The primary transcriptome, small RNAs and regulation of antimicrobial resistance in *Acinetobacter baumannii* ATCC 17978

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Received December 20, 2017; Revised June 21, 2018; Editorial Decision June 24, 2018; Accepted June 26, 2018

**ABSTRACT**

We present the first high-resolution determination of transcriptome architecture in the priority pathogen *Acinetobacter baumannii*. Pooled RNA from 16 laboratory conditions was used for differential RNA-seq (dRNA-seq) to identify 3731 transcriptional start sites (TSS) and 110 small RNAs, including the first identification in *A. baumannii* of sRNAs encoded at the 3′ end of coding genes. Most sRNAs were conserved among sequenced *A. baumannii* genomes, but were only weakly conserved or absent in other *Acinetobacter* species. Single nucleotide mapping of TSS enabled prediction of −10 and −35 RNA polymerase binding sites and revealed an unprecedented base preference at position +2 that hints at an unrecognized transcriptional regulatory mechanism. To apply functional genomics to the problem of antimicrobial resistance, we dissected the transcriptional regulation of the drug efflux pump responsible for chloramphenicol resistance, *craA*. The two *craA* promoters were both down-regulated >1000-fold when cells were shifted to nutrient limited medium. This conditional down-regulation of *craA* expression renders cells sensitive to chloramphenicol, a highly effective antibiotic for the treatment of multidrug resistant infections. An online interface that facilitates open data access and visualization is provided as ‘AcinetoCom’ (http://bioinf.gen.tcd.ie/acinetocom/).

**INTRODUCTION**

In 2017, the World Health Organization (WHO) ranked carbapenem-resistant *Acinetobacter baumannii* as the top priority critical pathogen in the WHO’s first-ever list of priority antimicrobial-resistant pathogens (1,2). It is estimated that one million *A. baumannii* infections occur worldwide each year, causing 15 000 deaths (3). In the United States, *A. baumannii* is responsible for more than 10% of nosocomial infections, and causes a variety of diseases such as ventilator-associated pneumonia, bacteraemia, skin and soft tissue infections, endocarditis, urinary tract infections and meningitis (4). Despite the urgency to develop new antimicrobial drugs, we know little about *A. baumannii* infection biology and virulence mechanisms because only a few *A. baumannii* virulence genes and their regulation have been functionally studied (5).

Characterizing the transcriptional landscape and simultaneously quantifying gene expression on a genome-wide scale using RNA sequencing (RNA-seq) is a cornerstone of functional genomic efforts to identify the genetic basis of cellular processes (6). RNA-seq identifies transcripts for an entire genome at single-nucleotide resolution in a strand-specific fashion, making it an ideal tool to un-
cover transcriptome features. Recent technical enhancements to RNA-seq enable improved delineation of transcriptional units to localize TSS, transcript ends, sRNAs, antisense transcription and other transcriptome features (6,7). The ability to quantify gene expression and characterize transcripts from any organism is particularly valuable for the study of emerging bacterial pathogens, where research knowledge lags behind the global spread, health burden and economic impacts of disease.

To address the lack of basic biological insight of A. baumannii, we used a functional genomics approach to investigate the transcriptome architecture of A. baumannii ATCC 17978 and to investigate virulence and antibiotic resistance gene expression in this priority pathogen. A. baumannii ATCC 17978 is one of the best-studied strains of Acinetobacter, and is a useful model for genetic manipulation due to its natural sensitivity to most antibiotics used in the laboratory (8,9). Differential RNA-seq (dRNA-seq) facilitates the precise identification of TSS by distinguishing 5′ triphosphorylated (i.e. newly synthesized, ‘primary’ transcripts from the dephosphorylated 5′ termini) of processed RNA species (10). dRNA-seq analysis of transcription in virulence-relevant conditions, antimicrobial challenge, and standard laboratory culture generated a high-resolution map of TSS and small RNAs in the A. baumannii chromosome, revealing the precise location of 3731 promoters and 110 small RNAs. This comprehensive view of TSS identified two promoters that regulate chloramphenicol resistance by the CraA efflux pump and we discovered growth conditions that stimulate low craA expression and render A. baumannii sensitive to chloramphenicol. To facilitate similar discoveries by the research community, we created the online resource AcinetoCom (http://bioinf.gen.tcd.ie/acinetocom/) for free and intuitive exploration of the A. baumannii ATCC 17978 transcriptome.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions**

Acinetobacter baumannii ATCC 17978 was obtained from LGC-Standards-ATCC. We used the reference strain containing all three plasmids (pAB1, pAB2, pAB3) for dRNA-seq analysis. Cells were cultured overnight in 5 ml Lennox (L-) broth (10 g l\(^{-1}\) tryptone, 5 g l\(^{-1}\) yeast extract, 5 g l\(^{-1}\) NaCl; pH 7.0). For routine cultures, cells were subcultured (1:1000) in 250 ml flasks containing 25 ml of growth medium and grown with shaking at 220 RPM in a waterbath. For experiments in non-standard conditions or when exposing cells to specific challenges, a fresh L-broth culture was grown for 2–3 h to achieve steady-state exponential growth physiology before inoculation into an alternate growth condition or challenge.

To maximize the number of TSS identified, cells were exposed to a diversity of growth conditions (detailed below) to induce expression from as many gene promoters as possible. RNA extracts from cells in each of 16 conditions were pooled together into a single sample, creating the ‘Pool’ sample summarized in Table 1. To capture all TSS in the different growth stages of routine L-broth culture, cells were grown in L-broth at 37°C and sampled at select culture densities (OD\(_{600}\) values of 0.25, 0.75, 1.8, 2.5 and 2.8). To identify TSS expressed in the presence of disinfectants and antimicrobial agents, L-broth cultures of exponentially-growing cells (OD\(_{600}\) of 0.3) at 37°C were subjected to 10-min shocks, containing either a 1:100,000 dilution of disinfectant (Distel ‘High Level Medical Surface Disinfectant’, non-fragranced), hydrogen peroxide to a final concentration of 1 mM, ethanol to a final concentration (v/v) of 2%, kanamycin to a final concentration of 50 μg ml\(^{-1}\), NaCl to a final concentration of 0.3 M or 2,2′-dipyridyl to a final concentration of 0.2 mM. To mimic growth in environmental conditions, cells were cultured in L-broth at low temperatures (25°C) until an OD\(_{600}\) of 0.3; these same cultures were transferred to 37°C for 10 min. To evaluate gene expression in response to alternative nutrient environments, overnight cell cultures were sub-cultured in liquid M9 minimal medium supplemented with 1 mM MgSO\(_4\), 0.1 mM CaCl\(_2\) and a carbon source of either 31.2 mM fumarate or 33.3 mM xylose, and grown at 37°C to an OD\(_{600}\) of 0.3. For growth on solid media, A. baumannii ATCC 17978 was plated on L-agar plates and grown for 16 hours at 37°C.

Acinetobacter baumannii ATCC 17978 was also used for early stationary phase (ESP) transcriptome analysis via RNA-seq. For this analysis, cells were sub-cultured in 250 ml flasks containing 25 ml of L-broth and grown with shaking at 37°C to an OD\(_{600}\) of 2.0. RNA-seq revealed that pAB3 was absent in the strain used for this analysis, indicating that pAB3 was lost during shipping of ATCC 17978 between our laboratories. A comparison of RNA-seq reads to the reference genome did not detect any point mutations in the derivative strain lacking pAB3.

**RNA extraction, ribosomal RNA depletion, DNase digestion and the RNA pool**

Total RNA was isolated from cultures of A. baumannii ATCC 17978 cells using TRizol as described previously for Salmonella enterica serovar Typhimurium (11). The RNA quality was analyzed using an Agilent Bioanalyzer 2100 and RNA concentration was measured on a NanoDrop™ spectrophotometer or the Qubit™ (Invitrogen). To generate the pooled RNA sample for dRNA-seq analysis, equal amounts of RNA from each growth condition were combined into a single sample. For ESP transcriptome analysis, three biological replicates were generated. These ESP samples were depleted for ribosomal RNA using the RiboZero rRNA Removal Kit Bacteria (Illumina). Contaminating DNA was removed from ESP and pooled RNA samples via DNase I (TURBO DNA-free™ kit, Ambion) digestion; the pooled RNA sample was also DNase I treated (TURBO DNase I, Ambion) as part of sample workflow by vertis Biotechnologie AG (Freising, Germany).

**Library preparation for next generation sequencing**

The pooled RNA sample was divided into two separate aliquots prior to library preparation. Libraries of both aliquots were prepared by vertis Biotechnologie AG (Freising, Germany) and sequenced on an Illumina NextSeq machine (single reads). For dRNA-seq, one of the two aliquots were digested with Terminator Exonuclease (TEX) prior to cDNA library preparation (10,12). Libraries of the ESP
RNA samples were prepared using the NEBNext™ Ultra RNA Library Prep Kit for Illumina (NEB) according to the manufacturer’s instructions. These libraries were sequenced on an Illumina MiSeq (paired-end reads). We noted that for very highly-expressed genes in the ESP samples, there were distinct transcripts mapped to the opposite strand that perfectly mirrored the expected transcript. This is best explained by incomplete depletion of non-template strand (second strand incorporated dUTP) by the USER enzyme during library preparation with NEBNext™ Ultra. Therefore, we considered only the pooled RNA-seq data from vertis Biotechnologien AG, which is generated by an RNA adaptor ligation step before cDNA synthesis, to determine the amount of antisense transcription.

**Mapping of sequencing reads, statistics and analysis**

The original genome sequence for *A. baumannii* ATCC 17978 identified two plasmids (pAB1 and pAB2) (8). However, it was subsequently revealed that ATCC 17978 carries a third plasmid (pAB3) that was incorrectly assembled as chromosome sequence in the first assembly (13), thus the *A. baumannii* ATCC 17978-mff genome (accession: NZ_CP012004.1) was used as a reference sequence in our study. Quality control was carried out using FastQC and showed excellent quality for the sequencing data. DNA sequencing reads were mapped using bowtie2 with the --very-sensitive–local settings for use of soft-clipping and maximum sensitivity (14). Conversion into BAM and BigWig files was carried out using SAMtools (15) and Galaxy at galaxy.org (16). Mapped reads were visualized in the Integrated Genome Browser (IGB) (17) and Jbrowse (18). Number of sequence reads mapped were the (under-score indicates the biological replicate) ESP.1 (10,588,403; mapped uniquely: 8,403,658), ESP.2 (9,374,114; mapped uniquely: 6,769,516), ESP.3 (11,121,847; mapped uniquely: 7,891,371), RNA_pool (66,853,984; mapped uniquely: 19,607,931) and dRNA-seq_Pool (77,237,102; mapped uniquely: 30,041,320). The difference in percentage of uniquely mapped reads of the ESP conditions and the Pool samples is caused by the fact that RNA from the ESP condition was depleted for rRNA, while the RNA samples of the RNA Pool were not. FeatureCounts was used to summarize read counts for each annotated feature (19). These values were transformed into transcripts per million reads (TPM) following the formula provided by Li *et al.* (20); (Supplementary Table S4).

**Identification and categorization of TSS**

Before the identification of TSS, the coverage files for the RNA Pool and TEX-digested RNA Pool sample were normalized to account for different library sizes in the following way: the number of mapped nucleotides for each position was divided by the number of uniquely mapped reads of the smallest library (ESP.2) and then multiplied by the number of reads for the library being normalized. The locations of TSS were predicted with TSSpredator (21), which is used to assess enrichment of reads in the TEX-digested sample over the non-TEX-digested control; enrichment is reflective of a 5' triphosphate protecting the transcript from TEX digestion. Default settings were used, with the exception of changing the step height option to 0.2, the 5' UTR (untranslated region) length to 400 nt and the enrichment factor to 1.5. The locations of the predicted TSS were manually inspected in IGB and Jbrowse. TSS that exist ≤400 nt upstream of the start of a coding region and TSS of non-coding RNA genes were classified as Primary or Secondary TSS; in cases where multiple candidate TSS are upstream, the TSS associated with the strongest expression was designated as the Primary TSS, while all additional TSS were designated Secondary TSS. TSS with intragenic location on the sense strand were classified as Internal TSS and when located on the antisense strand as Antisense TSS. TSS were

| Condition name | Growth description |
|----------------|-------------------|
| OD 0.25 | Growth in Lennox broth to OD600 0.25 |
| OD 0.75 | Growth in Lennox broth to OD600 0.75 |
| OD 1.8 | Growth in Lennox broth to OD600 1.8 |
| OD 2.5 | Growth in Lennox broth to OD600 2.5 |
| OD 2.8 | Growth in Lennox broth to OD600 2.8 |
| 25°C | Growth in Lennox broth to OD600 0.3 at 25°C |
| Temp10 | Growth in Lennox broth to OD600 0.3 at 25°C; then transfer to 37°C for 10 min. |
| NaCl shock | Growth in Lennox broth to OD600 0.3; then addition of NaCl (Sigma, Cat. S3014) to a final concentration of 0.3 M for 10 min. |
| LowFe²⁺ shock | Growth in Lennox broth to OD600 0.3; then addition of 2,2'-dipyridyl (Sigma, Cat. D21630) to a final concentration of 0.2 mM for 10 min. |
| H₂O₂ shock | Growth in Lennox broth to OD600 0.3; then addition of H₂O₂ (Merck, Cat. 822287) to final concentration of 1 mM H₂O₂ for 10 min |
| Kan shock | Growth in Lennox broth to OD600 0.3; then addition of kanamycin to final concentration of 50 μg/ml for 10 min. |
| EtOH shock | Growth in Lennox broth to OD600 0.3; then addition of ethanol to final concentration of 2% (v/v) for 10 min. |
| Disinfectant | Growth in Lennox broth to OD600 0.3; then addition of 250 μl disinfectant Distel 'High Level Medical Surface Disinfectant', non-fragranced (1:100 dilution, VWR, Cat. 141-0933) for (1:100 dilution) for 10 min |
| L-agar | Growth on an Lennox broth agar plate for 16 h at 37°C |
| M9+xyl | Growth in M9 minimal medium (incl. 1 mM MgSO₄, 0.1 uM CaCl₂, 33.3 mM D-xylose (Sigma, Cat. X3877) to OD600 0.3) |
| M9+fum | Growth in M9 minimal medium (incl. 1 mM MgSO₄, 0.1 uM CaCl₂, 31.2 mM sodium fumarate (Sigma, Cat. F1506) to OD600 0.3) |
| ESP | Growth in Lennox broth to OD600 2.0 |

*Note: The table values are rounded for presentation.*
classified as Orphan if they could not be assigned to any other category. Occasionally, manual curation changed the classification of TSS, e.g. five genes with a 5′ UTR > 400 nt were classified as Primary or Secondary (Supplementary Table S2).

Rapid amplification of cDNA ends (5′ RACE)

5′ RACE experiments were carried out as described earlier (11,22) with the following modifications: Twenty-four μg of DNaseI-treated total RNA isolated from A. baumannii cells grown in L-broth to ESP was split into two 12 μg aliquots of which one was treated with 10 U pyrophospho-hydrolase RppH (NEB). The other aliquot remained untreated as a negative control. Following phenol-chloroform extraction and sodium-acetate precipitation, a single strand RNA oligonucleotide was ligated to the RNA samples using single strand RNA ligase (NEB). Following phenol–chloroform extraction and Na-acetate precipitation, two μg of linker-ligated RNA (RppH- and mock-treated) was used to synthesize cDNA using random hexamer priming and Superscript III reverse transcriptase (Invitrogen). Taq polymerase was used for PCR amplification of RACE products to facilitate cloning and sequencing of RACE products using the TOPO cloning vector pCR2.1 (Invitrogen). After transformation of Escherichia coli TOPO cells with pCR2.1 containing RACE products, plasmid DNA of five clones was isolated and inserts sequenced. All five sequences agreed with the TSS identified by dRNA-seq.

Identification of −10 and −35 sigma factor motifs

DNA sequences upstream of all transcription start sites were extracted in two blocks of nucleotides: between positions −3 to −18 (−10 block) and between −16 to −50 (−35 block). Each block was searched with the unbiased motif finding program MEME 4.11.2 (23). Each sequence block was searched with the following settings: one occurrence per sequence with a width of 6–12 bp (−10 motif) or 4–10 bp (−35 motif). Sequence logos were generated by WebLogo (24).

Identification of candidate sRNA genes and prediction of transcription terminators

The identification of candidate sRNA genes was carried out by searching mapped sequence reads in IGB and Jbrowse for short unannotated transcripts as well as by using the output from the TSSpredator software, which annotates orphan and internal TSS that may represent new sRNA candidates. A new candidate sRNA gene was assigned if a short (≤500 nt) unannotated transcript was observed in the RNA-seq data. A minimum cutoff for expression was set at TPM > 10, as previously described (12). The borders of the transcript were informed by the location of TSS determined by dRNA-seq data and the location of predicted Rho-independent transcription terminators using ARNold with the default setting (25).

sRNA conservation analysis

The conservation of candidate sRNA genes was investigated using GLSEARCH (26). The genome sequences were obtained from NCBI (A. baumannii Ab04-mff (NZ_CP012006.1), A. baumannii AB030 (NZ_CP009257.1), A. baumannii AB0057 (NC_011586.1), A. baumannii ABS0T5-UW (NZ_CP008706.1), A. baumannii ACICU (NC_010611.1), A. baumannii strain ATCC 17978 (NZ_CP018664.1), A. baumannii ATCC 17978-mff (NZ_CP012004.1), A. baumannii str. AYE (NC_010410.1), A. baumannii IOMTU 433 (NZ_AP014649.1), A. baumannii LAC-4 (NZ_CP007712.1), A. baumannii A1 (NZ_CP010781.1), A. baumannii USA15 (NZ_CP020595.1), A. baumannii XH386 (NZ_CP010779.1), A. baumannii TYTH-1 (NC_018706.1), A. baumannii XDR-BJ83 (NZ_CP018421.1), Acinetobacter baylyi DSM 14961 (NZ_KB849622.1-NZ_KB849630.1), Acinetobacter equi 114 (NZ_CP012808.1), Acinetobacter haemolyticus CIP 64.3 (NZ_KB849798.1-KB849812.1), Acinetobacter lwofvii NCTC 5866 (NZ_KB851224.1-NZ_KB851239.1), Acinetobacter nosocomialis 6411 (NZ_CP010368.1), Acinetobacter pittii PHEA-2 (NC_016603.1), Acinetobacter venetianus VE-C3 (NZ_CM01772.1), Moraxella catarrhalis BBH18 (NC_014417.1), Pseudomonas aeruginosa PAO1 (NC_002516.2), Pseudomonas fluorescens F113 (NC_016830.1), Pseudomonas putida KT2440 (NC_002947.4), Pseudomonas syringae pv. syringae B728a (NC_007005.1). The score shows percentage sequence identity with the reference sequence (A. baumannii 17978-mff).

Northern blotting

Northern blotting was performed using the DIG Northern Starter Kit (Roche) using DIG-labelled riboprobes generated by in vitro transcription as described earlier and according to the manufacturer’s instructions (11). Templates for in vitro transcriptions using T7 RNA polymerase were generated by PCR using DNA oligonucleotides listed in Supplementary Table S1. Five microgram of total RNA was separated on a 7% urea–polyacrylamide gel in 1× Tris-borate-EDTA (TBE) buffer cooled to 4°C. Band sizes on the northern blots were compared with Low Range single strand RNA ladder (NEB). After blotting and UV crosslinking, the lane with the ladder was cut off, stained with 0.1% methylene blue solution for 1 min at room temperature, destained in sterile water, and re-attached to the membrane before detection of chemiluminescent bands using an ImageQuant LAS4000 imager (GE Healthcare).

Chloramphenicol sensitivity and craA expression analysis

Cells were cultured overnight in L-broth containing 25 μg ml−1 chloramphenicol and the next morning inoculated to OD600 0.05 in 20 ml fresh medium and grown to OD600 of 0.3 in a 250-ml flask with shaking at 37°C. Cells were captured on 0.2 μm analytical test filter funnels (Nalgene), washed with M9 containing 20 mM pyruvate as the carbon source and the filter (retaining the cells) was transferred to M9 plus pyruvate. The cells were washed off the filter and inoculated in fresh 20 mL M9 plus pyruvate medium at
an OD₆₀₀ of 0.1 and incubated at 37°C with shaking for 5
days. Sampling for qPCR and chloramphenicol resistance
was performed at t = 0, 4, 24, 48 and 96 h and transcription
was stopped by the addition of phenol:ethanol stop solution
(27,28). For qPCR, total RNA was isolated from cultures using
the EZ-10 Spin Column Total RNA Mini-Preps Super Kit (BioBasic)
and purity and quality was assessed using the EZ-10 Spin Column Total
RNA kit (BioBasic). Total RNA was treated in a 50 μl reaction using
the TURBO DNA-free kit (AMBION), and cDNA templates
were synthesized by random priming 0.5 μg RNA in a 20
μl reaction using Verso cDNA synthesis kit (Fisher). PCR
reactions were carried out in duplicate with each primer
set on an ABI StepOnePlus PCR System (Applied Biosys-
tems) using Perfecta SYBR Fastmix with ROX (Quanta
Biosciences). Standard curves were included in every qPCR
run; standard curves were generated for each primer set us-
ing five serial ten-fold dilutions of A. baumannii genomic
DNA. Quantitative PCR (qPCR) primers are listed in Sup-
plementary Table S1. Viable counts were performed by se-
persive SYBR Fastmix with ROX (Quanta
Biosciences). Standard curves were included in every qPCR
run; standard curves were generated for each primer set us-
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DNA. Quantitative PCR (qPCR) primers are listed in Sup-
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RESULTS AND DISCUSSION

Global identification of A. baumannii TSS

To generate a comprehensive list of TSS for A. baumann-
nii ATCC 17978, we adopted the technique developed for
Salmonella Typhimurium in which diverse laboratory con-
ditions were used to elicit transcription activation from as
many gene promoters as possible (12). Thus, we used 16
conditions that reflect a diversity of environments expe-
rienced by A. baumannii during infection, environmental
survival, and general stress (Table 1). Specifically, A. baum-
nannii was cultured in L-broth and sampled throughout
growth at OD₆₀₀ 0.25, 0.75, 1.8, 2.5, 2.8; in liquid M9 mini-
imal medium with different sole carbon sources (xylose, fu-
murate), and grown on an L-agar plate for 16 h at 37°C,
and in liquid L-broth at OD₆₀₀ 0.3 at 25°C. These condi-
tions encompass high nutrient (L-broth) and low nutri-
ent (M9) conditions; amino acids (L-broth) and carbono-
drates (M9) as primary carbon sources; solid (L-agar) and
liquid media; host (37°C) and external (25°C); and a full
range of growth states, including stages of rapid growth
in which bulk transcription rates are very high, to station-
ary phase in both rich and energy poor media, when tran-
scriptional output is low and favors long-lived transcripts
(29). We also isolated RNA from several ‘shock’ condi-
tions, including disinfectant shock, osmotic shock (addi-
tion of sodium chloride), limitation of iron availability (addi-
tion of 2,2′-dipyridyl), translational stress (addition of
kanamycin), oxidative stress (addition of hydrogen pero-
oxide), temperature shift from ambient to body temperature
(25-37°C), and the addition of ethanol (which increases
virulence in A. baumannii infection of Dictyostelium dis-
coideum and Caenorhabditis elegans (30)). Total RNA was
isolated from the 16 conditions, pooled, and used as a single
sample.

To enrich for primary transcripts, an aliquot from the
pooled sample was digested with Terminator Exonucle-
ase (TEX) (10). Over 20 million mapped sequencing reads
were analyzed with TSSpredator software (21) and manu-
ally inspected with Integrated Genome Browser (IGB) and
Jbrowse (17,18) to define the genomic location of 3731 TSS
(Figure 1). The TSS of ACX60_RS00325 encoding a pu-
tative 4-hydroxyphenylpyruvate dioxygenase was indepen-
dently confirmed by 5′ RACE and agreed with the TSS
determined by RNA-seq (Supplementary Figure S1). In
addition, three biological replicates of RNA isolated from
OD₆₀₀ 2.0 (early stationary phase (ESP)) yielded over 6.7
million mapped reads for each of the ESP biological repli-
cates and the >20 million mapped reads for the remaining
aliquot from the pooled RNA sample (i.e. the aliquot not
treated with TEX) provided sufficient depth to cover the
bacterial transcriptome (31).

TSS were categorized according to their locations rela-
tive to coding sequences (Figure 2A). Of 3731 TSS, 1079
(~29%) were primary and 153 (~4%) were secondary TSS
(Figure 2B and Supplementary Table S2). Manual curation
of the TSS predictions determined that five TSS had a 5′
UTR longer than 400 nt (Figure 2C). It is likely that
these cases represent unusually long 5′ UTRs; however, it
is possible that these regions encode small open reading
frames missed during automated gene annotation (Figure
2C). About 18% (679) of transcripts were initiated from in-
side of coding regions (i.e. internal TSS). An internal TSS
can drive expression of downstream genes, e.g. the
promoter of DNA gyrase B-encoding gene (gyrB) lies within
the ORF located upstream encoding DNA recombination
protein RecF. The biological role of internal promoters that
do not drive the expression of a downstream gene is unclear,
however, a subset initiates the expression of small RNAs lo-
cated at the 3′ end of coding genes (see below). TSSpredator
predicted that almost half (~47%, 1764/3731) of the iden-
tified TSS are located antisense of coding regions. Manual
inspection revealed that the large majority of antisense TSS
produce very short (<100 nt) and low-copy transcripts. The
function of these short antisense transcripts is debated and
a portion might be considered as non-functional transcripts
that are initiated at promoter-like sequences (32,33). How-
ever, an increasing number of studies suggest that pervasive
transcription (including short antisense transcripts) exhibit
biological roles (34). The ~1.5% (56/3731) of TSS that do
not have a clear association with an annotated genomic fea-
ture were categorized as orphan TSS.

A. baumannii promoter architecture

To initiate transcription, a sigma factor directs binding of
RNA polymerase to DNA within 50 nt upstream of a TSS
(35). We determined the consensus promoter structure up-
stream of the 3731 TSS using unbiased motif searching
to detect TSS-proximal and TSS-distal motifs correspond-
ing to the −10 and −35 locations of sigma factor bind-
ing sites. Because the spacing between −10 and −35 bind-
ing sites differs between promoters, we searched for each
motif separately; constraining a specific spacing between
−10 and −35 elements prevents resolution of the weaker
motifs located around the −35 position. MEME searching
identified canonical RpoD (sigma 70) binding site motifs
in both the −10 and −35 locations (Figure 2D), consistent
with the expectation that RpoD is responsible for a large majority of transcription initiation in *A. baumannii*. Aligning all 3731 *A. baumannii* TSS revealed that adenine is the most common initiating nucleotide (A = 47%) (Figure 2C). The majority of transcripts initiate with a purine nucleotide triphosphate (ATP = 47%, GTP = 23%); these are the most abundant nucleotides and are the primary energy storage molecules, thus provide a mechanism to reduce transcription initiation when nucleotide and energy pools are depleted. Yet it is position +2 that has the strongest sequence bias (T = 57%). Pyrimidine nucleotides are less abundant in cells, and the preference for pyrimidine nucleotides at the −1 (72% C+T) and +2 (81% C+T) positions immediately flanking the TSS may represent a mechanism to focus transcription initiation at the purine-requiring position +1 (11). In other words, preference for pyrimidines at positions −1 and +2 arises because of selection against having purines at these positions.

UTR of mRNA perform multiple biological functions, including providing binding sites for ribosomes and the potential to fold into mRNA secondary structures that regulate transcription and RNA stability (36). The median length of 5′ UTR for primary TSS is 37 nt and secondary TSS is 100 nt (Figure 2C). Similar UTR analyses have not been performed for the *E. coli* transcriptome; the median length of 5′ UTR in *Salmonella* Typhimurium, another Gamma-proteobacterium, is 55 nt for primary TSS and 124 nt for secondary TSS (11).

**Identification of *A. baumannii* ATCC 17978 small RNAs**

Small, regulatory RNAs are a class of post-transcriptional regulators that modulate gene expression of many cellular processes (37,38). Functions have been determined for some sRNAs in *Enterobacteriaceae* (e.g. *E. coli*, *Salmonella* Typhimurium), but no sRNA functions have been experimentally determined in *A. baumannii* (5). Two previous studies used bioinformatic and experimental approaches to locate small RNAs in *A. baumannii*. In *A. baumannii* MTCC 1425 (ATCC 15308), 31 sRNAs were predicted bioinformatically, of which three of which were verified by Northern blotting (39). In *A. baumannii* AB5075, RNA-seq analysis of exponential growth in lysogeny broth (LB) identified 78 sRNAs, six of which were verified by Northern blotting (40). Here, we employed dRNA-seq on pooled growth con-
Figure 2. Characterization of TSS. (A) Schematic explaining TSS categories. P: Primary TSS, S: Secondary TSS, I: Internal TSS, A: Antisense TSS, O: Orphan TSS. (B) Pie chart showing the number of TSS per category. (C) Histogram showing the number and length of 5’ UTRs of primary (red) and secondary (black) TSS. The inset illustrates the frequency of occurrence of nucleotides around the TSS. (D) Meme-derived motifs showing −35 and −10 region of A. baumannii ATCC 17978 promoters.

Figure 3. sRNA in A. baumannii ATCC 17978. (A) Normalized, mapped sequence reads from RNA-seq show the expression of sRNAs 17, 37, 75,
ing genes are increasingly recognized as genomic locations that can harbor sRNA genes (6,41,42). In *A. baumannii*, we identified 22 sRNAs that mapped to the 3′ regions of coding genes, all of which possess their own promoters located within the upstream coding gene. We anticipate that there will be additional regulatory sRNAs produced by endo-
nucleolytic processing of an mRNA, as observed in other bacteria (42). However, additional experiments are required to annotate such sRNAs effectively (42). Only a small number (14/110; 13%) of sRNAs were located antisense of coding genes.

We used Northern blots to validate sRNA annotations (Figure 3). Sequence-specific riboprobes were designed to hybridize to two families of homologous sRNAs and five randomly selected unique sRNA candidates, including one sRNA (sRNA17) located in the 3′ region of ACX060_RS03050, a predicted TonB-dependent receptor protein. Eighteen of the 110 sRNA candidates were homologs (i.e. present as 18 copies with highly similar nucleotide sequence), which we classified as ‘group I sRNAs’ (Figure 3B). Gene duplications are common amongst sRNA species and may benefit the bacterium by allowing for rapid evolution and diversification of gene regulation (37). The transcriptomic data suggested a variable length of the group I sRNAs and Northern blotting with a riboprobe hybridizing to the homologous region of group I sRNAs displayed multiple bands between 100–250 nt in length. The group I family of *A. baumannii* ATCC 17978 sRNAs corresponds to the ‘C4-similar’ group in *A. baumannii* AB5075, reported previously to share regions of homology with bacteriophage P1 and P7 (40). Two of the group I *A. baumannii* ATCC 17978 sRNAs contained a 5′ region with low sequence homology; the diversity in this region may provide these particular sRNAs with additional species-specific functions (Figure 3B).

We next considered orthologs of sRNA that have been characterized in model species like *E. coli*. sRNA37 (119 nt) is predicted to be the 4.5S RNA of the signal recognition particle that is involved in membrane protein targeting. sRNA84 (182 nt) is predicted to be the 6S RNA that can bind and inhibit RNA polymerase in *E. coli* (5).

The RNA binding proteins Hfq, CsrA or ProQ coordinate binding of sRNAs to target mRNAs in *Enterobacteriaceae* (43–45). Only Hfq has been studied functionally in *Acinetobacter* spp. (46,47) and it possesses an unusually long, glycine-rich C-terminus that could alter Hfq function compared to what has been observed in model species (47). Thus, post-transcriptional regulation by Hfq and other RNA-binding proteins should be explored in *Acinetobacter* species.

### Conservation of *Acinetobacter* small RNAs

A gene conservation analysis was carried out by comparing the nucleotide sequence identity of the sRNAs identified in 17978-mff with other bacterial genomes. The search approach started by comparing members of the same species, then was expanded to representative members of the genus, and finally among other members of the order Pseudomonadales. The suite of 27 genomes contained fifteen *A. baumannii* strains, seven species in the genus *Acinetobacter*, and five more distantly related members of the order Pseudomonadales (Figure 4). The most highly conserved sRNA across all species were 4.5S RNA (sRNA37), 6S RNA (sRNA84) and to a lesser extent tmRNA (sRNA89), but the level of conservation drops rapidly outside of the genus. The conservation of *A. baumannii* sRNAs was much higher in the opportunistic pathogens *A. pittii* and *A. nosocomialis* showing a mean sequence identity across all 110 sRNAs of ~87% for *A. pittii* and ~84% for *A. nosocomialis* compared to other *Acinetobacter* species. Environmental, non-pathogenic *Acinetobacter* species had lower mean sequence identity. Using a 70% sequence identity cutoff, five sRNAs are present only in *A. baumannii* L9778 (sRNA11, sRNA23, sRNA52, sRNA82, and sRNA83). Searching for sRNA homologs in other members of the order Pseudomonadales revealed that only a very small number of sRNA with sequence identity >70% are found outside of the genus *Acinetobacter*. A broader search revealed that no *A. baumannii* sRNA are present in *E. coli* or *Salmonella* Typhimurium. sRNAs conserved in *A. baumannii* sRNA but absent from other species may have evolved for roles in *A. baumannii* virulence or antibiotic resistance, making these good targets for future studies. Unfortunately, the biological functions of *Acinetobacter* sRNAs are hard to predict due to the low level of sequence conservation between *Acinetobacter* sRNAs and well-characterized sRNAs from relatively closely-related *Pseudomonas* species.

### Functional genomics for the study of virulence and antimicrobial resistance in *A. baumannii*

*A. baumannii* is responsible for an increasing number of hospitalized cases of pneumonia leading to death, which has fueled efforts to identify genes that are important for virulence, host colonization and antimicrobial resistance. In a mouse model of *A. baumannii* pneumonia, genome-wide transposon mutagenesis followed by DNA sequencing (insertion sequencing) identified 157 genes associated with *A. baumannii* persistence in the lung confirming the importance of known virulence genes and identifying several genes with no previous connection to *A. baumannii* infection (48). Our dataset builds upon this analysis by offering detailed molecular insights into the transcriptional architecture of important *A. baumannii* virulence factors. This dataset is useful to the study of other *A. baumannii* strains because many promoter regions are highly conserved between clinically-relevant strains. For example, the TSS and promoter sequences for the primary virulence factor *ompA*

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76, 77, 84, 99 and 100 (yellow arrows). Curved arrows depict TSS identified in this study and lollipop structures are predicted rho-independent terminators. Northern blotting of selected sRNAs are shown to the right. RNA was isolated from ESP and five μg of total RNA was loaded per lane. The sRNA sizes below the individual blots have been predicted from dRNA-seq data. (B) Sequence alignment of Group I and Group III sRNAs created with the Geneious Software (v. 8.1.8); colored bases indicate conservation in at least 50% of aligned sequences (A, red; C, blue; G, yellow; T, green). The riboprobes used in Northern blotting are depicted as black bars atop the sRNA alignments.
Figure 4. sRNA conservation in representative members of the order Pseudomonadas. Genomes of multiple *Acinetobacter* species, *Pseudomonas* species, and *Moraxella catarrhalis* were compared using GLSEARCH. The colour scale shows the percentage sequence identity of the 110 sRNAs compared to the reference sequence from *A. baumannii* ATCC 17978.
are conserved across \textit{A. baumannii} strains, suggesting the same TSS is used by all members of the species (Supplementary Figure S2). Here, we provide examples of how AcinettoCom can be used by researchers to evaluate transcriptional features of both well-characterized and novel virulence factors, and discuss the importance of \textit{in vitro} growth conditions in the study of virulence gene expression (Supplementary Table S4).

\textbf{Regulation of zinc acquisition and the Znu ABC transporter.} Metal acquisition genes are a critical component in \textit{A. baumannii} virulence as they are required to wrest essential co-factors from host chelators (5,49,50). Zinc limitation has been demonstrated as a central feature in controlling bacterial replication in the lung and dissemination to systemic sites in the murine model of \textit{A. baumannii} pneumonia (49,51). In \textit{A. baumannii}, the Zur (zinc uptake regulator, ACX60_RS17350) transcriptional regulator is responsible for mediating the expression of several zinc-associated functions, including the repression of an ABC family zinc transporter encoded by \textit{znuABC}. In \textit{A. baumannii} ATCC 17978, the \textit{zur} gene is co-transcribed as part of an operon with \textit{znuCB}, which encode the cognate ATPase and permease protein of the ABC transporter (49). The third component, \textit{znuA}, is found on the opposite DNA strand and encodes a substrate-binding protein (49). During growth in zinc-replete conditions, the Zur protein binds to a 19-bp operator site located 30 nucleotides upstream from the \textit{znuA} translation start site and represses the expression of both \textit{znuA} and the \textit{zurznuCB} operon (49,51).

\textit{TSSpredator} identified a total of 8 TSS within the chromosomal region that includes both the \textit{znuA} gene and \textit{zurznuCB} operon (Figure 5A). Two of the TSS were located 29 and 93 nucleotides upstream of the \textit{znuA} ORF. While both TSS were annotated as antisense to the \textit{zur} gene, their location within the intergenic region also make them prime candidates as primary or secondary TSS to \textit{znuA}. The \textit{zurznuCB} operon was expressed in our test conditions. Although a potential TSS could be observed at position (3 647 395) in the ESP and Pool samples, it was not enriched in the TEX sample relative to the Pool RNA sample. This loss of a TSS-like peak in the TEX sample suggests that the TSS could be a substrate of a cellular 5′ pyrophosphohydrolase (52). When this enzyme converts 5′ triphosphates into monophosphates, TEX will digest this unprotected TSS. Based on the above predictions, the TSS for \textit{znuA} or \textit{zurznuCB} are found on either side of the Zur-binding site and may represent an important regulatory feature for Zur-based transcription repression (49,51). An additional TSS was identified within the \textit{znuC} ORF, located 239 nucleotides upstream of \textit{znuB}. To our knowledge, this TSS has not been previously identified in the well-characterized \textit{zurznuCB} operon and suggests the potential for transcription of \textit{znuB} independently from other members within the operon. Such a format of expression may be a mechanism for cells to accommodate modest increases in the intracellular level of zinc while avoiding simultaneous increases in the Zur protein. Further, there are no reports of a Zur box associated with the region surrounding this internal TSS. A Rho-independent transcriptional terminator is also present in this region, located within the \textit{znuB} ORF or 113 nucleotides downstream of \textit{znuC}. Several additional internal TSS were also identified within the \textit{znuA} gene, but did not demonstrate a clear association with downstream genes or with an sRNA transcript. The remaining TSS within the re-
region were annotated as antisense TSS and contribute to the overall theme of pervasive low-level antisense transcription within the *A. baumannii* transcriptome.

**The membrane lipid asymmetry (Mla) transport system.** Several of the genes responsible for *A. baumannii* persistence in the mouse pneumonia model were identified as belonging to efflux pump systems (48). The authors highlighted the Mla ATP-binding cassette (ABC) transport system because this system was annotated as conferring a tolerance phenotype (53), raising the question of why resistance to organic solvents may be important during infection of a mammalian host. However, recent studies have since uncovered a central role for Mla-mediated transport in maintaining the integrity of the outer membrane of Gram-negative bacteria (54,55). Wang et al. reported a 15-fold decrease in *in vivo* persistence of mutants lacking *mlaA* (48), which correlates with roles for the Mla transport system in iron limitation and outer membrane vesicle formation as well as resistance to antibiotics such as doripenem and colistin (56–58).

Our transcriptomic data reveal that genes encoding the Mla ABC transporter components and periplasmic binding protein are co-transcribed in the *mlaFEDCB* operon (Figure 5B). Similar to the *zurznuCB* operon, TSSpredator did not call a TSS upstream of this operon because of a lack of enrichment compared to the Pool sample, but manual inspection of the mapped reads suggest a single TSS located 29 nt upstream of *mlaF* at position 419,650. We noted greater transcript abundance of *mlaDCB* (most notably *mlaCB*) compared to *mlaFE* in ESP, an expression pattern that was also observed for an LPS-deficient mutant of *A. baumannii* strain 19606 (56). As there is no additional promoter within the operon, this suggests that the *mlaDCB* portion of the transcript may turnover more slowly or is protected from degradation resulting in elevated production of the periplasmic substrate binding domain MlaC. A Rho-independent terminator was identified downstream of the operon and provides a potential 3′ border for the transcript; however, multiple rho-independent terminators were also identified within the operon, with two terminators present early in the *mlaF* and *mlaE* ORFs. Like *E. coli* and other Gram-negative bacteria, the *A. baumannii* *mlaA* homolog is found at a separate location on the chromosome (Figure 5C) and we identified a single TSS for *mlaA*, located 74 nt upstream of the start codon. An additional antisense TSS was also identified, located opposite of the 3′ end of the *mlaA* ORF. Both the *mlaFEDCB* and *mlaA* loci were expressed during ESP, suggesting that this growth condition could be used for further investigation of the transport system.

**Chloramphenicol resistance is regulated by transcription initiation.** We discovered that *A. baumannii* ATCC 17978 cultured in M9 medium loses resistance to chloramphenicol (Figure 6). All *A. baumannii* strains contain the chromosomally-encoded efflux pump CraA (*ACX60_RS01760*), for chloramphenicol resistance *Acinetobacter*, also called MdfA. Efflux by CraA appears to be highly specific to chloramphenicol, conferring a minimum inhibitory concentration >256 μg mL⁻¹ chloramphenicol (59). Inhibition of CraA in *A. baumannii* strain 19606 increases sensitivity by 32-fold while deletion of *craA* increases sensitivity by 128-fold, respectively (59). Expression of *craA* is highly consistent between strains of multi-drug resistant *A. baumannii* (60), but it is not known if this expression is constitutive in nature (61). In *A. baylyi*, a point mutation 12 bp upstream of the *craA* start codon caused increased stability of the *craA* mRNA resulting in higher *craA* expression and increased resistance to chloramphenicol (62).

We used dRNA-seq and quantitative PCR to examine the promoter architecture and regulation of *craA* in *A. baumannii* ATCC 17978, and tested whether loss of chloramphenicol resistance is coupled to altered expression of *craA* (Figure 6A). dRNA-seq revealed two promoters at the *craA* gene, the gene-proximal TSS-1 promoter (position 367,680, Supplementary Table S2) and the gene distal TSS-2 promoter (position 367,564, Supplementary Table S2). Phylogenetic comparisons confirmed that these promoter elements are conserved across *A. baumannii* strains (Supplementary Figure S3). To examine how *craA* expression is regulated in response to growth conditions, we used qPCR to differentiate transcripts originating at TSS-1 from TSS-2. Because TSS-1 is contained within TSS-2 transcripts, TSS-1 transcripts were calculated by subtracting TSS-2 transcripts from total *craA* transcripts. During steady-state rapid growth (exponential phase) in nutrient-rich L-broth, 92% of *craA* transcription originates at TSS-1 (Figure 6A). Conversely, in the nutrient-poor medium M9 plus pyruvate, TSS-1 activity was undetectable and all transcription was driven by TSS-2. Next, we tested how the two promoters responded to nutrient downshift. Within four hours of transferring exponentially-growing cells from L-broth to M9 plus pyruvate, TSS-1 was down-regulated by 50-fold, whereas TSS-2 was down-regulated only 1.4-fold (Figure 6B). Therefore, the promoter driving TSS-1 is highly responsive to nutritional quality of the growth medium. Over the following days, both promoters continued to decline in activity: TSS-1 has a poor match to the RpoD −35 consensus, suggesting that protein transcription factors control TSS-1 by recruiting RNAS to the promoter. The TSS-2 promoter contains an archetypical −35 sequence, suggesting that RNAS can bind this promoter without assistance from an activator protein, which could explain the consistent expression of TSS-2 in L-broth and M9 medium.

We hypothesized that down-regulation of *craA* in M9 medium would result in a chloramphenicol-sensitive phenotype akin to that of a *craA* mutant strain. Thus, we evaluated the proportion of resistant and sensitive *A. baumannii* cells in liquid cultures. During exponential growth in rich medium (L-broth), all cells were resistant to chloramphenicol (Figure 6B). All cells remained resistant at 4 h after transfer to M9 medium, but 24 h after transfer to minimal medium, 50% of cells were susceptible. Cell viability was high after 96 h in M9 medium, but chloramphenicol resistance had declined by >3000-fold (Figure 6B). Taken together, these results suggest that antibiotic resistance is conditional, especially when cells are instantly deprived of amino acids upon transfer from Lennox medium to M9 plus pyruvate medium. Although chloramphenicol resistance is ubiquitous in *A. baumannii* isolates due to drug efflux by CraA,
we have discovered that *craA* expression—and thus chloramphenicol resistance—is conditional on nutritional quality of the environment. This lack of endogenous activation or repression of drug resistance presents a potential target for the effective treatment of multi-drug resistant infections with an inexpensive and widely available antibiotic.

**CONCLUSION**

As an emerging pathogen in the ‘omics’ age, *A. baumannii* pathobiology is defined largely by high-throughput technologies that can guide molecular studies of pathogenicity mechanisms and antimicrobial resistance. We have conducted the first dRNA-seq analysis of the model strain *A. baumannii ATCC 17978* to identify several thousand TSS at single nucleotide resolution, and generated a comprehensive map of the transcriptome that includes 110 sRNAs. Although 3731 TSS is the largest and most precise dataset yet for *Acinetobacter*, it is important to note that computational identification of TSS can overlook true sites. TSS are only called where a sharp increase in read depth in the TEX-treated sample exceeds the normalized read depth in the Pool sample. Overall, we identified an average of 5.1 sense strand TSS/10 kb in *A. baumannii ATCC 17978*, which is similar but fewer than the 7.0 sense strand TSS/10 kb identified in *S. Typhimurium 4/74* (12). Because manual curation of *S. Typhimurium 4/74* identified more TSS per bp, we suspect the TSSpredator program used here did not identify...
all TSS. Thus, scientists using AcinetetoCom are encouraged to examine the RNA-seq read depth at their gene(s) of interest. A sharp increase in read depth in the TEX track alone can be highly suggestive of a TSS. Thus, investigators are advised to scrutinize cases where there is high expression in the ESP tracks but no TSS is called in the TEX track.

A growing body of evidence suggests a positive relationship between conditions that slow bacterial growth and the expression of virulence factors. For notable pathogens such as Legionella, Yersinia and Salmonella, key virulence traits are induced during the early stationary phase in laboratory culture (11,63–66). Expression of virulence determinants in standard laboratory conditions has permitted the study of gene function and characterization of the regulatory networks that control virulence gene expression in other model pathogens (67). It was fortuitous to discover that A. baumannii expresses key pathogenicity genes in standard laboratory conditions, including the three loci identified in the mouse model of pneumonia (5,48) highlighted in Figure 5. Similarly, simple laboratory conditions revealed an environmental dimension to the control of drug resistance in A. baumannii. Although diagnostic techniques that test for the presence of antibiotic resistance genes can predict resistance, our finding of chloramphenicol sensitivity in a species that is considered highly chloramphenicol resistant illustrates the importance of characterizing gene expression and gene regulatory mechanisms. Reduced chloramphenicol efflux due to rapid down-regulation of cra expression presents two intriguing leads for combating this ubiquitous resistance mechanism in A. baumannii. First, characterization of the regulatory mechanism and identification of the environmental cues that stimulate or repress cra expression will raise the potential to predict stages of infection and locations in the host that A. baumannii may be sensitive to chloramphenicol. Second, elucidation of the transcription factors controlling cra may reveal additional drug targets that could be targeted synergistically. This could enhance the effectiveness of a widely available and inexpensive antibiotic that is being used effectively in the treatment of other multi-drug resistant infections.

DATA AVAILABILITY

The raw data and normalized mapped reads are available from the Gene Expression Omnibus (GEO) at accession no: GSE103244. The AcinetetoCom database can be accessed under http://bioinf.gen.tcd.ie/acinetocom/.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

FUNDING

Saskatchewan Health Research Foundation [Establishment grant 2867 to A.D.S.C.]; Natural Sciences and Engineering Research Council of Canada [Discovery grant RGPIN-435784-2013 to A.D.S.C.]; Saskatchewan Health Research Foundation [3866 to K.D.M.]; Irish Research Council Government of Ireland Postdoctoral Fellowship [GOIPD/2016/292 to A.A.M.C.L.]. Funding for open access charge: University of Regina Research Office.

Conflict of interest statement. None declared.

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mRNA decay is mediated by RrpH via the formation of a Kin-dependent complex with Hfq. This complex then targets the mRNA for degradation. 

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