

Brief Report

# Effect of Subinhibitory Concentrations of Antibiotics and Disinfectants on IS*Aba*-Mediated Inactivation of Lipooligosaccharide Biosynthesis Genes in *Acinetobacter baumannii*

Héctor Olmeda-López , Andrés Corral-Lugo <sup>†</sup> and Michael J. McConnell <sup>\*,†</sup>

Intrahospital Infections Laboratory, National Centre for Microbiology, Instituto de Salud Carlos III (ISCIII), 28221 Madrid, Spain; hectorolmeda97@gmail.com (H.O.-L.); acorral@isciii.es (A.C.-L.)

\* Correspondence: michael.mcconnell@isciii.es; Tel.: +34-918-223-896

<sup>†</sup> A.C.-L. and M.J.M. contributed equally to this work and should be considered co-senior authors.

**Abstract:** Inactivation of the lipooligosaccharide (LOS) biosynthesis genes *lpxA*, *lpxC* and *lpxD* by IS*Aba* insertion elements results in high-level resistance to colistin in *A. baumannii*. In the present study, we quantify the rate of spontaneous insertional inactivation of LOS biosynthesis genes by IS*Aba* elements in the ATCC 19606-type strain and two multidrug clinical isolates. Using insertional inactivation of *lpxC* by IS*Aba*11 in the ATCC 19606 strain as a model, we determine the effect of several subinhibitory concentrations of the antibiotics, namely tetracycline, ciprofloxacin, meropenem, kanamycin and rifampicin, as well as the disinfectants ethanol and chlorhexidine on IS*Aba*11 insertion frequencies. Notably, subinhibitory concentrations of tetracycline significantly increased IS*Aba*11 insertion, and rifampicin completely inhibited the emergence of colistin resistance due to IS*Aba*11 inactivation of *lpxC*. Sequencing of IS*Aba*11 insertion sites within the *lpxC* gene demonstrated that insertions clustered between nucleotides 382 and 618 (58.3% of unique insertions detected), indicating that this may be a hotspot for IS*Aba*11 insertion. The alignment of insertion sites revealed a semi-conserved AT-rich consensus sequence upstream of the IS*Aba*11 insertion site, suggesting that IS*Aba*11 insertion sites may be sequence-dependent. This study explores previously uncharacterized aspects regarding the acquisition of colistin resistance through insertional activation in LOS biosynthesis genes in *A. baumannii*.

**Keywords:** *Acinetobacter baumannii*; insertion sequence; IS*Aba*; colistin; antibiotic resistance



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## 1. Introduction

Multidrug-resistant strains of *Acinetobacter baumannii* have complicated the clinical management of infections caused by this pathogen and are a significant cause of morbidity in the healthcare setting [1]. The peptide antibiotic colistin is one of the few antimicrobials that retains activity against many multidrug-resistant *A. baumannii* strains, although the emergence of pandrug-resistant isolates with decreased susceptibility to all clinically available antibiotics, including colistin, has been described [2,3]. The main bacterial target for colistin is the lipooligosaccharide (LOS) on the Gram-negative outer membrane, which interacts with positively charged colistin molecules due to its high negative charge [4]. This interaction results in membrane disruption and bacterial cell death in a wide range of Gram-negative species [4].

*A. baumannii* can acquire resistance to colistin via the modification of LOS through the addition of ethanolamine moieties to lipid A mediated by horizontally acquired Mcr-1 or mutations resulting in increased activity of endogenous PmrC [3]. Additionally, *A. baumannii* can acquire resistance to colistin (MIC values of  $\geq 128$  mg/L) through the complete loss of LOS biosynthesis due to a mutation in the genes that encode the first three steps of lipid A biosynthesis, *lpxA*, *lpxC* and *lpxD* [5,6]. Since LOS is the bacterial component that binds colistin through electrostatic interactions, the complete loss of LOS results in high-level

resistance to colistin in *A. baumannii* [3–5]. Inactivation of these genes can occur through spontaneous point mutations, deletions and insertions, including the insertion of endogenous *ISAbA* insertion elements present in the *A. baumannii* genome [3]. *ISAbAs* are genetic elements present in some *A. baumannii* strains that typically encode a transposase and are flanked by sequence repeats, facilitating their spontaneous insertion in the *A. baumannii* genome [7].

In the present study, we aimed to characterize the frequency of spontaneous emergence of colistin resistance in different *A. baumannii* strains through *ISAbA*-mediated insertional inactivation of LOS biosynthesis genes. In addition, we characterized the effect of sub-inhibitory concentrations of clinically relevant antibiotics and disinfectants on the frequency of *ISAbA* insertion and identified a semi-conserved sequence motif at *ISAbA* insertion sites.

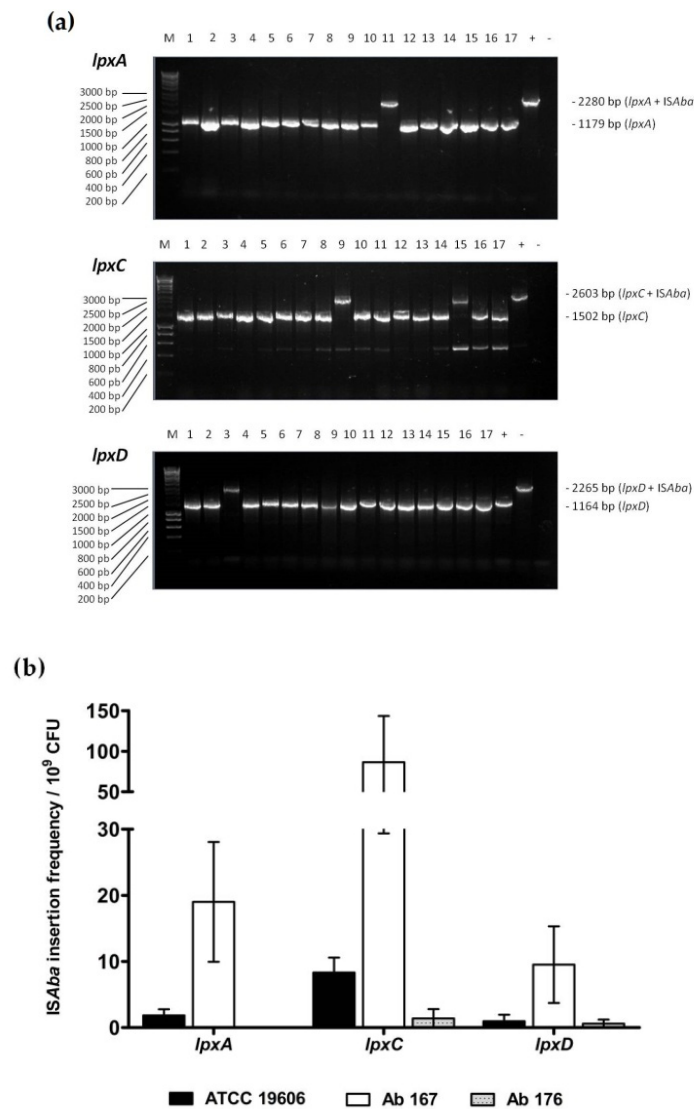
## 2. Results

### 2.1. Development of an Assay for Quantifying *ISAbA* Insertion Frequency in LOS Biosynthesis Genes

In order to characterize the spontaneous emergence of colistin resistance via *ISAbA*-mediated insertional inactivation of LOS biosynthesis genes under different conditions, a PCR-based assay was developed that permitted the identification and quantification of *lpxA*, *lpxC* and *lpxD* mutants harboring *ISAbA* elements. In this assay, *A. baumannii* strains are cultured under defined conditions (e.g., in the presence or absence of an antibiotic; see Materials and Methods) for 18 h and then plated on media containing 10 mg/L of colistin or media without antibiotic. Colistin-resistant derivatives that grow in the presence of 10 mg/L of colistin are isolated and subjected to PCR with primers flanking the *lpxA*, *lpxC* and *lpxD* genes (Table S1) in order to identify genes harboring the characteristic insertion of approximately 1.1 Kb that corresponds to *ISAbA* elements (Figure 1a). The presence of *ISAbA* elements in these amplicons is confirmed by DNA sequencing. The quantification of colistin-resistant derivatives containing *ISAbA* insertions and total bacteria in the culture permits calculation of the frequency of spontaneous *ISAbA* insertion in each gene under the conditions tested, and is expressed as the number of isolates harboring an *ISAbA* element/ $10^9$  colony-forming units (CFU).

### 2.2. Characterization of *ISAbA* Insertion Frequencies in LOS Biosynthesis Genes in Different *A. baumannii* Strains

Using the assay described above, we first aimed to characterize the baseline frequency of *ISAbA* insertion in *lpxA*, *lpxC* and *lpxD* for the ATCC 19606-type strain, which harbors *ISAbA11* [6], and two multidrug-resistant clinical isolates that have previously been shown to harbor *ISAbA* elements, Ab-167 and Ab-176 [8,9]. All strains were grown in the absence of antibiotics, and *ISAbA* insertion frequencies were quantified as described above in three independent biological replicates. As can be seen in Figure 1b, insertion frequencies for the ATCC 19606 strain were  $1.85 \pm 0.93$ ,  $8.34 \pm 2.25$  and  $0.98 \pm 0.98 /10^9$  CFU for *lpxA*, *lpxC* and *lpxD*, respectively. For the multidrug-resistant clinical isolate Ab-167, there was a tendency towards higher insertion frequencies in all genes compared to the ATCC 19606 strain, with frequencies of  $19.02 \pm 9.06$ ,  $86.41 \pm 57.04$  and  $9.52 \pm 5.79 /10^9$  CFU for *lpxA*, *lpxC* and *lpxD*, respectively. Finally, the Ab-176 strain demonstrated a tendency towards the lowest insertion frequencies of the three strains tested with no insertions detected in *lpxA*,  $1.41 \pm 1.41 /10^9$  CFU in *lpxC* and  $0.61 \pm 0.61 /10^9$  CFU in *lpxD*. Interestingly, there was a tendency towards higher insertion frequencies in *lpxC* compared to *lpxA* and *lpxD* for all three strains tested.

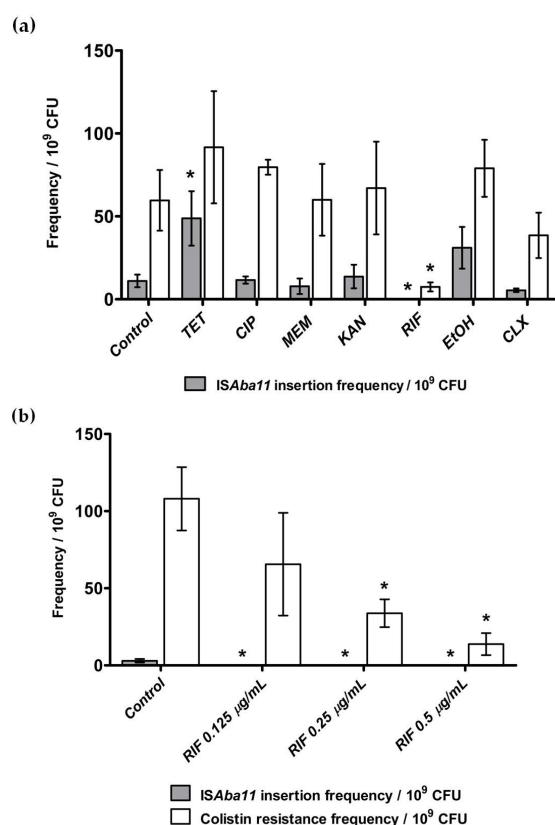


**Figure 1.** ISAbA insertion frequencies in *lpxA*, *lpxC* and *lpxD* genes in three *A. baumannii* strains. (a) Representative agarose gels showing PCR products obtained after amplifying *lpxA*, *lpxC*, and *lpxD* genes in order to screen colistin-resistant *A. baumannii* mutants for the presence of ISAbA insertions using the newly developed method described in the Materials and Methods section. (b) ISAbA insertion rates in *lpxA*, *lpxC* and *lpxD* genes in the *A. baumannii* strains ATCC 19606, Ab-167 and Ab-176 determined using the method described in the Materials and Methods section. M, DNA marker; +, positive control with a sequence-confirmed ISAbA11 insertion; −, negative control; bp, base pairs.

### 2.3. Effect of Antibiotics and Disinfectants on ISAbA11 Insertion Frequencies in *lpxC*

We next aimed to determine how subinhibitory concentrations of clinically relevant antibiotics and disinfectants affected ISAbA insertion frequencies in LOS biosynthesis genes. Based on the finding that insertion frequencies in *lpxC* tended to be higher than for *lpxA* and *lpxD* (Figure 1b), we used ISAbA insertion in *lpxC* in the ATCC 19606 strain for these studies. The ATCC 19606 strain harbors ISAbA11 in its genome [6], and its susceptibility profile permits the use of clinically relevant concentrations of multiple antimicrobials. The minimum inhibitory concentrations of five antibiotics (tetracycline, ciprofloxacin, meropenem, kanamycin and rifampicin) and two disinfectants (ethanol and chlorhexidine) were determined for the ATCC 19606 strain by broth microdilution. Subsequently, the effect of subinhibitory concentrations of these compounds was quantified by incubating cultures of the ATCC 19606 strain with one-quarter of the MIC of each compound and

determining *ISAb*11 insertion frequencies in *lpxC* using the assay described above. As can be seen in Figure 2a, the frequency of *ISAb*11 insertion in *lpxC* (grey bars) and the frequency of emergence of colistin resistance (white bars) was similar to control samples incubated without an antibiotic for ciprofloxacin, meropenem, kanamycin, ethanol and chlorhexidine. In contrast, incubation with tetracycline resulted in significantly increased *ISAb*11 insertion in *lpxC* ( $p = 0.04$ ), whereas incubation with rifampicin resulted in the complete absence of isolates with *ISAb*11 insertions in *lpxC* ( $p = 0.02$ ). The effect of subinhibitory concentrations of rifampicin on *ISAb*11 insertion in *lpxC* was further explored by characterizing the effect of 1/8 to 1/2 of the MIC of rifampicin. As can be seen in Figure 2b, all concentrations of rifampicin tested resulted in the complete absence of isolates with *ISAb*11 insertions in *lpxC*.



**Figure 2.** Effect of subinhibitory concentrations on *ISAb*11 insertion frequency in *lpxC*. (a) Frequency of spontaneous emergence of colistin resistance (by any mechanism; white bars) and the emergence of colistin-resistant mutants harboring *ISAb*11 insertions in *lpxC* (grey bars) after exposure to subinhibitory concentrations of different antibiotics and disinfectants at concentrations of one-quarter of the MIC; TET, tetracycline (0.5  $\mu$ g/mL); CIP, ciprofloxacin (1  $\mu$ g/mL); MEM, meropenem (0.5  $\mu$ g/mL); KAN, kanamycin (2  $\mu$ g/mL); RIF, rifampicin (0.5  $\mu$ g/mL); EtOH, ethanol (1.5%) and CLX, chlorhexidine (8 mg/mL). (b) Spontaneous emergence of colistin resistance (by any mechanism; white bars) and frequency of colistin-resistant mutants harboring *ISAb*11 insertions in *lpxC* (grey bars) after exposure to the indicated concentrations of rifampicin. Data represented as mean  $\pm$  standard error of the mean (SEM) of three independent experiments. (\*,  $p < 0.05$  compared to untreated controls).

#### 2.4. Analysis of *ISAb*11 Insertion Sites in *lpxC*

In order to characterize *ISAb*11 insertion sites in *lpxC*, amplicons containing *ISAb*11 in *lpxC*, obtained via PCR with primers flanking the *lpxC* gene as described in Supplementary Table S1, were subjected to DNA sequencing. Amplicons from a total of 32 derivatives of the ATCC 19606 strain containing *ISAb*11 insertions in *lpxC* were sequenced, resulting in the identification of 12 unique insertion sites within the *lpxC* gene (Figure 3). Interestingly,





hibitory concentrations of rifampicin, which inhibits bacterial RNA synthesis, completely inhibited the emergence of mutants with IS*Aba* insertions in *lpxC* at all concentrations tested (Figure 2b). We previously demonstrated that *A. baumannii* strains acquiring resistance to colistin via insertional inactivation of LOS biosynthesis genes become highly susceptible to rifampicin, with MIC values  $\leq 0.125$  mg/L [16]. These previous results may suggest that spontaneous mutants with IS*Aba*-mediated inactivation of LOS biosynthesis genes are not viable in the presence of the concentrations of rifampicin used in this study, thus explaining the complete absence of these mutants observed here (Figure 2).

The isolation of a large number of independent mutants with IS*Aba* insertions in *lpxC* allowed us to sequence multiple IS*Aba* insertion sites and assess insertion sites within *lpxC*. Our findings that IS*Aba* insertions clustered between nucleotides 382 and 618 of *lpxC* are consistent with a previous study suggesting two possible insertional hot spots within *lpxC* between nucleotides 390 and 393 and nucleotides 420 and 421 [6]. Sequence analysis of insertion sites also showed a bias towards A at the  $-3$  and  $-8$  positions and T at the  $-11$  position. These results may suggest that IS*Aba* insertion is sequence-biased in *A. baumannii*.

Taken together, the results of this study elucidate previously uncharacterized aspects related to the emergence of resistance to a last-resort antibiotic in a clinically important pathogen due to the insertional activation of genes by mobile genetic elements.

## 4. Materials and Methods

### 4.1. Strains and Growth Conditions

The colistin-susceptible *A. baumannii* ATCC 19606 strain and two previously described multidrug-resistant (colistin susceptible) clinical isolates, Ab-167 and Ab-176, which were previously shown to harbor IS*Aba* elements, were included in this study [8,9]. *A. baumannii* strains were routinely grown at 37 °C and maintained in Mueller–Hinton Broth II (MHBII) or Luria Bertani media (LB), unless otherwise stated.

### 4.2. Antibiotics, Disinfectants and Minimum Inhibitory Concentration Determination

Colistin sulfate (COL) and ethanol (EtOH) were purchased from PanReac AppliChem ITW Reagents. Ciprofloxacin (CIP), chlorhexidine (CLX), kanamycin sulfate (KAN), meropenem trihydrate (MEM), rifampicin (RIF) and tetracycline hydrochloride (TET) were purchased from Sigma-Aldrich. All antimicrobials were prepared in sterile milliQ water at the desired concentration. MIC values were determined for disinfectants and antibiotics. Overnight cultures of each strain were adjusted in MHBII to  $5 \times 10^5$  CFU/mL and MIC values were determined according to the recommendations of the Clinical and Laboratory Standards Institute guidelines [17].

### 4.3. Quantification of IS*Aba* Insertion in LOS Biosynthesis Genes

For determining IS*Aba* insertion frequencies, frozen stocks of *A. baumannii* strains were plated on LB media and incubated overnight at 37 °C, and colonies were used to inoculate a pre-culture of LB broth before incubation at 37 °C for 18 h. Pre-cultures were adjusted to an OD<sub>600</sub> of 2, and a 1:100 dilution was prepared in LB broth (with or without antibiotics, as indicated) before incubation for 18 h at 37 °C with agitation at 200 rpm. Spontaneous colistin-resistant mutants were identified by plating 100  $\mu$ L of the culture on MH agar plates containing 10 mg/L of colistin followed by incubation overnight at 37 °C. Colistin-resistant colonies were quantified and individually struck onto MH plates containing 10 mg/L of colistin for the detection of IS*Aba* insertion by PCR. Cultures were also quantitatively plated on LB agar with no antibiotics in order to determine the number of colony-forming units (CFU)/mL in the culture.

For the detection of IS*Aba* insertions in colistin-resistant isolates, genomic DNA was obtained from the colistin-resistant mutants by incubating a cellular pellet in 20  $\mu$ L of water at 98 °C for 10 min, followed by centrifugation at 13,000 rpm for two minutes. Supernatants were used to amplify the LOS biosynthesis genes *lpxA*, *lpxC* and *lpxD* using the primers

described by Moffat et al. [5] (Table S1). PCR products were separated on a 1% agarose gel to identify mutants with IS*Aba* insertions. Amplicons demonstrating the expected size corresponding to IS*Aba* insertions were sequenced in order to confirm the presence of an IS*Aba*. The IS*Aba* insertion rate was calculated using Equation (1).

$$\text{IS}Aba \text{ Insertion frequency} = \frac{\text{CFU/mL with IS}Aba \text{ insertion}}{\text{Total CFU/mL}} \times 10^9 \quad (1)$$

For assays assessing the effect of antibiotics and disinfectants, the following concentrations were employed: COL, 10 mg/L; TET, 0.5 mg/L; CIP, 1 mg/L; MEM, 0.5 mg/L; KAN, 2 mg/L; RIF, 0.5 mg/L; EtOH, 1.5%; and CLX, 8 mg/L.

#### 4.4. Sequencing of IS*Aba*11 Insertions in *lpxC*

Amplicons containing an IS*Aba*11 insertion in the *lpxC* gene from assays employing the ATCC 19606 strain were subjected to sequencing with the primers IS*Aba*11\_Fw\_Seq and IS*Aba*11\_Rev\_Seq (Table S1), and the insertion site was determined using the BioEdit sequence alignment editor.

#### 4.5. Statistical Analysis

The distribution of all datasets was assessed using the Shapiro-Wilk test. IS*Aba* insertion and colistin resistance frequencies between strains were compared using a one-way ANOVA, and differences between groups were determined using the Tukey post hoc test. A Mann–Whitney U-test was performed to assess differences in the frequencies of the emergence of colistin-resistant mutants harboring IS*Aba*11 insertions and of the colistin-resistance emergence. A *p*-value  $\leq 0.05$  was considered significant.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/article/10.3390/antibiotics10101259/s1>, Table S1: List of primers used in the present work.

**Author Contributions:** Conceptualization, H.O.-L., A.C.-L., and M.J.M.; methodology, H.O.-L., A.C.-L. and M.J.M.; formal analysis, H.O.-L., A.C.-L. and M.J.M.; investigation, H.O.-L., A.C.-L. and M.J.M.; resources, M.J.M.; data curation, M.J.M.; writing—original draft preparation, H.O.-L., A.C.-L. and M.J.M.; writing—review and editing, H.O.-L., A.C.-L. and M.J.M.; funding acquisition, M.J.M. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** Not applicable for studies not involving humans or animals.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author.

**Conflicts of Interest:** M.J.M. is a founder, shareholder, and has received research support from the biotechnology company Vaxdyn, S.L. Vaxdyn played no role in the present study. No other competing interest is declared.

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