Roles of proteins containing immunoglobulin-like domains in the conjugation of bacterial plasmids.

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Abstract

Horizontal transfer of bacterial plasmids generates genetic variability and contributes to the dissemination of the genes that enable bacterial cells to develop antimicrobial resistance (AMR). Several aspects of the conjugative process have long been known, namely, those related to the proteins that participate in the establishment of cell-to-cell contact and to the enzymatic processes associated with the processing of plasmid DNA and its transfer to the recipient cell. In this work, we describe the role of newly identified proteins that influence the conjugation of several plasmids. Genes encoding high-molecular-weight proteins that contain one or several immunoglobulin-like domains (Big) are located in the transfer regions of several plasmids that usually harbor AMR determinants. These Big proteins are exported to the external medium and target two extracellular organelles: the flagella and the conjugative pili. The plasmid-encoded Big proteins facilitate conjugation by reducing cell motility and facilitating cell-to-cell contact by binding both to the flagella and to the conjugative pilus. They use the same export machinery as that used by the conjugative pilus components. In the examples characterized in this paper, these proteins influence conjugation at environmental temperatures (i.e., 25°C). This suggests that they may play relevant roles in the dissemination of plasmids in natural environments. Taking into account that they interact with outer surface organelles, they could be targeted to control the dissemination of different bacterial plasmids carrying AMR determinants.

Introduction

Bacterial infectious diseases, despite the availability of antibiotics, remain an important public health issue, representing the second leading cause of death worldwide [1]. The gradual increase in the resistance rates of several important bacterial pathogens represents a serious threat to public health [2–4]. Indeed, multidrug-resistant bacteria are the cause of a slow-growing pandemic. The dissemination of multiple antimicrobial resistance (AMR) genes has been largely attributed to the acquisition of plasmids by horizontal gene transfer (HGT), especially in Gram-negative bacteria [5–7], as well as in Gram-positive bacteria [8]. Plasmids can confer resistance to the major classes of antimicrobials [9].

For decades, plasmid incompatibility [10] has been a useful tool for grouping bacterial plasmids. In recent years, other approaches have been considered. Based on phylogenetic analysis of conjugative relaxase, the protein required to initiate plasmid mobilization through conjugation, plasmids can be grouped into different relaxase families [11]. Plasmids belonging to the incompatibility group (Inc) HI (MOB_H relaxase family) are widespread in *Enterobacteriaceae* and

55 most commonly include genetic elements encoding multiple AMR determinants [12]. IncHI
56 plasmids, often >200 kb in size, share a common core of approximately 160 kb. The differences
57 in size are due to the presence of distinct insertion elements, including many AMR determinants
58 [13]. IncHI-encoded AMR can be present in enterobacterial such as *Salmonella, Escherichia coli*
59 [14], *Klebsiella pneumoniae* [15] and *Citrobacter freundii* [16]. Plasmids of the IncHI2 subgroup
60 predominate in antibiotic-resistant *Salmonella* isolates. In *S.* Typhi*,* more than 40% of isolates
61 harbor an IncHI plasmid [17]. In recent years, a novel role of IncHI plasmids in AMR spread has
62 been reported. The emergence of Gram-negative bacteria with AMR, especially those producing
63 carbapenemases, led to reintroduction of colistin as a last resort antibiotic for the treatment of
64 severe infections [18]. In contrast to its limited clinical use, colistin is widely used in veterinary
65 medicine [19]. In the past, colistin resistance was associated with chromosomal mutations only
66 [20]. Nevertheless, plasmid-mediated resistance, conferred by the mobilized colistin resistance
67 gene (*mcr-1*), has emerged recently. Since its discovery in 2016 in China [21], *mcr* genes, have
68 been detected in animals, food, the human microbiota, and clinical samples in over thirty
69 countries [22–26]. IncHI2 plasmids represent 20.5% of the overall plasmids encoding the *mcr-1*
70 gene worldwide but up to 41% in Europe [27]. Of special concern is the presence of the *mcr-1*
71 resistance determinant in *Enterobacteriaceae* carrying carbapenem resistance genes, such as
72 bla_{NDM} and bla_{KPC}. The combination of these AMR determinants seriously compromises the
73 treatment of infections caused by pathogenic strains harboring these plasmids [28,29]. An
74 example of this is the recent report of an AMR clone of the highly virulent *E. coli* ST95 lineage
75 [14]. *E. coli* ST95 clones underlie neonatal meningitis and sepsis. They are usually sensitive to
76 several antibiotics. This clone harbors an IncHI2 plasmid that encodes, among other factors,
77 determinants of resistance to colistin and multiple other antibiotics (including the extended-
78 spectrum beta-lactamase *blaCTX-M-1*). The spread of such an AMR ST95 clone could pose a
79 threat to human health worldwide [14].

80 IncHI plasmid conjugation has a distinctive feature: while optimal conjugation rates are obtained
81 at temperatures found outside the host (30°C and below), conjugative transfer is repressed at
82 temperatures encountered within the host (37°C) [30,31]. The plasmid R27 is the prototype of
83 IncHI1 plasmids. It harbors the Tn*10* transposon, which confers resistance to Tc, and has been
84 exhaustively studied. The R27 replication and conjugation determinants are well characterized
85 [32,33], and its complete nucleotide sequence is available [34]. Several ORFs from the plasmid
86 R27 (66%) do not show similarity to any known ORFs.

87 IncA/C plasmids belong to the same MOB_H relaxase family as IncHI plasmids. They were originally
88 identified in the 1970s among multidrug-resistant *Aeromonas hydrophila* and *Vibrio* spp. isolates

89 that infected cultured fish [35,36]. Since the 1990s, these plasmids have received increasing
90 interest because of their role in mobilizing AMR in enterobacteria and other Gram-negative
91 microorganisms [37–40]. They have an extremely broad host range that includes members of
92 Beta-, Gamma- and Deltaproteobacteria [41] and play a relevant role in the global spread of
93 AMR [42,43]. They represented 50% of all plasmids isolated from *bla*$_{NDM}$-producing *Klebsiella*
94 *pneumoniae* of clinical origin characterized in a recent study [44].
95 Proteins containing an immunoglobulin (Ig)-like domain contain several chains of approximately
96 70-100 amino acid residues present in antiparallel β-strands and organized in two β-sheets that
97 are packed against each other in a β-sandwich. The Ig-like domain has been identified in a large
98 number of proteins with diverse biological functions, is widely distributed in nature, and is
99 present in vertebrates, invertebrates, plants, fungi, parasites, bacteria, and viruses [45].
100 Bacterial proteins containing Ig-like domains (Big) exhibit a wide range of functions. They include
101 fimbrial subunits, adhesins, membrane transporters and several enzymes (as reviewed in [46]).
102 In a previous report, we studied a high-molecular-weight extracellular protein (the RSP protein)
103 that contains a Big domain and plays an essential role in IncHI plasmids conjugation. Among
104 other targets, the RSP protein appears to be associated with flagella, reducing cell motility.
105 Under specific mating conditions, it could be shown that binding of the RSP protein to the flagella
106 influences conjugation [47]. In this report, we present novel data about the roles of these
107 plasmid-encoded Big proteins. We show that two Big proteins bind both flagella and the
108 conjugative pilus to favor conjugation of the IncHI1 plasmid R27. Furthermore, we also show
109 that other groups of plasmids such as IncA/C and IncP2 also encode these proteins. We provide
110 evidence for their role in the conjugation of IncA/C plasmids. The role of plasmid-encoded Big
111 proteins in plasmid conjugation is discussed.

112 **Results**

113 **The RSP protein interacts in vitro both with a new R27-encoded Big protein and with a protein**
114 **involved in plasmid conjugation.**

115 To gain further insight into the role of the RSP protein in the conjugation of the R27 plasmid, we
116 decided to assess whether this protein interacts with other proteins expressed by the *Salmonella*
117 strain SL1344 (R27). We performed immunoprecipitation of a cellular extract of strains SL1344
118 (R27 RSP-Flag) and SL1344 (R27 Δ*rsp*) and analyzed the proteins that specifically coprecipitated
119 with the RSP protein. Two R27-encoded proteins were found to specifically coprecipitate with
120 the RSP protein (Table S1). The protein showing the highest score (187.77) and coverage (58.56)
121 was the R27_p055 protein. The *R0055* gene was mapped between transfer regions 2 and 1 of
122 the R27 plasmid (Fig. 1A). The *R0055* gene product is a 794 AA protein with a molecular mass of

123 86.75 kDa. As with the RSP protein, the protein encoded by *R0055* also contains bacterial Ig-like
124 domains (Big_1 and _3): a Big_1 domain spanning amino acid residues 143 to 254, and a Big_3
125 domain spanning residues 537 to 693. The R27_p055 protein, herein termed RSP2, also contains
126 a DUF4165 domain of unknown function (amino acids 23 to 142) (Fig. 1B).

127 The second protein showing a high score (59.46) and coverage (39.92) was the R27-encoded
128 TrhH protein. This protein shares 26% of identity with the IncF TraH protein, which is involved
129 in plasmid conjugation [32].

130 **The RSP2 protein shows RSP-dependent expression in the external medium.**

131 To determine whether the RSP2 protein is also present in the cell-free secretome of strain
132 SL1344 (R27), we analyzed the cell-free secreted protein profile of this strain by SDS-PAGE (Fig.
133 1C). In addition to the band corresponding to the already characterized RSP protein, a second
134 band corresponding to a protein of molecular mass equivalent to that of the RSP2 protein, was
135 observed. The band was isolated and analyzed by LC-MS/MS. It corresponded to the RSP2
136 protein (Fig. 1C). To identify the RSP2 protein in the different cellular compartments, a Flag-tag
137 was added to the *rsp2* gene (see Materials and Methods section for details). Cultures of strains
138 SL1344 wt and SL1344 (R27 RSP2-Flag) were grown in LB medium at 25°C to an OD_{600nm} of 2.0.
139 Samples were then collected, and the different cellular fractions were obtained. The RSP2
140 protein was detected in the different fractions by Western blotting, using anti-Flag-specific
141 antibodies (Fig. 2A-C). The protein was identified in the same cell compartments as the RSP
142 protein (i.-e., periplasm, inner membrane, cytoplasm, and cell-free secreted proteins).

143 As the above reported data show that the RSP2 protein can be exported to the external medium,
144 we decided to study whether this protein is exported by the R27-encoded type IV secretion
145 system that is also used by RSP [47]. To address this point, we first used the SSPred program
146 (http://www.bioinformatics.org/sspred/html/sspred.html) for *in silico* prediction of whether
147 the RSP2 protein, similar to the RSP protein, could be exported through a type IV secretion
148 system (Fig S1). To provide evidence supporting this hypothesis, we used strains SL1344 (R27
149 RSP2-Flag) and SL1344 (R27 Δ*trhC* RSP2-Flag) and analyzed the presence of RSP2 protein in the
150 protein profile of the cell-free secreted fractions (secretome). This protein could not be detected
151 in the secretome of strain SL1344 (R27 Δ*trhC*) (Fig. 3A). Immunodetection of RSP2-Flag by using
152 anti-Flag specific antibodies confirmed the requirement for TrhC expression for RSP2 export (Fig.
153 3B). We next checked whether RSP2 export in strain SL1344 (R27 Δ*trhC*) could be made to occur
154 by providing the gene encoding the TrhC ATPase *in trans*, cloned in the plasmid pBR322 (plasmid
155 pBR322-*trhC*). Complementation of RSP2 export was observed (Fig. 3B), suggesting that, as was

156 the case for the RSP protein, the R27-encoded type IV secretion system mediates export of the
157 RSP2 protein.

158 We next addressed the question whether there is co-dependence in protein export between the
159 RSP and the RSP2 proteins. To that end, a Flag-tag was added to the *rsp2* gene in the strain
160 SL1344 (R27 Δ*rsp*). Expression of both RSP and RSP2 proteins was assessed in the strains SL1344
161 (R27 RSP2-Flag), SL1344 (R27 Δ*rsp* RSP2-Flag) and SL1344 (R27 Δ*rsp* RSP2-Flag pLG338-rsp). The
162 results obtained show that export of the RSP2 protein is dependent on the RSP expression (Fig.
163 4A and 4B).

164 **Expression of the RSP2 protein influences the motility and conjugation of strain SL1344 (R27).**

165 We previously showed that the expression of the RSP protein is essential for R27 plasmid
166 conjugation, and that SL1344 cells that express RSP show reduced motility compared to plasmid-
167 free cells [47]. Considering the observed interaction of both RSP and RSP2 proteins, we studied
168 whether, as is the case for the RSP protein, expression of the RSP2 protein influences cell motility
169 and/or conjugation.

170 After constructing an R27 derivative lacking the *rsp2* gene (plasmid R27 Δ*rsp2*), we performed a
171 comparative motility assay with the *Salmonella* strain SL1344 and its derivatives harboring the
172 R27, R27 Δ*rsp2*, and R27 Δ*rsp2* pLG338-*rsp2* plasmids. The results obtained (Fig.5A) showed that
173 the RSP2 protein influences the motility of strain SL1344 (R27).

174 We were able to show previously that the RSP protein is associated with the flagella synthesized
175 by the Salmonella strain SL1344 [47]. We decided next to assess whether RSP2 also targets the
176 flagella. In an attempt to detect the RSP2 protein by transmission electron microscopy, we used
177 strain SL1344 (R27 RSP2-Flag) and gold-labelled anti-Flag monoclonal antibodies (Fig 5B and 5C).
178 As was the case for RSP, RSP2 also exhibited binding to the flagella.

179 To assess the role of the RSP2 protein on plasmid conjugation, we constructed a R27 derivative
180 lacking the *rsp2* gene (plasmid R27 Δ*rsp2*) and compared the conjugation frequencies of strains
181 SL1344 (R27), SL1344 (R27 Δ*rsp2*) and SL1344 (R27 Δ*rsp2* pLG338-*rsp2*) growing at 25°C in liquid
182 media. We also compared the conjugation frequencies of strains SL1344 (R27) and SL1344 (R27
183 Δ*rsp2*) growing cells on nitrocellulose filters placed on LB plates. When cells were grown in liquid
184 medium, transfer of the R27 Δ*rsp2* plasmid was detected at a frequency that was approximately
185 two logs lower than that of wt R27 (Fig.5D). The presence *in trans* of the RSP2 protein encoded
186 by the pLG338-*rsp2* plasmid, restored the conjugation frequency of the wt R27 plasmid. When

187 cells were grown on solid medium, we also detected a significant decrease in the conjugation
188 frequency of the R27 Δ*rsp2* plasmid compared to that of the wt R27 plasmid (Fig. 5E).

189 **Relationship between the RSP and RSP2 proteins and the conjugative machinery of the R27**
190 **plasmid.**

191 As mentioned above, immunoprecipitation of the RSP protein indicated interactions with both
192 the RSP protein and the R27 *trhH* gene product. The TrhH protein of the plasmid R27 shares
193 identity with the TraH protein encoded by IncF plasmids [32]. The TraH protein is a component
194 of the outer membrane complex involved in conjugation [48] and has been shown to be required
195 for pilus assembly [49]. On the other hand, the *trhA* gene product encoded in IncHI plasmids has
196 been considered to be the pilin subunit itself [50]. We decided to analyze whether the
197 expression of the R27 TrhH and/or TrhA proteins encoded by IncHI plasmids influences the
198 expression of the RSP and RSP2 proteins. After inactivation of the *trhH* or *trhA* genes of strain
199 SL1344 (R27), the expression of the RSP and RSP2 proteins in the external medium was analyzed.
200 As both the RSP and RSP2 proteins were copurified with flagella when a conventional flagella
201 purification protocol was used (see the Material and Methods section), we obtained fractions
202 containing flagella from the SL1344 wt, SL1344 (R27), SL1344 (R27 Δ*trhA*) and SL1344 (R27
203 Δ*trhH*) isogenic derivatives. Proteins were analyzed by SDS-PAGE (Fig. 6A). The expression of
204 both the RSP and RSP2 proteins in the extracellular medium is dependent upon the function of
205 TrhH or TrhA. We also analyzed the intracellular expression of the RSP and RSP2 proteins in the
206 *trhH* and *trhA* genetic backgrounds. Lack of either TrhH (Fig. 6B and 6C) or TrhA (Fig. 6D and 6E)
207 function resulted in intracellular accumulation of either RSP or RSP2. Considering that
208 expression of the RSP and RSP2 proteins reduces cell motility in strain SL1344 (R27) and that
209 inactivation of the *trhH* gene interferes with the presence of these proteins in the extracellular
210 medium, it could be expected that *trhH* inactivation would also result in increased motility in
211 SL1344 cells harboring plasmid R27 Δ*trhH*. We then performed a mobility assay of the plasmid-
212 free SL1344 strain and of its SL1344 (R27) SL1344 (R27 Δ*rsp*), SL1344 (R27 Δ*rsp2*) and SL1344
213 (R27 Δ*trhH*) derivatives. In accordance with (i) the observed requirement of TrhH function for
214 RSP and RSP2 export and (ii) the effect of the RSP/RSP2 proteins on cell motility, the motility of
215 strain SL1344 (R27 Δ*trhH*) was significantly reduced compared to that of the wt strain (Fig. 7).

216 **The RSP and RSP2 proteins bind the R27 conjugative pili.**

217 Taking into account the observed relationship between elements of the conjugation machinery
218 of the R27 plasmid and the RSP and RSP2 proteins, we decided to assess whether these proteins,
219 in addition of binding the flagella, they also target the conjugative pilus. To that end, we blocked

220 flagella expression and used electron microscopy on order to detect either the RSP or RSP2
221 proteins bound to the conjugative pili. To prevent flagella expression, we constructed Δ*fliC/fljB*
222 (flagellin subunit) derivatives of strains SL1344 (R27) and SL1344 (R27 RSP2-Flag). Immunogold
223 transmission electron microscopy imaging of these strains by using either polyclonal anti-RSP
224 antibodies [47] or monoclonal anti-Flag antibodies showed that in both examples, gold particles
225 were associated with tubular structures that likely corresponded to the conjugative pilus (Fig.
226 8A and 8C). When the Δ*trhH* allele was introduced into the mutant derivatives lacking flagella,
227 the tubular structures were no longer detected, thus supporting the hypothesis that they
228 corresponded to the conjugative pilus (Fig. 8B and 8D).

229 **The RSP2 protein is specific for IncHI1 plasmids.**

230 The RSP protein is restricted to the IncHI plasmids, from both the IncHI1 and IncHI2 subgroups
231 [47]. Upon having shown the relevant role of the RSP2 protein in the conjugation of the IncHI1
232 plasmid R27, we performed a BLAST search to identify the *rsp2* gene in other bacterial plasmids
233 (Table S2). In contrast to the RSP protein, the RSP2 protein is present in IncHI1 plasmids but not
234 in IncHI2 plasmids. Interestingly, a group of IncN plasmids also contains an homolog of the R27
235 *rsp2* gene.

236 **Distribution of proteins containing the Big domain among bacterial plasmids.**

237 We next addressed the question of whether proteins containing Big domains are a feature of
238 only IncHI plasmids or whether they are also encoded by plasmids of other incompatibility
239 groups. We used for this analysis the genome viewer integrated into the NCBI database,
240 configuring it to show the features and domains of the annotated proteins. The search was
241 performed in assembled and sequenced plasmids of all incompatibility groups, selecting those
242 that presented proteins with annotated Big domains. Among all the plasmids analyzed, proteins
243 with these domains were also found in the plasmids of the IncA/C and IncP2 incompatibility
244 groups. Big proteins from IncA/C plasmids show a high degree of similarity (Fig. S2) and can be
245 mapped to the corresponding *tra* regions (Fig. S3 and Fig. S4). Big proteins from IncP2 plasmids
246 also show a very high degree of similarity (Fig. S5) and can be mapped close to a pilin gene (Fig.
247 S4).

248 **The ALG87338.1 gene product of the IncA/C plasmid pKAZ3 is required for plasmid conjugation**
249 **at low temperature.**

250 Upon having identified genes encoding proteins containing Big domains harbored on plasmids
251 different from those of the IncHI group, we also aimed to assess whether a protein containing a

252 Big domain encoded by a plasmid different from the IncHI group is also found in the secretome
253 and influences conjugation. For this study, we selected the IncA/C plasmid pKAZ3, encoding the
254 ALG87338.1 gene, the product of which contains a Big domain. The plasmid pKAZ3 was isolated
255 from an antibiotic-contaminated lake and confers multiple-antibiotic resistance [51]. The
256 ALG87338.1 gene product is an 1843 AA protein (200.75 kDa) that contains Big (3_2 and 3_3)
257 and DUF4165 domains (Fig. S6). Plasmid pKAZ3 was first transferred to strain SL1344, and the
258 secretomes of strains SL1344 and SL1344 (pKAZ3) were compared. A band corresponding to a
259 protein of a molecular mass corresponding to the ALG87388.1 gene product was identified (Fig.
260 9A). This was confirmed by LC-MS/MS analysis of the protein excised from the SDS-PAGE gel.
261 We next generated an ALG87388.1 knockout mutant derivative of plasmid pKAZ3. Considering
262 that expression of either the RSP or RSP2 protein reduces SL1344 cell motility, we also compared
263 the motility of strains SL1344, SL1344 (pKAZ3) and SL1344 (pKAZ3 ΔALG87388.1) (Fig. 9B). We
264 observed that, while the acquisition of the pKAZ3 plasmid reduced cellular motility, there were
265 no significant differences in motility between strains SL1344 (pKAZ3) and SL1344 (pKAZ3
266 ΔALG87388.1). We also compared the conjugation frequencies of strains SL1344 (pKAZ3) and
267 SL1344 (pKAZ3 ΔALG87388.1), at both 37°C and at 25°C (Fig. 9C). ΔALG87388.1 did not influence
268 the conjugation frequency at 37°C, but it had a very strong effect at 25°C.

269 **Discussion**

270 The conjugative process has been studied for decades, and a detailed picture of the molecular
271 mechanism of conjugational DNA transfer is available (as reviewed in [50,52,53]). Plasmids such
272 as the F factor, RP4, R388 or pTi have been studied as reference models to determine the
273 function of several Tra proteins in plasmid conjugation by using genetic and biochemical
274 approaches [50]. Comprehensive information is available on processes such as conjugative pilus
275 biosynthesis, the establishment of donor-recipient cell contact or the assembly and activity of
276 the relaxosome, which are key aspects of bacterial conjugation that must occur prior to the DNA
277 transfer process. Nevertheless, several key questions, such as those regarding the function of
278 several Tra proteins or the pilus´s ability to transport DNA between distant donor and recipient
279 cells, remain to be answered [50]. In this report, we have elaborated on the role of plasmid-
280 encoded high-molecular-weight Big proteins in the conjugation process of different plasmids.

281 In the IncHI plasmid R27, two genes that were mapped to the Tra2 and Tra1-Tra2 intergenic
282 regions (*rsp* and *rsp2*, respectively) [32,33], encode proteins containing Big domains that
283 participate in the conjugation process. Both the RSP and RSP2 proteins bind flagella, this
284 resulting in a reduced cell motility. Export of the RSP2 protein to the external medium appears

285 to be dependent upon RSP export. This may explain that, whereas loss of RSP function
286 completely compensates for the effect of R27 acquisition on bacterial cell motility, loss of RSP2
287 function only shows a partial effect on cell motility.

288 The relationship between the RSP and RSP2 proteins, and the conjugative machinery of the R27
289 plasmid is shown in this work. Expression of key elements required for the synthesis of the
290 conjugative pilus of incHI1 plasmids (TrhH protein) and of the pilin subunit itself (TrhA protein)
291 is required for the correct translocation of the RSP and RSP2 proteins to the external surface of
292 the cells. In addition, imaging of cells lacking flagella showed short filaments that likely
293 corresponded to the conjugative pilus. These filaments were targeted both by the RSP and RSP2
294 proteins. Hence, there is an interaction between the RSP and RSP2 proteins and the R27
295 conjugative pilus.

296 IncHI plasmids and *Salmonella* participate in regulatory crosstalk that, is based on the expression
297 of both the tetracycline determinant and other plasmid-encoded genes [54,55]. One of the
298 effects of the crosstalk is the alteration in cellular motility, which is mediated by both plasmid-
299 borne regulators [56] and the binding of the RSP and RSP2 proteins to the flagellar structure
300 ([47], this work). Nevertheless, these proteins also play a second and critical role in IncHI plasmid
301 conjugation. They also bind the conjugative pilus, which is likely required to facilitate
302 transmission of the conjugative plasmid. Plasmid-encoded Big proteins binding to the flagella
303 and thus reducing motility may favor cell-to-cell contact and hence conjugation. Binding to the
304 conjugative pilus may contribute to the stabilization of the pilus structure. The overall effect of
305 the expression of these proteins is to significantly favor the transfer of the plasmid that encode
306 them.

307 Genes encoding proteins containing Big domains are not restricted to IncHI plasmids. The
308 genomic analysis performed in this work has shown that such proteins are also present in IncA/C
309 and IncP2 plasmids. Both groups of plasmids have a wide host range. IncA/C plasmids are also
310 key players in the dissemination of AMR in *Enterobacteriaceae* and other Gram-negative
311 microorganisms. IncP2 plasmids are high-molecular-weight plasmids that are prevalent in
312 *Pseudomonas* and contribute to the dissemination of AMR within this genus [57,58]. As the
313 search that we performed had some limitations, it cannot be ruled out that other groups of
314 plasmids also express Big proteins. By using the genome viewer integrated into the NCBI
315 database, only those plasmids that were well-annotated and characterized were considered. In
316 addition, proteins that contain Big domains were annotated because of the confirmation that
317 this domain, was present, not because they shared amino acid sequence similarity. Notably, the

318 RSP2 protein is specific of a subgroup of IncHI plasmids, incHI1. IncHI2 plasmids that encode the
319 *rsp* gene lack the *rsp2* gene. Interestingly, our BLAST analysis also showed that the *rsp2* gene has
320 jumped to plasmids of a different Inc group, IncN. This may indicate that the *rsp2* genes are
321 spreading and may also influence conjugation in these plasmids.

322 The identified high-molecular-weight Big proteins encoded by IncA/C plasmids show a very high
323 degree of homology, which suggests that they play similar roles in these plasmids. The genes
324 encoding these proteins were also mapped to the *tra* region *(traV/A/W/F/N* genes) and, in some
325 instances, were adjacent to the pilin genes. A recent study focused on performing a
326 comprehensive analysis of IncC plasmid conjugation [59], and the gene encoding the identified
327 Big protein encoded by the pMS6198A plasmid (the product of the gene MS6198_A094) was
328 considered not to influence conjugation, despite mapping within the Tra1 region, between the
329 *dsbC*A and *traL* genes. The likely reason for this was that the conjugation experiments were
330 performed only at 37°C. We showed here that the ALG87338.1 gene product of the IncA/C
331 plasmid pKAZ3 positively influenced conjugation at 25°C (i.e., at environmental temperature).
332 IncHI and IncA/C plasmids belong to the same relaxase family (MOB_H). Interestingly, they also
333 share the requirement for a high-molecular-weight Big protein for efficient conjugation at
334 environmental temperatures. The identified Big proteins of IncP2 plasmids also show a high
335 degree of similarity. While the molecular mass of these proteins is lower than that of the Big
336 proteins of IncHI and IncA/C plasmids, they map close to a pilin, which also suggests that they
337 may play a role in the conjugative process.

338 Out of the IncHI plasmids, which have classically been characterized as being conjugative only
339 at temperatures below 30°C, plasmids harbored by *Enterobacteriaceae* have usually been
340 studied in cells growing at the optimal growth temperature for these microorganisms, that is,
341 37°C. Our study also highlights the importance of studying plasmid conjugation at other
342 temperatures, closer to those that most microorganisms encounter outside their warm-blooded
343 hosts. Indeed, AMR transmission occurs mainly in natural environments at temperatures that
344 rarely reach 37°C.

345 The relevance of the Big proteins studied in this work is not only their role as elements favoring
346 plasmid conjugation but also the fact that, as they are associated with extracellular appendages
347 of the bacterial cells, they can be targeted with specific antibodies either to restrict the
348 dissemination of the plasmids that encode them or, when expressed within the human body, to
349 control infections caused by bacteria that express the plasmid that encode these proteins.

350 **Materials and Methods**

351 **Bacterial strains, plasmids, and growth conditions.**

352 The bacterial strains (Table S3) were routinely grown in Luria-Bertani (LB) medium (10 g l^{-1} NaCl,
353 10 g l^{-1} tryptone and 5 g l^{-1} yeast extract) with vigorous shaking at 200 rpm (Innova 3100, New
354 Brunswick Scientific). The antibiotics used were chloramphenicol (Cm) (25 µg ml^{-1}), tetracycline
355 (Tc) (15 µg ml^{-1}), carbenicillin (Cb) (100 µg ml^{-1}) and kanamycin (Km) (50 µg ml^{-1}) (Sigma-Aldrich).

356 **Oligonucleotides.**

357 The oligonucleotides (from 5' to 3') used in this work are listed in Table S4.

358 **Genetic manipulation.**

359 All enzymes used to perform standard molecular and genetic procedures were used according
360 to the manufacturer's recommendations. To introduce plasmids into *E. coli* and *Salmonella*,
361 bacterial cells were grown until an $OD_{600\ nm}$ of 0.6. Cells were then washed several times with
362 10% glycerol, and the respective plasmids or DNA was electroporated by using an Eppendorf
363 gene pulser (Electroporator 2510).

364 Deletions of the *rsp2* (ORF *R0055), fliC, fljB, trhH* and *trhA* genes were performed in strain SL1344
365 (R27) by using the λ Red recombination method, as previously described [60]. The antibiotic
366 resistance determinant of the plasmids pKD3/pKD4 was amplified using the corresponding
367 oligonucleotides (P1/P2 series, see Table S4). The mutants were confirmed by PCR using the
368 corresponding oligonucleotides (P1up/P2down series, see Table S4). We used phage P22 HT for
369 combining mutations by transduction [61]. When necessary, the antibiotic resistance cassette
370 was eliminated using the FLP/FRT-mediated site-specific recombination method as previously
371 described [62].

372 For deletion of the ALG87338.1 gene from the pKAZ3 plasmid, we first constructed the plasmid
373 pKD46-Km^R following the strategy described in [63]. Briefly, kanamycin resistance from the pKD4
374 plasmid was amplified using the PkmXmnIFW/PkmXmnIRv oligonucleotides together with
375 Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific) following the
376 manufacturer's recommendations. The corresponding XmnI-flanked kanamycin resistance
377 fragment was cloned into the vector pKD46 previously digested with the same enzyme. The
378 resulting plasmid was termed pKD46-Km^R. Deletion of ALG87338.1 was performed in strain
379 SL1344 (pKAZ3) pKD46-Km^R by using the λ Red recombination method. The antibiotic resistance
380 determinant of the plasmid pKD3 was amplified using the corresponding oligonucleotides

381 (P1/P2 series, see Table S4). The mutants were confirmed by PCR using the corresponding
382 oligonucleotides (P1up/P2down series, see Table S4).

383 Recombinational transfer of the Flag sequence into the *rsp2* gene was achieved by following a
384 previously described methodology [64]. The template vector encoding Flag and Km^r used was
385 the pSUB11 plasmid. The primers used for construction of the Flag-tagged derivative were
386 R27_p0553XP1 and R27_p0553XP2 (Table S4). The correct insertion of the Flag-tag was
387 confirmed by PCR using oligonucleotides R27_p0553XP1UP and R27_p0553XP2DOWN (Table
388 S4).

389 To construct the plasmid pLG338-*rsp2*, the ORF *R0055* (*rsp2*) (GenBank accession number
390 AF250878.1, position 56639-59970) was amplified using the oligonucleotides R27_R55 pLG322
391 ECORI fw/R27_R55 pLG322 Bam rv (see Table S4 for the sequences) together with Phusion Hot
392 Start II High-Fidelity DNA Polymerase (Thermo Scientific) following the manufacturer's
393 recommendations. The corresponding EcoRI/BamHI fragment was cloned into the vector
394 pLG338-30 previously digested with the same enzymes. The resulting plasmids were Sanger
395 sequenced and termed pLG338-*rsp2*.

396 **Plasmid conjugation.**

397 The R27 and pKAZ3 plasmids were conjugated as described previously [47]. The mating
398 frequency was calculated as the number of transconjugants per donor cell. Student´s *t*-test was
399 used to determine statistical significance, and the values were obtained by using GraphPad
400 Prism 8 software. A *P* value of less than 0.05 was considered significant.

401 **Motility assays.**

402 The motility assay was performed as previously described [47]. The experiments were repeated
403 three times with three plates of each strain in each experiment. The colony diameter was
404 measured and plotted, and standard errors were calculated. Student´s t-test was used to
405 determine statistical significance, and the values were obtained by using the GraphPad Prism 8
406 software. A P value of less than 0.05 was considered significant.

407 **Flagellum Isolation.**

408 Flagellum isolation was prepared as previously described [47].

409 **Cell-free secreted proteins (secretome).**

410 Cell-free supernatants were prepared as previously described [47].

411 **Cell fractionation.**

412 Cell fractionation was performed as previously described [47].

413 **Immunogold electron microscopy.**

414 Immunogold microscopy experiments were performed as previously described [47].

415 **Electrophoresis and Western blotting analysis of proteins.**

416 Protein samples were analyzed by 10% and 12.5% SDS-PAGE [65]. Proteins were transferred
417 from the gels to PVDF membranes using the Trans-Blot Turbo system (Bio-Rad). Western blot
418 analysis was performed with a monoclonal antibody raised against the Flag-epitope (Sigma)
419 diluted 1:10.000 in a solution containing PBS, 0.2% Triton, and 3% skim milk and incubated for
420 16 hours at 4°C. The membranes were washed for 20 minutes each with PBS and 0.2% Triton
421 solution. The washing step was repeated three times. Thereafter, the membranes were
422 incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Invitrogen) diluted
423 1:5.000 in a solution containing PBS and 0.2% Triton for 1 hour at room temperature. Again,
424 three washing steps of 45 minutes with PBS and 0.2% Triton solution were performed, and
425 detection was performed by enhanced chemiluminescence using ImageQuant LAS 54000
426 imaging system software (GE Healthcare Lifesciences).

427 **RSP-Flag Immunoprecipitation.**

428 For RSP-Flag protein immunoprecipitation, the strains SL1344 (R27 RSP-Flag) and SL1344 (R27
429 Δ*rsp*, negative control) were grown in LB medium for 16 hours at 37ºC. One hundred milliliters
430 of fresh LB medium was inoculated 1:100 with both overnight-cultured strains and grown at
431 25°C until an $OD_{600\ nm}$ of 2.0 was reached. Then, the cells were centrifuged at 9.000 rpm for 30
432 minutes at 4°C, the pellets were discarded, and the supernatants were filtered through 0.22 µm
433 filters. For each immunoprecipitation protocol, we used 100 µl of Anti-Flag M2 Affinity Gel
434 (Sigma-Aldrich) and 100 ml of each supernatant and incubated the mixture under slow rotation
435 at 4°C for 16 hours. Each supernatant was loaded onto a Poly-Prep chromatography column (Bio-
436 Rad), and the flowthrough was stored at -20°C for further analysis. Each column was washed out
437 with 100 ml of washing buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl), and elution was
438 performed 3 times with 0.3 ml of elution buffer (0.1M glycine pH 3.5). Elution fractions were
439 concentrated using a trichloroacetic acid (TCA) precipitation protocol, briefly, 1 ml of the
440 samples was mixed with 0.5 ml of a 45% TCA solution (w/v). The samples were kept on ice for
441 30 minutes and centrifugated for 30 minutes at 13,400 rpm. The supernatants were carefully

442 discarded, acetone was added to the protein pellets, and the pellets were again centrifugated
443 for 30 minutes at 13,400 rpm. The supernatants were carefully discarded. The protein pellets
444 were dried and solubilized with 1x Laemmli Sample Buffer (Bio-Rad). Samples were boiled for 10
445 minutes and loaded onto a 12.5% SDS-PAGE gel. When the protein samples entered the stacking
446 phase, the gel run was stopped, and samples were excised from the gel and sent to the
447 Proteomic Platform (Barcelona Science Park, Barcelona, Spain) for protein identification, as
448 previously described [55]. Immunoprecipitation and protein identification experiments were
449 repeated twice.

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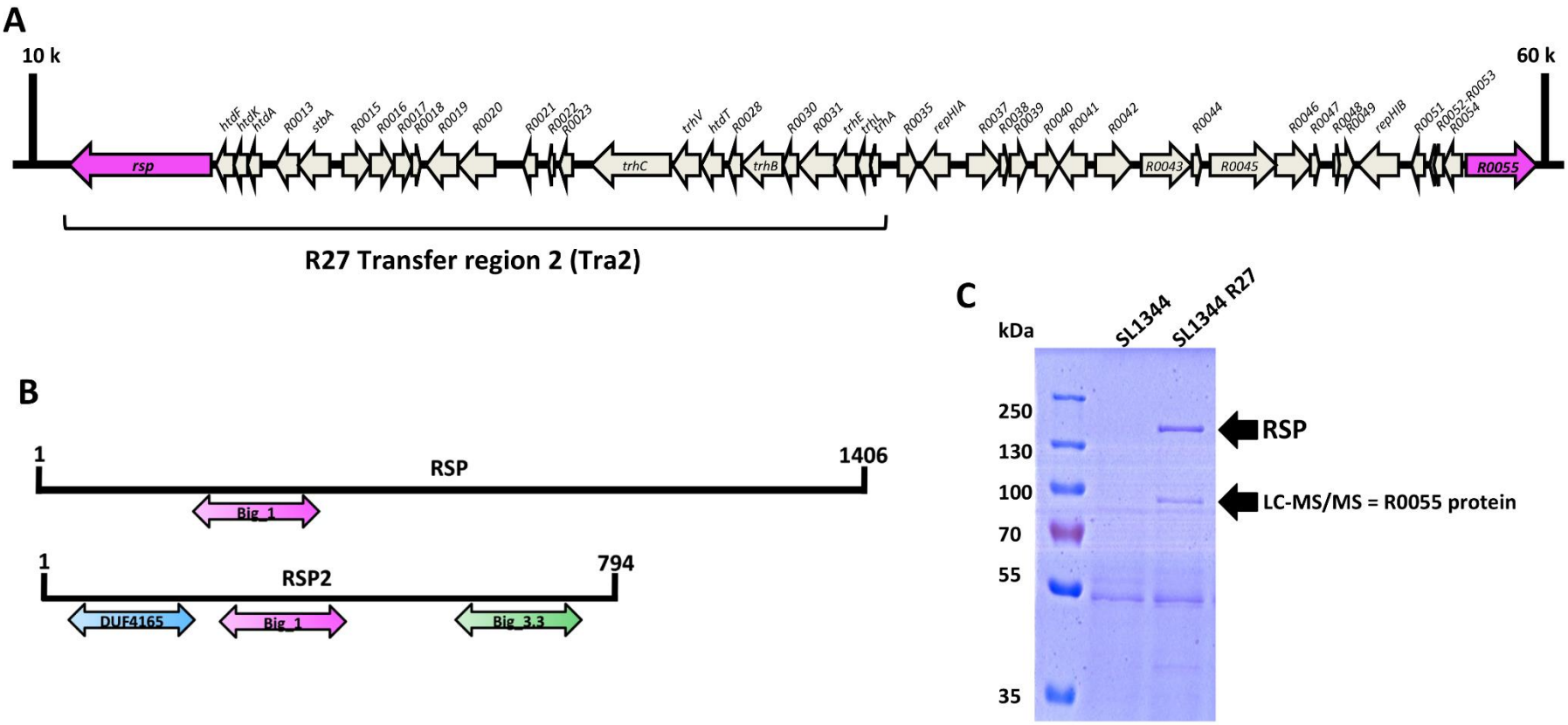
672 **Figures**



673

674 **Figure 1.** Identification of the RSP2 protein. (A) Genetic map of the R27 plasmid region where both the *rsp* and *rsp2* (*R0055*) genes were mapped. (B)
675 Comparison of the Big domains of the RSP and RSP2 proteins. (C) Detection of the RSP2 protein in the cell-free secretome of the SL1344 (R27) strain. Arrows
676 point to the bands corresponding to the RSP and RSP2 proteins, the latter of which was confirmed by LC-MS/MS analysis.

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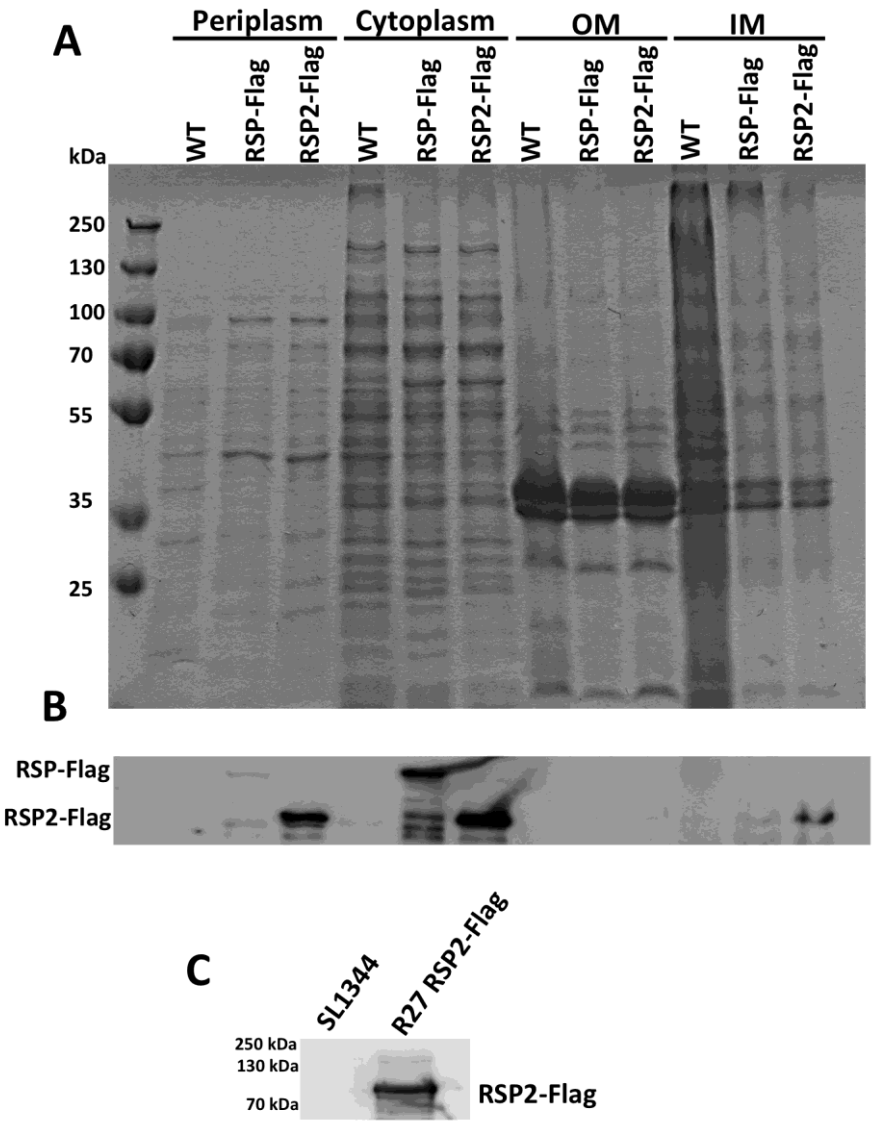

681

682 **Figure 2.** Immunodetection of the RSP2-Flag protein in different cellular compartments. (A)
683 Coomassie blue staining of the different cellular fractions obtained from strains SL11344 wt,
684 SL1344 (R27 RSP-Flag) and SL1344 (R27 RSP2-Flag). (B) Immunodetection of the RSP-Flag and
685 RSP2-Flag proteins in the periplasm, cytoplasm, and outer and inner membrane fractions of
686 strains SL1344 (R27 RSP-Flag) and SL1344 (R27 RSP2-Flag), respectively. (C) Immunodetection of
687 the RSP2-Flag protein in the cell-free secretome of strain SL1344 (R27 RSP2-Flag) grown at 25ºC
688 until $OD_{600\,nm}$ of 2.0.

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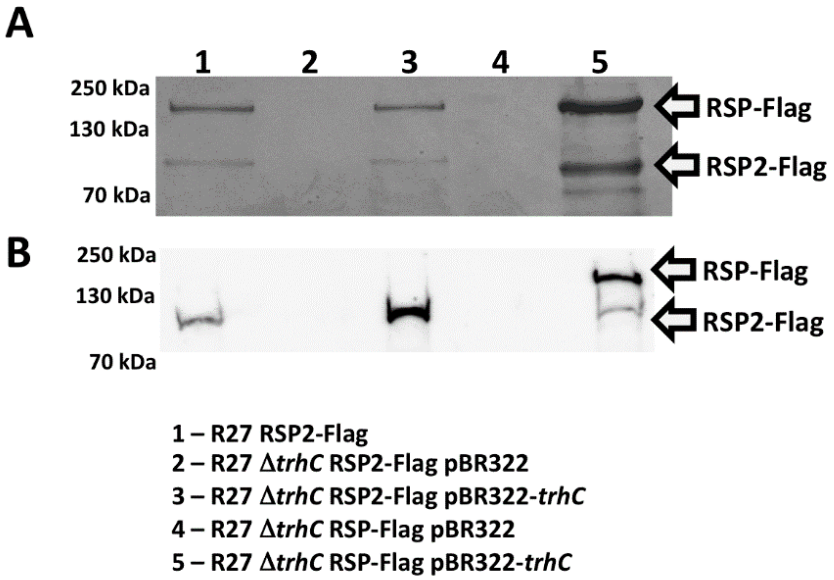

693

694 **Figure 3.** RSP2 export requires the type IV secretion system encoded by the R27 plasmid. (A)
695 Coomassie blue staining for SDS-PAGE analysis of the cell-free secretome of the strains SL1344
696 (R27 RSP2-Flag), SL1344 (R27 Δ*trhC* RSP2-Flag pBR322), SL1344 (R27 Δ*trhC* RSP2-Flag pBR322-
697 *trhC*), (R27 Δ*trhC* RSP-Flag pBR322) and (R27 Δ*trhC* RSP-Flag pBR322-*trhC*) grown at 25ºC until
698 $OD_{600\ nm}$ of 2.0. (B) Immunodetection of the RSP-Flag and RSP2-Flag proteins with anti-Flag
699 antibodies. Arrows point to the RSP-Flag and RSP2-Flag proteins. The experiment was repeated
700 three times. A representative experiment is shown.

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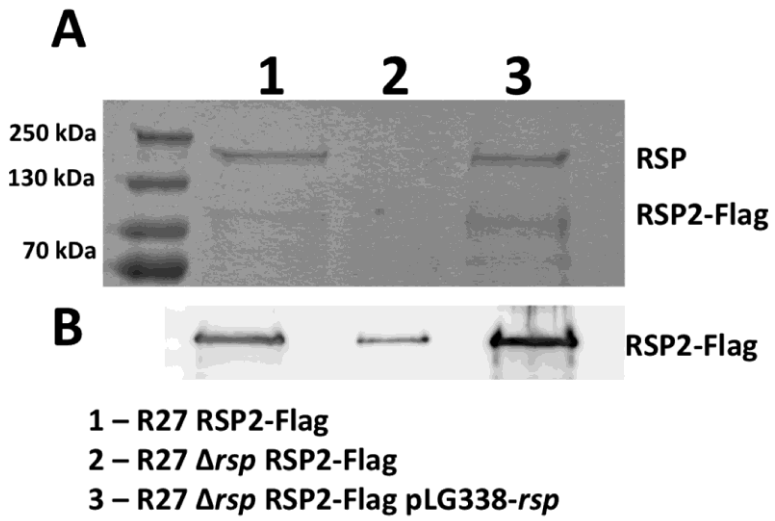

704

705 **Figure 4.** RSP2 export is dependent on the expression of the RSP protein. (A) Coomassie blue
706 staining for SDS-PAGE analysis of the cell-free secretomes of the strains SL1344 (R27 RSP2-Flag),
707 SL1344 (R27 Δ*rsp* RSP2-Flag) and SL1344 (R27 Δ*rsp* RSP2-Flag pLG338-*rsp*) grown at 25ºC until
708 $OD_{600\ nm}$ of 2.0. (B) Immunodetection of the RSP2-Flag protein with anti-Flag antibodies. RSP2-
709 Flag protein is indicated. The experiment was repeated three times. A representative
710 experiment is shown.

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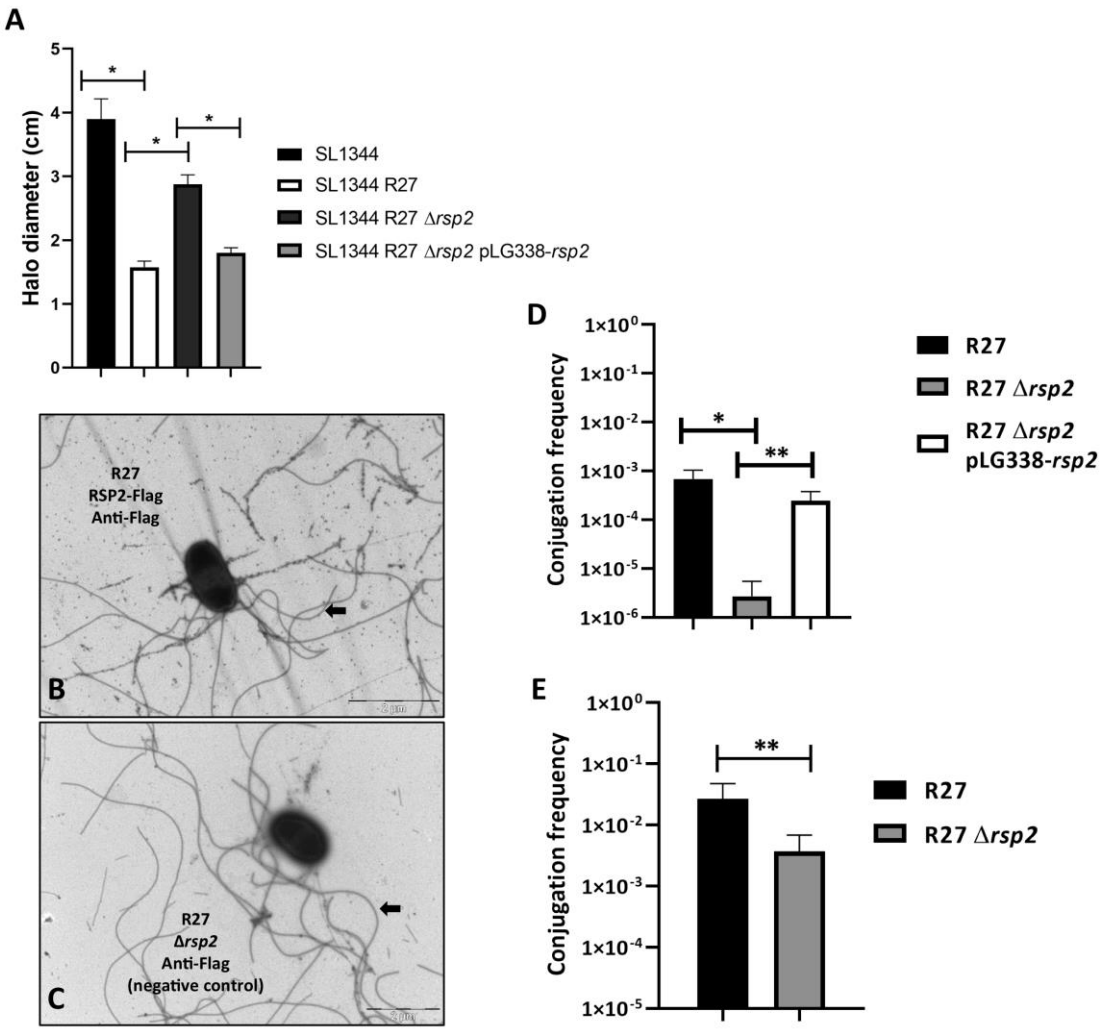

714

715 **Figure 5.** The RSP2 protein influences the motility of strain SL1344 (R27) and the conjugation of
716 the R27 plasmid. (A) Effect of the *rsp2* allele on the motility of strain SL1344 (R27). The results
717 are the means of three independent experiments. Standard deviations are shown. Statistical
718 analysis showed significant differences, analyzed by unpaired two-sided Student´s t-test (**P*-
719 value < 0.0001). (B and C) The RSP2 protein binds to the flagella of strain SL1344 (R27).
720 Immunogold electron microscopy of cells from strains SL1344 (R27 RSP2-Flag) (B) and SL1344
721 (R27 Δ*rsp2*) (C) using monoclonal anti-Flag antibodies and goat anti-mouse IgG conjugated to 12
722 nm gold particles. Arrows point to the RSP protein associated with the flagella in (B) and only to
723 the flagella in (C). Bars represent 2 µm. (D and E) Effect of the *rsp2* allele on the conjugation
724 frequency of the R27 plasmid in liquid (D) and solid (E) media, respectively. The data shown are
725 the means and standard deviations of three independent experiments. Statistical analysis
726 showed significant differences, analyzed by unpaired two-sided Student´s t-test (D) (**P*-value
727 0.0082, ***P*-value 0.0095) and (E) (***P*-value 0.0428).

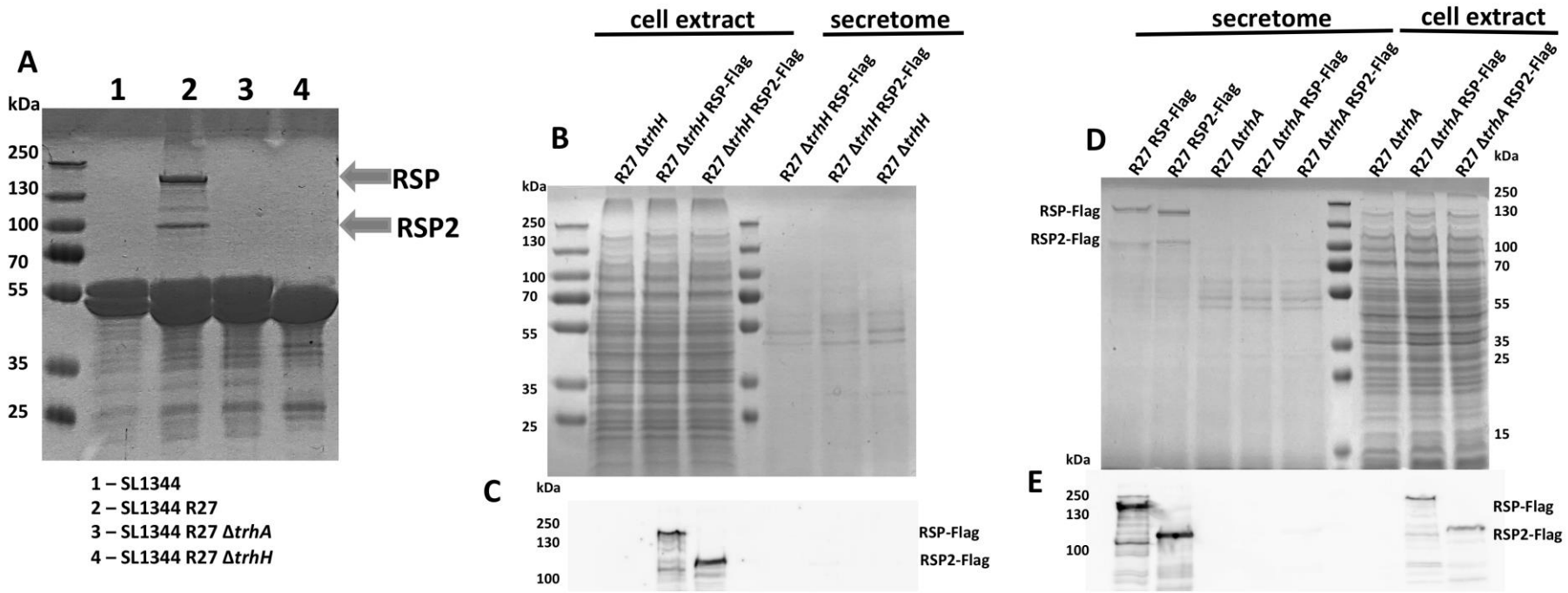

Figure 6. Expression of the RSP and RSP2 proteins depends on the R27 TrhA and TrhH functions. (A) SDS-PAGE analysis of the purified flagellar fractions of the plasmid-free strain SL1344 and strain SL1344 harboring (R27) and the (R27 Δ*trhA*) and (R27 Δ*trhH*) derivatives. (B) SDS-PAGE analysis of the cell extract and cell-free secretome of the strains SL1344 (R27 Δ*trhH*), SL1344 (R27 Δ*trhH* RSP-Flag) and SL1344 (R27 Δ*trhH* RSP2-Flag), respectively. (C) Immunodetection of RSP-Flag and RSP2-Flag in the intracellular compartments of strain SL1344 harboring the corresponding *trhH* derivatives of the R27 plasmid. (D) SDS-PAGE analysis of the cell extract and cell-free secretome of the strains SL1344 (R27 RSP-Flag), SL1344 (R27 RSP2-Flag), SL1344 (R27 Δ*trhA*), SL1344 (R27 Δ*trhA* RSP-

734 Flag) and SL1344 (R27 Δ*trhA* RSP2-Flag), respectively. (E) Immunodetection of RSP-Flag and RSP2-Flag in the intracellular compartments of strain SL1344
735 harboring the corresponding *trhA* derivatives of the R27 plasmid. The experiments were repeated three times. A representative experiment is shown.

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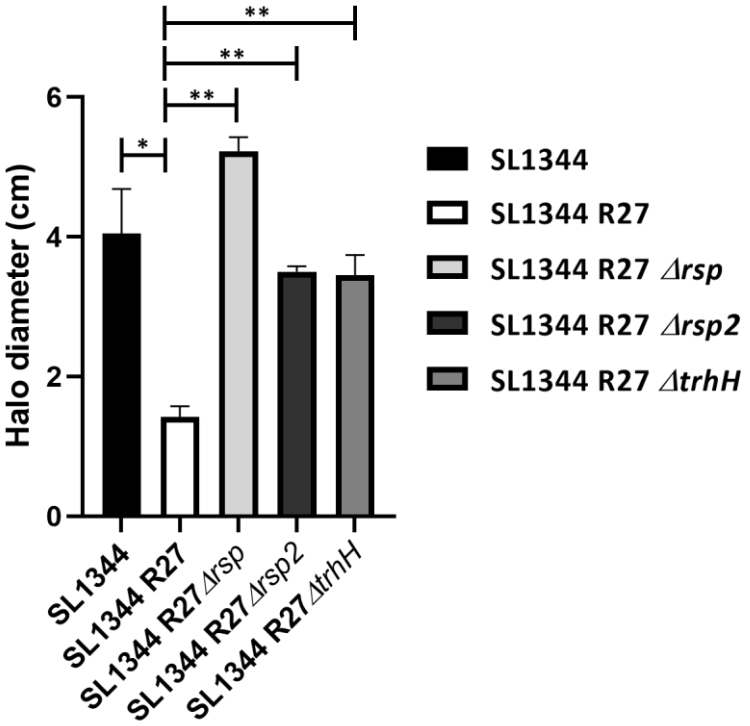

740

741 **Figure 7.** Loss of TrhH function impairs the effect of the RSP and RSP2 proteins on SL1344 cell
742 motility. The motility of the different strains was measured as the halo diameter of the different
743 colonies growing on motility agar. The results are the means of three independent experiments.
744 Standard deviations are shown. Statistical analysis showed a significant difference analyzed by
745 unpaired two-sided Student´s t-test (**P*-value 0.0009, ***P*-value <0.0001).

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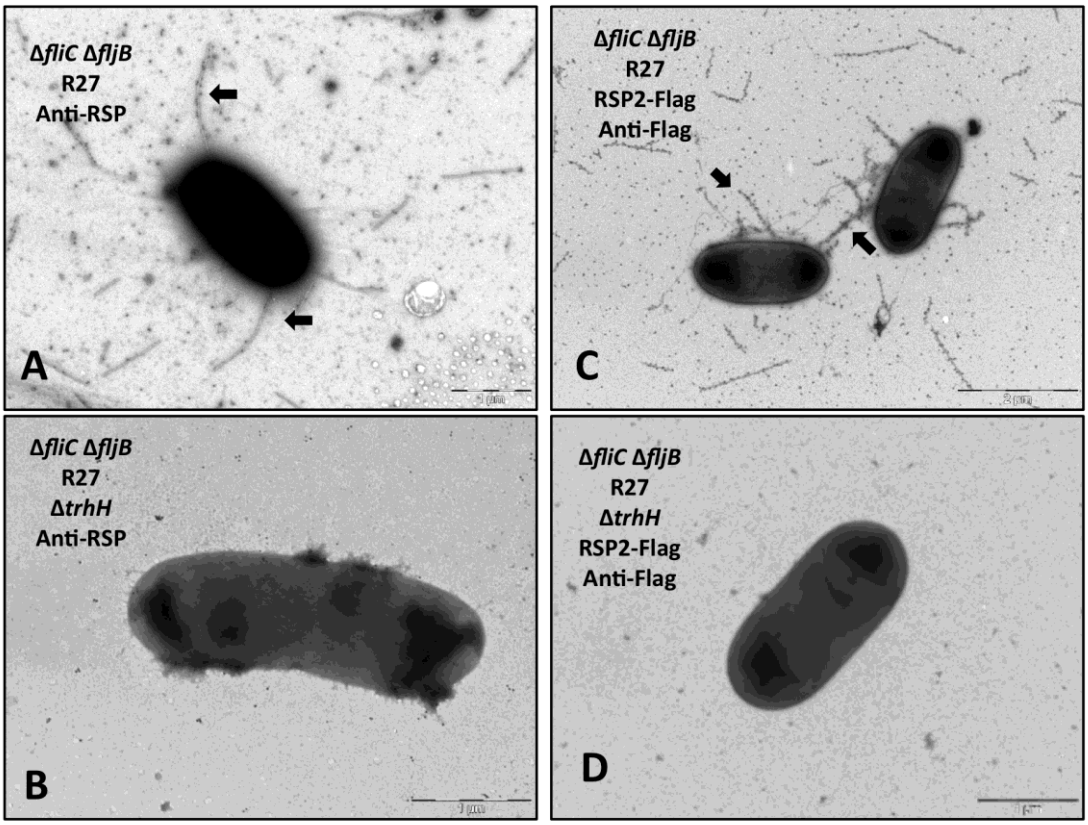

749

750 **Figure 8.** The RSP and RSP2 proteins bind to the conjugative pilus encoded by the R27 plasmid.
751 Immunogold electron microscopy of cells from strains SL1344 Δ*fliC* Δ*fljB* (R27) (A) and SL1344
752 Δ*fliC* Δ*fljB* (R27 Δ*trhH*) (B) using polyclonal anti-RSP antibodies and goat anti-rabbit IgG
753 conjugated to 12 nm gold particles. Arrows point to the RSP protein associated with the
754 conjugative pilus. Immunogold electron microscopy of cells from strains SL1344 Δ*fliC* Δ*fljB* (R27
755 RSP2-Flag) (C) and SL1344 Δ*fliC* Δ*fljB* (R27 RSP2-Flag Δ*trhH*) (D) using anti-Flag monoclonal
756 antibodies and goat anti-mouse IgG conjugated to 12 nm gold particles. Arrows point to the
757 RSP2 protein associated with the conjugative pilus. Bars represent 1 µm (in A, B and D) and 2
758 µm (in C).

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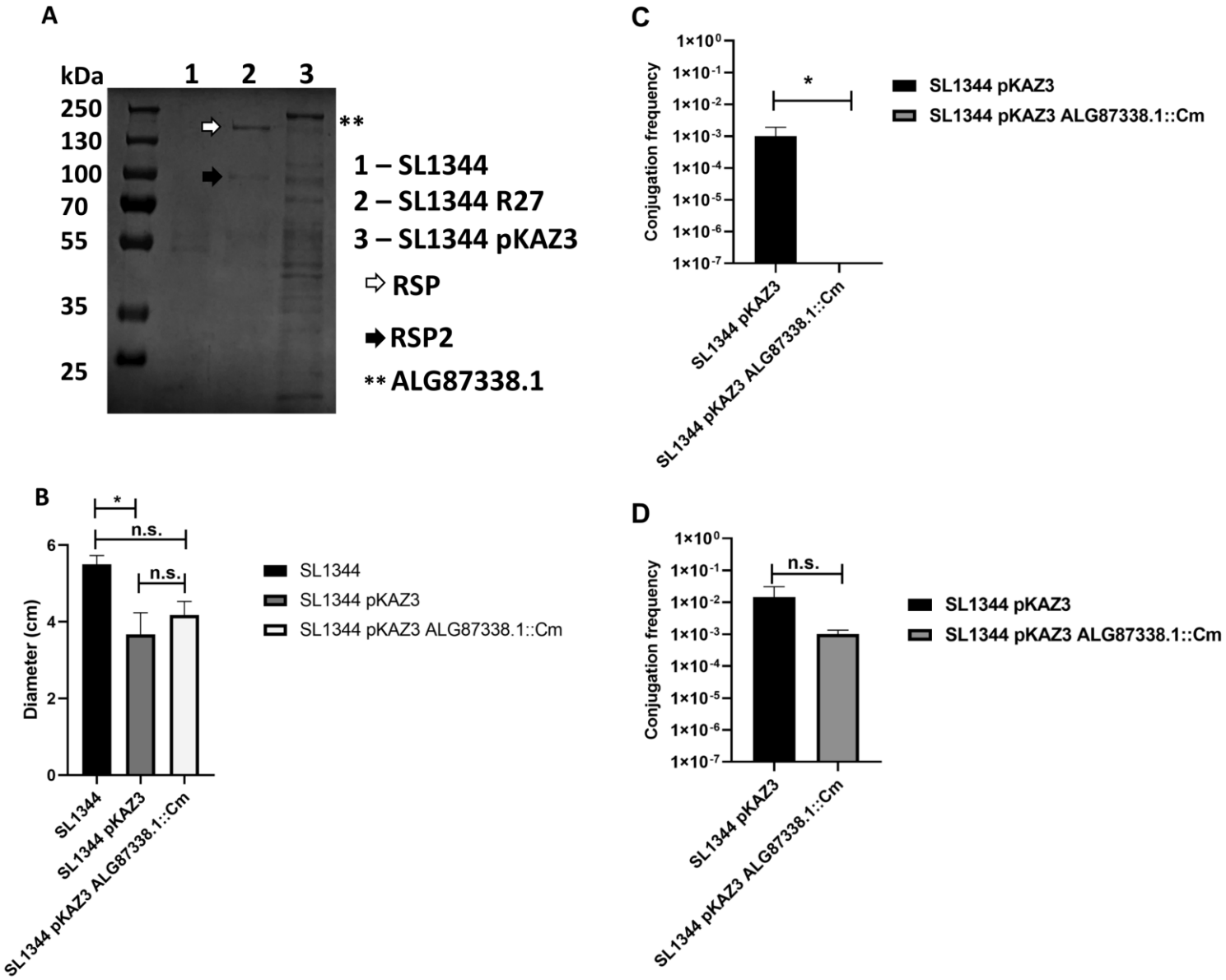
A
kDa
1
2
3
250
130
100
70
55
35
25
**
1 – SL1344
2 – SL1344 R27
3 – SL1344 pKAZ3
RSP
RSP2
** ALG87338.1
B
*
n.s.
n.s.
Diameter (cm)
6
4
2
0
SL1344
SL1344 pKAZ3
SL1344 pKAZ3 ALG87338.1::Cm
C
*
Conjugation frequency
1×10^0
1×10^-1
1×10^-2
1×10^-3
1×10^-4
1×10^-5
1×10^-6
1×10^-7
SL1344 pKAZ3
SL1344 pKAZ3 ALG87338.1::Cm
D
n.s.
Conjugation frequency
SL1344 pKAZ3
SL1344 pKAZ3 ALG87338.1::Cm

760

761 **Figure 9.** The ALG87388.1 gene product of the pKAZ3 plasmid influences conjugation at 25°C. (A) SDS-PAGE analysis of the cell-free secretomes of strains
762 SL1344, SL1344 (R27) and SL1344 (pKAZ3) grown at 25ºC until $OD_{600\ nm}$ of 2.0. Asterisks indicated the detection of the ALG87388.1 protein in the secretome
763 of strain SL1344 (pKAZ3) confirmed by LC-MS/MS. Arrows indicate the RSP and RSP2 protein. (B) Motility of the SL1344, SL1344 (pKAZ3) and SL1344 (pKAZ3
764 ΔALG87388.1) strains. The data shown are the means and standard deviations of three independent experiments. Statistical analysis showed significant
765 differences analyzed by unpaired two-sided Student´s t-test (**P*-value 0.0469; n.s. not significant). (C and D). Effect of the ΔALG87388.1 allele on the
766 conjugation frequency of the pKAZ3 plasmid at 25°C (C) and 37°C (D) in liquid media. The data shown are the means and standard deviations of three
767 independent experiments. Statistical analysis showed significant differences analyzed by unpaired two-sided Student´s t-test (**P*-value 0.042; n.s. not
768 significant).

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