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Running title: ECC clinical isolates, MFS efflux pump repressor, chlorhexidine

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ABSTRACT

Major Facilitator Superfamily (MFS) efflux pumps have been shown to be important for bacterial cells to cope with biocides such as chlorhexidine (CHX), a widely used molecule in hospital settings. In this work, we evaluated the role of two genes: *smvA* and *smvR* in CHX resistance in *Enterobacter cloacae* complex (ECC). *smvA* encodes an MFS pump whereas *smvR*, located upstream of *smvA*, codes for a TetR-type transcriptional repressor. To this aim, we constructed corresponding deletion mutants from the ATCC 13047 strain (MIC = 2 mg/L) as well as strains overexpressing *smvA* or *smvR* both in ATCC 13047 and three clinical isolates exhibiting elevated MICs of CHX (16-32 mg/L). Determination of MICs revealed that *smvA* played a modest role in CHX resistance, contrarily to *smvR* that modulated the ability of ECC to survive in the presence of CHX. In clinical isolates, the overexpression of *smvR* significantly reduced MICs of CHX (2-8 mg/L). Sequences analyses of *smvR* and promotor regions pointed out substitutions in conserved regions. Moreover, transcriptional studies revealed that SmvR acted as a repressor of *smvA* expression even if no quantitative correlation between the level of *smvA* mRNA and MICs of CHX could be observed. On the other hand, overproduction of *smvA* was able to complement the lack of the major resistance-nodulation-cell division (RND) superfamily efflux pump AcrB and restored resistance to ethidium bromide and acriflavine. Although SmvA could efflux biocides such as CHX, other actors, of which the expression is under SmvR control, should play critical role in ECC.

INTRODUCTION

Biocidal agents are wildly used to limit the spread of antibiotic-resistant bacteria in hospitals. However, numerous compounds that help for disinfection in healthcare may enhance the risk of emergence of antibiotic resistance, especially during a long period of low-level exposure (1). The bisguanide chlorhexidine (CHX) is frequently used in healthcare environments for hospitalized patients skin (catheter maintenance) or mucous (mouthwash) disinfection (2). Bacterial decreased susceptibility to biocides involve one or several mechanisms such as activation of efflux pumps, modification or overexpression of targets (3). For example, some efflux pumps as AceI in *Acinetobacter baumannii* and CepA in *Klebsiella pneumoniae* have been described to be involved in decrease of CHX susceptibility (4,5). Moreover, Wand *et al.* demonstrated that increased tolerance to CHX in *K. pneumoniae* was associated with an overexpression of the *smvA* gene encoding an efflux pump belonging to the major facilitator superfamily (MFS) and that the latter gene was under the control of the TetR-family transcriptional regulator SmvR (6).

The species of the *Enterobacter* genus, mainly *E. cloacae* complex (ECC) and *E. aerogenes*, are responsible for 7% of infections among patients hospitalized in intensive care units (ICUs) (7). Actually, ECC is composed of 14 clusters (designated C-I to C-XIV) among which three (C-III, VI, and VIII) are the most frequently recovered from human clinical specimens (8,-10). In addition, ECC are capable of 'escaping' the antibacterial action of several antibiotics and were classified among the multidrug-resistant (MDR) ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.) (11).

The purpose of the study was 1) to evaluate the *in vitro* antibiotic and biocide susceptibilities of the ATCC 13047 reference strain and clinical strains belonging to ECC, 2) to characterize the role of ECL_1895/ECL_01896 locus (homologous to *smvAR*) by constructing the

corresponding knock-out mutants and overexpressing strains. Our data revealed that the SmvR-like regulator played an important role in CHX resistance and no correlation was observed between MICs of CHX and transcriptional levels of *smvA*.

MATERIALS AND METHODS

Strains, media and growth conditions

Bacterial strains and plasmids used in this study are listed in Table S1. The reference strain was *E. cloacae* subp. *cloacae* ATCC 13047 [ECL13047] for which the genome sequence is available (GenBank accession numbers CP002886, FP929040 and AGSY00000000) and belonging to the cluster XI (12). *Escherichia coli* and *E. cloacae* strains were cultured with shaking (200 rpm) at 37°C in Luria-Bertani (LB) medium. Besides the reference strain, three unrelated ECC clinical isolates (belonging to cluster III, VI and VIII) with different CHX resistance levels collected from the University Hospital of Caen (France) were included in the study. Identification at the species level was performed by using the MALDI-TOF mass spectrometry method (Microflex, Bruker Daltonik, Bremen, Germany) while delineation to the cluster membership was obtained regarding *hsp60* gene sequence as previously described (8).

Drug susceptibility testing

MICs of CHX, crystal violet (CV), cetyltrimethylammonium bromide (CTAB), benzalkonium chloride (BL), ethidium Bromide (EtBr), acriflavine (ACR), cefotaxime (CTX), gentamicin (GEN), amikacin (AMK), chloramphenicol (CHL), erythromycin (ERY), norfloxacin (NOR) and tetracycline (TET) were determined by the broth microdilution (BMD) reference method using the cation-adjusted Mueller-Hinton broth (CA-MHB) (Becton-Dickinson, Le Pont de Claix, France), in accordance with EUCAST guidelines (http://www.eucast.org/). MICs of CHX was also determined by BMD with or without reserpine (20 mg/L). The minimal

bactericidal concentration (MBC) was determined only for CHX by sub-culturing the last tube showing visible growth and all the tubes in which there was no growth on already prepared plates containing Mueller Hinton agar medium. The plates were then incubated at 37 °C for 24 h and the lowest concentration showing no growth was taken as the MBC.

Construction of the knockout mutants

Disruption of the genes encoding MFS transporter (*smvA*) and its putative TetR regulator (*smvR*) was performed using the method previously described using the Red helper plasmid pKOBEG (13,-15). The primers used in the study are reported in Table S2.

Construction of multicopy plasmid library containing putative efflux pump or regulator ORFs

The regulator and the MFS efflux pump encoding genes (*smvA*/*smvR*) including their own promoters were amplified by PCR using primers listed in Table S2. Each amplicon was then TA-cloned into the overexpression plasmid, pBAD202 (low-copy number plasmid: ~20 copies/cell) Directional TOPO (Invitrogen, Villebon sur Yvette, France). *E. coli* TOP-10 cells (Invitrogen) carrying pBAD202 recombinants containing correctly oriented inserts were selected on LB plates with 40 mg/L of kanamycin. After purification, each plasmid carrying the regulator or MFS efflux pump encoding genes was used to transform ECC ATCC 13047 strain and clinical isolates (Table S1).

RNA extraction and qRT-PCR

Total RNAs were extracted from bacterial cells grown to the late-exponential phase in presence or not of CHX (1/4 MIC) by using the Direct-Zol RNA mini-prep kit (Zymo Research, Irvine, CA). Residual chromosomal DNA was removed by treating samples with the TURBO DNA-*free* kit (Life Technologies, Saint-Aubin, France). Samples were quantified using the NanoDrop One spectrophotometer (Thermo Fisher Scientific, Courtaboeuf, France). cDNA was synthesized from total RNAs (~1 µg) using the QuantiTect reverse transcription

kit (Qiagen) according to the manufacturer's instructions. For each RNA sample, transcript levels were determined by the DeltaCt method using the expression of the corresponding *rpoB* gene as a housekeeping control gene from the same RNA extraction (Table S2). The expression ratio were determined by comparison with the level of transcription measured for the ATCC 13047 strain. Experiments were performed at least three times and Student t-test was performed. *p*-values less than 0.05 were considered as statistically significant.

WGS and bioinformatic analysis

Genomic DNA was isolated from ECC isolates using the using the Quick-DNA fungal/bacterial miniprep kit (Zymo Research, Irvine, CA) according to the manufacturer's recommendations. After DNA shearing, the DNA libraries were prepared using the NEBNext Ultra DNA library prep kit for Illumina (New England Biolabs, Ipswich, MA) and sequenced as paired-end reads (2 x 300 bp) using an Illumina MiSeq platform and the MiSeq reagent kit version 3. The Illumina reads were trimmed using Trimmomatic (Bolger Bioinformatics 2014), quality filtered with the Fastx-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and then assembled using the SPAdes and plasmidSPAdes softwares (16,17). The nucleotide sequences were also submitted to ResFinder server (www.genomicepidemiology.org/) to identify acquired antimicrobial resistance genes (18). The presence of *qac* and *teh* genes was exanimed using Blast software on a home-made database containing 59 genes recovered from NCBI database. These genes are listed in Table S3.

RESULTS AND DISCUSSION

ECL_01896 (*smvA*) is a putative efflux pump involved in CHX decreased susceptiblity

One of the main mechanism underlying CHX decreased susceptibility in *Enterobacteriaceae* is related to the expression of efflux pumps allowing the expulsion of antiseptic molecules

(19). The gene coding for the quaternary ammonium (*qac*) compound efflux SMR (Small Multidrug Resistance) transporter QacE delta 1 has been recovered among 68% of CHX decreased susceptibility extended-spectrum β-lactamase producing (EBSL) *E. cloacae* clinical isolates (20). However, the prevalence of this gene, located into an integron, did not seem to correlate with the decreased susceptibility to biocides in MDR enterobacterial species including *E. cloacae* and no *qac* gene was recovered in any strain of this study (21). Analysis of the genome of *E. cloacae* ATCC 13047 and that of clinical isolates ECLJO_031, ECLJO_32 and ECLJO_38, allowed us to identify a locus (ECL_01896, henceforth named *smvA*) encoding a major facilitator transporter of the MFS family (12). This last showed 91% and 72% amino acid identities with the SmvA protein of *Klebsiella aerogenes* and of *K. pneumoniae*, respectively (data not shown). To evaluate the role of this SmvA-like protein, we constructed the *smvA* deletion mutant strain and showed that the MIC of CHX was 4-fold reduced compared to the ATCC 13047 parental strain (0.5 mg/L and 2 mg/L, respectively) (Table 1) indicating that *smvA* is intrinsically able to efflux CHX in ECC.

ECL_01895 (*smvR*) regulates the transcription of *smvA*

Interestingly, the gene ECL_01895 (henceforth named *smvR*), located upstream *smvA* and divergently transcribed, coded for a TetR-family transcriptional regulator homologous to *smvR* of *K. aerogenes* and *K. pneumoniae*. Recently, Wand *et al*. showed that SmvR acted as a repressor of the *smvA* transcription and mutation into the sequence of the regulator was correlated with the CHX adaptation (22). Unexpectedly, in ECC ATCC 13047 strain that exhibited a low MIC of CHX (2 mg/L), the total deletion of *smvR* led to an overexpression of *smvA* by 6-fold but only modestly increased CHX MIC (4 mg/L) ($p < 0.05$) (Figure 1, Table 1). On the other hand, CHX susceptibility of the ECL_JO04 strain, genetically very close to the susceptible strain ECL_JO40) but totally deleted of both *smvA* and *smvR*, was more reduced (0.25 mg/L) than single *smvA* or *smvR* mutants (Table 1). In order to further analyze

Table 1. MICs of biocides and antimicrobials for the different strains

	MIC (mg/L)												
Strains	CHX[1]	CV	CTAB	CB	EtBr	ACR	GEN	AMK	CTX	CHL	ERY	NOR	TET
E. cloacae ATCC 13047	2/2	32	16	32	512	64	0.25	2	1	8	256	0.12	1
ATCC 13047Δ*smvA*	**0.5/0.5**[2]	32	16	32	256	32	0.25	2	1	8	256	0.25	1
ATCC 13047Δ*smvR*	4/4	32	32	32	1024	64	0.25	1	1	8	256	0.12	1
ATCC 13047 pBAD202	2/2	32	16	32	512	64	0.25	2	1	8	256	0.12	1
ATCC 13047 pBAD202Ω*smvA*	4/4	32	16	32	**2048**	**256**	0.12	1	1	4	256	0.12	1
ATCC 13047 pBAD202Ω*smvR*	**0.5/1**	32	32	32	256	64	0.25	2	1	8	128	0.12	1
ATCC 13047Δ*acrB*	1/1	0.5	16	4	16	4	0.25	2	1	0.5	8	0.06	0.5
ATCC 13047Δ*acrB* pBAD202	1/1	0.5	16	4	16	4	0.25	2	1	0.5	8	0.03	0.5
ATCC 13047Δ*acrB* pBAD202Ω*smvA*	2/4	1	8	4	**256**	**64**	0.12	1	1	0.5	8	0.03	0.5
ATCC 13047Δ*acrB* pBAD202Ω*smvR*	0.5/0.5	1	16	4	8	4	0.25	2	1	1	8	0.03	1
Cluster III													
ECL_JO04 Δ*smvA*/*smvR*	0.25/0.25	64	32	32	1024	128	0.25	2	1	8	256	0.06	2
ECL_JO32	32/32	64	32	32	1024	128	0.25	2	1	8	256	0.06	2
ECL_JO32 pBAD202	32/32	64	32	32	1024	128	0.25	2	1	8	256	0.06	2
ECL_JO32 pBAD202Ω*smvA*	64/64	32	32	32	2048	256	0.25	2	1	8	256	0.06	2
ECL_JO32 pBAD202Ω*smvR*	**4/8**	32	32	32	512	64	0.25	2	1	8	256	0.06	2
Cluster VI													
ECL_JO31	16/16	32	16	16	512	64	0.12	1	1	4	256	0.06	1
ECL_JO31 pBAD202	16/16	32	16	16	512	64	0.12	1	1	4	256	0.06	1
ECL_JO31 pBAD202Ω*smvA*	32/32	32	16	16	1024	128	0.12	1	1	4	256	0.06	1
ECL_JO31 pBAD202Ω-*smvR*	**2/4**	32	16	16	256	32	0.12	1	1	4	256	0.06	1
Cluster VIII													
ECL_JO38	16/16	64	32	32	1024	128	0.25	2	1	4	256	0.06	2
ECL_JO38 pBAD202	16/16	64	32	32	1024	128	0.25	2	1	4	256	0.06	2
ECL_JO38 pBAD202Ω*smvA*	32/32	32	32	32	2048	256	0.25	2	1	4	256	0.06	2
ECL_JO38 pBAD202Ω*smvR*	**2/4**	32	32	32	**256**	**32**	0.25	2	1	4	256	0.06	2

CHX : Chlorhexidine; CV: Crystal violet; CTAB: Cetyl trimethylammonium bromide; CB: Benzalkonium chloride; EtBr : Ethidium Bromide ; ACR: Acriflavine ; GEN : Gentamicin; AMK: Amikacin; CTX : Cefotaxime; CHL: Chloramphenicol ; ERY: Erythromycin; NOR : Norfloxacin; TET : Tetracycline.

[1] MIC/MBC

[2] In bold are values with significant changes (>4 fold change) compared to the parental strain

the role of SmvR regulator three clinical isolates (ECL_JO31, ECL_JO38, and ECL_JO32) with higher MICs of CHX (16-32 mg/L) were also analyzed and complemented with *smvR* cloned with its own promoter into the pBAD202 vector (Table 1). ECL_JO32 was categorized as decreased susceptibility because the MICs was < 64 mg/L. This cut-off value also corresponded to the MBC and was determined for *Enterobacter* spp. based on the analysis of 54 clinical isolates (23). As shown in Table 1, all these strains reverted to a susceptible phenotype to CHX with MICs of 2-4 mg/L. In parallel, the transcriptional levels of *smvA* highly decreased (non-detectable) in these complemented strains (Figure 1). These results strongly pointed out the role of SmvR in CHX susceptibility in *E. cloacae*, which are in agreement with those observed in *K. pneunoniae* (22). Analysis of SmvR sequences revealed that 14 to 15 amino acids substitutions were recovered in clinical isolates compared to the sequences of the two susceptible ATCC and ECL_JO40 strains. Among them, 11 were at the same positions (Figure 2A). Note that one and three substitutions were specific for ECL_JO38 and ECL_JO32, respectively. These data contrast with the absence of SmvR mutations in *Enterobacter* sp. upon CHX exposure while it has been observed in *Klebsiella*, *Citrobacter* and *Salmonella* (6). Because ~~two~~ one substitution (G52A) was present into one of the two highly conserved region of the SmvR sequence in clinical isolates, it is tempting to speculate that it could play a role in high CHX MICs. We also compared the shared promotor region for *smvAR* especially into the palindromic DYAD motifs which are typical for Tet-repressors. Two interesting nucleotide-changes were identified in the sequences from clinical isolates: one in the -35 box located into the well conserved motif P3, another into the P2 motif (Figure 2B). The impact of such modifications in decreased susceptibility to CHX needs to be further studied.

In order to verify whether CHX may constitute an inducer of *smvR* expression, we measured its transcriptional level in the presence of a sub-MIC concentration of CHX (1/4 of MIC). As

shown in Figure 3, the expression of *smvR* was induced from 2.5 to 9 fold after 20, 40 and 60 minutes in presence of CHX in ATCC 13047, ECL_JO32 and ECL_JO38 strains. As for other Tet-family repressors, it appears that when a signal molecule (here CHX) binds to SmvR, a conformational change takes place that renders the repressor protein unable to bind DNA (24). As a consequence, *smvR* (likely under its own regulation) was overexpressed (Figure 3). Concomitantly, increased expression of *smvA* was observed in ECL_JO32 and ECL_JO38 strains but not in ATCC 13047 (Figure 4). It may be suggested that, in ATCC strain, the structurally modified SmvR by CHX, may still interact with the promotor region of *smvA*.

The CHX susceptibility level is not quantitatively correlated to the level of *smvA* transcription.

We showed that an almost undetectable level of *smvA* transcription led to a low MIC of CHX and overexpression of *smvA* in all *Enterobacter* strains was coupled with a two fold increase of MICs arguing for a role of SmvA (Figure 1, Table 1). However, no strict quantitative correlation between the transcriptional level of *smvA* and MICs were observed. Indeed, the transcription of *smvA* in ECL_JO31 (with a MIC of 16 mg/L) was 4.3 fold higher than in ECL_JO38 (with also a MIC of 16 mg/L) and 2-fold lower than in *smvR* mutant of the ATCC 13047 strain that has a MIC of 4 mg/L (Figure 1). Likewise, we measured 3 fold more *smvA* transcript in the *smvR* mutant of ATCC 13047 than in ECL_JO32, whereas MIC of CHX was 8 fold lesser (Figure 1, Table 1). Recently, it has been shown that heterologous expression of *smvA* from *K. pneumoniae* or *S. enterica* in *E. coli* (which do not possesses *smvAR* homologs) modestly increased MICs of CHX (from 0.5 to 2-4 mg/L) (6). Noteworthy, contrarily to these *E. coli* strains, we did not observe any growth defect for our ECC transconjugants (data not shown).

Thus, in *Enterobacter*, it may be hypothesis that SmvR may have other targets than *smvA* whose products are involved in CHX reduced susceptibility. Such of targets should be

other(s) MFS-family efflux pump(s) because the addition of 20 mg/L reserpine (MFS pumps inhibitor) dramatically reduced the MICs of CHX (2-4 mg/L) in clinical isolates and strains overexpressing *smvA* (Table S4).

Role of SmvA and SmvR in resistance to other biocide compounds.

We previously showed that the ArcB efflux pump of the RND family was involved in the resistance of *E. cloacae* to the biocides such as BC and tetraphenylphosphonium and to the dyes ACR, CV, EtBr and rhodamine (25). On the other hand, the lack of AcrB did not impact the resistance to CHX or cetyltrimethylammonium bromide (CTAB) (Table 1) (25). To test whether SmvA and/or SmvR can play a role in the resistance to such of molecules, MICs were determined for the different constructions of ECC previously used as well as in transcomplemented strains of *acrB* mutant of *Enterobacter*. Towards CV, CTAB and CB, no difference was observed for the *smvA* and *smvR* mutant or transcomplemented strains compared to the wild-type or parental counterparts (Table 1). On the other hand, a 4-fold increase of MICs to EtBr and ACR was observed when *smvA* was overexpressed into the ATCC 13047 strain and a 4-fold decrease when *smvR* was overproduced in the ECLJO_038 clinical isolate (Table 1). However, the most important effects were obtained when *smvA* was expressed in the *acrB* mutant strain susceptible to EtBr and ACR. Indeed, complementation by *smvA* in this mutant almost completely restored levels of resistance to EtBr and ACR until those of the wild type ECC strain (Table 1). This clearly show that, according to biocide, the MFS-family efflux pump SmvA was able to compensate the lack of the major RND family pump AcrB.

SmvA and SmvR are not involved in antibiotic resistance.

The question if biocides select for antibiotic resistance has been asked since long time (3). This possible link may be due to the selective pressure constituted by the presence of antiseptics leading to the emergence of mechanisms of resistance that are common with other

antimicrobial compounds. In this context, the overexpression of efflux pumps may be of great interest. In *E. cloacae*, the *acrB* mutant is more susceptible to several biocides as well as fluoroquinolones, tetracyclines, cotrimoxazole, chloramphenicol, fusidic acid, and erythromycin (25). In order to evaluate the putative role of SmvA and SmvR in antibiotic resistance, MICs of GEN, AMK, CTX, CHL, ERY, NOR and TET of the different strains used in this study were determined. As shown in Table 1, no significant difference was observed neither for *smvA* or *smvR* mutant strains or cells overproducing SmvA or SmvR compared to their isogenic parental strains. In contrast to AcrB, these results argue for a minor involvement of the SmvA efflux pump in the acquisition of antibiotic resistance.

Concluding remark.

The transcriptional regulator SmvR of *Enterobacter* acted as repressor of the expression of *smvA* (encoding a MFS family efflux pump). However, other(s) member(s) of its regulon may be involved in the resistance to CHX observed in some clinical isolates. In addition, the overproduction of MFS-type SmvA was able to partially complement the lack of the AcrB RND-family efflux pump by restoring the resistance to EtBr and ACR biocides. Despite that *smvRA* locus did not seem implicated in the antimicrobial resistance of ECC, it appears as an important opportunistic trait. Characterization and survey of such mechanisms enabling this pathogen to cope with biocides are important especially in hospital environment to avoid the emergence and persistence of virulent isolates.

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FIGURE LEGENDS

Figure 1: Expression ratios of *smvA* in ATCC 13047, *smvA* mutant, *smvR* mutant, clinical isolates and complemented strains by pBADΩ0-*smvR*. Asterix indicates *p*-value < 0.05 compared to the data obtained with the ATCC 13047 strain.

Figure 2: Sequence alignments of SmvR (A) and *smvR-smvA* intergenic region (B). (A) In bold are the amino acids substitutions compared to the sequences of the two susceptible strains ATCC 13047 and ECL_JO40. Conserved regions in *Enterobacteriaceae* in SmvR proteins are indicated in boxes. (B) Potential conserved motifs P2 and P3 are in boxes. Grey boxes indicate start codons of *smvR* and *smvA* ORFs. Putative -10 and -35 boxes are indicated in italic. Putative interesting nucleotide changes are in bold.

Figure 3: Expression ratios of the *smvR* gene in ATCC 13047 and clinical isolates without and with ¼ MIC of CHX during 20, 40 and 60 minutes. Asterix indicates *p*-value < 0.05 compared to the data obtained with the ATCC 13047 strain without CHX.

Figure 4: Expression ratios of the *smvA* gene in ATCC 13047 and clinical isolates without and with ¼ MIC of CHX during 20, 40 and 60 minutes. Asterix indicates *p*-value < 0.05 compared to the data obtained with the ATCC 13047 strain without CHX.

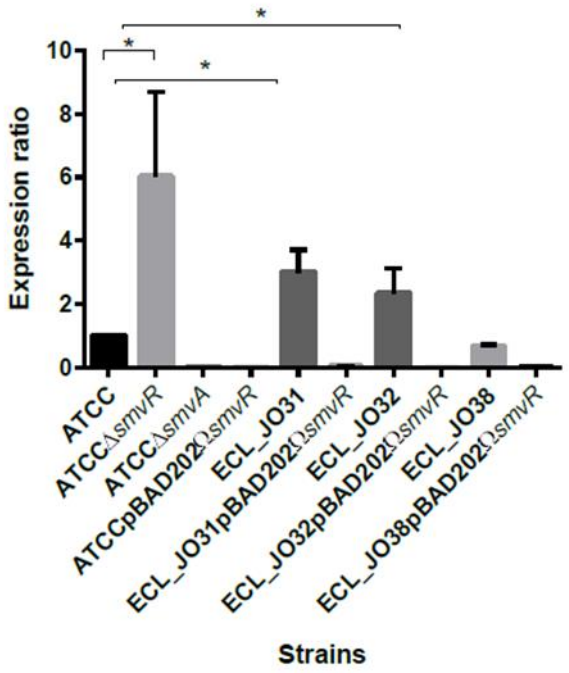

Figure 1: Expression ratios of *smvA* in ATCC 13047, *smvA* mutant, *smvR* mutant, clinical isolates and complemented strains by pBAD202Ω*smvR*. Asterix indicates *p*-value <0.05 compared to the data obtained with the ATCC 13047 strain.

A

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ATCC13047 MGYLTRDERREEILQAAMRVALAEGFSAMTVRRIASEAGVATGQVHHHFTSGGELKSLAFIRLIRELLDADVVGEHASWRARLHAMLGSDDGRFEPYIRLWREAQILATRDAEIKGAYVL
ECL_JO40  MRYLSKEERREAILQAAMRVALSEGLAAMTVRRIAAEAGVATGQVHHHFASGGELKSLAFVRVIRELLDAEVVGENASWRERLHSMLGSDDGGFEPYIRLWREAQILASRDPEIKAAYVL
ECL_JO31  MRYLSKDERREEILQAAMRVALTEGFAAMTVRRIATEAGVATGQVHHHFASAGELKSLAFVRLIRNLLDAEIVGENAGWRERLHAMLGSDDGGFEPYIRLWREAQILASRDSDIKGAYVL
ECL_JO32  MRYLSKDERREEILHAAMRVALSEGLSAMTVRRIATEAGIATGQVHHHFASAGELKALAFVRLIRDLLDAEVVGENAGWRERLHAMLGSDDGGFEPYIRLWREAQILASRDSDIKGAYVL
ECL_JO38  MRYLSKDERREEILQAAMRVALTEGFAAMTVRRIATEAGVATGQLHHHFASAGELKSLAFVRLIRDLLDAEIVGENAGWRERLHAMLGSDDGGFEPYIRLWREAQILASRDSDIKGAYVL

ATCC13047 TMEMWHQETVALIEAGRNAGVFRSGDPAASIAWRLIGLVCGLDGIYLLNMPEMDDAAFNNHLDKLISLELF      191
ECL_JO40  TMEMWHQETVAIIRAGATACAFTLNDRAENIAWRLIGLVCGLDGITILNMPEMDDAAFNKHLDKLITLELF      191
ECL_JO31  TMEMWHQETVAIIRAGAEANAFTLADQPENIAWRLIGLVCGLDGIYVLNMPEMDDAAFNKHLDKLISLELF      191
ECL_JO32  TMEMWHQETVAIIRAGAEANVFTLTDRPENIAWRLIGLVCGLDGIYVLNMPEMDDAAFNKHLDKLISLELF      191
ECL_JO38  TMEMWHQETVAIIRAGAEANAFTLADQPENIAWRLIGLVCGLDGIYVLNMPEMDDAAFNKHLDKLISLELF      191
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B

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                                                          -10          P3        -35          P2
ATCC3047 <smvR CATTTTCTCTCACTCTTAAATAGCAATGAAACGAGTGTAACAAAGAGCTGGACAAGTGTTCAACATTCCGTAGGATCGCATCCTGGACATGTG
ECL_JO40 <smvR CATTTTTTTCTACTCTC.AATGGGAATGAAACGAGTGTAACAAAAAGCTGGACATGCGTTCAACATTCCGTAGGATCGCATCCTGGACATGTG
ECL_JO31 <smvR CATAATTTCTCACTCTC.AATGGCAACGAAACGAGTGTAACAAAGAGCTGGACAAGTGTTCAACTTTACGTAGGATCGCAATCTGGACATGTG
ECL_JO32 <smvR CATATTTCCTCACTCTC.AATGGCAACGCAACGAGTGTAACAAAGAGCTGGACAAGCGTTCAACTTTACGTAGGATCGCATTCTGGACATGTG
ECL_JO38 <smvR CATAATTTCTCACTCTC.AATGGCAACGAAACGAGTGTAACAAAGAGCTGGACAAGTGTTCAACTTTACGTAGGATCGCAATCTGGACATGTG
         ***::*  ********  ***.**** *.*************************** *******:**.************: ***********

ATCC3047 TCCA.TTTTTA.TATAAAAAGGAAATTTATG> smvA
ECL_JO40 TCCATGAATGAAAATGATAAGGAAATTTATG> smvA
ECL_JO31 TCCAGTTTTT.AAATGAAAAGGAAATTTATG> smvA
ECL_JO32 TCCAGTTTTT.AACTGAAAAGGAAATTTATG> smvA
ECL_JO38 TCCAATTTTT.AACTGAAAAGGAAATTTATG> smvA
         **** ****: :.*.****************
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Figure 2: Sequence alignments of SmvR (A) and *smvR-smvA* intergenic region (B). (A) In bold are the amino acids substitutions compared to the sequences of the two susceptible strains ATCC 13047 and ECL_JO40. Conserved regions in *Enterobacteriaceae* in SmvR proteins are boxed. (B) Potential conserved motifs P2 and P3 are in boxes. Grey boxes indicate start codons of *smvR* and *smvA* ORFs. Putative -10 and -35 boxes are indicated in italic. Putative interesting nucleotide changes are in bold.

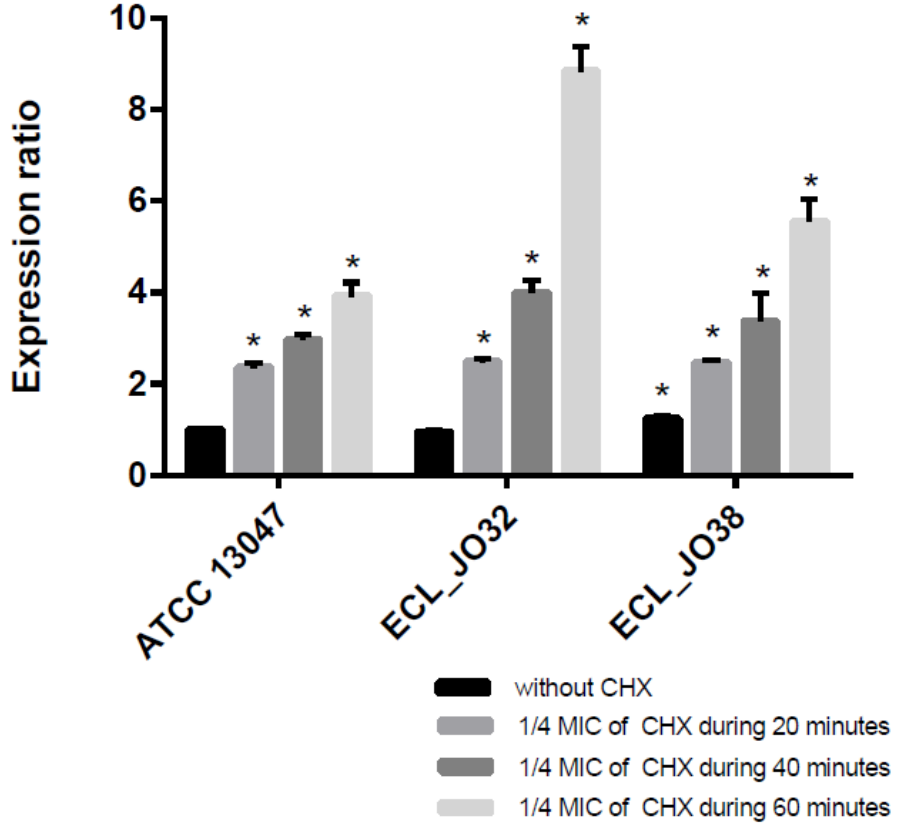

Figure 3: Expression ratios of the *smvR* gene in ATCC 13047 and clinical isolates without and with ¼ MIC of CHX during 20, 40 and 60 minutes. Asterix indicates *p*-value <0.05 compared to the data obtained with the ATCC 13047 strain without CHX.

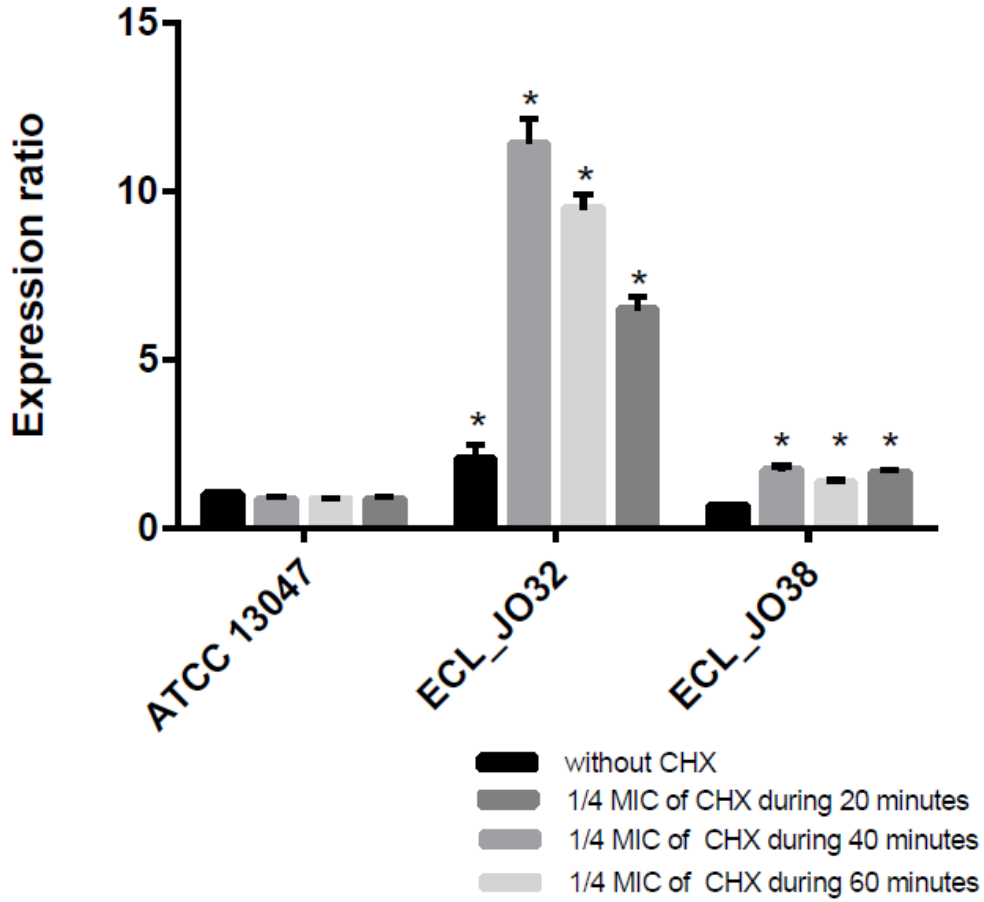

Figure 4: Expression ratios of the *smvA* gene in ATCC 13047 and clinical isolates without or with ¼ MIC of CHX during 20, 40 and 60 minutes. Asterix indicates *p*-value <0.05 compared to the data obtained with the ATCC 13047 strain without CHX.