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RESEARCH ARTICLE

The cost of resistance to colistin in *Acinetobacter baumannii*: a proteomic perspective

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Colistin resistance in *Acinetobacter baumannii*, a pathogen of clinical concern, was induced in the susceptible strain ATCC 19606 by growth under increasing pressure of the antibiotic, the only drug universally active against multi-resistant clinical strains. In 2-D difference gel electrophoresis (DIGE) experiments, 35 proteins with differences in expression between both phenotypes were identified, most of them appearing as down regulated in the colistin-resistant strain. These include outer membrane (OM) proteins, chaperones, protein biosynthesis factors, and metabolic enzymes, all suggesting substantial loss of biological fitness in the resistant phenotype, as substantiated by complementary experiments in the absence of colistin. Results shed light on the scarcity of widespread clinical outbreaks for resistant phenotypes.

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1 Introduction

The successful thriving of bacteria in the antibiotic era has set off an alarm situation in the therapeutic community, as the antimicrobial armamentarium against many species of clinical importance is nearing exhaustion. These disquieting prospects, however, must be offset against the fact that induction of resistance entails an additional metabolic burden to the resistant organism, frequently resulting in loss of fitness and thus limited dissemination in the absence of antibiotic [1]. Successful microbes, on the other hand, overcome this problem through compensating and/or free-cost mutations, together with allelic exchange between susceptible and resistant strains [2]. This scenario is particularly true for antibiotics with a well-defined single target, but the situation becomes more complicated for those with not fully defined and possibly multiple targets, such as colistin (polymyxin E).

Polymyxins (PMXs), a family of cyclic decapeptides produced by *Bacillus polymyxa*, have an N-terminal fatty acid chain and over several (>50%) 2,4-diaminobutyric acid residues conferring them, respectively, the amphipathicity and cationic character that underlie their antibiotic properties [3, 4]. PMXs show strong bactericidal activity on Gram negatives, together with anti-endotoxic activity [5, 6]. Since cytotoxicity precluded systemic administration of PMXs, they were reserved for topical applications while other antibiotics with better toxicity profiles were promoted. However, as the efficacy of the latter was again eroded by the appearance of

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Abbreviations: OM, outer membrane; **PMXs,** polymyxins; **SRP,** signal recognition particle

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resistance, interest in the use of PMXs, particularly colistin, against severe Gram negative infections has revived, helped along by a reassessment of the clinical toxicity of PMXs, which recent work has shown to be lower than found in earlier studies [7–9].

Acinetobacter baumannii is an opportunistic Gram-negative coccobacillus that is the causative agent of a broad set of clinical conditions such as bacteremia, pneumonia, and soft tissue or urinary infections. Nosocomial outbreaks of *A. baumannii* are a serious concern for their high morbidity and mortality rates, particularly in intensive care units [10]. Underscoring the clinical threat posed by this microorganism are its high metabolic versatility and environmental adaptability, along with a remarkable ability to develop resistance against most antibiotics commonly used in clinical settings [8, 11, 12]. This has caused an alarming decline in the chemotherapeutic resources available, including drugs such as imipenem, an inhibitor of peptidoglycan biosynthesis that until recently was considered the golden standard for *Acinetobacter* treatment [10, 13]. More recent reports have described sporadic episodes of *A. baumannii* resistant to colistin (polymyxin E), the last universally active drug against this microorganism, used only as a last-resort antibiotic due to its toxicity [13–20]. That such outbreaks have not yet become more clinically widespread is intriguing, in view of the attested ability of *Acinetobacter* to thrive in harsh environments, including noxious antibiotics. While prophylactic measures may partially account for such a success in containment, the fact remains that other antibiotic-resistant phenotypes are rather adept at trespassing such barriers while colistin-resistant *A. baumannii* is not, suggesting that microorganism-specific features underlie this fortunate loss of fitness. Examples of loss of fitness associated to antibiotic resistance have been reported [2]. In general, the biological cost inherent to the acquisition of such resistance traits is advantageous to the organism only in the presence of antibiotic pressure.

Over the last decade, proteomics has emerged as a powerful approach to investigate at the molecular level a wide variety of biological problems, including antibiotic resistance in bacteria [21–24]. For *A. baumannii*, some studies [21, 25], including a recently completed comprehensive cataloguing of the proteome [26], have unveiled several differences in protein expression associated to a multi-resistant pattern. On the whole, though, proteomic data on *A. baumannii* antibiotic resistance remain scarce, in particular regarding colistin, for which, to the best of our knowledge, no data on differential expression inherent to resistance are yet available.

The definition of a specific proteomic signature for antibiotic resistance faces two major challenges: (i) misleading expression differences associated with resistance to other previously failed antibiotics − a not uncommon situation in the clinics of *A. baumannii* [10], for which colistin is usually a last-resort drug, and (ii) sorting out early, transient proteome differences common to any antibiotic-induced stress and facilitating bacterial survival before stable re-adaptation (*i.e.*, resistance) is actually achieved [27]. Thus, while we initially considered analyzing the proteome of multi-resistant clinical isolates of *A. baumannii*, using reference strain ATCC 19606 for comparison, on further reflection opted for inducing such resistance in a reference strain and investigating by 2-D difference gel electrophoresis (DIGE) the differences in protein expression patterns between resistant and susceptible phenotypes. Of a total of 64 spots with significantly altered expression in either total lysate or membrane-enriched fractions, 35 proteins could be identified. The differential set included several outer membrane (OM) proteins, symptomatic of altered membrane structures in the resistant strain, and also differences in chaperones and proteins involved in protein biosynthesis, which in the resistant strain appeared mostly under-expressed, suggesting that the colistin-resistant strain has reduced metabolic fitness relative to the wild type. This lower fitness in the absence of colistin, which we have corroborated in separate experiments, causes the resistant mutant to be rapidly outgrown by the wild type, once the antibiotic pressure is released. This may in turn prevent the massive dissemination that characterizes many multi-drug-resistant strains, and helps to explain the fact that no pervasive episodes of colistin-resistant *A. baumannii* have been yet described.

2 Materials and methods

2.1 Bacteria

A. baumannii strain ATCC 19606 (gift of Prof. M. López-Brea, Hospital de la Princesa, Madrid, Spain), sensitive to colistin (MIC = 2 μM), was grown in Luria Bertani medium at 37°C. MIC were determined as described [28]. Colistin resistance was achieved by liquid culturing under increase in the concentration of colistin sulfate (Sigma España, Madrid, Spain). Once a maximal level of resistance was obtained (MIC = 36 μM), bacteria were grown for 2 months at constant colistin concentration. A single clone was isolated, cultivated, and aliquots stored in storage buffer (65% glycerol, 0.1 M $MgSO_4$, 25 mM Tris-HCl, pH 8.0) at −80°C. For each growth experiment an aliquot of the frozen stock was used for seeding.

2.2 Microbial fitness in the absence of colistin

MICs of 2 and 32 μM for ATCC 19606 and its isogenic colistin-resistant strain were determined as described [29]. Equal amounts from both strains were mixed and allowed to grow (10^6 CFU/mL, 37°C, shaking) in Müller-Hinton broth devoid of colistin. After 24 h, a sample of the mixed culture was taken and the number of resistant and susceptible colonies evaluated by serial dilution and final plating on solid medium with or without colistin, respectively, at 32 μM. The resulting culture was diluted 1000-fold in the same medium,

growth allowed to resume and colony counting performed as above. The same procedure was carried out daily during the time-frame of the experiment [30]. A similar protocol was followed to evaluate the spontaneous loss of colistin resistance, except that the resistant strain was cultured alone, and the ratio of resistant *versus* susceptible colonies evaluated every other days using plates containing 8 and 32 μM of colistin, respectively.

2.3 Other functional assays

Outer membrane permeability was monitored by the inhibition of bacterial growth in the presence of increasing concentrations of Triton X-100 (0.01–0.1% v/v). To this end, bacteria were incubated with detergent at 4°C for 1 h, washed thoroughly with growth medium to remove unbound detergent, resuspended in new medium and allowed to proliferate for 24 h at 37°C. Then the plate was read at 650 nm. Affinity of LPS for colistin was measured using the displacement of dansyl-PXB previously bound to the corresponding LPS by PXB as described [31]. Differences in global metabolism were monitored with a GN2 Gram-negative identification panel microplate (Biolog, Hayward, CA), with bacteria allowed to grow on a single carbon source for each well [32]. Biofilm formation was assessed on polystyrene 96-well microplates (MicroTest 96, Becton Dickinson, Franklin Lanes, NJ) by staining as described [33].

2.4 Total protein extract

Cells resulting from the growth of 25 or 35 mL culture medium for sensitive or resistant clones, respectively, were harvested at exponential growth phase (OD_{600} = 1.5 or 1.0 for ATCC 19606 and its colistin resistant isolate, respectively) by centrifugation (20 min, 4°C, 4 500 × g), washed twice with (PBS, 10 mM Na_2HPO_4, 1 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl, pH 7.0), and once with doubly distilled water. Pellets were stored at −80°C until protein extraction. The protein lysate was obtained by mechanical disruption of the cells. Briefly, pellets were thawed in 300 μL of lysis buffer (10 mM SDS, 190 mM DTT, 80 mM Tris, 4 mM $MgCl_2$), 17 μg/mL protease-free RNAase (Sigma) and 7 μg/mL of protease-free DNase I (Roche Diagnostics, Sant Cugat del Vallès, Spain). Cells were lysed in a Hybaid Ribolyser (Hampshire, UK) using lysing matrix B tubes (Q-Biogene, Irvine, CA) as described [34], with minor modifications for *A. baumannii* cell lysis (1 single cycle, speed 6, 45 s, 4°C). Matrix was removed by centrifugation (13 000 × g, 20 min, 4°C), supernatants were cleaned with Ready-Prep 2-D cleanup kit (BioRad, Hercules, CA), following manufacturer instructions, then resuspended in rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG buffer pH 4.0–7.0 (GE Healthcare, Madrid, Spain), 16.2 mM DTT, 0.002% bromophenol blue). Protein concentration was measured by the bicinchoninic acid method using a commercial kit (Pierce, Rockford, IL).

2.5 Membrane-enriched protein fraction

The protocol of Molloy *et al.* [35] was followed. Briefly, cells were harvested as above, then disrupted at 12 000 psi in a French press (Thermo Fisher Scientific, Waltham, MA). EDTA potassium salt was added to the resulting lysate to a final 15 mM concentration. Unbroken cells were removed by centrifugation at 4500 × g for 20 min, 4°C, and the resulting pellet re-extracted with the same buffer and combined with the previous supernatant. The crude membrane fraction was obtained by ultracentrifugation (140 000 × g, 50 min, 4°C); contamination by soluble material removed by two consecutive washes with 10 mL of the same buffer. The pellet was next resuspended in 20 mL of 0.1 M Na_2CO_3, pH 11, and gently shaken for 1 h at 4°C, then ultracentrifuged as described above. The resulting pellet was resuspended in 2 mL of 50 mM Tris-HCl pH 7.3 containing 1% of the zwitterionic sulfobetaine detergent 3-[N,N-dimethyl(3-myristoylaminopropyl)ammonio]propanesulfonate (ASB-14, Sigma) and 2 mM tributylphosphine. The protein content was measured as above, prior to cleaning up the supernatant with the Ready Prep 2D kit (BioRad), according to manufacturer's directions.

2.6 Two-dimensional gel electrophoresis

For total lysate samples, 70 μg protein aliquots were solubilized in 300 μL of rehydration buffer. The protein was applied to a 17 cm, pH 4.0–7.0, IPG strip placed in a Protean IEF cell system (BioRad), rehydrated at 50 V overnight at 20°C, followed by three IEF steps: 300 V for 45 min, gradient to 3500 V for 22 h 45 min, 5000 V for 30 min; (max mA/gel = 99). The strips were equilibrated in 2 mL of equilibration buffer (0.12 M Tris-acetate, 6 M urea, 30% glycerol, 5% SDS, pH 7.0) containing first 52 mM DTT and then 135 mM iodoacetamide, for 15 min each. Polyacrylamide (12%) gels were cast according to manufacturer's protocol in a Protean II XI cell (BioRad); strips were placed on top of a 12% polyacrylamide gel and standard continuous SDS–PAGE was performed at 70 mA/gel in running buffer (25 mM Tris-HCl, 192 mM glycine; 0.1% SDS, pH 8.3). Gels were stained with silver MS-compatible staining solution [36]. A GS-800 calibrated densitometer (BioRad) was used for imaging. Protein spots were quantified using PDQuestTM 2D analysis 7.1.0 software (BioRad). For this quantification, 136 stained spots were matched in all gels and used for normalization of the average intensity.

For membrane-enriched fractions, 150 μg protein aliquots solubilized in 300 μL of rehydration buffer were used in duplicate runs. First and second dimension were as above, except that a nonlinear IPG from pH 3.0 to 10.0 was used.

2.7 2D-DIGE analysis

For protein labeling and gel analysis, manufacturer's protocols (GE Healthcare) were followed. Briefly, 50 μg of cytosolic extract and membrane-enriched fraction from either colistin-

resistant (R) and susceptible (S) cell lines were minimally labeled with 400 pmol Cy3 or Cy5 fluorescent dye dissolved in 99.8% dimethylformamide (Sigma) on ice for 30 min in the dark. The labeling reaction was quenched with 10 μL of 10 mM lysine (Sigma) and additionally incubated for 10 min. An internal pool was generated by combining equal amounts of R and S extracts. This pool was labeled with Cy2 fluorescent dye and was included in all gel runs. Following the labeling reaction, R and S extracts, together with a pool aliquot, were mixed according to the experimental design and separated by 2DE, with both IEF and SDS–PAGE steps performed as described above.

Proteins were visualized using a Typhoon 9400 fluorescence scanner (GE Healthcare) at appropriate wavelength for the Cy2, Cy3, and Cy5 dyes. Scans were acquired at 100 μm resolution. Total protein was detected by subsequent silver staining of the gels. Staining was stopped with 5% acetic acid, and protein patterns were digitized using a GS-800 calibrated densitometer (BioRad).

Image analysis was carried out with the DeCyder 5.01 software (GE Healthcare). The differential in-gel analysis module was used for pair-wise comparisons of each R and S extract to the mixed standards in each gel, and for calculation of normalized spot volume/protein abundance. Replicate gels were used to calculate average abundance differences and paired Student's *t*-test *p*-values for each protein across the four replica gels, using the DeCyder biological variation analysis module and the Cy3:Cy2 and Cy5:Cy2 ratios for each individual protein.

2.8 In-gel digestion and peptide extraction

Protein spots from silver-stained gels were manually excised and in-gel digested in a 96-well ZipPlate placed in a Multiscreen vacuum manifold (Millipore Ibérica, Madrid, Spain). Proteins were reduced, alkylated, and digested with sequence grade trypsin (Promega, Alcobendas, Spain). Peptides were eluted with 15–25 μL of 1.0% formic acid in 60% methanol.

2.9 MS analysis and protein identification

For MALDI-TOF MS, 2.5 μL of tryptic digest were deposited onto Mass·Spec·Turbo 192 type 1 peptide chips prespotted with CHCA (Qiagen, Barcelona, Spain) and left for 3 min for peptide adsorption. Then each spot was washed for 5 s with 1 μL of finishing solution (Qiagen) and left until dryness. MALDI-TOF MS was performed in a Voyager DE-STR instrument (Applied Biosystems, Foster City, CA) using a 337 nm nitrogen laser operated in the reflector mode, with an accelerating voltage of 20 kV. Samples were analyzed in the m/z 800–3000 range and were calibrated externally with the Sequazyme Peptide Mass Standards kit (Applied Biosystems). Peptides from trypsin autolysis were used for the internal calibration. Peak lists were generated with the MoverZ software (Knexus edition, Genomic Solutions, Ann Arbor, MI). Monoisotopic peaks with S/N >3 were selected and common contaminants were eliminated using the Peak Erazor software (v 2.0.1; Lighthouse data, Odense, Denmark).

Samples for nanoLC-MS were run on a Q-Star Pulsar (Applied Biosystems) instrument fitted with a nanoESI source, employing either direct nanoESI infusion or previous nanoLC separation in an Ultimate II system (LC Packings, Bannockburn, IL). In the direct injection mode, an MS scan was recorded and the more intense doubly and triply charged ions were fragmented. Prior to LC-MS analysis, tryptic peptide mixtures were desalted and concentrated on RP-C18 Stage tips (Proxeon Biosystems, Odense, Denmark) as described [37]. Peptides were separated in a RP Atlantis dC18 NanoEase Column, 75 μm × 150 mm (Waters, Milford, MA), using a linear 5–50% ACN gradient into 0.1% formic acid over 30 min. An electrospray voltage of 2400 V was used. For LC MS/MS analysis samples were directly injected and analyzed in the information-dependent acquisition (IDA) mode. Peak lists were generated from the raw data using the Analyst QS 1.1 software (Applied Biosystems), with the MASCOT script 1.6 b21 (MatrixScience, Boston, MA). MS/MS data was centroided and deisotoped, and only precursor charge states +2, +3, and +4 were considered.

2.10 Database searches

Protein identification from either MALDI-TOF or nanoLC-MS/MS results was done with the MASCOT search engine (Matrix Science), using a custom database of all *A. baumannii* genus proteins (15 949 sequences) available in the NCBI nonredundant database. For database searches, Cys carbamidomethylation and one missed cleavage, plus Met oxidation and deamidation were allowed as fixed and variable modifications, respectively. Mass tolerance was set at 50 ppm for MALDI-TOF-derived searches, whereas for LC-MS/MS-based searches tolerance was set at 0.15 Da for both MS and MS/MS data. In all cases, a significant MASCOT probability score (p>0.05) was used as condition for positive identification. Homology searches were done with BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Names of the identified proteins were chosen by means of the Proxeon software, v. 1.2.4 (Proxeon Bioinformatics A/S, (http://www.proxeon.com/home/index.html). For interpretation of proteomic differences in metabolism, the KEGG pathways (http://www.genome.ad.jp/kegg/pathway.html) were used.

3 Results

3.1 Differential expression analysis

With a view to obtaining a proteomic signature strictly correlated to colistin resistance (see Introduction), an 18-fold increase in resistance was induced in the colistin-sensitive strain by growing it under antibiotic pressure, as described

in Materials and Methods. DIGE was used to investigate differences in protein expression between susceptible and resistant strains. Prior to this, a total protein extract and a membrane-enriched protein fraction of the reference strain ATCC 19606 had been analyzed by 2-DE and MS, resulting in the identification of 119 and 28 proteins out of 145 and 62 gel spots investigated, respectively (Figs. S1 and S2, and Tables S1 and S2 of Supporting Information). In the DIGE experiments, both total lysate (Fig. 1A) and membrane-enriched (Fig. 1B) protein fractions were examined. In the total lysate, 42 proteins of interest [*i.e.*, showing confirmed ($p<0.05$) average ratios of expression variation above 3 or below −3 in 6 out of 12 gels] could be detected, of which 24 were identified by either MALDI-TOF PMF or LC-MS/MS (Table 1) using MASCOT search engines on an *A. baumannii*-specific sequence database. Similarly, in the membrane fraction, 22 differentially expressed proteins were detected, of which 11 were unambiguously identified by the above MS techniques (Table 1). Altogether, 35 *A. baumannii* proteins have been associated to the generation of a resistant phenotype in this study. A substantial subset of these proteins is primarily related to metabolic events such as regulation of nitrogen assimilation, tricarboxylic acid cycle, or biosynthesis of proteins. Other cellular functions seemingly affected by the induction of resistance, albeit to a lesser extent, are the chaperone system and the induction of pili formation, which is linked to biofilm formation [38]. Concerning the latter, and despite the enhanced expression of the Csu operon, preliminary experiments showed the resistant strain to be impaired relative to the parental one as regards biofilm formation in culture plates. Finally, one of the proteins differentially expressed in the membrane fraction (OsmY) can be specifically related to osmotic stress and has been directly correlated to the mechanism of action of colistin [39–41].

3.2 Characterization of the resistant phenotype

To further account for the multiplicity of factors that the proteomic analysis suggests as contributing to the *A. baumannii* colistin-resistant phenotype, whether produced by genetic differences or by adaptation to physiological levels, further characterization of (i) biological fitness, and (ii) differences in global metabolism were performed for this clone. First, in an in vitro competition experiment with the susceptible strain, the resistant trait was practically lost in just six days after removal of antibiotic pressure (Fig. S3 of Supporting Information). This could be due to (i) lower fitness of the resistant strain in the absence of colistin pressure, or (ii) colistin resistance being associated to an inherently unstable phenotype, easily reverted in the absence of antibiotic pressure, or (iii) a mixture of the previous two. To clarify this point, the resistant strain was cultured alone in the absence of antibiotic pressure and, at different time points, challenged with two different antibiotic concentrations (8 and 32 μM). A steady decay in resistance was observed (Fig. S3 of Supporting Information, inset), though not reaching zero activity, thus suggesting option (iii) above as the most feasible. Other indicators of the decreased fitness of the resistant strain when colistin is not present were a longer generation time (62 *vs.* 40 min) and an increased susceptibility to detergent lysis (LD_{50} 0.005% and >0.1% Triton X-100, respectively), a trait also found in colistin-resistant *Pseudomonas aeruginosa* [42].

Next, in order to explore changes in global metabolism, the ability of both strains to grow on different molecules as single carbon sources was examined by means of the GN2 microplate, which screens 95 different substrates as single carbon sources in a simultaneous assay. The most remarkable difference between susceptible and resistant strain was the significantly enhanced growth of the latter on citrate (Fig. S4 of Supporting Information).

4 Discussion

Genome mining in *Salmonella* has led to the conclusion that the number of potentially lethal targets for drug development is smaller than expected, due to the robustness of its metabolism [43], a fact which may be extrapolated to *A. baumannii*. Thus, rather than exploring new bactericidal targets [27, 44], it may be fitting to focus on adaptation mechanisms enabling the bacteria to strive under antibiotic challenge. In this regard proteomic techniques, so useful in obtaining a global view of physiological adaptation, can allow identification of bacterial proteins which, by preventing antibiotic resistance, might expand the conventional armamentarium [23, 45, 46].

In the specific case of colistin, currently the last-resort drug against *A. baumannii*, preliminary microbiological characterization of the resistant phenotype (see Results) suggests that two factors, *i.e.*, reduced fitness and intrinsic instability, might be responsible for the phase-out of the colistin-resistant strain in the absence of antibiotic pressure. In order to gain further insight on this phenomenon at the protein level, we have investigated differences in protein expression between susceptible and resistant strains. A slightly reductionistic approach has been adopted to avoid the limitations posed by clinical isolates (see Introduction) and thus better to define a specific colistin-resistant signature. In the following subsections, we relate differences in protein expression revealed by DIGE analysis with the biological traits of this resistant phenotype.

4.1 Membrane architecture and permeability

DIGE annotation of the resistant phenotype includes three OM porins, the channel-forming proteins that allow antibiotic influx into the periplasm. All three belong to the OmpA family, and only #885 (gi|40287452) has MW and p*I* consistent with theory (Table 1). The other two (#1085, gi|21666310; #1384, gi|109675218), we assume to be degradation products from a pre-existing species or, perhaps, from

Table 1. Differentially expressed proteins in colistin-resistant *A. baumannii*

Spot[a)]	Name	MW	p*I*	Function	NCBInr accession no.	No. of matched peptides[b)]	Coverage (%)	Score	Ri′
1085 (M)	Outer membrane protein HMP	37 793	5.2	Porin	gi|21666310	2	7	78	2.88
885 (M)	Outer membrane protein A	38 397	5.3	Porin	gi|40287452	5	14	146	−3.18
1384 (M)*	Outer membrane protein A	26 290	4.7	Porin	gi|109675218	5 (5)	19	84	−2.86
1556 (M)*	Putative outer membrane protein (CarO)	25 581	4.5	Porin	gi|72535027	5 (11)	25	85	−2.35
218 (M)	CsuD P pilus assembly protein, porin PapC	92 841	6	Fimbriae component	gi|126642259	4	5	153	3.06
1578 (M)	CsuA/B Uncharacterized secreted protein	18 807	5.2	Fimbriae component	gi|126642263	1	7	70	3.04
1436 (M)	Putative signal peptide (OsmY)	16 557	4.8	Hyperosmotic shock	gi|126386984	1	7	51	2.36
1033 (M)	30S ribosomal protein S2	27 507	9.0	Protein biosynthesis and folding	gi|49531348	3	8	113	2.59
1086 (M)	Leader peptidase	23 242	67	Metabolism	gi|126388440	4	20	112	3.45
295 (T)	Protein chain elongation factor EF-G	79 089	5.0	Protein biosynthesis and folding	gi|49530070	15	21	161	3.17
1402 (T)	Protein chain elongation factor EF-Tu	43 213	5.2	Protein biosynthesis and folding	gi|49529548	2	5	64	−3.37
1521 (T)	50S Ribosomal protein L15	15 472	10.9	Protein biosynthesis and folding	gi|126388961	4	33	128	−3.33
1263 (T)	30S Ribosomal protein S3	27 964	10.3	Protein biosynthesis and folding	gi|49532185	5	21	194	3.13
1272 (T)	30S Ribosomal protein S3	22 047	10.3	Protein biosynthesis and folding	gi|126388974	7	32	295	4.78
1272 (T)	Triosephosphate isomerase	26 508	4.7	Metabolism	gi|126386296	6	30	291	4.78
620 (T)	Chaperone Hsp60 (GroEL)	57 193	4.9	Protein biosynthesis and folding	gi|126642698	14	21	281	−9.41
650 (T)	Chaperone Hsp60 (GroEL)	57 193	4.9	Protein biosynthesis and folding	gi|126642699	7	11	77	−6.86
667 (T)	Chaperone Hsp70 (DnaK)	69 563	4.7	Protein biosynthesis and folding	gi|126642981	11	15	178	−3.13
765 (M)	Putative signal peptide (chaperone HsiJ)	31 301	5.9	Protein biosynthesis and folding	gi|126386961	3	16	52	−2.69
285 (T)	Isocitrate dehydrogenase	82 876	5.3	Metabolism	gi|126642513	10	13	114	3.21
286 (T)	Isocitrate dehydrogenase	82 876	5.3	Metabolism	gi|126642513	17	17	234	3.3
644 (T)	3-Hydroxyacyl-CoA dehydrogenase	49 207	5.7	Metabolism	gi|126387273	16	34	605	3.16
284 (T)	MaeB hypothetical protein	73 653	5.4	Metabolism	gi|126388262	9	14	326	3.16
285 (T)	MaeB hypothetical protein	82 788	5.3	Metabolism	gi|126388263	10	10	268	3.21
1636 (T)	Nitrogen assimilation regulatory protein P-II 2	10 529	5.1	Metabolism	gi|126386195	3	40	125	−8.32
1028 (T)	Glycyl-tRNA synthetase alpha chain	37 200	5.0	Protein biosynthesis and folding	gi|126643133	7	18	188	5.72

Table 1. Continued

Spot[a)]	Name	MW	p*I*	Function	NCBInr accession no.	No. of matched peptides[b)]	Coverage (%)	Score	Ri′
877 (T)	Serine hydroxymethyl-transferase	40 519	6.2	Metabolism	gi|126388232	4	12	140	−3.61
1355 (T)	Putative ubiquinone biosynthesis protein	18 059	6.0	Metabolism	gi|126388063	10	55	293	−3.32
1075 (T)	Putative iron-dependent peroxidase	30 322	4.7	Metabolism	gi|126387550	4	13	105	−4.37
1275 (T)	Thiamine biosynthesis protein	22 793	4.8	Metabolism	gi|126388190	4	20	190	7.47
1188 (T)	Molybdopterin biosynthesis protein	29 293	5.0	Metabolism	gi|126388075	2	11	56	−3.4
1538 (T)*	aac(6′)-Ik gene product	16 685	5.5	Metabolism	gi|847647	4 (8)	28	61	−4.83
1135 (T)*	Hypothetical protein A1S_1372	27 170	5.6	Unknown	gi|126641418	5 (13)	36	95	−4.94
1468 (T)	Hypothetical protein A1S_3155	18 057	6.1	Unknown	gi|126389054	7	37	238	−5.91
1385 (M)	Conserved hypothetical protein	19 383	4.9	Unknown	gi|49531733	1	5	62	−2.95

a) (M) and (T) refer to membrane-enriched fraction and total extract, respectively. Asterisk refers to MALDI-TOF identified proteins.
b) In parenthesis, number of unmatched peptides (MALDI-TOF-identified proteins).

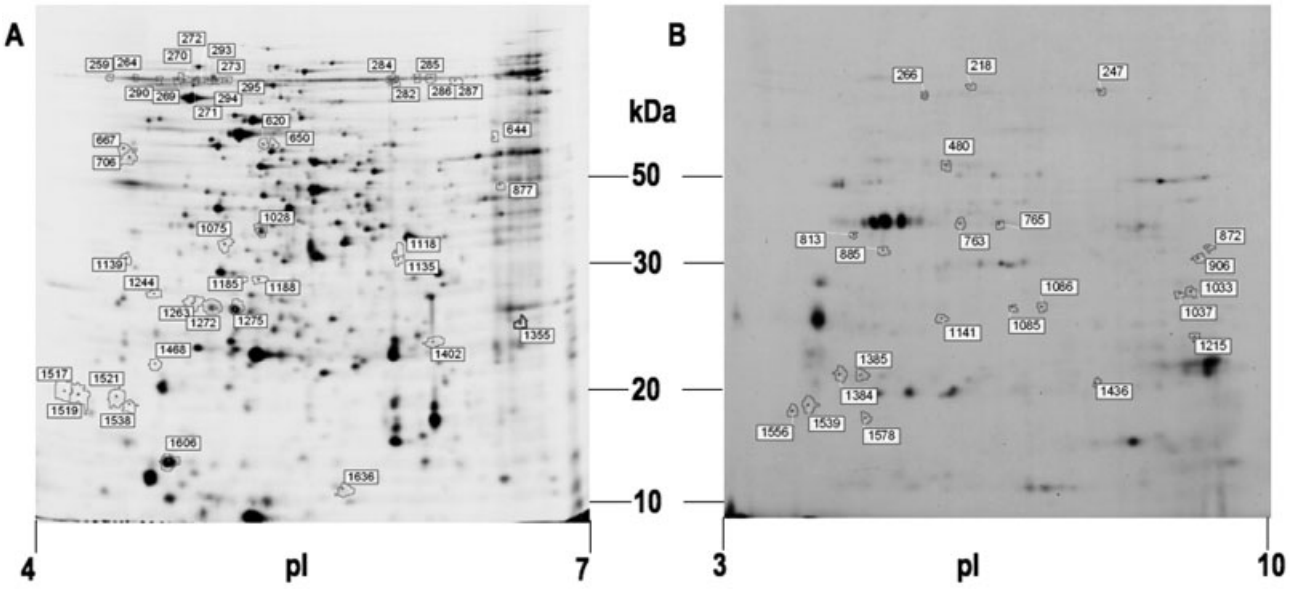

Figure 1. Differential gel electrophoresis of total protein extract (panel A) and membrane-enriched fraction (panel B) of *A. baumannii* ATCC 19606 and colistin-resistant derived strain. Numbered spots indicate proteins with differential expression (within 99% confidence level) between both strains.

a defective protein resulting from the impaired chaperone system of the resistant strain (see below). One of them is annotated as heat modifiable protein (spot #1085, gi|21666310 Table 1), the most abundant porin [21, 47]. Altered porin expression has been related to resistance against carbapenems (including imipenem) in *A. baumannii* [47–49]. Colistin, on the other hand, does not appear to require porins for passage through the OM; instead, it reaches the periplasmic space through self-promoted uptake, by causing rearrangement of the OM structure [41]. None-

theless, differential porin expression associated to colistin resistance has been described in *S. tiphymurium* [50] and in *A. baumannii*, where decreased levels of OmpW [46] were found. Also, increased OM permeability associated to colistin resistance has been described for *Pseudomonas* [42] and *E. coli* [51], with some controversy in the latter case [52].

Interestingly, spot #1556, gi|72535027, is annotated as CarO protein, reported to facilitate the entrance of imipenem and to cause resistance when deficient [53]. In the present case, however, when both strains were tested for imipenem susceptibility in a disk diffusion test, inhibition halos of similar size were observed (data not shown). A plausible explanation for the similar susceptibility is that an alternative pathway for imipenem influx exists in the resistant strain, enhancing OM permeability despite the CarO deficiency. Indeed, the lower LD_{50} of Triton X-100 needed to lyse the resistant strain is suggestive of a faulty packing of LPS in the membrane. This could in turn be due to a loss of negative charge caused by Mg^{2+} ion cross-linking of neighbouring LPS molecules, and/or to the lack of palmitoylation of lipid A [54] (see below). An increased OM permeability may also account for the higher susceptibility to antibiotics described recently for *A. baumannii* colistin–resistant strains [55].

Of comparable interest is the identification of protein CsuA/B (#1578, gi|126642263), involved in fimbriae formation, and its usher protein CsuD (#218, gi|126642259), encoded by the same operon [38] and thus probably linked to a concomitant up-regulation. Fimbriae formation, which improves bacterial ability to stick to surfaces and is therefore considered a virulence factor [26], is in tune with the ability of *Acinetobacter* to colonize a wide range of environments and capacity to form biofilms [33]. Nonetheless, the resistant strain showed an impaired biofilm formation ability compared to the wild type.

The mainstay of colistin resistance in *Enterobacteriaceae* [56, 57] consists in the remodeling of LPS, the major component of the OM external leaflet, in order to prevent colistin association with LPS and further translocation across the OM into the periplasmic space [58]. In a resistant strain, colistin's self-promoted uptake mechanism is impaired mainly by conjugation of additional palmitic acid units causing an increase in LPS hydrophobicity [54], and/or by esterification of phosphate groups in lipid A causing a decrease in negative charge. Since no proteins related to this LPS modification were directly identified, BLAST protein mining of the annotated *A. baumannii* ATCC 17978 genome [59] was performed, using the *P. aeruginosa* and *S. tiphymurium* enzymes involved in LPS modification as reference. No homologue for PagP (a palmitoyl transferase that incorporates an additional palmitic acid into lipid A) was found in *A. baumannii*, as somehow expected given the narrow, restricted distribution of this enzyme [54]. On the other hand, two hypothetical *A. baumannii* counterparts (gi|49530287, and gi|126388544) of ArnT (4-amino-4-deoxy-L-arabinose lipid A transferase, catalyzing esterification of 4-amino-4-deoxy-L-arabinose to the 4' phosphate group of the core of lipid A) [60] and two *A. baumannii* orthologs (gi|126388667 and gi|126642783) for EptA (a transferase that incorporates an ethanolamine residue into the 1' phosphate group of lipid A) were also found. The existence of enzymes decrease in the anionic character in LPS would be consistent with the resistant pattern induced in the *A. baumannii* strain. In addition, one cannot discard that other unknown modifications in *A. baumannii* LPS and/or their regulatory systems are involved in colistin resistance at levels below the DIGE detection limits. In this regard, it is worth mentioning a study on polymyxin resistance in *Salmonella tiphymurium* [50] where none of the enzymes directly involved in LPS modification could be identified in 2-D gels. Instead, PhoP, a component of the PhoP–PhoQ phosphorelay system that serves as master regulator for LPS modification in *Enterobacteriaceae* was identified, along with its partially subordinate PmrA–PmrB system (reviewed in [61]).

Perhaps the most revealing finding of the proteomic signature of colistin resistance was the up-regulation of #1436 (gi|126386984), again inadequately annotated as putative signal peptide but exhibiting a significant homology to OsmY, a small periplasmic protein [62] whose induction is associated to hyperosmotic shock and to polymyxin activity in *E. coli* [39–41, 63]. A marker of the stress which the continued presence of antibiotic exerts on the bacterium, the OsmY class of proteins is defined by its task of providing mechanisms that restore, even if partially, cellular homeostasis so that bacteria can survive under harsh conditions. Furthermore, OsmY, somewhat like polymyxin, was hypothesized to provide connections between the periplasmic layers of the IM and OM [41, 63]. If so, OsmY over-expression may compete with colistin (polymyxin E), thereby preventing prolonged stasis and eventual death. Furthermore, while PMXs have been demonstrated to promote phospholipid exchange between these two monolayers [3, 64] and consequent loss of membrane asymmetry, a similar process has not, to the best of our knowledge, been yet described for OsmY. Interestingly, OsmY expression in *E. coli* is intimately associated to RpoS [65, 66], an alternative sigma factor for RNA polymerase and a master regulator of resistance to stress and survival in stasis [67], which in *S. tiphymurium* is up-regulated in the presence of colistin [49] and other types of stress. Nevertheless, this sigma factor is absent in *Acinetobacter* [68], making comparisons between *Acinetobacter* and other *Enterobacteriaceae* more risky.

4.2 Protein biosynthesis and folding

The DIGE results also reveal variations in the expression levels of proteins involved in protein biosynthesis. Thus, in the membrane-enriched fraction an up-regulation of ribosomal S2 protein (#1033, gi|49531348) is evidenced for colistin-resistant strains. The increased expression, which persisted despite repeated washes of the membrane fraction to eliminate cytoplasmic contamination, might in turn be plausibly related to up-regulation of the leader peptidase (#1086,

gi|126388440), a component of the ribosome-nascent protein signal recognition particle (SRP) involved in correct protein secretion. Interestingly, in *E. coli* it was demonstrated that this ribosomal S2 protein belonging to the 30S ribosomal subunit, interacts with the N-terminal chain of the nascent OmpA [69], and in cross-linking experiments it was shown to interact with the 4.5 S RNA of the SRP, alongside other ribosomal proteins of this subunit [70]. As both this SRP component and ribosomal S2 proteins were in this case up-regulated, the finding may be interpreted as an attempt by the cell to ensure as much as possible the proper translocation of periplasmic molecules across the inner membrane. This would prevent or minimize the effect of defective, energetically wasteful metabolic cycles resulting from down-regulated chaperone systems (see below) which cause protein misfolding and thus overall deterioration of protein biosynthesis. Similarly, in *Listeria monocytogenes* over-expression of signal peptidase is associated to mammalian cell invasion, which ensures correct processing of virulence factors [71].

Colistin-resistant *A. baumannii* is also characterized by longer generation times. Thus, after continuous growth for months in the presence of colistin, the generation time of the resistant strain rose to 62 min, *vs.* 40 min for the wild type. Bacterial growth is directly linked to protein synthesis, and this in turn to the amount of active ribosomes [72]. This was again revealed by DIGE analysis, where colistin resistance resulted in unbalanced levels of elongation factors EF-G (#295, gi|49530070) and EF-Tu (#1402, gi|49529548; up- and down-regulated, respectively), and of ribosomal proteins such as L15 (#1521, gi|126388961), a late-assembly component of the large ribosomal subunit, involved in the integration of 5S rRNA into the ribosome [72]. Although L15 does not play an essential role in protein biosynthesis [73]—as evidenced by the existence of *E. coli* strains lacking this protein—, it has been reported to increase generation time by a factor of two [74], in agreement with our data. Another over-expressed ribosomal protein, S3, was identified in two spots (#1263, gi|49532185 and #1272, gi|126388974), in the latter co-migrating with triosephosphate isomerase (gi|126386296). Since no PTMs have hitherto been described for S3, nor for the other two differentially expressed ribosomal proteins S2 and L15 [75], one might conjecture a minor trimming of S3 as the cause of its double appearance. As both S2 and S3 belong to the late-assembly step of the small ribosomal subunit−the presence of S2 being mandatory for subsequent integration of S3 into the ribosome [76]−, their increased expression may contribute to the fine-tuning of the final steps of protein translation.

A down-regulation of the major chaperones is another remarkable trait revealed by DIGE analysis, with significant decreases in Hsp60 (GroEl) (#620, gi|126642698), Hsp70 (DnaK) (#650; gi|126642699) and the hypothetical (see below) HslJ (#765, gi|126386961). Under-expression of these chaperones is bound to have more adverse effects on protein biosynthesis than the slight perturbations in ribosomal protein levels discussed above. Thus colistin, despite its lack of permeabilizing effect on the IM [77, 78], will nonetheless decrease the biological fitness of its *A. baumannii* target, since chaperone down-regulation will cause wasteful (misfolded, nonfunctional) protein biosynthesis and thus global deterioration of bacterial homeostasis. This takes place most likely in a nonspecific manner, in contrast to pyrrhocoricin, another antimicrobial peptide that kills *E. coli* by inhibiting the ATPase activity of DnaK and thus prevents chaperone-assisted protein folding [79].

Finally, the down-regulated spot #765, gi|126386961, annotated as signal peptide despite a molecular weight of 16 657, is shown by BLAST analysis to have homology to the *meta* domain associated to bacterial motility, as well as with the chaperone HslJ of other bacteria. True to its name, *Acinetobacter* is motionless, though twitching associated to the presence of fimbriae has been described in *A. calcoaceticus* [80]. In any event, with the currently available data no obvious task can be assigned to this under-expressed protein.

4.3 Metabolism

The DIGE results have also revealed a large number of metabolic enzymes with altered expression. To complement proteomic data, the metabolic signature of the strain was obtained by a GN2 microplate system, which tests the growth of a given bacteria on a wide variety of single carbon sources. The most significant differences between the colistin susceptible and resistant strain was the enhanced ability of the latter to grow on citrate as a single carbon source compared with the parental strain, with a more modest growth on this carbon source; (Fig. S4 of Supporting Information), whereas for other citric acid analogs the differences between both strains are considerably lower. This would suggest that it increasingly relies on the TCA cycle for its metabolic requirements, in agreement with (i) the existence of a functional citrate-proton symporter in the *Acinetobacter* genome (gi|126386938 and gi|126641054)[81], and (ii) the DIGE finding of up-regulated expression of isocitrate dehydrogenase (#285 and #286, gi|126642513) [82] in the resistant strain. Another enzyme whose up-regulated expression in the later strain might be significant in this same context is 3-hydroxyacyl-CoA dehydrogenase (#644, gi|126387273), involved in the production of redox equivalents by transformation of hydroxylated substrates into ketoacyl-CoA products with formation of NADH. This enzyme is highly homologous to 3-hydroxybutyryl-CoA dehydrogenase, which produces acetyl-CoA from the catabolism of either fatty acids or amino acids such as lysine or tryptophan. The acetyl-CoA thus generated would also feed the TCA cycle, or be otherwise employed in anabolic pathways.

The up-regulated spots #284 (gi|126388262) and #285 (gi|126388263) in the colistin resistant strain were annotated as hypothethical protein MaeB (malic enzyme), with high homology with other bacterial malic enzymes. MaeB catalyzes the oxidative decarboxylation of malate, linked to the reduction of adenine nucleotides. Its role in the present

context might be to divert part of the malate generated in the TCA cycle to pyruvate which in turn would feed the gluconeogenic pathway.

An enhanced functional TCA cycle may presumably increase the levels of oxoglutarate which, together with glutamine, are sensed by the cell in order to monitor nitrogen assimilation and thus adjust its uptake. The DIGE experiments show a strongly depressed expression of spot #1636 (gi|126386195), annotated as nitrogen assimilation regulatory system PII. This is made up of two small proteins, GlnK, and GlnB, which control the balanced uptake of nitrogen and carbon so as to avoid wasting of metabolic energy [83]. This regulation is mediated through the functionality of AmtB, a membrane protein acting as an ammonium pore, which becomes inactivated on GlnK binding, thereby preventing ammonium intake and its eventual incorporation into glutamine [84]. In the resistant strain, down-regulation of GlnK will thus favour glutamine synthesis, in tune with the presumably upgraded oxoglutarate levels expected from an accelerated TCA cycle.

The sizable over-expression in the colistin-resistant strain of the alpha subunit of glycyl-tRNA synthetase (#1028, gi|49532234), one of few tRNA-synthetases with two rather than a single polypeptide chain, is surprising given the lack of concomitant up-regulation of other tRNA synthetases. A straightforward explanation would be the need to maintain adequate levels of glycyl-tRNA for protein biosynthesis in the face of a glycine shortage. This could be caused by serine hydroxymethyltransferase (#877, gi|126388232), a key enzyme in glycine biosynthesis, typical of the stringent response [85], which is down-regulated in the resistant strain. Given the key role of glycine in the metabolism of one-carbon fragments, its shortage would affect, among others, the biosynthesis of purines and glutathione, with detrimental consequences on global metabolism.

Another DIGE finding of metabolic significance is the under-expression of gi|126388063 (spot #1355, gi|126388063), an enzyme involved in the biosynthesis of ubiquinone, a lipid-soluble component of membrane-bound electron-transport chains. As ubiquinone levels are directly related to the oxygen content of the medium [86], and given the aerobic character of *Acinetobacter*, a deficit in ubiquinone will severely hamper the metabolic capacity of the bacteria, and will result in increased production of harmful oxygenated metabolites [86]. This would be further compounded by the down-regulation of the peroxidase gi|126387550 (spot #1075), and consequent increase in peroxide species. The glycine shortage just discussed above, with its ensuing depletion of glutathione levels, would compromise the scavenging of such ROS and contribute to the decrease of metabolic fitness of the resistant strain. Antibiotics that boost the production of ROS have been described in other bacteria [87, 88].

The colistin-resistant strain also shows a strong up-regulation of the protein encoded by ThiG (#1275, gi|126388190), involved in thiamine biosynthesis. In its diphosphate form, thiamine is a cofactor in metabolic decarboxylations and, as triphosphate, works as a signal for optimal growth of *E. coli* under amino acid starvation [89].

Finally, the DIGE analysis also reveals a down-regulation of #1188 (gi|126388075), a protein involved in the biosynthesis of molybdopterin, a cofactor of oxido-reductases. In facultative anaerobes such as *E. coli*, molibdoenzymes help bacteria thrive under low oxygen environments [86]. For a strict aerobe as *Acinetobacter*, however, such function is unlikely. On the other hand, a nitrate reductase with a molybdopterin cofactor has been described in *A. calcoaceticus* [90], and both a nitrate (gi|126387933) and a nitrite reductase (gi|126387934), as well as a nitrate transporter (gi|1263879369) are annotated in the *A. baumannii* genome, suggesting that *Acinetobacter* is able to reduce nitrate to ammonium. Even if this process is slowed down by faulty molybdopterin synthesis, it might be compensated by the above-mentioned down-regulation of the GlnK protein, with ensuing AmtB-mediated increased ammonium uptake.

4.4 Colistin resistance and antibiotic susceptibility

An important observation in the literature covering the spread of colistin resistance in *A. baumannii* is the increased susceptibility to antibiotics described for other colistin-resistant strains [55]. In the present DIGE study, two modifications in the protein pattern may be linked to antibiotic susceptibility. One is the significant down-regulation of the product of the aac (6′)-Ik gene, an N-acetyltransferase involved in the inactivation by acetylation of position 6′ of aminoglycosides such as amikacin, kanamycin, netilmicin, and tobramycin, but not gentamycin [91]. Under-expression of aac (6′)-Ik has only mild functional impact on resistance, as no statistically significant difference in the MICs of either amikacin or gentamycin was found between the parental and resistant strains (data not shown). In fact, *A. baumannii* does not seem to depend exclusively on this gene for aminoglycoside resistance, but since it has a battery of other inactivating enzymes capable of aminoglycoside inactivation [92], even basal and parallel expression of such enzymes might compensate for the deficiency in aac (6′)-Ik. Another difference relatable to antibiotic susceptibility is the over-expression of CarO protein (#1556), involved in imipenem entrance through the OM [52]. However, as no increase in imipenem sensitivity was found for the resistant strain, the bearing of this change on antibiotic susceptibility remains to be ascertained.

Finally, for three DIGE spots no functional assignment could be found. For #1468 (gi|126389054) and #1385 (gi|49531733), high homology with other bacterial proteins was found, but none of them has yet been assigned a function. For #1135 (gi|126641418), the poor homology with hydroxymethylglutaryl CoA lyase precludes any reliable interpretation.

Taken together, all the hitherto described readjustments of the colistin-resistant strain clearly point towards a substantially handicapped phenotype, which either reverts to the sensitive one in the absence of antibiotic pressure or must face the consequences of its reduced fitness, *i.e.*, being easily outgrown by the sensitive wild-type strain in the absence of colistin. Similar examples of loss of fitness associated to antibiotic resistance have been reported in other bacteria [2, 93]. Fitness, in sum, entails a trade–off between survival under antibiotic pressure and a surplus of metabolic expenditure required for survival, as shown by the recent a multi-resistant *A. baumannii* strain has been shown to be less virulent in a *Dictyostelium* model of virulence [59].

5 Conclusions

Colistin remains the last resort against the serious clinical threat posed by multi-resistant *A. baumannii*, and no new, safe antibiotic is expected to be available for some years, as tigecycline resistance has been described [94, 95] and other alternative strategies are still at preliminary stages [16, 28, 96].

Assuming that the present DIGE results can be translated to clinical scenarios, some of the adaptation mechanisms unveiled in this work might be proposed as targets to combat colistin resistance [27]. This might include tRNA-synthethases, key steps in the TCA cycle, and/or chaperones. Inhibitors for these systems have been described for other bacteria [97–99]. Still, two main caveats must be recognized. First, the inherent reductionism of in vitro approaches *vis-à-vis* the complexity of colistin resistance in clinical settings (see Introduction) and the heterogeneity of colistin-resistant isolates [100], likely to entail a diversity of adaptation mechanisms. Second, difficulties in target validation, given the limited development of metabolomic and microarray techniques for *A. baumannii*, albeit the recent release of the *A. baumannii* genome for four isolates with different human pathogenicity [59, 68, 101, 102] must be counted as a positive factor in this direction.

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This article has been previously published online with preliminary pagination, which has now been corrected.

