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Uncovering the Functions of Genes in the Pathogen *Acinetobacter baumannii*

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Abstract

Currently, only approximately 2% of the genome of the multi-drug resistant organism *Acinetobacter baumannii* has been experimentally characterised. Unfortunately, this is not uncommon. The experimental verification of gene functions is now lagging behind the rapidly increasing amount of sequence data generated via high-throughput next generation sequencing technology. However, using cellular fluorescence as a proxy for phenotypic data, TraDISort (Transposon-Directed Insertion Site Sequencing and Fluorescence Activated Cell Sorting) is a novel high-throughput protocol able to provide phenotypic data for a multitude of mutants in one experiment. In this thesis, data from a TraDISort experiment performed on an *A. baumannii* transposon mutant library treated with a sub-inhibitory concentration of the DNA/RNA intercalating fluorescent biocide ethidium bromide was bioinformatically analysed. TraDISort uncovered significant ethidium bromide efflux systems in *A. baumannii*, exposed a novel gene potentially involved with ethidium bromide resistance, and consistently selected genes related to DNA, transcription, and translation. FACS gating procedures also predisposed TraDISort to select for mutants with altered morphologies, including genes involved with shape-regulation and cell-division, confirmed via phase-contrast microscopy. Combined with alternative compounds possessing different cellular targets and/or sites of accumulation, TraDISort possesses great potential in probing the functions of numerous genes with diverse functions.

Declaration

This thesis is written in the form of a journal article from PLOS ONE

I certify that the work presented in this thesis has not been submitted to any university besides Macquarie University, Sydney, Australia. The work presented in this thesis is my own original work, and any outside contributions have been acknowledged and cited appropriately.

This thesis is in fulfilment of the requirements for the award of the degree of Master of Philosophy

Brendan Wilson-Mortier

22/10/17

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1. Introduction

1.1 From Genes to Gene Functions: The Genomics of Bacterial Pathogens

Frederick Sanger and colleagues completed the sequencing of a full genome for the very first time in 1977. The 5386 base pair sequence of bacteriophage DNA ushered in a new era, one now often termed the "genomic era".[1, 2, 3] This era was characterised by a rapid increase in the quantity of sequencing data, and was cemented at the turn of the century with the success of the international human genome project.[3, 4] Due to the invention, commercialisation, and continually decreasing costs of high-throughput next generation sequencing technologies, genome sequences are being generated at an increasingly rapid rate.[5, 4] Today, the genomes of almost 100,000 prokaryotic, and 5,000 eukaryotic organisms have been sequenced.[6] This is an impressive feat. However, the important task of identifying gene functions has proven more difficult than genome sequencing.[7] The functions of most genes in a newly sequenced genome are simply predicted using sequence similarities to genes in well-studied model organisms, such as *Escherichia coli*, *Drosophila melanogaster*, or *Candida albicans*.[5, 7] Currently, inadequate automatic-annotation pipelines combined with human-error has led to many incorrect or misleading annotations. The need for the experimental characterisation of gene products and functions is becoming apparent, but this is a labour-intensive process.[5]

Experimental characterization of genes is known as functional genetics, but there is now a stronger emphasis being placed on this field due to the rapid explosion of sequence data provided by NGS technologies. There is also a paucity of protocols to deal with the large datasets generated by genomics.[5, 8] Even extremely well-studied and established model organisms such as *E. coli* and *Bacillus subtilis*, still lack functional data for approximately 25% and 50% respectively, of their genomes.[8, 9, 10] This lack of knowledge is a problem for research into prokaryotic organisms, especially when studying pathogenic bacteria that possess multi-drug resistance.[11] Multi-drug resistant pathogens are a major threat to global health, food security and economic development. Resistance often arises shortly after commercial release of the corresponding antibiotic for a particular infection or pathogen, as seen with linezolid and daptomycin.[12, 13, 14]

To address the resistance problem, there has been a steady increase in genomics-based studies on bacterial pathogens, particularly those which present the highest risk to human health, such as the

"ESKAPE" pathogens designated by the Infectious Diseases Society of America (IDSA). The ESKAPE pathogens are *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species.[15, 16] Each of these species have shown a remarkable ability to develop resistance to frontline antibiotics, has displayed either multi-drug resistance, extensive drug resistance, or pan-drug resistance, and possess global distributions.[17, 18] In recent decades, multi-drug resistance has intensified, and species such as *A. baumannii* have emerged as significant nosocomial pathogens on a global scale. Therefore, it is a critical need to develop novel treatment agents for emergent nosocomial pathogens.

1.2 *A. baumannii*, an Emerging Multi-Drug Resistant Pathogen

A. baumannii is a critically significant, but understudied pathogen.[19, 20] It was first isolated from soil in 1911 and described taxonomically in 1971. The *Acinetobacter* genus is a family of Gram-negative coccobacilli, currently comprised of more than 50 validly named species.[21, 22, 23] *Acinetobacter* are ubiquitous in soil and water samples, on vegetables, human skin, and most strains pose little human safety risk.[23] The species significantly involved in human infections are *Acinetobacter nosocomialis*, *Acinetobacter pittii*, *Acinetobacter dijkshoorniae*, and *A. baumannii*. They all share a close genetic relationship with *Acinetobacter calcoaceticus*, and together have been termed the "ACB complex".[24, 25] Of these, *A. baumannii* represents the most significant clinical threat, with some strains displaying pan-drug resistance.[26]

A. baumannii was first formally named in 1986. It is rarely isolated from environments outside hospitals, and its natural environmental reservoir is puzzlingly unknown.[22, 23] *A. baumanni* can cause different types of infections, including ventilator-associated pneumonia, soft-tissue infections, urinary tract infections, bacteraemia, endocarditis, and blood stream infections.[26] In the 1970s, *A. baumannii* infections were susceptible to most antibiotics, but since the 1990s, resistance to first-line antibiotics such as carbapenems have risen sharply, and mortality rates can reach as high as 40-60%.[15, 22, 27] Most recently, *A. baumannii* strains have been implicated with resistance to last-resort antibiotics for multi-drug resistant Gram-negative organisms, such as colistin and tigecycline.[28, 29] Genomic evidence shows that there are three major clonal lineages of *A. baumannii* associated with global outbreaks. These are known as International Clonal Lineages 1, 2 and 3.[30, 31, 32]

The success of *A. baumannii* in hospital environments is due to several physical characteristics, including resistance to desiccation, the ability to survive on abiotic surfaces for long periods of time, and effective iron-uptake, quorum-sensing, and biofilm formation systems.[26, 33, 34] Virulence factors aid host-invasion, and a naturally reinforced Gram-negative membrane architecture coupled with porin loss helps to reduce membrane permeability.[22, 35] Isolates can also possess an array of antibiotic resistance genes, including those encoding beta-lactam hydrolysing enzymes such as extended spectrum beta-lactamases (ESBLs). *A. baumannii* possessing either oxacillinase or metallo-beta-lactamase enzymes pose the greatest threat, since they are resistant to almost all beta-lactams.[26, 36] Many of these traits are encoded within a large and transferrable accessory genome.[37] This can encode for the enzymatic degradation of other classes of antibiotics, the modification of drug targets, and highly effective efflux pump systems.

Membrane efflux proteins are part of ubiquitous cellular export and homeostasis systems distributed across all domains of life, and contribute to quorum sensing and biofilm formation in prokaryotes.[38, 39] However, they have also been recognised in pathogens to efflux a wide variety of structurally unrelated antimicrobial substances, including biocides, dyes, detergents, disinfectants and antibiotics, and are now known as clinical resistance-determinants.[40, 41] The most clinically significant family of efflux pumps is the Resistance Nodulation Division Superfamily (RND), displayed in Table 1.[42] Unlike most families of efflux pumps, RND type efflux pumps are “tripartite”, possessing three protein components that assemble to span the entire cellular envelope and extrude substances outside the cell, a feat which single protein component pumps residing in the inner membrane cannot achieve.[13,39,41] Nonetheless, single protein component efflux pumps remain clinically significant, and may also work synergistically with tripartite efflux pumps in producing resistance in gram-negative bacteria, which are known to be able to extrude substances from either the cytosol, or the periplasmic space.[40,41]

Table 1: Families of Efflux Pumps, their Structures, Energy Sources, and Cell Types.

Family	# Protein Components	Energy Source	Present Within
Resistance Nodulation Division Superfamily (RND)	Three	Proton Motive Force (H+)	Prokaryotes and Eukaryotes
ATP-binding Cassette Superfamily (ABC)	One to Three	ATP Hydrolosis	Prokaryotes and Eukaryotes
Major Facilitator Superfamily (MFS)	One to Three	Proton Motive Force (H+)	Prokaryotes and Eukaryotes
Small Multi-Drug Resistance Family (SMR)	One	Proton Motive Force (H+)	Prokaryotes
Multiple Antimicrobial Extrusion Protein Family (MATE)	One	Proton Motive Force (Na+ / H+)	Prokaryotes and Eukaryotes
Proteobacterial Antimicrobial Compound Efflux Family (PACE)***	One	Proton Motive Force (Unknown, likely H+)	Prokaryotes
Multidrug Endosomal Transporter Family (MET)	One	Proton Motive Force (Na+ / H+)	Eukaryotes

- *** = Newly Discovered Efflux Pump Family

Along with the possession of multiple chromosomally encoded and genetically mobile single component efflux pumps, three chromosomal RND-type efflux pumps, AdeABC, AdeIJK and AdeFGH, contribute significantly to *A. baumannii's* resistance profile.[43, 44] AdeIJK is constitutively expressed in all *A. baumannii* strains and contributes a basal level of broad resistance. AdeABC and AdeFGH are both tightly regulated, but also contribute to the MDR phenotype when overexpressed, especially AdeABC.[43, 45, 46] In addition, *A. baumannii* strains usually possess many efflux pumps for specific classes of antibiotics that have been acquired by lateral gene transfer, such as efflux for aminoglycosides or fluoroquinolones.[26] Bioinformatic analyses also reveal that strains of *A. baumannii* typically possess more than 50 putative efflux pump genes, in some cases up to 70, suggesting that efflux is a common defence mechanism in the pathogen.[44, 47] Because of the clinical relevance of *A. baumannii*, it is the most studied *Acinetobacter* species, and there is an urgent need to characterise its genome functions.[21] The latest data suggest that 26% of the genes in the well-studied *E. coli* K12 MG1655 genome still encode unknown functions (Figure 1), whilst 74% possess some form of experimental evidence.[6,9,10] For understudied organisms such as *A. baumannii*, much fewer proteins, and mostly those with clinical relevance, such as virulence factors and efflux pumps, have been experimentally characterized (Personal Communication with Professor Ian Paulsen, June 2017, unreferenced).[15,27,33,48] Identification of gene functions in *A. baumannii* is therefore of critical significance.

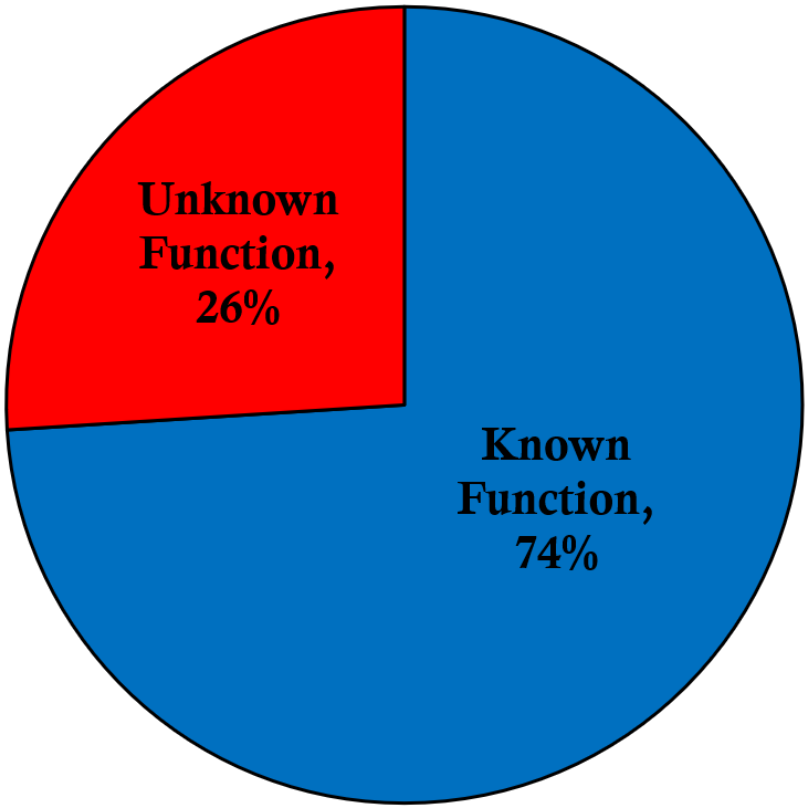

Figure 1: A Graphical Representation of Recent Data Regarding the Proportion of Genes within the Genome of Model Organism *E. coli K12 MG1655* that have been Functionally Verified, and the Proportion of Genes Possessing Unknown Functions. [6, 10, 48]

1.3 Techniques in Functional Genomics

Due to the recent explosion in sequence data, bioinformatic and computational analyses have become the predominant method used to annotate genomes.[49] However, current bioinformatic approaches are not able to assign functions to some 30% to 40% of genes in a newly sequenced genome. This figure may increase in the future as more phylogenetically distant bacterial genomes are sequenced.[50, 51] Surprisingly, one third of the synthetic minimal genome of *Mycoplasma mycoides* possessed completely uncharacterised genes, highlighting that we still have much to understand about the basic requirements of a living cell.[51] Although automatic annotation pipelines have improved greatly over recent years, bioinformatics is still a developing field, and suffers from drawbacks due both to its rapid ascension, and data-heavy nature.[49, 52] Unfortunately, misannotated genes are estimated to be highly prevalent within genomic databases, as homologous genes (orthologs and paralogs) do not always possess similar or identical functions.[51,

[53] Further complicating factors include the prevalence of older and inaccurate genome sequences constructed via short-read length sequencing technology, or current limitations such as issues faced by predictive software in identifying genomic repeat sequences, dealing with guanine and cytosine content, and horizontally acquired genomic regions.[6,54,55,56,57] Thus, incorrect annotations are perpetuated and propagated both within and across species, making functional genetics a vital tool in correct gene-annotation.[51, 52]

In the field of functional genetics, the standard methodology followed to uncover the function of a protein is via gene knockout, essentially deleting the gene for the protein, followed by observation of the mutant's phenotypic characteristics.[58, 59] Gene functions are then hypothesised via complementation of the mutant with the wildtype gene; If the wildtype phenotype is restored, the gene knockout may be deduced as the cause of the observed phenotypic irregularity.[60, 61] Gene knockouts are the most common method of mutation, and traditionally are generated using random chemical or UV mutagenesis, followed by the painstaking phenotypic screening of mutants and mapping of mutant alleles, one by one.[50, 60] This method, known as forward genetics, utilises information derived from mutant phenotypes to define the genetic basis of a phenotype.[7, 8] Forward-genetics based screens in bacteria do possess advantages, such as not requiring prior knowledge about the organism's genome, but phenotypic screenings can be laborious, and it can prove difficult to deduce the location of random genomic mutations.[60, 62] In contrast to forward genetics, 'reverse genetics' is made possible by next generation sequencing technologies.[60] Reverse genetics-based techniques begin with the sequenced genome of organisms. Using the knowledge of the proteins encoded within them, specific genes may be targeted to understand the phenotypes that arise due to their disruptions.[7, 8] Unlike forward genetics, this eliminates the need for identifying the exact locations of mutant alleles of interest, which becomes a steep task when dealing with large datasets.[8, 63] Figure 2 displays a visual representation of the main differences between these two processes. However, not all genes are dispensable, and some are unable to be knocked out, thus are termed "essential genes" due to an inability to survive without them.[64, 65] For genes like these, and those genes for which phenotypic screens cannot provide clues to functionality, other techniques have been established.

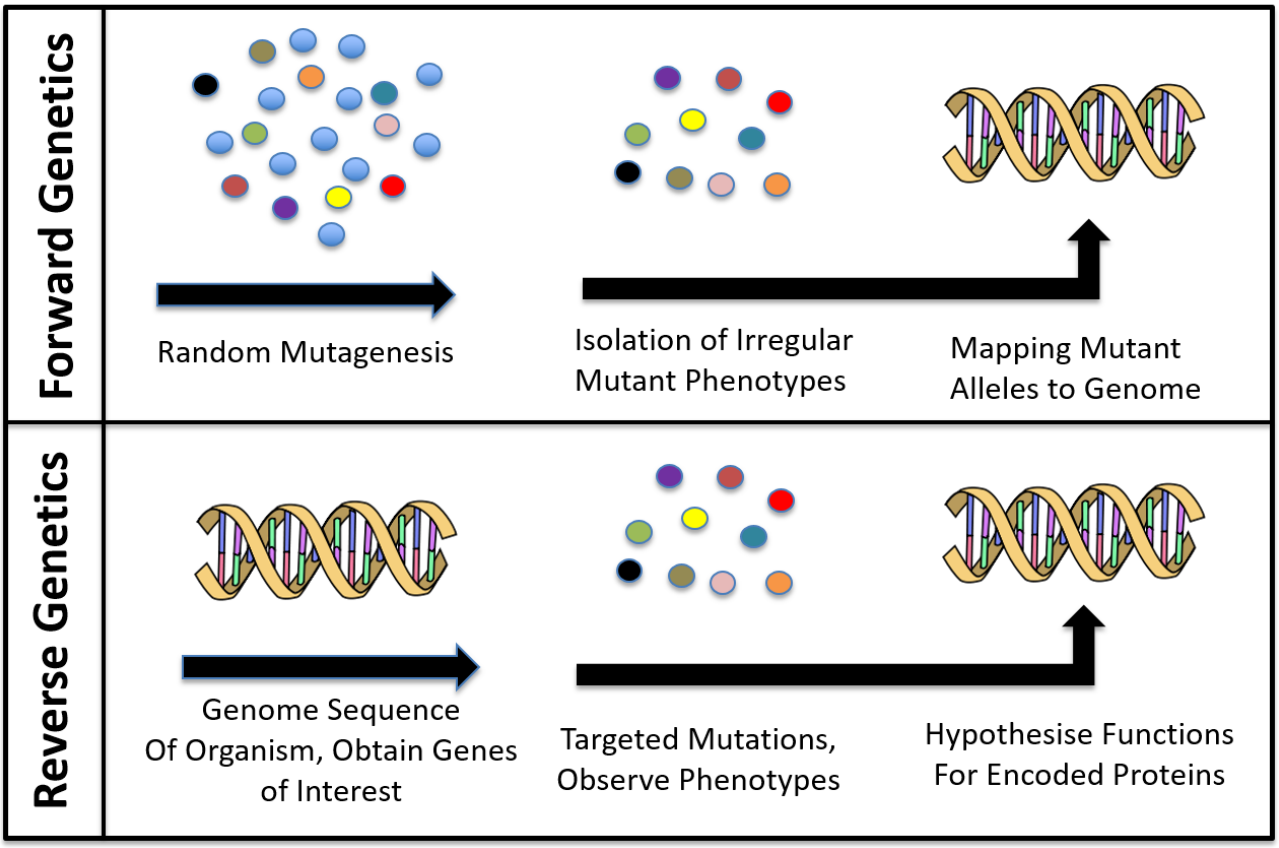

Figure 2: A Graphical Representation of the Main Differences Between Forward, and Reverse Based Genetics Workflows (Image Credit for DNA Molecule: Wikimedia Commons < https://commons.wikimedia.org/wiki/File:DNA_simple.svg>

Modulation of transcription, or the under or over-expression of target genes cloned into expression vectors such as *E. coli* is a common method, as is protein purification and biochemical characterisation.[66, 67] However, while data from these experiments is beneficial, specific gene-targeted experiments can be time consuming.[68] Circumventing this disadvantage, next generation sequencing technologies now permit the use high-throughput protocols in the "omics" fields, allowing rapid and efficient whole-genome analyses.[69, 70] The "omics" fields, transcriptomics, proteomics, and metabolomics, take advantage of high-throughput technologies to analyse the transcriptome, proteome, and metabolome of biological systems.[68, 71] Transcriptomics is a useful tool when assigning functions to hypothetical or putative genes.[72] For bacteria, modulation of transcription is one of the first regulatory steps in response to external stimuli, and much of this is directed by transcriptional regulators, of which *Acinetobacter* species possess between 33 and 35 families of.[68, 73] Transcriptomic protocols like RNA-Seq, now standard for expression analyses, are

able to provide static genome-wide surveys of gene expression in bacteria under normal, varied or stressed conditions, such as growth in different media, or exposure to antibiotics.[74] Furthermore, by analysing transcriptomic data from multiple conditions it is possible to deduce which genes are being activated in response to particular stimuli and which genes are being co-expressed, or suppressed.[50] Protocols such as Real-time PCR can then confirm the quantitative transcriptomic data from RNA-Seq. Such analyses have already been performed in *A. baumannii* to examine genes involved in virulence and in resistance to various antibiotics.[75, 76] However, the main drawback of data interpretation from transcriptomic analyses is that cellular mRNA abundance is not a direct representation of protein expression, but represents a complex balance between messenger RNA (mRNA) synthesis, stability, translation and degradation.[49, 77]

To overcome this problem, the proteome can be characterized. The main high-throughput technique used to characterise proteins in proteomics is liquid or size exclusion chromatography followed by tandem mass spectrometry.[68] In genome annotation, a new branch of proteomics is emerging, deemed "proteogenomics".[78, 79] Proteomic data from tandem mass spectrometry may be used to confirm genome annotations with the use of reference genomes or custom "translatome databases".[78, 80] Subsequently, proteogenomics based techniques can identify expressed proteins beyond the annotated "translatome", and confirm the true boundaries of incorrectly annotated genes.[49, 79] However, proteogenomics based techniques are currently limited by a high false discovery rate due to the sheer mass of data generated. Extensive proteogenomic experiments may identify >1 million individual peptides for a single prokaryotic organism, with a p-value of <.01 still providing approximately 10,000 false positive peptide identifications.[49, 80] Subsequently, the implementation of data analyses able to control for this disadvantage remains a primary concern in all proteogenomics based studies.[49, 81] Furthermore, unlike transcriptomics, proteogenomics is not an established field. Mass spectrometry based proteogenomics requires expensive equipment, software, and expertise, and there is a lack of established bioinformatic frameworks to guide users through analyses.[49, 82] Due to these complexities, the reverse-genetics based creation of extensive mutant-libraries has emerged as an ideal method utilised to determine bacterial gene functions.[83, 84, 85]

1.4 The Advantages of Whole-Genome Mutagenesis

Whole-genome mutagenesis techniques possess two advantages over other protocols. They permit the simultaneous identification of the approximate number of essential genes, and allow phenotypic screens to be applied to a mutant library to identify condition-dependent genes.[85, 86] Termed "knockout collections", the most well-known commercially available mutant libraries were created using systematic single-gene deletions via homologous recombination. Such libraries have been generated for the model organisms *Saccharomyces cerevisiae* and *E. coli*.[70, 87] Both libraries have proven very useful. The yeast knockout collection has been used in over 1000 experiments, and the Keio *E. coli* library has been used to investigate a multitude of cellular processes.[84, 88] However, although these systematic single-gene deletion collections are informative, this methodical approach requires a significant amount of time, technical capability, expense and human resources.[70, 83, 89] For example, construction of the yeast deletion collection began in 1998 and finished in 2002. Additionally, although a highly-efficient homologous recombination method was used to create the Keio *E. coli* collection, the systematic deletion of thousands of genes is still a labour-intensive task.[70, 90, 91] There are only a few other systematic single-gene deletion prokaryotic mutant libraries available, for example in *B. subtilis,* and in the soil bacterium *Acinetobacter baylyi* ADP1. The latter collection generated the first approximation of essential genes in an *Acinetobacter* species.[65, 89]

In contrast to the systematic single-gene deletion method, transposon mutagenesis can be used to knock out genes, with massively parallel sequencing used to determine insertion sites.[92, 93] Transposons are mobile genetic elements that can move within and between genomes through a "cut-and-paste" or "copy-and-paste" mechanism, facilitated by a self-encoded transposase enzyme.[93, 94] In the 1970's researchers realised transposons could be used to manipulate genomes in a variety of ways, and today, global transposon mutagenesis is one of the main methods used to connect phenotypes to genotypes.[50, 93] Global transposon mutagenesis was first coupled with Sanger-sequencing of transposon-insertion sites by Hutchison et al[95] in 1999 to successfully identify the minimal essential gene-set of *Mycoplasma genitalium,* and has spawned several similar established protocols utilising next generation sequencing technologies.[50, 63, 96] In 2009, four different protocols for genome-wide transposon-insertion mapping were published, Transposon Sequencing (Tn-Seq), Insertion Sequencing (INSeq), High-Throughput Insertion Tracking by Deep Sequencing (HITS), and Transposon-Directed Insertion-Site Sequencing (TraDIS).[63, 64, 93] The four

protocols differ in the transposon used, library preparation and sequencing methods, but all rely upon the same approach; global or "saturation" transposon mutagenesis followed by high-throughput sequencing and mapping of the genomic DNA flanking the transposon-insertion sites.[63, 93, 97] After genomic DNA from a mutant pool is purified, it is cleaved and adaptors are used to allow the PCR amplification of fragments which contain transposon-insertions, after which the insertion densities of these amplified genes is calculated. [63, 93]

Given the wide genomic coverage of transposon mutagenesis, the earliest use of transposon-insertion-site sequencing was to identify the minimal essential gene set for *M. genitalium*. More recently it has mostly been used to identify condition-dependent required genes by imposing a fitness selection upon mutant libraries.[92, 98] After a genome-wide mutant library of all non-essential genes is constructed and validated via insertion-site sequencing, the mutant pool is then subjected to one or multiple permissive and/or selective growth conditions depending on the set of genes being investigated.[93, 98] After conditioning the mutant pool, genomic DNA is extracted, sequenced, and transposon-insertion sites are mapped back to the genome.[98, 99] Conditional gene essentiality is then determined via comparing the read-count of transposon-insertions after mutant conditioning to the read-counts of the original mutant pool.[98, 100] Essentially, if a viable-mutant is present in the mutant input pool, but no mutants with this insertion are present in the conditioned pool, this gene is then considered conditionally essential.[93, 99] Additionally, if a viable mutant is present in equivalent quantities in both the input and output mutant pools, this gene is considered to not play a significant role in responding to the imposed condition.[93, 99]

Global transposon mutagenesis studies have been useful in identifying the sets of genes required for growth in certain types of media. They have also been useful in studies on virulence and pathogenesis, and for identifying antimicrobial resistance mechanisms.[64, 93] Transposon mutagenesis experiments have already been performed in a variety of bacterial pathogens, with a non-exhaustive selection displayed in Table 2. In *A. baumannii*, these have been performed to analyse genes involved in processes such as virulence, motility, antibiotic resistance, iron and zinc acquisition, adherence to abiotic surfaces, and exogenous DNA uptake.[20, 101, 102, 103, 104, 105] Furthermore, being based on the presence or absence of mutants in input and output pools, transposon mutagenesis experiments are essentially fitness tests. Mutant libraries require

conditioning to be of significant utility due to a lack of high-throughput methods able to efficiently screen the phenotypes of thousands of single-gene mutants in one experiment.[98, 107]

Table 2: A Compilation of Recent Transposon Mutagenesis Experiments

Organism	Method	Fitness Tested Via
A. baumannii	TraDIS	Bloodstream infection[108]
	EZ-Tn5 (KAN-2) Transposome System	Surface Motility[109]
	EZ-Tn5 (KAN-2) Transposome System	Pathogenesis, Multiple Conditions[110]
	EZ-Tn5 (KAN-2) Transposome System	Rat-Soft Tissue Infection, LPS Biosynthesis[111]
Campylobacter jejuni	STM-Protocol + NGS	Invasion, Adhesion, Motility[112]
Clostridium perfringens	Mariner-Based Transposon Mutagenesis	Gliding Motility[113]
E. coli	STM-Protocol + NGS	Biofilm Development[114]
Mycobacterium marinum	TraDIS	Virulence, Host-Specific Responses[115]
Mycobacterium tuberculosis	Pristinamycin I-Inducible Himar1 Transposon Derivative System: Tn-*pptr*/Tn-*pip*/*pptr*	Hygromycin Resistance[116]
	Himar1-Based Transposon Mutagenesis	Growth on 7H10 Media[117]
P. aeruginosa	Tn-Seq	Exposure to Tobramycin[118]
Salmonella enteritidis	Mini-Tn5 Transposon Mutagenesis	Invasiveness: Human Colon Adenocarcinoma (Caco-2) Cells[119]
Salmonella typhimurium	Tn-Seq	Nutrient Limitation, Response to Bile Acid, Heat Shock[120]
	MudJ Mutagenesis	Virulence and Pathogenicity[121]
S. aureus	Tn-Seq	Osteomyelitis (Bone Infection)[122]
	Mariner-Based Transposon Mutagenesis	Antibiotic Exposure, Persister Cell Formation[123]
	Mariner-Based Transposon Mutagenesis	Biofilm Formation[124]
Vibrio cholerae	Tn-Seq	Competition: Type VI Secretion System Assembly and Immunity[125]

1.5 TraDISort, A Novel High-Throughput Protocol for the Functional Characterisation of Gene Products

Ideally, an experiment enabling a sensitive and rapid screening of a multitude of mutants would provide a broader level of data than a conditional fitness test, where the output data relies on mutants falling out of the population.[93, 98] To combat this drawback to transposon mutagenesis experiments, Hassan et al[47] recently pioneered a novel protocol combining saturation mutagenesis via transposon directed insertion-site sequencing (TraDIS) and fluorescence activated cell-sorting (FACS), in 2016 named TraDISort.[47, 107] The TraDIS protocol was chosen as it displayed a higher degree of genomic coverage than similar protocols published in 2009.[93]

FACS is a form of flow cytometry, a technique which was developed in the 1960s and has been continuously developed over the past 50 years.[126, 127] The origins of flow cytometry began with quantifying cell populations flowing through a fluidic chamber under a microscope. Today, flow cytometers are capable of sorting heterogeneous cell populations based on their physical parameters, including light absorbance and scattering, size, shape, granularity (internal complexity) and fluorescence.[126, 127] The three main components of a flow cytometer are the flow chamber, the optical systems, and light detectors.[128] Sample material is usually injected into a sheath buffer circuit, which then focuses the stream of cells into the flow chamber where they cross the machine's two laser beams one by one.[129] The flow cytometers optical detectors then measure the forward and side scatter of each cell, subsequently capturing measurements relating to cell size, shape, internal complexity, and if using a fluorescent marker, fluorescence intensity.[129, 130] Given that flow cytometers possess the ability to purify rare sub-populations of cells from a sample (less than 0.01%), they are now essential instruments in both clinical diagnostics, and in molecular biology.[126, 131, 132]

The TraDISort protocol, published in 2016, was performed on *A. baumannii* BAL062, which is classified as a multi drug resistant strain, and demonstrated rapid induced resistance to colistin, a drug of last resort for Gram-negative organisms.[28, 134] A dense mutant-library of over 100,000 *A. baumannii* BAL062 cells was created with the Tn5 transposon in an attempt to identify major efflux systems and regulators.[47] Following recovery of the mutants and insertion-site sequencing, the mutant-library was exposed to 1/16th of the minimum inhibitory concentration (MIC) of ethidium

bromide (approximately 40µm), a toxic quaternary ammonium compound which fluoresces upon entry into the cytoplasm and intercalation with DNA or RNA.[135, 136] ethidium bromide was chosen as it is known to be a common substrate for multi drug resistant efflux pumps (especially those of the RND family), whilst the sub-inhibitory concentration allowed for excellent differentiation between the fluorescence intensities of mutants without imposing a direct fitness selection on the cell population.[47, 137] FACS was performed, and using fluorescence intensity as a proxy for ethidium bromide permeation, the flow sorter was programmed to purify only the top and bottom 2% (4% in total) of differentially fluorescent cells within the mutant library, or just those mutants displaying the most aberrant fluorescence intensities.[47] After collection of mutant cells with abnormal cellular fluorescence, TraDISort detected three efflux pumps (AdeABC, AdeIJK and AmvA) as the main efflux systems for ethidium bromide in *A. baumannii* BAL062.[47] In addition, the protocol identified and confirmed a novel negative transcriptional regulator controlling the pump action of AmvA (Figure 3) named the AmvR protein.[47, 107]

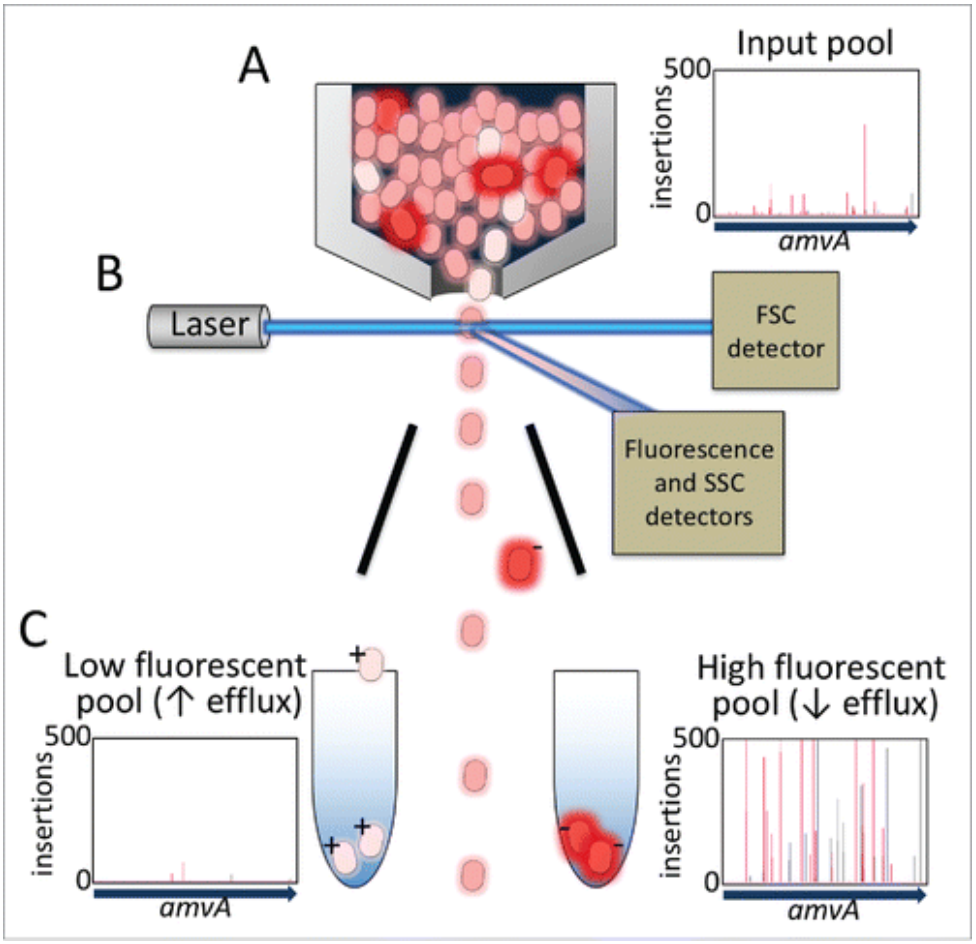

Figure 3: A Diagram Representing the Flow-Sorting Stage of the TraDISort Protocol Using the Insertion Densities of the Ethidium Bromide Binding Efflux Pump AmvA. The Mutant of this Pump Component Exhibited Significant Positive Selection (Increased Abundance and Increased Insertion Reads) in the High Fluorescence Pool, Indicative of a Deficiency in Ethidium Bromide Efflux.

However, findings from the published TraDISort data[47] also identified both negatively and positively selected non-efflux related transposon-insertions affecting intracellular ethidium bromide concentration. Some of these genes were annotated as possessing functions involved with cell-division, morphology, aggregation, DNA methylation, transcription, translation, capsule assembly, and membrane proteins.[47, 107] Many of these genes were hypothesised to have been negatively selected by TraDISort due to the gating procedures performed before flow-sorting, or their contributions toward fitness in response to ethidium bromide or cellular intercalation levels, despite not being directly related to cell survival.[47, 107] The FACS procedure performed on *A. baumannii* BAL062 was gated to target the collection of cells with uniform forward and side scatter (or standard morphology) to reduce the sorting of dead or aggregated cells which would complicate downstream analyses, and potentially clog the nozzle of the flow sorter.[47] A corollary of this discovery was that TraDISort may be used to uncover genes involved in a diverse array of cellular functions.

In this thesis, an unpublished TraDISort data-set performed on *A. baumannii* BAL062 exposed to 1/16th of the minimum inhibitory concentration of ethidium bromide was analysed to determine the functions of selected genes. In this dataset, the flow-sorter was programmed to purify a larger percentage of the cell population to capture a broader level of data. The top and bottom 5% (10% in total) of cells within the flow-sorted mutant library, or those displaying the lowest and highest fluorescence intensities, were purified. As with the published dataset, transposon-insertions causing a log fold change in insertion densities of ≥2 were extracted from raw TraDISort data for analysis. Several bioinformatic tools and orthologous genes from *E. coli* K12 MG1655 and *A. baumannii* ATCC 17978 were used to elucidate functional trends associated with the 5% TraDISort dataset. Genes with strong shifts in insertion densities (changes in mutant abundance) compared to the input mutant pool following flow-sorting were also analysed, and several mutants were chosen for follow-up tests to further probe functionality. Given trends from the published dataset, it was

hypothesized that in addition to efflux pump components and regulators, selected mutants would possess gene disruptions affecting processes such as membrane integrity (ethidium bromide permeation), DNA/RNA content (intercalation level), or cause a size and/or shape defect (reduction or increase of space within the cytoplasm, or potential rejection by gating procedures).

2. Methods

2.1 Extracting Biologically Significant Data from the TraDISort Dataset

In TraDIS and other transposon-insertion sequencing protocols, the sequenced insertion densities following mutant library selection, and therefore the abundance of each mutant, are tabulated and represented as $\log^2$-fold changes.[139] Insertion densities are generally considered to be biologically significant if a $\log^2$-fold change of >2 in the insertion density of a transposon mutant is displayed, indicating a 4-fold decrease or increase in mutant abundance.[139] Therefore, in TraDISort, the insertion density of the *A. baumannii* BAL062 transposon-insertion library was measured on a $\log^2$-fold scale, and the fluorescence values for mutants were considered biologically significant if they occurred with a $\log^2$-fold change in mutant abundance of >2.[47]

The unpublished, raw TraDISort dataset analysed here (3361 genes) contained fluorescence intensities and insertion densities (proportional abundances) for the highest and lowest 5% (10% in total) of cells displaying aberrant fluorescence intensities. These genes were analysed, and transposon-insertions causing a $\log^2$FC shift in insertion density of >2 were extracted for a total of 656 genes. Transposon-insertions were then separated into four quartiles, those causing low fluorescence with decreased insertion density, low fluorescence with increased insertion density, high fluorescence with decreased insertion density, and high fluorescence with increased insertion density. Mutants possessing strong shifts in insertion densities (mutant abundances) were also extracted (Q value, <0.1) for further analyses to deduce the biological significance of these selections. Additionally, mutants possessing cells which were flow sorted into both the low and high fluorescence pools, hereon termed “duplicates”, were excluded from individual quartile analyses to reduce interpretive errors related to mutant phenotypes (225 genes), for a total of 431 genes.

Results from this experiment were also compared to the published TraDISort dataset published by Hassan et al[47] which examined cells with the highest and lowest fluorescence intensities with a 2% cut-off (4% in total), as well as the associated mutant pool grown in ¼-MIC ethidium bromide, analysed via TraDIS. This was done to analyse the reproducibility of the method, and to elucidate which genes selected by TraDISort were explicitly related to survival in response to ethidium bromide.

2.2 Deciphering Functional Trends Associated with TraDISort Data

An ortholog list between *A. baumannii* BAL062 and *E. coli* K-12 MG1655 was created via OrthoMCL with minimal protein identity set at 60%.[140] Ortholog lists between *A. baumannii* BAL062, *A. baumannii* ATCC 17978 and *A. baumannii* AB5075 UW were also created via OrthoMCL, with minimal protein identities set at 80%.[140] KEGG.jp: Kyoto Encyclopedia of Genes and Genomes was then used to assign molecular pathways to ortholog genes *in E. coli* K-12 MG1655 and *A. baumannii* ATCC 17978 via the search and colour pathway mapper.[141]

Pathways assigned to *A. baumannii* BAL062 were then used to deduce the most prevalent biological pathways implicated with the TraDISort data, or how many genes from each pathway were being selected. Orthologous genes from *E. coli* K-12 MG1655 were also used to extract GO (Gene Ontology) terms via the Panther Classification System[142] along with sequence data extracted from Interpro: Sequence Analysis and Classification.[143] These were assigned to their corresponding genes in *A. baumannii* BAL062. Gene pathways from Kegg, GO terms from Panther Classification, Interpro, and orthologous gene function data from Biocyc[10] were used to elucidate the potential function of genes selected by TraDISort. Genes were then categorised based on putative functions.

2.2.1 Genes Lacking Functional Data: Sequence Analyses

Genes selected by TraDISort without GO terms and/or annotated pathways were also extracted from the dataset, and analysed separately. These were divided into two groups, non-hypothetical genes and hypothetical genes. Their amino acid sequences were searched on InterPro to confirm gene annotations for non-hypothetical genes, and provide potential gene functions for hypothetical genes via the identification of family or domain sequences.

Additional tools used for analysis of genes identified by the TraDISort analysis included PredictProtein.org[144] and I-Tasser: Protein Structure and Function Prediction[145], (used to predict potential functions, molecular processes, biological processes, and cellular compartments for hypothetical genes with strong shifts in insertion densities following flow-sorting) Loctree3: Protein Subcellular Localization Prediction system[146] and Cello V2.5: Subcellular Localization Predictor[147] (used to predict and confirm the subcellular localizations of non-duplicate hypothetical and non-hypothetical genes analysed without family and/or domain data). The Kyte & Doolittle hydropathy

tool on ExPASy[149], and the TMHMM Server 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) were also used to construct hydropathy and transmembrane segment plots to probe the structure of genes selected by TraDISort with strong shifts in insertion densities following flow-sorting. All search parameters for bioinformatic tools were set to default.

Furthermore, the transport database (TransportDB 2.0)[149] was used to gain locus tags for the genes of efflux pumps in the model organism *A. baumannii* ATCC 17978. These genes were then matched to their orthologs in *A. baumannii* BAL062 via the ortholog lists created via OrthoMCL for the identification of known efflux pump genes selected by TraDISort.

2.3 Bacterial Strains and Growth Conditions

Orthologous mutants for genes in *A. baumannii* BAL062 (NCBI Reference Sequence: NZ_LT594095.1) from a single gene transposon-insertion knockout library of a related *A. baumannii* strain (*A. baumannii* AB5075 UW, NCBI Reference Sequence: NZ_CP008706.1) obtained from the Manoil lab were used for follow-up tests.[150] Specific mutants used from the Manoil knockout library are detailed in Table 3. Mutants were grown at 37°C overnight in cation adjusted Mueller Hinton broth (with shaking) and cultured on agar plates. Single colonies (colony forming units) were picked from the agar plates, and grown to exponential phase (OD = 0.6) for subsequent testing.

Table 3: Mutant Library Strains from the *A. baumannii* AB5075-UW Knockout Library Used for Confirmation Tests

A. *baumannii* BAL062 Gene Locus Tags	A. *baumannii* AB5075 UW Ortholog	Manoil Knockout Library Mutant
BAL062_00029	ABUW_0033	AB00070
BAL062_00030	ABUW_0034	AB00071
BAL062_00031	ABUW_0035	AB00076
BAL062_00032	ABUW_0036	AB00078
BAL062_00146	ABUW_0153	AB00419
BAL062_00713	ABUW_0715	AB01948
BAL062_00714	ABUW_0716	AB01951
BAL062_01106	ABUW_1191	AB03244
BAL062_02646	ABUW_2840	AB07437
BAL062_03143	ABUW_3115	AB08151
BAL062_03687	ABUW_3651	AB09595
BAL062_03688	ABUW_3652	AB09596
BAL062_03912	ABUW_3875	AB10245

2.4 Mutants Selected by TraDISort with Strong Shifts in Insertion Densities

Mutants flow-sorted into one pool only and possessing strong shifts in insertion densities (mutant abundances) following flow-sorting (Q value =<0.01) were extracted for further experimental analyses. A selection of mutants hypothesised to possess morphological alterations causing them to be negatively selected by the TraDISort gating procedures were observed under a microscope via phase-contrast microscopy (BAL062_00713, BAL062_00714, BAL062_03143, BAL062_03688 and BAL062_02646). Mutants of the negatively selected and unannotated hypothetical proteins (BAL062_00032, BAL062_00146, BAL062_01106) and the negatively selected lipoprotein BAL062_03912 were also subjected to minimum inhibitory concentration (MIC) testing to discover if they conferred resistance to ethidium bromide in orthologous gene knockout mutants in *A. baumannii* AB5075 UW.

MIC testing was also performed on four genes not selected by TraDISort. It is well known that RND-type transporters can bind and extrude various structurally unrelated compounds, and the three main RND-type efflux systems of *A. baumannii* strains (AdeABC, AdeFGH and AdeIJK) have all been reported to accept ethidium bromide as a substrate.[45, 151, 152] Therefore, a putative RND-operon consisting of BAL062_00029, BAL062_00030 and BAL062_00031 (a transcriptional repressor, periplasmic fusion protein, and an AcrB/AcrD/AcrF-like inner membrane protein immediately preceding the negatively selected BAL062_00032) was tested to elucidate whether BAL062_00029—BAL062_00031 (a novel RND system with a currently unclear substrate profile) constituted a functional RND operon implicated with ethidium bromide resistance.[153, 154] The fourth gene selected for MIC testing but not selected by TraDISort was BAL062_03687, a DNA modification methylase protein significantly negatively selected in the published 2% TraDISort dataset, hypothesised to potentially protect DNA from ethidium bromide induced damage.[47]

2.4.1 Searching for Morphological Abnormalities in Mutants Negatively Selected by TraDISort, with Strong Shifts in Insertion Densities

Transposon-insertion mutants for microscopy were grown to the exponential phase (OD = 0.6) and 783uL of each sample was added to a 217uL pre-mixed fixative solution containing 30mM (pH of 7.5) NaPO4, 2.4% formaldehyde and 0.04% glutaraldehyde in 5ml Eppendorf tubes, for a total volume of 1ml of fixative solution per sample. Samples were then incubated within the fixative

solution for 15 minutes at room temperature, and put on ice for 45 minutes. Fixed mutant cells were harvested from the fixative solution via centrifugation at 3500rpm for 10 minutes.

The resulting mutant cell pellets were washed twice in PBS, and resuspended to an appropriate density (OD = 0.6) for microscopic imaging. Glass slides for mutant samples were decontaminated via an autoclave before being submerged in sterile petri dishes with 0.01% sterile-filtered poly-L-lysine solution for five minutes. Glass slides were then removed from the poly-L-lysine solution with sterile forceps, placed in a sterile covered container to prevent contamination, and dried at room temperature for two hours. After glass-slide preparation, one drop of each resuspended cell sample was then spread on individual dried poly-L-lysine coated microscope slides and incubated for 2 minutes each. Glass-slides were then aspirated and washed once more with PBS, followed by a second aspiration before samples were coated with mounting media (50% glycerol in distilled water) and covered with a glass slide. Approximately 20 fields of view were inspected for each slide, and a representative image of each cell population was taken.

2.4.2 Minimum Inhibitory Concentration (MIC) Testing

The MICs of 8 mutants were tested against ethidium bromide in flat-bottom polystyrene 96-well microtitre plates using the two-fold serial dilution technique. Starting concentrations were double the MIC (500ug/ml) of wildtype *A. baumannii* BAL062 MIC to ethidium bromide. The lowest concentrations were 0.9765ug/ml, as shown in Table 4. The 96-well plates were incubated overnight at 37°C with shaking, and after 24 hours the absorbance was measured at 600nm using a PHERAstar FSX plate reader (BMG LABTECH, Ortenberg, Germany). MIC levels were determined as the minimum concentration of ethidium bromide able to fully inhibit the *A. baumannii* AB5075 UW cells.

Table 4: MIC testing 96-well plate layout. Highest concentration = 500ug/ml, Lowest Concentration = 0.98ug/ml. (GC = Growth Control: Contained Bacterial Strain without Ethidium Bromide, SC = Sterile Control: Contained MH Media but no Antibiotics or Bacteria)

Units		1	2	3	4	5	6	7	8	C9	10	11	12
ug/ml	A	GC	GC	GC	GC	GC	GC	GC	GC	GC	GC	GC	SC
ug/ml	B	500	250	125	62.5	31.25	15.62	7.81	3.91	1.95	0.98	GC	SC

ug/ml	C	500	250	125	62.5	31.25	15.62	7.81	3.91	1.95	0.98	GC	SC
ug/ml	D	500	250	125	62.5	31.25	15.62	7.81	3.91	1.95	0.98	GC	SC
ug/ml	E	500	250	125	62.5	31.25	15.62	7.81	3.91	1.95	0.98	GC	SC
ug/ml	F	500	250	125	62.5	31.25	15.62	7.81	3.91	1.95	0.98	GC	SC
ug/ml	G	500	250	125	62.5	31.25	15.62	7.81	3.91	1.95	0.98	GC	SC
ug/ml	H	GC	GC	GC	GC	GC	GC	GC	GC	GC	GC	GC	SC

3. Results and Discussion

3.1 TraDISort Summary: Mutant Fluorescence and Insertion Densities

Most genes within the *A. baumannii* genome (87% of 431 selected genes) showed decreased insertion densities, or a decreased proportional abundance of mutants following flow sorting, indicative of potential decreases in fitness for these mutants compared to the rest of the flow-sorted mutant population, or altered morphologies. Furthermore, a slightly smaller proportion but still the majority of non-duplicate transposon-insertions selected by TraDISort (69% of 431 selected genes) resulted in high fluorescence, indicating again that gene loss largely results in abnormal and less-fit phenotypes.[47, 155] These results are displayed in Figure 4, which displays the general statistics regarding each quartile in the TraDISort dataset for non-duplicate genes.

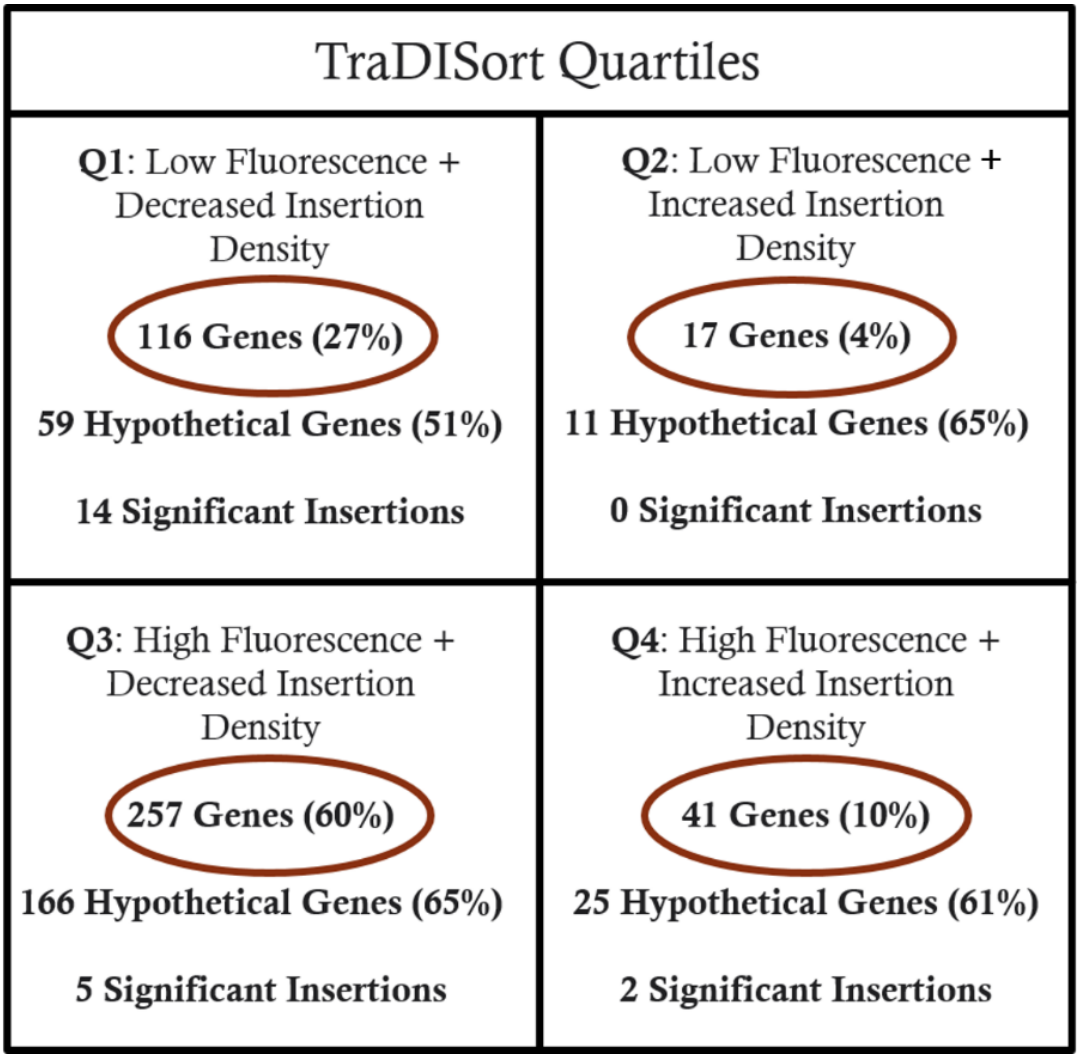

Figure 4: A Graphical Representation of the Basic TraDISort Summary Statistics. Overall, most Transposon-Insertions within non-Duplicate Genes (87%) that were Selected by TraDISort Resulted in

Decreased Mutant Abundances Following Flow-Sorting. Additionally, 69% of Transposon-Insertions in non-Duplicate Genes Resulted in Higher, as Opposed to Lower Fluorescence Following Flow-Sorting. Both Statistics are Indicative of Potential Decreases in Fitness, as is Usually Expected with Gene Loss.[47]

In contrast, only a small proportion of mutants displayed increased insertion densities following flow-sorting, regardless of fluorescence intensity. Only 17 mutants (4% of transposon-insertions) resulted in low fluorescence with increased insertion densities, and 41 mutants (10% of transposon-insertions) resulted in high fluorescence with increased insertion densities. Similarly, most mutants (76%) with strong shifts in insertion densities, or mutant abundances, (Q value =<0.01) decreased in proportional abundance following flow-sorting. Furthermore, 261 (61% of selected genes) were annotated as hypothetical or with only putative functions, indicating a lack of knowledge regarding genes with potentially significant cellular functions.

A further 225 genes were duplicates (flow sorted into both the low and high fluorescence mutant pools), providing a total of 656 individual genes. The presence of these mutants in more than one mutant pool following flow-sorting were also confirmed as legitimate via an analysis of the raw transposon-insertion plots in Artemis 16.0.0.[156] Summary statistics for these genes followed the same trends as non-duplicate transposon-insertions. 191 (85% of duplicate genes) were selected in both low and high fluorescence pools, with decreased insertion densities (mutant abundances). In contrast, only 13 transposon-insertions (6% of duplicate genes) resulted in mutants flow-sorted into both low and high fluorescence pools with increased insertion densities. A further 21 genes (9% of duplicates) resulted in mutants flow-sorted into both low and high fluorescence pools, and possessing opposite insertion densities. Summary statistics regarding duplicate genes selected by TraDISort are displayed in Table 5.

Table 5: A Statistical Summary on Duplicate Genes Selected by TraDISort

Mutant Pools	# Genes Selected	# Hypothetical Genes	# Genes with Strong Shifts in Abundance	# Duplicates with Strong Shifts in Abundance in Both Pools

Low and High Fluorescence, with Decreased Insertion Densities	191 (85%)	84 (44%)	31	12 (39%)
Low Fluorescence and High Fluorescence, with Increased Insertion Densities	13 (6%)	9 (69%)	8	6 (75%)
Low and High Fluorescence, with Opposite Insertion Densities	21 (9%)	16 (76%)	1	1 (100%)
Total	225	109 (48%)	40 (18%)	19 (8%)

Of the 31 transposon-insertions resulting in strong negative shifts in insertion densities, only 12 possessed strong shifts in pools of both low and high fluorescence. These genes possessed functions in homologous recombination, RNA degradation, amino acid metabolism and biosynthesis, membrane biosynthesis, 2-oxocarboxylic acid metabolism, and iron transport. In transposon-insertions causing enrichment of mutants in both pools, five of seven sequential genes (BAL062_01328—BAL062_01334) annotated as a TetR-type transcriptional regulator followed by a putative CsuA/B-csuD/E operon (the chaperone-usher secretion system with roles in pili assembly, aggregation and biofilm formation) possessed strong shifts in insertion densities in both pools with increased insertion densities. Besides hypothetical uncharacterised genes, the only other genes in this group were annotated with functions in nickel/cobalt utilisation regulation, terpenoid backbone biosynthesis, polysaccharide deacetylation, and acetylation. A mutant of the *adeB* gene, encoding an RND-type cytoplasmic transporter component, was the only mutant with strong and opposite shifts in insertion densities, in pools of both low and high fluorescence.

The biological significance of these duplicates, or mutants flow sorted into pools of both low and high fluorescence, within the TraDISort dataset is not yet fully known. However, as noted by Hassan et al[47], the significant enrichment of the Csu type 1 pilus biosynthesis and regulatory gene cluster (BAL062_01328 — BAL062_01334) in pools of both low and high fluorescence in the published dataset may be due to these mutants being less likely to aggregate, resulting in a phenotype favourable to the flow-sorting process[47]. The additional selection of the gene cluster within this TraDISort dataset may corroborate this hypothesis.

As for most duplicates (resulting in decreased abundance in both pools of low and high fluorescence compared to the input mutant pool), there is a possibility that these mutants may

have possessed morphological abnormalities causing them to be rejected by the flow-sorter due to gating procedures. Additionally, *A. baumannii* BAL062 has been observed undergoing flocculation within media during the exponential phase (Personal Communication with Dr. Karl Hassan, June 2017, unreferenced). This leads to the possibility that duplicates may have been negatively selected in both pools due to the formation and rejection of aggregates during the flow-sorting process, regardless of gene functions. A further possibility is that some duplicate genes decreasing in abundance following flow-sorting may play yet undiscovered roles in processes such as aggregation, biofilm formation and flocculation, but this is outside the scope of this thesis. Regarding AdeB, the inner membrane component of the AdeABC efflux system, these mutants possessed strong negative shifts in abundance in the low fluorescence pool, and strong positive shifts in abundance in the high fluorescence pool. These selections make sense given that the AdeABC efflux system is known to accept ethidium bromide as a substrate, whilst the occurrence of this mutant in both pools of low and high fluorescence may the reflect the fact that efflux systems in *Acinetobacter* species may display functional redundancy.[157, 158]

3.1.1 Genomic Locations of Efflux Systems in *A. baumannii* BAL062

Using data from orthologs in *A. baumannii* ATCC 17978 on TransportDB, 322/408 (79%) orthologous cytoplasmic transport proteins were identified in the genome of *A. baumannii* BAL062. However, in the TraDISort dataset (656 genes), just 28 of these orthologs were selected, with only *adeB* and *smvA* strictly implicated with antimicrobial resistance.[46, 47]

Orthologs from *A. baumannii* ATCC 17978 were then used to uncover the genomic locations of the main RND-type efflux pump components and regulators in *A. baumannii* BAL062, along with the ethidium bromide specific pump AmvA (annotated in BAL062 as SmvA) selected in the published TraDISort dataset, displayed in Table 6. As with the published dataset, mutants with insertions in genes encoding the RND-type efflux systems AdeABC and AdeIJK, and the MFS transporter SmvA were overrepresented in the high fluorescence pool, indicating that these efflux pumps all play a role in ethidium bromide extrusion.[47] The selection of only the outer membrane components of AdeIJK (positively selected) and AdeFGH (negatively selected) is puzzling given the functional redundancy displayed in the efflux systems of *Acinetobacter* species.[153, 157, 158] This could suggest that these outer membrane proteins may associate with other efflux systems, or that these

deletions possess potentially pleiotropic phenotypes.[159] However, mutants of the RND-type outer membrane gene *tolC* in *E. coli* K-12 MG1655 have been shown to cause cellular elongation, shape, and/or growth defects.[160, 161] This lends credence to the further possibility that these efflux components may have possessed altered abundances via rejection by the flow-sorter, due to aberrant morphologies.

Table 6: Main RND-type Efflux Systems of *A. baumannii* BAL062 (+ AmvA/SmvA)

Locus Tag	Name	Function	Family	Selection
BAL062_00759	*adeI*	AdeI, AdeIJK inner membrane component	RND	N/A
BAL062_00758	*mexB_1*	AdeJ, AdeIJK membrane fusion component	RND	N/A
BAL062_00757	*oprM_1*	AdeK, AdeIJK outer membrane component	RND	+
BAL062_01623	N/A	AdeN, AdeIJK transcriptional repressor	TetR	N/A
BAL062_01237	*oprM_2*	AdeH, AdeFGH outer membrane component	RND	—
BAL062_01238	*bepE*	AdeG, AdeFGH inner membrane component	RND	N/A
BAL062_01239	*mdtE*	AdeF, AdeFGH membrane fusion component	RND	N/A
BAL062_01241	*dmlR_5*	AdeL, AdeFGH transcriptional repressor	LysR	N/A
BAL062_01897	*adeA*	AdeA, AdeABC membrane fusion component	RND	N/A
BAL062_01898	*acrB*	AdeB, AdeABC inner membrane component	RND	+/—
BAL062_01899	*adeC*	AdeC, AdeABC outer membrane component	RND	N/A
BAL962_01896	*srrA*	AdeR, AdeRS two-component system protein	TCS	N/A
BAL062_01895	*adeS*	AdeS, AdeRS two-component system protein	TCS	—
BAL062_01496	*smvA*	AmvA efflux protein	MFS	+
BAL062_01495	N/A	AmvR, regulatory protein of AmvA	TetR	—

- RND = Resistance Nodulation Division Superfamily
- TCS = Two-component System Regulator
- + = Positive Selection, — = Negative Selection, N/A = Not Selected by TraDISort

3.2 Interpreting Gene Interactions in Biological Networks: Biological Pathways Associated with Genes Selected by TraDISort

Pathway analysis of genes in *A. baumannii* BAL062 selected during TraDISort using orthologs from *E. coli* K-12 MG1655 and *A. baumannii* ATCC 17978 provided 135 genes with at least one annotated biological pathway. 54 pathways were assigned to duplicates, whilst 59 pathways were assigned to non-duplicates. Most pathways associated with non-duplicate genes were assigned to mutants falling within either pool of decreased insertion densities (mutant abundances), as opposed to the increased insertion density pools. This was likely due both to the smaller size, and the larger proportion of uncharacterised hypothetical genes in the increased insertion densities pools. Pathways associated with mutants resulting in low fluorescence and high fluorescence are detailed in Tables S1 and S2 (see supplementary data).

Multiple genes in several biological pathways were selected by TraDISort. Pathways possessing >1 corresponding gene within the low fluorescence and decreased insertion density pool were purine metabolism, homologous recombination, quorum sensing, two-component system regulators, carbon metabolism, the citrate cycle, glycolysis/gluconeogenesis, pyrimidine metabolism and pyruvate metabolism. Pathways involving >1 gene in the high fluorescence with decreased insertion densities pool were ABC transporters, carbon metabolism, glutathione metabolism, homologous recombination, phenylalanine, tyrosine and tryptophan biosynthesis, 2-oxocarboxylic acid metabolism, bacterial secretion systems, the degradation of aromatic compounds, glycine, serine and threonine metabolism, histidine metabolism, nicotinate and nicotinamide metabolism, and valine, leucine and isoleucine biosynthesis. No pathways involving >1 gene in were identified in the low fluorescence with increased insertion densities pool. Finally, only one pathway (beta-lactam resistance) was present in >1 gene (2 genes in total) within the high fluorescence and increased insertion densities pool, assigned to genes *oprM_1* and *smvA* (the AdeK and SmvA efflux proteins). However, it should be noted that it these genes were selected by TraDISort in response to ethidium bromide, and that these pathway annotations perhaps reflect observed cross-resistances between ethidium bromide and beta-lactams in *A. baumannii.*

Overall, the most highly selected pathways were homologous recombination (DNA repair) and purine metabolism (nucleotide synthesis), with 6 and 5 genes, respectively, in these pathways being

negatively selected in both pools of low and high fluorescence. This agrees with the mode of action of ethidium bromide as a DNA intercalating mutagen, and it is likely that deficiencies in nucleotide synthesis and DNA-repair systems caused these mutants to possess aberrant fluorescence intensities.[135] Furthermore, *A. baumannii* ATCC 17978 has been shown to possess an increased susceptibility to ethidium bromide and other DNA damaging agents upon disruption of the homologous recombination pathway, supporting this hypothesis.[162] Besides homologous recombination, most highly selected pathways were involved with amino acid biosynthesis and metabolism, and energy assimilation. This indicates that disruption in significant metabolic processes led to either aberrant ethidium bromide permeation, or perhaps potential growth abnormalities, which are known to occur with some dispensable metabolic genes, even in rich media.[163]

All pathways assigned to either pools of increased insertion densities besides beta-lactam resistance possessed only one corresponding gene in *A. baumannii* BAL062, as the small sample size of these pools impeded adequate pathway annotations. However, 8/17 (53%) of pathways assigned to these pools were involved with the metabolism or degradation of aromatic compounds. Interestingly, aromatic degradation pathways possess a recently recognised role in the production of metabolic exchange factors for cell-to-cell communication, and quorum sensing was also shared by both pools of increased insertion densities.[164, 165] Quorum sensing systems are known to mediate processes such as aggregation and biofilm formation, and these mutants may have also been less likely to aggregate, causing enrichment in both pools of increased insertion densities.[166, 167]

3.2.1 Pathways Associated with Duplicate Mutants Flow-Sorted into Both Low, and High Fluorescence Pools

Pathway analysis of duplicate genes within the TraDISort data provided 54 cellular pathways, with pathway disruptions in various significant biological processes, such as pyrimidine metabolism, homologous recombination, 2-oxocarboxylic acid metabolism, biotin metabolism and the citrate cycle consistently resulting in both low and high fluorescence, with decreased insertion densities (displayed in Table S3, see supplementary data). Other pathways commonly associated with >1 duplicate gene causing decreased insertion densities were annotated as being involved in amino acid and carbon metabolism, pantothenate and CoA biosynthesis, folate biosynthesis, DNA replication, and RNA degradation. The absolute reasons behind the presence of duplicates, and a

substantial portion of which decreasing in abundance in both pools within the TraDISort dataset, are not entirely known. However, as previously stated, these mutants may have formed aggregates, or possessed growth and/or morphological defects causing decreases in abundances in both pools due to rejection by the TraDISort gating procedure.[47] Additionally, these mutants may have possessed pleiotropic and less-fit phenotypes, causing sorting into both pools, and decreases in abundance in both.

Only one pathway was returned for duplicates with increased insertion densities (13 genes in total), terpenoid backbone biosynthesis (BAL062_01629). Interestingly, this pathway is known to be involved with the biosynthesis of peptidoglycan, a major cell envelope component.[168, 169] This may be indicative of a compromised morphology for these mutants, allowing enrichment by the flow sorter in both pools of increased insertion densities. Only one pathway, beta-lactam resistance, was returned for duplicates with opposite insertion densities (21 genes in total), and this belonged to AdeB, the inner membrane protein component of the RND-type efflux system AdeABC. Furthermore, most genes within this group were annotated as completely hypothetical proteins.

20 pathways were shared between duplicate and non-duplicate genes, accounting for 37% of pathways assigned to duplicate genes, and 35% of pathways assigned to non-duplicate genes. When compared to duplicate genes within the published 2% TraDISort dataset, over half (65%) were also present within the current dataset, indicating a modest level of consistency in the selection of these genes, and their gene functions. On the contrary, only 23% of non-duplicate genes within the 5% dataset were present as non-duplicates within the published 2% TraDISort dataset, yet the reasoning for this discrepancy is currently unknown.

3.3 Probing the Functions of Genes Selected by TraDISort

In total, 199 (46%) non-duplicate genes could be provided with GO terms and/or pathways indicative of biological functions. This included 73 genes which resulted in low fluorescence, with decreased or increased insertion densities. Genes were then categorised based on biological functions.

3.3.1 DNA, Transcription and Translation: Transposon- Insertions Resulting in Low Fluorescence

Of the 73 genes resulting in low fluorescence (displayed in Table S4, see supplementary data) twenty transposon mutants negatively selected by TraDISort (experiencing a decrease in abundance after flow-sorting) appeared to possess disruptions in genes involved with either DNA, transcription, translation, or DNA repair, constituting the largest cohesive functional category of negatively selected genes resulting in low fluorescence. Genes affecting DNA or transcription were annotated as *purC*/*E*/*K* (nucleotide synthesis), *pyrE* (nucleotide synthesis), *hiuH* (purine degradation), *recB*, *recG* and *ruvC* of cellular DNA repair systems, RNA-polymerase subunit omega *(rpoZ*), DNA Topoisomerase 1 (*topA*) a nucleotide hydrolase (BAL062_02074) and *greA*, a transcription elongation factor. This agrees with the mechanism of action of ethidium bromide, and it is likely that these mutants may have possessed aberrant fluorescence intensities due to the altered availability and accessibility of DNA and mRNA for ethidium bromide intercalation.[135, 170] For example, genes within the purine and pyrimidine pathways in *E. coli* are essential for virulence, (ie:, *purC*), and mutants cannot survive in vivo without them.[171] Additionally, another possibility remains that mutants possessing decreases in insertion densities may have experienced decreases in fitness resulting in damaged and/or unviable cells due to the fluidic pressure of the capillaries within the flow-sorter.[172]

Genes affecting translation included BAL062_02990 (phage TraR/DksA family protein, potentially involved with rRNA transcription initiation), *prfC* (peptide chain release factor III), *rsmG* and *rsmH* (small ribosomal subunit methyltransferases involved in ribosomal RNA processing), *ybeB* (ribosomal silencing), *hslR* (ribosomal recycling), *rluD* (ribosomal large subunit pseudouridine synthase D) and the protein folders *slyD* and *ppiB*. A possibility for the selection of multiple genes related to translational processes is that alterations in rates of translation may affect steady-state mRNA levels, thus ethidium bromide intercalation.[173, 174, 175] Furthermore, these genes function in a significant biological process, and orthologous gene knockouts of *ybeB* and *rluD* in *E. coli* cause growth defects due to inefficient ribosomal biogenesis and translation.[176, 177] Again, this suggests the possibility of growth disadvantages causing the negative selection of these mutants.

3.3.2 Transcriptional Regulation: Transposon-Insertions Resulting in Low Fluorescence

Twelve transposon-insertions in transcriptional regulators were negatively selected, constituting the second largest category of mutants resulting in low fluorescence. These genes were annotated

as *gacA* (biofilm formation), *barA* (annotated as *gacS* of *gacAS* in *A. baumannii* ATCC 17978), *arsR* (biofilm growth-associated repressor), *hmrR_1* (copper export regulator), *vfr* (an ortholog of *E. coli's* cyclic AMP receptor protein), *fadR_1* (fatty acid metabolism), *phoU* (phosphate transport), *nahR* (choline sulfate utilisation), *dmlR_12* (D-malate degradation), *lrp_6* (a leucine-responsive regulatory protein), BAL062_01811 (putative betaine synthesis repressor) and BAL062_02779 (unknown regulon), whilst *marA* (multiple antibiotic resistance protein) was positively selected.

These regulators possessed various significant functions, however the *gacA*/barA two-component system, *arsR* and *marA* regulators are implicated with aggregation and biofilm formation, and the expression of efflux pumps, respectively.[178, 179] A possibility for the GacA/S system not being enriched in one or both pools due to deficiencies in aggregation and biofilm formation, is that this two-component system also contributes to pili synthesis, motility, and cellular structure.[180] A combination of these deficiencies, particularly in structure, may have caused rejection by the flow-sorter. On the contrary, the biofilm-growth associated repressor protein ArsR decreased in abundance in this pool, suggesting potential rejection due to the overexpression of genes related to this process. Furthermore, the MarA protein is known as a positive regulator of drug efflux in *A. baumannii*, thus the enrichment of *marA* mutants in the low fluorescence pool may be suggestive of up-regulation of further global regulators, such as SoxS.[181] A further possibility for the multiple selections of transcriptional regulators resulting in low fluorescence may be altered cellular mRNA content, but given the variability in the regulons of selected genes, this is not likely.[173, 174]

3.3.3 Amino Acid Biosynthesis, and Metabolic Processes: Transposon-Insertions Resulting in Low Fluorescence

Five amino acid biosynthesis and metabolism related genes, annotated as *folP_1*, *fabG_4*, *gcvH*, BAL062_01774 and *tdcB_2*, and were all negatively selected. This may indicate aberrant ethidium bromide permeation within these mutants, or potential growth abnormalities, not uncommon for disruptions within genes related to amino acid biosynthesis and metabolism, especially under potentially non-favourable conditions, such as within the flow-sorter.[163, 172, 182] A further eight transposon-insertions resulting in low fluorescence were in genes involved with cellular metabolism, including *azR* (xenobiotic metabolism), *aceE* (pyruvate dehydrogenate), BAL062_00149 (an ortholog of *E. coli's aceF*), *fadD_2* (fatty-acid metabolism), *yfcF* (glutathione metabolism), and resulted in decreased insertion densities, whilst three, in *catC* (benzoate degradation),

BAL062_01652 (esterase) and *fabG_2* (geraniol degradation), resulted in increased insertion densities. This suggests that these metabolic genes possess significant cellular functions, affecting either the permeation or intercalation of ethidium bromide, or cellular shape/morphology. For example, orthologous mutants of both *aceE* and *fadD_2* in *E. coli* experience decreased fitness, and potential growth defects.[183, 184] Furthermore, as aforementioned, the positive selection of genes related to the degradation of aromatic compounds (*catC*) may hint at deficiencies in quorum sensing systems.[164, 165] Thus, the propensity of these mutants to aggregate within media may have been influenced, resulting in the increased sorting of these mutants.

3.3.4 Transporters and Membrane / Membrane Associated Proteins: Transposon-Insertions Resulting in Low Fluorescence

Four genes annotated as transporters decreased in abundance in the low fluorescence pool, annotated as a chromate transporter (BAL062_02230), amino-acid transporter (BAL062_02288), biopolymer transporter (ExbD_2) and a cation transporter (CzcD_2). Two transporters positively selected, BAL062_01827 (a putative conserved inner membrane exporter, YdcZ), and TolQ_2 (colicin uptake) were positively selected. Though none of these transporters were strictly related to antimicrobial resistance, transporters still play ubiquitous integral roles in cellular homeostasis, and disruption must have compromised these processes, leading to aberrant fluorescence intensities.[38, 39]

Mutants in four membrane proteins of unknown function also resulted in low fluorescence (BAL062_01425, *yhgN_1*, *dedA_2* and *yqaA*), with only BAL062_01425 being positively selected. Insertions in these components were assumed to result in increased permeation of ethidium bromide, and increased intercalation. However, ethidium bromide is a hydrophilic compound, thus preferring to enter cells via hydrophilic passages such as porins, the deletion of which decreases membrane permeability.[39, 106, 185] Subsequently, the decreased abundance of these mutants may indicate compromised morphologies for these mutants. Though BAL062_01425 was enriched in the low fluorescence pool, the function of this gene is unclear apart from the fact that it is integral to the plasma membrane. Three genes were implicated with membrane synthesis or stability were also negatively selected within this pool, annotated as *blc* (membrane biogenesis), *galU* (lipopolysaccharide core region biosynthetic process) and *mltD* (cell-wall maturation). Again, it was assumed that insertions within these components would theoretically increase intercalation levels

due to their roles in the maintenance of the membrane. However, it should be noted that not all gene knockouts produce obvious phenotypes, nor contribute equally to their biological processes.[186]

3.3.5 Transposon-Insertions Resulting in Low Fluorescence: Miscellaneous Functions

A further two genes involved with cell division were negatively selected, annotated as the rod shape determining protein *mreB* and *scpB*, involved with chromosome segregation and condensation. These were hypothesised to possess morphological abnormalities due to the role of *mreB* in cellular-shape determination, and the common cellular elongation noted in *scpB* mutants.[187] The remaining genes resulting in low fluorescence possessed varying functions, including riboflavin, molybdopterin, pyrroloquinoline, and siderophore biosynthesis (*ribD*, *yibN*, *pqqB* and *pqqC*), transposition (BAL062_01585), a predicted secreted protein (BAL062_00684), siderophore biosynthesis (*iucA*) and metal-ion binding (BAL062_03669), a sensor of blue light (*ycgF*), and ADP-ribose pyrophosphatase (BAL062_01857). Though the functional contribution of most of these genes in response to ethidium bromide is unclear, all decreased in abundance except for the transposase BAL062_01585, indicating either fitness decreases following sorting, or negative selection due to aberrant morphologies. This would be as expected for genes encoding ubiquitous coenzymes such as *ribD*, an essential gene in *A. baylyi,* or disruptions within metal-ion binding genes, as free metals within the cytoplasm can be toxic.[188, 189]

3.4 Probing the Functions of Genes Selected by TraDISort, Resulting in High Fluorescence

The remaining genes with annotated GO terms and/or pathways indicative of biological functions were assigned to 126 non-duplicate genes resulting in high fluorescence, with increased or decreased insertion densities (mutant abundances) following flow-sorting. Genes were then categorised based on biological functions.

3.4.1 DNA, Transcription and Translation: Transposon-Insertions Resulting in High Fluorescence

As with transposon-insertions resulting in low fluorescence, most transposon-insertions (23 genes) resulting in high fluorescence (Table S5, see supplementary data), were annotated as being involved in either DNA, transcription, translation or DNA repair. Again, these gene disruptions were hypothesised to affect DNA/mRNA availability and accessibility for ethidium bromide

intercalation.[135, 179] Annotations potentially affecting DNA content and transcription rates included *cyaA* (nucleotide biosynthesis), *umuc_4* (DNA Polymerase V subunit), *recF*, *recR*, *priA* and *xerD_2* of DNA repair systems, *greB* (a transcriptional elongation factor) and *rob* (a DNA gyrase inhibitor). The multiple selections of genes with functions in DNA repair resulting in high fluorescence, such as the DNA Polymerase V subunit, is not surprising as deficiencies in these systems must have altered the amount of double stranded DNA available for intercalation by ethidium bromide.[135, 162, 190] Furthermore, all these mutants decreased in abundance besides *rob*, suggestive of decreases in fitness for most of these gene disruptions. All remaining mutants also decreased in abundance following flow-sorting, and possessed various functions related to translation, including ribosome assembly and modification, tRNA processing and modification, and the proper folding of proteins (BAL062_00126, *ypeA*, *prmB*, *rlmN*, *rbfA*, *rlmB*, *lepA*, *rnpA*, *truC*, *tadA*, *trmE*, *csaA*, *dsbC_2*, *fkpB*, *tiG*). Again, it is possible that altered rates of translation due to disruption in these significant genes may have affected cellular content available for intercalation.[173, 174, 175] For example, in *E. coli*, mutants of the translational elongation factor *lepA* and ribosomal methyltransferase *prmB* accumulate immature rRNA assembly intermediates, and abnormal ribosomal particles, respectively.[191, 192]

3.4.2 Transcriptional Regulation: Transposon-Insertions Resulting in High Fluorescence

As with insertions resulting in low fluorescence, insertions in transcriptional regulators were the second most prevalent group of genes resulting in high fluorescence. Again, these regulators possessed control over various processes, and seventeen of these mutants decreased in abundance following flow-sorting, including *recX*, *hmrR_2*, BAL062_01079, BAL062_01495, BAL062_02752, *pdtaR*, *iclR_2*, *gbpR*, *ethR*, *gltC_4*, *rhaS_2*, BAL062_02079, *sixA*, *modE*, *aseR*, and *lrp*. Due to the variation of processes potentially controlled by these regulators, they cannot be said to form a cohesive functional group. However, it is not surprising that *recX* disruption resulted in high fluorescence due to its role in the regulation of DNA repair, and the increased sensitivity of *A. baumannii* mutants to DNA damaging agents upon the disruption of DNA repair systems.[162] The SmvA efflux repressor protein also resulted in high fluorescence, BAL062_01495 (see 3.1.1), which could indicate that de-repression of this pump possesses lethal consequences, as with AdeIJK.[43] The Rhas_2 protein, which regulates the biosynthesis of the polysaccharide sugar L-rhamnose, potentially affecting membrane integrity, also resulted in high fluorescence. Additionally, the ortholog of BAL062_02079 in *E. coli* K-12 MG1655 (r*raA*) is known to be involved with mRNA

metabolism, with deletions increasing cellular mRNA content via impeding RNA turnover.[193, 194] If this gene possesses similar functions in *A. baumannii* BAL062, it is likely that mutants in this gene resulted in high fluorescence due to altered mRNA metabolism. Additionally, four mutants with transposon-insertions in transcriptional regulators increased in abundance, *adeS* (see3.1.1), *gltR_3*, *rstA*, and *yxaF_4*. Interestingly, *gltR_3* was annotated as a repressor for chromosomal replication, and was enriched in this pool, suggesting some of these mutants may have had increased DNA content.

3.4.3 Amino Acid Biosynthesis and Metabolic Processes: Transposon- Insertions Resulting in High Fluorescence

Mirroring the low fluorescence pool, transposon-insertions resulting in high fluorescence and in genes annotated as either being involved with amino acid biosynthesis or amino acid metabolism were all negatively selected (*thrB*, *astB*, *aroE*, *thiE*, *psdhT*, *hdC*, *aroA*, *hpcD*, *argF*, *puuB_3*, *antC*, *aroC*, *hisG*, *ilvC*), indicating either aberrant ethidium bromide accumulation for these mutants, or potential growth abnormalities.[163] Nine genes annotated as being involved with metabolism negatively selected (*ureD*, *icD_2*, BAL062_03527, *ligE*, *hapE_1*, *nadX*, *ndoA*, *gstB_1* and *pgi*) whilst three were positively selected (*cbdB*, BAL062_02059 and *benB*). Again, genes related to metabolism possessed various functions, and it is likely that these metabolic genes resulted in aberrant fluorescence intensities or growth abnormalities due to perturbations in metabolic networks, for example, *nadX* participates in NAD+ biosynthesis, whilst *pgi* is a major gene in the glycolosis pathway.[195, 196] Few genes resulted in increased insertion densities, but one, *benB*, was associated with the degradation of aromatic compounds, which again, may have compromised the formation of aggregates, resulting in enrichment of these mutants.[164, 165]

3.4.4 Transporters and Membrane Proteins / Membrane Associated Proteins: Transposon-Insertions Resulting in High Fluorescence

Thirteen transporters resulted in high fluorescence and were negatively selected, annotated as BAL062_00257 (hypothetical ABC transporter), *corA_2* (magnesium transport), *pitA* (phosphate transporter), *oprM_2* (AdeFGH outer membrane component), *yhhT* (autoinducer II transport system), *exbD_1* (biopolymer transport), *bauE* (acinetobactin transport), *yddG* (amino acid transport), *yclQ_2* (iron ABC transporter), *naiP_1*, *potA*, BAL062_02350, and *lolD_2* (lipoprotein releasing ABC system). Six transporter genes were positively selected, annotated as *oprM_1*,

BAL062_01850, BAL062_01420, *smvA* , *rhtB_2*, and BAL062_02400, a hypothetical DMT family (drug/metabolite) efflux protein. Again, most of these transporters possessed various significant functions such as iron, amino acid or lipoprotein transport, and deficiencies in these processes might have caused homeostatic imbalances and fitness decreases, resulting in high fluorescence.[38, 39] Unlike the low fluorescence pool, three transporters or transport components related to antimicrobial resistance were selected. These were *oprm_2*, *oprM_1* and *smvA* (see 3.1.1).[45, 46] Additionally, two putative transporters were also selected in this pool (BAL062_00257 and BAL062_02400), suggesting that they may accept ethidium bromide as a substrate.

Eight transposon-insertions within genes involved with the biosynthesis of membrane components resulted in high fluorescence and decreased mutant abundances (BAL062_00344, *tesA*, *smpA*, *htrB_3*, *lspA*, BAL062_03862, BAL062_02080 and BAL062_02190). Two additional genes implicated with membrane processes were also negatively selected, including *mscL* (mechanosensitive channel), BAL062_02272 (putative effector of murein hydrolase) whilst BAL062_01801 (phospholipase D activity) was positively selected. It is likely not a coincidence that more genes involved with membrane biosynthesis, and maintenance resulted in high, and not low fluorescence, as disruption in these genes may increase ethidium bromide permeation through the membrane.[135, 170] Furthermore, four genes annotated as being involved with pilus assembly (*pilA, pilF, pulG*, BAL062_02116) resulted in high fluorescence, and decreased in abundance. Why these mutants resulted in increased fluorescence is uncertain, given mutants of pili synthesis genes, such as *pilA* in *A. baumannii* exhibit no other known phenotype than loss of pili.[197]

3.4.5 Miscellaneous Gene Functions: Transposon-Insertions Resulting in High Fluorescence

One gene involved with cell division (the rod-shape determining protein *mreC*) resulted in high fluorescence with decreased abundance following flow-sorting. As noted, these mutants may have been rejected by the flow-sorter as *mreB* mutants invariably result in phenotypes resembling small dark spheres.[187, 198] Again, the remaining negatively selected transposon-insertions resulting in high fluorescence were annotated with various functions, such as conserved hypothetical genes with only vague functions, transposition-related genes and metal-ion binding genes, and it is not certain why all these genes were selected (*yqeY, ybaB, sohB, yhbU*, BAL062_01349, BAL062_01584, *yddE*, BAL062_02836, *viuB, rubA*, BAL062_00506, BAL062_01682, BAL062_01684, *dcaC, ahpC_2, arsC*).

However, BAL062_02836 was annotated with functions involved with the response to stress, and this response is integral to prokaryotic survival in response to antimicrobial substances.[11, 16]

3.5 Genes Lacking Functional Data: Non-Hypothetical Genes

The remaining transposon-insertions in non-hypothetical genes resulting in low fluorescence (12 in total) could not be assigned GO terms and/or pathways via ortholog or sequence analyses. For these genes, family and domain data from Interpro were then used to confirm current annotations, whilst subcellular localizations were predicted via LocTree3 and confirmed via Cello V2.5 for genes returning no family or domain data, and only unintegrated signatures. All except *mdcC* resulted in decreased insertion densities, displayed in Table 7. Interestingly, within this group existed a multidrug ABC transporter with no orthologous genes in either *E. coli K12* MG1655, or *A. baumannii* ATCC 17978 and *A. baumannii* AB5075-UW. This may indicate that it accepts ethidium bromide as a substrate, as other ABC-type transporters are known to do.[40] Sequence analyses also confirmed BAL062_01142 to possess a HNH endonuclease domain, and that *soJ_1* possessed a structure consistent with functions in the DNA replication process.[199] As hypothesised by Hassan et al[47] for the selection of BAL062_03687 (a DNA methylase) in the published TraDISort dataset, the selection of these genes is likely to have caused an alteration in the availability or accessibility of DNA, resulting in alterations of rates of ethidium bromide intercalation.[135, 170] Additionally, BAL062_02364 returned sequence results indicative of amino acid biosynthesis. The selection of this gene is not surprising given the over-representation of genes with this function with GO terms and/or annotated pathways, and the potential for these genes to cause growth abnormalities and perturbations in other cellular pathways.[163, 200, 201]

Table 7: Non-Duplicate and Non-Hypothetical Genes Selected by TraDISort Without GO Terms and/or Pathways Resulting in Low Fluorescence

Locus Tag	Annotated Function	LogFC	Sequence Analysis
BAL062_00876	Multidrug ABC transporter permease	↓↓ -2.67962	Cytoplasm (100%)
BAL062_00941	Acyl-ACP thioesterase	↓↓ -2.84224	Potential thioesterase/thiol-ester dehydratase-isomerase
BAL062_01142	HNH endonuclease	↓↓ -2.33749	HNH Endonuclease domain
BAL062_01258	hemin uptake protein	↓↓ -2.51853	Hemin uptake protein, HemP
BAL062_01390	17 kDa surface antigen family protein	↓↓ -2.35668	Extracellular region (86%)

BAL062_01666	Alpha/beta hydrolase	↓↓ -2.59901	Alpha/beta hydrolase, conserved
BAL062_02128 *SoJ_1*	Chromosome partitioning protein, Sporulation initiation inhibitor	↓↓ -3.84714	P-loop containing nucleoside triphosphate hydrolase homologous superfamily
BAL062_02364	Protein of unknown function (DUF1185)	↓↓ -3.49425	Putative amino acid synthesis protein family
BAL062_02477	Signal peptide	↓↓ -3.11121	Cytoplasm (36%)
BAL062_02789	Phage nucleotide-binding protein	↓↓ -2.07763	Cytoplasm (89.6%)
BAL062_02957	Oligophrenin 1	↓↓ -9.72314	Extracellular region (68%)
BAL062_02264 *mdcC*	Malonate decarboxylase, delta subunit	↓↑ 2.12887	Cytoplasm (92.6%)

- ↓↓ = Decreased fluorescence + decreased insertion density
- ↓↑ = Decreased fluorescence + increased insertion density
- Percentages Indicate Prediction Confidence

The majority of the remaining 34 transposon-insertions in non-duplicate genes causing high fluorescence resulted in decreased insertion densities (mutant abundances), with only 5 transposon-insertions (17%) causing increased insertion densities, displayed in Table 8.

Table 8: Non-Duplicate and Non-Hypothetical Genes Selected by TraDISort Without GO Terms and/or Pathways Resulting in High Fluorescence

Locus Tag	Annotated Function	LogFC	Sequence Analysis
BAL062_00335	Signal peptide	↑↓ -2.23886	Periplasm (40%)
BAL062_00360	YCII-related domain protein	↑↓ -9.95817	YCII-related domain, unknown function
BAL062_00466	Unknown function, (DUF2789)	↑↓ -8.21806	DUF2789, unknown function
BAL062_00545	Unknown function, (DUF2511)	↑↓ -2.39738	DUF2511, unknown function
BAL062_00604	Acid shock protein	↑↓ -2.24577	Periplasm (98.4%)
BAL062_00879	Multidrug ABC transporter permease	↑↓ -2.56572	ABC transporter
BAL062_01006	Domain of unknown function (DUF20), permease	↑↓ -2.14302	Transmembrane protein, tqsA-like, family. AI-2E transport.
BAL062_01500	Acyl-ACP thioesterase	↑↓ -9.96626	Potential thioesterase/dehydratase-isomerase
BAL062_01592 cmpC	Bicarbonate transport ATP-binding protein	↑↓ -2.19801	Periplasmic binding protein-like II superfamily protein

BAL062_01711	NADH: flavin oxidoreductase	↑↓ -8.46793	Nuclear transport factor 2-like domain. Polyketide cyclase SnoaL-like domain
BAL062_01737	Predicted transporter component	↑↓ -9.772	Sulphur transport domain
BAL062_01816	IS66 Orf2 family protein, Transposase and inactivated derivatives	↑↓ -9.99222	Cytoplasm (38%)
BAL062_01821	Di- and tricarboxylate transporter	↑↓ -2.24471	Dicarboxylate carrier, matC N-terminal domain
BAL062_01978 hxlr_2	HTH-type transcriptional regulator	↑↓ -2.51105	HTH-type transcriptional regulator domain
BAL062_01995	Pyrroloquinoline quinone biosynthesis protein	↑↓ -4.34496	PqqC-like protein family
BAL062_02183	Predicted membrane protein	↑↓ -8.59145	Chlorhexidine efflux transporter domains
BAL062_02289	Acetyltransferase (GNAT) family	↑↓ -7.78625	Acetyltransferase, NAGS-type GNAT domain
BAL062_02373 lrtA	Retron-type reverse transcriptase, Group II intron-encoded protein	↑↓ -3.38698	Reverse transcriptase domain
BAL062_02492	Protein of unknown function (DUF559)	↑↓ -2.07837	Restriction type II endonuclease core structure domain
BAL062_02540	Ferredoxin, Domain of Unknown Function (DUF326)	↑↓ -2.75198	Unknown function (DUF326), Small cysteine-rich repeat
BAL062_02590	Signal peptide	↑↓ -4.4998	Periplasmic (74%)
BAL062_02616	Glycine/D-amino acid oxidase (deaminating)	↑↓ -6.77062	Cytoplasm (37%)
BAL062_02622 podJ_1	Polar organelle development protein, Sel1 repeat	↑↓ -9.18950	Sel1 repeat, HCP-like superfamily
BAL062_02689	Phage head maturation protease	↑↓ -3.05188	Prohead protease family.
BAL062_02750	Glutamate 5-kinase	↑↓ -2.25713	Cytoplasm (33%)
BAL062_03085	Predicted metal-dependent hydrolase	↑↓ -11.6824	Uncharacterised conserved protein, (UCP07580)
BAL062_03280	Long-chain fatty acid outer membrane transporter	↑↓ -2.1309	Outer membrane transport protein family
BAL062_03497	Predicted membrane protein (DUF2061)	↑↓ -3.96399	Unknown function (DUF2750)
BAL062_03912	Lipoprotein	↑↓ -8.28013	Fimbrium (73%)
BAL062_01481	RDD family protein	↑↑ 2.04479	RDD family protein
BAL062_01677	Unknown function (DUF2750)	↑↑ 2.36166	Unknown function (DUF2750)
BAL062_02004	Transcriptional regulator	↑↑ 2.19798	Extracellular region (46%)

BAL062_02285 peb1A	Cystine transporter subunit	↑↑ 2.22326	Solute-binding protein family 3/N-terminal domain of MltF domain
BAL062_03481 mfpsA	Glycosyltransferase	↑↑ 2.43642	Glycosyltransferase, family 1 domain

- ↑↑ = Increased fluorescence + increased insertion density
- ↑↓ = Increased fluorescence + decreased insertion density
- Percentage indicates prediction confidence

As with genes possessing GO terms and/or annotated pathways, genes encoding transporters were more prevalent in this pool, or transposon-insertions resulting in high fluorescence. This group possessed five transport related proteins. Sequence analysis confirmed BAL062_00879 as a multidrug ABC-type transporter, whilst the predicted membrane protein (BAL062_02183) possessed a chlorhexidine transporter domain. Again, this may be suggestive of potential roles in the extrusion of ethidium bromide for these transporters, as multiple families of efflux pumps are known to accept ethidium bromide as a substrate.[40] Furthermore, BAL062_01006 may possess roles in quorum sensing, and bears structural resemblance to the transport protein YddG of *E. coli*, with deletions implicated with increased biofilm formation.[202] Subsequently, these mutants may have been negatively selected due to an increased propensity to form aggregates. The gene BAL062_01737 possessed a sulphur-transport domain, whilst BAL062_03280 was confirmed as an outer membrane protein required for long-chained fatty-acid transport. Disruption of the latter could potentially be indicative of altered membrane permeability and increased ethidium bromide permeation, as exogenous fatty acids may be taken up and incorporated into the membrane in *A. baumannii,* however it is more likely that this gene has been selected for alternative reasons.[203] Additionally, sequence analysis revealed the negatively selected BAL062_02492, possessing a domain of unknown function, may be a restriction type II endonuclease-related protein, and that BAL062_00360 may possess functions in transcription initiation. Again, it is not surprising that mutants in these gene functions may have resulted in increased ethidium bromide intercalation. Both seem to be involved with significant processes possessing the ability to affect the state or cellular content of DNA (digestion), or mRNA (transcriptional initiation).[204, 205]

Positively selected transposon-insertions (those increasing in abundance following flow-sorting) resulting in high fluorescence possessed unclear functions. This included an RDD-type

transmembrane protein, a cysteine transporter, a glycosyltransferase, a protein of unknown function (BAL062_01677) and a transcriptional regulator of unknown specificity (BAL062_02004).

3.5.1: Sequence Analyses of Transposon-Insertions in Hypothetical Genes Resulting in Low Fluorescence: Functional Trends

186 mutants flow-sorted into one pool only, without GO terms and/or pathways, possessed transposon-insertions in hypothetical genes, or genes with only putative functions. Interpro sequence analyses provided family and/or domain data for 46 of these (25%). Several completely hypothetical genes returned family and/or domain sequence results, again highlighting the complexities involved with gene annotation via automatic annotation pipelines.[50, 51]

Transposon-insertions in 13 of these 46 genes resulted in low fluorescence. Sequence analysis revealed many genes with domains of unknown function, or genes belonging to uncharacterised protein families. Another potential endonuclease (BAL062_01149) was selected within this group, (Table 9). This bore similarity to the Vsr protein of *E. coli*, which functions within a DNA repair pathway, and may possess similar function in *A. baumannii* BAL062, thus affecting ethidium bromide intercalation upon disruption.[206] Additionally, three hypothetical HTH-type transcriptional regulators decreased in abundance following flow-sorting, thus these regulators may mediate significant biological processes. Furthermore, sequence analysis confirmed BAL062_02159 as an OsmC/Ohr family protein, and OsmC and orthologs seem to function in cellular stress responses.[207, 208] Stress responses are known to be activated in bacteria, even in response to sub-inhibitory concentrations of antimicrobial substances, and disruption may be a reason for aberrant fluorescence for this mutant.[209] Besides BAL062_00756 (belonging to an uncharacterised protein family) it is unknown why BAL062_02043 (a conserved siderophore biosynthesis protein) increased in abundance in this pool. However, this may be indicative of increased fitness compared to the rest of the flow-sorted mutant library, given that *A. baumannii* genomes typically encode for multiple siderophores.[210] Subsequently, a siderophore knockout mutant in *A. baumannii* may not suffer substantial decreases in fitness.

Table 9: Interpro Sequence Analysis Results of Transposon-insertions in Hypothetical or Putative Genes Without GO Terms and/or Pathways Resulting in Low Fluorescence.

Locus Tag	Annotated Function	LogFC	Sequence Analysis Result
BAL062_00146	Hypothetical protein	↓↓ -3.3253572920	Uncharacterised protein family: UPF0232
BAL062_01107	Putative signal-peptide containing protein	↓↓ -2.1757649820	DUF3015, unknown function
BAL062_01149	Hypothetical protein	↓↓ -4.522150346	VSR Endonuclease Superfamily
BAL062_01512	Hypothetical protein	↓↓ -10.21987866	HTH-type transcriptional regulator domain
BAL062_02721	Hypothetical protein	↓↓ -9.630103452	HTH-type transcriptional regulator domain
BAL062_02946	Hypothetical protein	↓↓ -3.232229135	HTH-type transcriptional regulator domain
BAL062_02159	putative redox disulphide bond formation, OsmC	↓↓ -9.068760736	OsmC/Ohr family protein
BAL062_02835	putative esterase	↓↓ -5.09838812	Uncharacterised protein family, UPF0227
BAL062_02727	putative secretion activating protein	↓↓ -4.400427271	DUF847, unknown function
BAL062_02855	putative sulfatase modifying factor 1 precursor	↓↓ -2.932411134	Sulfatase-modifying factor enzyme domain. C-type lectin fold homologous superfamily.
BAL062_03270	Uncharacterised protein conserved in bacteria	↓↓ -2.42449	Uncharacterised conserved protein, family UCP028291
BAL062_00756	Domain of unknown function (DUF74)	↓↑ 2.65229	Uncharacterised protein family UPF0145
BAL062_02043	Putative acetyltransferase. Siderophore biosynthesis protein domain	↓↑ 2.253179502	Acyl-CoA N-acetyltransferase homologous superfamily. Siderophore biosynthesis protein, conserved domain.

- ↓↓ = Decreased fluorescence + decreased insertioty density
- ↓↑= Decreased fluorescence + increased insertion density

3.5.2 Sequence Analyses of Transposon-Insertions in Hypothetical Genes Resulting in High Fluorescence: Functional Trends

Sequence analyses of hypothetical genes resulting in high fluorescence revealed several bacteriophage-related proteins, the functions and significance of which are unknown (Table 10). As with previous trends in transposon-insertions resulting in high fluorescence, this group possessed multiple genes related to the synthesis of membrane components, hypothesised to affect ethidium bromide permeation through the membrane, thus intercalation (BAL062_02057, BAL062_02833, and BAL062_03858).[135, 170] This was in addition to other commonly selected gene functions within the dataset, such as components of DNA replication machinery (BAL062_01184), translational initiation inhibition (BAL062_03352), a ribosomal maturation factor (BAL062_00534), an

endonuclease (BAL062_01126) and a potential plasmid partitioning protein (BAL062_00340). Disruptions in genes with these functions (related to DNA accessibility or availability, transcription, or translation) as previously hypothesised, must have altered cellular DNA or mRNA content available for ethidium bromide intercalation, causing aberrant fluorescence intensities within these mutants.[173, 174, 175] Two insertions within hypothetical transporters resulted in high fluorescence, an ABC-transporter component (BAL062_01345) and a putative amino acid transporter (BAL062_02212). It may be that these are transporters that accept ethidium bromide as a substrate, but again, it is not surprising that transporter disruptions resulted in aberrant phenotypes given their significant cellular functions.[38, 39] Again, few mutants resulted in increased insertion densities, and the function of most was not fully clear.

Table 10: Interpro Sequence Analysis Results of Transposon-insertions in Hypothetical or Putative Genes Resulting in High Fluorescence.

Locus Tag	Annotated Function	LogFC	Sequence Analysis Results
BAL062_00218	Putative beta-lactamase hcpC precursor	↑↓ -2.20775	Uncharacterised tetratricopeptide repeat
BAL062_00340	Putative partition-related	↑↓ -7.75289	MinD/ParA nucleotide binding domain.
BAL062_00440	MICS metal-binding protein	↑↓ -2.71009	Hypothetical protein family CHP02443
BAL062_00474	Hypothetical protein	↑↓ -9.99006	ATP-phosphribosyltransferase (C-terminal domain)
BAL062_00534	Hypothetical protein	↑↓ -3.45192	Ribosome maturation factor, RimP, C-terminal domain.
BAL062_00750	SAM-dependent methyltransferase	↑↓ -2.06634	S-adenosyl-L-methionine-dependent methyltransferase homologous superfamily
BAL062_01010	Hypothetical protein	↑↓ -4.72912	Potential thioesterase/thiol ester dehydratase-isomerase
BAL062_01057	Putative enzyme of the cupin superfamily	↑↓ -8.58871	RmlC-like cupin domain homologous superfamily
BAL062_01106	Integral membrane protein	↑↓ -8.56184	DUF817, unknown function
BAL062_01126	Hypothetical protein	↑↓ -7.53253	HNH nuclease domain
BAL062_01165	Putative bacteriophage protein	↑↓ -2.02100	Bacteriophage rv5, orf52 family
BAL062_01184	DNA polymerase V component	↑↓ -3.23752	Peptidase S24/S26A/S26B/S26C domain
BAL062_01277	Putative metal-dependent HD phosphohydrolase domain	↑↓ -4.64619	Predicted HD phosphohydrolase family

BAL062_01345	Signal peptide, ABC-type transport system	↑↓ -3.38294	ABC-type transport auxillary lipoprotein component domain
BAL062_01686	Predicted outer membrane lipoprotein	↑↓ -8.82382	Membrane bound ybgT-like family protein
BAL062_01968	Uncharacterised protein conserved in bacteria	↑↓ -9.2058	DUF971, unknown function
BAL062_02057	Putative ubiquinone biosynthesis protein UbiB	↑↓ -3.62279	Ubib domain/Ubib domain, ADCK3-like
BAL062_02212	Putative amino acid transporter	↑↓ -9.78252	Branched-chain amino acid transport, AzlD family
BAL062_02407	Putative beta-lactamase HcpC precursor	↑↓ -2.75803	Uncharacterised tetratricopeptide repeat
BAL062_02485	Phage uncharacterized protein	↑↓ -2.88585	Archaeophage psiM2 terminase large subunit family
BAL062_02686	Hypothetical protein	↑↓ -9.04807	Phage gp6-like head-tail connector protein family
BAL062_02745	Hypothetical protein	↑↓ -2.52751	DUF4128, potential bacteriophage origin
BAL062_02761	Putative phage-like protein	↑↓ -6.18479	Ribosomal protein L9 (C-terminal) domain
BAL062_02833	Putative signal peptide-containing protein	↑↓ -2.89889	Capsule assembly protein, group 1 capsule biosynthesis family
BAL062_02944	gp54 protein,Uncharacterized phage-encoded protein	↑↓ -2.87213	BRO N-terminal domain, DNA binding viral proteins
BAL062_03030	Hypothetical protein	↑↓ -2.01955	Ead/Ea22-like, probable integrated phage protein family
BAL062_03352	YjgF family translation initiation inhibitor, putative endoribonuclease	↑↓ -8.14982	YigF/YER057c/UK114 family
BAL062_03858	Putative UDP-galactose--lipooligosaccharide galactosyltransferase	↑↓ -2.94043	Nucleotide-diphospho-sugar transferases homologous superfamily
BAL062_03891	Integral membrane protein	↑↓ -7.52321	Nickel/Cobalt homeostatis protein RcnB family.
BAL062_01557	Hypothetical protein	↑↑ 2.13576	DUF4128, potential bacteriophage origin.
BAL062_01683	Signal peptide-containing protein	↑↑ 2.14225	Uncharacterised protein family UPF0319
BAL062_02278	Putative rhodanese-related sulfurtransferase	↑↑ 2.84054	Rhodanese-like domain
BAL062_02914	Inner membrane protein YbaN	↑↑ 2.16542	DUF454, unknown function

- ↑↑ = Increased fluorescence + increased insertion density
- ↑↓ = Increased fluorescence + decreased insertion density

3.6 Mutants Selected by TraDISort with Strong Shifts in Insertion Densities

A total of 21 non-duplicate genes (5% of non-duplicate genes) were selected by TraDISort in response to ethidium bromide, with strong shifts in insertion densities, or mutant abundances (Q value = <0.01) (Table 11). In contrast, approximately 41% (113 out of 271 non-duplicate genes) possessed strong shifts in insertion densities in the published 2% TraDISort dataset, indicating that a wider collection range may lower the statistical confidence of TraDISort. A total of 19/21 of these transposon-insertions resulted in strong negative shifts in insertion densities, accounting for 90% of mutants with strong shifts in abundance following flow-sorting, and agreeing with most transposon-insertions in the full dataset. Additionally, NCBI databases revealed all genes to be conserved in *A. baumannii* strains, indicating that these genes may play roles in significant cellular functions.[6]

Table 11: Non-Duplicate Genes Selected by TraDISort, with Strong Shifts in Insertion Densities

Locus Tag	Function	Ortholog (*E. coli,* K12 MG1655)	Conserved in *A. baumannii ?*	LogFC
BAL062_00032	Hypothetical protein	N/A	Yes	↓↓ -13.5027
BAL062_00146	Hypothetical protein	N/A	Yes	↓↓ -7.11446
BAL062_00148 aceE	Pyruvate dehydrogenase	N/A	Yes	↓↓ -2.25631
BAL062_00149	Pyruvate/2-oxoglutarate dehydrogenase	B0115	Yes	↓↓ -2.16413
BAL062_00309 rpoZ	RNA Polymerase subunit – omega	B3649	Yes	↓↓ -11.1893
BAL062_00351 astB	N-succinylarginine dihydrolase	B1745	Yes	↑↓ -5.22611
BAL062_00713 mreB	Rod shape-determining protein	B3251	Yes	↓↓ -15.3805
BAL062_00714 mreC	Rod shape-determining protein	B3250	Yes	↑↓ -11.0558
BAL062_00757 oprM_1	Outer membrane protein	B0572	Yes	↑↑ 2.08502
BAL062_01106	Hypothetical integral membrane protein	N/A	Yes	↑↓ -8.56184
BAL062_01496 smvA	MFS-type efflux pump	N/A	Yes	↑↑ 3.46703
BAL062_02646 mltD	Soluble lytic murein transglycosylase	B0211	Yes	↓↓ -4.14519
BAL062_03143 scpB	Chromosomal segregation and condensation protein	N/A	Yes	↓↓ -2.80849
BAL062_03342	Signal transduction histidine-protein	B2786	Yes	↓↓ -3.16187

barA				
BAL062_03363 prfC	Peptide chain release factor 3	B4375	Yes	↓↓ -2.19295
BAL062_03671 dsbC_2	Protein-disulfide isomerase	B2893	Yes	↑↓ -2.0055
BAL062_03675 gacA	Response regulator	B1914	Yes	↓↓ -2.40045
BAL062_03688 ribD	Riboflavin biosynthesis protein	B0414	Yes	↓↓ -11.3207
BAL062_03722 fadD_2	Long-chain-fatty acid-CoA ligase	B1805	Yes	↓↓ -2.28378
BAL062_03856 galU	UTP glucose-1-phosphate uridylyltransferase	B1236	Yes	↓↓ -10.5205
BAL062_03912	Lipoprotein	N/A	Yes	↑↓ -8.28013

- ↑↑ = Increased fluorescence + increased insertion density
- ↑↓ = Increased fluorescence + decreased insertion density
- ↓↓ = Decreased fluorescence + decreased insertion density
- Bold Locus Tag = Hypothetical gene product

As with most selected genes, transposon-insertions causing strong shifts in insertion densities were associated with genes with varying functions. Of these 21 genes, five were annotated as hypothetical proteins, with three being annotated as hypothetical membrane proteins, one being the chromosome condensation and segregation protein (ScpB), and the last annotated as *barA* (annotated as *gacS* in *A. baumannii* ATCC 17978). As previously noted, *scpB* mutants were likely to have been negatively selected due to the common elongation noted in *scpB* mutants, and the GacA/S system due to its roles in aggregation, cellular structure and biofilm formation.[178, 180, 211] In addition to these hypothetical genes, the efflux proteins OprM_1 and SmvA (though not OprM_2) also possessed strong shifts in insertion densities, and were the only mutants with positive shifts in mutant abundances which resulted in high fluorescence. Overrepresentation of these deletions within the high fluorescence mutant pool was not unexpected at the sublethal concentration of ethidium bromide used during the TraDISort experiment, given the fact that Oprm_1 and SmvA are known to accept ethidium bromide as a substrate.[45, 47] The remaining genes were involved with carbon, fatty-acid and amino acid metabolism, rod-shape maintenance (*mreB* and *mreC*), the synthesis of cell envelope components and membrane maintenance (*fadD_2, galU* and *mltD*), RNA polymerase subunit omega (*rpoZ*), translational processing (*prfC*), riboflavin biosynthesis (*ribD*), and a lipoprotein (BAL062_02912).

As noted, it is possible that disruptions in genes with functions related to transcription (*rpoZ*), or translation (*prfC)* may have affected cellular DNA and mRNA available for ethidium bromide to intercalate with, resulting in aberrant fluorescent intensities.[173, 174, 175] Mutants of the rod-shape determining proteins MreB and MreC were also hypothesised to possess morphological alterations causing negative selection by the flow-sorter, as this is a common observation for many mutants in these conserved genes.[187] The strong negative shifts in the insertion densities of mutants with transposon-insertions in genes contributing to membrane synthesis or maintenance (*fadD_2, galU and mltD*) might have, as hypothesised, disrupted membrane integrity. However, mutants of *mltD* (a conserved bacterial gene) in *H. pylori* display separation defects, or a chaining morphology, and *galU* mutants in *A. baumannii* ATCC 17978 possess deficiencies in capsule synthesis and express a partially truncated LPS.[186, 212] This suggests another potential negative selection by the flow-sorter due to aberrant morphology, and raises the possibility that perhaps most mutants with strong negative shifts in insertion densities following flow-sorting were caused by altered morphologies. This is further supported by the fact that disruptions in genes related to significant metabolic processes, such as *aceE* (pyruvate dehydrogenase) have been shown to cause growth abnormalities in *E. coli,* and may be a reason for the strong negative shift in the insertion density of the *aceE* mutant within this dataset.[183]

Mutants with transposon-insertions within 3 genes (*oprM_1*, BAL062_01106, and *smvA*) selected by TraDISort with strong shifts in insertion densities were present within the ¼ MIC ethidium bromide TraDIS data published by Hassan et al[47]*; oprM_1* and *smvA* were significantly negatively selected by TraDIS, owing to their roles as efflux systems for ethidium bromide, whilst BAL062_01106, a hypothetical integral membrane protein, was positively selected.[45, 46] Given the higher concentration of ethidium bromide used during this published TraDIS experiment, this suggests that the hypothetical integral membrane protein BAL062_01106 may play a role in fitness in response to ethidium bromide treatment.

3.6.1 Analysing Selected Hypothetical Genes with Strong Shifts in Insertion Densities for Potential Transporter Activity

Hydropathy plots and the prediction of transmembrane helices for hypothetical proteins selected by TraDISort with strong shifts in insertion densities, or mutant abundances (Q Value =<0.1),

indicated that BAL062_01106 (Figure 5) possessed the most transmembrane segments (8 in total), indicative of a membranous localisation, and hence could potentially represent a novel transporter protein.[39]

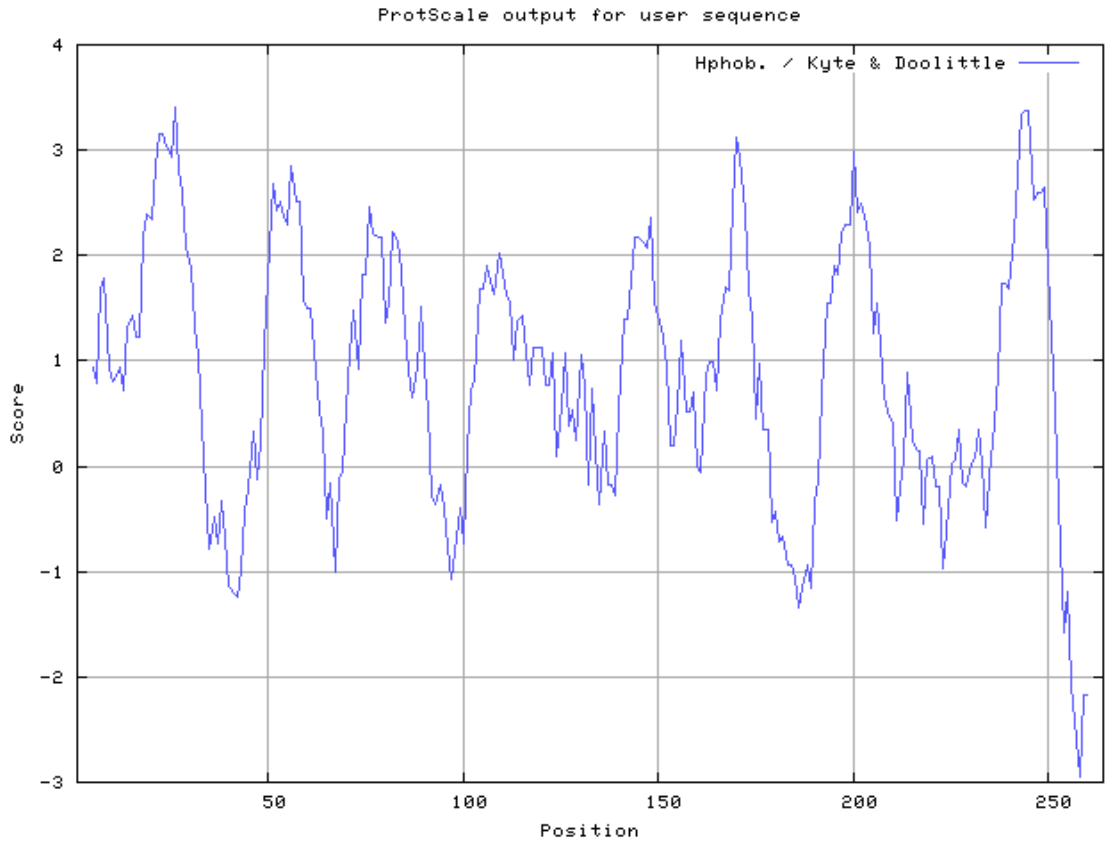

Figure 5: A Hydropathy Plot of BAL062_01106, a Hypothetical Integral Membrane Protein (Gasteiger et al, 2005). Segments Above 0 on the X-Axis Represent Hydrophobic Sequence Regions. Segments Below 0 on the X-Axis Represent Hydrophilic Regions. The Number of Spikes above 0 on the X-Axis is Indicative of the Number of Transmembrane Segments within this Protein.

Additionally, PredictProtein, and I-Tasser: Protein Structure & Function Prediction, predicted BAL062_00146 as a cytoplasmic protein most likely involved with the cyclic nucleotide metabolic process, BAL062_00032 as an integral inner membrane protein with unspecific functions, and BAL062_01106 as an inner membrane protein with potential transport activity (Table 12). This suggests that BAL062_01106 may potentially be an efflux pump which accepts ethidium bromide as a substrate, but this cannot be confirmed without further testing.

Table 12: Prediction of Subcellular Localizations and GO Term/s for Hypothetical Genes

Locus Tag	Predicted Localization	Predicted GO Term/s
BAL062_00032	Inner Membrane (99%)	MF: Protein Binding (35%) BP: Oxidation-reduction (14%) CC: Integral to membrane
BAL062_00146	Cytoplasm (83%)	MF: Adenyl ribonucleotide binding (31%) MF: Purine ribonucleoside triphosphate binding (31%) BP: Cellular response to stimulus (54%) BP: Cyclic nucleotide metabolic process (54%) CC: Cytoplasm
BAL062_01106	Inner Membrane (100%)	MF: Protein transporter activity (53%) MF: RNA binding (35%) BP: RNA transport (40%) CC: Integral to membrane

- Percentages indicates PredictProtein prediction confidence.
- MF = Molecular Function, BP = Biological Process, CC = Cellular Compartment

3.6.2 MIC Testing: Analysing the Connection Between TraDISort Phenotypes and Ethidium Bromide Resistance

MIC results for the putative RND-operon not selected by TraDISort (BAL062_00029—BAL062_00031) in orthologous gene knockouts of *A. baumannii* AB5075 UW (ABUW_0033—ABUW_0035) displayed identical MIC's to that of the wildtype control (250ug/ml) except ABUW_0035, the inner membrane component of this putative RND-system, which possessed very low growth at 125ug/ml (less than half that of all other mutants). Given that these proteins are the supposed substrate capturing components of tripartite efflux systems, the lowered MIC of this mutant might occur if this pump accepts ethidium bromide as a substrate.[42, 152] Furthermore, although TraDISort did not pick up this efflux system, a role in ethidium bromide efflux cannot be ruled out due to the functional overlap of efflux systems in *Acinetobacter* species.[157, 179]

MIC results for orthologous gene knockout mutants of, BAL062_01106 (hypothetical integral membrane protein, with potential transporter activity), BAL062_03687 (DNA methylase) and BAL062_03912 (lipoprotein) in *A. baumannii* AB5075 UW also displayed identical MICs to the wildtype control. However, unlike TraDIS, TraDISort is sensitive enough to identify the fitness contributions of genes not directly related to cell survival, and these genes may still contribute in some way to ethidium bromide intercalation.[47, 107]

Furthermore, the addition of further MIC tests with more sensitive concentration increments, or fluorescence shift assays, may have been able to detect changes in MICs or intracellular ethidium bromide accumulation for these mutants, and will be investigated in the future. However, growth for the last mutant ABUW_0153 (BAL062_00146) ceased at 125ug/ml, indicating that this hypothetical protein with predicted functions in cyclic nucleotide metabolism contributes to resistance to ethidium bromide in *A. baumannii* (Figure 6).

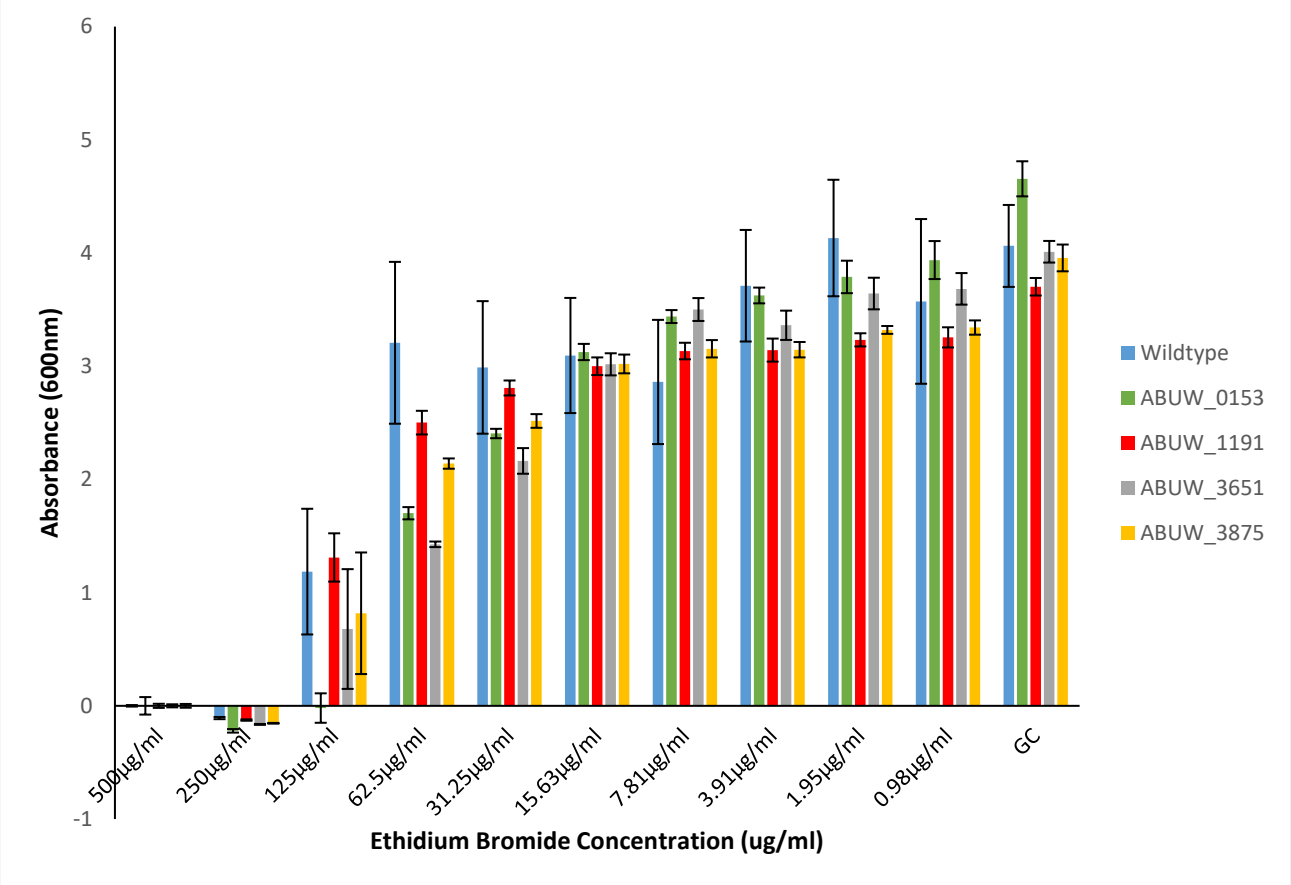

Figure 6: A Graph Representing the MIC's to Ethidium Bromide of A. *baumannii* AB5075 UW Mutants with Transposon-Insertions within ABUW_0153, ABUW_1191, ABUW_3651 and ABUW_3875. The

MIC of Wildtype *A. baumannii* AB5075 UW to Ethidium Bromide is 250ug/ml.[47] Only one Mutant Possessed an MIC Lower than that of Wildtype *A. baumannii* AB5075, ABUW_0153.

3.6.3 Confirmation of Morphological Abnormalities for Mutants Selected by TraDISort, with Strong Negative Shifts in Insertion Densities

Phase-contrast microscopy of a selection of mutants with strong negative shifts in insertion densities, or mutant abundances (Q. Value =<0.1) following flow-sorting were examined via phase-contrast microscopy to confirm if TraDISort was able to identify mutants with aberrant morphologies. BAL062_00713 (*mreB*), BAL062_00714 (*mreC*), BAL062_03143 (*scpB*), BAL062_02646 (*mltD*) and BAL062_03688 (*ribD*) displayed that all mutant cells exhibited, to a degree, abnormal morphologies (Figure 7).

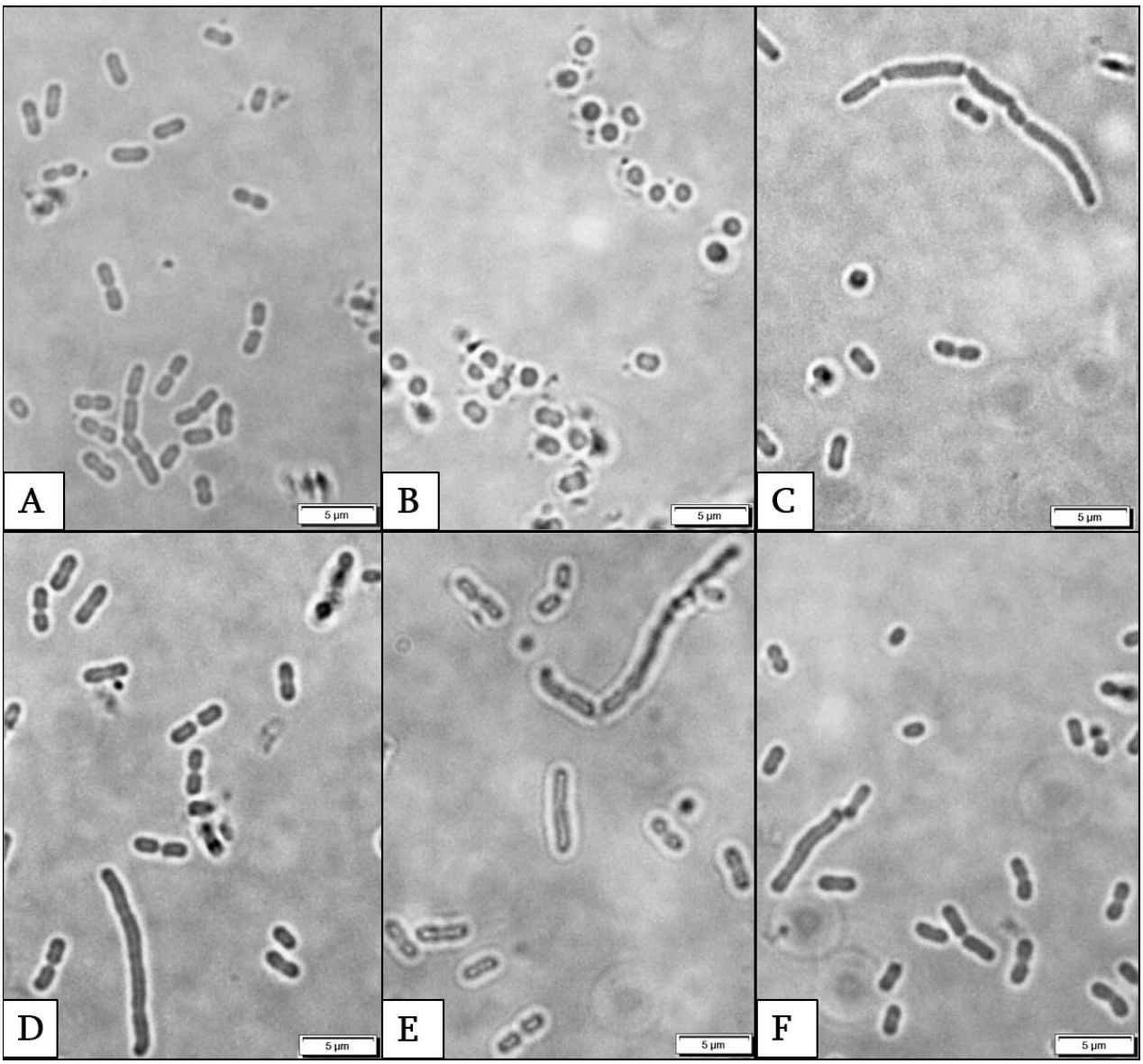

Figure 7: Phase-Contrast Microscopy Images of Wildtype and Transposon-mutant A. *baumannii* AB5075-UW Cells Grown to the Exponential Phase, Taken at 100x Magnification. (A) Wildtype A. *baumannii* AB5075-UW (B) Δ *mreB* cells (C) Δ *mreC* cells (D) Δ *scpB* cells (E) Δ *mltD* cells (F) Δ *ribD* cells

Wildtype *A. baumannii* AB5075-UW tended to form rod-shaped cells up to approximately 2.5µm in length. Mutants of the rod-shape determining proteins MreB and MreC both displayed aberrant morphologies. MreB mutants all appeared as small, dark-coloured and spherical cells, which was expected given data on this deletion in other bacterial strains.[187, 198]. Mutants of the MreC protein also formed small, dark-coloured and spherical cells approximately 1µm in diameter. However, they also formed cells of varying lengths growing in chains, some >5µm long, though both abnormalities

were outnumbered by cells with apparent wildtype morphology. This was unexpected given that this phenotype is not usually reported for mutants of the MreB/C/D proteins.[187, 198]

Mutants of the chromosome segregation and condensation protein ScpB exhibited mostly wildtype morphology, though many cells appeared significantly elongated, with many being over 10µm long. This was expected given that mutants in this gene are known to possess frequent cellular elongation, but also anucleation (a depletion of cellular DNA) which may also be a reason why these mutants also resulted in low fluorescence.[213] Mutants of the murein hydrolase, a peptidoglycan maturation enzyme, possessed morphology ranging from small spherical cells, to cells >5µm in length growing in chains, as *mreC* mutants appeared. This also satisfied the hypothesis that mutants possessing strong negative shifts in insertion densities may display growth abnormalities, as is seen in mutants of *mltD* (a gene with conserved bacterial functions) in *H. pylori*.[212] Finally, given that the riboflavin biosynthesis protein RibD is an essential gene in *A. baylyi*, it was not surprising that transposon-insertions within this gene in *A. baumannii* BAL062 also compromised cellular morphology, causing apparent growth defects, as well as cellular elongation.

Given the results from phase-contrast microscopy, the possibility remains that further mutants with strong negative shifts in insertion densities following flow-sorting compared to the mutant input pool may have possessed altered morphologies. Furthermore, the observation that some of these non-duplicate mutants (those flow-sorted into one pool only) did not possess population-wide phenotypic abnormalities is significant. This suggest that phenotypically-wildtype versions of these mutant cells should have been flow-sorted, whilst mutants with aberrant morphologies could have been a plausible reason for negative selection. As for the overwhelming number of duplicate transposon-insertions (mutants possessing cells flow-sorted into both pools of low and high fluorescence, and decreasing in abundance in both), these deletions may have also caused pleiotropic phenotypes. This could account for cells being apparently flow-sorted into both pools (potentially wildtype morphology), whilst a substantial proportion were rejected by the flow-sorter, causing negative selection in both pools.

4. Conclusions and Future Directions

Results from this 5% TraDISort dataset align with the original results of the published 2% TraDISort dataset in 2016.[47] The in-depth analysis in this thesis reconfirmed the AdeABC, AdeIJK and AmvA efflux systems as the main ethidium bromide efflux pumps of *A. baumannii*, as the published 2% TraDISort dataset also revealed. However, this dataset also detected that AdeFGH, another RND-type efflux system in *A. baumannii* may also play a regular role in the efflux of ethidium bromide. Additionally, the overrepresentation of mutants with transposon-insertions within genes related to DNA, RNA, transcription, and translation within this dataset aligns with the hypothesis that TraDISort would detect mutants with transposon-insertions potentially affecting levels of cellular ethidium bromide intercalation. Though these mutants are not detected by TraDIS and other standard transposon mutagenesis protocols, their occurrence in this dataset provides evidence that TraDISort is able to detect differences in the phenotypes of mutants within mutant populations when not imposed under a direct fitness selection. [107]

The selection of the hypothetical gene BAL062_01106 in both the published ¼ MIC ethidium bromide TraDIS dataset and this dataset also suggests that this protein may play a role in the response to ethidium bromide.[47] Interpro protein sequence analyses also revealed numerous mutants with transposon-insertions within hypothetical transporters and transcriptional regulators, resulting in both low and high fluorescence, and these genes may be of interest regarding future research on *A. baumannii.*[143] This is especially the case when viewed in the context of cross resistance; resistance elements tend to cluster together in genomes on mobile genetic elements, and those such as efflux pumps may possess broad substrate specificities.[11,12] Therefore, transposon-insertions within genes detected by TraDISort potentially affecting ethidium bromide permeation and/or intercalation may potentially contribute to fitness under a number of other conditions.[11,12,94] Additionally, phase-contrast microscopy confirmed that the TraDISort procedure possesses the ability to select for mutants with transposon-insertions in genes causing shape and/or growth abnormalities.

The results from this analysis are of utility, but the discrepancies between the published 2% TraDISort dataset and the 5% TraDISort dataset must be noted. For example, many duplicate genes (65%) yet fewer non-duplicate genes from this dataset (23%) were present within the 2% dataset.

As stated, the scientific reasoning behind this discrepancy is currently unknown, but may be related to the potential predisposition of TraDISort to select for mutants with morphological irregularities. This dataset also possessed 21 (5% of non-duplicate genes) transposon-insertions causing strong shifts in insertion densities, whilst the published 2% dataset possessed 113 (41%). This might suggest that widening the collection range of the flow-sorter may lower the confidence of the protocol, however the exact biological significance of insertion density reads within the TraDISort protocol is yet unclear. Additionally, the potentially overwhelming negative selection of mutants with growth and/or shape defects by the protocol may be regarded as "false positives" due to rejection by gating procedures, and not alterations in abundance related to the condition imposed, which may be interpreted as a drawback. However, future procedures may take advantage of this information to improve results analyses, and potentially aid cell-gating procedures. Furthermore, the fact remains that this thesis represents only the second analysis of the TraDISort protocol, and that no replicates were used. Unlike protocols such as TraDIS, TraDISort is not an established procedure. Therefore, this work should not be interpreted as factually specific, but serves to generate new hypotheses regarding the analysis of future TraDISort projects.

Though the presence of many mutants within the TraDISort dataset is unclear, as aforementioned, TraDISort is an evolving protocol and is currently undergoing refinement. Multiple steps must be reconsidered before the protocol is optimised, such as compound exposure time, optimal sample subculturing for efficient flow-sorting, and when to sequence the flow-sorted samples. Indeed, it is likely that these factors also affected the analysis of TraDISort in this thesis, as noted by the potential for many mutants to have suffered from growth defects, perhaps due to a combination of exposure to ethidium bromide, the physical stress within the flow-sorters capillaries, and competition within the flow-sorted mutant pool. Nonetheless, the potential future applications of TraDISort, detailed extensively by Paulsen et al[107] include the identification of substrates for multi drug resistant efflux pumps, the identification of efflux pump inhibitors, and the measurement of gene expression via fluorescent reporter constructs.[107] Combined with alternative compounds that possess different mechanisms of action and intracellular sites of accumulation, TraDISort possesses the potential to infer the phenotypic differences exhibited by mutant libraries in responses to an array of conditions. Furthermore, unlike TraDIS and other transposon mutagenesis protocols, TraDISort is not limited by fitness, and once fully optimised, represents a promising future high-throughput method of probing bacterial genome functions.

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6. Supplementary Data

Table S1: Pathways Associated with Mutants Detected by TraDISort via Ortholog Analyses, with Transposon-Insertions in Genes Resulting in Low Fluorescence

Pathway	Insertion Density	# Genes in Pathway
Purine Metabolism	Decreased	5 Genes
Homologous Recombination	Decreased	3 Genes
Quorum Sensing	Decreased	3 Genes
Two-Component System	Decreased	3 Genes
Carbon Metabolism	Decreased	2 Genes
Citrate Cycle	Decreased	2 Genes
Glycolysis/Gluconeogenesis	Decreased	2 Genes
Pyrimidine Metabolism	Decreased	2 Genes
Pyruvate Metabolism	Decreased	2 Genes
ABC Transporter	Decreased	1 Gene
Amino Sugar and Nucleotide Metabolism	Decreased	1 Gene
Biofilm Formation	Decreased	1 Gene
Fatty Acid Biosynthesis	Decreased	1 Gene
Fatty Acid Degradation	Decreased	1 Gene
Fatty Acid Metabolism	Decreased	1 Gene
Folate Biosynthesis	Decreased	1 Gene
Galactose Metabolism	Decreased	1 Gene
Amino Sugar and Nucleotide Metabolism	Decreased	1 Gene
Biofilm Formation	Decreased	1 Gene
Fatty Acid Biosynthesis	Decreased	1 Gene
Fatty Acid Degradation	Decreased	1 Gene
Fatty Acid Metabolism	Decreased	1 Gene
Folate Biosynthesis	Decreased	1 Gene
Galactose Metabolism	Decreased	1 Gene
Glutathione Metabolism	Decreased	1 Gene
Glycine, Serine and Threonine Metabolism	Decreased	1 Gene
Glyoxylate and Dicarboxylate Metabolism	Decreased	1 Gene
Pentose and Glucose Interconversions	Decreased	1 Gene
Riboflavin Metabolism	Decreased	1 Gene
RNA Polymerase	Decreased	1 Gene
Starch and Sucrose Metabolism	Decreased	1 Gene
Benzoate Degradation	Increased	1 Gene
Degradation of Aromatic Compounds	Increased	1 Gene
Geraniol Degradation	Increased	1 Gene
Quorum Sensing	Increased	1 Gene

Table S2: Pathways Associated with Mutants Detected by TraDISort via Ortholog Analyses, with Transposon-Insertions in Genes Resulting in High Fluorescence

High Fluorescence with Decreased Insertion Density	Insertion Density	# Genes in Pathway
ABC Transporter	Decreased	3 Genes
Carbon Metabolism	Decreased	3 Genes
Glutathione Metabolism	Decreased	3 Genes
Homologous Recombination	Decreased	3 Genes
Phenylalanine, Tyrosine and Tryptophan Biosynthesis	Decreased	3 Genes
2-Oxocarboxylic Acid Metabolism	Decreased	2 Genes
Bacterial Secretion System	Decreased	2 Genes
Degradation of Aromatic Compounds	Decreased	2 Genes
Glycine, Serine and Threonine Metabolism	Decreased	2 Genes
Histidine Metabolism	Decreased	2 Genes
Nicotinate and Nicotinamide Metabolism	Decreased	2 Genes
Valine, Leucine and Isoleucine Biosynthesis	Decreased	2 Genes

Amino Sugar and Nucleotide Metabolism	Decreased	1 gene
Arginine and Proline Metabolism	Decreased	1 Gene
Arginine Biosynthesis	Decreased	1 Gene
Biosynthesis of Amino Acids	Decreased	1 Gene
Biosynthesis of Antibiotics	Decreased	1 Gene
Citrate Cycle	Decreased	1 Gene
Glycolysis/Glycogenesis	Decreased	1 Gene
Lipopolysaccharide Biosynthesis	Decreased	1 Gene
Microbial Metabolism in Diverse Environments	Decreased	1 Gene
Novobiocin Biosynthesis	Decreased	1 Gene
Pantothenate and CoA Biosynthesis	Decreased	1 Gene
Pentose Phosphate Pathway	Decreased	1 Gene
Phenylalanine Metabolism	Decreased	1 Gene
Protein Export	Decreased	1 Gene
Starch and Sucrose Metabolism	Decreased	1 Gene
Thiamine Metabolism	Decreased	1 Gene
Tyrosine Metabolism	Decreased	1 Gene
Beta-Lactam Resistance	Increased	2 Genes
ABC Transporters	Increased	1 Gene
Benzoate Degradation	Increased	1 Gene
Beta-Alanine Metabolism	Increased	1 Gene
Carbon Metabolism	Increased	1 Gene
Degradation of Aromatic Compounds	Increased	1 Gene
Fatty Acid Degradation	Increased	1 Gene
Fatty acid Metabolism	Increased	1 Gene
Fluorobenzoate Degradation	Increased	1 Gene
Propanoate Metabolism	Increased	1 Gene
Quorum Sensing	Increased	1 Gene
Two-Component System	Increased	1 Gene
Valine, Leucine and Isoleucine Degradation	Increased	1 Gene
Xylene Degradation	Increased	1 Gene

Table S3: Known pathways associated with Duplicate Genes Selected by TraDISort via Ortholog Analyses. Pathways are Listed in Order of Prevalence, Alphabetised from Top to Bottom.

Pathway	# Genes in Pathway	Mutant Abundance
Pyrimidine Metabolism	9	Decreased Insertion Densities in Both Pools
Homologous Recombination	7	Decreased Insertion Densities in Both Pools
2-Oxocarboxylic Acid Metabolism	6	Decreased Insertion Densities in Both Pools
Biotin Metabolism	6	Decreased Insertion Densities in Both Pools
Citrate Cycle	6	Decreased Insertion Densities in Both Pools
Carbon Metabolism	5	Decreased Insertion Densities in Both Pools
Glycine, Serine & Threonine Metabolism	5	Decreased Insertion Densities in Both Pools
Purine Metabolism	5	Decreased Insertion Densities in Both Pools
C5-Branched Diabasic Acid Metabolism	4	Decreased Insertion Densities in Both Pools
Pantothenate and CoA Biosynthesis	4	Decreased Insertion Densities in Both Pools
Porphyrin and Chlorophyll Metabolism	4	Decreased Insertion Densities in Both Pools
RNA Degradation	4	Decreased Insertion Densities in Both Pools
Cysteine and Methionine Metabolism	3	Decreased Insertion Densities in Both Pools

DNA Replication	3	Decreased Insertion Densities in Both Pools
Folate Biosynthesis	3	Decreased Insertion Densities in Both Pools
Oxidative Phosphorylation	3	Decreased Insertion Densities in Both Pools
Phenylalanine, tyrosine and tryptophan biosynthesis	3	Decreased Insertion Densities in Both Pools
Valine, Leucine & Isoleucine Biosynthesis	3	Decreased Insertion Densities in Both Pools
Alanine, Aspartate and Glutamate Metabolism	2	Decreased Insertion Densities in Both Pools
Amino Sugar and Nucleotide Sugar Metabolism	2	Decreased Insertion Densities in Both Pools
Beta-Lactam Resistance	2	Decreased Insertion Densities in One Pool Increased Insertion Densities One Pool
Butanoate Metabolism	2	Decreased Insertion Densities in Both Pools
Glutathione Metabolism	2	Decreased Insertion Densities in Both Pools
Glycerophospholipid Metabolism	2	Decreased Insertion Densities in Both Pools
Glyoxylate and Dicarboxylate Metabolism	2	Decreased Insertion Densities in Both Pools
Lysine Biosynthesis	2	Decreased Insertion Densities in Both Pools
Mismatch Repair	2	Decreased Insertion Densities in Both Pools
Peptidoglycan Biosynthesis	2	Decreased Insertion Densities in Both Pools
Propanoate Metabolism	2	Decreased Insertion Densities in Both Pools
Pyruvate Metabolism	2	Decreased Insertion Densities in Both Pools
Terpenoid Backbone Biosynthesis	2	Decreased Insertion Densities in One Pool Increased Insertion Densities in One Pool
Two-Component System	2	Decreased Insertion Densities in Both Pools
Vitamin B6 Metabolism	2	Decreased Insertion Densities in Both Pools
ABC Transporter	1	Decreased Insertion Densities in Both Pools
Aminoacyl-tRNA Biosynthesis	1	Decreased Insertion Densities in Both Pools
Arginine and Proline Metabolism	1	Decreased Insertion Densities in Both Pools
Ascorbate and Alderate Metabolism	1	Decreased Insertion Densities in Both Pools
Base Excision Repair	1	Decreased Insertion Densities in Both Pools
Biofilm Formation	1	Decreased Insertion Densities in Both Pools
Biosynthesis of Secondary Metabolites	1	Decreased Insertion Densities in Both Pools
D-Glutamine and D-Glutamate Metabolism	1	Decreased Insertion Densities in Both Pools
Fructose and Mannose Metabolism	1	Decreased Insertion Densities in Both Pools
Glycerolipid Metabolism	1	Decreased Insertion Densities in Both Pools
Glycolysis/Gluconeogenesis	1	Decreased Insertion Densities in Both Pools
Histidine Metabolism	1	Decreased Insertion Densities in Both Pools
Inositol Phosphate Metabolism	1	Decreased Insertion Densities in Both Pools

Lipoid Acid Metabolism	1	Decreased Insertion Densities in Both Pools
Nucleotide Excision Repair	1	Decreased Insertion Densities in Both Pools
One Carbon Pool by Folate	1	Decreased Insertion Densities in Both Pools
Pentose and Glucuronate Interconversions	1	Decreased Insertion Densities in Both Pools
Ribosome	1	Decreased Insertion Densities in Both Pools
Streptomycin Biosynthesis	1	Decreased Insertion Densities in Both Pools
Sulfur Metabolism	1	Decreased Insertion Densities in Both Pools
Thiamine Metabolism	1	Decreased Insertion Densities in Both Pools

Table S4: GO Terms and/or Pathways via Ortholog Analyses Assigned to Mutants with Transposon-insertions Resulting in Low Fluorescence

Locus Tag	Annotated Function	LogFC	Pathways/Go Term/s
BAL062_00055 *purC*	Phosphoribosylaminoimidazole-succinocarboxamide synthase	↓↓ -2.45938	PW: Purine metabolism. GT: Purine nucleotide biosynthetic process.
BAL062_00123 *hiuH*	5-hydroxyisourate hydrolase precursor	↓↓ -2.46425	PW: Purine metabolism. GT: Purine nucleobase metabolic process.
BAL062_00135 *pyrE*	Orotate phosphoribosyltransferase	↓↓ -2.22072	PW: Pyrimidine metabolism. GT: Pyrimidine nucleotide biosynthetic process.
BAL062_00148 *aceE*	Pyruvate dehydrogenase E1 component	↓↓ -2.23631	PW: Carbon metabolism, Citrate cycle, Glycolysis/Gluconeogenesis, Pyruvate Metabolism. GT: Glycolytic process.
BAL062_00149	Pyruvate/2-oxoglutarate dehydrogenase complex	↓↓ -2.16413	PW: Carbon metabolism, Citrate cycle, Glycolysis/Gluconeogenesis, Pyruvate metabolism. GT: Acetyl CoA biosynthetic process from pyruvate, Glycolytic process.
BAL062_00279 *rsmH*	Ribosomal RNA small subunit methyltransferase	↓↓ -3.40153	GT: rRNA base methylation, rRNA processing, Methylation
BAL062_00309 *rpoZ*	DNA-directed RNA polymerase subunit omega	↓↓ -11.2892	PW: Purine metabolism, Pyrimidine metabolism, RNA polymerase. GT: Protein complex assembly, Transcription, DNA-templated
BAL062_00390 *recG*	ATP-dependent DNA helicase recG	↓↓ -3.03228	PW: Homologous recombination. GT: DNA repair, DNA recombination, Cellular response to DNA damage stimulus, RNA secondary structure unwinding
BAL062_00684	Predicted small secreted protein, entericidin EcnA/B family	↓↓ -4.10598	GT: Response to toxic substances.
BAL062_00713 *mreB*	Rod shape-determining protein	↓↓ -5.38054	GT: Regulation of cell-shape, FtsZ-dependent cytokinesis, Negative regulation of cell division, Regulation of chromosome segregation, Cell Morphogenesis
BAL062_00805 *greA*	Transcription elongation factor	↓↓ -2.46782	GT: Regulation of DNA templated transcription, Elongation
BAL062_00813 *folP_1*	Dihydropteroate synthase	↓↓ -2.32500	PW: Folate biosynthesis. GT: Folic acid biosynthetic process, Response to drugs, Tetrahydrofolate biosynthetic process.
BAL062_00963 *Blc*	Outer membrane lipoprotein	↓↓ -2.47878	GT: Cellular response to DNA damage stimulus, Transport
BAL062_00983 *slyD*	Peptidyl-prolyl cis-trans isomerase	↓↓ -2.57101	GT: Response to heat, Protein maturation by protein folding.
BAL062_01069 *yhgn_1*	Multiple antibiotic transporter	↓↓ -2.82287	GT: Integral component of membrane.
BAL062_01082 *tdcB_2*	Threonine ammonia-lyase	↓↓ -6.18747	GT: Cellular amino acid biosynthetic process.
BAL062_01319 *ycgF*	Sensors of blue-light using FAD family protein	↓↓ -4.74874	GT: Blue light photoreceptor activity, FAD binding.

BAL062_01398 fabG_4	3-hydroxyacyl-CoA dehydrogenase	↓↓ -2.37896	PW: Isoleucine I degradation. GT: Oxioreductase activity.
BAL062_01439 ppiB	Peptidyl-prolyl cis-trans isomerase precursor	↓↓ -3.15277	GT: Protein peptidyl-prolyl isomerisation, Protein folding.
BAL062_01643 hslR	Ribosome-associated heat shock protein	↓↓ -5.45227	GT: Response to heat, RNA modification.
BAL062_01774	Fumarylacetoacetate (FAA) hydrolase family protein	↓↓ -2.45275	GT: Metabolic process, Catalytic activity
BAL062_01797 hmrR_1	Copper export regulator	↓↓ -2.76555	GT: Regulation of transcription, DNA-templated
BAL062_01811	Putative transcriptional regulator	↓↓ -2.49139	GT: DNA binding.
BAL062_01857	ADP-ribose pyrophosphatase	↓↓ -5.77701	GT: Hydrolase activity.
BAL062_01928 pqqC	Pyrroloquinoline quinone biosynthesis protein	↓↓ -2.0824	GT: Pyrroloquinoline quinone biosynthetic process.
BAL062_01929 pqqB	Pyrroloquinoline quinone biosynthesis protein	↓↓ -2.51903	GT: Pyrroloquinoline quinone biosynthetic process, Transport
BAL062_02054 iucA	Putative siderophore biosynthesis protein	↓↓ -2.3724	GT: Siderophore biosynthetic process.
BAL062_02074	Histidine triad protein,HIT domain	↓↓ -2.04237	GT: Catalytic activity
BAL062_02096 *exbD_2*	Biopolymer transport protein	↓↓ -6.40818	GT: Transporter activity.
BAL062_02129 *rsmG*	Ribosomal RNA small subunit methyltransferase	↓↓ -3.56231	GT: rRNA base methylation, rRNA processing.
BAL062_02141 *arsR*	Biofilm growth-associated repressor	↓↓ -4.84121	GT: Regulation of transcription, DNA templated.
BAL062_02148 *gcvH*	Glycine cleavage system protein H	↓↓ -2.70856	PW: Glycine, serine and threonine metabolism, Glyoxylate and dicarboxylate metabolism. GT: Glycine decarboxylation via glycine cleavage system.
BAL062_02168 *ruvC*	Holliday junction resolvase	↓↓ -2.34122	PW: Homologous recombination. GT: Recombinational repair, Response to radiation, Replication fork reversal, DNA repair.
BAL062_02174 *fimF*	Fimbrial protein	↓↓ -3.82833	GT: Cell adhesion.
BAL062_02230	Putative chromate transport protein	↓↓ -5.48494	GT: Transporter activity.
BAL062_02274 *yfcF*	Glutathione S-transferase	↓↓ -2.08432	PW: Glutathione metabolism. GT: Glutathione transferase activity.
BAL062_02283 *Dmlr_12*	Putative transcriptional regulator, D-malate degradation	↓↓ -2.88314	GT: Regulation of transcription, DNA templated.
BAL062_02288 *yecS*	Amino acid ABC transporter	↓↓ -9.39705	PW: ABC transporter. GT: Glutamine transport, Amino acid transmembrane transport.
BAL062_02293 *Lrp_6*	Putative transcriptional regulator	↓↓ -8.04465	GT: Regulation of transcription, DNA templated.
BAL062_02316 *fadR_1*	Fatty acid metabolism regulator protein	↓↓ -3.19516	GT: DNA binding.
BAL062_02577 *Vfr*	Cyclic AMP receptor protein	↓↓ -2.8354	PW: Quorum sensing, Two-component system. GT: Carbon catabolite repression of transcription.
BAL062_02646 *mltD*	Soluble lytic murein transglycosylase	↓↓ -4.14519	GT: Positive regulation of oxioreductase activity, Peptidoglycan metabolic process, Cell wall organisation.
BAL062_02657 *czcD_2*	Co/Zn/Cd efflux system	↓↓ -4.3218	GT: Cation transport, Transmembrane transport, Metal-ion binding
BAL062_02779	Hypothetical protein	↓↓ -6.81546	GT: DNA binding
BAL062_02825 *nahR*	Putative LysR family transcriptional regulator	↓↓ -2,89199	GT: Regulation of transcription, DNA templated.
BAL062_02990	putative phage TraR/DksA family protein	↓↓ -8.64309	GT: Zinc ion binding.
BAL062_03114 *rluD*	Ribosomal large subunit pseudouridine synthase D	↓↓ -2.42227	GT: Ribosomal large subunit assembly, Enzyme directed rRNA pseudouridine synthesis, rRNA processing, RNA modification.
BAL062_03143 *scpB*	*Segregation and condensation protein B*	*↓↓ -2.80849*	*GT: Chromosome segregation.*
BAL062_03323 *yqaA*	Inner membrane protein yqaA	↓↓ -2.10921	GT: Movement of cell or subcellular component.

BAL062_03342 *barA*	Signal transduction histidine-protein kinase	↓↓ -3.16187	PW: Two-component system. GT: Phosphorelay signal transduction system.
BAL062_03346 *ybeB*	Iojap-like ribosome-associated protein	↓↓ -2,74784	GT: Protein binding, Ribosomal large subunit binding.
BAL062_03363 *prfC*	Peptide chain release factor 3	↓↓ -2.19295	GT: Translational termination.
BAL062_03383 yibN_2	Rhodanese-related sulfurtransferase	↓↓ -2.31203	GT: Cytosolic protein.
BAL062_03473 topA	DNA topoisomerase 1	↓↓ -4.67745	GT: DNA topological change.
BAL062_03555 recB	Exodeoxyribonuclease V beta chain	↓↓ -3.16244	PW: Homologous recombination. GT: Double-strand break repair, DNA recombination.
BAL062_03654 phoU	Phosphate transport system regulatory protein	↓↓ -2.06747	GT: Regulation of cell growth, Cellular response to phosphate starvation, Negative regulation of ion transmembrane transporter
BAL062_03669	Protein FeoA,FeoA domain	↓↓ -2.96574	GT: Metal-ion binding
BAL062_03675 gacA	Response regulator	↓↓ -2.40045	PW: Biofilm formation, Two component system. GT: Phosphorelay signal-transduction system.
BAL062_03688 ribD	Riboflavin biosynthesis	↓↓ -11.3207	PW: Quorum sensing, Riboflavin metabolism. GT: Riboflavin biosynthetic process, RNA modification.
BAL062_03722 fadD_2	Long-chain-fatty-acid--CoA ligase	↓↓ -2.28378	PW: Fatty acid biosynthesis, degradation, and metabolism, Quorum sensing. GT: Lipid metabolism, fatty acid metabolism, Phospholipid biosynthetic process.
BAL062_03786 deaA_2	Rrotein DedA	↓↓ -2.13398	GT: Plasma membrane.
BAL062_03856 galU	UTP--glucose-1-phosphate uridylyltransferase	↓↓ -10.5205	PW: Amino sugar and nucleotide sugar metabolism, Galactose metabolism, Pentose and glucuronate interconversions, Starch and sucrose metabolism. GT: UDP-glucose metabolic process, Lipopolysaccharide core region biosynthetic process.
BAL062_03905 Azr	NADPH-dependent FMN reductase	↓↓ -3.23801	GT: Xenobiotic metabolic process, Oxidation-reduction process.
BAL062_03921 purK	N5-carboxyaminoimidazole ribonucleotide synthase	↓↓ -2.03284	PW: Purine metabolism. GT: Purine nucleotide biosynthetic process.
BAL062_03932 purE	N5-carboxyaminoimidazole ribonucleotide mutase	↓↓ -2.9215	PW: Purine metabolism. GT: Purine nucleotide biosynthetic process
BAL062_00479 fabG_2	Dehydrogenase,3-oxoacyl-[acyl-carrier-protein] reductase	↓↑ 2.21997	PW: Geraniol degradation. GT: rRNA base methylation.
BAL062_01322 marA	Multiple antibiotic resistance protein mar	↓↑ 2.01518	GT: Regulation of transcription, DNA-templated
BAL062_01425	Protein of unknown function (DUF441)	↓↑ 2.1097	GT: Integral component of plasma membrane.
BAL062_01585	Transposase	↓↑ 3.02126	GT: Transposition, DNA-mediated, Transposase activity.
BAL062_01652	Esterase/lipase,alpha/beta hydrolase fold	↓↑ 2.17087	GT: Metabolic process, Hydrolase activity.
BAL062_01767 catC	Muconolactone Delta-isomerase	↓↑ 2.30512	PW: Benzoate degradation, Degradation of aromatic compounds.
BAL062_01827	Uncharacterized protein conserved in bacteria	↓↑ 2.04891	PW: Quorum Sensing.
BAL062_02095 tolQ_2	MotA/TolQ/ExbB proton channel	↓↑ 2.15175	GT: Transporter activity.

- **A bold locus tag indicates a hypothetical gene product.**
- **PW = Pathway/s, GT = GO Term/s**
- **↓↓ = Decreased fluorescence + decreased insertion density, ↓↑ = Decreased fluorescence + increased insertion density**.

Table S5: GO Terms and/or Pathways via Ortholog Analyses Assigned to Mutants with Transposon-insertions Resulting in High Fluorescence

Locus Tag	Annotated Function	LogFC	Pathway/s and/or GO Term/s
BAL062_00126	S30EA ribosomal protein	↑↓-3.64668	GT: Primary metabolic process
BAL062_00235 *thrB*	Homoserine kinase	↑↓-3.16328	PW: Glycine, Serine & Threonine Metabolism.
BAL062_00249 *ypeA*	Ribosomal-protein-alanine acetyltransferase	↑↓-3.96426	GT: Peptide alpha-N-acetyltransferase activity.
BAL062_00257	Putative ABC transporter	↑↓-3.97284	GT: Transporter activity.
BAL062_00302 *pilA*	Tfp pilus assembly protein, major pilin PilA	↑↓-3.50611	GT: Pilus.
BAL062_00344	SAM-dependent methyltransferase, biotin biosynthesis protein bioC	↑↓-3.012	GT: Methyltransferase activity.
BAL062_00351 *astB*	N-succinylarginine dihydrolase	↑↓-5.22611	PW: Arginine and proline metabolism. GT: Arginine catabolic process, Arginine metabolic process.
BAL062_00375 *aroE*	Shikimate 5-dehydrogenase	↑↓-5.52109	PW: Phenylalanine, tyrosine and tryptophan biosynthesis. GT: Cellular amino acid biosynthetic process, Chorismate biosynthetic process
BAL062_00506	Sulfide dehydrogenase	↑↓-4.2776	GT: Hydrolase activity.
BAL062_00655 *mscL*	Large-conductance mechanosensitive channel	↑↓-4.16278	GT: Ion transport, Cellular water homeostasis.
BAK062_00714 *mreC*	Rod shape-determining protein	↑↓-11.0558	GT: Regulation of cell shape, Establishment or maintenance of cell polarity regulating cell shape.
BAL062_00842 *greB*	Transcription elongation factor GreB	↑↓-3.49312	GT: Oxidation-reduction process.
BAL062_00895 *yhhT*	Putative permease, pheromone autoinducer 2 transporter, sporulation integral membrane protein	↑↓-2.06614	GT: Transport activity.
BAL062_00908 *ExbD_1*	Biopolymer transport protein exbD, colicin uptake protein	↑↓-5.18843	GT: Protein stabilisation, Protein transport, Bacteriocin transport.
BAL062_00942 *lepA*	GTP-binding protein LepA, Elongation factor 4	↑↓-2.60691	GT: Translational elongation, Response to pH, Response to cold, Response to salt stress, Translation.
BAL062_00989 *Icd_2*	Isocitrate dehydrogenase	↑↓-2.09797	PW: Carbon metabolism, 2-oxocarboxylic acid metabolism, Citrate cycle, Glutathione metabolism. GT: Response to oxidative stress, Electron transport chain, Glyoxylate cycle, Tricarboxylic acid cycle, Oxidation-reduction process.
BAL062_00995 *yddE_1*	Phenazine biosynthesis protein	↑↓-3.22697	GT: Biosynthetic process.
BAL062_01001 *thiE*	Phenazine biosynthesis protein	↑↓-4.98165	PW: Thiamine metabolism.GT: Thiamine biosynthetic process, Thiamine diphosphate biosynthetic process.
BAL062_01023 *sohB*	SohB, Probable protease, putative inner membrane peptidase	↑↓-3.46107	GT: Proteolysis.
BAL062_01079	TetR regulator	↑↓-3.75804	GT: DNA binding
BAL062_01081 p*sdhT*	Putative threonine ammonia-lyase	↑↓-5.90849	PW: Carbon metabolism, Glycine, serine and threonine metabolism, Valine, leucine and isoleucine biosynthesis.
BAL062_01084 *viuB*	BauF, Vibriobactin utilization protein	↑↓-2.10079	GT: Oxidation-reduction process.
BAL062_01091 *bauE*	Ferric acinetobactin transport system	↑↓-2.34779	PW: ABC transporter. GT: Ferric-hydroxamate transport.
BAL062_01098 *Hdc*	Histidine decarboxylase	↑↓-3.57616	PW: Histidine metabolism.
BAL062_01237 *oprM_2*	Outer membrane efflux protein for the RND-type adeFGH efflux system	↑↓-2.1709	GT: Transport activity
BAL062_01248 *yhbU*	Protease, Uncharacterized protease YhbU precursor	↑↓-2.04348	GT: Proteolysis.
BAL062_01269 *aroA*	3-phosphoshikimate 1-carboxyvinyltransferase	↑↓-3.68526	PW: Novobiocin biosynthesis, Phenylalanine, tyrosine and tryptophan biosynthesis. GT: Cellular amino acid biosynthetic process, Chorismate biosynthetic process.
BAL062_01288 *sixA*	Phosphohistidine phosphatase	↑↓-3.84137	GT: Cellular protein modification process, Protein dephosphorylation.

BAL062_01298 *yqeY*	Yqey-like family protein	↑↓-4.16014	GT: Carbon-nitrogen ligase activity, with glutamine as amido-N-donor.
BAL062_01347 *yddG_2*	Permeases of the drug/metabolite transporter (DMT) superfamily	↑↓-4.42324	GT: Amino acid transport.
BAL062_01349 *tnpA_1*	IS6 family transposase,Integrase core domain	↑↓-7.67709	GT: DNA integration, Nucleic acid binding
BAL062_01430 *truC*	Pseudouridine synthase	↑↓-2.00408	GT: Pseudouridine synthesis, RNA modification
BAL062_01450 *hpcD*	Putative 5-carboxymethyl-2-hydroxymuconate delta isomerase	↑↓-5.60561	PW: Degradation of aromatic compounds, Tyrosine metabolism. GT: Aromatic compound catabolic process.
BAL062_01456 *ligE*	Putative glutathione S-transferase	↑↓-3.33626	PW: Glutathione metabolism. GT: Protein binding.
BAL062_01463	Probable dihydroxyacetone kinase regulator	↑↓-2.10635	GT: DNA binding.
BAL062_01495	TetR regulator	↑↓-10.3344	GT: DNA binding
BAL062_01580	Hypothetical protein	↑↓-2.86379	GT: Binding.
BAL062_01584	Transposase-like protein, Integrase core domain	↑↓-3.84273	GT: Transposition, DNA recombination, DNA integration.
BAL062_01593 *pdtaR*	Response regulator protein	↑↓-2.02557	GT: Phosphorelay signal-transduction system
BAL062_01622 *argF*	Ornithine carbamoyltransferase	↑↓-2.52284	PW: Biosynthesis of amino acids, Arginine biosynthesis, Biosynthesis of antibiotics.GT: Cellular amino acid biosynthetic process, Cellular amino acid metabolic process.
BAL062_01641 *recX*	RecX, recombination regulator	↑↓-11.037	GT: Regulation of DNA repair.
BAL062_01682	Putative oxidoreductase, Nitro reductase family	↑↓-9.10578	GT: Cellular response to oxidative stress.
BAL062_01684	Putative acetyltransferase	↑↓-3.91406	GT: N-acetyltransferase activity.
BAL062_01828 *iclR_2*	Transcriptional regulatory protein	↑↓-4.13706	GT: Regulation of transcription, DNA-templated
BAL062_01841 *gbpR*	LysR family regulator	↑↓-4.05473	GT: Regulation of transcription, DNA-templated
BAL062_01849 *yclQ_2*	Iron ABC transporter	↑↓-7.03726	PW: ABC transporter. GT: Cobalamin binding.
BAL062_01904 *ethR*	Transcriptional regulator	↑↓-9.21757	GT: DNA binding
BAL062_01905 *Puub_3*	Putative oxidoreductase, Glycine cleavage system T protein (aminomethyltransferase)	↑↓-2.39031	GT: Oxidation-reduction process.
BAL062_01938 *modE*	ModE molybdate transport repressor	↑↓-4.56112	GT: Molybdate ion transport.
BAL062_01941 *antC_1*	Anthranilate dioxygenase reductase	↑↓-6.67142	GT: Oxidation-reduction process.
BAL062_01943 *rhaS_2*	Putative transcriptional regulator,L-rhamnose operon regulatory protein rhaS	↑↓-4.51045	GT: Regulation of transcription, DNA-templated.
BAL062_01997 *aroC*	Chorismate synthase	↑↓-9.48188	PW: Phenylalanine, tyrosine and tryptophan biosynthesis. GT: Cellular amino acid biosynthetic process, Chorismate biosynthetic process.
BAL062_01998 *prmB*	50S ribosomal protein L3 glutamine methyltransferase	↑↓-9.6108	GT: Protein methylation, Peptidyl-glutamine methylation, DNA methylation on adenine.
BAL062_02008 *recR*	Recombination protein RecR	↑↓-2.69193	PW: Homologous recombination. GT: DNA repair, DNA recombination, Cellular response to DNA damage stimulus.
BAL062_02009 *ybaB*	YbaB/EbfC family DNA-binding protein	↑↓-5.77015	GT: DNA binding.
BAL062_02073 *cyaA*	Adenylate or guanylate cyclase	↑↓-3.74002	GT: Cyclic nucleotide biosynthetic process.
BAL062_02079	RraA,Putative regulator of ribonuclease activity	↑↓-10.9417	GT: Protein binding, Ribonuclease inhibitor activity.
BAL062_02080	Nucleoside-diphosphate-sugar epimerase	↑↓-11.2645	GT: Oxidation-reduction process
BAL062_02102 *hapE_1*	Flavoprotein, Flavin-binding monooxygenase-like	↑↓-5.86808	GT: Oxidation-reduction process.
BAL062_02110 *tadA*	Deaminase, tRNA-specific adenosine deaminase	↑↓-7.58751	GT: tRNA wobble adenosine to inosine editing, tRNA processing.

BAL062_02116	Tfp pilus assembly protein PilV	↑↓-9.39262	PW: Bacterial secretion system.
BAL062_02117 *pulG*	Putative general secretion pathway protein G	↑↓-10.5139	PW: Bacterial secretion system. GT: Protein secretion.
BAL062_02190	1-acylglycerol-3-phosphate O-acyltransferases	↑↓-3.13174	GT: Metabolic process.
BAL062_02234 *aseR*	Arsenite inducible repressor	↑↓-2.73568	GT: Regulation of transcription, DNA-templated.
BAL062_02272	Putative effector of murein hydrolase LrgA,holin-like protein	↑↓-2.44026	GT: Integral component of plasma membrane.
BAL062_02273 *gltC_4*	Regulatory protein	↑↓-2.13010	GT: Regulation of transcription, DNA-templated
BAL062_02334 *dcaC*	3-ketoacyl-ACP reductase, pyridoxal 4-dehydrogenase	↑↓-2.16183	GT: Oxioreductase activity.
BAL062_02344 *naiP_1*	Putative niacin/nicotinamide transporter	↑↓-2.07576	GT: Transmembrane transport.
BAL062_02349 *potA*	Spermidine/putrescine ABC transporter	↑↓-2.81708	GT: ATP binding atpase activity.
BAL062_02350	ABC transporter periplasmic substrate-binding protein	↑↓-4.49307	PW: ABC transporter.
BAL062_02433 *Lrp_7*	Putative leucine-responsive regulatory protein	↑↓-3.01617	GT: Regulation of transcription, DNA-templated.
BAL062_02527 *cspE*	Cold shock protein	↑↓-2.19289	GT: Transcription antitermination.
BAL062_02542 *hmrR_2*	HTH-type transcriptional regulator, Copper export regulator	↓↓↑↓-96944	GT: Cellular response to metal ions.
BAL062_02555 *ahpC_2*	alkyl hydroperoxide reductase C22 subunit	↑↓-2.34988	GT: Iron assimilation by chelation and transport, response to oxidative stress, Cell redox homeostasis, Cellular oxidant detoxification.
BAL062_02561 *gstB_1*	Putative glutathionine S-transferase	↑↓-4.42983	PW: Glutathione metabolism. GT: Glutathione transferase activity.
BAL062_02752	Putative stress-responsive nuclear envelope protein,HeH/LEM domain	↑↓-10.5512	GT: DNA-templated transcription, Termination.
BAL062_02821 *ureD*	Urease accessory protein	↑↓-2.17115	GT: Nitrogen compound metabolic process, Nickel cation binding
BAL062_02836	Activator of Hsp90 ATPase homolog 1-like protein	↑↓-8.86237	GT: Response to stress
BAL062_02837 *rubA*	anaerobic nitric oxide reductase	↑↓-11.3167	GT: Metal ion binding, Electron carrier activity.
BAL062_02846 *lolD_2*	ABC transporter, Lipoprotein-releasing system	↑↓-2.13376	GT: Transporter activity.
BAL062_02847 *tesA*	Multifunctional acyl-CoA thioesterase I/protease I/lysophospholipase	↑↓-5.12479	PW: Nicotinate and nicotinamide metabolism. GT: Proteolysis, Lipid metabolic process.
BAL062_02859 *pitA*	Phosphate/sulfate permease	↑↓-2.94817	GT: Phosphate ion transmembrane transport, Phosphate ion transport.
BAL062_02877 *nadX*	Putative L-aspartate dehydrogenase	↑↓-2.30425	PW: Nicotinate and nicotinamide metabolism. GT: NADP catabolic process, NAD biosynthetic process.
BAL062_02887 *ndoA*	Ferredoxin,Naphthalene 1,2-dioxygenase system	↑↓-4.73727	PW: Phenylalanine metabolism, Microbial metabolism in diverse environments, Degradation of aromatic compounds.
BAL062_02951 *umuC_4*	Nucleotidyltransferase/DNA polymerase involved in DNA repair	↑↓-2.3371	GT: DNA repair.
BAL062_03059 *smpA*	SmpA, Small protein A precursor, outer membrane biogenesis protein	↑↓-6.28806	GT: Gram-negative-bacterium-type cell wall outer membrane assembly, Response to antibiotic, Protein insertion into the membrane.
BAL062_03287 *hisG*	ATP phosphoribosyltransferase	↑↓-2.44223	PW: Histidine metabolism. GT: Histidine biosynthetic process, Cellular amino acid biosynthetic process.
BAL062_03369 *ilvC*	Ketol-acid reductoisomerase	↑↓-2.56404	PW: 2-oxocarboxylic acid metabolism, Pantothenate and CoA biosynthesis, Valine, leucine and isoleucine biosynthesis. GT: Cellular amino acid biosynthetic process.
BAL062_03412 *pilF*	Type 4 fimbrial biogenesis protein	↑↓-2.62437	GT: Protein binding
BAL062_03143 *rlmN*	Ribosomal RNA large subunit methyltransferase	↑↓-3.31806	GT: rRNA base methylation, tRNA modification.
BAL062_03435 *Tig*	Trigger factor	↑↓-2.07428	GT: Protein complex assembly, Chaperone mediated protein folding, Protein peptidyl-prolyl isomerisation, Cell cycle, Protein transport, Cell division.

BAL062_03480 *htrB_3*	HtrB, Lipid A biosynthesis lauroyl acyltransferase	↑↓-2.3973	PW: Lipopolysaccharide biosynthesis.
BAL062_03527	Glutathione-dependent formaldehyde-activating enzyme family protein	↑↓-4.20987	GT: Carbon-sulfur lyase activity.
BAL062_03540 *csaA*	tRNA-binding protein, export-related chaperone	↑↓-7.53146	GT: tRNA binding.
BAL062_03544 *priA*	Primosomal protein N, primosome assembly protein	↑↓-4.74322	PW: Homologous recombination. GT: DNA replication, DNA unwinding involved in DNA replication, Double strand break repair, DNA recombination, Response to gamma radiation, Response to antibiotic, Synthesis of RNA primer.
BAL062_03550 *corA_2*	Magnesium transport protein	↑↓-2.69175	GT: Metal ion transport
BAL062_03556 *recC*	Exodeoxyribonuclease V gamma chain, exonuclease V subunit gamma	↑↓-7.06019	PW: Homologous recombination. GT: DNA recombination, DNA repair, Cellular response to DNA damage stimulus, DNA duplex unwinding, Nucleic acid phosphodiester bond hydrolysis.
BAL062_03573 *rbfA*	Hypothetical protein, ribosome-binding factor	↑↓-2.68897	GT: Ribosomal small subunit binding, rRNA processing, Response to cold, Cellular response to DNA damage stimulus.
BAL062_03587 *rlmB_2*	tRNA/rRNA methyltransferase	↑↓-2.05722	GT: Enzyme directed rRNA '2' O-methylation, rRNA processing, RNA processing, Methylation.
BAL062_03670 *xerD_2*	Tyrosine recombinase XerD, site-specific tyrosine recombinase	↑↓-8.50035	GT: Plasmid maintenance, Response to radiation, Resolution of recombination intermediates, DNA recombination, Cell cycle, Chromosome segregation, DNA integration, Cell division.
BAL062_03671 dsbC_2	Protein disulphide isomerase II	↑↓-2.0055	GT: Cell-redox homeostasis, Oxidation-reduction process.
BAL062_03854 *Pgi*	Glucose-6-phosphate isomerase	↑↓-9.44996	PW: Carbon metabolism, Amino sugar and nucleotide sugar metabolism, Glycolysis and gluconeogenesis, Pentose phosphate pathway, Starch and sucrose metabolism. GT: Glycolytic process, Cellular response to oxidative stress, Gluconeogenesis.
BAL062_03862	Putative polysaccharide biosynthesis protein	↑↓-11.1155	GT: Integral membrane protein.
BAL062_03903 *lspA*	Prolipoprotein signal peptidase	↑↓-7.87389	PW: Protein export. GT: Lipoprotein biosynthetic process via signal peptide cleavage, Proteolysis.
BAL062_03904 *fkpB*	FKBP-type peptidyl-prolyl cis-trans isomerase	↑↓-4.09372	GT: Chaperone-mediated protein folding.
BAL062_03939 *trmE*	tRNA modification GTPase	↑↓-7.73789	GT: tRNA wobble uridine modification, Response to pH, tRNA methylation, tRNA processing.
BAL062_03942 *RnpA*	Ribonuclease P protein component	↑↓-8.23283	GT: tRNA 5' leader removal, RNA processing, tRNA processing, Nucleic acid phosphodiester bond hydrolysis, Endonucleolytic.
BAL062_00599 *psiE*	Phosphate-starvation-inducible protein	↑↑ 2.04705	GT: Cellular response to phosphate starvation.
BAL062_00757 *oprM_1*	Outer membrane efflux protein	↑↑ 2.08502	PW: Beta-lactam resistance, Quorum sensing. GT: Transport activity.
BAL062_01292 *cvpA*	Putative membrane protein required for colicin V production	↑↑ 2.25636	GT: Toxin biosynthetic process.
BAL062_01420	VIC family potassium channel protein	↑↑ 2.24103	GT: Potassium ion transport
BAL062_01451 *roB*	DNA gyrase inhibitor	↑↑ 2.11943	GT: Regulation of transcription, DNA-templated.
BAL062_01496 *smvA*	MFS superfamily permease	↑↑ 3.46703	GT: Transmembrane transport
BAL062_01753 *cbdB*	Putative Aromatic-ring-hydroxylating dioxygenase small subunit	↑↑ 3.34238	GT: Cellular aromatic compound metabolic process, Oxidation-reduction process.
BAL062_01801	Metal-dependent hydrolase,Beta-lactamase superfamily domain	↑↑ 2.1373	GT: Zinc ion binding, N-acylphosphatidylethanolamine-specific phospholipase D activity
BAL062_01850	ABC-type Fe3+-siderophore transport system	↑↑ 2.17674	PW: ABC transporter. GT: Ferric-hydroxamate transport.
BAL062_01895 *adeS*	AdeS, Signal transduction histidine-protein kinase	↑↑ 3.18774	PW: Beta-lactam resistance.

BAL062_01933 *dhbB_2*	Isochorismate hydrolase	↑↑ 2.2956	GT: Isochorismatase actibity.
BAL062_02059	Acyl-CoA dehydrogenase	↑↑ 2.08571	PW: Carbon metabolism, Beta-alanine metabolism, Fatty acid degradation, Fatty acid metabolism, Propanoate metabolism, Valine, leucine and isoleucine degradation. GT: Oxioreductase activity, acting on the ch-ch groups of donors.
BAL062_02235 *arsC*	Arsenate reductase	↑↑ 2.55822	GT: Cellular response to DNA damage stimulus, Response to arsenic-containing substance, Oxidation-reduction process.
BAL062_02400	DMT superfamily, carboxylate/amino acid/amine transporter	↑↑ 2.48085	GT: Integral component of membrane.
BAL062_02414 *Gltr_3*	Chromosome replication initiation inhibitor protein ,transcriptional regulator	↑↑ 2.07352	GT: Regulation of transcription, DNA-templated.
BAL062_02428 *yxaF_4*	Mycofactocin system transcriptional regulator	↑↑ 2.1143	GT: DNA binding.
BAL062_02528 *rhtB_2*	Homoserine/homoserine lactone efflux protein	↑↑ 2.48247	GT: Amino acid transport
BAL062_02547 *benB*	Benzoate 1,2-dioxygenase subunit beta	↑↑ 2.44549	PW: Degradation of aromatic compounds, Benzoate degradation, Fluorobenzoate degradation, Xylene degradation.
BAL062_03207 *rstA*	Transcriptional regulatory protein RstA	↑↑ 2.31084	PW: Two-component system. GT: Phosphorelay signal-transduction system, Transcription, DNA-templated.

- **A bold locus tag indicates a hypothetical gene product**
- **PW = Pathway/s**
- **GT = GO Term/s**
- **↑↑ = Increased fluorescence and increased insertion density, ↑↓ = Increased fluorescence and decreased insertion density**

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