MICROBIOLOGY

RESEARCH ARTICLE

Cardoso *et al.*, *Microbiology*

DOI 10.1099/mic.0.000725

MICROBIOLOGY SOCIETY

Comparative transcriptome analyses of magainin I-susceptible and -resistant *Escherichia coli* strains

Marlon H. Cardoso,[1,2,3]† Keyla C. de Almeida,[1,2]† Elizabete S. Cândido,[1,3] Gabriel da R. Fernandes,[1,4] Simoni C. Dias,[1,5] Sérgio A. de Alencar[1] and Octávio L. Franco[1,2,3,*]

Abstract

Antimicrobial peptides (AMPs) have attracted considerable attention because of their multiple and complex mechanisms of action toward resistant bacteria. However, reports have increasingly highlighted how bacteria can escape AMP administration. Here, the molecular mechanisms involved in *Escherichia coli* resistance to magainin I were investigated through comparative transcriptomics. Sub-inhibitory concentrations of magainin I were used to generate four experimental groups, including magainin I-susceptible *E. coli*, in the absence (C) and presence of magainin I (CM); and magainin I-resistant *E. coli* in the absence (R) and presence of magainin I (RM). The total RNA from each sample was extracted; cDNA libraries were constructed and further submitted for Illumina MiSeq sequencing. After RNA-seq data pre-processing and functional annotation, a total of 103 differentially expressed genes (DEGs) were identified, mainly related to bacterial metabolism. Moreover, down-regulation of cell motility and chaperone-related genes was observed in CM and RM, whereas cell communication, acid tolerance and multidrug efflux pump genes (ABC transporter, major facilitator and resistance-nodulation cell division superfamilies) were up-regulated in these same groups. DEGs from the C and R groups are related to basal levels of expression of homeostasis-related genes compared to CM and RM, suggesting that the presence of magainin I is required to change the transcriptomics panel in both C and R *E. coli* strains. These findings show the complexity of *E. coli* resistance to magainin I through the rearrangement of several metabolic pathways involved in bacterial physiology and drug response, also providing information on the development of novel antimicrobial strategies targeting resistance-related transcripts and proteins herein described.

INTRODUCTION

Antimicrobial agents have long been developed as a flagship in medical modernization. During the mid-20th century, more precisely starting from 1940, the golden era of antibiotic discovery and application was established [1]. However, since the 1970s this reality has gone from boom to bust [1]. Bacterial resistance has gained huge ground over the years, and difficult-to-treat infections are among the greatest threats to human health [2]. In the United States, it is estimated that 23 000 people die annually due to multidrug-resistant bacterial infections, and more than two million require hospital treatment [1]. In addition, looking from another perspective, the bacterial resistance crisis is estimated to impact the global economy by up to US$100 trillion [1]. In this scenario, a race to comprehend the molecular panel involved in bacterial resistance to antibiotics has been in progress.

Received 24 February 2018; Accepted 6 September 2018

Author affiliations: [1]Pós-Graduação em Ciências Genômicas e Biotecnologia, Centro de Análises Proteômicas e Bioquímicas, Universidade Católica de Brasília, Brasília- DF, Brazil; [2]Programa de Pós-Graduação em Patologia Molecular, Faculdade de Medicina, Universidade de Brasília, Brasília-DF, Brazil; [3]S-Inova Biotech, Pós-graduação em Biotecnologia, Universidade Católica Dom Bosco, Campo Grande-MS, Brazil; [4]Oswaldo Cruz Foundation, René Rachou Research Center, Belo Horizonte, MG, Brazil; [5]Programa de Pós Graduação em Biologia Animal, Universidade de Brasília, Brasília, Brazil.

***Correspondence:** Octávio L. Franco, ocfranco@gmail.com

Keywords: RNA-seq; antimicrobial peptides; bacterial resistance; magainin I; comparative transcriptomics.

Abbreviations: AcrA and B, multidrug efflux pump subunit; *adk*, adenilate kinase; C, magainin I-susceptible *E. coli* in the absence of magainin I; CM, magainin I-susceptible *E. coli* in the presence of magainin I; CysA, P, U and W, sulfate/thiosulfate import ATP-binding protein; *cysG*, siroheme synthase; DEG, Differentially expressed genes; DNA *gyrB*, gyrase subunit B; dicF, small regulatory RNA; FlgF, H, G and K, flagellar hook-associated proteins; *frr*, ribosome recycling factor; *fumC*, fumarate hydratase; GadA, B and C, glutamate decarboxylase alpha, beta and gamma-aminobutyrate, respectively; *gyrA*, DNA gyrase subunit A; IbpA and B, small heat shock proteins; KEGG, Kyoto Encyclopedia of Genes and Genomes; MacAB, macrolide export ATP-binding/permease protein; *metG*, methionyl-tRNA synthetase; MIC, minimum inhibitory concentration; MqsR, mRNA interferase toxin; MSF, major facilitator superfamily; mtr, tryptophan-specific transport protein; PrpB, methylisocitrate lyase; PrpC, 2-methylcitrate synthase; PrpD, 2-methylcitrate dehydratase; PrpE, propionyl-CoA synthetase; R, magainin I-resistant *E. coli* in the absence of magainin I; *recA*, DNA recombination and repair protein; RM, magainin I-resistant *E. coli* in the presence of magainin I; RND, resistance-nodulation cell division; SecM, secretion monitor; SRP, signal recognition particle receptor; TolC, outer membrane protein channel; ZraP, R and S, zinc-associated proteins.

†These authors contributed equally to this work.

Four supplementary tables are available with the online version of this article.

The manner in which bacteria resist antibiotics is diverse, and may be intrinsic to each strain or acquired via horizontal gene transfer and chromosomal gene mutation. In general, resistance mechanisms are mainly divided into five strategies [3], including (i) the prevention of access to target through reduced permeability of bacterial outer membrane and increased efflux via overexpression of multidrug efflux pumps [4]; (ii) changes in antibiotic target by mutation, thus leading to non-specific binding between antibiotics and mutated targets; (iii) target modification and/or protection, including bacterial surface remodelling [5] and methylation events [6]; (iv) direct modification/inactivation of antibiotics by hydrolysis [7] and chemical transfer [8]; and finally (v) changes in bacterial lifestyle from free-swimming to biofilm, thus enhancing both physical and chemical barriers toward antibiotics [9].

In an attempt to counter this resistance phenomenon, the search for and design of antimicrobial peptides (AMPs) have increased greatly. AMPs are relatively short (<50 amino acid residues), usually positively charged and amphipathic, showing diverse and complex antimicrobial activities [10]. However, even though they represent a promising alternative to conventional antibiotics, an increasing number of reports have highlighted that bacteria can resist AMP treatment. As observed by Beceiro and coworkers [11], *Acinetobacter baumannii* strains are capable of resisting polymyxin E administration by adding phosphoethanolamine to the lipid A hepta-acylated form [11]. Bacteria can also produce a series of proteinases involved in AMP degradation [12, 13]. Studies have shown that *Pseudomonas aeruginosa* expresses elastases, a typical metalloprotease, in order to resist LL-37 and alpha-defensin treatment [12]. Moreover, *Staphylococcus aureus*, *Streptococcus pyogenes* and *E. coli* can also degrade LL-37 by producing metallo- [13], serine- [12] and cysteine proteases [14], respectively. Interestingly, AMP trapping and neutralization events have also been described, suggesting that surface-associated proteins can either bind and neutralize AMPs or bind host-derived proteins, forming complexes to trap AMPs [15]. In addition, bacteria can secrete outer membrane vesicles to bind AMPs, thus decreasing their concentration in the bacterial micro-environment [16]. If AMPs escape extracellular degradation and/or overcome physical barriers, bacteria can also express efflux pumps to expel them [17]. This resistance mechanism has been well studied in the antimicrobial field and allows bacteria to resist different AMPs, including LL-37, polymyxin B [18], cecropin P1, melittin [19], colistin [20] and protamine [21].

In previous studies, we reported the cytosolic sub-proteome of magainin I-resistant *E. coli* strains by classic proteomics, revealing intense metabolic response by magainin I-resistant strains, including energy and nitrogen intake, stress response and amino acid conversion [22]. More recently, we also performed a comparative nanoUPLC-MS^E analysis between magainin I-susceptible and -resistant *E. coli* strains, describing a series of resistance-related proteins in magainin I-resistant strains, including biofilm formation and multidrug efflux pumps [4]. In both works we highlighted the importance of studying magainin I resistance. The magainin family is composed of amphipathic α-helical, linear, cationic peptides originally isolated from the African clawed frog *Xenopus laevis* [23]. Several magainin analogues have been studied regarding their biological function, and some of these have reached advanced clinical trials and have been further submitted for approval by the Food and Drug Administration (FDA) (e.g. pexiganan, brand name: Locilex).

Here, we examined the expression profile of magainin I-susceptible and -resistant *E. coli* strains using RNA sequencing (RNA-seq) technology, providing useful information on the biological pathways and main transcripts involved in this resistance. RNA-seq allows far more precise measurements of transcript levels than other technologies used for transcriptomics studies, including DNA microarrays [24]. Moreover, to the best of our knowledge, this is the first report regarding comparative transcriptomics of AMP-susceptible and -resistant bacterial strains.

METHODS

Magainin I synthesis

Magainin I was purchased from Peptide 2.0 Incorporated (USA), and was used to synthetize the peptide by the solid-phase method using N-9-fluorenylmethyloxycarbonyl (Fmoc) chemistry on a Rink amide resin, with 95 % purity following removal of trifluoroacetic acid (TFA). High-pressure liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) analyses were provided.

Bacterial strains

Magainin I-susceptible and -resistant strains were prepared from *E. coli* (ATCC 8739) by 10 successive propagations in Luria–Bertani (LB) medium in the absence or presence of magainin I at 0.5× minimum inhibitory concentration (MIC=31 µM), as previously described [25]. Briefly, a single colony of *E. coli* (ATCC 8739) was cultivated in LB broth at 37 °C and 240 r.p.m., overnight. This pre-inoculum was further used to prepare bacterial stocks until the middle of its exponential phase in LB broth, at 37 °C and 240 r.p.m. Subsequently, 1×10^5 c.f.u.ml^{-1} of this inoculum were incubated in 10 ml of LB medium in the absence and presence of magainin I 0.5× MIC at 37 °C and 240 r.p.m, for 16 h. This step was repeated 10 times (successive propagations). After the 10th propagation, the bacterial suspensions corresponding to the susceptible and resistant groups were spread on LB magainin I-free agar plates with incubation at 37 °C and used as magainin I-susceptible and -resistant *E. coli* stocks for further experiments. The experiments were performed in triplicate (biological and technical replicates). In order to evaluate the molecular response of magainin I-susceptible and -resistant *E. coli* in the absence and presence of magainin I, four experimental groups were here proposed and

generated from our bacterial stocks (as described above). These groups are: (i) magainin I-susceptible *E. coli* in the absence of magainin I (control; C); (ii) magainin I-resistant *E. coli* in the absence of magainin I (resistant; R); (iii) magainin I-susceptible *E. coli* in the presence of magainin I (control+magainin I; CM); and (iv) magainin I-resistant *E. coli* in the presence of magainin I (resistant+magainin I; RM). To generate these experimental groups, 500 µl of the magainin I-susceptible and -resistant bacterial suspensions were inoculated in 5 ml of LB broth and incubated at 37 °C and 240 r.p.m, for 16 h. Further, 1 ml of each pre-inoculum was transferred to 4 ml of magainin I-free LB broth, as well as to 4 ml of the same medium containing magainin I 0.5× MIC. The samples were incubated at 37 °C and 240 r.p.m, for 7 h. Subsequently, 1×10^5 c.f.u.ml^{-1} from each inoculum were transferred to 10 ml of LB medium in the absence and presence of magainin I 0.5× MIC and incubated at 37 °C and 240 r.p.m, for 16 h. All experiments were performed in independent triplicate. The total volume of three samples representing biological replicates from each colony (C; CM; R; RM) was centrifuged separately at 3200 ***g*** and 4 °C, for 5 min. The supernatants were discarded and the cellular sediments used for total RNA extraction. Strain identification was performed using automated microbiological systems (MicroScan and VITEK) and MALDI-ToF (BioTyper and MSP dendrogram); antimicrobial susceptibility assays (VITEK and MicroScan) and AMP susceptibility assays (broth microdilution) were carried out using 27 antibiotics used in clinical practice and four AMPs, including magainin I, cecropin P1, PMAP-23 and PR-39. Previously published data [25] confirmed the magainin I-resistant *E. coli* strains' specific resistance used in the present study.

RNA extraction

Total RNA from each sample was extracted using the RNeasy Protect Bacteria (Qiagen) kit, according to the manufacturer's guidelines. Contaminating DNA was removed from the RNA preparations using DNase I (Sigma-Aldrich) and RDD buffer (Qiagen). Ribosomal RNA was removed using the Ribo-Zero Magnetic Gram-Negative Bacteria (Epicentre) kit. Quantity, quality and integrity of the total RNA obtained for each sample were evaluated through Qubit RNA BR (Invitrogen) assays and Agilent Bioanalyser 2100 (Agilent Technologies), respectively. This analysis resulted in four samples in triplicate (3×C, 3×CM, 3×R and 3×RM), which were further submitted for complementary DNA (cDNA) library preparation.

cDNA library preparation and sequencing

An Illumina TruSeq RNA Sample Preparation kit was used to construct the cDNA libraries of the purified mRNA samples, following the manufacturer's protocol. Library quantity and quality were assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies). Finally, sequencing data were generated from paired-end reads (2×150 bp) on a MiSeq platform (Illumina) at Universidade Católica de Brasília, Brazil. After sequencing, samples were individualized according to their indices and converted to fastq format using the MiSeq Reports (Illumina) program. Raw data from the sequencing runs were submitted to the Sequence Read Archive (SRA) repository of the National Center of Biotechnology (NCBI) under accession number SRA: SRR6760835.

RNA-seq data pre-processing

Initially, the FastQC tool (http://www.bioinformatics.babraham.ac.uk) was used to check the quality of the raw reads, including analysis of sequence quality, GC content, presence of adaptors, over-represented k-mers and duplicated reads [26]. To perform quality trimming of the fastq files obtained from Illumina MiSeq sequencing, pre-processing was carried out using the Trimmomatic tool with the following parameters: sliding window: 4 : 30; leading: 10; trailing: 10; minlen: 40 [27]. All sequences smaller than 40 bases were eliminated on the assumption that small reads might represent sequencing artefacts. Moreover, Trimmomatic verified the presence of adapter sequences and removed any that matched entries in a FASTA file containing all known Illumina adapters. Quality assessment of the Trimmomatic pre-processed data was carried out using the FastQC tool (http://www.bioinformatics.babraham.ac.uk), which confirmed that poor-quality bases had been removed.

Identification of differentially expressed genes

The FASTA file corresponding to the *E. coli* (ATCC 8739) reference genome (GenBank accession number NC_010468.1) was downloaded from the NCBI genome database (http://www.ncbi.nlm.nih.gov/genomes). After processing and trimming, all the clean reads were aligned to the reference genome using Rockhopper [28]. In this study, an absolute value of $\log^2$ fold change >1 (up-regulated genes) or <−1 (down-regulated genes) and a q-value <0.01 was set to declare statistically significant differentially expressed genes (DEGs) between the C-CM and R-RM interactions in response to magainin I 0.5× MIC. Volcano plots comparing P-value versus fold change for each condition studied were also generated.

Functional annotation of differentially expressed genes

To obtain an overview of the gene biological network of the different *E. coli* strains studied, pathway data were downloaded from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg) [29]. A MySQL relational database (https://www.mysql.com) was then constructed to cross the KEGG Orthology IDs of the transcripts with their pathway annotations.

RESULTS

Raw data analysis and RNA-Seq data statistics

In order to understand the mechanisms of *E. coli* resistance to magainin I, four cDNA samples (magainin I-susceptible *E. coli* in the absence (C) and presence of magainin I (CM); and magainin I-resistant *E. coli* in the absence (R) and presence of magainin I (RM)) were prepared from *E. coli*

(ATCC 8739). A total of 12 RNA-seq libraries were constructed (including three replicates for each sample) and sequenced using Illumina MiSeq. As result, more than nine million reads from each experimental group were generated (Table 1). High-quality reads were obtained and used for further analyses after stringent quality assessment using the FastQC tool and data pre-processing, including the removal of adapter sequences and the filtering of low-quality data. High-quality reads were aligned using the package Rockhopper, a system parameterized for computational analysis of bacterial RNA-Seq data [28]. The alignments showed that more than 97 % of the reads from all experimental groups could be aligned to the reference genome (Table 1). In addition, reads aligned to protein-coding genes represented over 29 % of all reads aligned to the reference genome in each sample analysed, and less than 2.3 % could not be aligned to any position in the genome (Table 1).

To confirm the quality of the RNA-Seq *in silico* analysis, eight *E. coli* housekeeping control genes coding for siroheme synthase (*cysG*), fumarate hydratase (*fumC*), adenilate kinase (*adk*), DNA gyrase subunit B (*gyrB*), methionyl-tRNA synthetase (*metG*), DNA recombination and repair protein (*recA*), ribosome recycling factor (*frr*) and DNA gyrase subunit A (*gyrA*) were selected to evaluate gene expression in all samples analysed (Table 2). When comparing the differentially expressed genes (DEGs) between groups C and R (here described as C-R interaction), C and CM (here described as C-CM interaction), R and RM (here described as R-RM interaction), as well as the groups CM and RM (here described as CM-RM interaction) we observed that none of these reference genes were significantly differentially expressed. Their *q*-values were not below the 0.01 cut-off, and their log2 fold change values were not higher than/equal to 1.0, or lower than/equal to −1.0 for up-regulated and down-regulated genes, respectively (Table 2). Hence, this result suggests that the transcript expression values obtained in this study are qualified for further transcriptome analyses.

Table 1. Summary of susceptible (control) and magainin I-resistant *E. coli* RNA-Seq reads aligned to the *E. coli* (ATCC 8739) reference genome

Summary	Control (%)	Control-magainin I (%)	Resistant (%)	Resistant-magainin I (%)
Raw data (number of reads)	9.176.378	12.198.784	11.598.992	13.584.298
Reads discarded after filtering	1.789.430	2.806.926	2.743.230	2.244.342
No. of reads after filtering	7.386.948	9.391.858	8.855.762	11.339.956
Reads aligned to *E. coli* ATCC 8739 genome	7 364 786 (99.7 %)	9 229 0042 (98.3 %)	8 650 704 (97.7 %)	11 104 754 (97.9 %)
Reads aligned to protein-coding genes	2 305 522 (31.3 %)	4 031 126 (42.9 %)	2 585 334 (29.9 %)	4 248 134 (38.3 %)
Unmapped reads	22 162 (0.3 %)	162 854 (1.7 %)	205 058 (2.3 %)	235 202 (2.1 %)

Control represents the magainin I-susceptible strain in the absence of magainin I; control-magainin represents the magainin I-susceptible strain in the presence of magainin I; resistant represents the magainin I-resistant strain in the absence of magainin I; resistant-magainin represents the magainin I-resistant strain in the presence of magainin I.

Functional annotation of differentially expressed genes

A total of 103 DEGs were identified, a biological pathway-based analysis was used to classify all DEGs and volcano plots were built for each interaction to identify the genes with greatest fold and/or significance change (Fig. 1). The majority of the DEGs uniquely found in the C-CM (54.3 %) and R-RM (48.9 %) interactions encoded proteins involved in metabolism (Tables S1 and S2, available in the online version of this article). In the C-CM interaction, there was a significantly higher proportion (16.0 %) of proteins involved in genetic information processing in comparison to R-RM (2.2 %) (Table S1). However, the proportion of uncharacterized proteins with unknown functions was higher in R-RM (31.1 %) than in C-CM (17.3 %), and important proteins involved in the mechanism of magainin I resistance may be present in this category (Table S2). A similar pattern of KEGG category distribution was observed when analysing DEGs common in the C-CM and R-RM interactions, except for the absence of proteins involved in genetic information processing.

Differentially expressed genes in magainin I-susceptible (C) and magainin I-resistant (R) *E. coli* in the absence of magainin I

Initially, the C-R interaction (absence of magainin I) was investigated in order to identify DEGs probably related to magainin I resistance events after the *E. coli* strains resistance induction. Interestingly, however, only four genes were differentially expressed in this analysis (Fig. 1a and Table S1). Among these, a conserved hypothetical protein was up-regulated in R. A conserved hypothetical protein was also found down-regulated in R, as well as a methylisocitrate lyase (prpB) and a small regulatory RNA (dicF) (Fig. 1a and Table S1). These findings may have indicated that the presence of magainin I is necessary to significantly modify the transcriptomics panel in both magainin I-susceptible and -resistant *E. coli* strains. In this context, further analyses were performed to evaluate the response of *E. coli* strains to the absence/presence of magainin I.

Differentially expressed genes in magainin I-susceptible *E. coli* in the absence (C) and presence of magainin I (CM)

A total of 78 and 49 DEGs were identified for the C-CM and R-RM interactions, respectively. Among these, 54 are exclusive to the C-CM interaction (Table S2); 25 are exclusive to the R-RM interaction (Table S3); and 24 are shared between C-CM and R-RM (Table S4). In the C-CM interaction, the majority of the genes (73.1 %) are up-regulated in

Table 2. Expression levels of eight housekeeping control genes in the *E. coli* C-CM and R-RM interactions

Locus tag	Gene	Description	Log2 (FC)	*q*-value C vs. CM	Significant	Log2 (FC)	*q*-value R vs. RM	Significant
EcolC_0344	*cysG*	Siroheme synthase	−0.46	1	No	0.21	0.864	No
EcolC_2019	*fumC*	Fumarate hydratase	0.16	1	No	0.05	0.994	No
EcolC_3142	*adk*	Adenylate kinase	−0.02	1	No	−0.14	1.000	No
EcolC_0004	*gyrB*	DNA gyrase subunit B	−0.31	1	No	−0.47	0.888	No
EcolC_1533	*metG*	Methionyl-tRNA synthetase	−0.04	1	No	0.22	1.000	No
EcolC_1013	*recA*	Recombinase A	−0.17	1	No	0.19	0.994	No
EcolC_3488	*frr*	Ribosome recycling factor	0.21	1	No	0.16	1.000	No
EcolC_1420	*gyrA*	DNA gyrase subunit A	−0.04	1	No	0.19	1.000	No

CM. In addition, all up- and down-regulated genes are mainly related to bacterial metabolism (54.3 %), followed by unknown genes (17.3 %), genetic information processing (16.0 %), environmental information processing (7.4 %), cellular processes (2.5 %) and others (2.5 %) (Table S2). Among the up-regulated genes in CM we observed a high investment in energy, carbohydrate and amino acid metabolism, as well as in ribosome biogenesis and translation processes (Fig. 1b and Table S2). Moreover, up-regulated genes related to drug transport and bacterial defence were also identified (Fig. 2 and Table S2). Furthermore, genes involved in flagellum assembly were down-regulated in CM (Fig. 2 and Table S2). A total of nine genes with greatest fold and/or significance change were identified in the volcano plot (Fig. 1b). Among the up-regulated genes in CM two hypothetical proteins, a methylisocitrate lyase (carbohydrate metabolism) and a sulfate ABC transporter (CysP) (Fig. 2), were identified (Fig. 1b and Table S2). In addition, a zinc-associated protein (ZraP) and a small heat shock protein (IbpA) were down-regulated in CM in the C-CM interaction (Figs 1b, 2; Table S2).

Differentially expressed genes in magainin I-resistant *E. coli* in the absence (R) and presence of magainin I (RM)

A similar proportion of genes found up-regulated in CM in the C-CM interaction was also observed in RM in the R-RM interaction (75.5 %). Among all genes identified in the R-RM interaction, 48.9 % are associated with metabolism, 31.1 % are unknown, 8.9 % are 'others', 6.7 % are related to environmental information processing and 2.2 % to cellular process and genetic information processing (Table S3). Interestingly, differently to what was observed for those DEGs in the C-CM interaction, up-regulated genes in RM in the R-RM interaction are not primarily involved in energy processes (Fig. 3 and Table S3). Here, quorum sensing, cell community and prokaryote defence system-related genes were up-regulated in RM (Fig. 3 and Table S3). Moreover, drug transport and drug resistance-related genes coding multidrug efflux systems were also up-regulated in RM (Fig. 3 and Table S3). In regard to the CM in the C-CM interaction (Table S2), RM molecular chaperone genes are down-regulated in the R-RM interaction (Table S3). Furthermore, seven genes were highlighted in the volcano plot (Fig. 1c). Among the up-regulated genes, two hypothetical proteins and a glutamate decarboxylase (GadA and B) were identified (Fig. 1c and Fig. 3). Moreover, in regard to CM in the C-CM interaction, the small heat shock proteins IbpA and IbpB (Fig. 3) were down-regulated in RM. A tryptophan-specific transport protein (mtr) and a hypothetical protein were also down-regulated in RM (Fig. 1c and Table S3).

Shared genes between the C-CM and R-RM interactions

Twenty-four DEGs were common to both CM and RM groups (Table S4), and almost all showed the same expression changes (up- or down-regulation). However, significant fold change variations were observed among the samples for a few DEGs. Among the 24 shared genes, nine could be annotated according to KEGG orthology and classified as drug resistance (K08224 and K13650), amino acid metabolism (K02858 and K00557), transcription factors (K07726), transporters (K04758), ribosome biogenesis (K05808), chaperones and folding catalysis-related genes (K04080 and K04081) (Table S4). Moreover, according to the volcano plot, a putative selenate reductase FAD-binding subunit was up-regulated in RM in the CM-RM interaction with highest fold and/or significance change (Fig. 1d). Furthermore, the volcano plot also revealed six genes down-regulated in RM in the CM-RM interaction, including a methylisocitrate lyase (PrpB), a 2-methylcitrate synthase (PrpC), a 2-methylcitrate dehydratase (PrpD) and a propionyl-CoA synthetase (PrpE), all related to propanoate metabolism. In addition, a gene coding for a protein of unknown function and an ABC transporter (CysP) were also down-regulated in RM (Fig. 1d and Table S4).

Exclusive genes in the C-CM and R-RM interactions

As described above, 54 genes are exclusive to the C-CM interaction whereas 25 are exclusive to the R-RM interaction. Among the 54 exclusive genes in C-CM, 38 that were up-regulated in CM are mainly involved in energy (K02045, K00390, K02046, K00381, K01738, K02047 and K00957), carbohydrate (K01659, K01720 and K01908), fatty acid (K00632) and amino acid metabolism (K01484, K06447, K00673 and K00840), as well as being related to transcription factors (K03704) and translation processes

(K02879 and K02886) (Table S2). Sixteen exclusive genes were down-regulated in CM and classified as genes related to other amino acid metabolism (K12527 and K12529), cell motility (K02396) and hydrolases (K01104 and K13694) (Table S2). Otherwise, among the 25 exclusive genes from R-RM, 18 are up-regulated in RM and were annotated as transporters (K07490), oxidoreductases (K13979), cellular community (quorum sensing; biofilm formation) (K01580) and drug resistance/multidrug efflux pump system (K18898) (Fig. 3 and Table S3). Seven exclusive genes were down-regulated in RM; however, only two (K02774 and K03835) could be annotated and classified into carbohydrate metabolism and transporter pathways (Table S3).

DISCUSSION

Currently, the advent of multidrug bacterial resistance is considered one of the most alarming situations worldwide. As a response to these events, the development of novel antimicrobial compounds represents an urgent and critical reality [1]. In this context, AMPs appear as an alternative to drugs no longer effective because of their multiple and complex modes of action. Indeed, this class of antimicrobials has been widely studied over the years, and promising activities have been found against Gram-negative and -positive, susceptible and resistant bacterial strains and biofilms. Nevertheless, reports have increasingly shown that bacteria can evade AMP treatment through a range of resistance-related mechanisms. Thus, here we report the comparative transcriptome analyses of magainin I-susceptible and -resistant *E. coli* strains through RNA-Seq. Four experimental groups were studied, and a total of 103 DEGs were identified in the C-CM and R-RM interactions. Moreover, 25 DEGs were shared between the two interactions, 54 DEGs were exclusive to the C-CM interactions and 25 DEGs exclusive to the R-RM interaction.

Among the up-regulated DEGs in both C-CM and R-RM interactions, intense metabolic responses (>48 %) in the presence of magainin I were observed, mainly related to energy metabolism. It is known that, under non-stress conditions, bacteria maintain basal energy levels required for essential cellular processes. The presence of antimicrobial agents, however, seems to drastically change this situation. In a robust study performed by Holden and co-workers, the genomes of two methicillin-resistant *S. aureus* (hospital-acquired and invasive community-acquired strains) were sequenced [30]. As result, a large number of protein-coding sequences related to energy metabolism were observed, mainly for hospital-acquired methicillin-resistant *S. aureus* [30]. Therefore, in our previous findings regarding the cytosolic subproteome of magainin I-susceptible and -resistant *E. coli*, 49 % of the differentially expressed proteins in the latter group were related to energy metabolism [22], thus creating a clear parallel between both proteomics and transcriptomics data.

Interestingly, it has been reported that *E. coli* strains are capable of directing energy investment to search out better living conditions [31]. In this context, Zhao and co-workers [31] reported the adaptation of *E. coli* bacterial flagellar and motility systems [31]. Among their findings the authors concluded that, depending on the availability of carbon sources, *E. coli* strains might carry out tactical responses by increasing flagellar operation, but decreasing the synthesis of a costly flagellar subunit. Here, we found that a flagellar assembly subunit (FlgK) was down-regulated in CM in the C-CM interaction, indicating low investment in the synthesis of flagella-related proteins, but did not discard the possibility of intense flagellar operation to escape magainin I presence. This can also be supported by the up-regulation (in both CM and RM) of a GCU-specific mRNA interferase toxin of the MqsR-MqsA toxin–antitoxin system and motility regulator (MqsR). MqsR has been shown to play a crucial role in the control of cell motility, as well as the induction of persister cells in the presence of antibiotics, including ciprofloxacin, ampicillin and ofloxacin [32, 33]. Interestingly, studies have suggested that down-regulation of flagellar subunits, allied to the up-regulation of MsqR, may be related to the initial stages of biofilm formation, a well-described bacterial consortium that may be developed to respond to antibiotic treatment [34]. However, future comparative proteomics/transcriptomics studies regarding AMP-susceptible and -resistant bacteria in their biofilm lifestyle are encouraged to support these hypotheses.

Studies have shown that cell density, starvation and antibiotic presence may modulate acid adaptation in several bacterial strains [35, 36]. It is expected that pore-forming AMPs, including magainin I, cause an imbalance in the periplasmic and intracellular pH, directly interfering in the function of proteins essential for bacterial survival. In response, bacteria can express a series of proteins involved in pH stability within the cell. Here, glutamate decarboxylase protein family members (GadA, B and C) were up-regulated in CM (GadA and B) and RM (GadA, B and C). GadA and B convert glutamate to gamma-aminobuyrate (GABA) in the cytosol, consuming one proton, whereas GadC simultaneously exports GABA to the periplasm while importing glutamate to the cytosol. This cycle has played a crucial role in the maintenance of a near-neutral intracellular pH in acidic conditions, assisting bacterial cell homeostasis [37, 38].

Multidrug efflux pumps have been widely described in the literature as the major contributors to intrinsic resistance in bacteria. Moreover, when overexpressed, these transporter proteins can confer high levels of resistance to previously effective antibiotics and AMPs [3, 4]. Among the five families of efflux transporters we found that two were up-regulated in CM, including adenosine triphosphate (ATP)-binding cassette (ABC) transporters and the major facilitator superfamily (MFS); and two were up-regulated in RM, including MFS and the resistance-nodulation cell division (RND) superfamily. Moreover, here the ABC transporters CysA, CysP, CysU and CysW were up-regulated in CM and associated with the transport of sulfate and thiosulfate from

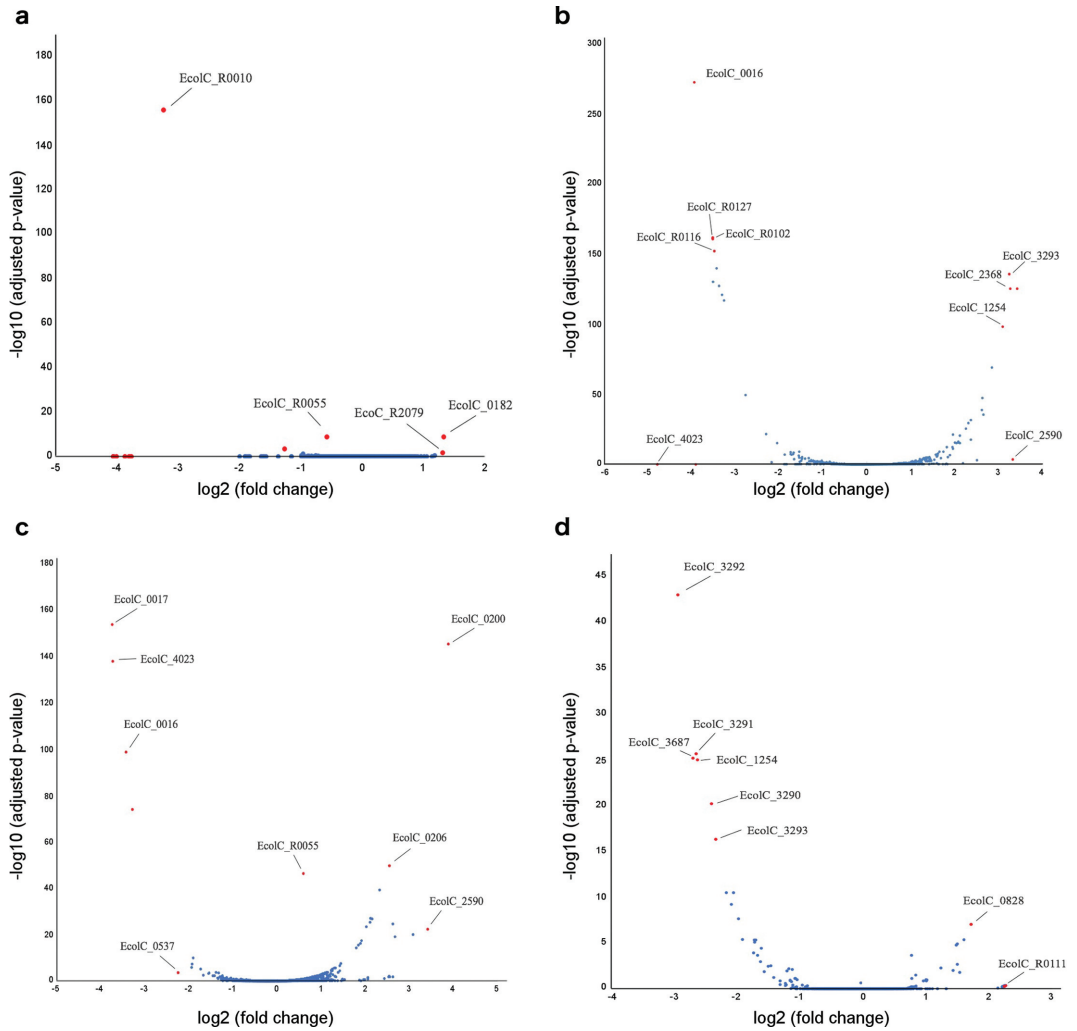

Fig. 1. Volcano plots for the interaction C-R (a), C-CM (b), R-RM (c) and CM-RM (d). Differently expressed genes with greatest fold and/or significance change are highlighted. The −log10 (*P*-value) is plotted on the vertical (y) axis.

the medium to the bacterial cytosol. Sulfur is an essential element for bacterial development and is usually obtained from cysteine [39]. However, under stress conditions, where high concentrations of sulfur are required, bacteria can use sulfate and oxidized inorganic sulfur compounds as a source of sulfur [39]. Based on that, our data suggest that *E. coli* strains from the CM group present high investment in sulfur metabolism in response to magainin I.

Unlike the ABC transporters identified in this study, MFS and RND efflux pumps are directly involved in drug efflux processes driven by proton motive force. A MFS member was found up-regulated in both CM and RM groups, whereas membrane fusion proteins from RND were up-regulated only in RM. MFS efflux pumps represent ~25 % of all transporters in prokaryotes, playing a crucial role in bacterial physiology and resistance to antimicrobials. Studies have shown that MFS members contribute to multidrug resistance in emerging nosocomial pathogens, including *Stenotrophomonas maltophilia* and *E. coli* [40, 41]. Moreover, key transporters from MFS have been crystallized and suitable targets for putative modulation have been proposed [42].

Also in the context of efflux pumps, membrane fusion proteins from the RND superfamily were found up-regulated in RM, corroborating our previous findings regarding the comparative nanoUPLC-MS^E analysis between magainin I-susceptible and -resistant *E. coli* strains [4]. In that work, the multidrug efflux pump subunit (AcrA) was up-regulated in the latter [4]. Differently to the mechanisms of MFS members, RND superfamily efflux pumps are constituted by an outer membrane protein channel (TolC) and membrane fusion proteins, allowing the expulsion of antimicrobials from the cytosol to the outside of the bacterial cell [43]. In this context, drugs have been developed to target both TolC

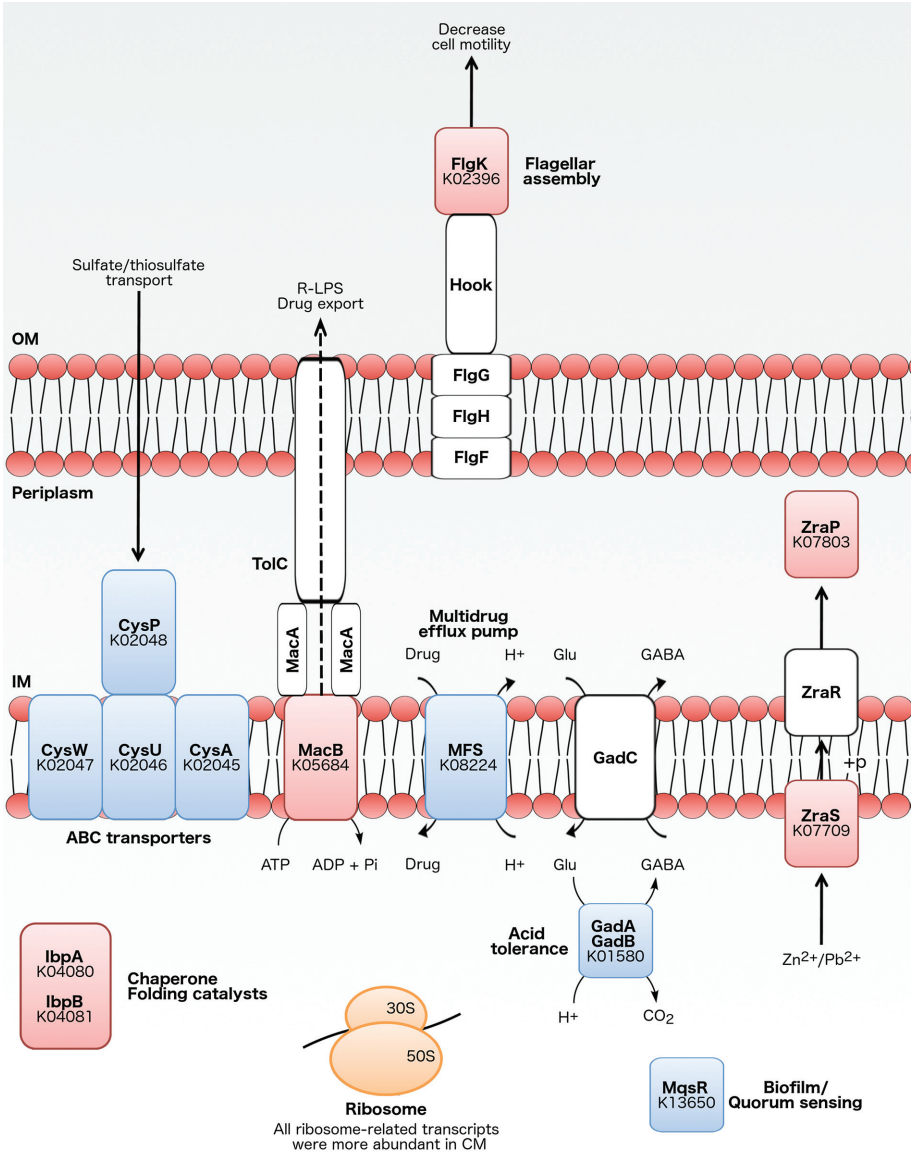

Fig. 2. Schematic representation of an *E. coli* cell highlighting DEGs (blue: up-regulated; red: down-regulated) in CM in the C-CM interaction. DEGs illustrated here are mainly involved in bacterial resistance and, therefore, not all DEGs from Table S2 were included. Abbreviations: OM: outer membrane; IM: inner membrane; CysA, P, U and W: sulfate/thiosulfate import ATP-binding protein; IbpA and B: small heat shock proteins; MacAB: macrolide export ATP-binding/permease protein; TolC: outer membrane protein channel; MFS: major facilitator superfamily; GadA, B and C: glutamate decarboxylase alpha-, beta- and gamma-aminobutyrate, respectively; MqsR: mRNA interferase toxin; FlgF, H, G and K: flagellar hook-associated proteins; ZraP, R and S: zinc-associated proteins.

and fusion proteins and, more importantly, studies have already revealed how bacteria can resist these drugs [44, 45]. For instance, membrane fusion proteins, including AcrA and AcrB subunits, have been related to ciprofloxacin therapy failure due to single mutation (G288D substitution), thus compromising the binding of this drug to AcrB [44]. Altogether, these findings clearly indicate the relevance of both MFS and RND members to the resistance of *E. coli* strains (RM) to magainin I.

Interestingly, in the CM group, an ABC transporter-related gene (MacB) was down-regulated. MacB belongs to the MacAB-TolC ABC-type tripartite multidrug efflux pump, whose functions include the active extrusion of macrolide antibiotics and polypeptide virulence factors [46]. However, even down-regulated, its fold change was considerably lower and *q*-value considerably higher compared to MFS efflux pump-related genes found up-regulated in CM in the C-CM interaction.

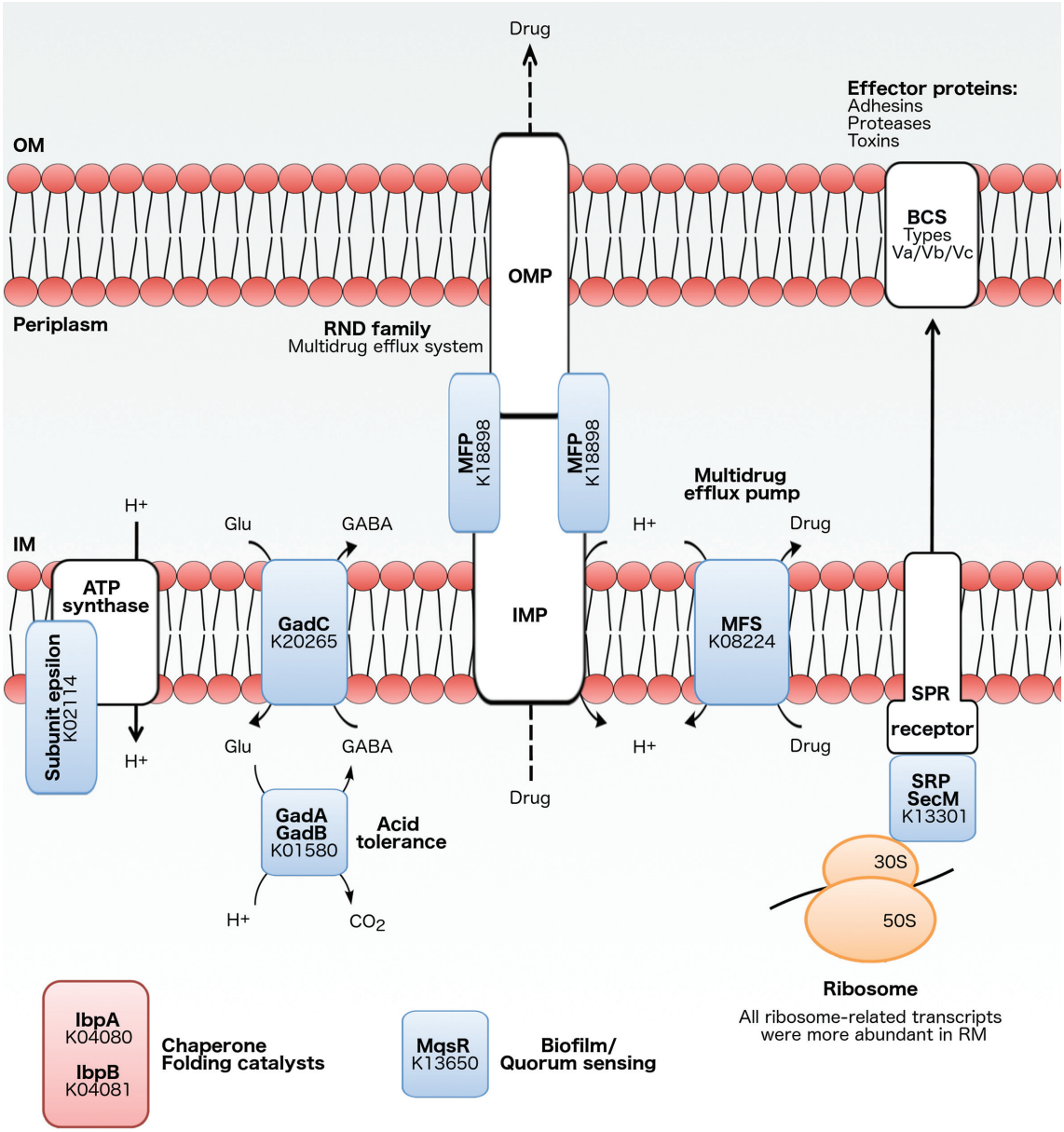

Fig. 3. Schematic representation of an *E. coli* cell highlighting DEGs (blue: up-regulated; red: down-regulated) in RM in the R-RM interaction. DEGs illustrated here are mainly involved in bacterial resistance and, therefore, not all DEGs from Table S3 were included. Abbreviations: OM: outer membrane; IM: inner membrane; IbpA and B: small heat shock proteins; MFS: major facilitator superfamily; GadA, B and C: glutamate decarboxylase alpha-, beta- and gamma-aminobutyrate, respectively; MqsR: mRNA interferase toxin; RND: resistance-nodulation cell division superfamily; OMP: outer membrane protein; IMP: inner membrane protein; MFP: membrane fusion protein; BCS: bacterial secretion system; SecM: secretion monitor; SRP: signal recognition particle receptor.

Two genes encoding zinc-associated proteins (ZraS and ZraP) were also down-regulated in the CM group. These proteins seem to play a crucial role in zinc homeostasis, mainly in the periplasmic space, as well as chaperone and regulatory functions [47], thus protecting enzymes from high concentrations of zinc [48]. Interestingly, ZraP was up-regulated in magainin I-resistant *E. coli* strains in our previous nanoUPLC-MS^E work, indicating that resistant *E. coli* strains express ZraP in order to maintain homeostasis in the periplasmic space, thus favouring the proper function of other magainin I resistance-related proteins [4].

Similarly, two small heat shock chaperone-related genes (IbpA and B) were down-regulated in both CM and RM groups. During heat shock and oxidative stress, bacteria can

produce the complex IbpAB to stabilize and protect proteins from irreversible denaturation and proteolysis [49]. Interestingly, however, *E. coli* strains from groups CM and RM were not submitted to heat stress, and resistance-related proteins involved in acid tolerance, cell motility and multidrug efflux pumps found in the present work are not Zn^{2+} dependent, which may explain the low investment by these strains in heat shock chaperones and zinc-associated proteins. Similar findings were observed in our previous works, where the heat shock protein DnaK was down-regulated in magainin I-resistant *E. coli* strains [4].

Among the up-regulated genes in the RM group, a secretion monitor-related gene (SecM) was identified. The up-regulation of SecM directly interferes in the translational levels of SecA, a major component of the bacterial Sec pathway and responsible for driving protein export through the SecYEG system [50]. Studies have shown that high levels of SecM and SecA are expected when the Sec pathway is compromised [50]. Moreover, SecM is also related to outer membrane proteins involved in the secretion of effector proteins, including adhesins, toxins and proteases. Proteases are among the most common bacterial resistance mechanisms in response to AMPs [17], and the up-regulation of SecM may help the secretion of these enzymes for further magainin I degradation. All these processes mediated by the SecYEG system are driven by ATP hydrolysis energy. Interestingly, the ATP-synthase epsilon subunit was also up-regulated in RM, reinforcing the investment in energy generation for the proper function of AMP resistance-related proteins identified in this study.

In summary, here we observed high investment in *E. coli* energy metabolism, translational processes and bacterial defence in *E. coli* strains in the presence of magainin I (CM and RM) compared to magainin I-susceptible and -resistant strains in the absence of magainin I (C and R). In general, DEGs from the C group are related to basal levels of expression of homeostasis-related genes compared to CM, whereas DEGs from the R group suggest that, even if presenting resistance to magainin I, the presence of this AMP is required to change the transcriptomics panel in *E. coli* strains. Moreover, as previously described [4], these data strongly support the hypothesis of an overexpression of different multidrug efflux pumps for the *E. coli* response to magainin I in both CM and RM groups. Furthermore, this work complements our previous findings regarding comparative proteomics studies between magainin I-susceptible and -resistant *E. coli* strains, revealing the complexity of the molecular mechanisms of AMP resistance, as well as providing information for the future development of antimicrobial strategies targeting resistance-related transcripts and proteins here described.

Funding information
This study was supported by grants from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), FUNDECT (Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul), FAPDF (Fundação de Apoio à Pesquisa do Distrito Federal), UnB (Universidade de Brasília) and UCB (Universidade Católica de Brasília). This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. Arias CA, Murray BE. A new antibiotic and the evolution of resistance. *N Engl J Med* 2015;372:1168–1170.
2. Crofts TS, Gasparrini AJ, Dantas G. Next-generation approaches to understand and combat the antibiotic resistome. *Nat Rev Microbiol* 2017;15:422–434.
3. Blair JM, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ. Molecular mechanisms of antibiotic resistance. *Nat Rev Microbiol* 2015; 13:42–51.
4. Cardoso MH, de Almeida KC, Cândido ES, Murad AM, Dias SC *et al.* Comparative NanoUPLC-MSE analysis between magainin I-susceptible and -resistant *Escherichia coli* strains. *Sci Rep* 2017;7: 4197.
5. Taneja NK, Ganguly T, Bakaletz LO, Nelson KJ, Dubey P *et al.* D-alanine modification of a protease-susceptible outer membrane component by the *Bordetella pertussis dra locus* promotes resistance to antimicrobial peptides and polymorphonuclear leukocyte-mediated killing. *J Bacteriol* 2013;195:5102–5111.
6. Long KS, Poehlsgaard J, Kehrenberg C, Schwarz S, Vester B. The Cfr rRNA methyltransferase confers resistance to Phenicols, Lincosamides, Oxazolidinones, Pleuromutilins, and Streptogramin A antibiotics. *Antimicrob Agents Chemother* 2006;50:2500–2505.
7. Voulgari E, Poulou A, Koumaki V, Tsakris A. Carbapenemase-producing Enterobacteriaceae: now that the storm is finally here, how will timely detection help us fight back? *Future Microbiol* 2013;8:27–39.
8. Romanowska J, Reuter N, Trylska J. Comparing aminoglycoside binding sites in bacterial ribosomal RNA and aminoglycoside modifying enzymes. *Proteins* 2013;81:63–80.
9. Drenkard E, Ausubel FM. Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature* 2002;416:740–743.
10. Fjell CD, Hiss JA, Hancock RE, Schneider G. Designing antimicrobial peptides: form follows function. *Nat Rev Drug Discov* 2011;11: 37–51.
11. Beceiro A, Llobet E, Aranda J, Bengoechea JA, Doumith M *et al.* Phosphoethanolamine modification of lipid A in colistin-resistant variants of *Acinetobacter baumannii* mediated by the pmrAB two-component regulatory system. *Antimicrob Agents Chemother* 2011; 55:3370–3379.
12. Schmidtchen A, Frick IM, Andersson E, Tapper H, Björck L. Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37. *Mol Microbiol* 2002;46:157–168.
13. Sieprawska-Lupa M, Mydel P, Krawczyk K, Wójcik K, Puklo M *et al.* Degradation of human antimicrobial peptide LL-37 by *Staphylococcus aureus*-derived proteinases. *Antimicrob Agents Chemother* 2004;48:4673–4679.
14. Ulvatne H, Haukland HH, Samuelsen Ø, Krämer M, Vorland LH. Proteases in *Escherichia coli* and *Staphylococcus aureus* confer reduced susceptibility to lactoferricin B. *J Antimicrob Chemother* 2002;50:461–467.
15. Smyth D, Cameron A, Davies MR, McNeilly C, Hafner L *et al.* DrsG from *Streptococcus dysgalactiae* subsp. *equisimilis* inhibits the antimicrobial peptide LL-37. *Infect Immun* 2014;82:2337–2344.
16. Manning AJ, Kuehn MJ. Contribution of bacterial outer membrane vesicles to innate bacterial defense. *BMC Microbiol* 2011;11:258.
17. Fleitas O, Agbale CM, Franco OL. Bacterial resistance to antimicrobial peptides: an evolving phenomenon. *Front Biosci* 2016;21: 1013–1038.

18. Tzeng YL, Ambrose KD, Zughaier S, Zhou X, Miller YK *et al.* Cationic antimicrobial peptide resistance in *Neisseria meningitidis*. *J Bacteriol* 2005;187:5387–5396.
19. Bengoechea JA, Skurnik M. Temperature-regulated efflux pump/potassium antiporter system mediates resistance to cationic antimicrobial peptides in Yersinia. *Mol Microbiol* 2000;37: 67–80.
20. Srinivasan VB, Rajamohan G. KpnEF, a new member of the *Klebsiella pneumoniae* cell envelope stress response regulon, is an SMR-type efflux pump involved in broad-spectrum antimicrobial resistance. *Antimicrob Agents Chemother* 2013;57:4449–4462.
21. Weatherspoon-Griffin N, Yang D, Kong W, Hua Z, Shi Y. The CpxR/CpxA two-component regulatory system up-regulates the multidrug resistance cascade to facilitate *Escherichia coli* resistance to a model antimicrobial peptide. *J Biol Chem* 2014;289:32571–32582.
22. Maria-Neto S, Cândido ES, Rodrigues DR, de Sousa DA, da Silva EM *et al.* Deciphering the magainin resistance process of *Escherichia coli* strains in light of the cytosolic proteome. *Antimicrob Agents Chemother* 2012;56:1714–1724.
23. Zasloff M. Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proc Natl Acad Sci USA* 1987; 84:5449–5453.
24. Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet* 2009;10:57–63.
25. de Almeida KC, Lima TB, Motta DO, Silva ON, Magalhães BS *et al.* Investigating specific bacterial resistance to AMPs by using a magainin I-resistant *Escherichia coli* model. *J Antibiot* 2014;67: 681–687.
26. Conesa A, Madrigal P, Tarazona S, Gomez-Cabrero D, Cervera A *et al.* A survey of best practices for RNA-seq data analysis. *Genome Biol* 2016;17:13.
27. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 2014;30:2114–2120.
28. McClure R, Balasubramanian D, Sun Y, Bobrovskyy M, Sumby P *et al.* Computational analysis of bacterial RNA-Seq data. *Nucleic Acids Res* 2013;41:e140.
29. Kanehisa M, Sato Y, Kawashima M, Furumichi M, Tanabe M. KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Res* 2016;44:D457–D462.
30. Holden MT, Feil EJ, Lindsay JA, Peacock SJ, Day NP *et al.* Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. *Proc Natl Acad Sci USA* 2004;101:9786–9791.
31. Zhao K, Liu M, Burgess RR. Adaptation in bacterial flagellar and motility systems: from regulon members to 'foraging'-like behavior in *E. coli*. *Nucleic Acids Res* 2007;35:4441–4452.
32. Maisonneuve E, Shakespeare LJ, Jørgensen MG, Gerdes K. Bacterial persistence by RNA endonucleases. *Proc Natl Acad Sci USA* 2011;108:13206–13211.
33. Wood TK, Knabel SJ, Kwan BW. Bacterial persister cell formation and dormancy. *Appl Environ Microbiol* 2013;79:7116–7121.
34. González Barrios AF, Zuo R, Hashimoto Y, Yang L, Bentley WE *et al.* Autoinducer 2 controls biofilm formation in *Escherichia coli* through a novel motility quorum-sensing regulator (MqsR, B3022). *J Bacteriol* 2006;188:305–316.
35. Li YH, Hanna MN, Svensäter G, Ellen RP, Cvitkovitch DG. Cell density modulates acid adaptation in *Streptococcus mutans*: implications for survival in biofilms. *J Bacteriol* 2001;183:6875–6884.
36. McNeill K, Hamilton IR. Acid tolerance response of biofilm cells of *Streptococcus mutans*. *FEMS Microbiol Lett* 2003;221:25–30.
37. de Biase D, Tramonti A, Bossa F, Visca P. The response to stationary-phase stress conditions in *Escherichia coli*: role and regulation of the glutamic acid decarboxylase system. *Mol Microbiol* 1999;32:1198–1211.
38. Ma Z, Gong S, Richard H, Tucker DL, Conway T *et al.* GadE (YhiE) activates glutamate decarboxylase-dependent acid resistance in *Escherichia coli* K-12. *Mol Microbiol* 2003;49:1309–1320.
39. van der Ploeg JR, Eichhorn E, Leisinger T. Sulfonate-sulfur metabolism and its regulation in *Escherichia coli*. *Arch Microbiol* 2001;176:1–8.
40. Dulyayangkul P, Charoenlap N, Srijaruskul K, Mongkolsuk S, Vattanaviboon P. Major facilitator superfamily MfsA contributes to multidrug resistance in emerging nosocomial pathogen *Stenotrophomonas maltophilia*. *J Antimicrob Chemother* 2016;71:2990–2991.
41. Lomovskaya O, Lewis K. Emr, an *Escherichia coli* locus for multidrug resistance. *Proc Natl Acad Sci USA* 1992;89:8938–8942.
42. Ranaweera I, Shrestha U, Ranjana KC, Kakarla P, Willmon TM *et al.* Structural comparison of bacterial multidrug efflux pumps of the major facilitator superfamily. *Trends Cell Mol Biol* 2015;10: 131–140.
43. Nikaido H. Structure and mechanism of RND-type multidrug efflux pumps. *Adv Enzymol Relat Areas Mol Biol* 2011;77:1–60.
44. Blair JM, Bavro VN, Ricci V, Modi N, Cacciotto P *et al.* AcrB drug-binding pocket substitution confers clinically relevant resistance and altered substrate specificity. *Proc Natl Acad Sci USA* 2015; 112:3511–3516.
45. Venter H, Mowla R, Ohene-Agyei T, Ma S. RND-type drug efflux pumps from Gram-negative bacteria: molecular mechanism and inhibition. *Front Microbiol* 2015;6:377.
46. Fitzpatrick AWP, Llabrés S, Neuberger A, Blaza JN, Bai XC *et al.* Structure of the MacAB-TolC ABC-type tripartite multidrug efflux pump. *Nat Microbiol* 2017;2:17070.
47. Appia-Ayme C, Hall A, Patrick E, Rajadurai S, Clarke TA *et al.* ZraP is a periplasmic molecular chaperone and a repressor of the zinc-responsive two-component regulator ZraSR. *Biochem J* 2012;442:85–93.
48. Maruthamuthu MK, Ganesh I, Ravikumar S, Hong SH. Evaluation of zraP gene expression characteristics and construction of a lead (Pb) sensing and removal system in a recombinant *Escherichia coli*. *Biotechnol Lett* 2015;37:659–664.
49. Tao J, Sang Y, Teng Q, Ni J, Yang Y *et al.* Heat shock proteins IbpA and IbpB are required for Nlpl-participated cell division in *Escherichia coli*. *Front Microbiol* 2015;6:51.
50. Nakamori K, Chiba S, Ito K. Identification of a SecM segment required for export-coupled release from elongation arrest. *FEBS Lett* 2014;588:3098–3103.

Edited by: T. Schneiders and M. Whiteley