Activation of the Cpx regulon destabilizes the F plasmid transfer activator, TraJ, via the HslVU protease in Escherichia coli

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Summary

The Escherichia coli CpxAR two-component signal transduction system senses and responds to extracytoplasmic stress. The cpxA101* allele was previously found to reduce F plasmid conjugation by post-transcriptional inactivation of the positive activator TraJ. Microarray analysis revealed upregulation of the protease–chaperone pair, HslVU, which was shown to degrade TraJ in an E. coli C600 cpxA101* background. Double mutants of cpxA101* and hslV or hslU restored TraJ and F conjugation to wild-type levels. The constitutive overexpression of nlpE, an outer membrane lipoprotein that induces the Cpx stress response, also led to HslVU-mediated degradation of TraJ and repression of F transfer. However, Cpx-mediated TraJ degradation appears to be growth phase-dependent, as induction of nlpE in mid-log phase cells did not appreciably alter TraJ levels. Further, His7-TraJ was sensitive to HslVU degradation in vitro only when it was purified from cells over-expressing nlpE. Thus, TraJ appears to become resistant to HslVU during normal growth, with this resistance mapping to the F transfer region. Extracytoplasmic stress prevents this modification of TraJ, leaving it susceptible to HslVU. Thus, the CpxAR stress response indirectly controls the synthesis of the F mating apparatus, a complex transenvelope type IV secretion system, by degrading TraJ.

Introduction

F conjugation involves the transfer of the F plasmid from a donor to a recipient cell. This process requires the synthesis of the conjugative pilus, which contacts a recipient bacterium and retracts, leading to the formation of a stable mating pair (Frost et al., 1994). In F and F-like plasmids, conjugation and the synthesis of pili are highly regulated by different host- and plasmid-encoded factors. F TraJ is a positive activator of the 33.3 kb tra operon transcribed from the P7 promoter, which contains all the tra genes necessary for pilus synthesis and DNA transfer except traM and traJ (Finnegan and Willetts, 1972; Frost et al., 1994). TraJ and the host factor, ArcA (SfrA), are important for P7 transcription. In the absence of either ArcA or TraJ, P7 expression is reduced by 10- or 30-fold respectively, leading to reduced mating efficiency (Buxton and Drury, 1983; Silverman et al., 1991). TraJ also indirectly activates the expression of traM through TraY, the first gene product encoded on the P7 transcript (Penfold et al., 1996). TraM, TraY and the TraI relaxase-helicase, along with host factor IHF form the relaxosome, a nucleoprotein complex at the origin of transfer that is essential for DNA transfer (Frost et al., 1994; Nelson et al., 1995).

Escherichia coli has evolved several mechanisms to control the metabolically expensive process of conjugation by F-like plasmids. These include silencing of the transfer region in stationary phase by the host-encoded H-NS protein with TraJ being an antagonist of H-NS repression at P7, Pm and P7 in early exponential phase growth (Will and Frost, 2006). Transfer is repressed in the absence of recipient cells via the plasmid-encoded FinOP antisense RNA system (Mullineaux and Willetts, 1985; Arthur et al., 2003). FinP antisense RNA transcription is, in turn, activated by Dam methylation at GATC sites near its promoter in the F-like plasmids pSLT and R100 (Camacho et al., 2005a). Lrp is an activator of P7 in pSLT and R100 but not F, and binding of Lrp to P7 is also inhibited by Dam methylation (Camacho et al., 2005b). F plasmid transfer gene expression also responds to nutritional signals through CRP and catabolite repression (Starcic et al., 2003).

The Cpx (conjugative plasmid expression) locus was first identified by isolating mutations (cpx) that reduced levels of F transfer efficiency by preventing the accumulation of TraJ (Sambucetti et al., 1982). The Cpx regulon was later found to be an extracytoplasmic stress response
system that responds to misfolding or overproduction of cell envelope components (Raivio and Silhavy, 2001). It has also been shown to be involved in cell surface composition, biogenesis of pili, adhesion and growth (Raivio and Silhavy, 2001; Raivio, 2005; Zahrli et al., 2006). Thus, the F transfer operon, which produces a complex transenvelope type IV secretion apparatus (Lawley et al., 2003), is an excellent candidate for regulation by the Cpx system.

The CpxA and CpxR proteins constitute a typical two-component regulatory system that senses stress and conveys this signal from the envelope to the cytoplasm via a phosphotransfer reaction. The inner membrane sensor kinase, CpxA releases CpxP (Buelow and Raivio, 2005; Fleischer et al., 2007), autophosphorylates at a conserved histidine in the cytoplasmic domain and transfers the phosphate group to a conservsed aspartate in the cytoplasmic response regulator CpxR (Raivio and Silhavy, 1997). Phosphorylated CpxR (CpxR-P) acts as a transcriptional activator by binding to the promoters of target genes at the consensus sequence 5'-GTA N2 GTA N3' (Pogliano et al., 1997; De Wulf et al., 2002). Approximately 100 promoters that are potentially regulated by CpxR-P were identified by screening the E. coli genome with a 15 bp weighted matrix corresponding to the consensus CpxR-P binding site (De Wulf et al., 2002). Examples of known CpxR-P target genes are cpxP (Danese and Silhavy, 1998), degP (Cosma et al., 1995; Danese et al., 1995), ppiA and dsbA (Danese and Silhavy, 1997; Pogliano et al., 1997), encoding chaperones, proteases and other enzymes for maintaining envelope protein integrity. CpxR-P has also been found to repress promoters of chemoreceptor and motility genes (De Wulf et al., 1999). Currently, the number of confirmed Cpx-regulated promoters is 25 (De Wulf et al., 2002; Dorel et al., 2006), not all of which are related to envelope stress.

In the absence of a stress signal, CpxA acts as a phosphatase to catalyse the dephosphorylation of CpxR-P, thereby downregulating the Cpx pathway. Some cpxA* mutants, such as cpxA101*, retain autokinase and kinase functions but lose phosphatase activity (Raivio and Silhavy, 1997). As a consequence, the levels of CpxR-P are elevated in cpxA* cells, causing constitutive activation of the Cpx regulon. The original cpxA point mutation that led to reduced Pγ activity and F conjugation was later characterized as cpxA2*. That CpxA is not required for tra operon transcription was further confirmed by the finding that a deletion within cpxA had little effect on transfer ability (Rainwater and Silverman, 1990). The cpxA101* mutation, which involves a single amino acid change from threonine to proline at position 253 (Raivio and Silhavy, 1997), affects F conjugation via a post-transcriptional mechanism that reduces TraJ levels (Gubbins et al., 2002). We hypothesized that a cytoplasmic protease or a chaperone partner is upregulated when the Cpx system is activated. Taking advantage of the strong phenotype of the cpxA101* mutant, we performed microarray analysis to identify the HslVU heat shock protease–chaperone pair as being involved in TraJ degradation. TraJ, which is stable throughout the growth cycle (Frost and Manchak, 1998), is degraded only in the presence of stress (nlpE expression) or perceived stress (cpxA101*) both in vivo and in vitro. TraJ also appears to be a substrate for HslVU throughout the growth cycle, but is protected or modified by a factor encoded by the F transfer region in the absence of stress.

### Results

**Proteases are upregulated in E. coli cpxA101*/pOX38-Km**

We hypothesized that a protease or a chaperone is upregulated in cpxA* leading to the degradation of TraJ. Accordingly, preliminary microarray analyses were performed to compare the gene profile in E. coli MC4100 (wild type) and TR189 (cpxA101*), both containing pOX38-Km, an F derivative (Table 1). The microarray results were considered valid as known Cpx-regulated genes such as cpxR, -A, -P, degP and dsbA were upregulated in TR189 as expected (data not shown). The cpxA101* mutation appeared to stimulate the heat shock regulon as many heat shock genes were upregulated in TR189 (wild type) and TR189 (cpxA101*), both containing the F derivative, pOX38-Km. We tested the involvement of cpxA101* (rpoH) in the Cpx-mediated

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<th>Blattner no.</th>
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a. Signal log2 ratio of transcript levels for TR189 (cpxA101*) relative to the MC4100 (wild type) strain. Both strains contain the F derivative, pOX38-Km.
inhibition of conjugation, the levels of TraJ were measured by immunoblot analysis of wild-type and rpoH mutant (KY1621) cells with and without pLD404. pLD404 induces stress by constitutively overexpressing nlpE, which encodes an outer membrane protein that activates the CpxAR regulon (Snyder et al., 1995). Although the overall levels of TraJ were lower in KY1621, they were further reduced in the presence of pLD404, suggesting that CpxAR does not regulate TraJ via its native promoter, cloned into pBR322. When HslVU was supplied in trans, TraJ was reduced to undetectable levels (Fig. 2) as were TraY and TraM (data not shown). Suppling HslVU in trans in C600/pOX38-Km pl13 also resulted in the degradation of TraJ (Fig. 2), indicating that TraJ or a protein required for TraJ stability, was a substrate for HslVU in vivo.

**Effect of hslV and hslU mutations on TraJ stability in cpxA101* cells**

SG12064 (hslV) and SG12065 (hslU) were generous gifts from Dr Susan Gottesman (NIH). pOX38-Km was mated into the double mutants IL1 (cpxA101* hslV) and IL2 (cpxA101* hslU) to provide TraJ, which was detected by immunoblot analysis (Fig. 1). All experiments were performed at 30°C, because of the temperature-sensitive phenotype of cpxA101* strains, which is known to decrease mating ability 10-fold (Frost and Simon, 1993). TraY and TraM levels were also assayed by immunoblot to monitor the P, and P promoters, which require TraJ directly or indirectly for activation. Levels of TraJ, -Y and -M were significantly reduced in IL9 (C600 cpxA101*)/pOX38-Km, but were restored in the double mutants IL1 (cpxA101* hslV)/pOX38-Km (Fig. 1) and IL2 (cpxA101* hslU)/pOX38-Km (data not shown). Thus both the chaperone (HslU) and the protease (HslV) are required for TraJ reduction in cpxA101* cells. We also performed the same experiments in *E. coli* MC4100, which was used in the microarray experiments, and found that TraJ was restored in the double mutants MC4100 cpxA101* hslV or hslU (data not shown).

To further confirm that the restoration of TraJ in IL1/ pOX38-Km and IL2/pOX38-Km was due solely to the mutations in hslV, -U, the double mutants IL1 and IL2 were complemented with pIL13, which expresses hslVU from its native promoter, cloned into pBR322. When HslVU was supplied in trans, TraJ was reduced to undetectable levels (Fig. 2) as were TraY and TraM (data not shown). Supplying HslVU in trans in C600/pOX38-Km pl13 also resulted in the degradation of TraJ (Fig. 2), indicating that TraJ or a protein required for TraJ stability, was a substrate for HslVU in vivo.

**pOX38-Km transfer ability is rescued in an cpxA* hslV double mutant**

Mating assays, which are sensitive over a 6–7 log range, were used to confirm that the restoration of TraJ also restored mating ability using C600/pOX38-Km and *E. coli* XK1200 as the background donor and recipient strains respectively (Table 2). The mating efficiency of IL9 (C600 cpxA101*)/pOX38-Km decreased to 5.4% of wild-type levels whereas the mating efficiency of IL1 (C600 cpxA101* hslV)/pOX38-Km was restored to 75% of wild type (C600/pOX38-Km). These results were consistent with the levels of TraJ, -Y and -M detected by immunoblot analyses (Fig. 1).
TraJ levels were reduced in wild-type cells (C600/pOX38-Km) expressing hslVU from pIL13 (Fig. 2). This was also reflected in the reduced mating efficiency of these cells (0.6%). Mating efficiency was also low for IL1 (cpxA101 hslV)/pOX38-Km (1.1%), compared with IL1/pOX38-Km (75%; Table 2). Thus, constitutively expressed hslVU appears to efficiently reduce TraJ levels and, concomitantly, conjugation.

An hslVU mutation restores TraJ and F conjugation in cells exposed to envelope stress

Overproduction of the outer membrane lipoprotein NlpE, which activates the Cpx pathway (Snyder et al., 1995), more closely resembles extracytoplasmic stress than the pleiotropic cpxA101 mutation. When NlpE is overproduced from multicopy plasmids, F conjugation and TraJ levels are both reduced (Gubbins et al., 2002). To determine whether HslVU is involved, NlpE was constitutively expressed from the plasmid pLD404 in C600/pOX38-Km and SG12064 (hslV)/pOX38-Km (Table 3). The mating efficiency of wild-type cells decreased 5.7-fold in the presence of pLD404, whereas the mating efficiency in SG12064/pOX38-Km/pLD404 was unaffected. The levels of TraJ and TraY reflected these results as shown by immunoblot analysis (data not shown). Thus, HslVU was implicated in the reduction of TraJ levels and mating efficiency during induction of stress by NlpE overproduction.

CpxAR influences TraJ degradation in cells overexpressing nlpE

Our results could be explained by the activation of another stress regulon that acts in parallel or in conjunction with the CpxAR response system. To test this possibility, the levels of TraJ were monitored in the presence (pLD404) or absence (pBR322) of NlpE in a cpxRA mutant. The levels of TraJ were comparable to wild type in a cpxRA mutant, TR51, as were the mating efficiencies (data not shown). This result suggests that the absence of CpxR and CpxA in TR51 prevents the degradation of TraJ. As a control, an hslVU::lacZ transcriptional fusion on plasmid pIL18 was introduced into MC4100 or TR51 carrying pLD404, and was found to be induced 4.3-fold from 600 MU to 2600 MU in MC4100 but not TR51. Thus, it appears that the reduction in TraJ and conjugation in the presence of nlpE overexpression is CpxR-dependent.

In vivo degradation of TraJ requires stress or synthesis of fresh TraJ

We next asked whether existing TraJ levels decreased when stress was induced during exponential growth by expressing nlpE from the arabinose-inducible promoter in pBAD18 (pND18). TR49/pOX38-Km/pND18 was assayed for TraJ and TraY levels after 0.05% arabinose induction for 2 h (Fig. 3A). TraJ or TraY levels remained constant and mating efficiency did not decrease. Cells were monitored for activation of the Cpx regulon by following the induction of a degP::lacZ transcriptional fusion in TR49 (Raivio and Silhavy, 1997; Fig. 3B). The half-life of TraJ, measured using the procedure described in Gubbins et al. (2002), was greater than 8 h, indicating that previously synthesized TraJ was stable (Fig. 3C and D). This is in agreement with the results of Frost and Manchak (1998).

We then asked whether freshly synthesized TraJ was susceptible to degradation by HslVU, using a transcriptional fusion of traja to the araBAD promoter in pBAD33 (pLJ14). pLJ14 was introduced into MC4100/F lac traja90 cells containing pBR322, pLD404 (nlpE) or pIL13 (hslVU). TraJ production was induced by the addition of 0.05% arabinose for 50 min, followed by removal of the arabinose and addition of 0.4% glucose and 0.2 mg ml\(^{-1}\).
rifampicin to halt further transcription. The levels of TraJ were monitored over 4 h by immunoblot, and were estimated by densitometry of the bands (Fig. 4A and B). TraJ was found to be stable in the presence of pBR322 (97%), and partially degraded in the presence of pIL13 (81%) and pLD404 (41%). Thus, TraJ appears to be stable in wild-type cells, and is degraded in the presence of excess HslVU or stress. These experiments were carried out in the presence of the F_{lac} traJ90 plasmid (Achtman et al., 1971) that carries an amber mutation in traJ that maintains the H-NS binding sites required for silencing (Will et al., 2004). We then tested the effect of the F plasmid on TraJ stability by repeating the experiment in the presence or absence of F_{lac} traJ90 using pLJ15, a derivative of pLJ14 that is KmR rather than CmR (Fig. 4C). Interestingly, the presence of F_{lac} traJ90 increased TraJ stability. Thus, TraJ appears to exist in two forms, which we denote TraJ and TraJ* (see Discussion), with TraJ*, which is dependent on the presence of F, being resistant to HslVU degradation.

In vitro degradation of His_{f}TraJ by purified HslV and HslU

The previous results suggest that TraJ could be either a direct or indirect substrate for HslVU. To demonstrate direct proteolysis, purified TraJ was incubated with purified HslV and HslU (kindly provided by Dr. Eyoung Park, Seoul National University) in the presence of ATP and detected by immunoblot analysis. MBP-SuLA, a known substrate of HslVU (also provided by Dr. Park), was used as a positive control. MBP-SuLA was partially degraded
A arabinose-inducible plasmid encoding His 6-TraJ (pILJ16) was constructed and shown to complement the traJ amber mutation in Flac traJ90 (data not shown). pILJ16 was induced with 0.05% arabinose and purified by Ni-NTA agarose chromatography. One microlitre of His 6-TraJ, which was set at 100% at time 0, was incubated with HsiV and HsiU at 37°C and monitored over 4 h (Fig. 5B). A control digestion was performed in the absence of HsiV and HsiU for 4 h to ensure that His 6-TraJ was not degraded by contaminating proteases (Fig. 5C). His 6-TraJ was stable when purified from cells carrying the pBR322 vector control (Fig. 5B, pBR322). In contrast, His 6-TraJ was degraded to 62% of the original amount when it was purified from MC4100/pILJ16/pLD404 (NlpE; Fig. 5B and D). Thus the presence of envelope stress, generated by pLD404, appears to alter the susceptibility of TraJ to HsiVU.

**Discussion**

Our results suggest that TraJ, the activator of F transfer operon transcription, is a substrate for the host protease HsiVU during the envelope stress response mediated by the Cpx regulatory system. Microarray analysis of a cpxA101* mutant of *E. coli* MC4100 revealed that the protease–chaperone pair hsiVU was upregulated and, based on its role in degrading other regulators, was investigated further. Mutations in hsiVU restored or increased TraJ levels in the presence of stress or in wild-type cells. The introduction of HsiVU in trans complemented these mutations and also led to decreased TraJ levels and mating ability in wild-type cells. While these results seemed straightforward, the intransigence of intracellular TraJ to degradation upon controlled induction of the Cpx response suggested a more complex mechanism (Fig. 3A). This result prompted us to investigate TraJ degradation under various conditions, including in the presence and absence of F, and under in vitro conditions (Fig. 5). Our results suggest that: (i) TraJ appears to be present in two forms, only one of which is active (TraJ*), and is susceptible to HsiVU; (ii) a factor encoded on F modifies TraJ such that it is more stable and (iii) TraJ is susceptible to degradation in the presence of stress, for instance by the overproduction of NlpE, in a CpxR-dependent manner. Other interpretations could involve alterations in the activity of HsiVU or the presence of other chaperones such as GroESL (Table 1) that are upregulated in response to stress that alter the susceptibility of TraJ to HsiVU.

These observations agree with what is known about TraJ function and activation during growth. TraJ is present at high levels in stationary F+ cells (Frost and Manchak, 1998), but is unable to rescue the F transfer region from H-NS silencing, suggesting that it is inactive (TraJ*) and is modified (or requires modification) in some way. Upon

**Fig. 4.** Monitoring the in vivo degradation of TraJ in the presence and absence of the F plasmid (Flac traJ90). pILJ14 (TraJ) was induced with 0.05% arabinose for 50 min prior to the addition of 0.4% glucose and 3 µM rifampicin to prevent further rounds of traJ transcription. TraJ levels were assayed by immunoblot at the times (min) indicated after rifampicin addition. Uninduced refers to a sample that was not treated with arabinose.

A. MC4100 Flac traJ90/pILJ14 with pBR322 (vector control, I), pLD404 (NlpE, II) or pIL13 (HsiVU, III).

B. TraJ levels detected in A were quantified with AlphaEase software and a FluorChem IS-5500 imaging system as described in Experimental procedures, and were plotted versus time after the addition of rifampicin and glucose. The percentages of TraJ remaining in MC4100 Flac traJ90/pILJ14 with pBR322 (I, diamond), pLD404 (II, square) or pIL13 (III, triangle) are shown on the right.

C. The levels of TraJ in MC4100/pILJ15 with (+; upper panel) or without (−; lower panel) Flac traJ90 over 5 h (min) after addition of rifampicin is shown.

**Fig. 5.** Degradation of F TraJ by HsiVU

An arabinose-inducible plasmid encoding His 6-TraJ (pILJ16) was constructed and shown to complement the traJ amber mutation in Flac traJ90 (data not shown). pILJ16 was induced with 0.05% arabinose and purified by Ni-NTA agarose chromatography. One microlitre of His 6-TraJ, which was set at 100% at time 0, was incubated with HsiV and HsiU at 37°C and monitored over 4 h (Fig. 5B). A control digestion was performed in the absence of HsiV and HsiU for 4 h to ensure that His 6-TraJ was not degraded by contaminating proteases (Fig. 5C). His 6-TraJ was stable when purified from cells carrying the pBR322 vector control (Fig. 5B, pBR322). In contrast, His 6-TraJ was degraded to 62% of the original amount when it was purified from MC4100/pILJ16/pLD404 (NlpE; Fig. 5B and D). Thus the presence of envelope stress, generated by pLD404, appears to alter the susceptibility of TraJ to HsiVU.
resumption of growth, for instance by dilution of a stationary culture into fresh medium, either existing TraJ* is activated by reversing this modification, or fresh TraJ is produced by stimulation of the traJ promoter. If stress (NlpE) or perceived stress (cpxA101*) is present during resumption of growth, for instance, in lag phase, the newly translated, active TraJ could predominate and be immediately degraded, leading to continued silencing by H-NS. This would be an efficient mechanism for reducing F transfer potential during unfavourable physiological conditions. The induction of stress in mid-exponential phase cells did not cause an appreciable change in the level of TraJ (Fig. 4A), suggesting that once transfer gene expression is activated and the transfer apparatus is synthesized, excess TraJ becomes modified/stabilized by an F-encoded factor, and is no longer subject to HslVU degradation.

Our data may explain why TraJ binding to DNA has been difficult to demonstrate previously. Purified His6-TraJ does not bind DNA (W.R. Will and L.S. Frost, unpubl. obs.), although ‘fresh’ TraJ from the F-like plasmid R100-1 bound DNA at pH 5.5 (Taki et al., 1998). Our data suggest that the majority of TraJ molecules visible in immunoblots or in TraJ preparations might be inactive (TraJ*), and the small portion of TraJ synthesized to activate transfer region gene expression at each cell division could be masked by this pool of inactive TraJ*. Why TraJ* accumulates in wild-type cells is unclear at this time.

The presence of the F plasmid appeared to protect TraJ from degradation by HslVU in vivo in the absence of stress. Our preliminary results suggest that the stabilizing element maps to an EcoRI fragment containing trbG, -R and -V (Frost et al., 1994). The hypothesis that TraR may play such a role is currently under investigation. Interestingly, TraR is thought to be a homologue of DksA, a suppressor of defects in DnaK, an Hsp70 family member (Doran et al., 1994).

HslV (ClpQ) is an ATP-dependent protease with a threonine in its active site that requires the adjacent gene product, HslU (ClpY), a chaperone, for activity (Rohrwild et al., 1996; Gottesman, 2003). Substrates of HslVU include the cell division inhibitor SulA (Wu et al., 1999), and the capsule synthesis regulatory protein RcsA (Kuo et al., 2004), with both proteins being coregulated by the Lon protease. Aberrant cell division and randomized FtsZ ring assembly have been observed in cpxA101* cells by Pogliano et al. (1998) possibly as a result of the rapid degradation of SulA, a substrate of HslVU, which is activated by the Cpx system. Interestingly, the filamentous phenotype of cpxA101* cells is suppressed by the hslV mutation in IL1 (cpxA101* hslV) as revealed by electron microscopy (I.C. Lau-Yong and L.S. Frost, unpubl. obs.). Thus, TraJ is a member of a select group of regulators that are subject to HslVU control, and is the first to be
shown to be degraded in response to extracytoplasmic stress.

The susceptibility of TraJ to HslVU appears to be affected by other factors including the presence of the F plasmid, with TraR being an intriguing candidate. Recently, GroEL has been shown to interact with TraJ and target it for degradation during the heat shock response (Zahrli et al., 2007). Using anti-GroEL antibodies kindly provided by Dr Gunther Koraimann, we determined that GroEL was present in nearly equivalent amounts in His6-TraJ preparations purified from cells in the presence or absence of pLD404 (NlpE; data not shown), arguing against a role for GroEL in altering TraJ susceptibility to HslVU, although further investigation of this possibility is warranted. GroEL could participate in TraJ degradation in response to other inducing cues such as heat shock, or by affecting the stability of TraJ modifiers such as TraR, suggesting a multifactorial mechanism for fine control of TraJ levels and F plasmid transfer potential.

A model incorporating our observations is summarized in Fig. 6. An inducing cue is sensed in the envelope to activate CpxAR yielding CpxR-P that activates the Cpx regulon, including hslVU and possibly other factors that affect TraJ susceptibility to HslVU, such as GroEL. TraJ could be modified in some way, for instance, through dimerization, or by being bound to DNA, and is converted to TraJ* that is protected from degradation. Furthermore, in the absence of stress, an F-encoded factor such as TraR also appears to affect the stability of pools of TraJ in the cell, perhaps by promoting dimerization or binding to the dimmer form. These two possibilities are not mutually exclusive, and could indicate that TraJ is degraded only when it is actively opposing H-NS silencing. Our results also suggest that F transfer gene expression is repressed if envelope stress is present in lag phase at the beginning of the growth cycle. However, once the transfer apparatus has been synthesized and exponential growth is occurring, this control mechanism is of less importance and other mechanisms, such as conformational changes in TraM (Lu et al., 2006), that provide a quick response to physiological changes such as temperature or pH, become central to regulating conjugation.

Experimental procedures

Bacterial strains and plasmids

All strains and plasmids used in this study are described in Table 4. Standard genetic techniques were employed to construct strains (Silhavy et al., 1984). The double mutants IL1 (cpxA101* hslV) and IL2 (cpxA101* hslU) were constructed by P1 transduction of the cpxA101* allele from TR189 into SG12064 (hslV) and SG12065 (hslU) using the linked zii::Tn10 (TcR) as a marker. The double mutants were confirmed by polymerase chain reaction (PCR) to show the pes-
ence of the antibiotic resistance insertion and their resistance to amikacin, which is conferred by the cpxA101* allele.

**Media, antibiotics and growth conditions**

All cultures were grown and maintained in Luria–Bertani (LB) broth or on agar plates at 30°C (for experiments involving cpxA101*, rpmH or hsiV) or 37°C. Antibiotics were used at the following concentrations in selective media: ampicillin (Amp), 100 μg ml⁻¹; kanamycin (Km), 25 μg ml⁻¹; chloramphenicol (Cm), 20 μg ml⁻¹; streptomycin (Sm), 100 μg ml⁻¹; nalidixic acid (Nal), 20 μg ml⁻¹; rifampicin (Rif), 200 μg ml⁻¹; and spectinomycin (Spc), 100 μg ml⁻¹. cpxA* strains were supplemented with 3 μg of amikacin per millilitre to prevent reversion (Raivio et al., 1999).

**Construction of plL13, 17, 18, plLJ14–16 and pRWJ2**

*Escherichia coli* MC4100 genomic DNA was isolated using standard methods (Moore, 1994), and was used as the template for PCR amplification of the hsiVU coding region for plL13 and the hsiV promoter region for plL17. plL13 was constructed by using the upstream primer ILA19

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<th>Strain or plasmid</th>
<th>Genotype</th>
<th>Source or reference</th>
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<tr>
<td><strong>Bacterial strains</strong></td>
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<tr>
<td>MC4100</td>
<td>F⁻ araD139Δ(argF-lac)U169 rpsL150 relA1 thi6501 deoC1 ptsF25 rbsR</td>
<td>Casadaban (1976)</td>
</tr>
<tr>
<td>XK1200</td>
<td>F⁻ lacZD124Δ (nadA araG gal attL)</td>
<td>Moore et al. (1981)</td>
</tr>
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<td>C600</td>
<td>supE44 thi-1 thr-1 leuB6 lacY1 tonA21</td>
<td>Laboratory Collection</td>
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<tr>
<td>SG12064</td>
<td>C600 hsiV::cm</td>
<td>Susan Gottesman</td>
</tr>
<tr>
<td>SG12065</td>
<td>C600 hsiU::cm</td>
<td>Susan Gottesman</td>
</tr>
<tr>
<td>KY1621</td>
<td>rpmH::km</td>
<td>Klein et al. (2003)</td>
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<tr>
<td>TR20</td>
<td>MC4100 cpxA101*</td>
<td>Raivio et al. (1999)</td>
</tr>
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<td>TR51</td>
<td>MC4100 cpxR1::spc (polar on cpxA) Spc'</td>
<td>Raivio and Silhavy (1997)</td>
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<td>TR189</td>
<td>MC4100 cpxA101* zii::Tn10 λRS88[degP-lacZ]</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>Flac traJ90</td>
<td>traJ lac⁺ F derivative</td>
<td>Achtman et al. (1971)</td>
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<td>pOX38-Km</td>
<td>Km', F tra region, Rep FIA replicon</td>
<td>Chandler and Galas (1983)</td>
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<tr>
<td>pACYC184</td>
<td>Cmr', Tc', general cloning vector</td>
<td>Chang and Cohen (1978)</td>
</tr>
<tr>
<td>pBR322</td>
<td>Amp', Tc', general cloning vector</td>
<td>New England Biolabs</td>
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<td>pLD104</td>
<td>Amp', nlpE cloned into pBR322</td>
<td>Snyder et al. (1995)</td>
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<tr>
<td>pBAD18</td>
<td>Amp', general cloning vector</td>
<td>Guzman et al. (1995)</td>
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<td>Danese et al. (1995)</td>
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<td>pBAD24</td>
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<td>Guzman et al. (1995)</td>
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<td>pBAD33</td>
<td>Cm', general cloning vector</td>
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<td>plL17</td>
<td>Amp', Cmr', hsiVU::lacZ in pJLac101</td>
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<td>plL18</td>
<td>Cmr', hsiV::lacZ in pACYC184</td>
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<tr>
<td>plLJ14</td>
<td>Cmr', traJ cloned into pBAD33</td>
<td>This work</td>
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<tr>
<td>plLJ15</td>
<td>Km', traJ cloned into pBAD33 with KIXX</td>
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<td>plLJ16</td>
<td>Cmr', his-traJ cloned into pBAD33</td>
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<tr>
<td>pRS27</td>
<td>Tc', 9 kb partial EcoRI fragment of F in pSC101</td>
<td>Skurray et al. (1978)</td>
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<tr>
<td>pRWJ2</td>
<td>Amp', his-traJ cloned into pBAD24</td>
<td>This work</td>
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5‘-GGATTTCCTGACGCAGCCAAAACCG-3’ and the downstream primer ILA20 5‘-CCGGATCCCGCCGATAATTGCGA GC-3’ to introduce an EcoRI site and a BamHI site at the 5’ and 3’ ends respectively. Vent polymerase (NEB) was used to amplify the 2386 bp PCR product, which was cloned into EcoRI/BamHI-digested pBR322. pIL17 was constructed using the upstream primer ILA49 5‘-AGATCTGTACCAGCGC CAAGACCGAGC-3’ and downstream primer ILA50 5‘-GG TACCCGAGTCCCCCTTGTAC-3’ to amplify the hsiV promoter region and to introduce the BglII and KpnI sites. The PCR product was cloned into BglII/KpnI-digested pJLac101, an RK2-replicon-based promoter assessment plasmid. pIL18 was constructed by digesting pIL17 with BglII and XbaI and ligating the hsiV::lacZ fragment into BamHI/XbaI-digested pACYC184 T4 using DNA ligase (Roche Diagnostics, same as below). Positive clones were sequenced using the DYEnamic ET fluorescent sequencing system (Amersham Pharmacia Biotech) to confirm that the hsiV genes and the hsiVU promoter region were correctly cloned into the plasmids. pRWJ2 was constructed using upstream primer RWI34 5‘-CCATGGTCATTCTCAATTTCCTATTATGACACATAGCCCG-3’ to amplify the F traJ gene and to introduce an NcoI and a
six-histidine tag at the 5′ end as well as a PstI site at the 3′ end. The PCR product was cloned into Ncol/PstI-digested pBAD24. pLJ14 was constructed by digesting pBADTraJ (Gubbins et al., 2002) with ClaI and HindIII, and ligating the traJ fragment into pBAD33. pLJ15 was constructed by digesting pUC4-KIXX with Smal and ligating the Kmr fragment into PvuII-digested pLJ14 to confer kanamycin resistance. Positive clones of pLJ15 were selected based on their phenotypes (KmrCmS) and sequenced. pILJ16 was constructed by digesting pRWJ2 with ClaI and HindIII, and ligating the his6-traJ fragment into pBAD33 to confer Cm resistance. The traJ coding region on pLJ14, pLJ15 and pLJ16 was sequenced and the plasmids were tested in a complementation experiment using a Flac traJ90 mutant to ensure they were functional in vivo.

**Microarray analysis**

*Escherichia coli* MC4100/pOX38-Km and TR189/pOX38-Km cells were inoculated in LB broth containing the appropriate antibiotics and were grown overnight at 30°C with aeration. The next day, cultures were diluted 1:50 into 10 ml of fresh LB broth and grown at 30°C with aeration to an optical density at 600 nm (OD600) of 1.0. Total RNA was isolated from the cells using the MasterPure™ RNA Purification kit (Epicentre). Enrichment and direct labelling of mRNA were done as described in the GeneChip Expression analysis technical manual (Affymetrix) and as described elsewhere (Masuda and Church, 2002). Pelleted RNA was dissolved in 20 μl of nuclease-free water, and hybridized to an *E. coli* genome array (Affymetrix). Hybridization was done as described in the GeneChip Expression analysis technical manual. The array was scanned at 570 nm with a resolution of 3 μm with a GeneArray scanner. Data analysis was performed using Affymetrix Microarray Suite 5.0 software (http://www.affymetrix.com/support/technical/whitepapers.affx).

**Immunoblot analysis**

Volumes of cell pellets corresponding to 0.1 OD600 were collected from cultures that were grown to an OD600 of 0.5–1 for all immunoblot analyses. Samples were boiled in 10 μl of sodium dodecyl-sulphate (SDS) loading buffer (Laemmli, 1970) for 5 min, and separated by SDS-12% PAGE using the Bio-Rad Protean Minigel system. Proteins were transferred to Immobilon-P membranes (Millipore) using Towbin buffer (Towbin et al., 1979). Membranes were blocked for 2 h at room temperature or overnight at 4°C with 10% (w/v) skim milk (Difco) dissolved in TBST (50 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.1% (v/v) Tween 20 (Caledon Laboratories)). Rabbit polyclonal antisera were diluted (anti-TraJ, 1:40 000 or 1:25 000 for older antiserum; anti-TraM, 1:10 000; and anti-TraY, 1:2000) in the blocking solution and incubated overnight at 4°C. Blots were washed at room temperature (four times 10 min) with TBST, and incubated with the secondary antibody (1:10 000) horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G (Amersham Life Sciences), washed as described above, and then developed with Western Lightning™ Chemiluminescence Reagent Plus (PerkinElmer Life Sciences) and exposed to Kodak X-Omat R film. For in vivo and in vitro TraJ degradation, proteins analysed by immunoblotting were quantified with an AlphaEase software package and a FluorChem IS-5500 imaging system (Alpha Innotech, Fisher Scientific). The densities along each lane of the immunoblots were measured by using the 1D-Multi autogrid function. The peak area corresponding to the level of TraJ or His6-TraJ was normalized to bovine serum albumin as a standard.

**Stability of TraJ in vivo**

Cultures of *E. coli* containing the arabinose-inducible plasmids pLJ14 and pLJ15 were grown at 37°C with 0.4% glucose and appropriate antibiotics to an OD600 of 0.4. Samples were collected before and after induction, and the cell pellets were frozen at −20°C until required. Three millilitres of the cultures were centrifuged and washed to remove glucose. 0.05% arabinose in 3 ml of fresh LB was added to induce the expression of TraJ. Induction was carried at 37°C for 50 min with agitation. The zero time sample was collected, and the induced culture was centrifuged and washed to remove arabinose. Three millilitres of fresh LB containing 0.4% glucose and 200 μg ml−1 rifampicin was added to prevent further expression from the arabinose promoter Pbad. Samples were collected at 30, 60, 120, 180, 240 or 300 min post induction and subjected to immunoblot analysis as described above. Induction of nlpE from the arabinose-inducible promoter in pND18 was done in a similar manner to pLJ14 and 15.

**β-Galactosidase assays**

β-Galactosidase assays were performed as previously described (Gubbins et al., 2002).

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References


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