Confidential: for peer review only

1 # Differential impact of *ramRA* mutations on both *ramA*
2 # transcription and decreased antimicrobial susceptibility in
3 # *Salmonella* Typhimurium

4

5

6 Anna Fàbrega[1], Clara Ballesté-Delpierre[1], Jordi Vila[1,2*]

7

8 [1] ISGlobal, Barcelona Ctr. Int. Health Res. (CRESIB), Hospital Clínic - Universitat de
9 Barcelona, Barcelona, Spain
10 [2] Department of Clinical Microbiology, Hospital Clínic, School of Medicine, University
11 of Barcelona, Barcelona, Spain.

12

13

14 * Corresponding author:
15 Jordi Vila. Servei de Microbiologia, Centre de Diagnòstic Biomèdic, Hospital Clínic,
16 Facultat de Medicina, Universitat de Barcelona, Villarroel 170, Barcelona 08036, Spain.
17 Phone: +34 93 2275522. Fax: +34 93 2279372. E-mail: jvila@ub.edu.

18

19

20 **Running title**: *ramRA* mutations and impact on MDR
21 **Key words**: *Salmonella*, *acrB*, *tolC*, *acrF*, MDR

22

23

24

25

26 **SYNOPSIS**

27 **Objectives**: This study was focused on analysing the heterogeneity of mutations
28 occurring in the regulators of efflux-mediated MDR in *Salmonella* Typhimurium.
29 Moreover, the impact of such mutations on impairing the transcription of *ramA*, *acrB*,
30 *tolC* and *acrF* was also assessed as was the impact on the resistance or decreased
31 susceptibility phenotype.

32 **Methods**: Strains were selected *in vitro* under increasing ciprofloxacin concentrations.
33 Etest and broth microdilution tests were used to determine the MICs of several
34 unrelated compounds. Screening of mutations in the quinolone target genes and the
35 MDR regulators was performed. RT-PCR analysis was used to detect the levels of
36 expression of *acrB*, *tolC*, *ompF*, *acrF*, *emrB*, *acrR*, *ramA*, *soxS* and *marA*.

37 **Results**: All mutant strains showed increased MICs of most of the antimicrobials tested,
38 with the exception of kanamycin. Mutations in the quinolone target genes did not occur
39 in all the mutants, which all harboured mutations in the *ramRA* regulatory region. All
40 the mutants overexpressed *ramA*, *tolC* and *acrB* (when active) whereas differential
41 results were seen for the remaining genes.

42 **Conclusions**: Mutations in the *ramRA* region related to resistance and/or decreased
43 susceptiblity to antimicrobials predominate in *Salmonella*. There is heterogeneity in the
44 type of mutations, with deletions affecting the RamR binding sites having a greater
45 impact on *ramA* expression and the MDR phenotype.

46

47

48

49

Confidential: For Peer Review only

50 **INTRODUCTION**

51 The ever increasing levels of resistance to antimicrobial compounds are of great
52 concern, particularly for pathogens of clinical relevance. *Salmonella enterica* serovar
53 Typhimurium is a pathogen distributed worldwide which typically causes gastroenteritis
54 in humans.[1] Fluoroquinolones and cephalosporins are the current first-line treatments,
55 however, recent data have revealed that in particular geographic areas, such as China,
56 high percentages of resistance to compounds such as nalidixic acid (61.9%) and
57 cefepime, cefotaxime and ceftazidime (90%) have already been detected.[2]

58 Quinolone resistance has been widely studied in Enterobacteriaceae, particularly
59 in *Escherichia coli* and *S. enterica*.[3] In *E. coli* the mechanism which largely contributes
60 to resistance and/or decreased susceptibility to quinolones is the acquisition of
61 mutations located in the genes encoding the two quinolone targets: DNA gyrase (*gyrA*
62 and *gyrB*) and topoisomerase IV (*parC* and *parE*).[4,5] These mutations are usually
63 acquired in the quinolone resistance-determining regions (QRDRs) detected in each of
64 the target genes.[3] On the other hand, increased drug extrusion by means of the
65 overexpression of AcrAB-TolC, the main efflux pump described in Enterobacteriaceae,[3]
66 is also of great concern since it confers cross-resistance to several unrelated compounds,
67 including antimicrobial drugs.[6,7] To a lesser extent, other efflux systems, such as AcrEF
68 and EmrAB, have been reported to participate in the extrusion of antimicrobial
69 compounds.[8,9] In *Salmonella* increased efflux has been described as the primary
70 mechanism in quinolone resistance acquisition.[10] Alternatively, decreased production of
71 the OmpF porin has at times been related to the MDR phenotype[11,12] despite
72 controversial data suggesting no clear role in *S. enterica*.[13]

73 Several regulators have been reported to influence the expression of the *acrAB*
74 operon in *Salmonella*. AcrR is the local repressor encoded upstream of the *acrAB* genes

Confidential: For Review Only

75 and mutations within its coding sequence have been associated with increased
76 expression of the pump.[14] In addition, three homologous transcriptional activators,
77 RamA, SoxS and MarA, have been associated with increased *acrB* and *tolC* expression
78 levels. While clear associations have been reported for enhanced production of SoxS
79 and RamA and overexpression of *acrAB*,[11,15,16] only indirect results have associated
80 greater production of MarA with increased levels of resistance, supposedly mediated by
81 higher levels of AcrAB.[11,17] In terms of regulation, each of these three activators has its
82 own regulator: RamR, SoxR and MarR, respectively.[3] In terms of the MDR phenotype,
83 the clinical relevance of mutations located in the genes encoding for these latter
84 regulators has been clearly shown for RamR,[18,19] while there have been few reports for
85 mutations located in the *soxRS* region.[11,15] Concerning MarA, even though its
86 overexpression has been detected in MDR *S. enterica* strains,[8,20] the putative
87 responsible mutations in the *marRAB* region have not been mapped. Naturally-occurring
88 mutations in this region have been widely reported *in E. coli*,[21,22] whereas, to our
89 knowledge, such mutations in *S. enterica* have only been reported in a single study,
90 associating it with high MarA overexpression and an MDR phenotype.[12]
91 The aim of this study was to determine the mechanisms involved in increasing
92 the MICs of different antimicrobial agents in a collection of *S.* Typhimurium mutants
93 selected *in vitro*, particularly when studying strains with low MICs of ciprofloxacin and
94 their derivative mutants selected at the initial steps of drug exposure following a
95 stepwise procedure. The mechanisms studied included target gene mutations and the
96 expression of several genes involved in decreasing the intracellular concentration of the
97 drug. Moreover, and as a novel approach, we also assessed the role and heterogeneity of
98 *ramRA* mutations and their impact on increasing the expression of *ramA* and the
99 phenotype of decreased susceptibility to multiple antibiotics or MDR.

Confidential: for review only

100 **MATERIALS AND METHODS**

101 ***Bacterial strains and selection of resistant mutants***

102 Two *S.* Typhimurium clinical isolates, strains 59-wt and 60-wt, were recovered from
103 independent stool samples in the Department of Clinical Microbiology at the Hospital
104 Clinic of Barcelona, Spain. Strain 59-wt has previously been characterised as have its
105 derivative mutants displaying increasing ciprofloxacin MICs, including the highly
106 resistant mutant 59-64.[23] As indicated, the clinical isolate 59-wt was grown at 37ºC on
107 MacConkey agar plates in the presence of ciprofloxacin (Fluka) in a multi-step selection
108 process with doubling concentrations of the drug.[23] Single colonies were randomly
109 selected at different steps and previously characterised. In the present study we
110 characterised additional randomly-selected colonies during the process (59-mut1, 59-
111 mut2 and 59-mut3) to assess the occurrence of heterogeneity in the mechanisms of
112 resistance. Likewise, strain 60-wt was similarly treated and exposed to increasing
113 ciprofloxacin concentrations and two different mutants were randomly selected (60-
114 mut1 and 60-mut2).

115

116 ***Susceptibility testing***

117 The MICs of several quinolones and unrelated antimicrobial compounds were
118 determined by Etest (AB Biodisk) according to the manufacturer's recommendations
119 and interpreted according to CLSI guidelines.[24] The broth microdilution method was
120 used to evaluate the MICs of ciprofloxacin, norfloxacin and nalidixic acid when
121 maximum Etest values were reached. The compounds tested were: ciprofloxacin,
122 norfloxacin, nalidixic acid, chloramphenicol, tetracycline, erythromycin, amoxicillin,
123 ceftriaxone and cefoxitin.

124

Confidential: For Peer Review Only

125 ***Detection of mutations within the QRDRs and regulatory loci***

126 Mutations acquired in the QRDRs of the *gyrA*, *gyrB*, *parC*, and *parE* genes, as well as
127 in the MDR regulatory loci *soxRS*, *marRAB*, *acrR* and *ramR* were screened by PCR
128 amplification as described previously.[25] Amplicons were purified and sent to Beckman
129 Coulter Genomics (Essex, UK) for sequencing reactions. Detection of mutations was
130 carried out using the BioEdit® software (Ibis Biosciences, Carlsbad, CA) by comparison
131 with the genome of *S.* Typhimurium LT2 as the reference strain (RefSeq
132 NC_003197.1).

133

134 ***RNA extraction and real time PCR***

135 Bacterial pellets were obtained as described previously.[25] Briefly, strains were grown in
136 LB at 37ºC with shaking to reach the exponential phase (OD_{600}=0.6). Four mL of
137 bacterial cells were treated with 8 mL of RNA Protect Bacteria Reagent (Qiagen) and
138 subsequently incubated with Tris-EDTA (TE) buffer supplemented with lysozyme.
139 RNA extractions were obtained using the Maxwell ® 16 Research Instrument
140 (Promega) and the Maxwell® 16 LEV simplyRNA Blood Kit (Promega) following the
141 manufacturer's recommendations. Five independent RNA extractions were made.

142 The *acrB*, *tolC*, *ompF*, *acrF*, *emrB*, *ramA*, *marA*, *soxS* and *acrR* genes were
143 tested for RT-PCR analysis following previously described conditions.[26] The 16S rRNA
144 gene was used as an internal control for normalisation, and susceptible strains 59-wt and
145 60-wt were the reference strains for their respective derived mutants. The $2^{-\Delta\Delta CT}$ method
146 was used for relative gene expression calculations.[27] Five independent assays were
147 performed and each RNA sample was tested in triplicate. The primers used are reported
148 in Table 1. Mean values and standard deviation are detailed in Table 2.

149

Confidential: for peer review only

150 **RESULTS AND DISCUSSION**

151 ***Quinolone resistance and the MDR phenotype***

152 Three and two derivative mutants were selected from the quinolone-susceptible clinical
153 isolates 59-wt and 60-wt, respectively. Susceptibility testing to several unrelated
154 compounds was used to determine the acquisition of the quinolone resistance and MDR
155 phenotypes (Table 3). The term MDR has been defined as resistance to one agent in
156 three or more antimicrobial categories,[28] or to four or more antimicrobials in the
157 particular case of nontyphoidal *Salmonella*.[29] In the present study we used instead the
158 term decreased susceptibility to multiple antibiotics when increased MICs to more than
159 4 antimicrobial compounds were seen even though the resistance breakpoints were not
160 reached. Strain 59-64, already characterised in a previous study,[23] was also included in
161 the present work for comparison with the mutants.

162 The results showed that in comparison with their wild-type strain, all selected
163 mutants had increased MICs (1.5- to >8-fold) to all the drugs tested, except for
164 kanamycin, for which no increase was recorded. Only 59-wt derivative mutants showed
165 the acquisition of QRDR mutations (Table 4). Strains 59-mut1 and 59-mut2 showed a
166 similar genetic background in terms of target gene mutations. However, higher MIC
167 values were seen for 59-mut2 concerning all the drugs (except for amoxicillin and
168 chloramphenicol, which had already shown maximum Etest values in 59-wt, and
169 tetracycline). Likewise, on comparing strains 60-mut1 and 60-mut2 a similar conclusion
170 was obtained, with higher MIC results seen for 60-mut2 despite having background
171 similarity. In accordance with the fact that strains 59-mut3 and 59-64 were selected at
172 higher ciprofloxacin concentrations, these strains showed the highest MICs, mostly
173 concerning quinolones, being maximal for strain 59-64.

Confidential: for review only

174 Taking into account the increased MICs of most of these compounds in all the
175 mutants, and the fact that increased efflux confers a cross-resistance phenotype by
176 means of increased AcrAB or even a hitherto uncharacterised efflux pump,[6,23] enhanced
177 extrusion activity was the most likely mechanism underlying this phenotype. Moreover,
178 the results obtained from 60-wt and its derivative mutants strengthen the idea that efflux
179 is selected at primary stages of the process of quinolone resistance acquisition as
180 suggested previously,[10,25] and this mechanism is selected even before target gene
181 mutations. It should be noted that mutants selected in a single step-selection process,
182 usually performed at concentrations higher than the initial MIC, may follow a different
183 pattern of acquisition of resistance mechanisms.
184
185 ***Expression of structural genes involved in MDR***
186 Gene expression analysis was performed to determine the expression patterns of
187 genes related to bacterial efflux and permeability. The results were interpreted after
188 comparison of the expression levels of each clinical isolate with their respective mutant
189 derivatives. The genes studied were *acrB*, *tolC*, *ompF*, *acrF* and *emrB* (Figure 1)(Table
190 2). Overexpression of the AcrAB-TolC efflux pump has been reported as the most
191 relevant mechanism in terms of efflux.[3] In the present study *acrB* was only analysed in
192 60-wt and its derivates, which all overexpressed this gene (5.2- to 9.5-fold), since it was
193 reported that 59-wt has a mutation inactivating the *acrAB* operon.[23] The *tolC* gene was
194 found to be consistently overexpressed in all the mutants (>2.3-fold), particularly for
195 strains 59-mut2 and 60-mut2 (5.4- and 6.2-fold, respectively). On the contrary, *ompF*
196 always showed decreased expression with the strongest results being seen in strains 59-
197 64 (-3.3-fold) and 60-mut2 (-2.4-fold). With these results we suggest that AcrAB-TolC
198 was involved in the phenotype of decreased susceptibility to multiple antibiotics in the

Confidential: for review only

199 case of 60-wt derivatives whereas an unknown efflux system, likely acting in
200 conjunction with TolC, participated in the case of 59-wt derivatives.
201 Next, we assessed other efflux-related genes, such as *acrF* and *emrB*, (Figure
202 1)(Table 2) which may play a secondary role in antibiotic resistance.[8,9] Our results
203 showed that only two strains clearly overexpressed *acrF* [59-mut2 (6-fold) and 60-mut2
204 (4.9-fold)] whereas *emrB* showed a slightly decreased expression in all the mutants (-
205 1.2- to -1.9-fold). Thus, we can only suggest a role in increasing the MICs mentioned
206 for the AcrEF efflux system in these two particular mutants, one of which is also an
207 AcrAB-overproducer (60-mut2).
208
209 ***Expression of the MDR regulators: the key role of ramA***
210 In addition to the analysis of these structural genes, we also studied the levels of
211 expression of the AcrAB regulators: *acrR*, *ramA*, *soxS* and *marA* (Figure 1)(Table 2).
212 We could not find a clear interpretation for *acrR* expression. In contrast, *ramA* was
213 overexpressed in all the mutants thereby suggesting this regulator as the cause of the
214 increased MICs in both the mutants overexpressing *acrB* and those overexpressing an
215 unknown efflux system. Similar results have also highlighted the greater importance
216 and prevalence of increased RamA over that of the other regulators.[16,30] Maximal *ramA*
217 expression levels were seen for 59-mut2 and 60-mut2 (66- and 74.2-fold, respectively)
218 above the levels detected for the remaining mutants (13.4- to 19.6-fold). In line with
219 these results, these two strains also showed higher MICs and *acrB* and *tolC* expression
220 values in comparison with their closely related mutants 59-mut1 and 60-mut1,
221 respectively. In addition, as mentioned above, 59-mut2 and 60-mut2 were also reported
222 to clearly overexpress *acrF*. This latter association between high *ramA* expression (>60-

Confidential: for review only

223 fold in the present study) and *tolC* and *acrF* overexpression agrees with a previously
224 reported study.[31]
225 The *soxS* expression values detected in the present study were <2-fold higher in
226 most of the mutants *versus* the expression levels seen in the two clinical isolates (Figure
227 1). Only two mutants, strains 59-64 and 60-mut2 showed an overexpression of >4-fold.
228 However, it was not possible to consistently associate this trait with higher expression
229 values of *ramA* or *acrF* in both mutants. On the contrary, these two strains did show the
230 minimum levels of *ompF* expression (-3.3- and -2.2-fold, respectively). Similarly, *marA*
231 transcription also showed ≤2-fold increased expression in three mutant strains: 59-mut1,
232 59-mut3 and 60-mut1. On the contrary, the highest levels were seen in 59-mut2 (4.3-
233 fold), 59-64 (3.7-fold) and 60-mut2 (3.6-fold).
234 To understand our results it is worth mentioning that the RamA binding sites
235 have already been reported in *Salmonella* concerning the *acrAB* and *tolC* promoters.[32]
236 The 20-bp sequences recognised by this regulator resemble those initially reported to be
237 present in all members of the *marA*/*soxS*/*rob* regulon in *E. coli*.[33] It has been described
238 that most of the residues of the two helix-turn-helix motifs (important for DNA
239 sequence recognition) of MarA from *E. coli* are conserved in RamA from *Salmonella*
240 *enterica* serovar Paratyphi B.[34] Moreover, it has previously been reported that the
241 *marRAB* promotor contains its own marbox sequence.[33] In agreement with this, RamA
242 from *S.* Paratyphi B has been shown to bind the MarA operator of *E.coli*.[34] Thus, the
243 binding sites characterised for MarA and SoxS in *E. coli*, equally termed marbox or
244 soxbox, are similar to the already mentioned rambox in *Salmonella*.[31,32] Therefore,
245 increased levels of RamA (>60-fold) and/or SoxS (>4-fold) could bind to the
246 rambox/marbox located in the *marRAB* promoter and activate *marA* transcription, hence
247 explaining the increased levels of *marA* expression observed for strains 59-mut2

Confidential: for review only

248 [RamA-overproducer (>60-fold)], 59-64 [SoxS-overproducer (>4-fold)] and 60-mut2
249 [RamA-overproducer (>60-fold) and SoxS-overproducer (>4-fold)]. Nonetheless, lower
250 *ramA* overexpression values (13- to 20-fold) would not have the same effect, thereby
251 reinforcing the idea of an activator concentration-dependent response.[31,35]
252

253 ***Unravelling the mutations leading to the phenotype of decreased susceptibility to***
254 ***multiple antibiotics***

255 In order to determine the mutations underlying the resistance phenotypes, sequencing
256 and detection of mutations was performed in all the strains for all known regulators of
257 MDR (*acrRA*, *ramRA*, *soxRS*, *marRAB* and *acrSE*). The results revealed the acquisition
258 of mutations in the *ramRA* loci for all the mutants (Table 4). Mutations were located
259 within the *ramR* coding sequence, either leading to a single amino acid substitution
260 (Gln-19→Pro, strain 60-mut1) or even deletions of 44 and 6 nucleotides (strains 59-
261 mut1 and 59-mut3, respectively). Surprisingly, the two strains (59-mut2 and 60-mut2)
262 with the highest *ramA* overexpression values harboured a similar genotype: a 6- and 16-
263 nucleotide deletions, respectively, in the *ramA* promoter. Lastly, and as previously
264 reported[23] strain 59-64 showed a single-nucleotide change also located in the *ramA*
265 promoter.

266 Previous reports have revealed that mutations or gene interruptions can be either
267 acquired within *ramR* or in the *ramA* promoter.[11,16,30] However, no association has ever
268 been made between the type of mutation and transcription levels of *ramA*. The results
269 observed in the present study point out that severe nucleotide deletions located in the
270 *ramA* promoter have a higher impact on increasing the expression of this regulator,
271 whereas mutations within *ramR* or single nucleotide changes in the *ramA* promoter have
272 a lesser effect. We performed an exhaustive analysis of the literature looking for studies

Confidential: For Review Only

273 which determined both the *ramA* transcription levels and *ramRA* mutations in strains
274 with resistance or decreased susceptibility to fluoroquinolones. Studies conducted in
275 serovars Typhimurium,[36,37] Enteritidis,[11] Kentucky[38] and other serovars[30] were found to
276 report similar results (Table 4). In order to understand this situation, it is necessary to
277 note that RamR has been reported to bind as a homodimer to two RamR binding sites
278 located in the *ramA* promoter (Figure 2).[37] Thus, taking into account all this information
279 we hypothesize that important deletions occurring in these binding sites seriously impair
280 the RamR repressive activity by preventing RamR binding and lead to high levels of
281 *ramA* expression (>60-fold). On the contrary, mutations or deletions occurring in RamR
282 or single nucleotide modifications affecting one binding site do not seem to abolish
283 repression to the same extent and lead to moderate levels of *ramA* transcription (<~40-
284 fold). This latter situation would be supported by the capacity of the mutated form of
285 RamR to partially preserve its repressive activity or by the existence of other regulators
286 capable of binding to the *ramA* promoter even in the absence of a functional RamR
287 protein. Nonetheless, to our knowledge two exceptions have been reported, one *S.*
288 Kentucky strain[38] and one *S.* Paratyphi B mutant (Table4).[30] The former situation might
289 be explained by a large deletion detected at the very beginning of the repressor
290 (affecting the protein sequence from the amino acid at position 14), whereas no clear
291 explanation could justify the latter situation. Therefore, in order to elucidate the role of
292 these mutations and strengthen or not our hypothesis, a larger number of strains needs to
293 be analysed in further studies.
294 In no strain did we find any mutation in any of the other regulatory sequences
295 analysed in the present study. Consequently, we are unable to explain the increased *soxS*
296 transcription reported in 59-64 and 60-mut2. Concerning *acrF* overexpression, previous
297 results have associated it with mutations within the *acrS* gene or in the *acrEF*

Confidential: for peer review only

298 promoter.[9] However, in the present study no mutation in the *acrSE* regulatory region
299 could explain our findings. Instead, and as previously mentioned and reinforced by our
300 results, overexpression of this efflux component is related to the levels of *ramA*
301 transcription.[31] High levels of *ramA* expression trigger *acrF* overexpression whereas
302 intermediate levels do not. In line with these results, a previous study has also
303 associated nucleotide deletions in the *ramA* promoter with *acrEF* overexpression.[36] In
304 view of these findings, the regulatory network that controls the expression of genes
305 involved in the phenotype of decreased susceptibility to multiple antibiotics or MDR
306 still needs further research to completely understand the bacterial response for survival
307 under antimicrobial exposure. Nonetheless, we must keep in mind that our observations
308 have arisen from mutants selected in a stepwise process which may harbour additional
309 mutations with unknown influence. Additional experiments are required in order to
310 validate these results.
311
312 ***Conclusions***
313 The results of our study indicate that RamA overexpression leads to the
314 phenotype of decreased susceptibility to multiple antibiotics by using two different
315 efflux-related strategies: overexpression of AcrAB and overexpression of a hitherto
316 uncharacterised efflux pump. Moreover, we provide further evidence of the prevalence
317 of *ramRA* mutations versus other *acrB* regulators in the acquisition of MDR. However,
318 heterogeneity was observed in the types of mutations acquired, which may be associated
319 with different levels of *ramA* transcription. Large deletions affecting the RamR binding
320 sites in the *ramA* promoter were observed in strains with higher *ramA* transcription
321 levels, a trait which may account for the highest expression levels of *acrB*, *tolC*, *marA*

Confidential: For peer review only

322 and *acrF*, hence related to a major contribution to the phenotype of decreased
323 susceptibility to multiple antibiotics.
324
325
326 **ACKNOWLEDGEMENTS**
327 We are grateful to Lee Rosner for his general revision of the manuscript. AF is
328 sponsored by the Barcelona Institute for Global Health (ISGlobal).
329
330 **FUNDING**
331 This study was supported by the Ministerio de Economía y Competitividad,
332 Instituto de Salud Carlos III, co-financed by the European Regional Development Fund
333 (ERDF) "A Way to Achieve Europe," the Spanish Network for Research in Infectious
334 Diseases (REIPI RD12/0015), and the Spanish Ministry of Health (grant number FIS
335 PI11/02024). This study was also supported by grant 2014SGR0653 from the
336 Departament d'Universitats, Recerca i Societat de la Informació, of the Generalitat de
337 Catalunya and by funding from the Innovative Medicines Initiative (Translocation,
338 contract IMI-JU-6-2012-115525).
339
340 **TRANSPARENCY DECLARATION**
341 The authors declare no conflicts of interest.
342
343

Confidential: for peer review only

344 **REFERENCES**

345
346

347 1. Fàbrega A, Vila J. *Salmonella enterica* serovar Typhimurium skills to succeed in
348 the host: virulence and regulation. *Clin Microbiol Rev* 2013; **26**: 308-41.

349 2. Liang Z, Ke B, Deng X *et al*. Serotypes, seasonal trends, and antibiotic
350 resistance of non-typhoidal *Salmonella* from human patients in Guangdong
351 Province, China, 2009-2012. *BMC Infect Dis* 2015; **15**: 53.

352 3. Fàbrega A, Madurga S, Giralt E *et al*. Mechanism of action of and resistance to
353 quinolones. *Microb Biotechnol* 2009; **2**: 40-61.

354 4. Heisig P. Genetic evidence for a role of *parC* mutations in development of high-
355 level fluoroquinolone resistance in *Escherichia coli*. *Antimicrob Agents*
356 *Chemother* 1996; **40**: 879-85.

357 5. Vila J, Ruiz J, Goni P *et al*. Detection of mutations in *parC* in quinolone-
358 resistant clinical isolates of *Escherichia coli*. *Antimicrob Agents Chemother*
359 1996; **40**: 491-3.

360 6. Cohen SP, McMurry LM, Hooper DC *et al*. Cross-resistance to fluoroquinolones
361 in multiple-antibiotic-resistant (Mar) *Escherichia coli* selected by tetracycline or
362 chloramphenicol: decreased drug accumulation associated with membrane
363 changes in addition to OmpF reduction. *Antimicrob Agents Chemother* 1989; **33**:
364 1318-25.

365 7. Nishino K, Yamaguchi A. Analysis of a complete library of putative drug
366 transporter genes in *Escherichia coli*. *J Bacteriol* 2001; **183**: 5803-12.

367 8. Chen S, Cui S, McDermott PF *et al*. Contribution of target gene mutations and
368 efflux to decreased susceptibility of *Salmonella enterica* serovar Typhimurium
369 to fluoroquinolones and other antimicrobials. *Antimicrob Agents Chemother*
370 2007; **51**: 535-42.

371 9. Olliver A, Valle M, Chaslus-Dancla E *et al*. Overexpression of the multidrug
372 efflux operon *acrEF* by insertional activation with *IS1* or *IS10* elements in
373 *Salmonella enterica* serovar typhimurium DT204 *acrB* mutants selected with
374 fluoroquinolones. *Antimicrob Agents Chemother* 2005; **49**: 289-301.

375 10. Giraud E, Cloeckaert A, Kerboeuf D *et al*. Evidence for active efflux as the
376 primary mechanism of resistance to ciprofloxacin in *Salmonella enterica* serovar
377 typhimurium. *Antimicrob Agents Chemother* 2000; **44**: 1223-8.

378 11. O'Regan E, Quinn T, Pages JM *et al*. Multiple regulatory pathways associated
379 with high-level ciprofloxacin and multidrug resistance in *Salmonella enterica*
380 serovar Enteritidis: involvement of RamA and other global regulators.
381 *Antimicrob Agents Chemother* 2009; **53**: 1080-7.

Confidential: for peer review only

Confidential: For peer review only

382 12. Balleste-Delpierre C, Fabrega A, Ferrer-Navarro M *et al*. Attenuation of *in vitro*
383 host-pathogen interactions in quinolone-resistant *Salmonella* Typhi mutants. *J*
384 *Antimicrob Chemother* 2015. Epub ahead of print.

385 13. Piddock LJV, Griggs DJ, Hall MC *et al*. Ciprofloxacin resistance in clinical
386 isolates of *Salmonella typhimurium* obtained from two patients. *Antimicrob*
387 *Agents Chemother* 1993; **37**: 662-6.

388 14. Olliver A, Valle M, Chaslus-Dancla E *et al*. Role of an *acrR* mutation in
389 multidrug resistance of in vitro-selected fluoroquinolone-resistant mutants of
390 *Salmonella enterica* serovar Typhimurium. *FEMS Microbiol Lett* 2004; **238**:
391 267-72.

392 15. Koutsolioutsou A, Martins EA, White DG *et al*. A *soxRS*-constitutive mutation
393 contributing to antibiotic resistance in a clinical isolate of *Salmonella enterica*
394 (Serovar typhimurium). *Antimicrob Agents Chemother* 2001; **45**: 38-43.

395 16. Abouzeed YM, Baucheron S, Cloeckaert A. *ramR* mutations involved in efflux-
396 mediated multidrug resistance in *Salmonella enterica* serovar Typhimurium.
397 *Antimicrob Agents Chemother* 2008; **52**: 2428-34.

398 17. Sulavik MC, Dazer M, Miller PF. The *Salmonella typhimurium* mar locus:
399 molecular and genetic analyses and assessment of its role in virulence. *J*
400 *Bacteriol* 1997; **179**: 1857-66.

401 18. Giraud E, Baucheron S, Virlogeux-Payant I *et al*. Effects of natural mutations in
402 the *ramRA* locus on invasiveness of epidemic fluoroquinolone-resistant
403 *Salmonella enterica* serovar Typhimurium isolates. *J Infect Dis* 2013; **207**: 794-
404 802.

405 19. Ricci V, Tzakas P, Buckley A *et al*. Ciprofloxacin-resistant *Salmonella enterica*
406 serovar Typhimurium strains are difficult to select in the absence of AcrB and
407 TolC. *Antimicrob Agents Chemother* 2006; **50**: 38-42.

408 20. Tibbetts RJ, Lin TL, Wu CC. Phenotypic evidence for inducible multiple
409 antimicrobial resistance in *Salmonella* choleraesuis. *FEMS Microbiol Lett* 2003;
410 **218**: 333-8.

411 21. Oethinger M, Podglajen I, Kern WV *et al*. Overexpression of the *marA* or *soxS*
412 regulatory gene in clinical topoisomerase mutants of *Escherichia coli*.
413 *Antimicrob Agents Chemother* 1998; **42**: 2089-94.

414 22. Alekshun MN, Levy SB. Regulation of chromosomally mediated multiple
415 antibiotic resistance: the mar regulon. *Antimicrob Agents Chemother* 1997; **41**:
416 2067-75.

417 23. Fàbrega A, Soto SM, Ballesté-Delpierre C *et al*. Impact of quinolone-resistance
418 acquisition on biofilm production and fitness in *Salmonella enterica*. *J*
419 *Antimicrob Chemother* 2014; **69**: 1815-24.

420 421 422 24. Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing: Twenty-third Informational Supplement M100-S23*. CLSI, Wayne, PA, USA, 2013.

423 424 425 25. Fàbrega A, du Merle L, Le Bouguenec C *et al*. Repression of invasion genes and decreased invasion in a high-level fluoroquinolone-resistant *Salmonella* Typhimurium mutant. *PLoS One* 2009; **4**: e8029.

426 427 428 26. Balleste-Delpierre C, Sole M, Domenech O *et al*. Molecular study of quinolone resistance mechanisms and clonal relationship of *Salmonella enterica* clinical isolates. *Int J Antimicrob Agents* 2014; **43**: 121-5.

429 430 27. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2($^{-\Delta\Delta C}$T) method. *Methods* 2001; **25**: 402-8.

431 432 433 434 28. Magiorakos AP, Srinivasan A, Carey RB *et al*. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* 2012; **18**: 268-81.

435 436 29. Parry CM, Threlfall EJ. Antimicrobial resistance in typhoidal and nontyphoidal salmonellae. *Curr Opin Infect Dis* 2008; **21**: 531-8.

437 438 439 440 30. Kehrenberg C, Cloeckaert A, Klein G *et al*. Decreased fluoroquinolone susceptibility in mutants of *Salmonella* serovars other than Typhimurium: detection of novel mutations involved in modulated expression of *ramA* and *soxS*. *J Antimicrob Chemother* 2009; **64**: 1175-80.

441 442 443 31. Bailey AM, Ivens A, Kingsley R *et al*. RamA, a member of the AraC/XylS family, influences both virulence and efflux in *Salmonella enterica* serovar Typhimurium. *J Bacteriol* 2010; **192**: 1607-16.

444 445 446 32. Nikaido E, Yamaguchi A, Nishino K. AcrAB multidrug efflux pump regulation in *Salmonella enterica* serovar Typhimurium by RamA in response to environmental signals. *J Biol Chem* 2008; **283**: 24245-53.

447 448 449 33. Martin RG, Rosner JL. Genomics of the *marA/soxS/rob* regulon of *Escherichia coli*: identification of directly activated promoters by application of molecular genetics and informatics to microarray data. *Mol Microbiol* 2002; **44**: 1611-24.

450 451 452 453 34. Yassien MA, Ewis HE, Lu CD *et al*. Molecular cloning and characterization of the *Salmonella enterica* Serovar Paratyphi B *rma* Gene, which confers multiple drug resistance in *Escherichia coli*. *Antimicrob Agents Chemother* 2002; **46**: 360-6.

454 455 456 35. Martin RG, Bartlett ES, Rosner JL *et al*. Activation of the *Escherichia coli marA/soxS/rob* regulon in response to transcriptional activator concentration. *J Mol Biol* 2008; **380**: 278-84.

457 458 459 36. Zheng J, Cui S, Meng J. Effect of transcriptional activators RamA and SoxS on expression of multidrug efflux pumps AcrAB and AcrEF in fluoroquinolone-resistant *Salmonella* Typhimurium. *J Antimicrob Chemother* 2009; **63**: 95-102.

Confidential: For peer review only

460 37. Baucheron S, Coste F, Canepa S *et al*. Binding of the RamR repressor to wild-
461 type and mutated promoters of the RamA gene involved in efflux-mediated
462 multidrug resistance in *Salmonella enterica* serovar Typhimurium. *Antimicrob*
463 *Agents Chemother* 2012; **56**: 942-8.

464 38. Baucheron S, Le HS, Doublet B *et al*. *ramR* mutations affecting fluoroquinolone
465 susceptibility in epidemic multidrug-resistant *Salmonella enterica* serovar
466 Kentucky ST198. *Front Microbiol* 2013; **4**: 213.

467 39. Spengler G, Rodrigues L, Martins A *et al*. Genetic response of *Salmonella*
468 *enterica* serotype Enteritidis to thioridazine rendering the organism resistant to
469 the agent. *Int J Antimicrob Agents* 2012; **39**: 16-21.

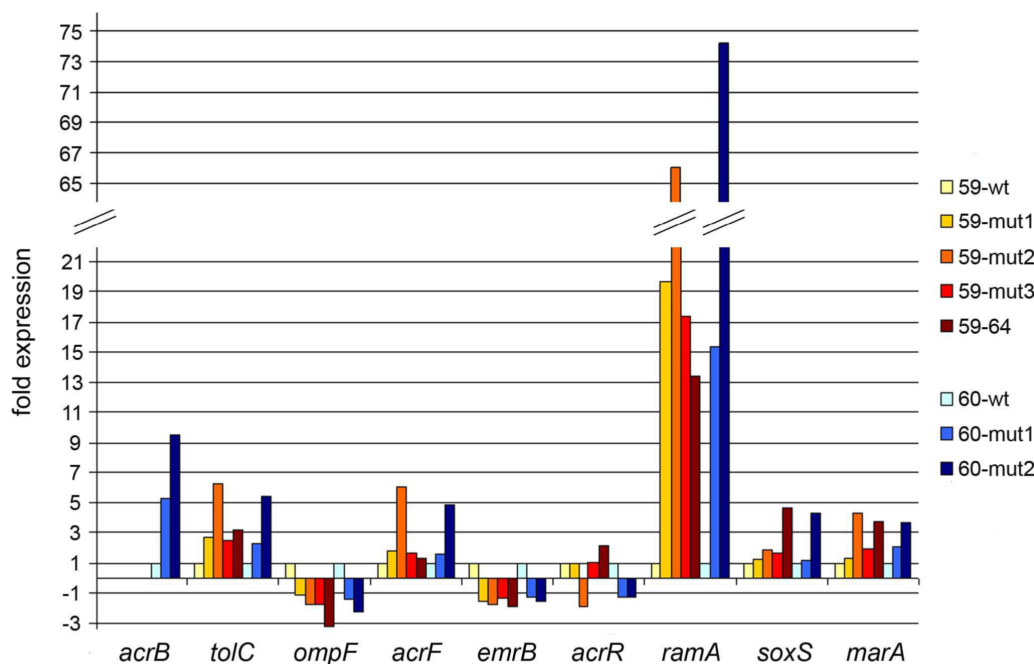
470
471

472

Confidential: for peer review only

473 **FIGURES:**

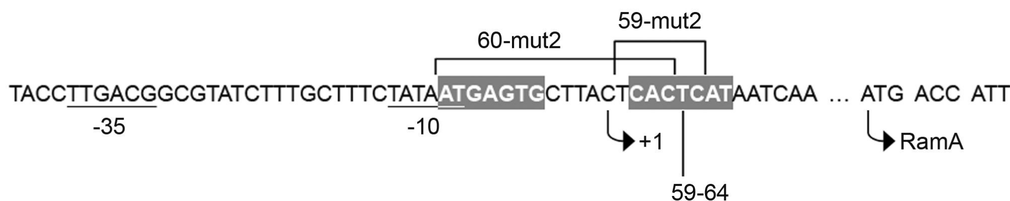
474 **Figure 1.** Expression levels obtained by RT-PCR analysis. Single and double asterisks
475 refer to high levels of *ramA* overexpression, 66- and 74.2-fold, respectively, which are
476 out of scale to facilitate the visualisation of the results.



477

478

479 **Figure 2.** Representative location of the RamR binding sites and the MDR-related
480 mutations detected in the *ramA* promoter. White letters and grey boxes indicate the two
481 RamR binding sites. The -35 and -10 boxes of the *ramA* promoter are underlined.
482 Ellipses indicate DNA sequences not shown. The black arrow is used for the *ramA*
483 transcriptional start site (+1) as well as for the initiation of translation (RamA).



484

485 **TABLES**:

486 **Table 1.** Primers used in the RT-PCR analysis.

Genes	Primers	Sequence 5'-3'	Reference
Internal control			
16S rRNA	16S_RT_F	GCGGCAGGCCTAACACAT	[39]
	16s_RT_R	GCAAGAGGCCCGAACGTC	
Structural genes			
acrB	AcrB_RT_F	TTTTGCAGGGCGCGGTCAGAATAC	[11]
	AcrB_RT_R	TGCGGTGCCCAGCTCAACGAT	
tolC	TolC_RT_F	GTGACCGCCCGCAACAAC	[26]
	TolC_RT_R	ATTCAGCGTCGGCAGGTGAC	
acrF	SacrF.RT.1	TACCCAGGACGACATCTCTGA	[26]
	SacrF.RT.2	CACACCATTCAGACGGCTGAT	
emrB	EmrB_RT_F	CCGTCGTCCTGATGACGTTA	[26]
	EmrB_RT_R	CCGTTCGGTATGCGTTTCAC	
ompF	SompF.RT1	GGGCGCGACTTACTACTTCAAC	This study
	SompF.RT2	TCGTTTTCGTCCAGCAGGTT	
Regulatory genes			
acrR	SacrR.RT1	AGAACGACGCCGCTTATTGA	[12]
	SacrR.RT2	GCGCCTGTTGAACCACAAC	
ramA	SramA.RT1	CTCGACACCGACCAGAAGGT	[12]
	SramA.RT2	GTAAAAATGCGCGTAAAGGTTTG	
soxS	SsoxS.RT1	CATATCGACCAACCGCTAAACA	[12]
	SsoxS.RT2	CGAAACATCCGCTGCAAATA	
marA	SmarA.RT1	ATTCCAAATGGCACCTGCAA	This study
	SmarA.RT2	CATTTTACGGCTGCGGATGT	

487

Confidential: for peer review only

Confidential: for peer review only

Table 2. Mean values of RT-PCR analysis obtained in five independent experiments.

Strains	Gene expression values[a]																	
	acrB		*tolC*		*ompF*		*acrF*		*emrB*		*acrR*		*ramA*		*soxS*		*marA*	
59-wt	---[b]		1		1		1		1		1		1		1		1	
59-mut1	---	---	2.7	(0.59)	-1.2	(0.23)	1.7	(0.27)	-1.5	(0.13)	1.0	(0.25)	19.6	(8.05)	1.2	(0.57)	1.3	(0.23)
59-mut2	---	---	6.2	(0.36)	-1.8	(0.12)	6.0	(1.43)	-1.8	(0.22)	-1.9	(0.16)	66.0	(12.68)	1.8	(1.77)	4.3	(4.13)
59-mut3	---	---	2.5	(0.49)	-1.8	(0.05)	1.7	(0.27)	-1.4	(0.35)	1.0	(0.41)	17.3	(9.44)	1.7	(0.85)	2.0	(0.51)
59-64	---	---	3.2	(1.11)	-3.3	(0.10)	1.4	(0.33)	-1.9	(0.27)	2.1	(1.57)	13.4	(4.71)	4.6	(2.06)	3.7	(0.48)
60-wt	1		1		1		1		1		1		1		1		1	
60-mut1	5.2	(1.33)	2.3	(1.02)	-1.4	(0.45)	1.6	(0.86)	-1.2	(0.57)	-1.3	(0.54)	15.4	(7.78)	1.1	(0.46)	2.0	(1.10)
60-mut2	9.5	(4.85)	5.4	(2.69)	-2.2	(0.27)	4.9	4.77	-1.6	(0.37)	-1.2	(0.49)	74.2	(30.19)	4.3	(4.28)	3.6	(2.35)

[a] Values in parenthesis represent ± the standard deviation (SD).

[b] ---, Not determined.

Confidential: for peer review only

495 **Table 3.** Susceptibility testing of all the strains and ciprofloxacin concentrations used for the selection of mutants.

Strain	[CIP] (mg/L) at selection	MICs (mg/L)[b]									
		CIP	NOR	NAL	AMX	CRO	FOX	TET	CHL	ERY	KAN
59-wt	---[a]	0.012	0.094	4	>256	0.094	2	64	>256	32	1.5
59-mut1	0.06	0.125	2	32	>256	0.190	6	128	>256	128	1.5
59-mut2	0.25	0.38	6	96	>256	0.5	12	128	>256	256	1.5
59-mut3	2	8	16	8128	>256	0.25	4	96	>256	128	1.5
59-64	64	256	512	8128	>256	1	96	256	>256	>256	1.5
60-wt	---	0.016	0.094	3	1	0.032	3	3	3	32	1
60-mut1	0.015	0.047	0.19	6	1.5	0.064	8	8	8	192	1
60-mut2	0.03	0.094	0.38	24	3	0.125	12	12	24	>256	1

496

497 [a] ---, clinical isolate not exposed to ciprofloxacin *in vitro*.

498 [b] CIP, ciprofloxacin; NOR, norfloxacin; NAL, nalidixic acid; AMX, amoxicillin; CRO, ceftriaxone; FOX, cefoxitin; CHL, chloramphenicol;

499 TET, tetracycline; ERY, erythromycin; KAN, kanamycin.

500

501

Confidential: for peer review only

502 **Table 4.** Mutations acquired in the quinolone target genes and the *ramRA* regulatory region. Comparison of the *ramA* transcriptional levels and

503 regulatory mutations with previously reported mutants.

Strains	QRDR mutations						*ramRA* mutations[a,b]		*ramA* expression levels[f]	*Salmonella* serovar	Reference
	GyrA		GyrB	ParC		ParE	*ramR*/RamR[c]	*ramA* promoter[d]			
59-wt	---	---	---	---	---	---	---	---	1	Typhimurium	This study
59-mut1	---	---	E466D	---	---	---	Del C$_{514}$-G$_{557}$	---	19.6	Typhimurium	This study
59-mut2	---	---	E466D	---	---	---	---	**Del T$_{-162}$/C$_{-157}$**	**66.0**	Typhimurium	This study
59-mut3	S83Y	---	E466D	S80R	---	---	Del A$_{346}$-G$_{352}$	---	17.3	Typhimurium	This study
59-64	S83Y	D87G	E466D	S80R	F115S	---	---	T$_{-158}$A	13.4	Typhimurium	This study
60-wt	---	---	---	---	---	---	---	---	1	Typhimurium	This study
60-mut1	---	---	---	---	---	---	Q19P	---	15.4	Typhimurium	This study
60-mut2	---	---	---	---	---	---	---	**Del A$_{-174}$/C$_{-159}$**	**74.2**	Typhimurium	This study
Previously reported mutants											
LTL	S83F	---	---	---	---	---	---	**Del A$_{-174}$/T$_{-166}$**	**69.1**	Typhimurium	36
BN10055	S83Y	---	---	---	---	---	---	Del T$_{-162}$/C$_{-161}$[e]	6.6	Typhimurium	16,37
5408-Cip	D87Y	---	E466D	---	---	V461G	G25A	---	33.7	Enteritidis	11
05-8560	S83F	D87N	---	S80I	---	---	Ins (1 nt) A$_{506}$	---	24.6	Kentucky	38
02-8141	S83F	---	---	---	---	---	**Del G$_{42}$-G$_{132}$**	---	**106.1**	Kentucky	38
02-2818	S83F	---	---	---	---	---	Dup (4 nt) C$_{508}$	---	29.1	Kentucky	38
5 mutant 3	D87Y	---	---	---	---	---	---	C$_{-157}$A	10.0	Paratyphi B	30
10 mutant 2	D87Y	---	---	---	---	---	**Del G$_{520}$-G$_{534}$; E160D**	---	**94.8**	Paratyphi B	30

3 mutant 2	S83Y	---	---	---	---	---	R46P	---	34.3	Livingstone	30
1 mutant 2	S83Y	---	---	---	---	---	---		41.6	Infantis	30

504

505

506 [a] Del, deletion. Ins, insertion. Dup, duplication

507 [b] Mutations leading to the maximum *ramA* expression values are represented in bold.

508 [c] Mutations are indicated by either the nucleotide positions deleted relative to the translation start site or the amino acid substitution.

509 [d] Numbers indicate the upstream positions relative to the translation start site.

510 [e] This is a 2-nucleotide deletion although only one nucleotide affects a RamR binding site.

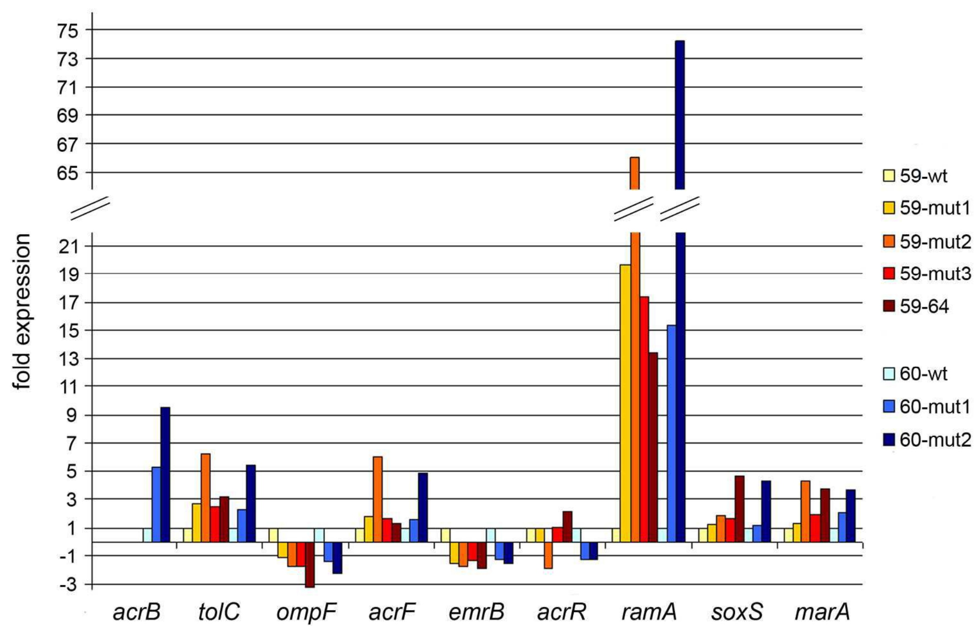
511 [f] Maximum *ramA* expression values are represented in bold.

512

Confidential: for peer review only

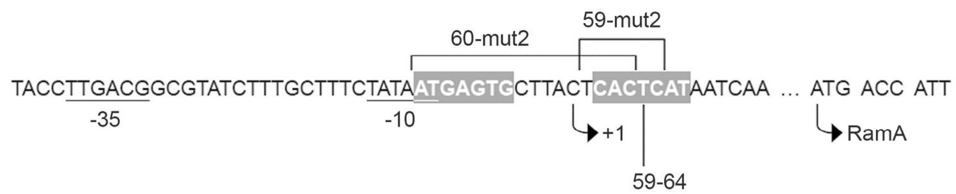
513
514
515

Confidential: for peer review only



180x117mm (300 x 300 DPI)

peer review only



180x39mm (300 x 300 DPI)

Confidential: for peer review only