contaminated medical products and direct patient-to-patient transmission, the source of an infection is not always clear. Unlike *P. aeruginosa*, which is carried by approximately 10% of the population, *B. cenocepacia* is not typically recovered from human sources other than sites of infection, and is thus likely acquired from the environment (287).

1.1.5.1 Cystic fibrosis

Cystic fibrosis is one of the most common genetic diseases in Caucasians, affecting approximately 1 in 3000 live births (301). The disease is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*cftr*) gene encoding a cyclic adenosine 5'- monophosphate (cAMP) regulated chloride channel (77, 384). Although the disease affects many organs, the most important site for morbidity and mortality is the lungs. In the lungs, CFTR defect results in thick mucus secretions which impair mucociliary clearance (81, 316), as well as an accumulation of salt in the airway surface liquid covering the epithelia, interfering with the bactericidal activity of defensins and lysozyme (175, 301). Bacteria like *P. aeruginosa,* the eventual predominant pathogen in CF patients, easily and sequentially colonize the CF lung causing chronic infection leading to bronchiectasis, respiratory failure, and death (116).

"P. cepacia" was recognized as an important pathogen in CF patients in 1984; throughout a ten year study, though the prevalence of *P. aeruginosa* remained stable at 70-80%, carriage of "*P. cepacia*" increased from 10% to 18%, with the latter infections associated with greater impairment in lung function (212).

Currently, approximately 3.5% of CF patients are infected with Bcc by age 18 (171), though regions of significantly higher prevalence exist (342); such patients exhibit a more rapid decline in lung function compared to those infected with other CF-related pathogens (100), with mean survival decreasing by between 12 and 25 years following Bcc infection (79, 455). Though many Bcc-infected individuals show asymptomatic carriage or chronic lung infection, one in five develop a condition known as 'cepacia syndrome', a generally fatal condition characterized by high fever, severe progressive respiratory failure, acute necrotizing pneumonia, and septicaemia (212). Resistance to almost all clinically relevant antibiotics (264) complicates treatment and creates great difficulties in eradicating infection. Though nosocomial acquisition is possible, patient-to-patient transmission has been demonstrated (177, 457), resulting in segregation of the CF community and discouragement of CF social opportunities such as summer camps; such isolation negatively affects psychological well-being (137).

Although nearly all species of the Bcc have been identified in the sputum of CF patients, *B. cenocepacia* and *B. multivorans* are the most prevalent species, accounting for 85-97% of all Bcc-CF infections (380, 495, 496). However, *B. cenocepacia* infection has far more severe clinical consequences than *B. multivorans*. *B. cenocepacia* is highly transmissible, containing the majority of epidemic strains identified thus far (134); indeed, two distinct sequence types of *B. cenocepacia* have shown intercontinental spread among CF patients (134, 218). *B. cenocepacia* is also associated with more severe infection, reduced survival, and increased risk of developing cepacia syndrome (102, 219, 292, 457), and can replace other Bcc species in the CF lung (293). Though lung transplantation is the only viable treatment for end-stage CF patients, infection with *B. cenocepacia* is a relative or absolute contraindication to transplantation in almost every centre outside of Toronto, where the prevalence of infection is over 15% (22, 342). Patients infected pre-operation with *B. cenocepacia* have significantly increased morbidity and mortality post-transplant, while those infected with other Bcc species fare no differently than *P. aeruginosa*-infected CF patients (22, 120). *B. cenocepacia* post-transplant death is generally due to

development of sepsis, or cepacia syndrome (120); there are only five reported cases in the literature of survival in septic patients with cepacia syndrome, all after extremely aggressive antibiotic therapy (342).

Aggressive CF management has increased the life expectancy of CF patients to mid-forties, revealing CF-related non-pulmonary conditions, including osteoporosis, diabetes, and liver disease (87, 137). Chronic colonization with Bcc, but not with *P. aeruginosa*, is associated with risk of developing CF liver disease, which itself is associated with requirement for lung transplantation, and death (87).

1.1.5.2 Chronic granulomatous disease

Chronic granulomatous disease (CGD) is a much less common genetic disease, conferring immunodeficiency in approximately one in 250 000 individuals with presentation varying from fatal septicaemia in infancy to mild symptoms late in life (489). CGD is genetically heterogeneous, caused by mutation to any of the five structural elements of the nicotinamide dinucleotide phosphate (NADPH) oxidase complex: membrane-bound cytochrome components $gp91^{phox}$ and $p22^{phox}$, and cytoplasmic components $p47^{phox}$, $p40^{phox}$ and $p67^{phox}$ (433). Defect to the NADPH oxidase leaves phagocytes unable to generate an oxidative burst, causing susceptibility to recurrent infection; CGD patients also suffer from a dysregulated Th-17-lymphocyte-controlled inflammatory response resulting in granuloma formation, and poor wound healing and dehiscence (387, 431). In a normally functioning oxidative burst, the NADPH oxidase generates superoxide anion, which is metabolized by superoxide dismutase to hydrogen peroxide, which is further metabolized by myeloperoxidase to hypohalous acid and hydroxyl anion; such reactive oxygen species (ROS) can function directly as antimicrobials (371). Activation of the NADPH oxidase is also necessary for the activation of microbicidal granule proteases and formation of neutrophil extracellular traps (NETs) (433). In the absence of a fully functional immune

response, CGD patients suffer from recurrent infections, most commonly with *Nocardia* and *Aspergillus* spp., *Staphylococcus aureus, Serratia marcescens,* and Bcc (433, 489, 523). Large cohort studies in Europe and North America have shown *Aspergillus* spp. to be the most common infectious agents and cause of death in CGD patients; however, the latter is a function of the former, as *Aspergillus*-mediated death occurs in only 8% (15/181) and 19% (23/120) of European and North American infections, respectively (489, 523). Less common, but far more serious, Bcc infections caused death in at least 43% (3/7) and 50% (12/24) of European and North American Bcc-infected CGD patients (489, 523).

Serious "*P. cepacia*" infection in CGD patients has frequently been linked with pneumonia, septicaemia and death in smaller studies (9, 56, 247, 456). It has been shown that CGD neutrophils, which can kill *P. aeruginosa* effectively, are unable to kill "*P. cepacia*" (456). *B. cenocepacia* is able to promote apoptosis in both normal and CGD human neutrophils; however, engulfed *B. cenocepacia* is also able to induce necrosis in CGD neutrophils lacking ROS (67). Among Bcc species, *B. cenocepacia* is most virulent in a gp91$^{phox\ -/-}$ murine model of CGD, causing septic death 3 days post-infection (p.i.) (453). In a p47$^{phox\ -/-}$ murine model of CGD, restoration of p47^{phox} through *ex vivo* gene transfer lowered "*B. cepacia*" bacteremia levels and conferred a survival advantage to the mouse (302), again linking the NADPH oxidase and Bcc infection. Interestingly, *B. cenocepacia* is also able to delay assembly of the NADPH oxidase complex in murine macrophages (230).

1.1.5.3 Nosocomial infection

Hospitalized patients are susceptible to nosocomial "*B. cepacia*" infection, especially in light of contaminated medical products. Nosocomial infections most commonly arise in the intensive care unit (ICU), and can result in pneumonia, bacteremia, surgical site infection, urinary tract infection, and skin-soft tissue infection (131). Typically, non-CF-related "*B. cepacia*" bacteremia occurs in

severely ill patients who require frequent health care interventions and invasive procedures, and thus have multiple exposures to antibiotic resistant nosocomial pathogens; specific risk factors include greater than two bronchoscopic procedures, tracheostomy, abdominal surgery, dialysis-requiring renal failure, and the presence of an indwelling vascular catheter (60). *B. cenocepacia* bacteremia has been seen in oncology patients (300, 311), haemodialysis patients (222, 271), and intensive care neonates (260), generally related to central venous catheter use. In patients who have undergone mitral valve replacement, "*B. cepacia*" infections lead to endocarditis, or inflammation of the inside lining of the heart chambers or valves; such infections can be fatal (8). "*P. cepacia*"-induced endocarditis has also been seen in heroin addicts both with and without histories of heart disease (345); in rare cases, these infections can exacerbate into skin and soft tissue involvement via ecthyma gangrenosum (299). "*B. cepacia*" has also caused postoperative bacterial endophthalmitis and keratitis leading to poor visual outcome (354, 398, 470). Bcc bacteria can also be transmitted between CF and non-CF patients, as seen in a large hospital outbreak where a single strain of "*B. cepacia*" infected 23 CF patients and 245 non-CF ICU patients; non-CF patients that survived the infection generally cleared the bacteria following discharge from the ICU, while CF patients remained persistently infected (201).

1.1.5.4 Immunocompetent individuals

Bcc infections in immunocompetent patients are rare. Community-acquired "*P. cepacia*" pneumonia and bacteremia have been reported on several occasions in previously-healthy adults (42, 111, 512), and children (368, 524). Hospital-acquired bacteremia has also been seen in immunocompetent children (174, 524). An individual with chronic suppurative otitis media developed intracranial infection and brain abscesses requiring multiple surgeries and resulting in loss of inner ear function in one ear (194). Less serious infection has presented in

individuals with sinonasal polyposis (59, 305), while "*B. cepacia*" infection in an immunocompetent individual resulted in infective endocarditis with subsequent cerebral involvement (234). "*P. cepacia*" was also found to cause 'trench foot', or macerated hyperkeratotic lesions of the feet, in soldiers working in swamp conditions (475). A single case showed evidence of Bcc transmission from an infected CF child to a healthy, non-CF mother, resulting in chronic colonization and acquired bronchiectasis (257).

1.1.6 Model systems to study *B. cenocepacia* pathogenesis

Various *in vivo* and *in vitro* systems have been developed to study *B. cenocepacia* pathogenesis. Though traditional murine models arguably provide the most relevant context for investigation relative to human disease, researchers have sought simpler, swifter, more economical models better suited to genetic manipulation (347). Several different models have since been developed, including plants, animals, and *in vitro* cell culture, which fit some or all of the criteria, allowing investigation into virulence factors, host response, and treatment options.

1.1.6.1 Murine models

CF patients infected with Bcc have three potential clinical presentations, including asymptomatic carriage, chronic infection with slow lung function decline, and acute infection leading to bacteremia and death. The latter two situations may be investigated by means of murine models. A chronic, nonlethal, pulmonary infection in rats established for CF pathogen *P. aeruginosa* (72) can also be applied to Bcc (446, 450). In this model, chronic infection is initiated by intratracheal insertion of agar beads containing the bacteria of interest; at 7 and 28 days p.i., the lungs are removed for bacterial enumeration and histopathological analysis (446). Lung inflammation in the model approximates

that seen in non-bacteremic pneumonia with mixed inflammatory cell infiltrate and foci of necrosis (72). An agar bead model of Bcc lung infection has also been utilized to examine systemic infection in C57/BL6 mice (91, 458). A second murine model of acute infection approximating bacteremia involves aerosol inoculation of neutropenic mice (106, 446). On days 0, 3, and 7 p.i., the mice are sacrificed and bacterial load in the lungs enumerated; though the bacteria are generally cleared, factors involved in colonization and short-term infection can be explored (446). Disease-specific murine models have been used with Bcc, including a cystic fibrosis *Cftr*$^{-/-}$ murine model of chronic pneumonia (407), and two murine models of chronic granulomatous disease: gp91$^{phox\ -/-}$ (453), and p47$^{phox\ -/-}$ (302). Aside from investigating pathogen virulence factors and host response, murine models are also useful in the development of *B. cenocepacia* vaccines (50, 294).

1.1.6.2 Other animal models

Simpler, less expensive animal models applied to Bcc infection include the zebrafish *Danio rerio* (502), the nematode *Caenorhabditis elegans* (241), the fruit fly *Drosophila melanogaster* (73), and larvae of the wax moth *Galleria mellonella*, or wax worms (429). For each of the models, members of the Bcc, as well as strains of the same species, often have different outcomes; however, the results do not always agree between models (485).

C. elegans is a simple, genetically-tractable model in which bacterial virulence in worms often parallels clinical data; *C. elegans* lacks adaptive immunity, but mounts an innate immune response to bacterial pathogens (347). *B. cenocepacia* is able to effectively kill *C. elegans* via two separate killing modes, termed slow killing and fast killing (241). In fast killing, *B. cenocepacia* grown on a high-osmolarity medium produces an extracellular toxin which paralyzes, then kills the nematodes within 24 h (241). When *B. cenocepacia* is grown on nematode growth medium, slow killing of *C. elegans* occurs over 2-3 days as the

bacteria accumulate in the intestinal lumen; the more pathogenic the bacteria, the more rapid the killing (241). To facilitate large scale analyses, a rapid screening method called the 48-well plate mortality assay was developed which correlates well with the standard slow killing assay (69). Additionally, lumenal infection can be visualized microscopically (241).

The innate immune system of *D. melanogaster* is similar to the mammalian version (152), and the genome has been fully sequenced (6), enabling a dissection of host-pathogen interactions. Infection can be induced naturally by feeding, or artificially by nicking; ingested Bcc colonize the fly without injury, while nicked flies are killed by internally-replicating Bcc, at strain-specific rates, with results comparable to those seen in murine models (73).

Virulence assays with *B. cenocepacia* have used zebrafish at different stages of the life cycle. Mortality assays and quantitation of bacterial survival have utilized 6-month-old zebrafish following intraperitoneal inoculation of bacteria (122). However, at the embryonic stage (28-32 h post-fertilization), zebrafish have a developed innate immune system resembling the mammalian equivalent, but are transparent, allowing infection to be visualized in real-time (502). Infection is more complicated than the previous models, requiring dechorionation – removal of the membrane surrounding the developing embryo – and anaesthetization of the embryo prior to microinjection of bacteria into blood circulation (502). *B. cenocepacia* are taken up by macrophages, replicate intracellularly, then disseminate throughout the embryo, leading to systemic infection and embryo death; however, virulence is species and strain dependent (502). While the ability to visualize infection in real-time is of great interest, *D. rerio* is temperature sensitive, a limitation common to *D. melanogaster* and *C. elegans*; none survive long at optimal temperatures for bacterial growth (73, 502).

G. mellonella possesses a fairly complex innate immune system (429), but differs from the three previous models in two key ways: Although it survives at 30^{o}C – amenable to bacterial growth – it lacks genetic tractability thus far (73). Similar to *D. melanogaster* and *C. elegans*, the model can be predictive of mammalian

response to pathogens; Bcc infection in *G. mellonella* corresponds well with results in murine models (429). The model itself is exceedingly simple, scoring larvae as alive or dead up to 72 h post-Bcc injection; dead larvae turn black as a result of melanization (429).

1.1.6.3 Plant models

Research with *P. aeruginosa* has demonstrated a conservation of virulence factors in the infection of plant and animal hosts (374), meaning preliminary work can be done in a simple, inexpensive model with no ethical considerations. Because "*B. cepacia*" was first identified as a phytopathogen in onions, *Allium cepa* remains a useful model for investigation of pathogenicity; tissue maceration, water soaking, and necrosis are quantified in wounded onion scales inoculated with Bcc (213). Alfalfa, or *Medicago sativa*, was the first non-murine model proposed for Bcc. Seedling leaves are wounded and inoculated with Bcc, then maintained at 37^{o}C under normal light/dark conditions; 7 days p.i., disease symptoms – yellow leaves, stunted roots, and brown necrotic regions – are assessed (48). Common plant model *Arabidopsis thaliana* has been little applied to Bcc work, though Bcc lipopolysaccharide (LPS) activates the innate immune response of the plant (286), and Bcc-produced volatiles promote its growth (504).

1.1.6.4 *In vitro* cell culture models

Although Bcc are predominantly extracellular bacteria, they have been isolated from intracellular environments in infected patients and *in vivo* models (80). "*B. cepacia*" has been isolated from the tracheal epithelial cells of a septicaemic CF patient (80). *B. cenocepacia* has also been found in the respiratory epithelium, alveolar septae, inflammatory cell infiltrates, and luminal and parenchymal macrophages of CF patients (406). "*B. cepacia*" invades respiratory epithelial

cells and macrophages in a murine model of infection (80), while *B. cenocepacia* is preferentially taken up by macrophages in a zebrafish embryo model (502).

Numerous cells and cell lines have been used to investigate interactions between Bcc and host cells (412). *In vitro, B. cenocepacia* has been shown to survive within free-living amoebae (253, 308), phagocytes (252), and epithelial cells (409). In *Acanthamoeba polyphaga*, *B. cenocepacia* survives without replication in an acidic vacuole distinct from the lysosomal compartment (253). The interactions between *B. cenocepacia,* and macrophages and epithelial cells have been much more closely studied.

It is not known whether *B. cenocepacia* is able to actively invade macrophages or whether the bacteria are taken up by phagocytosis, though internalization at a higher rate than *P. aeruginosa* or *S. aureus* has been demonstrated (321). Once internalized, *B. cenocepacia* is able to delay phagosomal maturation (252) and assembly of the NADPH oxidase complex (230), and induce interleukin (IL)-1β production (242); each of these phenotypes requires viable bacteria, and is exacerbated in the absence of a functional CFTR (230, 242, 254). *B. cenocepacia* is able to inactivate the Rab7 GTPase, a marker found on the cytosolic face of the phagosome which is important for phagolysosomal fusion (210). *B. cenocepacia* is also able to alter the actin cytoskeleton of the macrophage in a process requiring a functional Type VI secretion system (T6SS) (25, 390). Despite the delay in phagosomal maturation, the bacteria-containing vacuole (BCV) has been shown to fuse with lysosomes and become acidified by 6h p.i. (252), and replication of intracellular bacteria by 24h p.i. has been demonstrated (185), suggesting *B. cenocepacia* may be able to replicate within an acidic environment.

B. cenocepacia invasion of pulmonary epithelial cells is mediated by binding to glycosphingolipid receptors (335), and involves bacterial lipase (336), and host microfilaments and microtubules, but not CFTR (477). In CF-derived epithelial cells, F-actin polymerization into stress fibres is seen upon bacterial uptake into the endocytic pathway; live bacteria escape the classical endocytic pathway to

non-acidified autophagosomes and eventual replication in the endoplasmic reticulum (ER) (409). Infection of epithelial cells with *B. cenocepacia* upregulates expression of matrix metalloprotease-9, which inhibits wound repair (525), and proinflammatory cytokine IL-8, which enhances growth of intracellular bacteria (229). *B. cenocepacia* is able to access lung capillaries to initiate septicaemia by traversing the respiratory epithelium via disruption of tight junctions (236) or induction of epithelial cell death (107).

The sputum of Bcc-infected CF patients has less viable granulocytes, and a higher proportion of secondary necrotic granulocytes, typically seen when phagocytic clearance of apoptotic cells is ineffective (513). In cell culture models, *B. cenocepacia* interacts with neutrophils, promoting apoptosis post-internalization (67). *B. cenocepacia* is also able to interfere with normal maturation and induce necrosis in dendritic cells (285).

1.1.7 Genetics of *B. cenocepacia*

B. cenocepacia is a remarkably adaptable bacterium exhibiting nutritional versatility and inhabiting a wide variety of niches ranging from the CF lung to agricultural soil; the same sequence type has been isolated from clinical, industrial & natural sources (287). It is thought that the adaptability of Bcc bacteria is due to both larger than average coding potential arranged in a multireplicon structure, and the presence of many insertion sequences yielding genomic plasticity & adaptability (265).

1.1.7.1 Genetic organization

The genome of the type strain of *B. cenocepacia*, J2315, encodes 7261 putative coding sequences (CDS) in 8 Mbp of genetic material with an average G+C content of 66.9% (195). The genome is divided into three circular chromosomes

and a plasmid, with sizes of approximately 3.9 Mbp, 3.2 Mbp, 0.9 Mbp, and 92 kbp, respectively; genes are designated with a replicon-specific prefix – BCAL, BCAM, BCAS, or pBCA – and a number. The genome contains a perfect gene duplication of 57 putative CDSs on chromosome 1, as well as 79 insertion sequences, and 126 partial or pseudogenes; fourteen putative genomic islands – likely a result of horizontal gene transfer – account for 9.3% of chromosomal DNA and may contain mobile genetic elements including bacteriophage and transposons (195).

1.1.7.2 Genetic manipulation

B. cenocepacia is not readily tractable genetically, and until recently, genetic manipulation was greatly hindered by few tools and limited selection markers, mainly trimethoprim and tetracycline; while the latter has not changed, development of the homing endonuclease pGPI-SceI system enables sequential, non-polar, unmarked gene deletions in *B. cenocepacia* (156). The system uses two separate plasmids, suicide plasmid pGPI-SceI, and replicative but unstable plasmid pDAI-SceI, each sequentially transferred via tri-parental mating. Two small – 200 bp is sufficient – regions of homology flanking the sequence to be deleted are cloned into mutagenesis plasmid pGPI-SceI, which contains a unique I-SceI recognition site in the plasmid backbone. Because *B. cenocepacia* does not produce Pir, required for maintenance of pGP plasmids with a Pir-dependent ori_{R6K} (154), the mutagenesis plasmid is incorporated into the genome by homologous recombination, producing an intermediate mutant. Introduction of the second plasmid, pDAI-SceI, constitutively expressing the I-SceI nuclease, creates a double strand break in the integrated plasmid backbone, which must be repaired for survival of the bacterium. Recombination within the first region of homology restores the wild-type chromosome, but recombination within the second region of homology results in a deletion of all chromosomal DNA between the two regions of homology (156). Deletion mutants are confirmed by

polymerase chain reaction (PCR) with primers external to the deletion, and/or Southern blot hybridization. The plasmid expressing the I-SceI nuclease also contains a *sacB* site, allowing it to be cured by growth on sucrose (185). Because the resultant deletion is unmarked, application of the pGPI-SceI strategy allows continued re-use of the same two antibiotic selection markers to create sequential deletions in the same strain.

1.1.7.3 Strain differences

B. cenocepacia as a species designation takes in four different *recA* lineages, IIIA to D; thus far, all IIIC are environmental, while there are no environmental strains of IIID and few for IIIA, suggesting possible niche-specific adaptations (287). Though most CF epidemic strains in the United States, including PHDC – so designated based on the two American cities in which the strain was first isolated from CF patients (92) – and Midwest, belong to the IIIB group, Canada and Europe are dominated by IIIA strains; sequenced strain J2315 belongs to a *rec*IIIA lineage known as Edinburgh Toronto (ET)-12, or ST-28 (134). There are significant genetic differences between lineages, as comparison of the IIIA J2315 sequence with IIIB lineage PHDC strains AU1054 and HI2424 showed regions of difference encompassing 21% of the total genome, including lack of all fourteen genomic islands in PHDC (195). There are also genetic differences within the same lineage: ST-28 and ST-32 are closely related IIIA strains with intercontinental distribution, but differing pathogenicity; the less-virulent ST-32 undergoes extensive genomic rearrangement and transposition events when exposed to ROS, while the genome of the more-virulent ST-28 appears stable under the same conditions (134).

The work described in this thesis uses *B. cenocepacia* K56-2, or ST-30, isolated from the lungs of a Canadian CF patient. K56-2 belongs to the ET-12 lineage, and is assigned to the same clonal complex as J2315 (290). Because K56-2 has yet to be sequenced, the published sequence of J2315 was applied to K56-2 for

the purposes of this work. However, there are differences between K56-2 and J2315 both genetically (195), and phenotypically, including LPS structure (356), ability to infect macrophages (411), and virulence in *G. mellonella* (429), *D. melanogaster* (73), *C. elegans* (69), and *D. rerio* (502). Because K56-2 has lower resistance to useful antimicrobial selection agents than does J2315, the former is more amenable to genetic manipulation, and was thus the strain of choice for this work.

Recently, MH1K, an aminoglycoside-sensitive strain of K56-2, was developed; though sensitive to concentrations of gentamicin as low as 10 µg/mL, MH1K behaves as wild-type when internalized by murine macrophages (185). This strain is a very useful tool for *in vitro* studies of *B. cenocepacia*. Prior to MH1K, classical studies of the interaction between bacteria and eukaryotic cells were hampered by the extreme resistance of *B. cenocepacia* to antibiotics, requiring researchers to use high concentrations of gentamicin and ceftazidime to kill extracellular bacteria; the presence of such high levels of aminoglycosides had the potential to affect intracellular bacteria, as well as the eukaryotic cells. With the use of MH1K, extracellular bacteria can now be killed, facilitating more accurate and longer-term investigation of the internalization and intracellular behaviour of *B. cenocepacia*.

1.1.8 Virulence factors in *B. cenocepacia*

B. cenocepacia possesses a wide range of virulence factors putatively involved in the ability of the bacterium to cause disease [reviewed in (278)]. Previous work employed a signature-tagged transposon mutagenesis (STM) to identify 84 unique genes essential for *in vivo* survival in a rat model of chronic lung infection; these genes were spread across all four genetic elements of *B. cenocepacia* and included metabolic, regulatory, transport, cell surface-associated, and information processing genes, as well as conserved genes of unknown function (207). Transposon mutation screens have also been done in a *C. elegans* model,

identifying several genes involved in virulence (451). Recently, the metabolic network was modelled for J2315, demonstrating biosynthesis pathways for virulence factors including lipopolysaccharide, quorum sensing molecules, and putatively, rhamnolipids (145). Comparative transcriptomics has also been applied to the identification of virulence factors; global expression studies have examined the response of clinical and environmental isolates of *B. cenocepacia*, to synthetic CF sputum (133, 535, 536), although only four genes are common to all three studies. Many putative virulence factors have been specifically targeted for investigation through a mutation-based approach; other studies have utilized these mutants to test the contribution of putative virulence factors in multiple models of infection.

1.1.8.1 Genomic islands

B. cenocepacia IIIA strain J2315 contains fourteen genomic islands, comprising 9.3% of the total genome, which are not found in sequenced IIIB strains; because most IIIA strains are clinical isolates, it is possible that the genomic islands contain genes promoting adaptation to the CF lung (195). Many of the islands are prophages, or are of phage or plasmid origin, while others contain metabolic genes; genomic island 11, formerly referred to as the *B. cenocepacia* island (cci), encodes 43 CDS involved in a wide variety of functions, including quorum sensing, stress response, antibiotic and arsenic resistance, and ion and amino acid transport (195). The cci also includes the "*B. cepacia* epidemic strain marker" (BCESM), unique to strains capable of epidemic spread among CF patients (291); genes within the cci are linked with persistence and virulence in a murine model of chronic lung infection (32).

1.1.8.2 Antimicrobial resistance

Pan-resistant *B. cenocepacia* strains – that is, resistant to most, if not all, clinically useful antibiotics – are common; where treatment is possible, it often requires the concurrent use of multiple antibiotics (1). Isolates from CF patients suffering from pulmonary exacerbation are generally more resistant to antibiotics than those isolated from stable, chronically-infected patients (460). Antibiotic resistance in *B. cenocepacia* is undoubtedly multifactorial, as the bacteria possess mechanisms for alteration of cell wall permeability, alteration of drug targets, enzymatic inactivation, and active efflux (134). Transcriptomic studies have suggested a role for efflux pumps in antimicrobial resistance (417), which has been borne out in the increased antibiotic susceptibility of deletion mutants (40, 64, 185). Surface structure lipopolysaccharide also plays a role in resistance to antimicrobial peptides (277). Genomic analysis has also demonstrated the presence of beta-lactamases, fosmidomycin resistance protein, and trimethoprim resistance factor dihydrofolate reductase, among others (195).

1.1.8.3 Quorum sensing

Quorum sensing (QS) is a form of interbacterial communication that regulates gene expression relative to cell density; a signalling molecule – typically *N*-acyl-homoserine-lactone (AHL) – is produced by a synthase and detected by a transcriptional regulator (167). *B. cenocepacia* is able to synthesize at least three different types of signalling molecules, including putative intergenus signal 2-heptyl-4(1*H*)-quinolone (HHQ), which can be processed into a QS signal by *P. aeruginosa* (129). Epidemic strains of *B. cenocepacia* carry two copies of the AHL-mediated quorum sensing system: The first, with AHL-synthase CepI and regulator CepR, is common to all *B. cenocepacia* strains and produces primarily *N*-octanoyl-L-homoserine lactone (C8-HSL) (266); the second, designated CciIR, is located within the cci and synthesizes mainly *N*-hexanoyl-L-homoserine lactone (C6-HSL) (32, 297). Though both systems function as global regulators

of quorum sensing gene expression, the two participate in reciprocal regulation of many genes, including motility- and iron transport-associated genes, synthesis of exoenzymes, and the nematocidal protein AidA (349). Accordingly, mutation of *cepI* reduces virulence in *C. elegans*, but mutation to *cciI* has no effect (485). CepIR QS-deficient mutants were less virulent in both the rat agar bead model of chronic lung infection, and intranasal infections in wild-type and *Cftr*$^{-/-}$ mice (449); CciIR mutants were also attenuated for virulence in the rat model (32). A third CepR homolog exists in *B. cenocepacia* which lacks an associated AHL synthase; CepR2 participates in QS regulation, but can function in the absence of AHL signals (298).

A third quorum sensing system in *B. cenocepacia* uses cis-2-dodecenoic acid (BDSF), a non-homoserine lactone diffusible signal factor detected by a histidine kinase encoded by BCAM0227; mutation of the sensor decreases virulence in both *G. mellonella* larvae and the mouse agar-bead model of chronic lung infection (318). Mutation of a gene involved in the synthesis of BDSF results in decreased virulence in a *D. rerio* model of infection (122).

1.1.8.4 Iron acquisition

Though an essential nutrient, free iron is limited in a host environment, and must thus be scavenged by the infecting pathogen. *B. cenocepacia* produces iron-chelating siderophores salicylic acid (SA), ornibactin, and pyochelin (446, 506). In a survey of "*P. cepacia*" isolates from CF patients, pyochelin-producing strains were associated with more severe pulmonary disease and death, while pyochelin-negative strains were associated with more mild infections (444). Though the pyochelin biosynthesis genes in J2315 contain a frameshift mutation, the transport and utilization genes are intact (195); in a rat model of chronic lung infection, exogenously supplied pyochelin increased the virulence of pyochelin-negative "*B. cepacia*" strains (450), though pyochelin alone is insufficient for iron acquisition *in vivo* (506). SA, originally referred to as azurechelin, is also

produced by clinical isolates of "*P. cepacia*"; similar to pyochelin, SA is able to promote growth of "*B. cepacia*" under iron-limiting conditions, and compete with transferrin for iron *in vitro* (444, 447). Though SA and ornibactin are the predominant siderophores produced by clinical isolates of "*B. cepacia*" (114), SA does not appear to be able to compete with host iron binding proteins and promote iron acquisition *in vivo* (445), but may instead play a role in inducing antibiotic resistance (339). Ornibactin contributes to colonization, bacterial persistence, and lung pathology in murine models of acute and chronic lung infection (446); ornibactin-deficient mutants also display decreased virulence in *C. elegans*, and are avirulent in *G. mellonella* (485). *B. cenocepacia* is also able to utilize some xenosiderophores, including ferrichrome and ferrioxamine B (478).

Iron sequestration in the lung typically involves molecules of transferrin, lactoferrin, and ferritin (484). Compared to a normal lung, the CF lung contains elevated levels of ferritin, which are elevated further in the presence of an inflammatory response (379, 465). *B. cenocepacia* is able to acquire iron from ferritin in a serine-protease-dependent manner (521), and also capable of utilizing oxidized haem as an iron source (478, 521).

1.1.8.5 Secretion systems

Many pathogens interfere with eukaryotic host cell processes by employing large, specialized secretion systems to translocate effector molecules into the host (168). In particular, types III, IV, and VI secretion systems – all of which are present in *B. cenocepacia* – have been implicated in interactions between prokaryotic and eukaryotic cells. The pathogenic potential of the type III secretion system (T3SS) of *B. cenocepacia* has been examined in several models: Previous studies have shown that a mutant bearing a polar insertion in the T3SS gene cluster delays phagosomal maturation similar to wild type (252), but an insertionally-activated T3SS mutant was cleared more rapidly from the lungs of infected mice (480), and displayed reduced virulence in *C. elegans* (306, 485).

B. cenocepacia encodes two type IV secretion systems (T4SS), the first a chimera of VirB/D4 and F-specific subunits on the plasmid (T4SS-1), and the second a VirB/D4 homolog on chromosome two (T4SS-2) (86, 195). *B. cenocepacia* T4SS-1 has been previously examined for *in vivo* relevance; insertional inactivation of a putative T4SS coupling protein affected intracellular persistence in macrophages (403). T4SS-1 is also required to induce plant tissue water soaking (141). The only known role for T4SS-2 is in plasmid mobilization (539). Mutations in the type VI secretion system (T6SS) of *B. cenocepacia* render the bacteria deficient in survival in a murine model of chronic lung infection (207) and susceptible to predation by the amoeba *Dictyostelium discoideum* (25). An active T6SS mediates actin rearrangement in *B. cenocepacia*-infected macrophages (25).

B. cenocepacia also encodes a type II secretion system (T2SS) (195), generally responsible for movement of toxins, proteases, cellulases and lipases across the bacterial membrane [reviewed in (217)]. A type II secretion pseudopilin was found to be involved in the pathogenesis of *B. cenocepacia* in *C. elegans* (451). Finally, *B. cenocepacia* possesses four type V secretion systems (T5SS); all four autotransporters appear to be involved in adhesion, two containing pertactin domains and two containing hemagglutinin repeat domains (195).

1.1.8.6 Exoenzymes

B. cenocepacia produces various exoenzymes which may be involved in host-pathogen interactions. Early studies of "*P. cepacia*" clinical isolates revealed production of lipase, protease, lecithinase, phospholipase C, and, in a small proportion of isolates, hemolysin (340, 497). More recent genomic studies have shown that the *B. cenocepacia* J2315 genome encodes five phospholipase C homologs with putative functional specificity, and a phosphatidylinositol-specific phospholipase C, which, in gram-positive pathogens, is involved in virulence;

J2315 also encodes pectin degradation enzymes, involved in the breakdown of plant tissue (195).

Functional studies with *B. cenocepacia* have revealed a wide variety of exoenzymes. *B. cenocepacia* J2315 produces a haemolytic protein with strong biosurfactant properties that is able to induce apoptosis and degranulation in phagocytic cells; the toxin is capable of inhibiting *S. aureus* and, at high concentrations, *P. aeruginosa*, but does not inhibit *B. cenocepacia* (209). *B. cenocepacia* also secretes a homologue of the redox enzyme azurin which is capable of inducing caspase-dependent apoptosis in macrophages (370), and a purple alkaline phosphatase highly active against phosphorylated proteins, and, to a lesser extent, phosphoenolpyruvate and ATP (533). The invasion of epithelial cells by *B. cenocepacia* involves a secreted lipase that does not affect the eukaryotic plasma membrane or tight junction integrity (336). The best studied exoenzymes of *B. cenocepacia* are the QS-regulated ZmpA and ZmpB zinc metalloproteases (99, 240). Both enzymes can degrade casein, gelatin, tissue components type IV collagen and human fibronectin, protease inhibitors α_2-microglobulin and α-1 proteinase inhibitor, and a variety of antimicrobial peptides, though the manner of cleavage is generally enzyme-specific (238-240, 322); ZmpA also cleaves gamma interferon (238), and ZmpB degrades lactoferrin, transferrin, and immunoglobulins IgA, IgG, and IgM (240). Though both enzymes contribute to lung pathology in a murine model of chronic lung infection (99, 240), ZmpA also contributes to persistence in the lung (99) and, when instilled in a purified form, induces bronchopneumonia in rats (322).

1.1.8.7 Cell surface structures

Structures present on the bacterial cell envelope are frequently involved in the interactions between the bacterium and host cells, modulating adherence, invasion, and cytotoxicity, as well as bacterial motility. Alterations to the cell surface can affect persistence in murine models of infection (89). *B. cenocepacia*

possesses polar flagella, which have been implicated in pathogenesis. Non-motile flagellar mutants are deficient in invasion of, but not in adherence to, respiratory epithelial cells, a phenotype dependent on flagellar-mediated motility (481). Flagellar mutants are also non-lethal in a murine model of chronic lung infection, and do not stimulate a strong immune response, whereas wild type *B. cenocepacia* elicits a strong immune response, partially by initiating a signalling cascade through the interaction of bacterial flagellin with Toll-like receptor (TLR) 5 (487). There has been speculation that the flagella are important in establishment of an infection, while adaptation to chronic lung infection involves a loss of motility (417), as is seen for *P. aeruginosa* isolates from chronically-infected CF patients (289).

One of the two hallmarks of the highly transmissible ET-12 lineage of *B. cenocepacia* is the presence of the *cblA* gene encoding the major subunit of surface cable (Cbl) pili (469). A 22-kDa Cbl pilin-associated adhesin mediates bacterial binding to cytokeratin 13 (CK13) and mucin, both of which are abundant in the CF airway epithelia (404, 408); binding of *B. cenocepacia* to CK13 promotes bacterial invasion into epithelial cells and cell damage (405). Cbl pili are cytotoxic, able to activate caspases and induce apoptosis in epithelial cells (78). J2315 also encodes components of a type IVa pilus, and two clusters of Flp-type pili (195).

Other proteins are also involved in mediating bacterial adherence to host cells. A trimeric autotransporter adhesin is involved in *in vitro* adherence to extracellular matrix proteins and virulence against *G. mellonella* larvae (326). Genomic studies have shown that J2315 encodes eight BuHA family proteins, autotransporting membrane proteins with homology to hemagglutinin and invasin proteins (195).

In contrast, bacterial adherence to epithelial cells is inhibited by the presence of O-antigen, the outermost portion of lipopolysaccharide (LPS) (411). LPS, which forms the major surface component of gram negative bacteria, is a complex glycolipid composed of lipid A, a core oligosaccharide, and, in some strains, O-

antigen (373). Unlike other bacteria, *B. cenocepacia* constitutively incorporates 4-amino-4-deoxy-L-arabinose (Ara4N) residues into the lipid A and core oligosaccharide, resulting in a reduction in the net negative charge of the LPS molecule (358). Lipid A anchors the LPS molecule in the membrane, maintaining membrane integrity while serving as an endotoxin (373). *B. cenocepacia* LPS induces cytokine production by human monocytes in a TLR4- and CD-14-dependent manner (35, 119); the immune response to "*B. cepacia*" LPS is over nine-fold greater than that elicited by *P. aeruginosa* LPS (434). The O-antigen also prevents phagocytosis by macrophages (411) and serum-mediated killing (356). Mutations to the O-antigen synthesis cluster result in attenuated survival in a rat model of chronic lung infection (207), as do mutant strains unable to synthesize the complete LPS core oligosaccharide (277). The latter also show impaired virulence in *C. elegans* and *G. mellonella* models, fail to traffic properly in murine macrophages, and are more sensitive to antimicrobial peptides (357, 485).

The production of exopolysaccharide (EPS), leading to a mucoid phenotype, has been noted in some strains of *B. cenocepacia*; typically, EPS production appears more common in environmental isolates and strains belonging to the *recA* IIIB lineage (541). In clinical isolates, there is speculation that EPS masks bacterial surface ligands, reducing the virulence of mucoid isolates and increasing bacterial persistence in the murine lung (97); however, EPS-deficient mutants show reduced virulence in a CGD mouse model of infection (453). The most common EPS produced by Bcc species is designated cepacian (443). Though the ET-12 lineage cannot produce cepacian due to a deletion in the biosynthesis cluster, other *B. cenocepacia* strains can produce EPS in response to carbohydrates in onion tissue and alcohol sugars (38). *B. cenocepacia* is capable of producing mucoid material consisting of multiple polysaccharides, including polysaccharide-1 (PS-1), cepacian (formerly PS-2), and dextran; EPS produced by *B. cenocepacia* can inhibit neutrophil chemotaxis and the production of reactive oxygen species (66).

1.1.8.8 Resistance to oxidative stress

In the course of a respiratory infection, *B. cenocepacia* is exposed to a toxic array of reactive oxygen species; intracellular bacteria are subject to the oxidative burst of phagocytic cells, while extracellular bacteria are exposed to the highly oxidative environment of the CF lung, characterized by persistent neutrophil infiltration and a sustained inflammatory response. *B. cenocepacia* possesses numerous mechanisms to resist damage due to ROS. Two separate catalase/peroxidase enzymes have been identified in *B. cenocepacia*, the first a classical enzyme participating in global resistance to oxidative stress, and the second a specialized enzyme employed in the metabolism of carbon sources through the tricarboxylic acid cycle, and in hydrogen peroxide resistance under conditions of iron limitation (263). Intracellular *B. cenocepacia* is able to delay assembly of the NADPH oxidase complex, responsible for producing ROS (230); a periplasmic superoxide dismutase SodC is required for resistance to extracellular superoxide, mutations to which result in rapid NADPH oxidase-dependent killing by murine macrophages (232).

Some strains of *B. cenocepacia* produce a melanin-like pigment which also contributes to resistance to oxidative stress *in vitro* and intracellular survival within murine macrophages (231), while EPS produced by *B. cenocepacia* is able to scavenge ROS from activated phagocytes (66). Genes involved in resistance to oxidative stress are upregulated when *B. cenocepacia* is grown in cystic fibrosis sputum (133). Transcriptomic analysis of the response of sessile *B. cenocepacia* to ROS shows upregulation of pathways involved in scavenging and neutralizing ROS, and repair of cellular damage resulting from ROS exposure (363).

1.1.8.9 Biofilm

B. cenocepacia is capable of forming biofilms – complex multicellular communities protected from antibiotics and host defences – on both abiotic (98) and biotic (425) surfaces. The invasion of the airway epithelium by *B. cenocepacia* is mediated by biofilm formation; biofilm-associated bacteria are able to degrade the glycocalyx barrier, rearrange the actin cytoskeleton of the underlying epithelial cells, and induce necrosis of the luminal cells (425). Biofilm-associated *B. cenocepacia* produces cyanide, which may participate in destruction of host tissue (397); accumulation of cyanide in the sputum of *P. aeruginosa*-infected CF patients is associated with a decline in lung function (396).

Reported regulators of *B. cenocepacia* biofilm formation, structure, and stability include all three quorum sensing systems (123, 206, 482), RpoN (410), ShvR (47), and AtsR, a hybrid sensor kinase-response regulator that also regulates the T6SS (25); formation and structure are also affected by iron availability (46), and EPS production (108).

During co-infection of the CF lung, *B. cenocepacia* and *P. aeruginosa* can form mixed-species biofilms, where interspecies communication occurs (383). *P. aeruginosa* is able to enhance the binding of "*P. cepacia*" to epithelial cells (401), and produces QS molecules that can upregulate the production of virulence factors in *B. cenocepacia* (320, 383).

Biofilm-associated bacteria are more resistant to disinfectants (362), and antibiotics, including double antibiotic combinations (112). However, treating biofilms with a combination of antibiotic and quorum-sensing inhibitors (QSI) has proven effective in killing biofilm-associated *B. cenocepacia*; compared to treatment with antibiotic alone, the combination with QSI also increases survival of infected *C. elegans* and *G. mellonella*, and decreases bacterial burden in a murine model of lung infection (58).

1.1.8.10 Other virulence determinants

Degradation of many aromatic compounds converges at the catabolic pathway for phenylacetic acid (PA) prior to entering the Krebs cycle (280). The PA pathway is upregulated when *B. cenocepacia* is grown in synthetic CF medium (186); transposon mutants in the PA pathway are attenuated for survival in *C. elegans* (256), as well as a rat model of chronic lung infection (207), suggesting aromatic compounds may be an important nutrient source in host-pathogen interactions.

MgtC is a protein of unknown function which is required for growth under magnesium-limiting conditions and for intramacrophage survival in intracellular pathogens such as *Salmonella enterica* (381) and *Brucella suis* (255). MgtC fulfills the same role in *B. cenocepacia* (296), where it is also necessary for virulence in a rat model of chronic lung infection (207). There are two additional homologs of MgtC in the *B. cenocepacia* genome that have not yet been investigated; since the original mutant was not susceptible to *in vitro* intramacrophage-like conditions such as reactive oxygen and nitrogen species, cationic antimicrobial peptides, or low pH (296), it is possible the two additional MgtC proteins can complement certain phenotypes or under certain conditions.

ShvR, a LysR-type transcriptional regulator, is necessary for virulence in an alfalfa model, as well as full lung histopathology in a murine model of chronic lung infection (47). ShvR also regulates colony morphotype, biofilm formation, exoenzyme production, type II secretion, and production of an anti-fungal agent (348).

HtrA is a periplasmic protein involved in the extracytoplasmic stress response; necessary for growth under thermal and osmotic stress, HtrA is also essential for *in vivo* survival in a murine model of chronic lung infection (154). HtrA-like proteases are regulated by the alternative sigma factor RpoE, which in *B. cenocepacia*, is also necessary for growth under thermal and osmotic stress, as well as proper trafficking in murine macropahges (157). A second alternative

sigma factor, RpoN, also plays a role in intra-macrophage trafficking, as well as motility and biofilm formation (410).

Rhamnolipids are amphiphilic molecules which can act as biosurfactants; in *P. aeruginosa*, rhamnolipids are involved in biofilm development (115) and swarming motility (483), and can inhibit macrophage phagocytosis (319), disrupt the respiratory epithelium (543), and interfere with normal functioning of the tracheal cilia (378). Rhamnolipid production has been seen in non-Bcc *Burkholderia* spp. (19, 136, 187), where it functions as an endotoxin in pathogenic strains. Orthologs to the rhamnolipid biosynthetic genes of *B. thailandensis* and *P. aeruginosa* have been identified in *B. cenocepacia*, suggesting a capacity to generate rhamnolipids (145).

1.2 Secretion systems

Secretion in gram-negative bacteria requires transport across two hydrophobic membranes separated by a peptidoglycan-containing aqueous periplasm. The movement of large macromolecules across this cellular envelope poses challenges in terms of maintaining the integrity of the membrane, as well as regulation of cross-membrane movement. Estimates place at least 25-30% of the typical gram-negative total protein content in the cell envelope or secretome; as such, gram negative bacteria have developed six known large multi-gene secretion systems – types I through VI – including both one-step “tunnels” and two-step processes separated by a periplasmic stage (198). A type VII secretion system has also been identified in gram-positive bacteria (2). Secretion is particularly important in intercellular signalling for cooperation or competition, and in virulence effector delivery to eukaryotic cells, and thus some secretion systems are contact-dependent (188).

1.2.1 The Sec and Tat systems

The general secretory (Sec) pathway and the twin-arginine translocation (Tat) pathway both function to move proteins across the inner membrane (IM); the former transports unfolded proteins, while the latter translocates folded proteins (261). Protein substrates are targeted for export via an amino (N)-terminal signal sequence called a signal peptide (SP), which is cleaved when the protein reaches the periplasm. The composition of the Tat sytem is varied, minimally consisting of a TatABC substrate-binding receptor complex which associates with a separate TatA complex to form an active translocon, through which tranport is driven by the proton motive force (385). Proteins targeted to the Sec system bind to cytosolic motor protein SecA, an ATPase that drives translocation through a SecYEG channel; in the periplasm, the SP is cleaved by signal peptidase, allowing folding of the mature protein (135).

1.2.2 Type I

The type I secretion system (T1SS) is a simple trimeric complex spanning the entire cell envelope (199). The translocon consists of an IM ATP-binding cassette (ABC) transporter, with a nucleotide binding domain (NBD) fused to a transmembrane domain, and an outer membrane (OM) pore linked by an adaptor protein, or membrane fusion protein. Unfolded substrates are targeted to the T1SS, where a non-cleavable carboxy (C)-terminal signal sequence binds to the NBD of the ABC transporter to induce translocation.

1.2.3 Type II

The type II secretion system (T2SS), often referred to as the general secretory pathway, mediates a two-step process. First, exoproteins with an N-terminal SP are transported to the periplasm by either the Sec or Tat systems; following

cleavage of the SP, fully folded proteins are translocated across the OM by the secreton (217). The secreton is a large complex composed of an OM pore called a secretin, and an IM pilus-like structure postulated to act as a piston to force substrates through the secretin (90). The T2SS is closely related to the type IV pili (217), and secretes a wide variety of exoenzymes (90).

1.2.4 Type III

The type III secretion system (T3SS) is a generally contact-dependent system found in pathogenic bacteria for the delivery of effectors to the cytosol of eukaryotic cells. It is characterized by an injectisome, or needle-like structure capable of injecting substrates directly into a eukaryotic cell through a cell-envelope-spanning channel; outside the bacterial OM, the T3SS is capped with a needle, a tip complex, and a translocation pore forming a channel in the eukaryotic membrane (188). In structure and assembly, the T3SS is homologous to the flagellar export system (143), though secreted effectors are varied.

1.2.5 Type IV

The type IV secretion system (T4SS) is a mostly contact-dependent system able to transport substrates directly into both prokaryotic and eukaryotic cells. Homologous to bacterial conjugation machinery, T4SSs are able to translocate both DNA and protein substrates; the former contribute to rapid inter-bacterial spread of resistance genes and fitness traits, while the latter contribute to virulence against eukaryotic cells (86). The main structures of the T4SS comprise a cell surface adhesin or pili for intercellular contact, a transenvelope channel, and a Type IV coupling protein (T4CP), which mediates substrate entrance into the channel (188). There are two general lineages of T4SS, one homologous to the VirB/D4 system of *Agrobacterium tumefaciens*, and one

homologous to the Dot/Icm system of *L. pneumophila,* although there are T4SSs that fit well into neither lineage (86).

1.2.6 Type V

The simplest of the secretion systems, Type V secretion systems (T5SS) also use a two-step secretion mechanism with a periplasmic intermediate for the secretion of very large proteins. Unfolded proteins are transported to the periplasm through the Sec system, where a β-barrel translocator domain is needed for transport across the OM (189). T5SS can be divided into autotransporters (AT) and two-partner secretion systems (TPS). ATs consist of a signal domain and a β-barrel domain linked by a passenger domain; the signal domain directs the protein to the periplasm where the β-barrel domain forms a pore in the OM for secretion of the passenger domain. The passenger domain may remain associated with the OM or be released into the extracellular milieu by proteolysis. AT proteins often contribute to cellular adhesion, aggregation, biofilm formation, invasion, and toxicity (519). TPSs are very similar to ATs, but the passenger domain, or exoprotein, and the pore-forming β-barrel domain, or transporter, are translated as two separate proteins (189). TPSs are implicated in contact-dependent growth inhibition (188).

1.2.7 Type VI

The type VI secretion system (T6SS) is the most recently discovered of the large multi-gene secretion systems, and as such, has not been as well characterized. Some proteins of the T6SS are homologous to bacteriophage proteins, including tube-like hexameric ring protein Hcp, and tailspike-like protein VgrG, and the T6SS can participate in contact-dependent effector delivery (215). However, the purpose of the T6SS is a hotly debated topic, with phenotypes ranging from

restriction of *H. hepaticus* colonization and downregulation of the host immune response in infected epithelial cells (83), to *P. aeruginosa* toxin-mediated killing of neighbouring prokaryotic cells (204), *B. thailandensis* resistance to cell contact-induced growth inhibition (427), *B. mallei* virulence in hamsters (421), and translocation of an actin cross-linking effector into host cells by intracellular *Vibrio cholerae* (284).

1.3 Prokaryotic interactions with macrophages

As professional phagocytic cells, macrophages are key elements of the innate immune response uniquely suited to engulf large particles, including microorganisms. Particles are generally taken up by a receptor-mediated process into plasma-membrane derived vacuoles; these phagosomes mature through successive interactions with the endosomal pathway, a series of membrane bound organelles responsible for the degradation, recycling, and sorting of molecules from both the cellular membrane and the extracellular milieu. As the phagosome matures, both the membrane composition and the internal contents are altered through interaction with a multitude of cellular and membrane proteins, changing the phagosome from a relatively innocuous environment to a highly acidic and oxidative environment rich in degradative enzymes suitable for microbe destruction (55). While most bacteria are successfully eliminated by the macrophage, some pathogens have developed strategies for preventing internalization or interfering with normal host cell processes. Still others have adapted to the intracellular environment, which has advantages for pathogens able to overcome host defenses. Macrophages are fairly long-lived cells, and thus provide a competition-free, nutrient-rich niche protected from extracellular host defenses for microbes able to subvert normal macrophage degradative processes.

1.3.1 Bacterial internalization

The most common means of bacterial uptake into macrophages is via receptor-mediated, actin-driven, zipper-like phagocytosis. Macrophages express a wide range of receptors recognizing both pathogen-associated molecules and host serum opsonins coating the bacterial surface. Opsonins such as complement protein C3b and immunoglobulin G (IgG) are recognized by complement receptor 3 and Fcγ receptors, respectively; because Fcγ receptors are relatively low affinity, activation of phagocytosis requires simultaneous binding of multiple receptor-ligand complexes (55). While opsonization is a non-specific process enabling detection of a vast array of microbes (490), phagocytes also express a wide variety of pattern recognition receptors (PRRs) which recognize pathogen-associated molecular patterns (PAMPs) (479). Plasma membrane-localized PRRs, including those belonging to the scavenger receptor, C-type lectin, and Toll-like receptor (TLR) families, are able to recognize diverse bacterial PAMPs, including simple and complex lipids, carbohydrates, proteins and peptides, and nucleic acids (364). There is evidence of synergy between both opsonic and non-opsonic receptors. Binding of microbial ligands to phagocytic receptors activates complex signalling cascades, cytoskeletal remodelling, and membrane delivery from intracellular compartments, leading to engulfment of the pathogen into a vacuole composed of the plasma membrane (55). Receptor ligation and actin polymerization are linked by the action of Rho family small G proteins, including Rac1, RhoA, and Cdc42, which function as molecular switches (71). Inactive Rho proteins are bounding to guanine dissociation inhibitors (GDIs); guanine nucleotide exchange factors (GEFs) activate Rho proteins by exchanging GDP for GTP, while GTPase-activating proteins (GAPs) mediate GTP hydrolysis and inactivation (71). Rho-family GTPases recruit and activate downstream effectors, which in turn activate the actin-nucleating Arp2/3 complex to mediate actin remodelling (181).

1.3.1.1 Preventing bacterial internalization

Bacteria unequipped to survive within phagocytic cells have developed methods to avoid internalization. *S. aureus*, *E. coli*, and *P. aeruginosa* can all prevent opsonic phagocytosis; *S. aureus* interferes with the activation of complement via a secreted inhibitor (389), *E. coli* produces an outer membrane protein which binds to a regulator of complement (367), and *P. aeruginosa* secretes enzymes able to directly degrade complement proteins (203). Group B streptococci prevent scavenger receptor-mediated non-opsonic phagocytosis by producing a polysaccharide capsule which masks a surface lipoprotein ligand (21). Pathogenic *Yersinia* spp. employ a T3SS to translocate multiple Yop effector proteins into phagocytic cells; YopO acts as a GDI, while YopE functions as a GAP, both of which modulate the activity of Rho GTPases to prevent bacterial uptake (7). Other bacteria, including *Clostridium* spp., produce binary toxins capable of self-mediated delivery to the cytosol and ADP-ribosylation of actin, which inhibits actin polymerization and destroys the actin cytoskeleton, preventing uptake of bacteria (11). *Clostridium* spp. also produce toxins capable of covalent modification and inactivation of Rho (37). *P. aeruginosa* and *Aeromonas salmonicida* produce bifunctional T3-secreted effectors with both RhoGAP and ADP-ribosyltransferase activity (269, 303), while the T6SS effector VgrG1 from *Aeromonas hydrophila* is also able to ADP-ribosylate actin, leading to depolymerization of actin and apoptosis (467).

1.3.1.2 Promoting bacterial internalization

Bacteria have also developed methods to enter non-phagocytic cells, either by direct invasion – the zipper mechanism – or by promoting phagocytosis through manipulation of host signalling – the trigger mechanism. *Listeria monocytogenes* employs the zipper mechanism, mimicking natural phagocytosis through the binding of bacterial surface proteins to cell-adhesion-associated receptors; the clustering of ligand-receptor complexes induces actin polymerization and

membrane extension, leading to engulfment of the pathogen (101). While the zipper mechanism is dependent on adhesion, bacteria utilizing the trigger mechanism rely on translocation of effectors which directly activate the actin cytoskeleton remodelling necessary for phagocytic uptake. *Salmonella* mediates internalization into non-professional phagocytes via T3-secreted effector SopB, which mediates actin remodelling by activating SGEF, an exchange factor for RhoG (361). Similarly, *B. pseudomallei*, *Shigella flexneri,* and *E. coli* (both EHEC and EPEC) secrete effectors capable of acting as GEFs for the Rho family GTPases, while *Bordetella* spp., *E. coli*, and *Y. pseudotuberculosis* produce toxins that covalently modify Rho proteins such that they are constitutively active (10).

1.3.2 Phagosomal maturation

Following internalization of a bacterium into a plasma-membrane-derived vacuole, the nascent phagosome matures from early phagosome to late phagosome to phagolysosome; the progression is mediated by sequential interactions with subcompartments of the endosomal pathway (155). Interaction between the nascent phagosome and the early, or sorting, endosome creates the early phagosome, characterized by a mildly acidic pH (6.1 – 6.5) and an active form of the Rab5 GTPase. Rab5 recruits effector proteins, including tethering protein early endosome antigen 1 (EEA1), which facilitates the fusion of phagosome and early endosome, and Vps34, a kinase that generates phosphatidylinositol-3-phosphate (PI(3)P); PI(3)P anchors EEA1 to the cytosolic face of the phagosomal membrane (155). Active Rab5 also recruits Mon1, which in turn recruits Ccz1; the Mon1-Ccz1 complex displaces the Rab7 GDI, recruiting Rab7 to the phagosomal membrane and marking the transition to the late phagosome. As markers of the early phagosome are lost, the late phagosome becomes characterized by a lower pH (5.5 - 6.0) due to the acquisition of V-ATPases, as well as enrichment in hydrolytic enzymes, lysobisphosphatidic acid

(LBPA), mannose-6-phosphate receptors (M6PR), and lysosomal-associated membrane proteins (LAMPs). Active Rab7 recruits Rab-interacting lysosomal protein (RILP), an adaptor for microtubule-associated motor protein dynein, which can bring together late phagosomes and lysosomes. Late endosomal markers such as LBPA and M6PR are removed during the transition to a fully formed phagolysosome, a highly acidic (pH < 5.5) vacuole characterized by LAMPs and V-ATPases which employs both oxidative and non-oxidative killing mechanisms. The phagolysosome is rich in antimicrobial peptides and active hydrolases, including lipases, proteases, nucleases, and glycosidases; recruited NADPH oxidase and inducible nitric oxide synthase generate reactive oxygen and nitrogen species, and nutrients are scavenged by molecules such as lactoferrin, creating an environment hostile to continued microbial presence (55).

1.3.3 Bacterial strategies for intracellular survival

Pathogens have developed unique strategies for surviving within phagocytic cells. True intracellular pathogens survive and replicate either in the cytosol or within a membrane-bound organelle, in most cases attempting to avoid the harsh lysosomal environment. Bacteria remaining within a membrane-bound vacuole utilize several different survival strategies to evade or adapt to the lysosome.

1.3.3.1 Escape to the cytosol

Bacteria such as *B. pseudomallei* (464), *S. flexneri* (359), *L. monocytogenes* (372), and *Rickettsia prowazekii* (509) rapidly escape the nascent phagosome and invade the cytosol, where the pH is relatively neutral, nutrients are plenteous, and host cell defenses are less effective (428). Generally, this exit strategy is environmentally triggered, but bacterially driven, caused by destabilization of the phagosome membrane by bacterial gene products and/or permeabilization via pores and enzymes (377). Once in the cytosol, these bacteria can replicate, and,

by polymerizing eukaryotic actin, form comet tails to aid in intracellular and intercellular locomotion (377). Bacterial actin-based motility (ABM) can force membrane protrusions into neighbouring cells, leading to the engulfment of the bacteria into a double-membrane vacuole in a process termed paracytophagy; rapid ABM facilitates evasion of host immune responses within the cell, as well as facilitating intercellular spread without exposure to the extracellular milieu (463).

1.3.3.2 Arrest of phagosomal maturation

Some bacteria, such as *Mycobacterium tuberculosis* (162), and *S. enterica* serovar Typhimurium (28), arrest the maturation of the phagosome, rendering the environment more hospitable for bacterial growth. *M. tuberculosis*-containing vacuoles maintain an early-endosome-like environment, preventing the transition to late phagosome by retaining the Rab5 GTPase while excluding EEA1 and Rab7 (162). The lipoglycan mannose-capped lipoarabinomannan can also affect vacuole dynamics (438). In contrast, *S.* Typhimurium halts phagosome maturation at a later stage through the action of T3-secreted effectors; though the lumen acidifies and acquires late endosomal markers Rab7 and LAMP1, the *Salmonella*-containing vacuole (SCV) does not accumulate mature hydrolases, nor does it fuse with lysosomes (27). Although a multiplicity of effectors are likely involved in SCV trafficking, a T3-secreted phosphoinositide phosphatase reduces the negative charge on the phagosomal membrane, which affects the trafficking of endocytic proteins and prevents lysosomal fusion (28). Thus, by arresting phagosomal maturation, *Mycobacterium* spp. and *Salmonella* spp. are spared the full microbicidal effects of the lysosome.

1.3.3.3 Diversion from the endosomal pathway

While some bacteria modify the phagocytic vacuole such that it is no longer recognized as part of the endosomal pathway, others manipulate the host cell

and take up residence in an existing organelle such as the endoplasmic reticulum (ER). Following phagocytosis, *Legionella pneumophila* persists within a vacuole that transiently recruits mitochondria, followed by ER exit vesicles; eventually, the *Legionella*-containing vacuole (LCV) takes on the character of the rough ER, suitable for bacterial replication (211). Successful remodeling of the LCV requires T4-mediated secretion of a multitude of effectors affecting host signaling, membrane trafficking, and protein synthesis (182). The *Brucella abortus*-containing vacuole (BCV) acquires early endosomal markers EEA1 and Rab5, acidifies, and acquires LAMP1, but not Rab7, thus avoiding lysosomal fusion; similar to the LCV, the BCV interacts with ER exit vesicles in a T4SS-dependent manner, acquiring characteristics of the ER permissive to bacterial replication (364). *Chlamydia trachomatis* produces proteins that insert into the nascent phagosomal membrane, interacting with Rab proteins to direct intracellular trafficking (68); *C. trachomatis* induces Golgi fragmentation, forming Golgi ministacks that accumulate around the vacuole to promote sphingolipid acquisition and bacterial replication (192).

1.3.3.4 Delay of phagosomal maturation for adaptation to the phagolysosome

Finally, some organisms, including *Coxiella burnetti* (507) and *Leishmania donovani* (273), delay the maturation of the phagosome while evolving to a form suitable for survival and replication within an acidic environment. Previous work in our laboratory has examined the maturation of the *B. cenocepacia*-containing vacuole (BcCV) in the presence of metabolically-active bacteria, demonstrating rapid association with the early endosome, followed by prolonged absence of phagosomal markers, then eventual fusion with the late endosome and lysosome (252); bacterial replication has been noted 24h p.i. (185). In other organisms, mechanisms of delay are thought to include regulation of surface glycoconjugants, release of effectors through a secretion system, and blebbing of outer membrane vesicles (OMV). Though not a bacterium, *L. donovani* delay of

phagosomal maturation has been well characterized. *L. donovani* employs lipophosphoglycan (LPG) to alter lipid microdomains in the phagosomal membrane, inhibiting fusion with late endocytic organelles (124, 125); LPG also prevents assembly of a functional NADPH oxidase complex on the phagosomal surface (274). In the presence of LPG, the *L. donovani*-containing vacuole retains RhoGTPase Cdc42, causing an accumulation of periphagosomal F-actin (272). Assembly of an F-actin coat delays phagosomal maturation by physically preventing vesicle docking; this association is transient, as F-actin is not found on mature phagolysosomes (267). *C. burnetii* is the only true bacterium known to delay phagolysosomal fusion as a prelude to replication in an acidified, proteolytically-active environment (205). Early, transient interactions with the autophagy pathway have been postulated to contribute to *C. burnetii* delay of phagosomal maturation (388). During early stages of infection, *C. burnetii* upregulates expression of a T4SS (332) capable of translocating effectors into the host (76); many effectors contain eukaryotic-like domains capable of modifying host proteins and modulating signalling pathways (76). Although *L. pneumophila* employs multiple strategies for intracellular survival, the bacterium has been shown to delay autophagosomal maturation (221); it is thought that this delay involves developmental regulation and shedding of LPS (430) and the release of OMV (151), though modulation of phagosomal membrane composition (436) and host trafficking via T4-secreted effectors (142, 528) are also likely.

1.3.4 The immune response

Ligand binding to PRRs initiates the immune response by activating a signalling cascade, leading to gene expression, biosynthesis, and secretion of inflammatory mediators, including cytokines and chemokines. In particular, bacterial ligands binding to TLRs activate type I interferons (IFN) and/or transcription factor NF-κB (165), which controls expression of a wide variety of genes involved in the immune response, including cytokines TNF-α, IL-6, IL-8, and IL-2 (33).

Membrane-associated receptors, both surface and endosomal, are complemented by a family of cytosolic PRRs, deemed NOD-like (NLRs), which detect microbial products present in the cytosol following effector injection or escape from the phagosome (127). NLRs form a critical component of the inflammasome, a complex containing a NOD-like receptor linked to pro-caspase-1 by an adaptor protein; signalling through the NLR results in cleavage of pro-caspase-1 to the active form (127). Cross-talk between membrane-associated and cytosolic signalling systems is seen in the activation of IL-1β; TLR-mediated NF-κB activation produces pro-IL-1β, which is cleaved to form active IL-1β by NLR-activated caspase-1 (226). Caspase-1 also promotes maturation of other cytokines, including IL-18, and IL-33, an inducer of IL-4, IL-5, and IL-13 (165); caspase 1 also contributes to unconventional protein secretion and pyroptosis (251). Since potential host responses to infection include oxidative stress, inflammasome activation, autophagy, apoptosis, pyroptosis, and necrosis, host survival depends on both control of the infection and activation of a protective response, including downregulation of sensory receptors and induction of stress response mechanisms (364).

1.3.4.1 Pathogen response to inflammation

Controlling or evading the host immune response is essential to the survival of intracellular pathogens. Some bacteria have modified forms of TLR ligands, thereby preventing activation of the immune response; *Campylobacter jejuni, Helicobacter pylori,* and *Bartonella bacilliformus* all produce flagellin that is not recognized by TLR5 (17). Other bacteria exploit TLR2 signalling to induce immunosuppression: *M. tuberculosis* produces a lipoprotein that persistently activates TLR2, thus inhibiting IFN-γ production (160), whereas *Yersinia* spp. secrete V antigen to stimulate production of IL-10, and resultant suppression of IFN-γ and TNF (441). Inflammasome activation can be inhibited by microbial products, including *M. tuberculosis* zinc metalloprotease Zmp1 (315), and *P.*

aeruginosa T3-secreted exoenzymes ExoU (471) and ExoS (169). *Yersinia* spp. also produce a range of T3-secreted effectors that can interfere with the immune response; YopP and YopJ prevent activation of NF-κB (394, 540), while YopE and YopT inactivate Rac1, causing inhibition of caspase-1 activation and IL-1β secretion (424), and YopK prevents NLR recognition of the T3SS, thus preventing inflammasome activation (61). *L. pneumophila* manipulates expression of the inflammasome adaptor ASC (3), thereby downregulating inflammasome activation; ASC is also able to modulate expression of NF-κB (462). *L. pneumophila* also inhibits host protein synthesis via T4-secreted effectors, causing prolonged activation of NF-κB due to decreased synthesis of inhibitor IκB (159). *Chlamydia* spp. produce a protease which degrades a subunit of NF-κB (248), thereby reducing host sensitivity to proinflammatory stimuli (85).

1.3.5 Cell death

The three principal mechanisms of host cell death are apoptosis, necrosis, and pyroptosis, which differ in initiation and outcome; under certain conditions, autophagy can also lead to cell death. Apoptosis is an immunologically silent form of programmed cell death characterized by chromosome fragmentation and nuclear condensation, cytoplasmic shrinkage, plasma membrane blebbing, and eventual cell dispersion in membrane-bound fragments known as apoptotic bodies with exposed phosphatidylserine residues (355). Apoptosis may be induced by both intrinsic and extrinsic pathways, the former caused by the release of pro-apoptotic proteins from the mitochondria, and the latter involving ligand binding to cell surface-associated death receptors; both pathways activate a caspase-signalling cascade, ultimately leading to the activation of executioner caspase 3, which cleaves cellular substrates and induces apoptosis (250). Release of pro-apoptotic proteins through the mitochondrial outer membrane occurs through the Mitochondrial Apoptosis-induced Channel, and is regulated by

the Bcl-2 family of proteins; the Bcl-2 family includes anti-apoptotic effectors such as Bcl-2, pro-apoptotic effectors such as Bax, Bak, and Bim, and pro-apoptotic BH3-only sentinels such as Bad (121).

Necrosis is a caspase-independent mode of cell death characterized by increased cell volume and permeability, but uncondensed DNA content; necrosis is also characterized by an oxidative burst, mitochondrial hyperpolarization, and lysosomal membrane permeabilization, releasing hydrolytic enzymes (44). Due to membrane permeabilization, necrotic cells spill intracellular contents into the extracellular milieu, triggering a significant inflammatory response and tissue damage (250). Though originally considered an accidental form of death due to severe cellular insult, necrosis can be regulated by ligand-bound death receptors in the absence of caspase activation; such a mechanism, employing the RIP1 kinase, is termed necroptosis (494).

Pyroptosis is a form of programmed cell death initiated by excessive inflammasome activation, and thus typically induced by infection (54). Pyroptosis is characterized by cellular DNA damage, cell lysis, and inflammatory cytokine release; activation of caspase-1 causes membrane perturbations and an influx of extracellular calcium, leading to lysosome exocytosis (45). Pyroptosis is highly inflammatory, due to the release of inflammatory cytokines, antimicrobial lysosomal contents, and potentially degraded microbial products suitable for presentation as antigens by neighbouring cells (45).

Though autophagy is typically a protective response to cellular starvation and stress, when excessive, the process can result in cell death. The kinase mTOR and ATG/Beclin proteins regulate autophagosome formation, resulting in the engulfment of a portion of the cytoplasm into a double membrane-bound organelle. The autophagosome, characterized by marker LC3-II, fuses with lysosomes to degrade phagosomal contents, including damaged organelles or intracellular bacteria (54). Autophagy can be induced by TLR ligands, and can thus be used to control bacterial infections, including those that can alter the classical phagosomal pathway (54). In comparison to necrosis and pyroptosis,

by very nature, autophagy is an immunologically silent death process, similar to apoptosis.

1.3.5.1 Bacterial influence on host cell death

Bacterial modulation of host cell death is common; intracellular pathogens seek to inhibit host death to promote bacterial survival and replication, while other pathogens evade macrophage-mediated killing by rapidly inducing host cell death. Bacterial targetting of caspases, Bcl-2 family proteins, and survival pathways (52) can inhibit cell death. Intracellular *C. burnetii* blocks apoptosis via T4-secreted effector AnkG, which prevents cytochrome c release from mitochondria (281, 282). *C. burnetii* also recruits anti-apoptotic Bcl-2 (498) and activates prosurvival kinases Akt and Erk1/2 (508). T4SS effectors of *L. pneumophila* are capable of specific interaction with and neutralization of proapoptotic Bcl-2 family proteins (36), induction of NF-κB nuclear translocation (5), and upregulation of antiapoptotic genes (276). *C. trachomatis* prevents apoptosis by degrading multiple pro-apoptotic BH3-only proteins (534), and activating Akt, leading to sequestration of proapoptotic Bad (501).

Some pathogens may induce host cell death to eliminate host cells required to mount an immune response. *Clostridium septicum* produces a pore-forming α-toxin which induces programmed necrosis (233), while *H. pylori* secretes a protein which induces apoptosis by binding to the death receptor Fas (15). *Yersinia* and *Salmonella* promote apoptosis in naïve macrophages by T3-secreted effector inhibition of NF-κB activation (422) (94); however, infection of activated macrophages with the same bacteria results in pore formation in the host membrane, calcium influx, and lysosome exocytosis in a caspase-1 dependent manner (45). Following escape from the phagosome, intracellular *S. flexneri* induces pyroptosis in macrophages (544, 545) via a T3SS-secreted effector IpaB that activates caspase-1 (193). *L. monocytogenes* induces pyroptosis through the activation of multiple inflammasomes by bacterial ligands,

including DNA (526); oddly, *L. monocytogenes* infection also causes metabolic signalling which inhibits activation of caspase-1 (542), potentially balancing pro- and anti-pyroptotic effects in macrophage infection. *L. pneumophila* induces apoptosis in a T4SS-dependent manner at late stages of infection (414); however, Legionnaire's disease is typically characterized by extensive inflammation, and *L. pneumophila* can also induce pyroptosis (440), and necrotic macrophage death, especially with high bacterial load (333). Intracellular pathogens may induce cell death at the end of their replication cycle to facilitate escape from the macrophage. In *M. tuberculosis*, induction of caspase-independent necrosis occurs in response to a high bacterial burden (259, 518). Similarly, *Francisella tularensis*, which escapes to the cytosol, prevents inflammasome activation and modulates caspase-3 activity to inhibit apoptosis until late stage infection (23, 415).

Bacteria have also developed strategies to counteract autophagy. *M. tuberculosis* is able to non-specifically inhibit autophagy, which also inhibits necrotic-like host cell death (437). VirG, an intracellular ABM protein produced by *S. flexneri* binds Atg5 to induce autophagy; *S. flexneri* prevents induction of the autophagy signalling cascade by T3-secretion of an effector to competitively bind VirG (351). *L. monocytogenes* evades autophagy by recruiting host cytoskeleton proteins to the bacterial surface via ABM protein ActA (537). Conversely, *Y. pseudotuberculosis* activates autophagy, but prevents acidification to survive and replicate within an autophagosome (330).

1.4 Identification of intracellular gene expression by prokaryotic cells

While many studies have examined the transcriptional response of bacteria to *in vitro* host-like conditions, true understanding of bacterial response to internalization by macrophages requires analysis of global transcription by intracellular bacteria. Such studies are inherently challenging, being hampered

by the quality and quantity of microbial mRNA compared to host RNA, especially at a lower multiplicity of infection (MOI) and early time points p.i.. However, a lower MOI is desirable for physiological relevance, and though pathogen numbers are low and transcriptional changes often subtle, early time points p.i. represent the greatest potential for therapeutic intervention (466). Typical bacteria contain 0.05-0.10 pg of RNA, of which mRNA comprises only 4% and has a half-life of mere minutes (246, 376). Because bacterial gene expression can change rapidly, chemical disruption of the host cell and/or bacterial purification from the cell is impractical; thus, total RNA from both host and pathogen is extracted, from which bacterial mRNAs must be purified.

1.4.1 Bacterial mRNA purification and enrichment

In the last decade, a wide variety of strategies have been employed to separate mixed populations of prokaryotic and eukaryotic transcripts in an infectious context (246). Some enrichment strategies employ commercial kits to selectively remove host ribosomal transcripts and polyadenylated eukaryotic mRNA; though simple and relatively swift, such practices risk loss of pathogen RNA, particularly small transcripts (466). Other strategies, including differential expression using customized amplification libraries (DECAL) (13), coincidence cloning (CC) (26), and selective capture of transcribed sequences (SCOTS) (180), rely on purification of bacterial sequences from a pool of mixed eukaryotic and prokaryotic cDNAs.

DECAL first requires construction of a customized amplification library (CAL) containing size-fractionated genomic DNA lacking ribosomal sequences; each library fragment is ligated to PCR adaptors, allowing total library amplification. Total RNA from the infection system of interest is reverse-transcribed with biotinylated primers; CAL fragments that hybridize to cDNA are retained with streptavidin-coated magnetic beads, and can be PCR amplified for infection-proportional representation of prokaryotic-specific cDNA sequences (13).

DECAL has been applied to analysis of the *Borrelia burgdoferi* transcriptome in the central nervous system of infected non-human primates (341).

Coincidence cloning is a recent development wherein total cDNA from infected tissue is hybridized with a great excess of bacterial genomic DNA, followed by selective PCR amplification. RNA from the system of interest is reverse-transcribed using nonanucleotides linked to a 5' 25-nucleotide constant region containing a restriction endonuclease site. Both genomic DNA and cDNA are digested with the restriction endonuclease, and each pool is ligated to a separate suppression adaptor. When the two pools are hybridized together, DNA fragments unique to one set – eukaryotic sequences – can only self-hybridize, forming homo-duplexes, while sequences common to both pools – presumably prokaryotic – form hybrid duplexes. While hybrid duplexes can be PCR-amplified, the matching 5' adaptors in homo-duplexes cause PCR-selective suppression. CC has been used with *M. tuberculosis*-infected murine lung tissue; random sequencing of CC-selected amplicons showed 30% murine sequences and 70% *M. tuberculosis* sequences, while pyrosequencing demonstrated greater than 1000-fold increase in bacterial transcripts following a single round of CC (26).

SCOTS shares features with both DECAL and CC. Transcripts isolated from the system of interest are reverse-transcribed and double-stranded with random nonanucleotides linked to a 5' 25-nucleotide linker sequence containing a restriction site. Microbial cDNAs can be positively selected by hybridization to biotinylated bacterial genomic DNA fragments pre-blocked with ribosomal sequences; hybrids retained via streptavidin-coated magnetic beads can be PCR amplified with a linker-specific primer (180). Successive rounds of SCOTS amplification can enrich low abundance transcripts. SCOTS can also be used to create multiple libraries for a single species under different conditions or at different infection time points, each library bearing a unique linker sequence. This technique has been used to identify genes expressed by several intracellular

pathogens, including *M. tuberculosis* (180), *S. enterica* serovar Typhi (109, 149), *E. coli* O157:H7 (365), *Ehrlichia ruminantium* (140), and *L. pneumophila* (148).

1.4.2 Analysis of intracellular transcription

Following purification and amplification of the infectious prokaryotic transcriptome, there are different methods available for transcript identification. Several techniques, including SCOTS competitive enrichment (SCOTS-CE) (180) and suppressive subtractive hybridization (128), allow isolation and enrichment of sequences preferentially transcribed *in vivo*; this requires creation of a second cDNA pool representing sequences transcribed *in vitro*. For SCOTS-CE, the capturing chromosome is pre-blocked with both rDNA and an excess of cDNA from non-macrophage-exposed bacteria, such that only infection-specific cDNAs will be captured. By using different linkers for each cDNA pool, infection-specific cDNAs can be selectively PCR-amplified. Restriction sites present in the linker allow cDNA cloning into a vector for single-transcript analysis.

Practical global transcriptome analysis requires a high-throughput method. Bacterial DNA microarrays hybridized to initial macrophage-infected cDNA show few and faint signals; however, purification and amplification of prokaryotic-specific transcripts through one of the above methods produces cDNA probes suitable for microarray analysis (149, 341). Recently, *B. cenocepacia* J2315 genomic microarrays have been developed, leading to an explosion in *B. cenocepacia* transcriptomic research (133). More recently, high-throughput massively parallel sequencing technologies have led to direct sequencing of prokaryotic cDNA pools in a technique known as RNA-seq (43, 105), which has been used to examine the intra-amoebae transcriptome of *L. pneumophila* (517); despite removal of ribosomal sequences, of the total sequences from two broth cultures and two infection time points, nearly half represented amoebae transcripts. RNA-seq has been applied to *in vitro* investigation of *B. cenocepacia* niche-specific gene expression (535). High-throughput pryosequencing has also

been applied to the downstream analysis of the CC amplicon derived from *M. tuberculosis* infecting murine lung tissue (26). Both DNA hybridization-based microarrays and massively parallel next-generation sequencing present advantages in the analysis of cDNA pools: Microarrays are low cost, and have rapid multiple-sample throughput, low requirements for initial starting material, and a well-established bioinformatic infrastructure (466); sequence-based analyses are less subject to experimental design artefacts, and have digital rather than analog signal quantitation, offering greater sensitivity and inter-platform reproducibility (435).

1.5 Hypothesis and research objectives

The overall hypothesis of this work is that *B. cenocepacia* adapts to persist within murine macrophages; this adaptation is a result of changing gene expression, and may involve classical multi-gene secretion systems. The first hypothesis was addressed by surveying intracellular transcription at a global level, and the second by a reductionist approach involving the deletion of secretion systems to examine individual and combined contributions to the intracellular behaviour of *B. cenocepacia*.

The results of this work are divided into two separate sections. The first section details the optimization of the SCOTS technique for *B. cenocepacia*, and its subsequent application to *B. cenocepacia*-infected murine macrophages. Global gene expression is described at an early post-infection stage in an attempt to identify gene products involved in avoidance of macrophage microbicidal activity and adaptive response to the phagosomal environment. The second section describes the contribution of the T2SS, T3SS, T4SS-1, T4SS-2, and T6SS both individually and collectively, in internalization by macrophages, intracellular replication, and host-directed cytotoxicity.

Chapter 2

2 Materials and Methods

2.1 Bacterial strains, plasmids, media, and growth conditions

Bacterial strains and plasmids used in this study are listed in Tables 1 and 2, respectively. Bacteria were grown at 37^{o}C in Luria-Bertani (LB; Difco) broth with agitation or on LB plates with 1.6% Bacto agar. *Escherichia coli* cultures were supplemented as required with 20 µg/ml tetracycline, 50 µg/ml trimethoprim, and 40 µg/ml kanamycin; *B. cenocepacia* cultures were supplemented as required with 100 µg/ml tetracycline and 100 µg/ml trimethoprim. Plasmids were conjugated into *B. cenocepacia* by triparental mating at 37^{o}C using *E. coli* DH5α carrying helper plasmid pRK2013 (153). *E. coli* donor and helper strains were selected against with 50 µg/ml gentamicin or 100 µg/ml ampicillin and 25 µg/ml polymyxin B. To determine growth rates of parental and mutant strains of *B. cenocepacia*, overnight cultures were inoculated into fresh medium at a starting optical density at 600 nm (OD_{600}) of 0.01. Growth rates with high-amplitude shaking were determined in 100-well microtiter plates via Bioscreen C automated microbiology growth curve analysis system (MTX Lab Systems).

2.2 General molecular techniques

DNA manipulations were performed as in (413). DNA was amplified by PCR using a PTC-221 DNA engine (MJ Research) with *Taq* DNA polymerase or HotStar DNA polymerase (Qiagen). Amplification of *B. cenocepacia* DNA was aided by the inclusion of Qiagen Q solution. DNA sequencing was performed at the York University Core Molecular Biology and DNA Sequencing Facility in Toronto, Ontario, Canada. Sequence analysis was done by BLAST. Restriction enzymes, T4 DNA ligase (Roche Diagnostics) and Antarctic alkaline

Table 1: Bacterial strains and cell lines

Strain or cell line	Relevant characteristics	Source or reference
Escherichia coli		
DH5α	F^-, f80 *lacZ* DM15 D(*lacZYA-argF*) *U169 endA1 recA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1* D*gyrA96 relA1*	Laboratory stock
SY327	*araD* D(*lac pro*) *argE*(*Am*) *recA56 rifr nalA*, l *pir*	(328)
Burkholderia cenocepacia		
J2315	epidemic strain ET12 clone, CF clinical isolate	P.A. Sokol
K56-2	epidemic strain ET12 clone, CF clinical isolate	BCRRC[a]
MH1K	K56-2 ΔBCAL1674-6	(185)
JST19	MH1K ΔBCAL0124	This study
JST71	MH1K ΔBCAS0186	This study
JST75	MH1K ΔBCAM2837	This study
JST128	MH1K ΔBCAL1726	This study
JST130	MH1K ΔBCAM0276	This study
JST132	MH1K ΔBCAM0411	This study
JST134	MH1K ΔBCAM2446	This study
JST136	MH1K ΔBCAM0434-5	This study
JST190	MH1K ΔBCAL0340	This study
JST194	MH1K ΔBCAM1679	This study
JST89	K56-2 ΔT2SS ΔBCAL1674-6	This study
JST40	MH1K ΔT3SS	This study
JST17	K56-2 ΔT4SS-1 ΔBCAL1674-6	This study
JST39	MH1K ΔT4SS-2	This study
JST53	K56-2 ΔT6SS ΔBCAL1674-6	This study
JST112	K56-2 ΔT2SS ΔT3SS ΔT4SS-1 ΔT4SS-2 ΔT6SS ΔBCAL1674-6	This study
JST178	MH1K ΔBCAL3516	This study
JST182	MH1K ΔpBCA025	This study
JST184	MH1K ΔBCAM0333	This study
JST162	K56-2 ΔBCAL0343 ΔBCAL1674-6	This study
Macrophage cell lines		
ANA-1	C57BL/6 murine bone marrow-derived macrophage cell line	(103)
RAW 264.7	BALB/c murine macrophage cell line	ATCC[b]

[a] *B. cepacia* complex Research and Referral Repository for Canadian CF Clinics
[b] American Type Culture Collection

Table 2: Plasmids

Plasmid	Relevant characteristics	Source or reference
pRK2013	RK2 derivative, Kan^R, *mob*$^+$, *tra*$^+$, ori_{colE1}	(153)
pUC19	ori_{pMB1}, Ap^R, *mob*$^+$, P_{lac}	(532)
pJT25	pUC19 with K56-2 rDNA (23S, 16S, 5S), Ap^R	This study
pGPI-SceI	ori_{R6K}, Tp^R, *mob*$^+$, carries I-SceI cut site	(156)
pDAI-SceI -SacBN	ori_{pBBR1}, Tet^R, *mob*$^+$, P_{dhfr}, *sacB*, encodes I-SceI endonuclease	(185)
pDA12	ori_{pBBR1}, Tet^R, *mob*$^+$, P_{dhfr}	(25)
pDA42	pDA12, *eGFP*	(25)
pJT31	pGPI-SceI with regions flanking BCAL0124	This study
pJT41	pGPI-SceI with regions flanking BCAS0186	This study
pJT42	pGPI-SceI with regions flanking BCAM2837	This study
pJT45	pGPI-SceI with regions flanking BCAL1726	This study
pJT46	pGPI-SceI with regions flanking BCAM0276	This study
pJT47	pGPI-SceI with regions flanking BCAM0411	This study
pJT48	pGPI-SceI with regions flanking BCAM2446	This study
pJT49	pGPI-SceI with regions flanking BCAM0434-5	This study
pDelbcsM	pGPI-SceI with regions flanking BCAL0340	(24)
pJT72	pGPI-SceI with regions flanking BCAM1679	This study
pMH306	pGPI-SceI with regions flanking *gspDEF* (T2SS)	M. Hamad
pJT43	pGPI-SceI with regions flanking T3SS (BCAM2040-57)	This study
pJT30	pGPI-SceI with regions flanking T4SS-1 (pBCA017-59)	This study
pJT44	pGPI-SceI with regions flanking T4SS-2 (BCAM0324-35)	This study
pDelT6SS	pGPI-SceI with regions flanking T6SS (BCAL0333-52)	(24)
pJT59	pGPI-SceI with regions flanking BCAL3516	This study
pJT61	pGPI-SceI with regions flanking pBCAL025	This study
pJT62	pGPI-SceI with regions flanking BCAM0333	This study
pMH304	pGPI-SceI with regions flanking BCAL1674-6	(185)
pJT57	pDA12, BCAL0124	This study
pJT71	pDA12, BCAL0340	This study
pJT52	pDA12, BCAL1726	This study
pJT54	pDA12, BCAM0411	This study
pJT62	pDA12, BCAL3516	This study
pJT67	pDA12, BCAM0333	This study
pJT69	pDA12, pBCA025	This study
pHcp	pDA12, BCAL0343	(25)

phosphatase (New England Biolabs) were used as recommended by manufacturers. Transformation of *E. coli* SY327 and DH5α was done by the calcium chloride protocol (93).

2.3 Cell culture and infection

Macrophage cell lines listed in Table 1 were maintained in Dulbecco's modified Eagle medium (DMEM; Wisent) supplemented with 10% fetal bovine serum (FBS; Wisent) at 37^{o}C in a 95% humidified atmosphere with 5% CO_2. For macrophage infection, cells were seeded in a 12-well plate at 1.25 x 10^5 cells per well or a 6-well plate at 2.5 x 10^5 cells per well and grown 15 h. Bacterial cultures grown at 37^{o}C for 16 h were washed twice and resuspended in DMEM-10% FBS. Macrophage monolayers were washed with phosphate-buffered saline (PBS; Wisent). Bacteria were added at a multiplicity of infection (MOI) of 50:1 or 10:1 in 1-2 ml DMEM-10% FBS. Plates were centrifuged 1 min at 300 x *g* and incubated at 37^{o}C, 95% humidity and 5% CO_2.

2.4 RNA extraction, cDNA synthesis, and amplification

RNA was extracted from two 6-well plates containing equal numbers of *B. cenocepacia*, differing only in the presence (intracellular (I) RNA) or absence (non-macrophage-exposed (NME) RNA) of macrophages. At 4 h post-infection, macrophage monolayers were washed three times with PBS and lysed in 1 ml cold deionized H_2O. NME cells were resuspended and collected. For each condition, cells from 6 wells were resuspended in 1 mg/ml lysozyme (Roche). Total RNA was extracted using TRIzol (Invitrogen) and treated with RNase-free DNase (Roche) according to the manufacturer's instructions. RNA purity, integrity, and concentration were determined by PCR, agarose gel electrophoresis, and A_{260}/A_{280} spectrophotometer readings, respectively. A 5 µg

RNA sample from each condition (“intracellular” and “NME”) was converted to first-strand cDNA by random priming with Transcriptor reverse transcriptase (Roche) according to the manufacturer's specifications. Primers had a defined 5' terminal sequence and a 3' random nonamer; different terminal sequences were used for intracellular (I-3025) and NME RNA (N-3017) (Table 3). cDNAs were double-stranded using Klenow fragment (Roche) as described in (164); cDNA libraries were amplified for 25 cycles using defined primers I-3032 (intracellular) or N-3033 (NME).

2.5 Selective capture of transcribed sequences

The SCOTS protocol was carried out as described (110). In brief, 0.3 µg of denatured, sonicated, biotinylated *B. cenocepacia* K56-2 chromosome were mixed with 5 µg denatured ribosomal DNA fragments (sonicated pJT25; Table 2) and hybridized 30 min at 68^{o}C to pre-block rRNA-encoding DNA regions. 3 µg of cDNA were denatured and re-annealed 30 min at 68^{o}C to remove abundant transcripts. cDNA and chromosomal DNA were combined and hybridized 24 h at 68^{o}C. Chromosome-cDNA hybrids were removed from solution with streptavidin-coated magnetic beads (Invitrogen). Captured cDNA was eluted, precipitated, and amplified using library-specific defined primers (Table 3). For each condition, 10 parallel first-round reactions were done to maximize the cDNA sample diversity; the resultant cDNA was pooled for each condition and two subsequent rounds of SCOTS carried out. The final cDNA libraries were used both for competitive enrichment hybridization and as probes for *B. cenocepacia* microarrays. A detailed protocol may be found in Appendix 2.

Table 3: Oligonucleotide primers

Primer name	5'-3' primer sequence[a]	Restriction enzyme[b]
Cloning primers		
16S-F	TTTTTCTAGAGAACTGAAGAGTTTGATCCTGGCTCAG	XbaI
16S-R	TTAATCTAGAAAGGAGGTGATCCAGCCGCACC	XbaI
23S-F	TTTTGAATTCGGTCAAGCGAACAAGTGCATGTG	EcoRI
5S-R	TTTTTGGATCCGCCTGACGATTACCTACTTTCACAC	BamHI
SCOTS primers		
I-3025	TGCTCTAGACGTCCTGAATTCCGTANNNNNNNNN	XbaI
N-3017	GATGTGTCGACTCAGGATCCATAGGNNNNNNNNN	BamHI
I-3032	TGCTCTAGACGTCCTGAATTCCGTA	XbaI
N-3033	GATGTGTCGACTCAGGATCCATAGG	BamHI
M13F	GTAAAACGACGGCCAGT	N/A
M13R	CAGGAAACAGCTATGAC	N/A
SCOTS test genes		
T1-I-F	TGCTCTAGACGTCCTGAATTCCGTAATTCTGCTTGTCGAAGAT	XbaI
T1-I-R	TGCTCTAGACGTCCTGAATTCCGTAAGCCGTACATCTTCTCCT	XbaI
T2-I-F	TGCTCTAGACGTCCTGAATTCCGTAACAACCATGACTTTCGAT	XbaI
T2-I-R	TGCTCTAGACGTCCTGAATTCCGTAAAATGCTTTTCTCCATGCT	XbaI
T1-N-F	GATGTGTCGACTCAGGATCCATAGGATTCTGCTTGTCGAAGAT	BamHI
T1-N-R	GATGTGTCGACTCAGGATCCATAGGAGCCGTACATCTTCTCCT	BamHI
T2-N-F	GATGTGTCGACTCAGGATCCATAGGACAACCATGACTTTCGAT	BamHI
T2-N-R	GATGTGTCGACTCAGGATCCATAGGAAATGCTTTTCTCCATGCT	BamHI
T3-N-F	GATGTGTCGACTCAGGATCCATAGGAACGCGAACATCTGGTT	BamHI
T3-N-R	GATGTGTCGACTCAGGATCCATAGGGAAGAATGAATGAACTGC	BamHI
RNA check primers		
cblB F	CGCAATACGTGAAGGCAAT	N/A
cblB R	GCGATACAGCACCTCCTTCT	N/A

β-actin F	CATCGTGGGCCGCTCTAGGCAC	N/A
β-actin R	CCGGCCAGCCAAGTCCAGACGC	N/A
qPCR primers		
BCAM0314-F	GCATCATGGGATTTATGTCG	N/A
BCAM0314-R	GGCCTCTGTTCCGTCGTA	N/A
BCAM2141-F	GAGCAGGTCGGATACAGCTT	N/A
BCAM2141-R	GCGTGTCGTTGAACAGGAT	N/A
BCAM0276-F	AGCATCATGGTCGCAGTAGA	N/A
BCAM0276-R	GCAGGATCGTCTTGCCATA	N/A
BCAS0186-F	TCTCGCAGAAGGATGTGTTG	N/A
BCAS0186-R	AGCCACGTTTCCATGTAAGG	N/A
BCAM1928-F	GTCACGATGAACTCGCAGAT	N/A
BCAM1928-R	GCTGCATCACCTTGACCAT	N/A
rpoD-F	GAGATGAGCACCGATCACAC	N/A
rpoD-R	CCTTCGAGGAACGACTTCAG	N/A
Deletion primers		
BCAL0124-UF	TCCTCTAGAGGTTTACAGACAAATCCTGC	XbaI
BCAL0124-UR	TTTAATCGATAGGTCGAAGGCGTCCGCTG	ClaI
BCAL0124-DR	TTTTGAATTCCTTGTACAGCTTGATCAGAC	EcoRI
BCAL0124-DF	TTTTATCGATCACTGAGCATTTCGCTGGTA	ClaI
BCAS0186-UF	TTTTTCTAGAGTGCTCTATATCGAAGGCTC	XbaI
BCAS0186-UR	TTTAATCGATAGGCATCGGCAGATCCCA	ClaI
BCAS0186-DF	TTTTATCGATCTTACATGGAAACGTGGCTC	ClaI
BCAS0186-DR	TTTTGAATTCGGCGACGTCAGAACGTG	EcoRI
BCAM2837-UF	TTTTTCTAGACTTTGCCGATTGTAATTGCC	XbaI
BCAM2837-UR	TTTTATCGATTGTTTCCAGGACCTGATATC	ClaI
BCAM2837-DF	TTTTATCGATTCGATCCTCGACGAACTC	ClaI
BCAM2837-DR	TTTAGAATTCATCAGCCGGATCGAATCCT	EcoRI
BCAM1679-UF	TTTATCTAGACTGAACACGTTATGTCTTCC	XbaI
BCAM1679-UR	TATTATCGATGACGAGCGCGAGATAGCTGA	ClaI
BCAM1679-DF	TATAATCGATACGCAGTCGGTGATGCTGGT	ClaI
BCAM1679-DR	TATAGAATTCGAAGCGCCAGACCATCAGG	EcoRI
BCAL1726-UF	TTTTTCTAGATATAGTCAAGCATCGGTGGT	XbaI
BCAL1726-UR	TATAATCGATACACGAAACACGACGCACT	ClaI
BCAL1726-DF	CGCACGATTGGTCGCATCGAT	ClaI[c]
BCAL1726-DR	TATAGAATTCCGATTCGATTCCTGCGT	EcoRI
BCAM0276-UF	TAATTCTAGATGGCACACGACCGAACAC	XbaI
BCAM0276-UR	TATTATCGATTCTACTGCGACCATGATGCT	ClaI

BCAM0276-DR	TATAGAATTCCCATTGACTGCGGACGAA	EcoRI
BCAM0411-UF	TATTTCTAGATCATCGAGTGGAACCGCA	XbaI
BCAM0411-UR	TTTTATCGATGACACGACCATCACGAG	ClaI
BCAM0411-DF	TTTTATCGATTTCATCATCGAGCAGGAC	ClaI
BCAM0411-DR	TTTAGAATTCAATGTCCTCGGAAATCTTC	EcoRI
BCAM2446-UF	TTTTTCTAGATCAGAACTTCTGGCGCAG	XbaI
BCAM2446-UR	TTTTATCGATCAGTATCACAACGTCACGC	ClaI
BCAM2446-DF	TTTAATCGATAGATCCTCGGAACCCTTGA	ClaI
BCAM2446-DR	TTCAGAATTCACATACAACATGAACAAGC A	EcoRI
BCAM0434-5-UF	TTTTTCTAGATGACAAGGCAATCACTGAT G	XbaI
BCAM0434-5-UR	TTTTATCGATTTTGTCGAAGTGCTGGTT	ClaI
BCAM0434-5-DF	TTTTATCGATGAAGTCATGCAGCGCAT	ClaI
BCAM0434-5-DR	TTTTGAATTCTCATCTTCATCGTGATTCTC	EcoRI
M2040-T3-UF	TTTATCTAGAATGCCCGCCGCTTCCC	XbaI
M2040-T3-UR	TTTAATCGATAGCAACGCCACGCGCTG	ClaI
M2057-T3-DF	TTTTATCGATTCGACACCGTCAGCAGCA	ClaI
M2057-T3-DR	TTTAGAATTCACTGGCAGCGGTTGTTTTC C	EcoRI
p017-T41-UF	TTTTTCTAGATCGCAGCCGAGCTTGTGCT G	XbaI
p017-T41-UR	TATAATCGATGGCGAAAAACTCGGCCTTG C	ClaI
p059-T41-DF	TTTTATCGATGGAAGAGCCCGTTTGGCTT T	ClaI
p059-T41-DR	TTTTGAATTCCGAATATGCCCGACGTCTC A	EcoRI
M0324-T42-UF	TACATCTAGAGACCACGCAGGACTTCGT	XbaI
M0324-T42-UR	TTTTATCGATTTGTGGATGGATATGAATGA	ClaI
M0335-T42-DF	TTTTATCGATTGTGCGAGCGGACTATCTG	ClaI
M0335-T42-DR	TTTTGAATTCCATTGAACACCACCACTTGC	EcoRI
BCAL3516-UF	TTTATCTAGATGAAAACCGAACAACTGAA C	XbaI
BCAL3516-UR	TAATATCGATCAGCACCGAATACGCGAT	ClaI
BCAL3516-DF	TTTAATCGATACCCAGTGGCTCGACGAT	ClaI
BCAL3516-DR	TTTTGAATTCCTATTTCTGGACGGAAGGTT	EcoRI
BCAM0333-UF	TTTTTCTAGAGCAGATATGGATACAGGTAT	XbaI
BCAM0333-UR	TTTTATCGATTATTAGCGCAAGTACGATGA	ClaI
BCAM0333-DF	TTTTATCGATGTACTCGATCAGACCATCAA T	ClaI
BCAM0333-DR	TTTTGAATTCCTGAAGTGTTGCCTCGAAC	EcoRI
pBCA025-UF	TTTTTCTAGATGCGCCCTACGCTTCCCATT	XbaI

pBCA025-UR	TTTTATCGATGTCGCAGTACCAGTTGGTGCG	ClaI
pBCA025-DF	TTTTATCGATGATGGCCGTGCACAGGCT	ClaI
pBCA025-DR	TTTTGAATTCTGTCCCGGTCAGCACGAACA	EcoRI
Complementation Primers		
BCAL3516-cF	TTTACATATGACACGGGCAAATGGACGATC	NdeI
BCAL3516-cR	TTTTTCTAGACCTTCGTTCGCGTCATCCAC	XbaI
BCAM0333-cF	TTTTCATATGGTGTGACGGTCGACGGC	NdeI
BCAM0333-cR	TTTTTCTAGAGTTTGCGGCTGAAGTGTTGC	XbaI
pBCA025-cF	TTTTCATATGGAACATCTGCGCGGACGGTA	NdeI
pBCA025-cR	TTTTTCTAGAGTTGGTCGGAATACATGCGA	XbaI
BCAL0124-cF	TTTTTTCATATGCCAAAGCAAAGCTCTTTAAC	NdeI
BCAL0124-cR	TTTTTCTAGAGACAGACGCAAGGCGGGA	XbaI
BCAL0340-cF	TTTTTTCATATGTCTCAACGATTTAAAACTCA	NdeI
BCAL0340-cR	TTTTTCTAGAGTGGTTATCGCGTCAGTTGC	XbaI
BCAL1726-cF	TTTTCATATGCCCGATTCGATTCCTGCG	NdeI
BCAL1726-cR	TTTTTCTAGAGTAGATTTCCTGCCCGTC	XbaI
BCAM0411-cF	TTTTTTCATATGGAGTGGAACCGCAGGACAA	NdeI
BCAM0411-cR	TTAATCTAGAAATGTCCTCGGAAATGTTCG	XbaI

[a] Restriction endonuclease sites incorporated into the oligonucleotide sequences are underlined
[b] N/A indicates the absence of a restriction site
[c] native ClaI site in chromosome

2.6 Competitive enrichment

To preferentially enrich for phagosomally-expressed or -upregulated transcripts, 0.3 µg of *B. cenocepacia* K56-2 chromosome was pre-blocked with both 5 µg rDNA and 10 µg of denatured triple-SCOTS enriched NME cDNA (above). 3 µg of triple-SCOTS enriched intracellular cDNA (above) was denatured and re-annealed 30 min at 68^{o}C to remove abundant transcripts. cDNA and blocked chromosomal DNA were combined and hybridized 18 h at 68^{o}C. Chromosome-cDNA hybrids were removed from solution with streptavidin-coated magnetic beads. Captured cDNA was eluted, precipitated, and amplified using intracellular library-specific defined primer I-3032. Following three rounds of enrichment, cDNA were digested with restriction enzyme XbaI, cloned into XbaI-digested, dephosphorylated pUC19, and transformed into *E. coli* DH5α. Individual cDNA inserts were screened by Southern blot for hybridization to DIG-labelled cDNA libraries. cDNA which hybridized to intracellular cDNA, but not to NME cDNA, were sequenced and identified by BLAST analysis.

2.7 Microarray experimental design and analysis

SCOTS cDNA or *B. cenocepacia* J2315 genomic DNA was labelled with CyScribe™ Array CGH Labeling Kit (GE Healthcare) according to the manufacturer's protocols. SCOTS cDNA was labelled with Cy5 and genomic DNA with Cy3; cDNA samples were mixed, hybridized to custom *B. cenocepacia* microarrays (Agilent) according to the Agilent 60-mer oligonucleotide microarray processing protocol, and scanned. Labelling of cDNA, hybridization, and scanning of arrays were performed by the Mahenthiralingam Laboratory, Cardiff University, Wales.

Microarray data analysis was done using GeneSpring GX 7.3.1 (Agilent). Raw data was preprocessed via the enhanced Agilent FE import prior to per-spot and per-chip normalizations for each array. Feature intensity varied across the six

arrays. Genes were selected as “expressed” at a threshold of 1.0 relative to genomic DNA. Statistical analysis between intracellular and non-macrophage-exposed conditions was performed using a paired Student’s *t*-test; genes were considered differentially expressed at an I:NME ratio of ±2-fold with a $p < 0.05$.

The microarray dataset has been deposited in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress/) under accession number E-MEXP-3408.

2.8 Quantitative reverse transcriptase PCR (qRT-PCR)

To validate microarray data, five genes with altered expression (4 upregulated: BCAM0314, BCAM2141, BCAM0276, and BCAS0186; and 1 downregulated: BCAM1928) were examined individually using qPCR. Sigma factor gene *rpoD* (BCAM0918) was used as a reference gene. Oligonucleotide primers for each gene (Table 3) were designed with Primer3 (393). Total RNA was isolated as described above. cDNA was synthesized using random hexamers (Invitrogen) and Transcriptor reverse transcriptase. Quantitation and melting curve analyses for qPCR were performed using the Rotor Gene 6000 (Corbett Life Science) with FastStart SYBR Green (Roche) according to the manufacturer's instructions. Expression of the target gene was normalized to the reference gene for each condition, allowing inter-condition comparison. Data shown are representative of at least two independent experiments.

2.9 Mutagenesis of *B. cenocepacia* K56-2

The I-SceI homing endonuclease system was used for all deletions of genes or gene clusters in *B. cenocepacia* K56-2 (156, 185). In brief, regions flanking the gene or region to be deleted were amplified with gene-specific primers containing restriction sites (Table 3). Upstream amplicons were digested with restriction enzymes XbaI-ClaI; downstream amplicons were digested with ClaI-EcoRI. Both

upstream and downstream amplicons were cloned into XbaI-EcoRI digested, de-phosphorylated pGPI-SceI, giving rise to mutagenesis plasmids (Table 2). Mutagenesis plasmids were introduced to either *B. cenocepacia* K56-2 or MH1K by triparental mating. A plasmid - pDAI-SceI-SacBN – carrying the homing endonuclease was conjugated into single crossover mutants, causing a double-strand break resolved by either a second crossover, yielding mutant genotype, or by reversion to wild-type. Exconjugants were screened by PCR, and confirmed mutants were plated on LB with 5% sucrose to cure pDAI-SceI-SacBN. For the T2SS, T4SS-1, and T6SS, deletions were made in K56-2, followed by deletion of BCAL1674-6 to confer gentamicin sensitivity. For the T3SS and T4SS-2, deletions were made directly in MH1K. For JST112 (Table 1), lacking all secretion systems, deletions were performed sequentially. PCR and Southern blot hybridization confirmed that the deletion had occurred, yielding gentamicin-sensitive mutants (Table 1). In the same manner, single gene deletions in MH1K were made in the T2SS (BCAL3516), T4SS-1 (pBCA025), T4SS-2 (BCAM0333), and T6SS (BCAL0343), as well as SCOTS-identified genes BCAL0124, BCAL0340, BCAM1679, BCAS0186, BCAM2837, BCAL1726, BCAM0276, BCAM0411, BCAM2446, and BCAM0434-5.

2.10 Complementation experiments

To complement single gene deletions, wild-type genes were PCR amplified from *B. cenocepacia* K56-2 with gene-specific primers (Table 3) and the following thermal cycling conditions: 95^{o}C for 5 minutes, 30 cycles of 95^{o}C for 45s, 60^{o}C for 45s, and 72^{o}C for 90s, and a final extension at 72^{o}C for 10 min. The resulting amplicon was digested with restriction enzymes XbaI and NdeI, and ligated into similarly-digested, dephosphorylated pDA12, giving rise to a complementation plasmid (Table 2) which could be introduced to the mutant strain through conjugation. pDA12 was used as a vector control in all experiments.

2.11 Motility plate assays

Swimming and swarming motility were assessed by the appearance of rings of growth away from the site of inoculation on specialized agar plates; motility was quantified by measuring the diameter (in mm) of the bacterial growth. Swimming motility was determined following stab inoculation of LB plates with 0.3% agar. Swarming motility was determined following drop inoculation onto nutrient broth plates with 0.2% glucose and 0.5% agar.

2.12 Exoenzyme secretion assays

Secretion of protease, lipase, and phospholipase C (PLC) was measured via plate assays. Protease secretion was measured as a zone of clearing on dialyzed brain-heart infusion agar with 1.5% skim milk (448). Lipase secretion was measured as the width of the insoluble precipitate halo formed upon inoculation of Tween 80 agar (82). PLC activity was measured as the zone of clearing on LB plates containing one hen egg yolk per 100 mL (227); egg yolk plates also show activity of lipase and lecithinase, the former by the appearance of an iridescent sheen on the surface of the bacterial growth, and the latter by the production of an insoluble opaque precipitate surrounding bacterial growth.

2.13 Lipopolysaccharide analysis

LPS was extracted as described previously (307, 309). Briefly, bacteria were resuspended in PBS, and boiled for 10 min in lysis buffer containing 2% sodium dodecyl sulfate (SDS), 4% β-mercaptoethanol, 10% glycerol, and 1M Tris (pH 6.8). The lysate was treated with proteinase K at 60^{o}C for 12h, and stored at -20^{o}C. LPS was separated on a 14% polyacrylamide gel using Tricine-SDS buffer and silver-stained as described previously (309).

2.14 Gentamicin protection assays

30 min post-infection, *B. cenocepacia*-infected cells were washed three times with PBS and DMEM-50 μg/ml gentamicin was added to kill extracellular bacteria. After 30 min, the media was replaced with DMEM-10 μg/ml gentamicin. Infected monolayers were washed with PBS, and lysed with 0.1% Triton X-100 (Sigma) in PBS at 1 and 24 h post-infection. Surviving bacteria were enumerated by bacterial plate count (CFU). Bacterial entry was calculated as a percentage of initial inoculum. Intracellular replication was calculated as a percentage of bacterial entry for each strain. To compare between experiments, percentage recovery was normalized against the parental control, set as 100% entry and replication.

2.15 Flow cytometry

B. cenocepacia expressing eGFP from the plasmid pDA42 (25) were infected as above in 12-well plates at a multiplicity of 50:1. At 2 h post-infection cells were washed three times with PBS and DMEM-50 μg/ml gentamicin was added to kill extracellular bacteria. After 30 min, the media was replaced with DMEM with and without 10 μg/ml gentamicin. Infected monolayers were washed with PBS, and removed with cold 0.04% EDTA in PBS at 24 h post-infection. Cells maintained in media with gentamicin represented initial infection, while cells maintained in antibiotic-free media represented uncontrolled infection. Macrophages were collected by centrifugation for 5 min at 400 x *g* and 4^{o}C . Cells were resuspended in cold PBS with 2.5 μg/mL 7-aminoactinomycin D (7AAD; Invitrogen) and maintained on ice. Samples were enumerated using a FACSCalibur with CellQuest Pro acquisition software (Becton Dickinson). Data analysis was done via FlowJo (Tree Star, Inc.). All results were normalized against uninfected controls.

2.16 Fluorescence microscopy

The *B. cenocepacia*-containing vacuole (BcCV) in macrophages was characterized 24 h post-infection. Macrophages were infected with *B. cenocepacia* MHK1 at a moi of 50:1; 2h post-infection, extracellular bacteria were killed as above, and the infection maintained in DMEM-10 μg/ml gentamicin. Labelling of the endocytic pathway, including lysosomes, was done using 250 μg/ml tetramethylrhodamine-dextran (TMR-dextran; Invitrogen). To label lysosomes, macrophages were pulsed with TMR-dextran for 2 h, followed by a 1 h chase in dextran-free media prior to bacterial infection. To label the endocytic pathway, 21 h post-infection, macrophages were pulsed with TMR-dextran for 2 h, followed by a 1 h chase in dextran-free media. Acidic compartments were labelled with 0.5 μM Lysotracker Red (Invitrogen) for 30 s prior to visualization. Live images were acquired using a Qimaging cooled charged-coupled device camera (Burnaby) on an Axioscope 2 (Carl Zeiss) microscope with a 100x oil immersion objective and a 50W mercury arc lamp. Images were digitally processed using Northern Eclipse imaging software (Empix Imaging).

Chapter 3

3 Global changes in gene expression by the opportunistic pathogen *B. cenocepacia* in response to internalization by murine macrophages

3.1 Construction of SCOTS components for *B. cenocepacia*

Application of the SCOTS technique to any system requires the creation of two organism-specific elements: The first is a library of biotinylated bacterial chromosome fragments with ribosomal blocking sequences; the second is a cDNA library containing the transcripts of interest, where both ends of each cDNA contain a 25-nt defined “linker” sequence not found within the bacterial chromosome.

B. cenocepacia J2315 encodes four complete rRNA operons on the first chromosome, and one on each of the second and third chromosomes; all six operons are 99-100% identical (195). The 16S rRNA gene, and 23S/5S rRNA genes were each amplified from the final operon on chromosome 1, which does not contain any tRNA sequences. All genes were cloned into pUC19, a high-copy number plasmid, to form pJT25 (Figure 1A), the source of ribosomal blocking sequences. Chromosomal DNA extracted from *B. cenocepacia* K56-2 was biotinylated (Figure 1B) and sonicated together with an excess of pJT25 to form the chromosomal library (Figure 1E).

Two linker primers were created, each containing a different restriction site (Table 3). When added to a random nonamer, the linker primer can be used for both single-strand cDNA synthesis and double-stranding to create a pool of cDNAs with the linker sequence at both ends; this allows the entire cDNA pool to be amplified with a single primer (Figure 1E). Neither linker primer binds to the K56-2 chromosome, as PCR amplification occurs only in the presence of linkered cDNA.

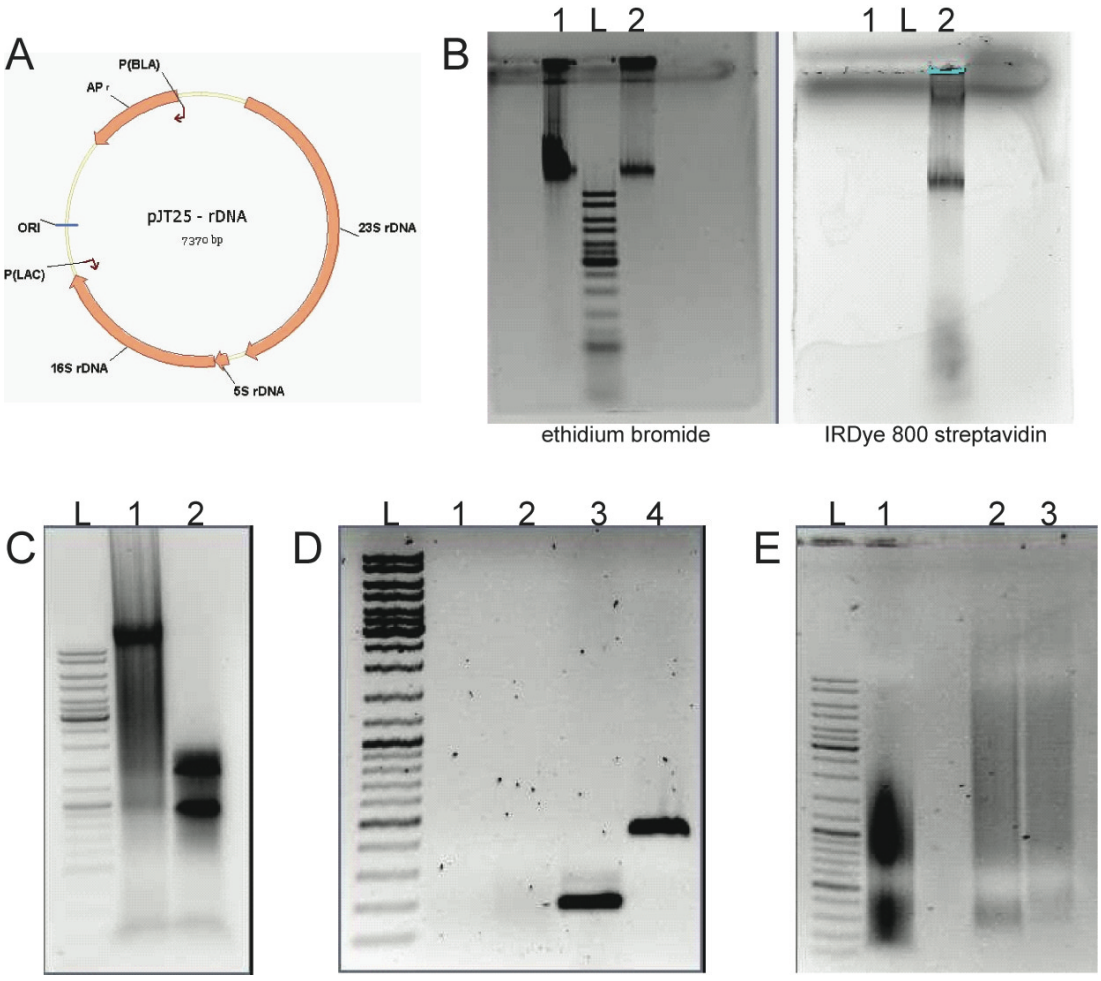

Figure 1. SCOTS components for *B. cenocepacia.*

(A) Ribosomal blocking plasmid pJT25. (B) Biotinylated *B. cenocepacia* chromosome. Biotinylated (2) and unbiotinylated (1) K56-2 chromosome were electrophoresed, and the gel stained with IRDye800 streptavidin (right), labeling biotinylated DNA, followed by ethidium bromide (left), labeling all DNA. (C) RNA isolated from K56-2 shown pre- (1) and post- (2) DNase treatment. (D) DNase treatment is effective, as neither bacterial nor eukaryotic sequences can be PCR-amplified from RNA template. 1 – I-RNA, 2 – NME-RNA, 3 – K56-2 DNA, 4 – macrophage cDNA. (E) Complete *B. cenocepacia* chromosomal library fragments (1) and cDNA libraries from intracellular (2) and NME (3) *B. cenocepacia*. L – GeneRuler Ladder mix.

3.2 Optimization of the SCOTS protocol for *B. cenocepacia*

The SCOTS protocol is complex, with no readout of functionality until the final screening step. Thus, a simple artificial system was created to test the individual steps of the protocol, including capture of cDNA with biotinylated chromosomal fragments, cDNA amplification with a linker primer, and blocking the capture of duplicate sequences during competitive enrichment. We created this artifical system using three test genes of different sizes: 500bp BCAL2831 (T1), 700bp *mgtC* (T2), and 900bp *arnA1* (T3), all easily separated and visualized by simple gel electrophoresis. All three genes were amplified with linker sequence 2 (N) at either end; the first two genes were also amplified with linker sequence 1 (I) at either end (Figure 2). These artificial cDNA constructs enabled testing and optimization of every aspect of the *B. cenocepacia*-specific SCOTS protocol in five separate tests; cDNA captured from each test library was divided in half and amplified separately with each linker primer (I or N) (Table 3). Sonicated herring sperm DNA was used to represent eukaryotic sequences, to which the test genes were added in known quantities to form the test cDNA libraries (L1-L5). L1 contained only genes I1 and I2, while L2 contained N1, N2, and N3; these two libraries tested the capture of bacterial sequences and the specificity of linker primer amplification. L3 contained the five test genes, but capture was carried out in the absence of biotinylated library fragments to ascertain the necessity of hybridization for cDNA capture. The fourth test pre-hybridized a ten-fold excess of L2 to the chromosome prior to the capture of L1 to examine the efficacy of blocking during competitive enrichment; in a successful test, pre-hybridization of L2 would prevent the capture of L1. The fifth test pre-hybridized a ten-fold excess of T1-N and T3-N to the chromosome prior to capture of L1; in this case, successful competitive enrichment would prevent binding of T1-I while capturing T2-I.

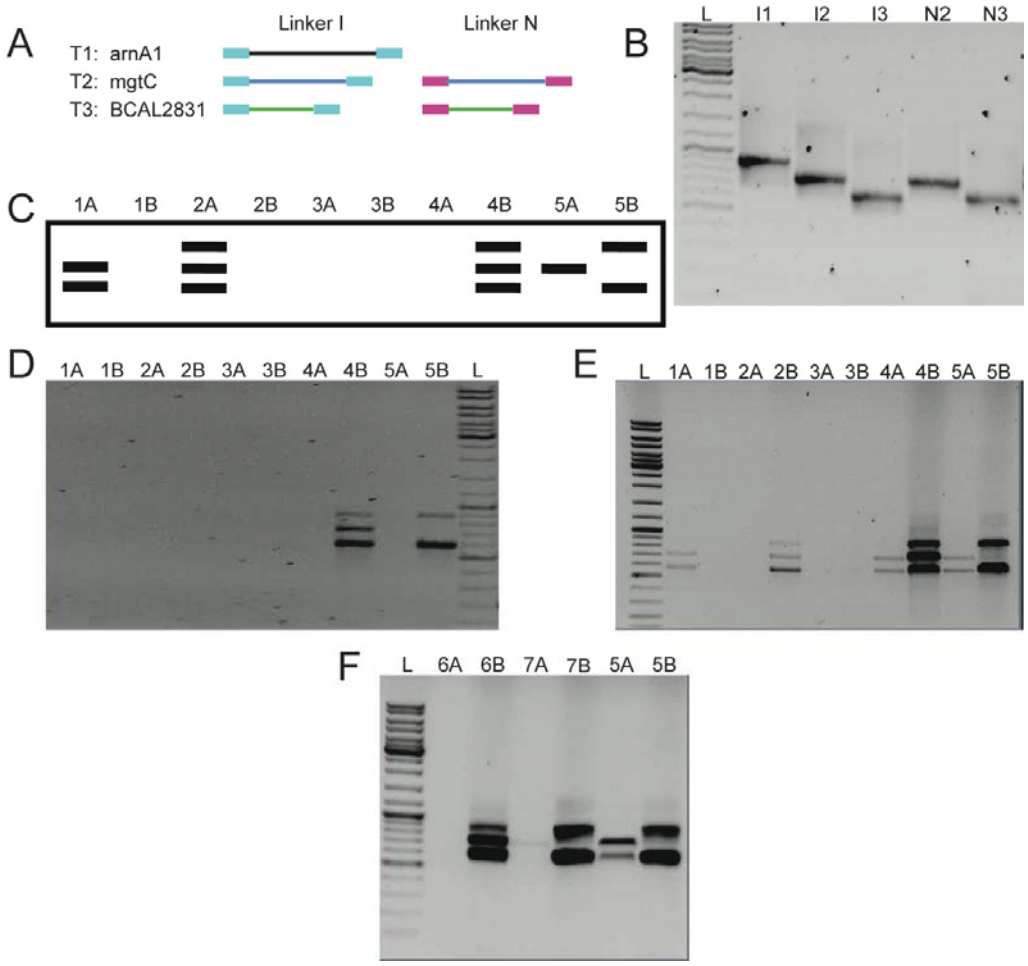

Figure 2. An artificial system to optimize SCOTS for *B. cenocepacia*

Test genes (A) can be clearly distinguished by size via gel electrophoresis (B). (C-E) Optimization of SCOTS conditions. (C) Expected results. (D) Pre-hybridization effectively removes abundant sequences. (E) Genes can be effectively captured and amplified, but blocking sequences are insufficient. (F) 100x NME genes are required to effectively prevent capture of I genes.

Legend: L – GeneRuler Ladder mix
PCR amplification with I linker primer (A) or N linker primer (B)
1 – Capture of I2 and I3 ; 2 – Capture of N1, N2, and N3
3 – Capture of all five genes in the absence of biotinylated chromosome
4 – Capture of I2 and I3 following pre-hybridization with 10x N1, N2, and N3
5 – Capture of I2 and I3 following pre-hybridization with 10x N1 and N3
6 – Capture of I2 and I3 following pre-hybridization with 100x N1, N2, and N3
7 – Capture of I2 and I3 following pre-hybridization with 100x N1 and N3

The initial experiment revealed the efficacy of the pre-hybridization step to remove abundant transcripts (Appendix 2), as only cDNA hybridized to the chromosome immediately following denaturation could be amplified (Figure 2D). After eliminating pre-hybridization of the cDNA pools, the second experiment demonstrated successful capture in the presence of biotinylated bacterial chromosome fragments and linker-specific amplification of cDNA; however, blocking for competitive enrichment was ineffective (Figure 2E). Successive experiments demonstrated successful competitive enrichment by decreasing the hybridization temperature, and increasing both the duration of pre-hybridization with blocking sequences and the quantity of blocking sequences (Figure 2F).

3.3 Preliminary applications of SCOTS: Transcriptional changes during growth in sub-inhibitory concentrations of tetracycline

Sub-inhibitory concentrations of tetracycline modulate outer membrane protein expression in *B. cenocepacia* J2315, implying changes in gene transcription (295); SCOTS and CE were applied to identify these transcriptional changes while optimizing later steps in the SCOTS-CE protocol. cDNA from J2315 grown to log phase in LB with and without 25 μg/mL Tet was subjected to three rounds of SCOTS, followed by three rounds of CE. The resultant selectively captured sequences (*scs*) were cloned into pUC19 (Table 2) for individual screening by Southern blot hybridization; cDNAs were identified that hybridized to DIG-labelled cDNA from tetracycline-grown J2315 more strongly than to cDNA from broth-grown J2315 (Figure 3). Sequencing of tetracycline-enhanced transcripts identified an imidazole glycerol phosphate synthase, a putative exported protein with a coagulation factor domain, an extracellular solute-binding protein, a putative cell wall lysis peptidase, and several hypothetical proteins. Previous studies in protein expression changes also identified an extracellular solute-binding protein, as well as a peptidoglycan-associated lipoprotein, and multiple

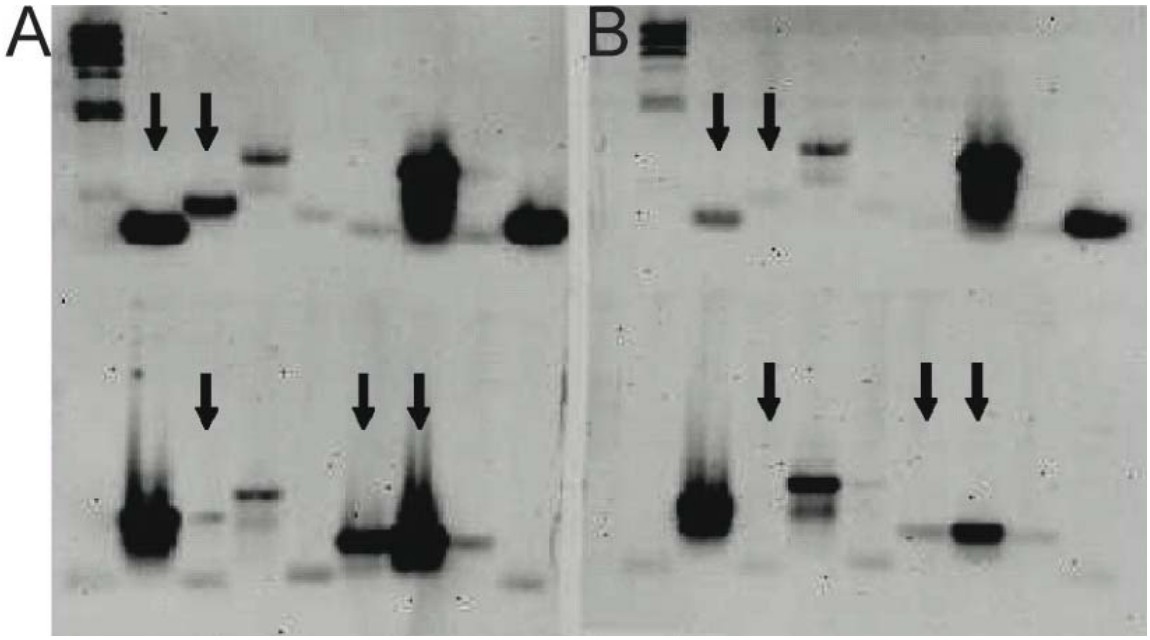

Figure 3. Modulation of bacterial transcript levels during growth in sub-inhibitory concentrations of tetracycline.

Each lane contains captured cDNAs amplified from a single recombinant plasmid, transferred to a membrane, and probed with SCOTS-amplified DIG-labelled cDNA from growth in tetracycline (A) or LB (B). Arrows indicate genes upregulated during growth in tetracycline.

conserved hypothetical proteins (295).

Originally, cDNA inserts were amplified with M13 vector primers (Table 3), such that empty vectors would show a small product. However, PCR amplification with linker primers revealed the presence of multiple cDNAs within a single vector (Figure 4). Such a situation could lead to false-negatives during Southern blot screening, should both probes detect one of several cDNAs in a single amplicon. Modification to the ligation procedure decreased the incidence of multiple cDNA inserts within a single vector.

3.4 Intracellular gene expression by *B. cenocepacia* in murine macrophages

SCOTS was used to examine gene expression by intracellular *B. cenocepacia* in murine macrophages at 4 h p.i.. We have previously demonstrated that at 4 h p.i., most intracellular bacteria reside in vacuoles that do not fuse with the lysosome (230, 252, 296). In addition, by 4 h p.i., infected macrophages display observable alterations to the actin cytoskeleton caused by the bacterial T6SS, which is not well-expressed under normal culture conditions in LB medium (25). These observations suggest that at 4 h p.i., there are changes in bacterial gene expression reflecting response and adaptation to the intracellular environment. As a control, we used bacteria grown for 4 h under the same conditions in macrophage growth medium (DMEM-10% FBS), but without macrophages. To isolate and enrich bacterial transcripts, three rounds of SCOTS were performed on both intracellular and non-macrophage-exposed (NME) bacterial cDNA samples. The resultant cDNA pools were subjected to competitive enrichment for transcripts specific to the phagosome, and to microarray analysis with *B. cenocepacia* J2315 DNA arrays.

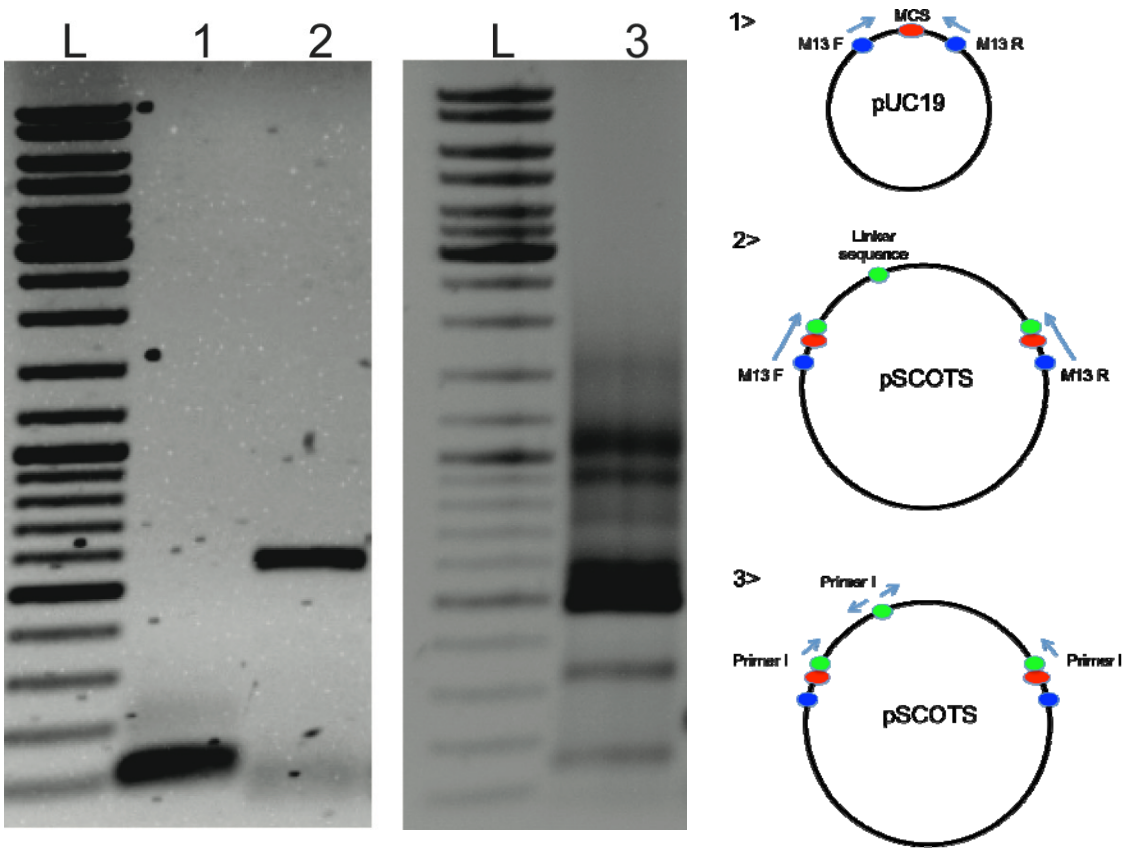

Figure 4. Multiple cDNAs may be present in a single recombinant plasmid. PCR amplification with M13 vector primers shows a negative product from an empty vector (1), or a single product containing all cDNA inserts (2). PCR amplification with I linker primers reveals multiple cDNA inserts present in a single recombinant plasmid (3). L – GeneRuler Ladder Mix

3.4.1 Low abundance transcripts identified by competitive enrichment

Competitive enrichment is similar to the initial SCOTS technique, but pre-blocks the chromosome with both rDNA and control NME cDNA prior to capture of intracellular cDNA. This encourages the capture of sequences specifically or more highly expressed within the phagosome. Following three rounds of competitive enrichment, cDNA sequences were cloned into pUC19, a high-copy-number vector, and introduced into *E. coli* DH5α by transformation. DNA inserts from colonies were individually amplified by PCR and screened by Southern blot for differential hybridization to DIG-labelled cDNA libraries. A number of recombinant plasmids contained multiple cDNA inserts; however, these could be differentiated during Southern blot analysis. Following competitive enrichment, many *scs* clones were obtained which hybridized to labelled intracellular cDNA but not to NME cDNA (Figure 5). One hundred eighteen unique *scs* sequences, encoding 124 proteins, were mapped to the *B. cenocepacia* genome (Table 4); of these sequences, four were secondary sequences in an already-identified gene, two were within a duplicated region on chromosome one, and one was a transposase with nine identical copies distributed throughout the genome. Identified sequences were proportionally represented in each of the three chromosomes and the plasmid: 57 *scs* mapped to chromosome one, 51 to chromosome two, 14 to chromosome three, and 2 to the plasmid. For the latter three genetic elements, this represents approximately 2 percent of coding sequences, whereas for chromosome one, only 1.5 percent of the genes were identified. While this is a small difference, it is interesting to note that the core functions of the cell, including cell division, central metabolism, and "housekeeping genes" are overrepresented on chromosome one, while the latter three genetic elements carry a higher proportion of accessory functions and unknown proteins (195). Identified *scs* were classified into functional categories based on clusters of orthologous groups of proteins (COG) designations (Figure 6). The COGs database (http://www.ncbi.nlm.nih.gov/COG/) predicts the

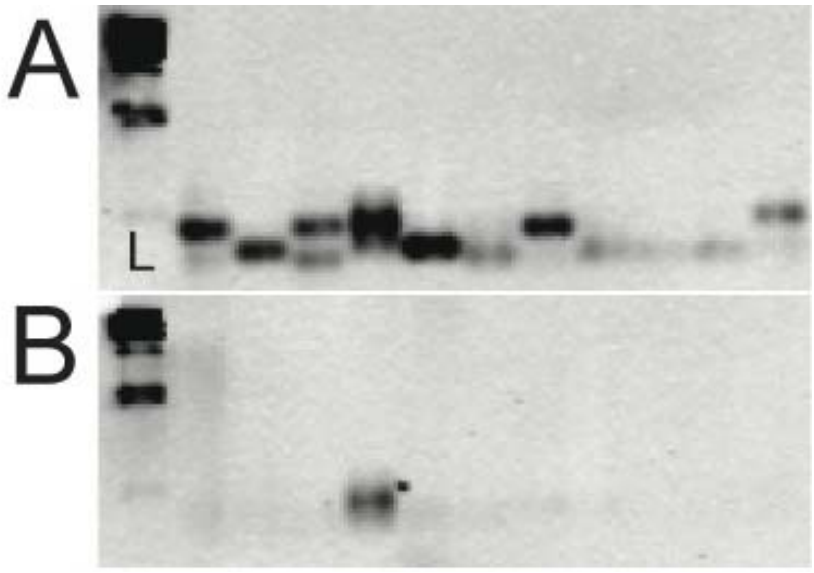

Figure 5. Modulation of bacterial transcript levels by intracellular *B. cenocepacia*

Selectively captured sequences (*scs*) isolated from intracellular bacteria were hybridized with DIG-labelled SCOTS-derived *B. cenocepacia* cDNA pools from (A) intracellular bacteria or (B) non-macrophage-exposed bacteria. Each lane represents cDNA amplified from a single recombinant plasmid. L indicates equally loaded DIG-labelled ladder.

Table 4: Intracellular-specific selectively captured sequences

Gene[a]	Known or putative function	COG
BCAL0017	putative branched-chain amino acid ABC transporter periplasmic protein	E
BCAL0032	ATP synthase B chain	C
BCAL0044	putative transposase	-
BCAL0110	putative aminotransferase	M
BCAL0124	flagellar regulon master regulator subunit FlhD	-
BCAL0133 **BCAL0134**	putative chemoreceptor glutamine deamidase cheD chemotaxis response regulator protein-glutamate methylesterase 1	NT NT
BCAL0205	NAP-dependent malic enzyme	C
BCAL0343 ***BCAL0344***	putative T6SS protein TssD putative T6SS protein TssE	R S
BCAL0351	putative T6SS protein TssM	S
BCAL0420	type I restriction component of type I restriction-modification system	L
BCAL0434	putative exported protein	-
BCAL0566	basal-body rod modification protein FlgD	N
BCAL0571	flagellar P-ring protein precursor	N
BCAL0576	flagellar hook-associated protein 1	N
BCAL0594	putative exported protein	-
BCAL0770	error-prone DNA polymerase	L
BCAL0772	AraC family regulatory protein	R
BCAL0850 BCAL0851	glycolate permease putative iron-sulphur cluster containing protein	C C
BCAL0983 BCAL0984	ribosomal large subunit pseudouridine synthase C 2 haloacid dehalogenase-like hydrolase	J R
BCAL1010	beta-hexosaminidase 2	G
BCAL1165	conserved hypothetical protein	S
BCAL1182	TetR family regulatory protein	K
BCAL1203	conserved hypothetical protein (BcenGI5)	-
BCAL1403 BCAL1403A	LysR family regulatory protein putative membrane protein	K -
BCAL1526 ***BCAL1527***	putative flp type pilus assembly protein flp type pilus assembly protein	- -
BCAL1535	putative membrane protein	-
BCAL1536	putative sigma-54 related transcriptional regulatory protein	T
BCAL1599b BCAL1601	hypothetical phage protein (BcenGI7) hypothetical phage protein (BcenGI7)	T -
BCAL1643 BCAL1644	putative ATP/GTP binding protein permease protein	R R

BCAL1647	putative oligosaccharide ABC transporter ATP-binding protein	Q
BCAL1664	conserved hypothetical protein	S
BCAL1822	putrescine transport system permease protein	E
BCAL1975	putative diguanylate cyclase	T
BCAL2208	dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	C
BCAL2224	glutamine synthetase	E
BCAL2339	NADH dehydrogenase I chain F	C
BCAL2860	beta-hexosaminidase 1	G
BCAL2886 BCAL2887	haloacid dehalogenase-like hydrolase ribosomal large subunit pseudouridine synthase C 2	R J
BCAL2978	NAD-dependent formate dehydrogenase alpha subunit	R
BCAL3023	putative chloride-channel protein	P
BCAL3029	putative alkane monooxygenase	-
BCAL3054	6,7-dimethyl-8-ribityllumazine synthase	H
BCAL3077	putative membrane protein (BcenGI10)	-
BCAL3239	glucosyltransferase	M
BCAL3240	putative capsular polysaccharide transporter ATP-binding protein	G
BCAL3244	glycosyltransferase	M
BCAL3263	conserved hypothetical protein	E
BCAL3371	putative DNA polymerase III delta subunit	L
BCAL3458	cell division protein FtsA	D
BCAL3474	long-chain-fatty-acid—CoA ligase	IQ
BCAL3478	putative RNA polymerase sigma factor	K
BCAM0068	Major Facilitator Superfamily protein	GEPR
BCAM0151	conserved hypothetical protein (fragment)	I
BCAM0164	putative lipoprotein	-
BCAM0187	putative 2-isopropylmalate synthase	E
BCAM0374	conserved hypothetical protein	-
BCAM0435	cation efflux system protein	P
BCAM0520	conserved hypothetical protein (pseudogene)	-
BCAM0779	putative methyl-accepting chemotaxis protein	N
BCAM0780	putative helicase	L
BCAM0949	exported lipase LipA	R
BCAM0992	putative DNA methylase	L
BCAM1053C	hypothetical phage protein (BcenGI12)	-
BCAM1081	hypothetical phage protein (BcenGI12)	-
BCAM1082A BCAM1083	putative exported phage protein putative transmembrane phage protein	- S
BCAM1160	putative cyclic-di-GMP signaling protein	T

BCAM1172	gamma-glutamylputrescine oxidoreductase	E
BCAM1245	putative phosphoenolpyruvate phosphomutase/sugar nucleotidyltransferase	G
BCAM1357	putative gluconate 2-dehydrogenase flavoprotein subunit	N
BCAM1460	Major Facilitator Superfamily protein	GEPR
BCAM1518	conserved hypothetical protein	-
BCAM1569 BCAM1570	putative BNR/Asp-box protein alcohol dehydrogenase	- R
BCAM1572 IG	upstream region from methyl-accepting chemotaxis protein	N
BCAM1810	putative cold shock protein	K
BCAM1848 BCAM1849	IclR family regulatory protein putative hydrolase	K R
BCAM1890	hypothetical phage protein (BcenGI13)	-
BCAM1892	hypothetical phage protein (BcenGI13)	-
BCAM1893	hypothetical phage protein (BcenGI13)	-
BCAM1901	hypothetical phage protein (BcenGI13)	-
BCAM1904	hypothetical phage protein (BcenGI13)	-
BCAM1910 BCAM1911	hypothetical phage protein (BcenGI13) hypothetical phage protein (BcenGI13)	- -
BCAM1918	putative phage lysozyme (BcenGI13)	-
BCAM2123 BCAM2124	putative 4-oxalocrotonate decarboxylase 2-keto-4-pentenoate hydratase	Q Q
BCAM2126	putative outer membrane porin	-
BCAM2169	putative outer membrane autotransporter	-
BCAM2237	putative 2,2,-dialkylglycine decarboxylase	E
BCAM2253	RHS-family protein	M
BCAM2254 IG	upstream region from putative exported protein	S
BCAM2274a	conserved hypothetical protein	-
BCAM2276	putative FAD dependent oxidoredutase	E
BCAM2348	putative lipoprotein	-
BCAM2433 IG	upstream region from putative acyl-CoA dehydrogenase	I
BCAM2486 BCAM2487	putative membrane protein putative membrane protein	E -
BCAM2502	3-dehydroquinate dehydratase	E
BCAM2540	fenitrothion hydrolase protein FedB	-
BCAM2563	methyl-accepting chemotaxis protein	N
BCAM2564	putative aerotaxis receptor	NT
BCAM2586	putative lactonase	G
BCAM2609	putative exported protein	-
BCAM2654	putative acetyltransferase	-
BCAM2764	putative exported lipase	-
BCAS0094	putative membrane protein	-

BCAS0104 IG	upstream region from A-type flagellar hook-associated protein 2	N
BCAS0186	putative acyl carrier protein phosphodiesterase	I
BCAS0199 BCAS0201	putative transporter protein-Dct family putative FAD dependent oxidoreductase	S C
BCAS0249 BCAS0250	chromate resistance transport protein conserved hypothetical protein	P -
BCAS0252	DJ-1/PfpI family protein	R
BCAS0258	GntR family regulatory protein	K
BCAS0371	conserved hypothetical protein	Q
BCAS0633	hypothetical protein	-
BCAS0661B	conserved hypothetical protein	-
BCAS0667	conserved hypothetical protein	S
BCAS0684 BCAS0686	conserved hypothetical protein (fragment) conserved hypothetical protein	- -
pBCA055	putative membrane protein	T
pBCA080	hypothetical protein	-

[a] **Genes** are strongly upregulated (log2 > 2, $p < 0.05$)
Genes are in a transcriptional unit with strongly upregulated genes (log2 > 2, $p < 0.05$)
Genes are upregulated in intracellular bacteria

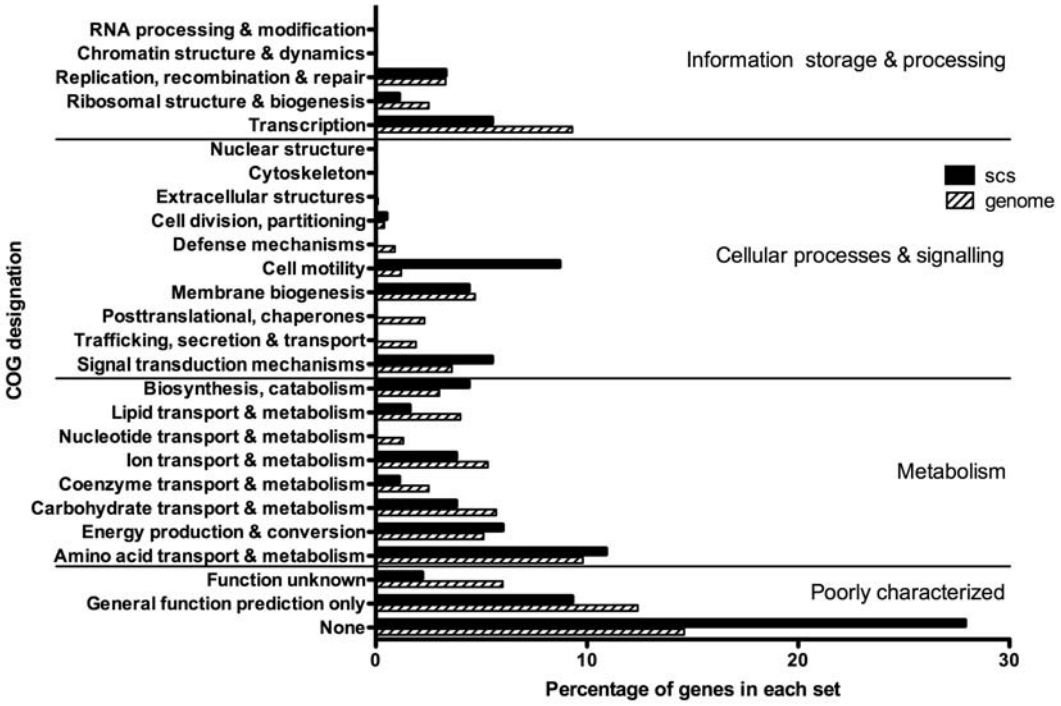

Figure 6. Functional classification of selectively captured sequences.

Profile of each COG functional class is shown as a percentage of all *scs*. Also shown is the functional profile of the genome as a whole.

function of a protein based on orthology, or homology among species (476). Almost half (46%) of identified *scs* represented poorly characterized genes, of which two-thirds had no functional classification. Several major COGs were not represented in the set, including nucleotide metabolism and transport, trafficking, secretion and transport, posttranslational modification and chaperones, and defense mechanisms; however, genes classified as cell motility were overrepresented relative to genome content. This COG profile is representative of 115 genes; *B. cenocepacia* J2315, a sequenced clonal isolate of K56-2, has over 7000 identified genes (195). Therefore, to determine if these trends hold true across the entire cDNA library, SCOTS-derived cDNA pools were applied to *B. cenocepacia*-specific microarrays to gain a more complete overview of intracellular gene expression.

3.4.2 Global intracellular gene expression identified by microarray analysis

Each cDNA pool was hybridized against reference genomic DNA; expression was set as a value of 1.0 or greater versus the genomic control. Using this threshold, 55 percent of all genes, or approximately 4000 genes, were expressed under each condition. Of the 115 unique genes identified by competitive enrichment, only 6 failed to meet the arbitrary level for expression by intracellular bacteria; of the remainder, more than half were upregulated by intracellular bacteria. Twenty percent of sequences identified by competitive enrichment showed significant intracellular upregulation (> 4-fold, $p < 0.05$) of the gene itself or of a putative transcriptionally-associated gene. Gene expression was compared between intracellular and non-macrophage-exposed bacteria; seven hundred sixty-six microarray genes or intergenic sequences showed significant changes in expression ($-2 > \log2 > 2$, $p < 0.05$), of which 329 were induced and 437 were repressed. Distribution of these genes among and throughout the four genetic elements of *B. cenocepacia* was relatively proportionate to the size of the genetic element, regardless of G+C content (Figure 7), with the exception of the

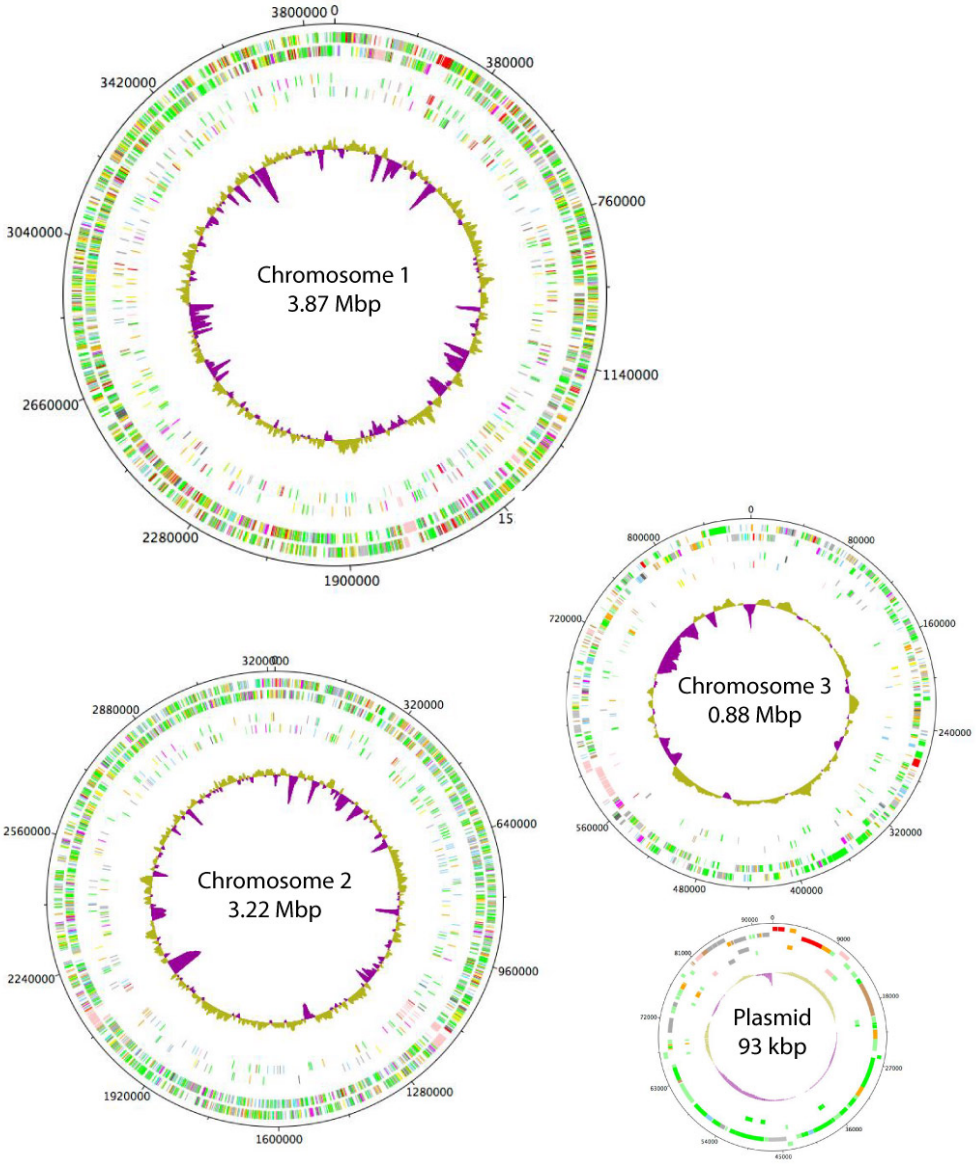

Figure 7. Genes differentially expressed by intracellular bacteria are distributed among the four genetic elements of *B. cenocepacia*

The outer pair of concentric circles represents both coding strands of the *B. cenocepacia* genome. The second pair of concentric circles represents gene induced (outer) and repressed (inner) by intracellular *B. cenocepacia*. Percentage of G+C is also shown, with above average in yellow and below average in purple. Chromosome 3 is 3x scale; the plasmid is 17.5x scale.

plasmid, where 20% of the plasmid-encoded genes were repressed in intracellular bacteria. Among the differentially expressed genes, 145 were regulated greater than ± 10-fold ($p < 0.05$) (Figure 8); many of these genes are poorly characterized (Tables 5 & 6), suggesting a novel survival strategy.

Differentially regulated genes were classified into functional categories based on COG designations (Figure 9). Expression was altered in every COG designation represented in *B. cenocepacia.* Metabolic adaptation accounts for one-third of differentially regulated genes in intracellular bacteria, though more metabolic genes were induced than were repressed. Genes involved in amino acid, lipid, and carbohydrate transport and metabolism, and energy production and conversion account for 25% of all induced genes. The large categories of information storage and processing, and cellular processes and signaling each accounted for one-sixth of differentially regulated genes; in particular, significant changes were seen in the expression of transcriptional regulators, accounting for 9% of all differentially regulated genes. Similar to the over-representation seen in the SCOTS-CE profile (Figure 6), cell motility (flagellar) genes were mainly induced. Interestingly, genes involved in the synthesis and transport of phospholipid precursor *sn*-glycerol-3-phosphate were induced by intracellular bacteria, as were genes involved in the production and transport of cholesterol-like hopanoids, exoenzymes, ion transporters, an osmolarity-dependent two-component regulatory system, and catalase *katB*. Unexpectedly, many genes involved in the acquisition of iron were repressed, including ornibactin receptor *orbA*, the transcriptional activator of the pyochelin receptor, and a putative pyochelin biosynthesis protein. Other repressed genes of note include *B. cenocepacia*-specific quorum sensing regulator *cciR*, adhesin *adhA*, and many transport genes, including the Sec and Tat systems, and elements of the type II and both type IV secretion systems. However, at least one-third of both induced and repressed genes fall into the poorly characterized classification; of these, greater than half lack a COG designation. In total, 25% of all differentially regulated genes are annotated only as hypothetical proteins.

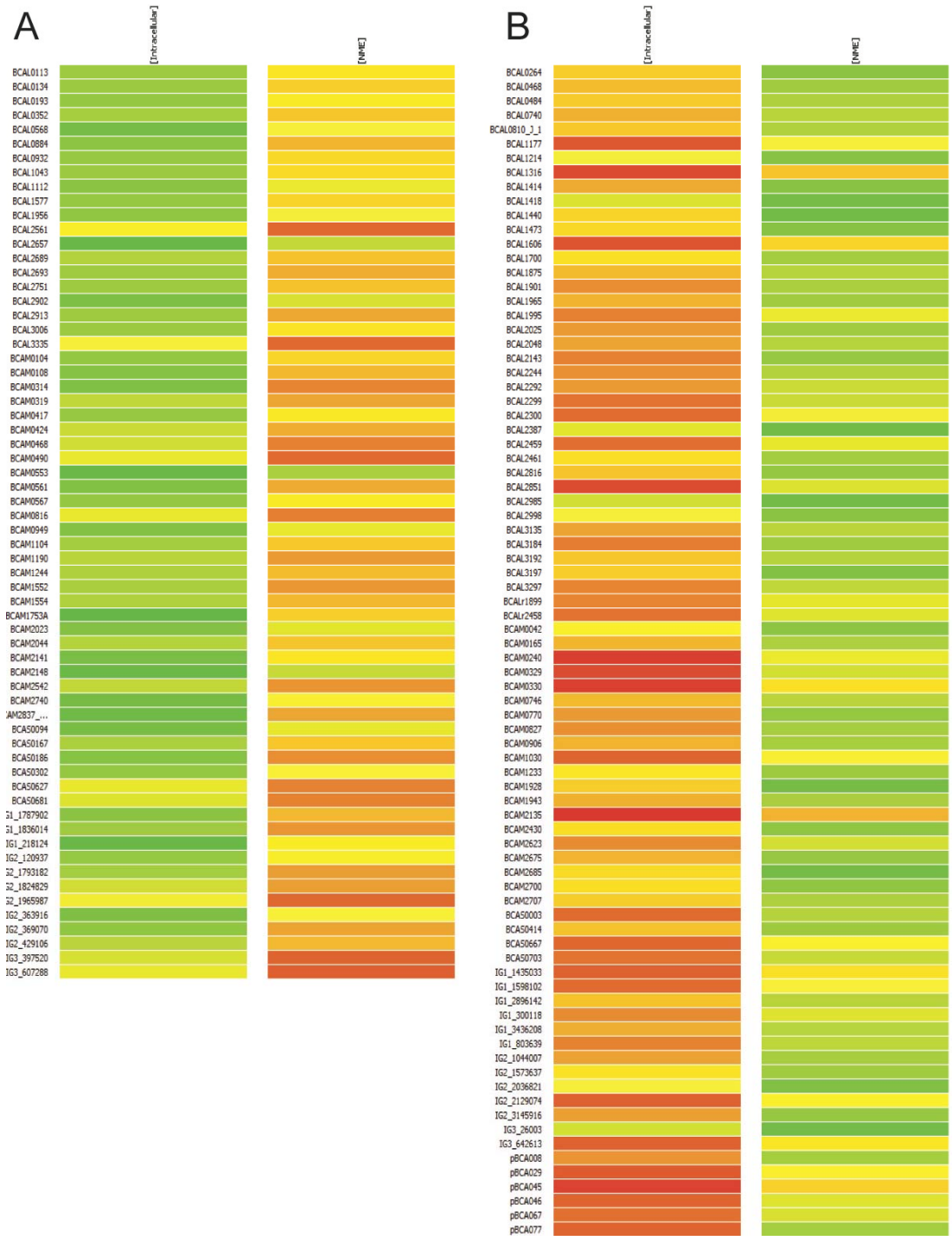

Figure 8. Relative expression of genes strongly regulated by intracellular *B. cenocepacia*.

Heat map of the I:NME expression ratio of genes induced (A) or repressed (B) greater than 10-fold ($p < 0.05$). Colour scale shows green as high expression, red as low expression, and yellow as intermediate.

Table 5: Genes strongly induced by intracellular *B. cenocepacia*

	COG	Gene	Closest Gene	Known or putative function	p-value	Fold Change
Metabolism	Amino Acid Transport & Metabolism	BCAL2902		putative branched-chain amino acid transport protein	4.14E-02	11.68
		BCAM0424		putative polyamine transport protein	3.10E-02	10.27
		BCAM1190		aspartate ammonia-lyase (aspA)	3.56E-02	20.60
		BCAM2044		putative asparagine synthetase	1.83E-02	11.07
		IG1_218124	BCAL0195	putative amino acid transport protein	1.82E-02	102.51
		BCAM0104		putative dihydrodipicolinate synthase	1.51E-02	19.41
	Energy Production & Conversion	BCAM0490		putative NADH oxidoreductase	1.40E-02	17.31
		BCAM1104		putative glycerol kinase	3.24E-02	14.74
		BCAM1552		putative dehydrogenase, iron-sulfur binding subunit	3.02E-02	31.41
		BCAM2148		putative L-lactate dehydrogenase	3.44E-02	23.33
		IG2_1824829	BCAM1648	putative monooxygenase	2.65E-02	10.89
		IG2_1965987	BCAM1756	putative molybdopterin oxidoreductase	2.80E-03	14.42
	Carbohydrate Transport & Metabolism	BCAM0108		Major Facilitator Superfamily protein	6.31E-03	51.61
		BCAM0567		Major Facilitator Superfamily protein	4.18E-02	13.40
		IG2_120937	BCAM0108	Major Facilitator Superfamily protein	1.08E-02	11.60
		IG3_397520	BCAS0343	Major Facilitator Superfamily protein	2.05E-02	28.77
	Coenzyme Transport & Metabolism	BCAL2657		putative bifunctional cobalamin biosynthesis	3.20E-02	28.98
		BCAL2751		2-dehydropantoate 2-reductase	2.31E-03	20.84
	Ion Transport & Metabolism	BCAM0553		putative ABC transporter system permease	2.42E-02	14.44
		BCAM1753A		putative sulfate transporter	5.42E-03	406.44
	Nucleotide Transport & Metabolism	IG2_363916	BCAM0310	putative ribonucleotide reductase	2.29E-02	23.59

	Lipid Transport & Metabolism	BCAL0884		putative acyl-CoA dehydrogenase oxidoreductase	9.80E-03	40.51
		BCAS0167		squalene-hopene cyclase	2.35E-02	12.84
		BCAS0186		putative acyl carrier protein phosphodiesterase	1.98E-02	110.18
		IG1_1787902	BCAL1636	putative exported endonuclease	1.58E-02	41.70
	Biosynthesis & Catabolism	BCAL2689		putative short-chain type dehydrogenase/reductase	2.91E-02	11.84
Cellular Processes & Signalling	Signal Transduction Mechanisms	BCAL2913		sigma-54 interacting regulatory protein	2.37E-02	31.92
		BCAM0319		putative universal stress protein	4.04E-02	14.07
		BCAM1554		putative diguanylate cyclase	1.45E-02	14.79
		BCAM2837		two-component regulatory system, response regulator	2.30E-02	210.68
		BCAL0134		chemotaxis-specific methylesterase (cheB1)	8.99E-03	17.39
	Trafficking, Secretion & Transport	IG1_1836014	BCAL1678	putative outer membrane usher protein precursor	1.70E-02	41.61
		BCAM0561		transport protein	3.57E-03	73.84
	Posttranslational Modification, Chaperones	BCAS0302		conserved hypothetical protein	4.95E-02	11.69
	Membrane Biogenesis	BCAM0104		putative dihydrodipicolinate synthase	1.51E-02	19.41
		BCAL1043		glucarate dehydratase (gudD)	3.64E-02	14.23
	Cell Motility	BCAL0113		B-type flagellar hook-associated protein 2 (fliD1)	3.96E-02	11.59
		BCAL0568		flagellar basal-body rod protein FlgF	3.09E-02	35.69
		BCAL0134		chemotaxis-specific methylesterase (cheB1)	8.99E-03	17.39

		IG1_1836014	BCAL1678	putative outer membrane usher protein precursor	1.70E-02	41.61
	Defense Mechanisms	BCAM2141		ABC transporter ATP-binding membrane protein	2.42E-02	37.63
Information Storage & Processing	Transcription	BCAL2561		LysR family regulatory protein	9.30E-03	10.55
		BCAL2693		LysR family regulatory protein	4.99E-02	27.77
		BCAL3006		cold shock-like protein (cspA)	1.09E-02	12.83
		BCAM0417		LuxR superfamily regulatory protein	1.30E-02	13.08
		BCAL3335		DNA-binding protein Fis	3.63E-02	12.84
	Replication, Recombination & Repair	BCAL3335		DNA-binding protein Fis	3.63E-02	12.84
Poorly Characterized	General Function Prediction only	BCAL1043		glucarate dehydratase (gudD)	3.64E-02	14.23
		BCAL2689		putative short-chain type dehydrogenase/reductase	2.91E-02	11.84
		BCAL1112		putative phosphodiesterase	4.90E-02	10.87
		BCAM0949		lipase	1.89E-02	12.84
		BCAM1244		putative phosphonopyruvate decarboxylase	6.19E-03	14.19
		BCAS0627		putative lipoprotein	1.69E-02	10.80
		IG2_429106	BCAM0382	putative lipoprotein	1.06E-02	11.66
	Function Unknown	BCAL1956		putative lipoprotein	4.55E-02	11.44
		BCAM0816		conserved hypothetical protein	2.71E-02	11.56
	None	BCAL0193		putative exported protein	1.33E-02	14.01
		BCAL0352		metallo peptidase, subfamily M15C	4.93E-02	14.56
		BCAL0932		conserved hypothetical protein	3.84E-02	16.90
		BCAL1577		hypothetical phage protein	4.41E-02	18.63
		BCAM0314		conserved hypothetical protein	1.04E-03	163.22
		BCAM0468		conserved hypothetical protein	6.23E-03	19.90

		BCAM2023		conserved hypothetical protein	4.39E-02	11.56
		BCAM2542		fenitrothion hydrolase protein FedA	3.13E-02	19.10
		BCAM2740		conserved hypothetical protein	4.14E-02	34.59
		BCAS0094		putative membrane protein	1.35E-02	20.16
		BCAS0681		putative transposase (pseudogene)	2.54E-02	13.57
		IG2_1793182	BCAM1611	putative exported protein	9.90E-03	31.41
		IG2_369070	BCAM0314	conserved hypothetical protein	5.60E-03	51.99
		IG3_607288	BCAS0550	hypothetical phage protein - BcepMu5	4.55E-02	22.11

Table 6: Genes strongly repressed by intracellular *B. cenocepacia*

	COG	Gene	Closest Gene	Known or putative function	p-value	Fold Change
Metabolism	Amino Acid Transport & Metabolism	BCAL2244		urocanate hydratase (hutU)	1.90E-02	28.10
		BCAL3197		serine hydroxymethyltransferase (glyA)	7.81E-03	39.82
		BCAM0746		arginosuccinate synthase (argG)	7.33E-03	11.03
		IG3_26003	BCAS0024	GntR family regulatory protein	2.56E-02	17.55
		IG1_3436208	BCAL3157	putative ornithine cyclodeaminase	1.16E-02	15.25
	Energy Production & Conversion	BCAL1214		branched-chain alpha-keto acid dehydrogenase subunit E2 (bkdB)	3.40E-02	15.59
		BCAL2143		ubiquinol oxidase polypeptide I (cyoB)	4.27E-03	80.60
		BCAM0042		putative aldo/keto reductase	3.61E-02	15.01
		BCAM2675		putative cytochrome oxidase subunit II	2.54E-02	31.91
	Carbohydrate Transport & Metabolism	BCAM2135		major facilitator superfamily protein	2.25E-02	11.71
	Coenzyme Transport & Metabolism	BCAL0264		delta-aminolevulinic acid dehydratase (hemB)	3.94E-03	29.36
		IG2_3145916	BCAM2784	aminotransferase	1.03E-03	41.88
	Ion Transport & Metabolism	BCAL0740		hypothetical protein	4.60E-03	13.60
		BCAL1700		ornibactin receptor OrbA	3.29E-02	10.01
		BCAL2299		putative permease	2.30E-02	29.76
		BCAL3297		putative ferritin DPS-family DNA binding protein	2.40E-02	25.61

		BCAM0827		putative voltage gated chloride channel membrane protein	3.43E-02	37.03
	Nucleotide Transport & Metabolism	BCAL2387		uracil phosphoribosyltransferase (upp)	6.46E-03	27.83
	Lipid Transport & Metabolism	BCAL1473		succinyl-CoA:3-ketoacid-coenzyme A transferase subunit B (scoB)	8.15E-04	27.84
		BCAM2430		putative biotin carboxylase	1.55E-02	18.92
	Biosynthesis & Catabolism	BCAL3184		homogentisate 1,2-dixoygenase (hmgA)	1.82E-02	65.59
		BCAM0906		putative dienelactone hydrolase family protein	3.14E-02	18.93
		BCAM2707		putative FAA-hydrolase family protein	3.21E-02	10.79
Cellular Processes & Signalling	Signal Transduction Mechanisms	IG1_1435033	BCAL1318	putative nitrate regulatory protein	6.16E-03	10.06
		IG2_1573637	BCAM1422	putative cyclic nucleotide binding protein	1.82E-02	10.46
	Trafficking, Secretion & Transport	BCAL2292		putative biopolymer transport protein	3.20E-02	13.59
	Posttranslational Modification, Chaperones	BCAL0468		M48 family peptidase	7.53E-03	20.30
		BCAL1995		ATP-dependent protease ATP-binding subunit ClpX	4.87E-02	10.68
		BCAL3192		putative oxidoreductase	3.75E-02	10.44
	Membrane Biogenesis	BCAL3135		dTDP-D-glucose 4,6-dehydratase (rmlB)	2.15E-02	16.63
		IG3_642613	BCAS0591	efflux system transport protein	4.70E-02	10.87

Information Storage & Processing	Transcription	BCAL1440		LysR family regulatory protein	5.77E-03	77.50
		BCAL1901		transcription termination factor Rho	1.80E-02	35.58
		BCAL2048		GntR family regulatory protein	4.11E-02	18.16
		BCAL2985		GntR family regulatory protein	9.85E-03	17.08
		BCAM0240		N-acylhomoserine lactone dependent regulatory protein (cciR)	1.37E-02	46.13
		BCAM1928		putative transcription elongation factor	1.23E-02	69.67
		BCAM1943		MarR family regulatory protein	2.54E-02	19.08
		IG3_26003	BCAS0024	GntR family regulatory protein	2.56E-02	17.55
	Ribosomal Structure & Biogenesis	BCAL0484		aspartyl/glutamyl-tRNA amidotransferase subunit A (gatA)	3.38E-02	10.93
		IG1_1598102	BCAL1448	valyl-tRNA synthetase (valS)	2.14E-02	11.04
Poorly Characterized	General Function Prediction only	BCAL1414		putative hydrolase	1.45E-03	57.89
		BCAL1965		putative lipoprotein	1.77E-02	24.22
		BCAL2459		putative O-methyltransferase	1.25E-02	16.73
		BCAL2816		S-formylglutathione hydrolase	4.86E-02	25.61
		BCAL2851		putative hydrolase	3.98E-02	44.20
		BCAM0165		hypothetical protein	3.52E-02	15.56
		BCAS0703		putative short chain dehydrogenase	1.96E-02	31.93
	Function Unknown	BCAL1177		putative fusaric acid resistance transporter protein	2.96E-02	18.42
		BCAL1606		hypothetical protein	1.90E-02	10.88
		BCAM0770		hypothetical protein	2.69E-03	50.40
		BCAM1233		hypothetical protein	1.07E-02	11.73
		BCAM2700		hypothetical protein	6.22E-04	19.09
		BCAS0667		hypothetical protein	4.98E-02	11.40
		IG2_1044007	BCAM0492	putative transmembrane peptidase	1.34E-02	27.15

		IG2_2036821	BCAM1818	hypothetical protein	3.53E-02	21.39
		pBCA008		hypothetical protein	4.52E-03	35.68
	None	BCAL0810		putative PTS system, EIIA 2	2.78E-03	11.22
		BCAL1316		hypothetical protein	1.73E-02	10.64
		BCAL1418		major facilitator superfamily protein	2.33E-02	19.25
		BCAL1875		hypothetical protein	2.24E-02	11.90
		BCAL2025		hypothetical protein	1.91E-02	37.61
		BCAL2300		hypothetical protein	1.22E-02	13.82
		BCAL2461		hypothetical protein	4.14E-02	10.33
		BCAL2998		transglycosylase associated protein	4.66E-03	14.72
		BCAM0329		hypothetical protein	9.26E-03	52.46
		BCAM0330		putative lipoprotein	2.10E-02	22.02
		BCAM1030		hypothetical protein	1.90E-02	12.71
		BCAM2623		hypothetical protein	2.44E-02	14.17
		BCAM2685		hypothetical protein	1.70E-03	59.50
		BCAS0414		hypothetical protein	2.68E-02	20.91
		IG1_2896142	BCAL2633	putative DNA-binding protein	1.01E-03	10.43
		IG1_300118	BCAL0272	putative lipoprotein	1.98E-02	11.50
		IG1_803639	BCAL0740	hypothetical protein	5.37E-03	28.34
		IG2_2129074	BCAM1916	transposase	2.64E-02	13.36
		pBCA029		hypothetical protein	3.19E-03	14.73
		pBCA045		hypothetical protein	3.08E-02	15.11
		pBCA046		putative traE conjugative transfer protein	1.76E-02	25.95
		pBCA067		hypothetical protein	2.75E-02	22.44
		pBCA077		hypothetical protein	1.03E-02	90.16
		BCALr1899		signal recognition particle RNA	2.05E-02	11.02

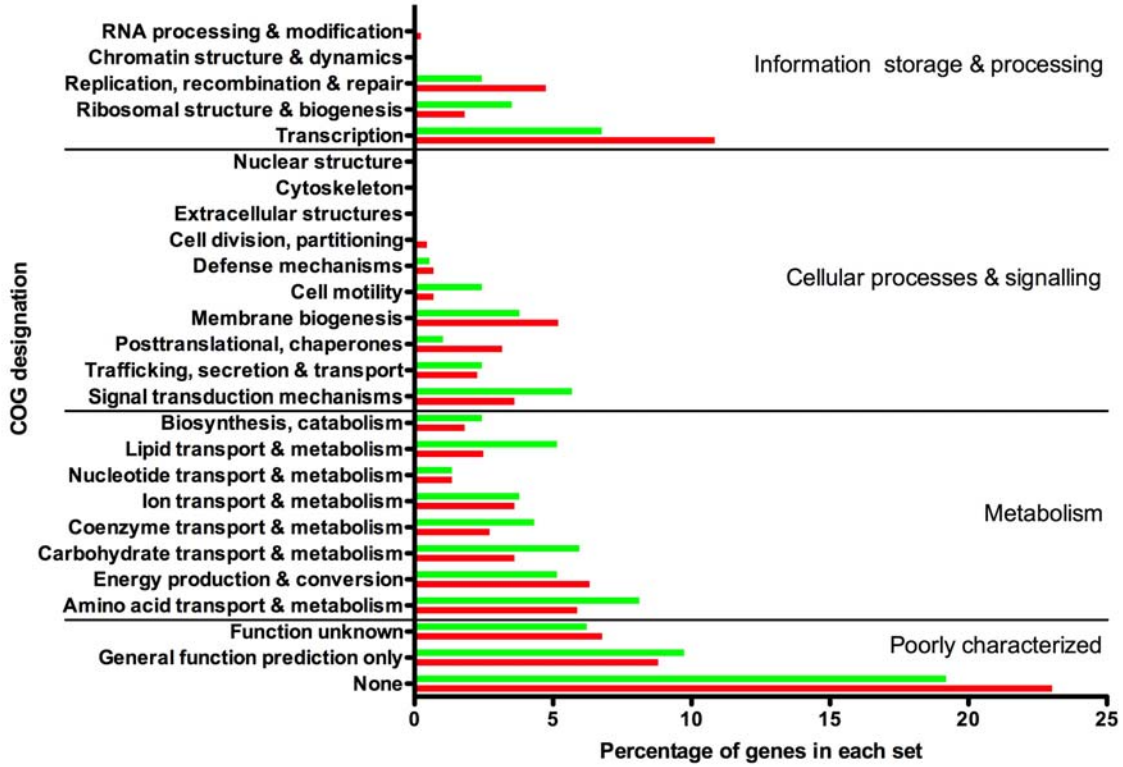

Figure 9. Functional classification of genes differentially expressed by intracellular *B. cenocepacia*

Profiles of functional classes are shown as a percentage of all induced (green bar) or repressed (red bar) genes ($-2 > \log2 > 2$, $p < 0.05$).

3.5 SCOTS-identified changes in gene expression validated by other means

A previous study identified *B. cenocepacia* K56-2 signature-tagged transposon mutants (STM) attenuated for survival in a rat agar bead model of chronic lung infection (207); in total, the study mapped 84 unique insertions within genes essential for *in vivo* survival. Four of these genes were identified directly by SCOTS competitive enrichment; another three SCOTS-identified genes were found in genes located within a putative transcriptional unit with an STM insertion (Table 7). SCOTS microarray analysis identified 26 of the 84 STM genes as being expressed by intracellular bacteria (Table 7), a significant finding considering the lung environment exposes the bacteria to many factors other than macrophages.

Quantitative RT-PCR was performed for a subset of genes to experimentally validate trends seen in the microarray data in the absence of any capture or enrichment. Total RNA was extracted and DNase-treated as was done for SCOTS, and reverse transcribed to cDNA using random hexamers. Microarray data indicates conserved hypothetical protein BCAM0314, ABC transporter ATP-binding protein BCAM2141, putative universal stress protein BCAM0276, and putative acyl carrier protein phosphodiesterase BCAS0186 should be induced by intracellular bacteria, while putative transcription elongation factor BCAM1928 should be repressed. Sigma factor BCAM0918, or *rpoD* - also designated *sigA* (297) or *sigE* (297, 349) – was chosen as an internal control. An internal reference gene allows comparison across samples; despite reverse transcribing equal amounts of RNA, expression of the reference gene averaged 60-fold less in intracellular samples as compared to NME samples due to eukaryotic content. Expression of the gene of interest was first related to the internal control; this ratio was compared between samples to give fold increase in expression in intracellular bacteria. qRT-PCR confirmed the expected trends of higher

Table 7: *In vivo* essential genes expressed by intracellular bacteria

Tag[a]	Gene designation[b]	Known or putative function
Selectively captured sequences		
7C6	BCAL0770	putative DNA polymerase III alpha subunit
37G5	BCAL0345[c]	conserved hypothetical protein
34C6	BCAL0352[c]	metallo peptidase, subfamily M15C
34A1	BCAL1536[c]	putative sigma-54 related transcriptional regulatory protein
36B4	BCAL3240	putative capsular polysaccharide transporter ATP-binding protein
31B3	BCAM0992	putative DNA methylase
31C5	BCAS0258	GntR family regulatory protein
Microarray		
4G5	BCAL0154	histone-like nucleoid-structuring (H-NS) protein
34C4	BCAL0339	putative type VI secretion system protein TssJ
1A5	BCAL0347	protease associated ATPase ClpB
34C6	**BCAL0352**	metallo peptidase, subfamily M15C
15B2	BCAL0423	chromosomal replication initiation protein
38H2	BCAL1122	conserved hypothetical protein
1H4	BCAL1318	putative nitrate regulatory protein
9C1	BCAL1850	putative dehydrogenase
38C6 39H4	BCAL2732	cold shock-like protein
32D2	BCAL3122	glycosyltransferase
38C2	**BCAL3125**	glycosyltransferase
3A6	BCAL3173	xanthine dehydrogenase
4H4	BCAL3178	LysR family regulatory protein
10F1 33F9	**BCAL3228**	hypothetical protein
40H2	BCAL3258	tetracycline repressor protein
20D2	BCAL3287	putative FAD-binding oxidase
33G2	BCAM0020	putative membrane protein
33B1	BCAM0179	putative mechanosensitive ion channel protein
28D8	**BCAM1011**	putative acetyltransferase
33G4	BCAM1478	aromatic amino acid aminotransferase
28D5	BCAM1679	putative lysylphosphatidylglycerol synthetase
40C1	BCAM1861	calcineurin-like phosphoesterase
6E3	BCAM1867	putative Mg(2+) transport ATPase
18D2	**BCAM2150**	transcriptional activator FtrA
31C5	BCAS0257	putative acetyltransferase
31G1	BCAS0695	putative phospholipid-binding exported protein

[a]Tag assigned to mutant in STM *in vivo* study (207)
[b]Gene names in bold were strongly induced (>3.5-fold, $p<0.05$)
[c]Immediately adjacent gene identified by SCOTS

expression of BCAM0314, BCAM2141, BCAM0276, and BCAS0186, and negligible expression of BCAM1928 in intracellular bacteria (Figure 10).

3.6 SCOTS-identified genes are involved in bacterial entry and intracellular replication

Because survival within macrophages may contribute to the pathogenesis of *B. cenocepacia*, the role of several SCOTS-identified genes in intracellular survival was examined. Ten genes were deleted in the gentamicin-sensitive *B. cenocepacia* MH1K, allowing intracellular survival to be assessed via the gentamicin protection assay (185). The ten deleted genes encoded the following proteins: a subunit of the flagellar regulon master regulator (BCAL0124), a putative acyl carrier protein phosphodiesterase (BCAS0186), a cation efflux system (BCAM0434-5), the response regulator component of a two-component regulatory system (BCAM2837), a putative oxidoreductase (BCAL1726), a putative universal stress protein (BCAM0276), an MgtC family protein (BCAM0411), a putative lipoprotein (BCAL0340), a putative lysylphosphatidylglycerol synthetase (BCAM1679), and a putative Gram-negative porin (BCAM2446). These genes were chosen based on high intracellular expression by microarray data; the first three genes were also identified by competitive enrichment.

The ability of mutant strains to enter and replicate within macrophages was assessed relative to the parental strain (Figure 11). While six of the ten mutant strains showed a defect in macrophage entry, the defect was statistically significant only for BCAL0124 ($p < 0.01$) and BCAM0411 ($p < 0.05$). In contrast, deletion of BCAL1726 resulted in a significant increase in macrophage entry ($p < 0.001$). While none of the deletions negatively affected intracellular survival, deletions in BCAL0340 and BCAM0411 increased the ability of the bacteria to replicate intracellularly. Statistically significant differences could be complemented by introducing the deleted gene encoded in a plasmid under the

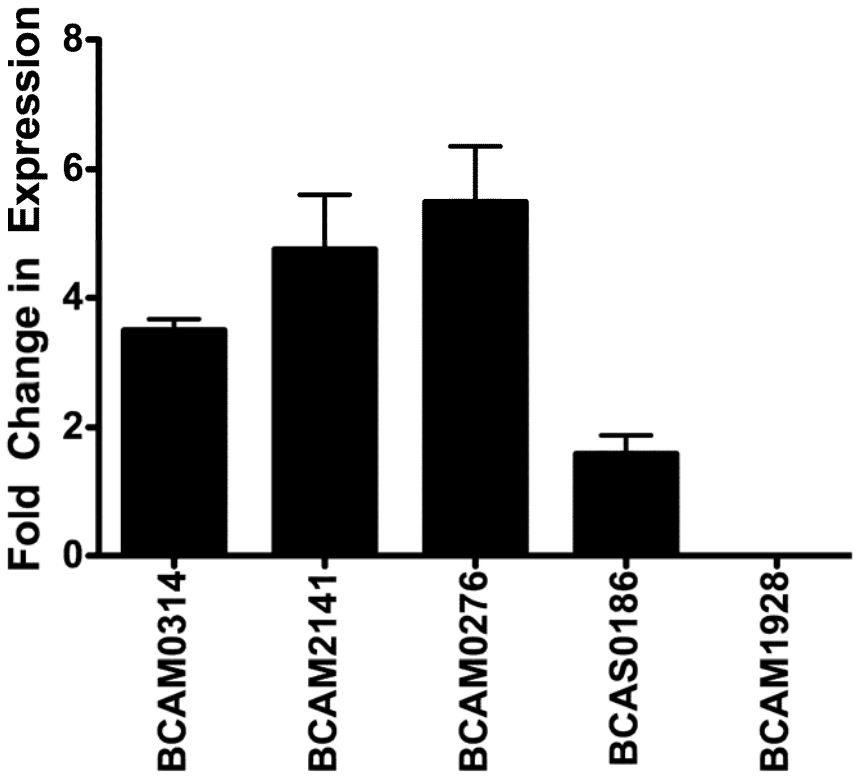

Figure 10. *n*-Fold changes in the intracellular expression of SCOTS-identified *B. cenocepacia* genes as measured by qRT-PCR.

Fold change represents the induction of expression by intracellular bacteria over NME bacteria, each relative to a reference gene presumed to have stable transcriptional levels.

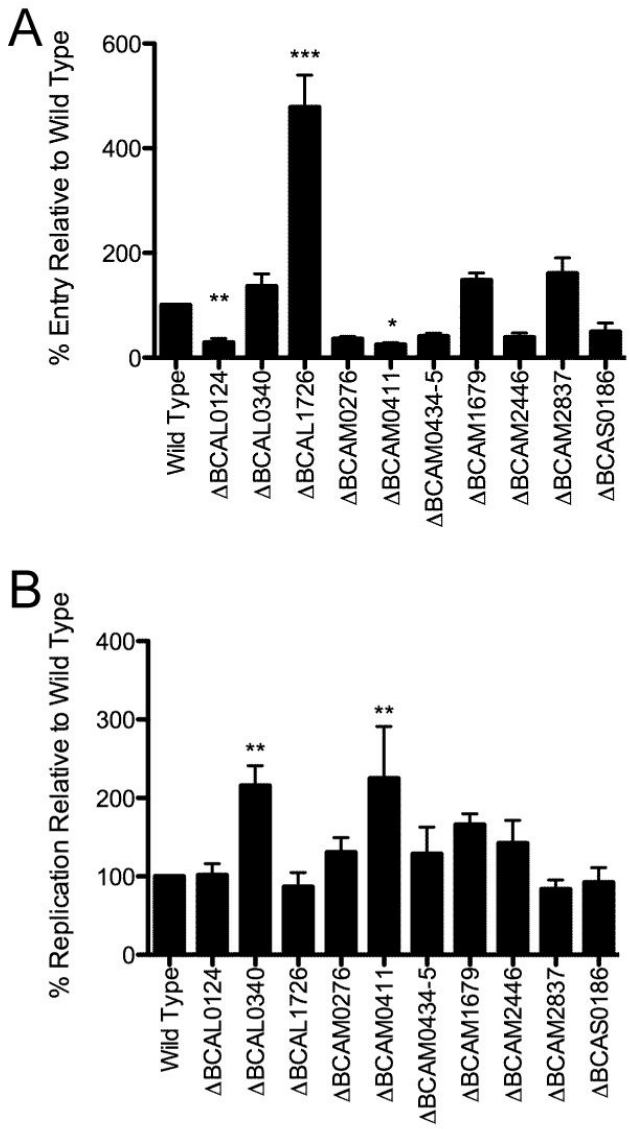

Figure 11. Comparative macrophage entry and intracellular replication of parental *B. cenocepacia* and *scs* mutants in murine macrophages. Entry (A) was calculated relative to initial inoculum, and replication (B) was calculated relative to entry; both were normalized relative to the parental control, set at 100% entry and 100% replication. Standard error bars are indicated. Significance was determined using one-way ANOVA and Dunnett's Multiple Comparison Test. Mutants demonstrating significant difference from the wild-type are indicated (*p<0.05, **p<0.01, ***p<0.001).

control of a constitutive promoter (Figure 12). Among the deletion strains, the only gene with an obvious phenotype is JST19, or ΔBCAL0124, which is defective in both swimming and swarming motility; complementation restored motility to the deletion strain (Figure 13). JST75, or ΔBCAM2837, is impaired in swarming motility, but not in swimming motility (Figure 14).

3.7 SCOTS-identified genes are involved in bacterial spread and macrophage cytotoxicity

The gentamicin protection assay allows the enumeration of intracellular bacteria. However, the results may be misleading if the survival of the host cell is not considered, as bacterial toxicity associated with membrane damage of the host cell will allow entry of the antibiotic, which will in turn result in the killing of intracellular bacteria. Analysis of toxicity of the host cell can be performed via flow cytometry. The membrane impermeant fluorescent dye 7-AAD binds DNA only in macrophages with permeabilized membranes. Macrophages were infected with GFP-expressing bacteria and assessed on three different parameters: entry, cytotoxicity, and spread. Parental *B. cenocepacia* was found in 3% of all macrophages, and was cytotoxic to macrophages at a 1:1 ratio, killing approximately 3% of all cells (Figure 15A). Despite infecting nine-fold better than parental, ΔBCAM2837 was also cytotoxic at a 1:1 ratio, killing 27% of all macrophages. Though ΔBCAL0340 infected two-fold better than the parental isolate, it was only half as cytotoxic, whereas ΔBCAL0124 demonstrated the opposite trend with two-fold less infection, but seven-fold greater cytotoxicity. Other mutants, including ΔBCAM0276 and ΔBCAM2446, appear to prevent permeabilization of the macrophage membrane, decreasing cytotoxicity below the level seen in uninfected macrophages (Figure 15). In total, of the ten mutants, only four were cytotoxic, defined as permeabilization of the host cellular membrane greater than seen in uninfected cells. However, a permeable membrane would permit gentamicin to enter the cell, thereby killing intracellular

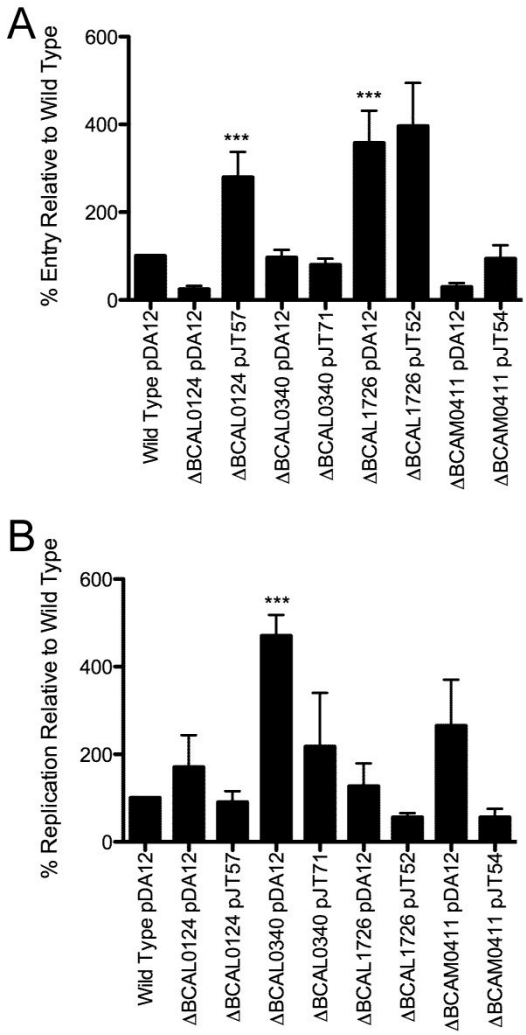

Figure 12. Comparative macrophage entry and intracellular replication of parental *B. cenocepacia* and complemented *scs* mutants in murine macrophages.

Plasmid pDA12 is a vector control; pJTx is a constitutively active complementation plasmid (Table 2). Entry (A) was calculated relative to initial inoculum, and replication (B) was calculated relative to entry; both were normalized relative to the parental control, set at 100% entry and 100% replication. Standard error bars are indicated. Significance was determined using one-way ANOVA and Dunnett's Multiple Comparison Test. Mutants demonstrating significant difference from the wild-type are indicated (*p<0.05, **p<0.01, ***p<0.001).

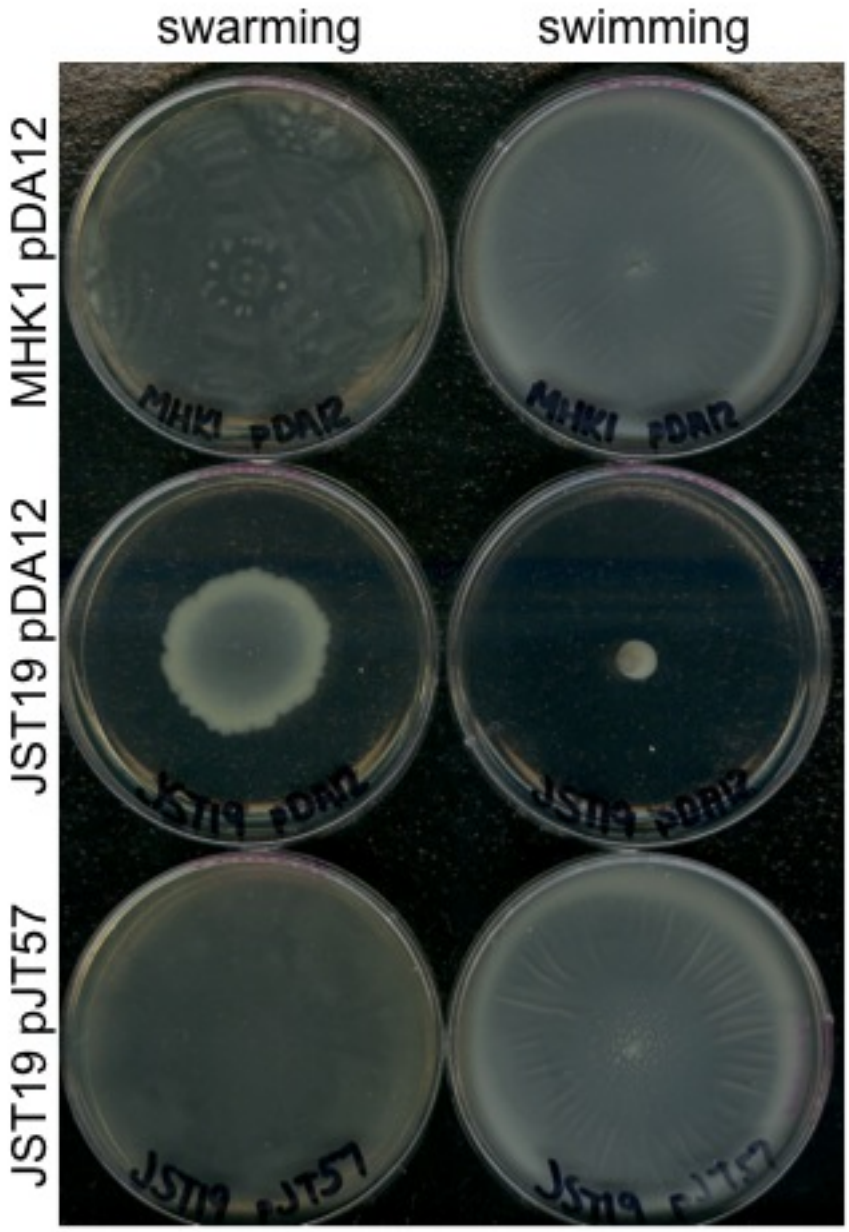

Figure 13. A mutation in the flagellar regulon master regulator results in a strain (JST19) defective in both swimming and swarming motility.

This defect can be restored by complementation on a constitutively active plasmid pJT57. pDA12 is a vector control. Bacterial cultures were adjusted to an OD_{600} of 0.1; 2 μL was stab-inoculated into LB 0.3% agar to observe swimming motility, and 2 μL was drop-inoculated onto NB 0.2% glucose, 0.5% agar to observe swarming motility.

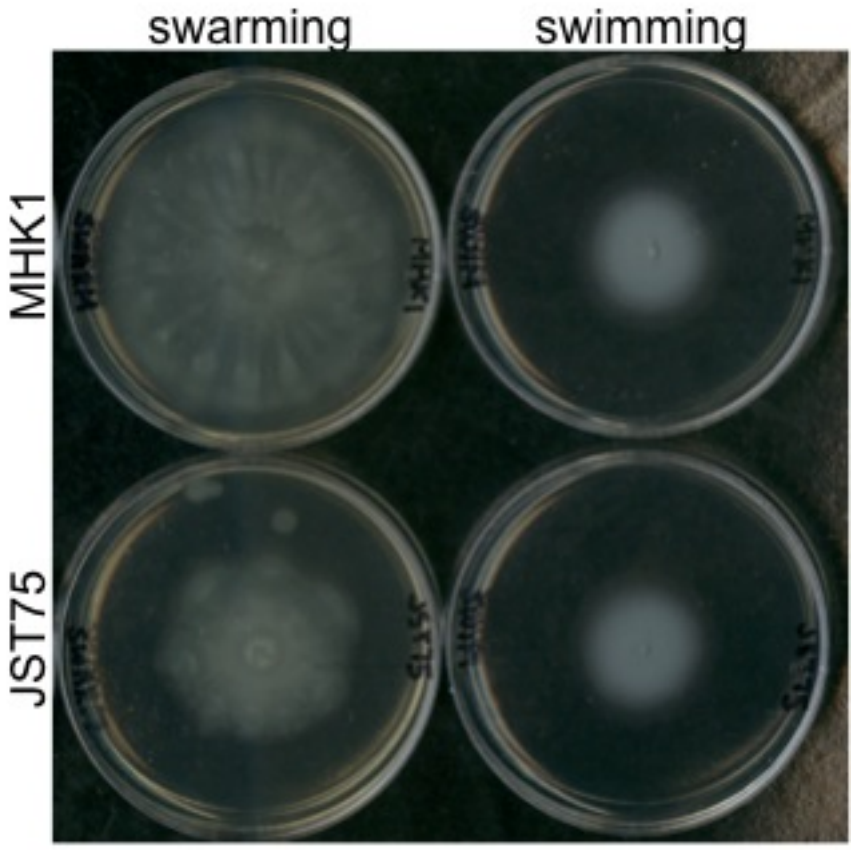

Figure 14. Deletion of a putative response regulator results in a strain (JST75) defective in swarming, but not in swimming motility.

Bacterial cultures were adjusted to an OD_{600} of 1.0; 2 μL was stab-inoculated into LB 0.3% agar to observe swimming motility, and 2 μL was drop-inoculated onto NB 0.2% glucose, 0.5% agar to observe swarming motility.

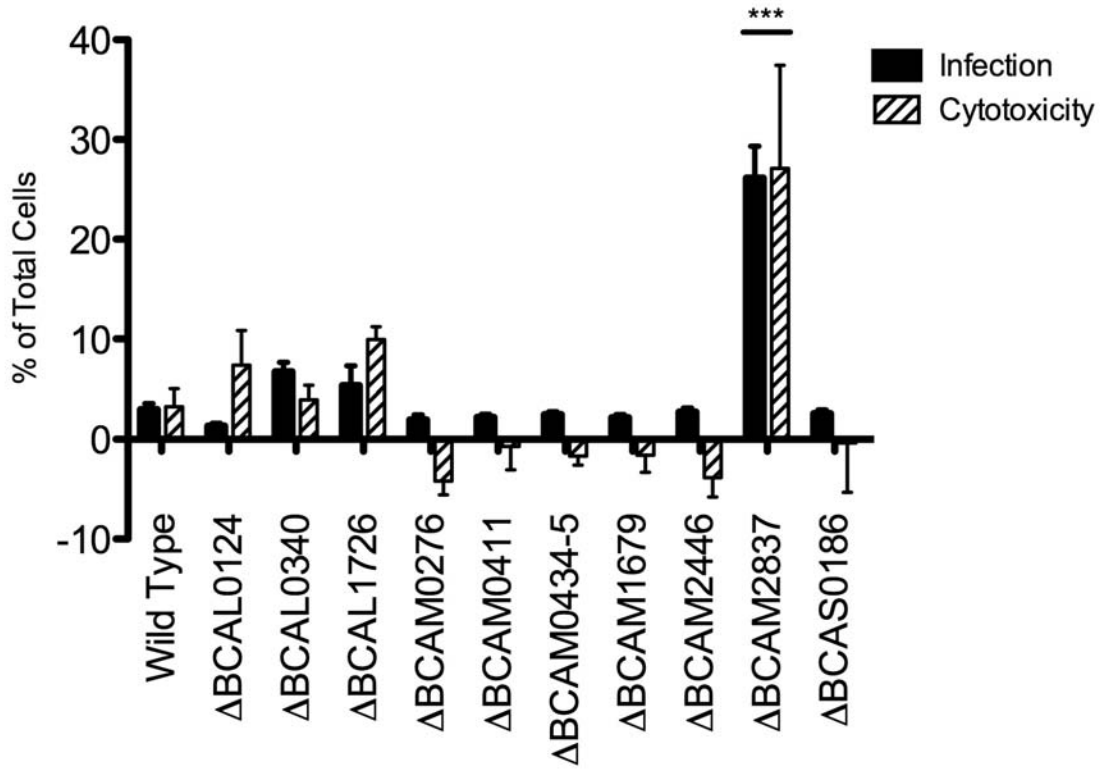

Figure 15. *B. cenocepacia* entry and host cytotoxicity in murine macrophages.

Bacterial entry and cytotoxicity are shown as a percentage of total cells. Cytotoxicity was normalized against cell death in uninfected cells. 50 000 cells were counted in each of three independent experiments. Standard error bars are indicated. Significance was determined using one-way ANOVA and Dunnett's Multiple Comparison Test. Mutants demonstrating significant difference from the wild-type are indicated (*p<0.05, **p<0.01, ***p<0.001).

bacteria and preventing quantification in the gentamicin protection assay. Thus, intracellular replication was normalized for cytotoxicity (Figure 16), revealing significant intracellular replication by ΔBCAL0124. In contrast, normalization revealed that ΔBCAL0340 replicates at a rate similar to the parental isolate, but is protected from gentamicin because it is less cytotoxic. Mutants displaying a cytotoxic ratio (Figure 16A) less than zero were not normalized, as in the absence of membrane permeability, intracellular bacteria would not be affected by gentamicin.

Flow cytometry also permits investigation of the spread of *B. cenocepacia* in an unchecked infection. Following killing of extracellular bacteria, infected macrophages were maintained in antibiotic-free media; any bacteria able to escape the macrophage, whether through lysis or otherwise, were able to infect neighbouring macrophages. Under such conditions, the number of macrophages infected with the parental strain increased six-fold (Figure 17), indicating that *B. cenocepacia* was able to escape the macrophage by some means. With the exception of ΔBCAM2837, which spread with half the efficiency of parental, the *scs* mutants spread as well or better than parental. ΔBCAL1726 and ΔBCAM1679 spread efficiently, increasing macrophage infection by 15-fold in an uncontrolled environment. The ratio of dead:infected cells remained approximately 1:1 in an unchecked infection, suggesting that viable *B. cenocepacia* escaped from dead macrophages. The two exceptions to this ratio were seen in ΔBCAL0124, which killed 2.5 cells for every cell infected at the time of analysis, and ΔBCAM2837, which killed only 30% of infected cells.

3.8 *B. cenocepacia* remains in an acidic vacuole associated with the endocytic pathway

Despite using 24 h as an endpoint in gentamicin protection assays, the BcCV has not been extensively characterized beyond 6 h p.i., where greater than 80%

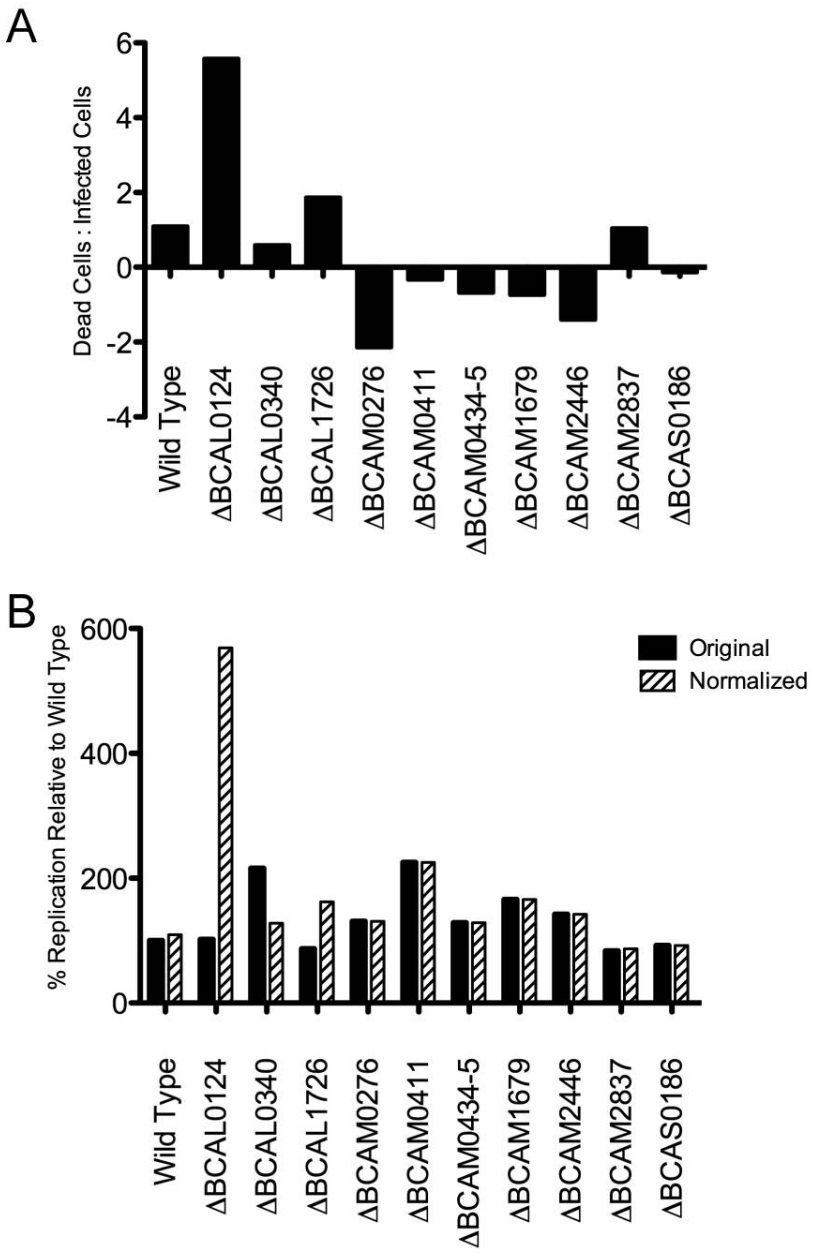

Figure 16. Intracellular replication normalized for host cytotoxicity of *B. cenocepacia* parental strain and *scs* mutants.

(A) Cell death was normalized against infection, determined by flow cytometry. (B) Intracellular replication, determined by the gentamicin protection assay, was then multiplied by this ratio to give the total expected ratio of intracellular bacteria in the absence of cell death. Mutants with a ratio in (A) less than zero were unchanged in (B).

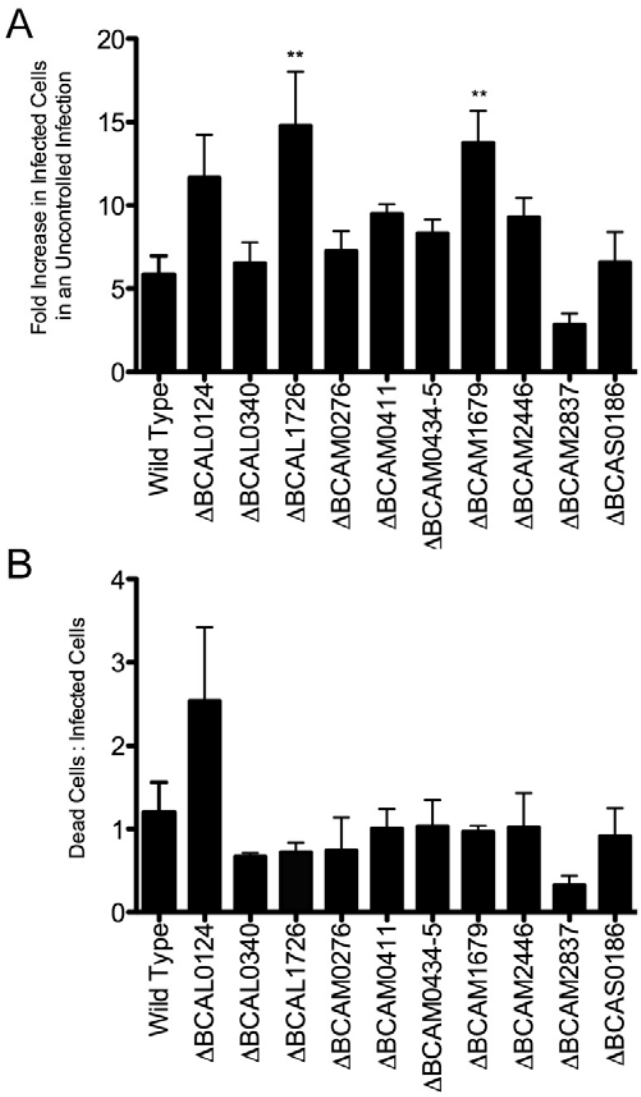

Figure 17. *B. cenocepacia* spread of infection and host cytotoxicity in an unchecked infection of murine macrophages.

Capacity to spread (A) is calculated as the fold increase in infected macrophages when maintained in antibiotic-free media rather than 10 µg/mL gentamicin in the same experiment. Host cytotoxicity (B) is calculated as the ratio of total dead cells to infected cells. 50 000 cells were counted in each of three independent experiments. Standard error bars are indicated. Significance was determined using one-way ANOVA and Dunnett's Multiple Comparison Test. Mutants demonstrating significant difference from the wild-type are indicated (*p<0.05, **p<0.01, ***p<0.001).

of BcCVs remain associated with the endocytic pathway, and approximately 50% of BcCVs are acidic phagolysosomes (252). At 24 h p.i., B. cenocepacia is still found within a vacuole, and a proportion of BcCVs contain numerous bacteria (Figure 18). 92% of BcCVs are acidic, indicated by colocalization with the acidotropic dye LysoTracker Red; in some vacuoles, rapidly-moving bacteria are strongly-labelled with Lysotracker Red, while the surrounding vacuole is only weakly-stained (Figure 18B). The BcCV remains associated with the endocytic pathway, as TMR-dextran added 21 h p.i. is delivered to 90% of BcCVs during a 2 h pulse followed by a 1 h chase (Figure 19). A fluid phase marker internalized via endocytosis, dextran follows the typical endocytic pathway, ultimately and rapidly accumulating in the lysosome. A dextran pulse-chase prior to bacterial infection is often used to pre-label lysosomes (252). However, 24 h post-B. cenocepacia infection, the dextran signal is weak in infected macrophages (Figure 19B); while some BcCVs are labelled with dextran, the weakness of the signal precludes designation of non-labelled BcCVs as non-lysosomal.

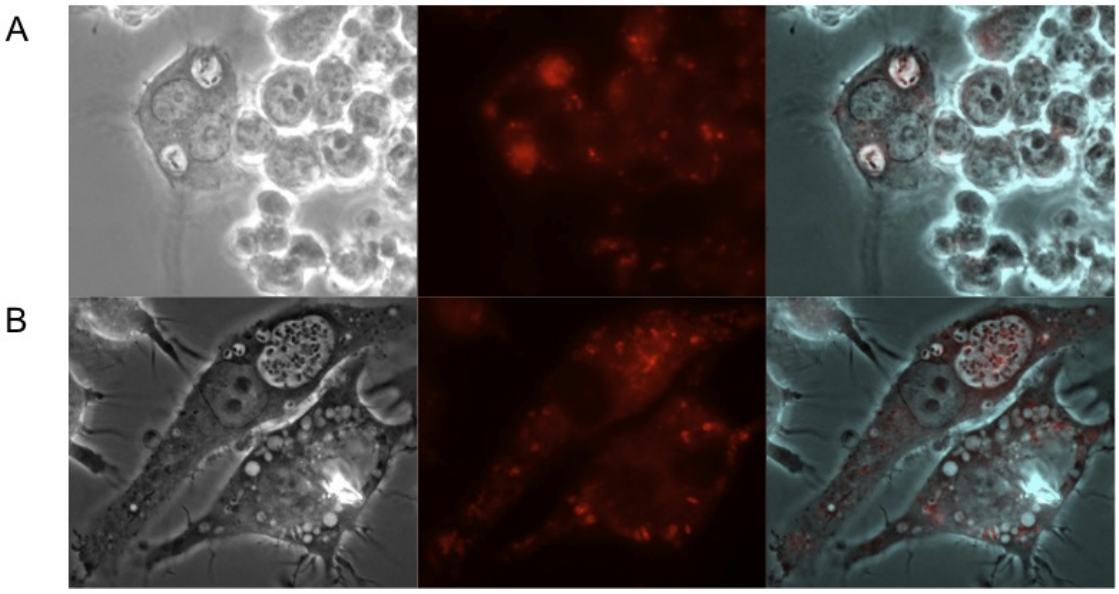

Figure 18. *B. cenocepacia* remains within a membrane-bound vacuole that accumulates acidotropic probe Lysotracker Red.

RAW 264.7 macrophages infected with *B. cenocepacia* were observed at 24 h p.i. after treatment with Lysotracker red. Panel A shows accumulation of Lysotracker in the BcCV, while panel B shows accumulation of Lysotracker by *B. cenocepacia*. From left to right, panels show the phase contrast image, red fluorescence (Lysotracker red), and a merged image. Arrow indicates representative BcCVs.

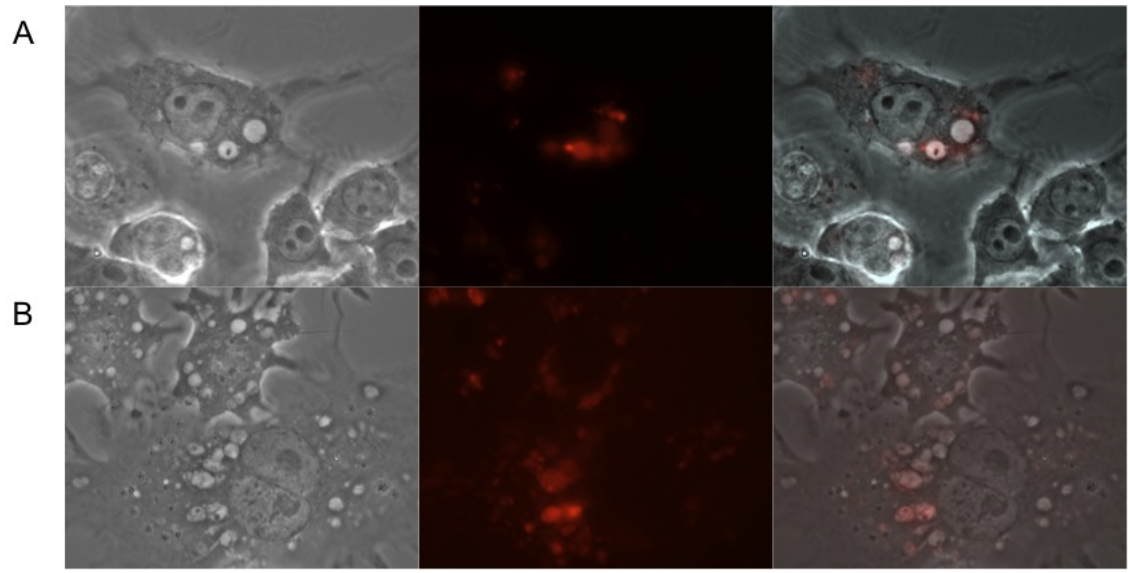

Figure 19. The *B. cenocepacia*-containing vacuole remains associated with the endocytic pathway.

Panel A shows RAW 264.7 macrophages infected with *B. cenocepacia* that were pulsed with dextran at 21 h p.i. and observed 3 h later. Panel B shows the opposite experiment, where RAW 264.7 macrophages were pulsed with dextran, then infected with *B. cenocepacia* and observed at 24 h p.i. From left to right are the phase contrast image, red fluorescence, and a merged image. Arrows indicate representative BcCVs.

Chapter 4

4 The role of secretion systems in the intracellular behaviour of *B. cenocepacia* in murine macrophages

4.1 Identification and deletion of secretion systems in *B. cenocepacia* K56-2

Large, specialized, multi-protein secretion systems are a common device employed by intracellular pathogens to interact with eukaryotic cells (188). The specific contribution of secretion systems to the intracellular lifestyle of *B. cenocepacia* is not well understood. The recent development of a system to create unmarked deletions in *B. cenocepacia* (156) permits construction of large, sequential deletions in the same strain. For each of the various secretion systems in *B. cenocepacia*, we deleted the entire gene cluster; we also created combination mutant strains with deletions in multiple secretion systems. This strategy served a dual purpose in preventing both any potential cross-complementation from paralogous genes, as well as a potentially toxic build-up of inactive or partial secretion systems. Because the clusters encode numerous proteins, deletion entailed removal of large segments of DNA: 28.7 kbp of chromosome 2 for the T3SS, 41.5 kbp of the plasmid for T4SS-1, 11.3 kbp of chromosome 2 for T4SS-2, and 22.3 kbp of chromosome 1 for the T6SS. For the plasmid, in particular, deletion of all putative T4SS-1-associated genes – a stretch of DNA including 45 genes - represented 45% of the entire plasmid sequence. We also deleted multiple essential genes of the T2SS, totalling 5 kbp of chromosome 1. In the strain JST112, lacking all secretion systems, this totals deletion of 108.7 kbp of genetic material. Despite these large deletions, growth of all strains in culture was unaffected (Figure 20).

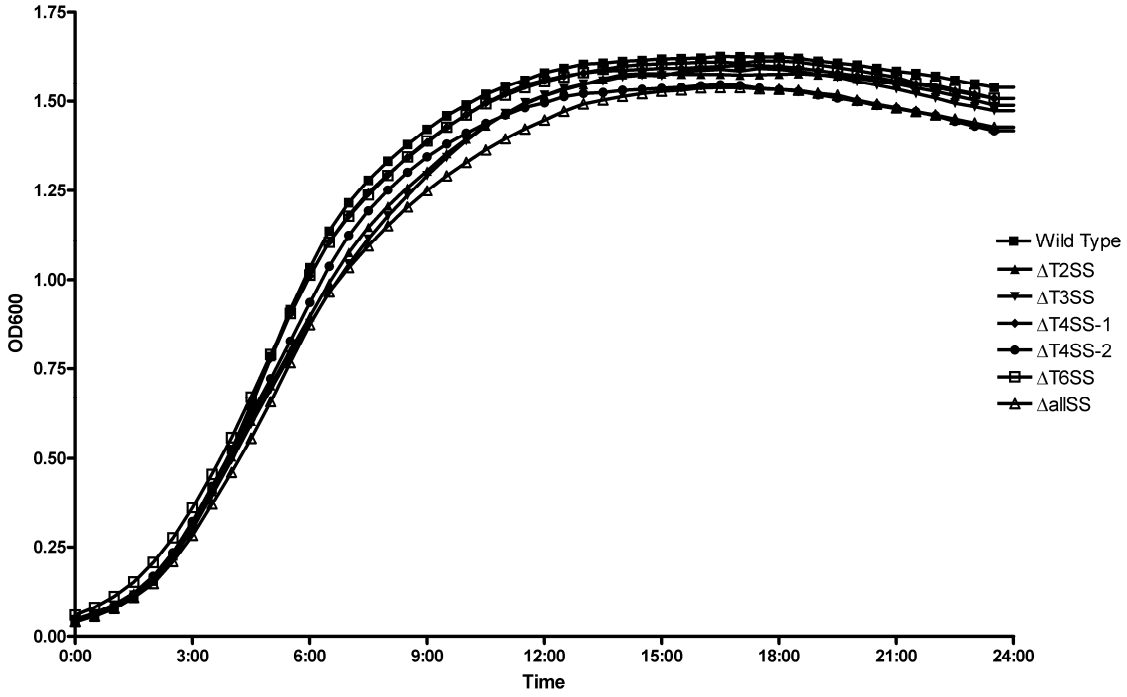

Figure 20. Deletion of secretion system clusters does not affect growth in culture.

Data represents the mean of two independent experiments, each performed in triplicate.

4.2 Characterization of secretion system deletion mutants

Secretion system mutants were examined for *in vitro* phenotypes, including exoenzyme secretion and LPS production. Among the secretion system mutants, ΔT2SS and ΔallSS were defective in secretion of casein-degrading protease, and lipase; ΔT3SS was also attenuated for secretion of protease, but not lipase (Figure 21). Results on the egg yolk agar were less clear; ΔT2SS and ΔallSS could not clear the media – characteristic of phospholipase A or protease secretion – nor form an opaque precipitate – characteristic of phospholipase C secretion (84, 275). The ΔT3SS mutant cleared the media more slowly than the parental strain; however, bacterial growth resembled neither ΔT2SS nor the parental strain, appearing flat, rough, dry, and white (Figure 21). None of the deletions had any effect on LPS production (Figure 22).

4.3 Secretion systems are involved in bacterial entry and intracellular replication

B. cenocepacia has previously been shown to enter (252, 402) and replicate within murine macrophages (185). Early studies of intracellular *B. cenocepacia* either relied on microscopic techniques (252), or utilized high concentrations of ceftazidime and gentamicin to overcome high aminoglycoside resistance and kill extracellular bacteria (409). Recent construction of an aminoglycoside-sensitive strain of *B. cenocepacia* with intramacrophage behaviour similar to the parental isolate (185) has enabled use of the gentamicin protection assay to reliably quantify intracellular survival using only low doses of antibiotic. We utilized the gentamicin protection assay to examine whether any of the secretion systems play a role in the entry and intracellular replication of *B. cenocepacia* in murine macrophages (Figure 23). After an initial infection period, extracellular bacteria were killed with gentamicin, allowing quantitation of intracellular bacteria at 1h (entry) and 24h (replication) post-infection. Because the generation time of

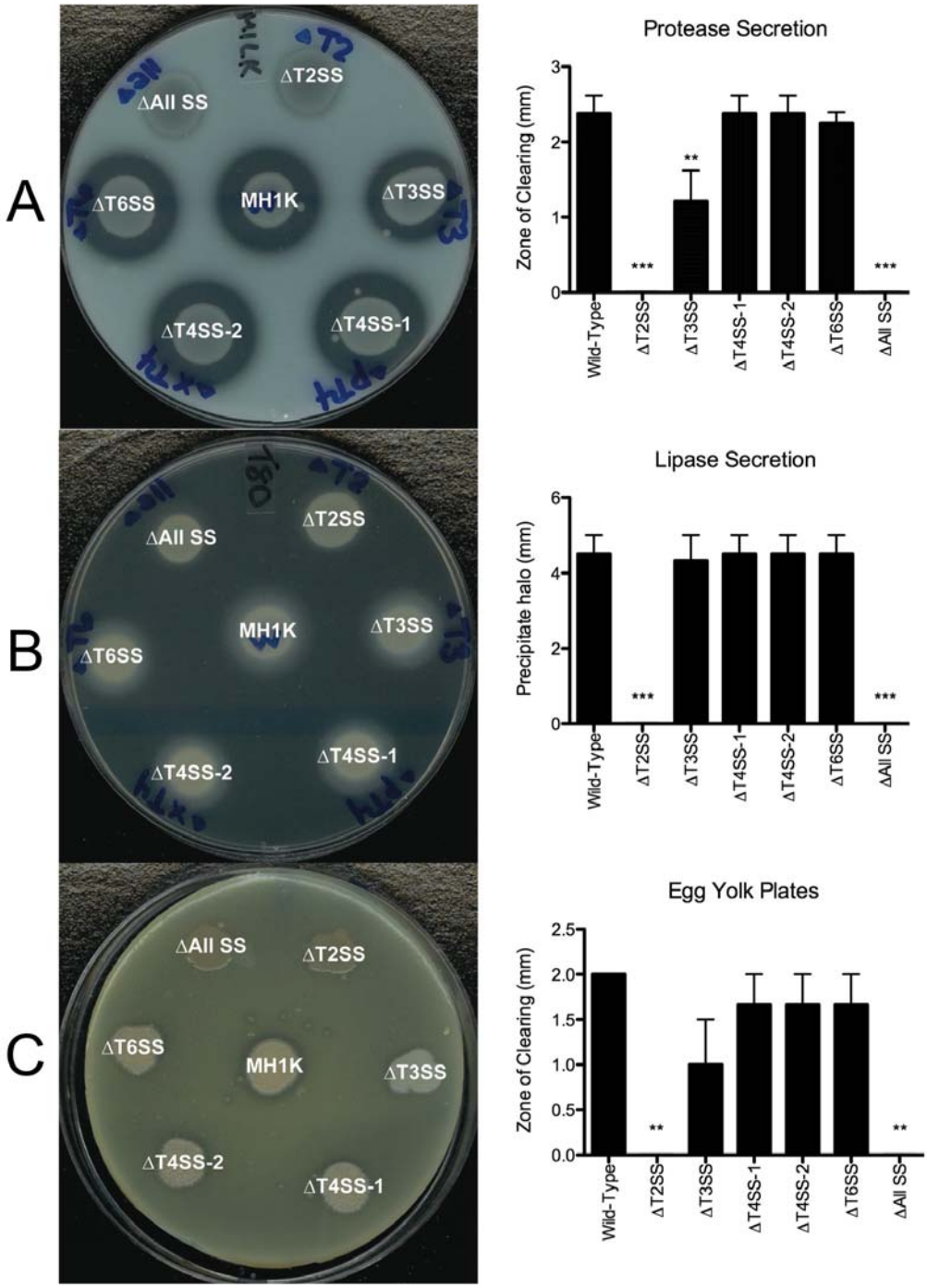

Figure 21. Deletion of secretion system clusters affects exoenzyme activity. Protease secretion (A) was measured as the zone of clearing on skim milk agar. Lipase secretion (B) was measured as the zone of precipitation on Tween 80 agar. Phospholipase C secretion (C) was measured as the zone of clearing on egg yolk agar.

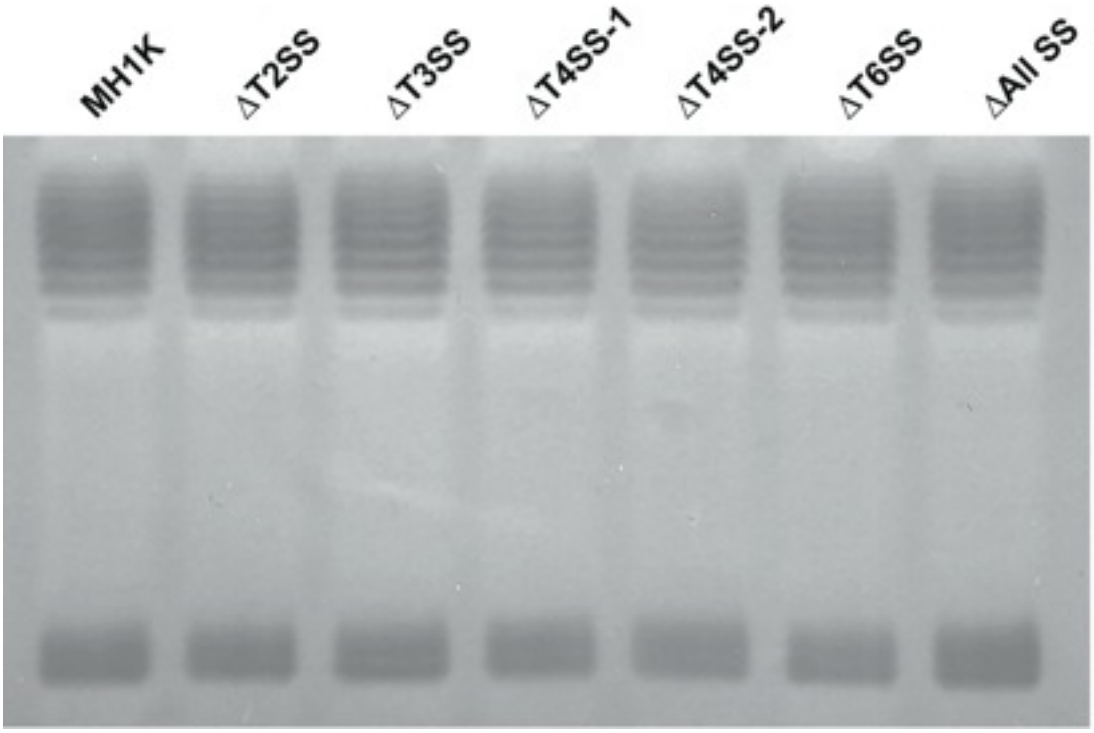

Figure 22. Lipopolysaccharide production is unaffected in secretion system deletion mutants.

Samples were separated on a 14% Tricine-SDS-PAGE gel, and stained with silver nitrate.

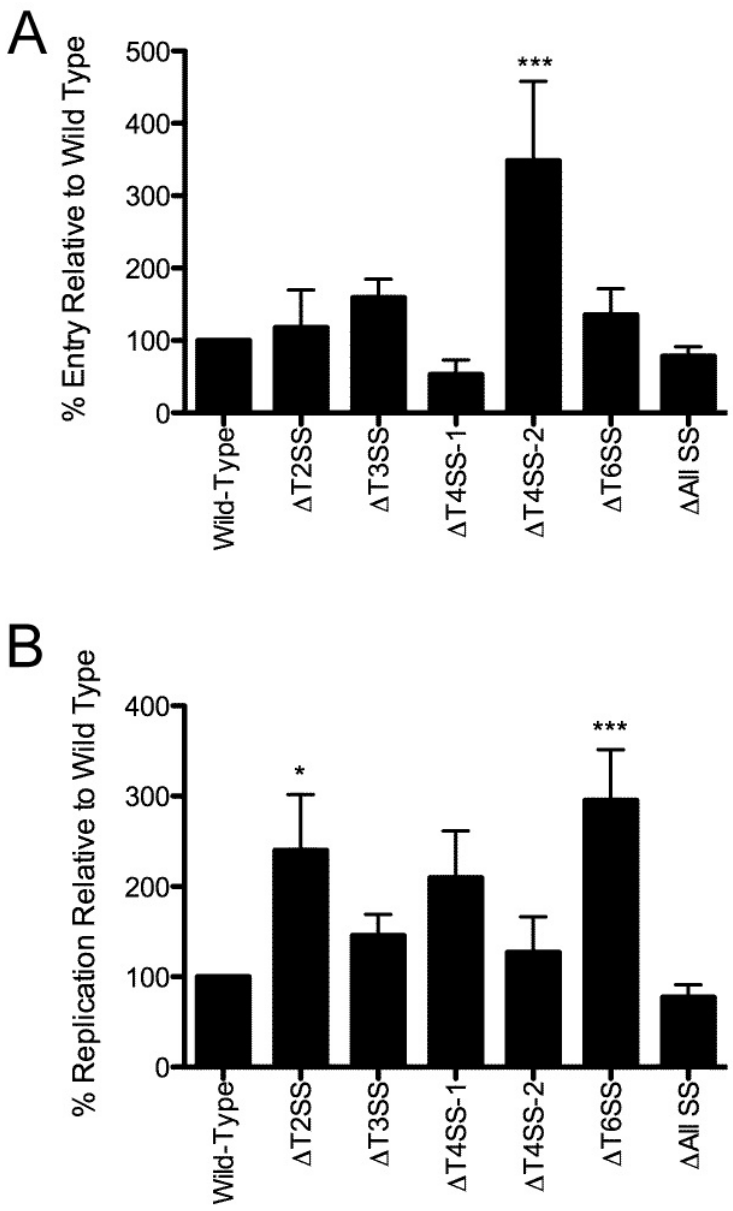

Figure 23. Secretion systems play a role in *B. cenocepacia* entry into murine macrophages and intracellular replication.

Entry was calculated relative to initial inoculum, and replication was calculated relative to entry; both were normalized relative to the parental strain, set at 100% entry and 100% replication. Standard error bars are indicated. Significance was determined using one-way ANOVA and Dunnett's Multiple Comparison Test. Mutants demonstrating significant difference from the wild-type are indicated (*p<0.05, **p<0.01, ***p<0.001).

B. cenocepacia in growth medium is approximately 1 h, the quantitation data at this time point after macrophage infection were considered to reflect amount of bacteria engulfed by macrophages prior to significant intracellular replication. Deletion of the T3SS had no apparent effect on bacterial entry or intracellular replication. Deletion of T4SS-1 resulted in slight, non-significant differences – a two-fold decrease in bacterial entry coupled with a two-fold increase in intracellular replication. Deletion of T4SS-2 resulted in a significant increase in bacterial entry; however, once internalized, ΔT4SS-2 replicated at the same rate as the parental strain. Deletion of the T2SS and the T6SS each showed the opposite trend with a significant increase in intracellular replication despite no effect on bacterial entry. Deletion of all secretion systems together showed no demonstrable phenotype in terms of either bacterial entry or intracellular replication.

Due to the large size of the cluster deletions, complementation of the original secretion system mutants was not practical. Instead, we created complementable single gene deletions in key structural components of each secretion system demonstrating a role in bacterial internalization or intracellular replication. To inactivate the T2SS, we deleted BCAL3516 (GspM), a key inner membrane protein component of the T2 apparatus (225, 283). Successful complementation by expression of the gene from plasmid pJT62 was evidenced by restored secretion of lipase, protease, and phospholipase C (Figure 24). Overexpression of GspM did not affect internalization of the bacteria, but significantly reduced intracellular replication (Figure 25), thereby complementing the phenotype seen in deletion of the entire cluster. To inactivate the T6SS, we deleted BCAL0343, or Hcp, a structural and secreted protein critical to the function of the T6SS in *B. cenocepacia* (25). Complementation of the deletion reduced intracellular replication to parental strain levels (Figure 25B). To inactivate the T4SS, we deleted pBCA025 (TraF) for T4SS-1, and BCAM0333 (VirB10) for T4SS-2. VirB10 is an essential inner membrane protein for the formation of the core complex of the T4SS-2 (86, 214); TraF is the homologous protein in the T4SS-1 (163). Deletion of VirB10 affected neither internalization

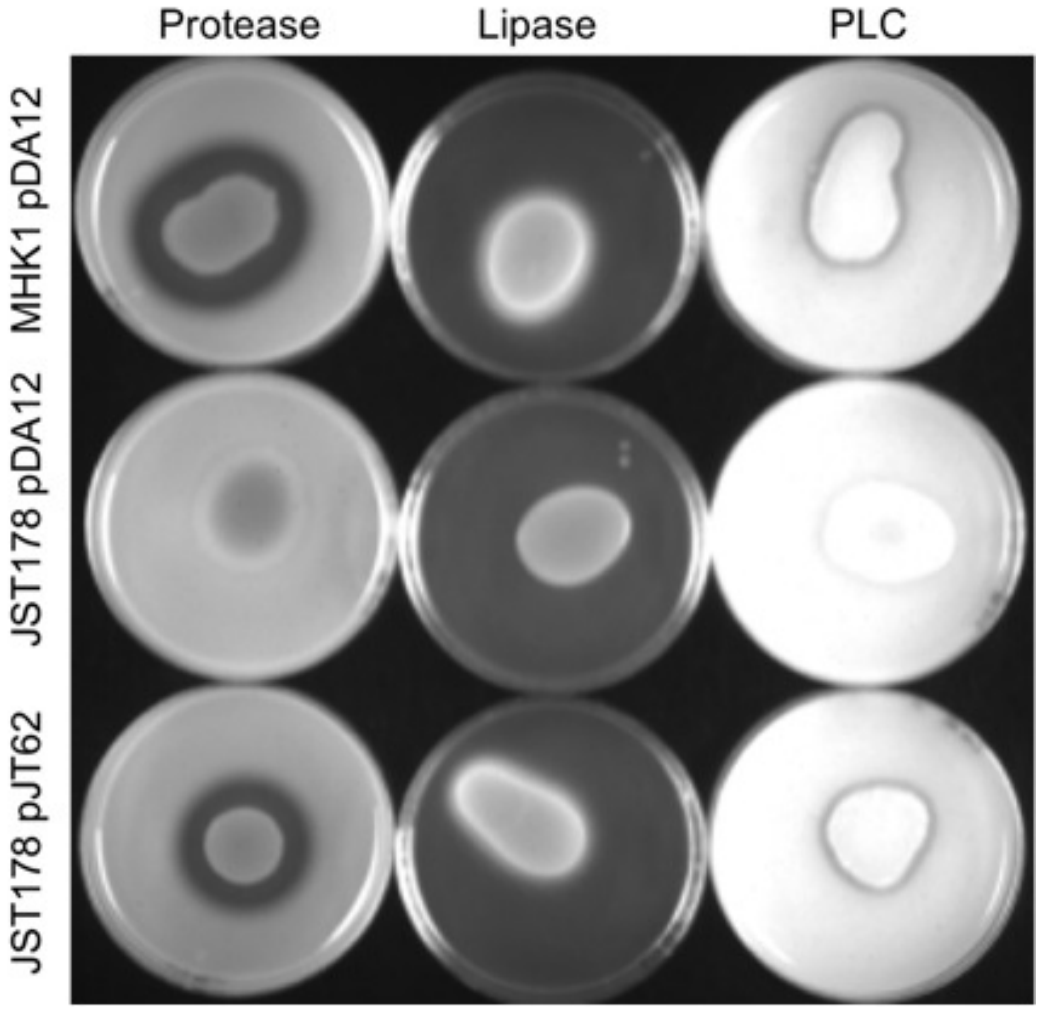

Figure 24. Defects in exoenzyme release in a T2SS mutant can be complemented.

Protease secretion was measured as the zone of clearing on skim milk agar. Lipase secretion was measured as the zone of precipitation on Tween 80 agar. Phospholipase C (PLC) secretion was measured as the zone of clearing on egg yolk agar. pDA12 is a vector control.

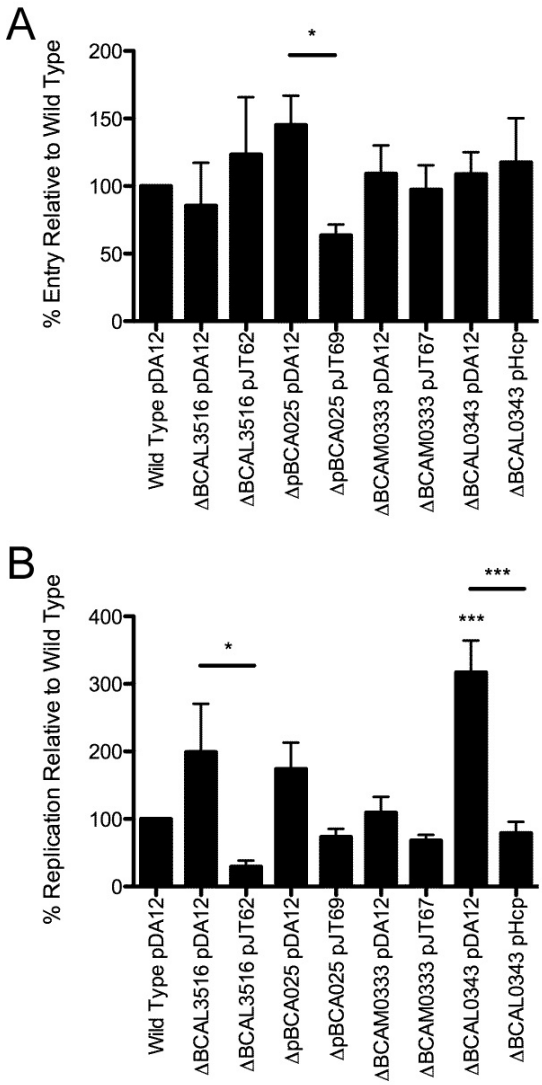

Figure 25. Defects in macrophage entry (A) and intracellular replication (B) of *B. cenocepacia* secretion system mutants can be complemented. Complementation plasmids are indicated as pJTx (Table 2); pDA12 is a vector control. Entry was calculated relative to initial inoculum, and replication was calculated relative to entry; both were normalized relative to the parental strain, set at 100% entry and 100% replication. Standard error bars are indicated. Significance was determined using one-way ANOVA and Dunnett's Multiple Comparison Test. Mutants demonstrating significant difference from the wild-type are indicated (*p<0.05, **p<0.01, ***p<0.001).

nor replication (Figure 25). However, deletion of *traF* alone yielded marginal increases in bacterial entry into the macrophage and intracellular replication, phenotypes that could be complemented.

4.4 Secretion systems are involved in bacterial spread and macrophage cytotoxicity

The fate of the host cells is not usually investigated in cellular infection models. However, it is reasonable to assume that intracellular bacteria may exert various effects on the host cells that could alter the gentamicin protection assay. Therefore, we also utilized flow cytometry to examine bacterial infection and host cell viability. Infected macrophages were identified by green fluorescence due to *B. cenocepacia* expression of GFP from a constitutively-active plasmid. Host cell viability was determined based on the permeability of cellular membranes; dead macrophages were identified by red fluorescence from the marker 7-AAD, a DNA-binding compound which cannot cross membranes. For initial infection, the data produced by the gentamicin protection assay (Figure 23) correlated well with the data produced by flow cytometry (Figure 26A). The single exception to this correlation was seen with ΔT4SS-1. The gentamicin protection assay showed two-fold less viable intracellular bacteria than the parental isolate, while flow cytometry showed two-fold more infected cells than the parental strain; however, neither difference was statistically significant from the parental isolate. It is important to note that the two assays have different measures; whereas the gentamicin protection assay quantifies the total number of intracellular bacteria, flow cytometry quantifies the total number of infected eukaryotic cells. This data, then, would suggest that, compared to infections with the parental strain, a greater number of macrophages internalize a lower number of bacterial cells in the absence of T4SS-1. Deletion mutants in T2SS, T3SS, and T6SS all showed infection levels similar to those of the parental strain, whereas deletion of T4SS-2 resulted in a statistically significant ($p < 0.05$) four-fold increase in infected cells. Deletion of all secretion systems caused a non-significant reduction in the

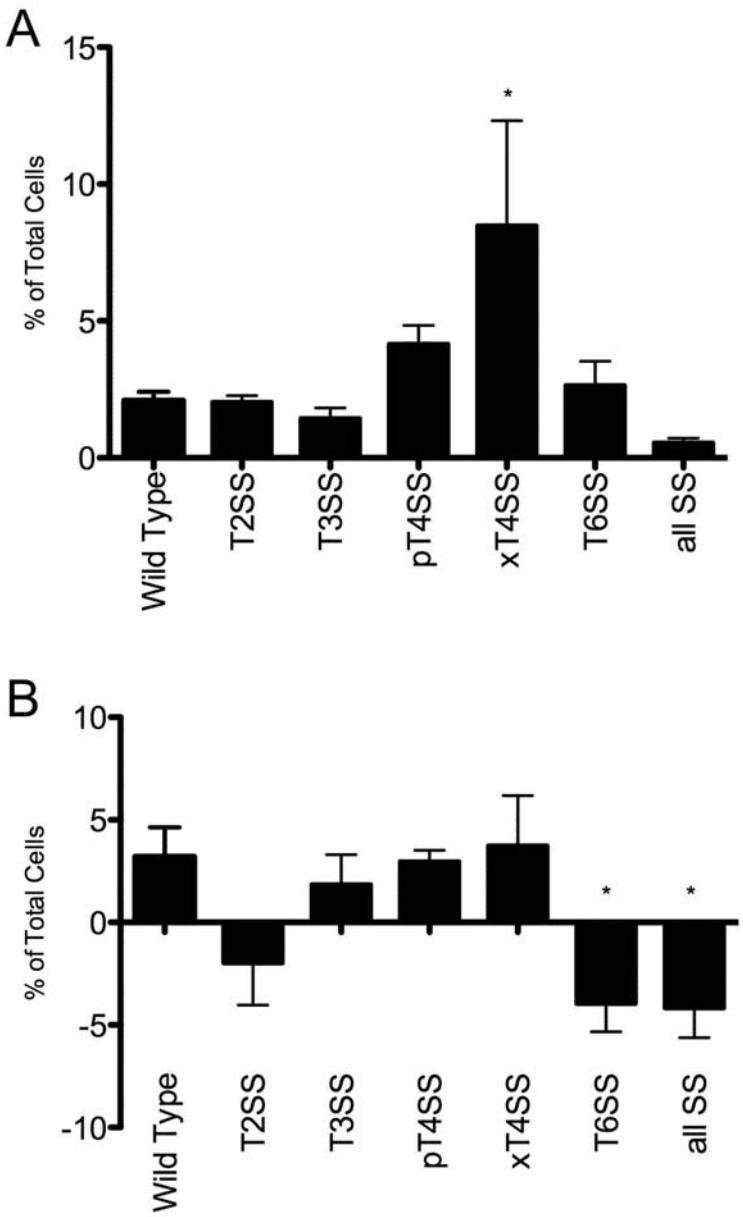

Figure 26. Secretion systems are involved in *B. cenocepacia* entry and host cytotoxicity in murine macrophages.

Bacterial entry (A) and cytotoxicity (B) are shown as a percentage of total cells. Cytotoxicity was normalized against cell death in uninfected cells. 50 000 cells were counted in each of three independent experiments. Standard error bars are indicated. Significance was determined using one-way ANOVA and Dunnett's Multiple Comparison Test. Mutants demonstrating significant difference from the wild-type are indicated (*p<0.05, **p<0.01, ***p<0.001).

number of infected macrophages (Figure 26A). However, deletion of all secretion systems also resulted in a significant decrease in cytotoxicity, equivalent to that seen with deletion of the T6SS. In both these mutant strains, as well as the T2SS mutant, membrane permeability was lower than that seen in natural cell death in uninfected cells, one of the controls for flow cytometry experiments. It is possible that these strains induce an alternate form of cell death in which the membrane is not permeabilized, which would prevent 7-AAD access to the host DNA. The cytotoxicity of *B. cenocepacia* towards the host macrophage was relatively unaffected by deletion of the T3SS, nor by deletion of either of the T4SSs (Figure 26B). However, because permeabilization of the membrane in the presence of *B. cenocepacia* allows gentamicin into the host cell, a measure of host cytotoxicity could be used to correct intracellular replication. When the ratio of dead cells to infected cells at 24 hours was taken into account (Figure 27A), intracellular replication was higher for both the parental isolate and for ΔT3SS, but lower for both T4SSs (Figure 27B); while ΔT4SS-1 appears to replicate at the same pace as the parental isolate, intracellular ΔT4SS-2 is three-fold less.

In the gentamicin protection assay, extracellular bacteria are killed via 30 minute incubation with a higher dose of gentamicin. In the classical assay, cells are then maintained in a low concentration of gentamicin such that bacteria that escape the macrophage cannot replicate in the media or re-infect neighbouring cells. However, by maintaining parallel cells in antibiotic-free media – an unchecked infection – bacteria which permeabilize the host membrane and escape from the macrophage are free to re-infect neighbouring cells, and the capacity of the bacteria to spread can be examined. The number of cells infected with the parental strain increased eight-fold in an unchecked infection (Figure 28A), and two-thirds of all infected cells had permeabilized membranes, and were presumably dead (Figure 28B). Deletion of the T3SS, which showed no discernable phenotype in gentamicin protection assays, appeared to facilitate the spread of infection at more than three times the speed of the parental isolate (27.4-fold increase for ΔT3SS versus 8.6-fold for parental). Deletion of all

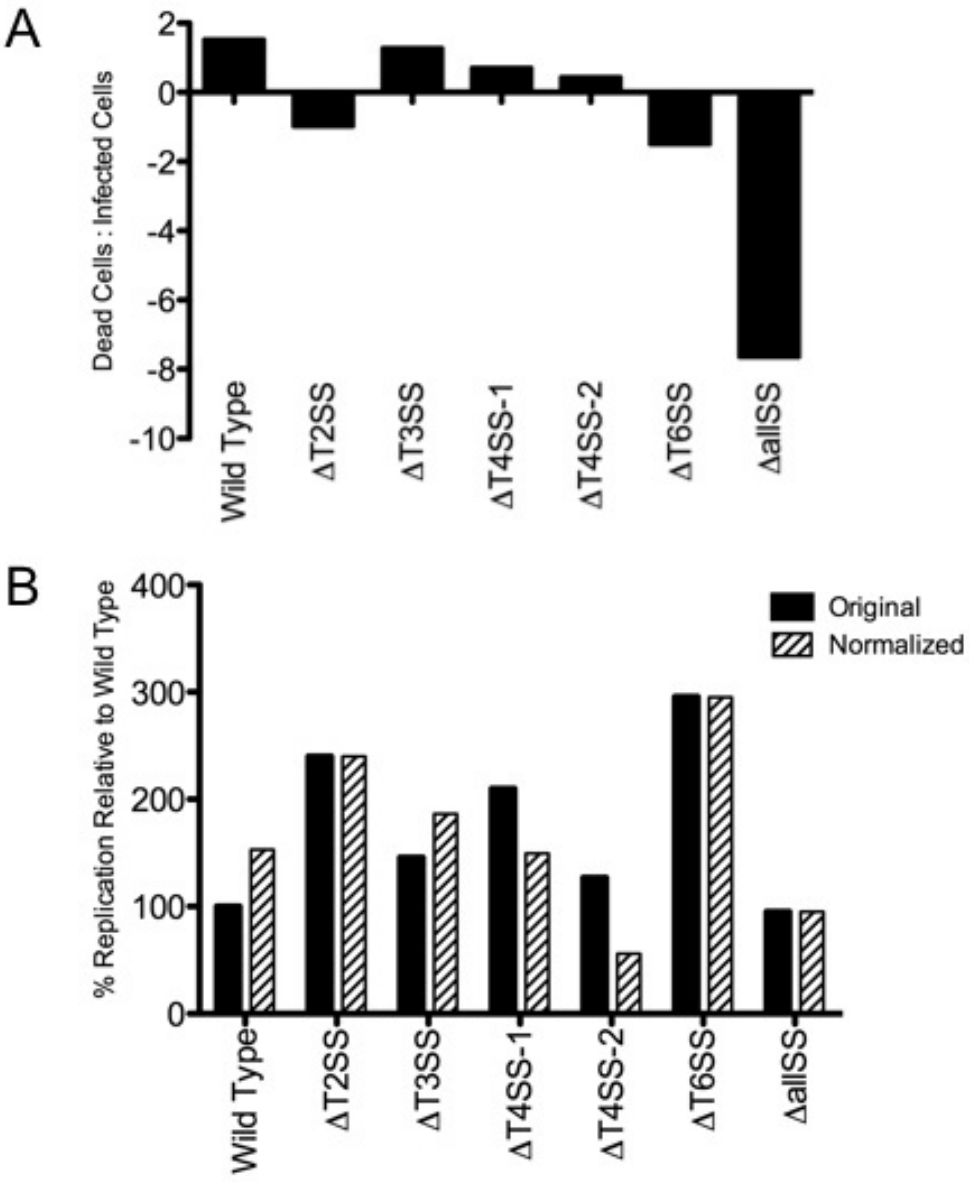

Figure 27. Intracellular replication normalized for cytotoxicity of *B. cenocepacia* parental strain and secretion mutants in murine macrophages.

(A) Cell death was normalized against infection, determined by flow cytometry. (B) Intracellular replication, determined by the gentamicin protection assay, was then multiplied by this ratio to give the total expected ratio of intracellular bacteria in the absence of cell death. Mutants with a ratio in (A) less than zero were unchanged in (B).

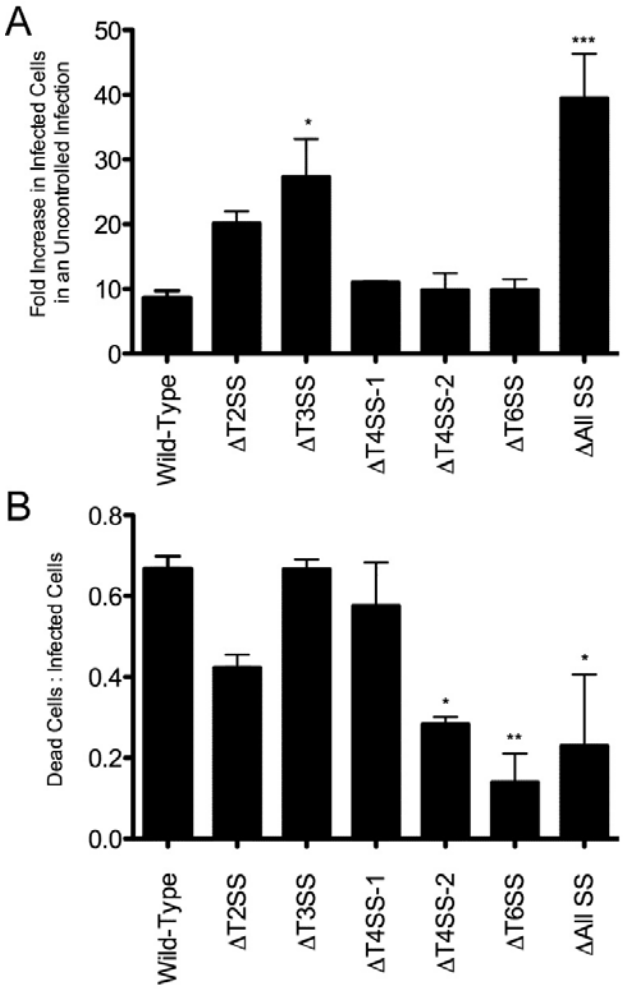

Figure 28. Secretion systems are involved in *B. cenocepacia* spread of infection (A) and host cytotoxicity (B) in an unchecked infection of murine macrophages.

Capacity to spread (A) is calculated as the fold increase in infected macrophages when maintained in antibiotic-free media rather than 10 μg/mL gentamicin in the same experiment. Host cytotoxicity (B) is calculated as the ratio of total dead cells to infected cells. 50 000 cells were counted in each of three independent experiments. Standard error bars are indicated. Significance was determined using one-way ANOVA and Dunnett's Multiple Comparison Test. Mutants demonstrating significant difference from the wild-type are indicated (*p<0.05, **p<0.01, ***p<0.001).

secretion systems significantly increased the ability of the bacterium to spread to neighbouring macrophages, resulting in a 40-fold increase in infected cells (Figure 28A). In the T3SS mutant, this increase in infected cells was accompanied with a proportionate increase in cell death, such that the ratio of dead:infected cells was equivalent to parental strain (Figure 28B). However, ΔT4SS-2, ΔT6SS, and ΔallSS all showed a significant decrease in cytotoxicity in an unchecked infection, killing only 28%, 14%, and 23% of infected macrophages, respectively.

Chapter 5

5 Discussion

5.1 Transcriptional changes by intracellular *B. cenocepacia*

B. cenocepacia is a bacterium with a large genome and an ability to survive in diverse environments, including within macrophages and other eukaryotic cells. In this study, we sought to identify genes expressed by intracellular *B. cenocepacia*. Microbial transcripts were initially isolated and amplified using the SCOTS technique, which removes eukaryotic cell transcripts and amplifies bacterial transcripts while discarding bacterial ribosomal RNA. Previous studies in other bacteria have shown that application of SCOTS enriches microbial transcripts without introducing a significant bias in gene expression data (148). SCOTS-purified cDNA was subjected to both microarray analysis and a competitive enrichment approach to identify gene regulation by intracellular *B. cenocepacia*, producing a wealth of information towards understanding the behaviour of intracellular *B. cenocepacia* following internalization by macrophages.

The distribution of upregulated classes of genes was similar between both analysis techniques, and 86% of the genes identified by SCOTS-CE also showed upregulation in the microarray data. However, while competitive enrichment identified 115 genes putatively upregulated by intracellular bacteria, microarray analysis showed expression of over 4000 genes and significant upregulation of 329 genes by intracellular bacteria. In some instances, the analysis techniques provided complementary information. For example, though CE-identified flagellar regulon master regulator BCAL0124 itself is not significantly upregulated by intracellular bacteria 4 h p.i., flagellar genes controlled by BCAL0124 show increased expression, and BCAL0124 clearly plays a role in the interaction of *B. cenocepacia* with macrophages (Figures 11-12, 15-17). Combining the SCOTS

technique with microarray analysis yields global gene profiles of genes induced and repressed in intracellular bacteria.

5.1.1 The gentamicin protection assay and flow cytometry: Complementary analyses for cell culture infection models

The classical measure of bacterial intracellular survival in eukaryotic cells is the gentamicin protection assay (139, 185), which is based on intracellular bacterial colony forming unit (CFU) counts. Extracellular bacteria are eliminated by treatment with gentamicin, which poorly permeates eukaryotic cells; subsequently, host cells are lysed to quantify intracellular bacteria. However, should the infecting bacterium be toxic to the host cell, resulting in a potentially compromised host cell membrane, antibiotics present in the cell culture medium would also kill intracellular bacteria, confounding plate counts. In addition, total intracellular bacteria are quantified, rather than the actual number of host cells infected. Population analysis by flow cytometry addresses some of these issues. Flow cytometry links macrophage infection with cell death on a per cell basis. Thus, combining the gentamicin protection assay with flow cytometry reveals trends in bacterial entry into macrophages, including the numbers of bacteria taken up per macrophage, as well as the capacity of bacteria to replicate within the cell and induce host cell death.

5.1.2 The role of SCOTS-identified genes in interactions with macrophages

The contribution of SCOTS-identified genes to macrophage entry, intracellular replication, and host-directed cytotoxicity was examined through deletion of genes upregulated by intracellular bacteria. In comparing initial infection data between the gentamicin protection assay and flow cytometry assay, those *scs* mutants that showed a defect in entry with the former assay generally show the same trends with the latter. However, while the gentamicin protection assay

shows a striking increase in infection by ΔBCAL1726, flow cytometry shows a more modest increase in infection, coupled with a higher level of cell death. This suggests two potentially-complementary possibilities: Either more mutant bacteria may be present per macrophage, or ΔBCAL1726 is more cytotoxic than the parental strain and, despite initially infecting at a higher level, has been killed by gentamicin entering dying host cells. The enhanced ability of the mutant to spread to neighbouring cells in an unchecked infection could support either hypothesis; a greater number of bacteria per macrophage potentiates re-infection of a greater number of host cells, while increased cytotoxicity could lead to more rapid spread. However, complementation of the BCAL1726-mediated increase in bacterial entry was unsuccessful (Figure 12). While complementation in *B. cenocepacia* can prove generally difficult, it is possible that the lack of complementation for BCAL1726 may be due to a secondary mutation, or to a lack of regulation. BCAL1726, annotated as a putative oxidoreductase, is located in a precorrin synthesis cluster, and is orthologous to precorrin-3B synthetase, involved in aerobic cobalamin biosynthesis (224, 225). This gene is one of five in *B. cenocepacia* regulated by a cobalamin riboswitch (195), which modulates the expression of associated genes – all involved in cobalamin biosynthesis or transport - in response to changing concentrations of the cofactor (338). Although the complementing plasmid pJT52 encodes the complete sequence of BCAL1726, it does not include the riboswitch found immediately upstream, resulting in constitutive expression regardless of cobalamin concentration. It is possible that overexpression of a single key enzyme in the biosynthetic pathway disrupts cellular homeostasis in a manner equivalent to absence of the enzyme. Cobalamin is an important cofactor, and has been shown to act as an intracellular antioxidant (51), to inhibit nitric oxide synthase (516), and to regulate cytokines IL-6 (419) and TNF-α (418), thus indirectly regulating transcription factor NFκB (499).

A disparity between flow cytometry and gentamicin protection assays was also seen for ΔBCAM2837. While the gentamicin protection assay shows a modest increase in initial infection, flow cytometry shows a dramatic increase in infection,

coupled with an equivalent increase in cell death; the failure of this particular mutant to spread effectively suggests that host cell death may be rapid – accounting for the more modest increase seen with the gentamicin protection assay – and that the bacterium may not be able to escape the dying macrophage as efficiently as parental strain, or may be killed during host cell death. BCAM2837 is annotated as the response regulator component of a two-component regulatory system (TCS). Though it is indicated as a pseudogene in the Artemis visualization of the J2315 genome (195, 395), it is evidently active in K56-2, as deletion of the gene demonstrates a clear phenotype in macrophage infection. Bioinformatic analysis shows that the protein consists of two distinct domains, one homologous to the chemotaxis protein CheC, and the second homologous to the response regulator CheY. Che proteins are typically involved in chemotaxis, where phosphorylated CheY (CheY-P) binds to the flagellar switch to alter rotation, affecting the ability of the bacterium to swim smoothly rather than tumbling (366). CheC is a phosphatase for the dephosphorylation of CheY (473). CheYC proteins – having an N-terminal CheY domain and a C-terminal CheC domain – are also found in organisms such as *Pseudomonas syringae* (334), although the function is unclear; CheYC proteins may function as standard TCS phosphatases, as phosphate sinks, or as completely novel proteins (334). Unlike the typical CheYC protein, BCAM2837 is likely cotranscribed with a putative diguanylate cyclase and is found together with a histidine kinase, suggesting a role in signal transduction. *B. cenocepacia* carries three other genes annotated as CheY (224, 225), all of which show expression by intracellular bacteria. The only recognized CheY-phosphatase gene within *B. cenocepacia* is a single CheZ – BCAL0136 – which does not show expression by intracellular bacteria. If the CheYC gene encoded by BCAM2837 does indeed function as a phosphate sink, its inactivation will lead to increased levels of CheY-P and thus increased bacterial tumbling; as bacterial reorientation becomes more frequent, unidirectional smooth swimming decreases, slowing bacterial migration; ΔBCAM2837 shows a defect in swarming motility, but not in swimming motility (Figure 14). Such impaired motility may enhance bacterial uptake by simply

decreasing the ability to move away from the phagocytic cell, and may also make escape from a dying macrophage more difficult.

The contribution of bacterial motility to interaction with macrophages can also be seen in ΔBCAL0124, which encodes FlhD, a subunit of the flagellar regulon master regulator necessary for expression of the flagellar structural components (270). The regulator is essential for both swimming and swarming motility (Figure 13), and appears to be required for efficient bacterial internalization (Figure 11). Loss of the bacterial flagella through deletion of *fliCD* also results in a severe defect in bacterial internalization by macrophages (data not shown). Related strain *B. pseudomallei* requires flagellin for efficient invasion of macrophages (88), while in CF-pathogen *P. aeruginosa*, loss of swimming motility results in a resistance to phagocytosis (16). Mouse studies demonstrate a requirement of *B. cenocepacia* flagella for lethality, but not for bacterial dissemination; survivors showed no differences in bacterial load between non-flagellated versus parental strain infection (487). Deletion of BCAL0124 increases cytotoxicity and dissemination of the bacteria to neighbouring cells. In the aforementioned mouse study, *B. cenocepacia* lacking flagellin was unable to activate NFκB or elicit secretion of IL-8 through TLR signalling (487). ΔBCAL0124 may avoid activation of classical proinflammatory signaling cascades, allowing it to replicate undetected until it overwhelms the macrophage and escapes in large numbers to re-infect neighbouring cells.

Modifications to membrane proteins of *B. cenocepacia* affect interactions with macrophages. BCAM0411 is one of three genes in *B. cenocepacia* encoding an MgtC family protein. Deletion of this gene, which has multiple transmembrane helices, resulted in a defect in bacterial internalization, but an increase in intracellular replication (Figure 11). This phenotype was gene-specific, as it could be complemented (Figure 12). A second MgtC family protein in *B. cenocepacia* was previously shown to be important for growth under magnesium-limiting conditions and for intramacrophage survival (296), phenotypes common to those identified for MgtC family proteins in intracellular pathogens such as *Salmonella*

enterica (381) and *Brucella suis* (255). However, because *B. cenocepacia* encodes three MgtC family proteins, it is possible that each protein serves a different role within the bacterium. BCAL0340 encodes a putative lipoprotein located within the Type VI secretion cluster of *B. cenocepacia*; deletion of this protein promotes replication of intracellular bacteria (Figure 11) but does not inactivate the secretion system itself, as actin protrusions are still seen in infected macrophages (data not shown). Thus, the contribution of BCAL0340 to the intracellular persistence of *B. cenocepacia* remains unclear. ΔBCAM1679, lacking a putative lysylphosphatidylglycerol (Lys-PG) synthetase, shows a minor decrease in host cell cytotoxicity, but paradoxically, also shows a significant increase in infected cells in an unchecked infection, demonstrating a capacity for rapid spread. The addition of lysine to PG lowers the net negative charge of the cellular envelope, already lowered in *B. cenocepacia* by the constitutive expression of 4-amino-4-*deoxy*-L-arabinose linked to lipid A (358). Lys-PG may also decrease permeability to cations and protons by raising the surface potential of the membrane, increase membrane fluidity, inhibit genomic DNA replication, and decrease activity of the host defense factor phospholipase A2 (PLA2) [reviewed in (392)]. Deletion of the Lys-PG synthetase may enhance intracellular replication – a small increase is seen in the gentamicin protection assay (Figure 11B) – leading to more rapid host cell death, possibly potentiated by an increase in PLA2 activity. Interestingly, secretory PLA2 is capable of hydrolyzing the membrane of cells undergoing early stages of apoptosis (343), possibly providing a more rapid exit for intracellular bacteria.

5.1.3 Gene expression in intracellular *B. cenocepacia*

Internalization of *B. cenocepacia* by macrophages causes changes in bacterial gene expression. In particular, large changes are seen in the expression of genes associated with signal transduction mechanisms and transcription, showing that *B. cenocepacia* senses the phagosomal environment and activates mechanisms necessary for survival in a new niche. Repression of many

transcriptional regulators may indicate downregulation of non-essential genes and a shifting of resources towards survival. This hypothesis is supported by the repression of genes associated with invasion or entry, which would be no longer required by internalized bacteria.

Though motility clearly plays a role in the intracellular behaviour of *B. cenocepacia*, based on the phenotypes seen for ΔBCAL0124 and ΔBCAM2837, it is possible that upregulation of flagellar genes may be a stress response. Escape is a logical response to exposure to a harsh environment, and indeed, similar upregulation of motility-associated genes has been found in microarray analyses of *B. cenocepacia* response to other stressors (data not shown). Strong upregulation of flagellar genes may be a common response by intracellular bacteria, as it is also seen in intracellular *L. pneumophila* (148).

The largest category of characterized genes affected by bacterial internalization is that of metabolism suggesting metabolic adaptation to the phagosomal environment is key to survival and replication of the bacterium. Genes involved in carbohydrate transport and metabolism were prominently upregulated in intracellular bacteria, accounting for 6% of all differentially upregulated genes (Figure 9). In species of the protozoan parasite *Leishmania*, which also delays phagosomal maturation and replicates in an acidified phagolysosome, metabolic pathways involved in carbohydrate metabolism are important for intracellular survival; studies with metabolic mutants of *Leishmania* have suggested that the phagolysosome contains a variety of carbon sources and essential nutrients, but is lacking in carbohydrates (337). Some of the most strongly induced genes are involved in amino acid transport (Table 5); many pathogens upregulate amino acid transport during intracellular growth, including *L. pneumophila* (148), *S.* Typhi (149), and *Y. pestis* (166), suggesting amino acid acquisition from the host may be a general carbon and nitrogen acquisition strategy.

B. cenocepacia is able to occupy a diverse array of niches, and more than one-third of *B. cenocepacia*'s 7261 genes are involved in metabolism (195). Thus, it is possible that there are redundant nutrient uptake systems to function under

different conditions and exploit multiple forms of the same metabolite. The changes seen in expression of metabolic genes (Figure 9) may demonstrate the ability of *B. cenocepacia* to utilize phagosomal resources. Production of cholesterol-like hopanoids is catalyzed by squalene-hopene cyclase Shc; *B. cenocepacia* encodes two *shc* genes. Of these, BCAS0167 is found alone on the third chromosome and, although contributing little to *in vitro* hopanoid production (423), was strongly upregulated by intracellular bacteria. However, the theme of genetic redundancy and niche specialization carries beyond metabolism. One of the most strongly repressed genes was *cciR*, the transcriptional regulator for the *B. cenocepacia*-specific second quorum sensing system. A deletion mutant in *cciR* has lower protease activity than the parental isolate (297), suggesting that production or secretion of proteases is regulated by CciR, and is thus repressed by intracellular bacteria. Interestingly, a number of genes involved in protein export are also strongly repressed, including the first gene of the Tat system, several genes in the Sec system, and the signal recognition particle RNA.

Poorly characterized genes account for one-third of the *B. cenocepacia* genome. This trend holds true when examining differential regulation of genes by intracellular bacteria, where at least one-third of identified genes are poorly characterized.

5.1.4 The BcCV at 24 hours post-infection

During late-stage infection, *B. cenocepacia* appears to reside within acidic vacuoles (Figure 18) that remain associated with the endocytic pathway (Figure 19), suggestive of acidified phagolysosomes. Lysosomes loaded with TMR-dextran pre-infection showed only a weak signal in infected macrophages 24 h p.i. (Figure 19), suggesting that *B. cenocepacia* can alter the dextran in some way. Molecular Probes dextran conjugates are hydrophilic polysaccharides with an α-1,6-poly-glucose linkage; of the three polysaccharides produced as EPS by *B. cenocepacia*, one is a dextran consisting of α-1,6-glucans (66). Since *B.*

cenocepacia can produce dextran, it may also possess mechanisms to degrade or alter dextran; thus, long-term co-infections of *B. cenocepacia* and fluorescent dextran are not feasible.

During the delay of phagosomal maturation, *B. cenocepacia* alters transcription of many metabolic genes. The low pH of the phagolysosome can be used to drive high-affinity uptake of metabolite:proton symporters to bring nutrients into the cell (62). Such a mechanism could account for the labeling of intraphagosomal bacteria by the acidotropic dye Lysotracker red during late-stage infection (Figure 18); as the bacteria take up nutrients from the phagosome, the corresponding increase in intracellular proton concentration would cause the bacteria to be more acidic than the surrounding vacuole, leading to Lysotracker labeling of the bacteria rather than the vacuole. The SCOTS microarrays show intracellular upregulation of a putative sodium:solute symporter and a putative Na^+/H^+ antiporter. This suggests a cyclic system whereby the low vacuolar pH drives sodium out of the cell through the antiporter, which returns with a metabolite through the symporter.

5.1.5 Bacterial cytotoxicity towards murine macrophages

Previous studies have demonstrated *B. cenocepacia* cytotoxicity in epithelial cells (78, 107); however, this study is the first to demonstrate *B. cenocepacia* cytotoxicity towards macrophages. *B. cenocepacia* infection of macrophages results in secretion of proinflammatory cytokines IL-1β (242), IL-6, IL-8, and TNF-α, (104). Elevated levels of the inflammatory mediators IL-6, TNF-α, macrophage inflammatory protein MIP-2, and IL-8 homolog KC are also found in the bronchoalveolar lavage of mice infected with *B. cenocepacia* (487, 500). Production of proinflammatory cytokines, in particular TNF-α, can lead to both apoptosis and necrosis (4). Pilin-mediated apoptosis of *B. cenocepacia*-infected epithelial cells depends on the bacterial load (78). Similarly, macrophage cell death can be induced by *M. tuberculosis* when the intracellular bacillary load

exceeds a certain threshold (258); this atypical cell death freely releases viable bacteria to the extracellular space (259). Non-apoptotic cell death in monocytes is associated with higher bacterial burden for infections with both *E. coli* and *Klebsiella pneumoniae*; in both instances, cell death results in membrane permeabilization (515). The capacity of *B. cenocepacia* to spread in an unchecked infection suggests either pyroptosis or necrosis, both non-apoptotic forms of host cell death in which the macrophage membrane is permeabilized. Production of IL-1β by infected cells is indicative of inflammasome and caspase-1 activation; excessive caspase-1 activation can lead to pyroptosis. The inflammasome is activated by NLR-detection of bacterial ligands in the cytosol, and many bacterial elements – putative NLR-ligands – are dispersed throughout the macrophage by late stages of infection (Figure 39). Pyroptosis also induces lysosome exocytosis, whereby any bacteria residing within a lysosome are freely delivered to the extracellular milieu as the lysosome fuses with the macrophage plasma membrane. Thus, further investigation into bacterially-induced pyroptosis as the putative mechanism of macrophage cell death is warranted. Where the mutant strain appeared to prevent cytotoxicity (Figure 15), it is possible that infected macrophages instead underwent non-membrane-permeabilizing apoptosis, which would not be detected with 7-AAD.

5.2 Secretion systems and *B. cenocepacia*

The objective of this study was to determine the role played by classical multi-gene secretion systems in the interaction of *B. cenocepacia* with phagocytic cells. Because *B. cenocepacia* is a significant pathogen within cystic fibrosis patients, leading to chronic infection, and increased morbidity and mortality, an understanding of pathogenesis is critical. Survival and replication within host cells may contribute to the establishment and persistence of chronic infection, while induction of host cell death and stimulation of a strong inflammatory response may lead to deterioration of the lung. Studies have shown that *B. cenocepacia* is able to modulate the endocytic pathway of phagocytic cells,

delaying phagosomal maturation (210, 252). Furthermore, the transcriptomic studies above suggest that *B. cenocepacia* utilizes the delay to metabolically adapt to the phagosomal environment for persistence and replication in the phagolysosome; differential regulation of a number of secretion-associated genes by intracellular bacteria emphasizes the involvement of bacterial secretion in interactions with macrophages.

Classical multi-gene secretion systems have been shown to have many differing roles in the interaction of intracellular bacteria with host cells, ranging from inhibition of phagocytosis to alteration of phagosomal maturation, to induction of host cell death. Thus, secretion systems may play a role in the internalization, intracellular replication, and host-directed cytotoxicity of *B. cenocepacia*. The ability of *B. cenocepacia* to inactivate Rab7 (210), located on the cytosolic face of the phagosome, suggests that the bacterium must be able to secrete one or more effectors into the host cell cytosol. Though direct translocation remains to be shown, a functional T6SS is required for intracellular *B. cenocepacia* to alter the actin cytoskeleton of macrophages (25); in *Vibrio cholerae* (369) and *Aeromonas hydrophila* (467), a T6SS has been shown to secrete effectors into the host cell capable of affecting actin. Thus, it is likely that intra-vacuolar *B. cenocepacia* is able to secrete effectors from the phagosome into the host cell.

5.2.1 Type II secretion

B. cenocepacia employs a T2SS for secretion of a variety of enzymes, including protease, lipase, and phospholipase C (Figure 21). Deletion of the major structural components of the T2SS did not affect internalization of *B. cenocepacia*, but increased intracellular replication. This phenotype could be complemented in a single-gene deletion (Figure 24). Deletion of the T2SS also decreased host-directed cytotoxicity (Figure 26), and marginally increased bacterial spread (Figure 28); the latter is likely accounted for by higher intracellular replication yielding an increased number of bacteria available to re-

infect. It is also possible that less host-directed cytotoxicity could account for, at least in part, the increase seen in intracellular replication, as preservation of intact host cells protects intracellular bacteria from the antibiotic-containing cell culture media. Lower host-directed cytotoxicity in ΔT2SS could indicate a role for type II-secreted enzymes in interactions with eukaryotic cells. Inactivation of the T2SS-essential gene *gspD* in *B. pseudomallei* caused a small increase in LD_{50} in a hamster model of infection (126), but had no effect on nematode killing (350). A *B. cenocepacia* AU1054 transposon mutant in *gspJ*, a pseudopilin of the T2SS, was less pathogenic in a nematode model of infection (451), whereas four different transposon mutants in the T2SS apparatus of *B. cenocepacia* H111 had no effect on nematode killing (241). In *B. vietnamiensis*, the T2SS is not involved in the intracellular survival in *Acanthamoeba polyphaga* (150). Thus, it seems probable that the membrane-compromising induction of cell death by the T2SS and T2-secreted effectors is specific to macrophages.

5.2.2 Type III secretion

Many intracellular pathogens utilize a T3SS in diverse interactions with eukaryotic cells. In *Yersinia* spp., effectors secreted by the T3SS are able to prevent phagocytosis (144). In *Edwardsiella tarda*, lack of a functional T3SS allows acidification of the bacteria-containing vacuole, which precludes intracellular replication (352). *Salmonella enterica* also utilizes T3SS-secreted proteins to alter intracellular trafficking, directing the BCV away from the endosomal pathway to a compartment more suitable for replication (191). An effector secreted through the T3SS of *Shigella flexneri* induces IL-8 secretion in epithelial cells (147). In contrast, the T3SS of *Y. pseudotuberculosis* is responsible for restricting the intracellular survival of the bacterium while inducing apoptosis in host macrophages (539). The gentamicin protection assay showed no real contribution of the *B. cenocepacia* T3SS to bacterial entry or intracellular replication (Figure 23), with 1.6- and 1.5-fold increases respectively, nor does it appear to contribute significantly to host-directed cytotoxicity. However, deletion

of the T3SS significantly increases spread of *B. cenocepacia* in an unchecked infection, while maintaining a parental strain rate of host cell death, suggesting that a functional T3SS is necessary to restrict *B. cenocepacia* to the phagosome. Previous studies regarding the role of the T3SS in *B. cenocepacia* have had mixed results. A polar insertion in the T3SS cluster did not impair intracellular trafficking of *B. cenocepacia* in macrophages (252). Insertional inactivation of T3SS gene *bcscN* resulted in reduced lung tissue inflammation and more rapid bacterial clearance from the lung in a mouse model of lung infection, but did not affect invasion of epithelial cells (480). Mutations in the T3SS also affect the rate of killing in *C. elegans* in a strain-dependent manner only seen at certain time points (306).

5.2.3 Type IV secretion

T4SSs are able to translocate both DNA and protein substrates, usually in a cell-contact dependent manner [reviewed in (188)]. Together with the T3SS, T4SSs are the most common secretion systems involved in the interplay between prokaryotes and eukaryote hosts. Many intracellular pathogens shown to delay or alter phagosomal maturation utilize T4SS to translocate effectors into the host. *Legionella pneumophila* requires a functional Dot/Icm T4SS for intracellular replication and evasion of BCV fusion with lysosomes (472). *Brucella* spp. also utilize a T4SS to subvert the endosomal pathway (95), with recent work showing translocation of a Rab2-interacting effector into the host cytosol through the VirB-type T4SS (117). *Coxiella burnetii*, which is able to delay phagolysosomal fusion and subsequently replicate within an acidified compartment (317), possesses a functional T4SS capable of secreting effectors containing eukaryotic-like domains into the cytosol of the host cell (76). Some bacteria utilize T4SSs to induce inflammation, including *L. pneumophila* (172), which, along with *C. burnetii,* also produces a T4-secreted effector capable of preventing host cell apoptosis (36, 281); in *Bartonella* spp., T4SSs lead to host adaptability (400).

Previous research in *B. cenocepacia* has identified two T4SS which contain different subunits and carry out different functions (141, 538). T4SS-1 consists of a combination of VirB/D4 and F-specific subunits, the genes for which are spread across half of the 92 kb plasmid of *B. cenocepacia*; also referred to as the Ptw T4SS, it secretes a cytotoxic effector protein responsible for plant tissue water soaking, thought to provide the bacteria with nutrients through the leakage of plant cell cytosol during soft rot (141). T4SS-2, located on chromosome 2, is homologous to the VirB/D4 T4SS of *Agrobacterium tumefaciens* (141), and is involved in plasmid mobilization (538). A single study linked T4SS-1 with intracellular survival in epithelial cells and macrophages, showing reduced intracellular replication and more rapid maturation through the endosomal pathway in the absence of a functional T4SS coupling protein (403). We found that absence of the complete T4SS-1 cluster halved the number of total internalized bacteria in the gentamicin protection assay, but doubled the number of total infected cells with flow cytometry, suggesting internalization of fewer bacteria per cell. The intracellular replication of ΔT4SS-1 was increased relative to the parental strain, though correction for cytotoxicity lowered replication to parental strain levels (Figure 27). This apparent contradiction with the previous study may be explained by differences in calculation method. While we related intracellular replication to bacterial entry, the former study reported total numbers of intracellular bacteria at each time point; since the mutant bacteria are not internalized as well, there are less initial bacteria present to replicate, lowering the final CFU counts. Additionally, it is possible that other genes present within the 42 kbp deletion contribute to virulence. Deletion of the plasmid-encoded T4SS required deletion of almost half of the entire plasmid; in other intracellular pathogens with a large plasmid, including *Salmonella enterica* (527), *R. equi* (202), and *Y. pestis* (130), the plasmid contributes to intracellular survival and virulence.

Deletion of the complete T4SS-2 cluster resulted in an increase in both internalized bacteria and infected cells, but a decrease in intracellular replication, when corrected for cytotoxicity. Deletion of a single gene in the T4SS-2 operon

had no effect on bacterial entry or replication, while deletion of the homologous gene in T4SS-1 increased bacterial entry and replication, both of which could be complemented by restoration of the gene on a complementation plasmid (Figure 25). It is possible that subunits of each T4SS are able to cross-complement, explaining the lack of phenotype seen for JST184. *L. pneumophila* contains two separate T4SSs; the *dot/icm* T4SS is required for intracellular growth in macrophages, and is involved in conjugation, while the *lvh* T4SS is involved in plasmid mobilization; elements of the *lvh* system are able to substitute for homologous components of the *dot/icm* system (432).

The significant increase in bacterial internalization in the absence of T4SS-2 suggests some involvement of the secretion system with the inhibition of phagocytosis. *Bartonella henselae* translocates effector BepG through a VirB/D4 T4SS to inhibit endocytosis by human endothelial cells (382). *Yersinia* spp. (144), *P. aeruginosa* (170), and enteropathogenic *E. coli* (EPEC) (176) are all able to inhibit phagocytosis by macrophages dependent on the action of a T3SS. Interestingly, the plant water soaking phenotype linked to T4SS-1 in *B. cenocepacia* is also found in *Erwinia* spp. and *Pantoea stewartii* subsp. *stewartii,* where it is caused by T3SS-mediated secretion of effectors DspE/A (197), and WtsE/F (184), respectively. Thus, it is conceivable that an antiphagocytic effector delivered by a T3SS in other bacteria may be delivered by T4SS-2 in *B. cenocepacia*. Of note, *B. cenocepacia* J2315, which belongs to the ET12 epidemic clone of *B. cenocepacia* along with strain K56-2, is internalized by macrophages at an increased rate and in increased numbers per cell (411), and contains a frameshift mutation in VirD4 of the T4SS-2 cluster (195).

In flow cytometry assays, neither T4SS deletion affected cell spread, but ΔT4SS-2 was less cytotoxic. We determined cytotoxicity by means of 7-AAD, a fluorescent compound that binds to DNA but cannot readily cross cell membranes, thus technically measuring membrane permeability. *Legionella* spp. require the *dot/icm* T4SS to permeabilize macrophage membranes, leading to

pyroptosis (522), thus is it possible that inactivation of T4SS-2 in *B. cenocepacia* prevents release of a membrane-permeabilizing effector.

5.2.4 Type VI secretion

The recently identified T6SS, the most common of the gram-negative large specialized secretion systems, plays a diverse role in interbacterial interactions; however, it is also linked with host interactions in certain pathogens [reviewed in (426)]. In a previous study, three separate transposon mutants in the T6SS cluster of *B. cenocepacia* were attenuated for virulence in a rat model of chronic lung infection (207), showing a definite role in pathogenesis. *B. cenocepacia* upregulates expression of T6SS genes during macrophage infection. Deletion of the T6SS in *B. cenocepacia* did not alter the number of bacteria internalized by macrophages (Figure 23), nor the number of eukaryotic cells infected (Figure 26), but significantly increased intracellular survival of the bacteria, a phenotype clearly complemented in a single gene deletion model (Figure 25B). An increase in observed intracellular replication could be due to the fact that cytotoxicity was significantly decreased in ΔT6SS infected-macrophages. As with the T2SS, maintaining the integrity of the macrophage membrane protects intracellular bacteria from the antibiotic-laden culture media, thus leading to an increase in viable bacteria at 24 h post-infection. However, the unchecked spread of the mutant at a rate similar to the parental strain suggests either a true increase in intracellular replication - with less macrophages dying, more bacteria must be released per macrophage to maintain the rate of spread – or a non-membrane damaging method of bacterial release. Comparison with ΔBCAL0340 – examined during the transcriptomic study above – lends credence to the former hypothesis. BCAL0340, a putative lipoprotein, is the first gene in the second transcriptional unit of the T6SS (24); macrophages infected with ΔBCAL0340 still show evidence of an altered actin cytoskeleton (data not shown), while macrophages infected with deletions in BCAL0342, BCAL0343, or the entire T6SS cluster do not (24, 25). ΔBCAL0340 does not show decreased

macrophage-directed cytotoxicity (Figure 15), but does show increased intracellular replication (Figure 11), suggesting that ΔT6SS truly replicates faster than the parental strain bacteria within the phagosome. The ability of the T6SS to limit intracellular replication is also seen in *Y. pestis*, although the corresponding promotion of phagocytosis in *Y. pestis* was not seen in *B. cenocepacia* (386). In *B. pseudomallei*, inactivation of T6SS-1 resulted in decreased cytotoxicity in macrophages, as well as decreased virulence in a hamster model of melioidosis (65). The role of the T6SS in macrophage-directed cytotoxicity has also been demonstrated in *A. hydrophila*; expression of T6-essential gene *hcp* resulted in epithelial cell apoptosis (468). Thus, there is a clear role for the T6SS of *B. cenocepacia* in interactions with macrophages, especially in limiting intracellular replication and promoting host-cell directed cytotoxicity.

5.2.5 The contribution of secretion systems as a whole

B. cenocepacia is somewhat unusual in that it encodes at least one of every known gram-negative multi-gene secretion system, including both T3SS and T4SS (195). Among pathogenic *Burkholderia* spp., however, it lacks the large redundancy of secretion systems seen with *B. pseudomallei* and *B. mallei*, which have three and two T3SSs, and six and four T6SSs, respectively (196, 344). Deletion of each secretion system, entailing deletion of large segments of DNA, does not affect the growth of the bacterium in rich media; indeed, deletion of all secretion systems in a single strain – accounting for a total of 109 kbp – does not cause any growth defect (Figure 20). In gentamicin protection assays, JST112 behaved as the parental isolate with regards to bacterial entry and intracellular replication (Figure 23). However, flow cytometry revealed a four-fold reduction in infection for JST112, suggesting internalization of a greater number of bacteria per cell by a decreased number of cells. Interestingly, though T4SS-2 is absent, there is no increase in bacterial internalization, suggesting that bacterial uptake by macrophages may be more complex than simple phagocytosis. In *B.*

cenocepacia J2315, the internalization of more bacteria per cell has been linked to the absence of a complete lipopolysaccharide (LPS) O antigen (411), demonstrating a role in for surface structures in the interactions between bacteria and macrophage. However, J2315 also infects a greater number of cells, which is not seen for JST112. The means by which *B. cenocepacia* enters macrophages is unknown; it is possible that there is a threshold level of stimulation for phagocytosis which is more difficult to reach in the absence of the large outer membrane structures unique to specialized secretion systems, thus requiring the presence of increased numbers of JST112 for a single event.

Flow cytometry also showed a decrease in host-directed cytotoxicity by a mutant lacking large secretion systems; though JST112 spreads rapidly in an unchecked infection, it remains much less cytotoxic than the parental strain (Figure 28). The cause of such bacterial spread – 40-fold in 24 hours – in the absence of increased intracellular replication or increased host cell death is source for speculation. Lack of large specialized secretion systems does not preclude all secretion from the cell – at the minimum, types I and V remain possibilities (195) – but it does prevent expression, or at least translocation, of a vast array of effectors. Effectors of the T2SS, T4SS-2, and T6SS all contribute to host-directed cytotoxicity (Figure 26). It is possible that JST112, likely unable to secrete effectors to the host cytosol, avoids activating a strong proinflammatory response or permeabilizing the cell membrane, and simply resides within the phagosome. In the absence of a functional T6SS, the host cytoskeleton is unaffected, allowing normal motility and replication of the macrophage. Infected macrophages typically show multiple BcCVs, especially at later time points post-infection (Figure 18); the rapid spread of the bacteria could be due to normal proliferation of infected macrophages. Of note, though the ratio of dead to infected host cells is lower in a JST112-infection, the total number of dead host cells is actually increased due to the rapid spread. Thus, secretion systems may function as an infection-limiting factor in *B. cenocepacia*, enabling bacterial persistence. Such a strategy is exemplified by *Brucella* spp., which utilize a T4SS to stimulate an inflammatory response carefully balanced between

checking uncontrolled intracellular replication and preventing host cell death, thus allowing the bacteria to establish a chronic infection within a granuloma (118). Similarly, *B. cenocepacia*-infected macrophages attract other non-infected cells in zebrafish embryos, forming cellular aggregates within which local bacterial spreading occurs 15 to 20 h post-infection (502). Aggregate formation and kinetics of embryo death differ between *B. cenocepacia* J2315 and K56-2.

B. cenocepacia strains K56-2 and J2315 are clonal isolates belonging to the highly transmissible lineage known as ET12; J2315, first isolated from a CF patient in Scotland, was the index strain for epidemic spread, while the more genetically-tractable K56-2 was isolated from a CF patient in Canada (290). Though closely related, K56-2 and J2315 have accumulated different genetic mutations. Apart from the aforementioned frameshift mutation in T4SS-2, *B. cenocepacia* J2315 also contains a 110-bp deletion in *gspL*, an essential component of the T2SS, and a frameshift mutation in BCAM2043, located in the T3SS cluster (195), putatively inactivating three of the five large, specialized secretion systems. Though there are undoubtedly other genetic differences between K56-2 and J2315, it is interesting to link the presence of functional secretion systems with pathology. In our study, absence of the T3SS allowed the bacteria to spread more widely, while absence of T4SS-2 was linked with increased bacterial uptake, and both the T2SS and T4SS-2 were associated with host-directed cytotoxicity. Thus, in the absence of these three secretion systems, one could expect to see increased bacterial uptake, widespread bacterial spreading, and slower host killing. As mentioned previously, J2315 infects a greater number of macrophages with a greater number of bacteria than an equivalent infection with K56-2 (411). As an aside, this would tend to suggest a role for T4SS-1 in balancing the anti-phagocytic activity of T4SS-2, as this increase is not seen in JST112. In a *C. elegans* nematode model, though K56-2 killed rapidly, J2315 was also pathogenic at a slower rate, a phenotype not dependent on the LPS O antigen (69). Differences in virulence may also be seen in a zebrafish embryo model, where killing by K56-2 occurred within 48h, but J2315 required an additional 24h to induce significant host death; microscopic

examination revealed widespread dissemination of J2315 bacterial aggregates throughout the vasculature of the embryo prior to death, a phenotype not seen in K56-2 infection (502). Thus, anecdotal evidence would tend to support the role of secretion systems in the pathogenicity of *B. cenocepacia*.

5.3 Proposed future experiments

There are many interesting avenues to pursue, beginning with further investigation into genes upregulated by intracellular *B. cenocepacia* during phagosomal adaptation 4 h p.i.. Transcriptomic analysis of *B. cenocepacia* and further characterization of the BcCV during late stage infection would contribute to a greater understanding of the bacteria-macrophage interaction. The role of bacterial secretion systems should be investigated in models more relevant to human infection, and an attempt made to identify effectors contributing to interactions with eukaryotic cells.

5.3.1 Further investigation of genes regulated by intracellular bacteria

One area for further study is the role of iron in the intracellular life of *B. cenocepacia.* Intracellular bacteria may limit siderophore-mediated iron acquisition, as the receptor for ornibactin is downregulated 10-fold, as is the transcriptional activator for the pyochelin receptor; the putative pyochelin biosynthetic protein is also repressed. However, three transport proteins predicted to be involved in TonB-dependent ferric iron uptake are upregulated.

Another aspect of future studies is further characterization of metabolic adaptation to the phagosome, including the upregulation of amino acid transporters, and the role of cobalamin in the intracellular life of *B. cenocepacia*. Other avenues to pursue include quorum sensing - *B. cenocepacia*-specific regulator *cciR* is downregulated 46-fold by intracellular bacteria - and changes to

the membrane and surface structures; a squalene-hopene cyclase necessary for hopanoid biosynthesis is strongly upregulated, as are genes found in the clusters of both the Flp type pilus and type IVa pilus, while a number of adhesins, including the 22 kDa adhesin encoded by BCAM2143 and several BuHA family proteins, are differentially regulated. Lipase has proven important to infection in epithelial cells (336), suggesting that strongly upregulated lipase BCAM0949 may play a role in the interactions between *B. cenocepacia* and macrophages. Other specific genes meriting further characterization include CheYC protein BCAM2837 and conserved hypothetical protein BCAM0314, both among the most strongly upregulated by intracellular bacteria; BCAM2837 displays a strong phenotype in macrophages, but little is known regarding its true function, while BCAM0314 is homologous to a putative cell wall-associated hydrolase.

5.3.2 The transcriptional response of macrophage-adapted *B. cenocepacia*

With the development of gentamicin-sensitive *B. cenocepacia* MH1K, the transcriptional response of *B. cenocepacia* at later stages of infection can now be analyzed with SCOTS and microarrays or next-generation sequencing. Many of the genes upregulated at 4h p.i. are involved in signal transduction and transcription, suggesting adaptation is only beginning; as the phagosomal environment continues to evolve, *B. cenocepacia* must continue to adapt. In *L. pneumophila*, the number of upregulated genes increases with time post-infection (148). Following adaptation to the phagosome, *B. cenocepacia* is capable of replicating, inducing host cell death, and escaping the macrophage. Thus, transcriptome analysis of *B. cenocepacia* throughout a time-course of infection could significantly contribute to a more complete understanding of the interaction between *B. cenocepacia* and murine macrophages.

5.3.3 The role of secretion systems in CF infection

Large, specialized, multigene secretion systems play a clear role in the interaction of *B. cenocepacia* with murine macrophages; however, there are many steps between relevance in murine macrophages and relevance to human infection. The panel of secretion system mutants should be examined in more relevant cell culture models, including $CFTR^{-/-}$ macrophages and primary cells, and in higher organisms, including zebrafish and murine models. In addition, because it is strongly upregulated by intracellular bacteria, a deletion mutant in the T1SS should be added to the panel of secretion system mutants. Furthermore, analysis of the secretome for each of the mutants may prove useful in identifying effectors important in interactions with macrophages.

The contribution of secretion systems to the dispersal of bacterial elements throughout the macrophage could be investigated by *B. cenocepacia* Ab labelling of macrophages infected with JST112; should the dispersed bacterial elements be absent in JST112 infection, further experiments with the entire panel of secretion system mutants could identify the specific system responsible. Identification of the bacterial components would require isolation of macrophage membranes during late-stage infection. If the bacterial component is a protein, it could be detected in a Western blot using *B. cenocepacia* Ab and identified by mass spectrometry; likewise, bacterial LPS could be detected by Western blot. Effectors translocated in the host cell cytosol by other bacteria have been identified by fusing genomic fragments to adenylate cyclase and assaying for an increase in host cell cAMP levels (70). Once identified, putative effectors would be screened through the panel of secretion system mutants to determine which system is responsible for translocation.

5.3.4 Further characterization of late stage macrophage infection by *B. cenocepacia*

Preliminary characterization suggests that the BcCV is an acidified

phagolysosome 24h p.i., but further studies are necessary. Immunolabelling with compartment-specific markers such as LAMP-1 and cathepsin D, and colocalization with Lysosensor dextran would contribute to confirmation; the possibility of autophagy should also be investigated. Because experiments with Lysotracker red suggest that intracellular bacteria themselves become acidified, measurement of intraphagosomal pH over time would show whether *B. cenocepacia* is able to decrease the pH of the vacuole.

While we have shown that *B. cenocepacia* can induce host cell death, the mode of cell death is unclear. Apoptosis could be investigated via flow cytometry analysis of infected cells labelled with specific markers. The ability of *B. cenocepacia* to affect host cell signalling, including caspase activation, should also be examined.

It would also be interesting to isolate and characterize phagosomally-adapted *B. cenocepacia*. It has been shown that *E. coli* is capable of transcriptional adaptation in anticipation of natural environmental change (329); such bacterial "learning" suggests that bacteria which escape the macrophage may be better suited to re-infect neighbouring cells. It would be interesting to investigate the infection rate, intracellular trafficking, and replication rate of *B. cenocepacia* isolated from late-stage macrophage infection. Perhaps *B. cenocepacia* would not need to delay phagosomal maturation, being already adapted to the phagosomal environment. It could be hypothesized that previously intracellular *B. cenocepacia* would display increased infection and intracellular replication, and more rapid host cell-directed cytotoxicity.

5.4 Significance and concluding remarks

Altogether, it appears that there are many elements contributing to the intracellular survival and behaviour of *B. cenocepacia*; if there is a single gene or gene cluster that is absolutely essential for intracellular survival, it has yet to be

discovered. *B. cenocepacia* has an extremely large genome and broad metabolic range, potentially explaining the ability of the bacteria to survive in a wide variety of niches, including the hostile environment of the phagolysosome. Indeed, the intracellular survival of *B. cenocepacia* in macrophages is associated with profound changes in metabolic activity and motility rather than the presence of more specific traditional "virulence" genes. Delay of phagosomal maturation provides time for the bacteria to alter gene expression conferring a survival advantage for persistence and replication in a phagolysosomal compartment.

The role of secretion systems in the intracellular behaviour of *B. cenocepacia* is complex. Large, specialized secretion systems - predominantly the T4SSs - are involved in bacterial entry into macrophages, in terms of both numbers of host cells infected and numbers of bacteria per host cell. Secretion systems, particularly the T2SS and T6SS, also play a role in intracellular replication, and host-directed cytotoxicity, though it is likely that the latter two are intertwined, as preservation of the host protects intracellular bacteria. Together, secretion systems function to limit the spread of *B. cenocepacia* and increase pathogenicity during cell culture infection.

B. cenocepacia is an opportunistic pathogen, not an obligate intracellular pathogen, and as such, simply adapts to survive in the niche in which it finds itself until it can be released back into the extracellular milieu by dying macrophages. The mechanism by which *B. cenocepacia* is internalized and induces host cell death, whether purposeful or incidental, and the role of the varied secretion systems in this interaction, is a subject for further study as it relates to the establishment and maintenance of a chronic infection in host cells of cystic fibrosis patients.

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Appendices

Appendix 1

1 The role of outer membrane vesicles in the intracellular behaviour of *B. cenocepacia*

1.1 Introduction

Vesiculation is a nearly ubiquitous process in Gram-negative bacteria. Vesiculation may allow bacteria to interact with and alter surrounding prokaryotic and eukaryotic cells (11, 16). Indeed, vesiculation has been referred to as a secretion mechanism, allowing delivery of potentially large and complex mixtures of proteins and lipids to the extracellular milieu (11). Although the actual mechanism of vesiculation is elusive, it is thought that periplasmic cargo is encapsulated within an outer membrane (OM) bleb (20, 23) when the OM bulges and pinches off at sites where cross-links between peptidoglycan and the OM are decreased (9); vesiculation is not indicative of membrane instability (17), but appears to be directed, as specific proteins, lipids, and sugars may be enriched or excluded in these vesicles (11). As such, vesicles have the capacity to act as potent virulence factors for pathogenic bacteria, delivering toxins, enzymes, and immunomodulatory compounds to a host or neighbouring cell; indeed, vesicles from *B. cepacia* have been found to possess LPS, proteases, lipases, PLC, and peptidoglycan hydrolases, all putative virulence factors which could have a role in intracellular survival (1). Vesicles have been observed within the context of infection: *Borrelia burgdorferi*, the causative agent of Lyme disease, produces vesicles following invasion of the dermis (3), vesiculating *Neisseria meningitidis* has been identified in the cerebrospinal fluid and blood of meningitis patients (21), and, *Helicobacter pylori* vesicles containing vacuolating cytotoxin have been detected in gastric epithelium biopsies (7). Vesiculation has been suggested as a response to stress, which would certainly be present in an intracellular setting. *P. aeruginosa* alters LPS and increases production of membrane vesicles under conditions of oxidative stress (22), and *Escherichia coli* appears to vesiculate in

response to envelope stress (18). *L. pneumophila* releases natural membrane vesicles which are capable of delaying phagosome-lysosome fusion independently of the T4SS (5). Thus, for pathogenic bacteria, including *B. cenocepacia*, vesicles may be important tools in establishing and maintaining infection.

1.2 Materials and Methods

1.2.1 Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 8. Bacteria were grown at 37^{o}C in Luria-Bertani (LB; Difco) broth with agitation or on LB plates with 1.6% Bacto agar. For vesicle isolation, large scale broth cultures of bacteria were grown in either trypticase soy broth (TSB) or 3-(*N*-morpholino) propanesulfonic acid (MOPS) – minimal salts – tryptose (MMST) medium [3 mM KCl, 12 mM $(NH_4)_2SO_4$, 3.2 mM $MgSO_4$, 0.02 mM $FeSO_4$, 3 mM NaCl, 20 mM glucose, 0.1% w/v tryptose (Difco)]. *Escherichia coli* cultures were supplemented as required with 20 μg/ml tetracycline, 50 μg/ml trimethoprim, and 40 μg/ml kanamycin; *B. cenocepacia* cultures were supplemented as required with 100 μg/ml tetracycline and 100 μg/ml trimethoprim. Plasmids were conjugated into *B. cenocepacia* by triparental mating at 37^{o}C using *E. coli* DH5α carrying helper plasmid pRK2013 (6). *E. coli* donor and helper strains were selected against with 50 μg/ml gentamicin.

1.2.2 Vesicle isolation

Outer membrane vesicles were isolated as in Allan *et al.* (1). Briefly, one litre of *B. cenocepacia* C5424 was grown to an OD_{600} of 1.0-1.5. Cells were removed by centrifugation at 6000 x *g*, and the cell-free supernatant was filtered

Table 8: Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Escherichia coli		
DH5α	F^-, f80 *lacZ* DM15 D(*lacZYA-argF*) *U169 endA1 recA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1* D*gyrA96 relA1*	Laboratory stock
Burkholderia cenocepacia		
J2315	epidemic strain ET12 clone, CF clinical isolate	P.A. Sokol
K56-2	epidemic strain ET12 clone, CF clinical isolate	BCRRC[a]
C5424	epidemic strain ET12 clone, CF clinical isolate	BCRRC[a]
Plasmid		
pRK2013	RK2 derivative, Kan^R, *mob*$^+$, *tra*$^+$, ori_{colE1}	(6)
pDA17	ori_{pBBR1}, Tet^R, *mob*$^+$, P_{dhfr}, FLAG epitope	(8)
pRF132	pDA17, BCAL2829 HtrA	(8)
pJRL1	ori_{pBBR1}, Tp^R, *mob*$^+$, P_{dhfr}, mRFP1	(12)

[a] *B. cepacia* complex Research and Referral Repository for Canadian CF Clinics

sequentially through 0.45 μm and 0.2 μm polyethersulfone (PES) membranes. OMVs were collected by centrifugation at 150 000 x *g* for 3h at 5°C, resuspended in 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) pH 6.8, and retained at -20°C. OMVs were purified through a 30% - 60% sucrose gradient in 50 mM HEPES pH 6.8. Gradients were centrifuged at 183 000 x *g* for 18h; no brake was applied to slow the centrifuge. Fractions were removed and protein concentration determined via absorbance at 280 nm.

OMV were also isolated as in McBroom *et al*. (17). Bacteria were pelleted from 250 mL overnight broth cultures by centrifugation at 10 000 x *g* for 10 min at 4°C. The supernatant was filtered twice through a 0.45 μm cellulose ester filter and centrifuged at 38 400 x *g* for 1h at 4°C. Pelleted vesicles were resuspended in Dulbecco's PBS with 0.2 M NaCl and retained at -20°C.

1.2.3 Outer membrane and periplasmic protein isolation

Outer membrane proteins were isolated via a modified version of the Sarkosyl insolubility protocol of Carlone *et al*. (4). Bacterial cells were resuspended in 10 mM Tris/HCl pH 8.0 and disrupted by sonication until clear. The lysate was pelleted, and the supernatant centrifuged at 31 191 x *g* for 30 min at 4°C. The pellet was resuspended in 1.5% w/v Sarkosyl in 20 mM Tris/HCl pH 8.0 and incubated at RT for 20 min. Samples were centrifuged as above, and the OM-containing pellet resuspended in sterile water.

Periplasmic proteins were isolated as described previously (10). Bacterial cells were pelleted at 9000 x *g* for 10 min and resuspended in lysis buffer (20% w/v sucrose, 30 mM Tris/HCl pH 8.0, 1 mM EDTA pH 8.0, 3.5 mg/mL lysozyme). Cell suspensions were incubated at 37°C for 3h, and spheroplast formation confirmed by light microscopy. Spheroplast preparations were centrifuged as above, and supernatants containing periplasmic proteins were retained at -20°C.

1.2.4 Protein analysis

Protein concentration was determined using the Bradford assay (Bio-Rad). For a slot blot (BioRad), protein adherence to a nitrocellulose membrane was mediated by vacuum according to the manufacturer's instructions. Both SDS-14% PAGE and protein transfer to nitrocellulose membranes were performed as described previously (2). Gels were stained with Coomassie [0.1% (w/v) Coomassie blue, 10% (v/v) acetic acid, and 50% (v/v) methanol] or with silver. Silver staining required gel fixation [40% (v/v) 95% ethanol, 10% (v/v) acetic acid] and sensitization [0.02% (w/v) $Na_2S_2O_3 \cdot 5H_2O$] prior to staining [0.1% (w/v) $AgNO_3$] and developing [0.04% (v/v) CH_2O, 2% (w/v) Na_2CO_3]. Membranes for slot blot and Western blot were incubated with our own rabbit-raised *B. cenocepacia* J2315 antibodies or FLAG M2 monoclonal antibodies (Sigma) and AlexaFluor IRDye800 CW affinity-purified anti-rabbit IgG (Rockland) or AlexaFluor 680 goat anti-mouse IgG (Molecular Probes) secondary antibodies, respectively; reacting bands were fluorescently detected with the Odyssey infrared imaging system (Licor Biosciences).

1.2.5 Lipopolysaccharide analysis

LPS was extracted as described previously (14, 15). Briefly, bacteria or OMV were resuspended in PBS, and boiled for 10 min in lysis buffer [2% sodium dodecyl sulfate (SDS), 4% β-mercaptoethanol, 10% glycerol, and 1M Tris (pH 6.8)]. The lysate was treated with proteinase K at 60°C for 12h, and stored at -20°C. LPS was separated on a 14% polyacrylamide gel using Tricine-SDS buffer and silver-stained as described previously (15). LPS was quantified using the Purpald assay (13).

1.2.6 Lipid quantitation

OMVs were incubated with 3.3 μg/mL FM4-64 (Molecular Probes) in PBS at 37°C for 10 min. Following excitation at 506 nm, emission at 750 nm was measured with a Cary Eclipse spectrofluorometer (Varian). Vesicle production was calculated as fluorescence units (FU) relative to the CFU of the initial culture.

1.2.7 Electron microscopy

OMV preparations and *B. cenocepacia* C5424 grown overnight in TSB were negatively stained with 1% uranyl acetate and imaged on a Phillips EM300 electron microscope; imaging was done in the Transmission Electron Microscope (TEM) facility in the Department of Microbiology and Immunology at the University of Western Ontario.

1.2.8 Cell culture and infection

RAW 267.4 macrophages (ATCC) were maintained in DMEM (Wisent) supplemented with 10% FBS (Wisent) at 37°C in a 95% humidified atmosphere with 5% CO_2. For macrophage infection, cells were seeded in a 6-well plate at 5 x 10^5 cells per well and grown 15 h. Bacterial cultures grown at 37°C for 16 h were washed twice and resuspended in DMEM-10% FBS. Macrophage monolayers were washed with PBS (Wisent). Bacteria were added at a multiplicity of infection (MOI) of 50:1 in 2 ml DMEM-10% FBS. Plates were centrifuged 1 min at 300 x *g* and incubated at 37°C, 95% humidity and 5% CO_2.

1.2.9 Gentamicin protection assay

RAW267.4 macrophages were infected as above with *E. coli* DH5α at an MOI of 30. *E. coli* was infected alone, with HEPES buffer, or with *B. cenocepacia* OMV.

30 min post-infection, macrophage monolayers were washed three times with PBS and DMEM-50 µg/ml gentamicin was added to kill extracellular bacteria. Infected monolayers were washed with PBS, and lysed with cold sterile water at 30, 60, and 90 min p.i.. Surviving bacteria were enumerated by bacterial plate count for CFU.

1.2.10 Bead labelling and infection

Protein A Dynabeads (Invitrogen) were coated with *B. cenocepacia* J2315 Ab according to the manufacturer's instructions; 40 µL of antibody was added to 10 µL of 0.5 M $NaPO_4$ buffer pH 8.1, and incubated with 50 µL of Dynabeads for 10 min. 75 µL of *B. cenocepacia* or 500 µL of *B. cenocepacia* OMV were captured by the Ab-coated beads. Macrophages were infected as above at an MOI of 10 beads/macrophage.

1.2.11 Immunolabelling and confocal microscopy

Immunolabelling was performed as in Lamothe *et al.* (12). Briefly, coverslip-grown infected macrophage monolayers were fixed in 4% (v/v) paraformaldehyde for 30 min at RT, followed by a 10 min incubation with 100 mM glycine. Cells were permeabilized with 0.1% (v/v) Triton X-100 and blocked with 5% (w/v) skim milk powder for 1h at RT. Cells were sequentially labelled with 1:500 rabbit anti-*B. cenocepacia* J2315 and 1:1000 AlexaFluor 488 goat anti-rabbit (Molecular Probes) antibodies, each for 1h at RT in 5% milk. Rat anti-mouse LAMP-1 (ID4B; Developmental Studies Hybridoma Bank) was used at a dilution of 1:50, with 1:1000 secondary antibody AlexaFluor 488 chicken anti-rat (Molecular Probes). Coverslips were mounted onto glass slides using fluorescent mounting medium (Dako Cytomation). Images were acquired on a Zeiss LSM 510 laser scanning confocal microscope with a 100x oil immersion objective.

1.3 Results

1.3.1 Vesicle isolation and characterization

OMVs were isolated from late-exponential phase rich medium cultures of *B. cenocepacia* using the protocol of Allan *et al*. (1). The presence of bacterial components in the vesicle fraction was confirmed by a slot blot with *B. cenocepacia* J2315 antibodies (Figure 29), which revealed higher putative vesicle production by *B. cenocepacia* C5424 than by J2315. *B. cenocepacia* C5424 is effectively labelled with J2315 Ab, but not with K56-2 Ab, and delays phagosomal maturation similar to J2315. Neither the Bradford assay for protein nor the purpald assay for LPS were able to accurately quantify OMV production, despite the presence of LPS core and several major proteins in OMV preparations (Figure 30).

TEM imaging of *B. cenocepacia* C5424 showed vesiculation by TSB-grown bacteria (Figure 31A); imaging of isolated OMVs (Figure 31B) demonstrated the efficacy of vesicle isolation, but also showed significant contamination by flagella and pili. OMV purification through a sucrose gradient showed protein content only in the final fractions (Figure 32), of which most contained only a single 45-kDa protein suggestive of flagellin. Consequently, the OMV isolation protocol of McBroom *et al*. (17) was adopted, as lower centrifugation speeds would be less prone to pelleting contaminating structures.

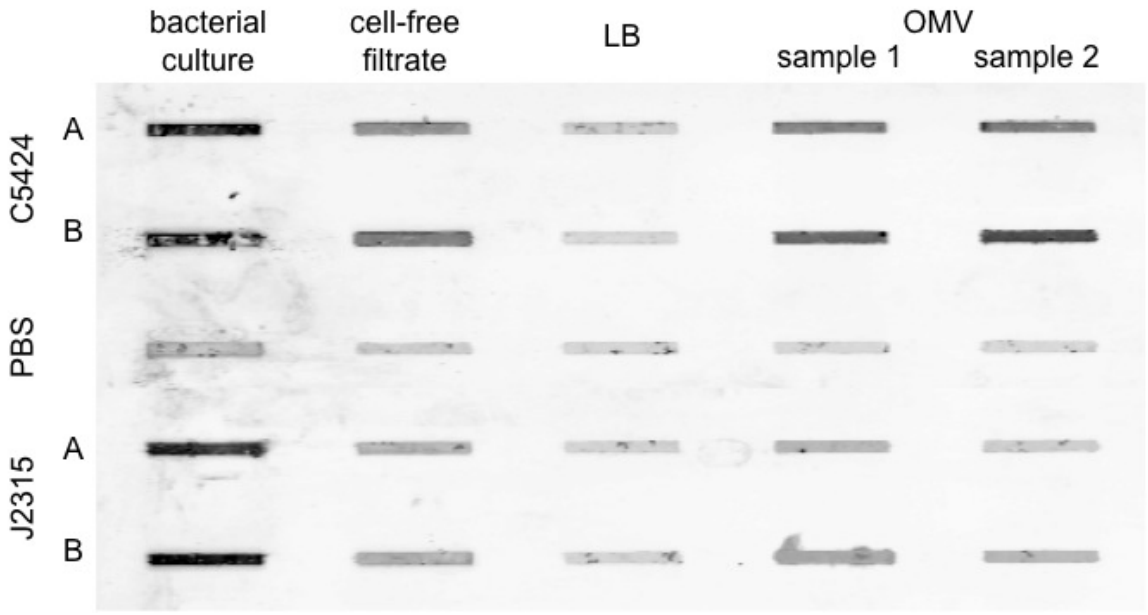

Figure 29. Bacterial components are detectable in outer membrane vesicle fractions.

Samples were adsorbed to a nitrocellulose membrane via vacuum. Rows contain 50 μL (A) or 200 μL (B) of indicated sample. Membranes were blotted with anti-*B. cenocepacia* J2315.

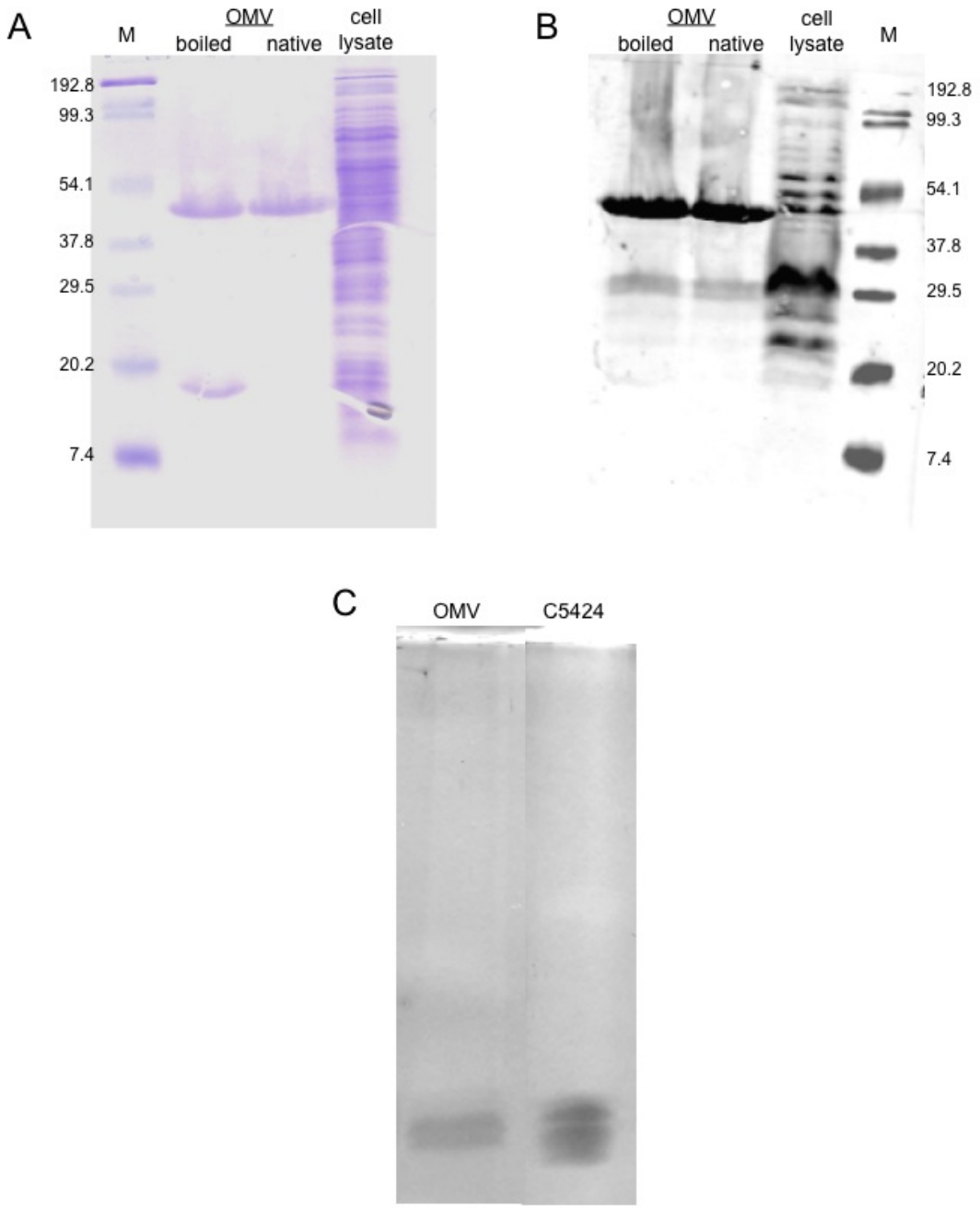

Figure 30. Vesicle fractions contain proteins and lipopolysaccharide.

Native or boiled OMV fractions and whole cell lysates were separated on a 14% polyacrylamide gel by SDS-PAGE and stained with Coomassie blue (A) or transferred to a nitrocellulose membrane and blotted with anti-*B. cenocepacia* J2315 (B). Molecular weight marker (M) masses in kDa. LPS extracted from isolated OMV or *B. cenocepacia* C5424 was separated on a 16% Tricine-SDS-PAGE gel and stained with silver nitrate.

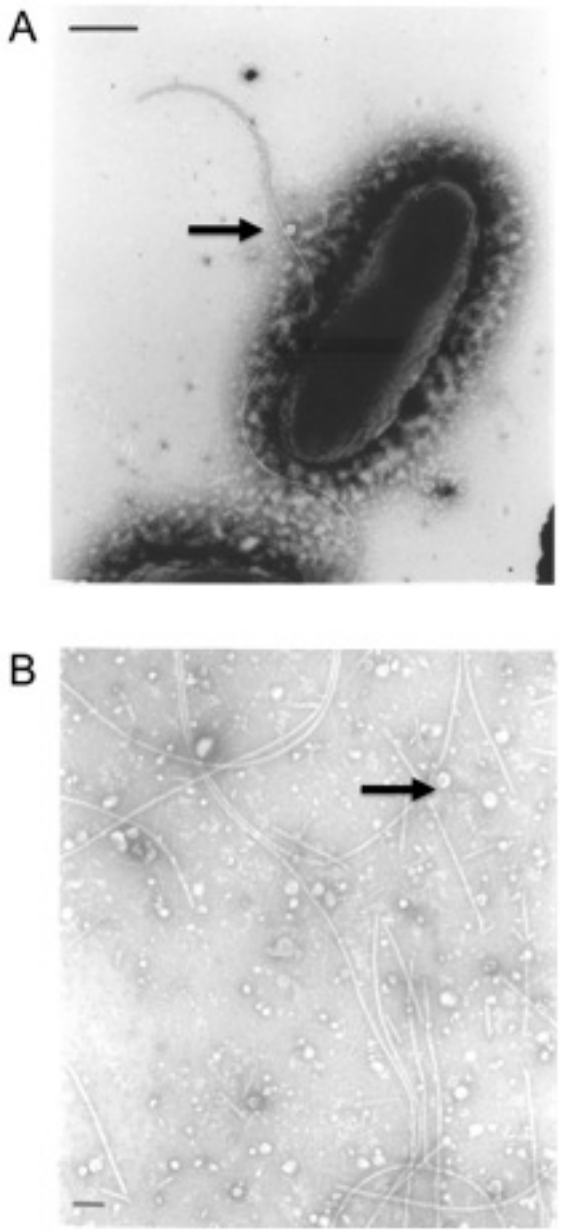

Figure 31. *B. cenocepacia* produces vesicles.

(A) Negative-stained *B. cenocepacia* C5424 grown in TSB. Arrow indicates vesicle. Bar = 500 nm. (B) Negative-stained OMV preparations without sucrose gradient purification. Contaminating flagella and pili are present. Arrow indicates OMV. Bar = 100 nm.

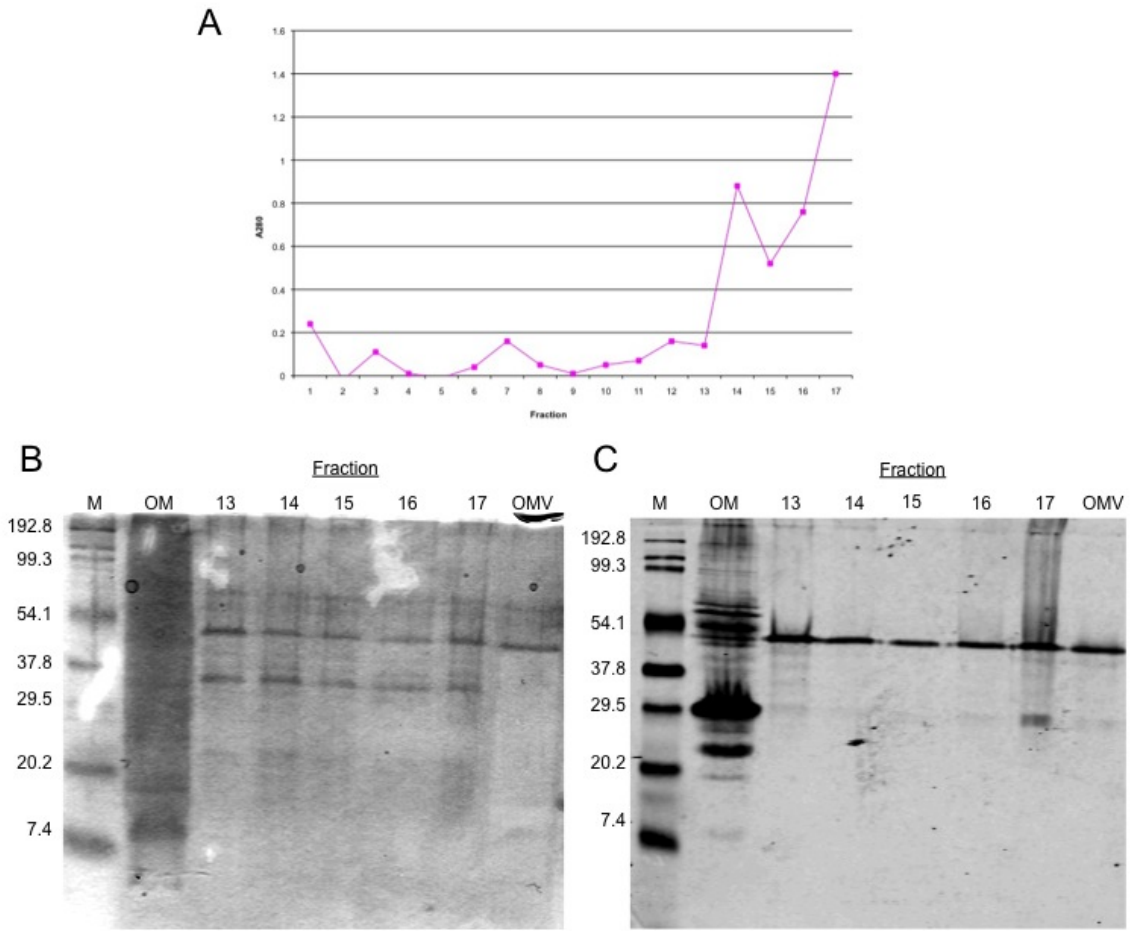

Figure 32. OMV purification through sucrose gradient ultracentrifugation.

Protein concentration in each fraction of the sucrose gradient was quantified at a 1 in 5 dilution via absorbance at 280 nm (A). Later fractions were separated on a 14% polyacrylamide gel by SDS-PAGE and stained with silver nitrate (B) or transferred to a nitrocellulose membrane and blotted with anti-*B. cenocepacia* J2315 (C). Molecular weight marker (M) masses in kDa.

1.3.2 Increasing vesiculation *in vitro*

Bacteria increase vesiculation under conditions of stress, including oxidative (22) and envelope stress (18). We overexpressed the periplasmic protein HtrA from constitutively active plasmid pRF132 in *B. cenocepacia* C5424. OMV produced by *B. cenocepacia* C5424 pRF132 are enriched in protein relative to OMV produced by wild type bacteria (Figure 33). While the FLAG-tagged protein is present in isolated periplasmic protein, a lesser amount is also present in the OM; though this may be due to the high level of overexpression, the FLAG signal in isolated OMV cannot be conclusively linked to the packaging of periplasmic proteins (Figure 34).

Nutrient limitation and growth rate have been linked to the regulation of virulence factor production in "*B. cepacia*" (19); previous studies have shown an enrichment in OMV-packaging of enzymes during growth in minimal media (1). Bacterial growth in minimal medium MMST significantly increased relative vesiculation compared to growth in rich medium TSB (Figure 35); the protein composition of MMST-OMV was different from TSB-OMV (Figure 36). TEM imaging of MMST-grown C5424 revealed smaller bacteria, many of which were associated with OMV (Figure 37); TEM imaging of isolated OMV showed a decrease in contaminating protein.

1.3.3 Intramacrophage vesiculation

An antibody against whole formalin-fixed *B. cenocepacia* J2315 was raised in rabbits; this antibody could be used for effective (Figure 38A), and specific (Figure 38B) immunolabelling of *B. cenocepacia*. In infected macrophages, the *B. cenocepacia*-specific Ab labels elements unassociated with intact mRFP1-labelled bacteria; over the course of an infection, these bacterially-derived elements increase in number and in distance from intracellular bacteria (Figure 39).

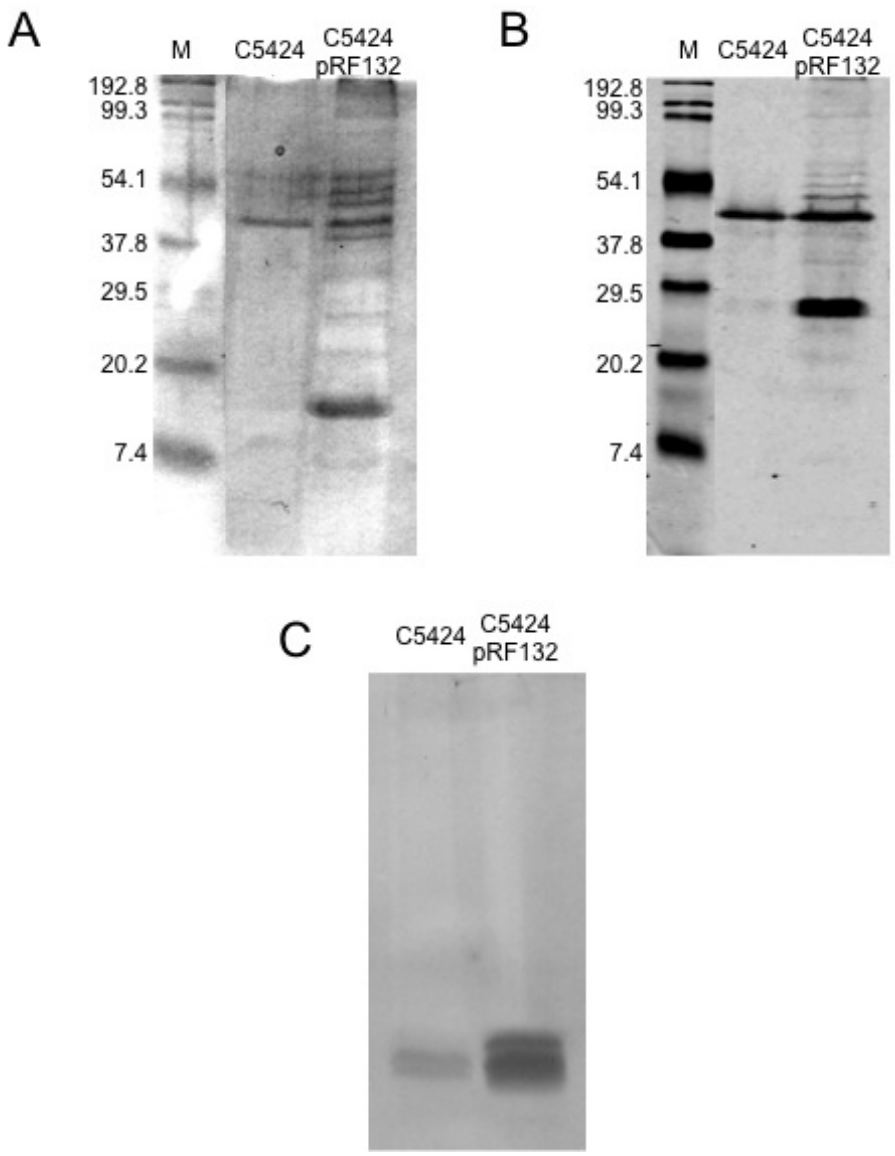

Figure 33. Overexpression of a periplasmic protein results in a greater concentration of protein and LPS in isolated OMV fractions.

Equivalent volumes of OMV isolated from *B. cenocepacia* C5424 and *B. cenocepacia* C5424 pRF132 were separated on a 14% polyacrylamide gel by SDS-PAGE and stained with silver nitrate (A) or transferred to a nitrocellulose membrane and blotted with anti-*B. cenocepacia* J2315 (B). Molecular weight marker (M) sizes are listed in kDa. LPS prepared from equivalent volumes of OMV isolated from *B. cenocepacia* C5424 and *B. cenocepacia* C5424 pRF132 were separated by 16% Tricine-SDS-PAGE and stained with silver nitrate (C).

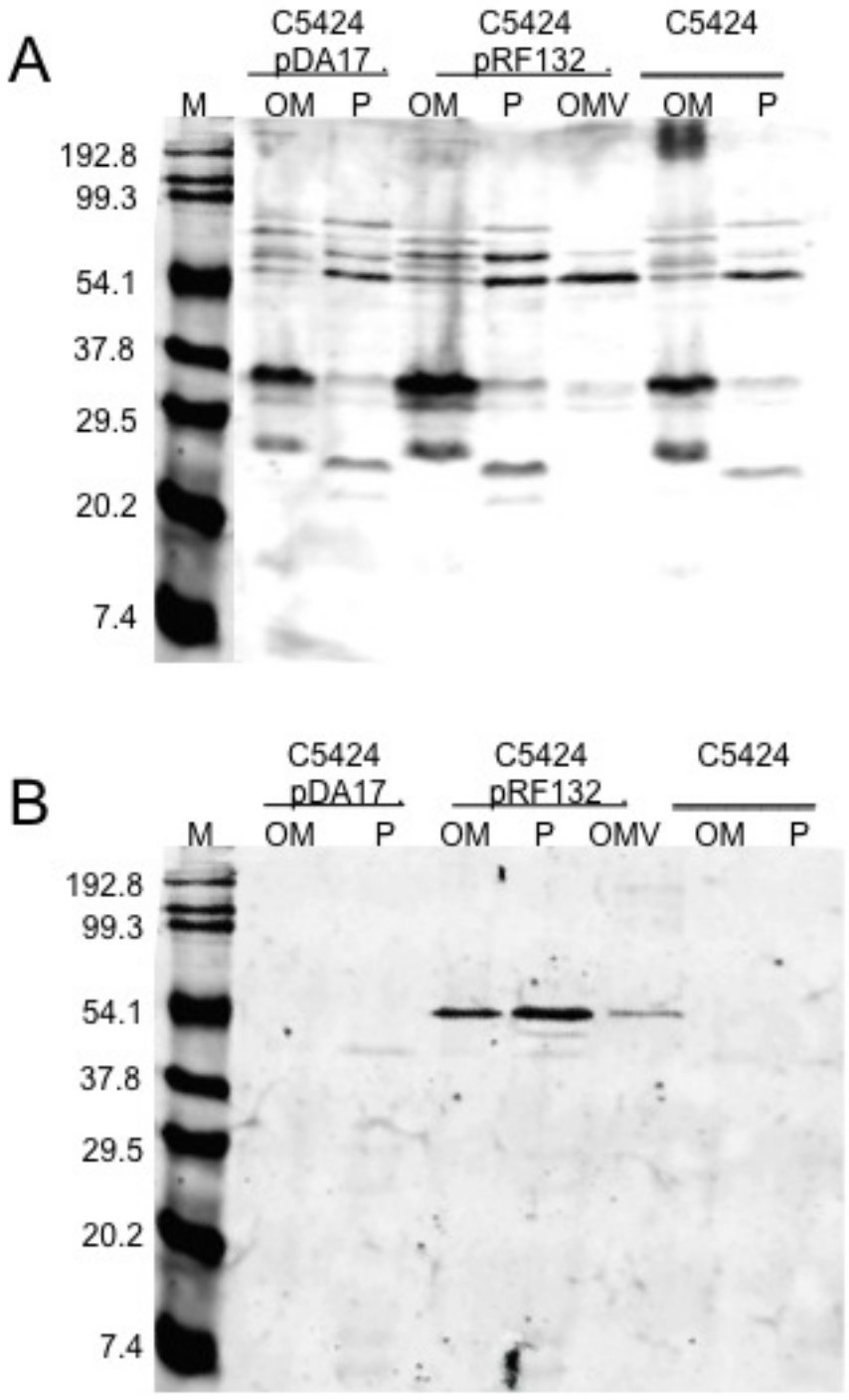

Figure 34. Periplasmic protein can be detected in isolated OMV.

Outer membrane (OM), periplasmic protein (P), and isolated OMV (V) were separated on a 14% polyacrylamide gel by SDS-PAGE, transferred to a nitrocellulose membrane and blotted with anti-*B. cenocepacia* J2315 (A) or anti-FLAG (B). Molecular weight marker (M) masses in kDa.

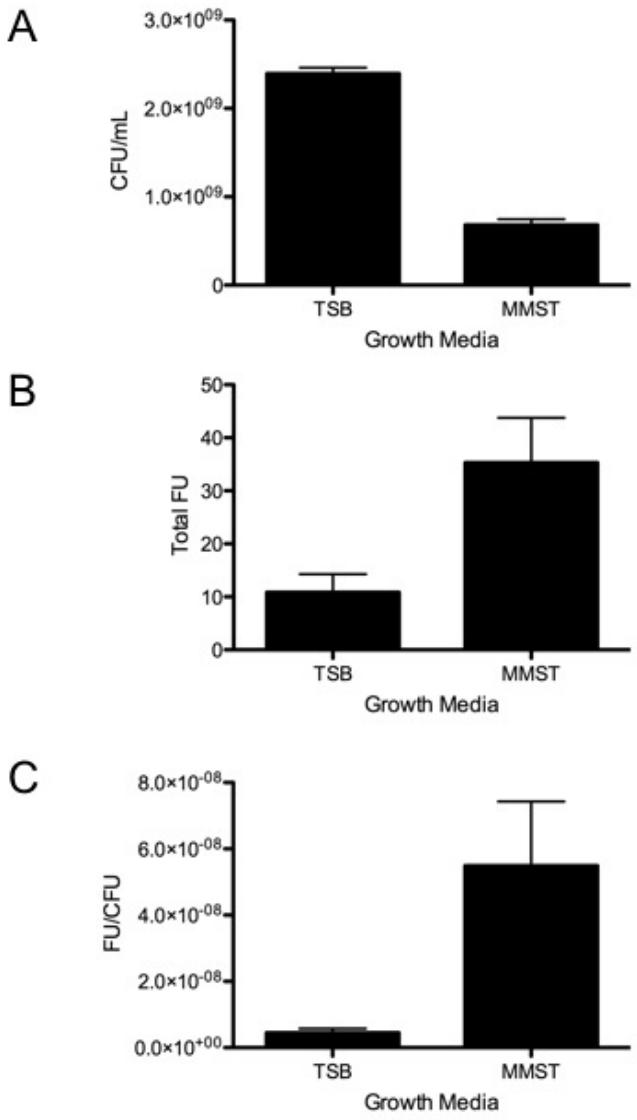

Figure 35. Nutrient stress increases relative vesiculation.

B. cenocepacia growth is slowed in minimal media MMST when compared to growth in rich media TSB, but vesiculation is increased. (A) Bacterial concentration of overnight cultures from which OMV were isolated. (B) Total OMV obtained in an equivalent isolation procedure, as measured in fluorescence units. Fluorescence units quantify the lipid content of OMV fractions via lipophilic probe FM4-64. (C) Relative vesiculation, defined as fluorescence units in isolated OMV fractions per CFU of culture. Standard error of 2 (TSB) and 4 (MMST) independent experiments is indicated.

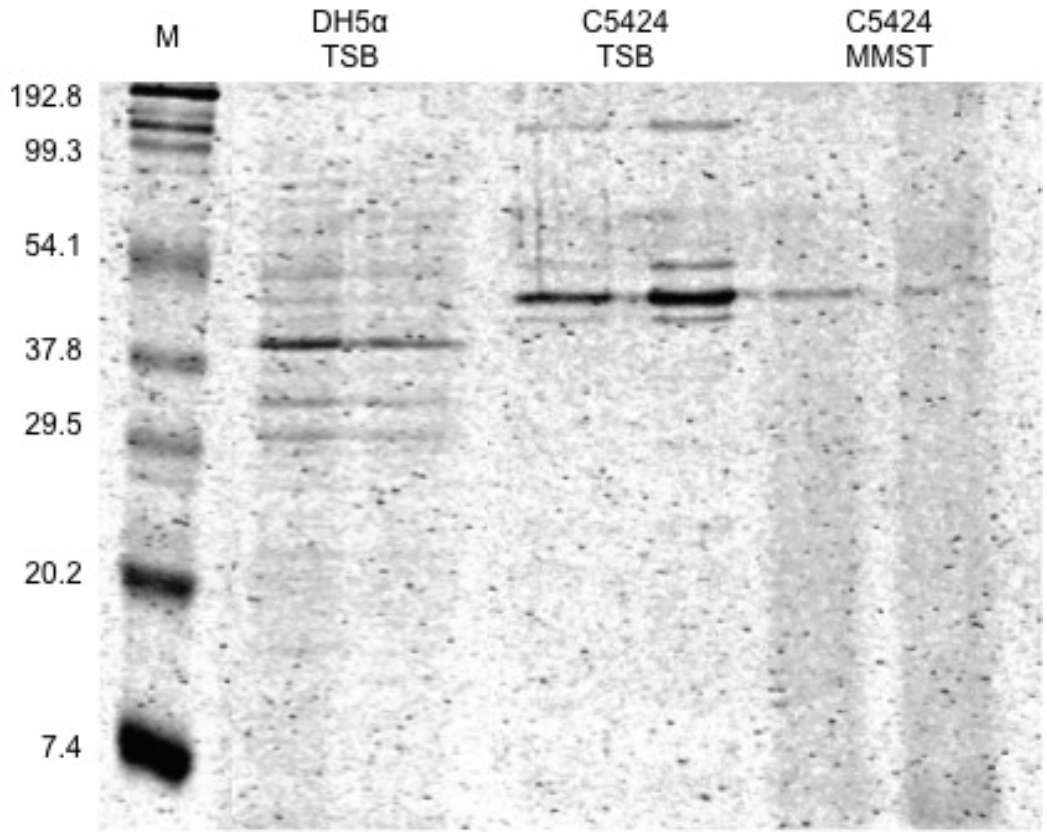

Figure 36. OMV isolated from nutrient-stressed bacteria display an altered protein profile.

OMV isolated from *E. coli* DH5α, *B. cenocepacia* C5424 grown in rich media TSB, and *B. cenocepacia* C5424 grown in minimal media MMST were separated on a 14% polyacrylamide gel by SDS-PAGE and stained with Coomassie blue. Molecular weight marker (M) masses in kDa.

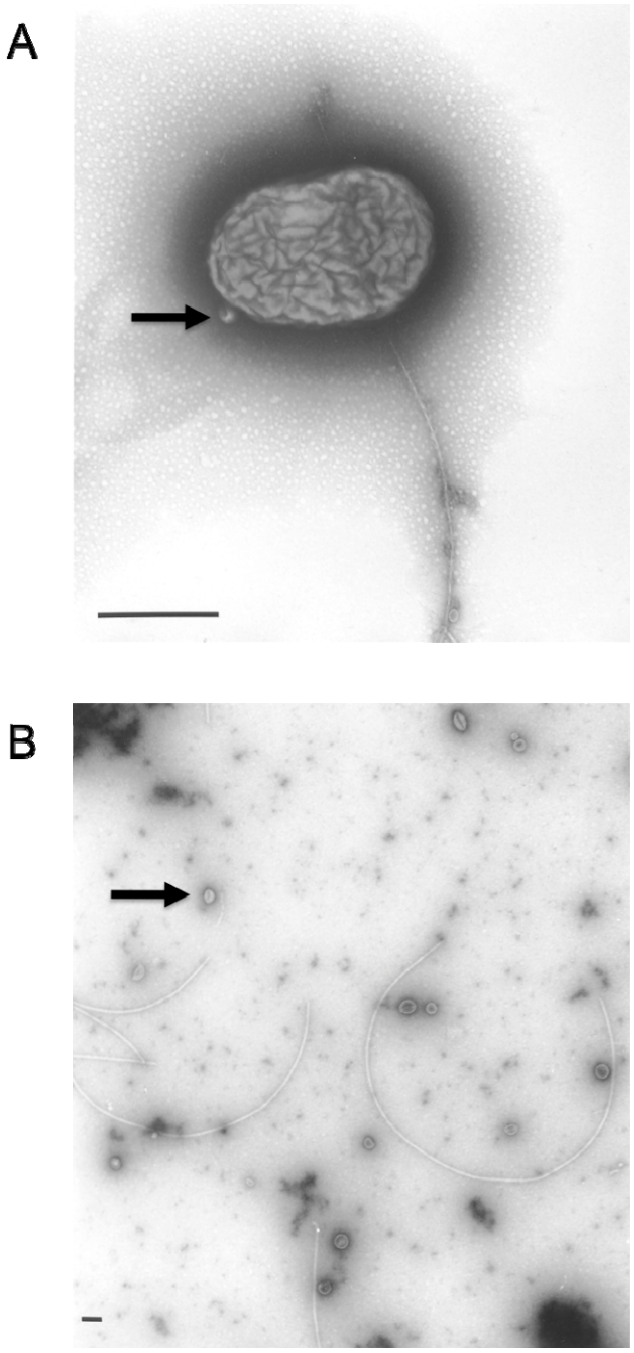

Figure 37. Growth in minimal media alters the size, shape, and vesiculation of *B. cenocepacia.*

(A) Negative-stained *B. cenocepacia* C5424 grown in MMST. Arrow indicates vesicle. Bar = 500 nm. (B) Negative-stained OMV preparations isolated with slower centrifugation. Arrow indicates vesicle. Bar = 100 nm.

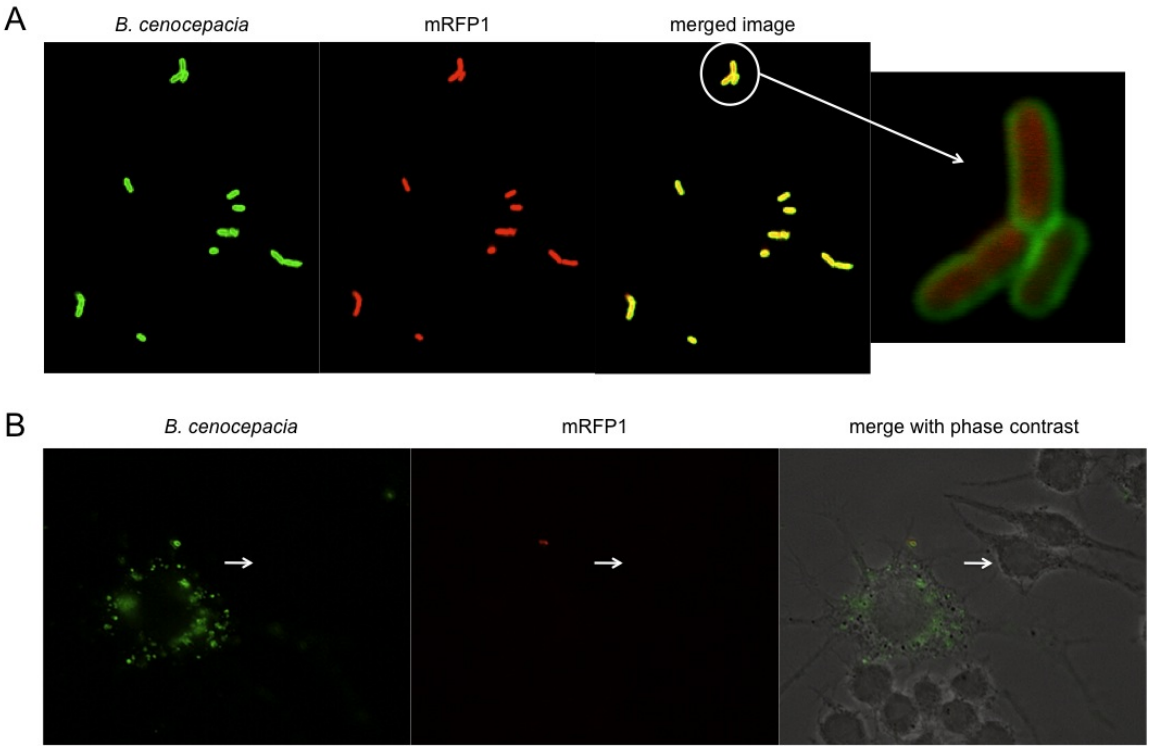

Figure 38. *B. cenocepacia* antibodies effectively label the bacteria (A), but do not bind to uninfected macrophages (B).

RAW264.7 macrophages were infected with *B. cenocepacia* J2315 expressing mRFP1, immunolabelled with anti-*B. cenocepacia* J2315, and observed with confocal (A) or fluorescence light (B) microscopy. Arrow indicates an uninfected cell.

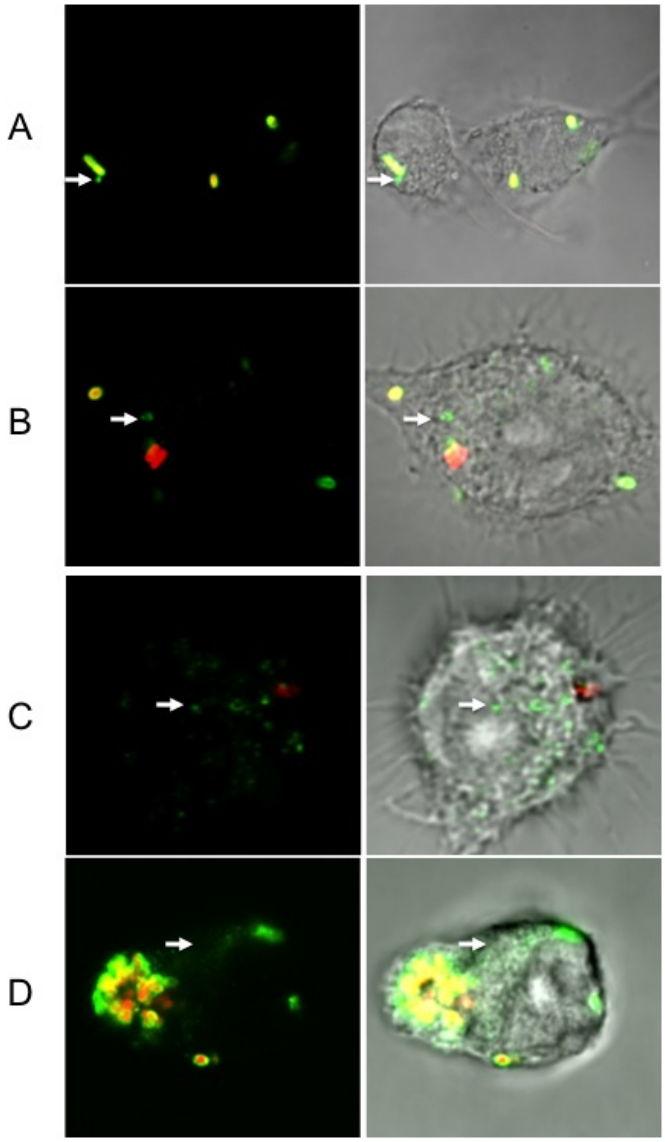

Figure 39. Anti-*B. cenocepacia*-reacting particles dispersed throughout infected macrophages increase in number and in distance from intact bacteria over time.

RAW264.7 macrophages were infected with *B. cenocepacia* J2315 expressing mRFP1, immunolabelled with anti-*B. cenocepacia* J2315, and observed with confocal microscopy. From left to right, panels show merged green anti-J2315 and red mRFP1-expressing J2315 fluorescence, and fluorescence merged with the DIC image at 30 min (A), 2h (B), 4h (C), and 6h (D) post-infection. Arrows indicate anti-*B. cenocepacia*-reacting particles that are unassociated with intact bacteria.

1.3.4 The effect of *B. cenocepacia* OMV on the intracellular trafficking of inert particles

If the OMV shed by *B. cenocepacia* are the mechanism by which phagosomal maturation is delayed, then the OMV alone should be able to delay delivery of an inert particle to the lysosome. Protein A Dynabeads are magnetic particles to which IgG will strongly bind; thus, *B. cenocepacia* bacteria or OMV can be linked to the particle via *B. cenocepacia* J2315 Ab. In theory, such a protocol would allow magnetic purification of the BcCV. However, the beads are poorly internalized in the absence of bacterial components (Figure 40), immunolabelling antibodies bind directly to the beads, antibody binding is inconsistent, and linked bacteria can be sheared from the beads (Figure 41).

1.3.5 The effect of *B. cenocepacia* OMV on the intracellular survival of *E. coli*

If the OMV shed by *B. cenocepacia* are the mechanism by which phagosomal maturation is delayed, then co-infection of *E. coli* with *B. cenocepacia* OMV should prolong the intracellular survival of *E. coli* by delaying delivery to the lysosome. Macrophages were infected with *E. coli* alone, in the presence of buffer, or in the presence of *B. cenocepacia* OMV. In the presence of HEPES buffer, macrophages took up more *E. coli*, a phenotype abrogated by the presence of *B. cenocepacia* OMV in HEPES (Figure 42). However, in macrophages infected with *E. coli* alone or with buffer, the population of intracellular bacteria decreased over time to less than 40% of the initial population by 90 min p.i.. In contrast, following an initial decrease, in the presence of *B. cenocepacia* OMV, intracellular *E. coli* began to increase in number, suggesting intracellular replication by living bacteria. Thus, it appears that *B. cenocepacia* OMV are able to affect the intracellular fate of *E. coli*.

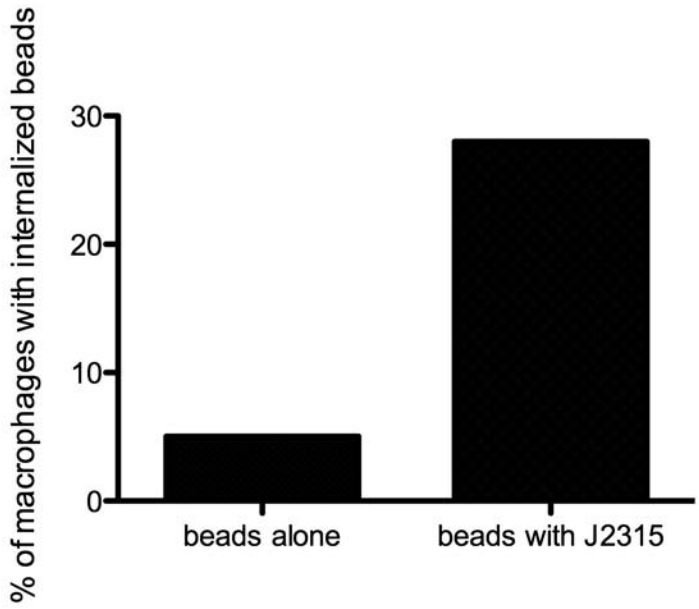

Figure 40. Dynabeads are poorly internalized by macrophages in the absence of bacteria.

RAW264.7 macrophages were infected with Dynabeads alone or in the presence of *B. cenocepacia* J2315. 1 h p.i., a total of 1575 macrophages were examined for the presence of internal Dynabeads in a single experiment.

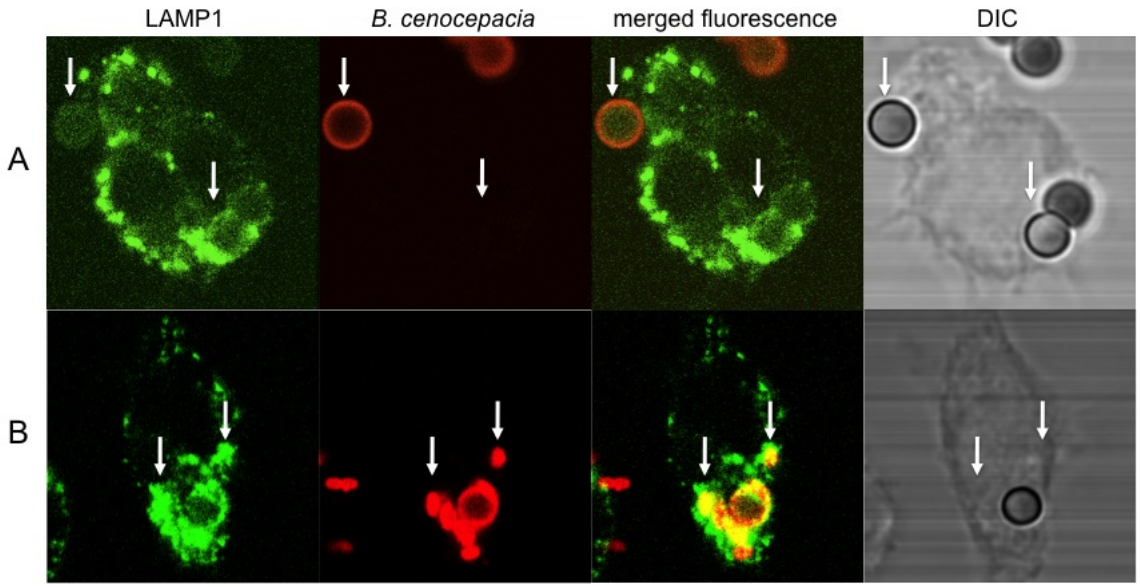

Figure 41. Substrate binding to Dynabeads is inconsistent and incomplete. RAW264.7 macrophages were infected with Dynabeads coated with *B. cenocepacia* OMV (A) or whole bacteria (B). 4 h p.i., macrophages were labeled with anti-LAMP1 (green) and anti-J2315 (red) and observed with confocal microscopy. Arrows in (A) indicate an extracellular bead with bound antibody for endosomal marker LAMP1, and an intracellular OMV-coated bead unlabelled with anti-J2315. Arrows in (B) indicate intracellular bacteria distinct from any bead.

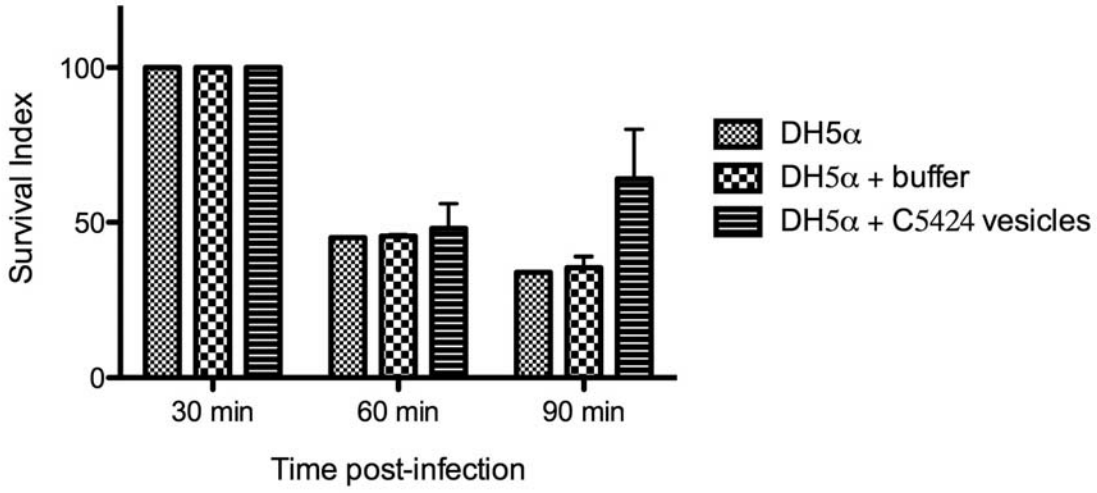

Figure 42. *B. cenocepacia* OMV affect the intracellular fate of *E. coli*. RAW264.7 macrophages were infected with *E. coli* DH5α alone, in the presence of buffer, or in the presence of *B. cenocepacia* OMV. Viable intracellular bacteria were quantified 30, 60, and 90 min p.i.. The survival index was calculated relative to bacteria present 30 min p.i., set as 100%. Data represents the mean of two independent experiments. Standard error bars are indicated.

1.4 Summary

B. cenocepacia produces natural outer membrane vesicles than can be pelleted from cell-free supernatants. Strain C5424 appears to produce more OMV than J2315. Biochemical characterization demonstrates LPS and protein components in isolated OMV. Growth in minimal media – nutrient limitation and slowed growth rate – greatly increases relative vesiculation, as quantified by lipophilic dye, and alters the protein composition of the OMV. Bacterial components are shed into macrophages during infection; further study is necessary to characterize both the bacterial elements and the mechanism of dispersal. Preliminary experiments show that *B. cenocepacia* OMV are able to increase the intracellular survival of *E. coli* during co-infection, suggesting that further investigation into the relevance of OMV to intracellular trafficking is warranted.

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Appendix 2

2 Selective capture of transcribed sequences for *B. cenocepacia*

2.1 rDNA blocking plasmid isolation

- Grow *E. coli* DH5α pJT25 8h in 5 mL LB Ap100
- Add 25 μL of O/N culture to 100 mL LB Ap100
  - Grow to 3-4 x 10^9 cells/mL (12-16 hours – OD_{600} = 35)
- Extract pJT25 with Qiagen midi-plasmid kit (re-use column with 2 batches)
  - Spin 6000 x *g* 15 min 4°C
  - Resuspend in 4 mL Buffer P1
  - Add 4 mL Buffer P2, mix by vigorous inversion 4-6 times
    - 5 min at RT
  - Add 4 mL chilled Buffer P3, mix by immediate vigorous inversion 4-6 times
    - 15 min on ice
  - Spin > 20 000 x *g* for 30 min at 4°C
    - Remove supernatant containing plasmid promptly (to glass bottles)
  - Apply 4 mL buffer QBT to Qiagen-tip 100 column & empty by gravity flow
  - Filter supernatant through glass wool (in pasteur pipet) into column
  - Wash column with 2 x 10 mL Buffer QC
  - Elute DNA with 5 mL Buffer QF
    - It may be stored O/N at 4°C
  - Add 3.5 mL RT isopropanol
    - Mix & centrifuge immediately at 15 000 x *g* for 30 min at 4°C
    - Carefully decant supernatant
  - Resuspend pellet in 200 μL water & transfer to eppendorf
    - Add 20 μL 3M Na acetate pH 5.2
    - Add 1 mL 100% EtOH
    - Leave at -20°C for > 45 min (O/N)
    - Spin 10-15 min 13.2 krpm
  - Wash pellet in 1 mL of RT 70% ethanol
  - Spin 1 min
  - Air dry pellet 5-10 min
  - Resuspend plasmid in appropriate volume of 10mM EPPS – 1mM EDTA (EE)

- Quantify with NanoDrop spectrophotometer

2.2 Chromosomal library preparation

- Grow *B. cenocepacia* K56-2 to mid-log phase (OD_{600} 0.4-0.6)
- Extract DNA
  - Pellet 1.5 mL of O/N culture
  - Resuspend pellet in 560 μL TE buffer, 30 μL 10% SDS, 10 μL Proteinase K (20 mg/mL)
  - Incubate at 37°C at least 1 h (until mix is clear)
  - Add 300 μL chloroform:isoamyl alcohol (24:1)
    - Mix well & spin 10 min at 13.2 krpm
  - Transfer ~ 400 μL of upper phase to new tube
  - Add 0.1 volume (= 40 μL) 3M sodium acetate & 2.5 volumes (=1.2 mL) 100% EtOH
    - Mix & spin 15 min at 13.2 krpm
  - Discard supernatant & wash twice with 500 μL 70% EtOH
  - Dry pellet at 45°C (~ 10 min)
  - Resuspend in 50 μL water by vortexing vigorously
  - Add 1 μL RnaseA (10 mg/mL)
  - Incubate at 37°C 30 min
  - Check A_{260} (NanoDrop)
  - Aliquot 15 μL with 0.5 – 1.0 μg/mL DNA into clear 0.6 mL thin-walled PCR tube
- photobiotinylate
  - Add 15 μL of 1μg/mL photobiotin acetate in sterile distilled water
  - Incubate on ice 2-3 cm below 250W incandescent bulb for 30 min
    - Switch ice buckets & rotate tube every 5 minutes
  - Add 15 μL fresh photobiotin acetate solution
  - photoactivate on ice an additional 30 min
    - Switch ice buckets & rotate tube every 5 minutes
  - Dilute to 50 μL with water
  - Add 50 μL 0.1x TE buffer [pH 9.0] (10 mM Tris-1mM EDTA)
  - Butanol extract DNA (removes unbound biotin – repeat 2x or as required)
    - Add 100 μL isobutanol & mix well
    - Centrifuge 2 min
    - Remove & discard organic (upper) layer
  - Evaporate any residual isobutanol

  - Add 1/10 volume 3M sodium acetate pH 5.2
  - Add 1 μL glycogen
  - Precipitate with 2 volumes 100% EtOH >3h
  - Spin at 4°C 15-30 min
  - Resuspend DNA in EE
- For each chromosomal library, include 0.3 μg of biotinylated chromosome and 5 μg of pJT25 (1 chromosome: 17 rDNA) – typical SCOTS-CE requires 30 aliquots
  - Use microtip and sonicate in minimal volume
  - Sonicate 150 μg pJT25 with 2-2s pulses at 10% amplitude
  - Add 9 μg biotinylated chromosome
  - Sonicate 2s at 10% amplitude
  - Check fragments on gel, determine final volume & quantitate
- Divide into 30 aliquots of 4 μL containing 5.4 μg of DNA

2.3 RNA extraction

- Prepare RNA from 2-6 well plates per condition
  - Resuspend & collect bacteria from non-macrophage-exposed condition
  - Lyse macrophages in 1mL cold water / well
  - Spin 8 min at 8000 rpm in a 50mL Falcon tube
- Resuspend in 100 μL lysozyme (1 mg/mL in TE buffer)
  - 5 min at room temperature (RT)
- Add 1 mL TRIzol
  - Vortex until homogeneous (~ 1 min)
  - 5 min on ice
- Add 200 μL chloroform
  - Mix ~ 1 min
  - 5 min on ice
- Spin 15 min at 4°C & 12 000xg
- Recover ~ 600 μL RNA-containing supernatant
- Add 500 μL isopropanol & mix
  - 30-40 min on ice
- Spin 10 min at 4oC & 12 000xg
  - Discard supernatant
- Wash with 500 μL cold 75% EtOH
  - Vortex
- Spin 5 min at 4oC & 7500xg

  - Discard EtOH
  - respin ~ 30 s & draw out residual EtOH
- air-dry pellet
  - 5-10 min at 45°C
- Resuspend gently in 10 μL RNase-free water
  - 5 min at RT
- add 5 μL DNase
  - 60 min at 37°C
  - 15 min at 75°C
- check DNA removal by PCR with RNA as template:
  21.2 μL water
  8 μL Q solution
  4 μL 5x PCR buffer
  0.8 μL dNTPs
  0.8 μL / primer (3550+3551: cblB, 3743+3744: β-actin)
  0.8 μL Taq polymerase
  - divide into 3 x 9 μL aliquots
  - add 1 μL of template (two RNA samples + water control)
  3 min at 95 °C
  28 cycles of:
    45s at 95°C
    30s at 55°C
    30s at 72°C
  5 min at 72°C
  check by agarose gel
- quantify clean RNA with NanoDrop

2.4 cDNA synthesis

2.4.1 First strand cDNA synthesis

- heat RNA & primer 75°C 5 min
  1 μL random primer (I-3025; NME-3017)
  x μL DNase-trasted RNA (5 μg)
  (11-x) μL RNase-free water
- set up RT reactions:
  8 μL RT buffer

1 µL RNase inhibitor
4 µL dNTPs
1 µL DMSO
1 µL Q Solution
- divide into 2 x 7.5 µL
- add RNA mixture
    0.75 µL RT enzyme

- 15 min at 25^{o}C
- 40 min at 55^{o}C
- 5 min at 85^{o}C
- immediately on ice

2.4.2 Second strand cDNA synthesis

- to 20 uL RT rxn add (for 50 uL Klenow rxn):
  - 5 uL 10x Klenow buffer (random priming)
  - 1.25 uL dNTP
  - 4 uL Klenow fragment
  - 19.75 uL water
- 45 min at 37^{o}C
- clean with PCR product purification kit
  - elute in 62 µL water

2.4.3 cDNA amplification

- 100 uL cDNA PCR:
  - 4 µL primer (I-3032; NME-3033)
  - 2 µL dNTP
  - 10 µL 10x buffer
  - 20 µL Q solution
  - 2 µL Taq
  - 62 uL template
- 95^{o}C for 5 min
- 25 cycles of:
  - 95^{o}C for 30 s
  - 49^{o}C for 90 s
  - 72^{o}C for 50 s
- 72^{o}C for 5 min
- 4^{o}C for 10 min

- purify cDNA & elute in 42 µL EE
- quantify with NanoDrop
- divide into 10 x 4 µL aliquots of 750 ng/µL

2.5 Selective capture of transcribed sequences

- heat 1 chromosomal DNA aliquot (5.3 µg in 4 µL) to 98^{o}C (boiling) under mineral oil for 3 min
  - add 1 µL 1M NaCl
  - incubate at 68^{o}C 30 min
- boil cDNA (3 µg) 3 min in 4 µL hybridization buffer (10 mM EPPS – 1 mM EDTA)
  - add 1 µL 1M NaCl
  - incubate at 68^{o}C 30 min (if desiring to enrich for low abundance transcripts)
- mix chromosomal DNA with cDNA
  - continue hybridization at 68^{o}C for 20-24 h with oil overlay
- wash Dynabeads-streptavidin (10 mg/mL)
  - shake vial to resuspend beads
  - transfer 60 µg (6 µL) of beads to eppendorf
  - place on magnet 1-2 min
  - remove supernatant with pipette while tube remains on magnet
  - remove tube from magnet
  - resuspend in equal volume capture buffer
  - repeat wash
    - resuspend in 1 mL capture buffer
- add cDNA to capture buffer with beads
  - incubate at 43^{o}C 30 min
  - place tube on magnet 1-2 min
  - remove supernatant with pipette
- 3 washes in wash buffer (20mM NaCl-0.5% SDS)
  - > 5 minutes at 65^{o}C
- elute with 100 µL elution buffer (0.25M NaOH-0.1M NaCl)
  - 15 minutes at 65^{o}C
  - place tube on magnet 1-2 min
  - remove supernatant to fresh eppendorf with pipette
- neutralize with 20 µL neutralization buffer (1M Tris pH 7.4)
- ethanol precipitate in the presence of 10 µg (0.5 µL) glycogen
- PCR amplify with linker primers
  - resuspend pellet in 124 µL water

    - 8 µL primer (I-3032; NME-3033)
    - 4 µL dNTP
    - 20 µL 10x buffer
    - 40 µL Q solution
    - 4 µL Taq
  - divide into 2 x 100 µL PCR reactions
  - 95^{o}C for 5 min
  - 25 cycles of:
    - 95^{o}C for 30 s
    - 49^{o}C for 90 s
    - 72^{o}C for 50 s
  - 72^{o}C for 5 min
  - 4^{o}C for 10 min
- clean PCRs
  - elute both together in hybridization buffer
- 10 parallel first round captures of each cDNA mixture
  - combine amplified cDNAs for second round
- at least 3 rounds of SCOTS with cDNA mixtures from each condition

Hybridization Buffer:	10 mM EPPS – 1 mM EDTA
Capture Buffer:	1 M NaCl – 5 mM EPPS – 0.5 mM EDTA
Wash Buffer:	20 mM NaCl – 0.5% SDS
Elution Buffer:	0.25 M NaOH – 0.1 M NaCl
Neutralization Buffer:	1 M Tris pH 7.4

2.6 Competitive enrichment

- prehybridize chromosomal DNA with both rDNA & SCOTS-treated NME cDNA
  - use 10 µg NME-cDNA / chromosomal aliquot
- 24 hour hybridization with 0.5 µg I-cDNA at 68^{o}C
- recover by binding to streptavidin-coated beads as above
- PCR amplify with I-3032 linker primer
- Repeat twice for a total of 3 rounds of competitive enrichment

2.7 Screening

- prepare 50 µL pUC19 using Qiagen MiniPrep kit

- digest vector & cDNA with XbaI
  - 10 µL vector / cDNA
  - 1.5 µL Buffer H
  - 1.5 µL XbaI
  - 2 µL water
- incubate at 37^{o}C O/N
- clean digested cDNA with MinElute kit
  - elute in 15 µL water
- treat vector with alkaline phosphatase
  - 35 µL digested pUC19
  - 4 µL 10x AP buffer
  - 1 µL Antarctic alkaline phosphatase
- incubate at 37^{o}C at least 1 h
- clean vector with Qiagen kit
- ligate O/N
  - 15 µL vector/cDNA
  - 2 µL 10x ligation buffer
  - 1 µL T4 DNA ligase
  - 2 µL water
- incubate at 16^{o}C O/N
- transform plasmid into *E. coli* DH5α
  - heat shock or electroporation
- screen colonies using linker primers (I-3032) or vector primers (3035+3037)
  - with vector primers, negative results will have ~100 bp product
  - with linker primers, multiple inserts of different sizes will be obvious
- screen clones using parallel Southern blot hybridization with DIG-labelled cDNA libraries
  - dilute PCRs 1/20 to run on gel

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Tolman, J.S. and M.A. Valvano. 2011. Elucidation of intracellular gene expression by *Burkholderia cenocepacia* within murine macrophages. International *Burkholderia cepacia* Working Group Conference, Prague, Czech Republic.

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