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1 **The transcriptional repressor SmvR is important for chlorhexidine**
2 **decreased susceptibility in *Enterobacter cloacae* complex**

3
4 Running title: ECC clinical isolates, MFS efflux pump repressor, chlorhexidine

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28 **ABSTRACT**

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

47

48 INTRODUCTION

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

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

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

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

76

77 **MATERIALS AND METHODS**

78 **Strains, media and growth conditions**

79 Bacterial strains and plasmids used in this study are listed in Table S1. The reference strain
80 was *E. cloacae* subsp. *cloacae* ATCC 13047 [ECL13047] for which the genome sequence is
81 available (GenBank accession numbers CP002886, FP929040 and AGSY000000000) and
82 belonging to the cluster XI (12). *Escherichia coli* and *E. cloacae* strains were cultured with
83 shaking (200 rpm) at 37°C in Luria-Bertani (LB) medium. Besides the reference strain, three
84 unrelated ECC clinical isolates (belonging to cluster III, VI and VIII) with different CHX
85 resistance levels collected from the University Hospital of Caen (France) were included in the
86 study. Identification at the species level was performed by using the MALDI-TOF mass
87 spectrometry method (Microflex, Bruker Daltonik, Bremen, Germany) while delineation to
88 the cluster membership was obtained regarding *hsp60* gene sequence as previously described
89 (8).

90 **Drug susceptibility testing**

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

98 bactericidal concentration (MBC) was determined only for CHX by sub-culturing the last tube
99 showing visible growth and all the tubes in which there was no growth on already prepared
100 plates containing Mueller Hinton agar medium. The plates were then incubated at 37 °C for
101 24 h and the lowest concentration showing no growth was taken as the MBC.

102 **Construction of the knockout mutants**

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

106 **Construction of multicopy plasmid library containing putative efflux pump or regulator**

107 **ORFs**

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

116 **RNA extraction and qRT-PCR**

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

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

129 **WGS and bioinformatic analysis**

130 Genomic DNA was isolated from ECC isolates using the using the Quick-DNA
131 fungal/bacterial miniprep kit (Zymo Research, Irvine, CA) according to the manufacturer's
132 recommendations. After DNA shearing, the DNA libraries were prepared using the NEBNext
133 Ultra DNA library prep kit for Illumina (New England Biolabs, Ipswich, MA) and sequenced
134 as paired-end reads (2 x 300 bp) using an Illumina MiSeq platform and the MiSeq reagent kit
135 version 3. The Illumina reads were trimmed using Trimmomatic (Bolger Bioinformatics
136 2014), quality filtered with the Fastx-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and
137 then assembled using the SPAdes and plasmidSPAdes softwares (16,17). The nucleotide
138 sequences were also submitted to ResFinder server (www.genomicepidemiology.org/) to
139 identify acquired antimicrobial resistance genes (18). The presence of *qac* and *teh* genes was
140 examined using Blast software on a home-made database containing 59 genes recovered from
141 NCBI database. These genes are listed in Table S3.

142

143

144 **RESULTS AND DISCUSSION**

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

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

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

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

163 Interestingly, the gene ECL_01895 (henceforth named *smvR*), located upstream *smvA* and
164 divergently transcribed, coded for a TetR-family transcriptional regulator homologous to
165 *smvR* of *K. aerogenes* and *K. pneumoniae*. Recently, Wand *et al.* showed that SmvR acted as
166 a repressor of the *smvA* transcription and mutation into the sequence of the regulator was
167 correlated with the CHX adaptation (22). Unexpectedly, in ECC ATCC 13047 strain that
168 exhibited a low MIC of CHX (2 mg/L), the total deletion of *smvR* led to an overexpression of
169 *smvA* by 6-fold but only modestly increased CHX MIC (4 mg/L) ($p < 0.05$) (Figure 1, Table
170 1). On the other hand, CHX susceptibility of the ECL_JO04 strain, genetically very close to
171 the susceptible strain ECL_JO40) but totally deleted of both *smvA* and *smvR*, was more
172 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

Strains	MIC (mg/L)												
	CHX ¹	CV	CTAB	CB	EtBr	ACR	GEN	AMK	CTX	CHL	ERY	NOR	TET
<i>E. cloacae</i> ATCC 13047	2/2	32	16	32	512	64	0.25	2	1	8	256	0.12	1
ATCC 13047 Δ <i>smvA</i>	0.5/0.5²	32	16	32	256	32	0.25	2	1	8	256	0.25	1
ATCC 13047 Δ <i>smvR</i>	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 pBAD202 Ω <i>smvA</i>	4/4	32	16	32	2048	256	0.12	1	1	4	256	0.12	1
ATCC 13047 pBAD202 Ω <i>smvR</i>	0.5/1	32	32	32	256	64	0.25	2	1	8	128	0.12	1
ATCC 13047 Δ <i>acrB</i>	1/1	0.5	16	4	16	4	0.25	2	1	0.5	8	0.06	0.5
ATCC 13047 Δ <i>acrB</i> pBAD202	1/1	0.5	16	4	16	4	0.25	2	1	0.5	8	0.03	0.5
ATCC 13047 Δ <i>acrB</i> pBAD202 Ω <i>smvA</i>	2/4	1	8	4	256	64	0.12	1	1	0.5	8	0.03	0.5
ATCC 13047 Δ <i>acrB</i> pBAD202 Ω <i>smvR</i>	0.5/0.5	1	16	4	8	4	0.25	2	1	1	8	0.03	1
Cluster III													
ECL_JO04 Δ <i>smvA</i> / <i>smvR</i>	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 pBAD202 Ω <i>smvA</i>	64/64	32	32	32	2048	256	0.25	2	1	8	256	0.06	2
ECL_JO32 pBAD202 Ω <i>smvR</i>	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 pBAD202 Ω <i>smvA</i>	32/32	32	16	16	1024	128	0.12	1	1	4	256	0.06	1
ECL_JO31 pBAD202 Ω - <i>smvR</i>	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 pBAD202 Ω <i>smvA</i>	32/32	32	32	32	2048	256	0.25	2	1	4	256	0.06	2
ECL_JO38 pBAD202 Ω <i>smvR</i>	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

173 the role of SmvR regulator three clinical isolates (ECL_JO31, ECL_JO38, and ECL_JO32)
174 with higher MICs of CHX (16-32 mg/L) were also analyzed and complemented with *smvR*
175 cloned with its own promoter into the pBAD202 vector (Table 1). ECL_JO32 was categorized
176 as decreased susceptibility because the MICs was < 64 mg/L. This cut-off value also
177 corresponded to the MBC and was determined for *Enterobacter* spp. based on the analysis of
178 54 clinical isolates (23). As shown in Table 1, all these strains reverted to a susceptible
179 phenotype to CHX with MICs of 2-4 mg/L. In parallel, the transcriptional levels of *smvA*
180 highly decreased (non-detectable) in these complemented strains (Figure 1). These results
181 strongly pointed out the role of SmvR in CHX susceptibility in *E. cloacae*, which are in
182 agreement with those observed in *K. pneumoniae* (22). Analysis of SmvR sequences revealed
183 that 14 to 15 amino acids substitutions were recovered in clinical isolates compared to the
184 sequences of the two susceptible ATCC and ECL_JO40 strains. Among them, 11 were at the
185 same positions (Figure 2A). Note that one and three substitutions were specific for ECL_JO38
186 and ECL_JO32, respectively. These data contrast with the absence of SmvR mutations in
187 *Enterobacter* sp. upon CHX exposure while it has been observed in *Klebsiella*, *Citrobacter*
188 and *Salmonella* (6). Because ~~two~~ one substitution (G52A) was present into one of the two
189 highly conserved region of the SmvR sequence in clinical isolates, it is tempting to speculate
190 that it could play a role in high CHX MICs. We also compared the shared promoter region for
191 *smvAR* especially into the palindromic DYAD motifs which are typical for Tet-repressors.
192 Two interesting nucleotide-changes were identified in the sequences from clinical isolates:
193 one in the -35 box located into the well conserved motif P3, another into the P2 motif (Figure
194 2B). The impact of such modifications in decreased susceptibility to CHX needs to be further
195 studied.

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

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

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

208 We showed that an almost undetectable level of *smvA* transcription led to a low MIC of CHX
209 and overexpression of *smvA* in all *Enterobacter* strains was coupled with a two fold increase
210 of MICs arguing for a role of SmvA (Figure 1, Table 1). However, no strict quantitative
211 correlation between the transcriptional level of *smvA* and MICs were observed. Indeed, the
212 transcription of *smvA* in ECL_JO31 (with a MIC of 16 mg/L) was 4.3 fold higher than in
213 ECL_JO38 (with also a MIC of 16 mg/L) and 2-fold lower than in *smvR* mutant of the ATCC
214 13047 strain that has a MIC of 4 mg/L (Figure 1). Likewise, we measured 3 fold more *smvA*
215 transcript in the *smvR* mutant of ATCC 13047 than in ECL_JO32, whereas MIC of CHX was
216 8 fold lesser (Figure 1, Table 1). Recently, it has been shown that heterologous expression of
217 *smvA* from *K. pneumoniae* or *S. enterica* in *E. coli* (which do not possesses *smvAR* homologs)
218 modestly increased MICs of CHX (from 0.5 to 2-4 mg/L) (6). Noteworthy, contrarily to these
219 *E. coli* strains, we did not observe any growth defect for our ECC transconjugants (data not
220 shown).

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

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

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

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

244 **SmvA and SmvR are not involved in antibiotic resistance.**

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

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

257

258 **Concluding remark.**

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

268

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276

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360

361 **FIGURE LEGENDS**

362

363 **Figure 1:** Expression ratios of *smvA* in ATCC 13047, *smvA* mutant, *smvR* mutant, clinical
364 isolates and complemented strains by pBAD Ω 0-*smvR*. Asterix indicates *p*-value < 0.05
365 compared to the data obtained with the ATCC 13047 strain.

366

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

373

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

377

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

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382

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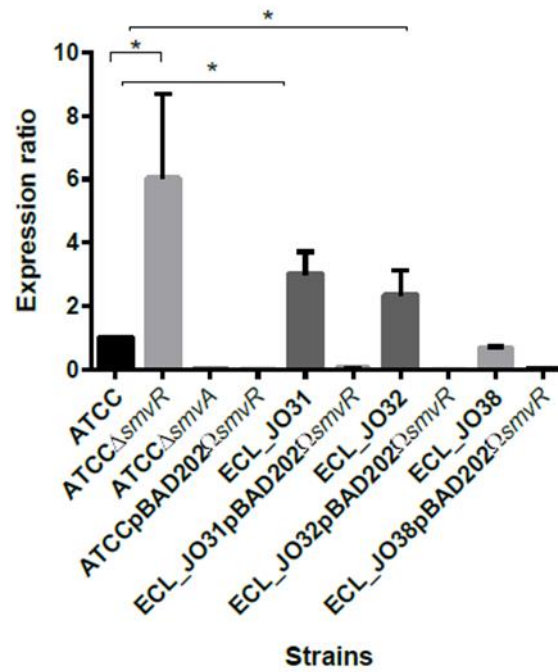


Figure 1: Expression ratios of *smvA* in ATCC 13047, *smvA* mutant, *smvR* mutant, clinical isolates and complemented strains by pBAD202 Ω *smvR*. Asterix indicates *p*-value < 0.05 compared to the data obtained with the ATCC 13047 strain.

A

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ATCC13047 MGYLTRDERREEILQAAMRVALAEGFSAMTVRRIASEAGVATGQVHHHFTSGGELKSLAFIRLIRELLDADVVEGASWRARLHAMLGSDDGRFEPYIRLWREAQILATRDAEIKGAYVL
ECL_JO40  MRYLSKEERREAILQAAMRVALSEGLAAMTVRRIAAEAGVATGQVHHHFASGGELKSLAFVVRVIRELLDAEVEVGENASWRERLHSMGLSDDGGFEPYIRLWREAQILASRDPEIKAAAYVL
ECL_JO31  MRYLSKDERREEILQAAMRVALTEGFAAMTVRRIATEAGVATGQVHHHFASAGELKSLAFVRLIRNLLDAEIVGENAGWRERLHAMLGSDDGGFEPYIRLWREAQILASRDSDIKGAYVL
ECL_JO32  MRYLSKDERREEILHAAMRVALSEGLSAMTVRRIATEEAGIATGQVHHHFASAGELKALAFVRLIRDLLDAEVEVGENAGWRERLHAMLGSDDGGFEPYIRLWREAQILASRDSDIKGAYVL
ECL_JO38  MRYLSKDERREEILQAAMRVALTEGFAAMTVRRIATEAGVATGQLHHHFASAGELKSLAFVRLIRDLLDAEIVGENAGWRERLHAMLGSDDGGFEPYIRLWREAQILASRDSDIKGAYVL

ATCC13047 TMEMWHQETVALIEAGRNAGVFRSGDPAASIAWRLIGLVCGLDGIYLLNMPEDDAAFNNHLDKLISLELF 191
ECL_JO40  TMEMWHQETVAIIRAGATACAFTLNDRAENIAWRLIGLVCGLDGIITLNMPEDDAAFNKHLDKLITLELF 191
ECL_JO31  TMEMWHQETVAIIRAGAEANAFTLADQPENIAWRLIGLVCGLDGIYVLNMPEDDAAFNKHLDKLISLELF 191
ECL_JO32  TMEMWHQETVAIIRAGAEANVFTLTDRPENIAWRLIGLVCGLDGIYVLNMPEDDAAFNKHLDKLISLELF 191
ECL_JO38  TMEMWHQETVAIIRAGAEANAFTLADQPENIAWRLIGLVCGLDGIYVLNMPEDDAAFNKHLDKLISLELF 191

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B

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                -10                P3                -35                P2
ATCC3047 <smvR CATTTTTCTCTCACTCTTAAATAGCAATGAAACGAGTGTAACAAGAGCTGGACAAGTGTTCAACAATTCCGTAGGATCGCATCCTGGACATGTG
ECL_JO40 <smvR CATTTTTTCTACTCTC.AATGGGAATGAAACGAGTGTAAACAAAAAGCTGGACATGCGTTCAACAATTCCGTAGGATCGCATCCTGGACATGTG
ECL_JO31 <smvR CATAATTTCTCACTCTC.AATGGCAACGAAACGAGTGTAAACAAAGAGCTGGACAAGTGTTCAACTTTTACGTAGGATCGCAATCTGGACATGTG
ECL_JO32 <smvR CATAATTTCTCACTCTC.AATGGCAACGCAACGAGTGTAAACAAAGAGCTGGACAAGCGTTCAACTTTTACGTAGGATCGCATTCTGGACATGTG
ECL_JO38 <smvR CATAATTTCTCACTCTC.AATGGCAACGAAACGAGTGTAAACAAAGAGCTGGACAAGTGTTCAACTTTTACGTAGGATCGCAATCTGGACATGTG
          ***.:.*  *****  ***.***  *.*****  *****.:.*.*****.: *****

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ATCC3047 TCCA.TTTTTT.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.

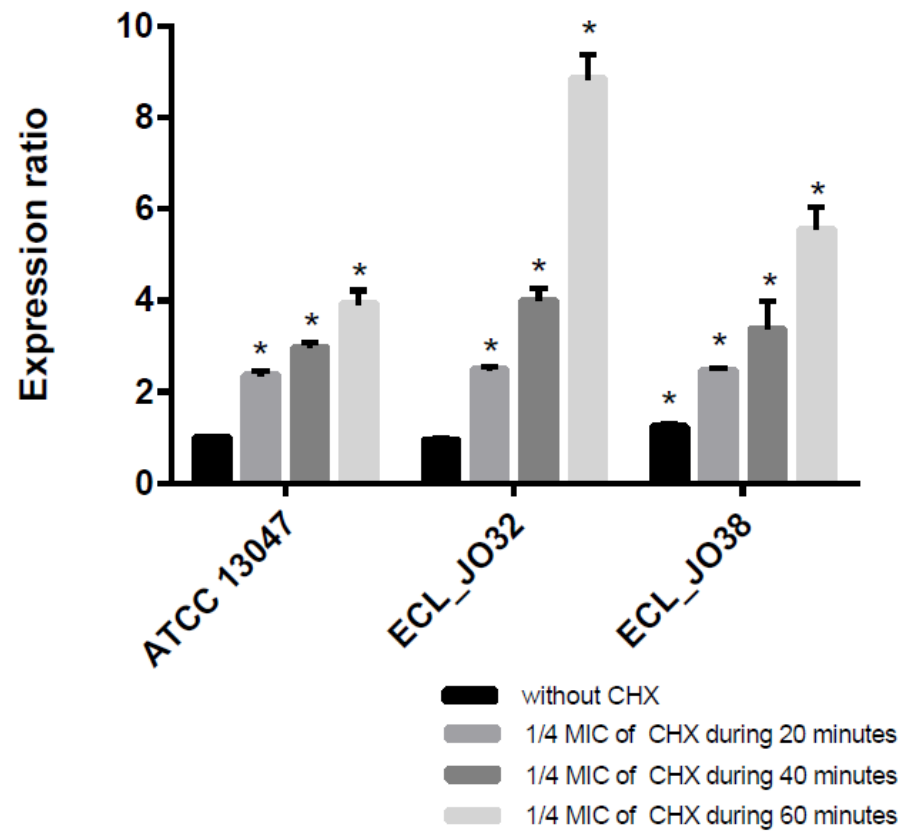


Figure 3: Expression ratios of the *smvR* gene in ATCC 13047 and clinical isolates without and with 1/4 MIC of CHX during 20, 40 and 60 minutes. Asterisk indicates p -value < 0.05 compared to the data obtained with the ATCC 13047 strain without CHX.

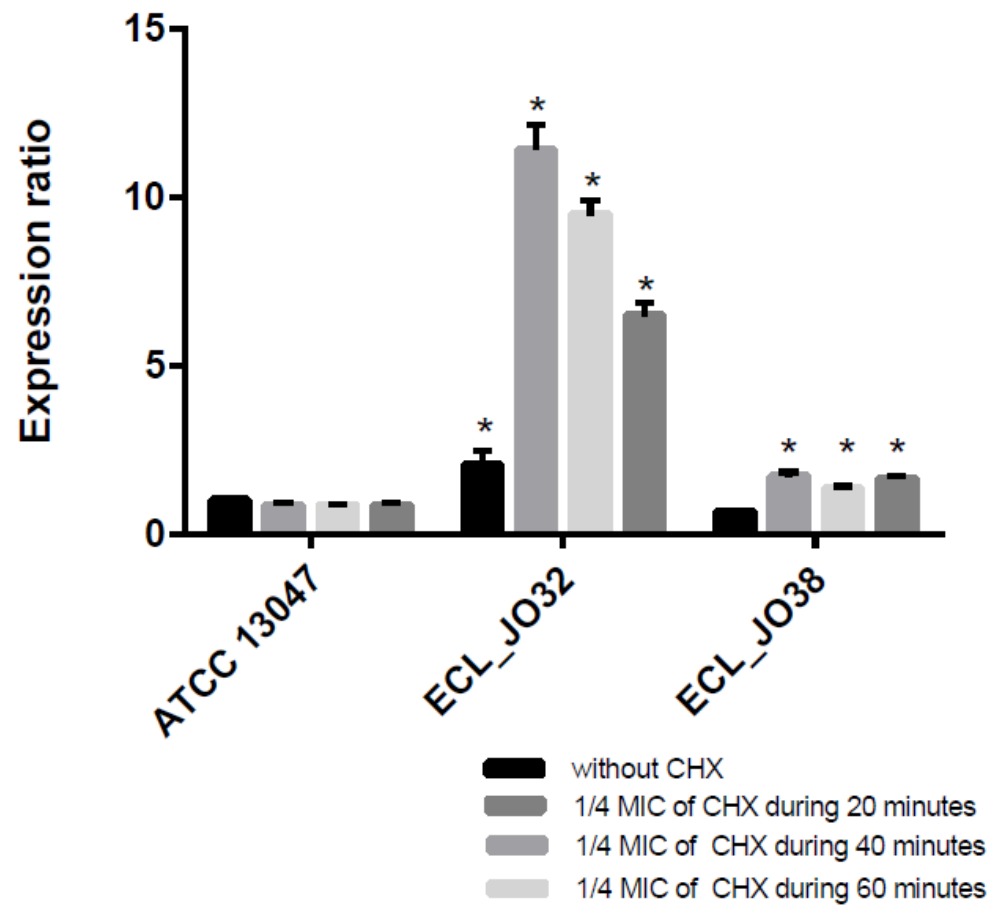


Figure 4: Expression ratios of the *smvA* gene in ATCC 13047 and clinical isolates without or with 1/4 MIC of CHX during 20, 40 and 60 minutes. Asterisk indicates p -value < 0.05 compared to the data obtained with the ATCC 13047 strain without CHX.