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Serum Albumin and Ca^{2+} Are Natural Competence Inducers in the Human Pathogen *Acinetobacter baumannii*

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The increasing frequency of bacteria showing antimicrobial resistance (AMR) raises the menace of entering into a postantibiotic era. Horizontal gene transfer (HGT) is one of the prime reasons for AMR acquisition. *Acinetobacter baumannii* is a nosocomial pathogen with outstanding abilities to survive in the hospital environment and to acquire resistance determinants. Its capacity to incorporate exogenous DNA is a major source of AMR genes; however, few studies have addressed this subject. The transformation machinery as well as the factors that induce natural competence in *A. baumannii* are unknown. In this study, we demonstrate that naturally competent strain A118 increases its natural transformation frequency upon the addition of Ca^{2+} or albumin. We show that *comEA* and *pilQ* are involved in this process since their expression levels are increased upon the addition of these compounds. An unspecific protein, like casein, does not reproduce this effect, showing that albumin's effect is specific. Our work describes the first specific inducers of natural competence in *A. baumannii*. Overall, our results suggest that the main protein in blood enhances HGT in *A. baumannii*, contributing to the increase of AMR in this threatening human pathogen.

*A*cinetobacter baumannii has emerged as a severe nosocomial pathogen over the course of the last few decades, with high levels of morbidity and mortality associated with infections by this pathogen (1, 2). *A. baumannii* is considered to be a paradigm of multidrug resistance since it has developed resistance to almost all available antibiotics, leaving few or no treatment options left. The ability of *A. baumannii* to persist in the clinical setting even under desiccation and nutrient starvation, as well as its ability to accumulate several antibiotic resistance determinants, allowed its evolution as a successful pathogen in the hospital environment (3).

The large number of available *A. baumannii* genomes (*n* = 1,289) shows that foreign DNA is acquired at high frequencies (4–7).

The transformation process has been well described for some species, such as *Streptococcus pneumoniae*, *Vibrio cholerae*, *Neisseria meningitidis*, and *Helicobacter pylori* (8–12). However, how natural competence is regulated has not been thoroughly studied, particularly for Gram-negative bacteria. Some well-characterized competence inducers are DNA damage in *H. pylori* (13), starvation, as was suggested for *Haemophilus influenzae*, and chitin metabolism in *Vibrio cholerae* (14, 15). In most known examples, natural competence is a transitory state that is regulated by different internal and external signals. Often, these regulation networks are not completely understood. For *Acinetobacter* spp., most of the studies focusing on this issue were performed by using *Acinetobacter baylyi* strain ADP1 (16–20), a bacterium not as threatening to human health as *A. baumannii* (18–23). Data regarding *A. baumannii* and competence inducers are scarce (24–27). Wilharm et al. showed previously that several *A. baumannii* clinical isolates were able to acquire exogenous DNA depending on the expression of *pilT*, encoding an ATPase involved in type IV pilus retraction, and *comEC*, encoding the DNA uptake channel (26). Another study showed that the natural competence of *Acinetobacter nosocomialis* strain M2 relies on the presence of *pilA*, *pilD*, and *pilT* (28).

We recently demonstrated that *A. baumannii* strain A118,

which was recovered from a patient's blood sample and was shown to be susceptible to several antibiotics, has the capacity to acquire exogenous DNA (24, 25).

In the present study, we focused on identifying inducers that enhance or trigger natural competence in *A. baumannii*. Careful comparison of media led us to identify two specific signals, bovine serum albumin (BSA) and Ca^{2+}, as transformation inducers. These compounds increased the natural competence frequency while inducing *comEA* and *pilQ* expression. Albumin induction was protein specific, since human serum also exhibited an effect on natural transformation, and other proteins, such as casein, did not enhance the rate of DNA acquisition.

Our results show that albumin, the main blood protein, and Ca^{2+} are specific inducers of natural competence in *A. baumannii*, a mechanism that could be implicated in the increasing frequency of emergence of antimicrobial resistance (AMR) in this threatening pathogen.

MATERIALS AND METHODS

Strains, media, and plasmids. *Acinetobacter baumannii* A118, ATCC 17978, and A42 and *Escherichia coli* TOP10 cells harboring pDsRedAK were cultured on Luria-Bertani (LB) broth and LB agar at 37°C. When appropriate, antibiotics (12.5 μg/ml kanamycin and/or 6 μg/ml amika-

cin) were added to the bacterial cultures. Total genomic DNA (gDNA) from *A. baumannii* Ab144, Ab155, and A118 was obtained by using a Wizard genomic DNA purification kit according to the manufacturer's instructions (Promega, Madison, WI). Plasmid DNA (pDsRedAK) was extracted from *E. coli* TOP10 cells by using a Highway ADN Puriprep-P kit (Inbio Highway).

Natural transformation assays. Standard natural transformation assays were performed as previously described (24). Briefly, 50 μl of late-stationary-death-phase cultures of *A. baumannii* A118 was transferred to 50 μl of sterile LB medium with 100 ng of pDsRed plasmid DNA and/or gDNA. These cultures were incubated for 1 h at 37°C and then plated onto LB agar with 12.5 μg/ml kanamycin. Transformation events were scored by counting Kan^r colonies, while total CFU were assessed by plating serial dilutions onto LB agar (see Fig. S1 in the supplemental material). Transformation events were confirmed by PCR targeting *aac(6′)-Ib*, *aac(3)-IIa*, and *aph(3′)-Ia* as well as by measuring the level of resistance to aminoglycosides (MICs) and macroscopic observation of red-pigmented colonies. MICs were determined by the gradient diffusion method (Etest method) (29) with commercial strips (bioMérieux) according to procedures recommended by the supplier. An average of 15 colonies (transformants) were checked in every independent transformation experiment.

In order to test different conditions, assays were carried out as described above while altering each physical parameter one at a time. The temperature was switched to 25°C, 30°C, 35°C, 37°C, or 42°C. The pH varied from 2 to 14. The effect of osmolarity was tested by changing the concentration of sodium chloride. Different media were assayed, including brucella broth, lactose broth, complete transformation medium (CTM), minimal medium M9 (20% [wt/vol] α-D-glucose and 20% [wt/vol] sucrose), Terrific broth, and human serum. Individual effects of BSA (0.2%), human serum albumin (HSA) (0.2%), casein (0.2%), and CaCl$_2$ (1 mM) were also tested (Sigma-Aldrich). Finally, the concentration of transforming DNA was studied by using total genomic DNA of *A. baumannii* strains Ab155^{KanR} and Ab144^{KanR}. All experiments were performed in triplicate, and statistical analysis was performed. Transformation events were scored as mentioned above.

Kinetics of natural transformation. To determine the kinetics of natural transformation, 50 μl of the late-stationary-phase culture was transferred to 50 μl of sterile LB medium with 100 ng of pDsRed plasmid DNA. The transformation experiments were done at various time points (0 to 180 min) and scored by plating onto selective agar. Termination of transformation was done by adding 0.0017 U of DNase I (Promega, Madison, WI) to the mixture of transforming DNA and the bacterial inoculum.

Denaturation and digestion of BSA and casein. To denature BSA and casein, the proteins were exposed to heat treatment at 100°C for 1 min. BSA and casein digestions were done with trypsin (2 μg) as previously described (30).

RNA procedures and transcriptional analysis. Strain A118 was grown in LB broth, LB broth with 1 mM CaCl$_2$, LB broth with 0.2% BSA, LB broth with 1 mM CaCl$_2$ and 0.2% BSA, and CTM for 24 h at 37°C under agitation. Cell pellets were immediately lysed by using a solution containing 0.3 M sodium acetate (pH 4), 30 mM EDTA, and 3% SDS (wt/vol) and incubated for 3 min at 100°C. RNAs were extracted according to protocols described previously by Ramirez et al. (31). The integrity of the RNA samples was checked by agarose electrophoresis. RNA concentrations were determined by spectrophotometry. Reverse transcription was carried out by using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, WI) according to the manufacturer's instructions. Specific oligonucleotides were designed for retrotranscription coupled to quantitative PCR (RT-qPCR) with Primer3 (see Table S1 in the supplemental material). Samples containing no reverse transcriptase or template RNA were included as negative controls to ensure that RNA samples were free of DNA contamination. The 16S rRNA gene was used as an internal control for relative quantification. The RT-qPCRs were completed by using 5× Hot FIREPol EvaGreen qPCR Mix Plus (Solis Biodyne, Tartu, Estonia) with a two-step reaction protocol

consisting of 40 cycles of 94°C for 15 s and 52°C for 20 s, followed by a dissociation phase for quality control. The 20-μl qPCR mixtures contained 0.2 μM specific primers and 2 μl of cDNA (10 ng/μl).

The *comEA* and *pilQ* transcript levels of each sample were normalized to the 16S rRNA transcript levels for each cDNA sample. Each cDNA sample was run in triplicate, and experiments were repeated with at least three independent sets of samples. The relative quantification of gene expression was performed by using the $2^{-\Delta\Delta C_T}$ comparative threshold method. The ratios obtained after normalization were expressed as fold changes compared to values for cDNA samples isolated from bacterial cultures on LB medium.

Comparative analyses of BSA and HSA. The amino acid sequence comparison was performed by using BLAST (V2.0) and ClustalX. Moreover, the structure comparison between BSA and HSA was performed by using the jFATCAT-rigid (java flexible structure alignment by chaining aligned fragment pairs allowing twists) method using UCSF Chimera software.

Bioinformatic analyses of presumptive competence genes. Forty-two genome sequences of *A. baumannii* and 8 reference genomes of other *Acinetobacter* species (*Acinetobacter* sp. strain ADP1, *A. baylyi*, *A. calcoaceticus*, *A. haemolyticus*, *A. johnsonii*, *A. junii*, *A. lwoffii*, and *A. nosocomialis*) were used to generate an amino acid similarity matrix for the 16 presumptive natural competence genes (*comEA*, *pilQ*, *pilD*, *pilF*, *pilP*, *pilA*, *comEC*, *pilW*, *pilH*, *pilO*, *pilN*, *pilY1* [*comC*], *pilE*, *pilR*, *pilM*, and *pilT*) previously identified in the A118 strain (25). Comparison and analyses of these genes were performed by blastp and represented by using a heat map. The heat map was done by using R project software (version 3.0.2).

Statistical methods. Data were expressed as means ± standard deviations (SD). The Student *t* test was used to analyze differences between two groups, and one-way analysis of variance with Bonferroni correction was used to study differences between three or more groups. Statistical analyses were performed by using R project software (version 3.0.2).

RESULTS

Natural transformation is optimal at blood pH but is not affected by temperature or osmolarity. Abundant evidence from the literature points out that physical environment factors, such as temperature, pH, and osmolarity, impact natural competence in different bacterial species (11, 17, 32, 33). Therefore, we inquired about the effect of different physical conditions on the transformation frequency of the A118 strain. Natural transformation assays with A118 at 26°C, 30°C, 37°C, and 42°C showed no significant differences, indicating that temperature does not influence transformation in *A. baumannii* A118 (see Fig. S2A in the supplemental material).

Variations of the NaCl concentration (0, 0.25, 0.5, 0.75, 1.0, and 1.25%) showed no significant differences in transformation frequencies, suggesting that osmotic stress does not affect transformation (see Fig. S2B in the supplemental material).

We next investigated the effect of pH on natural competence by measuring bacterial survival and the natural transformation frequency of *A. baumannii* A118 at a range of pHs from 2 to 14. The bacterium survived at a pH range of 5.5 to 7.5, while natural transformation was optimal at pH 7.5 and occurred at a lower frequency at pH 5.5 (see Fig. S2C in the supplemental material). These results show that although growth can be sustained at a pH interval ranging from 5.5 to 7.5, the pH for optimal transformation frequency corresponds to the highest pH supporting bacterial culture. Interestingly, this value coincides with the pH found in human blood (pH 7.35 to 7.45).

Transformation in *A. baumannii* A118 occurs readily and is independent of the amount of DNA. For some species, such *A. calcoaceticus* and *S. pneumoniae*, it has been shown that transfor-
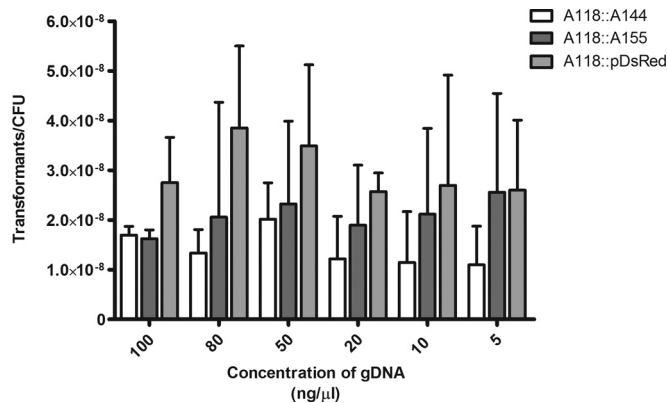
FIG 1 Natural transformation frequencies using different concentrations of genomic and plasmid DNAs. The transformation assay was performed with gDNAs of two *A. baumannii* strains (A144^{KanR} and A155^{KanR}) and plasmid pDsRed. The cultures were then plated onto selective media, and the number of CFU per plate was determined. Three independent experiments were performed. Data are presented as the means, and error bars represent the SD ($n = 3$).

FIG 2 Natural transformation frequency with LB broth, LB broth plus BSA, LB broth plus CaCl$_2$, LB broth plus BSA and CaCl$_2$, and CTM. Transformation assays were performed with LB broth with 0.2% (wt/vol) BSA, LB broth with 1 mM CaCl$_2$, LB broth with 0.2% (wt/vol) BSA and 1 mM CaCl$_2$, and CTM. The experimental control was transformed in LB broth. The cultures were then plated onto selective media, and the number of CFU per plate was determined. Data are presented as the means, and error bars represent the SD. Six independent experiments were performed. Asterisks indicate that the observed difference in transformability is statistically significant (*, $P < 0.05$ by the Student *t* test; $n = 6$).

mation follows saturation kinetics (17, 18, 34). For *E. coli*, Baur et al. obtained higher transformation frequencies with increasing amounts of DNA (35).

To address this point, transformation assays were performed by adding plasmid DNA (pDsRed) at various concentrations ranging from 5 ng/μl to 100 ng/μl (Fig. 1). Meanwhile, we also tested transformation using increasing quantities of gDNA in A118. For this purpose, we carried out experiments by adding gDNAs of two different *A. baumannii* clinical strains (A144^{KanR} and A155^{KanR}) that contain aminoglycoside resistance genes [*aac(6′)-Ib*, *aac(3)-IIa* (*aacC2*), and *aph(3′)-Ia*] within their genomes (GenBank accession numbers JQSF01000000 and JXSV01000000, respectively). The acquisition of A155 and A144 gDNAs was confirmed phenotypically by measuring the level of resistance to aminoglycosides and by PCRs using specific primers that amplified the *aac(3)-IIa* and/or *aph(3′)-Ia* gene.

No significant differences were detected in the transformation frequency independently of the concentration or the source of the DNA (Fig. 1). The same amount of DNA resulted in a similar transformation frequency when plasmid DNA or gDNA was used. However, in the latter case, the generation of resistant clones required a double-recombination event for the integration of the resistance marker into the A118 chromosome. This suggests that the entry of DNA into the bacterial cytoplasm is the limiting step, rather than further recombination steps.
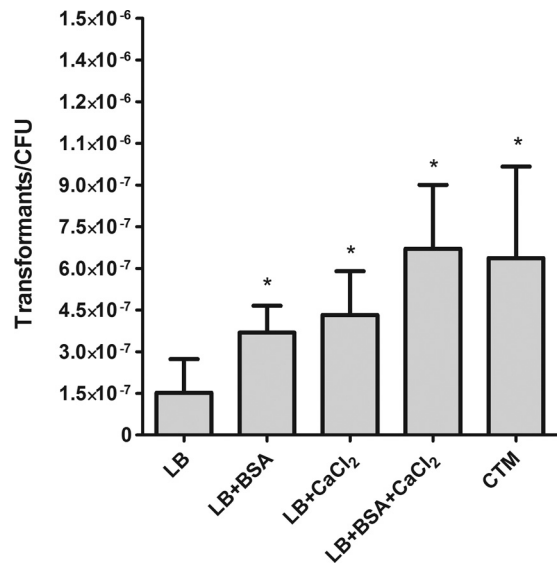
We next studied natural transformation kinetics by monitoring this process over time (see Fig. S3 in the supplemental material). Surprisingly, we already obtained transformant colonies after 30 s. The frequency of transformation was then constant over further time points (see Fig. S3 in the supplemental material).

Identification of Ca^{2+} and BSA as competence inducers for natural transformation through comparison of media. Data from previous studies support the notion that specific chemical compounds influence natural competence in various species (17, 35–39). A well-studied example is the case of chitin in the Gram-negative species *V. cholerae* (40, 41).

Information regarding the *Acinetobacter* genus is scarce, as *A. baylyi* ADP1 is the most studied strain (16–19). Most of the data obtained by using this strain support the hypothesis that an up-shift of nutrients could have a role in competence induction (18, 42). Moreover, in 1993, Palmen et al. proved that transformation in *A. calcoaceticus* BD413 (also know as *A. baylyi* ADP1) was Mg^{2+}, Mn^{2+}, and Ca^{2+} dependent, not observing a dependency on the growth medium and pH (18). Previously reported conclu-

TABLE 1 Natural transformation frequencies of *A. baumannii* strain A118 challenged with different culture media[a]

| Medium	Mean transformation frequency ± SD	Mean CFU/ml ± SD
LB	$4.81 \times 10^{-8} \pm 4.67 \times 10^{-8}$	$2.79 \times 10^{5} \pm 2.10 \times 10^{5}$
Brucella broth	$1.17 \times 10^{-8} \pm 4.55 \times 10^{-9}$	$1.93 \times 10^{6} \pm 1.40 \times 10^{6}$
Lactose broth	$2.82 \times 10^{-9} \pm 2.87 \times 10^{-9}$	$3.02 \times 10^{6} \pm 1.47 \times 10^{6}$
Terrific broth	$2.00 \times 10^{-8} \pm 5.61 \times 10^{-8}$	$3.00 \times 10^{6} \pm 1.03 \times 10^{6}$
M9 broth supplemented with 20% (wt/vol) α-D-glucose	$9.25 \times 10^{-8} \pm 9.47 \times 10^{-8}$	$1.33 \times 10^{5} \pm 1.20 \times 10^{5}$
M9 broth supplemented with 20% (wt/vol) sucrose	$4.94 \times 10^{-8} \pm 2,84 \times 10^{-8}$	$1.58 \times 10^{5} \pm 2.04 \times 10^{5}$
CTM	$4.67 \times 10^{-7} \pm 4.46 \times 10^{-7}$	$5.26 \times 10^{5} \pm 3.7 \times 10^{2}$

[a] $n = 3$ biological replicates.
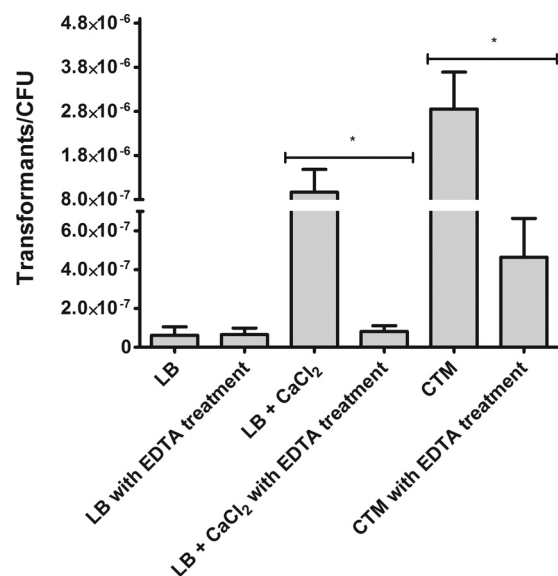
FIG 3 Transformability of *A. baumannii* cells under $CaCl_2$ induction. Transformation assays were carried out by using different media: LB broth, LB broth plus $CaCl_2$, and CTM. The cells were treated with EDTA to remove Ca^{2+}. The cultures were then plated onto selective media, and the number of CFU per plate was determined. Data are presented as the means, and error bars represent the SD. Three independent experiments were performed. Asterisks indicate that the observed difference in transformability is statistically significant (*, $P < 0.05$ by the Mann-Whitney test; $n = 3$).

sions and data proving natural competence for *A. baylyi* ADP1 have often been applied to the entire genus. However, putative key inducer chemical compounds remain to be identified.

In this study, we challenged the natural transformation of A118 with six different media: brucella broth, lactose broth, CTM, minimal medium M9 supplemented with 20% (wt/vol) α-D-glucose, minimal medium M9 supplemented with 20% (wt/vol) sucrose, and Terrific broth. Transformation assays using the above-mentioned media were performed in parallel with assays using LB medium. Among these media, CTM showed a significant increase in the transformation frequency compared to that with LB medium (Table 1). The differential components in CTM compared to LB medium are 1 mM $CaCl_2$ and 0.2% BSA (Fig. 2). Therefore, medium composition impacts the transformation frequency.

To gain insight into the role of $CaCl_2$ in this process, we performed natural transformation experiments by adding $CaCl_2$ or EDTA, a Ca^{2+}-chelating agent. On one hand, a significant $CaCl_2$ addition enhanced the transformation frequency (Fig. 3 and Table 2). Meanwhile, EDTA treatment reduced transformation lev-

els. These results demonstrate that Ca^{2+} ions are specifically involved in promoting competence in *A. baumannii* A118. Indeed, divalent cations seem to play a role in transformation in several bacterial systems (35, 36).

Since BSA is the other differential component in CTM, we first carried out transformation experiments using LB medium supplemented with 0.2% BSA, at the same concentration as that in CTM, to determine if this protein directly induces competence (Fig. 2).

We observed an ~2.5-fold increase in the frequency of transformation upon the addition of BSA. However, these levels are still below the ones observed with CTM (Fig. 4). Assays with the addition of $CaCl_2$ and BSA to the same levels as those in CTM showed a frequency of transformation equal to that observed with CTM (Fig. 2). These two inducers showed added effects rather than synergistic or antagonistic modes of action, suggesting that they may act through different mechanisms or at different stages of the transformation process.

To determine if the increase in the frequency of transformation observed with BSA is specific to this protein or rather due to the mere consequence of a higher peptide concentration, we carried out transformation assays by adding 0.2% (wt/vol) casein to LB medium. Notably, casein did not induce natural competence, showing that the effect of BSA is specific (Fig. 4A and Table 3).

To determine dosage dependence, we performed the experiment with a wide range of BSA concentrations (0.002 to 3.2%, wt/vol). The lowest concentration of BSA tested (0.002%) was already able to induce a significant increase in the transformation frequency, and a concentration of 0.2% was enough to reach maximal competence induction (see Fig. S4 in the supplemental material). Moreover, a posttest for linear trends showed a significant linear trend between the amount of BSA added and the transformation frequency, indicating dose dependence for the observed biological effect ($P < 0.0001$; slope, 0.1169; $R^2 = 0.4$).

Since albumin is a main component of serum, we performed transformation assays by supplementing LB medium with human serum and compared the effect of this mixture with the effect of BSA. Human serum triggered an increase in natural transformation (Fig. 4B). Moreover, the addition of HSA independently to LB medium also showed a significant 12.65-fold increase in the transformation frequency. This result, as well as results with BSA and serum, strongly supports our above-described hypothesis, showing the role of albumin as an inducer of competence.

We next inquired how BSA could induce this physiological process. To test if its structure was necessary, BSA was heat denatured. To determine if the whole molecule is necessary for com-

TABLE 2 Natural transformation frequencies of *A. baumannii* A118 in the presence or absence of $CaCl_2$ in LB medium and CTM with or without EDTA treatment[a]

Treatment	Mean transformation frequency $\pm$ SD	Mean CFU/ml $\pm$ SD
LB	$1.522 \times 10^{-7} \pm 4.952 \times 10^{-8}$	$1.025 \times 10^8 \pm 9.874 \times 10^7$
LB + EDTA	$6.560 \times 10^{-8} \pm 3.348 \times 10^{-8}$	$4.11 \times 10^5 \pm 2.08 \times 10^5$
LB + $CaCl_2$[b]	$5.620 \times 10^{-7} \pm 4.140 \times 10^{-7}$	$4.762 \times 10^6 \pm 4.471 \times 10^6$
LB + $CaCl_2$ + EDTA[b]	$2.146 \times 10^{-7} \pm 1.482 \times 10^{-7}$	$1.502 \times 10^7 \pm 1.354 \times 10^7$
CTM	$1.433 \times 10^{-6} \pm 7.375 \times 10^{-7}$	$1.010 \times 10^8 \pm 5.050 \times 10^7$
CTM + EDTA	$4.640 \times 10^{-7} \pm 1.999 \times 10^{-7}$	$1.861 \times 10^8 \pm 1.641 \times 10^8$

[a] $n = 5$ biological replicates.
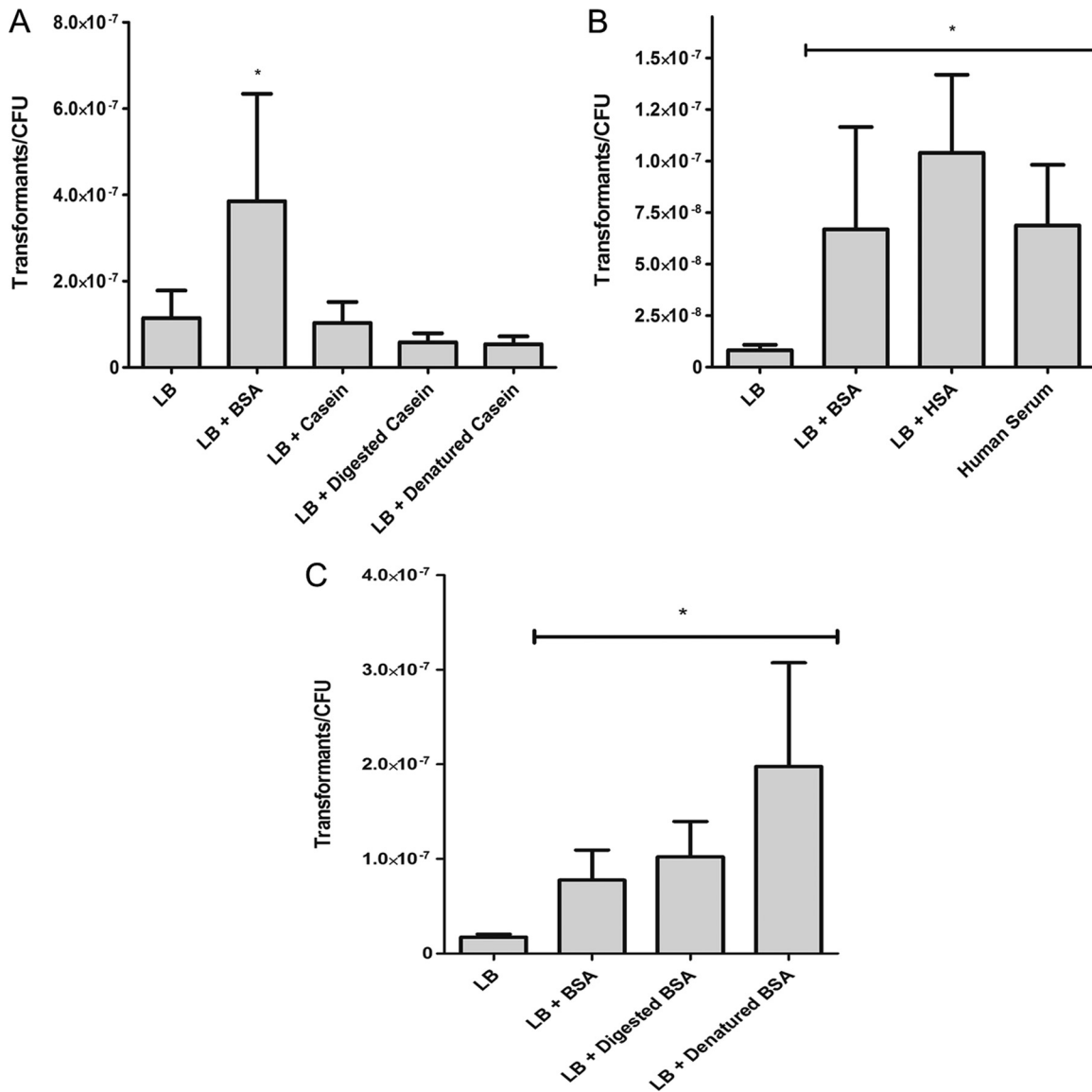[b] A total of 0.001 M $CaCl_2$ was used.

FIG 4 Transformability of *A. baumannii* A118 cells under different conditions. (A) Transformation assays with the addition of 0.2% (wt/vol) casein, heat-denatured 0.2% (wt/vol) casein, trypsin-digested 0.2% (wt/vol) casein, and 0.2% (wt/vol) BSA to LB medium (*, $P < 0.05$ by the Mann-Whitney test; $n = 3$). (B) Comparison of natural transformation frequencies with LB broth, LB broth and 0.2% (wt/vol) BSA, LB broth and HSA, and human serum (*, $P < 0.05$ by the Mann-Whitney test; $n = 3$). (C) Transformation assays with denatured and digested 0.2% (wt/vol) BSA added to LB broth. Simultaneously transformation assays using LB broth were performed to allow comparison. We compared transformation with BSA treatment to transformation without treatment in LB broth (*, $P < 0.05$ by the Mann-Whitney test; $n = 3$). For all assays, cultures were plated onto selective media, and the number of CFU per plate was determined. Data are presented as the means, and error bars represent the SD. Three independent experiments were performed in each case. Asterisks indicate that the observed difference in transformability is statistically significant.

petence induction, we digested BSA with trypsin. The samples were then used for transformation assays. Assays in which cultures are treated with BSA or trypsin-digested BSA showed similar increases in their frequencies of natural transformation. Interestingly, competence levels were even higher when denatured BSA was employed than when BSA, LB plus BSA, or LB plus trypsin-digested BSA were employed (Fig. 4C and Table 4). Our results indicate that neither BSA's native full-length structure nor its tertiary structure is needed to enhance competence. We hypothesized that some of the peptides obtained after trypsin digestion maintain the capacity to prompt the effect and that a masked

protein motif may serve as a specific enhancer of natural competence. Importantly, no variations in natural transformation were observed when denatured casein was used, showing that BSA possesses a specific motif capable of inducing natural competence (Fig. 4A). In the case of *Streptococcus thermophilus*, it was observed that competence is induced by casein-derived octapeptides (43). This was not observed with *A. baumannii* A118, suggesting a different regulatory mechanism and reinforcing the idea of the specificity exerted by albumins.

BSA and Ca^{2+} induce *comEA* and *pilQ* transcription in *A. baumannii* strain A118. The recent sequencing of the A118 ge-

TABLE 3 Natural transformation frequencies of *A. baumannii* A118 in the presence of casein, denatured and digested casein, and BSA in LB medium[a]

Treatment	Mean transformation frequency ± SD	Mean CFU/ml ± SD
LB	$1.146 \times 10^{-7} \pm 6.362 \times 10^{-8}$	$4.784 \times 10^{6} \pm 2.168 \times 10^{6}$
LB + BSA[b]	$3.849 \times 10^{-7} \pm 2.492 \times 10^{-7}$	$5.534 \times 10^{7} \pm 5.433 \times 10^{7}$
LB + casein[c]	$1.030 \times 10^{-7} \pm 4.854 \times 10^{-8}$	$1.737 \times 10^{7} \pm 4.105 \times 10^{6}$
LB + denatured casein	$1.818 \times 10^{-8} \pm 2.553 \times 10^{-8}$	$2.944 \times 10^{7} \pm 2.315 \times 10^{7}$
LB + digested casein	$3.800 \times 10^{-8} \pm 1.384 \times 10^{-8}$	$1.087 \times 10^{8} \pm 2.935 \times 10^{7}$

[a] $n = 3$ biological replicates.
[b] A total of 0.2% (wt/vol) BSA was used.
[c] A total of 0.2% (wt/vol) casein was used.

nome shows the conservation of genes that could be related to competence, such as *comEA*, which encodes a small DNA-binding periplasmic protein, and *pilQ*, which encodes the outer secretin protein found in type IV pili (11, 44–46). We investigated whether Ca^{2+} could induce the expression of such genes. Retrotranscription coupled to quantitative PCR (RT-qPCR) was performed in the presence or absence of 1 mM $CaCl_2$. We observed 2-fold increases in the expression levels of both *comEA* and *pilQ* in the presence of $CaCl_2$ (Fig. 5), suggesting that divalent cations, specifically Ca^{2+}, imposed an effect on the frequency of natural transformation of the A118 strain by inducing competence-related genes. We also observed that upon the addition of BSA, the levels of *comEA* and *pilQ* transcripts were increased by 1.4- and 2.5-fold, respectively (Fig. 5). Most prominently, the addition of BSA and $CaCl_2$ to the media increased the expression levels of the *comEA* and *pilQ* transcripts by 2.5- and 3.1-fold, respectively (Fig. 5).

BSA and Ca^{2+} induce competence among several *A. baumannii* strains. To rule out the possibility that BSA-dependent competence enhancement is limited to A118, two other *A. baumannii* strains (ATCC 17978 and A42) were tested. ATCC 17978 is one of the most studied *A. baumannii* strains (5, 47–52), and A42 is a multidrug-resistant clinical isolate exhibiting susceptibility to kanamycin (53). We performed transformation assays with and without BSA, and we observed an increase in the transformation frequency for both strains (Fig. 6A). Upon the addition of BSA and using plasmid DNA as the DNA source, we observed 23- and 1.5-fold increases for ATCC 17978 and A42, respectively (see Table S2 in the supplemental material).

We carried out the transformation assay by adding Ca^{2+}, and we also observed a significant increase in the frequency of transformation for both strains (Fig. 6B). Increases of 5.96- and 7.17-fold were observed for strains ATCC 17978 and A42, respectively (see Table S3 in the supplemental material).

These results strongly suggest that induction by BSA and Ca^{2+} is not limited to the A118 strain, thereby extending the relevance of our findings to other *A. baumannii* strains. Additionally, these findings suggest that transformation is a common trait of *A. baumannii*.

Moreover, for clinical strain A42, we observed a significantly higher transformation frequency when plasmid DNA was used as the DNA source. As is known for *A. baumannii*, transformation rates can vary depending on the isolates. These results suggest that this strain can acquire plasmid DNA more efficiently. Other plasmids and gDNA sources can be tested to see if the same results occur with other types of DNA.

Bioinformatic analysis of putative competence genes in *A. baumannii* genomes and other non-*A. baumannii* species. We recently identified the presence of 17 presumptive competence genes (*comM*, *comEA*, *pilQ*, *pilD*, *pilF*, *pilP*, *pilA*, *comEC*, *pilW*, *pilH*, *pilO*, *pilN*, *pilY1* [*comC*], *pilE*, *pilR*, *pilM*, and *pilT*) in strain A118 (25). To confirm whether this set of genes is conserved within *A. baumannii* species and to determine the extent to which natural competence is preserved among members of this genus, we decided to perform a comparative sequence analysis using 42 genomes of *A. baumannii* and 8 genomes of species within the *Acinetobacter* genus available in GenBank. Since *comM* was interrupted by an insertion of a resistance island (AbaR-type genomic island) in most of the *A. baumannii* genomes, this gene was excluded from the analysis. We found that 15 of the 16 presumptive competence gene-encoded proteins are highly conserved (>80% similarity) among *A. baumannii* isolates, whereas 12 of the 16 genes were present in all members of the *Acinetobacter* genus included in the present study (Fig. 7). Of the 16 genes, *pilA*, *pilY*, *pilE*, and *pilT* were not present in all *Acinetobacter* spp. *A. nosocomialis*, a species closely related to *A. baumannii* and an important pathogen in hospital-acquired infections, possessed all 16 genes, with a considerable degree of average similarity (86.47%). Our results, as well as those reported in the literature, support the notion that most *Acinetobacter* species strains are likely to be transformable under certain conditions and that they share common mechanisms of DNA uptake described for other transformable bacteria (7, 25).

TABLE 4 Natural transformation frequencies of *A. baumannii* A118 in the presence of native, denatured, and digested BSA[a]

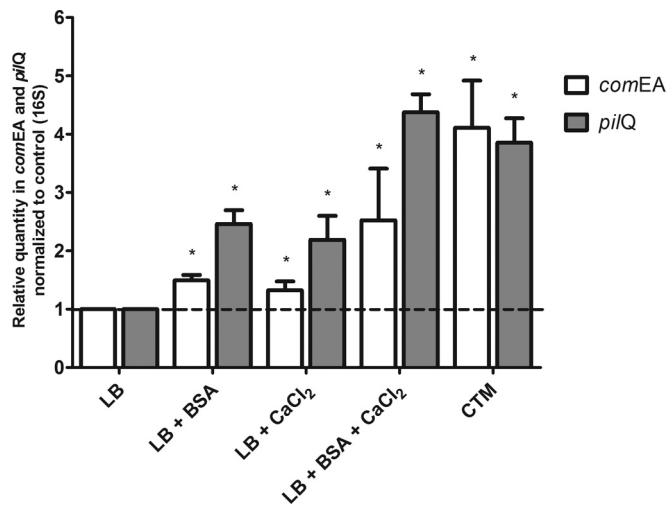| Treatment	Mean transformation frequency ± SD	Mean CFU/ml ± SD
LB	$2.607 \times 10^{-8} \pm 7.441 \times 10^{-9}$	$1.250 \times 10^{6} \pm 7.05 \times 10^{5}$
LB + BSA	$7.049 \times 10^{-8} \pm 3.807 \times 10^{-8}$	$2.881 \times 10^{6} \pm 2.560 \times 10^{6}$
LB + denatured BSA	$2.620 \times 10^{-7} \pm 1.540 \times 10^{-7}$	$1.544 \times 10^{6} \pm 1.168 \times 10^{6}$
LB + digested BSA	$6.332 \times 10^{-8} \pm 5.684 \times 10^{-8}$	$5.708 \times 10^{6} \pm 4.100 \times 10^{6}$

[a] $n = 3$ biological replicates.

FIG 5 BSA and Ca^{2+} induction of *comEA* and *pilQ* transcription in *A. baumannii* strain A118. Bacterial cells grown in LB, LB plus $CaCl_2$, LB plus BSA, LB plus $CaCl_2$ and BSA, and CTM were used. Determination of relative expression levels of *comEA* and *pilQ* was performed by using RT-qPCR. Fold changes were calculated from threshold cycle (C_T) values after normalization to 16S rRNA. Data are presented as the means and SD. (*, $P < 0.05$ compared with the absence of 1 mM $CaCl_2$ and 0.2% [wt/vol] BSA in medium, as determined by the Kruskal-Wallis test; $n = 4$).

DISCUSSION

Antibiotic-resistant bacteria, such as *A. baumannii*, pose a serious threat to human health, and the increase in the number of infections caused by such pathogens is a growing concern worldwide. The CDC reports 12,000 infections with multidrug-resistant *A. baumannii* annually (54). This pathogen is responsible for a wide variety of nosocomial infections, including pneumonia, blood infections, meningitis, and skin infections (1, 55). In some cases, the patient outcomes associated with extensively drug-resistant *A. baumannii* infections can be deadly (2).

Recent analysis of the *A. baumannii* genome suggests that

transformation plays a key role in the development of antibiotic resistance in *A. baumannii*, but the mechanisms by which it occurs and the factors or signals that play a role in transformation are poorly understood.

Here we identified environmental conditions and factors that could trigger and enhance transformation in this dangerous pathogen. Transformation could help *A. baumannii* acquire DNA and increase its potential to be a deadly nosocomial pathogen. We employed *A. baumannii* strain A118, which was previously shown to be naturally competent (24), for this purpose.

We observed that temperature and osmolarity did not exert any effect on the level of natural transformation of A118. In contrast, we observed that different alkaline or acidic conditions impacted transformation levels. A higher level of transformation was obtained at pH 7.5, a pH that corresponds to the pH found in human blood (pH 7.35 to 7.45). It is known that one of the most severe infections caused by this pathogen is septicemia (1, 56); therefore, this result shows that blood pH could be a signal that could have an impact on transformation during the course of *A. baumannii* infection.

Our results also showed that transformation is not dependent on the amount of DNA or on its source. As has been observed for other species, it is a prompt process that occurs rapidly during the first seconds of DNA-cell exposure, proceeding continuously thereafter over time.

The capacity to undergo natural competence has been studied for many bacterial species. However, how natural competence is triggered, enhanced, or blocked is less understood, particularly for Gram-negative bacteria. In some species, such as *H. influenzae*, starvation of required carbon sources acts as an inducer of competence (14, 15); in *Moraxella catarrhalis*, iron limitation affects natural competence (57); and in *V. cholerae*, chitin induces natural competence (11, 40). In other cases, replication arrest generated by stress can induce competence when competence genes are located near the replication origin (58). For *Acinetobacter* spp., using *A. baylyi* strain ADP1, it was observed that an upshift of nutrients has a role in competence induction (18, 42). However,
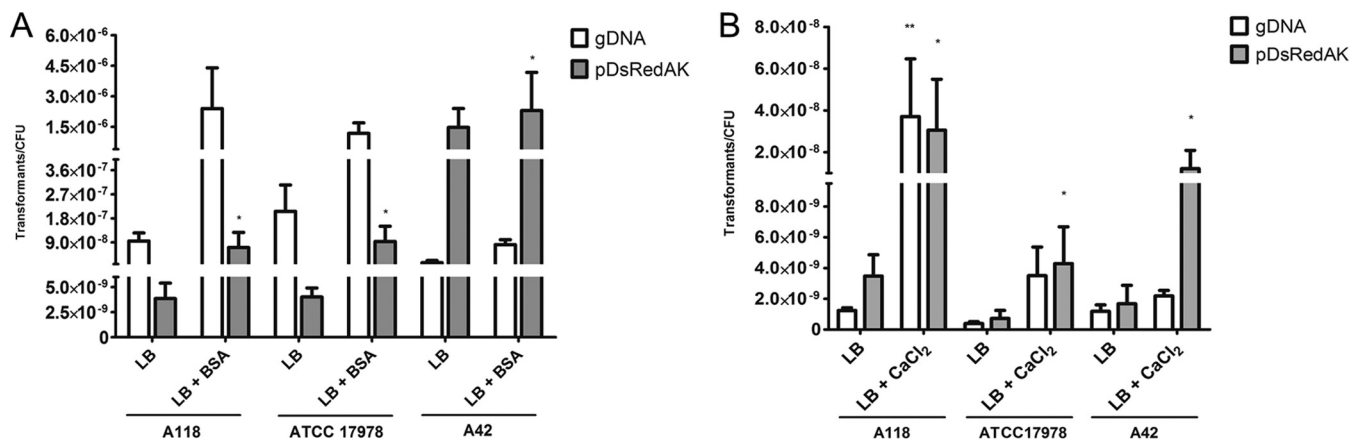

FIG 6 Transformability of *A. baumannii* A118, ATCC 17978, and A42. Transformation using gDNA (A144^{KanR}) and plasmid DNA (pDsRed) was performed under two different conditions. (A) LB medium with 0% BSA and LB medium with 0.2% (wt/vol) BSA. Cultures were plated onto selective media, and the number of CFU per plate was determined. Data are presented as the means, and error bars represent the SD. Four independent experiments were performed. Asterisks indicate significant differences in transformability (*, $P < 0.05$ by the Mann-Whitney test; $n = 4$). (B) LB medium and LB medium with 0.001 M $CaCl_2$. Cultures were plated onto selective media, and the number of CFU per plate was determined. Data are presented as the means, and error bars represent the SD. Five independent experiments were performed. Asterisks indicate significant differences in transformability (*, $P < 0.05$ by the Mann-Whitney test; **, $P < 0.01$ by the Mann-Whitney test; $n = 5$).
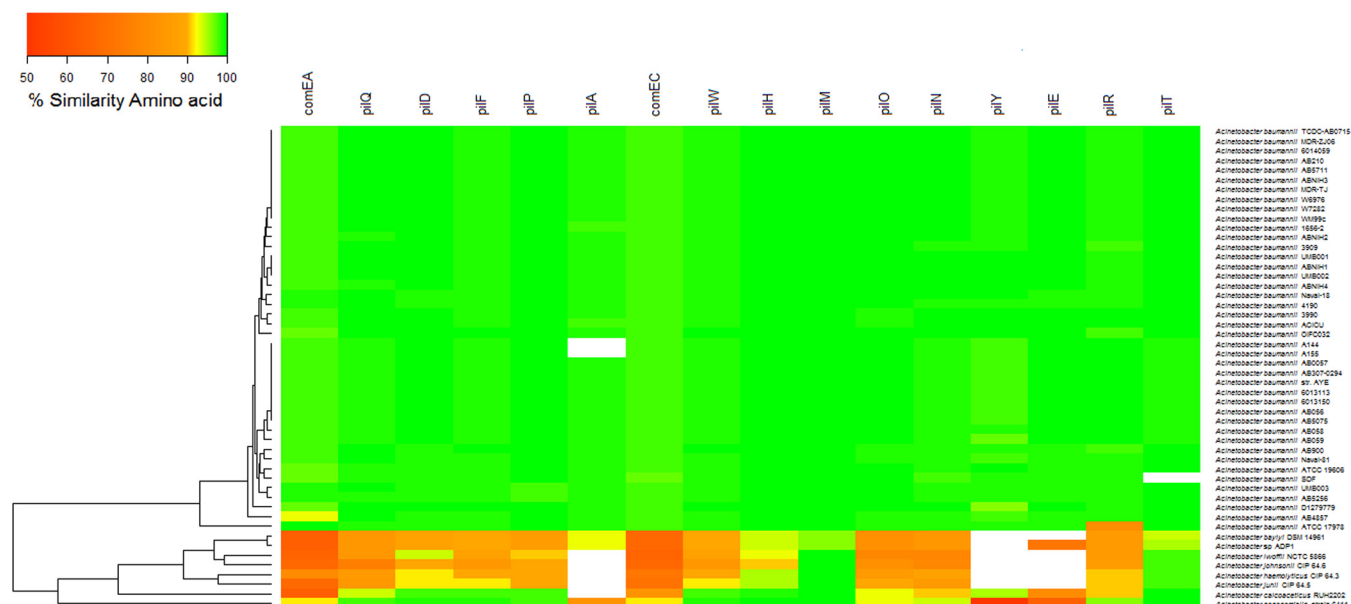
FIG 7 Comparative sequence analysis of known competence genes. The heat map representation shows similarity between 15 competence genes of 42 genomes of *Acinetobacter baumannii* and 8 other species of the genus against the *Acinetobacter baumannii* A118 genome. The values are represented from 50% (red) to 100% (green) amino acid similarities, as indicated by the color scale. Clustering was carried out by using the complete-linkage method together with Euclidean distance.

the effect of individual compounds was not clearly defined in the literature. Our results obtained by comparing different media revealed that two distinctive components, Ca^{2+} and BSA, produced an increase in the level of transformation observed for strain A118 as well as the other two *A. baumannii* strains tested. Each component induced transformation independently and presented added effects when included in LB medium. Importantly, the addition of BSA did not cause transformation induction simply by increasing the nutrient concentration or protein availability, since the addition of casein at the same concentration did not enhance the rate of transformation. Interestingly, such specificity is not provided by the tertiary structure of the protein but rather by a motif that is exposed when the protein is denatured. We are currently searching this motif and investigating if it is borne by other human proteins within blood or the extracellular matrix. On the other hand, a specific receptor might exist on the bacterium surface.

Another important element supporting the notion that BSA induces natural transformation is that it induces the transcription of *comEA* and *pilQ*, two competence genes. Meanwhile, these two genes are also overexpressed upon the addition of Ca^{2+}. Taken together, these results and the observed decrease in the transformation frequency upon EDTA treatment provide evidence that demonstrates that Ca^{2+} is actively involved in the induction of competence rather than just aiding in this process by mitigating electrostatic repulsion between the negative charges present in the DNA phosphate backbone and on the cell surface. In accordance with our results, Palmen et al. also showed that divalent ions, such as Mg^{2+}, Ca^{2+}, or Mn^{2+}, are required during the transformation process in *A. baylyi* strain BD413 (18).

Serum albumin is the main protein of mammalian blood plasma, and it is involved in the regulation of the colloidal osmotic pressure of blood as well as serving as a carrier for molecules that have low solubility in water. Calcium is among the elements carried by this protein (59). In particular, BSA (also known as "fraction V") is a serum albumin protein derived from cows, and its crystal structure showed three calcium ions bound to it (60). As shown by our results, the association of calcium with BSA could play a role in the observed effect on natural transformation, since added effects were observed when both elements were present in the medium. These results possessed relevant significance, since it is known that serum albumins interact with Ca^{2+} (acid dissociation constant $[Ka] = 1.5 \times 10^3 \, M^{-1}$ for human albumin) and that almost 45% of the 2.4 mM flowing Ca^{2+} in serum is albumin bound (60, 61).

A. baumannii A118 showed an increase in the transformation frequency with BSA. Similar results were obtained for two other *A. baumannii* clinical strains tested under the same conditions. Therefore, it is highly likely that induction of natural transformation by BSA could be present in the whole species or even along the genus, greatly increasing the significance of our finding.

Jacobs et al. reported increased expression levels of various genes when *A. baumannii* ATCC 17978 was grown in human serum by using microarrays (51). Among some of the genes that showed upregulation were DNA uptake genes (51). Our experiments are in line with those results, since we also observed an increase of natural transformation when we used human serum as well as HSA in the transformation experiments. Comparison of BSA and HSA proteins showed a high level of similarity between the BSA and HSA structures (root mean square deviation [RMSD], 1.372; Q score, 0.811). Taken together, the results that we provide are evidence that serum albumins could serve as a competence inducer.

Our work provides a description of the first specific inducers, albumins and Ca^{2+}, of natural competence and also shows some aspects of this process in *A. baumannii*. We observed that natural transformation is a prompt and continuous process that is af-

fected by the pH of the environment. The competence genes *comEA* and *pilQ* showed high levels of expression in medium containing both Ca^{2+} and BSA, conditions under which higher levels of natural transformation were obtained.

Overall, our results suggest that the main blood proteins enhance a primary mechanism of horizontal gene transfer (HGT) in *A. baumannii* and therefore contribute to the emergence of AMR in this threatening human pathogen. Remarkably, the A118 strain, as well as the other two strains tested here (ATCC 17978 and A42), easily acquired resistance determinants from related bacteria, showing the high flux of AMR across different isolates from this species.

Therefore, albumins and Ca^{2+} can directly contribute to the acquisition and dispersion of antimicrobial resistance determinants among clinical *A. baumannii* strains.

REFERENCES

1. **Roca I, Espinal P, Vila-Farres X, Vila J.** 2012. The *Acinetobacter baumannii* oxymoron: commensal hospital dweller turned pan-drug-resistant menace. Front Microbiol **3:**148. http://dx.doi.org/10.3389/fmicb.2012.00148.
2. **Falagas ME, Kopterides P, Siempos II.** 2006. Attributable mortality of *Acinetobacter baumannii* infection among critically ill patients. Clin Infect Dis **43:**389. http://dx.doi.org/10.1086/505599. (Reply, **43:**389-390, http://dx.doi.org/10.1086/505607.)
3. **Mussi MA, Gaddy JA, Cabruja M, Arivett BA, Viale AM, Rasia R, Actis LA.** 2010. The opportunistic human pathogen *Acinetobacter baumannii* senses and responds to light. J Bacteriol **192:**6336–6345. http://dx.doi.org/10.1128/JB.00917-10.
4. **Fournier PE, Vallenet D, Barbe V, Audic S, Ogata H, Poirel L, Richet H, Robert C, Mangenot S, Abergel C, Nordmann P, Weissenbach J, Raoult D, Claverie JM.** 2006. Comparative genomics of multidrug resistance in *Acinetobacter baumannii*. PLoS Genet **2:**e7. http://dx.doi.org/10.1371/journal.pgen.0020007.
5. **Smith MG, Gianoulis TA, Pukatzki S, Mekalanos JJ, Ornston LN, Gerstein M, Snyder M.** 2007. New insights into *Acinetobacter baumannii* pathogenesis revealed by high-density pyrosequencing and transposon mutagenesis. Genes Dev **21:**601–614. http://dx.doi.org/10.1101/gad.1510307.
6. **Li H, Liu F, Zhang Y, Wang X, Zhao C, Chen H, Zhang F, Zhu B, Hu Y, Wang H.** 2015. Evolution of carbapenem-resistant *Acinetobacter baumannii* revealed through whole-genome sequencing and comparative genomic analysis. Antimicrob Agents Chemother **59:**1168–1176. http://dx.doi.org/10.1128/AAC.04609-14.
7. **Touchon M, Cury J, Yoon EJ, Krizova L, Cerqueira GC, Murphy C, Feldgarden M, Wortman J, Clermont D, Lambert T, Grillot-Courvalin C, Nemec A, Courvalin P, Rocha EP.** 2014. The genomic diversification of the whole *Acinetobacter* genus: origins, mechanisms, and consequences. Genome Biol Evol **6:**2866–2882. http://dx.doi.org/10.1093/gbe/evu225.
8. **Johnston C, Martin B, Fichant G, Polard P, Claverys JP.** 2014. Bacterial transformation: distribution, shared mechanisms and divergent control. Nat Rev Microbiol **12:**181–196. http://dx.doi.org/10.1038/nrmicro3199.
9. **Engelmoer DJ, Donaldson I, Rozen DE.** 2013. Conservative sex and the benefits of transformation in *Streptococcus pneumoniae*. PLoS Pathog **9:**e1003758. http://dx.doi.org/10.1371/journal.ppat.1003758.
10. **Humbert O, Dorer MS, Salama NR.** 2011. Characterization of *Helicobacter pylori* factors that control transformation frequency and integration length during inter-strain DNA recombination. Mol Microbiol **79:**387–401. http://dx.doi.org/10.1111/j.1365-2958.2010.07456.x.
11. **Seitz P, Blokesch M.** 2013. Cues and regulatory pathways involved in natural competence and transformation in pathogenic and environmental Gram-negative bacteria. FEMS Microbiol Rev **37:**336–363. http://dx.doi.org/10.1111/j.1574-6976.2012.00353.x.
12. **Woodhams KL, Benet ZL, Blonsky SE, Hackett KT, Dillard JP.** 2012. Prevalence and detailed mapping of the gonococcal genetic island in *Neisseria meningitidis*. J Bacteriol **194:**2275–2285. http://dx.doi.org/10.1128/JB.00094-12.
13. **Dorer MS, Fero J, Salama NR.** 2010. DNA damage triggers genetic exchange in Helicobacter pylori. PLoS Pathog **6:**e1001026. http://dx.doi.org/10.1371/journal.ppat.1001026.
14. **Herriott RM, Meyer EM, Vogt M.** 1970. Defined nongrowth media for stage II development of competence in *Haemophilus influenzae*. J Bacteriol **101:**517–524.
15. **Redfield RJ.** 1991. *sxy-1*, a *Haemophilus influenzae* mutation causing greatly enhanced spontaneous competence. J Bacteriol **173:**5612–5618.
16. **Carr EL, Kampfer P, Patel BK, Gurtler V, Seviour RJ.** 2003. Seven novel species of *Acinetobacter* isolated from activated sludge. Int J Syst Evol Microbiol **53:**953–963. http://dx.doi.org/10.1099/ijs.0.02486-0.
17. **Lorenz MG, Wackernagel W.** 1994. Bacterial gene transfer by natural genetic transformation in the environment. Microbiol Rev **58:**563–602.
18. **Palmen R, Vosman B, Buijsman P, Breek CK, Hellingwerf KJ.** 1993. Physiological characterization of natural transformation in *Acinetobacter calcoaceticus*. J Gen Microbiol **139:**295–305. http://dx.doi.org/10.1099/00221287-139-2-295.
19. **Young DM, Parke D, Ornston LN.** 2005. Opportunities for genetic investigation afforded by *Acinetobacter baylyi*, a nutritionally versatile bacterial species that is highly competent for natural transformation. Annu Rev Microbiol **59:**519–551. http://dx.doi.org/10.1146/annurev.micro.59.051905.105823.
20. **Gerischer U, Ornston LN.** 2001. Dependence of linkage of alleles on their physical distance in natural transformation of *Acinetobacter* sp. strain ADP1. Arch Microbiol **176:**465–469. http://dx.doi.org/10.1007/s00203-001-0353-7.
21. **Nielsen KM, Bones AM, Van Elsas JD.** 1997. Induced natural transformation of *Acinetobacter calcoaceticus* in soil microcosms. Appl Environ Microbiol **63:**3972–3977.
22. **Nielsen KM, van Weerelt MD, Berg TN, Bones AM, Hagler AN, van Elsas JD.** 1997. Natural transformation and availability of transforming DNA to Acinetobacter calcoaceticus in soil microcosms. Appl Environ Microbiol **63:**1945–1952.
23. **Utnes AL, Sorum V, Hulter N, Primicerio R, Hegstad J, Kloos J, Nielsen KM, Johnsen PJ.** 2015. Growth phase-specific evolutionary benefits of natural transformation in Acinetobacter baylyi. ISME J **9:**2221–2231. http://dx.doi.org/10.1038/ismej.2015.35.
24. **Ramirez MS, Don M, Merkier AK, Bistue AJ, Zorreguieta A, Centron D, Tolmasky ME.** 2010. Naturally competent *Acinetobacter baumannii* clinical isolate as a convenient model for genetic studies. J Clin Microbiol **48:**1488–1490. http://dx.doi.org/10.1128/JCM.01264-09.
25. **Traglia GM, Chua K, Centron D, Tolmasky ME, Ramirez MS.** 2014. Whole-genome sequence analysis of the naturally competent *Acinetobacter baumannii* clinical isolate A118. Genome Biol Evol **6:**2235–2239. http://dx.doi.org/10.1093/gbe/evu176.
26. **Wilharm G, Piesker J, Laue M, Skiebe E.** 2013. DNA uptake by the nosocomial pathogen *Acinetobacter baumannii* occurs during movement along wet surfaces. J Bacteriol **195:**4146–4153. http://dx.doi.org/10.1128/JB.00754-13.
27. **Ramirez MS, Merkier AK, Quiroga MP, Centron D.** 2012. *Acinetobacter baumannii* is able to gain and maintain a plasmid harbouring In35 found in *Enterobacteriaceae* isolates from Argentina. Curr Microbiol **64:**211–213. http://dx.doi.org/10.1007/s00284-011-0052-9.
28. **Harding CM, Tracy EN, Carruthers MD, Rather PN, Actis LA, Munson RS, Jr.** 2013. *Acinetobacter baumannii* strain M2 produces type IV pili which play a role in natural transformation and twitching motility but not surface-associated motility. mBio **4:**e00360-13. http://dx.doi.org/10.1128/mBio.00360-13.
29. **Jorgensen JH, Ferraro MJ.** 2009. Antimicrobial susceptibility testing: a review of general principles and contemporary practices. Clin Infect Dis **49:**1749–1755. http://dx.doi.org/10.1086/647952.
30. **Turapov OA, Mukamolova GV, Bottrill AR, Pangburn MK.** 2008.

Digestion of native proteins for proteomics using a thermocycler. Anal Chem **80:**6093–6099. http://dx.doi.org/10.1021/ac702527b.

31. **Ramirez MS, Traglia GM, Perez JF, Muller GL, Martinez F, Golic AE, Mussi MA.** 2015. White and blue light induce reduction in susceptibility to minocycline and tigecycline in *Acinetobacter* sp. and other bacteria of clinical importance. J Med Microbiol **64:**525–537. http://dx.doi.org/10.1099/jmm.0.000048.

32. **Chen I, Dubnau D.** 2004. DNA uptake during bacterial transformation. Nat Rev Microbiol **2:**241–249. http://dx.doi.org/10.1038/nrmicro844.

33. **Vegge CS, Brondsted L, Ligowska-Marzeta M, Ingmer H.** 2012. Natural transformation of *Campylobacter jejuni* occurs beyond limits of growth. PLoS One **7:**e45467. http://dx.doi.org/10.1371/journal.pone.0045467.

34. **Saunders CW, Guild WR.** 1981. Monomer plasmid DNA transforms *Streptococcus pneumoniae*. Mol Gen Genet **181:**57–62. http://dx.doi.org/10.1007/BF00339005.

35. **Baur B, Hanselmann K, Schlimme W, Jenni B.** 1996. Genetic transformation in freshwater: *Escherichia coli* is able to develop natural competence. Appl Environ Microbiol **62:**3673–3678.

36. **Hisano K, Fujise O, Miura M, Hamachi T, Matsuzaki E, Nishimura F.** 2014. The *pga* gene cluster in *Aggregatibacter actinomycetemcomitans* is necessary for the development of natural competence in Ca(2+)-promoted biofilms. Mol Oral Microbiol **29:**79–89. http://dx.doi.org/10.1111/omi.12046.

37. **Huddleston JR, Brokaw JM, Zak JC, Jeter RM.** 2013. Natural transformation as a mechanism for horizontal gene transfer among environmental *Aeromonas* species. Syst Appl Microbiol **36:**224–234. http://dx.doi.org/10.1016/j.syapm.2013.01.004.

38. **Kang F, Wang H, Gao Y, Long J, Wang Q.** 2013. Ca2+ promoted the low transformation efficiency of plasmid DNA exposed to PAH contaminants. PLoS One **8:**e58238. http://dx.doi.org/10.1371/journal.pone.0058238.

39. **Liu X, Liu L, Wang Y, Wang X, Ma Y, Li Y.** 2014. The study on the factors affecting transformation efficiency of E. coli competent cells. Pak J Pharm Sci **27:**679–684.

40. **Meibom KL, Blokesch M, Dolganov NA, Wu CY, Schoolnik GK.** 2005. Chitin induces natural competence in *Vibrio cholerae*. Science **310:**1824–1827. http://dx.doi.org/10.1126/science.1120096.

41. **Marvig RL, Blokesch M.** 2010. Natural transformation of *Vibrio cholerae* as a tool—optimizing the procedure. BMC Microbiol **10:**155. http://dx.doi.org/10.1186/1471-2180-10-155.

42. **Porstendorfer D, Gohl O, Mayer F, Averhoff B.** 2000. ComP, a pilin-like protein essential for natural competence in *Acinetobacter* sp. strain BD413: regulation, modification, and cellular localization. J Bacteriol **182:**3673–3680.

43. **Fontaine L, Goffin P, Dubout H, Delplace B, Baulard A, Lecat-Guillet N, Chambellon E, Gardan R, Hols P.** 2013. Mechanism of competence activation by the ComRS signalling system in streptococci. Mol Microbiol **87:**1113–1132. http://dx.doi.org/10.1111/mmi.12157.

44. **Burkhardt NY, Baldridge GD, Williamson PC, Billingsley PM, Heu CC, Felsheim RF, Kurtti TJ, Munderloh UG.** 2011. Development of shuttle vectors for transformation of diverse *Rickettsia* species. PLoS One **6:**e29511. http://dx.doi.org/10.1371/journal.pone.0029511.

45. **Charpentier X, Kay E, Schneider D, Shuman HA.** 2011. Antibiotics and UV radiation induce competence for natural transformation in Legionella pneumophila. J Bacteriol **193:**1114–1121. http://dx.doi.org/10.1128/JB.01146-10.

46. **Salzer R, Joos F, Averhoff B.** 2015. Different effects of MglA and MglB on pilus-mediated functions and natural competence in *Thermus thermophi*

lus. Extremophiles **19:**261–267. http://dx.doi.org/10.1007/s00792-014-0711-4.

47. **Aranda J, Poza M, Pardo BG, Rumbo S, Rumbo C, Parreira JR, Rodriguez-Velo P, Bou G.** 2010. A rapid and simple method for constructing stable mutants of *Acinetobacter baumannii*. BMC Microbiol **10:**279. http://dx.doi.org/10.1186/1471-2180-10-279.

48. **Giles SK, Stroeher UH, Eijkelkamp BA, Brown MH.** 2015. Identification of genes essential for pellicle formation in *Acinetobacter baumannii*. BMC Microbiol **15:**116. http://dx.doi.org/10.1186/s12866-015-0440-6.

49. **Hare JM, Ferrell JC, Witkowski TA, Grice AN.** 2014. Prophage induction and differential RecA and UmuDAb transcriptome regulation in the DNA damage responses of *Acinetobacter baumannii* and *Acinetobacter baylyi*. PLoS One **9:**e93861. http://dx.doi.org/10.1371/journal.pone.0093861.

50. **Heindorf M, Kadari M, Heider C, Skiebe E, Wilharm G.** 2014. Impact of Acinetobacter baumannii superoxide dismutase on motility, virulence, oxidative stress resistance and susceptibility to antibiotics. PLoS One **9:**e101033. http://dx.doi.org/10.1371/journal.pone.0101033.

51. **Jacobs AC, Sayood K, Olmsted SB, Blanchard CE, Hinrichs S, Russell D, Dunman PM.** 2012. Characterization of the *Acinetobacter baumannii* growth phase-dependent and serum responsive transcriptomes. FEMS Immunol Med Microbiol **64:**403–412. http://dx.doi.org/10.1111/j.1574-695X.2011.00926.x.

52. **Johnson TL, Waack U, Smith S, Mobley H, Sandkvist M.** 2016. *Acinetobacter baumannii* is dependent on the type II secretion system and its substrate LipA for lipid utilization and *in vivo* fitness. J Bacteriol **198:**711–719. http://dx.doi.org/10.1128/JB.00622-15.

53. **Vilacoba E, Almuzara M, Gulone L, Traglia GM, Figueroa SA, Sly G, Fernandez A, Centron D, Ramirez MS.** 2013. Emergence and spread of plasmid-borne *tet*(B)::IS*CR2* in minocycline-resistant *Acinetobacter baumannii* isolates. Antimicrob Agents Chemother **57:**651–654. http://dx.doi.org/10.1128/AAC.01751-12.

54. **CDC.** 2013. Antibiotic resistance threats in the United States, 2013. CDC, Atlanta, GA.

55. **Doughari HJ, Ndakidemi PA, Human IS, Benade S.** 2011. The ecology, biology and pathogenesis of *Acinetobacter* spp.: an overview. Microbes Environ **26:**101–112. http://dx.doi.org/10.1264/jsme2.ME10179.

56. **Seifert H, Strate A, Pulverer G.** 1995. Nosocomial bacteremia due to *Acinetobacter baumannii*. Clinical features, epidemiology, and predictors of mortality. Medicine (Baltimore) **74:**340–349.

57. **Luke NR, Howlett AJ, Shao J, Campagnari AA.** 2004. Expression of type IV pili by *Moraxella catarrhalis* is essential for natural competence and is affected by iron limitation. Infect Immun **72:**6262–6270. http://dx.doi.org/10.1128/IAI.72.11.6262-6270.2004.

58. **Slager J, Kjos M, Attaiech L, Veening JW.** 2014. Antibiotic-induced replication stress triggers bacterial competence by increasing gene dosage near the origin. Cell **157:**395–406. http://dx.doi.org/10.1016/j.cell.2014.01.068.

59. **He XM, Carter DC.** 1992. Atomic structure and chemistry of human serum albumin. Nature **358:**209–215. http://dx.doi.org/10.1038/358209a0.

60. **Majorek KA, Porebski PJ, Dayal A, Zimmerman MD, Jablonska K, Stewart AJ, Chruszcz M, Minor W.** 2012. Structural and immunologic characterization of bovine, horse, and rabbit serum albumins. Mol Immunol **52:**174–182. http://dx.doi.org/10.1016/j.molimm.2012.05.011.

61. **Peters T.** 1995. All about albumin: biochemistry, genetics, and medical applications. Academic Press, San Diego, CA.