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New insights in the role of HtdA in the regulation of R27 conjugation

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ABSTRACT

R27 is the prototype of the IncHI group of conjugative plasmids, which are associated with multidrug resistance in several relevant pathogens. The transfer of this plasmid is thermodependent and all transfer-related genes are encoded in six operons (*tra* operons). Very little is known about the factors involved in the regulation of the R27 conjugation. This report describes transcriptional studies of the six *tra* operons. Our results indicate that HtdA, encoded in the R27 plasmid, is involved in the transcriptional repression of four *tra* operons (F, H, AC and Z). Although HtdA plays a pivotal role in the transcriptional regulation of those operons, it does not exert its effect as a classical repressor. The data indicate the existence of a crosstalk between HtdA and other unknown regulatory factors. The HtdA-mediated regulation of conjugation is independent of the R27 H-NS protein.

1. Introduction

Horizontal gene transfer (HGT), the transfer of genes between organisms, is a quantitatively relevant driving force of evolution in bacteria. In prokaryotes, the acquisition of new genes by HGT occurs via mobile genetic elements such as transposable elements, phages and plasmids. Metabolic capabilities, virulence-related functions and resistance to antimicrobial compounds are properties that often spread among bacteria through HGT. The term "mobilome" refers to all the mobile genetic elements present in the genome (Frost et al., 2005). In some bacterial species the percentage of the genome attributed to the mobilome is very high and it represent a highly dynamic gene pool that may vary notably between particular isolates (Toussaint and Chandler, 2012).

The extensive comparative genome analyses performed during the last fifteen years have confirmed the long-held belief that conjugative plasmids are important vehicles promoting the dissemination of genetic material between bacteria (Smillie et al., 2010). For instance, genes coding for metabolic pathways, toxins, secretion systems, adhesins, siderophores and antimicrobial-resistances have often been found in conjugative plasmids present in natural isolates (Schubert et al., 2004; Ong et al., 2008; Burgos et al., 2009; Nojiri, 2012). HGT, and particularly conjugative plasmid-mediated HGT, is responsible for rapid and broad dissemination of numerous antibiotic resistance determinants throughout diverse bacterial species (Bennett, 2008). As a result, bacterial conjugation has serious implications for public health. A broader knowledge of the regulatory pathways that control this process might help to design new therapeutic strategies and prevention measures to avoid the dissemination of antibiotic resistance among pathogens.

Conjugative plasmids of the incompatibility group (Inc) HI are often associated with multidrug resistance in major pathogens such as various serovars of *Salmonella enterica*. Nevertheless, IncHI plasmids have a broad host range including several enterobacteria and other Gram negative bacteria which are of environmental significance (Maher and Taylor, 1993). Plasmids from the IncHI group are large

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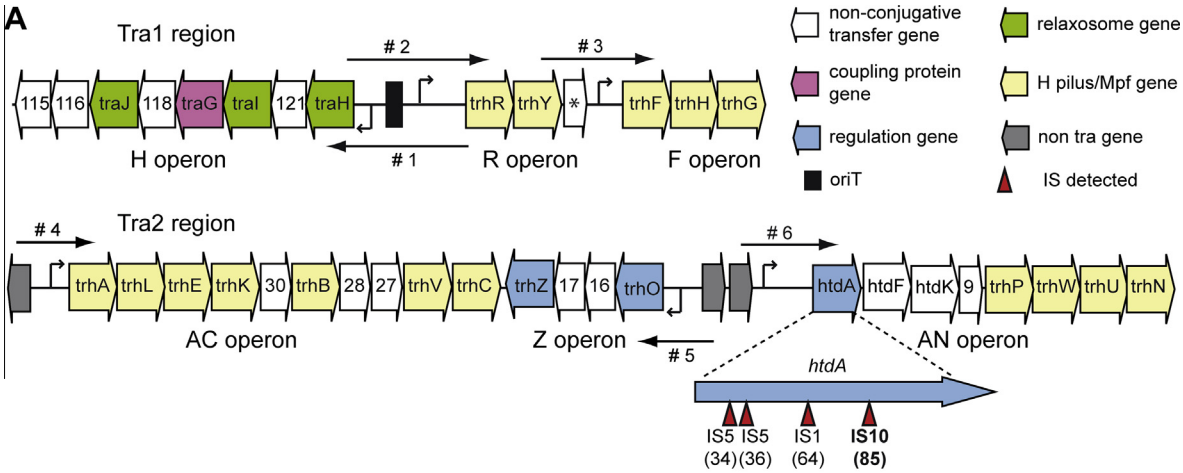

B

	fusion	From	To	UTR (nt)	Fw primer	Rv primer
# 1	H::*lacZ*	-767	+82	15	**gaattc**gcgtgatgttatctgattcgt	**ggatcc**gaacatcgatttgtgagcgc
# 2	R::*lacZ*	-690	+162	137	**gaattc**gaacatcgattttgtga	**ggatcc**gcgtgatgttatctga
# 3	F::*lacZ*	-627	+106	21	**ggatcc**ggcaatagcacggtattccc	**ggatcc**tgctcttcatttgcatgg
# 4	AC::*lacZ*	-571	+208	35	**ggatcc**gaatggacccataaaaggagc	**ggatcc**tcatgttaggcccagatatc
# 5	Z::*lacZ*	-620	+51	20	**gaattc**gagtttgattcagccacagc	**ggatcc**gatcaattgtctggacgtgc
# 6	AN::*lacZ*	-702	+166	138	**gaattc**gcaaacctcgcaaatcttgc	**ggatcc**gtgtattagtgagttgcgtg

Fig. 1. (A) Schematic representation of the genetic organization of the six *tra* operons in the Tra1 and Tra2 regions of R27 (adapted from (Alonso et al., 2005)). The defined ORF are indicated as boxes with arrowheads and the color code indicates the attributed functional category. The numbered arrows indicate the regions PCR-amplified with the primer pairs (indicated in B) used to construct the *lacZ* transcriptional fusions with the promoter sequences of the six *tra* operons. In the R operon the third ORF labelled with an asterisk corresponds to a short putative ORF of 56 bp, originally defined as *trhX*. However, experimental determination of the transcripts of the putative ORFs by Alonso et al. (2005) did not detect any mRNA containing the sequence of *trhX*. The *htdA* gene of the Tra2 region has been magnified to indicate different mutations caused by the insertion of ISs described in this study. The specific IS is designated and the codon where the insertion was found is shown in brackets. The IS*10* at codon 85 is labelled in bold to highlight that it was found in three independent clones. (B) Information regarding the *lacZ* transcriptional fusions with the promoter sequences of the six *tra* operons constructed and transferred to the *attB* locus of AAG1 strain. For each fusion (1–6, as labelled in A) the operon promoter sequence fused to *lacZ* is indicated, along with the coordinates of the fragment cloned in pRS551, using as +1 the transcriptional start described by 5′-RACE in (Alonso et al., 2005), the UTR defined for each operon and the primer pair used for the PCR-amplification. The sequence of the restriction site introduced during PCR amplification for cloning purposes is shown in bold. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

molecules, commonly larger than 150 kb, and their conjugation is thermosensitive. The optimal conjugation ratio is observed at low temperatures (22–30 °C) under standard experimental conditions (Taylor and Levine, 1980). This may suggest that dissemination of these plasmids is promoted during the transit of the pathogens in the environment outside the host (Maher et al., 1993).

The prototype of the IncHI1 group is the plasmid R27, which was isolated from a tetracycline-resistant isolate of *S. enterica* serovar Typhimurium in 1961 in the UK. Similar plasmids have also been isolated from Typhi and Paratyphi serovars (Phan and Wain, 2008). R27, a large plasmid of approximately 180 kb, contains 210 putative ORF. Interestingly, the function of more than 65% of the R27 ORFs is unknown, and no similarities with other described genetic elements have been found (Sherburne et al., 2000). One might expect that the acquisition of this huge amount of genetic material of unknown function by the recipient cells might affect the host physiology and provide new phenotypic properties. Further studies are required to elucidate the impact of conjugative plasmids uptake on bacterial cell biology and fitness. All genes coding for the functions related to R27 conjugation are clustered in six polycistronic operons distributed in two separate regions, named Tra1 and Tra2 (Fig. 1), which are 64 kb apart. The Tra1 region carries the origin of transfer (oriT) and operons H, R and F. The Tra2 region carries the AC, Z and AN operons and genes involved in plasmid partitioning and stability (Alonso et al., 2005; Sherburne et al., 2000).

Very little is known about the regulatory factors involved in the modulation of IncHI plasmids conjugation. R27 carries genes coding for paralogues of H-NS and Hha, two proteins involved in the regulation of gene expression in response to changes in environmental factors. In a previous study we reported that the H-NS and Hha proteins encoded by the R27 plasmid, as well as their chromosomal paralogues, play an important role in maintaining low ratios of R27 conjugation at non-permissive temperatures (Forns et al., 2005). In addition, *htdA*, a gene located in the R27 Tra2 region, was shown to be involved in conjugation repression during a genetic screen searching for mutants with an increased conjugation rate (Whelan et al., 1994).

In this report, transcriptional studies of the six *tra* operons were performed. The results indicate that the

expression of four of the six *tra* operons is tightly regulated by factors encoded in the R27 plasmid, whereas the transcriptional expression of both R and AN operons is R27-independent. The characterization of spontaneous randomly selected mutants with derepressed conjugation suggests that HtdA plays a pivotal role in the transcriptional repression of the four R27-dependent *tra* operons, although it does not act as a classical repressor. In addition, the data show that other R27-encoded regulators are involved in the HtdA-mediated pathway that controls R27 conjugation.

2. Material and methods

2.1. Bacterial strains, plasmids and growth conditions

The *Escherichia coli* strains used are MG1655 (Guyer et al., 1981) and its Δ*lac* derivative AAG1 (Aberg et al., 2008). Plasmid R27 (Grindley et al., 1972) and drR27 (a spontaneous *htdA* derivative, randomly selected) were a generous gift from Taylor's group. Bacteria were routinely grown in LB (10 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract). For conjugation experiments, strains were grown in PB medium (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 $K_2HP_4{\cdot}3H_2O$). M9 minimal media plates were used with the following composition: 1 × M9 salts (Sambrook et al., 1989), 0.2% lactose, 10 μM thiamine and 1.5% bactoagar. When needed, antibiotics were added at the following concentrations: tetracycline 15 μg/mL, chloramphenicol 20 μg/mL, kanamycin 25 μg/mL. LB agar plates were supplemented with 40 μg/mL Xgal and 0.5 mM IPTG, when required, to monitor the *lac* phenotype.

2.2. Conjugation assays

The conjugation assays were performed as in (Forns et al., 2005). Briefly, donor and recipient strains were grown statically in PB medium at 25 °C for 16 h. Bacterial cells were washed to eliminate antibiotics. Recipient and donor cell suspensions were mixed at a ratio of 4:1, and two volumes of PB medium were added. After two hours static incubation at 25 °C, serial dilutions were plated in appropriate media. The conjugation rate was calculated as transconjugants/donor cells. The average of three independent experiments plotted with standard deviation is shown.

The experiment layout used in conjugation assays to differentiate donor from transconjugant cells was based on the *lac* phenotype. In all cases, AAG1 (MG1655 Δ*lacZ*) strain was used as the donor and its *lacZ*$^+$ counterpart, MG1655, as the recipient. Therefore, transconjugants were selected as *lacZ*$^+$ Tc^R colonies either on LB agar plates supplemented with X-gal and IPTG or on M9 minimal medium containing lactose.

2.3. β-Galactosidase assay

β-Galactosidase assays were performed as described by Miller (1992). Data are mean values of duplicate determinations in at least three independent experiments plotted with standard deviations.

2.4. Genetic techniques

Basic molecular genetic manipulations were performed essentially as described previously (Sambrook et al., 1989). All genetic constructions were confirmed by DNA sequencing. *lacZ* fusions were constructed with each of the six promoters described to control the expression of the *tra* genes. PCR amplification of each of the six intergenic regions was performed using the primer pairs indicated in Fig. 1. For cloning purposes, primers were designed to incorporate either an *Eco*RI or a *Bam*HI restriction site (forward primers) and *Bam*HI restriction sites (reverse primers). The PCR-amplified fragments were cloned in pGEM-T and subsequently in pRS551, either in *Bam*HI or in *Eco*RI-*Bam*HI sites. The resulting constructs were transferred to the chromosome of the AAG1 strain, in the *attB* lambda site, using previously described protocols (Simons et al., 1987). Controls to confirm single gene fusion insertions in the *attB* locus were performed for all fusions. The *htdA* gene from the R27 plasmid was PCR-amplified using the primer 5′-GGATCCGTCAGCTTACCAGCTGGC-3′ and 5′-AAGCTTCCATTAAAGGG GGATTACC-3′, which introduced one *Bam*HI and one *Hin*dIII restriction site (underlined) respectively. The *htdA* allele containing the promoter sequences (519 bp upstream of the ATG are included in the PCR-amplified fragment) was cloned in pGEM-T and subsequently the *Bam*HI-*Hin*dIII fragment was cloned in the pACYC184 vector; the resulting plasmid was pACYC*htdA*.

For *htdA* genotyping the primers htdA-fw (5′-CCAGTTCCTCTCCAATCAAG-3′) and htdA-rv (5′-GTTACCCTAACCCATAAGATC-3′) which PCR-amplify a fragment of 550 bp of the wild type *htdA* allele were used.

2.5. Chemotaxis assay

The chemotaxis response was analyzed on TB (1% tryptone, 0.5% NaCl) containing 0.25% agar and supplemented with maltose at 2 mM final concentration. Overnight LB bacterial cultures at 37 °C were spotted (5 μL) on the centre of the plates and incubated 12 h at 25 °C. The data are representative of two independent experiments with four replica plates for each strain.

2.6. HgaI1 phage infection assays

The IncH-specific phage Hgal1 was propagated in an *E. coli* strain harbouring the R27 plasmid as described previously (Kannoly et al., 2012). The phage sensitivity assay, which provides a simple method to estimate the H-pili production, was performed using standard overlay methodology at 25 °C.

3. Results and discussion

3.1. Expression profile of the tra operons either in the presence or in the absence of the R27 plasmid

Transcription studies were performed to determine whether the R27 plasmid carries genes encoding regulatory factors that are relevant in the modulation of *tra*

operon expression. The expression of each of the six *tra* operons was monitored under permissive conjugation conditions (25 °C) in the absence/presence of either the R27 plasmid or a spontaneously selected derepressed R27 derivative (drR27), which showed a high frequency of conjugation. Transcriptional studies were performed using chromosomal *lacZ* fusions with each of the six promoter regions of the *tra* operons (see Section 2 and Fig. 1). In all fusions, to ensure that the amplified and cloned sequences contained the whole intergenic region, the 5′ end of the amplified fragment was positioned within the coding sequence of the upstream located gene.

Transcription results, measured as β-galactosidase activity, are shown in Fig. 2. When comparing the expression of the six *tra* operons in the presence or absence of R27 (grey and white bars), no important differences were observed. Interestingly, four of the six operons (H and F from Tra1 and AC and Z from Tra2) showed very low levels of expression (below 60 MU) whereas the R (Tra1) and AN (Tra2) operons had a very high level of expression (above 3000 MU) even in the absence of the R27 plasmid. Interestingly, major changes were detected in the expression profile in the presence of drR27. The four operons with a low level of expression either in the presence or absence of R27 were significantly induced in the presence of drR27. The expression rose more than 10-fold when drR27 was present for the F, H and AC operons, whereas the increase observed for the Z operon was only four-fold. On the other hand, the R and AN operons, which have a very high level of expression even in the absence of the plasmid, did not share the same expression profile as the F, H, AC and Z operons. The presence of drR27 did not cause a further increase in their expression: in fact R operon expression was reduced by up to 50% in the presence of drR27, while AN operon expression was not significantly affected. As for the conjugation experiments, the six reporter strains were grown statically during 16 h (Fig. 2). Nevertheless, an identical expression profile was observed when the reporter strains were grown with intense aeration up to an OD_{600nm} of 2.0 (data not shown).

The fact that the presence of the R27 derivative with an increased frequency of conjugation (drR27) significantly induced the expression of four *tra* operons suggests that the spontaneous mutation(s) accumulated in the drR27 plasmid affects the transcriptional regulation of F, H, AC and Z operon expression. This prompted us to further characterize the drR27 plasmid.

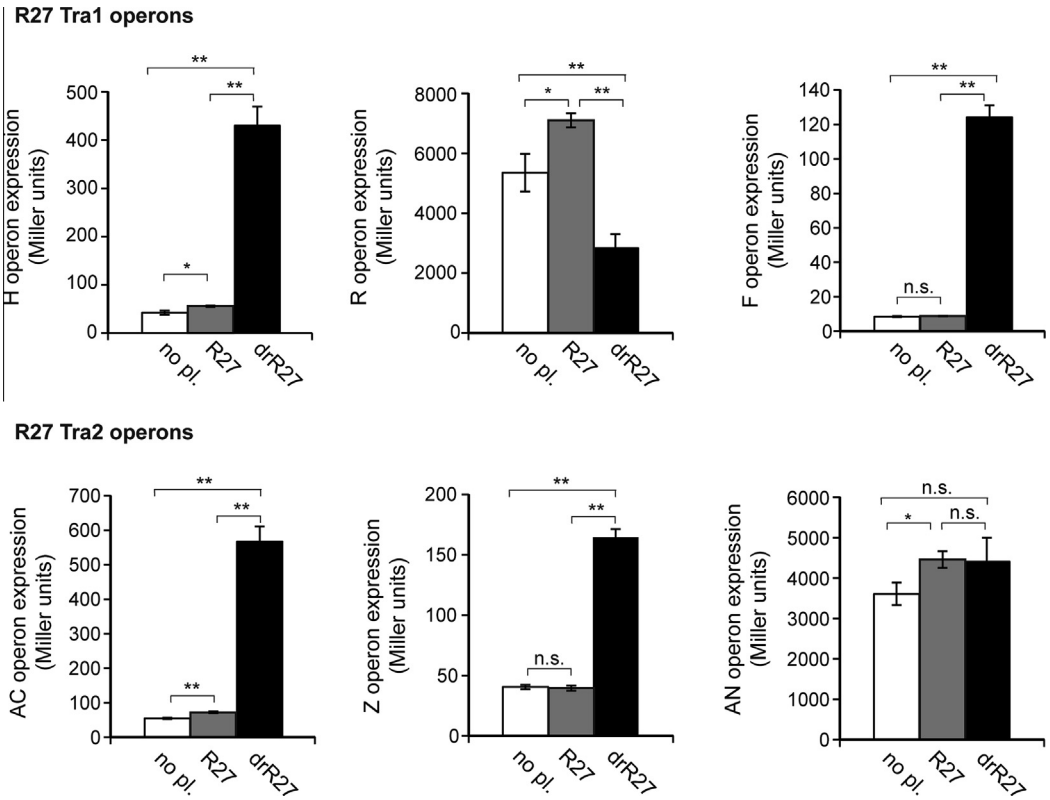

Fig. 2. Transcriptional expression of the promoter sequences of the six *tra* operons. The strains used were derivatives of AAG1 (MG1655 Δ*lacZ*) containing each of the *lacZ* fusions with one of the six *tra* operons, as indicated. Cultures of the strains lacking any conjugative plasmid or carrying either the R27 or drR27 (a spontaneous derepressed derivative randomly selected and discovered serendipitously) were grown statically at 25 °C in LB for 16 h and transcriptional expression was monitored as β-galactosidase activity (Miller units). Data are mean values of duplicate determinations of at least three independent cultures plotted with standard deviations. Statistical differences between average values were tested for high significance (p-value < 0.005) by performing an unpaired, two-tailed Student's t test. The resulting p-values are presented by the following symbols: n.s. = non-significant ($p > 0.05$); $^{*}p < 0.05$; $^{**}p < 0.005$.

3.2. The drR27 plasmid carries an IS10 insertion in the htdA gene located in the AN operon

Due to the low frequency of R27 conjugation, derepressed R27 variants have been extensively used to study different aspects of IncHI plasmid biology (Alonso et al., 2005; Lawley and Taylor, 2003; Kannoly et al., 2012). The drR27 plasmid used in this study was randomly selected. A previous study reported that a mutation in the *htdA* locus of the AN operon causes derepression of R27 conjugation (Whelan et al., 1994). Therefore, the *htdA* genotype of the drR27 derivative used in this study was tested by PCR amplification using specific primers (see Section 2). The PCR-amplified fragment from the drR27 plasmid had a molecular size of 1.8 Kb, higher than the *htdA* gene, which is 0.5 Kb (Fig. 3A and (Sherburne et al., 2000)). Sequence analyses of the PCR-amplified fragment demonstrated that the *htdA* gene in the drR27 plasmid carries an IS*10* insertion in the codon 85 (Fig. 1). Therefore, the drR27 plasmid used was an *htdA* mutant derivative, most probably randomly selected during previous conjugation experiments.

Conjugation frequency analyses were performed with drR27 and R27 derivatives. The results, shown in Fig. 3B, corroborate the previous data suggesting that HtdA acts as a repressor of R27 conjugation (Whelan et al., 1994). Trans-complementation of the *htdA* mutation of the drR27 plasmid using a pACYC184 derivative carrying the *htdA* gene significantly reduced the conjugation ratio, although the levels of conjugation were higher than with the R27 plasmid. This may be a consequence of the high *htdA* gene dose when cloned in the pACYC184 vector. Moreover, bearing in mind that *htdA* is the first gene in the AN polycistronic operon (Fig. 1), we cannot rule out a possible polar effect of the IS*10* insertion. Nor can we dismiss at this point the existence of additional mutations accumulated in the drR27 plasmid. Nevertheless, these data clearly indicate that the drR27 plasmid has a high frequency of conjugation due to the *htdA*::IS*10* insertion.

Several reports indicate negative crosstalk between the expression of conjugative apparatus and motility (Barrios et al., 2006; Reisner et al., 2012). Swimming motility of the strains carrying R27, drR27 and drR27/pACYC*htdA* was tested (Fig. 3C); comparing *htdA*$^+$ and *htdA*$^-$ genetic backgrounds, motility was notably repressed when conjugation was derepressed (*htdA*$^-$).

By using infection assays with Hgal1 phage, an H pilus specific bacteriophage, we were able to monitor H pilus production. Sensitivity to Hgal1 phage was determined for the strains carrying R27, drR27 and drR27/pACYC*htdA*. The low conjugation frequency with an *htdA*$^+$ background

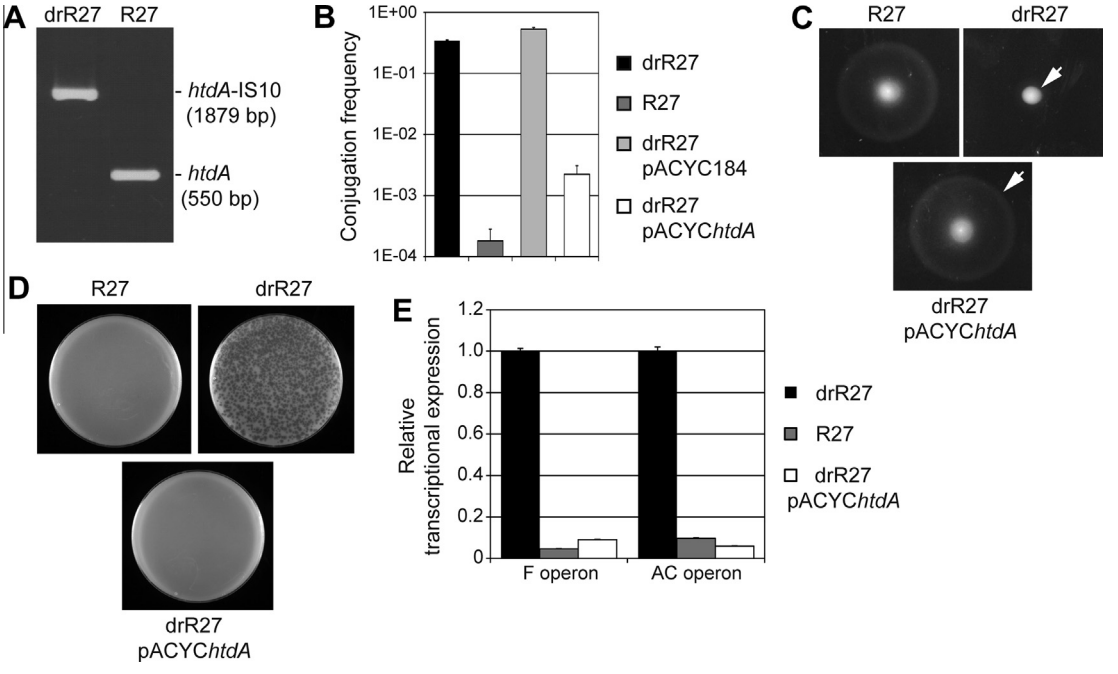

Fig. 3. Phenotypic characterization of the *htdA* derivative of R27. (A) *htdA* PCR genotyping using primers htdA-fw and htdA-rv and the strains containing drR27 and R27 as template. The nucleotide sequence of the PCR-amplified fragments was determined, and the size of the fragments and its remarkable features are indicated on the right side of the picture. (B) *htdA* mutation causes a significant increase in the conjugation frequency of R27 at 25 °C. Donor strains were derivatives of AAG1 carrying the plasmids, as indicated by the color code. The recipient strain was MG1655. Conjugation frequencies of three independent experiments were calculated and average values with standard deviations are shown. (C) Movement on 0.25% agar TB plates containing maltose after 12 h incubation at 25 °C was tested for the following strains: AAG1/R27 (*htdA*$^+$), AAG1/drR27 (*htdA*$^-$) and AAG1/drR27 + pACYC*htdA* (*htdA*$^+$). The limit of the bacterial growth is indicated with a white arrow. (D) Hgal1 bacteriophage infection test of cultures of the same strains as in C. (E) Transcriptional expression of the F (Tra1 region) and AC (Tra2 region) operons was determined in LB cultures grown under shaking conditions (200 rpm) at 25 °C up to an OD600 nm of 2.0 of the AAG1 derivatives strains carrying the F::*lacZ* and AC::*lacZ* fusions respectively. Assays were performed for each derivative carrying either the drR27 (*htdA*$^-$), R27 (*htdA*$^+$) or drR27 + pACYC*htdA* (*htdA*$^+$). Data are mean values of duplicate determinations of at least three independent cultures plotted with standard deviations.

correlated with a drastic decrease in the production of conjugative pili in those strains (Fig. 3D). Taken together, these results indicate that the IS*10* insertion in the *htdA* locus causes a considerable derepression of H pilus production, and consequently of R27 conjugation.

3.3. Mutation in the htdA locus causes a substantial derepression of the H, F, AC and Z tra operons

In view of the results shown in Figs. 2 and 3, we hypothesized that the *htdA* mutation detected in the drR27 plasmid was responsible for the F, H, AC and Z operon derepression. To test this hypothesis, we performed trans-complementation experiments. Transcriptional analysis of the F (Tra1 region) and AC (Tra2 region) operons in the presence of drR27 plasmid (*htdA*$^-$) complemented in *trans* with the pACYC*htdA* plasmid corroborated this *htdA* dependency (Fig. 3E). The presence of *htdA* causes a drastic reduction in the transcriptional expression of the two operons. In this case, full complementation was observed, since the presence in trans of *htdA* reduces the expression to similar levels as when the R27 plasmid was present.

HtdA was already identified as a repressor of R27 conjugation by transposon mutagenesis experiments searching for mutants with higher HgaI sensitivity, that is, mutants with increased expression of the H pilus, which results in increased conjugation (Whelan et al., 1994). However, the level at which HtdA was regulating the conjugation process remained unclear. Here we report that HtdA is involved in the transcriptional regulation of four of the *tra* operons (F, H, AC and Z) which are essential for R27 conjugation. Moreover, when comparing the transcriptional expression of these *tra* operons in the absence of R27 or in the presence of either the *htdA*$^+$ or *htdA*$^-$ R27 derivatives (Fig. 2), we conclude that the repressive effect of HtdA on the transcriptional expression cannot be attributed to classical direct repression. It should be stressed that in the absence of any R27 derivative (absence of HtdA) there is no derepression of the four *tra* operons (white bars in Fig. 2). Induced expression was only detected in the presence of a R27 *htdA*$^-$ plasmid (black bars in Fig. 2). On the basis that HtdA is a repressor, as originally described, our data would suggest that a positive regulator encoded in the R27 plasmid is required for full expression of the four *tra* operons and the consequent induction of R27 conjugation. In this case, we postulate that either the expression or the activity of this unknown regulator would be repressed by HtdA. At this stage the exact interplay between HtdA and the putative positive regulator remains unknown. HtdA may repress the expression of the positive regulator of the *tra* operons, or it may also counteract the effect of the positive regulatory pathway either by binding the positive regulator, causing its inactivation, or by binding to the DNA and avoiding its stimulatory effect on transcription. Alternatively, with the data available, we cannot rule out the possibility that HtdA might act as an activator of gene expression; if so, it would be stimulating the expression or activity of a repressor of the transcriptional expression of the *tra* operons.

In silico studies of the HtdA sequence have shown that HtdA is a 150 amino acid protein with an estimated molecular weight of 16.6 kDa and a theoretical pI of 4.63, which does not have any putative DNA binding domain. Further studies will be required to elucidate the molecular mechanism by which HtdA regulates transcriptional expression of the *tra* operons, modulating the transference of R27 between bacterial cells.

As mentioned above, the AN and R operons have a distinct transcriptional expression profile. Their expression does not rely heavily on the presence of either HtdA or any other R27-encoded factor since in the absence of any R27 derivative, a high level of transcriptional expression was detected. The *htdA* mutation merely causes a two-fold reduction in the R operon expression. The biological significance of this down-regulation is unknown. Moreover, no relevant effect was observed for the AN operon. Bearing in mind that *htdA* is located in the AN operon, this indicates that HtdA does not regulate its own expression.

There are common features that allow classification of the six *tra* operons into two groups according to the expression profiles observed. Group I comprises the F, H, AC and Z operons, which require directly or indirectly R27-encoded factor(s) in order to achieve a high level of expression (R27-dependent operons), factor(s) which are somehow counteracted by HtdA. Group II comprises the R and AN operons, which are fully induced in the absence of the conjugative plasmid (R27-independent operons). Therefore, our results suggest the presence of a regulatory pathway encoded totally or partially in R27 that would control the expression of the four R27-dependent *tra* operons. In this pathway, HtdA plays a crucial role promoting the silencing of the R27-dependent operons.

3.4. The repressive effect of HtdA is independent of H-NS

In a previous study, we reported that the H-NS/Hha complex regulates R27 conjugation. In enterobacteria, in response to environmental factors such as temperature, H-NS and Hha form a nucleoprotein complex that represses transcriptional expression (Nieto et al., 2000). The H-NS and Hha paralogues encoded in the R27 plasmid have been reported to repress R27 conjugation under non-permissive conditions (37 °C) (Forns et al., 2005). To test whether the H-NS/Hha complex might be involved in HtdA-mediated regulation, R27 conjugation frequencies in wild type and *hns* derivatives were measured either in the presence or in the absence of HtdA at the permissive temperature of conjugation (25 °C). The results shown in Fig. 4 clearly indicate that HtdA regulation is independent of H-NS, since no significant differences were found when comparing the effect of the *htdA* mutation on R27 conjugation in both wild type and *hns* mutant strains.

3.5. Random insertions in the htdA locus suggest that HtdA is a pivotal factor in the transcriptional control of the conjugative apparatus

Interestingly, when examining at the *lac* phenotype of the R27 transconjugants for the strains carrying the R27-dependent operon fusions on Xgal plates, darker blue colonies appeared at low frequency, indicating that these clones have a derepressed expression of the *lacZ* fusions.

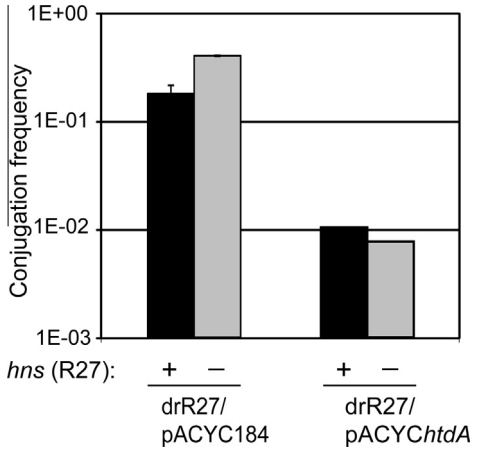

Fig. 4. At 25 °C the effect of *htdA* on the frequency of conjugation was independent of the H-NS paralogue encoded in R27. Donor strains were AAG1/pACYC184 and AAG1/pACYC*htdA* carrying either the plasmid drR27 (*htdA*$^-$) or drR27*hns* (*htdA*$^-$ *hns*$^-$). The recipient strain was MG1655. Conjugation frequencies of three independent experiments were calculated and average values with standard deviations are shown.

Bearing in mind the previous observation that an IS*10* insertion in *htdA* causes derepression of the *tra* operons mentioned (Fig. 2), the *htdA* genotype was determined for five independent clones showing a dark blue phenotype. Interestingly, the *htdA* gene was mutated by transposition of ISs in all of those clones. Sequence analyses revealed two IS*5* insertion mutants (codons 34 and 36), one IS1 mutant (codon 64) and two IS*10* mutants (in codon 85) (Fig. 1A). In the IS*10* insertion mutants isolated, IS*10* was located at the same site as the original drR27 plasmid used. The fact that all five independent clones with deregulated *tra* operon expression have mutations located in the *htdA* locus suggests that HtdA is the master regulator of *tra* operon transcriptional expression and is consequently a crucial modulator of R27 conjugation.

4. Concluding remarks

The conjugation of IncHI plasmids is a tightly regulated process that depends on environmental factors such as temperature. However, very little is known about the cellular factors involved in the regulation of the *tra* operons, which encode for the type IV secretion apparatus and other proteins involved in R27 transfer. In this report, we demonstrate the presence of a complex regulatory pathway that includes regulatory factors encoded by R27. The HtdA protein is a crucial factor in this regulatory pathway, which is involved in the repression of conjugation, although it does not act as a classical repressor. Moreover, other regulators, also encoded by R27, are involved in *tra* operon regulation. Further studies including random mutagenesis experiments, screening of overexpressing libraries to identify these regulators and biochemical studies with purified HtdA protein will be required to understand the regulatory mechanisms that are involved in the control of the expression of the *tra* operons and which therefore modulate the transfer of IncHI plasmids between bacteria.

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