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1 H-NS Plays a Role in Expression of *Acinetobacter baumannii* Virulence Features

2 Running title: The *A. baumannii* H-NS protein

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15 ABSTRACT

16 *Acinetobacter baumannii* has become a major problem in the clinical setting with the prevalence
17 of infections caused by multi-drug resistant strains on the increase. Nevertheless, only a limited
18 number of molecular mechanisms involved in the success of *A. baumannii* as a human pathogen
19 have been described. In this study, we examined the virulence features of a hyper-motile
20 derivative of *A. baumannii* strain ATCC 17978, which was found to display enhanced adherence
21 to human pneumocytes and elevated levels of lethality towards *Caenorhabditis elegans*
22 nematodes. Analysis of cellular lipids revealed modifications to the fatty acid composition,
23 providing a possible explanation for the observed changes in hydrophobicity and subsequent
24 alteration in adherence and motility. Comparison of the genome sequences of the hyper-motile
25 variant and parental strain revealed that an insertion sequence had disrupted a *hns*-like gene in
26 the variant. This gene encodes a homologue of the histone-like nucleoid structuring (H-NS)
27 protein, a known global transcriptional repressor. Transcriptome analysis identified the global
28 effects of this mutation on gene expression, with major changes seen in the autotransporter Ata, a
29 type VI secretion system and a type I pili cluster. Interestingly, isolation and analysis of a second
30 independent hyper-motile ATCC 17978 variant revealed a mutation to a residue within the DNA
31 binding region of H-NS. Taken together, these mutants indicate that the phenotypic and
32 transcriptomic differences seen are due to loss of regulatory control effected by H-NS.

33

34 INTRODUCTION

35 *Acinetobacter baumannii* is widely recognized as a highly multi-drug resistant pathogen that
36 causes major problems in hospitals globally (1, 2). Antimicrobial resistance in *A. baumannii* has
37 been extensively studied and resistance determinants novel to *Acinetobacter* are being identified
38 regularly. These resistance genes can often be found on horizontally-acquired genetic material,
39 increasing the risk of generating pan-resistant clones. As such, global dissemination of these
40 multi-drug resistant clones poses a serious threat. Furthermore, biofilm formation has also
41 previously been associated with increased resistance and survival as well as immune evasion and
42 may therefore play a significant role in *A. baumannii* pathogenicity. Adherence characteristics,
43 such as biofilm formation and binding to eukaryotic cells, have been studied in a large number of
44 *A. baumannii* strains (3-9) and are but one of many potential factors involved in virulence. An
45 additional factor that can influence virulence potential is motility; two distinct forms of surface
46 migration have been described for this species (3, 7, 10-12). Expression of these phenotypes
47 varies between strains and to a degree clonal groups, such as the conserved expression of
48 twitching motility by isolates in the international clone I group (3). Although motility has been
49 associated with increased biofilm formation and virulence in other bacteria, such as
50 *Pseudomonas aeruginosa* and *Dichelobacter nodosus* (13-18), the significance of motility in the
51 virulence potential of *A. baumannii* remains to be confirmed.

52 Various molecular mechanisms contributing to the virulence potential of *A. baumannii* have been
53 identified (19). For example, one of the most extensively studied virulence determinants in *A.*
54 *baumannii* is OmpA, which not only facilitates adherence to eukaryotic cells and abiotic surfaces
55 but also mediates invasion and promotes cell death of lung epithelial cells (20-24). The penicillin

56 binding protein-7/8, phospholipase D, lipopolysaccharides and production of K1 capsule in *A.*
57 *baumannii* have also been shown to play important roles in survival in a host environment (25-
58 28). Furthermore, phospholipase D facilitates bacterial crossing of the blood-lung barrier, as
59 shown in a rat model system (26). More recently, siderophore mediated iron-acquisition
60 mechanisms have been demonstrated to be essential for lethality in mice (29). Many virulence
61 determinants described in other Gram-negative pathogens have been identified in the genome
62 sequences of *A. baumannii*, such as the type VI secretion system (30, 31). However, to date, their
63 role in virulence and persistence of *A. baumannii* has not been characterized.

64 The regulatory networks controlling expression of *A. baumannii* virulence determinants, such as
65 iron acquisition, motility, attachment and biofilm formation, remain largely unknown.
66 Transcriptomic studies have indicated that the ferric uptake regulator (FUR) is the primary
67 regulator of *A. baumannii* iron-acquisition mechanisms (10, 32) and a number of studies have
68 shown that quorum-sensing influences both motility and biofilm formation (12, 33, 34).
69 Additionally, a two-component regulatory system, encoded by *bfmRS*, plays a critical role in the
70 regulation of the Csu type I pili, which may influence attachment and motility (35).

71 It is likely that *A. baumannii* has also acquired virulence factor genes via exogenous DNA
72 uptake. In other bacterial genera, transcriptional regulation of horizontally-acquired genetic
73 material is in part controlled by the histone-like nucleoid structuring (H-NS) protein and
74 provides a level of protection against expression of genes that encode products with detrimental
75 effects to the host bacteria (36, 37). Although *Acinetobacter* spp. are known to possess large
76 quantities of horizontally-acquired genetic material, to date, the function of H-NS in *A.*
77 *baumannii* has not been described.

78 This study describes the comprehensive characterization of a hyper-motile derivative of *A.*
79 *baumannii* strain ATCC 17978. Whole genome sequencing of this strain revealed the insertional
80 inactivation of a gene encoding the global regulator H-NS. Beyond the dramatic changes
81 observed with respect to motility, the mutant strain also displayed altered adherence phenotypes
82 to biotic surfaces, as well as an increase in virulence using a *Caenorhabditis elegans* model
83 system. In summary, this study describes the importance of H-NS in expression of *A. baumannii*
84 persistence and virulence genes.

85 MATERIALS AND METHODS

86 *Bacterial strains*

87 *Acinetobacter* strain ATCC 17978 (CP000521) was obtained from the American Type Culture
88 Collection (ATCC) and *E. coli* OP50 was kindly provided by Hannah Nicholas (The University
89 of Sydney). Freeze-dried *Acinetobacter* cells were revived as recommended by the ATCC and
90 colony material from the overnight cultures grown on Luria-Bertani (LB) media were transferred
91 to a cryopreservation tube containing 20% glycerol in LB broth and placed at -80°C for long term
92 storage. The isolated variants, i.e. 17978hm and HNSmut88 were similarly stored. For
93 subsequent culturing purposes, material was scraped from the top of the frozen stocks and
94 streaked onto LB media.

95 *Biofilm assays*

96 The static biofilm formation assay was performed as described previously (3). In brief, overnight
97 cultures were diluted 1:100 in fresh Mueller-Hinton (MH) broth in polystyrene microtiter trays
98 and incubated overnight at 37°C. Adherent cells were washed once with phosphate buffers saline

99 (PBS), stained by incubation with 0.1% crystal violet for 30 minutes at 4°C, and washed three
100 times with PBS. Dye was released from the cells using ethanol:acetone (4:1) and shaking at 200
101 rpm for 30 minutes at room temperature. Absorbance was measured at 595 nm on a Fluostar
102 Omega spectrometer (BMG Labtech, Offenburg, Germany).

103 *Pellicle formation assays*

104 Pellicle formation assays were based on a method used previously (6). Overnight bacterial
105 cultures in LB broth were diluted in fresh LB broth containing 100 mM NaCl. Pellicle formation
106 assays were performed in polypropylene tubes and were incubated at room temperature without
107 shaking for 72 hours. The pellicle film was separated from the tube by the addition of methanol.
108 The pellicle biomass was measured (OD₆₀₀) after resuspending pelleted cells in 1 ml PBS. The
109 experiments were performed at least three times.

110 *Eukaryotic cell adherence assays*

111 The adherence of *A. baumannii* cells to A549 human pneumocytes was investigated as described
112 previously (3). Cell lines were grown in Dulbecco's Modified Eagle medium (Invitrogen,
113 Australia) supplemented with 10% fetal bovine serum (Bovogen, Australia). Prior to use, the cell
114 monolayer was examined microscopically to ensure >95% coverage. Washed A549 monolayers
115 in 24-well tissue culture plates were subsequently infected with a bacterial inoculum containing
116 $\sim 1 \times 10^7$ colony forming units (CFU). After incubation at 37°C for 4 hours, culture medium was
117 removed, and the monolayers washed three times with 1 ml of PBS. Cell monolayers were
118 detached from the plate by treatment with 100 μ l of 0.25% trypsin and 0.02% EDTA in PBS.
119 Eukaryotic cells were subsequently lysed by the addition of 400 μ l 0.025% Triton X-100 and
120 serial 10-fold dilutions thereof were plated on LB agar to determine the number of CFU of

121 adherent bacteria per well. Collated data for adherence assays were obtained from at least three
122 independent experiments and represent the data points for each experiment of quadruplicate
123 wells. The CFU of the cell culture medium after 4 hours incubation was determined to ensure
124 that strains did not display differences in their respective growth rates during the adherence
125 assay.

126 *Caenorhabditis elegans* killing assays

127 *C. elegans* N2 nematodes were synchronized in their development by initially placing nematode
128 eggs onto *E. coli* OP50 seeded nematode growth media (NGM) (38). The larval L3/L4 stage
129 nematodes were harvested and placed on NGM agar plates seeded with *A. baumannii* strains.
130 Viability of at least 200 individual nematodes found in random fields of view were determined
131 by microscopic examination. The viability was determined at 24 hours intervals for up to 144
132 hours and expressed as a percentage of live nematodes. The results (n=4) represent duplicate
133 experiments performed on two different days. Two independent researchers determined the
134 viability of the nematodes in a 'blind' experiment.

135 *Cell surface hydrophobicity tests*

136 Cell surface hydrophobicity was examined as described previously (39). The OD of the cell
137 suspensions before (OD_{initial}) and after addition of xylene (OD_{final}) was measured at 600 nm.
138 Experiments were performed three times. The hydrophobicity index (HI), expressed in
139 percentage, was calculated using the following formula; $HI (\%) = (OD_{\text{initial}} -$
140 $OD_{\text{final}}/OD_{\text{initial}})*100$. Experiments were performed at least three times.

141 *Phenotype MicroArray analysis*

142 *A. baumannii* ATCC 17978 WT and 17978hm mutant strains were cultured on LB agar
143 overnight. Cells were transferred to 1x IF-0 inoculation fluid (BIOLOG, Inc.) to 42%
144 transmittance. Cells were then diluted 1:5 in 1x IF-0 containing dye A (BIOLOG, Inc.) with a
145 final transmittance of 85%. 100 µl of the cell suspensions were transferred to each well of Biolog
146 MicroPlate™ PM01 and PM02A plates. Plates were incubated in the OmniLog® Phenotype
147 MicroArray System (Biolog, Inc.) at 37°C for 48 hours, with readings taken every 15 minutes.
148 Data from the WT and mutant strains were overlaid using the OmniLog® File
149 Management/Kinetic Analysis software v1.20.02 and analyzed using OmniLog® Parametric
150 Analysis software v1.20.02 (Biolog, Inc.).

151 *Fatty acid analyses*

152 The strains for fatty acid analysis were grown for 8 hours on MH plates containing 0.25% agar.
153 Cells were collected and washed with pre-chilled PBS. Total cellular lipids were extracted using
154 chloroform/isopropanol and were subsequently methylated using a 1% sulphuric acid solution in
155 methanol, as described previously (40). The fatty acid methyl esters were separated by gas
156 chromatography at the School of Agriculture, Food and Wine, University of Adelaide. The
157 abundance of each fatty acid was expressed as the percentage of the total of all fatty acids. All
158 analyzed bacterial strains were prepared on two separate occasions and the data represent the
159 average over those two experiments.

160 *RNA isolation*

161 Cells used for measurement of transcription levels were harvested from semi-solid MH media
162 (0.25% agar) using pre-chilled PBS. The cells were subsequently pelleted by centrifugation and

163 lysed in TRIzol[®] reagent (Invitrogen, Australia) and chloroform. Following phase separation by
164 centrifugation, RNA was extracted from the aqueous phase using a RNA isolation kit (Bioline).
165 DNaseI (Promega) treatment was performed as per the manufacturer's recommendations.

166 *Transcriptome analysis*

167 We used a custom genomic microarray for *A. baumannii* ATCC 17978, as previously described
168 (10). The results represent data obtained from three biological replicates and a dye-swap
169 experiment. The array was designed to harbor at least four probes per gene. The transcriptomic
170 data have been deposited into the gene expression omnibus database
171 (<http://www.ncbi.nlm.nih.gov/geo/>) and can be accessed using the accession number GSE40681.

172 *Quantitative Reverse Transcription PCR*

173 cDNA was synthesized using random hexamers (GeneWorks, Australia) and M-MLV reverse-
174 transcriptase (Promega) as per the manufacturer's recommendations. Oligonucleotides used in
175 this study were designed using Primer3 (41) as an integral part of UGENE v1.6.1 (Unipro) and
176 are listed in Table S1. qPCR was performed on a Rotor-Gene RG-3000 (Corbett Life Science,
177 Australia) using DyNAmo SYBR[®] green qPCR kits (Finnzymes, Australia). Internal qPCR
178 controls used primers designed to 16s rRNA (A1S_r01) to ensure differences seen are due solely
179 to alterations in the target gene expression and not due to mRNA quality or quantity.
180 Transcriptional differences were calculated using the $\Delta\Delta C_t$ method (42) and the data represent
181 experiments performed in biological triplicates.

182 *Genome sequence analysis*

183 *A. baumannii* ATCC 17978 and 17978hm were sequenced using Illumina BeadArray
184 technology, performed by the Ramaciotti Centre for Gene Function Analysis, University of New
185 South Wales, Australia. The whole genome shotgun sequence reads were assembled using
186 Velvet 1.1 (43).

187 *Genetic complementation of the hyper-motile A. baumannii mutant strains*

188 The *hns* PCR product was cloned into *Bam*HI digested pWH1266 (44). Electro-competent *A.*
189 *baumannii* cells freshly prepared on the day of use were incubated on ice with plasmid DNA for
190 5 minutes followed by electroporation using a MicroPulser (Bio-Rad) at 2.5 kV, 200Ω and 25
191 μF. After recovery in 1 ml of LB media for at least 1 hour at 37°C, cells were cultured overnight
192 at 37°C on LB media containing 200 μg/ml ampicillin.

193 RESULTS AND DISCUSSION

194 *Isolation of hyper-motile variant strains*

195 Contrary to their original designation as non-motile, a number of *A. baumannii* strains have been
196 shown to participate in various forms of motility when grown under appropriate conditions (3).
197 Motility of strain *A. baumannii* ATCC 17978 is evident on semi-solid Luria-Bertani (LB) media
198 containing <0.5% agar (3, 10, 12), with concentrations of agar above this level inhibiting the
199 phenotype. However, we observed two distinct morphologies in a cryopreserved ATCC 17978
200 stock cultured on LB medium containing 1% agar. The colony morphology of one variant
201 appeared non-motile as per wild-type (WT) ATCC 17978, whereas the other displayed a motile
202 appearance, which was designated as a hyper-motile phenotype. To examine the motility of these

203 variants, five individual hyper-motile colonies were inoculated in the center of both LB and
204 Mueller-Hinton (MH) media containing different concentrations of agar. The phenotype was
205 most distinct using MH containing 0.25% agar, conditions non-permissive for motility of WT
206 cells. Whereas four out of five hyper-motile variants were equally motile and covered the entire
207 surface of the plate within eight hours (migration ~45 mm), one hyper-motile variant was found
208 to be delayed by approximately two hours compared to the other hyper-motile variants. This
209 indicated that at least two distinct variants of the parental strain were isolated. To examine
210 potential genotypic differences that may explain the altered phenotypic characteristics, one of the
211 hyper-motile strains displaying motility on semi-solid MH media, designated 17978hm, and the
212 parent WT ATCC 17978 strain were selected for further analyses.

213 *Sequence analysis of the hyper-motile A. baumannii ATCC 17978 derivatives*

214 The genetic differences between the *A. baumannii* strain 17978hm and ATCC 17978 were
215 assessed by sequencing both their genomes using Illumina BeadArray technology. Sequence
216 reads were assembled using Velvet 1.1 (43), generating 245 contigs for strain 17978hm and 292
217 contigs for strain ATCC 17978. Whole genome alignments were generated using Mauve, and the
218 ATCC 17978 genome sequence reported by Smith *et al.*, 2007 (CP000521) was included for
219 comparative purposes.

220 The most striking finding from comparative analyses of the parental and 17978hm genome
221 sequences was an insertion sequence (IS) element found in the *hns* (A1S_0268) locus of strain
222 17978hm (Fig. 1A). In other bacteria, H-NS has been shown to act as a global repressor that
223 preferentially binds AT-rich DNA sequences (36, 37). Spontaneous insertional inactivation of
224 *hns*, as observed in strain 17978hm, has also been described in *Mycobacterium smegmatis* (45).

225 Interestingly, the *M. smegmatis* H-NS mutant strain was also found to display a hyper-motile
226 phenotype (39). The H-NS protein is well conserved between *Acinetobacter* strains; the ATCC
227 17978 H-NS protein sequence is 100% identical to that from strains AYE and SDF, and 92%
228 identical to H-NS from *Acinetobacter baylyi* ADP1. The ATCC 17978 genome only encodes a
229 single copy of *hns* and to date, strain AB058 is the only *A. baumannii* strain found to harbor two
230 *hns*-like genes (data not shown).

231 Analysis of the DNA sequence revealed that transposition of an IS element into *hns* in strain
232 17978hm had occurred with nine nucleotides of the *hns* target sequence being duplicated and
233 now flanking the termini of the IS element (Fig. 1A). The sequence of this IS element exhibited
234 >99% identity to an IS element harboring A1S_0628 which encodes a putative transposase in
235 strain ATCC 17978, raising the possibility that the IS element identified within the *hns* locus of
236 strain 17978hm was generated by a transposition event. Two similar transposases found in strain
237 17978hm and ATCC 17978, designated A1S_2554 and A1S_1172, showed 97% and 84%
238 sequence identity to A1S_0628, respectively, and as such are unlikely to be the originator of the
239 insertion.

240 As mentioned above, a total of five hyper-motile variants were isolated in this study. The *hns*
241 gene of the four other hyper-motile strains was PCR amplified and sequenced. Three of these
242 were found to possess an insertion disruption identical to that seen in strain 17978hm and also
243 displayed a hyper-motile phenotype similar to 17978hm (data not shown). The *hns* gene of the
244 variant displaying a less pronounced level of hyper-motility (motility delayed by two hours on
245 semi-solid MH media compared to strain 17978hm) contained a single nucleotide polymorphism,
246 resulting in a lysine to isoleucine substitution at position 88 of the H-NS protein (H-NS_{K88I}).
247 Interestingly, lysine 88 is part of the DNA-binding domain of H-NS-like proteins (46) (Fig. 1B).

248 Lacking this positively-charged residue may have resulted in an altered affinity for regulatory
249 binding sites and consequently reduced repression of the genes encoded downstream. As
250 mentioned, the *A. baumannii* strain expressing the H-NS_{K88I} protein, designated HNSmut88,
251 displayed an intermediate hyper-motile phenotype compared to strain 17978hm, which was
252 investigated by comparing the positively charged residues in the DNA binding domain to those
253 in other H-NS-like proteins, such as Lsr2 from *Mycobacterium tuberculosis*, H-NS from
254 *Salmonella* and Ler from *Escherichia coli* (46, 47). Whereas the *Salmonella* H-NS and *E. coli*
255 Ler proteins contain a single positively charged residue in the DNA binding domain, the *M.*
256 *tuberculosis* Lsr2 and *A. baumannii* H-NS contain two; in *A. baumannii* H-NS these are lysine
257 and arginine at position 88 and 86, respectively (Fig. 1B). Previous mutagenesis studies in *M.*
258 *tuberculosis* have shown a significant level of Lsr2 DNA binding in mutants where only a single
259 positive charged residues in the DNA binding domain was removed (47). However, double
260 mutants in Lsr2, or mutation of the single charged residue present in the DNA binding domain of
261 the *Salmonella* H-NS resulted in a complete lack of binding (47). Therefore, arginine 86 may be
262 sufficient to maintain significant binding affinity of H-NS_{K88I} to certain regulatory targets
263 explaining the intermediate motility phenotype observed in this strain. Although the *A.*
264 *baumannii* H-NS and *E. coli* Ler proteins possess an arginine adjacent to the DNA-binding
265 domain (Fig. 1B), when present as the sole positively charged residue in this region of the
266 protein it does appear to be insufficient for maintaining the ability to bind its targets (46).

267 To confirm that the altered phenotypes observed in the hyper-motile *A. baumannii* variants were
268 a result of mutations in *hns*, the WT *hns* gene was cloned into the *E. coli*-*Acinetobacter* shuttle
269 vector pWH1266, producing pWH0268, and used for complementation in the mutant strains.
270 Motility assays revealed that introduction of pWH0268 into *A. baumannii* strains 17978hm and

271 HNSmut88 resulted in loss of the hyper-motile phenotype on solid LB media and semi-solid MH
272 media, returning the phenotype to that observed for WT cells (data not shown). Due to the
273 reduced severity of phenotypic changes observed in HNSmut88 compared to 17978hm, only the
274 ATCC 17978 and 17978hm strains were assessed in subsequent phenotypic and transcriptional
275 analyses.

276 *Adherence characteristics*

277 Biofilm formation is a complex process initiated by attachment to a surface, followed by the
278 formation of a multilayered biomass containing secondary structures. It was hypothesized that
279 the adherence characteristics of strain 17978hm may differ to that of WT cells since changes in
280 motility could affect biofilm formation, as previously reported for *P. aeruginosa* (18). However,
281 adherence to the surface of a polystyrene microtiter tray showed no major differences between
282 the WT and hyper-motile strains (data not shown). In contrast, pellicle formation, i.e., the biofilm
283 at the air-liquid interface, appeared significantly higher in 17978hm as compared to the WT
284 strain or its complemented derivative 17978hm (pWH0268) (Fig. 2A). The level of pellicle
285 biomass in strain 17978hm (pWH0268) was higher than that observed for the WT strain, which
286 could be a result of loss of the complementation plasmid over the 72 hours incubation period.
287 Pellicle formation was only observed in LB media at 25°C and not at 37°C (data not shown),
288 which corroborates data from similar studies on *Acinetobacter* spp. (6). Attempts to demonstrate
289 pellicle formation by strains grown in MH media was unsuccessful. During static culturing for
290 pellicle formation, it was apparent that the planktonic growth of the pellicle forming 17978hm
291 cells was lower than that of the non-pellicle forming WT cells, $OD_{600} = \sim 0.15$ and ~ 0.50 ,
292 respectively. The differences in planktonic growth may be due to a reduction of oxygen levels in

293 the growth medium of strain 17978hm as a result of the high oxygen dependency of the pellicle,
294 a phenomenon described previously (48).

295 Adherence to abiotic and biotic surfaces appears to be mediated by different molecular
296 mechanisms in most *A. baumannii* strains and as such does not always correlate (3, 5, 49).
297 Therefore, despite similar abiotic adherence levels observed in microtiter trays, adherence to
298 biotic surfaces by strains ATCC 17978, 17978hm, 17978hm (pWH1266) and 17978hm
299 (pWH0268) was investigated. A549 pneumocytes were selected for these experiments to mimic
300 adherence to the epithelial layer of the human lung (50). After incubating the bacteria in
301 conjunction with pneumocytes for 4 hours, the number of 17978hm cells adhered to and
302 potentially intracellularly located in the washed A549 eukaryotic cells was enumerated and
303 found to be significantly higher than the number of ATCC 17978 cells (~1.6-fold; $p < 0.05$) (Fig.
304 2B). Interestingly, adherence levels of 17978hm (pWH0268) cells were lower than those for WT
305 cells, indicating a possible dose-dependent effect due to a difference in plasmid copy number
306 (35), where H-NS may be more abundant in the complemented cells. The increased adherence
307 potential of strain 17978hm to human pneumocytes may indicate that 17978hm cells have higher
308 persistence levels during infection and more specifically, pneumonia. Further work using a
309 mouse pneumonia model may reveal whether the differences seen in adherence to pneumocytes
310 play a biological significant role in the disease process.

311 *The hyper-motile variant possesses an increased virulence potential*

312 Since 17978hm showed a significantly increased ability to adhere to eukaryotic cells its disease
313 potential was investigated further, by examining ATCC 17978 and 17978hm strains for their
314 ability to kill *C. elegans* nematodes. Measureable death of the *C. elegans* nematodes was

315 observed between 72 and 144 hours incubation on either WT or 17978hm cells (Fig. 3). A
316 significant difference between the percentage of live nematodes incubated with either WT or
317 17978hm cells was observed at 120 hours and 144 hours (Fig. 3). At both time points,
318 approximately 20% higher death rates were observed for nematodes incubated with 17978hm
319 cells compared to those with WT cells (Fig. 3). The experiment was terminated after 144 hours
320 as the remaining live nematodes were consuming the disintegrated dead nematodes at time points
321 beyond 144 hours, making it difficult to accurately determine the percentage of live versus dead
322 nematodes during the latter part of the experiment (data not shown).

323 *Cell surface hydrophobicity and fatty acid composition*

324 The differences between the phenotypes of the WT and hyper-motile *A. baumannii* cells
325 described above may be related to changes at the cell surface. Therefore, the hydrophobicity of
326 WT and 17978hm cells was investigated using the microbial adhesion to hydrocarbons (MATH)
327 test (39); an increase in the hydrophobicity index (HI) of 17978hm cells (HI = 65%) compared to
328 WT cells (HI = 44%) was observed (Table 1). When a WT copy of *hns* (carried on pWH0268)
329 was introduced into 17978hm cells, the hydrophobicity decreased significantly, returning to
330 levels lower than those observed for ATCC 17978 cells.

331 To investigate a potential cause for the change in cell surface hydrophobicity, the total fatty acid
332 compositions of WT and 17978hm cells, and 17978hm (pWH1266) and 17978hm (pWH0268)
333 were determined. In a study on *Listeria innocua*, decreases in the ratio between C₁₅ and C₁₇
334 saturated fatty acids were linked to increases in cell surface hydrophobicity (51). Similarly, in
335 this study the percentage of C₁₇ fatty acid was significantly higher in the hyper-motile strains,

336 which may explain the differences observed in cell surface hydrophobicity (Table 1). Major
337 changes in the concentrations of other fatty acids were not observed.

338 *Transcriptomic analysis of the motile versus non-motile population*

339 Comparative analysis of the transcriptomes of cells in distinct life styles may provide
340 information about the molecular mechanisms and regulatory pathways responsible for driving a
341 population into a certain mode of living. Therefore, we employed a whole genome microarray to
342 examine the effect of the *hns* inactivation on the transcriptome of *A. baumannii*. Major
343 differences were observed between the transcriptomes of the hyper-motile 17978hm and non-
344 motile WT cells (Fig. 4). More than 4-fold differential expression was seen for 152 genes, of
345 which 91 were down- and 61 up-regulated (Table S2; GEO:GSE40681). The most striking
346 differences were observed in the quorum-sensing regulated genes homoserine lactone synthase
347 (A1S_0109) and the homoserine lactone responsive regulator (A1S_0111), which were both
348 heavily up-regulated. Furthermore, an adjacent cluster involved in the production of secondary
349 metabolites, putatively lipopeptides or polyketides, was up-regulated by more than 100-fold.
350 Both quorum-sensing and the adjacent cluster have been shown to play an important role in
351 motility of *A. baumannii* (12).

352 Various genes encoding surface-presented structures were also heavily up-regulated in *A.*
353 *baumannii* strain 17978hm. The autotransporter Ata (A1S_1032) was overexpressed by
354 approximately 10-fold. In a recent report, Ata was found to play a major role in adherence and
355 virulence of *A. baumannii* strain ATCC 17978 (52), potentially explaining the increased
356 virulence levels of strain 17978hm observed in the *C. elegans* killing assays described above.
357 This gene appears to be transcriptionally linked to A1S_1033 (8-fold up-regulation), the product

358 of which shares homology with putative OmpA-like proteins (20-24). Even though there is no
359 evidence of a role for type I pili in promotion of surface translocation in *A. baumannii*, four
360 genes encoding a novel type I pili cluster (A1S_1507-1510) were up-regulated by more than 10-
361 fold. Other phenotypes, such as increased adherence to human epithelial cells or increased
362 pellicle formation, may be associated with overexpression of type I pili in the hyper-motile
363 strain. A gene cluster predicted to encode a type VI secretion system (A1S_1292-1311) was
364 found to be up-regulated in the motile population. Type VI secretion systems can contribute to
365 bacterial pathogenicity as observed in *P. aeruginosa* (53), however, the role of these systems in
366 *A. baumannii* is yet to be elucidated (54). It would appear not to be essential for full virulence in
367 a number of isolates, as comparative genomic analyses revealed that four open reading frames
368 encoding proteins of unknown function have replaced the type VI secretion system gene cluster
369 in *A. baumannii* strain D1279779 (AERZ00000000). The type VI secretion system may also be
370 non-functional in strain 1656-2 (CP001921) (55), as it contains an insertion element in the gene
371 homologous to A1S_1302. Furthermore, the gene cluster coding for the type VI secretion system
372 can also be found in the non-pathogenic *A. baumannii* strain SDF (CU468230). Therefore, the
373 function of the type VI secretion system in *A. baumannii* requires further investigation.

374 Interestingly, genes functioning in fatty acid biosynthesis, including *fabG* (A1S_0524) and *fabF*
375 (A1S_0525), were found to be overexpressed by more than 2-fold in strain 17978hm. FabF is
376 responsible for fatty acid elongation, which occurs in steps of two carbon additions i.e., C₁₃ to
377 C₁₅ to C₁₇ (56). Thus, these increases in transcription levels may be related to the increase of the
378 C₁₇ observed in 17978hm cells as compared to WT cells (Table 1).

379 Numerous genes functioning in metabolism were found to be down-regulated in the motile
380 population, such as those encoding members of the phenylacetic acid degradation pathway (Fig.

381 4). Down-regulation of metabolic pathways was also observed in a transcriptional investigation
382 of swarming *P. aeruginosa* cells (57). A Phenotype MicroArray™ (BIOLOG, Inc.) analysis using
383 MicroPlates™ PM01 and PM02A was performed to obtain a comprehensive depiction of carbon-
384 source utilization by the *Acinetobacter* strains 17978hm and ATCC 17978. The most pronounced
385 dissimilarities between these strains were observed in the presence of L-threonine or D-malic
386 acid, in which WT ATCC 17978 cells showed more active respiration than 17978hm cells (Table
387 S3). The up-regulation of a putative threonine efflux transporter (A1S_3397) in 17978hm may be
388 associated with a reduction in available threonine and consequently lower respiratory levels seen
389 in this strain. Changes in transcription levels of genes encoding proteins involved in D-malic
390 acid metabolism were not observed (data not shown).

391 *Identification of potential H-NS targets in the ATCC 17978 genome*

392 Although not previously examined in *A. baumannii*, H-NS is a well-studied protein in many
393 bacterial genera (58, 59). Sequence homology between H-NS proteins from different bacteria is
394 often low (<30%), however, the targets appear to be similar. Horizontally-acquired genetic
395 material is often AT-rich and is therefore a likely target for H-NS proteins (59). Hence, the H-NS
396 proteins are also known as xenogeneic silencers. A common example of a horizontally-acquired
397 genomic region targeted by H-NS encodes the type VI secretion system (60). Indeed, this cluster
398 (A1S_1292-1312) was found to be heavily up-regulated in strain 17978hm (Fig. 4). Based on the
399 potential for H-NS to act as a xenogeneic silencer, other potential regulatory targets were
400 identified encoded within novel genomic regions. Transcriptomic data were overlaid with the
401 results of BLASTP comparisons of CP000521 (ATCC 17978) with six other *Acinetobacter*
402 genomes; *A. baumannii* AB0057, ACICU, AYE, AB307-0294 and SDF, and *A. baylyi* ADP1 on
403 a circular representation of the ATCC 17978 genome (Fig. 5). A correlation was seen between

404 up-regulation and lack of conservation of the respective genes/gene clusters. These non-
405 conserved up-regulated loci were subsequently analyzed using a whole genome alignment
406 generated in MAUVE (61) containing the genomes described above. Thirteen potential
407 horizontally-acquired H-NS targets (Fig. 5) were identified, which included a surface protein
408 (A1S_0745) and the autotransporter adhesin (A1S_1032) described above. Interestingly, in *E.*
409 *coli*, transcription of a gene encoding the autotransporter protein UpaC was recently found to be
410 repressed by H-NS (62), consistent with our findings. These surface presented proteins,
411 potentially regulated by H-NS, may play a role in the phenotypic alterations of strain 17978hm.
412 The S-adenosyl-L-methionine-dependent methyltransferase (A1S_2744), also potentially
413 regulated by H-NS, is involved in methylation of proteins, lipids, DNA and RNA, and therefore,
414 in controlling a wide range of cellular processes (63). The putative threonine efflux transporter
415 protein described above (A1S_3397), also appeared to be under regulatory control of H-NS (Fig.
416 5). Overall, these analyses suggested that various horizontally-acquired genome regions are
417 targets for transcriptional repression by H-NS in *A. baumannii* and inactivation of H-NS results
418 in up-regulation of the genes within these regions.

419 Although H-NS is known to bind high AT-percentage regions, the global GC-skew analysis
420 shown in the circular genome figure did not reveal such an association with transcriptional up-
421 regulation (Fig. 5). This may be due to the generally low GC-percentage of the *A. baumannii*
422 ATCC 17978 genome (~39%). However, when examining the upstream regions of heavily
423 overexpressed genes, a correlation between high AT-content and transcriptional up-regulation in
424 strain 17978hm was observed. The GC-percentage of various potential H-NS targets was as low
425 as 20% (Fig. 6), which is significantly lower than the average GC-percentage of the ATCC
426 17978 genome-wide intergenic regions (~35%).

427 The genomic region harboring the gene encoding homoserine lactone synthase (A1S_0109) and
428 the cluster involved in biosynthesis of polyketides/lipopeptides (A1S_0112-0119) possessed two
429 regulatory sites with a low GC-percentage (Fig. 6). In particular, the upstream region of the
430 biosynthesis cluster (GC content of 20%) may be targeted by H-NS. Previously, Clemmer *et al.*
431 showed that the A1S_0112-0119 cluster is under control of quorum-sensing signals in the form
432 of acyl-homoserine lactones via AbaR (12).

433 Other overexpressed genes with a putative H-NS binding region upstream included the above
434 described autotransporter Ata and putative *ompA*-like gene (A1S_1032-1033), the type VI
435 secretion system (A1S_1292-1312), a novel type I pili cluster (A1S_1510-1507), a cluster
436 involved in lipote synthesis and acetoin metabolism (A1S_1698-1704), and a small putatively
437 secreted protein (A1S_3273) (Fig. 6).

438 Alanine racemases are ubiquitous in prokaryotes and are responsible for racemization of L- and
439 D-alanine. Like most other prokaryotes, *A. baumannii* strains possess two alanine racemases,
440 DadX (A1S_0096) mediating conversion of D- to L-alanine and Alr (A1S_2176), which
441 facilitates L- to D-alanine racemization. However, *A. baumannii* ATCC 17978 also contains a
442 second Alr-like alanine racemase encoded by A1S_1357. This heavily up-regulated gene in
443 strain 17978hm (>70-fold) appeared to have been horizontally-acquired and also possessed an
444 AT-rich upstream region making it a likely target for transcriptional repression by H-NS (Fig. 5
445 and 6).

446 *Transcriptional analysis of putative H-NS regulated genes*

447 We investigated the transcription levels of nine differentially expressed genes in the
448 complemented strain pair, 17978hm (pWH1266) and 17978hm (pWH0268). These genes are

449 potential targets of H-NS based on previously described findings or the bioinformatic analysis
450 performed in this study. Seven of these genes were highly up-regulated (>4-fold); A1S_0111,
451 A1S_0112, A1S_1032, A1S_1292, A1S_1510, A1S_1699 and A1S_3273, and two highly down-
452 regulated (>4-fold); A1S_0095 and A1S_1336, as determined by our microarray data.
453 Comparative qRT-PCR analysis examining the control 17978hm (pWH1266) and complemented
454 17978hm (pWH0268) strains, using the oligonucleotides listed in Table S1, showed significant
455 up- or down-regulation of the respective genes that were found to be significantly differentially
456 expressed in strain 17978hm as compared to the WT strain by microarray (Table 2). Noteworthy,
457 three of the investigated genes are known to be co-regulated by other proteins; A1S_0111 and
458 A1S_0112 by AbaR and A1S_0095 by Lrp, and therefore it is difficult to assess the magnitude
459 of the effect that H-NS exerts on these genes. Nevertheless, successful complementation of the
460 hyper-motile variants at a phenotypic and transcriptional level was shown, confirming that
461 inactivation of H-NS resulted in various alterations including an increased virulence potential
462 and transcriptional changes observed in the hyper-motile mutants.

463 SUMMARY

464 This study describes for the first time the role of H-NS in *A. baumannii*. We employed a broad
465 range of phenotypic and genotypic characterization methods to gain insight into *A. baumannii*
466 virulence mechanisms and the role that H-NS plays in their regulation. Phenotypic
467 characterization showed that hydrophobicity, adherence and motility are likely to be co-regulated
468 in strain ATCC 17978. Furthermore, these features may be associated with virulence based on
469 data from a nematode killing assay and increased binding capacity to the A549 eukaryotic lung
470 cell line. Our analyses also provided evidence that the cellular fatty acid composition is linked to

471 changes in cell surface hydrophobicity, which subsequently may alter the adherence, motility and
472 virulence characteristics. Although these phenotypes appear to be co-regulated in strain ATCC
473 17978, it is known that *A. baumannii* strains show major variation in their motility and adherence
474 phenotypes (3, 7). Therefore, the role of H-NS is likely to be distinct in other *A. baumannii*
475 strains, which correlates with the function of H-NS as a regulator of non-conserved genomic
476 regions.

477 Transcriptomic analysis of 17978hm and its parent strain on semi-solid media identified
478 molecular mechanisms that may be responsible for the phenotypic changes described above. The
479 autotransporter encoded by *ata*, which has been proven to play a role in adherence and virulence
480 (52), was heavily up-regulated. Furthermore, the type VI secretion system and a type I pili were
481 found to be up-regulated and these surface presented protein structures are known to affect
482 adherence and virulence in other Gram-negative pathogens, such as *P. aeruginosa* and *E. coli*.
483 Significantly, up-regulation of genes encoding the fatty acid biosynthetic proteins FabG and
484 FabF may explain the increase of C₁₇ fatty acids in the hyper-motile mutant as compared to the
485 WT strain. Although FabG has previously been defined as a nucleoid-associated protein in other
486 bacterial genera (64), the regulatory control of *fabG* and *fabF* in the H-NS deficient strain
487 requires further examination.

488 Bioinformatic analyses using a number of fully sequenced *A. baumannii* genomes assisted in
489 identification of putative H-NS targets. Various genomic regions that were characterized as
490 horizontally-acquired were transcriptionally repressed by H-NS in the WT strain. Furthermore,
491 examination of the upstream regions of highly expressed genes showed that H-NS is likely to
492 bind AT-rich DNA regions, as observed for H-NS in other bacterial genera. The bioinformatics

493 approach applied here could also be of use when examining the role of H-NS in *A. baumannii*
494 strains other than ATCC 17978.

495 In summary, the phenotypic, genomic and transcriptomic analyses carried out using strain ATCC
496 17978 and its hyper-motile derivatives 17978hm and HNSmut88 revealed a significant role for
497 H-NS in the regulation of the *A. baumannii* persistence and virulence associated genes.

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505

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694

695 FIGURE LEGENDS

696 Fig 1

697 Genetic characteristics of the hyper-motile *A. baumannii* strains. (A) Using whole genome
698 sequencing of the ATCC 17978 and 17978hm strains, an insertion sequence (IS) element was
699 identified in a position unique to *A. baumannii* strain 17978hm. The sequence as found in strain
700 17978hm has been provided; the target sites of the IS element are shown in red and the inverted
701 repeat section of the IS element underlined. (B) Amino acid sequences of the DNA binding
702 region of the H-NS-like proteins from strain ATCC 17978, HNSmut88, *Mycobacterium*
703 *tuberculosis*, *Salmonella* and *E. coli* are displayed. Positively charged residues that may be
704 involved in DNA interactions are shown in red. The arginine (R) residues positioned outside of
705 the DNA binding domain are in bold. The lysine (K) in position 88 in strain ATCC 17978
706 mutated to an isoleucine (I) in strain HNSmut88 is boxed in blue.

707 Fig 2

708 Adherence characteristics of strains ATCC 17978 and 17978hm. (A) Pellicles of *A. baumannii*
709 strains ATCC 17978 and 17978hm were examined after incubation for 72 hours at 25°C and
710 statistically significant ($p < 0.001$; two-tailed Student's *t*-test) differences are indicated by
711 asterisks. Experiments were performed at least three times, error bars represent the standard
712 deviation. (B) The number of colony forming units (CFUs) in Log_{10} values of *A. baumannii*
713 ATCC 17978, 17978hm, 17978hm (pWH1266) and 17978hm (pWH0268) recovered from a
714 washed A549 cell culture after incubation for 4 hours were enumerated. Significant differences
715 between ATCC 17978 and 17978hm ($p < 0.05$), and 17978hm (pWH1266) and 17978hm

716 (pWH0268) ($p < 0.005$) were observed using a two-tailed Student's *t*-test and are indicated by
717 asterisks. Error bars show the standard error of the mean.

718 Fig 3

719 Examination of the virulence potential of ATCC 17978 and 17978hm. The virulence potential of
720 *A. baumannii* strains ATCC 17978 and 17978hm was examined by counting live versus dead *C.*
721 *elegans* nematodes grown on a lawn of the respective *A. baumannii* test strains. Significant
722 differences ($p < 0.001$) in killing were observed at 120 hours and 144 hours as determined by
723 student's *t*-test and are indicated by asterisks. The error bars show the standard deviation ($n=4$).

724 Fig 4

725 Overview of the transcriptional differences between motile and non-motile *A. baumannii* cells.
726 Comparative transcriptomics are displayed as the differential expression (in Log_2 -values) of
727 strains 17978hm (motile) and ATCC 17978 (non-motile) harvested from semi-solid MH media
728 (0.25% agar). Diamond markers indicate the differential expression levels of all predicted open
729 reading frames of the ATCC 17978 genome and are sorted on the X-axis according to the locus-
730 tag. The dashed lines indicate 4-fold ($\text{Log}_2=2$) differential expression; up-regulated genes in
731 motile populations are located above the green line and down-regulated genes below the red line.
732 Examples of differentially expressed genes have been indicated in the figure, such as the genes
733 involved in biosynthesis of quorum-sensing signals and those encoding proteins of the
734 phenylacetic acid degradation pathway.

735 Fig 5

736 Bioinformatic analysis of the putative H-NS targets in the ATCC 17978 genome. Transcriptome
737 results were mapped onto a circular representation of *A. baumannii* ATCC 17978 (CP000521)
738 using CGview. Up-regulated genes are represented in green and down-regulated genes in red;
739 half-sized bars equal 2-4-fold differential expression and full-sized bars >4-fold. To identify
740 potentially horizontally-acquired genomic regions, comparative Blastp analyses between
741 CP000521 (outer ring) and *A. baumannii* AB0057 (brown), ACICU (blue), AYE (purple),
742 AB307-0294 (orange), SDF (turquoise) and *A. baylyi* ADP1 (grey) are included. Various up-
743 regulated genes or gene clusters not fully conserved between CP000521 and other genomes were
744 identified; these were subsequently examined using Mauve. Based on CGview and Mauve
745 analyses, the following 13 up-regulated genes or gene clusters were found to be horizontally-
746 acquired and therefore putative H-NS target sites: (1) A1S_0519-0525 fatty acid biosynthesis;
747 (2) A1S_0745 bacterial surface protein; (3) A1S_1032-1033 Ata and a putative antigen; (4)
748 A1S_1078-1079 hypothetical protein and dichlorophenol hydroxylase; (5) A1S_1357 alanine
749 racemase; (6) A1S_2271 RNA splicing ligase; (7) A1S_2396 transcriptional regulator; (8)
750 A1S_2509 putative chaperone; (9) A1S_2648-2649 putative regulatory proteins; (10) A1S_2744
751 S-adenosyl-L-methionine-dependent methyltransferase; (11) A1S_2789 metallopeptidase; (12)
752 A1S_3273 putative secreted protein; and (13) A1S_3397 lysine (LysE) or homoserine/threonine
753 resistance (RhtB) protein.

754 Fig 6

755 GC-percentage of the putative H-NS targets. The GC-content plots are shown above schematic
756 representations of genes differentially expressed in the *hns* mutant strain, 17978hm, with their

757 respective locus-tag number displayed underneath. The average GC-percentage has been
758 calculated over the regions located upstream of these highly up-regulated genes and is
759 highlighted in blue. The GC-content plots were derived from the *A. baumannii* ATCC 17978
760 (CP000521) genome displayed in UGENE v1.10.1 (UniPro). These regions have a GC-
761 percentage significantly lower than the average genome-wide intergenic regions (~35%) and may
762 form suitable H-NS binding sites.

Table 1

Cell surface hydrophobicity and fatty acid composition.

Strains	Hydrophobicity index (StDev)	Fatty acid percentage		
		15:0	16:0	17:0
ATCC 17978	44 (13.1)	1.9	29.1	3.4
17978hm	65 (6.4)	2.8	25.4	6.9
17978hm (pWH1266)	64 (3.3)	2.4	24.4	5.2
17978hm (pWH0268)	14 (0.1)	0.7	31.4	2.6

Supplementary Table 1

Oligonucleotides used in this study.

Name/target	Forward 5' -> 3'	Reverse 5' -> 3'
A1S_r01 (16S rRNA) ^a	CAGCTCGTGTCTGAGATGT	CGTAAGGGCCATGATGACTT
A1S_0111	TTGGTCGAGTCAATCTGCAA	CTCGGGTCCCAATAAAATCA
A1S_0112	ACGCCAGTCTGGTGGTATTC	AGGTTTGAACAGCAATACGG
A1S_1032	AAGCCAGTCAAGCAACTGGT	TCAGAATCTGCTGCACCATC
A1S_1292	ACGCAACGCGTAATAAAGTG	TAAAGGGTCAAAGGCGAAC
A1S_1509	CCAAGGAAGGCGCTGT	TTGGGGAATGGCTTGC
A1S_1699	CAAAGACATTGCTGGTCGTG	AATCACGCTTGGACCTTCAC
A1S_3273	GGGTACACCTTCAGCAGAGC	GCACCATATTTACGGGCAAC
A1S_0095	CCGCAAAGTTATGCTGTGAA	GACGTAAACCCGTCCAGAAA
A1S_1336	CGTGCGATGGTACGTATTTG	ACGGTTCACTGCATCTTGTG
A1S_0268	GAGAGGATCCATAAATATTA AGAAAATATATTAC	GAGAGGATCCTTAGATTAAGAA ATCTTCAAG
A1S_2562	CACCATGAATATGCTCAAAGA CAT	GGTTGAAATGGTCTCACCAACTG G

^a Oligonucleotide sequences obtained from **Higgins et al.** 2004 J Antimicrob Chemother **54**: 821-823.

Supplementary Table 2

Genes up-regulated more than 4-fold in strain 17978hm		
Locus-tag	Gene product	Times-fold difference
AIS_0109	homoserine lactone synthase	30.7
AIS_0110	hypothetical protein	7.9
AIS_0111	cR transcriptional regulator	5.7
AIS_0112	Acyl-CoA synthetase/AMP-acid ligases II	588.4
AIS_0113	Acyl-CoA dehydrogenase	536.6
AIS_0114	Acyl carrier protein	727.2
AIS_0115	Amino acid adenylation	611.4
AIS_0116	RND superfamily-like exporters	240.6
AIS_0117	hypothetical protein	211.4
AIS_0118	hypothetical protein	53.8
AIS_0119	Phosphotransferase-kinase-protein transferase	47.1
AIS_0628	putative transposase	42.9
AIS_0739	putative transcriptional regulator	4.1
AIS_0745	hypothetical protein	21.7
AIS_0921	arginine/ornithine antiporter	4.5
AIS_0922	putative homocysteine S-methyltransferase family protein	4.3
AIS_1032	hypothetical protein	10.0
AIS_1033	putative antigen	8.0
AIS_1078	hypothetical protein	6.4
AIS_1079	dichlorophenol hydroxylase (EC:1.14.13.20)	10.6
AIS_1081	putative transcriptional regulator	6.2
AIS_1256	putative transcriptional regulator	21.4
AIS_1272	putative transcriptional regulator	4.8
AIS_1292	putative signal peptide	49.0
AIS_1293	hypothetical protein	35.7
AIS_1294	hypothetical protein	34.4
AIS_1295	hypothetical protein	13.5
AIS_1296	hypothetical protein	4.6
AIS_1297	hypothetical protein	5.8
AIS_1304	hypothetical protein	4.7
AIS_1357	alanine racemase	70.9
AIS_1384	CtnA-like protein	5.7
AIS_1404	putative cysteine desulfurase 1 (Csd)	4.7
AIS_1405	putative cysteine desulfurase 1 (Csd)	5.7
AIS_1406	major membrane protein I (MMP-I)	5.8
AIS_1407	serine acetyltransferase	4.4
AIS_1408	putative thiaminase-related sulfatransferase	5.2
AIS_1438	putative coenzyme F420-dependent NSN10-methylene tetrahydromethanopterin reductase	12.9
AIS_1439	putative coenzyme F420-dependent NSN10-methylene tetrahydromethanopterin reductase	16.8
AIS_1440	putative transporter (MFS superfamily)	4.2
AIS_1507	fimbrial protein	13.8
AIS_1508	fimbrial biogenesis outer membrane usher protein	18.3
AIS_1509	pili assembly chaperone	17.2
AIS_1510	fimbrial protein	12.2
AIS_1699	acetyl-26-dichlorophenolindophenol oxidoreductase alpha subunit	7.4
AIS_1700	acetyl-26-dichlorophenolindophenol oxidoreductase beta subunit (EC:1.2.4.1)	7.3
AIS_1701	dihydropyrimidine acetyltransferase	5.5
AIS_1702	dihydropyrimidine dehydrogenase	6.2
AIS_1703	dihydropyrimidine dehydrogenase	6.7
AIS_1751	AdA membrane fusion protein	8.7
AIS_1769	putative RND family drug transporter	5.1
AIS_1770	hypothetical protein	5.3
AIS_2396	putative transcriptional regulator	4.1
AIS_2554	putative transposase	27.6
AIS_2647	putative transcriptional regulator	4.1
AIS_2648	hypothetical protein	6.9
AIS_2649	putative regulatory protein	6.9
AIS_3104	putative ATP-dependent RNA helicase	4.5
AIS_3120	hypothetical protein	4.2
AIS_3146	multidrug efflux transport protein	4.1
AIS_3273	putative peptide signal	4.8

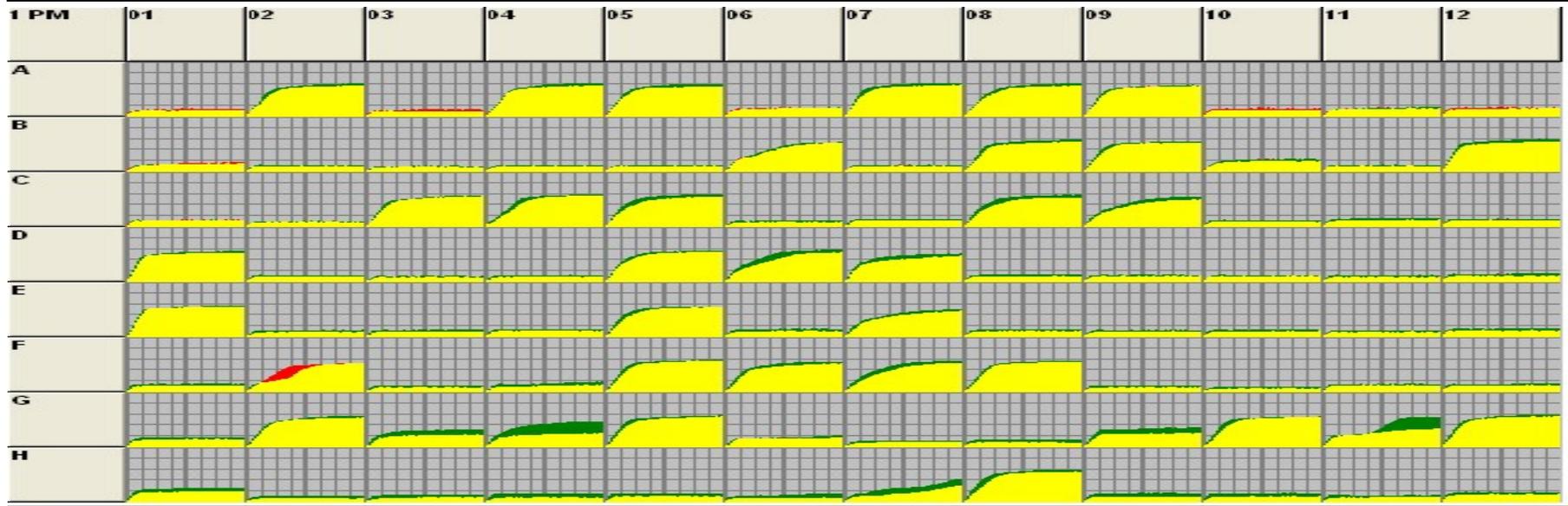
Genes down-regulated more than 4-fold in strain 17978hm

Locus-tag	Gene product	Times-fold difference
AIS_1186	ATP-dependent protease Hsp 100	-26.7
AIS_2183	putative signal peptide	-21.1
AIS_1950	putative universal stress protein	-19.9
AIS_0095	D-amino acid dehydrogenase (EC:1.4.99.1)	-17.4
AIS_0771	hypothetical protein	-16.6
AIS_3113	hypothetical protein	-16.0
AIS_3350	hypothetical protein	-14.4
AIS_2195	hypothetical protein	-12.6
AIS_1708	beta-lactamase-like protein	-12.2
AIS_3317	putative outer membrane protein	-12.2
AIS_1030	DNA-binding ATP-dependent protease La	-12.1
AIS_1932	hypothetical protein	-10.9
AIS_2960	chaperone Hsp70	-10.6
AIS_0096	alanine racemase 2 PLP-binding, catabolic	-9.7
AIS_2993	hypothetical protein	-8.9
AIS_0558	aconitate hydratase 1 (EC:4.2.1.3)	-8.8
AIS_1193	OmpA/MotB	-8.8
AIS_3175	bacterioferritin	-8.6
AIS_2070	P-type ATPase Mg ²⁺ -ATPase transporter (EC:3.6.3.2)	-8.4
AIS_1031	DNA-binding ATP-dependent protease La	-8.2
AIS_1687	transcriptional regulator	-8.1
AIS_1338	hypothetical protein	-8.1
AIS_0800	bacterioferritin	-8.0
AIS_1046	Lysine exporter protein (LysE/YggA)	-7.9
AIS_3023	hypothetical protein	-7.8
AIS_1984	D-amino acid dehydrogenase small subunit	-7.7
AIS_0363	hypothetical protein	-7.2
AIS_0683	putative sigma (S4) modulation protein RpoX	-6.9
AIS_2538	outer membrane protein CarO precursor	-6.8
AIS_1266	hypothetical protein	-6.8
AIS_1337	Phenylacetic acid degradation B	-6.7
AIS_2820	hypothetical protein	-6.7
AIS_1339	Phenylacetate-CoA oxygenase PaaJ subunit	-6.6
AIS_3277	putative pili-like protein	-6.5
AIS_0301	hypothetical protein	-6.5
AIS_1267	putative lactam utilization protein	-6.4
AIS_1910	ATP-binding protease component	-6.4
AIS_1246	putative universal stress protein	-6.1
AIS_0445	hypothetical protein	-6.1
AIS_0097	hypothetical protein	-6.1
AIS_1270	hypothetical protein	-5.9
AIS_3046	oligopeptidase A	-5.8
AIS_2296	putative protease	-5.8
AIS_1269	putative alkalylphosphate hydrolase subunit 1 and 2	-5.8
AIS_1340	Phenylacetate-CoA oxygenase/reductase PaaK subunit	-5.8
AIS_2809	bacteriolytic lipoprotein entericidin B	-5.8
AIS_1343	Paac	-5.7
AIS_1268	hypothetical protein	-5.6
AIS_2616	hypothetical protein	-5.5
AIS_0210	transposase	-5.5
AIS_2664	chaperone Hsp60	-5.4
AIS_2291	hypothetical protein	-5.3
AIS_1342	putative enoyl-CoA hydratase II	-5.3
AIS_2450	putative pyruvate decarboxylase	-5.2
AIS_2259	putative signal peptide	-5.2
AIS_2840	outer membrane protein A	-5.2
AIS_2959	Hsp 24 nucleotide exchange factor	-5.1
AIS_1925	cytochrome d terminal oxidase polypeptide subunit II	-5.1
AIS_1433	ubiquinol oxidase subunit II	-5.1
AIS_0207	hypothetical protein	-5.1
AIS_0646	kcaB protein	-5.0
AIS_2449	aromatic amino acid transporter (APC family)	-5.0
AIS_0172	hypothetical protein	-5.0
AIS_2072	putative universal stress protein family	-4.9
AIS_1518	putative suppressor of F exclusion of phage T7	-4.9
AIS_1390	hypothetical protein	-4.8
AIS_1862	hypothetical protein	-4.8
AIS_0496	putative phosphatidylglycerophosphatase B	-4.7
AIS_0412	catelase (EC:1.1.1.6)	-4.7
AIS_1926	hypothetical protein	-4.7
AIS_0884	putative outer membrane protein	-4.7
AIS_1726	aspartate ammonia-lyase (aspartase) (EC:4.3.1.1)	-4.6
AIS_2416	hypothetical protein	-4.6
AIS_3180	putative signal peptide	-4.5
AIS_1335	Phenylacetic acid degradation protein paaN	-4.5
AIS_1924	cytochrome d terminal oxidase polypeptide subunit I	-4.4
AIS_1861	benzoate dioxygenase large subunit	-4.4
AIS_0627	hypothetical protein	-4.4
AIS_2417	starvation-induced peptide utilization protein	-4.3
AIS_0642	hypothetical protein	-4.3
AIS_1336	hypothetical protein	-4.2
AIS_1859	aromatic-ring-hydroxylating dioxygenase beta subunit	-4.2
AIS_2696	hypothetical protein	-4.2
AIS_3122	hypothetical protein	-4.2
AIS_2092	aminopeptidase N	-4.1
AIS_0299	hypothetical protein	-4.1
AIS_2886	acyl-CoA dehydrogenase	-4.1
AIS_0465	Sac-independent protein translocase protein	-4.1
AIS_0634	hypothetical protein	-4.0
AIS_3246	hypothetical protein	-4.0
AIS_2504	excinuclease ABC subunit B	-4.0

Supplementary Table 3

PM1 MicroPlate™ CarbonSources

A1 Negative Control	A2 L-Arabinose	A3 N-Acetyl-D- Glucosamine	A4 D-Saccharic Acid	A5 Succinic Acid	A6 D-Galactose	A7 L-Aspartic Acid	A8 L-Proline	A9 D-Alanine	A10 D-Trehalose	A11 D-Mannose	A12 Dulcitol
B1 D-Serine	B2 D-Sorbitol	B3 Glycerol	B4 L-Fucose	B5 D-Gluconic Acid	B6 D-Gluconic Acid	B7 D,L- α -Glycerol- Phosphate	B8 D-Xylose	B9 L-Lactic Acid	B10 Formic Acid	B11 D-Mannitol	B12 L-Glutamic Acid
C1 D-Glucose- 6-Phosphate	C2 D-Galactonic Acid- γ -lactone	C3 D,L-Malic Acid	C4 D-Ribose	C5 Tween 20	C6 L-Rhamnose	C7 D-Fructose	C8 Acetic Acid	C9 α -D-Glucose	C10 Maltose	C11 D-Melibiose	C12 Thymidine
D1 L-Asparagine	D2 D-Aspartic Acid	D3 D-Glucosaminic Acid	D4 1,2-Propanediol	D5 Tween 40	D6 α -Keto-Glutaric Acid	D7 α -Keto-Butyric Acid	D8 α -Methyl-D- Galactoside	D9 α -D-Lactose	D10 Lactulose	D11 Sucrose	D12 Uridine
E1 L-Glutamine	E2 M-Tartaric Acid	E3 D-Glucose- 1-Phosphate	E4 D-Fructose- 6-Phosphate	E5 Tween 80	E6 α -Hydroxy Glutaric Acid γ -Lactone	E7 α -Hydroxy Butyric Acid	E8 β -Methyl-D- Glucoside	E9 Adonitol	E10 Maltotriose	E11 2-Deoxy Adenosine	E12 Adenosine
F1 Glycyl-L- Aspartic Acid	F2 Citric Acid	F3 M-Inositol	F4 D-Threonine	F5 Fumaric Acid	F6 Bromo Succinic Acid	F7 Propionic Acid	F8 Mucic Acid	F9 Glycolic Acid	F10 Glyoxylic Acid	F11 D-Cellobiose	F12 Inosine
G1 Glycyl-L- Glutamic Acid	G2 Tricarballic Acid	G3 L-Serine	G4 L-Threonine	G5 L-Alanine	G6 L-Alanyl- Glycine	G7 Acetoacetic Acid	G8 N-Acetyl- β -D- Mannosamine	G9 Mono Methyl Succinate	G10 Methyl Pyruvate	G11 D-Malic Acid	G12 L-Malic Acid
H1 Glycyl-L- Proline	H2 p -Hydroxy Phenyl Acetic Acid	H3 m -Hydroxy Phenyl Acetic Acid	H4 Tyramine	H5 D-Psicose	H6 L-Lyxose	H7 Glucuronamide	H8 Pyruvic Acid	H9 L-Galactonic Acid-Lactone	H10 D-Galacturonic Acid	H11 Phenylethylamine	H12 2-Aminoethanol



PM2A MicroPlate™ CarbonSources

A1 Negative Control	A2 Chondroitin Sulfate C	A3 α-Cyclodextrin	A4 β-Cyclodextrin	A5 γ-Cyclodextrin	A6 Dextrin	A7 Gelatin	A8 Glycogen	A9 Inulin	A10 Laminarin	A11 Mannan	A12 Pectin
B1 N-Acetyl-D-Galactosamine	B2 N-Acetyl-Neuraminic Acid	B3 β-D-Allose	B4 Amygdalin	B5 D-Arabinose	B6 D-Arabitol	B7 L-Arabitol	B8 Arbutin	B9 2-Deoxy-D-Ribose	B10 l-Erythritol	B11 D-Fucose	B12 3-O-β-D-Galactopyranosyl D-Arabinose
C1 Gentiobiose	C2 L-Glucose	C3 Lactitol	C4 D-Melezitose	C5 Maltitol	C6 α-Methyl-D-Glucoside	C7 β-Methyl-D-Galactoside	C8 3-Methyl Glucose	C9 β-Methyl-D-Glucuronic Acid	C10 α-Methyl-D-Mannoside	C11 β-Methyl-D-Xyloside	C12 Palatinose
D1 D-Raffinose	D2 Salicin	D3 Sedoheptulosa	D4 L-Sorbose	D5 Stachyose	D6 D-Tagatose	D7 Turanose	D8 Xylitol	D9 N-Acetyl-D-Glucosaminitol	D10 γ-Amino Butyric Acid	D11 d-Amino Valeric Acid	D12 Butyric Acid
E1 Capric Acid	E2 Caproic Acid	E3 Citraconic Acid	E4 Citramalic Acid	E5 D-Glucosamine	E6 2-Hydroxy Benzoic Acid	E7 4-Hydroxy Benzoic Acid	E8 β-Hydroxy Butyric Acid	E9 γ-Hydroxy Butyric Acid	E10 α-Keto Valeric Acid	E11 Itaconic Acid	E12 5-Keto-D-Gluconic Acid
F1 D-Lactic Acid Methyl Ester	F2 Malonic Acid	F3 Melibionc Acid	F4 Oxalic Acid	F5 Oxalomalic Acid	F6 Quinic Acid	F7 D-Ribono-1,4-Lactone	F8 Sebacic Acid	F9 Sorbic Acid	F10 Succinamic Acid	F11 D-Tartaric Acid	F12 L-Tartaric Acid
G1 Acetamide	G2 L-Alaninamide	G3 N-Acetyl-L-Glutamic Acid	G4 L-Arginine	G5 Glycine	G6 L-Histidine	G7 L-Homoserine	G8 Hydroxy-L-Proline	G9 L-Isoleucine	G10 L-Leucine	G11 L-Lysine	G12 L-Methionine
H1 L-Ornithine	H2 L-Phenylalanine	H3 L-Pyrogutamic Acid	H4 L-Valine	H5 D,L-Carnitine	H6 Sec-Butylamine	H7 D,L-Octopamine	H8 Putrescine	H9 Dihydroxy Acetone	H10 2,3-Butanediol	H11 2,3-Butanone	H12 3-Hydroxy 2-Butanone

