CHAPTER 3

INVESTIGATION OF THE HUMAN PATHOGEN *ACINETOBACTER BAUMANNII* UNDER IRON-LIMITING CONDITIONS

3.1 Introduction

Free iron is a limited micronutrient in many environments where it is typically tightly bound within a range of biomolecules, such as heme. As such, iron acquisition systems are important factors for the virulence of pathogenic organisms. Bacteria can adapt to iron-limited host environments through the expression of a range of iron acquisition mechanisms. One pathway for the uptake of iron involves direct binding of Fe^{2+} or heme to receptors or transport proteins on the cell surface (Koster 2005). A second more energy intensive mechanism of iron uptake involves the production and secretion of high-affinity iron chelating siderophores, which compete with host cells for iron (Section 1.2.1.2). The genes involved in the production of a siderophore are usually clustered within the genome of the producing organism. In addition to biosynthesis genes, many of these gene clusters also encode efflux pumps with putative roles in siderophore export. Transporters classified within the ABC superfamily, MFS and RND superfamilies have been associated with siderophore extrusion (Section 1.3.4).

The uptake and reduction of iron-loaded ferric siderophores involves the TonB-ExbB-ExbD energy transduction system in combination with a ferric siderophore complex receptor (Section 1.2.1.2). Bacteria often contain numerous ferric siderophore complex receptors. Some of these are encoded within siderophore biosynthesis gene clusters and are likely to display specificity for the locally encoded siderophore. However, various other ferric siderophore receptors can be scattered throughout the genome and may have the ability to recognise exogenously produced siderophores that are structurally unrelated to endogenous siderophores (Plessner *et al.* 1993; Poole and McKay 2003). A diversity in the number of iron acquisition mechanisms has been shown between different *Acinetobacter* strains. To date, three different siderophore biosynthesis gene clusters have been described in *A. baumannii*, of which the acinetobactin biosynthesis cluster has been studied most extensively (Section 1.2.1.3).

To comprehensively identify mechanisms of iron acquisition and low-iron adaptation in *A. baumannii*, the affect of iron starvation on *A. baumannii* cells was investigated at the global level. Transcriptomic responses of *A. baumannii* ATCC 17978 cells to low-iron conditions were examined using a whole genome microarray. The observations made at a genetic level were also studied using phenotypic assays.

3.2 Results and Discussion

3.2.1 Optimisation of test conditions for transcriptomic analysis

Iron is an essential micronutrient and depletion in the growth medium is likely to have an impact on cell viability. Therefore, growth of *A. baumannii* ATCC 17978 was investigated under varying iron concentrations to determine optimal conditions for whole transcriptome analysis. Reduction of available iron in MH medium was achieved by supplementation with 2,2'-dipyridyl, a synthetic iron chelator. Addition of this compound had no effect on the pH of the medium (data not shown). No significant change in the growth rate of *A. baumannii* strain ATCC 17978 was observed after addition of 100 µM 2,2'-dipyridyl, whereas, supplementation with 200 µM 2,2'-dipyridyl resulted in a growth delay of approximately 45 minutes at mid-log phase (OD_{600} = 0.7) (Figure 3.1). Moreover, the total biomass was reduced by more than 10% at stationary phase (>240 minutes). Addition of 300 µM 2,2'-dipyridyl had a major impact on growth and resulted in more than 70% biomass reduction compared to cultures under iron-replete conditions during stationary phase. Due to the moderate inhibitory, but non-lethal, effect of 200 µM 2,2'-dipyridyl, this concentration was chosen to study transcriptional changes under iron-limitation. Preliminary qRT-PCR (Section 2.4.15) was performed to confirm transcriptional adaptation in response to iron-limitation by assaying the level of transcription of *FUR* (A1S_0895) using oligonucleotides designed to A1S_0895 (Table 2.4). The genes *16srDNA* (A1S_r01) and *gapdh* (A1S_2501) were used as reference genes (Section 2.4.15) for which the oligonucleotide sequences can be found in Table 2.4. The protein encoded by *FUR* is known for auto-regulation when iron is limited as it contains a FUR binding site upstream of the gene (Daniel *et al.* 1999) (Section 1.2.1.3). Indeed, more than 2-fold increase in transcription of *FUR* was observed when *A. baumannii* ATCC 17978 cells were incubated with 200 µM 2,2'-dipyridyl as compared to ATCC 17978 cells grown without addition of 2,2'-dipyridyl (data not shown). Therefore, the genome wide transcriptional changes of ATCC 17978 grown in MH medium, and MH supplemented with 200 µM 2,2'-dipyridyl during mid log-phase, were compared by microarray analysis (Section 2.5).

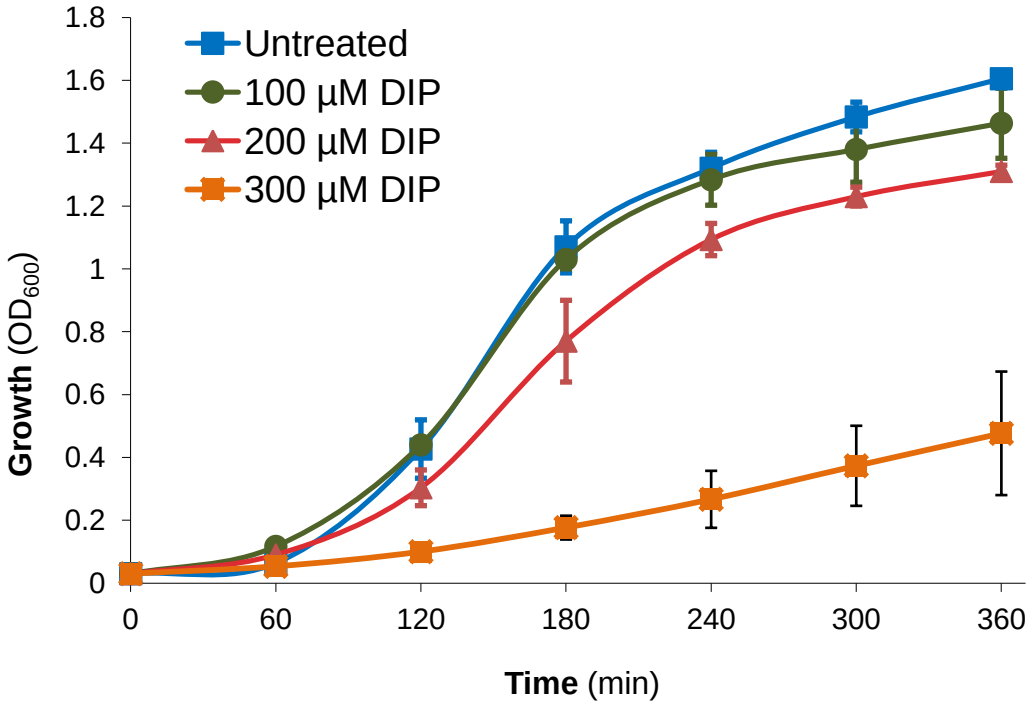

Figure 3.1: Growth curves of *A. baumannii* strain ATCC 17978 with varying iron concentrations

Growth under different iron concentrations was tested in MH broth (blue) and MH supplemented with 2,2'-dipyridyl (DIP) to final concentrations of 100 (green), 200 (red) and 300 µM (orange). Absorbance was measured every hour at OD_{600} for 6 hours; the data represent the average of three separate experiments and the error bars show the standard deviation.

3.2.2 Global transcriptional changes of *A. baumannii* ATCC 17978 to iron starvation

Iron limitation was found to have far-reaching transcriptional effects on *A. baumannii* cells (Figure 3.2). Significance analysis of the microarray data (Tusher *et al.* 2001) (Section 2.5) showed that 1207 genes were significantly differentially expressed under iron-limiting as compared to iron-replete conditions (Appendices A and B). Transcript levels were more than 2-fold higher for 463 genes, of which 95 genes were up-regulated more than 4-fold ($Log_2 \geq 2$) (Figure 3.2). The maximum overexpression observed was 165-fold for the siderophore biosynthesis gene *basD*. Fewer genes were down-regulated under iron-limitation; only 202 genes were more than 2-fold underexpressed with a maximum down-regulation of 29-fold (A1S_2297). The microarray results were validated by qRT-PCR analysis (Section 2.4.15) of a subset of differentially-expressed genes A1S_1647, A1S_2565, A1S_2562, A1S_2080, A1S_0895, A1S_3371, A1S_3420, A1S_0897, A1S_3195, A1S_2305, A1S_1509 and A1S_1925 (Table 3.1), using the oligonucleotides listed in Table 2.4. These 12 genes were selected based on their level of differential expression, ranging from heavily down-regulated to heavily up-regulated as determined by the microarray. A strong correlation between data from the qRT-PCR and the microarray analyses was observed (>0.99), although, the qRT-PCR data generally showed higher fold changes than the microarray expression data. The tendency for microarrays to underestimate fold changes relative to qRT-PCR is well established (Dallas *et al.* 2005).

Microarray data displayed by clusters of orthologous groups (COG) functional categories showed that 27% of the genes encoding proteins involved in secondary metabolite biosynthesis, transport and catabolism, were significantly up-regulated under iron-limited conditions (Figure 3.3). The majority of these genes are located within three large overexpressed gene clusters, each of which is known or predicted to synthesise a siderophore (Section 3.2.4; Figure 3.2). Siderophore cluster 1 (A1S_1647-1657) is a novel putative siderophore gene cluster, having not been previously identified. The two other highly overexpressed siderophore clusters identified, siderophore cluster 2 (A1S_2562-2581) and the acinetobactin cluster (A1S_2372-2392), have been described previously (Dorsey *et al.* 2003b; Zimbler *et al.* 2009). Many other overexpressed genes within this COG category encode ferric siderophore receptors, which are widely dispersed across the genome. In addition to

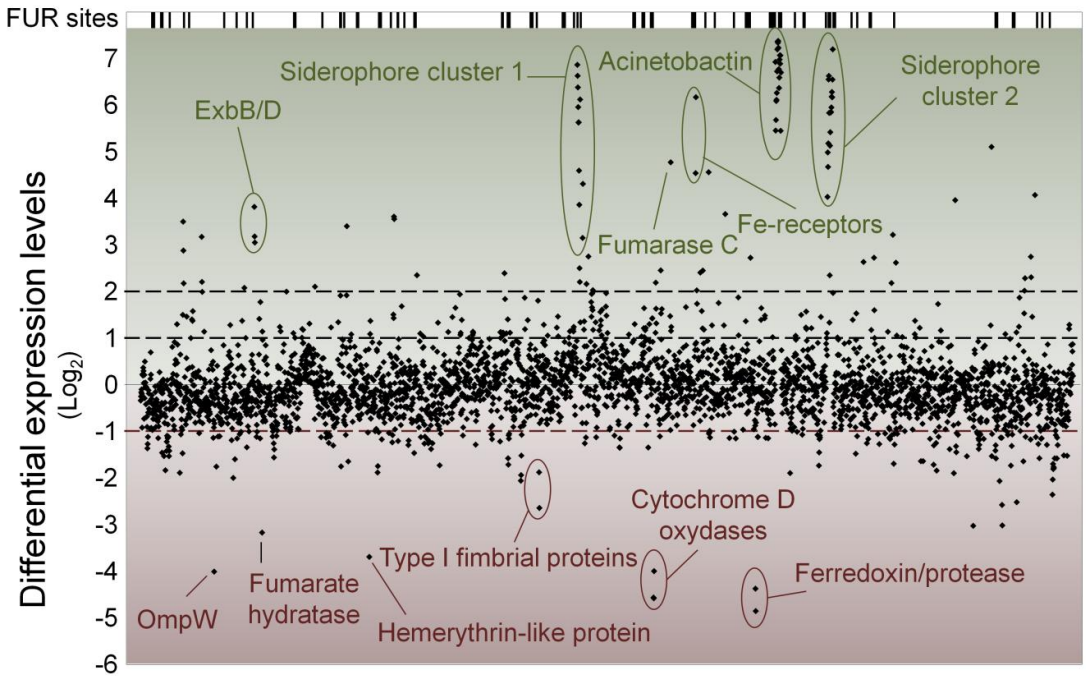

Figure 3.2: Overview of transcriptional responses of *A. baumannii* strain ATCC 17978 to iron starvation

The *A. baumannii* ATCC 17978 transcriptome was compared under iron-limiting (200 µM 2,2'-dipyridyl) and iron-replete conditions (Section 3.2.1). All 3367 genes of the *A. baumannii* ATCC 17978 genome were analysed using a microarray and are represented on the X-axis, ordered according to locus-tag (Smith *et al.* 2007). Differential expression levels between iron-replete and iron-limiting conditions are displayed in Log_2-values on the Y-axis. Up- and down-regulated genes under iron-limiting conditions are displayed in the green and red sections, respectively. The dashed lines indicate the level of differential expression; from top to bottom, 4-fold up-regulation (Log_2=2), 2-fold up-regulation (Log_2=1) and 2-fold down-regulation (Log_2=-1). Putative gene functions have been provided for various highly differentially expressed genes, such as the siderophore biosynthesis genes and fumarases. The putative FUR binding sites displayed in the top section were identified as described in Section 2.7.3 and the nucleotide sequences are listed in Appendix C.

Table 3.1: Validation of the transcriptomic data by comparison of expression levels determined by microarray and qRT-PCR analysis

Locus-tag	**Microarray** (Log_2)	**qRT-PCR (**Log_2)
A1S_1647	6.87	8.29
A1S_2565	5.18	6.05
A1S_2562	4.03	5.37
A1S_2080	2.03	2.64
A1S_0895	1.37	2.24
A1S_3371	-0.13	-0.11
A1S_3420	-0.43	-1.08
A1S_0897	-0.72	-1.55
A1S_3195	-0.96	-0.74
A1S_2305	-1.11	-1.65
A1S_1509	-1.87	-0.87
A1S_1925	-4.57	-6.10

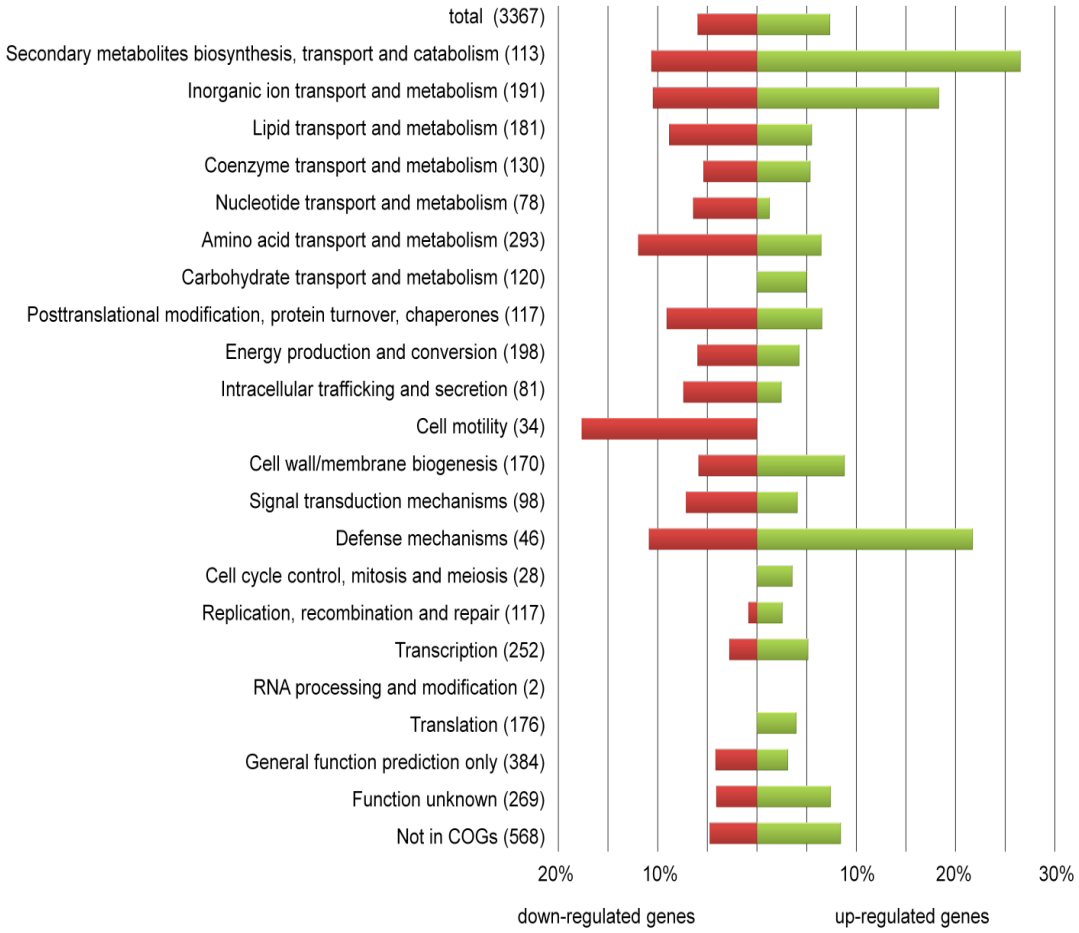

Figure 3.3: Microarray results of *A. baumannii* strain ATCC 17978 under iron-limiting and iron-replete conditions displayed by COG function

The statistical examination of the differential gene expression was analysed by significance analysis of the microarray (Tusher *et al.* 2001). The figure depicts the cluster of orthologous groups (COG) and the percentage of up-regulated (green) and down-regulated genes (red) under iron-limiting conditions within each group as determined by microarray. The total number of genes per COG is shown in parentheses. Three COG groups contained a large number of up-regulated genes; secondary metabolites biosynthesis, transport and catabolism, inorganic ion transport and metabolism, and defence mechanism. Various genes in the cell motility COG group were found to be significantly down-regulated.

siderophore-related genes, a high percentage, 22%, of genes up-regulated under iron-limitation, encoded proteins categorised within the defence mechanism COG. A number of these genes encode transporter proteins that are classified as defence proteins due to their predicted roles in the export of metabolic waste or other toxic compounds. However, analysis of several of these proteins, primarily those encoded within siderophore biosynthetic loci, suggested that they may function in the extrusion of siderophores (Hassan *et al.* 2011).

Various genes related to cell motility were down-regulated when *A. baumannii* was grown under iron-limiting conditions (Figure 3.2). These included biosynthesis genes homologous to both type IV pili and chaperone-usher pili assembly systems, or type I pili. Another heavily down-regulated gene encoded a hemerythrin-like protein (A1S_0891). Various functions have been suggested for these proteins with an iron-containing centre, including detoxification, transport and storage of iron and/or oxygen, or a role as a sensory protein (French *et al.* 2008). The cytochrome D genes, part of the respiratory system, and 4Fe-4S-ferredoxin, which facilitates electron transport in various metabolic processes, were also down-regulated more than 4-fold. The iron-dependent Class I fumarate hydratase was found to be 9-fold down-regulated. In contrast, fumarase C, which belongs to the iron-independent Class II was 27-fold up-regulated, suggesting a physiological shift to the Class II protein under iron-limitation (Figure 3.2). These findings correlated with results observed in a study on fumarase A (Class I) and fumarase C in the tri-carboxylic acid cycle of *Pseudomonas fluorescens* (Chenier *et al.* 2008). In conclusion, *A. baumannii* showed strong transcriptional responses to iron starvation, predominantly in up-regulation of iron acquisition mechanisms. However, many genes related to other processes than iron acquisition, such as respiration and motility, were also transcriptionally affected under the conditions tested.

3.2.3 *A. baumannii* FUR box optimisation

Bioinformatic analyses were performed to identify motifs within the promoter regions of iron-responsive genes that could serve regulatory functions. The multiple em (expectation maximisation) for motif elicitation (MEME) tool (Bailey and Elkan 1994) and the multiple alignment and search tool (MAST) (Bailey and Gribskov 1998) were used to identify motifs and search for these motifs across the *A. baumannii* ATCC 17978 genome, respectively (Section 2.7.3).

MEME-based analyses of the upstream regions of all genes overexpressed by at least 4-fold under iron limitation identified a motif bearing strong similarity to the FUR binding sites of *E. coli* and *Pseudomonas* spp. (Hassan *et al.* 2010). To confirm the importance of FUR in regulating iron-adaptation responses in *A. baumannii*, a scoring matrix was created using the experimentally determined *E. coli* FUR binding sites (Stojiljkovic *et al.* 1994). This scoring matrix was used to screen the ATCC 17978 genome using MAST. Hits obtained using MAST, which were found upstream of genes that were more than 4-fold up-regulated in response to iron-limitation and with a p-value less than $10E^{-5}$ (Section 2.7.3), were selected for iterative refinement of the scoring matrix, of which examples have been marked in Appendix C. This process was repeated until no new hits were obtained, the final results have been listed in Table 3.2. The resulting *A. baumannii* FUR box motif (Section 2.7.3) showed a 25 nucleotide palindromic sequence (Figure 3.4).

In a previous study, the upstream regions of *FUR* genes from different *A. baumannii* isolates were aligned to obtain a motif representing the FUR binding site (Daniel *et al.* 1999). Some differences exist between this sequence and the FUR box sequence shown in Figure 3.4. Most notably, the previously described motif lacked the typical FUR box palindromic structure. FUR is known to auto-regulate its own expression, however, as seen in this study, up-regulation of *FUR* under iron-limiting conditions is at lower levels (2.6-fold) than that of other FUR regulated genes, e.g., siderophore biosynthesis genes (>100-fold), a phenomenon that could reflect a lower binding affinity of the FUR protein for the *FUR* promoter region (Madsen *et al.* 2006; Ochsner *et al.* 2002; Palyada *et al.* 2004; Paustian *et al.* 2001). Therefore, the FUR binding sequences found upstream of the *FUR* gene may have predicted a less than optimal *A. baumannii* FUR box consensus motif, which does not show the typical palindromic structure. Moreover, FUR motifs of different bacterial genera show a high level of homology, whereas the previously described *A. baumannii* FUR motif is more distant. A MAST search (parameters; E<100, $p<10E^{-4}$) using the optimised *A. baumannii* FUR motif showed 81 hits to the *A. baumannii* ATCC 17978 genome (Appendix C). Over 80% of the genes with a well conserved FUR box upstream ($p<10E^{-5}$) showed more than 2-fold up-regulation (Appendix C). These studies highlight a significant correlation between the level of conservation of a putative FUR binding site and the level of up-regulation under iron-limiting conditions.

Table 3.2: Putative FUR binding sequences in the *A. baumannii* ATCC 17978 genome used for generating the FUR box motif

Locus-tag	Putative FUR binding sequence (5'-3')
A1S_0242	TTATTTGGTAATTATTCTCATTTAT
A1S_0416	GGATTTGTTAATGATTATCATTTGC
A1S_0474	GCGAATAATAATAATTCTTATTTAT
A1S_0980	GATATTGTTAATAATTATCATTATT
A1S_1647	TGAAATGATAATAATTATCATTAAT
A1S_1657	ATAATTGATAATGATAATCATTTTT
A1S_1667	GATAATGTAAATAATTCTCATTTAT
A1S_2077	TCATTTGATACTGATTATCAATATT
A1S_2080	ATAAATGAGAATGATTTTAATTAAT
A1S_2123	GATAATAAGAATTATTTTTATTTGT
A1S_2278	TTATTTGATAATGATTTTCATTTAT
A1S_2372	GTTATTGATAATAATAATCATTTGC
A1S_2382	GCAACTGGTAATCATTTTCATTTGT
A1S_2391	GTAATTGTAAATGATTATCATTTAT
A1S_2392	GTAAATAATAATCATTATTAATTGT
A1S_2567	TTACTTGAGAATGATTCTTGATAAC
A1S_2581	TTAAATGAGAATCATTTTCATTTAT
A1S_2582	TTAAATGAGAATCATTTTCATTTAT
A1S_2667	TTTTTTGAGAATTATTATTGATTAT
A1S_3174	ATTATTGATAATTATTATCGTTTGT
A1S_3324	GATAATGAGAATTATTTTAATTTAT
A1S_3339	TTAAATGATTATAATTATCATTTAT

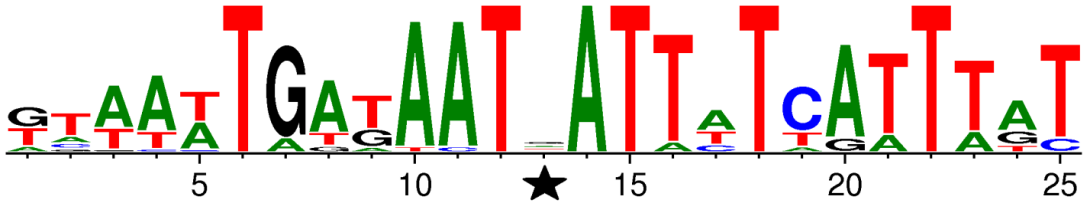

Figure 3.4: The optimised *A. baumannii* ATCC 17978 FUR motif

Per position, the size of the nucleotide (T in red, A in green, C in blue and G in black) indicates its prevalence in the 22 included sequences from Table 3.2. The motif shows a palindrome with a central non-conserved nucleotide in position 13, which is indicated by the asterisk. The figure of the *A. baumannii* ATCC 17978 FUR motif was created using WebLogo 3.0 (Crooks *et al.* 2004).

Extracytoplasmic function (ECF) transcription factors are σ^{70} family proteins that are responsive to environmental changes such as iron starvation (Helmann 2002). These proteins play an important role in regulating iron uptake mechanisms in several bacterial genera. For example, one of the best characterised ECF sigma factors, PvdS, controls the genes required for biosynthesis and transport of the siderophore pyoverdine in *P. aeruginosa* (Potvin *et al.* 2008). Expression of *pvdS* and various other σ^{70} factors in *Pseudomonas* is regulated by FUR (Potvin *et al.* 2008). However, in this study no predicted σ^{70} factors were identified in the list of genes containing a putative *A. baumannii* FUR binding site (Appendix C). Moreover, no significant differentially expressed σ^{70} factors were found under the iron-limited conditions (data not shown), suggesting that in *A. baumannii* strain ATCC 17978, these proteins do not function in iron uptake regulation. Another regulatory mechanism involved in iron homeostasis includes small RNA molecules, such as *ryhB* from *E. coli* or *prrF* from *P. aeruginosa.* However, sequences homologous to either of these small RNAs were not found in the *A. baumannii* ATCC 17978 genome. Nonetheless, a role for small RNAs in iron homeostasis cannot be ruled out, since the *A. baumannii* ATCC 17978 genome contains a gene encoding the RNA chaperone Hfq (A1S_3785), which is required for the functionality of numerous small RNAs involved in iron homeostasis in various Gram-negative bacteria (Gottesman 2004; Masse *et al.* 2003). The results of this study suggest that FUR is the primary regulator of iron uptake in *A. baumannii*.

3.2.4 Transcriptional profiling of the siderophore-mediated iron acquisition mechanisms

Genes involved in the biosynthesis, efflux and uptake of a siderophore are often clustered within bacterial genomes (Section 1.2.1.2). To date, three putative siderophore gene clusters have been identified in *A. baumannii* (Dorsey *et al.* 2003b; Mihara *et al.* 2004; Zimbler *et al.* 2009), of which two can be found in strain ATCC 17978. A significant finding from the microarray results was the detection of a novel putative *A. baumannii* siderophore gene cluster (Figure 3.2A). Several genes within siderophore cluster 1 were more than 100-fold over-produced under iron-limitation, highlighting their potential importance in iron uptake (Figure 3.5). Siderophore cluster 1 contains eight genes with a putative function in siderophore biosynthesis, A1S_1647, A1S_1648, A1S_1650-1654 and A1S_1657. Siderophore extrusion is most likely facilitated by an MFS efflux pump (A1S_1649). A receptor (A1S_1655)

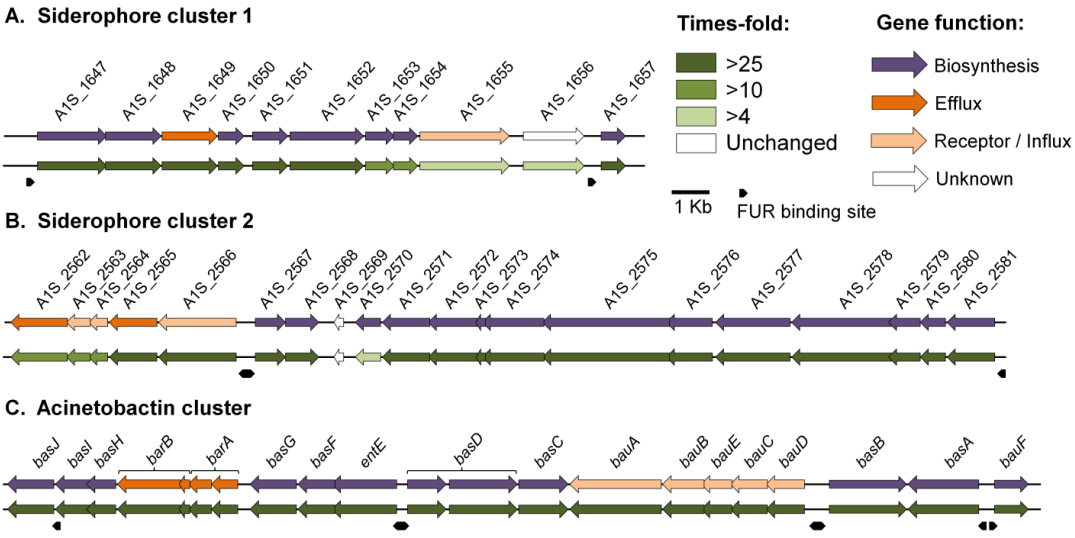

Figure 3.5: Transcriptional profiling of three siderophore gene clusters identified in *A. baumannii* strain ATCC 17978

Transcriptional alteration of the three siderophore gene clusters to low-iron conditions are shown, (A) siderophore gene cluster 1 (A1S_1647-1657), (B) siderophore cluster 2 (A1S_2562-2581) and (C) the acinetobactin gene cluster (A1S_2372-2392). The top arrows show predicted gene function; siderophore biosynthesis in purple, receptors and uptake mechanisms in light orange, efflux pumps in orange and genes of unknown function in white. The relative transcriptional differences between *A. baumannii* grown under iron-replete and iron-limiting conditions are depicted in the bottom set of arrows according to the green color scale bar, all values are in times-fold difference. Genes depicted in white were not differentially expressed and those in dark green were overexpressed more than 25-fold. No significant down-regulation was observed within the siderophore gene clusters. Putative FUR boxes are shown as black arrows.

and PepSY-associated transmembrane helix family protein (A1S_1656) are likely to be involved in recognition and reduction of ferric siderophores, respectively. FUR boxes for transcriptional regulation of the unidirectional, operon-like gene cluster could be identified upstream of A1S_1647 and A1S_1657 (Figure 3.5A). Siderophore gene cluster 2 (A1S_2562-2581) (Zimbler *et al.* 2009) showed similar high levels of overexpression as cluster 1 (Figure 3.5B). This cluster contains 15 genes involved in siderophore biosynthesis (A1S_2567-2581), three genes involved in recognition and uptake of the ferric siderophore (A1S_2563, A1S_2564 and A1S_2566) and two genes encoding putative efflux pumps. One efflux pump gene, A1S_2565, encodes a putative MFS efflux pump. As mentioned previously, members of this family have been identified in various siderophore gene clusters in other bacteria and have proven to play a role in the efflux of enterobactin (Furrer *et al.* 2002). The second efflux pump, A1S_2562 is a member of the MATE family (Sections 1.3.4.4 and 6.2.1). This is the first report of a bacterial MATE pump having a putative role in siderophore efflux. Similar to siderophore cluster 1, FUR appears to be the main transcriptional regulator, since binding sites could be identified upstream of A1S_2566, A1S_2567 and A1S_2581 (Figure 3.5B). The most extensively characterised *Acinetobacter* siderophore gene cluster is that responsible for the biosynthesis of acinetobactin (Mihara *et al.* 2004). Acinetobactin is synthesised from 2,3-dihydroxybenzoic acid, threonine and hydroxyhistamine, and contains catecholate and hydroxamate groups that provide a high affinity for iron (Yamamoto *et al.* 1994). The acinetobactin biosynthesis genes include *basA-D*, *basF-J* and *entE* (Figure 3.5C). Gene pair *barA* and *barB* encodes a siderophore efflux system of the ABC superfamily, the products of *bauA-F* form a receptor for recognition of ferric acinetobactin and the products of *bauB-E* are involved in translocation of ferric acinetobactin (Figure 3.5C). All of these genes showed high levels of overexpression, ranging from 43-fold to 165-fold. Putative FUR boxes upstream of *basJ*, *entE*/*basD*, *basA*/*bau* and *bauD*/*basD* could also be identified. These binding sites have also been experimentally identified using a FUR titration assay (Mihara *et al.* 2004), validating the FUR motif analysis described here. Although, most noticeable in siderophore cluster 1 (Figure 3.5A), transcriptional up-regulation gradually decreased in all three siderophore clusters when distance from the FUR box increased. This finding demonstrated the importance of FUR in regulating siderophore production at the level of gene transcription. This is the first time that a full transcriptional profile has been provided for the siderophore gene

clusters in *A. baumannii* under iron-limiting conditions. Most importantly, a novel putative siderophore gene cluster was identified.

Siderophore receptors expressed on the surface of the bacterial outer membrane play a crucial role in the recognition of iron-loaded siderophores and therefore iron uptake. These receptors are likely to recruit the TonB-ExbB-ExbD translocation system for transport of ferric siderophores from the extracellular space to the cytoplasm (Braun 1995; Braun 2003). The *A. baumannii* ATCC 17978 genome contains 20 putative siderophore receptors of which 13 were significantly up-regulated under iron-limiting conditions (Table 3.3). Analysis demonstrated that 14 of these are located downstream of a putative FUR box.

Under iron-limited conditions, high levels of overexpression were also determined for the *tonB-exbB-exbD* gene cluster (A1S_0452-0454) which contained a predicted FUR box. A second TonB-ExbB-ExbD energy transduction system in strain ATCC 17978 (A1S_1603-1605) has previously been described (Zimbler *et al.* 2009). This cluster restored enterobactin utilisation in *E. coli exbBD* mutants, but not in *tonB* mutants. However, in this study, no significant transcriptional up-regulation of the genes within this cluster (A1S_1603-1613) was observed under iron-limiting conditions. It is possible that the cluster is related to heme acquisition rather than siderophore-mediated iron uptake, since genes related to hemophore utilisation were located adjacent to this cluster. A heme receptor/reduction mechanism may not be required under the conditions tested in this study, since no hemophores are being synthesised by *A. baumannii* ATCC 17978 and no exogenous hemophores were present.

3.2.5 Investigation of motility under iron-limiting conditions

It is well established that pili play an important role in the pathogenicity of bacteria due to their roles in motility, adherence, invasion and resistance (Guerry 2007; Proft and Baker 2009). Grouping transcriptome results from this study by COG function showed that 18% of the genes related to motility were significantly down-regulated under iron-limiting conditions (Figure 3.3). The down-regulated genes from this group are part of the chaperone-usher pili assembly systems, or type I pili (*csu* cluster and A1S_1507-1510, Figure 3.6A) and type IV pili (Figure 3.6B), which have been previously identified in *A. baumannii* (Vallenet *et al.* 2008). Homologous features have been associated with biofilm formation and motility in

Table 3.3: Characteristics of the putative *A. baumannii* ATCC 17978 siderophore receptors

Locus-tag	FUR binding site	Significantly up-regulated under iron-limiting conditions [a]
A1S_0092	No	No
A1S_0474	No	Yes
A1S_0980	Yes	Yes
A1S_0981	Yes	Yes
A1S_1063	Yes	Yes
A1S_1607	Yes	No
A1S_1655	Yes	Yes
A1S_1667	Yes	Yes
A1S_1725	No	No
A1S_1921	Yes	Yes
A1S_2076	Yes	Yes
A1S_2077	Yes	Yes
A1S_2080	Yes	Yes
A1S_2357	Yes	No
A1S_2358	Yes	No
A1S_2385	Yes	Yes
A1S_2566	Yes	Yes
A1S_2829	No	No
A1S_2877	No	No
A1S_2892	No	Yes

[a] Significance was determined by SAM (Section 2.5).

various organisms, including *E. coli* and *P. aeruginosa* (Haussler 2004; Nudleman and Kaiser 2004; Waksman and Hultgren 2009).

In *A. baumannii*, biofilm formation on abiotic surfaces has been linked to a type I pili encoded by *csuAB-E* (Tomaras *et al.* 2003). The CsuA/B, CsuA, CsuB and CsuE proteins are predicted to form part of the type I pili rod (Proft and Baker 2009; Tomaras *et al.* 2003). CsuC forms a periplasmic chaperone protein that accelerates folding of the pilus rod subunits and CsuD is an OMP responsible for assembly and extension of the pilus (Proft and Baker 2009; Tomaras *et al.* 2003). CsuD shares 40% and 45% amino acid sequence similarity with the OMP of two other type I pili systems in strain ATCC 17978, A1S_1508 and A1S_2089, respectively. The genes *csuC* and homologue A1S_1509 were both shown to be down-regulated under iron starvation, by 2.0-fold and 3.7-fold, respectively (Figure 3.6A). The *csuB* and *csuE* homologues within this second cluster, A1S_1507 and A1S_1510, respectively, were also down-regulated by approximately 4-fold under iron-limitation.

There were no genes down-regulated in the third type I pili cluster (A1S_2088-2091) (data not shown). It has been shown that transcription of the *csu* cluster in *A. baumannii* is controlled by the BfmRS two-component regulator (Tomaras *et al.* 2008). However, no significant differential expression was observed for either gene encoding this system in this study.

Biofilm assays (Section 2.3.3) were performed under iron-replete and iron-limited conditions (200 µM 2,2'-dipyridyl) to assess the impact of down-regulation of the *csu* cluster in biofilm formation. However, no significant differences were observed between planktonic growth and biofilm formation under iron-limiting or iron-replete conditions (Section 5.2.8.3; Figure 5.14). Planktonic growth was assessed by measuring the OD_{600} of the growth medium, which was transferred to a new plate before analysing the adhering cells (Section 2.3.3). A similar study with *P. aeruginosa* on the effect of iron-limitation showed that biofilm formation was impaired to a greater extent than planktonic growth (Glick *et al.* 2010; Patriquin *et al.* 2008). Interestingly, it was also shown that twitching motility was enhanced when iron was less readily available (Glick *et al.* 2010; Patriquin *et al.* 2008). These phenotypic alterations were linked to the production of rhamnolipids, a biosurfactant that is not likely to be produced by *A. baumannii* strain ATCC 17978, as the gene involved in the biosynthesis of rhamnolipids can not be found in the ATCC

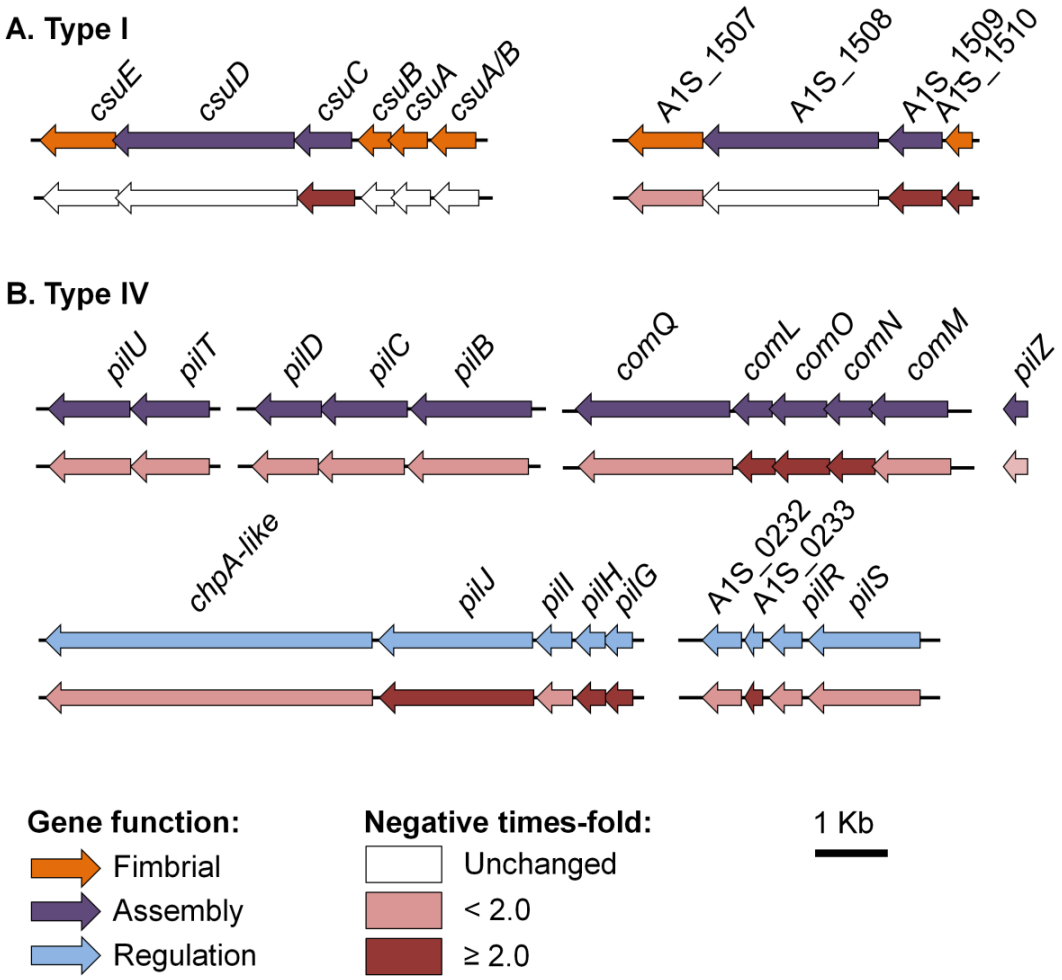

Figure 3.6: *A. baumannii* ATCC 17978 gene clusters with a putative role in motility

Many genes in the motility COG were found down-regulated including genes of (A) type I and (B) type IV pili. The top set of arrows show predicted gene function; pilus proteins in orange, pilus assembly proteins in purple, and regulatory proteins in light blue. The relative transcriptional differences (Section 2.5) between *A. baumannii* ATCC 17978 grown under iron-replete and iron-limiting conditions is depicted in the bottom set of arrows; all values are in times-fold. Genes depicted in white were not differentially expressed, those shaded in light red were down-regulated less than 2-fold, whereas those in dark red were down-regulated 2-fold or more. No significant up-regulation was observed within gene clusters related to motility.

17978 genome (data not shown). These findings indicate that binding and adherence characteristics follow different regulatory pathways in *A. baumannii* compared to other pathogens, such as *P. aeruginosa*.

Various genes involved in the biosynthesis of type IV pili, including *pilB-D, pilT, pilU, comM-O, comL, comQ* and genes that play a role in chemosensory and regulation of this complex, *pilG-J, pilR, pilS* and the *chpA*-like, were down-regulated under iron limitation in *A. baumannii* ATCC 17978 (Figure 3.6B). In *P. aeruginosa*, type IV pili have proven to play a role in swarming motility, a form of migration over nutrient rich semi-solid surfaces (Kaiser 2007; Overhage *et al.* 2007). Interestingly, type IV pili up-regulation has previously been observed in *Moraxella catarrhalis* under iron limitation (Luke *et al.* 2004). Previous studies on the type IV secretion mechanism in *Acinetobacter* have been predominantly related to its function in DNA acquisition (Herzberg *et al.* 2000; Porstendorfer *et al.* 1997). In this study, a swarming phenotype on LB medium containing a low percentage of agar (0.25%) was identified for *A. baumannii* strain ATCC 17978 (Section 2.3.4). The effect of iron-limitation on swarming motility was investigated by supplementation with 2,2'-dipyridyl to the swarming medium. *A. baumannii* strain ATCC 17978 was found to be incapable of migrating over the surface of the semi-solid medium when 200 µM 2,2'-dipyridyl was supplemented to the medium, whereas non-migrational growth remained largely unchanged (Figure 3.7). Since bacterial motility is a high energy consuming process, the inability to migrate may be a stress response of *A. baumannii* ATCC 17978 to low-iron levels.

3.2.6 Comparative analysis of the iron acquisition mechanisms of sequenced *Acinetobacter* isolates

The iron uptake machinery encoded by different *A. baumannii* strains may differ, as variation in the siderophore-mediated iron uptake proteins in the outer membrane has been shown in a previous study on different *Acinetobacter* strains (Dorsey *et al.* 2003a). Moreover, a siderophore gene cluster found in *A. baumannii* 8399 (*om73 – entD*) could not be identified in any other sequenced *Acinetobacter* strain using a Blastn search (Table 3.4). To explore this possibility in more detail, comparative analyses of siderophore gene clusters were conducted using Mauve (Section 2.7.2). The analyses were performed on 10 fully sequenced genomes; *A. baumannii* ATCC 17978, ATCC 19606, AYE, AB0057, ACICU, 307-0294, D1279779, WM99c and SDF, and *A. baylyi* strain ADP1 (Table 2.2).

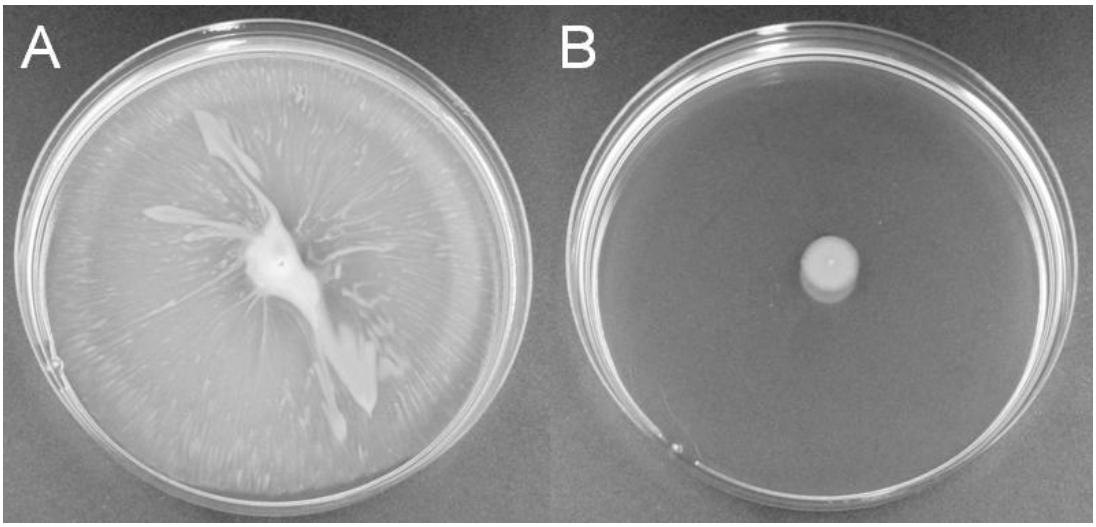

Figure 3.7: Swarming motility of *A. baumannii* strain ATCC 17978

A. baumannii colony material was spotted on LB medium containing 0.25% agar (Section 2.3.4). Swarming motility (A) is visible as the channel-like growth around the dense white colony material. The absence of halo growth around the colony (B) indicates a lack of swarming motility of *A. baumannii* when available iron is limited, which was experimentally tested by supplementation with 200 µM 2,2'-dipyridyl to the growth medium.

The novel putative siderophore cluster 1 (A1S_1647-1657) was indentified in *A. baumannii* strains ATCC 17978, ATCC 19606, AYE, AB0057, ACICU, 307-0294, D1279779 and WM99c, and in *A. baylyi* strain ADP1 using Mauve (Section 2.7.2; Table 3.4). Interestingly, the boundaries of this cluster, including genes orthologous to A1S_1647 and A1S_1657 that encode proteins with homology to the siderophore biosynthesis proteins IucA/IucC and acetyltransferase, respectively, were identified in strain SDF. However, the intervening genes appear to have been replaced by a 3.5 Kb transposon encoding a transposase of the IS*5* family (Figure 3.8). Well over 100 copies of this transposon are found throughout the SDF genome and, along with other insertion elements, are known to have played a major role in genome reduction in this non-pathogenic *A. baumannii* strain (Section 1.4.3). No other putative siderophore biosynthesis gene clusters were identified in strain SDF. Therefore, as a result of this insertion, this strain does not appear to encode any siderophore-mediated iron acquisition mechanisms. This may be one reason that strain SDF has a higher requirement for soluble iron than other *A. baumannii* strains, as outlined below.

In the 10 *Acinetobacter* strains surveyed, siderophore cluster 2 was only detected in ATCC 17978 and ADP1. Additionally, no positive hits for A1S_2562, a gene within this siderophore cluster, were identified in 54 clinical *Acinetobacter* isolates by PCR screening (Section 2.4.14; Section 6.2.1) using oligonucleotides designed to conserved regions within these genes as determined by whole genome alignments (Table 2.4). Therefore, this cluster appears to be relatively rare across the *Acinetobacter* genus. The average homology between the ATCC 17978 and ADP1 siderophore cluster 2 genes was 75%, which is high compared to orthologous genes elsewhere within these two genomes, e.g. 53% within siderophore cluster 1 as determined by a ClustalW2 alignment (Section 2.7.1). Transposons were found at the termini of siderophore cluster 2 in both ATCC 17878 and ADP1, suggesting that this gene cluster may have been horizontally acquired. Nonetheless, these transposons are distinct and have inserted into distinct genomic positions in the two strains, suggesting that siderophore cluster 2 was incorporated into the two genomes in separate transfer events. It remains unknown why this siderophore biosynthesis cluster has only been incorporated into the *A. baumannii* ATCC 17978 and *A. baylyi* ADP1 genomes.

Table 3.4: Genomic comparison of siderophore gene clusters in sequenced *Acinetobacter* isolates

		Cluster 1	**Cluster 2**	**Acinetobactin**	**Cluster 4**	**Cluster 5**
		A1S_1647–1657	A1S_2562–2581	A1S_2372–2392	*om73–entD*	ABAYE1888-1889
A. baumannii	**ATCC 17978**	+	+	+	-	-
	ATCC 19606	+	-	+	-	+
	AYE	+	-	+	-	+
	AB0057	+	-	+	-	+
	ACICU	+	-	+	-	+
	307-0294	+	-	+	-	+
	D1279779	+	-	+	-	+
	WM99c	+	-	+	-	+
	SDF	-	-	-	-	-
	8399	nd [a]	nd [a]	nd [a]	+	nd [a]
A. baylyi	**ADP1**	+	+	-	-	-

[a] nd= not determined.

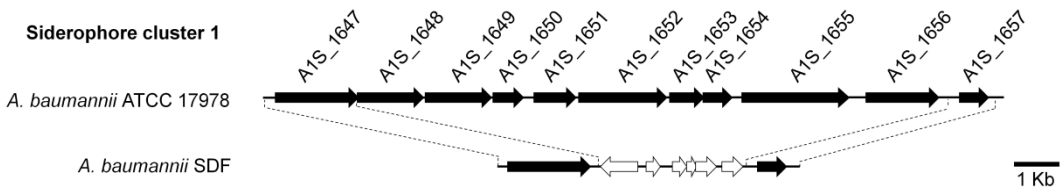

Figure 3.8: Comparison of siderophore cluster 1 between *A. baumannii* ATCC 17978 and SDF

The alignment of siderophore cluster 1 between *A. baumannii* strains ATCC 17978 and SDF is shown. Arrows indicate ORFs, in black, genes with high homology (>50% identity) and in white, genes representing a 3.5 Kb transposon insertion, which replaced the A1S_1648-1656 orthologs in strain SDF.

A high level of conservation was observed for the acinetobactin gene cluster among most *Acinetobacter* isolates (Table 3.4). However, this cluster was not seen in the genomes of strain SDF and ADP1. A fifth cluster, not present in strain ATCC 17978, was identified in several sequenced *A. baumannii* strains by Blastp searches (considering the *A. baumannii* 8399 siderophore cluster as the fourth *Acinetobacter* siderophore gene cluster) (Table 3.4). The genes are represented in *A. baumannii* strain AYE by ABAYE1888 and ABAYE1889. A putative FUR box could also be identified upstream of ABAYE1889 using the FUR scoring matrix generated from the putative FUR binding site sequences listed in Table 3.2 (data not shown). High expression levels of ABAYE1888 and ABAYE1889 were observed in *A. baumannii* strain AYE under iron-limiting conditions (>50-fold) using qRT-PCR analysis (Section 2.4.15) (data not shown). The two genes encode proteins that were found to be homologous to an isochorismatase and a 2,3-dihydro-2,3-hydroxybenzoate dehydrogenase. The product synthesised by these two enzymes in this cluster, 2,3-dihydroxybenzoate, is an iron-binding compound, but also a precursor component for more complex siderophores, like acinetobactin. This cluster is well conserved between strains AYE, AB0057, ACICU, 307-0294, ATCC 19606, D1279779 and WM99c.

The MIC of 2,2'-dipyridyl was determined for seven of the strains included in the genetic comparison (*A. baumannii* strains ATCC 17978, ATCC 19606, AYE, D1279779, WM99c and SDF, and *A. baylyi* strain ADP1) (Table 3.5). Growth of *A. baylyi* strain ADP1 and *A. baumannii* SDF was found to be inhibited at lower levels compared to most other *A. baumannii* strains, 200 µM versus ≥400 µM. ATCC 17978 did not show higher MIC values for 2,2'-dipyridyl compared to AYE, WM99c or D1279779, despite having three highly expressed siderophore gene clusters. Therefore, viability of *Acinetobacter* strains under varying iron concentrations does not appear to directly correlate with the presence or absence of siderophore gene clusters. Instead, strains growing to higher OD_{600} values under iron-replete conditions in general showed higher MIC levels for 2,2'-dipyridyl (data not shown). This indicates that the growth characteristics of the strains may be of more importance than the siderophore biosynthesis clusters present in their genome.

Table 3.5: The ability of different *Acinetobacter* strains to grow under iron-limiting conditions

Strain		**Minimal inhibitory concentration of 2,2'-dipyridyl** (μM)
A. baumannii	ATCC 17978	400
	ATCC 19606	400
	AYE	800
	D1279779	800
	WM99c	800
	SDF	200
A. baylyi	ADP1	200

3.2.7 A second FUR-like transcription repressor

Bacterial genomes often contain multiple genes encoding metal uptake regulators like FUR. However, they may possess a higher affinity for metals other than iron, such as zinc, manganese or nickel (Hantke 2001). Some *A. baumannii* genomes, but not the ATCC 17978 genome, harboured a second *FUR*-like gene that has not yet been characterised, which in *A. baumannii* strain AYE is represented by ABAYE1887. This gene in strain AYE was located adjacent to the genes encoding the putative siderophore biosynthesis described above (ABAYE1888 and ABAYE1889). Conserved domain (CD)-searches (Section 2.7.2) showed the highest homology with the domain called 'cd07153' at an expect value (E-value) of $10E^{-10}$. This domain can be found in FUR and other metallo-regulatory proteins. A putative zinc uptake regulator (ZUR) was identified in a zinc-uptake gene cluster in strain AYE (ABAYE3726). Pairwise alignment of ABAYE1887 and *FUR* (ABAYE2920), and ABAYE1887 and *ZUR* using ClustalW2 (Section 2.7.1), indicated higher homology for ABAYE1887 to *FUR* than to *ZUR*, showing 46% and 33% similarity, respectively (data not shown). Little is known about auto-regulation of *FUR* homologues. In the case of ABAYE1887, a FUR box with a low p-value (Section 2.7.3) can be found less than 200 bp upstream of the start codon using the ATCC 17978 optimised FUR motif. Moreover, qRT-PCR analysis (Section 2.4.15) using oligonucleotides specific to ABAYE1887 (Table 2.4), demonstrated that growth of *A. baumannii* AYE under iron-limiting conditions resulted in more than 100-fold up-regulation of ABAYE1887, whereas, *FUR* only showed 1.6-fold up-regulation (data not shown). This second *FUR*-like gene can also be found in strains ATCC 19606, D1279779 and WM99c, and a truncated form in SDF. Further experimental work is required to determine if ABAYE1887 plays a role in transcription of genes related to iron acquisition.

3.3 Conclusions

This study defined the global transcriptional response of *A. baumannii* to iron starvation. The up-regulation of three siderophore-mediated iron acquisition systems was the predominant feature of this transcriptional response. The high level of overexpression of these systems under iron-limitation, suggests that each is active in mediating iron uptake and therefore likely to be of importance to *A. baumannii* for survival in iron-limited environments, such as human hosts. However, several genes involved in other processes, such as respiration and electron transport were also

significantly differentially expressed. These data on the importance of iron in multiple cellular processes corroborate results from a recently published proteomic study of *A. baumannii* under iron-rich and iron-limiting conditions (Nwugo *et al.* 2011).

The *A. baumannii* FUR box motif was studied to investigate the regulatory control of iron homeostasis in *A. baumannii*. The motif was examined by combining the transcriptomic results obtained in this study and the results from bioinformatic motif-prediction analyses. A palindromic motif was obtained, which showed strong homology to the FUR box motif from other Gram-negative bacteria, including *P. aeruginosa*. The abundance of putative FUR binding sites identified upstream of up-regulated genes highlighted a major role for this regulator in transcriptional up-regulation under iron-limiting conditions.

Various genes of the type IV pili were down-regulated under iron-limiting conditions. This may in fact explain the inability of strain ATCC 17978 to migrate on semi-solid surfaces under low-iron concentrations. Overall, the results indicated that *A. baumannii* is adaptable to an environment with limiting iron availability.