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Molecular Analysis and Expression of *bap* Gene in Biofilm-Forming Multi-Drug-Resistant *Acinetobacter baumannii*

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Background: *Acinetobacter baumannii* is commonly resistant to nearly all antibiotics due to presence of antibiotic resistance genes and biofilm formation. In this study we determined the presence of certain antibiotic-resistance genes associated with biofilm production and the influence of low iron concentration on expression of the biofilm-associated protein gene (*bap*) in development of biofilm among multi-drug-resistant *A. baumannii* (MDRAB).
Methods: Sixty-five MDRAB isolates from clinical samples were collected. Molecular typing was carried out by random amplified polymorphism DNA polymerase chain reaction (RAPD-PCR). Biofilm formation was assayed by the microtiter method.
Results: The sequence of *bap* was determined and deposited in the GenBank database (accession no. KR080550.1). Expression of *bap* in the presence of low iron was analyzed by relative quantitative real time PCR (rqRT-PCR). Nearly half of the isolates belonged to RAPD-types A and B remaining were either small clusters or singleton. The results of biofilm formation revealed that 23 (35.4%), 18 (27.7%), 13 (20%), and 11 (16.9%) of the isolates had strong, moderate, weak, and no biofilm activities, respectively. *ompA* and *csuE* genes were detected in all, while *bap* and bla_{PER-1} were detected in 43 (66%) and 42 (64%) of the isolates that showed strong and moderate biofilm activities ($p \leq 0.05$), respectively. Analysis of *bap* expression by rqRT-PCR revealed five isolates with four-fold *bap* overexpression in the presence of low iron concentration (20 µM).
Conclusion: The results suggest that *bap* overexpression may influence biofilm formation in presence of low iron concentration.

Keywords: *Acinetobacter baumannii*, Biofilm, Biofilm-associated Protein (bap), Iron, rqRT-PCR

Introduction

Acinetobacter baumannii has gained considerable importance in the last decade due to its extensive antibiotic resistance and biofilm formation in hospitals worldwide (1, 2). Resistance of *A. baumannii* to different antibiotic classes is mainly mediated by biofilm formation; a specific antibiotic-resistance gene may not exist in this organism (3). Microbial biofilm is a community of one or more organisms attached to sessile substrates or live organs (4). Biofilm formation is thought to be an important pathogenic feature of *A. baumannii*, especially in cases of ventilator-associated pneumonias and catheter-related urinary tract infections, as it facilitates the survival of the bacterium in hostile environments (5).

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Common factors that influence biofilm formation are nutrient availability, the presence of outer membrane proteins and pili, and macromolecular secretions such as extracellular DNA (eDNA) (6). It has been shown that a blood stream isolate of *A. baumannii* produces a protein related to a staphylococcal biofilm-associated protein (Bap), which is also required for the development of biofilms on abiotic surfaces (7). Members of the Bap family are high-molecular weight proteins that present on bacterial cell surfaces (8).

The ability of *A. baumannii* to form biofilms is also largely dependent on pili, which mediate attachment and biofilm formation. Similarly, *csuE*, is a member of the usher-chaperone assembly system. The genes are cluster together in form of the *csu* operon, the products of which form a pilus-like bundle structure in this bacterium (9). This gene has proved to be an important factor of *A. baumannii* biofilm formation (10).

Among the outer membrane proteins identified in *A. baumannii*, OmpA (AbOmpA) is the most abundant surface protein (11). AbOmpA acts as a porin, is required for eukaryotic cell adhesion, and partially contributes to serum resistance and biofilm formation (12).

Biofilm formation in *A. baumannii* occurs in three stages; early development, matrix formation, and maturation (13). It has been shown that iron uptake contributes to biofilm formation. In *Pseudomonas aeruginosa*, intracellular iron levels are important in the first stage of biofilm formation (14). N-(3-oxododecanoyl)-L-homoserine lactone (AHL-12) activates defense-relevant functions of phagocytic cells, including enhancement of phagocytosis, increased expression of adhesion receptors, and induction of chemotaxis. This leads to the hypothesis that early recognition of developing biofilms might be the key to a successful host defense against biofilm infections (15). In *Stenotrophomonas maltophilia,* iron restriction induces biofilm formation; three-dimensional reconstructions obtained from confocal stacked images using the Image J program revealed that the amount of biofilm produced by *fur* mutant cells under iron-depleted conditions was greater than that of the wild type strain, and the addition of an iron chelator had no effect on biofilm production (16). Similar observations were previously reported for *Legionella pneumophila* and *Staphylococcus aureus* (17, 18).

Little information exists regarding the effect of iron concentration on *bap* expression in *Acinetobacter*; we reported previously that low iron plays an important role in biofilm formation by increasing the production of the autoinducer N-acyl homoserine lactone (AHL) (19). As the iron concentration decreased the amounts of siderophore and biofilm increased. Recently we showed that limited iron concentration results in overexpression of the *ade*ABC efflux pump and *lux*I and *lux*R quorum-sensing genes in clinical isolates of *A. baumannii* (20).

The aim of the present study was to evaluate the presence of certain antibiotic-resistance genes and influence of low iron concentration on *bap* expression and biofilm formation in multi-drug resistant *A. baumannii* (MDRAB).

Materials and Methods

Hospitals setting and bacterial isolation

From February to August 2013, 65 non-duplicate MDRABs were isolated from hospitalized patients in intensive care units (ICUs) of general hospitals A and B in Kerman, in southeast Iran. Hospital A has a pediatric, a neonatal, and an adult ICU. Hospital B has three ICUs with no age categories. Patients frequently transfer between the two hospitals. If multiple isolates were obtained from an individual, one was randomly selected for inclusion. The collected samples from both hospitals were inoculated on cysteine electrolyte-deficient (CLED) (Hi-Media, Mumbai, India) and Luria-Bertani (LB) agar (Merck, Darmstadt, Germany) medium and incubated overnight at 37 °C. Isolated colonies were then subjected to conventional diagnostic tests and identified to the species level by the API-20 NE (BioMérieux, Marcy-l'Etoile, France) system and the presence of $bla_{\text{OXA-51}}$ gene by PCR.

Antimicrobial susceptibility testing

Antimicrobial susceptibility of all the isolates was determined by the disk diffusion break point assay according to Clinical and Laboratory Standards Institute (CLSI) guidelines (21). All antibiotic disks were purchased from MAST Co. Ltd, UK and used as per manufacturer descriptions. The following

antibiotics disks were used in this study (concentration µg/ml): amikacin (AN: 30 µg), tetracycline (TE: 30 µg), gentamicin (GM: 10 µg), nalidixic acid (NA: 30 µg), levofloxacin (LE: 5 µg), cefotaxime (CTX: 30 µg), piperacillin (PIP: 100 µg), ciprofloxacin (CIP: 5 µg), amoxicillin + clavulanic acid (AMC: 30 µg), tobramycin (TOB: 10 µg), ceftazidime (CAZ: 30 µg), cefixime (CFM: 5 µg), rifampin (Rif: 30 µg), imipenem (IMP: 10 µg), and meropenem (MEM: 10 µg) with an inoculum of 104 CFU per spot. The minimum inhibitory concentrations (MICs) of ciprofloxacin, gentamicin, piperacillin/tazobactam, cefotaxime, ceftazidime, imipenem, and colistin were determined using the E-test (Mast Co. Ltd, UK). The results were interpreted according to the CLSI guidelines. A reference strain of Escherichia coli ATCC 25922 was used as a quality control.

Detection of bap, csuE, blaPER-1, and ompA by PCR

All isolates were initially inoculated in LB agar and incubated overnight at 37 °C. DNA was extracted from isolated colonies with a genomic DNA extraction kit (Thermo Scientific, Vilnius, Lithuania) according to the manufacturer's instructions, and used as the DNA template in all amplifications. The biofilm-formation-related genes *bap*, *csuE*, *ompA*, and *bla*$_{PER-1}$, were amplified by conventional PCR (10). A list of primers, their sequences, annealing temperatures, and sizes of the amplified products used in this study are shown in Table 1.

Table 1. List of primers, their sequences, annealing temperatures, and sizes of the amplified products used in this study.

Gene	Primer sequence (5'→3')	Annealing temperature (°C)	Product size (bp)	Reference
bap	F-ATGCCTGAGATACAAATTAT R-GTCAATCGTAAAGGTAACG	55	1449	(22)
ompA	F-GTTAAAGGCGACGTAGACG R-CCAGTGTTATCTGTGTGACC	56	578	(23)
***bla*$_{PER-1}$**	F- ATGAATGTCATTATAAAAGC R- AATTTGGGCTTAGGGCAGAA	56	925	(24)
csuE	F-CATCTTCTATTTCGGTCCC R- CGGTCTGAGCATTGGTAA	59	168	[a] This study
bap-rt	F-TAGACGCAATGGATAACG R- TTAGAACCGATAACGATACC	58	127	[a] This study

[a] Primers were designed by AlleleID software (v 7.5, Premier Biosoft International, Palo Alto, CA, USA).

bap sequencing

PCR products of *bap* were sent to Macrogen Inc., Seoul, Korea, for sequencing using an ABI prism 3730/3730x (Applied Biosystems, Foster City, CA, USA) DNA Analyzer. The 1449 bp *bap* amplicon was sequenced using the same primers used for the PCRs. The sequence was analyzed using the BLAST alignment search tool (http://www.ncbi.nlm.nih.gov/BLAST) and manually assembled using CLC main workbench software, version 5.5 (CLC Bio, Aarhus, Denmark). The *bap* sequence was deposited in GenBank under accession number KR080550.1.

Molecular typing by random amplified polymorphism DNA technique

The molecular typing was done by the random amplified polymorphism DNA (RAPD) technique using primers DAF4 (5'- CGGCAGCGCC-3') and M13 (5'-GAGGGTGGCGGTTCT-3') [25]. RAPD profiles were used to measure clonal relationships among *A. baumannii* isolates. Primers were purchased from Generay Biotech (Co., Ltd, Shanghai China). A 1-kb DNA ladder (Life Technologies, GIBCO BRL, Breda, Netherlands) as molecular size standards, a positive control consisting of *A. baumannii* ATCC 19606 DNA,

previously amplified using primer DAF4, and a negative control, which contained all the reaction components except template DNA, were included on the gels. Banding patterns were analyzed by the unweighted pair-group method with arithmetic averages (UPGMA) clustering using Gel Compare II software, version 4.0 (Applied Maths, Sint-Matens-latem, Belgium). Isolates with 96% or greater similarity were considered as identical, and a cut-off value of 80% similarity was used for clustering. The RAPD-PCR was repeated to confirm the results and 98.6% homology was obtained.

Biofilm formation measurement

Biofilm formation was quantified by the microtiter plate assay method as described previously (22). Briefly, *A. baumannii* strains were grown overnight in LB broth with 0.25% glucose (LBG) at 37 °C. The culture was diluted 1:50 in freshly-prepared LBG pre-warmed to 37 °C. Then, 200 ml of suspension was used to inoculate wells of sterile 96-well polystyrene microtiter plates, followed by incubation at 37 °C for 72 h. After three washes with phosphate-buffered saline (PBS), any remaining biofilm was stained with crystal violet 1 % (w/v) for 25 min and wells were washed again with PBS. The dye bound to the adherent cells was resolubilized with 200 ml of ethanol/acetone (80:20, V/V) and the optical density (OD) was quantified at 570 nm using an ELISA reader (Synergy4; BioTek). Each assay was performed in triplicate. The adherence capabilities of the test strains were classified into four categories; three standard deviations (SDs) above the mean OD of the negative control (broth only) was considered as the cut-off optical density (ODc). Isolates were classified as follows: if $OD \leq ODc$, the bacteria were non-adherent; if $ODc < OD \leq 2 \times ODc$, the bacteria were weakly adherent; if $2 \times ODc < OD \leq 4 \times ODc$, the bacteria were moderately adherent; if $4 \times ODc <$ OD, the bacteria were strongly adherent.

RNA extraction and relative quantitative real-time PCR of bap

RNA was extracted from isolated colonies with an AccuZol kit (Bioneer, Seoul, Korea) as described previously (20). Briefly, for amplification, 1 µl of each primer (Table 1), 25 µl of AccuPowerR® 2X Green Star Master Mix solution (Bioneer, Seoul, Korea), 1 µl of ROX dye, and 5 µl of cDNA template were added. Quantification of specific *bap* genes was performed using the real time PCR system (ABI Step One™, USA) with the following cycle profile: 1 cycle at 95 °C for 2 min followed by 35 cycles at 95 °C for 25 s, annealing at 55 °C for 5 min, and extension at 72 °C for 20 s. Real-time PCR performance was confirmed to be reproducible at the threshold cycles (Ct) < 35. To determine the sensitivity and specify of the primers, *A. baumannii* ATCC 19606 DNA was serially diluted from 100 ng to 1 fg and used as standards. In addition, one melting curve cycle was performed on the SYBR channel at gain 70 using a ramping rate of 0.5 ^{o}C/10 s for 65-95 ^{o}C. Melt (65-95 °C), hold 6 s on the 1st step, and hold 5 s on the next steps. The PCR amplification and one melting curve cycle were analyzed and optimized using Rotor Gene software (Qiagen) as described by the manufacturer. The experimental points were aligned in a straight line and correlation coefficients (R) ascertained at 0.91658 (R2 = 0.84), and the following calibration equation was obtained: y = 0.34908x + 29.66623. The slope of 0.34908 corresponded to an amplification efficiency of 99.8%. The Tm value of the *bap* products was 87 °C ± 00. Relative *bap* expression was calculated as a fold change (RQ) between the target genes and the matched reference 16SrRNA gene mRNA as follows: $RQ = 2^{-\Delta\Delta Ct}$, where Ct equals the difference between the Ct values for the analyzed gene and Ct for the 16SrRNA reference gene amplifications in 50 µM concentration as control and 20 µM tests. The primers used for real-time PCR are listed in Table 1**.**

Effect of iron on bap expression

Relative quantitative real-time PCR (rqRT-PCR) was performed to compare the effect of iron at concentrations of 20 µM as test and 50 µM as control on *bap* expression (20). Briefly, 1×10^6 CFU/mL of bacterial cells were inoculated into two separate 10 mL volumes of iron-limiting M9 medium with 20 and 50 µM $FeCl_3$ (Merck, Darmstadt, Germany), and incubated at 37 °C overnight with shaking at 200 rpm. The bacterial suspension was centrifuged at 10,000 rpm for 6 min at 4 ºC and RNA was extracted from the cell pellet. Pearson's $\chi 2$ or Fisher's exact tests were performed to compare the number of isolates that overexpressed *bap* gene.

Statistical analysis

Each value was expressed as a mean ± SD. SPSS software 17 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The prevalence of MDR and genes were compared using chi-squared or Fisher's exact tests. A *p*-value ≤ 0.05 was considered statistically significant for two-tailed tests. For descriptive statistics we calculated percentage, frequency, mean and median values, and 95% confidence intervals (95% CI).

Results

Demographic Analysis and Antimicrobial Susceptibility Testing

Sixty-five MDRABs were isolated from hospitalized patients in ICUs of two general hospitals (A and B) in Kerman, in southeast Iran. Twenty-eight (43.1%) of the isolates were recovered from sputum and bronchial alveolar lavage specimens of patients admitted with pulmonary and lower respiratory tract infections. Also, 18 (27.7%) of the isolates were collected from urine, 5 (7.7%) from blood, and 14 (21.5%) from patients who had undergone surgical procedures. Demographic information showed that 25 (38%) of the patients in the ICUs were ≤ 50 years of age, 12 (18.5%) had received either cephalosporins or aminoglycoside antibiotics, and 17 (26.2%) had underlying diseases such as diabetes or cancer. Forty-two patients (65%) were male and 23 (35%) were female.

The disk diffusion susceptibility testing revealed that all 65 (100%) of the isolates were resistant to piperacillin, cefixime, ciprofloxacin, levofloxacin, ceftazidime, and gentamicin, ticarcillin and 53 (81%) were resistant to imipenem. However, 56 (86.2%) of the isolates were susceptible to colistin (MIC_{90} = ≥ 4 µg/ml), according to E-test results (Table 2). All 65 isolates (100%) were defined as MDR based on their resistance to more than three antibiotic classes.

Table 2. Correlation of antibiotic resistance, biofilm intensity, and biofilm forming genes in clinical isolates of *A. baumannii*.

Biofilm intensity	Biofilm-associated genes					MIC90					
	blaPER-1	ompA	bap	csuE	blaPER-1 + bap	GEN	CIP	IPM	CAZ	TZP	CL
Strong (n=23)	18	23	21	23	17	256	256	>32	256	256	4
Moderate (n=18)	13	18	14	18	10	256	256	>32	256	256	1
Weak (n=13)	7	13	3	13	0	256	256	>32	256	256	1
Negative (n=11)	6	11	5	11	5	256	256	>32	256	256	1

Abbreviation: GEN, gentamicin; CIP, ciprofloxacin; CL, colistin; CAZ, ceftazidime; TZP, piperacillin/tazobactam; IPM, imipenem. MIC was determined by E-test.

Molecular typing by RAPD-PCR

To determine the genetic diversity of *A. baumannii* isolates and explore its putative relationship with biofilm formation and antibiotic resistance, all 65 isolates were analyzed by RAPD-PCR genotyping using the primers shown in Table 1. On the basis of RAPD profiles in agarose gels, the number of bands ranged from 10 to 19 and the bands ranged in size from 200–2000 bp (Fig. 1). Control samples for RAPD-types A and B were included in the gel to enable interpretation. Dendrogram analysis and clonal relationships of *A. baumannii* isolates revealed ten different RAPD types (Fig. 2).

Twenty-five isolates classified as RAPD-type A. The clonal lineages of RAPD-type A were 95% related and the band patterns indicated similar genetic backgrounds. This result indicates the possible spread of clones belonging to this cluster in the ICU of hospital A. Nine isolates classified as RAPD-type B and three isolates classified as RAPD-type C and D comprised most of the isolates from hospital B and were 62% similar to clones from RAPD-type A. Other isolates were either singleton (not related to any RAPD-type) or were small RAPD-type groups containing two identical clones.

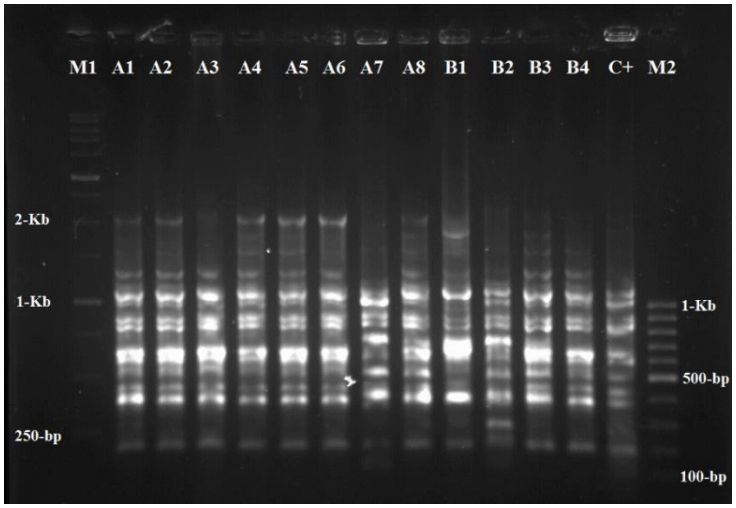

Fig. 1. Agarose gels following RAPD-PCR amplification with M13 and DAF4 primers. Fingerprint patterns obtained for *A. baumannii* isolates A1-A8 from hospital A and B1-B4 from hospital B. M1, 1-Kb DNA ladder. M2, 100-bp DNA ladder, C^+, positive control consisting of *A. baumannii* ATCC 19606.

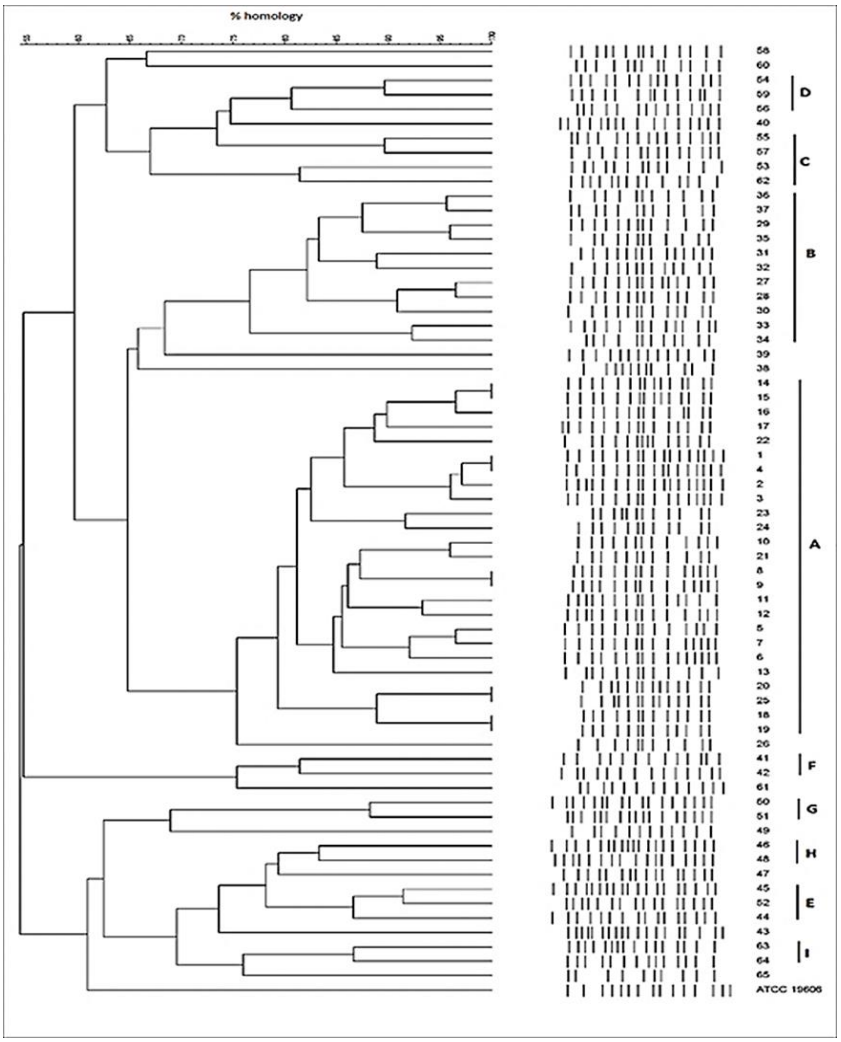

Fig. 2. Dendrogram of 65 *A. baumannii* isolates based on RAPD-PCR data. The fingerprints show genetic relationships among eleven clusters. Clustering was based on the unweighted pair group method with arithmetic mean. The vertical line is showing 80% similarity cut-off.

Biofilm formation assay and distribution of biofilm associated genes

Biofilm development revealed that 23 (35.4%), 18 (27.7%), 13 (20%), and 11 (16.9%) isolates had strong, moderate, weak, and non-adherent activity in the microplate assay, respectively (Fig. 3 and Table 2). We found that the measurements were consistent between the triplicate assays but varied considerably between isolates. PCR was performed to measure gene frequencies associated with biofilm formation, including *bap* (1449 bp), *csuE* (168 bp), bla_{PER-1} (925 bp), and *ompA* (578 bp). All 65 isolates encoded *ompA* and *csuE*, while 43 (66.2%) and 42 (64.6%) isolates encoded *bap* and bla_{PER-1}, respectively, and those isolates belonged to RAPD-type A (Table 2). *bap* and bla_{PER-1} were mainly detected in the isolates showing high biofilm activity. Correlations of RAPD-type, duration of stay, underlying diseases, presence of *bap*, *PER-1*, *csuE*, *ompA genes*, biofilm intensity, and minimum inhibitory concentrations (MICs) to colistin are shown in Table 3. Interestingly, *bap* was not detected in the isolates that did not produce biofilm. Amplification of *bap* from *A. baumannii* strains exhibiting strong biofilm activity was confirmed by DNA sequencing and the sequence was deposited in the GenBank database with the accession number KR080550.1.

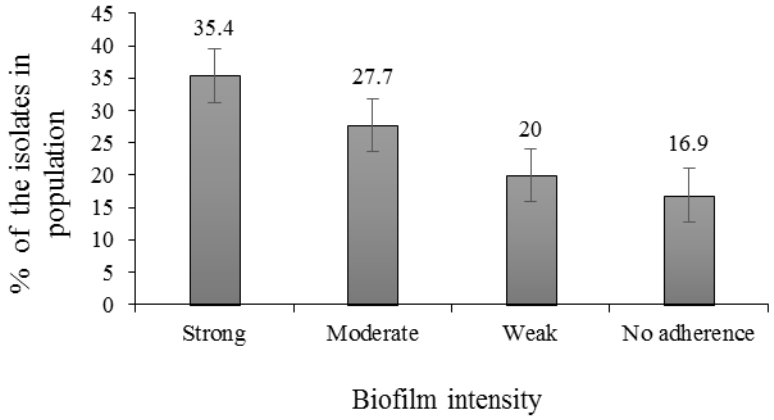

Fig. 3. Biofilm quantification by various groupings of the population of 65 clinical A. baumannii isolates. The above results are means of three experiments. SD = standard deviation. The biofilm intensity was determined by microplate assay as described in the text.

Table 3. RAPD analysis, duration of hospitalization, underlying diseases, and presence of *bap, PER-1, csuE, and ompA* genes, biofilm intensity, and MIC to colistin among *A. baumannii* isolates.

Isolate number	Hospital	Ward	Source	Underlying disease	Hospital stay (day)	RAPD-type	MIC of colistin (µg/ml)	*bap* expression (low Iron)	Biofilm intensity	Related genes			
										bap	PER-1	*csuE*	*ompA*
KR-AB2	A	PICU	T.A	Diabetes	<10	A	4	Twofold	Mod	+	+	+	+
KR-AB7	A	ICU	T.A	-	<10	A	6	Twofold	Mod	+	+	+	+
KR-AB12	A	ICU	T.A	Diabetes	<10	A	4	Twofold	Str	+	+	+	+
KR-AB14	A	ICU	T.A	Diabetes	22	A	6	Fourfold	Str	+	+	+	+
KR-AB15	A	NICU	U.C	UTI	11	A	4	Fourfold	Str	+	+	+	+
KR-AB20	A	ICU	T.A	Cancer	17	A	6	Twofold	Str	+	+	+	+
KR-AB27	A	ICU	U.C	UTI	12	A	4	Fourfold	Str	+	+	+	+
KR-AB28	A	ICU	T.A	Diabetes	<10	B	6	Fourfold	Str	+	+	+	+
KR-AB40	B	ICU1	T.A	COPD	<10	singleton	10	Twofold	Mod	+	+	+	+

Abbreviations: pediatric ICU, PICU; neonatal ICU, NICU; adults ICU, AICU; T.A, tracheal aspirates; UC, urine culture; COPD, chronic obstructive pulmonary disease; Mod, moderate; Str, strong.

Expression of bap gene in low iron

The results were normalized with 16SrRNA as a reference gene and the fold-change increase was measured by RQ = $2^{-\Delta\Delta Ct}$. Fig. 4 shows *bap* expression in the *A. baumannii* population grown in iron-limiting medium (M9 medium with 20 µM FeCl3). The result was compared with control strain *A. baumannii* ATCC 19606. In some *A. baumannii* isolates that exhibited strong biofilm formation, bap expression in 20 µM Fe was fourfold greater than in the same isolates grown in 50 µM Fe.

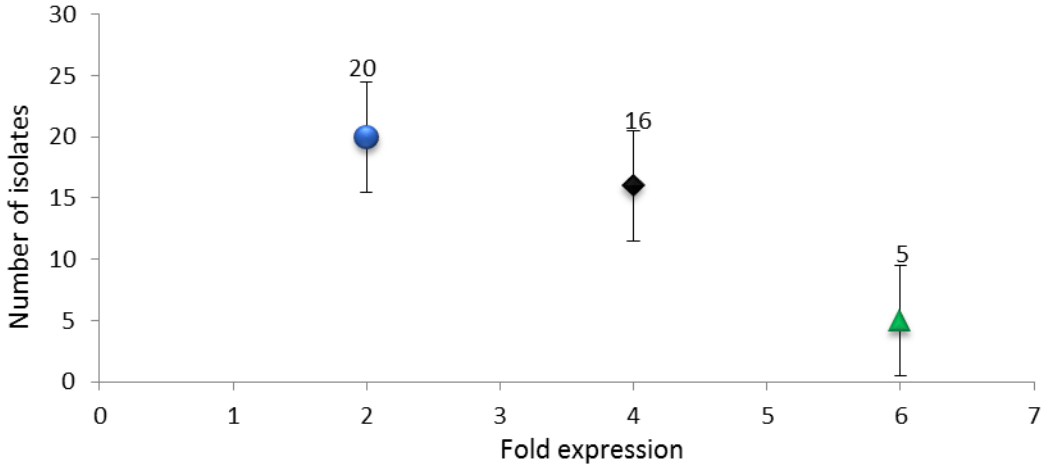

Fig. 4. RQ RT-PCR analysis of *bap* expression in 65 MDRAB isolates recovered from ICU patients in M9 medium containing 20 µM $FeCl_3$. Each analysis was performed three times.

Discussion

The ability of *A. baumannii* to persist in dry environments is a consequence of biofilm formation, and the presence of various antibacterial resistance genes makes this bacterium a successful pathogen among nosocomial bacteria (26). In *A. baumannii* the formation and maturation of biofilms depend on the complex interplay of environmental factors and cell-associated properties (27, 11). No information exists on the role of iron in *bap* expression and biofilm development in this bacterium; therefore, in this study we focused on the role of four biofilm-associated genes, *bap, csuE, bla*$_{PER-1}$, *and ompA*, and the effect of iron on bap expression and biofilm formation. Rodriguez-Baño *et al.*, (28) showed that biofilm-forming *A. baumannii* isolates were more susceptible to imipenem and ciprofloxacin than non-biofilm-forming counterparts, which suggests that the survival of these isolates in the hospital environment was less dependent on antibiotic resistance than on biofilm formation.

In the present study we found that *A. baumannii* isolates were resistant to drugs commonly used to treat *A. baumannii*. These drugs include cephalosporins, beta-lactam and beta-lactamase inhibitors, carbapenems, fluoroquinolones, aminoglycosides, tetracyclines, and rifampicin. We observed that the isolates that carried the *bla*$_{PER-1}$ extended-spectrum-resistant gene formed a significantly greater amount of biofilm than those that lacked *bla*$_{PER-1}$ ($p \leq 0.05$). Molecular analysis showed that 43 (66.2%) of the clinical isolates of *A. baumannii* in the current study encoded *bap* and formed strong biofilms ($p \leq 0.05$). Isolates that overexpressed *bap* and formed strong biofilms belonged mostly to RAPD-type A. Our PCR result was confirmed by sequencing *bap* gene. In the high-quality draft genomes AFDB, AFDK, AYOH, JFEL, and JFXM, *bap* coding sequences resided in one contig, but were interrupted by stop codons and split into two or more open reading frames (ORFs), as in many wholly sequenced genomes. Lee *et al.*, (10) and Badmasti *et al.*, (22) suggested that biofilm formation in *A. baumannii* was related to PER-1 production. A possible explanation for this significant characteristic of *A. baumannii* could be

that bla_{PER-1} may increase the adhesion of cells that carry this gene without necessarily contributing to biofilm formation as previously reported (29). It has been shown that micronutrients, including iron, may change the properties of *A. baumannii* cells, such as whole-cell hydrophobicity and altered bacterial cell-cell signaling, which can retard biofilm formation. This may explain why, for example, we observed some *bap* isolates with moderate biofilm activity.

To determine whether *bap* expression is actually regulated by the iron concentration, we performed rqRT-PCR of *bap* in low iron. Indeed, in 20 µM iron, biofilm formation in strains with *bap* was significantly greater than in strains without *bap* ($p \leq 0.05$). These data were supported by microscopic visualization of biofilms of three randomly-selected *A. baumannii* isolates that contained *bap* and exhibited strong biofilm formation, and suggest that the low iron concentration may play a role in early biofilm formation. Eijkelkamp *et al.*, (27) reported that in *A. baumannii*, under low iron conditions, transcription levels were more than 2-fold up-regulated for 463 genes, including 95 genes that were up-regulated more than 4-fold, some of which are critical to the virulence of relevant pathogenic strains, were up-regulated more than fourfold. Similarly, *A. baumannii* ATCC 19606 formed more biofilm on a plastic surface when cultured in a chemically-defined medium under iron-chelated conditions. In another investigation, the addition of 40 µM $FeCl_3$ to M9 minimal medium resulted in a greater number of attached cells than were seen in iron-free M9 (26).

We also found that nine (13.8%) of the *A. baumannii* isolates were resistant to colistin. Interestingly, most of the isolates were also resistant to imipenem. In nonfermenters, such as *A. baumannii*, resistance to colistin is rarely reported and demonstrates the gravity of emergence and the spread of new strains with pan-drug resistant (PDR) phenotypes. Such refractory isolates are problematic, because no other drugs are effective against this bacterium. Seven of the colistin-resistant isolates were placed in RAPD-type A and all were isolated from hospital A, which shows clonal distribution of this genotype.

From data presented in this study, we conclude that biofilm formation and quantity are influenced by bla_{PER1} and *bap* genes. The data also revealed that iron limitation affected *bap* expression in early stages of biofilm formation in certain isolates of *A. baumannii*.

Acknowledgement

The authors declare that they have no conflicts of interest.

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References

1. Doughari HJ, Ndakidemi PA, Human IS, Benade S. The ecology, biology and pathogenesis of *Acinetobacter* spp.: an overview. Microbes Environ 2011; 26: 101-12.

2. Shahcheraghi F, Abbasalipour M, Feizabadi MM, Ebrahimipour GH, Akbari N. Isolation and genetic characterization of metallo-β-lactamase and carbapenamase producing strains of *Acinetobacter baumannii* from patients at Tehran hospitals. Iran J Microbiol 2011; 3: 68-74.

3. Lin MF, Lan CY. Antimicrobial resistance in *Acinetobacter baumannii:* From bench to bedside. World J Clin Cases 2014; 2: 787-814.

4. McConoughey SJ, Howlin R, Granger JF, Manring MM, Calhoun JH, Shirtliff M, et al. Biofilms in periprosthetic orthopedic infections. Future Microbiol 2014; 9: 987-1007.

5. Mietto C, Pinciroli R, Patel N, Berra L. Ventilator associated pneumonia: evolving definitions and preventive strategies. Respir Care 2013; 58: 990-1007.

6. Sahu PK, Iyer PS, Oak AM, Pardesi KR, Chopade BA. Characterization of eDNA from the clinical strain *Acinetobacter baumannii* AIIMS 7 and its role in biofilm formation. Scientific World Journal 2012; 2012:973436.
7. Loehfelm TW, Luke NR, Campagnari AA. Identification and characterization of an *Acinetobacter baumannii* biofilm-associated protein. J Bacteriol 2008; 190: 1036-44.
8. De Gregorio E, Del Franco M, Martinucci M, Roscetto E, Zarrilli R, Di Nocera PP. Biofilm-associated proteins: news from *Acinetobacter*. BMC genomics 2015; 16: 933-937.
9. Tomaras AP, Flagler MJ, Dorsey CW, Gaddy JA, Actis LA. Characterization of a two-component regulatory system from *Acinetobacter baumannii* that controls biofilm formation and cellular morphology. Microbiology 2008; 154: 3398-409.
10. Lee HW, Koh Y, Kim J, Lee JC, Lee YC, Seol SY, et al. Capacity of multidrug-resistant clinical isolates of *Acinetobacter baumannii* to form biofilm and adhere to epithelial cell surfaces. Clin Microbiol Infec 2008; 14: 49-54.
11. Gaddy JA, Tomaras AP, Actis LA. The *Acinetobacter baumannii* 19606 OmpA protein plays a role in biofilm formation on abiotic surfaces and in the interaction of this pathogen with eukaryotic cells. Infect Immun 2009; 77: 3150-60.
12. Jyothisri K, Deepak V, Rajeswari MR. Purification and characterization of a major 40 kDa outer membrane protein of *Acinetobacter baumannii*. FEBS letters 1999; 443: 57-60.
13. Banin E, Vasil ML, Greenberg EP (2005) Iron and *Pseudomonas aeruginosa* biofilm formation. P Natl Acad Sci USA 102: 11076–11081).
14. Wang Y, Wilks JC, Danhorn T, Ramos I, Croal L, Newman DK. Phenazine-1-Carboxylic Acid Promotes Bacterial Biofilm Development via Ferrous Iron Acquisition. J Bacteriol 2011; 193: 3606-3617.
15. Wagner C, Zimmermann S, Brenner-Weiss GF, et al. The quorumsensing molecule N-3-oxododecanoyl homoserine lactone (3OC12-HSL) enhances the host defence by activating human polymorphonuclear neutrophils (PMN). Anal Bioanal Chem 2007;387:481–7.
16. García CA, Alcaraz ES, Franco MA, Passerini de Rossi BN. Iron is a signal for Stenotrophomonas maltophilia biofilm formation, oxidative stress response, OMPs expression, and virulence. Front Microbiol 2015; 6: 926-932.
17. Hindré T, Brüggemann H, Buchrieser C, Héchard Y. Transcriptional profiling of *Legionella pneumophila* biofilm cells and the influence of iron on biofilm formation. Microbiology 2008 Jan; 154(Pt 1):30-41.
18. Johnson M, Cockayne A, Williams PH, Morrissey JA. Iron-responsive regulation of biofilm formation in *Staphylococcus aureus* involves fur-dependent and fur-independent mechanisms. J Bacteriol 2005; 187:8211-5.
19. Modarresi F, Azizi O, Shakibaie MR, Motamedifar M, Mosadegh E, Mansouri S. Iron limitation enhances acyl homoserine lactone (AHL) production and biofilm formation in clinical isolates of *Acinetobacter baumannii*. Virulence 2015; 6: 152-61.
20. Modarresi F, Azizi O, Shakibaie MR, Motamedifar M, Valibeigi B, Mansouri S. Effect of iron on expression of efflux pump (adeABC) and quorum sensing (luxI, luxR) genes in clinical isolates of *Acinetobacter baumannii*. APMIS 2015; 123: 959-68
21. CLSI, Performance Standards for Antimicrobial Susceptibility Testing; Twenty fourth Informational Supplement (M100-S24). Clinical and Laboratory Standards Institute, Wayne, PA 2014.
22. Badmasti F, Siadat SD, Bouzari S, Ajdary S, Shahcheraghi F. Molecular detection of genes related to biofilm formation in multidrug-resistant *Acinetobacter baumannii* isolated from clinical settings. J Med Microbiol 2015; 64: 559-64.
23. Smani Y, Fabrega A, Roca I, Sanchez-Encinales V, Vila J, Pachon J. Role of OmpA in the multidrug resistance phenotype of *Acinetobacter baumannii*. Antimicrob Agents Chemother 2014; 58: 1806–1808.
24. Strateva T, Ouzounova-Raykova V, Markova B, Todorova A, Marteva-Proevska Y, Mitov I. Problematic clinical isolates of *Pseudomonas aeruginosa* from the university hospitals in Sofia, Bulgaria: current status of antimicrobial resistance and prevailing resistance mechanisms. J Med Microbiol 2007; 56: 956–963.
25. Wen H, Wang K, Liu Y, Tay M, Lauro FM, Huang H, et al., Population dynamics of an *Acinetobacter baumannii* clonal complex during colonization of patients. J Clin Microbiol 2014; 52; 3200–3208.
26. Farzan Modarresi, Omid Azizi, Mohammad Reza Shakibaie*, Mohammad Motamedifar,Shahla Mansouri. Cloning and expression of quorum

sensing N-Acyl-homoserine synthase (luxI) gene detected in *Acinetobacter baumannii*. Iran J Microbiol 2016; 8 (1): 1-7.
27. Eijkelkamp BA, Hassan KA, Paulsen IT, Brown MH. Investigation of the human pathogen *Acinetobacter baumannii* under iron limiting conditions. BMC genomics 2011; 12: 126.
28. Rodriguez-Baño J, Marti S, Soto S, Fernandez-Cuenca F, Cisneros JM, et al. Biofilm formation in *Acinetobacter baumannii*: associated features and clinical implications. Clin Microbiol Infect 2008; 14: 276–278.
29. Sechi LA, Karadenizli A, Deriu A, Zanetti S, Kolayli F, Balikci E, et al. PER-1 type beta-lactamase production in *Acinetobacter baumannii* is related to cell adhesion. Med Sci Monit 2004: 10: BR180-BR4.