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Crosstalk between bacterial conjugation and motility is mediated by plasmid-borne regulators.

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Summary

Plasmid conjugation is a major horizontal gene transfer mechanism. The acquisition of a plasmid may cause a perturbation of the cell functions in addition to provide advantageous properties for the recipient cell, such as the gaining of antibiotic resistances. The interplay between plasmid and chromosomal functions has been studied using the IncHI1 plasmid R27. Plasmids of the incompatibility group HI1, isolated from several Gram-negative pathogens, are associated with the spread of multidrug resistance. Their conjugation is tightly regulated by temperature, being repressed at temperatures within the host (37 ºC). In this report, we described that at permissive temperature, when conjugation of plasmid R27 is prompted, a reduction in the motility of the cells is observed. This reduction is mediated by the plasmid-encoded regulators TrhR/TrhY, which together with HtdA form a plasmid –borne regulatory circuit controlling R27 conjugation. TrhR/TrhY, required to induce R27 conjugation, are responsible of the downregulation of the flagella synthesis and the consequent decrease in motility. TrhR/TrhYrepress, direct or indirectly, the expression of the specific flagellar sigma subunit FliA and, consequently, the expression of all genes located bellow in the flagellar expression cascade.

Introduction

The dissemination of antimicrobial resistance in bacteria has been largely associated to DNA exchange by horizontal gene transfer (HGT) mechanisms. Plasmid conjugation is a major HGT mechanism. The acquisition of a plasmid may result advantageous for the host cell under specific conditions if it confers new phenotypes such as resistance to antibiotics and heavy metals. In other cases, the presence of the plasmid may cause a fitness loss due to perturbation of cell functions.

Plasmids of the incompatibility group HI1, isolated from several species of enterobacteria (*Escherichia coli*, *Salmonella enterica*, *Klebsiella pneumonia*…..) are important vectors of antibiotic resistance genes (Holt *et al.*, 2007; Phan and Wain, 2008, Holt *et al.*, 2011). The conjugative transfer of these plasmids is tightly regulated by temperature, being the conjugation frequency optimal in a temperature range of 22-30º C (Maher *et al.*, 1993). This behavior suggest that plasmid transfer is not promoted within the mammal host whereas is induced during the transit of *Salmonella* in water and/or soil environments. Plasmid R27, encoding resistance to tetracycline, is the prototype of IncHI1 plasmids and it has been intensively studied for over 20 years. Its complete nucleotide sequence is available and the replication and conjugation determinants are well characterized (Taylor *et al.*, 1985; Couturier *et al.*, 1988; Maher *et al.*, 1993; Gabant *et al.*, 1994; Newnham and Taylor, 1994; Sherburne *et al.*, 2000; Lawley *et al.*, 2002, 2003; Alonso *et al.*, 2005). The interplay between the R27 plasmid and the bacterial chromosome has been studied, and functional interactions between regulators from both genetic elements have been

characterized (Doyle *et al.*, 2007; Baños *et al.*, 2009; Dillon and Dorman, 2010; Gibert *et al.*, 2014, 2016). Moreover, the impact of the presence of R27 on the global expression profile of *Salmonella* under some environmental conditions has been described (Paytubi *et al.*, 2014). In previous studies we demonstrated that a regulatory circuit encoded in the R27 conjugative plasmid, formed by HtdA, TrhR and TrhY, tightly controls plasmid conjugation (Gibert *et al.*, 2014). TrhR and TrhY complex, from now named TrhR/Y, are essential to trigger conjugation by stimulating transcriptional expression of the *tra* genes, whereas HtdA has an overall repressor effect, presumably by acting as an antiactivator of TrhR/Y. We also observed that mutations in the *htd*A locus, derepressing R27 conjugation, cause a concomitant decrease in motility (Gibert *et al.*, 2013). Accordingly, the existence of a regulatory crosstalk between expression of conjugative apparatus and bacterial motility was previously anticipated by several authors (Bohlin and Burman, 1977; Maher *et al.*, 1993; Barrios *et al.*, 2006; Reisner *et al.*, 2012; Rösch *et al.*, 2014; Takahashi *et al.*, 2015).

In *Escherichia coli*, more than 50 genes are involved in the synthesis of a functional flagella, which are classified into three groups according to their temporal expression (Chilcott and Hughes, 2000). The master regulator of flagella synthesis, FlhD-FlhC, is encoded by the class 1 flagellar operon (*flhDC*). FlhD-FlhC activates the expression of class 2 genes, encoding the inner part of the flagellum, the flagellar sigma factor FliA (σ^{28}) and the anti- σ^{28} protein, FlgM. Class 3 genes are transcribed by a σ^{28}-RNAP and encode the outer components of the flagellum as well as the chemotaxis proteins.

In this report, we describe an inverse relationship between plasmid conjugation and motility in *E.coli*. Hence, when conjugation is prompted a reduction in motility is detected. Our studies indicate that TrhR/Y, plasmid-encoded factors that trigger expression of the conjugative apparatus genes, are required for the downregulation of the flagella synthesis and the concomitant decrease in motility.

Results

Mutations that alter the conjugation frequency of R27 plasmid affect the swimming motility.

To study the crosstalk between conjugation and motility, the plasmid R27 - prototype of the IncHI1 plasmids – was used as a model. The swimming ability on TB plates and the conjugation ratio at permissive temperature (25 ºC) was determined for *E. coli* strains carrying either the R27 plasmid. The presence of R27 plasmid causes an overall increase in the swimming ability. To establish if motility is affected when the plasmid conjugation is promoted, swimming ability was also monitored for strains carrying R27 or derivatives with mutations for different key regulators of bacterial conjugation: TrhR/Y that are required for induction of R27 conjugation and HtdA that acts as an antiactivator having an overall repressor effect on R27 conjugation. A decrease in the swimming ability of the cells is observed when a derepressed plasmid, drR27 (*htdA*), is present. In agreement with previous observations (Gibert *et al.*, 2013), absence of *htdA* (drR27) causes a drastic derepression of R27 conjugation frequency with more than 3 $\log_{10}$ increase as compared to wt (Fig. 1A). The presence of drR27 causes a significant reduction in the ability to swim (Fig. 1B and C). These results revealed a correlation between conjugation frequency and motility.. Next, the effect of the *trhR* mutation (R27 *trhR*-Tmp), that causes a decrease in the frequency of R27 conjugation as compared to wt (Fig 1A), was tested. . No effect of the *trhR* mutation was observed in the motility (Fig. 1B and C), which can be a consequence of the very low conjugation frequency (2.8E-5) of the R27 plasmid under the conditions used.. Thus, a further decrease in the

conjugation frequency, by introducing the *trhR* mutation (4.7E-6), would not cause any additive effect on swimming. Consistent with this explanation, the effect of the *trhR* mutation on motility was evident when using a drR27 plasmid. The *trhR* mutation (drR27 *trhR*-Tmp) causes a very severe drop in the frequency of conjugation (from 1.1E-1 to 2.3E-5, Fig. 1A) and a clear increase in the motility (Fig. 1B and C), as compared to drR27.

Our data indicate that when a cell trigger the conjugative process has the ability to modulate motility. Experiments were performed to determine whether motility can regulate R27 conjugation. The conjugation frequency of R27 and drR27 plasmids was determined in strains deficient in swimming motility by carrying a mutation in the *flhDC* operon, coding for the master regulator of the flagella biosynthesis cascade. The data obtained (Fig. 1A and B) show that the flhDC mutants are not motile and the conjugation frequency of both R27 and drR27 is not affected by the swimming ability of the donor cell.

TrhR/Y reduces bacterial motility and HtdA counteracts its action.

It can be hypothesized that the crosstalk between R27 conjugation and motility is due to either i) a side effect of the biosynthesis of the conjugational apparatus in the physiology and/or energetic status of the cell, or ii) a consequence of the involvement of specific regulatory circuits controlling both cellular processes, conjugation and motility. The possiblec involvement of the regulatory circuit TrhR/Y-HtdA, which modulates R27 conjugation, in the control of swimming motility was further characterized. The effect of the presence of the regulators TrhR/Y and HtdA on motility, in the absence of the whole R27 conjugative plasmid, was monitored (Figure 2A and 2B). A drop in motility (2.6- fold) was

observed in the presence of plasmid pBAD#3, carrying the *trhR* and *trhY* genes with its own promoter, as compared with the strain carrying the control vector (pBAD18). On the other hand, the presence of plasmid pACYC*htdA*, carrying the *htdA* gene in pACYC184, causes only a slight increase in the motility. This result suggests that TrhR/Y regulators interfere negatively with the chromosomal gene cascade responsible of flagella biosynthesis and motility.

Previously, we described that the regulatory activity of TrhR/Y on the control of R27 conjugation is counteracted by the presence of HtdA (Gibert *et al.*, 2014). This interplay TrhR/Y-HtdA is also involved in the motility control since addition of pACYC*htdA* plasmid restores motility in the strain carrying the pBAD#3 plasmid (Fig. 2A and 2B).

Further corroboration that the expression of *trhR* and *trhY* from the pBAD#3 plasmid causes a reduction in bacterial motility was obtained using plasmid pBAD*trhRY*. In plasmid pBAD*trhRY*, the expression of *trhR* and *trhY* genes is under the control of the arabinose inducible P_{BAD} promoter. As seen in figure 2C, a decrease in the motility was only observed when *trhR trhY* expression was induced by the addition of arabinose. Again, the negative effect of TrhR/Y was counteracted by the presence of HtdA. Previously, we described that both, TrhR and TrhY, are required for the transcriptional activation of R27 conjugation genes (Gibert *et al.*, 2014). Similarly, when *trhY* or *trhR* were expressed independently no effect on motility was detected, indicating that both, TrhR and TrhY, are required to cause motility reduction. Remarkably, our data also indicate that there is no need of any other R27-encoded factor to reduce motility.

The regulatory circuit TrhR/Y-HtdA is involved in the control of flagella biosynthesis.

Overall, our results indicated that the TrhR/Y-HtdA regulatory circuit, involved in the transcriptional control of the *tra* operons and, consequently, R27 conjugation, interferes with swimming motility. Swimming motility can be affected by altering either flagella synthesis or flagella functionality. To discriminate between the two options, flagella synthesis was monitored by direct observation using transmission electron microscopy (Fig. 3A). The data obtained reveals that the expression of *trhRY* (pBAD#3), causes a clear decrease in the number of flagella on the cell compared to the control strain (pBAD18). Most of the cells harboring pDAB#3 have no flagella. Consistent with the counteractivity described for HtdA, when pACYC*htdA* plasmid was introduced in cells carrying pBAD#3, the number of flagella per cell observed increases to similar numbers to those detected in the control strain (no plasmid). Our data clearly demonstrate that the conjugation regulators TrhR/Y-HtdA affect biosynthesis of flagella. Furthermore, the reduction in the number of flagella when the cell carries pBAD#3 plasmid correlates with a decrease in the expression of two motility-related genes, *fliC* and *motA* (Fig. 3B and C). Immunodetection of the flagelin FliC, main subunit of the flagellar filament, in total protein extracts shows a clear reduction in FliC levels (2.7-fold) when cells synthesize TrhR/Y (pBAD#3) (Fig. 3B). Expression of the *motA*, which encodes a motor subunit of the flagella, was monitored using a *lacZ* transcriptional fusion. A reduced activity of the *motA* promoter was detected in the strain that synthesizes TrhR/Y (1.7-fold) (Fig. 3C). Expression of both, *fliC* and *motA*, was

183 restored when pACYC*htdA* was introduced into the strains expressing *trhR/Y*
184 (Fig. 3B and C).

185 To further corroborate the effect of the regulatory circuit TrhR/Y-HtdA in the
186 transcriptional cascade that regulates flagella biosynthesis, the mRNA levels of
187 several flagellar genes were estimated by qRT-PCR assays (Fig. 3D). The
188 expression of early (*flhC*), middle (*fliA* and *flgM*) and late (*fliC* and *motA*) genes
189 in the flagella biosynthesis cascade was determined in cells expressing *trhR/Y*
190 (pBAD#3) and cells expressing *trhR/Y* and *htdA* (pDAB#3 and pACYC*htdA*).
191 FlhC, together with FlhD, is the master regulator required for transcriptional of
192 several middle genes. FliA is the sigma subunit (σ^{28}) that promotes the
193 transcription of late genes and FlgM is an antisigma factor that modulates FliA
194 activity. FliC and MotA are structural proteins of the flagella, encoded in late
195 genes whose expression is FliA dependent. Accordingly with the previous data,
196 the presence of plasmid pBAD#3 caused a harsh drop in the transcription of the
197 late genes *fliC* (10-fold), and *motA* (5-fold). Moreover, it causes a significant
198 decrease in the transcription of the middle gene *fliA* (3.3-fold). In contrast, the
199 expression levels of *flgM* and *flhC* were not affected. Again, HtdA counteracts
200 TrhR/Y-mediated repression of *fliC*, *motA* and *fliA* (see expression level in the
201 presence of pBAD#3 and pACYC*htdA*). These results indicate that the
202 regulatory circuit TrhR/Y-HtdA modulates cell motility by altering the
203 transcription of some of the middle and late genes required for flagella
204 synthesis.

Discussion

Conjugation is a major mechanism for genetic material transfer between bacteria, and represents one of the main processes involved in antibiotic resistance spread. Although acquisition of certain plasmids may provide advantages under specific growth conditions, for instance plasmids carrying genes encoding resistance to antibiotics or heavy metals and metabolic abilities (Frost *et al.*, 2005), the presence of large plasmids can affect certain cellular processes and cause an overall negative effect on the cell fitness. The effect of the presence of conjugative plasmids on bacterial metabolism has been studied in *Escherichia coli*, *Salmonella enterica*, *Pseudomonas* or *Bacillus* (Wang *et al.*, 2006; Shintani *et al.*, 2009; Paytubi *et al.*, 2014; Rösch *et al.*, 2014; Takahashi *et al.*, 2015; Jiang *et al.*, 2017). Cell motility was described among the cellular processes that can be altered by the presence of conjugative plasmids. It has been reported that both the R plasmid pUM5 in *Salmonella enterica* and the IncP-7 plasmid pCAR1 from *Pseudomonas* cause somehow motility inhibition (Bohlin and Burman, 1977; Takahashi et al., 2015). In *Bacillus*, the presence of pLS20 plasmid also represses motility. Transcriptional studies demonstrated that pSLP20 affects global transcription, being the expression of the *motA* gene reduced (2-fold) by the presence of this plasmid (Rösch *et al.*, 2014). In *E.coli*, a negative effect on motility by the presence of IncFII plasmids has been shown (Barrios *et al.*, 2006; Reisner *et al.*, 2012; Jiang *et al.*, 2017). Although the presence of R1*drd*19 plasmid does not alter the expression of flagella related genes (Barrios *et al.*, 2006), another IncFII plasmid, pHK01, causes a downregulation in the transcriptional expression of chemotaxis and flagellar assembly genes (Jiang *et al.*, 2017). In all the mentioned reports, the effect of

the presence of conjugative plasmids on motility was determined; however no specific regulators that mediate such plasmid-chromosome crosstalk were identified. It cannot be rule out that these conjugation/motility crosstalks are mediated by a similar mechanisms to that described in this report for R27 plasmid. However, different regulatory factors should be involved since TrhR/Y proteins have been only found encoded among IncHI plasmids (Gibert *et al*., 2014).

In this work, we show that conjugation of plasmid R27 affects motility in *E. coli*. The frequency of conjugation of the R27 plasmid is very low and, remarkably, when bacterial conjugation is promoted by the use of the derepressed plasmid drR27 a drastic drop in bacterial motility is detected. One might suggest that sudden biosynthesis of the conjugational apparatus may non-specifically affect cell physiology and/or the energetic status of the cell, which could influence as a side consequence the cellular motility. This is not the case, since we show that the TrhR/Y-HtdA complex, the pivotal regulatory circuit modulating R27 conjugation, is involved in the crosstalk between plasmid conjugation and motility. Ectopical expression of *trhR* and *trhY* genes, in the absence of R27 plasmid, causes a clear decrease on motility. TrhR/Y proteins are part of the plasmid-encoded regulatory circuit that controls expression of the *tra* genes and, consequently, the frequency of conjugation of the plasmid. TrhR and ThrY proteins are both required to induce *tra* operons expression. HtdA, another component of the regulatory circuit, acts as antiactivator of TrhR/Y having an overall repressor effect (Gibert *et al.*, 2014). TrhR/Y has a dual effect by inducing conjugation and repressing cell motility whereas HtdA counteracts TrhR/Y in both cellular processes. Overall, our data indicate that TrhR/Y is

required to repress motility and that there is no need of any other R27-encoded factor. This effect is also observed at the level of the expression of flagella-related genes. More than 50 genes are directly involved in motility in *E.coli*, and are expressed in a temporal-depending manner. The expression of the master regulator, FlhD-FlhC, triggers the expression of the specific sigma subunit (σ^{28}), encoded by *fliA*, that drives the transcription of the late operons (including *fliC* and *motA* genes). The expression studies indicate that *fliA* is repressed in the presence of TrhR/Y and consequently all the genes that are below in the flagellar expression cascade (*fliC* and *motA*). Whether TrhR/Y affects directly or indirectly the transcriptional expression of flagellar genes remains elusive. In silico data suggest that TrhY is a cytoplasmatic DNA binding protein and TrhR an inner membrane protein (Gibert *et al*., 2014). However, the molecular mechanism by which TrhR/Y regulate transcriptional expression of the *tra* genes is unknown.

Although the effect of large conjugative plasmids on motility was previously described, to our knowledge, this is the first report identifying a specific plasmid-encoded factor as responsible of this plasmid-chromosome crosstalk. Further research would be required to gain insight into the exact mechanism underlying this crosstalk.

Experimental procedures

Bacterial strains, plasmids and growth conditions

Escherichia coli strains and plasmids used in this study are listed in Table 1. Bacteria were grown in LB (10 g/l NaCl, 10 g/l tryptone, 5 g/l yeast extract), Penassay broth (1.5 g/l meat extract, 1.5 g/l yeast extract, 5 g/l peptone, 1 g/l glucose, 3.5 g/l NaCl, 1.32 g/l KH_2PO_4, 4.82 g/l $K_2HP_4 \cdot 3H_2O$), lactose M9 minimal media plates (1× M9 salts, 0.2% lactose, 10 µM thiamine and 1.5% agar), TB plates (10 g/l tryptone, 5 g/l NaCl, 2.5 g/l agar) and LB agar plates (LB plus 15 g/l agar), as indicated. When needed, antibiotics were added at the following concentrations: tetracycline (Tc) 15 µg/ml, chloramphenicol (Cm) 20 µg/ml, ampicillin (Amp) 50 µg/ml or kanamycin (Km) 50 µg/ml. For induction assays, arabinose was added at concentrations of 0.2 % (w/v).

To construct the AML strain, carrying a transcriptional fusion of the *motA* promoter with the *lacZ* gene, the *motA* promoter region was PCR amplified using the primer pair MotA-F-Eco and MotA-R-Bam. Next, the PCR-amplified fragment was cloned into EcoRI-BamHI sites of pRS551 (Simons *et al.*, 1987), resulting in plasmid pRS-motA. The *motA::lacZ* fusion was transferred to the *attB* lambda site in the chromosome of the AAG1 strain, using previously described protocols (Simons *et al.*, 1987). Controls to confirm single gene fusion insertion in the *attB* locus were performed as previously described (Powell *et al.*, 1994).

Plasmid conjugation

For conjugation experiments, cultures of donor (AAG1 derivatives, *lac*$^-$) and recipient (MG1655, *lac*$^+$) strains were grown in Penassay broth (PB) supplemented with the required antibiotics at 25 °C in static conditions for 16 h. Cells were washed with PB to eliminate the antibiotics. Aliquots of recipient strain suspension (50 µl) and donor strain suspension (25 µl) were added to a filter placed on a LB agar plate. After 2 h incubation at 25 °C, the filter was placed in a tube and washed with 1.5 ml of Penassay broth to free the bacteria from the filter. Serial dilutions were plated in LB agar plates supplemented with IPTG (0.5 mM) and Xgal (40 µg/ml) or lactose M9 minimal media plates to discriminate between donor (*lac*$^-$) and transconjugant (*lac*$^+$) cells, as previously described (Gibert *et al.*, 2013). When MG1655 *flhD*::Cm was used as a donor strain, strain AML was used as recipient, and transconjugants were selected in LB plates supplemented with tetracycline and kanamycin. The mating frequency was calculated as the number of transconjugants per donor cell. We show the average of three independent experiments plotted with standard deviation.

Motility Assays

The motility phenotype was analyzed on TB plates. Overnight LB bacterial cultures at 25 °C were spotted (5 µl) on the center of the plates and incubated at 25 °C. The colony diameter was measured and plotted, and standard deviations were calculated. The data are representative of two independent experiments with four replica plates for each strain.

Transmission electron microscopy

Bacterial strains used for flagella visualization were grown in LB at 25 °C in static conditions for 16 hours. Bacterial cells were centrifuged (1,500 x*g* for 5 min) and resuspended in filtered Ringer ¼ solution. Cu-Carbon grid (CF200-Cu Carbon Film On 200 Mesh Copper Grids, Electron Microscopy Sciences) was soaked for 60 sec on a 5 µl drop of each strain resuspension, washed three times with water for 20 sec and stained for 60 sec using a 2 % (w/v) uranyl acetate solution (Polysciences). Once stained, the grids were dried for at least 24 h before visualization under a JEOL JEM1010 transmission electron microscope. Images were obtained using the software analySIS (Soft Imaging System GmbH, Münster, Germany). Each sample was observed for at least 100 cells.

Total protein extracts

To obtain total protein extracts, bacterial strains were grown at 25 °C in shacking conditions for 16 hours and total protein was precipitated. Trichloracetic acid (TCA) was added to bacterial cultures at 20 % final concentration, mixtures were incubated for 1 h on ice and centrifuged (14,000 x*g*, 4 ºC). Precipitates were washed with 80 % cold acetone, air dried and resuspended in protein sample buffer. Final volume was adjusted to the original OD_{600} of each culture.

Gel electrophoresis and Western blot

Protein samples were analyzed by SDS-PAGE and immunoblotted by western blot upon transfer of proteins to PDVF membranes. Western blot analysis was performed with polyclonal antibodies raised against *E. coli* FliC protein [1:2000]

(Westerlund-Wikström *et al.*, 1997). Horseradish peroxidase-conjugated goat anti-rabbit IgG [1:10000] (Sigma) was used as secondary antibody. ECL Prime Western Blotting detection reagent (GE Healthcare) was used to immunodetect the transferred proteins. Visualization and analysis of the detected bands was performed using Molecular Imager ChemiDoc XRS System and Quantity One software (Bio Rad).

β-galactosidase assay

β-galactosidase assays were performed as described by Miller (Miller, 1992) using cultures grown in LB at 25 ºC until an OD_{600} of 2.0. Data are mean values of duplicate determinations in at least three independent experiments plotted with standard deviations.

Total RNA isolation

Total RNA was isolated from three independent cultures of the AAG1 derivative strains grown at 25 ºC in LB until an OD_{600} of 2.0, using a SV Total RNA Isolation System (Promega) according to manufacturer's directions. The purity and quality of the purified RNA was tested by Bioanalyzer 2100 (Agilent Technologies).

qRT-PCR

The expression level of *fliC*, *fliA*, *motA*, *flhC* and *flgM* genes was determined by real-time quantitative reverse transcription-PCR (qRT-PCR). Briefly, 1 µg of total RNA was reverse transcribed to generate cDNA using the "High-capacity cDNA Reverse Transcription kit" (Applied Biosystems) as recommended by the

363 manufacturer. As a control, samples in which reverse transcriptase was omitted
364 from the reaction mixture were used. Real-time PCR using "Power SYBR Green
365 PCR Master Mix kit" (Applied Biosystems) was carried out on the StepOne
366 Real-Time PCR System Thermal Cycling Block (Applied Biosystems).
367 Oligonucleotides to PCR amplification of the genes of interest were designed
368 using Primer3 online tool (Rozen and Skaletsky, 2000) provided by the
369 Whitehead institute (http://bioinfo.ut.ee/primer3/) (Table 2). The relative amount
370 of target cDNA was normalized using *gapdh* gene as an internal reference
371 standard. The relative expression was expressed as fold change using the
372 expression level in wild type strain set arbitrarily to 1.0.

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Conflict of interest

None to declare.

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Figure legends

Figure 1. High conjugation frequency correlates with a reduction in the ability to swim. A) Conjugation frequencies of R27 plasmid and its derivatives using AAG1 or MG1655 *flhD*::Cm as the donor and MG1655 or AML respectively as the recipient strains. B) Swimming motility on TB plates after 15 hours incubation at 25 ºC for strains carrying the indicated plasmids (n.p no plasmid)) C) The diameter of the perimeter of the bacterial growth in (B) was measured (dashed lines), and averages and standard deviations were plotted.

Figure 2. Effect of TrhRY and HtdA on the swimming phenotype. A) Motility on TB plates after 15 hours incubation at 25 ºC of strain MG1655 carrying indicated plasmids. B) The diameter of the perimeter of bacterial growth in (A) was measured (dashed lines), and averages and standard deviations were plotted. C) Motility on TB plates after 20 hours incubation at 25ºC of strain MG1655 carrying plasmids indicated in the X-axis, in the presence and absence of 0.2 % arabinose.

Figure 3. **Effect of TrhR/Y and HtdA on the flagella biosynthesis.** A) Visualization of MG1655 cells carrying the plasmids indicated (n.p, no plasmid) using transmission electron microscopy. B) Immunodetection of FliC on total protein extracts of strain MG1655 carrying the indicated plasmids (n.p, no plasmid). The relative amount of FliC was compared to that corresponding to the control without any plasmid (n.p.). OmpA immunodetection was used as a control. C) Transcriptional expression of the promoter sequence of *motA* in the absence or presence of regulators TrhRY (pBAD#3) or HtdA (pACYC*htdA*) monitored as β-galactosidase activity (Miller units). Data are mean values of

duplicate determinations of at least three independent cultures plotted with standard deviations. D) Expression level of *fliC*, *fliA*, *motA*, *flhC* and *flgM* determined by qRT-PCR in the absence (pBAD18) or the presence of TrhRY (pBAD#3) or TrhR/Y and HtdA (pBAD#3 pACYC*htdA*) regulators.

539 **Table 1**. Bacterial strains and plasmids

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Strain	**Genotype**	**Reference**
MG1655	F$^-$, *ilvG*, *rph1*	(Guyer *et al.*, 1981)
AAG1	MG1665 Δ*lac*	(Aberg *et al.*, 2008)
MG1655 *flhD*::Cm	*flhD*::Cm, Cm^R	(Fontaine *et al.*, 2008)
AML	AAG1 *motA*::*lacZ*^{R}	This work
Plasmids	**Genotype**	**Reference**
R27	IncHI1, Tc^R	(Grindley *et al.*, 1972)
drR27	R27 *htdA*::IS10, Tc^R	(Gibert *et al.*, 2013)
R27*trhR*-Tmp	R27 *trhR*::Tmp^R	(Gibert *et al.*, 2014)
drR27*trhR*-Tmp	drR27 *trhR*::Tmp^R	
pRS551	*lac* based transcriptional fusion vector, Amp^R Km^R	(Simons *et al.*, 1987)
pRS-motA	P_{motA} cloned into pRS551	This work
pACYC184	Cloning vector, Cm^R, Tc^R	(Rose, 1988)
pACYC*htdA*	pACYC184 + *htdA*	(Gibert *et al.*, 2014)
pDAB18	Cloning vector, Amp^R	(Guzman *et al.*, 1995)
pBAD#3	pBAD18 carrying an *Eco*RI fragment of R27 (*trhRtrhY*)	(Gibert *et al.*, 2014)
pBAD*trhR*	pBAD18 + *trhR*	(Gibert *et al.*, 2014)
pBAD*trhY*	pBAD18 + *trhY*	(Gibert *et al.*, 2014)
pBAD*trhRtrhY*	pBAD18 + *trhRtrhY*	(Gibert *et al.*, 2014)

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548 **Table 2.** Primers used in this work

Primer	**Sequence 5'→3'**
FliC_F	GCTATCGCATCTGTAGACAA
FliC_R	GTAGTGGTGTTGTTCAGGTT
FliA_F	CTGAGGTAGCGGAACGTTTA
FliA_R	AGAGCTGGCTGTTATTGGTG
motA_F	ATGCAGTGCGTCAAAGTCAC
motA_R	GCTGGAATAGAGCGTTTTGC
flhC_F	AGCTTATGTCAACCGCCATC
flhC_R	GTGGGATAATATCGGCAGGA
flgM_F	CAAGCAAAACTGATGCAACC
flgM_R	ACGAATCGCCAGTTTTAACG
zwf_F	CACGCGTAGTCATGGAGAAA
zwf_R	CCAAGATAGTGGTCGATACG
motA-F-Eco	CCGGAATTCCCTTGAACAGYGCCCACAAG
motA-R-Bam	CGCGGATCCACCGCCGAAAACTGTACCGA

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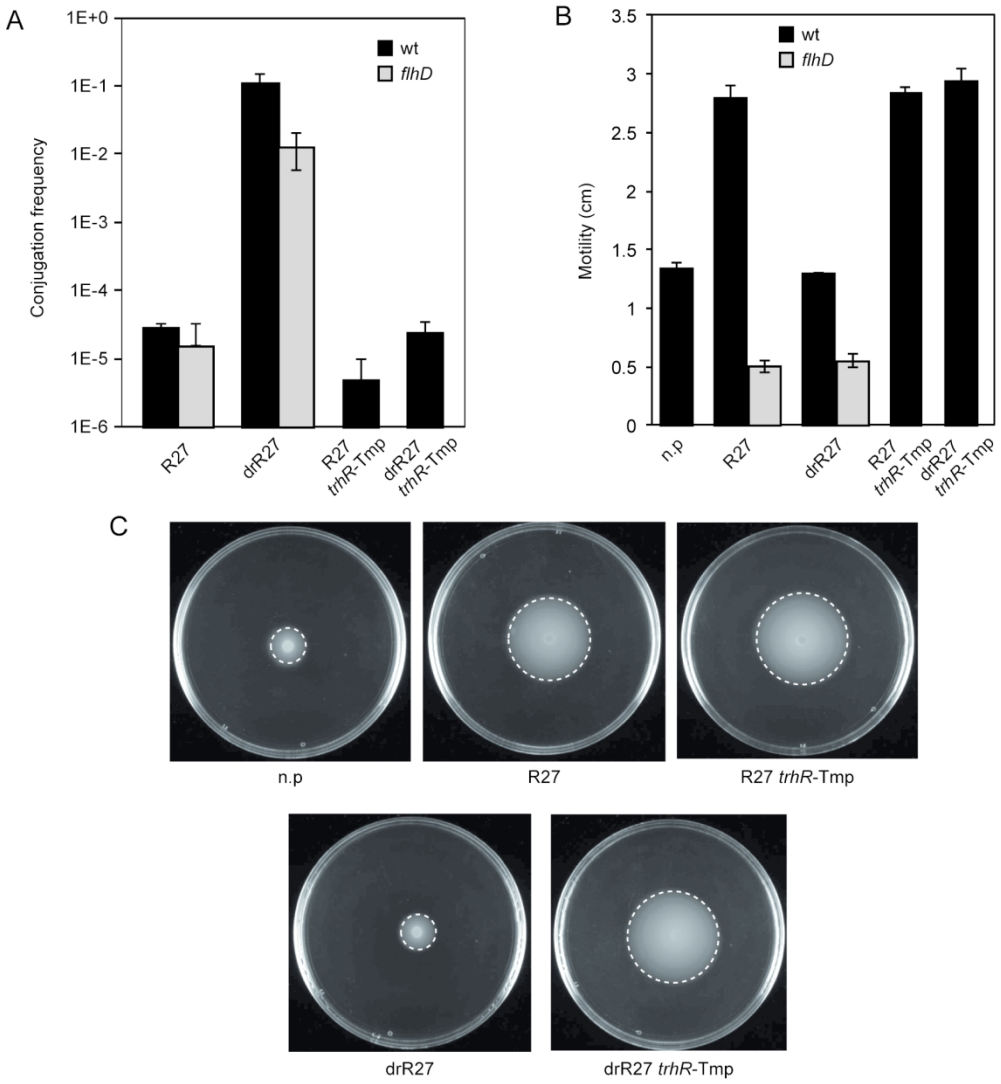

Figure 1

Figure 1. High conjugation frequency correlates with a reduction in the ability to swim

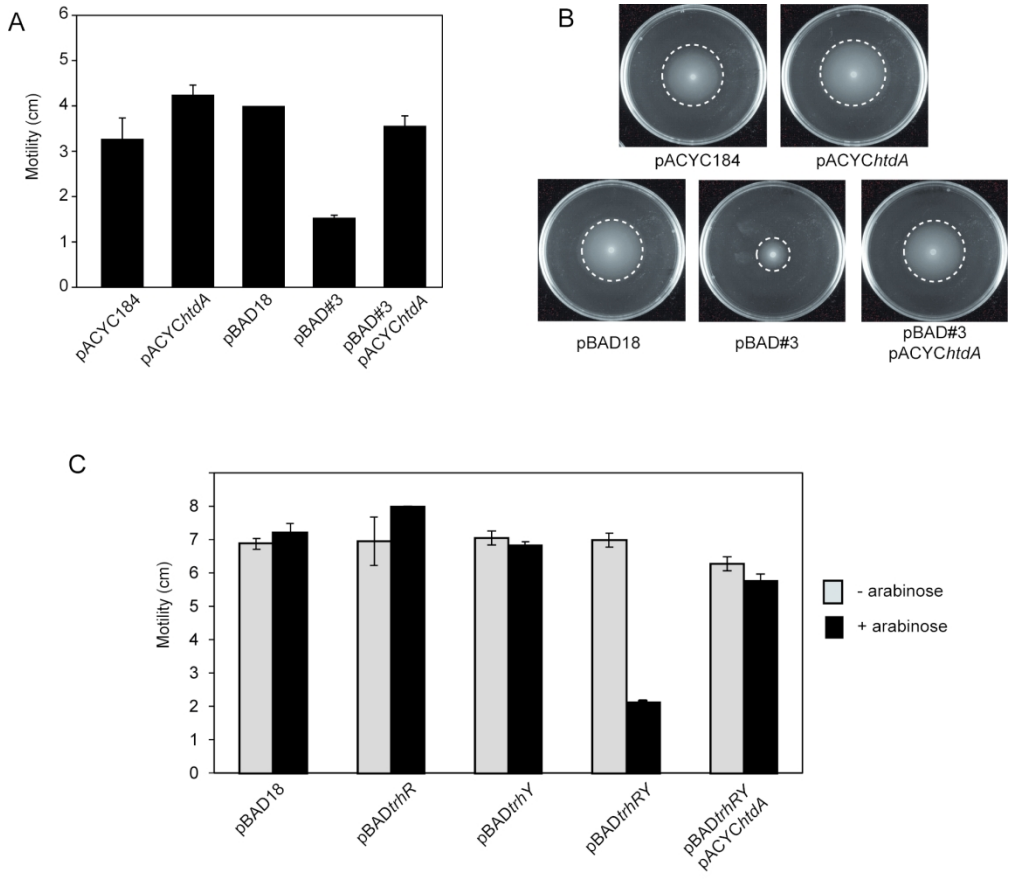

Figure 2

Figure 2. Effect of TrhRY and HtdA on the swimming phenotype

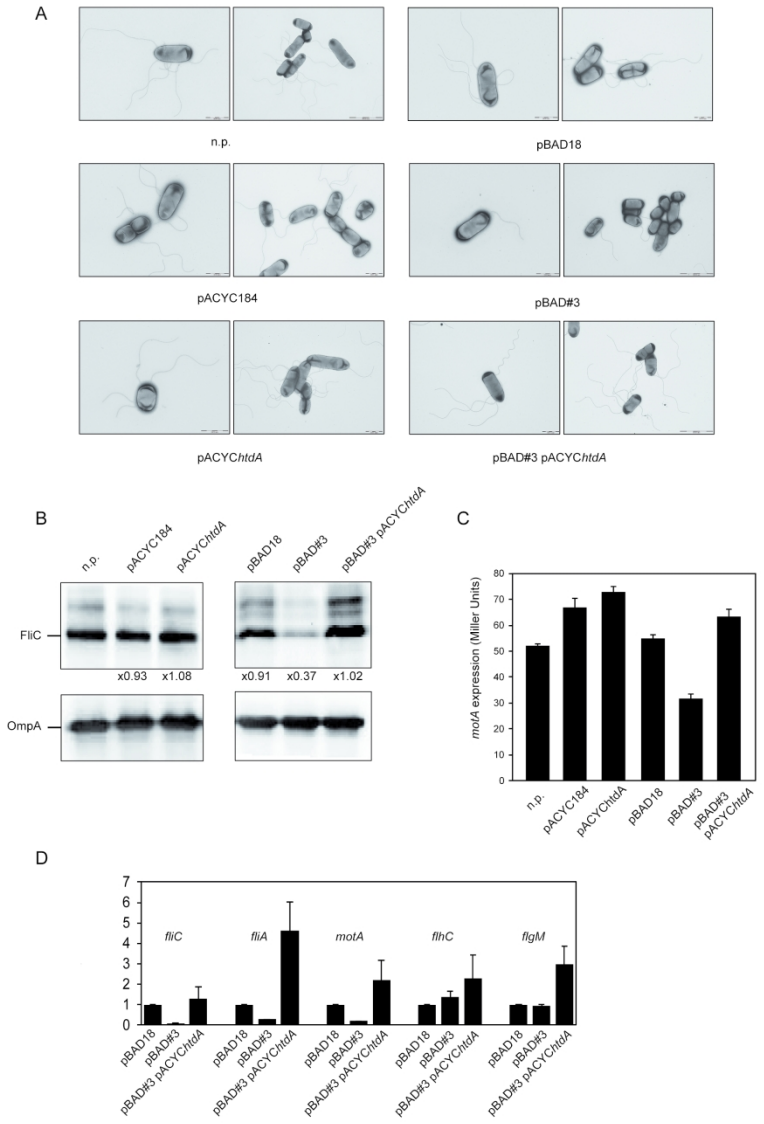

Figure 3

Figure 3. Effect of TrhR/Y and HtdA on the flagella biosynthesis