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Microbial interaction between a CTX_{M-15}-producing *Escherichia coli* and a susceptible *Pseudomonas aeruginosa* isolated from bronchoalveolar lavage: influence of cefotaxime in the dual-species biofilm formation

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Summary

Two isolates, *Escherichia coli* ella00 and *Pseudomonas aeruginosa* ella01, obtained from bronchoalveolar lavage, were found to be closely associated in clusters in agar medium. *Escherichia coli* ella00 was multidrug resistant and CTX_{M-15} extended-spectrum β-lactamase producer, while *P. aeruginosa* ella01 was susceptible to all antimicrobials tested. These observations impelled for further studies aimed to understand their microbial interaction. The *P. aeruginosa* ella01 biofilm-forming capacity was reduced and not affected when it was co-cultured with *E. coli* ella00 and *E. coli* ATCC 25922 respectively. Interestingly, the co-culture of ella isolates in the presence of high concentrations, such as 160 μg ml^{-1}, of cefotaxime allowed the formation of more biofilm than in the absence of the antibiotic. As revealed by fluorescence in situ hybridization, in co-culture, *P. aeruginosa* ella01 survived and subsequently flourished when exposed to this third-generation cephalosporin at a concentration 10 × higher than its minimum inhibitory concentration (MIC), and this was mostly due to β-lactamases production by *E. coli* ella00. In fact, it was demonstrated by high-performance liquid chromatography that cefotaxime was absent for the culture medium 4 h after application. In conclusion, we demonstrate that bacterial species can interact differently depending on the surrounding conditions (favourable or stressing), and that those interactions can switch from unprofitable to beneficial.

Introduction

Though a huge number of signalling and regulatory networks operate at every organizational level of living matter, from the cell to ecosystems (Aminov, 2009), little is currently known about bacterial multispecies interactions and how antimicrobial drugs shaped this continuous cross-talk, especially among clinical isolates. Interactions can be either positive or negative for the partakers (Faust and Raes, 2012), and are normally classified as antagonistic, when there is competition for nutrient resources, or cooperative, when production of molecules (Dimitriu *et al.*, 2014), transfer of metabolites or quorum sensing (QS) (Hibbing *et al.*, 2010) benefit the growth of neighboring cells. Bacteria that cohabit also easily share genes, even among distinct species (Dimitriu *et al.*, 2014). Regularly, a balance between competition and cooperation is established through the formation of biofilms (Nadell *et al.*, 2009). Most research on bacterial pathogenesis has focused on bacteria existing as single, independent cells (planktonic) causing acute infections; however, these infectious diseases are now being approached as chronic infections caused by bacteria growing in slime-enclosed aggregates, known as biofilms (Bjarnsholt, 2013). Within these biofilms, bacteria are well protected from antibiotics and other antimicrobial agents, as well as from the host immune defence (Valle

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et al., 2013), and thereby become extremely difficult or impossible to eradicate (Burmølle *et al*., 2010; Bjarnsholt, 2013). In nature (e.g. marine, oral and clinical microbial ecosystems), antibiotic-sensitive bacteria have been reported to coexist with antibiotic-producing bacteria in biofilms (Narisawa *et al*., 2008).

Today, we are in a situation where antimicrobial-resistant bacteria have been spreading rapidly worldwide, leading to antibiotic treatment failures. One potential strategy for delaying the spread of antimicrobial resistance would be to identify the infection-causing pathogen and perform antimicrobial susceptibility testing in order to appropriately select the treatment. Nevertheless, in many cases, testing bacterial isolates as single cultures may not mirror the real situation, since bacterial interactions are not taken into account.

The aim of this study was to characterize a microbial interaction between *Escherichia coli* and *Pseudomonas aeruginosa* that were isolated from a bronchoalveolar lavage of a dog with signs of chronic bronchitis. Antimicrobial susceptibility testing for each isolate was performed, and the ability to form biofilm in single culture or co-culture, and also in presence or absence of the antibiotic cefotaxime (CTX), was evaluated by biofilm biomass quantification and by microscopic visualization through the fluorescence in situ hybridization (FISH) technique.

Results and discussion

A bronchoalveolar sample obtained from a dog with signs of chronic bronchitis that was under amoxicillin plus clavulanic acid treatment for 7 weeks prior to the moment of bronchoalveolar lavage was processed for routinely microbiological analysis. The culture of that clinical sample revealed an interesting appearance of two closely associated distinct colonies on agar medium (i.e. one colony type always immediately adjacent to the other type) that caught our attention and led us to explore the identity of the isolates and how they interact (Fig. S1). The isolated strains were subsequently identified by biochemical tests as *E. coli*, named ella00, and *P. aeruginosa*, named ella01.

The resistance patterns of both clinical isolates were determined by the agar disk-diffusion method (CLSI, 2013). *Escherichia coli* ella00 was resistant to β-lactam/β-lactamase inhibitor combinations (except carbapenems and cephamycins), tetracycline, trimethoprim-sulfamethoxazole and fluoroquinolones and was susceptible to aminoglycosides, chloramphenicol and nitrofurantoin. On the contrary, *P. aeruginosa* ella01 was susceptible to all antimicrobials tested (ceftazidime, gentamicin, tobramycin, piperacillin, amikacin, aztreonam, cefepime, ciprofloxacin (CIP), levofloxacin, imipenem, meropenem, piperacillin-tazobactam and ticarcillin). Moreover, an extended-spectrum β-lactamase (ESBL) phenotype in *E. coli* ella00 culture was observed on plate agar after performing the phenotypic confirmatory test (CLSI, 2013). This outcome impelled us for the genetic characterization of the β-lactamase groups present by performing a multiplex polymerase chain reaction (PCR) according to Dallenne and colleagues (2010), which revealed that *E. coli* ella00 carried the β-lactamase groups OXA, CTX-M-1 and CIT. The $bla_{\text{CTX-M-15}}$ gene encoding the ESBL was identified after DNA sequencing. Based on this result and since CTX-M type ESBLs preferentially hydrolyses CTX over ceftazidime, we decided to use CTX for the further studies. Additionally, the phylogenetic group of *E. coli* was also determined by performing a multiplex PCR according to Clermont and colleagues (2013). *Escherichia coli* ella00 belonged to phylogenetic group C, which is closely related to phylogroup B1, considered to comprise non-pathogenic strains.

Both isolates are well-known respiratory pathogens, although the presence of *P. aeruginosa* in our case was unexpected due to the fact that the dog was being treated with a combination of drugs to which that isolate was susceptible. *Pseudomonas aeruginosa* is typically responsible for biofilm-associated infections (Marsh *et al*., 2014) and it has been demonstrated that multicellular organized assemblies in bacteria confer an advantage when facing antibiotics compared with planktonic cells (Stewart and Costerton, 2001; Lewis, 2007).

Therefore, it seemed appropriated to study the biofilm ability of these isolates. Biofilm formation of the clinical isolates, as well as of reference strains (ATCC strains) as single species or in dual-species combination, was induced in 96-well flat-bottomed microtiter plates for 24 h at 37°C after inoculation with fresh bacterial suspensions of 1×10^6 cfu ml^{-1} ($OD_{600} = 0.1$).

The ability to form a biofilm and the biofilm biomass quantification (Gomes *et al*., 2014), through the crystal violet assay, of both clinical isolates and reference strains (used for comparison studies) are shown in Fig. 1A. *Escherichia coli* strains did not form a biofilm within 24 h, whereas *P. aeruginosa* strains were fair producers of biofilm, with *P. aeruginosa* ATCC 27853 being a much stronger producer than *P. aeruginosa* ella01 (differences were statistically significant for a $P < 0.05$). Dual-species combinations using all four strains (Fig. 1B) revealed that when *P. aeruginosa* ella01 was present in a co-culture with *E. coli* (ATCC or ella00), little biofilm was formed in comparison to when *P. aeruginosa* ella01 was alone. Inversely, *P. aeruginosa* ATCC 27853 in co-culture with *E. coli* formed more biofilm, than when it was cultured alone.

Differences between the 24 h dual-species biofilm of ella isolates and ATCC strains, formed in 35 mm diameter polystyrene plates, could also be observed directly by

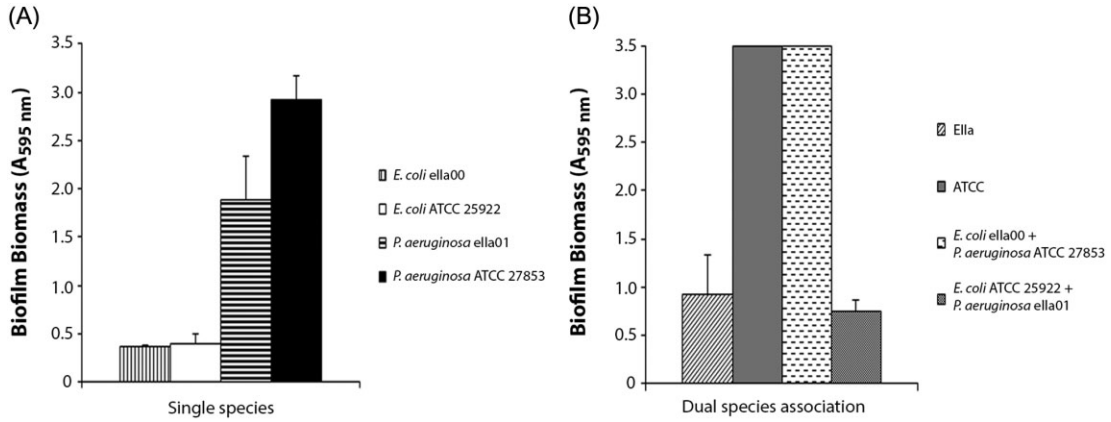

Fig. 1. Quantification and comparison of biofilm biomass produced after 24 h, using the crystal violet assay, by single species (A) or dual-species association (B).

microscopy after Live/Dead staining (Baffoni *et al.*, 2012) as shown in Fig. 2. The biofilm formed by ATCC strains was more consistent and organized and all cells were viable after 24 h, whereas the biofilm produced by ella isolates had very small aggregates and a reasonable number of dead cells; it could not be considered a well-established biofilm.

Subsequently, we selected two antibiotics (CTX and CIP) to which *E. coli* ella00 was resistant, determined their minimum inhibitory concentration (MIC) values (Table 1) and assessed the biofilm formation of single and dual species in presence of diverse concentrations of those antibiotics (added to the growth medium at time 0 h). *Pseudomonas aeruginosa* (both ella and ATCC) in the presence of sub-MICs of CTX (8 and 4 μg ml^{-1}) (Fig. 3A and B) formed more biofilm than in the absence of any antibiotic (control) (Fig. 1A). This result is not unexpected since there have been several reports of the increase in biofilm formation by Gram-negative bacteria in the presence of subinhibitory concentrations of antibiotics (Hoffman *et al.*, 2005; Kaplan, 2011).

However, the association of *P. aeruginosa* ella01 with *E. coli* ella00 (Fig. 3A) did not allow the production of such a biofilm (the biomass formed was significantly lower, $P < 0.05$), with the biofilm produced under these conditions being similar to the ella dual-species biofilm in absence of antibiotics (Fig. 1B).

Interestingly, it was observed that the association of both ella isolates in the presence of a range of concentrations of CTX that were higher than the MIC for *P. aeruginosa* ella01, but below the MIC value for *E. coli* ella00, formed more biofilm than in the absence of the antibiotic (Fig. 3C). In addition, when the planktonic phase from those dual-species biofilms was spread onto Hektoen Enteric agar, we observed the growth of both *E. coli* (yellow colonies) and *P. aeruginosa* (green to brown colonies) (data not shown). This outcome was confirmed using the FISH technique that demonstrated the survival of *P. aeruginosa* ella01 within the biofilm, when associated with *E. coli* ella00, in the presence of 160 μg ml^{-1} of CTX (Fig. 4). Indeed, under this condition, we observed an atypical morphology of the bacteria cells

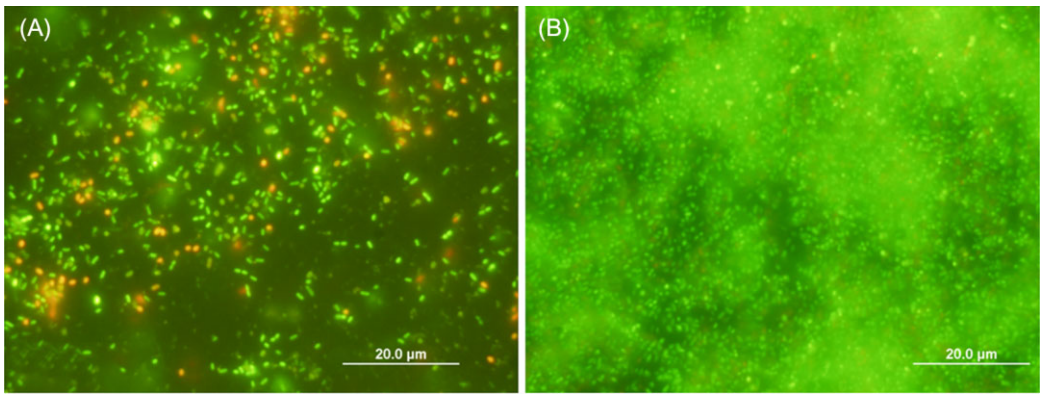

Fig. 2. Microscopic visualization of 24 h biofilms of dual-species association of ella isolates (A) or ATCC strains (B) after Live/Dead staining.

Table 1. Minimum inhibitory concentrations of cefotaxime (CTX) and ciprofloxacin (CIP) for the isolates and strains used in this study.

	MIC (μg ml^{-1})	
	CTX	CIP
E. coli ella00	>1500	128
P. aeruginosa ella01	16	0.5
Both ella species	>1500	128
E. coli ATCC 25922	<0.0625	0.0156
P. aeruginosa ATCC 27853	16	0.25
Both ATCC species	16	0.25

(more evident after 8 h), which is a common phenomenon observed when bacteria are under antibiotic stress (Horii *et al*., 1999; Yokochi *et al*., 2000). After 24 h, *P. aeruginosa* ella01 was completely predominant in the biofilm. In the absence of antibiotic, the formation of the dual-species biofilm evolved differently throughout the 24 h culture period; no morphological changes were observed and *E. coli* ella00 was involved in the biofilm of *P. aeruginosa* ella00, as shown in Fig. 4. However, ella dual-species biofilm was not so compact and was less viable (many bacterial cells stained red, Fig. 2) in comparison to the ATCC strains dual-species biofilm. These results might indicate that, in the absence of the antibiotic pressure, the ability of *P. aeruginosa* ella01 to form biofilm is negatively affected by the presence of *E. coli* ella00, revealing a probable antagonistic interaction. Why and how such an interaction might have occurred could not be clarified within the aims of this study, nonetheless, it can be hypothesized that the QS activity of *P. aeruginosa* ella01 was altered by the presence of *E. coli*. It has been established that the production of many virulence factors and the formation of biofilm by *P. aeruginosa* is regulated by QS (Hurley *et al*., 2012; Wang *et al*., 2013). According to Heurlier and colleagues (2006), clinical isolates of *P. aeruginosa* can also show huge variations in QS activity. Natural infections may often consist of mixed

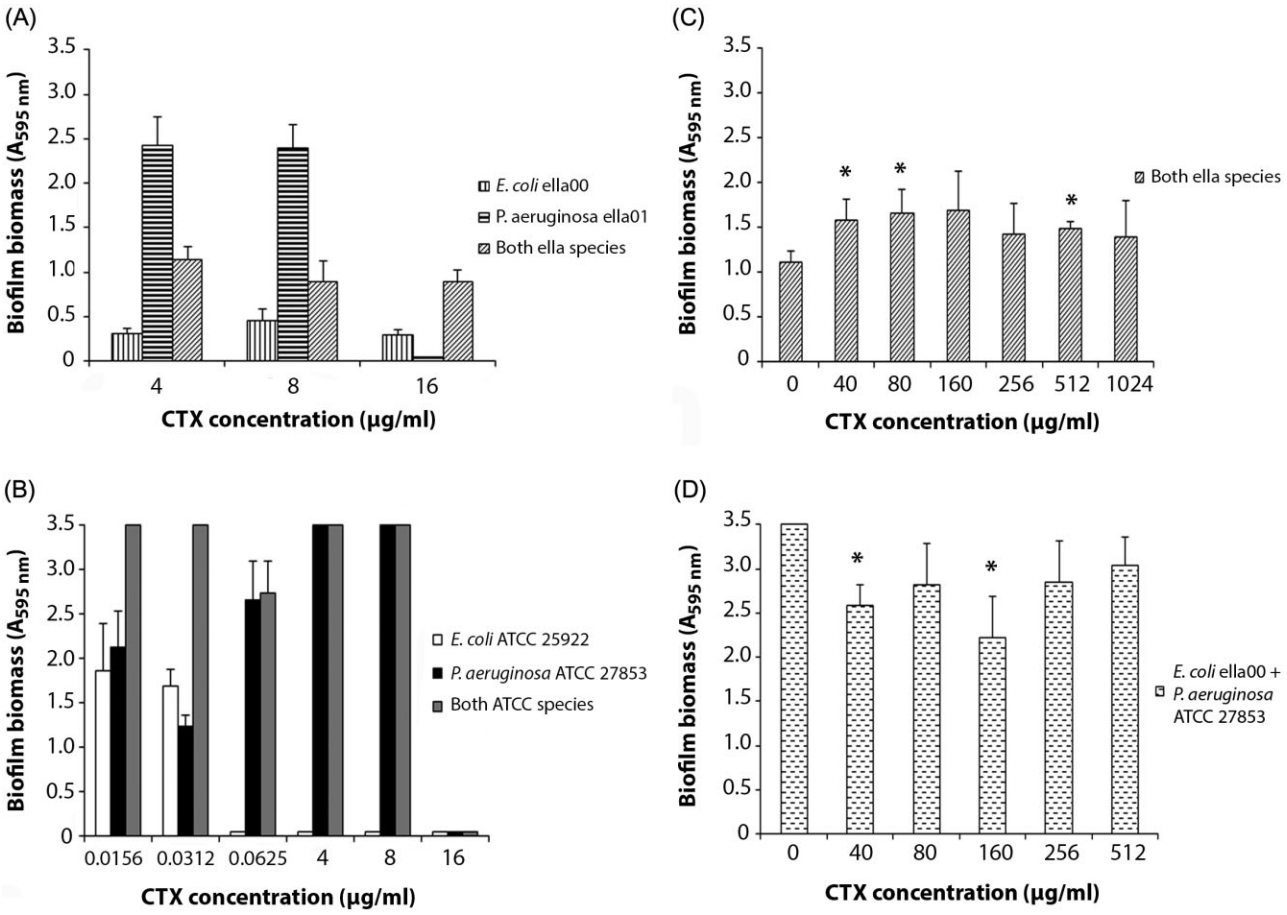

Fig. 3. Biofilm biomass quantification of ella isolates (A) or ATCC strains (B) alone or associated in presence of the MIC (16 μg ml^{-1}) and sub-MICs of CTX for *P. aeruginosa*. Biomass quantification of dual-species biofilm of ella isolates (C) or *E. coli* ella00 and *P. aeruginosa* ATCC 27853 (D) in presence of several sub-MICs of CTX for *E. coli* ella00. The asterisk indicates that the difference in comparison to the control (0 μg ml^{-1}) is statistically significant.

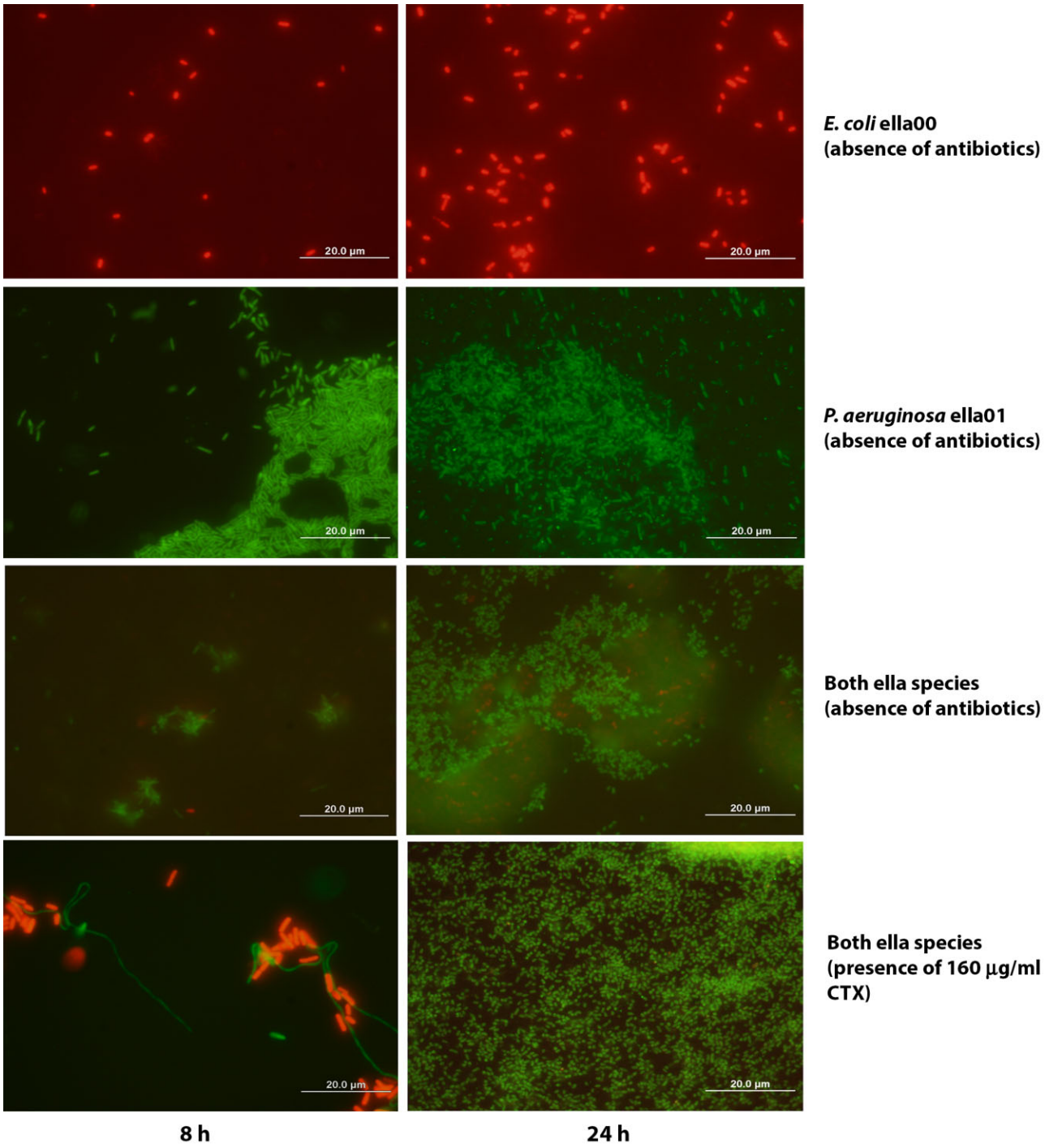

Fig. 4. Microscopic visualization of *E. coli* ella00 (using a red oligonucleotide fluorescent probe Eco440) and *P. aeruginosa* ella01 (using a green oligonucleotide fluorescent probe – PseaerA) during biofilm formation. Details about the oligonucleotide probes used can be seen in Table S1.

populations of QS-positive and QS-negative strains, able to affect and change the virulence and pathogenic behaviour of the consortium that, by this way, might have a selective advantage over pure cultures (Heurlier *et al.*, 2006). The negative impact of *E. coli* ella00 over *P. aeruginosa* ella01 is equally evident in Fig. 2A, where a high number of dead bacterial cells can be observed in the 24 h dual-species biofilm of ella isolates.

Moreover, the concentration of CTX was quantified by high-performance liquid chromatography throughout the

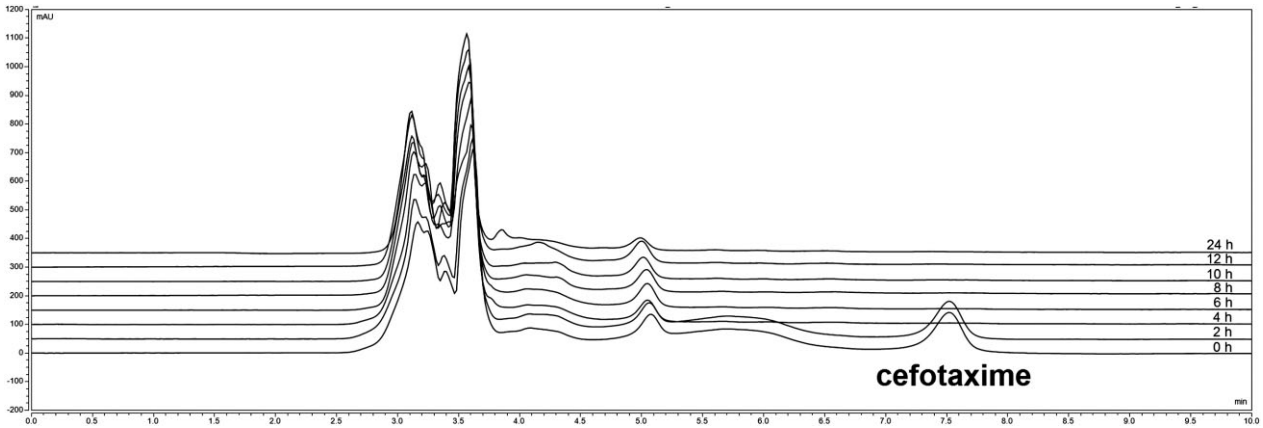

Fig. 5. Representative chromatograms [C18, 20 mM NaH_2PO_4, methanol and acetonitrile 83:7:10 (v/v/v); 1 ml min^{-1}, $\lambda = 254$ nm] of CTX samples in the *E. coli* ella00 planktonic culture (retention time 7.5) throughout time (0, 2, 4, 6, 8, 10, 12, 24 h).

E. coli ella00 planktonic culture period, and it was verified that CTX was no longer present 4 h after the initial addition in a concentration of 160 μg ml^{-1} (Fig. 5). The quick inactivation of CTX by *E. coli* ella00 assured the survival and further proliferation of *P. aeruginosa* ella01. This outcome indicates that *E. coli* ella00 rapidly produced β-lactamases to the surrounding medium to quickly inactivate the CTX (Walther-Rasmussen and Høiby, 2004). Moreover, our finding that *P. aeruginosa* ella01 maintained its MIC of 16 μg ml^{-1} indicates that horizontal transference of resistance genes (Kirchner *et al*., 2011) did not occur under co-culture.

In the presence of sub-MICs of CTX for *P. aeruginosa* and *E. coli* ATCC, these strains behaved differently from the ella isolates, forming more biofilm (Fig. 3B). When *E. coli* ella00 was associated with *P. aeruginosa* ATCC 27853, in the presence of sub-MICs of CTX for *E. coli* ella00, less biofilm was formed relatively to the amount produced in the absence of the antibiotic (Fig. 3D). A higher quantity of biofilm was always produced when *P. aeruginosa* ella01 was co-culture with *E. coli* (ATCC or ella) in presence of CTX comparatively to in absence of the antibiotic.

Regarding the susceptibility to CIP, *E. coli* ella00 was resistant while *E. coli* ATCC 25922 and *P. aeruginosa* ella01 and ATCC 27853 were susceptible. In the presence of sub-MICs of CIP for *E. coli* ella00, the co-culture of the two ella isolates was unable to form a biofilm, with a biofilm biomass (A_{595}) of less than 0.2, and *P. aeruginosa* ella01 could not be recovered in Hektoen agar medium. In the absence of any antibiotic (control), the co-culture of ella isolates produced a biofilm biomass of around 1.0 (data not shown). The presence of CIP did not cause the same microbial interaction as the third-generation cephalosporin, which highlights the importance of the type of resistance mechanism in promoting (favouring) synergistic interactions.

In conclusion, antibiotic usage has ecological and evolutionary consequences far beyond the fixation of mutations and horizontal gene transfer. The capacity of these drugs to switch microbial interactions from antagonistic or indifferent to cooperative deserves to be better examined, since these phenotypic responses (despite being reversible) either affect the clinical efficiency of antibiotics, due to the establishment of what we may call 'antibiotic-resistant environments', or may contribute to the dissemination of antibiotic resistance genes, as well as all genetic and cellular vehicles in which these genes are located.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Detailed experimental procedures.
Fig. S1. Morphologic aspect of associated colonies of *E. coli* ella00 and *P. aeruginosa* ella01 on blood agar medium.
Table S1. Oligonucleotide probes used in the study.
Appendix S1. Detailed experimental procedures.