Microbiology (2005), **151**, 3563–3573 DOI 10.1099/mic.0.28256-0

Transcriptional organization of the temperature-sensitive transfer system from the IncHI1 plasmid R27

Guillermina Alonso,[1] Kelly Baptista,[2] Trinh Ngo[2] and Diane E. Taylor[2]

[1]Instituto de Biología Experimental, Facultad de Ciencias, Universidad Central de Venezuela. Caracas, Venezuela

[2]Department of Medical Microbiology and Immunology, 1-63 Medical Sciences Building, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

Correspondence
Diane E. Taylor
diane.taylor@ualberta.ca

Received 10 June 2005
Revised 17 August 2005
Accepted 13 September 2005

One of the characteristic features of IncHI1 plasmids is a thermosensitive process of conjugation, which is optimal between 22 °C and 30 °C but inhibited at 37 °C. R27, the prototypical IncHI1 plasmid, contains transfer genes clustered in two regions of the plasmid, Tra1 and Tra2. In the present study, transcriptional analyses of the *tra* genes were undertaken at both 30 °C and 37 °C. Screening of 38 *tra* genes showed that *tra* genes are transcriptionally linked in six operons, three in each Tra region. RT-PCR analysis showed that gene expression was reduced at 37 °C relative to that observed at 30 °C. The transcription start sites of the six transcripts were identified, promoters and upstream regions were cloned, and transcription was tested at both temperatures. In cells grown at 37 °C, in the presence of R27, the promoters were inhibited, except for promoters of the H operon and AN operon. Conditions that influenced DNA topology, such as osmolarity, anaerobiosis, quorum sensing and acidity, showed no significant influence on transfer frequency. These results should facilitate future understanding of the basis of temperature-sensitive transfer in this large conjugative plasmid.

INTRODUCTION

Gene exchange in bacteria promotes adaptation to environmental challenges and the evolution of bacterial species. Conjugation is one of the main mechanisms responsible for horizontal gene transfer. This process provides competitive advantages for the bacteria and has serious implications for public health. It allows the maintenance and dispersal of genes that encode antimicrobial resistance in closely and distantly related bacteria. Conjugative plasmids transfer from donor to recipient bacteria using a structure termed the mating pair formation (Mpf) complex, also known as the type IV secretion system (T4SS). The T4SS is a membrane-associated protein complex, responsible for construction of the pilus and for DNA transfer. The inner-membrane-associated coupling protein connects the Mpf proteins to the cytoplasmic relaxosome, which is responsible for DNA processing prior to and during conjugative transfer (Cascales & Christie, 2004; Christie, 2001; Lawley *et al.*, 2003b).

Conjugative plasmids belonging to the incompatibility group HI1 (IncHI1) encode multiple antibiotic resistances; they have been implicated as a significant factor in the persistence and re-emergence of *Salmonella enterica* serovar Typhi, the causative agent of typhoid fever, and therefore contribute to the persistence of typhoid fever worldwide (Kariuki *et al.*, 2004; Wain & Kidgell, 2004). The continued prevalence of IncHI1 plasmids can be partially attributed to the conjugative transfer functions of these plasmids, since horizontal gene transfer maintains a plasmid within a bacterial population. Therefore, the conjugative system is a promising drug target to inhibit the dissemination of plasmid-encoded antibiotic resistance.

IncHI1 plasmids are low-copy-number, self-transmissible plasmids that have an unusual thermosensitive process of conjugation. Transfer occurs optimally between 22 °C and 30 °C, but is negligible at 37 °C (Rodriguez-Lemoine *et al.*, 1975; Taylor & Levine, 1980). Despite the fact that this phenomenon was known three decades ago, the molecular basis for this temperature dependence has not been widely studied.

Plasmid transfer requires the expression of plasmid-encoded transfer genes (*tra* genes). R27, the prototypical IncHI1

Abbreviations: CAT, chloramphenicol acetyltransferase; drR27, derepressed R27; RACE, rapid amplification of cDNA ends; T4SS, type IV secretion system; UTR, untranslated region.

Tables listing oligonucleotide primers (Table S1) and showing SD values for the effect of temperature and H-NS protein on the expression of *tra* promoters (Table S2) are available as supplementary data with the online version of this paper.

plasmid, has been sequenced (Sherburne *et al.*, 2000), and the transfer genes were found to be clustered in two separate regions on the plasmid, Tra1 and Tra2, which are separated by 64 kb. The Tra1 region contains the origin of transfer and 14 genes, nine of which are essential for transfer and encode Mpf components and the coupling and relaxosome proteins (Lawley *et al.*, 2002) (see Fig. 1a). The Tra2 region contains 24 genes, including 11 which are essential for transfer and encode Mpf proteins (Lawley *et al.*, 2003a; Rooker *et al.*, 1999) (see Fig. 2a). Although *tra* genes have been identified by sequencing and functional and mutational analyses, transcriptional regulation has not been examined and the promoter regions have not yet been determined.

Efforts to isolate a mutant of R27 showing temperature-independent transfer have been unsuccessful, implying that more than one mechanism could be present to thermally regulate plasmid transfer. Recent evidence indicates that at least one transfer gene, *trhC*, is not expressed at non-permissive transfer temperatures, suggesting that thermosensitive transfer may be imposed at the transcriptional level (Gilmour *et al.*, 2001). Nevertheless, the mechanism(s) underlying this phenomenon has not yet been revealed. In other conjugative plasmids it has been reported that transfer genes are often controlled by intricate mechanisms that involve both positive and negative regulation (Adamczyk & Jagura-Burdzy, 2003; Frost *et al.*, 1994).

Studies on other temperature-dependent expression systems, mainly virulence genes, have shown that bacterial genes usually become derepressed on a shift from an ambient low temperature to body temperature upon entering a human host (Madrid *et al.*, 2002; Prosseda *et al.*, 2002; Umanski *et al.*, 2002). In contrast, the effect of temperature on transfer of IncHI plasmids is the reverse, i.e. conjugation is repressed at 37 °C.

The goal of the present study was to analyse transcription of R27 transfer genes at 30 °C and 37 °C and to identify the operon organization and transcriptional start points. In addition, the promoters and regulatory regions were cloned and tested for expression. Our results suggest that R27 *tra* genes are controlled at the transcriptional level. This work provides new insights into the genetic structure, transcriptional organization and regulation of transfer genes on the R27 plasmid.

METHODS

Bacterial strains, growth conditions and plasmids. *Escherichia coli* strains and plasmids used in this study are listed in Table 1. *E. coli* strains were routinely grown at 30 °C or 37 °C in LB broth (Difco) with shaking, or on LB agar plates. Antibiotics were added at the following final concentrations when appropriate (in μg ml^{-1}): ampicillin 100, tetracycline 10, rifampicin 20, streptomycin 50, kanamacycin 50, novobiocin 20. In the mating experiment using the *fis* strain, RW1, the mutant strain, donated by W. R. Will and L. S. Frost (University of Alberta, unpublished), was made by P1 transduction of a *fis*::kan767 mutation from RLG1863 (Appleman *et al.*, 1998) into MC4100 as described elsewhere (Johnson *et al.*, 1988).

Various treatments were used on mating bacteria as follows. To test the effect of increased osmolarity, strains were grown in LB medium supplemented with 0·3 M NaCl. The effect of pH was tested by supplementing LB with 0·1 M MOPS buffer at pH 5·0. The effect of

Table 1. Bacterial strains and plasmids

Strain/plasmid	Genotype/relevant characteristics*	Source/reference
***E. coli* strains**		
DH5α	*supE44* Δ*lacU169* (ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan (1983)
RG192	*ara leu lac*, Rif^r	Lawley *et al.* (2003a)
MC4100	*araD139* Δ(*argF–lac*)*U169 rpsL150*(Str^r) *relA1 flb5301 deoC1 ptsF25 rbsR*	Casadaban (1976)
PD32	MC4100 *hns-206*::Ap^r	Dersch *et al.* (1993)
RW1	MC4100 *fis-767*::Kan^r	W. R. Will, University of Alberta
Plasmids		
R27	IncHI1 plasmid, Tc^r	Lawley *et al.* (2003a)
drR27	Tn*lacZ* insertion into *htdA*	Whelan *et al.* (1994)
pKK232-8	pBR322 derivative containing a promoterless CAT gene, Amp^r	Pharmacia LKB Biotechnology
pR	611 bp fragment of R operon promoter cloned in pKK232-8†	This study
pR300	310 bp fragment of R operon promoter cloned in pKK232-8†	This study
pR200	220 bp fragment of R operon promoter cloned in pKK232-8†	This study
pF	371 bp fragment of F operon promoter cloned in pKK232-8†	This study
pH	312 bp fragment of H operon promoter cloned in pKK232-8†	This study
pAC	382 bp fragment of AC operon promoter cloned in pKK232-8†	This study
pZ	384 bp fragment of Z operon promoter cloned in pKK232-8†	This study
pAN	480 bp fragment of AN operon promoter cloned in pKK232-8†	This study

*Abbreviations: Amp^r, ampicillin resistance; Rif^r, rifampicin resistance; St^r, streptomycin resistance; Tc^r, tetracycline resistance.
†Positions given refer to +1 of translation.

anaerobiosis was tested under an atmosphere of 5 % CO_2, 5 % H_2, 90 % N_2. Homoserine lactone (Sigma) was used at concentrations of 5, 10, 50 and 100 mM for the recipient and 5 mM for the donor.

DNA manipulations. R27 plasmid DNA was isolated using the Qiagen Plasmid Purification Kit. Standard recombinant DNA methods were performed as described by Sambrook & Russell (2001). Restriction endonucleases and T4 DNA ligase were used according to the manufacturers' instructions (Invitrogen, Roche Diagnostics) and digested DNA was analysed by agarose gel electrophoresis. DNA fragments were isolated from agarose gels using the QIAquick Gel Extraction Kit (Qiagen).

RNA extraction. *E. coli* cultures harbouring R27 were grown at either 30 °C or 37 °C. Cells were inoculated from overnight cultures grown at the same temperature. Cells were harvested when the culture reached 0·3 OD_{600} units. Total bacterial RNA was purified using the RNeasy Midi Kit (Qiagen), in accordance with the manufacturer's directions. To ensure that the RNA was devoid of contaminating DNA, the preparation was treated with Turbo DNase (Ambion). The RNA was precipitated with ethanol, dissolved in an appropriate volume of diethyl-pyrocarbonate-treated water, and quantified using an Ultraspec 4000 spectrophotometer (Pharmacia).

RT-PCR. For RT-PCR amplification of all transcripts, 2 μg total RNA was used as a template. RNA was retrotranscribed into cDNA utilizing the SuperScriptII RT with the random hexamers included in the kit (Invitrogen), generally according to the manufacturer's instructions. One-tenth of each product was subjected to PCR. Primer pairs specific for each two adjacent genes (see Table S1, provided as supplementary data with the online version of this paper) were designed using the R27 annotated sequence (GenBank accession no. AF250878). Typically, the annealing temperature was 5 degrees below the T_m of the primers. The PCR programme used was as follows: pre-incubation at 94 °C for 2 min, denaturing at 94 °C for 30 s, annealing for 30 s and extension at 72 °C for 1 min for every 1 kb of target sequence for 30 cycles, followed by 10 min further extension at 72 °C. Negative controls without template and negative control with RNA that had not been subjected to retrotranscription were included in each assay. A positive control using R27 DNA as the template was prepared with each pair of primers, generating products of the predicted sizes. The *E. coli pfkA* housekeeping gene, which encodes phosphofructokinase, was included as a positive control for the bacterial transcripts. Amplification products were resolved on an agarose gel and stained with ethidium bromide. RT-PCR amplifications were performed at least twice with total RNA preparations obtained from a minimum of three independent extractions and similar results were obtained in all experiments.

Rapid amplification of cDNA ends (RACE). To determine the transcription initiation sites, 5′RACE experiments were performed using the protocol provided by the manufacturer (Invitrogen). DNase-treated total RNA (4 μg) was reverse transcribed by using SuperscriptII RT with specific primers for each operon (see supplementary Table S1). cDNAs were tailed by adding cytosine residues (or adenosine when necessary) at the 3′ end by using Terminal Transferase and amplified by PCR with a second operon-specific primer. The PCR conditions used were 30 cycles consisting of 1 min denaturation at 94 °C, 1 min annealing at the appropriate temperature, and 1 min extension at 72 °C. The primary PCR products were amplified using a nested gene-specific primer. To precisely determine the 5′ ends of the transcripts, DNA sequencing reactions were done in the DNA Services Laboratory, Department of Biological Sciences, University of Alberta. At least 20 different final nested-PCR products for each one of the six promoters were sequenced.

Computer analysis. Prediction of intrinsic curvature and DNA sequence analysis was performed using the LASERGENE software package (DNASTAR, Inc.). Prediction of bacterial protein subcellular localization was performed using the PSORT program (Gardy *et al.*, 2003). Prediction of DNA-binding domains was done using DBS-PRED (Ahmad *et al.*, 2004). The BLAST program (Altschul *et al.*, 1990) was used for homology searches of amino acid sequences within the SWISS-PROT, GenBank/EMBL and PIR databases.

Construction of CAT-transcriptional fusions. Fragments containing each promoter were amplified by PCR (30 cycles) using R27 DNA as the template. The primer sequences used for cloning, as well as the sizes of the cloned regions, are listed in supplementary Table S1. The PCR products, which included terminal *Bam*HI–*Hin*dIII sites, were double digested and cloned in the low-copy-number vector pKK232-8 (Pharmacia LKB Biotechnology), which contains a promoterless chloramphenicol acetyltransferase (CAT) gene, digested with the same enzymes. Transcriptional fusion constructs were transformed into *E. coli* cells, after which ampicillin-resistant colonies were selected. The resulting plasmids (Table 1) were sequenced to confirm the fidelity of the PCR amplification.

Two serial deletion constructions of the R operon upstream region were created by PCR (Table 1, Fig. 4). The PCR products were digested with *Bam*HI/*Hin*dIII and the resulting fragments were cloned in the pKK232-8 vector, leading to the construction of plasmids pR300 and pR200. These clones included the ATG initiation codon. The resulting plasmids were sequenced and transformed into *E. coli* cells and also into *E. coli* cells harbouring R27. CAT expression of the resulting transformants was determined.

CAT assay. For measurement of CAT expression, cultures of relevant strains harbouring the transcriptional fusions were grown overnight at the selected temperature (30 °C or 37 °C). The resulting cultures were diluted 1 : 40 into fresh LB medium and incubated at the selected temperature to an OD_{600} of 0·3–0·4. The CAT Immunoassay Kit (CAT-ELISA, Roche Diagnostics) was used to quantify CAT expression, with some modifications for bacterial cells. Cells were lysed in 100 mM potassium phosphate buffer, pH 7·8, 2 mM EDTA, 1 % Triton X-100 and 1 mM DTT. Aliquots of cell extracts were assayed for CAT concentration and protein determination (BCA Protein Assay Kit, Pierce). The CAT readings were normalized to total protein. Assays were performed in duplicate with a minimum of three independent experiments for each fusion and/or growth condition. The standard deviations were calculated and were found insignificant (see supplementary Table S2 with the online version of this paper). Similar assays, using the vector alone and plasmid R27 were also included, to ensure that there was no intrinsic plasmid CAT expression.

Conjugation assay. Conjugal transfer of derepressed R27 (drR27) was performed as previously described (Taylor & Levine, 1980). A derepressed donor strain (Whelan *et al.*, 1994) was used to achieve a satisfactory degree of reproducibility, since consistently reproducible numbers of transconjugants were not obtained when a wild-type repressed donor was used. As transfer of R27 is dependent on the temperature at which the donor cells have been grown prior to mating, both donor and recipient cultures were pre-grown overnight at the appropriate temperature. Overnight cultures of *E. coli* donor and recipient strains were diluted 20-fold and incubated for 3 h with aeration at 30 °C or 37 °C. A mating mixture consisting of 0·1 ml of the donor, 0·4 ml of the recipient culture and 0·5 ml culture medium was incubated overnight at the tested temperature. Transconjugants were diluted and plated in duplicate on LB medium supplemented with the appropriate antibiotics. Each set of matings was repeated three times. Transfer frequencies were expressed as transconjugants per donor cell.

RESULTS

Operon organization

To determine the transcriptional organization of R27 transfer genes, RT-PCR was performed using total RNA extracted from cells harbouring R27 and primer sets specific for every adjacent gene in the two complete transfer regions (Figs 1b and 2b). In each run, DNA template controls were included to confirm the specificity of each primer pair to amplify the target sequence. No amplicons were detected in the RT-PCR analyses when reverse transcriptase was omitted, indicating the absence of contaminating genomic DNA in the RNA preparation. The systematic screening of the 38 *tra* genes revealed that the *tra* genes are transcriptionally linked in six operons, three in each Tra region (Figs 1a and 2a).

Within the Tra1 region, the following three operons were found: (1) The F operon, containing *trhF*, *trhH* and *trhG* genes, (2) the R operon, which contains *trhR* and *trhY* genes, and (3) the H operon, which comprises *traH*, orf121, *traI*, *traG*, orf118, *traJ*, orf116 and orf115 (Fig. 1a). The F operon and the R operon are transcribed in the same direction and away from *oriT*. The H operon is transcribed in the opposite direction and contains genes that encode relaxosome proteins, the coupling protein and other proteins not essential for transfer. These results were in agreement with the deduced operon organization assigned to this region by sequence analysis (Lawley *et al.*, 2002). Nevertheless, there was a discrepancy with the *trh*X gene, whose transcript could not be detected under the conditions used in the present work. Primer sets 1-3, 1-4 and 1-5 failed to amplify a product in the RT-PCR reaction (Fig. 1), supporting the assignment of the above three operons.

In the Tra2 region, the following three operons were found: (1) the AC operon, containing *trhA*, *trhL*, *trhE*, *trhK*, orf30, *trhB*, orf28, orf27, *trhV* and *trhC* genes, (2) the Z operon, which contains *trhO*, orf16, orf17 and *trhZ* genes, and (3) the AN operon, which contains *htdA*, *htdF*, *htdK*, orf9, *trhP*, *trhW*, *trhU* and *trhN* genes (Fig. 2a). The AC and AN operons encode proteins responsible for mating pair formation and pilus biosynthesis and are transcribed in the same direction. The Z operon, encoding entry exclusion proteins (J. Gunton & D. E. Taylor, unpublished), is transcribed in the opposite direction.

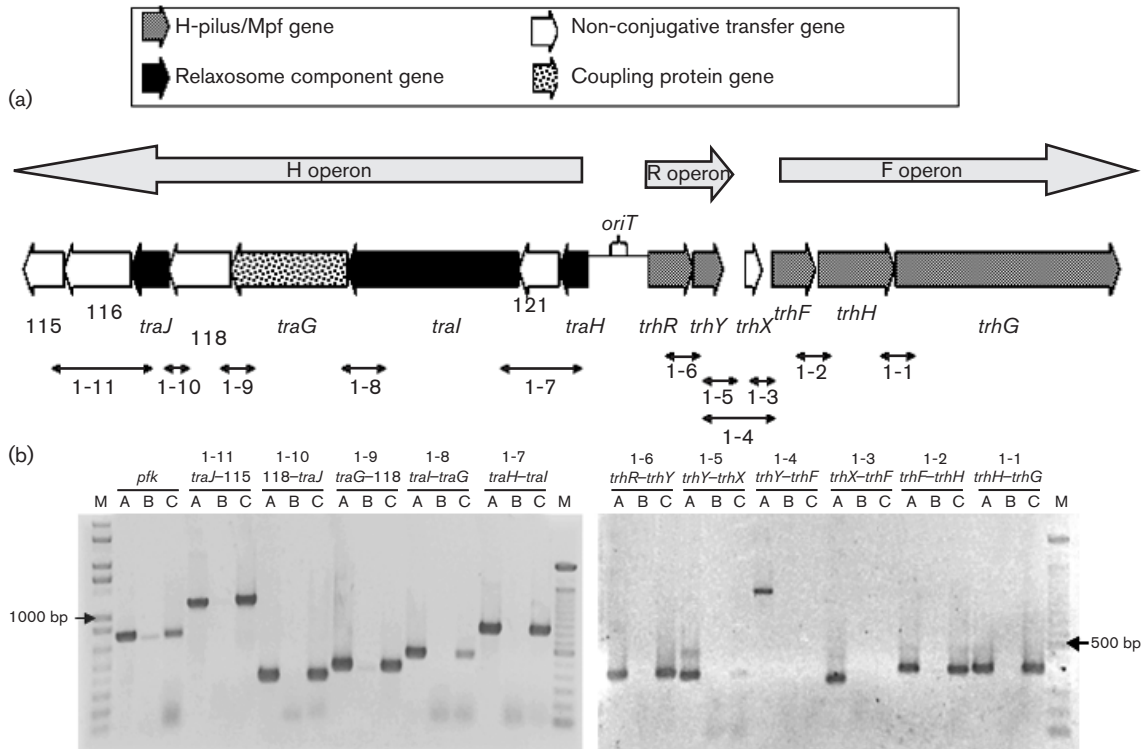

Fig. 1. RT-PCR analysis of the Tra1 region. (a) Organization of the Tra1 region. ORFs are represented as pattern boxes with arrowheads indicating their orientation. The double-ended arrows below the physical map indicate the regions amplified by the primer pairs. The grey arrows above the ORFs indicate the operon arrangements determined in this work. (b) Agarose gel of the RT-PCR amplification products. For each primer pair, three lanes are shown: A, positive control using DNA templates; B, negative control (RNA, no RT) to assess DNA contamination in RNA preparations; C, RT-PCR using as template RNA isolated from cells harbouring R27 grown at 30 °C. M, molecular mass marker; *pfk*, phosphofructokinase gene (control).

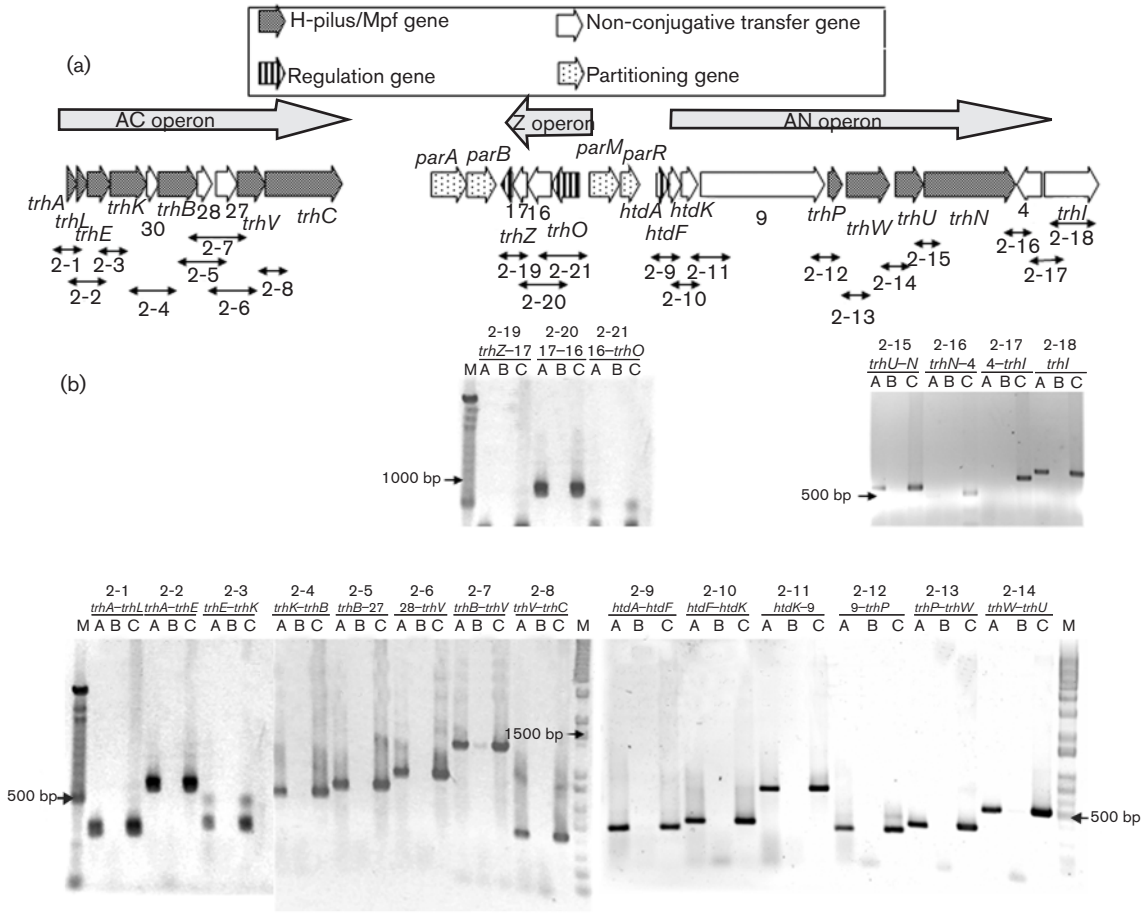

Fig. 2. RT-PCR analysis of the Tra2 region. (a) Organization of the Tra2 region. ORFs are represented as pattern boxes with arrowheads indicating their orientation. The double-ended arrows below the physical map indicate the regions amplified by the primer pairs. The arrows above the ORFs indicate the operon arrangements. (b) Agarose gel of the RT-PCR amplification products. For each primer pair, three lanes are shown: A, RT-PCR using as template RNA isolated from cells harbouring R27 grown at 30 °C; B, negative control (RNA, no RT) to asses DNA contamination in RNA preparations; C, positive control using DNA templates. M, molecular mass marker.

Transcriptional expression

Identification of *tra* operons enabled us to perform transcriptional studies for all the transfer genes. To determine if transcriptional control is responsible for temperature-sensitive R27 transfer, RT-PCR was carried out using RNA isolated from bacteria harbouring R27 grown at both 30 °C and 37 °C, using the same primer sets as above.

Results obtained from the F and R operons in the Tra1 region (Fig. 3a) and the AC operon in the Tra2 region (Fig. 3b) revealed that *tra* transcripts were consistently present at higher levels at 30 °C than 37 °C. The constitutively expressed *E. coli pfkA* gene, encoding phosphofructokinase, was used as a control. Transcripts of *tra* genes from cells growing at 37 °C were lower when compared to the levels of RNA isolated from cells grown at 30 °C. Although the control, *pfkA* (Fig. 3b, lane A), had significantly less of the total RT-PCR product loaded, this did not affect the result that the *tra* operons are transcriptionally down-regulated at 37 °C.

This result was consistent for all the *tra* operons (data not shown) in both the Tra1 and the Tra2 region. Although our RT-PCR analysis was semi-quantitative, the higher production of *tra* transcripts at 30 °C compared to that at 37 °C suggests that the thermosensitive regulation of R27 *tra* genes occurs at the transcriptional or post-transcriptional level. These results are consistent with previous findings from an earlier study of the *trh*C gene (Gilmour *et al.*, 2001).

Transcriptional start sites

To characterize each of the six *tra* operon promoters and putative regulatory regions, we verified the transcriptional start site by the 5′RACE procedure. The 5′ ends of these six transcripts, corresponding to the respective transcription initiation sites, were identified (Fig. 4). A single G residue

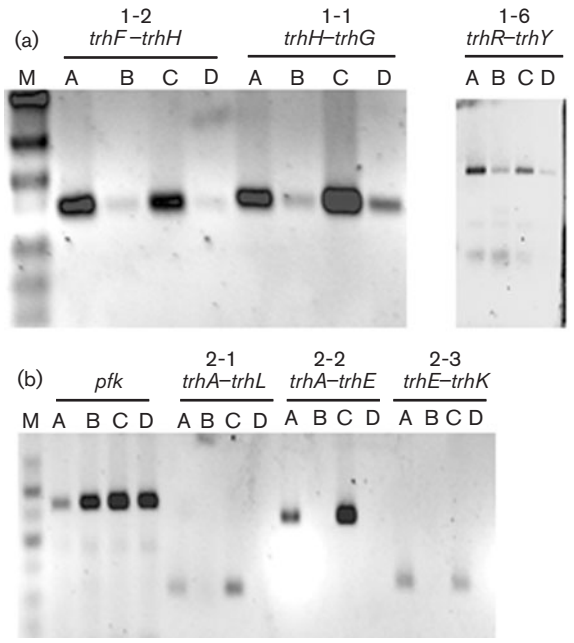

Fig. 3. Characterization of the transcriptional profile of R27 *tra* genes by RT-PCR analysis of (a) F and R operon transcripts in the Tra1 region anad (b) AC operon transcripts in the Tra2 region, from cells harbouring: A, wild-type R27 grown at 30 °C; B, wild-type R27 grown at 37 °C; C, derepressed (dr) R27 grown at 30 °C; D, drR27 grown at 37 °C. Primers used and their positions are shown in Fig. 1.

was detected as the start site for the AC and AN operons, whereas the other four operons had single A residues as the start site. The 5′ untranslated regions (UTR) are 14–34 bp long, except those of the R and AN operons, which are 136 bp and 137 bp, respectively (Fig. 4b, f). Analysis of the upstream regions of the R, H and AN operons revealed the presence of putative −35 and −10 boxes, with three to five of the six positions matching the *E. coli* consensus sequence. The positions of the transcriptional start sites are consistent with the locations of the deduced promoter sequence elements. The −35 and −10 boxes are separated by 17 or fewer base pairs, except those of the H and AN operons, which have longer spacer regions, 21 and 20 bp respectively (Fig. 4c, f). For the F, AC and Z operons, the promoter boxes could not be definitely assigned. The absence of consensus sequences and/or canonical spacing between the −10 and −35 boxes could have implications for the regulation of operon expression. The F and AC operons contain genes that encode major structural proteins; therefore these genes must be tightly regulated without canonical promoter regions. Also, the presence of several upstream AT tracts was found in all the sequences analysed.

Interestingly, the origin of transfer is contained within the 5′ UTR mRNA of *trhRY* (Fig. 4b). The TrhR and TrhY proteins are required for conjugation (Lawley *et al.*, 2002). These proteins have homologous counterparts in the IncHI2 R478 plasmid (GenBank accession no. BX664015), but show no homology to any component of other known conjugative systems. They may represent T4SS components that are unique to IncHI plasmids. Since genes that regulate conjugative transfer are generally present around *oriT* and are the least well-conserved components of transfer systems, *trhR* and *trhY* may play a role in regulating conjugative transfer. Consistent with this idea, overexpression of the *trhR* and *trhY* genes increased the transfer frequency of R27 (Lawley *et al.*, 2002). With this in mind, we searched for domains in TrhR and TrhY proteins. *trhR* encodes a protein, predicted to be an inner-membrane protein, with 50 % identity to the *Actinobacillus pleuropneumoniae* ApfC protein (GenBank accession no. AA064353), which is essential for type IV pilus production. In *Legionella pneumophila*, the homologous protein PilC has increased expression at 30 °C as opposed to 37 °C (Liles *et al.*, 1998). These proteins with functional similarity failed to complement mutants in related organisms (Johnston *et al.*, 1998), suggesting that they could be system-specific. *trhY* encodes a protein predicted to be a cytoplasmic protein with 85·5 % probability to bind DNA. TrhY shows 50 % identity with the transcriptional repressor of the xylose operon from *Bacillus halodurans* (GenBank accession no. F83994).

The origin of transfer is surrounded by the divergent promoters of the R and H operons, with A/T-rich regions, positioned as tracts. The presence of A tracts has been proposed to bend DNA in a way that is sensitive to temperature. The bend is stable below 30 °C, whereas the stability is reduced above 33 °C (Chan *et al.*, 1993; Falconi *et al.*, 1998; Rohde *et al.*, 1999). Analysis of upstream promoter sequences revealed potential H-NS and FIS binding sites in the region between the divergent promoters of the R and H operons. *In silico* analysis of the remaining upstream *tra* promoter regions demonstrated the presence of DNA curve regions in the three Tra1 operons and in the AC and Z operons (Fig. 4). The upstream region of the AN operon appears to be deficient in regions containing bends. Binding of H-NS to the *oriT* region has been recently reported (Forns *et al.*, 2005).

Functional analysis of the promoter regions

Identification of the transcriptional start sites of the *tra* operons enabled cloning of the promoters and potential regulatory regions. To address the question of how promoters are regulated, we used a CAT reporter gene fused to each *tra* promoter, including upstream regions (as described in Methods, Fig. 4). These clones were transformed into *E. coli* cells and *E. coli* cells harbouring the R27 plasmid grown at 30 °C or 37 °C, and CAT expression was determined. The expression values at 30 °C were normalized to 1 for comparative purposes (Table 2). At 37 °C, the constructs exhibited the same (AC and AN) or lower (R, F and Z) expression level, except for the H operon (relaxosome operon), which showed higher expression, compared to that seen at 30 °C.

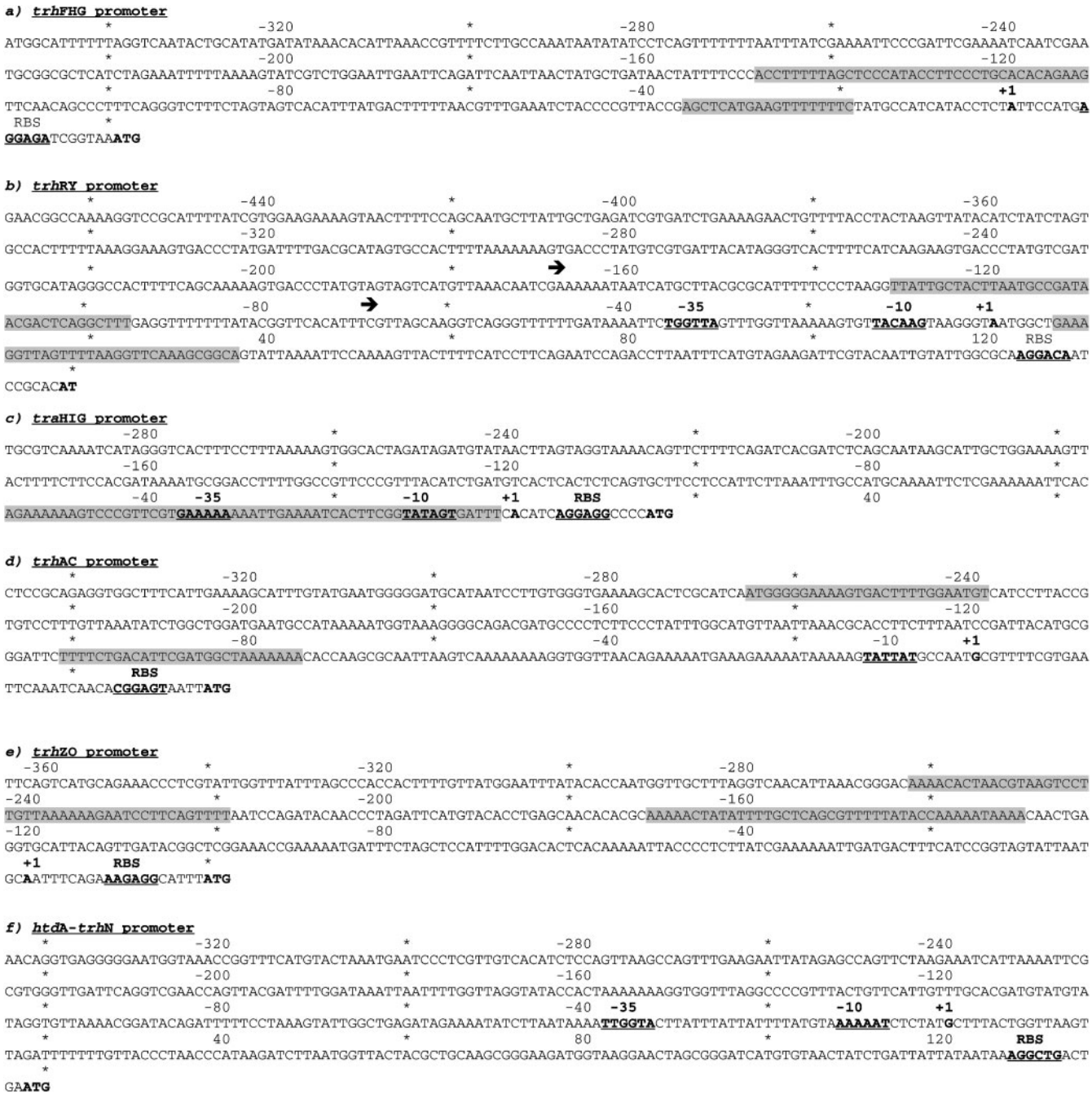

Fig. 4. Promoter and upstream regions of the *tra* operons. Nucleotide sequences contained in CAT-transcriptional fusions, numbered from the transcription start points (+1) are shown. Putative −35 and −10 promoter elements, ribosome-binding sites (RBS) and initiation codons are indicated. (a) Sequence cloned in pF. (b) Sequence cloned in pR; arrows indicate the end points of the upstream deletion clones pR300 and pR200, which included the ATG initiation codon. (c) Sequence cloned in pH. (d) Sequence cloned in pAC. (e) Sequence cloned in pZ. (f) Sequence cloned in pAN. The shadowed regions show the bent DNA.

In the presence of plasmid R27 *in trans*, the R, F, AC, Z and AN promoters were repressed at both temperatures (Table 2). R27 also had a strong silencing effect on the AN promoter when present at 30 °C.

Characterization of the upstream promoter regions containing intrinsic DNA curvature suggests that nucleoid-associated proteins can bind at this position. To test the influence of these proteins, transcriptional fusions

Table 2. Effect of temperature and H-NS protein on expression of *tra* promoters

Except where indicated, values relative to each construction in the wild-type strain grown at 30 °C are shown. ND, Not determined. A version of the table including SD values is provided as supplementary Table S2 with the online version of this paper.

Construct	Wild-type host				*hns* host			
	−R27*		+R27†		−R27*		+R27†	
	30 °C	37 °C	30 °C	37 °C	30 °C	37 °C	30 °C	37 °C
pR	1	0·68	0·70	0·29	0·97	0·86	1·81	1·30
pF	1	0·60	0·33	0·22	1·85	1·88	1·74	2·90
pH	1	4·67	1·23	6·55	1·36	3·11	4·92	6·01
pAC	1	1·30	0·31	0·11	1·62	2·99	1·95	1·59
pZ	1	0·13	0·40	0·27	0·43	0·55	0·54	0·29
pAN	1	1·14	0·05	1·21	1·33	1·27	1·18	1·28
pR300‡	ND	0·68	1·25	1·15	ND	1·19	ND	0·90
pR200‡	ND	0·60	3·29	1·63	ND	1·36	ND	2·10

*Absence of plasmid R27 *in trans*.
†Presence of plasmid R27 *in trans*.
‡Values relative to pR (−R27) at 30 °C.

were introduced into an isogenic *E. coli hns* mutant strain and also into the *hns* mutant carrying the R27 plasmid, and the transcriptional activities were measured (Table 2). Except for the Z promoter, in the presence of pR27, little difference was observed in transcription levels between 30 °C and 37 °C in the H-NS-deficient *E. coli* strain, compared with that obtained in wild-type background. These results indicated that the H-NS protein is involved in negative regulation of *tra* genes transcription, in agreement with recently published data (Forns *et al.*, 2005). The effect of the *hns* mutation is more obvious in the presence of the R27 plasmid than in its absence, except for the Z operon, whose transcription seemed to be H-NS independent. These results confirm that the H-NS protein is required for the thermosensitive repression activity of R27.

In the presence of R27, both AN and H operons exhibited higher expression at 37 °C than that observed at 30 °C in the wild-type host background. These results are contradictory to those obtained using RT-PCR. However, analysis of transcription directed by the cloned region excluded the possibility of detection of global regulation, or post-transcriptional processing or degradation. The increased expression of both operons at the temperature that is non-permissive for mating suggests that some factors, encoded by any one of the tested operons, are required to inhibit conjugation in a temperature-dependent fashion. The AN operon contains the *htdA* gene, which has been suggested to be a negative regulator (Whelan *et al.*, 1994). Nonetheless, the negative effect of pAN expression is influenced by the presence of H-NS protein, the temperature shift and the presence of R27, as well as perhaps some *cis* or *trans* element.

To determine if curved DNA is implicated in transcriptional regulation, we generated deletions that would eliminate the potential bend in the DNA. Two different deletions in the upstream region of the R operon were analysed (Fig. 4). Deletions of the curved regions caused derepression of transcription at the non-permissive temperature (Table 2), indicating that temperature sensitivity was lost. This occurred even in the presence of the parental plasmid, suggesting the presence of negative regulatory sites between bases −167 and +1. To investigate if H-NS might be involved in the loss of temperature-sensitive regulation due to deletions of the R operon upstream region, the constructs were also introduced into *hns*-deficient cells (Table 2). The results showed that H-NS repression is lost.

Some promoters that bind nucleoid-associated proteins are extremely sensitive to variations in the superhelical density of the DNA. To determine if DNA supercoiling is related to H-NS and curved DNA regulation, transcriptional analysis was performed using cells grown in media with subinhibitory concentrations of DNA gyrase inhibitor novobiocin. The *tra* promoters were inhibited to some extent at the same level at both temperatures (data not shown), but still responded to temperature regulation, indicating that DNA supercoiling does not modulate temperature-dependent regulation.

Mating

To confirm the *in vitro* results, we determined the frequency of transfer using different conditions known to play a role in the regulation of other bacterial genes and evaluated if these conditions altered the transfer frequency at both temperatures. The results showed that acidic pH, phase of growth, anaerobiosis, high osmolarity, as well as inhibition of

Table 3. Ability of drR27 to transfer via conjugation under different conditions

Conditions	Transfer frequency (T/D)*	
	30 °C	37 °C
drR27 (control)	$2{\cdot}55\times10^{-1}$	$9{\cdot}25\times10^{-8}$
Novobiocin	$1{\cdot}46\times10^{-2}$	0
LB + 0·3 M NaCl	$5{\cdot}53\times10^{-2}$	0
Microanaerobiosis	$3{\cdot}36\times10^{-1}$	0
LB pH 5·0	$1{\cdot}55\times10^{-2}$	0
LB + homoserine lactone	$6{\cdot}23\times10^{-1}$	0
drR27 in wild-type/recipient *hns*	$1{\cdot}42\times10^{-1}$	0
drR27 in *hns*/recipient wild-type	$1{\cdot}08\times10^{-1}$	0
drR27 in *fis*/recipient wild-type	$1{\cdot}25\times10^{-1}$	0

*Frequencies are expressed as the number of transconjugants per input donor cell. Values represent the mean of at least three independent experiments. 0, $<10^{-8}$ transconjugants per donor.

DNA gyrase and presence of quorum-sensing molecules (homoserine lactone), had no significant effect on transfer frequency at either temperature (Table 3). When the *hns* or *fis* mutant harbouring R27 was used as the donor in mating assays, the transfer frequency was slightly reduced.

DISCUSSION

Determination of the genetic organization of the genes responsible for the conjugation process in R27 is important in understanding the thermoregulation mechanism. The identification of regulatory factors and variations in regulation of conjugative transfer is crucial to the development of novel approaches to prevent the dissemination of plasmid-encoded genes, i.e. antibiotic resistance determinants. In this work, we have performed transcriptional analysis of the R27 *tra* genes. Our data demonstrated that the 38 R27 *tra* genes analysed are organized into six operons. However, we could not rule out that additional transfer genes could be present outside or in the vicinity of the two Tra regions. The organization of the genes in both Tra regions, which was previously suggested (Lawley *et al.*, 2002, 2003a), was confirmed in this study (Figs 1 and 2). The transcriptional start sites identified were consistent with the predicted −10 and −35 promoter elements (Fig. 4).

Like those of the plasmid RK2 (incompatibility group P) and the Ti plasmids (Farrand *et al.*, 1996; Zatyka *et al.*, 1994) the transfer genes of R27 are clustered into two regions, and they also share the same organization of two divergently expressed operons flanking the *oriT* region. One of these operons encodes TrhR and TrhY proteins, which might be a part of the regulatory system, involved in the control of transcription of the *tra* genes. Further studies are required to confirm or refute this idea.

The data presented here suggest that regulation of gene expression by nucleoid-structuring proteins may be more widespread in bacteria than previously documented. H-NS directly or indirectly affects the expression of several genes that are regulated in response to temperature or other environmental conditions (reviewed by Dorman, 2004). A recent publication has implicated Hha and H-NS proteins, both plasmid and chromosomal, in temperature-sensitive conjugative transfer of R27 (Forns *et al.*, 2005). Virulence and flagellar genes in *Yersinia enterocolitica* (Pepe *et al.*, 1994; Rohde *et al.*, 1994), virulence genes in *Shigella* (Falconi *et al.*, 1998; Prosseda *et al.*, 2004, 2002) and in enteropathogenic *E. coli* (Umanski *et al.*, 2002), lipid A myristoyl transferase and haemolysin in *E. coli* (Madrid *et al.*, 2002; Yoon *et al.*, 2004) and the urease gene cluster in *Proteus mirabilis* (Poore & Mobley, 2003) are also regulated by H-NS. Usually thermosensitive proteins that modulate expression bind DNA at low temperatures and permit expression at higher temperatures; however, the opposite pattern of thermosensitivity was observed in R27.

DNA superhelicity has been proposed to serve as a global regulator of gene expression (Hatfield & Benham, 2002). The absence of H-NS in the cell results in an increased degree of negative supercoiling in both plasmid and chromosomal DNA. An association between changes in DNA topology regulated by H-NS protein, regulation of gene expression and environmental conditions has been reported (Asakura *et al.*, 2004; Gallant *et al.*, 2004; Krishnan *et al.*, 2004). In other systems, DNA supercoiling is promoted by high temperature, high osmolarity and anaerobic conditions, and the addition of novobiocin and homoserine lactone. The data presented here show that these conditions do not significantly affect the transfer frequency of R27 plasmid at 37 °C.

The assembly of the transfer apparatus and coordinated transfer of plasmid DNA represent a complex biological process that is undoubtedly energetically demanding. It is therefore not surprising that conjugation is a tightly regulated process, which responds to environmental changes. Our data suggest that the *tra* promoters are strongly regulated, and their expression was repressed in the presence of plasmid R27. Literature reports suggest that the nucleoid-associated proteins do not usually exert regulatory functions alone. Each has at least one additional regulator. This is in agreement with our findings, which show that a putative R27-encoded repressor protein could be involved in the inhibition of the promoters at 37 °C. In plasmid RK2, it has been reported that relaxosome proteins repress *oriT* (Zatyka *et al.*, 1994). One of these proteins, TraK, binds to the *oriT* region, increasing the bending of this stretch of DNA. In plasmid R1162, promoters of the *mob* genes, required for conjugative mobilization, are negatively regulated at *oriT* by the Mob proteins.

Steck *et al.* (1993) showed that the length of the spacer region between the −35 and −10 elements is related to the optimal expression for promoters. Those promoters which have a maximum expression at high levels of supercoiling commonly have spacers that are larger than 17 bp. The H and AN promoters have the larger spacing region between the −35 and −10 elements identified (Fig. 4) and also exhibit higher expression at 37 °C (Table 2). While further investigation is required, it is tempting to suggest that any of the proteins encoded by these operons can repress the expression of other proteins essential for R27 transfer.

Bacteria harbouring H plasmids have been found in clinical environments as well as in soil, sewage and fresh water. These plasmids were shown to be promiscuous and to transfer at environmental temperatures (Maher & Taylor, 1993; Smith *et al.*, 1978). In addition to antibiotic resistance, other temperature-sensitive conjugative H plasmids encode determinants for resistance to a range of antimicrobial agents, including heavy metals, tellurium and channel-forming colicins (Alonso *et al.*, 2002; Rodriguez-Lemoine, 1992). This confers considerable selective advantages upon host cells, allowing for survival in hostile and rapidly changing environmental conditions and enhancing the dissemination of resistance among pathogenic and indigenous bacterial species in water and soil environments

(Maher & Taylor, 1993). Plasmid- and host-encoded proteins, such as H-NS, seem be part of the regulatory mechanism of IncH plasmid conjugation in response to temperature changes. This has implications for successful IncHI1 plasmid transfer and maintenance in the population. Knowledge of the regulation mechanism of plasmid transfer could be used to help reduce the transfer of conjugal plasmids in their natural niche.

ACKNOWLEDGEMENTS

The study was supported by grant MOP6200 to D. E. T. from the Canadian Institutes for Health Research. G. A. is a recipient of a Visiting Scientist Award from the Alberta Heritage Foundation for Medical Research (AHFMR). D. E. T. is an AHFMR Medical Scientist. We are grateful to J. Gunton and M. Gilmour for many useful discussions on bacterial conjugation and type IV secretion systems and for sharing unpublished results. We thank S. Patterson for performing the sequencing reactions, and R. W. Will for the gift of *E. coli* RW1.

REFERENCES

Adamczyk, M. & Jagura-Burdzy, G. (2003). Spread and survival of promiscuous IncP-1 plasmids. *Acta Biochim Pol* **50**, 425–453.

Ahmad, S., Gromiha, M. M. & Sarai, A. (2004). Analysis and prediction of DNA-binding proteins and their binding residues based on composition, sequence and structural information. *Bioinformatics* **20**, 477–486.

Alonso, G., Vilchez, G., Bruzual, I. & Rodriguez-Lemoine, V. (2002). Characterization of plasmid MIP233 (IncHI3) of the H complex. *Res Microbiol* **153**, 149–153.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.

Appleman, J. A., Ross, W., Salomon, J. & Gourse, R. L. (1998). Activation of *Escherichia coli* rRNA transcription by FIS during a growth cycle. *J Bacteriol* **180**, 1525–1532.

Asakura, H., Kawamoto, K., Shirahata, T. & Makino, S. (2004). Changes in *Salmonella enterica* serovar Oranienburg viability caused by NaCl-induced osmotic stress is related to DNA relaxation by the H-NS protein during host infection. *Microb Pathog* **36**, 147–151.

Casadaban, M. J. (1976). Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. *J Mol Biol* **104**, 541–555.

Cascales, E. & Christie, P. J. (2004). Definition of a bacterial type IV secretion pathway for a DNA substrate. *Science* **304**, 1170–1173.

Chan, S. S., Breslauer, K. J., Austin, R. H. & Hogan, M. E. (1993). Thermodynamics and premelting conformational changes of phased (dA)5 tracts. *Biochemistry* **32**, 11776–11784.

Christie, P. J. (2001). Type IV secretion: intercellular transfer of macromolecules by systems ancestrally related to conjugation machines. *Mol Microbiol* **40**, 294–305.

Dersch, P., Schmidt, K. & Bremer, E. (1993). Synthesis of the *Escherichia coli* K-12 nucleoid-associated DNA-binding protein H-NS is subjected to growth-phase control and autoregulation. *Mol Microbiol* **8**, 875–889.

Dorman, C. J. (2004). H-NS: a universal regulator for a dynamic genome. *Nat Rev Microbiol* **2**, 391–400.

Falconi, M., Colonna, B., Prosseda, G., Micheli, G. & Gualerzi, C. O. (1998). Thermoregulation of *Shigella* and *Escherichia coli* EIEC pathogenicity. A temperature-dependent structural transition of DNA modulates accessibility of *vir*F promoter to transcriptional repressor H-NS. *EMBO J* **17**, 7033–7043.

Farrand, S. K., Hwang, I. & Cook, D. M. (1996). The *tra* region of the nopaline-type Ti plasmid is a chimera with elements related to the transfer systems of RSF1010, RP4, and F. *J Bacteriol* **178**, 4233–4247.

Forns, N., Baños, R. C., Calsalobre, C., Juárez, A. & Madrid, C. (2005). Temperature-dependent conjugative transfer of R27: role of chromosome- and plasmid-encoded Hha and H-NS proteins. *J Bacteriol* **187**, 3950–3959.

Frost, L. S., Ippen-Ihler, K. & Skurray, R. A. (1994). Analysis of the sequence and gene products of the transfer region of the F sex factor. *Microbiol Rev* **58**, 162–210.

Gallant, C. V., Ponnampalam, T., Spencer, H., Hinton, J. C. & Martin, N. L. (2004). H-NS represses *Salmonella enterica* serovar Typhimurium *dsbA* expression during exponential growth. *J Bacteriol* **186**, 910–918.

Gardy, J. L., Spencer, C., Wang, K. & 8 other authors (2003). PSORT-B: improving protein subcellular localization prediction for Gram-negative bacteria. *Nucleic Acids Res* **31**, 3613–3617.

Gilmour, M. W., Lawley, T. D., Rooker, M. M., Newnham, P. J. & Taylor, D. E. (2001). Cellular location and temperature-dependent assembly of IncHI1 plasmid R27-encoded TrhC-associated conjugative transfer protein complexes. *Mol Microbiol* **42**, 705–715.

Hanahan, D. (1983). Studies of transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.

Hatfield, G. W. & Benham, C. J. (2002). DNA topology-mediated control of global gene expression in *Escherichia coli*. *Annu Rev Genet* **36**, 175–203.

Johnson, R. C., Ball, C. A. & Pfeffer, D. (1988). Isolation of the gene encoding the Hin recombinational enhancer binding protein. *Proc Natl Acad Sci U S A* **85**, 3484–3488.

Johnston, J. L., Billington, S. J., Haring, V. & Rood, J. I. (1998). Complementation analysis of the *Dichelobacter nodosus fim*N, *fim*O, and *fim*P genes in *Pseudomonas aeruginosa* and transcriptional analysis of the *fim*NOP gene region. *Infect Immun* **66**, 297–304.

Kariuki, S., Revathi, G., Muyodi, J., Mwituria, J., Munyalo, A., Mirza, S. & Hart, C. A. (2004). Characterization of multidrug-resistant typhoid outbreaks in Kenya. *J Clin Microbiol* **42**, 1477–1482.

Krishnan, H. H., Ghosh, A., Paul, K. & Chowdhury, R. (2004). Effect of anaerobiosis on expression of virulence factors in *Vibrio cholerae*. *Infect Immun* **72**, 3961–3967.

Lawley, T. D., Gilmour, M. W., Gunton, J. E., Standeven, L. J. & Taylor, D. E. (2002). Functional and mutational analysis of conjugative transfer region 1 (Tra1) from the IncHI1 plasmid R27. *J Bacteriol* **184**, 2173–2180.

Lawley, T. D., Gilmour, M. W., Gunton, J. E., Tracz, D. M. & Taylor, D. E. (2003a). Functional and mutational analysis of conjugative transfer region 2 (Tra2) from the IncHI1 plasmid R27. *J Bacteriol* **185**, 581–591.

Lawley, T. D., Klimke, W. A., Gubbins, M. J. & Frost, L. S. (2003b). F factor conjugation is a true type IV secretion system. *FEMS Microbiol Lett* **224**, 1–15.

Liles, M. R., Viswanathan, V. K. & Cianciotto, N. P. (1998). Identification and temperature regulation of *Legionella pneumophila* genes involved in type IV pilus biogenesis and type II protein secretion. *Infect Immun* **66**, 1776–1782.

Madrid, C., Nieto, J. M., Paytubi, S., Falconi, M., Gualerzi, C. O. & Juarez, A. (2002). Temperature- and H-NS-dependent regulation of a plasmid-encoded virulence operon expressing *Escherichia coli* hemolysin. *J Bacteriol* **184**, 5058–5066.

Maher, D. & Taylor, D. E. (1993). Host range and transfer efficiency of incompatibility group HI plasmids. *Can J Microbiol* **39**, 581–587.

Pepe, J. C., Badger, J. L. & Miller, V. L. (1994). Growth phase and low pH affect the thermal regulation of the *Yersinia enterocolitica inv* gene. *Mol Microbiol* **11**, 123–135.

Poore, C. A. & Mobley, H. L. (2003). Differential regulation of the *Proteus mirabilis* urease gene cluster by UreR and H-NS. *Microbiology* **149**, 3383–3394.

Prosseda, G., Falconi, M., Nicoletti, M., Casalino, M., Micheli, G. & Colonna, B. (2002). Histone-like proteins and the *Shigella* invasivity regulon. *Res Microbiol* **153**, 461–468.

Prosseda, G., Falconi, M., Giangrossi, M., Gualerzi, C. O., Micheli, G. & Colonna, B. (2004). The *vir*F promoter in *Shigella*: more than just a curved DNA stretch. *Mol Microbiol* **51**, 523–537.

Rodriguez-Lemoine, V. (1992). The incompatibility complex of H plasmids. *Rev Latinoam Microbiol* **34**, 115–127.

Rodriguez-Lemoine, V., Jacob, A. E., Hedges, R. W. & Datta, N. (1975). Thermosensitive production of their transfer systems by group S plasmids. *J Gen Microbiol* **86**, 111–114.

Rohde, J. R., Fox, J. M. & Minnich, S. A. (1994). Thermoregulation in *Yersinia enterocolitica* is coincident with changes in DNA supercoiling. *Mol Microbiol* **12**, 187–199.

Rohde, J. R., Luan, X. S., Rohde, H., Fox, J. M. & Minnich, S. A. (1999). The *Yersinia enterocolitica* pYV virulence plasmid contains multiple intrinsic DNA bends which melt at 37 degrees C. *J Bacteriol* **181**, 4198–4204.

Rooker, M. M., Sherburne, C., Lawley, T. D. & Taylor, D. E. (1999). Characterization of the Tra2 region of the IncHI1 plasmid R27. *Plasmid* **41**, 226–239.

Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sherburne, C. K., Lawley, T. D., Gilmour, M. W., Blattner, F. R., Burland, V., Grotbeck, E., Rose, D. J. & Taylor, D. E. (2000). The complete DNA sequence and analysis of R27, a large IncHI plasmid from *Salmonella typhi* that is temperature sensitive for transfer. *Nucleic Acids Res* **28**, 2177–2186.

Smith, H. W., Parsell, Z. & Green, P. (1978). Thermosensitive antibiotic resistance plasmids in enterobacteria. *J Gen Microbiol* **109**, 37–47.

Steck, T. R., Franco, R. J., Wang, J. Y. & Drlica, K. (1993). Topoisomerase mutations affect the relative abundance of many *Escherichia coli* proteins. *Mol Microbiol* **10**, 473–481.

Taylor, D. E. & Levine, J. G. (1980). Studies of temperature-sensitive transfer and maintenance of H incompatibility group plasmids. *J Gen Microbiol* **116**, 475–484.

Umanski, T., Rosenshine, I. & Friedberg, D. (2002). Thermoregulated expression of virulence genes in enteropathogenic *Escherichia coli*. *Microbiology* **148**, 2735–2744.

Wain, J. & Kidgell, C. (2004). The emergence of multidrug resistance to antimicrobial agents for the treatment of typhoid fever. *Trans R Soc Trop Med Hyg* **98**, 423–430.

Whelan, K. F., Maher, D., Colleran, E. & Taylor, D. E. (1994). Genetic and nucleotide sequence analysis of the gene *htd*A, which regulates conjugal transfer of IncHI plasmids. *J Bacteriol* **176**, 2242–2251.

Yoon, J. W., Minnich, S. A., Ahn, J. S., Park, Y. H., Paszczynski, A. & Hovde, C. J. (2004). Thermoregulation of the *Escherichia coli* O157 : H7 pO157 *ecf* operon and lipid A myristoyl transferase activity involves intrinsically curved DNA. *Mol Microbiol* **51**, 419–435.

Zatyka, M., Jagura-Burdzy, G. & Thomas, C. M. (1994). Regulation of transfer genes of promiscuous IncPα plasmid RK2: repression of Tra1 region transcription both by relaxosome proteins and by the Tra2 regulator TrbA. *Microbiology* **140**, 2981–2990.