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1 # Effects of siRNA silencing on the susceptibility of the
2 # fish cell line CHSE-214 to *Yersinia ruckeri*

3

4 **Running page head:** siRNA silencing vs *Y. ruckeri*

5

6 **Authors: Simon Menanteau-Ledouble[1#], Oskar Schachner[1], Mark L.
7 Lawrence[2], Mansour El-Matbouli[1]**

8 *[1] Clinical Division of Fish Medicine, University of Veterinary Medicine, Vienna,*
9 Austria
10 *[2] College of Veterinary Medicine, Mississippi State University, Mississippi State,*
11 *Mississippi, USA*

12

13 **Postal addresses:** Clinical Division of Fish Medicine, University of Veterinary
14 Medicine, Veterinärplatz 1, 1210 Vienna, Austria

15 **[#] Corresponding Author:** Dr. Simon Menanteau-Ledouble
16 **Email Address**: menanteaus@staff.vetmeduni.ac.at
17 **Tel. No.**: +431250775151
18 **Fax No.**: +431250775192

19

20 Additional email addresses:
21 M. El-Matbouli: mansour.el-matbouli@staff.vetmeduni.ac.at
22 M. L. Lawrence: lawrence@cvm.msstate.edu
23 O. Schachner : i107pc03@staff.vetmeduni.ac.at

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24

25 Abstract

26 Bacterial pathogens are known to co-opt mechanisms of the host cells' physiology to
27 gain intracellular entrance. Among the facultative intracellular bacteria is *Yersinia*
28 *ruckeri*, an enterobacterium mostly known as the causative agent of enteric redmouth
29 disease in salmonid fish. In the present study, we applied RNA inhibition to silence
30 twenty pre-selected genes on the genome of a fish cell line (CHSE-214) followed by
31 a gentamycin assay to quantify the effect of this silencing on the susceptibility of the
32 cells to infection. It was found that silencing of 16 out of 20 genes significantly
33 reduced the number of *Y. ruckeri* recovered at the end of the gentamycin assay.
34 Among the genes with the strongest impact were Rab1A, actin and Rac1, which is
35 consistent with our previous findings that N-acetylcysteine, a chemical inhibitor of
36 Rac1, completely prevented invasion of cells by *Y. ruckeri*. Conversely, silencing of
37 the Rho GTPase activating protein had no statistically significant effect, possibly
38 because *Y. ruckeri*, like some other members of the *Yersinia* genus is able to activate
39 Rho GTPase directly. Similarly, the effect of silencing E-cadherin was not statistically
40 significant, suggesting that this might not be a target for the adhesion molecules of *Y.*
41 *ruckeri*. Taken together, these findings improve our understanding of the infection
42 process by *Y. ruckeri* and of the interactions between this bacterial pathogen and
43 host cells.

44

45 Importance

46 Intracellular invasiveness is a mean for bacterial pathogen to gain shelter from the
47 immune system as well as access nutrients. The enterobacterium *Y. ruckeri* is well

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48 characterised as a facultative intracellular pathogen. However, the mechanisms of
49 invasion scrutiny. Investigations have mostly focused on the bacterial virulence rather
50 than on the host's mechanisms hicjacked during invasion. The present findings
51 therefore allow us to better understand the interaction between this important
52 potentially zoonotic pathogen of fish and host cells in vitro.

53

54 ## Keywords

55 Bacterial invasion, Gentamycin assay, Rac1, Rab1A, Actin, *Oncorhynchus*
56 *tshawytscha*

57

58 ## Introduction

59 The enterobacterium *Yersinia ruckeri* is a major fish pathogen world-wide; and has
60 mostly been studied has the causative agent of enteric red-mouth disease in
61 salmonid fish (1, 2). It is associated with acute outbreaks, especially in younger fish,
62 and causes septicaemia and haemorrhages leading to high levels of mortality in
63 infected fish (1–3), especially at temperature between 18 and 20°C. The bacterium
64 has zoonotic potential and has been associated with topical infections in humans (4).
65 Like several other enterobacteriaceae including other members of the genus
66 *Yersinia*, *Y. ruckeri* has demonstrated the ability to survive within macrophages (5) as
67 well as invade non-professional phagocytic cells (6, 7). This likely allows the access
68 to some restricted nutrients and protects it from the immune system, moreover it
69 might play a role in the bacterium's crossing of epithelial membranes as known from
70 other members of the genus *Yersinia* (8).

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71 Two main mechanisms of entry have been described in bacteria, and both are
72 present in *Enterobacteriaceae*. In the trigger mechanisms, effector proteins secreted
73 through the type three and type four secretion system (T3SS and T4SS) interact with
74 regulatory proteins, in particular members of the Rho family (RhoGTPases Rac,
75 Cdc42 or RhoG) (9). This leads to a rearrangement of the cytoskeleton of the host
76 cells resulting in the uptake of the bacterium (10, 11). Interestingly, while *Y. pestis* is
77 known to harbour a T3SS and some of its effector proteins have been shown to
78 target the cytoskeleton and actin filaments. However, these effectors seem to play a
79 role in preventing phagocytosis by professional phagocytic cells rather than to
80 promote intracellular invasion (12). However, the T3SS of *Y. ruckeri* actually belongs
81 to the Ysa family of T3SS, a different family than the one present in *Y. pestis*. It could
82 therefore play a different role in the virulence of *Y. ruckeri*. Indeed, Ysa is more
83 homologous to the T3SS carried on the *Salmonella* pathogenicity island 1 (SPI-1) of
84 *Salmonella enterica* (13, 14). Among the proteins carried on the SPI-1 is the
85 chaperon Invasion protein B (InvB) and the SPI-1 is known to play a role in the
86 intracellular invasion of *S. enterica* (15, 16) so the Ysa of *Y. ruckeri* could plausibly be
87 involved in intracellular invasion of *Y. ruckeri.* However, our knowledge of the *Ysa*
88 T3SS, and that of *Y. ruckeri* in particular is still incomplete (17) and no conclusion is
89 currently possible.
90 The other most studied mechanism of entry is the zipper mechanism that is
91 considered as the main mechanism of entry for bacteria belonging to the genus
92 *Yersinia.* It is initiated by the binding of the bacteria to specific molecules on the cell
93 membrane. For example, in *Y. pestis*, the attachment-invasion locus (ail) of *Yersinia*
94 *pestis* is a 17.5 kDa outer membrane protein that plays multiple roles in virulence
95 (18), including a minor role in binding to fibronectin (12, 19, 20). Similarly, the

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96 Plasminogen activator (Pla) has been shown to have adhesive property for laminin
97 (21) and likely moonlights as an adhesion factor. In *Y. pseudotuberculosis* and *Y.*
98 *enterocolitica*, the membrane proteins invasin (Inv) and Yersinia adhesin A (YadA)
99 interact with integrin receptors (22). Interactions of the bacteria with the cell surface
100 receptors lead to the recruitment of more receptors, activation of Rac1 and
101 cytoskeletal rearrangement culminating in the uptake of the bacterium (23).
102 An important feature of both invasion mechanisms is that they require active
103 uptake of the bacterium by the cell and it is possible to prevent host cells from
104 internalising the bacteria, for example by treating them with chemical blockers (24).
105 This is for example the case for *Y. ruckeri* and several chemical blockers have been
106 shown to prevent bacterial invasion (6, 7).
107 Beside chemical blockers, silencing of the genes involved with internalisation has
108 also been shown to have inhibitory effects. For example, a screening was performed
109 using 23300 dsRNA to silence over 95% of the annotated genes on the genome of
110 drosophila. This allowed to identify 305 genes whose inhibition interfered with the
111 ability of *Listeria monocytogenes* to invade cell cultures of drosophila SL2 cells and
112 86 genes necessary for invasion by *Mycobacterium fortuitum* (25, 26) . Interestingly,
113 a screening by Kumar *et al.* (27) on 18,174 genes from the genome of human
114 macrophages identified no less than 270 molecules whose silencing affected the load
115 of seven different field isolates of *Mycobacterium tuberculosis* within human
116 macrophages (27). Among these, 74 proteins were involved in the intracellular
117 survival of every isolate tested and over half of the genes identified played a role in
118 the regulation of autophagy, suggesting that this process was central in controlling
119 intracellular *M. tuberculosis*. Conversely, when Thornbrough *et al*. applied a
120 gentamicin assay to investigate the effect of a siRNA library targeting approximately

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121 22.000 genes, they found that a larger variety of genes were involved in the
122 intracellular survival of *Salmonella enterica* serovar *Typhimurium* within the human
123 epithelial cell line MCF-7 (28). Among the 252 genes whose silencing reduced the
124 survival of *S. enterica Typhimurium* more significantly were SEC22A, Rab1B and
125 VPS33B that were involved in vesicle trafficking as well as ATP6VOD1, an ATPase
126 involved in vacuole acidification and the iron transporter FTHL17. Nonetheless, the
127 authors noted a significant overlap between their findings and that reported by Kumar
128 *et al*. for *M. tuberculosis* (27) suggesting that many of these targets are well
129 conserved even between very evolutionary distant bacteria.
130 In the present study, we aimed to produce siRNA to silence 20 genes selected
131 because they are commonly associated with the intracellular survival of bacteria. The
132 fish cell line CHSE-214 was used and the effect of this silencing on the susceptibility
133 of the cells to intracellular invasion by the facultative intracellular fish pathogen *Y.*
134 *ruckeri* was investigated using a gentamicin assay.
135

136 Results and Discussion

137 **Several notable genes were found to be necessary for the intracellular**
138 **invasion process:** Silencing of every one of these genes resulted in reduction of the
139 number of bacteria recovered from Chinook Salmon Embryo (CHSE-214) cells at the
140 end of the assay; however, this reduction was only statistically significant in 16 out of
141 20 genes (Table 1).
142 Among the genes whose silencing had the strongest effect were the protein kinase
143 C, a regulatory protein overseeing a large number of varied functions, including
144 signal transduction as well as the expression of the cytoplasmic tyrosine kinase,
145 Focal Adhesion Kinase (FAK) and actin rearrangement (29). Previously, protein

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146 kinase C activity has shown an inverse correlation with the uptake of *L.*
147 *monocytogenes* by J774, a cell line of murine macrophage, as well as a positive
148 correlation with the escape of these bacteria from the macrophages endosomes (30).
149 Enteropathogenic *Escherichia coli* is also known to recruit protein kinase C to the
150 membrane of HeLa as well as the colon carcinoma cell line T84 (31) and more
151 recently, it has been shown that protein kinase C recruitment in lipid rafts was
152 induced by Enterohemorrhagic *E. coli* O157:H7. In the present study, silencing of the
153 gene encoding for protein kinase C resulted in a strong decrease of the cell
154 susceptibility to bacterial infection (P=0.000).

155 Another important gene was the Ras related protein Rab1A. The Rab family
156 constitute a subset of the Ras superfamily of proteins which are small GTPase acting
157 together as network involved in the regulation of vesicular transport (32). In humans,
158 more than 60 members of the Rab family have been identified (32) and about 52 in
159 the channel catfish (*Ictalurus punctatus*), although no census of Rab proteins has yet
160 been conducted for salmonid fish (33). Rab1A specifically is required for the
161 microtubule-based motility of murine endocytic vesicles (34) and silencing of Rab1b
162 was shown to lead to a reduced growth of *S. enterica Typhimurium* within MCF-7
163 cells (28).

164 Equilibrative nucleoside transporter 1 is a member of a well conserved family of
165 transmembrane proteins and silencing of the corresponding gene was found in the
166 present study to significantly decrease intracellular bacterial survival and
167 multiplication (P = 0.000). Pathogenic intracellular bacteria are known to scavenge
168 nucleotide from their environment and therefore, this difference could be explained by
169 a reduction in the nutrients available to the bacterium (35).

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170 Another molecule whose silencing had a significant effect oncell sensitivity to
171 infection (P = 0.000) was the precursor for integrin β-1. Integrins are trans-membrane
172 receptors and their role in cell adhesion to the extra-cellular matrix is well known.
173 Moreover, integrins β-1 have been well studied as target of the adhesin molecules of
174 *Yersinia* sp., including *Y. pseudotuberculosis* (36) and *Y. enterocolitica* (37). As such,
175 they play a central role in the use of the zipper mechanisms by these bacteria to gain
176 intracellular entry. The finding that this target is also important for *Y. ruckeri* suggests
177 that this bacterium might gain intracellular entry through the zipper mechanism
178 targeting of integrin β, in a manner reminiscent of that of *Y. pseudotuberculosis* and
179 *Y. enterocolitica*.

180 Similarly, silencing of the actin gene also had a very notable effect (P = 0.000).
181 Because actin is such a central component of the cytoskeleton and because of the
182 critical role of the cytoskeleton in both the trigger and zipper mechanism (11), this
183 finding was not unexpected.

184 Finally, another molecule of importance was Ras-related C3 botulinum toxin
185 substrate 1 (Rac1). It is a member of the Rho family of GTPases and plays a central
186 role regulating cytoskeleton rearrangement. It has previously been involved in
187 intracellular invasion of the enterobacteriaceae *Salmonella typhimurium* (38, 39) and
188 is targeted by the effector protein IpgB1 of *Shigella* (40). Among *Yersinia* spp., the
189 picture is more complex as Rac1 is inactivated by the T3SS effector protein YopE
190 expressed among others by *Y. pseudotuberculosis* (41) and *Y. enterocolitica* (42).
191 Notably, this Rac1 suppressive activity is most likely to hinder phagocytosis mediated
192 killing (43) while intracellular invasion with the zipper pathway relies on the CDC42-
193 independent activation of Rac1 (44). Moreover; in a previous experiment, we

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194 investigated the effect of multiple chemical and found out that the inhibitor of Rac1 N-
195 acetylcysteine completely inhibited entrance of *Y. ruckeri* into CHSE-214 cells (6).
196 **Conversely, several genes were identified for which silencing did not result**
197 **in a statistically significant decrease of the cells' sensitivity to infection.** Among
198 these genes were the Rho GTPase-activating protein (P = 0.795). Rho GTPases act
199 as regulator and facilitator for the activity of Rho-GTPase molecules such as Rac1
200 and CDC42. Several virulence factors of enterobacteriaceae are known to activate
201 Rho GTPase enzymes directly (45), bypassing RhoGTPase activating proteins in a
202 manner consistent with the present findings. An example of such a protein is the
203 cytolytic necrotising factor (CNFy) of *Y. pseudotuberculosis* (46). Interestingly, a
204 segment of the *Y. ruckeri's* genome is homologous to a section of the gene encoding
205 for *Y. pseudotuberculosis'* CNFy but it is not currently known if this molecule is active
206 in *Y. ruckeri*.
207 Another protein whose silencing did not result in a significant reduction of
208 sensitivity to infection was E-cadherin (P = 0.722). E-cadherin acts as the receptor for
209 Internalin used by *Listeria. monocytogenes* (47). However, members of the *Yersinia*
210 genus tend to use different surface receptors such as integrins (22). If E-cadherin is
211 not a target of *Y. ruckeri*'s adhesion molecules, then it would be expected that
212 silencing of the corresponding gene would not result in any significant change
213 susceptibility to intracellular invasion.
214 The next gene whose silencing had no significant effect on the bacterial invasion
215 was the caspase I precursor (P = 0.962). Caspases take part in the cellular immune
216 response and play an important role in regulating apoptosis (48). Caspase I is known
217 to be inhibited by the YopJ effector molecule of *Y. pestis* (49).

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218 Finally, the last gene whose silencing had no statistically significant effect was the
219 cyclin D1. Cyclin D1 regulates cellular proliferation (50) and its expression is known
220 to be affected by intracellular invasion of *Streptococcus pyogenes* (51). However,
221 there is no evidence suggesting it might play a role in the intracellular invasion of
222 Gram-negative bacteria. Moreover, cyclin D1 is most expressed during the G1 phase
223 of the cell life cycle (50).As our cells were confluent at the time of the intracellular
224 invasion procedure, it is expected that the expression of cyclin D1 was lowered even
225 in the absence of RNA inhibition.

226 To summarise, we used RNAi to silence 20 selected genes on the genome of
227 CHSE-214 cells. Silencing of 15 of these genes resulted in a significantly reduced
228 susceptibility to invasion of *Y. ruckeri*. The results of this study contribute to our
229 understanding of the invasion mechanisms in this important fish pathogen.
230

231 ## Materials and methods

232 **Gene selection and design of the siRNA sequences.** Twenty genes were
233 selected based on literature and the fact that homologs of these genes have been
234 implicated in the intracellular invasion of other bacterial pathogens. The selection
235 included genes commonly involved in invasion by bacterial pathogens, including
236 surface integrins as such molecules are often targeted by the invasin molecules
237 expressed by other Yersiniaceae (52–54). Other genes were cytoskeletal molecules
238 and genes involved in vacuolar trafficking and maturation (27, 28) as well as agents
239 of the cytoskeletal apparatus as this plays a central part in the internalization of
240 bacteria through both the zipper and trigger mechanisms (55). The selection also
241 included genes and pathways shared by *L. monocytogenes*, *M. tuberculosis* as well
242 as *S. typhimurium* (25–28). As these pathways were used by such diverse facultative

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243 intracellular pathogens, they might also be involved in the intracellular invasion of *Y.*
244 *ruckeri*. After selection of the genes, sequences for the siRNAs were designed by
245 Ambion Life Technology using the Silencer Select siRNA design algorithm. For the
246 genes for which no *Oncorhynchus tshawytscha* sequences were available,
247 sequences from other members of the *Oncorhynchus* genomes were used instead.
248 Upon reception, the siRNAs were resuspended to 20 μM and stored at -20°C until
249 use, according to the manufacturer's instruction.

250 **siRNA transfection was performed based on the manufacturer's guidelines.**
251 This study made use of CHSE-214 cells. The identity of this cell line derived from
252 Chinook Salmon Embryo has been confirmed by PCR and sequencing and their
253 suitability for gentamycin assay has been confirmed before (6). CHSE-214 cells were
254 grown in 24 well plates at 20°C supplied with 1250 μl Minimum Essential Medium
255 (MEM-glutaMAX^{TM}, Gibco) containing 2% FBS, where they reached about 80%
256 confluence one day after seeding.

257 On the day of the assay, 8 μl of the siRNA solution was added to 400 μl of Opti-
258 MEM medium (Gibco). At the same time, 72 μl of RNAiMax lipofectamine reagent
259 (Sigma Aldrich) was diluted in 1200 μl of Opti-MEM medium. The two solutions were
260 mixed together and incubated 5 minutes at room temperature. Afterwards, the culture
261 medium on top of two adjacent rows of the 24 wells (either rows 3 and 4 or 5 and 6; 8
262 wells in total) was replaced with 50 μl of the solution and, after 20 minutes, 450 μl of
263 fresh MEM-Glutamax medium was added.

264 In each plate, as negative control, cells from the 8 wells in column 1 and 2 were
265 processed in the same manner but without the siRNA, serving.

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266 The cells were then incubated at 20°C and due to the temperature lower than
267 other cell lines commonly used for transfection, incubation time was extended to
268 three days.

269 **Bacterial invasion assay.** The gentamycin assay was performed as previously
270 described (6). Briefly, *Y. ruckeri* ATCC 29473 was cultivated overnight in brain heart
271 infusion (BHI, Oxoid). Optical density of the culture was assessed by
272 spectrophotometry and adjusted to an optical density of 0.5 at 600 nm. The bacteria
273 were pelleted down by centrifugation at 3220 g and 20°C (in an Eppendorf Centrifuge
274 5810 R) before being resuspended in 10 times the original volume of MEM-
275 Glutamax. Then, the culture medium in the wells 1A-C, 3 A-C and 5 A-C was
276 replaced by this bacterial solution (for clarity, the plate loading plan is illustrated on
277 Fig. 1) and the bacteria were left to interact with the cells for five hours at room
278 temperature. In the wells 1D, 3D and 5D, the medium was replaced by fresh MEM-
279 glutamax without bacteria. After that time, the medium was removed, the cells were
280 washed three times with PBS and a fresh volume of MEM-glutamax, supplemented
281 with the antibiotic gentamycin (Sigma-Aldrich) at a concentration of 100 $\mu g.ml^{-1}$ was
282 added to the wells. The antibiotic was left to act for four hours. Afterwards the cells
283 were washed twice with PBS before replacing the medium supplemented with 1%
284 Triton-X (Sigma-Aldrich). After 10 minutes of exposure to the detergent, the cells
285 were triturated with a micropipette and serially diluted from 10^{-1} to 10^{-4} before being
286 plated onto Brain heart infusion agar (BHIA, Oxoid). Each of the three wells
287 represented a biological replicate and each dilution was plated in technical
288 quadriplicates, meaning that 48 plates were inoculated for each siRNA. The agar
289 plates were incubated at 22°C until clear colonies were visible and counted (generally
290 after 48 hours). The corresponding average CFU per ml value was then calculated.

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291 To minimise the effect of the variation between cell and bacterial culture, these
292 values were always compared to that of the control CHSE-214 cells from the same
293 plate (Fig 1).

294 **Controls.** For each siRNA, in the 24 well plate the cells of number 1D, 3D and 5D
295 were left un-inoculated then lysed and plated to act as a negative control and detect
296 any contamination of the reagents while the cells in 2D, 4D and 6D were exposed
297 tested using a trypan blue assay. Briefly, the cells were washed three times in PBS,
298 then 0.2% Trypan Blue (0.4% diluted 1:1 in PBS) was added. The cells were stained
299 for 1 minutes prior to fixation with 4% formalin for 10 minutes Afterwards they were
300 rinsed several times until any trace of blue dye had disappeared. The plates were
301 kept at 4°C until quantification of the cells using an inverted microscope (Leica DM
302 IRB). One hundred cells were counted and the number of blue stained cells among
303 them was recorded. The procedure was repeated 4 more times for each culture unit
304 to result in 5 percentage values for each siRNA. These were then compared to the
305 survival of the control cells without siRNA to confirm that the silencing procedure did
306 not have a toxic effect. In no instances did these numbers differ significantly from that
307 of the control.

308 Finally, the cells in the wells 2A-C, 4A-C and 5A-C were lysed in buffer RLT
309 (Qiagen). The cell solution/suspension? was homogenised using QIAshredder
310 columns (Qiagen) and centrifugation at 145000 RPM for two minutes at room
311 temperature using MiniSpin tabletop centrifuge (Eppendorf). Afterwards, the RNA
312 were extracted using the Rneasy mini kit (Qiagen) according to the manufacturer's
313 handbook. The RNA were immediately quantified using a Nanodrop machine
314 (ThermoFisher) and cDNA were immediately synthesised using Iscript kits (Bio-Rad)

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315 according to the manufacturer's instructions in a C1000 Touch thermocycler (Bio-
316 Rad). Resulting cDNAs were stored at 4°C until use.
317 **RTqPCRs.** The primer sequences used for confirmation of silencing are listed in
318 Table 2. Primers for ubiquitin and elongation factor 1-alpha designed by Peña *et al.*
319 (56) were used for the housekeeping reference genes, as these authors found them
320 among the most suitable when working with CHSE-214.
321 Genomic DNA was extracted from CHSE-214 cells using the DNeasy kit (Qiagen)
322 according to the manufacturer's instructions. End point PCR was performed using
323 these primers to confirm the optimal annealing temperature, afterwards the PCR
324 products were purified with a QIAquick gel purification kit (Qiagen). The products'
325 concentration were measured using a nanodrop, then adjusted to 10 ng.μl^{-1}.
326 Afterwards, serial dilution was performed to produce concentrations ranging from 10^{-1}
327 to 10^{-4} ng.μl^{-1}. qPCRs were then performed on these serial dilutions as well as the
328 cDNA produced as described in the previous section. Relative gene expression
329 levels of the silenced genes were calculated using the $2^{-\Delta\Delta Ct}$ method (57) to confirm
330 the efficacy of the silencing. The serial dilutions were used to calculate the R^2 and
331 efficiency of the qPCRs and confirm that these were above 0.9 and between 95 and
332 100% respectively for every qPCR.
333

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498

499 # Figure Legend

500

501 **Fig 1** Loading plan for the 24 wells plates used in the gentamycin assay. Each siRNA
502 was tested alongside a non-silenced control to minimise the variations between
503 plates. For each gene silenced, the three first wells of the leftmost row were infected
504 for the gentamycin assay while the bottom well was left uninfected as a control. On
505 the rightmost row, RNA was extracted from the first three wells to confirm silencing
506 by RTqPCR while the last well was stained using trypan blue to assess any toxic
507 effect of the RNA silencing.

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510 # Tables

511

512 **Tables 1** Gene investigated in this study and effect of the silencing on the number of
513 bacteria recovered at the end of the gentamycin assay. Greyed out cells represent
514 genes whose silencing did not significantly impact the gentamycin assay.

Gene name	Function	Significance
Protein kinase C	Signal transduction	P = 0.000
Rab-1A	Vesicular trafficking	P = 0.000
SEC22b-B	Vesicular trafficking	P = 0.002
Vacuolar ATP synthase subunit A	Vacuolar protein sorting	P = 0.003
VPS-associated protein 33A	Vacuolar acidification	P = 0.002
Rho1-GTPase	Cytoskeleton organisation	P = 0.006
Ubiquitin conjugating enzyme E2L3	Tagging of protein for ubiquitin degradation	P = 0.003
Sumo 2	Post-translational modification system	P = 0.005
Equilibrative nucleoside transporter 1	Cellular uptake of nucleosides	P = 0.000
Integrin β-1 precursor	Integrin	P = 0.000
Actin	Cytoskeletal apparatus	P = 0.000
Rac1	Cell growth & cytoskeletal reorganization	P = 0.000
SDC42	Rearrangement of actin filaments	P = 0.001
Rho GTPase-activating prot.	Rearrangement of actin filaments	P = 0.795
Laminin	Structural scaffolding of the tissues	P = 0.006
E-cadherin	Cell adhesion	P = 0.722
Myotubularin-related protein 2	Vesicular traffic and actin remodeling	P = 0.045
p38b1 mitogen activated protein kinase	Stress resistance and apoptosis	P = 0.000
Caspase I precursor	Role in apoptosis	P = 0.962
Cyclin D1	Coordination of mitosis	P = 0.948

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516

517 **Tables 2** Primer sequences for the RTqPCr confirmation of the siRNA silencing. The

518 two first (greyed out) primer pairs are the housekeeping genes and where designed

519 by Peña *et al.* (56)

520

Ubiquitin	GGAAAACCATCACCCTTGAG
	ATAATGCCTCCACGAAGACG
Elongation Factor 1 alpha	GTCTACAAAATCGGCGGTAT
	CTTGACGGACACGTTCTTGA
Protein kinase C	TTGTTTGGCGGACTCCTGAA
	CCACGCCGAAAACAATCTCC
Rab-1A	AACCCCACCAACACCTCTAC
	GCACACACACACAACTTCTTTC
SEC22b-B	AGAGAGGAGAGGAGATAGGG
	TGGATAGAATGTGAGACCAGA
Vacuolar ATP synthase subunit A	CCGCTGAGGACTTCCGATAAAC
	ACAGCCTTCCATAGCATTGCAC
VPS-associated protein	ATCCACAACCTGACTGCCTACC
	CTCTCGCTGCTCTTGATGAAC
Rho1-GTPase	TAGCCCCAGTTTGCTCTTCG
	GCTGTCTCGTTCACTGGTCA
Ube213	AACCCCACCTCCTTTCTCTC
	TTCACTAAGTCCCCTTGTTCC
Sumo 2	TCCACCACAGAACCAATACC
	CAAAACAGGAAGGAAACAAAGG
Equilibrative nucleoside transporter 1	ACGTCCGTGTTAAATGCTC

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	ACAAAATCTCTCCTCCCCTC
Integrin β-1 precursor	ACCAATCCTACCCCCTATCC
	AGACCTCCCTCATTGTGTCG
Actin	CCAAGCAGGAATACGACGAG
	GCGTGGCAAAAAAAGTCCAG
Rac1	ACTCACCCCCATTACCTACC
	CTGCTCCAATCTCTTTTGCC
SDC42	AGAGGGGAAGGAATCAACAG
	ATGACAGGCAGGTAGGAGAG
Rho GTPase-activating prot.	CAGAAGAAAATGAGCAAAGACC
	CCTGAGAACTACTGTCCACC
Laminin	GCGAATCAGAACCCTCAAC
	GCAGATACACATCCCTCCAAC
E-cadherin	TCCCTCAGTTCCCTCAACTC
	TCTCAATCCTCTCCTCCTCC
Myotubularin-related protein 2	TCAAGCGGAATACAAAAGACAG
	CCACTCTCTTCAACTCCTCATC
MaPK	TTCACTTCTCAAACCCCAAAC
	CGCTCAAAGGACAAACCAC
Caspase I precursor	GTGGAACAACAGGAAACAAAG
	TGATGGCAGTAGAACAGGG
Cyclin D1	GCTGTCCTGTATGCTCTCTC
	CCCAAACCATTCCATTCTTCTC

521

	1	2	3	4	5	6
A	No siRNA Infected	No siRNA RNA isol.	Gene 1 Infected	Gene 1 RNA isol.	Gene 2 Infected	Gene 2 RNA isol.
B	No siRNA Infected	No siRNA RNA isol.	Gene 1 Infected	Gene 1 RNA isol.	Gene 2 Infected	Gene 2 RNA isol.
C	No siRNA Infected	No siRNA RNA isol.	Gene 1 Infected	Gene 1 RNA isol.	Gene 2 Infected	Gene 2 RNA isol.
D	No siRNA Mock infected	No siRNA Trypan Blue	Gene 1 Mock infected	Gene 1 Trypan Blue	Gene 2 Mock infected	Gene 2 Trypan Blue