

1                   **Impact of Human Serum Proteins on Susceptibility of *Acinetobacter***  
2                   ***baumannii* to Cefiderocol: role of iron transport**  
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54 **Abstract**

55 Cefiderocol is a siderophore antibiotic that co-opts iron transporters to facilitate  
56 cell entry. We show that genes related to iron uptake systems and resistance to  
57  $\beta$ -lactams in *Acinetobacter baumannii* have altered expression levels in the  
58 presence of human serum, human serum albumin, or human pleural fluid.  
59 Cefiderocol MICs are also raised in the presence of the mentioned fluids.  
60 Clinical response in *A. baumannii* infections may be related to the interplay of  
61 these human factors.

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63                   Carbapenem-resistant *Acinetobacter baumannii* (CRAB), one of the most  
64                   feared pathogens in healthcare settings, driving the Centers for Disease Control  
65                   and Prevention (CDC) to categorize it as an “urgent threat” (1-5). As a  
66                   consequence of *A. baumannii*’s capability to develop multidrug resistance  
67                   (MDR), treatment strategies become extremely limited, with only a few active  
68                   antibiotics (6-8).

69                   The increasing number of CRAB infections translates into alarmingly high  
70                   morbidity and mortality (2, 9-11). Consequently, numerous efforts are focusing  
71                   on finding novel treatment options (12-19). Cefiderocol (CFDC), formerly known  
72                   as S-649266, approved by the FDA in November 2019 to treat nosocomial  
73                   pneumonia and urinary tract infections, is a hybrid molecule that consists of a  
74                   cephalosporin component that targets cell wall synthesis and a catechol  
75                   siderophore moiety that allows cell penetration by active ferric-siderophore  
76                   transporters (20-24). This novel synthetic compound uses a “Trojan horse”  
77                   strategy to improve antibiotic penetration and reach a high concentration at the  
78                   target site (21, 22). CFDC’s primary targets are cell wall synthetizing proteins  
79                   associated with  $\beta$ -lactam activity (PBP1 and PBP3) (25). Early research showed  
80                   promising results when treating Gram-negative carbapenem-resistant infections  
81                   (7, 26, 27). Recently, a study described a number of unfavorable outcomes in  
82                   patients with pulmonary or bloodstream *A. baumannii* infections compared to  
83                   other available therapies (7). Our goal was to determine if there is a molecular  
84                   basis for this observation.

85                   *A. baumannii* senses components of human fluids and responds by  
86                   modifying its transcriptional and phenotypic profiles (28-32). Human serum  
87                   albumin (HSA) as well as human pleural fluid (HPF) modulate the expression of

88 genes associated with iron-uptake systems, biofilm formation, antibiotic  
89 resistance, and DNA-acquisition among others (29, 33, 34). In previous studies  
90 we observed that CRAB AB5075 genes associated with iron uptake systems  
91 were down-regulated when exposed to HPF and 0.2% HSA (29), while genes  
92 associated with  $\beta$ -lactam resistance were up-regulated in the presence of  
93 physiological concentrations of HSA and human serum (HS) (33, 34). The  
94 modification in expression of iron-uptake systems and  $\beta$ -lactam resistance  
95 genes could be responsible for variations in *A. baumannii* susceptibility to  
96 CFDC, and concomitantly for the unfavorable outcomes of patients with  
97 pulmonary or bloodstream infections. In this work we describe the effect of HS,  
98 HSA, and HPF on representative CRAB strains.

99 CRAB AB0057 and AMA16 are clinical strains that belong to different  
100 clonal complexes and harbor OXA-23 and NDM-1, respectively (35, 36).  
101 Quantitative RT-PCR (qRT-PCR) assays were carried out using total RNA  
102 extracted from cells cultured in lysogeny broth (LB) or LB supplemented with  
103 4% HPF or 3.5% HSA, or cultured in HS (Fig. 1A and B), as previously  
104 described (29).

105 The expression of iron uptake related genes *bauA*, *basE*, *feoA* and *tonB*,  
106 was reduced in both strains under most of the conditions evaluated (Fig. 1 A  
107 and B). In addition, when the hypervirulent *A. baumannii* AB5075 strain was  
108 exposed to 3.5% HSA or HS, the expression levels of most genes analyzed  
109 were down regulated. Pimentel *et al.* previously showed that levels of  
110 expression of AB5075 iron related genes were down-regulated in cells cultured  
111 in iron-rich media (29); this finding is in agreement with level of expression of  
112 *basE* reported in this work (Fig. 1C). The *fhuE\_1*, *fhuE\_2*, *pirA* and *piuA* genes,

113 which are associated with iron-uptake (37), were also down-regulated in the  
114 presence of HPF (Fig. S1 A). Moreover, the transcriptional levels of these  
115 genes were further analyzed by qRT-PCR for the three strains (AMA16,  
116 AB0057, and AB5075) in the three tested conditions (HFP, HS, and HSA). In  
117 most of conditions evaluated, levels of expression were down-regulated (Fig. S1  
118 B).

119 Genes associated with  $\beta$ -lactam resistance, such as *bla*<sub>OXA-51-like</sub>, *bla*<sub>OXA-  
120 23</sub>, *pbp1* and *pbp3*(33, 34), were up-regulated in *A. baumannii* AB5075, AMA16,  
121 and AB0057 when cultured in the presence of 3.5% HSA. To further examine if  
122 changes in genes associated with  $\beta$ -lactam resistance are affected by HS or  
123 HPF in the CRAB strains, qRT-PCR was used to assess gene expression of  
124 *bla*<sub>OXA-51-like</sub>, *bla*<sub>OXA-23</sub>, *bla*<sub>NDM-1</sub>, *pbp1*, *pbp3*, and *carO* in cultures of strains  
125 AMA16 and AB0057 containing HS or HPF. This transcriptional analysis  
126 revealed a down-regulation in expression levels of *bla*<sub>NDM-1</sub> in *A. baumannii*  
127 AMA16 cultured in the presence of HS or HPF (Fig. 2 A). In this strain, the  
128 levels of expression of ISAb<sub>a</sub>125, *bla*<sub>PER-7</sub>, *pbp1*, and *pbp3* were up-regulated in  
129 the presence of HPF. HS also induced up-regulation of ISAb<sub>a</sub>125 (Fig. 2 A). In  
130 the case of the AB0057 strain, we observed up-regulation of *bla*<sub>OXA-23</sub>, *bla*<sub>OXA-51</sub>,  
131 *carO*, *bla*<sub>ADC</sub>, *pbp1* and *pbp3* in cultures containing HPF. In cultures containing  
132 HS the former four genes were up-regulated (Fig. 2 B). Assessment of the  
133 changes in  $\beta$ -lactam resistance-associated *A. baumannii* AB5075 genes in the  
134 presence of HPF showed down-regulation of the *carO* and *bla*<sub>GES-14</sub> genes (Fig.  
135 2 C). On the other hand, the expression levels of *bla*<sub>OXA-51</sub>, *pbp1*, *pbp3* and  
136 *bla*<sub>ADC</sub> were increased (Fig. 2 C).

137            The impact of human fluids in CFDC activity was further studied using a  
138            panel of twenty CRAB strains including AB5075, AMA16, and AB0057 (a strain  
139            isolated in the Walter Reed Army Medical Center (38). Cells cultured in LB, and  
140            LB supplemented with HPF or 3.5 % HSA were used to determine the MICs of  
141            CFDC. Ten strains had baseline resistance to CFDC (Table S1). An increase in  
142            the MIC values was observed in 14 strains when the cells were growing in the  
143            presence of HSA or HPF (Table S1 and Fig. 2 D). We draw attention to the  
144            occurrence of heteroresistant colonies with the inhibition ellipse in 10 of the  
145            tested strains (Fig. S2). Most of the strains that exhibited this phenomenon  
146            harbored *bla<sub>PER-7</sub>* (35). Our results agree with a recent report indicating that  
147            PER-like  $\beta$ -lactamases contribute to a decreased susceptibility to CFDC in *A.*  
148            *baumannii* (39). In addition, they observed that the combination of CFDC with  
149            avibactam may inhibit the activity of PER-type  $\beta$ -lactamases (39).

150            Overall, our results showed that *A. baumannii* responds to the interaction  
151            with human bodily fluids during the establishment of infection modulating the  
152            expression of iron-uptake genes and  $\beta$ -lactam associated genes. We  
153            hypothesize that the presence of iron binding proteins in the human fluids is  
154            sensed by *A. baumannii*, which in turn responds by down-regulating expression  
155            of iron uptake system genes impacting CDFC activity. The particular changes in  
156            gene expression of the aforementioned group of genes, which is not evaluated  
157            in traditional susceptibility tests, may contribute to unfavorable outcomes  
158            associated with iron-rich environments. These results also raise questions  
159            regarding the expression of other factors that may contribute to challenges in  
160            overcoming infections by *A. baumannii*.

161 The limitations inherent to performing MICs in the presence of bodily  
162 fluids (protein binding) is recognized. We are also aware that comparing these  
163 analysis does not take into account pharmacokinetics and pharmacodynamics  
164 (PK/PD) parameters that usure drug efficacy (40, 41). Laslty, we appreciate  
165 that these data must also be evaluated in the context of outcomes of animal  
166 models of infection (42-44). Nevertheless, the impact on iron transport  
167 mechanisms uncovered in these observations merits further analyses.

168

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173 conceived the study and designed the experiments. C.P., C.L., F.P., M.R.T.,  
174 T.S., J.E., B.N., S.A., A.C., A.J.V., L.A.A., M.E.T., R.A.B. and M.S.R. performed  
175 the experiments and genomics and bioinformatics analyses. C.P., C.L., F.P.,  
176 M.R.T., T.S., A.J.V., L.A.A., R.A.B., M.E.T. and M.S.R. analyzed the data and  
177 interpreted the results. R.A.B., M.E.T. and M.S.R. contributed  
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201

202 **Supplementary Materials**

203 **Table S1:** Minimal Inhibitory Concentrations of Cefiderocol (CFDC) for 22  
204 Carbapenem-resistant *Acinetobacter baumanii* representative strains performed  
205 using CFDC MTS strips ((Liofilchem S.r.l., Italy) on Mueller Hinton Agar (cation  
206 adjusted). *A. baumannii* cells were cultured in LB or LB supplemented with  
207 3.5 % HSA or HPF, respectively.

208 **Figure S1:** Differential expression of *fhuE\_1*, *fhuE\_2*, *pirA* and *piuA* genes  
209 associated with iron-uptake obtained for *A. baumannii* AB5075 cultured in the  
210 presence of HSA and HPF.

211 **Figure S2:** Intra-colonies in *A. baumannii* AMA40 representative of the tested  
212 strains in the presence of HPF.

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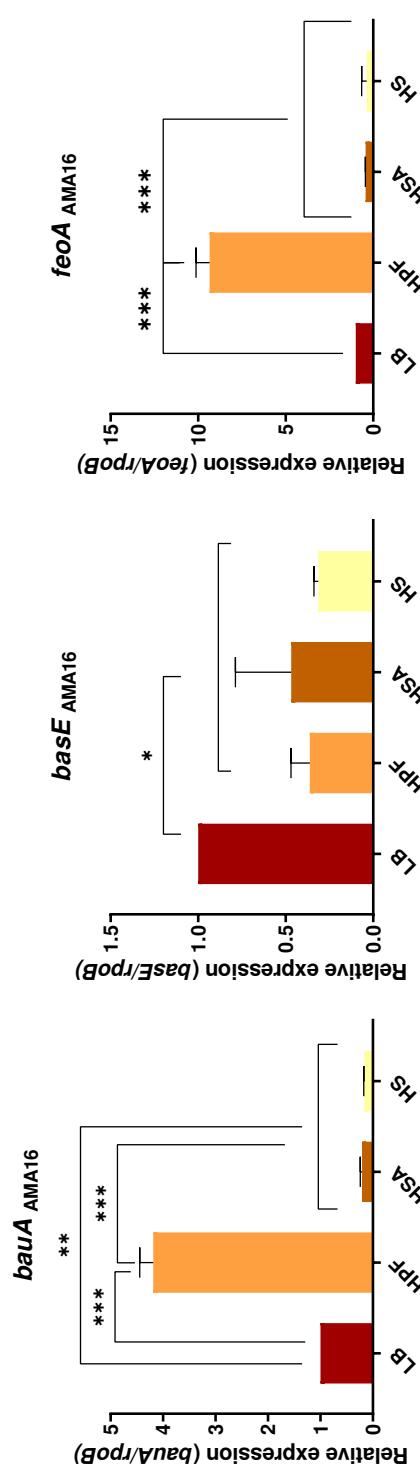
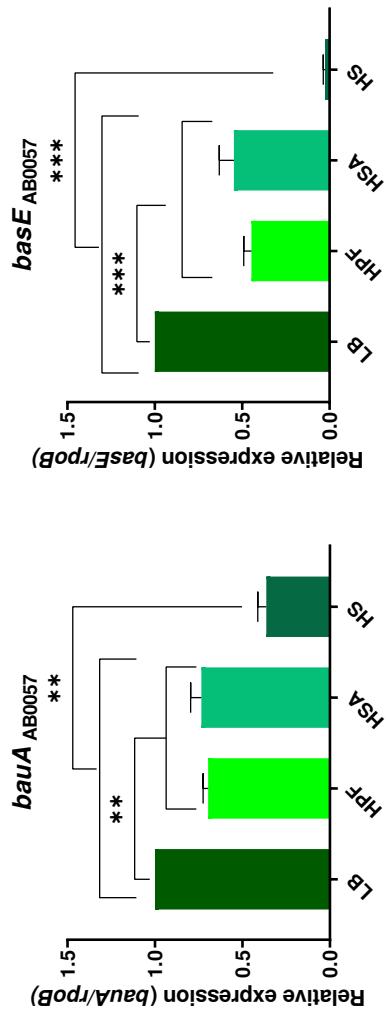
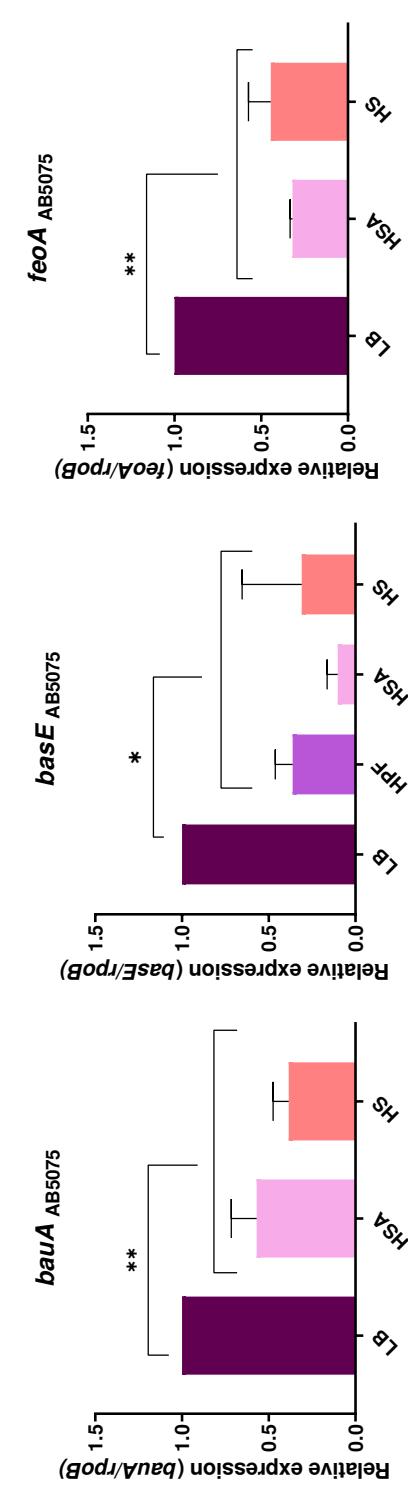
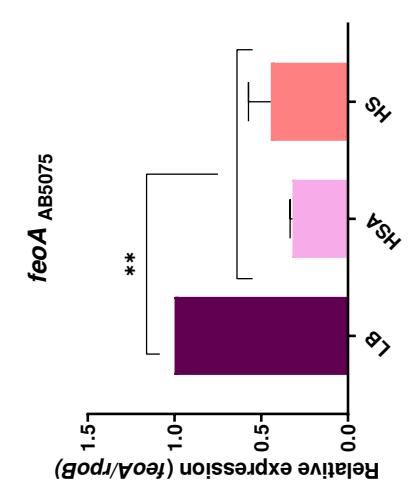
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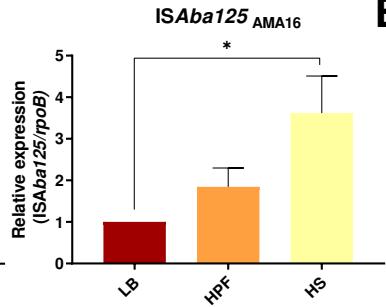
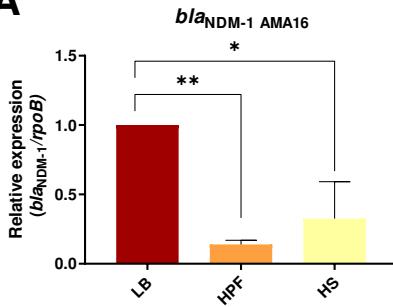
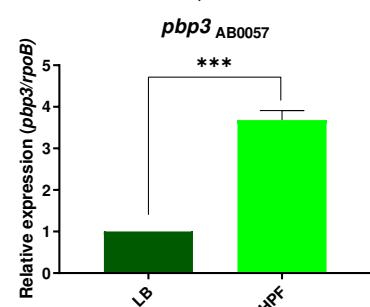
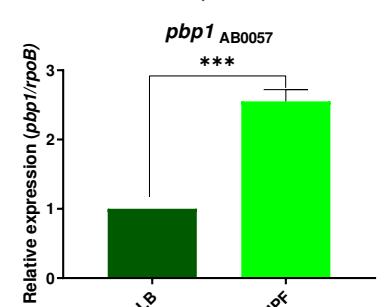
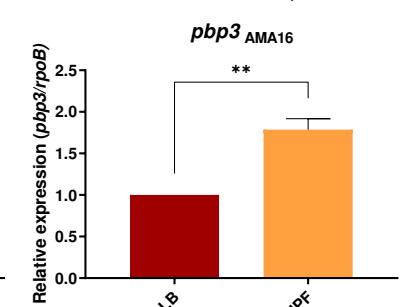
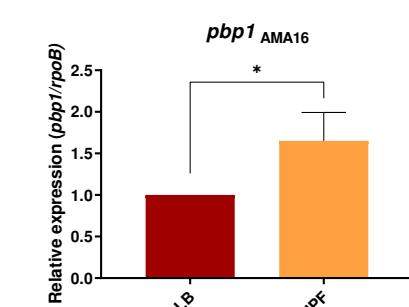
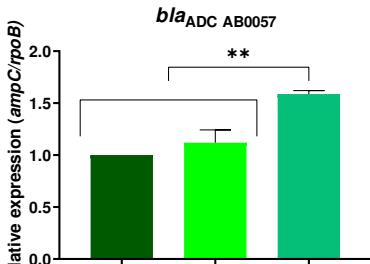
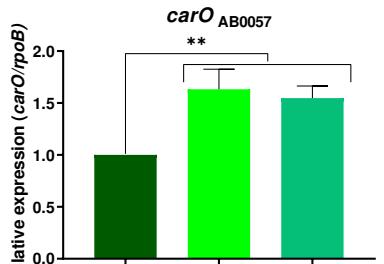
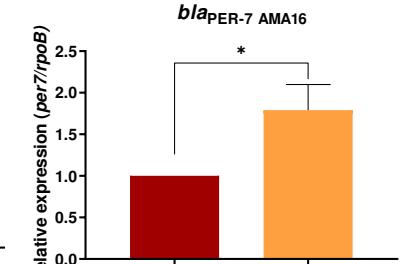
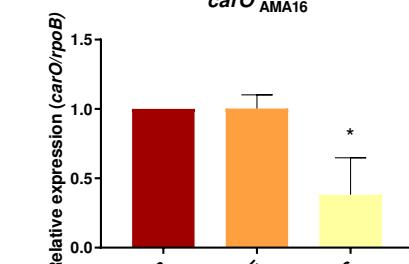
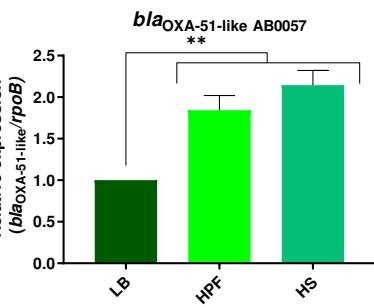
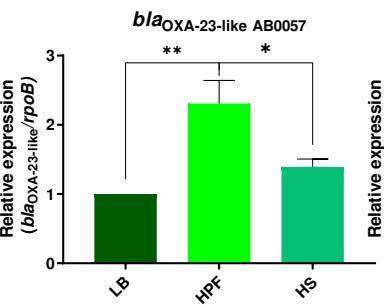
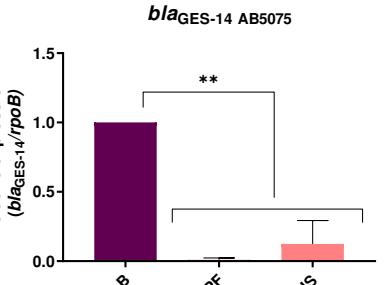
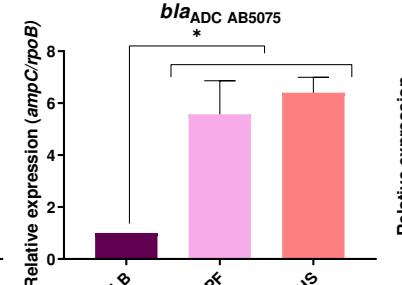
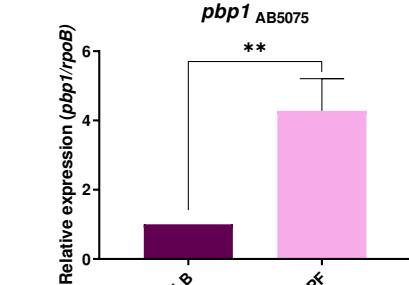
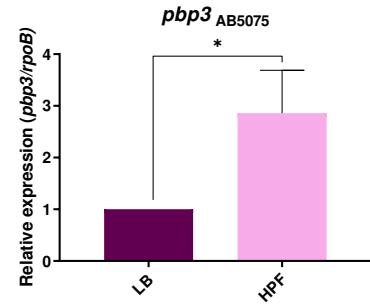
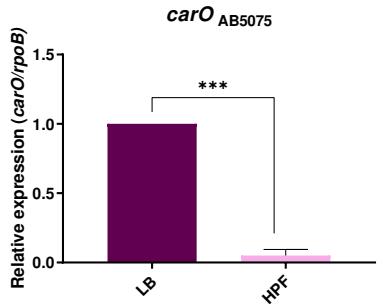
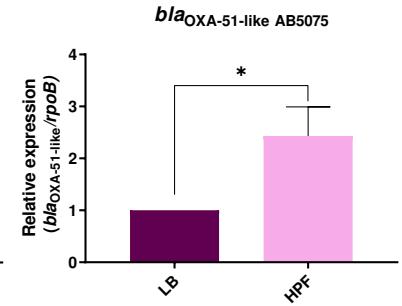
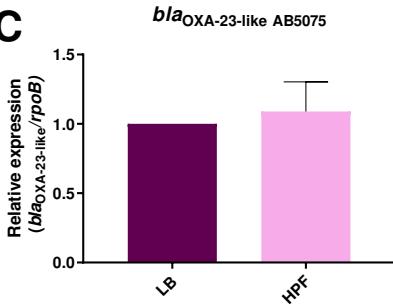
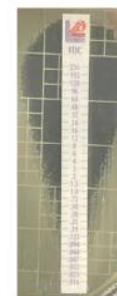
405 **Figure legends**

406 **Figure 1.** Genetic analysis of iron uptake genes of AMA16 (A), AB0057 (B) and  
407 AB5075 (C) *A. baumannii* strains. qRT-PCR of genes associated with iron  
408 uptake, *bauA*, *basE*, *feoA* and *tonB* expressed in LB or LB supplemented with  
409 HPF or HSA, or cultured in HS. Fold changes were calculated using double  $\Delta Ct$   
410 analysis. At least three independent samples were used, and four technical  
411 replicates were performed from each sample. The LB was used as reference.  
412 Statistical significance ( $p < 0.05$ ) was determined by ANOVA followed by  
413 Tukey's multiple-comparison test, one asterisks:  $p < 0.05$ ; two asterisks:  $p < 0.01$   
414 and three asterisks:  $p < 0.001$ .

415

416 **Figure 2.** (A-C) Genetic analysis of  $\beta$ -lactamase + PBP genes of AMA16 (A),  
417 AB0057 (B) and AB5075 (C) *A. baumannii* strains. qRT-PCR of genes  
418 associated with  $\beta$ -lactams resistance expressed in LB, LB supplemented with  
419 HPF, or in HS. Fold changes were calculated using double  $\Delta Ct$  analysis. At  
420 least three independent samples were used. LB was used as the reference  
421 condition. Statistical significance ( $p < 0.05$ ) was determined by ANOVA followed  
422 by Tukey's multiple-comparison test, one asterisks:  $p < 0.05$ ; two asterisks:  $p <$   
423  $0.01$ , and three asterisks:  $p < 0.001$ . (D) Effect of HSA and HPF on the  
424 antimicrobial susceptibility of *A. baumannii* representatives strains AMA40 and  
425 AMA113 strains grew in LB broth, LB broth plus 3.5 % HSA, or HPF were used  
426 to performed cefiderocol (CFDC) susceptibility. Minimum inhibitory  
427 concentration (MIC) on cation adjusted Mueller Hinton agar was performed by  
428 MTS (Liofilchem S.r.l., Italy) following manufacturer's recommendations.

**A****B****C****C**

**A****B****C****D** **AMA40** **AMA40 (HSA)** **AMA40 (HPF)****AMA40** **AMA16** **AMA16 (HSA)****AMA16** **AMA16 (HPF)****AMA113****AMA113** **AMA113 (HPF)**