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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 (by binding to flagella) and facilitating cell-to-cell contact (by binding 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. As they are located in 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 (Morens et al., 2004). The gradual increase in the resistance rates of several important bacterial pathogens represents a serious threat to public health (Meyer et al., 2010; Rossolini et al., 2007; Spellberg et al., 2008). 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 (Carattoli, 2013; Thomas & Nielsen, 2005; J. Wang et al., 2015), as well as in Gram-positive bacteria (Vrancianu et al., 2020). Plasmids can confer resistance to the major classes of antimicrobials (Carattoli, 2009).

For decades, plasmid incompatibility (Novick, 1987) 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

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conjugation, plasmids can be grouped into different relaxase families (Garcillán-Barcia et al., 2009). Plasmids belonging to the incompatibility group (Inc) HI (MOB_H relaxase family) are widespread in *Enterobacteriaceae* and most commonly include genetic elements encoding multiple AMR determinants (Phan & Wain, 2008). IncHI plasmids, often >200 kb in size, share a common core of approximately 160 kb. The differences in size are due to the presence of distinct insertion elements, including many AMR determinants (Gilmour et al., 2004). IncHI-encoded AMR can be present in enterobacterial such as *Salmonella, Escherichia coli* (Forde et al., 2018), *Klebsiella pneumoniae* (Villa et al., 2012) and *Citrobacter freundii* (Dolejska et al., 2013). Plasmids of the IncHI2 subgroup predominate in antibiotic-resistant *Salmonella* isolates. In *S.* Typhi, more than 40% of isolates harbor an IncHI plasmid (Holt et al., 2011). In recent years, a novel role of IncHI plasmids in AMR spread has been reported. The emergence of Gram-negative bacteria with AMR, especially those producing carbapenemases, led to reintroduction of colistin as a last resort antibiotic for the treatment of severe infections (Lim et al., 2010). In contrast to its limited clinical use, colistin is widely used in veterinary medicine (Catry et al., 2015). In the past, colistin resistance was associated with chromosomal mutations only (Olaitan et al., 2014). Nevertheless, plasmid-mediated resistance, conferred by the mobilized colistin resistance gene (*mcr-1*), has emerged recently. Since its discovery in 2016 in China (Liu et al., 2016), *mcr* genes, have been detected in animals, food, the human microbiota, and clinical samples in over thirty countries (Borowiak et al., 2017; Carattoli et al., 2017; Gao et al., 2016; Xavier et al., 2016; Yin et al., 2021). IncHI2 plasmids represent 20.5% of the overall plasmids encoding the *mcr-1* gene worldwide but up to 41% in Europe (Matamoros et al., 2017). Of special concern is the presence of the *mcr-1* resistance determinant in *Enterobacteriaceae* carrying carbapenem resistance genes, such as bla_{NDM} and bla_{KPC}. The combination of these AMR determinants seriously compromises the treatment of infections caused by pathogenic strains harboring these plasmids (Y. Wang et al., 2017; Zheng et al., 2016). An example of this is the recent report of an AMR clone of the highly virulent *E. coli* ST95 lineage (Forde et al., 2018). *E. coli* ST95 clones underlie neonatal meningitis and sepsis. They are usually sensitive to several antibiotics. This clone harbors an IncHI2 plasmid that encodes, among other factors, determinants of resistance to colistin and multiple other antibiotics (including the extended-spectrum beta-lactamase *blaCTX-M-1*). The spread of such an AMR ST95 clone could pose a threat to human health worldwide (Forde et al., 2018).

IncHI plasmid conjugation has a distinctive feature: while optimal conjugation rates are obtained at temperatures found outside the host (30°C and below), conjugative transfer is repressed at temperatures encountered within the host (37°C) (Alonso et al., 2005; Maher et al., 1993). The plasmid R27 is the prototype of IncHI1 plasmids. It harbors the Tn*10* transposon, which confers

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resistance to Tc, and has been exhaustively studied. The R27 replication and conjugation determinants are well characterized (Lawley et al., 2002, 2003), and its complete nucleotide sequence is available (Sherburne et al., 2000). Several ORFs from the plasmid R27 (66%) do not show similarity to any known ORFs.

IncA/C plasmids belong to the same MOB_H relaxase family as IncHI plasmids. They were originally identified in the 1970s among multidrug-resistant *Aeromonas hydrophila* and *Vibrio* spp. isolates that infected cultured fish (Aoki et al., 1971; Watanabe et al., 1971). Since the 1990s, these plasmids have received increasing interest because of their role in mobilizing AMR in enterobacteria and other Gram-negative microorganisms (Call et al., 2010; Fernández-Alarcón et al., 2011; Fricke et al., 2009; Welch et al., 2007). They have an extremely broad host range that includes members of Beta-, Gamma- and Deltaproteobacteria (Suzuki et al., 2010) and play a relevant role in the global spread of AMR (Eda et al., 2020; Rozwandowicz et al., 2018). They represented 50% of all plasmids isolated from bla_{NDM}-producing *Klebsiella pneumoniae* of clinical origin characterized in a recent study (Qamar et al., 2021).

Proteins containing an immunoglobulin (Ig)-like domain contain several chains of approximately 70-100 amino acid residues present in antiparallel β-strands and organized in two β-sheets that are packed against each other in a β-sandwich. The Ig-like domain has been identified in a large number of proteins with diverse biological functions, is widely distributed in nature, and is present in vertebrates, invertebrates, plants, fungi, parasites, bacteria, and viruses (Halaby & Mornon, 1998). Bacterial proteins containing Ig-like domains (Big) exhibit a wide range of functions. They include fimbrial subunits, adhesins, membrane transporters and several enzymes (as reviewed in (Bodelón et al., 2013)). In a previous report, we studied a high-molecular-weight extracellular protein (the RSP protein) that contains a Big domain and plays an essential role in IncHI plasmids conjugation. Among other targets, the RSP protein appears to be associated with flagella, reducing cell motility. Under specific mating conditions, it could be shown that binding of the RSP protein to the flagella influences conjugation (Mário Hüttener et al., 2019). In this report, we present novel data about the roles of these plasmid-encoded Big proteins. We show that two Big proteins bind both flagella and the conjugative pilus to favor conjugation of the IncHI1 plasmid R27. Furthermore, we also show that other groups of plasmids such as IncP2 and IncA/C also encode these proteins and provide evidence for their role in the conjugation of IncA/C plasmids. The role of plasmid-encoded Big proteins in plasmid conjugation is discussed.

Results

The RSP protein interacts with the *R0055* gene product from the plasmid R27.

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To better understand the role of the RSP protein in the conjugation of the R27 plasmid, we decided to assess whether this protein interacts with other proteins expressed by the *Salmonella* strain SL1344 (R27). We performed immunoprecipitation of a cellular extract of strains SL1344 (R27 RSP-Flag) and SL1344 (R27 Δ*rsp*) and analyzed the proteins that specifically coprecipitated with the RSP protein. Two R27-encoded proteins were found to specifically coprecipitate with the RSP protein (Table S1). The protein showing the highest score (187.77) and coverage (58.56) was the R27_p055 protein. The *R0055* gene was mapped between transfer regions 2 and 1 of the R27 plasmid (Fig. 1A). The *R0055* gene product is a 794 AA protein with a molecular mass of 86.75 kDa. As with the RSP protein, the protein encoded by *R0055* also contains bacterial Ig-like domains (Big_1 and _3): a Big_1 domain spanning amino acid residues 143 to 254, and a Big_3 domain spanning residues 537 to 693. The R27_p055 protein, herein termed RSP2, also contains a DUF4165 domain of unknown function (amino acids 23 to 142) (Fig. 1B). To determine whether this protein is also present in the secretome of strain SL1344 (R27), we analyzed the cell-free secreted protein profile of this strain by SDS-PAGE (Fig. 1C). In addition to the band corresponding to the already characterized RSP protein, a second band corresponding to a protein of a molecular mass equivalent to that of the RSP2 protein, was observed. Upon isolating the band and further analysis by LC-MS/MS, the band was confirmed to correspond to the RSP2 protein.

The second protein showing a high score (59.46) and coverage (39.92) was the R27-encoded TrhH protein. This protein shares 26% of identity with the IncF TraH protein, which is involved in conjugation (Lawley et al., 2002).

Immunodetection of the protein RSP2 in the different cellular compartments of the *Salmonella* SL1344 (R27) strain.

To identify the RSP2 protein in the different cellular compartments, a Flag-tag was added to the *rsp2* gene (see Materials and Methods section for details). Cultures of strains SL1344 wt and SL1344 (R27 RSP2-Flag) were grown in LB medium at 25°C to an OD_{600nm} of 2.0. Samples were then collected, and the different cellular fractions were obtained. The RSP2 protein was detected in the different fractions by Western blotting, using anti-Flag-specific antibodies (Fig. 2A-C). The protein was identified in the same cell compartments as the RSP protein (i.-e., periplasm, inner membrane, cytoplasm, and cell-free secreted proteins), suggesting that it can be translocated to the outer surface of the cell.

The *trhC* gene from the R27-encoded type IV secretion system is required for RSP2 export.

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As the above reported data show that the RSP2 protein can be exported to the external medium, we decided to study whether this protein is exported by the R27-encoded type IV secretion system that is also used by RSP (Mário Hüttener et al., 2019). To address this point, we first used the SSPred program (http://www.bioinformatics.org/sspred/html/sspred.html) for *in silico* prediction of whether the RSP2 protein, similar to the RSP protein, could be exported through a type IV secretion system (Fig S1). To provide evidence supporting this hypothesis, we used strains SL1344 (R27 RSP2-Flag) and SL1344 (R27 Δ*trhC* RSP2-Flag) and analyzed the presence of RSP2 protein in the protein profile of the cell-free secreted fractions (secretome). This protein could not be detected in the secretome of strain SL1344 (R27 Δ*trhC*) (Fig. 3A). Immunodetection of RSP2-Flag by using anti-Flag specific antibodies confirmed the requirement for TrhC expression for RSP2 export (Fig. 3B). We next checked whether RSP2 export in strain SL1344 (R27 Δ*trhC*) could be made to occur by providing the gene encoding the TrhC ATPase *in trans*, cloned in the plasmid pBR322 (plasmid pBR322-*trhC*). Complementation of RSP2 export was observed (Fig. 3B), suggesting that, as was the case for the RSP protein, the R27-encoded type IV secretion system mediates export of the RSP2 protein.

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

We previously showed that the expression of the RSP protein is essential for R27 plasmid conjugation, and that SL1344 cells that express RSP show reduced motility compared to plasmid-free cells (Mário Hüttener et al., 2019). Considering the observed interaction of both RSP and RSP2 proteins, we studied whether, as is the case for the RSP protein, expression of the RSP2 protein influences cell motility and/or conjugation.

After constructing an R27 derivative lacking the *rsp2* gene (plasmid R27 Δ*rsp2*), we performed a comparative motility assay with the *Salmonella* strain SL1344 and its derivatives harboring the R27, R27 Δ*rsp2*, and R27 Δ*rsp2* pLG338-*rsp2* plasmids. The results obtained (Fig.4A) showed that the RSP2 protein influences the motility of strain SL1344 (R27).

Then, after constructing an R27 derivative lacking the *rsp2* gene (plasmid R27 Δ*rsp2*), we compared the conjugation frequencies of strains SL1344 (R27), SL1344 (R27 Δ*rsp2*) and SL1344 (R27 Δ*rsp2* pLG338-*rsp2*) growing at 25°C in liquid media. We also compared the conjugation frequencies of strains SL1344 (R27) and SL1344 (R27 Δ*rsp2*) growing cells on nitrocellulose filters placed on LB plates. When cells were grown in liquid medium, transfer of the R27 Δ*rsp2* plasmid was detected at a frequency that was approximately two logs lower than that of wt R27 (Fig. 4B). The presence *in trans* of the RSP2 protein encoded by the pLG338-*rsp2* plasmid, restored

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the conjugation frequency of the wt R27 plasmid. When cells were grown on solid medium, we also detected a significant decrease in the conjugation frequency of the R27 Δ*rsp2* plasmid compared to that of the wt R27 plasmid (Fig. 4C).

Regulation of the expression of the RSP2 protein: the temperature and growth medium influence RSP2 transcription.

Considering that the RSP2 is involved in R27 conjugation, we also studied whether temperature influences RSP2 expression. To that end, we constructed an *rsp2::lacZ* transcriptional fusion and measured *rsp2* transcription at 25°C and 37°C in both rich (LB) and minimal (M9) media. In accordance with the observed effect of temperature on IncHI plasmids conjugation, low temperature influenced *rsp2* transcription, and this occurred in both culture media used (Fig. 5A and 5B). Nevertheless, transcription of the *rsp2* gene also occurred at significant levels at 37°C, especially when cells were grown in M9 medium (Fig. 5B). Notably, the growth phase also influenced RSP2 expression. In accordance with the observed effect of temperature on *rsp2* transcription, RSP2-Flag expression in the secretome was also higher at 25°C than at 37°C (Fig. 5C).

Expression of the RSP and RSP2 proteins depends on the functions of TrhH and TrhA.

As mentioned above, immunoprecipitation of the RSP protein indicated interactions with both the RSP protein and the R27 *trhH* gene product. The TrhH protein of the plasmid R27 shares identity with the TraH protein encoded by IncF plasmids (Lawley et al., 2002). The TraH protein is a component of the outer membrane complex involved in conjugation (Arutyunov et al., 2010) and has been shown to be required for pilus assembly (Frost et al., 1994). On the other hand, the *trhA* gene product encoded in IncHI plasmids has been considered to be the pilin subunit itself (Virolle et al., 2020). We decided to analyze whether the expression of the R27 TrhH and/or TrhA proteins influences the expression of the RSP and RSP2 proteins. After inactivation of the *trhH* or *trhA* genes of strain SL1344 (R27), the expression of the RSP and RSP2 proteins in the external medium was analyzed. Both the RSP and RSP2 proteins were copurified with flagella when a conventional flagella purification protocol was used (see the Material and Methods section). We obtained fractions containing flagella from the SL1344 wt, SL1344 (R27), SL1344 (R27 Δ*trhA*) and SL1344 (R27 Δ*trhH*) isogenic derivatives. Proteins were analyzed by SDS-PAGE (Fig. 6A). The expression of both the RSP and RSP2 proteins in the extracellular medium is dependent upon the function of TrhH or TrhA. We also analyzed the intracellular expression of the RSP and RSP2 proteins in these genetic backgrounds (Fig. 6B and 6C). Lack of either TrhH or TrhA function resulted in intracellular accumulation of either RSP or RSP2. Considering that

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expression of the RSP and RSP2 proteins reduces cell motility in strain SL1344 (R27) and that inactivation of the *trhH* gene interferes with the presence of these proteins in the extracellular medium, it could be expected that *trhH* inactivation would also result in increased motility in SL1344 cells harboring plasmid R27 Δ*trhH*. We then performed a mobility assay of the plasmid-free SL1344 strain and of its SL1344 (R27) SL1344 (R27 Δ*rsp*), SL1344 (R27 Δ*rsp2*) and SL1344 (R27 Δ*trhH*) derivatives. In accordance with (i) the observed requirement of TrhH function for RSP and RSP2 export and (ii) the effect of the RSP/RSP2 proteins on cell motility, the motility of strain SL1344 (R27 Δ*trhH*) was significantly reduced compared to that of the wt strain (Fig. 7).

Targeting of flagella and conjugative pili by the RSP and RSP2 proteins.

We were able to show previously that the RSP protein is associated with the flagella synthesized by the *Salmonella* strain SL1344 (Mário Hüttener et al., 2019). We decided next to assess whether RSP2 also targets the flagella. In an attempt to detect the RSP2 protein by transmission electron microscopy, we used strain SL1344 (R27 RSP2-Flag) and gold-labeled anti-Flag monoclonal antibodies for RSP2 detection. The RSP2 protein could be easily detected with gold-labeled anti-Flag monoclonal antibodies (Fig. 8A and 8B). As was the case for RSP, RSP2 also exhibited binding with the flagella.

We decided next to block flagellum expression and try to detect either the RSP or RSP2 protein bound to the conjugative pili. To prevent flagellum expression, we constructed Δ*fliC/fljB* (flagellin subunit) derivatives of strains SL1344 (R27) and SL1344 (R27 RSP2-Flag). Immunogold transmission electron microscopy imaging of these strains by using either polyclonal anti-RSP antibodies (Mário Hüttener et al., 2019) or monoclonal anti-Flag antibodies showed that in both examples, gold particles were associated with tubular structures that likely corresponded to the conjugative pilus (Fig. 9 A and 9B). When the Δ*trhH* allele was introduced into the mutant derivatives lacking flagella, the tubular structures were no longer detected, thus supporting the hypothesis that they corresponded to the conjugative pilus (Fig. 9C and 9D).

The RSP2 protein is specific for IncHI1 plasmids.

The RSP protein is restricted to the IncHI plasmids, from both the IncHI1 and IncHI2 subgroups (Mário Hüttener et al., 2019). Upon having shown the relevant role of the RSP2 protein in the conjugation of the IncHI1 plasmid R27, we performed a BLAST search to identify the *rsp2* gene in other bacterial plasmids (Table S2). In contrast to the RSP protein, the RSP2 protein is present in IncHI1 plasmids but not in IncHI2 plasmids. Interestingly, a group of IncN plasmids also contains an homolog of the R27 *rsp2* gene.

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Distribution of proteins containing the Big domain among bacterial plasmids.

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

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

Upon having identified genes encoding proteins containing Big domains harbored on plasmids different from those of the IncHI group, we also aimed to assess whether a protein containing a Big domain encoded by a plasmid different from the IncHI group is also found in the secretome and influences conjugation. For this study, we selected the IncA/C plasmid pKAZ3, encoding the ALG87338.1 gene, the product of which contains a Big domain. The plasmid pKAZ3 was isolated from an antibiotic-contaminated lake and confers multiple-antibiotic resistance (Flach et al., 2015). The ALG87338.1 gene product is an 1843 AA protein (200.75 kDa) that contains Big (3_2 and 3_3) and DUF4165 domains (Fig. S6). Plasmid pKAZ3 was first transferred to strain SL1344, and the secretomes of strains SL1344 and SL1344 (pKAZ3) were compared. A band corresponding to a protein of a molecular mass corresponding to the ALG87388.1 gene product was identified (Fig. 10A). This was confirmed by LC-MS/MS analysis of the protein excised from the SDS-PAGE gel. We next generated an ALG87388.1 knockout mutant derivative of plasmid pKAZ3. Considering that expression of either the RSP or RSP2 protein reduces SL1344 cell motility, we also compared the motility of strains SL1344, SL1344 (pKAZ3) and SL1344 (pKAZ3 ΔALG87388.1) (Fig. 10B). We observed that, while the acquisition of the pKAZ3 plasmid reduced cellular motility, there were no significant differences in motility between strains SL1344 (pKAZ3) and SL1344 (pKAZ3 ΔALG87388.1). We also compared the conjugation frequencies of strains SL1344 (pKAZ3) and SL1344 (pKAZ3 ΔALG87388.1), at both 37°C and at 25°C (Fig. 10 C).

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ΔALG87388.1 did not influence the conjugation frequency at 37°C, but it had a very strong effect at 25°C.

Discussion

The conjugative process has been studied for decades, and a detailed picture of the molecular mechanism of conjugational DNA transfer is available (as reviewed in (Cabezón et al., 2015; Smillie et al., 2010; Virolle et al., 2020)). Plasmids such as the F factor, RP4, R388 or pTi have been studied as reference models to determine the function of several Tra proteins in plasmid conjugation by using genetic and biochemical approaches (Virolle et al., 2020). Comprehensive information is available on processes such as conjugative pilus biosynthesis, the establishment of donor-recipient cell contact or the assembly and activity of the relaxosome, which are key aspects of bacterial conjugation that must occur prior to the DNA transfer process. Nevertheless, several key questions, such as those regarding the function of several Tra proteins or the pilus´s ability to transport DNA between distant donor and recipient cells, remain to be answered (Virolle et al., 2020). In this report, we have elaborated on the role of novel plasmid-encoded high-molecular-weight proteins in the conjugation process of different plasmids. In the IncHI plasmid R27, two genes that were mapped to the Tra2 and Tra1-Tra2 intergenic regions (*rsp* and *rsp2*, respectively) (Lawley et al., 2002, 2003), encode proteins containing Big domains that participate in the conjugation process. In a previous report, we showed that the R27-encoded RSP protein binds flagella and influences plasmid conjugation (Mário Hüttener et al., 2019). In the present report, the identification of the RSP2 protein as another protein present in the cell-free secreted protein profile (secretome) of *Salmonella* cells harboring the R27 plasmid enabled us to obtain a more accurate picture of the role of these proteins in the conjugation of IncHI plasmids. Similar to the RSP protein, the RSP2 protein also targets flagella and influences the motility and conjugation frequency of strains harboring the R27 plasmid. The RSP2 protein also seems to require the TrhC ATPase to be exported outside the cell. The fact that both the RSP and RSP2 interact *in vitro* suggests that they form multiprotein complexes in the cellular organelles that they target.

The genetic analysis that we performed in this work to better understand the interaction of both the RSP and RSP2 proteins with flagella has led to the finding of relevant information regarding the relationship between these proteins and the IncHI plasmid conjugative machinery. Expression of key elements required for conjugative pilus synthesis (TrhH protein) and of the pilin subunit itself (TrhA protein) are required for the correct translocation of the RSP and RSP2 proteins to the external surface of the cells. In addition, imaging of cells lacking flagella showed

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short filaments that likely corresponded to the conjugative pilus. These filaments were targeted both by the RSP and RSP2 proteins. Hence, there is an interaction between the RSP and RSP2 proteins and the R27 conjugative pilus.

IncHI plasmids and *Salmonella* participate in regulatory crosstalk that, is based on the expression of both the tetracycline determinant and other plasmid-encoded genes (M. Hüttener et al., 2018; Paytubi et al., 2014). One of the effects of the crosstalk is the alteration in cellular motility, which is mediated by both plasmid-borne regulators (Luque et al., 2019) and the binding of the RSP and RSP2 proteins to the flagellar structure ((Mário Hüttener et al., 2019), this work). Nevertheless, these proteins also play a second and critical role in IncHI plasmid conjugation. They also bind the conjugative pilus, which is likely required to facilitate transmission of the conjugative plasmid. Plasmid-encoded Big proteins binding to the flagella and thus reducing motility may favor cell-to-cell contact and hence conjugation. Binding to the conjugative pilus may contribute to the stabilization of the pilus structure. The overall effect of the expression of these proteins is to significantly favor the transfer of the plasmid that encode them.

Genes encoding proteins containing Big domains are not restricted to IncHI plasmids. The genomic analysis performed in this work has shown that such proteins are also present in IncA/C and IncP2 plasmids. Both groups of plasmids have a wide host range. IncA/C plasmids are also key players in the dissemination of AMR in *Enterobacteriaceae* and other Gram-negative microorganisms. IncP2 plasmids are high-molecular-weight plasmids that are prevalent in *Pseudomonas* and contribute to the dissemination of AMR within this genus (Cazares et al., 2020; Jacoby et al., 1983). As the search that we performed had some limitations, it cannot be ruled out that other groups of plasmids also express Big proteins. By using the genome viewer integrated into the NCBI database, only those plasmids that were well-annotated and characterized were considered. In addition, proteins that contain Big domains were annotated because of the confirmation that this domain, was present, not because they shared amino acid sequence similarity. Notably, the RSP2 protein is specific of a subgroup of IncHI plasmids, incHI1. IncHI2 plasmids that encode the *rsp* gene lack the *rsp2* gene. Interestingly, our BLAST analysis also showed that the *rsp2* gene has jumped to plasmids of a different Inc group, IncN. This may indicate that the *rsp2* genes are spreading and may also influence conjugation in these plasmids.

The identified high-molecular-weight Big proteins encoded by IncA/C plasmids show a very high degree of homology, which suggests that they play similar roles in these plasmids. The genes encoding these proteins were also mapped to the *tra* region *(traV/A/W/F/N* genes) and, in some instances, were adjacent to the pilin genes. A recent study focused on performing a

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comprehensive analysis of IncC plasmid conjugation (Hancock et al., 2020), and the gene encoding the identified Big protein encoded by the pMS6198A plasmid (the product of the gene MS6198_A094) was considered not to influence conjugation, despite mapping within the Tra1 region, between the *dsbC*A and *traL* genes. The likely reason for this was that the conjugation experiments were performed only at 37°C. We showed here that the ALG87338.1 gene product of the IncA/C plasmid pKAZ3 positively influenced conjugation at 25°C (i.e., at environmental temperature). IncHI and IncA/C plasmids belong to the same relaxase family (MOB_H). Interestingly, they also share the requirement for a high-molecular-weight Big protein for efficient conjugation at environmental temperatures. The identified Big proteins of IncP2 plasmids also show a high degree of similarity. While the molecular mass of these proteins is lower than that of the Big proteins of IncHI and IncA/C plasmids, they map close to a pilin, which also suggests that they may play a role in the conjugative process.

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

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

Materials and Methods

Bacterial strains, plasmids, and growth conditions.

The bacterial strains were routinely grown in Luria-Bertani (LB) medium (10 g l^{-1} NaCl, 10 g l^{-1} tryptone and 5 g l^{-1} yeast extract) or M9 minimal medium (Miller, 1992) supplemented with glucose at a final concentration of 0.4% (as indicated in the text) with vigorous shaking at 200 rpm (Innova 3100, New Brunswick Scientific). The antibiotics used were chloramphenicol (Cm) (25 µg ml^{-1}), tetracycline (Tc) (15 µg ml^{-1}), carbenicillin (Cb) (100 µg ml^{-1}) and kanamycin (Km) (50 µg ml^{-1}) (Sigma-Aldrich).

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Oligonucleotides.

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

Genetic manipulation.

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

Deletions of the *rsp2* (ORF *R0055), fliC, fljB, trhH* and *trhA* genes were performed in strain SL1344 (R27) by using the λ Red recombination method, as previously described (Datsenko & Wanner, 2000). The antibiotic resistance determinant of the plasmids pKD3/pKD4 was amplified using the corresponding oligonucleotides (P1/P2 series, see Table S4). The mutants were confirmed by PCR using the corresponding oligonucleotides (P1up/P2down series, see Table S4). We used phage P22 HT for combining mutations by transduction (Sternberg & Maurer, 1991). When necessary, the antibiotic resistance cassette was eliminated using the FLP/FRT-mediated site-specific recombination method as previously described (Cherepanov & Wackernagel, 1995).

For deletion of the ALG87338.1 gene from the pKAZ3 plasmid, we first constructed the plasmid pKD46-Km^R following the strategy described in (Doublet et al., 2008). Briefly, kanamycin resistance from the pKD4 plasmid was amplified using the PkmXmnIFW/PkmXmnIRv oligonucleotides together with Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific) following the manufacturer's recommendations. The corresponding XmnI-flanked kanamycin resistance fragment was cloned into the vector pKD46 previously digested with the same enzyme. The resulting plasmid was termed pKD46-Km^R. Deletion of ALG87338.1 was performed in strain SL1344 (pKAZ3) pKD46-Km^R by using the λ Red recombination method. The antibiotic resistance determinant of the plasmid pKD3 was amplified using the corresponding oligonucleotides (P1/P2 series, see Table S4). The mutants were confirmed by PCR using the corresponding oligonucleotides (P1up/P2down series, see Table S4).

A transcriptional *lacZ* fusion was made with the *rsp2* gene from the R27 plasmid. The antibiotic resistance determinant from the plasmid R27, *rsp2*::km, was eliminated using an FLP/FRT-mediated site-specific recombination method, as previously described (Cherepanov & Wackernagel, 1995), thus generating the plasmid R27 Δ*rsp2*. An FRT-generated site was used to

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integrate the plasmid pKG136 (Ellermeier et al., 2002), thereby generating a transcriptional R27 *rsp2*::*lacZ* fusion.

Recombinational transfer of the Flag sequence into the *rsp2* gene was achieved by following a previously described methodology (Uzzau et al., 2001). The template vector encoding Flag and Km^r used was the pSUB11 plasmid. The primers used for construction of the Flag-tagged derivative were R27_p0553XP1 and R27_p0553XP2 (Table S4). The correct insertion of the Flag-tag was confirmed by PCR using oligonucleotides R27_p0553XP1UP and R27_p0553XP2DOWN (Table S4).

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

Plasmid conjugation.

The R27 and pKAZ3 plasmids were conjugated as described previously (Mário Hüttener et al., 2019). The mating frequency was calculated as the number of transconjugants per donor cell. Student´s *t*-test was used to determine statistical significance, and the values were obtained by using GraphPad Prism 8 software. A *P* value of less than 0.05 was considered significant.

β-Galactosidase and motility assays.

β-Galactosidase activity measurements were performed as previously described (Miller, 1992). The values are given as Miller units. Two-way ANOVA was used to determine statistical significance, and the values were obtained by using GraphPad Prism 8 software. A *P* value of less than 0.05 was considered significant.

Motility assay.

The motility assay was performed as previously described (Mário Hüttener et al., 2019). The experiments were repeated three times with three plates of each strain in each experiment. The colony diameter was measured and plotted, and standard errors were calculated. One-way ANOVA was used to determine statistical significance, and the values were obtained by using GraphPad Prism 8 software. A *P* value of less than 0.05 was considered significant.

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Flagellum Isolation.

Flagellum isolation was prepared as previously described (Mário Hüttener et al., 2019).

Cell-free secreted proteins (secretome).

Cell-free supernatants were prepared as previously described (Mário Hüttener et al., 2019).

Cell fractionation.

Cell fractionation was performed as previously described (Mário Hüttener et al., 2019).

Immunogold electron microscopy.

Immunogold microscopy experiments were performed as previously described (Mário Hüttener et al., 2019).

Electrophoresis and Western blotting analysis of proteins.

Protein samples were analyzed by 10% and 12.5% SDS-PAGE (Sambrook J and Russell D, 2001). Proteins were transferred from the gels to PVDF membranes using the Trans-Blot Turbo system (Bio-Rad). Western blot analysis was performed with a monoclonal antibody raised against the Flag-epitope (Sigma) diluted 1:10.000 in a solution containing PBS, 0.2% Triton, and 3% skim milk and incubated for 16 hours at 4°C. The membranes were washed for 20 minutes each with PBS and 0.2% Triton solution. The washing step was repeated three times. Thereafter, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Invitrogen) diluted 1:5.000 in a solution containing PBS and 0.2% Triton for 1 hour at room temperature. Again, three washing steps of 45 minutes with PBS and 0.2% Triton solution were performed, and detection was performed by enhanced chemiluminescence using ImageQuant LAS 54000 imaging system software (GE Healthcare Lifesciences).

RSP-Flag Immunoprecipitation.

For RSP-Flag protein immunoprecipitation, the strains SL1344 (R27 RSP-Flag) and SL1344 (R27 Δ*rsp*, negative control) were grown in LB medium for 16 hours at 37ºC. One hundred milliliters of fresh LB medium was inoculated 1:100 with both overnight-cultured strains and grown at 25°C until an $OD_{600\ nm}$ of 2.0 was reached. Then, the cells were centrifuged at 9.000 rpm for 30 minutes at 4°C, the pellets were discarded, and the supernatants were filtered through 0.22 µm filters. For each immunoprecipitation protocol, we used 100 µl of Anti-Flag M2 Affinity Gel (Sigma-Aldrich) and 100 ml of each supernatant and incubated the mixture under slow rotation

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at 4°C for 16 hours. Each supernatant was loaded onto a Poly-Prep chromatography column (Bio-Rad), and the flowthrough was stored at -20°C for further analysis. Each column was washed out with 100 ml of washing buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl), and elution was performed 3 times with 0.3 ml of elution buffer (0.1M glycine pH 3.5). Elution fractions were concentrated using a trichloroacetic acid (TCA) precipitation protocol, briefly, 1 ml of the samples was mixed with 0.5 ml of a 45% TCA solution (w/v). The samples were kept on ice for 30 minutes and centrifugated for 30 minutes at 13,400 rpm. The supernatants were carefully discarded, acetone was added to the protein pellets, and the pellets were again centrifugated for 30 minutes at 13,400 rpm. The supernatants were carefully discarded. The protein pellets were dried and solubilized with 1x Laemmli Sample Buffer (Bio-Rad). Samples were boiled for 10 minutes and loaded onto a 12.5% SDS-PAGE gel. When the protein samples entered the stacking phase, the gel run was stopped, and samples were excised from the gel and sent to the Proteomic Platform (Barcelona Science Park, Barcelona, Spain) for protein identification, as previously described (M. Hüttener et al., 2018). Immunoprecipitation and protein identification experiments were repeated twice.

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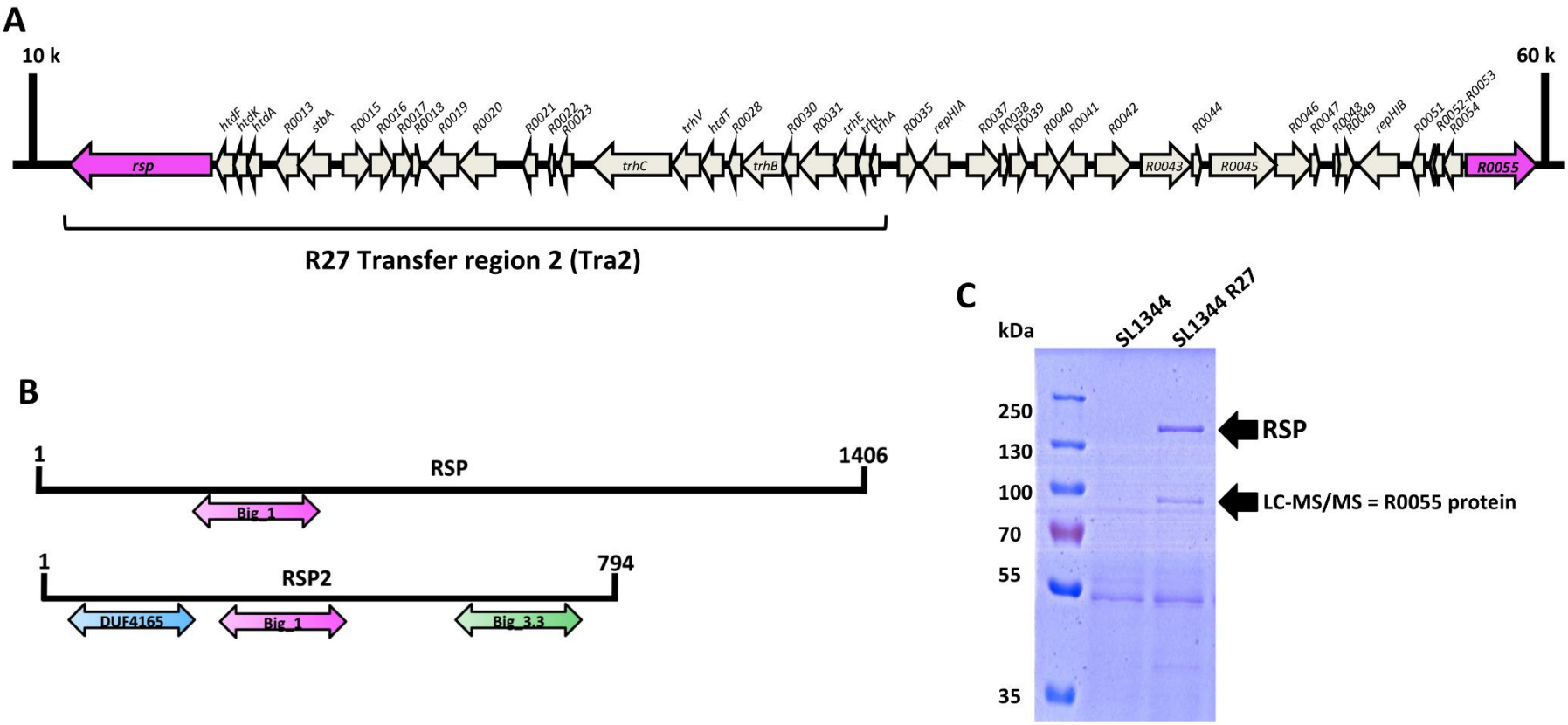
748 **Figures**



749

750 **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)
751 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
752 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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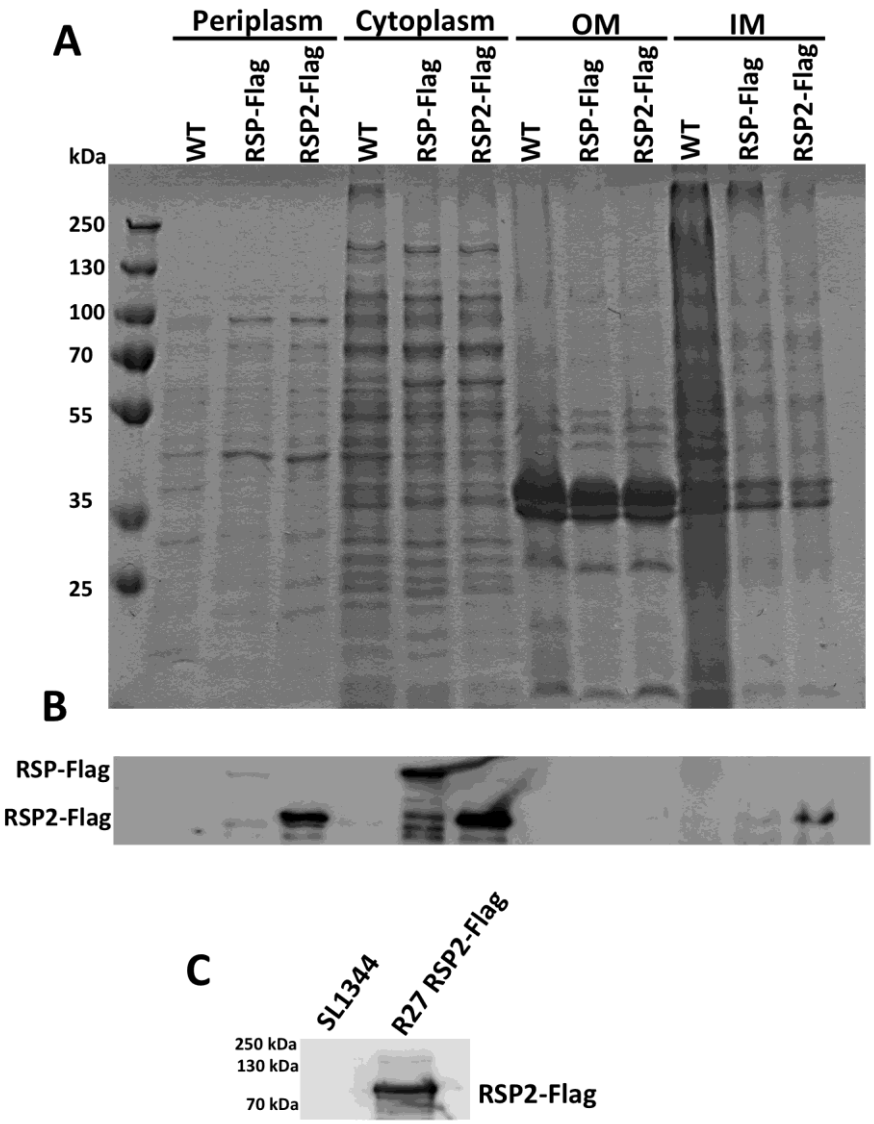

Figure 2. Immunodetection of the RSP2-Flag protein in different cellular compartments. (A) Coomassie blue staining of the different cellular fractions obtained. (B) Immunodetection of the RSP-Flag and RSP2-Flag proteins in the periplasm, cytoplasm, and outer and inner membrane fractions of strains SL1344 (R27 RSP-Flag) and SL1344 (R27 RSP2-Flag), respectively. (C) Immunodetection of the RSP2-Flag protein in the cell-free secretome of strain SL1344 (R27 RSP2-Flag).

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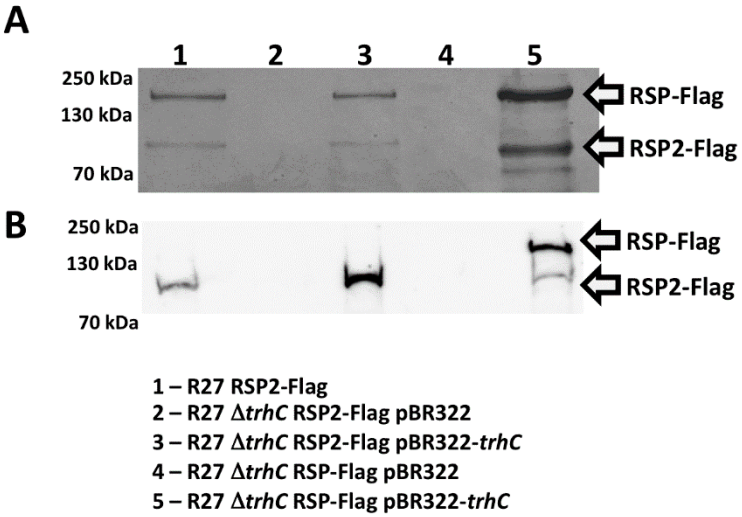
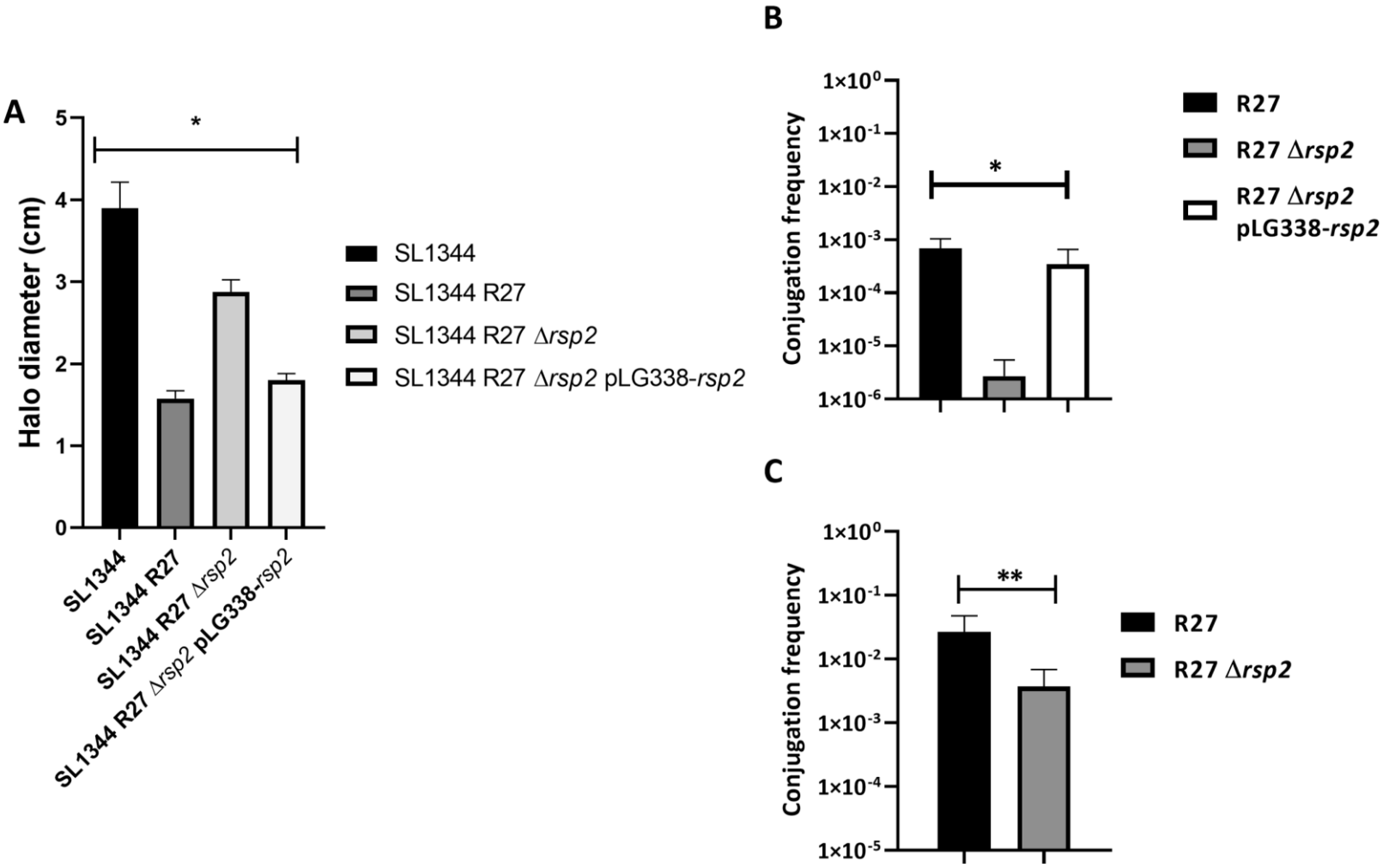


Figure 3. RSP2 export requires the type IV secretion system encoded by the R27 plasmid. Immunodetection of the RSP-Flag and RSP2-Flag proteins. (A) Coomassie blue staining for SDS-PAGE analysis of the secretomes of the different strains. (B) Immunodetection of the Flag-tagged proteins with anti-Flag antibodies. Arrows point to the RSP-Flag and RSP2-Flag proteins. The experiment was repeated three times. A representative experiment is shown.


A
Halo diameter (cm)
0
1
2
3
4
5
*
SL1344
SL1344 R27
SL1344 R27 Δrsp2
SL1344 R27 Δrsp2 pLG338-rsp2
B
Conjugation frequency
1×10^0
1×10^-1
1×10^-2
1×10^-3
1×10^-4
1×10^-5
1×10^-6
*
R27
R27 Δrsp2
R27 Δrsp2 pLG338-rsp2
C
Conjugation frequency
1×10^0
1×10^-1
1×10^-2
1×10^-3
1×10^-4
1×10^-5
**
R27
R27 Δrsp2

777

778 **Figure 4.** The RSP2 protein influences the motility of strain SL1344 (R27) and conjugation of the R27 plasmid. (A) Effect of the *rsp2* allele on the motility of
779 strain SL1344 (R27). The results are the means of three independent experiments. Standard deviations are shown. Statistical analysis showed significant
780 differences, one-way ANOVA (**P*-value < 0.0001). (B and C) Effect of the *rsp2* allele on the conjugation frequency of the R27 plasmid in liquid (B) and solid (C),
781 media, respectively. The data shown are the means and standard deviations of three independent experiments. Statistical analysis showed significant
782 differences (B) one-way ANOVA (**P*-value 0.0184) and (C) Student´s *t*-test (***P*-value 0.0428).

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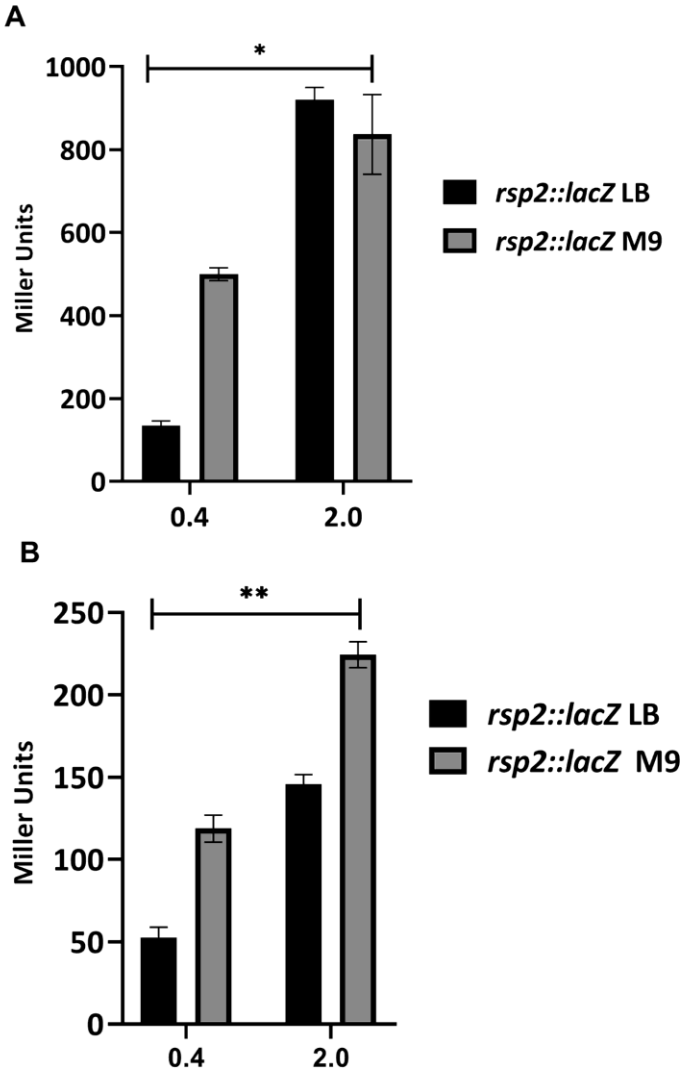
A
*
1000
800
600
400
200
0
Miller Units
0.4
2.0
rsp2::lacZ LB
rsp2::lacZ M9
B
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250
200
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100
50
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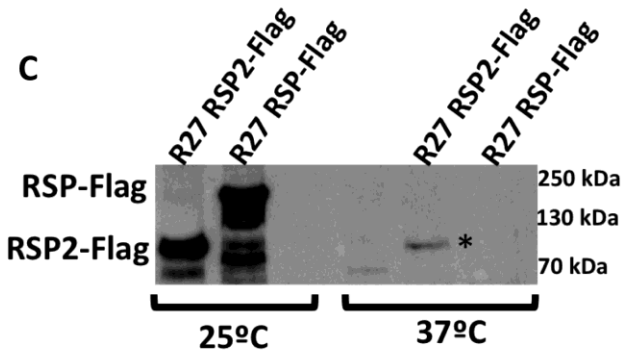
Miller Units
0.4
2.0
rsp2::lacZ LB
rsp2::lacZ M9


C
R27 RSP2-Flag
R27 RSP-Flag
R27 RSP2-Flag
R27 RSP-Flag
RSP-Flag
RSP2-Flag
250 kDa
130 kDa
70 kDa
*
25ºC
37ºC

784

785 **Figure 5.** Expression of the RSP2 protein is temperature and growth medium dependent. (A and B). Transcription of the *rsp2* gene measured as β-galactosidase
786 expression of a *rsp2::lacZ* fusion, in LB and M9 media, at 25°C (A) and 37°C (B). β-Galactosidase activity is expressed as Miller units. The data shown are the
787 means and standard deviations of three independent experiments. Statistical analysis showed a significant difference (**P*-value < 0.0001, ***P*-value 0.0332).
788 (C) Immunodetection of RSP-Flag and RSP2-Flag proteins in the cell-free secretomes of strains SL1344 (R27 RSP-Flag) and R27 (RSP2-Flag) at 25°C and 37°C.
789 Asterisk shows expression of RSP2-Flag at 37°C.

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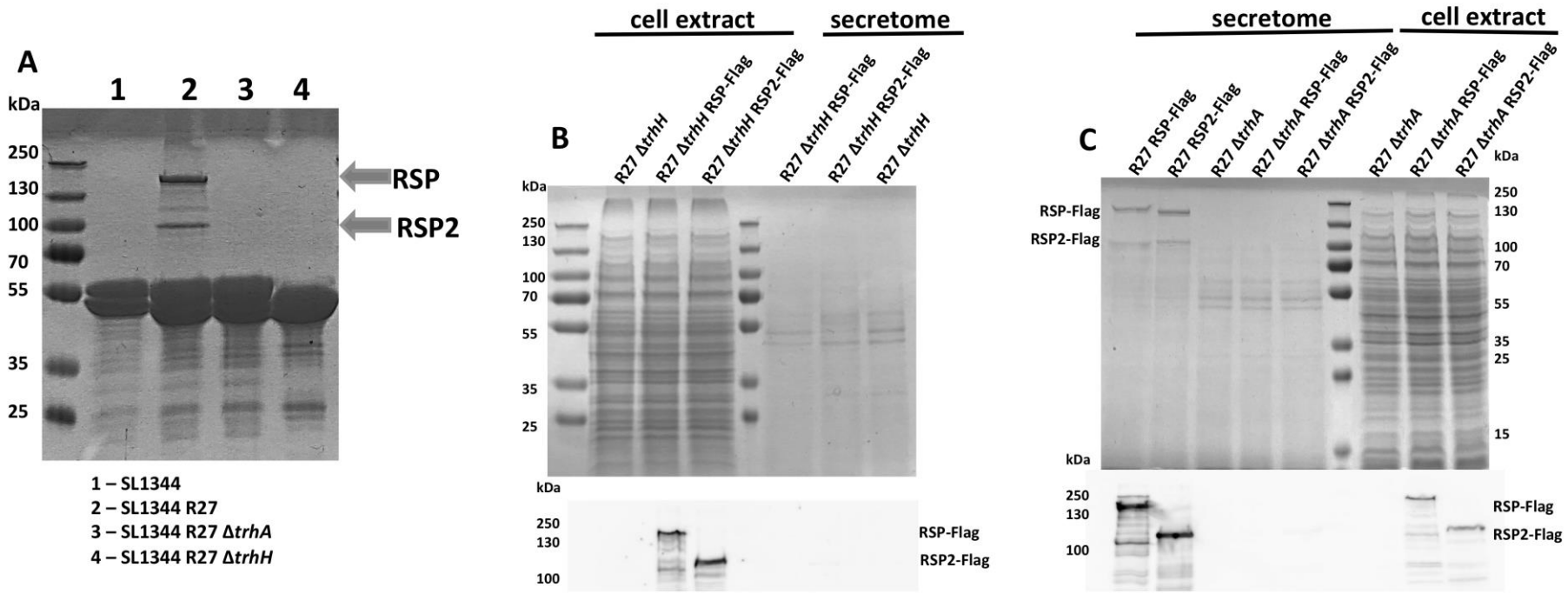

Figure 6. Expression of the RSP and RSP2 proteins depends on the R27 TrhA and TrhH functions. (A) SDS-PAGE analysis of the purified flagellar fractions of the plasmid-free strain SL1344 and strain SL1344 harboring R27 and the R27 Δ*trhA* and R27 Δ*trhH* derivatives. (B) Immunodetection of RSP-Flag and RSP2-Flag in the intracellular compartments of strain SL1344 harboring the corresponding *trhH* derivatives of the R27 plasmid. The upper part of the figure shows the Coomassie staining analysis of the SDS-PAGE gel containing the total cell extracts and the cell-free secretome, as indicated. The lower part shows to the

796 Western blot analysis. (C) Immunodetection of RSP-Flag and RSP2-Flag in the intracellular compartments of strain SL1344 harboring the corresponding *trhA*
797 derivatives of the R27 plasmid. The upper part of the figure shows to the Coomassie staining analysis of the SDS-PAGE gel containing total cell extracts and
798 the cell-free secretome, as indicated. The lower part corresponds to the Western blot analysis. The experiments were repeated three times. A representative
799 experiment is shown.

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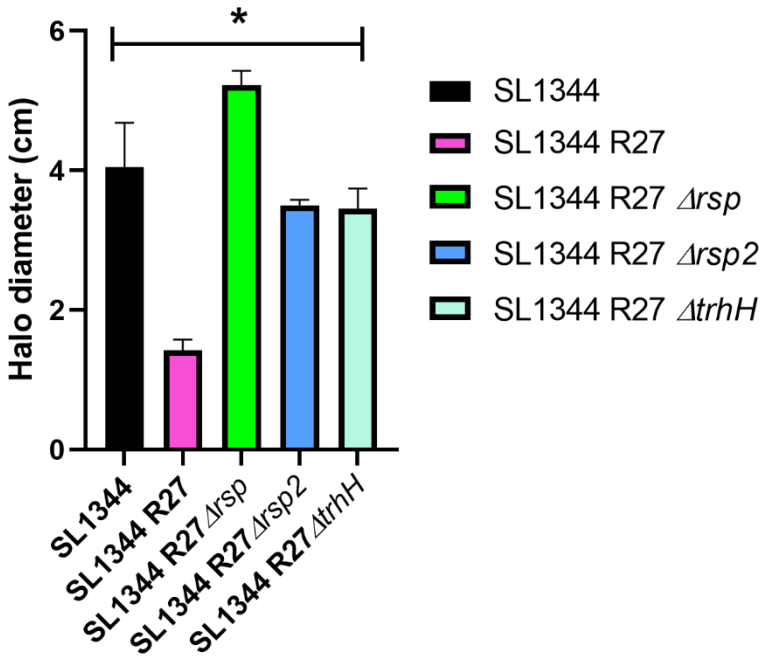

Figure 7. Loss of TrhH function impairs the effect of the RSP and RSP2 proteins on SL1344 cell motility. The motility of the different strains was measured as the halo diameter of the different colonies growing on motility agar. The results are the means of three independent experiments. Standard deviations are shown. (one-way ANOVA, *P-value <0.0001).

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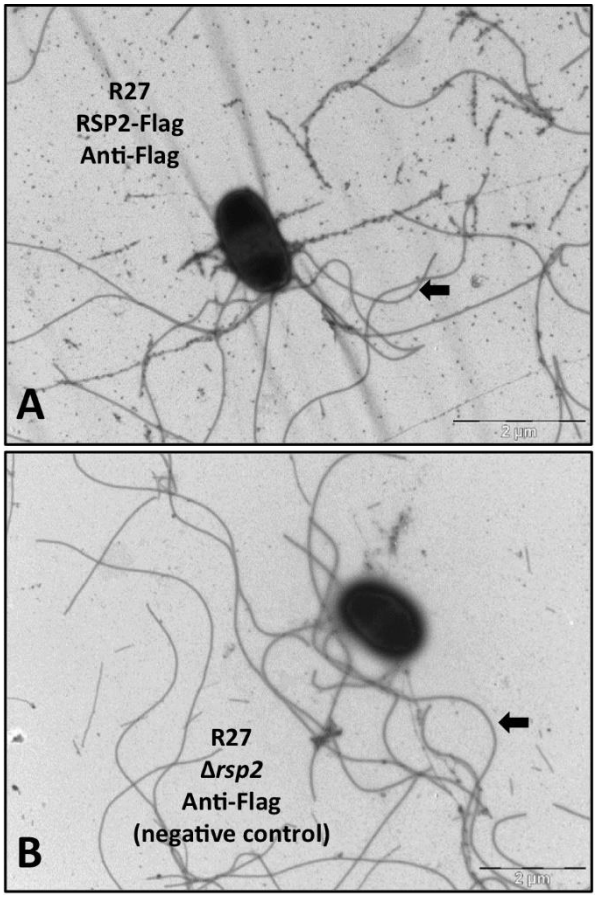

Figure 8. The RSP2 protein binds to the flagella of strain SL1344 (R27). Immunogold electron microscopy of cells from strains SL1344 (R27 RSP2-Flag) (A) and SL1344 (R27 Δ*rsp2*) (B) using monoclonal anti-Flag antibodies and goat anti-mouse IgG conjugated to 12 nm gold particles. Arrows point to the RSP protein associated with the flagella in (A) and only the flagella in (B). Bars represent 2 µm.

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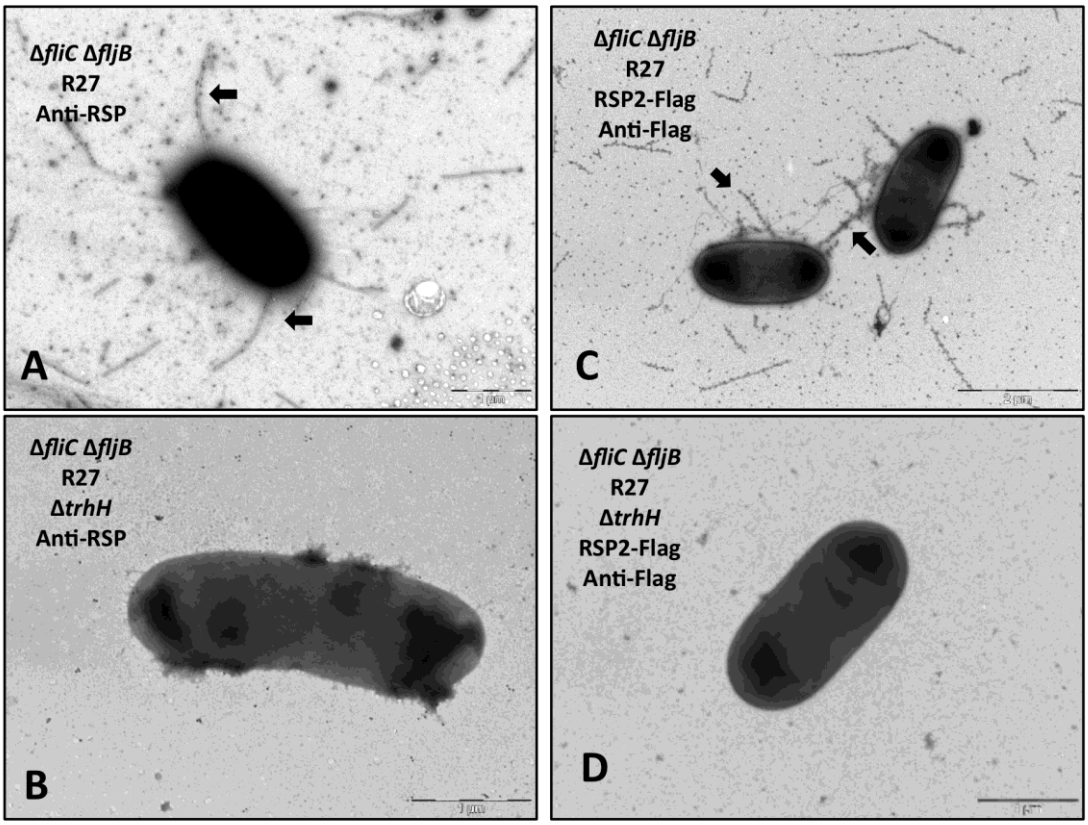
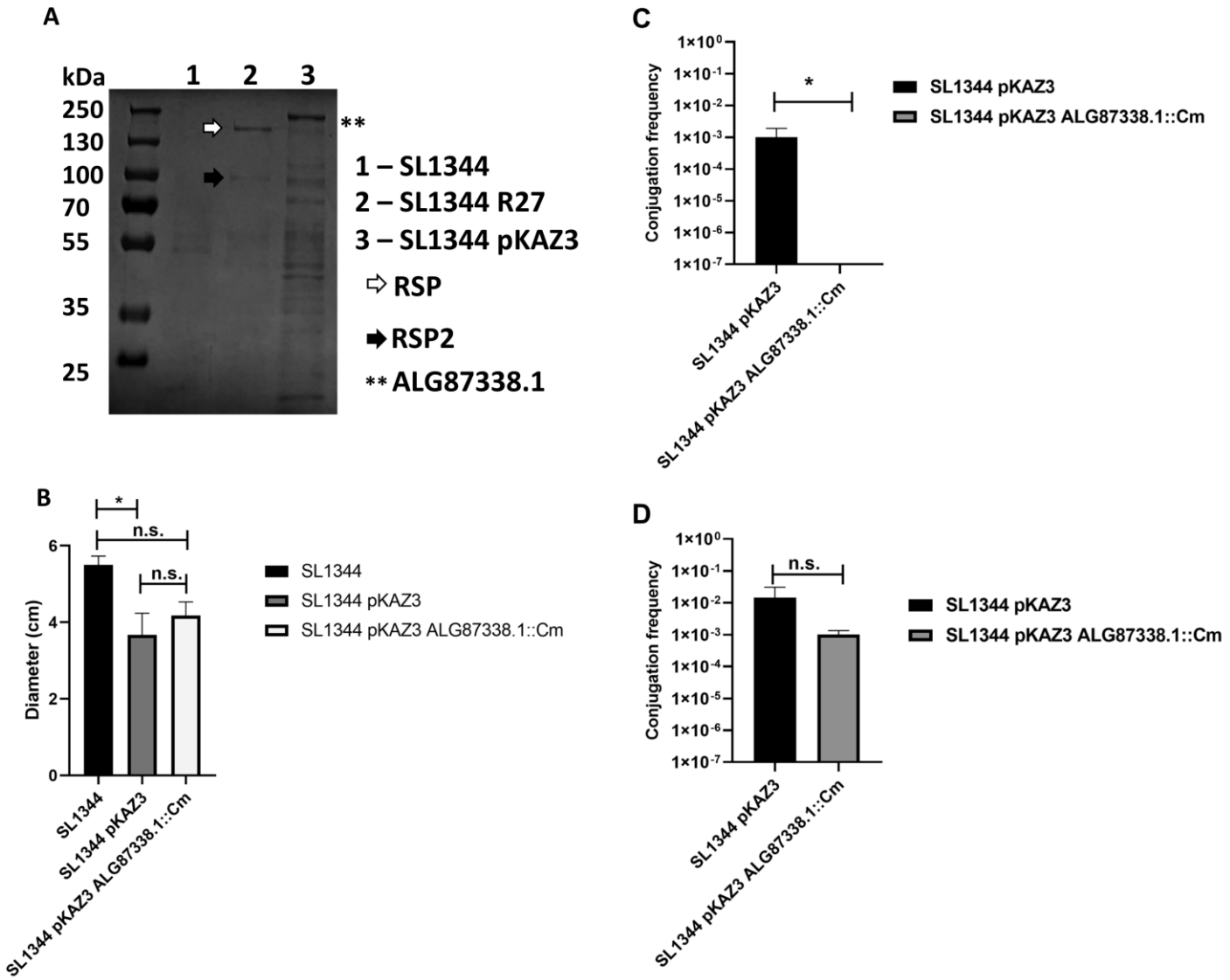


Figure 9. The RSP and RSP2 proteins bind to the conjugative pilus encoded by the R27 plasmid. Immunogold electron microscopy of cells from strains SL1344 Δ*fliC* Δ*fljB* (R27) (A) and SL1344 Δ*fliC* Δ*fljB* (R27 Δ*trhH*) (B) using polyclonal anti-RSP antibodies and goat anti-rabbit IgG conjugated to 12 nm gold particles. Arrows point to the RSP protein associated with the conjugative pilus. Immunogold electron microscopy of cells from strains SL1344 Δ*fliC* Δ*fljB* (R27 RSP2-Flag) (C) and SL1344 Δ*fliC* Δ*fljB* (R27 RSP2-Flag Δ*trhH*) (D) using anti-Flag monoclonal antibodies and goat anti-mouse IgG conjugated to 12 nm gold particles. Arrows point to the RSP2 protein associated with the conjugative pilus. Bars represent 1 µm (in A, B and D) and 2 µm (in C).


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
Diameter (cm)
*
n.s.
n.s.
SL1344
SL1344 pKAZ3
SL1344 pKAZ3 ALG87338.1::Cm
C
Conjugation frequency
*
SL1344 pKAZ3
SL1344 pKAZ3 ALG87338.1::Cm
D
Conjugation frequency
n.s.
SL1344 pKAZ3
SL1344 pKAZ3 ALG87338.1::Cm

833

834 **Figure 10.** The ALG87388.1 gene product of the pKAZ3 plasmid influences conjugation at 25°C. (A) Detection of the ALG87388.1 protein in the secretome of
835 strain SL1344 (pKAZ3). Arrows indicate the RSP and RSP2 protein, asterisk indicates ALG87388.1 protein. (B) Motility of the SL1344, SL1344 (pKAZ3) and SL1344
836 (pKAZ3 ΔALG87388.1) strains. The data shown are the means and standard deviations of three independent experiments. Statistical analysis showed significant
837 differences (**P*-value 0.0469; n.s. not significant). (C and D). Effect of the ΔALG87388.1 allele on the conjugation frequency of the pKAZ3 plasmid at 25°C (C)
838 and 37°C (D) in liquid media. The data shown are the means and standard deviations of three independent experiments. Statistical analysis showed significant
839 differences (**P*-value 0.042; n.s. not significant).

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