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ABSTRACT

Formation of bacterial biofilm communities leads to profound physiological modifications and increased physical and metabolic exchanges between bacteria. It was previously shown that bioactive molecules produced within the biofilm environment contribute to bacterial interactions. Here we describe new pore-forming colicin R, specifically produced in biofilms formed by the natural isolate *Escherichia coli* ROAR029 but that cannot be detected under planktonic culture conditions. We demonstrate that an increased SOS stress response within mature biofilms induces SOS-dependent colicin R expression. We provide evidence that colicin R displays increased activity against *E. coli* strains that have a reduced lipopolysaccharide length, such as the pathogenic enteroaggregative *E. coli* LF82 clinical isolate, therefore pointing to LPS size as an important determinant for resistance to colicins. We show that colicin R toxicity toward *E. coli* LF82 is increased under biofilm conditions compared to planktonic susceptibility, and that release of colicin R confers a strong competitive advantage in mixed biofilms by rapidly outcompeting sensitive neighboring bacteria.

This work therefore identifies the first biofilm-associated colicin that preferentially targets biofilm bacteria. Furthermore, it indicates that the study of antagonistic molecules produced in biofilm and multispecies contexts could reveal unsuspected, ecologically relevant bacterial interactions influencing population dynamics in natural environments.

INTRODUCTION

Biofilms are widespread mixed-species bacterial communities in which high cell density, reduced diffusion and heterogeneous structure favor physical and metabolic contact between bacteria. Biofilms are therefore ideal environments for development of antagonistic and synergistic interactions (Hibbing *et al.,* 2010; James *et al.,* 1995; Rendueles and Ghigo 2012). It was hypothesized early on that the particular conditions prevailing within biofilms could lead to the emergence of biofilm-specific competition mechanisms (Ghigo 2003; Valle *et al.,* 2008). Consistently, biofilm communities were shown to release molecules that affect bacterial access to space and resources. Examples of such compounds range from metabolites and amino acids (Kolodkin-Gal *et al.,* 2010; Kolodkin-Gal *et al.,* 2012; Valle *et al.,* 2008) to anti-adhesion molecules (Bendaoud *et al.,* 2011; Rendueles *et al.,* 2011; Rendueles *et al.,* 2013) and bacteriocins (Kreth *et al.,* 2004; Yan *et al.,* 2003).

Production of antagonistic molecules is a widespread trait across the microbial world (Cascales *et al.,* 2007; Chater 2006; Duquesne *et al.,* 2007; Hibbing *et al.,* 2010; Lee and Kim 2011; Wenzel and Muller 2009). In the specific context of biofilm development, bacteriocin production has been researched at length in oral biofilms (Bucci *et al.,* 2011; Kreth *et al.,* 2004; Merritt and Qi 2012) in which Gram-positive bacteria are predominant, however, little is known about the occurrence and efficacy of bacteriocins produced within Gram-negative biofilms.

It is estimated that at least 50% of all *E. coli* strains synthesize colicins, plasmid-encoded bacteriocins secreted by *Escherichia coli* and closely related enterobacteria, and microcins (chromosomally encoded low-molecular-weight bacteriocins). Secretion of

colicins is considered to provide an ecological advantage to colicin-producing bacteria compared to their colicin-sensitive neighbors (Cascales *et al.,* 2007; Gillor *et al.,* 2004). Colicinogenic bacteria are resistant to colicin biocidal action via a constitutively expressed immunity gene typically coded downstream the colicin gene. Colicin release into the extracellular medium is mediated by cell lysis, presumably encoded by the *kil* or lysis gene carried in the plasmid. For lethal activity, colicin interactions with target cells are mediated upon binding to receptors at the surface and subsequent colicin internalization. Colicins have two distinct mechanisms of action, they can either, form pores in the inner membrane (activity encoded in the C terminal of the colicin) or display an enzymatic activity that will degrade DNA or RNA, both of which will result in cell death (for review on colicin biology (Cascales *et al.,* 2007; Riley and Wertz 2002)). Colicin expression is tightly regulated by the SOS response (Gillor *et al.,* 2008). In neutral environments, the dimerized repressor LexA inactivates the SOS regulon by binding to a DNA sequence called the LexA box. Upon DNA damage, RecA activates LexA self-cleavage allowing gene expression of more than 80 genes under RecA/LexA control (Courcelle *et al.,* 2001; Fernandez De Henestrosa *et al.,* 2000).

Here we describe a new colicin detectable only within biofilms formed by natural *E. coli* ROAR029 and that was identified while screening for molecules inhibiting *E. coli* adhesion and growth (Rendueles *et al.,* 2011). We demonstrate that ROAR029 colicin (hereafter referred to as colicin R) is a pore-forming, plasmid-encoded colicin the production of which is not detected under planktonic conditions. We show that the increased SOS stress response induced in mature biofilms leads to induction of colicin R expression and to local concentration in biofilms. We demonstrate that lipopolysaccharides

(LPS) integrity is a determinant for resistance, since colicin R is mainly active toward bacteria producing short LPS. Finally, we show that release of colicin R confers a strong competitive advantage upon mixed biofilms and that colicin R toxicity toward the enteroaggregative *E. coli* LF82 clinical isolate is increased under biofilm conditions compared to planktonic susceptibility. This study therefore identifies the first biofilm-associated colicin targeting biofilm bacteria.

MATERIALS AND METHODS

Bacterial strains, plasmids, and liquid growth conditions. *E. coli* isolates used in this study originated from published collections (Escobar-Paramo *et al.,* 2006; Skurnik *et al.,* 2006), and compiled in (Rendueles *et al.,* 2011). Bacterial strains and plasmids used are listed in Supplementary Tables S1 and S2. Bacteria were cultured in 0.4% glucose M63B1 minimal medium (M63B1$_{Glc}$) or in LB lysogeny broth medium at 37°C. Antibiotics were used as follows: ampicillin, 100 μg mL^{-1}; apramycin, 30 μg mL^{-1}; chloramphenicol, 25 μg mL^{-1}; spectinomycin, 50 μg mL^{-1}; zeocin, 50 μg mL^{-1}; apramycin, 30 μg mL^{-1}; and kanamycin, 50 μg mL^{-1}.

Purification of planktonic and biofilm extracts. *Planktonic supernatants.* Bacteria were cultured for 24 h in M63B1$_{Glc}$ and adjusted to an optical density (OD$_{600nm}$) of 3. Cultures were centrifuged for 10 min at 7,500 rpm at 4°C and supernatants were filtered through 0.2 μm filters. *Biofilm extracts*. Sixty-milliliter microfermentors containing a removable glass stand were configured as continuous-flow culture bioreactors with a 40-mL h^{-1} flow rate, which maximizes biofilm development and minimizes planktonic growth of the bacteria (see at http://www.pasteur.fr/recherche/unites/Ggb/biofilmfermenter.html for more detailed

information) (Ghigo 2001; Rendueles *et al.*, 2011). Inoculation was performed by dipping the glass spatula for 5 min in a culture adjusted to an OD_{600nm} of 1 from overnight bacterial cultures. The spatula was then reintroduced into the microfermentor. After 72 h at 37°C, 45 mL of microfermentor medium ($OD_{600nm} < 0.1$) was carefully discarded, and biofilm biomass was rapidly resuspended in the remaining 15 mL medium by vortexing for 30 s. The final biofilm biomass was measured, adjusted to an OD_{600nm} of 3, centrifuged and filtered through 0.22-µm filters (this biofilm conditioned media is hereafter referred to as biofilm extract). Alternatively, microtiter plates were inoculated with overnight diluted cultures (1:100) and grown for 24 to 48 h at 37°C under static conditions. *E. coli* ROAR029 microtiter plate biofilms were resuspended in their own supernatant and directly spotted onto agar plates adequately supplemented with antibiotics to inhibit further growth of ROAR029.

Biofilm-related assays. *Biofilm inhibition assays.* Cultures diluted overnight in M63B1$_{Glc}$ were inoculated into 96-well polyvinyl chloride (PVC) plates and filter-sterilized biofilm extract or planktonic supernatant was added at a 1:1 ratio. Biofilms were grown for 24 h at 37°C before quantification. *Biofilm dispersion assays.* Static biofilms were grown in 96-well PVC microtiter plates for 48 h at 37°C. Free cells and used media were removed by inversion of the microtiter plate, biofilm was washed using M63B1 and 100 µL of ROAR029 biofilm extract were added to each biofilm for 2 h at 37°C before quantification. *Biofilm quantification.* Unbound cells were removed by inversion of the microtiter plate; biofilms were washed with water and adhering cells were then stained with 1% crystal violet for 20 min. Excess stain was removed by washing with deionized water 3 times.

Quantification of attached biofilms was performed by addition of acetone-ethanol (20:80) and the dissolved crystal violet was measured at an OD of 595 nm.

Colicin susceptibility assays on planktonic and biofilm cells. i. *Planktonic*. Bacterial cultures were grown around 6 h or 16 h (exponential and stationary, respectively) in M63B1$_{Glc}$. Bacteria were then harvested by centrifugation and resuspended in the same volume of M63B1$_{Glc}$ (control planktonic) or in a 1:1 mix of M63B1$_{Glc}$ and ROAR029 biofilm extract to obtain a final cell quantity of 1.10^8 (1.10^9 CFU/ml). One hundred μL were then transferred to microtiter plate wells. After 2 h of incubation at 37°C, serial dilution plating (CFU counts) was used to assess colicin susceptibility of the treated planktonic population versus a planktonic overnight untreated control.

ii. *Biofilm*. Biofilms were formed for 48 h in microtiter plates as described above. Unattached and planktonic bacteria were removed by inversion of the microtiter plates and gentle tapping. One hundred μL of either a 1:1 mix of M63B1$_{Glc}$ and ROAR029 biofilm extract or M63B1$_{Glc}$ (control) were added to each well in which the preformed biofilm (ca. 1.10^8 CFU, 1.10^9 CFU/mL) had developed. The treated biofilms were incubated for 2 h at 37°C, and then resuspended by vigorous pipetting. Cell survival was determined by CFU count. **iii. *Biofilm resuspended cells*.** Biofilms were formed for 48 h in microtiter plates as described above. Unattached and planktonic bacteria were removed, biofilms were washed by adding (without resuspending) and removing M63B1. Then, the biofilms were resuspended in 100 μL of either a 1:1 mix of M63B1$_{Glc}$ and ROAR029 biofilm extract or M63B1$_{Glc}$ (control). Cells were then incubated at 37°C for 2 h and bacterial survival was monitored as above.

Growth inhibition test. The inhibitory effect of filter-sterilized supernatants and biofilm extracts was evaluated by spotting 8 µL on an overlay of targeted cells on appropriate media. Plates were incubated overnight at 37°C. Batch growth in microtiter plates was also monitored by diluting overnight cultures to a final OD 600 nm of 0.1 and mixed at a 1:1 ratio with cell-free biofilm extract and grown in microtiter plates for 24 h at 37°C.

Generation of deletion mutants. Gene deletions in the different strains of *E. coli* were generated by the λ-red linear DNA gene replacement system (Chaveroche *et al.,* 2000; Derbise *et al.,* 2003) or by P1vir transduction from the Keio collection to *E. coli* MG1655 when indicated (Supplementary Table S2) (Baba *et al.,* 2006). Primers used are listed in Supplementary Table S3. ROAR029 constructs were checked by PCR and sequencing. Construction of ROAR029_KmRExTET_LexA were *lexA* is under the control of with an anhydrotetracycline inducible promoter was performed as described in (Da Re *et al.,* 2007).

Construction of an LPS reconstituted mutant in MG1655. Construction of O-antigen-reconstituted *E. coli* K-12 strains was performed using O-antigen trans-complementation between two related *E. coli* K-12 backgrounds, EMG2 and WG1 (Liu and Reeves 1994). Both backgrounds are O-antigen-deficient, either by IS insertion in *wbbL* for EMG2 (*rfb*-50) or by a deletion encompassing the end of *rfbB* for WG1 (*rfb*-51). The KmFRT selectable genetic cassette from the pKD4 plasmid (Datsenko and Wanner 2000) was inserted between the *gnd* and intact *wbbL* genes of WG1 strain using long primers (wbbL.KmFRT.long.L-5 and wbbL.KmFRT.long.L-3, Supplementary Table S3) and a λ-

red recombination. Primers hybridizing two-hundred base pairs before and after *wbbL* (wbbL200.ext-5 and wbbL200.ext-3 Supplementary Table S3) were then used to amplify the KmFRT-*wbbL* region that was used to replace the *wbbL*-deficient region of EMG2 derivatives MG1655 and TG1 strains. Restoration of functional *wbbL* gene and O-antigen production was verified by sequencing and LPS visualization on gel (Figure 5A).

Random mutagenesis. ***The ROAR029 genome.*** Transposon mutagenesis of ROAR029 was performed using random insertion of the EZ-Tn5™ <KAN-2> Transposome™ (Epicentre Biotechnologies). A total of 5,000 clones were analyzed. ***ROAR029 plasmid.*** Plasmid pColR was mutagenized by EZ-Tn5™ <KAN-2> transposition. Pools of mutagenized plasmids were electroporated into *E. coli* TG1 electrocompetent cells. In EZ-Tn5™ <KAN-2> the kanamycin gene is not followed by a transcription terminator allowing possible transcriptional read-through.

Mixed biofilm assays. ROAR029 bearing pColR_Km, pColR- or pColR+ and MG1655F' cells were grown overnight in M63B1$_{Glc}$, washed twice in M63B1 to remove traces of colicin R (if any) and diluted to OD$_{600nm}$ 0.1. Cells were mixed at different initial ratios of ROAR029 derivatives to MG1655F' (1:1, 5.10^7 CFU/mL : 5.10^7 CFU/mL ; 1:10, 5.10^6 CFU/mL : 5.10^7 CFU/mL ; 1:100, 5.10^5 CFU/mL: 5.10^7 CFU/mL and 1:10 000, 5.10^3 CFU/mL : 5.10^7 CFU/mL), and inoculated into 96-well PVC plates for 24 h at 37°C. We counted the proportion of each strain in the 24-h-grown biofilms after washing, resuspension and serial dilution plating in appropriate antibiotics.

SDS-polyacrylamide gel for LPS observation. *Cell preparation*. Cells were harvested by centrifugation, resuspended in deionized water and normalized to a final OD_{600nm} of 1. Samples were then heated for 10 min at 100°C and treated with proteinase K (2mg/mL) for 1 h at 37°C. *LPS gel and staining*. SDS-polyacrylamide gels were made according to (Lugtenberg *et al.,* 1975). Gels were run at 75 mA for 60 to 90 min. LPS were stained with silver nitrate as described previously (Tsai and Frasch 1982).

Sequence of ROAR029 colicin plasmid, colicin R sequence and structure analysis. The sequence of the pColR plasmid was deposited in Genbank (accession number KF137578). *Sequence alignments*. Alignments were performed by MultiAlin software described in (Corpet 1988). *Phylogenetic dendrogram*. Colicin sequences were directly obtained from GenBank (http://www.ncbi.nlm.nih.gov/gene). Sequences were aligned following ClustalW standards and further examined in order to create phylogenetic dendrograms with the program MEGA 5 software developed by (Tamura *et al.,* 2011). *Plasmid map*. pColR plasmid map was constructed using Seqgene software*. Colicin tertiary structure*. Colicin R structure was simulated using PHYRE software (Bennett-Lovsey *et al.,* 2008).

Statistical methods. Statistical analyses were performed using unpaired t-test with Prism v5.0 (GraphPad Software).

RESULTS

Commensal* E. coli *ROAR029 produces a biofilm-associated bacteriotoxic molecule

We had previously screened a collection of 122 *E. coli* natural isolates from different phylogenetic groups, hosts and origins for their ability to produce biofilm-specific molecules inhibiting biofilm formation of other competing strains (Rendueles *et al.,* 2011). That study revealed that biofilm extracts from commensal *E. coli* ROAR029 isolated from an impala antelope (*Aepyceros melampus)* in Gabon (phylogenetic group D) inhibited biofilm formation and planktonic growth of the biofilm-forming MG1655F'tetΔ*traD* strain (referred to as MG1655F') (Rendueles *et al.,* 2011; Valle *et al.,* 2006) (Figure 1A). While filter-sterilized planktonic supernatant of *E. coli* ROAR029 did not show biocidal activity, a filter-sterilized extract from ROAR029 biofilms (hereafter referred to as biofilm extract) produced a growth inhibition halo when spotted on an MG1655F' lawn (Figure 1B). Production of the bacteriotoxic compound was also detected in microtiter plates and colony biofilm models (Anderl *et al.,* 2000) (Figure 1C and data not shown). This biofilm-associated bacteriotoxic effect could not be attributed to the previously reported presence of valine in *E. coli* biofilms, since ROAR029 biofilm extract retained activity on the MG1655 *ilvG+* strain in which the corrected *ilvG* frameshift confers resistance to valine (Figure 1D) (Valle *et al.,* 2006). Finally, determination of colony-forming units of a MG1655F' planktonic culture upon exposure to ROAR029 biofilm extract in a 1:1 ratio for 2 h showed no surviving bacteria, indicating bacteriotoxic rather than bacteriostatic activity of the ROAR029 biofilm extract (data not shown).

The ROAR029 biofilm-associated bacteriotoxic molecule is a new pore-forming colicin

To identify the genetic determinants of ROAR029 bacteriotoxic activity, we performed random transposon mutagenesis and searched for ROAR029 mutants unable to produce active biofilm extract. We identified one transposon mutant inserted in *pcnB*, a gene coding for a poly-A polymerase involved in mRNA poly-A adenylation and plasmid copy number regulation (Liu and Parkinson 1989). Introduction of a *pcnB* deletion into ROAR029 abolished the bacteriotoxic activity of microtiter-plate- or microfermentor-grown biofilm extracts (Figure 2A). Since ROAR029 Δ*pcnB* did not show significant growth or biofilm defects (Supplementary Figure S1A), we hypothesized that the genetic determinants of the ROAR029 bacteriotoxin could be carried by a plasmid; indeed, we detected the presence of a ca. 5.8 kb plasmid in ROAR029 (Figure 2B). We subjected the ROAR029 plasmid to random *in vitro* mutagenesis and we electroporated pools of mutagenized plasmids into *E. coli* K-12 strain TG1. We found that 67% of kanamycin-resistant transformants produced active bacteriotoxic compound in microtiter plate biofilm extracts, demonstrating that this plasmid carries genes involved in synthesis of ROAR029 bacteriotoxin. The remaining 33% kanamycin-resistant TG1 transformants did not produce active bacteriotoxic compounds. Sequence analysis showed that inactive mutagenized plasmids carried transposon insertions in DNA regions homologous to pore-forming colicins U and Y encoding genes, including genes potentially coding for a 69.3 kDa colicin, as well as lysis and immunity proteins (Table 1 and Figure 2CD, Supplementary Figure S2, Supplementary Figure S3, Supplementary Figure S4) (Riley *et al.,* 2000; Smajs *et al.,* 1997). We further tested whether the immunity encoded by ROAR029 plasmid conferred

resistance to other colicins and indeed, it diminished the killing of colicin U and completely blocked growth inhibition of colicin Y (Supplementary Figure S4A), but had no effect against unrelated colicin E2. Sequence and phylogenetic analyses further showed that while immunity genes of pore-forming colicins U, Y and ROAR029 cluster together, key aminoacids for interaction between the immunity protein and its colicin partner (Smajs *et al.,* 2006) appeared more similar between ROAR029 and colicin Y than with colicin U (Supplementary Figure S4BC).

We then replaced the original marker-less ROAR029 colicin-encoding plasmid (pColR), hereafter referred to as colicin R for ROAR029, with an inactive kanamycin-resistant plasmid by transformation (pColR-). Plasmid maintenance upon antibiotic selection led to exclusion of the native plasmid. Biofilm extracts of ROAR029 strains with inactive kanamycin-resistant plasmid did not display bacteriotoxic activity (Figure 2A). Additionally, one mutagenized plasmid seemed to confer an increased killing effect, referred to as pColR+ (Supplementary Figure S5).

To further confirm the colicinogenic nature of the antagonistic molecule, we checked that TG1 transformed with pColR were resistant to biofilm extracts produced by pColR and pColR+ strains by virtue of the immunity protein carried in the plasmid (Supplementary Figure S6).

Taken together, our results demonstrated that ROAR029 biofilm extracts bacteriotoxic activity was associated with production of colicin R encoded by the 5.8 kb plasmid pColR.

Colicin R is a group A Tol-dependent colicin

Colicins are classified into groups A and B based on use of either Tol or Ton translocation systems, respectively (Cascales *et al.,* 2007). Although *E. coli* MG1655 Ton system mutants Δ*tonB,* Δ*exbB* and Δ*exbD* were sensitive to colicin R, a Δ*tolA* mutant displayed full resistance to colicin R as it did for colicin U (Figure 3). Consistently, we identified a conserved TolA box consensus motif (DGTGW), as well as several repeated TolA motifs involved in colicin internalization in the colicin R gene sequence (data not shown). Other Tol mutants (Δ*tolQ,* Δ*tolR,* Δ*tolC* and Δ*tolB*) showed various levels of tolerance to colicin R (Supplementary Figure S7). Residual activity of colicin R in other Tol mutants might be explained by partial phenotype complementation of Ton proteins. Indeed, the *E. coli* mutant strain carrying double *ton* and *tol* deletions displayed full resistance to colicin R (data not shown). Finally, we showed that, like for colicin U, an *ompA* mutation in *E. coli* MG1655 led to colicin R resistance (Figure 3 and Supplementary Figure S7). Interestingly, closely related colicins U and Y both used OmpF as a translocator (data not shown). However, colicin R activity remained unaffected in an *ompF* mutant (Supplementary Figure S7) as well as in an *ompC* mutant (data not shown), hence, we cannot exclude the possibility that OmpA acts as a colicin R receptor, translocator or both. Accordingly, *E. coli* TG1 transformed with pColR inhibited *E. coli* MG1655 but not OmpA and TolA mutants (data not shown).

Biofilm specificity of colicin R might be due to SOS regulatory activity

Colicins have been shown to be tightly regulated by the SOS system (Gillor *et al.,* 2008). Consistently, we identified two overlapping LexA boxes in the promoter region located upstream of the ROAR029 colicin gene (Figure 4A). LexA is a repressor of SOS-

regulated genes cleaved by RecA upon induction of the SOS response. We observed that overexpression of LexA correlated with decreased levels of colicin in *E. coli* ROAR029 biofilm extracts (Figure 4B), and addition of mitomycin C to either biofilm extracts or inactive *E. coli* ROAR029 planktonic cultures led to derepression of colicin R production (Figure 4C and data not shown, respectively).

We had previously shown that the SOS system is upregulated within *E. coli* biofilms (Beloin *et al.,* 2004; Bernier *et al.,* 2013). Thus, colicin R biofilm-specific production could be directly linked to an increased SOS response. To investigate this, we used three different plasmids derived from wild type pColR: (i) pColR-, unable to produce colicin R due to transposon-based interruption of the colicin gene, (ii) pColR+ in which colicin gene transcription is constitutive due to a probable combination of read-through from the promoter of the kanamycin gene from the EZ-Tn5TM <KAN-2> transposon and dirsruption of LexA binding to LexA box 1 caused by transposon insertion (Figure 4A) and (iii) pColR_Km, active kanamycin-resistant pColR (Supplementary Figure S5). These plasmids were transformed into *E. coli* TG1 and TG1*lexAind3*, with the latter unable to induce an SOS response due to a non-cleavable version of the LexA protein. As expected, biofilm extract from strains carrying pColR- did not show growth-inhibiting activity, whereas biofilm extracts from strains bearing pColR+ displayed strong SOS-independent growth-inhibiting activity (Figure 4D). In contrast, the growth-inhibiting activity of strains carrying the pColR_Km plasmid was totally dependent on a functional LexA protein (Figure 4D). Taken together, these results suggest that increased release of colicin R is a consequence of induction of the SOS response within biofilms.

Colicin R is only active toward* E. coli *strains with short LPS

To investigate the activity spectrum of colicin R, we tested a panel of pathogenic and commensal *E. coli* as well as other Gram-negative strains for their sensitivity to ROAR029 biofilm extract (Supplementary Table S1). We observed that all tested strains were resistant to colicin R except for *E. coli* K-12 EMG2 and B derivatives, and adherent-invasive *E. coli* LF82. *E. coli* K-12 EMG2 derivatives are characterized by an insH-7 insertion element in *wbbL* resulting in rough LPS (Liu and Reeves 1994). Reintroduction of a wild type *wbbL* allele in *E. coli* K-12 MG1655 (MG1655 *wbbL*+) and *E. coli* TG1 (TG1 *wbbL*+) restored O-antigen production (Figure 5A) and led to total resistance to colicin R in bacterial lawns, batch cultures and microtiter plate biofilms (Figure 5B and Supplementary Figure S8). Furthermore, *E. coli* LF82 naturally lacks O-antigen due to a deletion of *wbbLKIJH_glf_rfbX_rfbC* genes (Miquel *et al.,* 2010) (Supplementary Figure S9), which probably explains its colicin R sensitivity. We also tested whether colicins closely related to colicin R, i.e. colicins U and Y, or another colicin, colicin E2, had bacteriotoxic effects against *E. coli* K-12 MG1655 and *E. coli* MG1655 *wbbL*+; we observed that these three colicins were also inactive against the O-antigen LPS-restored MG1655 *wbbL* + strain (data not shown). Colicins U, Y and E2 as well as colicin R produced in TG1_pColR were also active against LF82 strain (data not shown).

We further investigated the influence of LPS length on colicin R activity using *E. coli* 536, a uropathogenic strain resistant to colicin R and its Δ*rfaH*, Δ*manB*, Δ*waaG* and Δ*waaC* mutants displaying various LPS sizes (Beloin *et al.,* 2006) (Figure 5C). Addition of ROAR029 biofilm extract to *E. coli* 536 mutants revealed that resistance increased with LPS size (Figure 5DE) and that restoration of whole LPS in a complemented *rfaH* mutant

(*rfaHc*) led to total resistance to colicin R (Figure 5DE). Interestingly, uropathogenic *E. coli* CFT073 and 536 mutants unable to synthesize other extracellular polymeric surface structures like colanic acid and capsules remained fully resistant to colicin R (Figure 5DE and data not shown). Taken together, these results showed that a reduction in LPS size correlated with increased sensitivity to colicin R.

ROAR029 colicin excludes other *E. coli* from mixed biofilms

To determine whether colicin R production confers a competitive advantage upon ROAR029 within structured communities, we compared the ability of ROAR029 carrying pColR_Km, pColR- or pColR+ plasmids to grow in mixed biofilms in competition with the biofilm-forming colicin-R-sensitive *E. coli* MG1655F' strain (Ghigo 2001). It was verified that ROAR029 strains expressing differing levels of colicin R did not display significantly different growth rates in liquid- and biofilm-forming abilities (Supplementary Figure S10). ROAR029 derivatives and MG1655F' cultures were adjusted to different initial ratios and grown as mixed biofilms for 24 h. Although ROAR029 pColR- was naturally able to outcompete MG1655F' due to a higher growth rate of ROAR029 derivatives (see Supplementary Figure S10), bacterial survival showed that ROAR029 pColR_Km and ROAR029 pColR+, colicin-producing strains, more efficiently outcompeted MG1655F' (Figure 6). In order to further confirm the prominent role of colicin R in the outcompetition within biofilm, transformed TG1 with pColR plasmids was competed against a streptomycin-resistant TG1 at different initial ratios. Accordingly, TG1 pColR outcompeted TG1. This effect was increased when TG1 carried pColR+ plasmid, and abolished when pColR- was used (Supplementary Figure S11).

We further characterized temporal dynamics of ROAR029/MG1655F' interactions by inoculating the two strains at a 1:1 ratio, frequencies at which bacteriocin production provides the greatest advantage (Inglis *et al.,* 2009). To avoid potential accumulation of colicin R in the medium, we used continuous flow microfermentors and observed a rapid reduction in MG1655F' and complete out-competition after 8 h (Supplementary Figure S12). This strong outcompetition was not due to higher ability of ROAR029 pColR_Km to initially adhere to glass spatula since, on the contrary, the initial adhesion of MG1655F', probably by virtue of the production of the strong F pilus adhesion factor (Ghigo 2001), was ~20 fold higher than the one of ROAR029 pColR_Km (adhesion of ROAR029_pColR_Km and MG1655F' are respectively 0.10 +/- 0.037 % and 2.22 +/- 0,76%, p= 0.00838, unpaired t-test). These results demonstrated that colicin R production and higher growth rate provide a strong competitive advantage to ROAR029 in mixed biofilms. This suggests that production of colicin R could play a role in population dynamics in natural situations.

Colicin R is more active against biofilms than planktonic cultures

The specific release of colicin R within *E. coli* ROAR029 biofilms led us to hypothesize that its production could correlate with increased sensitivity to colicin R in *E. coli* biofilms. To test this, we compared sensitivity to colicin R of planktonic and biofilm bacteria for two different biofilm-forming pathogens with smooth (full-length) and rough (lacking O-antigen) LPS, *E. coli* 55989 and *E. coli* LF82, respectively (Supplementary Figure S9A). Whereas *E. coli* 55989 in planktonic and biofilm conditions remained unaffected by colicin R, LF82 biofilms displayed increased sensitivity compared to LF82 planktonic cultures

(Figure 7). This increased sensitivity was not due to further reduction of LPS length during biofilm growth, since LF82 planktonic and biofilm LPS profiles were identical (Supplementary Figure S9A). In addition, we tested whether resistance to colicin R could be enhanced by the characteristic 3D structure and architecture displayed by biofilm cells by assessing the sensitivity of biofilm bacteria resuspended in liquid medium, maintaining the physiological specificities of biofilm bacteria, but not their characteristic structured environment. Resuspended LF82 biofilm bacteria were more sensitive to colicin R planktonic bacteria and marginally more sensitive than biofilms (Figure 7), indicating that colicin R preferentially targets biofilm cells of susceptible bacteria.

DISCUSSION

In the present study, we characterized an unknown antagonistic antimicrobial molecule released within biofilms formed by *E. coli* natural isolate ROAR029. We demonstrated that this biofilm-associated molecule corresponds to a new 5.8 kb group I plasmid-encoded colicin (colicin R), exhibiting group A colicin characteristics (Cascales *et al.,* 2007). Phylogenetic analysis of colicin-associated genes of ROAR029 showed that they cluster with two other pore-forming colicins, U and Y, isolated from *Shigella boydii,* and a natural *E. coli* isolate from the Amazonian region, respectively (Riley *et al.,* 2000; Smajs *et al.,* 1997). Additionally, colicin R immunity protein confers full cross-immunity to colicin Y and partial immunity to colicin U, but no immunity to colicin E2.

Biofilm-specificity of colicin R production

Here we demonstrate that induction of the SOS response, previously shown to be upregulated in *E. coli* biofilms (Beloin *et al.,* 2004; Bernier *et al.,* 2013), is directly correlated with increased production of colicin R in biofilms. Other reports have highlighted the fact that colicins are also upregulated in late stationary phase independently of the SOS system and are triggered by nutrient depletion in the medium (Eraso *et al.,* 1996; Kuhar and Zgur-Bertok 1999), a common situation in the deep layers of biofilms (Boles and Singh 2008; Stewart and Franklin 2008). Recently, Cornforth and Forster proposed that bacteriocin production is correlated with the stress response as a potential reaction to the presence of ecological competitors and probably plays an important role in ecological interference within structured bacterial populations (Cornforth and Foster 2013). In addition, several studies postulate that optimal efficiency of bacteriocins is achieved

within structured environments, such as biofilms (Chao and Levin 1981; Durrett and Levin 1997; Gardner *et al.,* 2004). In agreement with previous studies, we show that in mixed biofilm conditions, colicin R confers a competitive advantage against closely related direct-niche competitors consuming similar potentially limited resources.

In addition, enhanced colicin R production within biofilms could correspond to a specific stress-induced defense mechanism, since release of group A colicins is a lethal event for the cell (Cascales *et al.,* 2007). Induction of the SOS system in biofilms upon stresses such as waste product accumulation, microaerobic areas or increased oxidative stress, could lead to colicin R release and subsequent cell death. Hence, biofilm-associated colicin production could have several benefits, including eliminating the most stressed bacteria from biofilms without negatively impacting growth or subsequent biofilm development of the overall population (Figure S10). Other suicidal mechanisms have been reported in structured populations, including phage release (Carrolo *et al.,* 2010), nitric oxide production (Barraud *et al.,* 2009) and cannibalism toward *B. subtilis* cells (Lopez *et al.,* 2009).

Moreover, biofilm-specific colicin production could contribute to enabling kind discrimination mechanisms (Strassmann *et al.,* 2011). Biofilms rely on cooperative acts such as matrix production and communication signals that can be exploited by other non-cooperative individuals ("cheaters") who benefit from them, but do not share the associated cost. When uncontrolled, these cheaters can increase in frequency, undermine cooperation and even cause population crashes in situations where cooperation is important for survival (Hamilton 1964). In this context, biofilm-specific colicin production could ensure close relatedness between interacting bacteria and promote cooperation by excluding possible

cheaters and reducing social exploitation (kin selection) (Hamilton 1964; Nadell *et al.,* 2009).

Colicin R targets LPS-deficient and preferentially biofilm cells

Like previously described colicins, ROAR029 colicin has a narrow spectrum of action (Cascales *et al.,* 2007). Here we demonstrate that, in addition to receptor and translocator proteins on the surface of target bacteria, LPS may play an important role in determining sensitivity towards colicins. We show that *E. coli* strains with full-length LPS are resistant to colicin R, whereas strains without O-antigen display increased susceptibility. Interestingly, restoration of O-antigen led to resistance to two pore-forming colicins (U and Y) and to RNAse colicin E2 (data not shown), suggesting that intact O-antigen might be a general determinant of resistance to colicins. While the presence of O-antigen has been previously suspected to alter the effect of temperature or EDTA on colicin susceptibility of target cells by means of the change of receptor activity (Gado *et al.,* 1995) we postulate that colicin R resistance of full-length LPS strains could be due to charge or steric repulsion by O-antigen, therefore preventing colicin R interaction with its receptor at the cell surface. This phenomenon is consistent with observation that purified LPS fractions do not seem to interact directly with colicins (Law *et al.,* 2003; Weltzien and Jesaitis 1971). In the case of colicin R this potential steric or charge repulsion seems specific to O-antigen, since deletion of other large surface molecules such as colanic acid and capsules did not enhance susceptibility to colicin R of uropathogenic *E. coli* strains 536 and CFT073.

Additionally, we show that a reduction in the LPS lipid A core size was correlated with *E. coli* increased sensitivity to colicin R, probably because of an increased accessibility of colicin R to its receptor. This is in contrast to increased resistance to colicin U displayed by *E. coli* mutants with a shorter lipid A LPS core (Smajs *et al.,* 1997) or cellular resistance of deep rough mutants to colicin N (Sharma *et al.,* 2009) suggesting that lipid A core could play an important role in early interaction with certain colicins as shown for colicin E2 (Osada and Beppu 1985). Hence, colicins U, E2 and R have in common to be inhibited by the presence of O-antigen, but colicins U and E2 need an intact lipid A outer core for binding to the outer membrane and subsequent insertion while colicin R efficacy is reduced by the increased size of the LPS lipid A core.

Furthermore, we provide evidence that strains with naturally reduced LPS length display increased sensitivity to colicin R when grown as biofilms. In the clinical enteroaggregative *E. coli* LF82 strain, colicin R indeed killed biofilm cells more efficiently than planktonic cells. Despite this increased activity toward biofilm cells, colicin R was unable to dissolve biofilms (data not shown), suggesting that it can penetrate biofilms, but that the 3D structure of biofilms is conserved after the colicin R effect. However, biofilm 3D architecture and the presence of extracellular matrix slightly contributed to protecting *E. coli* LF82 biofilm from the effects of colicin R (Figure 7). Although the specific mechanism by which LF82 biofilm bacteria display a decreased survival ratio compared to planktonic cells remains to be elucidated, it does not seem to be linked to further LPS modifications. Indeed, LPS extracted from biofilms and planktonic *E. coli* LF82 appeared to be identical. Alternatively, the particular metabolic and physiological properties displayed by biofilm bacteria may account for their increased sensitivity. For instance, a

reduced growth rate resulting in decreased outer membrane component turnover or increased stress in biofilm layers or niches could lead to enhanced sensitivity to colicin R. Since biofilm bacteria typically display enhanced tolerance to antimicrobial agents (Hoiby *et al.,* 2010), this is, to our knowledge, the first example of biofilm bacteria being more sensitive to a bacteriotoxic compound than their planktonic counterparts.

Counterselection of LPS rough E. coli cells: ecological implications in species diversity

Production of antagonistic molecules has been proposed to play an important role in selection and evolution of strains (Kirkup and Riley 2004; Majeed *et al.,* 2011; Riley and Gordon 1999). Indeed, colicin production promotes microbial diversity, both theoretically (Czaran *et al.,* 2002) and empirically (Walker *et al.,* 2004). Here we demonstrated that colicin R can preferentially target strains with reduced LPS (absence of O-antigen and reduced lipid A core) probably by an increased ability in these strains to access its receptor. This suggests that, in mixed biofilm communities, selective pressure exerted by colicin R producers may favor bacteria with long LPS structures. In addition, it has been recently been shown that electrostatic interactions between LPS and other polysaccharides enhances and stabilizes biofilms (Amini *et al.,* 2009), and that LPS play a key role in mixed biofilm dynamics (Bandara *et al.,* 2010). Taken together, colicin R could therefore directly impact population evolution. Interestingly, in *P. aeruginosa* and *P. fluorescens* biofilms, reduction of LPS O-antigen has been reported (Ciornei *et al.,* 2010; Giwercman *et al.,* 1992; Spiers and Rainey 2005), suggesting that such LPS modifications could also occur in *E. coli* biofilms, increasing relevance of producing a colicin against strains with short LPS.

In conclusion, our study contributes to the role played by colicins in representative multispecies contexts. It further establishes biofilms as a source of bioactive antagonistic molecules, as well as being a crucial site for ecological interactions that influence *E. coli* evolution and population dynamics in complex and relevant multispecies environments.

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CONFLICT OF INTERESTS

The authors declared no conflict of interest

SUPPLEMENTARY INFORMATION

Supplementary information is available at ISMEJ's website

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FIGURE LEGENDS

Figure 1. Bacteriotoxic effect of ROAR029 extracts upon *E. coli* MG1655F'. A. Quantification of the effects of ROAR029 planktonic (ROAR029 Pk) and biofilm (ROAR029 Bf) extracts on biofilm formation and growth of MG1655F'. M63B1, control. *** $p < 0.001$. **B.** Growth inhibition of ROAR029 planktonic (ROAR029 Pk) and biofilm extracts (ROAR029 Bf) on a lawn of *E. coli* MG1655. **C.** Growth inhibition of ROAR029 colony on a lawn of *E. coli* MG1655. **D.** Effects of ROAR029 biofilm extract (ROAR029 Bf) on a lawn of valine-resistant *E. coli* MG1655 *ilvG+*.

Figure 2. ROAR029 bacteriotoxic activity is due to plasmid-encoded colicin regulated by PcnB. A. Addition of an 8 µL drop of ROAR029 wild type, ROAR029Δ*pcnB* or ROAR029 bearing a mutagenized plasmid unable to synthesize the bacteriotoxic molecule (ROAR029 pColR -) biofilm extract to a lawn of MG1655. **B**. DNA gel of ROAR029 pColR plasmid linearized by the *Eco*RV enzyme. **C.** ROAR029 pColR plasmid map. Arrows indicate transcription polarities of colicin genes. Unique-site restriction enzymes are indicated. **D.** Dendrogram of sequences alignment of the 200 last amino acids from colicin encoding genes C-terminal region. Phylogenetic analysis was carried out using the UPGMA method. Numbers represent bootstrap values above 75.

Figure 3. ROAR029 colicin is a group A colicin. A. Effect of addition of an 8 µL drop of ROAR029 (colicin R) or *S. boydii* M592 (colicin U) biofilm extracts to a lawn of *E. coli* MG1655 (Wild type), MG1655 Δ*tolA* and MG1655 Δ*ompA* cells.
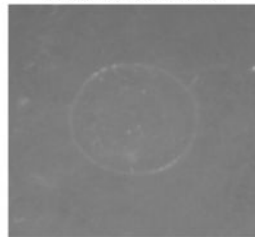
Figure 4. ROAR029 biofilm-specific production of colicin R is due to an SOS regulatory effect. A. Sequence analysis revealed two overlapping SOS boxes upstream of the ribosome binding sequence (rbs) and start codon ATG of ROAR029. Insertion site of transposon in pColR+ plasmid is indicated. Transcriptional read-through from kanamycin gene and disruption of LexA box1 binding site may explain constitutive expression of colicin R encoding gene. **B**. Effect of LexA induction upon ROAR029 bacteriotoxic activity was assessed using ROAR029 with LexA under control of an anhydrotetracycline-inducible (aTc) promoter. An 8 µL drop of ROAR029_KmRExTET_LexA treated with increasing concentrations of aTc ranging from 0 to 1000 ng/mL was added to a lawn of MG1655. **C.** Inhibition halo of *E. coli* MG1655 when treated with an 8 µL drop of ROAR029 biofilm extract (ROAR029 Bf) with and without induction of the SOS system by 0.1 µg/mL of mitomycin C added after inoculation. **D.** Growth inhibition of *E. coli* MG1655 by biofilm extracts produced by *E. coli* TG1 (black) and TG1 *lexAind3* (gray) transformed by different ROAR029 plasmids; active kanamycin-resistant pColR_Km, defective colicin mutant (pColR-, shaded) and constitutively expressed colicin (pColR+, dotted line).

Figure 5. ROAR029 colicin activity is dependent on LPS length. A. SDS-polyacrylamide (14%) gel showing LPS reconstruction of rough *E. coli* MG1655 and *E. coli* TG1 strains. **B**. Effect of ROAR029 biofilm extract on bacterial lawns of rough (MG1655 and TG1) and smooth (MG1655 *wbbL*+ and TG1 *wbbL*+) strains. **C**. SDS-polyacrylamide gel (14%) of *E. coli* 536 wild type and LPS (*rfaH, manB, waaG,* and
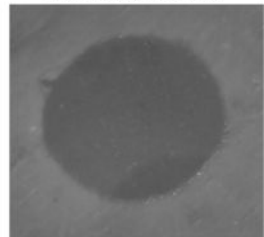
waaC), capsule (*kps*15) and colanic acid (*cpsG*) mutants. **D.** Effect of ROAR029 biofilm extract on *E. coli* 536 wild type and LPS mutants lawns. **E.** Growth curves showing the effect of ROAR029 biofilm extract addition to cultures of *E. coli* 536 and mutants with different LPS lengths. The graph shows means of 8 independent wells from a representative experiment.

Figure 6. ROAR029 excludes MG1655F' from mixed biofilms. Percentage of ROAR029 pColR_Km, pColR− or pColR+ and MG1655F' after 24 h of mixed biofilms inoculated at different initial ratios (1 :1, 1 :10, 1 :100 and 1 :10000).

Figure 7. **Colicin R has enhanced activity towards biofilms of LF82.** Differential effect of ROAR029 biofilm extract on planktonic (exponential and stationary phase), and biofilm cells (resuspended and non resuspended) of two pathogenic *E. coli* strains, 55989 with full LPS and LF82 with no LPS. Survival was determined by CFU counts after 2 h exposure to equal quantities of ROAR029 biofilm extract containing colicin R. *** p< 0.001.

A

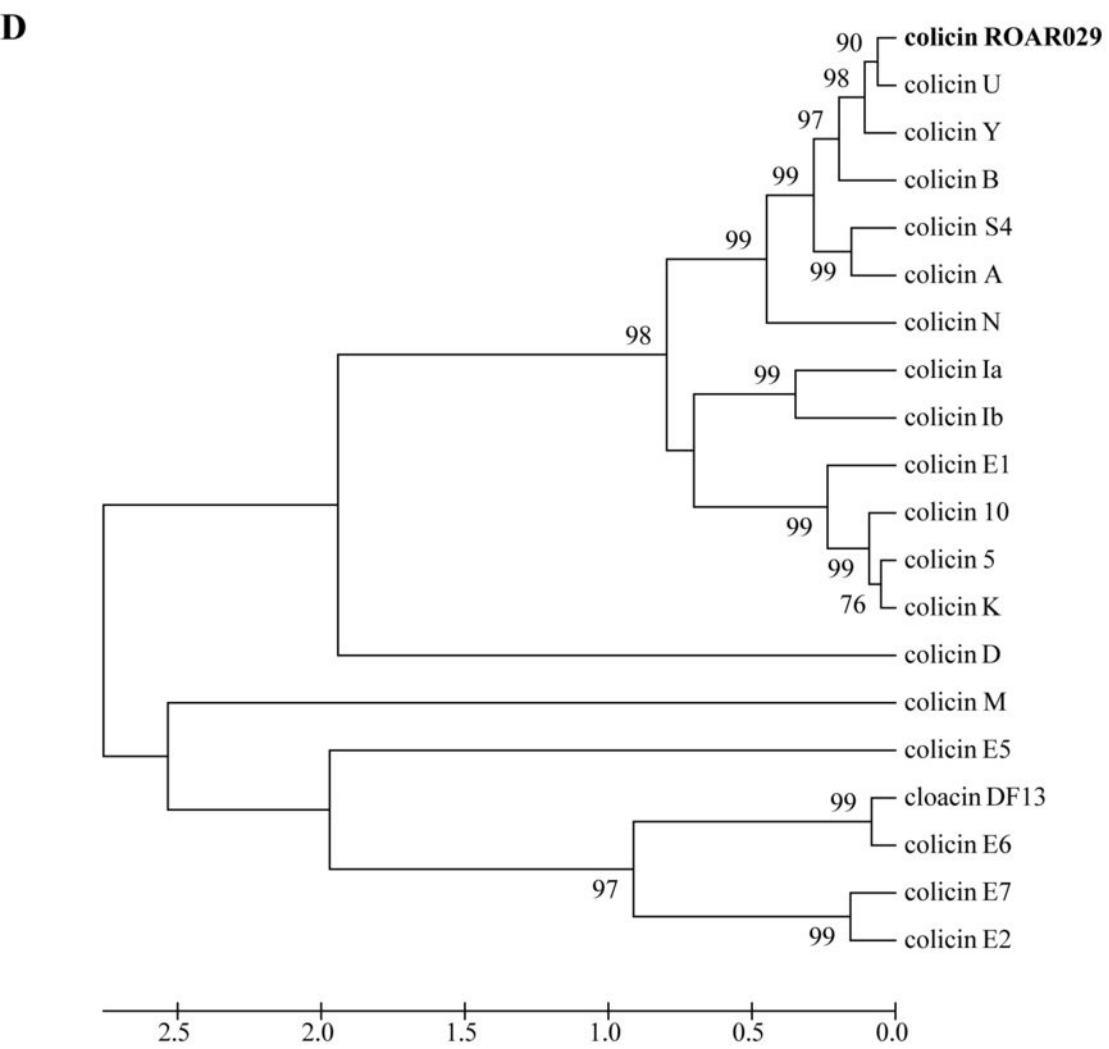**B**
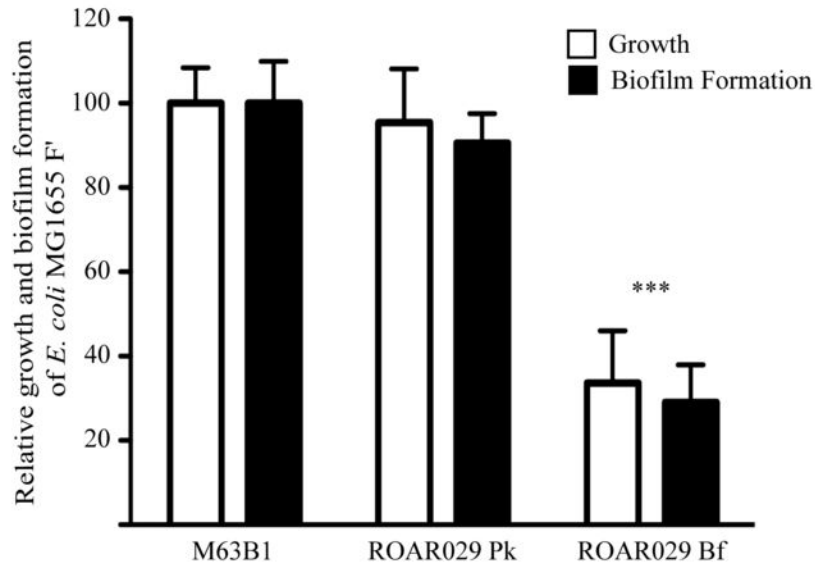
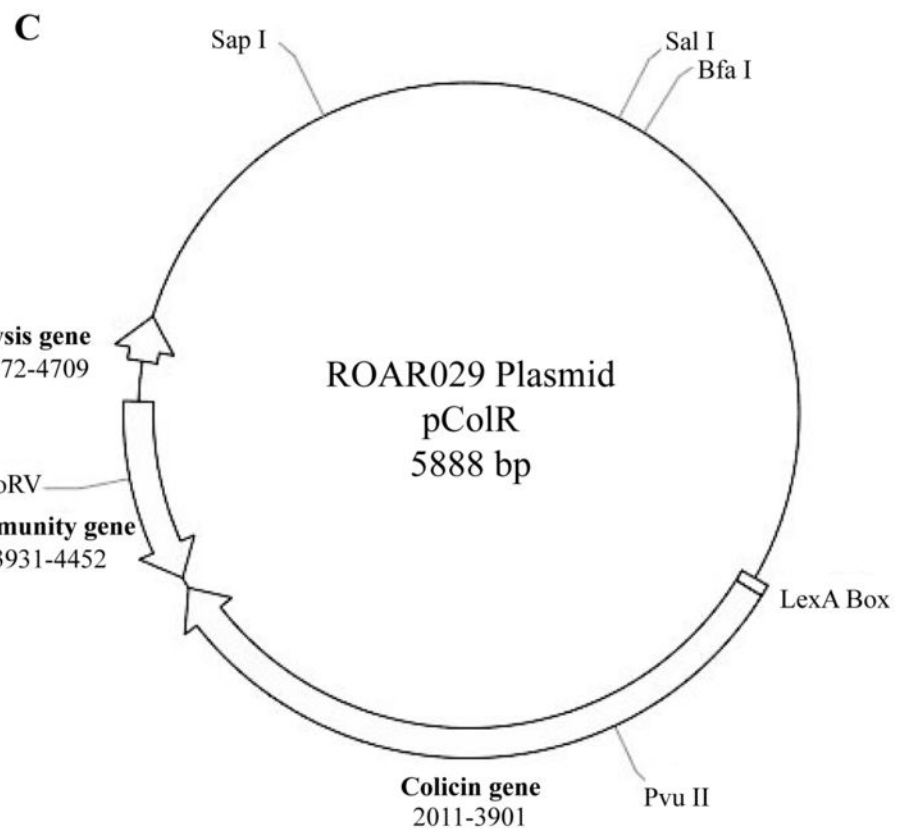E. coli MG1655   E. coli MG1655

ROAR029 Pk   ROAR029 Bf

C   E. coli MG1655
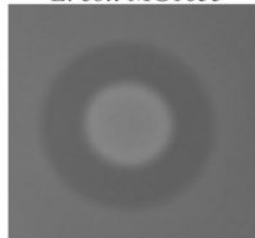
ROAR029 Colony Bf

D   E. coli MG1655 *ilvG+*

ROAR029 Bf

A ROAR029 Bf ROAR029 Δ*pcnB* Bf ROAR029 pColR- Bf

B

Linearized pColR Plasmid

C

Sap I Sal I Bfa I

ROAR029 Plasmid
pColR
5888 bp

Lysis gene
4572-4709

EcoRV

Immunity gene
3931-4452

LexA Box

Colicin gene
2011-3901

Pvu II

D

colicin ROAR029
colicin U
colicin Y
colicin B
colicin S4
colicin A
colicin N
colicin Ia
colicin Ib
colicin E1
colicin 10
colicin 5
colicin K
colicin D
colicin M
colicin E5
cloacin DF13
colicin E6
colicin E7
colicin E2
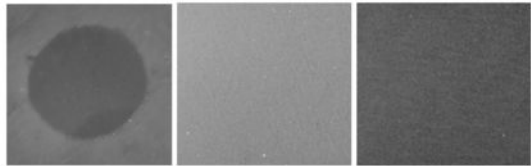
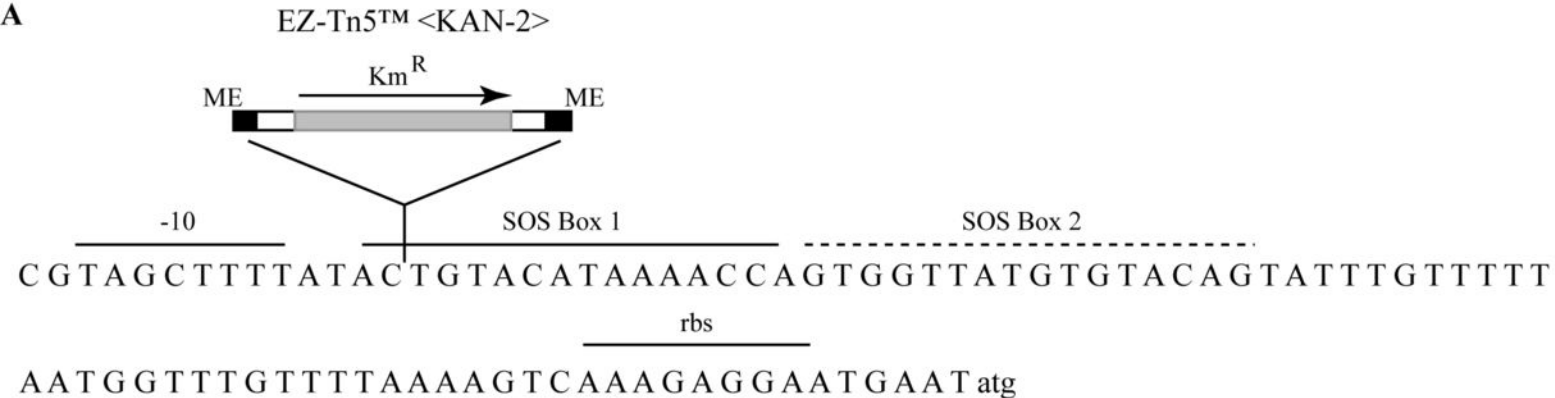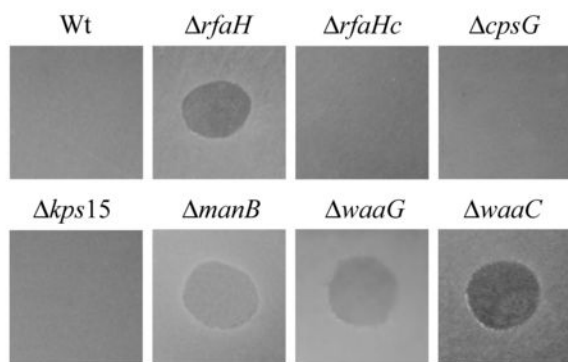| Wild type | $\Delta tolA$ | $\Delta ompA$ | Wild type | $\Delta tolA$ | $\Delta ompA$ |

+ ROAR029 Bf Extract + *S. boydii* M592 (colicin U) Bf Extract

A

EZ-Tn5™ <KAN-2>

ME   Km^R ME

-10   SOS Box 1   SOS Box 2

C G T A G C T T T T A T A C T G T A C A T A A A A C C A G T G G T T A T G T G T A C A G T A T T T G T T T T T

rbs

A A T G G T T T G T T T T A A A A G T C A A A G A G G A A T G A A T atg

B

+ aTc

200 ng/ml   500 ng/ml   1000 ng/ml

C

No mitomycin C   0.1 µg/ml mitomycin C

D

- + TG1 pColR+
- + TG1 pColR_Km
- + TG1 pColR -
- + TG1 *lexAind*3 pColR+
- + TG1 *lexAind*3 pCol_Km
- + TG1 *lexAind*3pColR-

A MG1655 | MG1655 *wbbL+* | TG1 | TG1 *wbbL+*

B + ROAR029

MG1655

MG1655 *wbbL+*

TG1

TG1 *wbbL+*

C Wt | Δ*rfaH* | Δ*rfaHc* | Δ*cpsG* | Δ*kps15* | Δ*manB* | Δ*waaG* | Δ*waaC*

D Wt | Δ*rfaH* | Δ*rfaHc* | Δ*cpsG* | Δ*kps15* | Δ*manB* | Δ*waaG* | Δ*waaC*

E OD 600 nm vs Time (min)
Δ*cpsG*, Δ*rfaHc*, Wild type, Δ*kps15*, Δ*manB*, Δ*rfaH*, Δ*waaG*, Δ*waaC*

TABLES

Table 1. Amino acid sequence identity of ROAR029 colicin system with related colicins, U and Y.

	Colicin Y	Colicin U
Lysis protein	97 %	86 %
Colicin protein	89 %	92.5 %
Immunity protein	85 %	85 %