Whole transcriptomic analysis of mechanisms of tolerance and resistance to chlorhexidine in clinical strains of *Klebsiella pneumoniae* producers of carbapenemase

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**Abstract:** Although the failure of antibiotic treatment is normally attributed to resistance, tolerance and persistence display a significant role in the lack of response to antibiotics. Due to the fact that several nosocomial pathogens show a high level of tolerance and/or resistance to chlorhexidine, in this study we analyzed the molecular mechanisms associated with chlorhexidine adaptation in two clinical strains of *Klebsiella pneumoniae* by phenotypic and transcriptomic studies. These two strains belong to ST258-KPC3 (high-risk clone carrying β-lactamase KPC3) and ST846-OXA48 (low-risk clone carrying β-lactamase OXA48). Our results showed that *K. pneumoniae* ST258-KPC3CA and ST846-OXA48CA strains exhibited a different behavior under CHLX pressure, adapting to this biocide through resistance and tolerance mechanisms, respectively. Furthermore, the appearance of cross-resistance to colistin was observed in the ST846-OXA48CA strain (tolerant to CHLX), using the broth microdilution method. Interestingly, this ST846-OXA48CA isolate contained a plasmid that encodes a novel type II toxin/antitoxin (TA) system, PemK/PemI. We characterized this PemK/PemI TA system by cloning both genes into the IPTG-inducible pCA24N plasmid, and found their role in persistence and biofilm formation. Accordingly, the ST846-OXA48CA strain showed a persistence biphasic curve in the presence of a chlorhexidine-imipenem combination, and these results were confirmed by the enzymatic assay (WST-1).

**Keywords:** Tolerance; Persistence; Cross-resistance; Toxin-antitoxin system; PemK/PemI; *Klebsiella pneumoniae*.

**Key Contribution:** Combination of resistance, tolerance and persistence mechanisms seen in clinical isolates under biocide and antimicrobial stress. PemK/PemI system TA system discovered in the plasmid carrying β-lactamase OXA48.
1. Introduction

The increase in antimicrobial resistance due to the emergence of multi-drug resistant (MDR) pathogens is one of the world’s greatest public health challenges, as it can lead to an era without effective antibiotics [1]. Recently, the World Health Organization (WHO) published a list of “priority pathogens”, which includes those microorganisms considered a serious threat to human health. Some members of this list are carbapenem-resistant pathogens and are known under the acronym of ESKAPE, including among other species, Klebsiella pneumoniae [1-3]. K. pneumoniae is a Gram-negative, opportunistic bacteria pathogen associated with a wide range of diseases such as urinary tract infections, pneumonias, septicemia, wounds and soft tissue infections [4]. Carbapenem resistance is increasing rapidly worldwide, particularly among K. pneumoniae. The main carbapenem-resistance mechanism is acquisition of plasmid-encoded carbapenemases, which may belong to the molecular class A (ie. KPC-type), B (ie. IMP-type, VIM-type, NDM-type) and D (ie. OXA-48-type). The high-risk clones of K. pneumoniae, in contrast to low-risk clones, have an extraordinary ability to persist and spread in the nosocomial environment, disseminating these carbapenemases and therefore being involved in nosocomial outbreaks [4].

Nevertheless, much less attention has been paid to the presence or occurrence of resistance to antiseptics and biocides, such as chlorhexidine (CHLX) [5], widely used in hospital settings. CHLX is a symmetric bis-biguanide molecule comprising two chloroguanide chains that are connected by a central hexamethylene chain, and carry two positive charges at physiological pH. CHLX is sparingly soluble in water, and thereby normally formulated with either acetate or gluconate to form water-soluble salts [6]. The antimicrobial effect of this compound is based on damaging the bacterial membrane, leading to the subsequent leakage of cytoplasmatic material. Therefore, mechanisms conferring resistance toward CHLX include multidrug efflux pumps and cell membrane changes [5]. Moreover, CHLX has been associated with the emergence of stable resistance to the last-resort antibiotic colistin (polymyxin E) [7-9].

In general, the failure of antibiotic treatments has been associated with resistance mechanisms. However, it has recently been noted that other mechanisms such as tolerance and persistence were also involved [10]. The recovery of persistent cells is one of the main causes of prolonged and recurrent infections, that can lead to the complete failure of antibiotic treatments.
In this context, it is important to distinguish between resistant, tolerant and persistent bacteria [12]. The term resistance is generally used to describe the inherited ability of a bacterial population to grow in the presence of high concentrations of antibiotics, regardless of the duration of treatment [12], due to active defense mechanisms associated with mutations [10]. Whereas, the term tolerance is used to describe the ability, inherited or not, of a bacterial population to survive the transient exposure of high concentrations of antibiotics without causing changes in MICs, due to the deceleration of essential biological processes [10-12]. It is important to emphasize that despite the slow-growth rate, tolerant bacteria keep a metabolically active state. In contrast to resistance and tolerance, persistence is characterized by the ability, not inherited, of a bacterial subpopulation (around 0.001%-1%) [10] to resist antibiotics by growth arrest due to the inactivation of their metabolism and their non-replicative state, thus entering into dormant state. Persistent bacteria exhibit transient levels of tolerance to antibiotics that do not affect their MICs, so once the drug pressure is removed and their metabolism is reactivated, they can rapidly re-grow. Nowadays, it is known that multiple molecular mechanisms are involved in the formation of persistent bacteria such as the stringent response molecule (p)ppGpp, stress response, SOS response, quorum sensing, toxin-antitoxin (TA) systems, efflux pumps, the ROS response and energy metabolism among others [11].

The involvement of TA systems in cell physiology, specifically in: (i) biofilm formation by regulating fimbriae [13], (ii) bacterial persistence, by generating slowly-growing cells tolerant to antibiotics and environmental changes [14, 15], (iii) plasmid maintenance [16], (iv) general stress response [17] and (v) phage inhibition [18] is becoming clearer [19]. A TA system is a module of two genes encoding a stable toxin and an unstable antitoxin. Under normal growth conditions the antitoxin inhibits the toxin, but under stress conditions the antitoxin is degraded, leaving the toxin free to inhibit the basic cellular processes like DNA replication or protein synthesis, and also promoting plasmid maintenance, slow growth and latency [19]. These systems are widely distributed and found in the bacterial chromosome, plasmids and bacteriophages [2].

In this context, this study provides a better comprehension of the molecular mechanisms associated with chlorhexidine adaptation (CA) in two clinical strains of K. pneumoniae, both of which produce carbapenemase: ST258-KPC3 (high-risk clone carrying β-lactamase KPC3) and
ST846-OXA48 (low-risk clone carrying β-lactamase OXA48), from a phenotypic and transcriptomic point of view. Moreover, this study aims to characterize a new toxin-antitoxin system (PemKI) located in a plasmid inside the ST846-OXA48CA strain, and to examine the possible role of this system in the establishing of persistence and in biofilm formation.

2. Results

2.1 Results subsection

2.1.1. Time-killing curve in presence of CHLX (10×MIC)

The time-killing curves of the strains ST258-KPC3CA and ST846-OXA48CA in presence of CHLX (10×MIC) showed two different growth patterns (Figure 1). The strain ST258-KPC3CA showed a slight reduction in its bacterial population, occurring in the first two hours of CHLX exposure, decreasing from $1.465 \times 10^7$ CFU/ml to $7.75 \times 10^5$ CFU/ml at 2 h (Figure 1A). In contrast, the ST846-OXA48CA strain, dramatically reduced its bacterial population during the first four hours of CHLX exposure, decreasing from of a bacterial population of $2.433 \times 10^7$ CFU/ml to $1.047 \times 10^3$ CFU/ml at 4 h (Figure1B). After this period, both bacterial strains grew again reaching respectively a bacterial population of $6.63 \times 10^7$ CFU/ml and $2.75 \times 10^6$ CFU/ml at 48 h. Both curves were characteristics of a resistant strain in the first case and a tolerant strain in the second one.

2.1.2. Transcriptomic study

All the transcriptomic results are deposited in the NCBI database as a GenBank BioProject (Code number: PRJNA609262) and GEO series (Code number: GSE147316). The transcriptomic profile from ST258-KPC3CA isolate, showed a CHLX resistant profile. Indeed, this strain has a higher number of overexpressed genes, specially efflux pumps such as methyl viologen resistance gene smvA (Log2FoldChange: 3.635). However, strain ST846-OXA48CA, showed a CHLX tolerant profile, with repressed genes for the energy metabolism, quorum network, efflux pumps, TA systems, SOS response and ppGpp mechanisms (Figure 2). This strain also showed high levels of expression of genes, pmrD and pmrK (Log2fold change 2.36 and 1.57, respectively), characteristics of the colistin resistance. Therefore, these transcriptomic results corroborated the results obtained by the time-killing curves, showing activation of molecular mechanisms of resistance and
tolerance molecular mechanisms in response to CHLX in ST258-KPC3CA and ST846-OXA48CA strains, respectively.

2.1.3. Antimicrobial susceptibility testing

The antimicrobial susceptibility test was done for strains ST258-KPC3 and ST846-OXA48 wild-type and the two CA strains. According to adaptation to CHLX, an increase in MICs of CHLX was observed in both CA strains. However, no differences in the minimum inhibitory concentration (MIC) values were observed for the antibiotics tested, except for the colistin, that in the ST846-OXA48CA strain showed an increase in the MIC value of 32-fold, that corresponds to a resistance value (Table 1).

2.1.4. Characterization of new TA system, PemK/PemI, present in a plasmid in the strain ST846-OXA48CA

A PemK/PemI TA system, whose closest relative is the type II toxin-antitoxin system PemK/MazF family toxin belonging to Enterobacteriacea (Query: 78%; Identity: 99.35%; Code number: WP_077688581.1) and which does not been previously described in K. pneumoniae, it was identified by transcriptomic analysis as encoded by a plasmid of K. pneumoniae ST846-OXA48CA. This system was composed of a 258 bp antitoxin gene (pemI) and a 333 bp toxin gene (pemK). To confirm that this system is a TA system, pemK/pemI and pemK genes alone were cloned into the overexpression vector pCA24N, widely used in the literature to overexpress TA systems [20, 21], and transformed into the cured plasmid strain ST846-OXA48CA CP (i.e. lacking plasmid and therefore the plasmid encodes PemK/PemI TA system). The toxicity of this TA system was tested by growth curves overexpressing both pemK/pemI and pemK (Figure 3). Overexpression of pemK in ST846-OXA48CA CP/pCA24N (pemK), inhibited bacterial growth during the first three hours, while overexpression of the pemK/pemI system (ST846-OXA48CA CP/pCA24N (pemKI)) in the strain led to normal bacterial growth, which was slightly impaired compared to the empty plasmid. Therefore plasmid-based PemK/PemI TA system found in K. pneumoniae ST846-OXA48CA is functional.
2.1.5. Biofilm formation assay

Since TA systems have been associated with the arrest of bacterial growth and the formation of biofilms, we studied the effect of the PemK/PemI TA system and the PemK toxin on biofilm formation (Figure 4). Production of PemK toxin resulted in a significant decrease in biofilm formation compared to the control (ST846-OXA48CA CP/pCA24N) (p-value < 0.001). Moreover, production of PemK/PemI restored a similar phenotype as the control, lacking a significant difference in biofilm formation (p-value < 0.05). Therefore, PemK/PemI TA system influences *K. pneumoniae* biofilm formation.

2.1.6. Time-killing curve in the presence of imipenem or in combination with chlorhexidine for ST846-OXA48, ST846-OXA48CA and ST846-OXA48CA CP

The time-killing curves of *K. pneumoniae* ST846-OXA48CA tolerant to CHLX (Figure 5) were performed in the presence of imipenem (IMP) (50×MIC) alone or in combination with CHLX (10×MIC) (Figure 5).

A drastic reduction in the number of CFU was observed during the first three hours in presence of IMP (50×MIC), decreasing from 2.13×10⁷ to 6×10⁴ CFU/ml. However, bacterial regrowth occurred after four hours, reaching the same level of CFU/ml as the control (2.425×10⁹ vs 3.375×10⁹ CFU/ml) at 28 h and exceeding it at 48 h (2.675×10⁹ vs 1.02×10⁹ CFU/ml). In the case of the combination of IMP and CHLX, a greater CFU reduction than IMP alone was observed, with no CFU detected after 4 h. Nevertheless, a regrowth of the bacterial population was observed after 28 h. Thus, ST846-OXA48CA in presence of the combination of IMP and CHLX showed a characteristic behavior of the persistent strains.

In the case of the ST846-OXA48CA CP strain, in which the plasmid was removed by means of a curing agent, 3% sodium dodecyl sulfate (SDS) (10% W/V pH = 7.4), a drastic reduction in the number of CFU was observed at 4 h with both IMP and CHLX, when no CFUs were recovered (Figure 5). Finally, the culture was considered dead as no regrowth was observed throughout the rest of the assay. Thus, the ST846-OXA48CA CP strain unlike the ST846-OXA48CA strain showed a sensitive curve pattern in the presence of the combination of IMP and CHLX. These results may suggest that the absence of the TA PemK / PemI system could be a factor responsible for the non-appearance of a persistent subpopulation.
2.1.7. **Enzymatic analysis using the cell proliferation reagent WST-1**

The results of the time-killing curve in the presence of IMP and CHLX combination was confirmed by enzymatic analysis using the cell proliferation reagent WST-1 (Figure 6), which measures the omnipresent reducing agents NADH and NADPH as biochemical marker to evaluate the metabolic activity of the cell [22]. Indeed, ST846-OXA48CA lacks metabolic activity/cell proliferation at 24 h (OD_{480} nm < 0.01), whereas it presents a significant increase at 48 h (OD_{480} nm > 0.4; p-value < 0.0001), confirming regrowth in the bacterial culture. In contrast, the ST846-OXA48CA CP strain, despite showing significant differences (p-value < 0.002) between 24 and 48 h in terms of metabolic activity/cell proliferation, it is considered as a dead culture since its OD_{480} nm is less than 0.1.
2.2 Figures and Tables.

**Figure 1.** Time-killing curve in the presence of CHLX (10×MIC) in *K. pneumoniae* CA strains ST258-KPC3CA (A) and ST846-OXA48CA (B). The same strains without being exposure to biocide pressure are used as controls.
Figure 2. Number of tolerance-related genes expressed (light grey) and repressed (dark grey) in the strain ST258-KPC3CA (A) and ST846-OXA48CA (B) K. pneumoniae strains. The transcriptomic results confirmed that ST258-KPC3CA and the ST846-OXA48CA were resistant and tolerant strains to CHLX, respectively, confirming the results obtained by time-killing curves.
**Table 1.** MIC values (μg/ml) of different antibiotics for ST258-KPC3, ST258-KPC3CA, ST846-OXA48 and ST846-OXA48CA.

<table>
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<th>TGC</th>
<th>TOB</th>
<th>IMP</th>
<th>MRP</th>
<th>GEN</th>
<th>CAZ</th>
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<td>4</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>1024</td>
<td>128</td>
<td>2</td>
<td>16</td>
<td>4</td>
<td>0.25</td>
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<tr>
<td>ST258-KPC3CA</td>
<td>1/1024</td>
<td>&gt;32</td>
<td>2</td>
<td>64</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>1024</td>
<td>128</td>
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<td>4</td>
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<tr>
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<td>8</td>
<td>8</td>
<td>32</td>
<td>16</td>
<td>&gt;32</td>
<td>32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>128</td>
<td>16</td>
<td>8</td>
<td>2</td>
<td>8</td>
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<td>ST846-OXA48CA</td>
<td>1/512</td>
<td>8</td>
<td>8</td>
<td>32</td>
<td>16</td>
<td>&gt;32</td>
<td>32</td>
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<td>&gt;32</td>
<td>128</td>
<td>16</td>
<td>8</td>
<td>2</td>
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<td>16</td>
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CHLX, Chlorhexidine; CIP, Ciprofloxacin; TGC, Tigecycline; TOB, Tobramycin; IMP, Imipenem; MRP, Meropenem; GEN, Gentamycin; CAZ, Ceftazidime; TZP, Piperacillin-tazobactam; SAM, Sulbactam; NET, Netilmicin; AMK, Amikacin; MIN, Minociclin; CST, Colistin.
Figure 3. Growth curve of ST846-OXA48CA CP containing pCA24N plasmids with pemKI (dark grey, dashed line) and pemK (light grey, dotted line) in the presence of 1 mM IPTG. The strain ST846-OXA48CA CP is used as a control as it carries the empty plasmid pCA24N (black).
Figure 4. Biofilm formation assay. (A) Biofilm strained with 10% crystal violet was dissolved in 30% acetic acid. (B) Optical density of Biofilm produced by the strain ST846-OXA48CA CP/pCA24N, ST846-OXA48CA CP/pCA24N (PemKI) and ST846-OXA48CA CP/pCA24N (PemK). The biofilm formation was expressed as the ratio between OD580/600nm, in order to normalize the data. *, p-value < 0.05.
Figure 5. Time-killing curve in the presence of IMP (50×MIC) for *K. pneumoniae* ST846-OXA48, and time-killing curve in presence of the combination of IMP (50×MIC) and CHLX (10×MIC) for the strains of *K. pneumoniae* ST846-OXA48CA and ST846-OXA48CA CP. The control is the strain ST846-OXA48CA without exposure to any stress (IMP, IMP+CHLX).
Figure 6. Enzymatic activity by colorimetric assay (WST-1 based) of the strain *K. pneumoniae* ST846-OXA48CA and ST846-OXA48CA CP in presence of the combination of IMP (50×MIC) and CHLX (10×MIC). The growth control is ST846-OXA48CA strain without antibiotic pressure. ***, p-value < 0.001 and ****, p-value < 0.0001.
<table>
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<th>Reference</th>
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<td></td>
</tr>
<tr>
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<td>5’-3’</td>
<td>This study</td>
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<td>PemK_Fow(BseRI)</td>
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<td>5’-3’</td>
<td>This study</td>
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<td><strong>Oligonucleotide for sequencing</strong></td>
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</table>
3. Discussion

Due to the emergence of MDR pathogens over the past few decades, public health faces new challenges, such as the alarming increase in antimicrobial resistance, as well as the emerging link between resistance strategies used by bacteria against antibiotics and biocides [19]. This last problem is even more worrisome due to the routinely and uncontrolled use of antiseptics and biocides in clinical practice [9]. One example of this is CHLX, a bis-biguanide antiseptic of cationic nature that has bactericidal activity through membrane disruption [23]. For these reasons is of great interest to decipher the molecular mechanisms involved in the adaptation to CHLX in clinical strains of *K. pneumoniae*, producers of carbapenemases.

In order to determine the molecular effect of the adaptation to CHLX in a strain of *K. pneumoniae*, we performed a phenotypic study in presence of CHLX (10×MIC), which showed that the adaptation to CHLX led to the activation of two different molecular mechanisms in the clinical strains of *K. pneumoniae* ST258-KPC3CA and ST846-OXA48CA. In effect, the ST258-KPC3CA strain presented a growth curve typical of resistant bacteria, where a slight reduction in the bacterial population occurs during the time of activation of defense mechanisms (e.g., efflux pumps, TA systems, quorum network), followed by a regrowth period similar to the control. In contrast, ST846-OXA48CA had a characteristic growth curve of tolerant bacteria, where the strain undergoes a drastic reduction or arrest of growth during the first four hours of exposure to the bactericide [23]. These results were corroborated by the transcriptomic study where the transcriptomic profile of the ST258-KPC3CA strain revealed the overexpression of a larger number of genes compared to ST846-OXA48CA strain, especially the overexpression of efflux pumps, which is generally considered as a basic molecular mechanism associated with resistance [25]. In our study, the efflux pump gene that was overexpressed in ST258-KPC3CA was the methyl viologen resistance gene *smvA*. *SmvA* is responsible for resistance to CHLX in *K. pneumoniae* strains previously adapted to CHLX, due to the interruption of the *smvR* gene (*smvA* repressor) [9].

The antimicrobial susceptibility test revealed the appearance of cross-resistance to colistin (polymyxin E), in the ST846-OXA48CA strain tolerant to CHLX, increasing its MIC value 32-fold. These results were also corroborated by the transcriptomic studies, in which a high level of expression of the colistin resistance genes, *pmrD* and *pmrK* could be observed. This phenomenon of cross-resistance was also described previously, where five out of six strains of *K. pneumoniae* adapted to CHLX also presented resistance to colistin, increasing their MIC values from 2-4 mg/l to 64 mg/l [9]. This phenotype is due to the common biochemical characteristics of CHLX and colistin: in fact, both are cationic compounds with hydrophobic function [6]. As for the other antibiotics
tested, no change in the MIC value was observed in the strain adapted to CHLX compared to the wild-types.

In the previous study of Fernández-García et al. (2018), a combination of β-lactam antibiotic IMP with the CHLX biocide caused in some strains of *Acinetobacter baumannii* the formation of a subpopulation of persistent bacteria [12]. This phenomenon could be observed in the ST846-OXA48CA strain in the presence of the same combination. However, we have seen that the combination of this β-lactam antibiotic and the biocide did not lead to the appearance of a persistent population in ST846-OXA48 CA CP, cured of the plasmid, but rather led to the death of the bacterial culture after 4 h. Thus suggests that the presence of the TA system PemK/PemI in the plasmid should be responsible of the emergence of a persistent subpopulation. As it has long been shown that TA systems are involved in the formation of persistent bacterial subpopulations [14]; indeed, TA systems are genetic elements composed of a toxin, which inhibits bacterial growth by interfering with essential cellular processes, and an antitoxin, which able to neutralize the effect of the toxin in normal growth conditions [11, 19, 26]. The pemK/pemI genetic module present in the plasmid of *K. pneumoniae* ST846-OXA48CA, has never been described in *K. pneumoniae*. This TA system was characterized and overexpression assays confirmed that this module corresponded to a TA system. The overexpression of the *pemK* gene led to the inhibition of bacterial growth during the first three hours; however, overexpression of the *pemKI* module led to normal bacterial growth. The same phenomenon was observed in *Bacillus anthracis* where the overexpression of *pemK* in the pHCMC05 vector was severely toxic to the growth of *B. anthracis* cells [27].

In recent decades many studies have shown that TA systems are associated with the formation of biofilm [17, 28]. Biofilm is characterized by a dense multicellular community of microorganisms, constituted after the attachment of bacteria to a biotic or an abiotic surface [29]. In fact, the first TA system linked to biofilm formation was the MqsR/MqsA system of *Escherichia coli* [30]. In this study, we have seen that the overexpression of the PemK toxin contributed to a significant decrease in biofilm formation. The effect that we observed for the toxin is supported by the data found by Ma et al. (2019), where the disruption of MazF toxin, in *Staphylococcus aureus*, led to an increase in biofilm formation [31].

4. Conclusions

In conclusion, this is the first study that describes the different effects of the adaptation to CHLX in two clinical strains of *K. pneumoniae*, producers of carbapenemase, becoming resistant (ST258-KPC3CA) and tolerant (ST846-OXA48CA) to CHLX. This adaptation has lead, in the case of ST846-OXA48CA strain, to the development of cross-resistance to colistin, an antibiotic of last resort in hospital’s infections.
Furthermore, this study is the first one to describe the relationship between the mechanisms of bacterial persistence and the combination of a β-lactam antibiotic (IMP) and a biocide (CHLX) in the clinical isolate of K. pneumoniae ST846-OXA48CA. And finally, a new PemK/PemI TA system was identified in a plasmid of the ST846-OXA48CA strain. Its subsequent characterization demonstrated it participation in the development of persister cell as well as the establishment of biofilm.

5. Materials and Methods

5.1. Bacterial strains and CHLX adaptation

Two clinical strains of K. pneumoniae, producers of carbapenemases harbouring in plasmids, ST258-KPC3 (high-risk clone) and ST846-OXA48 (low-risk clone) were used in this study. These clinical strains belonging to different sequence types (ST) came from urine and sputum samples, respectively. The attribution of the ST was carried out in the study of Esteban-Cantos et al. (2017) according to the scheme of the Pasteur Institute (http://bigsdb.web.pasteur.fr/Klebsiella)[32].

In order to obtain the CHLX adapted strains, the clinical strains of K. pneumoniae clinical ST258-KPC3 and ST846-OXA48, were exposed for two weeks to ¼ of their MIC of CHLX 1/4096 and 1/2048 μg/ml, respectively. After this exposure, two CA strains were obtained with MIC values of 1/1024 and 1/512 μg/ml, respectively.

Furthermore, to study the role of the plasmid encoded PemK/PemI TA system in the ST846-OXA48CA strain, plasmids were removed from the strain. The cured plasmid ST846-OXA48CA CP strain of K. pneumoniae was generated following the protocol of El-Mansi et al. (2000) [33], 3% sodium dodecyl sulfate (SDS) (10% W/V pH = 7.4) was used as a curing agent. To check the effective loss of the plasmid, PCR was carried out using the verification primers and, to corroborate the results, plasmid extractions were also performed and subsequently loaded on a 1% agarose gel.

5.2. Time-killing curve

The different time-killing curves were performed following the indications of Hofsteenge et al. (2016) [8] in low-nutrient Luria-Bertani (LN-LB) broth (2 g/l tryptone, 1 g/l yeast extract and 5g/l NaCl). The culture was incubated at 37°C with shaking (180 rpm) until it reached the optical density at 600 nm (OD̄600nm) of 0.6. At that moment CHLX digluconate (10×MIC) (Sigma-Aldrich), IMP (50×MIC) (Sigma-Aldrich) or the combination of IMP (50×MIC) and CHLX (10×MIC), were added. Bacterial concentrations (CFU/mL) were determined at 0,1,2,3,4,20,24,28 and 48 h by serial dilutions and plating on LB agar (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl and 20 g/l agar). All experiments were performed in triplicates.

5.3. Transcriptomic study
After RNA extraction of the four replicates of each strain ST258-KPC3, ST258-KPC3CA, ST846-OXA48 and ST846-OXA48CA, rRNA and tRNA were removed using the Ribo-Zero rRNA removal kits (bacteria) (Illumina). The rRNA depletion was checked using Agilent RNA ScreenTape Assay (TapeStation 4200 Agilent) and the RNA was quantified by Qubit TM RNA HS Assay Kit (Thermo Fisher Scientific). Then, the transcriptomic libraries were performed using the Ion RNAseq Kit v2 in combination with the Ion Xpress TM RNA-Seq Barcode 01-16 Kit. Sequencing was carried out by emulsion PCR with Ion Sphere Particles (IPs). The enrichment of the library carrying IPs and the subsequent loading of the chip was developed in the Ion Chef automated system using the Ion PI Hi-Q Chef kit (Thermo Fisher Scientific) and Ion PI chips v3 (Thermo Fisher Scientific). Chip analysis was carried out on the Ion Proton sequencer (Thermo Fisher Scientific). The generated data was analyzed using the specific software of the Torrent Suite 5.6.2 platform (Thermo Fisher Scientific). The resulting readings (approx. 60 million) were exported in a FASTQ file using the FileExporter 4.6.0.0 (Thermo Fisher Scientific) plugin. The alignment of the sequences against their respective controls was performed with the STAR software. Subsequently, the aligned readings were counted using the htseq-count software (HTSeq 0.6.1.p2). All data was normalized using DESeq2.

5.4. Antimicrobial susceptibility test

MIC values of chlorhexidine, ciprofloxacin, tigecycline, tobramycin, imipenem, meropenem, gentamycin, piperacillin-tazobactam, ceftazidime, sulbactam, netilmicin, doxycycline, amikacin, minocycline and colistin were determined by broth microdilution according to Clinical and Laboratory Standards Institute (CLSI) 2018 [34].

5.5. Construction of pCA24N (penKI) and pCA24N (penK)

The plasmids pCA24N (penKI) and pCA24N (penK) were constructed by amplifying the pemK/pemI module and the pemK gene in the expression vector pCA24N (cmR; LacIq) [35] inducible by IPTG (Fisher Scientific). The insertion of these genes was performed at the BseRI and NotI restriction sites under the control of T5-lac promotor (Table 2). Final constructions were verified by DNA sequencing.

5.6. Toxicity assay

The toxicity assay was performed as previously described Wood T.L. and Wood T.K. (2016) [36] with certain modifications. Overnight cultures of K. pneumoniae strains ST846-OXA48CA CP/pCA24N, ST846-OXA48CA CP/pCA24N (penKI) and ST846-OXA48CA CP/pCA24N (penK) were inoculated into 25 mL of LB broth medium with chloramphenicol (60 μg/ml; Sigma Aldrich) to maintain the plasmid. IPTG (1 mM) was added when OD<sub>600</sub>nm was 0.3. For 180 min, the OD<sub>600</sub>nm was measured to determine growth evolution.
5.7. Biofilm formation assay

The biofilm formation assay was performed in a 96-well polystyrene plate for 30 h. Briefly, cells of ST846-OXA48CA CP/pcA24N, ST846-OXA48CA CP/pCA24N (pemKI) and ST846-OXA48CA CP/pCA24N (pemK) were inoculated at OD\textsubscript{600}nm was 0.05 and incubated at 37ºC without shaking. After 6 h, IPTG at 1mM was added and cells were incubated at 37ºC without shaking. 0.4 mM of IPTG was added every 8 h to avoid its degradation and to be able to observe the action of either the TA system or the toxin, as they are under an IPTG inducible promoter. Then, the cell density (OD\textsubscript{600}nm) and total biofilm (OD\textsubscript{580}nm) were measured by using 10% crystal violet staining, and quantified in a NanoQuant plate reader. Normalized biofilm was calculated by dividing the total biofilm by the bacterial growth for each strain. Two biological replicates of each strain and five technical replicates were made.

5.8. Enzymatic assay using the cell proliferation reagent WST-1

The cell proliferation/metabolic activity of the ST846-OXA48CA and ST846-OXA48CA CP strains in presence of the combination of IMP (50×MIC) and CHLX (10×MIC) was analyzed using a colorimetric enzymatic assay based on the water soluble tetrazolium salt (WST-1) reagent and electron mediators (Roche, Germany). Tetrazolium salts have become some of the most widely used tools in cell biology for measuring the metabolic activity of cells ranging from mammalian to microbial origin [37, 38]. Briefly, the cultures of ST846-OXA48 and ST846-OXA48CA CP were incubated at 37ºC with shaking (180 rpm) until OD\textsubscript{600}nm was 0.6. At that moment the combination of IMP and CHLX, was added. After 24 and 48h of antibiotic exposition and two washing, the culture cell (OD\textsubscript{600}nm= 0.1) was put in 96-well polystyrene plate (Corning Incorporated) and 10 μl of the reagent was added. After 1h of incubation at 37ºC without shaking and 10 min with shaking (180 rpm), the optical density was measured at OD\textsubscript{480}nm. The ST846-OXA48CA strain without antibiotic addition was taken as a control. The OD\textsubscript{480}nm of the medium culture (LN-LB) in the presence of WST-1 reagent was used to normalize all data. Two biological replicates of each strain and five technical replicates were performed.

5.9. Statistical analysis

Statistical analysis was based on the number of populations and comparisons. A Student’s t-test was used to compare two populations. All statistical analysis were performed using the GraphPad (Prism 8) software.

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