University of Alberta

Regulation of F plasmid conjugation in times of envelope stress

by

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In loving memory of my father,

Thomas T.F. Lau,

who demonstrated a life of service and selfless love to our family

and inspired me to become courageous and persistence when life is tough.

Abstract

The *Escherichia coli* F plasmid is a paradigm for studying bacterial conjugation. The present study investigates the regulation of F conjugation in cells under extracytoplasmic stress. The Cpx two-component system senses and responds to extracytoplasmic stress by regulating the expression of proteins that aid in cell envelope protein folding or degradation. This study focuses on TraJ, an activator of the F transfer (*tra*) operon, which counters host H-NS repression. How extracytoplasmic stress is conveyed into a signal that shuts down conjugation through destabilization of TraJ is key to this project.

In vivo experiment showed that TraJ is degraded by HslVU, a host protease/ chaperone pair that is upregulated when cells are exposed to Cpx-mediated extracytoplasmic stress. Surprisingly, TraJ is susceptible to HslVU degradation *in vitro* only when it was purified from a Cpx-activated background. We proposed that TraJ exists in two forms, TraJ and TraJ*. As cells age, modified TraJ* is resistant to HslVU and unable to counter H-NS repression at the major transfer promoter, P_Y, thus leading to F^- phenocopies.

The alternative sigma factor, σ^H (encoded by *rpoH*), is required for F plasmid replication and conjugation. In an F^+ *rpoH* strain, transcription of *traJ* and F conjugation is diminished. Such an *rpoH* effect is suppressed by *hns*. At present σ^H is hypothesized to be responsible for synthesis of a de-repressor that antagonizes H-NS repression at P_J. Alternatively, σ^H could be important for initiation of *traM* transcription that reads through into *traJ*. The fate of TraJ is further elucidated.

When F and RP4 plasmids are co-harboured in a cell, F PifC inhibits RP4 conjugation. This inhibition has been shown to involve RP4 TraG protein ($TraG_{RP4}$). TraG is a type IV coupling protein that drives DNA transport during conjugation. With the bacterial two-hybrid system, interaction between $TraG_{RP4}$ and F PifC was shown the first time. Cross-linking and immunoprecipitation experiments further demonstrated $TraG_{RP4}$-PifC interaction *in vivo*. Our current model suggests that PifC binds to the bottom of the TraG hexamer and prevents the gate from opening for the relaxase-bound DNA strand, and thus inhibits RP4 conjugation.

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Last but not least, to Patrick, my best friend, my shelter, my comforter, thank you. Your love has made me grown into a stronger person than I could ever be.

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Abbreviations

α	alpha
A	adenine
A_{260}	absorbance at 260nm
ADP	adenosine diphosphate
Amp	ampicillin
ATP	adenosine triphosphate
β	beta
bp	base pair
BSA	bovine serum albumin
C	cytosine
cAMP	cyclic adenosine monophosphate
Cm	chloramphenicol
Cpx	conjugative plasmid expression
CRP	cAMP repressor protein
DEPC	diethyl-pyrocarbonate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
dNTP	deoxyribonucleoside triphosphate
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
F	F plasmid
Fin	fertility inhibition
Fis	factor for inversion stimulation
G	guanine
GTP	guanosine triphosphate
Hfq	host factor for phage Qβ replication
H-NS	histone-like nucleoid structuring
HRP	horseradish peroxidase
IHF	integration host factor
Inc	incompatibility
IPTG	isopropyl β-D-thiogalactopyranoside
IS	insertion sequence
Km	kanamycin
kb	kilobase

kDa	kilo Dalton
lac	lactose
LB	Luria-Bertani
Lrp	leucine responsive protein
μ	micro
M	molar
ml	milliliter
mg	milligram
mM	millimolar
mRNA	messenger RNA
MU	Miller Units
Nal	nalidixic acid
Ni^{2+}-NTA	nickel-nitrilotriacetic acid
nic	nick site
OD_{600}	optical density at 600nm
ONPG	*o*-nitrophenyl β-D-galactopyranoside
ORF	open reading frame
oriT	origin of transfer
P	promoter
P_J	promoter for *traJ*
P_M	promoter for *traM*
P_{M1}	promoter for *traM*, proximal
P_{M2}	promoter for *traM*, distal
P_Y	promoter for *traY-I*
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
Pif	phage inhibition
PNK	polynucleotide kinase
PVDF	polyvinylidene difluoride
R	resistant factor
RBS	ribosome binding site
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
σ	sigma
σ^D/σ^{70}	housekeeping sigma factor
σ^E/σ^{24}	sigma factor for envelope stress
σ^H/σ^{32}	heat shock sigma factor
SDS	sodium dodecyl sulphate

SL	stem-loop
Spc	spectinomycin
Sm	streptomycin
Tc	tetracycline
Tn	transposon
tra	transfer
T4SS	type IV secretion system
T4CP	type IV coupling protein
UV	ultraviolet
v/v	volume/volume
vir	virulence
w/v	weight/volume
°C	degrees Celsius

Chapter 1: General Introduction

1.1 Overview of bacterial conjugation

Historical perspective

In nature, extrachromosomal DNA molecule can be passed on from one bacterium to the other. One of the mechanisms of this phenomenon, termed bacterial conjugation, refers to the horizontal transfer of a plasmid from a donor to a recipient cell. Conjugation was first discovered by Tatum and Lederberg who studied mating in *Escherichia coli* K12 cells, in which two strains that were auxotrophic for different amino acids were able to grow as a mixed culture on a minimal medium (Lederberg and Tatum, 1946). Subsequently, the ability of the auxotrophs to recover was discovered by Hayes, which was due to a fertility factor that passed on from a donor to a recipient cell (Hayes, 1953). Mating was found to be unidirectional, and successful mating required continued viability of only the donors (Hayes, 1952). The F factor remains a paradigm for the understanding of the type IV secretion systems (T4SS) that are central to the propagation of genetic determinants between cells in Gram negative bacteria (Lawley *et al.*, 2003).

Bacterial conjugation requires contact of the recipient cell with the donor through a pilus or a mating bridge. After receiving the transferred plasmid, the recipient becomes what is known as the transconjugant and it is able to initiate another round of conjugation (reviewed in Frost *et al.*, 1994). Traits advantageous to the bacterium, such as antibiotic resistance, virulence factors, and bacteriocide production can be encoded within the plasmid and transferred to the recipient cell. Conjugation is ubiquitous in nature. It is found to be not only interspecies, but also intergeneric and interkingdom. The classic example of interkingdom conjugation is the *Ti* plasmid in *Agrobacterium tumefaciens*. *A. tumefaciens* is able to transfer and integrate the T-DNA into a higher plant's genome. Production of opines, a trait encoded within the T-DNA, provides compounds that can

serve as carbon and nitrogen sources for *A. tumefaciens*. Conjugation also gives advantages to the bacteria in nutrient-limiting environments (reviewed in Kado, 1994; Lessl and Lanka, 1994; White and Winans, 2007).

1.2 The fertility factor, F

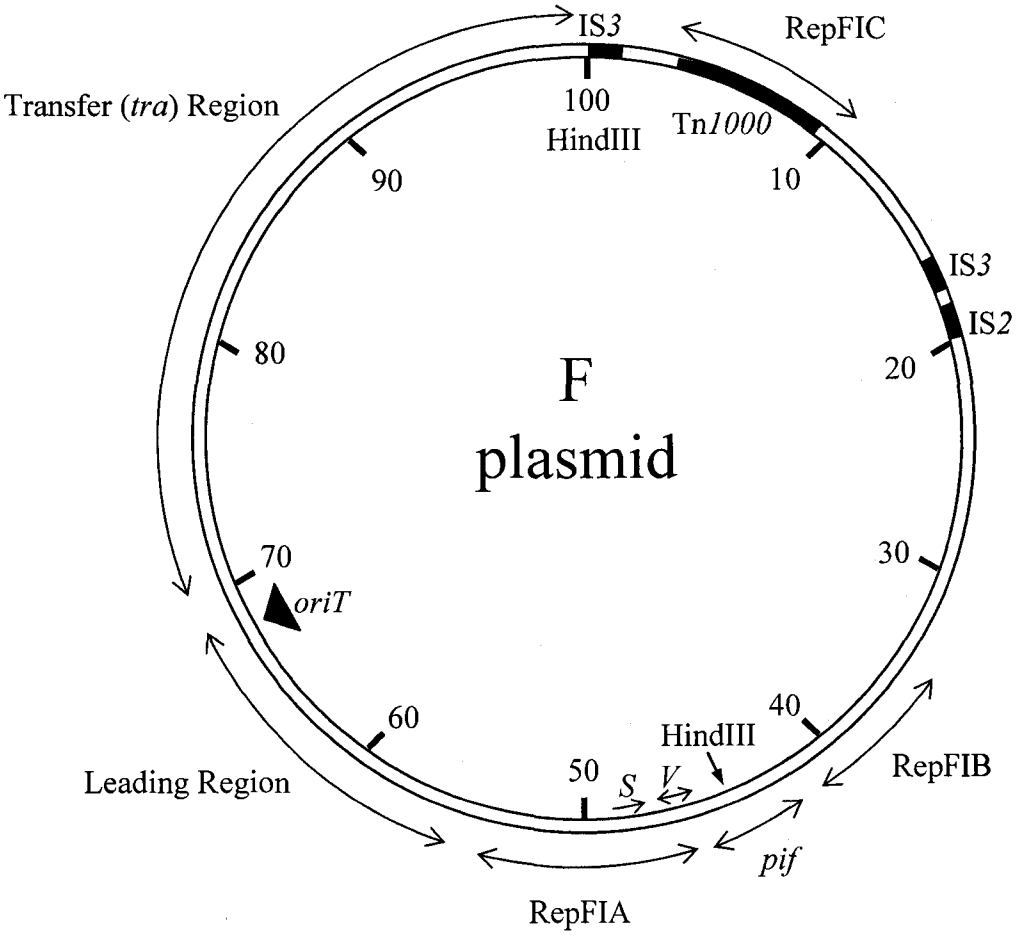
There are numerous plasmids that are able to undergo conjugation. These plasmids are all capable of autonomous replication (they contain the origin for replication) and self-mobilization (they can transfer themselves to other cells). These plasmids are classified into several incompatibility (Inc) groups base on their inability to coexist in a host (Datta, 1975). Generally, plasmids in the same Inc group exhibit similar plasmid size and gene organization.

The F fertility factor belongs to the IncF group. Other IncF plasmids, for example R-100 and ColB2, were found to possess F-related conjugative properties (Willetts and Maule, 1986). F is the best-studied conjugative system for over 60 years, and it remains a paradigm for plasmid-specific transfer systems. F is a 100kb, closed, circular, double-stranded plasmid (Figure 1.1; Willetts and Skurray, 1987). There are 115 genes mapped on F that occupy about 82 percent of the 100kb potential coding sequence (Shimizu *et al.*, 2000). The transfer (*tra*) region, bound by the *oriT* at 66.7kb and *finO* at 100kb in F, is one of the most intensively studied regions of F. The *tra* region and its regulation will be examined in detail in Subsections 1.3, 1.4 and the rest of this study.

The leading region

The leading region, between the boundary of RepFIA at 53.3kb and *oriT* at 66.7kb of F, is the first segment to enter the recipient cell during conjugation (Ray and Skurray, 1983). There are eight polypeptides encoded within this leading region. One of these genes, *ssb*, encoding a single-stranded DNA binding protein, is important in

Figure 1.1 Map of the 100-kb F plasmid. Coordinates are marked in the diagram in kilobases. Positions of the three origins of replication are indicated: RepFIA, RepFIB, and RepFIC. Insertion of Tn*1000* has led to disruption of RepFIC. There are two IS*3* and one IS*2* insertions on F, as indicated by the dark regions. The *pif* operon is responsible for both phage inhibition and inhibition of IncP plasmid transfer (See Fig. 1.2 in the following subsection and Chapter 6). The leading region is the region that first enters the recipient cell during mating. The origin of transfer (*oriT*) indicates the site where a nick is made during initiation of transfer. The transfer (*tra*) region is essential for the expression of pili and the process of conjugation. pOX38-Km (Table 2-1), an F derivative plasmid used extensively in this study, contains the region bound by two HindIII sites and thus is transfer proficient. The actual size of the F plasmid is 99,159 base pairs (GenBank accession number AP001918). This map is adapted from Willetts and Skurray (1987).


IS3
RepFIC
Transfer (tra) Region
100
HindIII
Tn1000
90
10
IS3
IS2
80
20
F
plasmid
70
oriT
30
60
40
HindIII
Leading Region
50
S
V
RepFIB
pif
RepFIA

maintaining the stability of the transferred strand (Chase *et al.*, 1983). The leading region is highly conserved among plasmids from other incompatibility groups (Golub and Low, 1986).

The replication regions

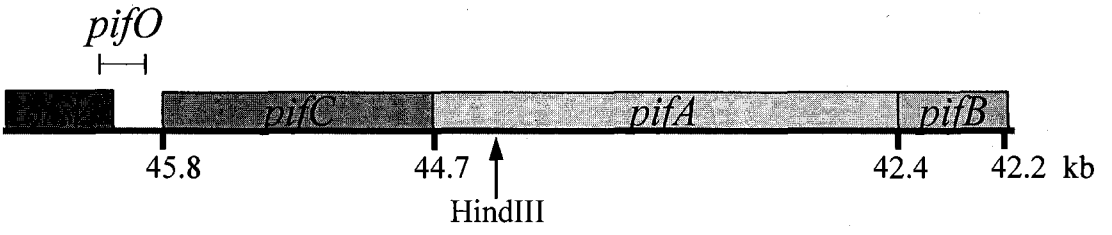
Autonomous DNA replication in the F plasmid is dependent on three replication regions: RepFIA, RepFIB, and RepFIC in conjunction with several host-encoded proteins including DnaB, DnaC, and PolC (Willetts and Skurray, 1987). RepFIA (45.9 kb to 53.3 kb) is the primary F replicon that governs F replication (Lane, 1981). Genes encoded within RepFIA region are responsible for the stability of F, its low-copy number, and its incompatibility with other F plasmids. Located within RepFIA, *oriS* initiates a unidirectional (to the left) replication, whereas *oriV* initiates a bidirectional replication. RepFIB (38.0 kb to 39.9 kb) is the secondary replication region, which functions independently to RepFIA and RepFIC.

The autonomous replication of F has been subject of several reviews (Willetts and Skurray, 1987; Kline, 1988). More recent analysis using a green fluorescent protein (GFP)-labeled replication origin proposed that F replication occurs near the middle of the cell division cycle when half of the host chromosome has been replicated (Cooper and Keasling, 1998; Gordon *et al.*, 2004). Sister plasmids migrate to the quarter positions in the cell, which become the septal regions following cell division.

The pif *operon*

The *pif* operon (phage inhibition by F) located at 43.3 kb to 47.2 kb in F encodes gene products PifA, PifB, PifC and the operator sequence *pifO* (Palchaudhuri and Maas, 1977). The *pif* operon is under the negative control of PifC (also named RepC) that overlaps within the RepFIA region (Figure 1.2). PifA has been shown to inhibit

Figure 1.2 Map of the *pif* region. The *pif* operon, extending from 42 kb to 46 kb in the F plasmid, is transcribed in an anti-clockwise direction. The *pifO* operator sequence is bound by PifC, which acts as an autorepressor for the regulation of *pif*. The *pifA* and *pifB* gene products inhibit reproduction of bacteriophages by different mechanisms. PifC is also named RepC, and functions as one of the replication proteins in the RepFIA replicon. Apart from this, the presence of *pifC* inhibits conjugation of RP4, an IncP plasmid. Chapter 6 examines the features of this protein. Numbers below indicate the position of the *pif* genes in the F coordinates. The map is not to scale.


pifO
pifC
pifA
pifB
45.8
44.7
42.4
42.2 kb
HindIII

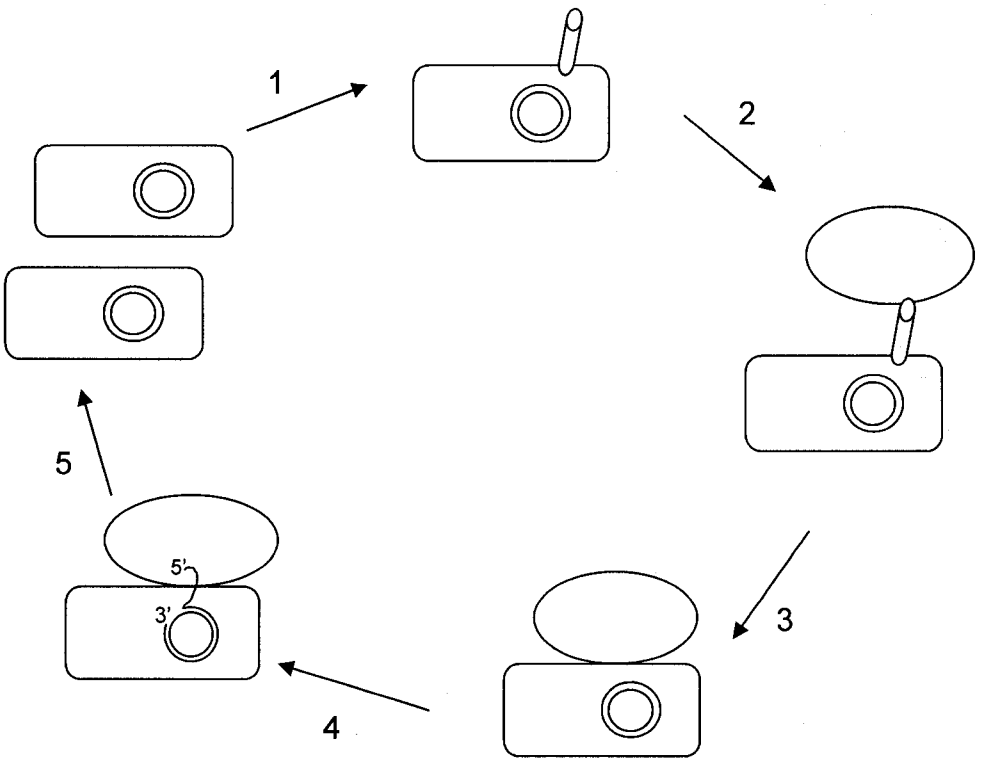
bacteriophage T7 in a F^+ cell at a late stage of infection by affecting the translation of the phage protein gp1-2, which is involved in replication and F targeting (Molineux *et al.*, 1989). PifB is responsible for causing membrane lesions in F^+ hosts, which leads to increased permeability (Blumberg *et al.*, 1975). PifC is an autorepressor that binds to the *pifO* site within the promoter (Miller and Malamy, 1983; 1986). PifC has also been shown to inhibit conjugation of an IncP plasmid, RP4, when F and RP4 co-exist in the same bacterial cell (Tanimoto and Iino, 1983). The proposed mechanisms for this inhibition have been contradictory. PifC was proposed to bind to one of the RP4 transfer gene promoters (Miller *et al.*, 1985). A more recent study, however, suggested that this inhibition was the result of a post-transcriptional control through sequestration of RP4 TraG, the coupling protein for transfer, by PifC (Santini and Stanisich, 1998). This interesting feature of F, which denotes one of its selfish but powerful characteristics, will be examined in Chapter 6. Our results suggested the latter model is correct.

1.3 F conjugation

Process of F conjugation

F plasmid conjugation has been the subject of previous reviews (Firth *et al.*, 1996; Lawley *et al.*, 2004). An overview of the process is outlined on Figure 1.3. The first step of conjugation involves a specific and direct contact of the pilus tip of the donor cell with the recipient cell surface (Achtman *et al.*, 1978). Upon pilus retraction, which results from the de-polymerization of the pilin subunits, the two cells are drawn together (Novotny and Fives-Taylor, 1974; Lawley *et al.*, 2004). A conjugative bridge is formed. Over time this bridge is stabilized, which is resistant to shear forces (Manning *et al.*, 1981). The relaxosome is formed by the binding of F-encoded TraI, TraM, TraY and the host-encoded integration host factor (IHF) to the origin of transfer (*oriT*; Firth *et al.*,

Figure 1.3 Schematic representation of the F mating cycle. The double circle inside the bacterial cell represents the F plasmid. For simplicity, the genomic DNA is omitted. The rectangular bacterium represents an F^+ *E. coli* cell, whereas the oval bacterium represents an F^- *E. coli* cell. 1. F conjugation starts requires synthesis of the F pilus, which is an extracytoplasmic appendage extending outward. 2. The tip of the pilus contacts an F^- recipient cell. 3. Retraction of the F pilus brings the two cells into close proximity, forming what is called a mating pair aggregate. 4. The plasmid is nicked at *oriT*, and transferred in a 5'-to-3' direction into the recipient cell. 5. Plasmid replication in each cell results in the formation of double stranded, complete F plasmids. The recipient cell, now referred to as a transconjugant, because it has received the full F plasmid, is capable of initiating the next round of conjugation by synthesizing the F pilus.

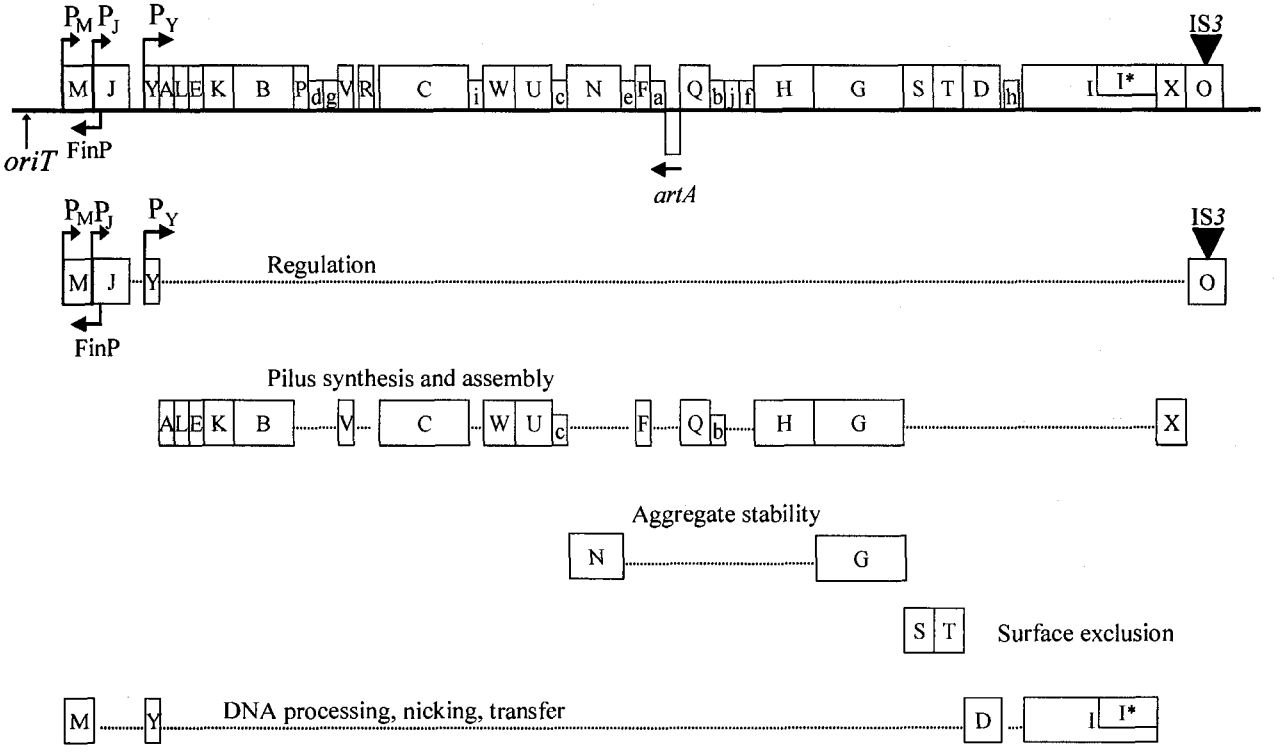

1
2
3
4
5
5'
3'

1996). F-encoded relaxase, TraI, binds to the *nic* site at *oriT* and nicks the DNA strand followed by covalently attaching to the 5' end. TraI also serves as a helicase and unwinds the dsDNA (Howard *et al.*, 1995). F-encoded TraD, the coupling protein, initiates DNA transfer by promoting the association between the relaxosome and the transferosome, which is established in the cell membrane and composed of F-encoded envelope proteins and the pilin (Fekete and Frost, 2002; Llosa *et al.*, 2002). Transfer of the single-stranded DNA into the recipient is in a 5'-to-3' direction. The entire process of transfer takes approximately 3 minutes (Lawley *et al.*, 2004). Both the complementary strand remaining in the donor cell and the transferred strand in the recipient are replicated (Willetts and Wilkins, 1984). The transconjugant becomes the donor, and both cells can initiate another round of conjugation.

F pilus expression

Expression of the F pilus requires the 33.3 kb transfer (*tra*) region on F (Figure 1.4). There are 37 *tra* gene products responsible for the regulation of *tra* proteins expression, the synthesis and assembly of F pilus, the stability of the mating aggregate, surface exclusion, origin nicking, unwinding, and transport (Frost *et al.*, 1994). *traM* and *traJ*, which encode regulatory proteins, are transcribed from their own promoters (Thompson and Taylor, 1982). Most of the *tra* genes are transcribed from the major operon preceded by the P_Y promoter. The *tra* mRNAs (upper case), the *trb* mRNAs (lower case), and the *finO* mRNA are transcribed in one direction. Both the *finP* antisense RNA and *artA*, a gene of unknown function, are transcribed in the opposite direction (Frost *et al.*, 1994). There is an IS*3* insertion within *finO* in F. *finO* encodes the protein product FinO, which stabilizes *finP* antisense RNA. The FinOP complex represses transcription of *tra* genes. Since the insertion of IS*3* disrupts *finO*, the expression of *tra*

Figure 1.4 Transfer (*tra*) region of the F plasmid. Conjugation is dependent on the 33.3-kb *tra* region. The upper case or lower case letters indicate the names of the *tra* or *trb* genes, respectively. The upward arrow indicates the site of *oriT*. The triangle at the right indicates the insertion site of IS*3* into *finO*. The arrows under P_M, P_J, and P_Y indicate the direction of transcription. The *finP* and *artA* mRNAs are transcribed in the opposite direction, as indicated by the arrows below. The *tra* region encodes 37 genes responsible for regulation, pilus synthesis and assembly, aggregate stability, surface exclusion, and DNA processing, nicking, and transfer. Adapted from Frost *et al.* (1994).


P_M P_J
P_Y
IS3
M J Y A L E K B P d g V R C i W U c N e F a Q b j f H G S T D h I I* X O
oriT
FinP
artA
P_M P_J
P_Y
IS3
M J Y
Regulation
O
FinP
Pilus synthesis and assembly
A L E K B V C W U c F Q b H G X
Aggregate stability
N G
S T
Surface exclusion
M Y
DNA processing, nicking, transfer
D I I*

genes is constitutive and F is said to be de-repressed (Cheah and Skurray, 1986). Although F *tra* genes are not subjected to the FinOP regulation system, they are regulated in many sophisticated ways. Examination of the effect of these regulatory systems on F *tra* gene expression constitutes a major part in this study.

1.4 Regulation of *tra* gene expression

Bacterial conjugation is sensitive to the environment. F conjugation is found to peak at exponential phase, decrease over time, and cease during stationary phase (Hayes, 1964; Frost and Manchak, 1998). Therefore, F^+ donor cells must sense the correct nutrients in order to maximize conjugation. Expression of the *tra* genes is highly regulated by host- and plasmid-encoded proteins. This section will examine the control mechanisms of these regulators, which interact with promoters of three key *tra* proteins, TraM, TraJ, and TraY.

Promoters and open reading frames

There are ten putative -35 and -10 binding sites and transcriptional start sites within the *tra* region. These include the major promoters P_M, the *traM* promoter; P_J, the *traJ* promoter (Thompson and Taylor, 1982); P_{finP}, the promoter for the antisense RNA *finP* (Frost *et al.*, 1994); P_Y, the major transfer operon promoter for the expression of *traY* gene to *traX* gene (Mullineaux and Willetts, 1985); P_{artA}, the promoter for the anti-*tra* oriented gene *artA* (Wu and Ippen-Ihler, 1989); P_{finO}, the *finO* promoter (van Biesen and Frost, 1992); and the distal promoters P_{trbF}, P_{traS}, P_{traT}, and P_{traD} which are the promoters for *trbF* (Ham *et al.*, 1989b), *traS* (Ham *et al.*, 1989a), *traT* (Ham *et al.*, 1989a), and *traD* (Jalajakumari and Manning, 1989). Studies have suggested that transcription of *traS* and *traT* can be entirely dependent on their distal promoters. However, with deletion of P_Y, transcription of these genes decreases, indicating their transcription is still P_Y-dependent

(Jalajakumari *et al.*, 1987). Since the P_Y promoter is dependent on TraJ (see below), a distal promoter for the surface exclusion proteins is desired for their expression immediately upon entry into the recipient cell. This can prevent entry of other conjugative plasmids into the recipient before TraJ is produced and might help disaggregate the mating pair aggregate. Studies have also suggested that a distal promoter for *traD* and *traI* transcripts may be important for establishing a new relaxosome complex in the new transconjugant, for terminating transfer, and for directing membrane-binding for the newly transferred plasmid (Frost *et al.*, 1994).

Regulation on traM gene

The *traM* gene encodes the cytoplasmic protein TraM, which is part of the relaxosome complex that initiates *oriT* nicking (Everett and Willetts, 1980; Kingsman and Willetts, 1978) TraM also relays the signal from the relaxosome to the transferosome by binding to the coupling protein, TraD, for DNA transfer (Lu and Frost, 2005). The P_M promoter is subject to autorepression: there are two high affinity binding sites for TraM, *sbmA* and *sbmB* that overlap the two *traM* promoters P_{M1} and P_{M2} (Penfold *et al.*, 1996). In a wild type cell containing F, the shorter transcript of *traM*, which is transcribed from P_{M1}, is more abundant than the transcript transcribed from P_{M2}. The amount of both transcripts increased dramatically in a *traM* mutant cell (Lu, Ph.D. Thesis, 2004). TraM is also up-regulated by TraY, the first protein encoded by the *traYX* operon transcribed from P_Y. Since the expression of P_Y is regulated by TraJ protein that is under FinOP regulation, it is not surprising that TraM is also negatively regulated by this system (Penfold *et al.*, 1996). Since there is an IHF binding site in between P_{M1} and P_{M2}, P_M is also considered to be under the control of host IHF. In R-100, an F-like plasmid, IHF can repress *traM* expression by 40% (Abo and Ohtsubo, 1993). However, Penfold *et al.* (1996) found that

an IHF mutant did not affect TraM expression in F. Binding of IHF at P_M may simply assist in the cooperative binding of TraM to *oriT*, without repressing P_M (Penfold *et al.*, 1996).

Regulation of the P_Y promoter - the promoter for traYX transcription

The P_Y promoter is responsible for transcription of the polycistronic *tra* operon that includes all the *tra* genes except *traJ* and *traM.* These genes are required for regulation (TraY), pilus synthesis (TraA, -Q, X), assembly (TraL, -E, -K, -B, -V, -C, -W, -F, -H, -G, and TrbC), *oriT* nicking (TraY and TraI), transfer of ssDNA (TraI and TraD) and surface exclusion (TraS and TraT; Frost *et al.*, 1994). Regulation of P_Y is important in maximizing conjugation output and saving energy since several steps during conjugation are ATP-dependent (Howard *et al.*, 1995). The P_Y promoter is controlled by several regulators: the plasmid-encoded TraJ and TraY proteins (Gaudin and Silverman, 1993); host encoded SfrA (ArcA), a member of the two-component response regulator family (Buxton and Drury, 1983; Lerner and Zinder, 1979; Silverman *et al.*, 1980); H-NS (Histone-like nucleoid-structuring; Will *et al.*, 2004); IHF (Integration host factor; Gamas *et al.*, 1987); Fis (Factor for inversion stimulation, Will, Ph.D. Thesis, 2006) and Hfq (Host factor for phage Qβ; Will and Frost, 2006b), global regulators in the host cell.

TraJ, the positive regulator of P_Y

TraJ is a 27.5 kDa cytoplasmic protein that is encoded immediately upstream of P_Y (Figure 1.4). The key function of F and F-like TraJ is to regulate expression of *tra* genes. The role of TraJ was first described by the Willetts lab (Gaffney *et al.*, 1983) and further supported by the work of Silverman and co-workers (Silverman *et al.*, 1991b). A low copy plasmid containing a *traY-lacZ* transcriptional fusion was used to determine the activity of the P_Y promoter. β-galactosidase activity was reduced 30-fold in cells with a

traJ deletion (Δ*traJ*) compared to that of the wild-type TraJ background. In addition, P_Y activity was rescued when Δ*traJ* was complemented with a wild-type TraJ. These data support the notion that initiation of transcription at P_Y is TraJ-dependent.

To-date, no TraJ binding site has been identified in the P_Y promoter region although Taki *et al.* (1998) have shown a site for R100 TraJ binding to its cognate P_Y using an electrophoretic mobility shift analysis (EMSA) assay. The mechanism of TraJ regulation was proposed to be sequence-dependent (Gaudin and Silverman, 1993). The importance of promoter context and structure dependence for TraJ and SfrA will be discussed below.

Sex factor regulator, SfrA

SfrA is a 27kDa host-encoded protein that has dual function. In the literature, *sfrA, dyeA, arcA, fexA, msp, seg, cpxC* all represent the same gene encoding for the same protein but different functions (Bachmann, 1983; Iuchi and Lin, 1988). SfrA functions to maximize expression of the F plasmid in the host (Buxton and Drury, 1984). During anaerobic growth, *arcA* encodes a two-component response regulator that activates anaerobic gene expression upon phosphorylation by its cognate sensor kinase ArcB (Iuchi *et al.*, 1989; Iuchi *et al.*, 1990; Iuchi and Lin, 1992). Regulation of P_Y by SfrA, however, is independent of ArcB (Iuchi *et al.*, 1989).

Using *lacZ* as a reporter gene for P_Y, β-galactosidase activity was found to be reduced 10-fold in *sfrA* cells as compared to cells expressing wild-type SfrA (Silverman *et al.*, 1991b). This result indicated that the effect of TraJ deprivation on the activity of P_Y (which was a 97% reduction) is more severe than that of SfrA. In addition, SfrA was found to act directly on P_Y and not through TraJ, since expression of TraJ is independent

of SfrA. Therefore the mechanism of SfrA control on P_Y is apparently not by altering TraJ levels. Although P_Y tended to be 1.8 times more active during anaerobic growth, regulation of P_Y by SfrA is thought to be independent of its ArcA activity, which prepares cells for anaerobic growth. In fact, the functions of SfrA and ArcA have been found to be separable (Silverman *et al.*, 1991a).

Like R100 TraJ, SfrA has been shown to bind upstream of R1 P_Y (Strohmaier *et al.*, 1998). Electrophoretic mobility shift analysis (EMSA) demonstrated site-specific binding of phosphorylated His_6-SfrA upstream of P_Y. DNaseI footprinting has identified the binding site of SfrA-P in the P_Y region, which overlaps the 3' end of the *traJ* coding sequence. SfrA-P binding was proposed to induce structural changes in the DNA (Strohmaier *et al.*, 1998). Although binding of SfrA to P_Y in the F plasmid has not been shown, a 10-bp consensus sequence for SfrA-P binding is found on F P_Y (Lynch and Lin, 1996). It is believed that SfrA-P and TraJ activate P_Y by inducing DNA bending and thus enhance the ability of RNA polymerase to initiate transcription (Gaudin and Silverman, 1993).

TraJ and SfrA activation on P_Y is sequence and context dependent

Activation of P_Y by TraJ and SfrA requires specific sequences at P_Y. TraJ and SfrA are needed to allow maximal expression at wild-type P_Y. When the sequence of this promoter was altered, P_Y became active in the absence of TraJ (Gaudin and Silverman, 1993). This unexpected result was resolved with the theory that TraJ and SfrA binding disrupts the nucleosome complex formed at P_Y that otherwise relaxes the promoter. Since a supercoiled state is required for σ^{70}-RNA polymerase to initiate transcription at P_Y (Gaudin and Silverman, 1993), binding of TraJ and SfrA could restore optimal topology

of the P_Y promoter; DNA is unwound to its negative supercoiling context to elicit transcription initiation. Therefore when the promoter sequence is altered, TraJ and SfrA are not needed for P_Y activation as the nucleosomal complex is unable to form and repress P_Y.

TraY- a plasmid protein that binds to P_Y

The plasmid-encoded TraY has been shown to bind P_Y (Nelson *et al.*, 1993). Although the binding of TraY to its own promoter is 5 times lower in affinity than the binding between TraY and *oriT*, it is believed to exert a positive effect in regulating its own transcription. The exact control mechanism of TraY on P_Y is unclear. A sequence homology search shows that TraY belongs to the ribbon-helix-helix family of transcriptional factors (Lum *et al.*, 2002). The structure of TraY has been determined and its kinetics was found to be complex (Schildbach *et al.*, 1998).

Global regulators: IHF, Fis, Hfq and H-NS

When reviewing the mechanism of H-NS and IHF control, the timing for pilus expression is important. In F^+ hosts, mating has been shown to peak in exponential phase, decrease over time, and cease as cells enter stationary phase. This phenomenon, termed F^- phenocopies (Jacob and Wollman, 1961), is characterized by a decrease in the transcript levels of *traA* (propilin), *traI* (*oriT* relaxase), and *traM* in early stationary phase (Frost and Manchak, 1998). Eventually these Tra proteins reach undetectable levels. However, the level of TraJ persists over time in stationary phase. Mating is restored when the culture is diluted with fresh broth indicating that repression of F piliation is a specific signal when the host enters stationary phase. It is of interest that P_Y transcription ceases in stationary phase although its positive regulator, TraJ, is present.

The role of H-NS became apparent when it was discovered to repress F *tra* promoters (Will *et al.*, 2004; Will and Frost, 2006a). H-NS is a 15.4 kDa protein that acts primarily as a transcriptional repressor (Dorman, 2004). It binds preferentially to DNA containing regions of intrinsic curvature at sub-saturating concentrations, but non-specifically at higher concentrations (Owen-Hughes *et al.*, 1992). Sequence analysis and binding studies showed that H-NS binds preferentially to the promoters of *traM*, *traJ*, and *traY*. In an F^+ *hns* mutant host, TraJ is not required to activate transcription from the P_Y promoter. H-NS is suggested to be involved in forming the nucleosomal complex at P_Y, repressing transcription when F^+ cells enter stationary phase growth. As such, it functions as a silencer for F *tra* genes and represses conjugation once cell density is high. There are several nucleation sites on P_M, P_J, and P_Y that are bound by H-NS. As the amount of bound H-NS reaches a certain threshold, H-NS begins to polymerize outward and repress transcription initiation, converting F^+ cells into F^- phenocopies in stationary phase (Will and Frost, 2006a). When growth is resumed, H-NS is antagonized by an as yet unknown protein. Since the presence of a functional TraJ is essential for activation of transcription at P_Y in a wild-type host, TraJ is viewed as one of the possible de-repressors that counteract H-NS repression (Will and Frost, 2006a).

IHF is an abundant DNA architectural protein that binds to sequence-specific DNA and relaxes the DNA duplex. It is important for processes that require DNA destabilization like DNA replication and transcription (Friedman, 1988). The effect of IHF on F P_Y is controversial, with suggestions for both positive and negative regulatory roles (Silverman *et al.*, 1991b; Gamas *et al.*, 1987). Transfer efficiency from a host strain containing a mutation in *himA*, which encodes one of the IHF subunits, was decreased throughout the growth cycle. IHF was found to bind P_Y using an EMSA (Will, Ph.D.

Thesis, 2006). Since its putative binding site overlaps that of H-NS, IHF has been proposed to be another possible antagonist that counteracts H-NS repression (Will and Frost, unpublished).

Fis is an 11kDa protein that is involved in the regulation of many systems, including DNA gyrase synthesis (Schneider *et al.*, 2000). Fis was found to bind F P_Y in a concentration-dependent manner and mutation of *fis* had a minor effect on F transfer (Will, Ph.D. Thesis, 2006). Since Fis also activates the expression of *hns* (Falconi *et al.*, 1996), its role on F regulation is complicated and might be indirect.

Hfq is a small, 11kDa global regulatory protein that preferentially binds AU-rich RNA that is flanked by structured regions (Moller *et al.*, 2002). Hfq has been found to regulate gene expression by influencing translation, RNA stability, and RNA bacteriophage replication. In F, the intergenic region between *traM* and *traJ*, encoding the *traJ* mRNA leader region as well as readthrough transcripts from P_M, has been shown to specifically bind Hfq in an EMSA (Will and Frost, 2006b). The transcripts of TraM and TraJ were found to be stabilized in the absence of Hfq. Accordingly, Hfq was proposed to repress TraM and TraJ through destabilization of their transcripts in an F^+ cell.

Regulation of traJ, the positive regulator in F

As stated previously, the primary function of TraJ is to positively regulate P_Y. TraJ also indirectly activates the P_M promoter through TraY. Several mechanisms affect the level of TraJ in an F^+ *E. coli* cell. Lrp is an activator of P_J in F-like plasmids pSLT and R100 but not F, and binding of Lrp to P_J is inhibited by Dam methylation (Camacho *et al.*, 2005). F plasmid transfer gene expression also responds to nutritional signals through CRP and catabolite repression (Starcic *et al.*, 2003). The plasmid-encoded FinOP

(Fertility inhibition) and host-encoded *cpx* (conjugative plasmid expression) systems are found to be distinct. FinOP impedes translation of TraJ protein from its mRNA transcript (Frost *et al.*, 1989; Gubbins *et al.*, 2003); Cpx system impairs accumulation of TraJ by a post-translational mechanism (Gubbins *et al.*, 2002).

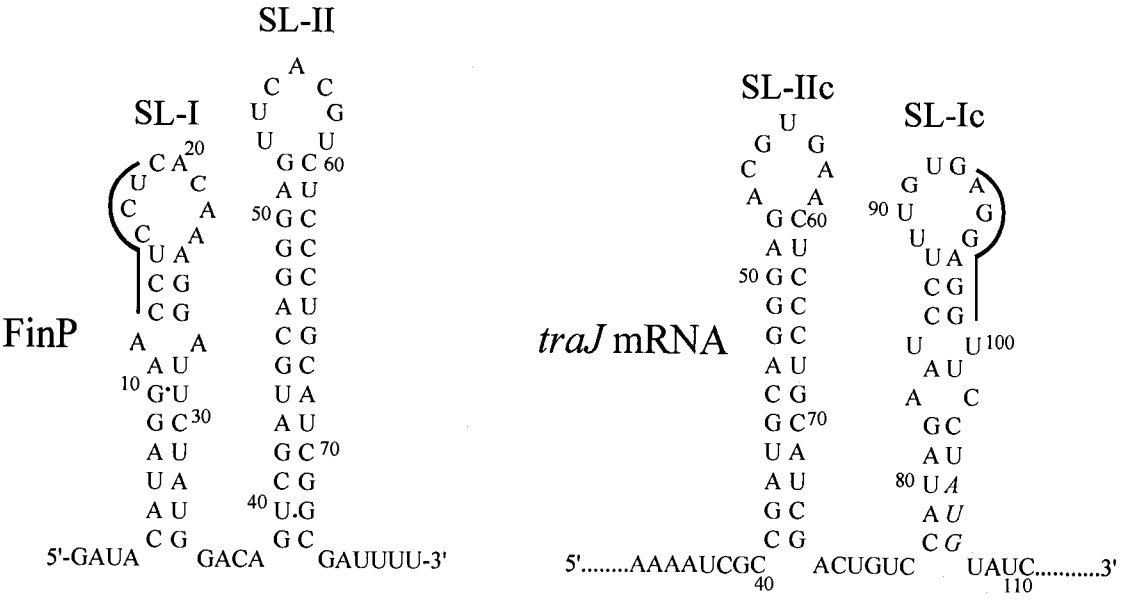
FinOP: The fertility inhibition factor that represses TraJ

F *finO* gene is located at the very end of the *tra* region. This position of *finO* is important to ensure that previous *tra* genes have been sufficiently transcribed before FinO is produced and able to repress their key regulator, TraJ (van Biesen and Frost, 1992). FinO is a 21.2kDa cytoplasmic, basic, RNA-binding protein (Yoshioka *et al.*, 1987). The functions of FinO are to stabilize *finP* antisense RNA and to catalyze FinP/*traJ* duplex formation (Jerome *et al.*, 1999). Binding of FinO to FinP RNA prevents the degradation of FinP by host RNaseE (Lee *et al.*, 1992). FinP is a 79-nucleotide RNA molecule that is transcribed from its own promoter, which is oriented opposite to the direction of *traJ* transcript (van Biesen and Frost, 1994; van Biesen *et al.*, 1993). FinP is therefore complementary to part of the 5' untranslated *traJ* leader sequence (Figure 1.5). Base pairing of FinP and *traJ* UTR prevents TraJ translation by sequestering its ribosome binding site (RBS) on stem-loop one complementary (SLIc). The proposed secondary structure of FinP illustrates that the spacer and the 3' tail of SLII were important for FinO binding (Figure 1.5; Jerome and Frost, 1999). In the F plasmid, *finP* RNA is degraded by RNaseE due to the absence of FinO (*finO* is disrupted by an IS3 insertion; Yoshioka *et al.*; Jerome *et al.*, 1999). Thus F is said to be "derepressed" as *tra* genes expression is constitutive in a *finO* background.

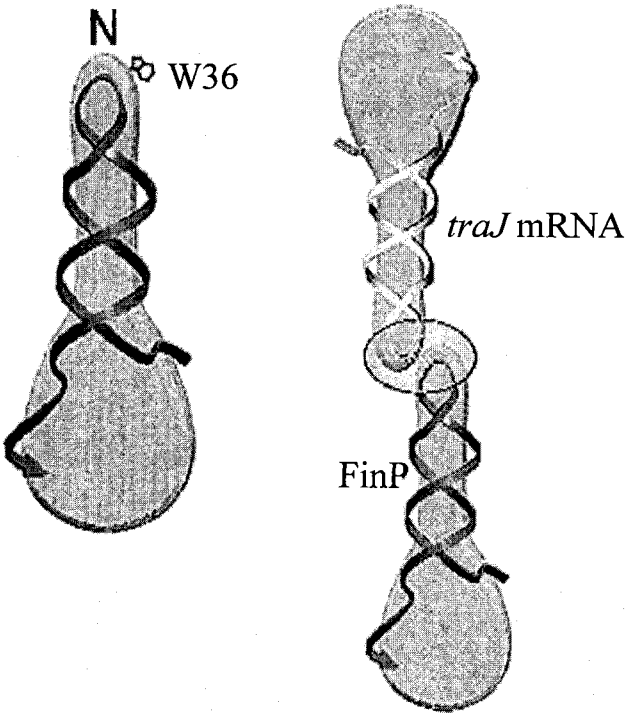
The crystal structure of FinO has been solved (Ghetu *et al.*, 2000) and found to comprise six α-helices. The positively charged N-terminal α-helix of FinO likely

Figure 1.5 FinOP: The fertility inhibition factor that represses TraJ. A. Secondary structure of FinP antisense RNA and a portion of the 5' UTR of the *traJ* mRNA transcript. The line indicates the RBS of *traJ* mRNA and the corresponding anti-RBS of FinP. The stem-loop numbers (SL) in FinP and their complementary SL structures in *traJ* mRNA are indicated. B i. A hypothetical model of FinO bound to SLII of FinP. The Trp 36 (W36) side chain is indicated. ii. A possible model for a FinO-stabilized kissing complex formed between FinP SLII and the complementary stem-loop structure of *traJ* mRNA. The N-terminal region of FinO is predicted to interact with and stabilize the RNA loop-loop interactions in the region circled. Figures were adapted from Ghetu *et al.* (2000).

A.


SL-II
SL-I
SL-IIc
SL-Ic
FinP
traJ mRNA
5'-GAUA GACA GAUUUU-3'
5'.....AAAAUCGC ACUGUC UAUC.....3'

B.


N
W36
traJ mRNA
FinP

interacts with SLII of FinP and SLIIc of *traJ* mRNA (Figure 1.5). Interestingly, the length of the N-terminal helix (45Å) matches the length of SLII. Tryptophan 36 is believed to play a role in stacking with unpaired residues in the loop. A model for FinO stabilized kissing complex between SLII and SLIIc has been proposed (Fig 1.5). The positively charged N-terminus of FinO is believed to interact with and stabilize RNA loop-loop interaction of the kissing complex (Ghetu *et al.*, 2000). Experimental details of FinOP regulation are discussed in Gubbins (Ph.D. Thesis, 2003).

Cpx-Conjugative plasmid expression

The effect of the host-encoded Cpx pathway on F conjugation was first discovered by McEwen and Silverman (McEwen and Silverman). Mutations in both *cpxA* and *cpxB* genes in the chromosome of the F^+ host resulted in decreased DNA donor activity and surface exclusion (McEwen and Silverman, 1980c). A single mutation in *cpxB*, however, did not decrease donor ability. Thus *cpxB* was considered to be cryptic. More than 60 percent of the wild-type F^+ cells were found to have at least one attached F-pilus, however less than 1 percent of the *cpxAcpxB* F^+ mutant was found to have attached pili (McEwen and Silverman, 1980b). Subsequently, F^+ *cpxA* hosts were shown to have reduced *tra* gene expression by reduced levels of TraJ (Silverman *et al.*, 1993). Identification of the *cpxR* gene (Dong *et al.*, 1993) revealed that CpxAR regulates gene expression as a two-component signal transduction pathway. In addition to down-regulating F conjugation, the Cpx system was found to regulate genes that are required for envelope stress response (Cosma *et al.*, 1995; Pogliano *et al.*). The next section will examine the historical findings of the function of the Cpx system, and how it may regulate pilus expression as well as stress response proteins.

1.5 The Cpx regulon in *E. coli*

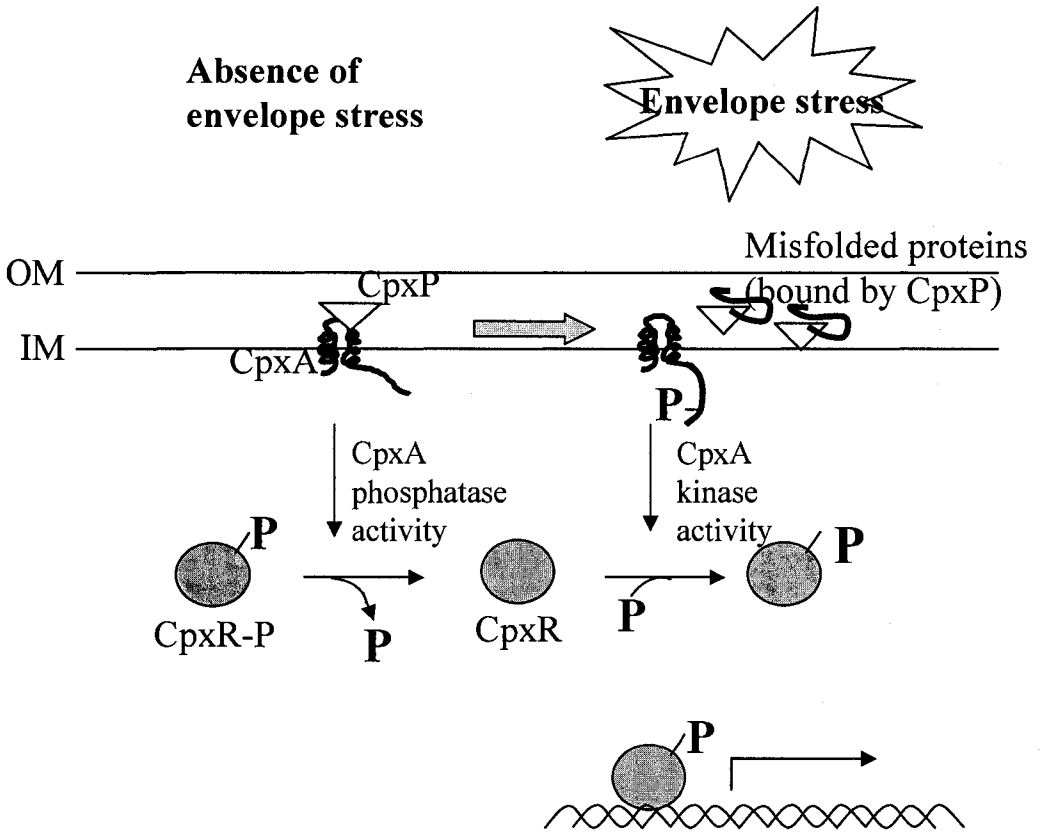
Overview of CpxA/CpxR signal cascade

CpxA/R is a member of the bacterial two-component transduction system (Figure 1.6). Upon sensing a signal from the environment, CpxA, an inner membrane histidine kinase, undergoes autophosphorylation at a conserved histidine residue. This phosphate is then transferred to a conserved aspartate residue in CpxR, its cognate response regulator (RR). CpxR-P functions as a transcriptional regulator and binds to promoter regions of Cpx-controlled genes, which are collectively called the Cpx modulon. As a typical histidine kinase (HK) of bacterial two-component systems, CpxA also possesses phosphatase activity. In the absence of an inducing cue, CpxA dephosphorylates CpxR-P, thereby keeping CpxR in an unphosphorylated state (Raivio and Silhavy, 1997). The amount of CpxR-P and the level of Cpx modulon up-regulation are thus dependent on a balance between CpxA kinase and phosphatase activities in a wild-type *E. coli* cell.

Cpx envelope stress response

CpxR regulates genes that encode proteins needed for the correct folding of extracytoplasmic proteins and the degradation of misfolded proteins (Danese *et al.*, 1995; reviewed in Raivio 2005). The Cpx regulon encodes DegP, the periplasmic protease, and PpiA/D, the peptidyl-prolyl-isomerase (Dartigalongue and Raina, 1998). Production of a protease/isomerase in times of stress maintains proper protein structure in the cell envelope. Other promoters which are activated by Cpx include *cpxP*, the proposed negative regulator of CpxA (Raivio *et al.*, 2000; Buelow and Raivio, 2005; Isaac *et al.*, 2005); *dsbA*, the enzyme that catalyzes disulfide bond formation and ensures proper protein folding (Pogliano *et al.*, 1997); and *cpxR* itself, which serves to amplify the stress response (Raivio *et al.*, 1999).

Figure 1.6 Schematic diagram of the Cpx two-component signal transduction system in *E. coli*. OM and IM indicate the outer membrane and inner membrane in the bacterium. In the absence of envelope stress, inner membrane-bound CpxA is not phosphorylated and is bound to the periplasmic protein, CpxP. CpxA phosphatase catalyzes the dephosphorylation of CpxR-P, thus reducing the amount of CpxR-P in the cell. In the presence of envelope stress, CpxP is sequestered by misfolded envelope proteins followed by degradation by DegP (Buelow and Raivio, 2005). Unbound CpxA undergoes conformational change and autophosphorylation. CpxA kinase catalyzes phosphorylation of CpxR. CpxR-P activates genes encoding protease / chaperone / isomerase proteins that together help alleviate the stress encountered in the envelope.



Cpx regulon

degP

dsbA

ppiA

cpxRA

spy etc.

Regulation of CpxAR pathway

The Cpx pathway requires an inner membrane bound component (CpxA) to relay the signal from the extracytoplasm to a cytoplasmic component (CpxR) which then activates the transcription of its modulons. CpxA has two transmembrane helices, the N-terminal and C-terminal cytoplasmic domains, and a periplasmic central region (Raivio and Silhavy, 1997). This periplasmic region is believed to contain the sensing domain. In the absence of envelope stress, the sensing domain is bound to CpxP, a small periplasmic protein that is strongly regulated by Cpx itself. Binding of CpxP is believed to inhibit CpxA, although no direct interaction between CpxP and CpxA has been shown (Raivio and Silhavy, 2001; Raivio *et al.*, 2000). Under a stress environment, misfolded/denatured proteins may induce degradation of CpxP. Recently, the release of CpxP inhibition was found to be associated with the proteolytic activity of DegP (Buelow and Raivio, 2005; Isaac *et al.*, 2005). It was proposed that misfolded proteins target CpxP for degradation by DegP. The unbound CpxA is believed to undergo a conformational change that results in its autophosphorylation at histidine 249 in the C-terminal cytoplasmic domain (Raivio and Silhavy, 2001) and subsequently elicits activation of the Cpx pathway.

The sequence of CpxR is homologous to nine two-component response regulators (RR; Dong *et al.*, 1993). CpxR is most homologous to OmpR, the RR that controls expression of outer membrane porins (OMP). A typical RR contains a N-terminal regulatory domain that harbors the conserved aspartate residue (D51 of CpxR) and the dimerization domain; and a C-terminal effector domain, which has a helix-turn-helix motif for DNA binding. Equilibrium between active and inactive states depends on the phosphorylation status of the RR. It is believed that with phosphorylation, the RR will shift to its active conformation. In some cases, dimerization is required for DNA binding

and the dimerized RRs function as transcriptional regulators. In other cases, dimerization is not required. A model of CpxR dimerization before DNA binding is favored since its closely related RR, OmpR, undergoes dimerization for gene regulation (De Wulf *et al.*, 1999).

Like many other HKs, CpxA can also catalyze dephosphorylation of its cognate RR, CpxR-P. Dephosphorylation is found to be the reverse of phosphorylation in most bacterial two-component signal transduction systems, in which the phosphoryl group is transferred from the conserved aspartate on the RR back to the conserved histidine on the HK. The phosphoryl group is then lost as inorganic phosphate (Pi). A balance between the kinase: phosphatase ratio of CpxA is required to maintain a proper amount of Cpx-controlled gene expression (Raivio *et al.*, 1999). A fast turnover rate of RR-P can elicit a strong signal during specific environmental changes and ensure efficient shut down of the pathway when not needed. This is probably the case for the Cpx pathway, in which CpxR itself is upregulated during envelope stress as a way to amplify the signal. The negative regulator of the Cpx pathway, CpxP, is also under Cpx regulation (Danese and Silhavy, 1998a). This suggests that Cpx is controlled by an autofeedback mechanism, in which overexpression of CpxP result in binding and repression of CpxA, resulting in shut down of the Cpx pathway.

Stimulation of Cpx pathways

Altered membrane protein content

During envelope stress, CpxAR conveys the signal from the environment to inside the cell to compensate for the damages in envelope composition. These envelope stress stimulations include elevated pH (Nakayama and Watanabe, 1995; Danese and Silhavy, 1998b), in which *cpx* null mutants were unable to survive in alkaline media. A change in

phospholipid composition can also stimulate the Cpx response. Mileykovskaya and Dowhan (1997) found that mutants lacking phosphatidylethanolamine (PE^-) exhibit phenotypes similar to cells under Cpx activation. In this PE^- mutant, transcription from the *degP* promoter is activated in a Cpx-dependent fashion. Thus a PE-deficient membrane can elicit a signal for CpxA activation in the cell membrane and activate the Cpx pathway (Mileykovskaya and Dowhan, 1997). Activation of Cpx may serve as a strategy for cells to restore a healthy homeostatic phospholipid membrane composition in time of stress.

Growth phase dependent activation

Cpx is activated when cells enter stationary phase as shown by examining CpxR transcription in a *cpxR-lacZ* fusion over a period of 12 hours (De Wulf *et al.*, 1999). Since the Cpx pathway is able to amplify itself by activating CpxR, an increase in β-galactosidase activity can serve as a marker for Cpx activation. In a *cpxR* deletion mutant this rise is diminished 9-fold. This suggests that the Cpx pathway is specifically activated during late exponential or early stationary phase. In addition, this dramatic increase in *cpxR* transcription is dependent on the *rpoS* gene product (De Wulf *et al.*, 1999). RpoS (σ^S) is a sigma factor that controls expression of proteins during stationary phase. The low expression of *cpxR* in a *rpoS* mutant confirmed its involvement in activating the Cpx pathway for cells entering stationary phase. This correlates with the idea that during stationary phase where there is starvation or energy depletion, Cpx signaling capacity is amplified (De Wulf *et al.*, 1999). A more recent study, however, found that activation of the Cpx pathway during growth is not *rpoS*-dependent. Furthermore, only CpxR, but not CpxA, is required to sense the signal for induction (DiGiuseppe and Silhavy, 2003).

NlpE, a new lipoprotein E

The *nlpE* gene encodes a 25-kDa outer membrane (OM) protein whose function is unclear. NlpE was discovered because of its ability to suppress periplasmic toxicity exerted by a LamB-LacZ-PhoA fusion strain in the presence of maltose (Gupta *et al.*, 1995; Snyder *et al.*, 1995). This phenomenon, referred to as maltose sensitivity, causes lysis because the fusion protein forms a high-molecular-weight aggregate in the periplasm. NlpE overproduction, which was achieved by expressing *nlpE* from an inducible promoter in the plasmid pBAD vector (Guzman *et al.*, 1995), was found to suppress maltose sensitivity (Snyder *et al.*, 1995). This resistance to maltose by overproducing NlpE was CpxR- and DegP-dependent. Over-expressed NlpE was found to accumulate in the inner membrane. Upon Cpx activation, DegP, the Cpx-upregulated protease degraded the toxic fusion protein.

The Cpx pathway was recently found to be important for surface adhesion in biofilm formation (Otto and Silhavy, 2002). *E. coli* cells attached to a hydrophobic surface were showed to activate the Cpx system. Cpx-regulated genes (*cpxR, cpxP, spy, dsbA, degP*) exhibit increased activities in attached but not planktonic cells. Moreover, CpxR and NlpE were found to be required for this Cpx activation. This suggests that NlpE may play a role in sensing and generating adhesion-specific signals to activate the Cpx pathway during contact to hydrophobic surfaces. Upregulation of the Cpx pathway can be important for cell-surface interactions, as most of the physiological changes made by Cpx activation are cell envelope associated.

CpxA the constitutive mutant*

Mutations in *cpxA* were originally found to impair F conjugation (McEwen and Silverman, 1980a). Since this discovery, other pleiotropic effects have been reported for

cpxA mutants. These include decreased production of murein lipoprotein and OmpF in the cell envelope (McEwen *et al.*, 1983); a random positioning of FtsZ ring during cell division (Pogliano *et al.*, 1998), a loss of ability to grow on succinate and L-lactose (McEwen and Silverman, 1980c), an increased sensitivity to high temperature (McEwen and Silverman, 1980b), and an enhanced ability to tolerate colicins A and K (De Wulf *et al.*, 1999). The *cpxA* mutations were characterized by Raivio and Silhavy (Raivio and Silhavy), who showed that some mutations in *cpxA* lead to a gain-of-function, rather than a null mutation, phenotype. Virtually all *cpxA* mutations, which are named *cpxA**, are constitutive mutations that lead to activation of the Cpx pathway and elicit a perceived extracytoplasmic stress response. There are two different kinds of mutations in *cpxA*. First, mutations that occur in the periplasmic or transmembrane two (TM2) regions, for examples, *cpxA102*, *cpxA24*, *cpxA744*, *cpxA17*, constitute a signal-blind CpxA protein, which shows elevated expression of Cpx-regulated genes. The periplasmic domain of CpxA is proposed to interact with CpxP under normal growth. Disruption of the normal amino acid sequence in this region results in a decreased ability to interact with CpxP and leads to a constitutively "turned-on" CpxA (Raivio and Silhavy, 1997).

The second kind of *cpxA* point mutation occurs in the C-terminal cytoplasmic region at a residue close to the conserved histidine residue. The *cpxA101* mutation, which has a T253P alteration, is an example of this category. This mutation did not abolish CpxA autokinase and kinase ability, but instead it was deficient in phosphatase ability for CpxR-P dephosphorylation. When incubated with CpxR-P that was phosphorylated by EnvZ115, a HK, CpxA101 was not able to catalyze dephosphorylation of CpxR-P. This leads to an elevated kinase/phosphatase ratio and an accumulation of CpxR-P in the *cpxA101* mutant. Therefore, the Cpx system in this mutant is constitutively active, as the

accumulated CpxR-P can constantly activate transcription of Cpx-controlled genes. These CpxA constitutive mutants, which are collectively named CpxA*, are useful in studying effect of Cpx up-regulation and in finding possible members of the Cpx modulon in time of stress.

1.6 Effect of Cpx on pili production

Cpx does not only regulate the envelope stress response, it also affects the ability of *E. coli* to express pili. Originally, the effect of Cpx on conjugation was studied on the F pilus. Cpx was later found to regulate expression of P pili (Hung *et al.*, 2001) and bundle-forming pili (Nevesinjac and Raivio, 2005), which are important for the pathogenicity of uropathogenic and enteropathogenic *E. coli*, respectively. Since pili are extracytoplasmic appendages, it is not surprising that the Cpx system, which is able to convey the signal from the envelope to the cytoplasm, serves as an excellent pathway to direct or repress the synthesis of pili.

Effect of cpx on F expression

McEwen and Silverman (1980b) were among the first to demonstrate the effect of chromosomal *cpx* mutations on F conjugation (Subsection 1.4). Subsequently, Silverman showed that this inhibition was due to repression of the P_Y promoter, as observed from the decrease in β-galactosidase activity in a *cpxA* mutant containing F and a plasmid with P_Y-*lacZ* fusion. Prior to the characterization of the *cpxA** mutation, it was generally believed that mutations in *cpxA* resulted in a loss-of-function phenotype. Early reviews suggested that CpxA is required for proper *tra* gene expression (Firth *et al.*, 1996). This is only partially correct, since a *cpxA* null mutant resembles a quasi-wild-type phenotype and did not result in decreased F mating ability (Silverman *et al.*, 1993).

Further examination of the effect of *cpx* on F *tra* gene expression revealed that the mutations in *cpxA* (*cpxA2* and *cpxA9*, which are in the periplasmic domain) had altered the function of CpxA rather than deleted its function. It was proposed that activation of Cpx inhibits F conjugation by reducing the amount of F TraJ protein (Silverman *et al.*, 1993). Recent experiments on Cpx supported this hypothesis. The findings that a *cpxA* point mutation leads to a signal-blind, constitutive CpxA (Raivio and Silhavy, 1997) supported the finding that the *cpxA* mutants used in the earlier studies were in fact gain-of-function mutations. Since there were two possible activators of P_Y, TraJ and SfrA, the decrease in P_Y transcription seen in *cpxA** may due to the absence of either regulator. Silverman *et al.* (1993) have shown such an effect did not work through SfrA. When an F^+ *cpxA* mutant was supplied with TraJ, the activity of P_Y was partially restored. If the effect of CpxA* on P_Y was dependent on SfrA, the same level of reduction in P_Y transcription would have resulted in the presence of TraJ (Silverman *et al.*, 1993). Therefore, Cpx activation impairs *tra* gene expression through its inability to accumulate TraJ in a *cpxA** mutant.

The loss of TraJ in a *cpxA** mutant decreases *tra* gene transcription and impairs F conjugation. The reduction in TraJ levels is thus key to the signal transduction pathway, which transmits extracytoplasmic stress sensed in the envelope to repress cytoplasmic *tra* gene transcription. The decrease in TraJ levels can be achieved by several mechanisms. Firstly, CpxR-P may bind directly to the P_J promoter and inhibit transcription of TraJ. Historically, CpxR-P has been found to function as a transcriptional repressor for the *motABcheAW* (mobility and chemotaxis) promoter (De Wulf *et al.*, 1999). Whereas deletion of *cpxR* increases the swarming rate (de-repression of *motABcheAW*), a *cpxA** mutation (increased CpxR-P) decreased swarming. This mechanism seems possible for

regulating F TraJ expression, in which increased CpxR-P binds and represses P_J directly. However, a more recent study demonstrated that this is not the case (Gubbins *et al.*, 2002) because a consensus CpxR-P recognition site is absent in the TraJ promoter region. Promoter assessment studies and Northern blot analysis showed that the P_J promoter is active and the *traJ* transcript is detectable in an F^+*cpxA** strain. Taken together, these data supported the notion that CpxR-P does not repress P_J directly.

The second possible mechanism by which Cpx can regulate TraJ is through inhibition of translation. This would resemble the inhibition by FinOP, in which the RBS in *traJ* mRNA is obstructed (van Biesen and Frost, 1992). As a result, the level of *traJ* transcript and P_J remain unaltered, but the amount of TraJ protein decreases. A small RNA (sRNA) molecule, named SraF, is transcribed in an intergenic region in the *E. coli* chromosome (Argaman *et al.*, 2001). SraF has an extensive secondary structure with its 3'-region complementary to the UTR in *traJ* mRNA. Chapter 3 presents data and discusses why this inhibition of translation did not appear to explain the decrease of TraJ and F conjugation in *cpxA**.

Lastly, Cpx could activate a proteolytic system to degrade TraJ proteins after they are translated. This would be similar to the degradation of misfolded proteins by the DegP protease during the envelope stress response. However, TraJ degradation would be expected to be independent of DegP, because they reside in different compartments in the cell. DegP is a periplasmic protease and TraJ is cytoplasmic. Evidence suggests that degradation by other proteases is possible. TraJ was found to be stable in wild-type cells over long periods of time. Whereas TraY, -M, -A levels decrease over time, TraJ persists well into stationary phase (Frost and Manchak, 1998). However, in a *cpxA** background, both wild-type TraJ and TraJ that is expressed from a foreign promoter decreased,

whereas TraY that was synthesized from a foreign promoter was relatively stable (Gubbins *et al.*, 2002). This data suggests that degradation of TraJ in *cpxA** cells is a specific phenomenon and it is likely that the Cpx pathway up-regulates a protease specific for the degradation of TraJ. Since CpxA* mimics Cpx activation for envelope stress response, reduction in F *tra* gene expression in a *cpxA** host illustrates a way for bacteria to sense and control the correct time for conjugation.

1.7 The heat shock regulon

The heat shock regulon is experimentally induced when bacteria are subjected to an increase in temperature from 30°C to 42°C. In *E. coli*, the alternate sigma factor, σ^H (gene product of *rpoH*), is responsible for the initiation of heat shock gene transcription (Grossman *et al.*, 1987; Straus *et al.*, 1987). The heat shock regulon encodes chaperones, for example DnaK, DnaJ, GrpE, and GroES and GroEL that assist in refolding proteins that have misfolded or aggregated as well as proteases, for example ClpAP/XP, ClpQY (also called HslVU), FtsH, and Lon that degrade abnormal proteins. Collectively, they are termed heat shock proteins (HSPs). The heat shock response is thus essential for the survival of cells under this stress environment.

The amount of cellular σ^H is crucial to the control of the heat shock response. At 30°C, the number of σ^H molecules is 50 per cell. At this basal level, minimal heat shock genes are being transcribed. When the temperature is shifted to 42°C, the number of σ^H molecules increases 20-fold to 1000 copies per cell (Grossman *et al.*, 1987; Craig and Gross, 1991). This sudden increase in σ^H is a result of increased transcription of *rpoH*, increased translation of the *rpoH* transcript, and stabilization of the σ^H protein. Under normal condition, σ^H is bound to DnaK and DnaJ (Liberek *et al.*, 1992; Gamer *et al.*, 1992) and degraded by FtsH and other proteases (Kanemori *et al.*, 1999b; Bertani *et al.*,

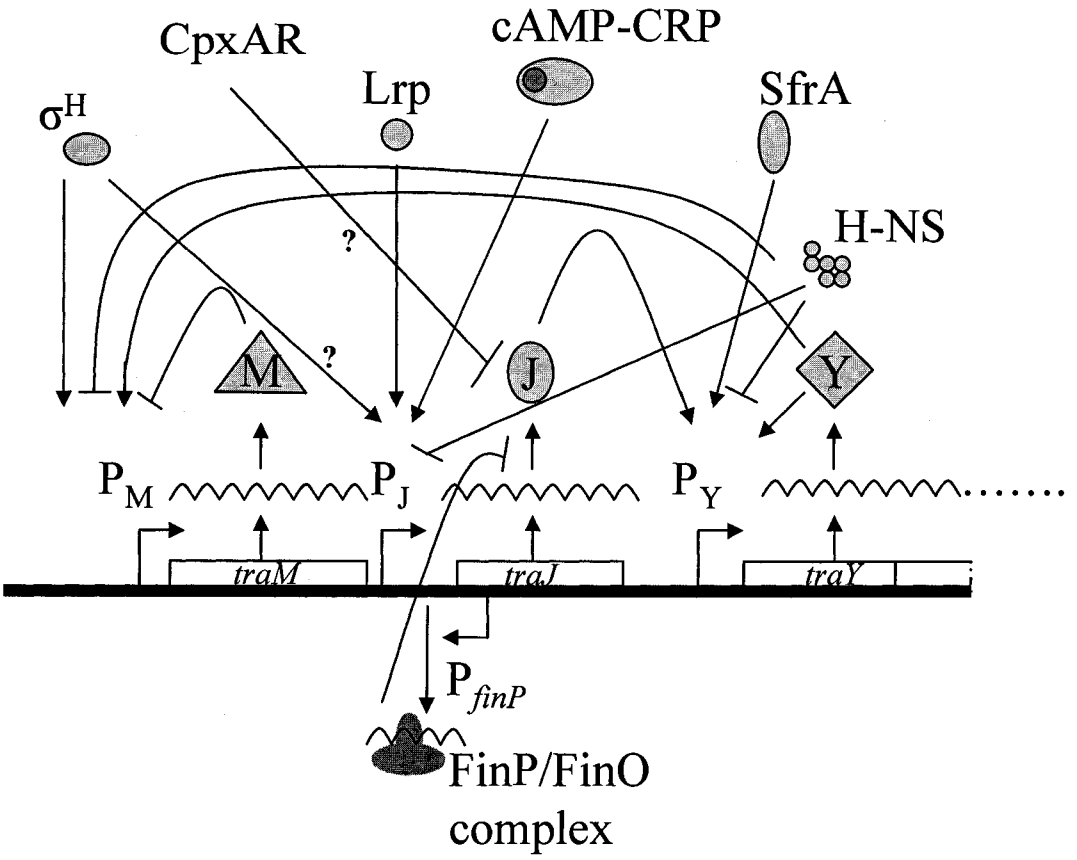
2001). Sequestration of HSPs to misfolded proteins at increased temperatures is believed to cause the sudden increase in the cellular levels of σ^H. Amplification of the heat shock regulon alleviates cells from heat stress. When the level of misfolded proteins decreases, the heat shock response is shut off as a consequence of reduced free σ^H in the cell. The heat shock response and proteolysis in bacteria have been subjects of several reviews (Ang *et al.*, 1991; Arsene *et al.*, 2000; Gottesman, 2003).

Interestingly, the heat shock sigma factor, σ^H, is also responsible for transcription initiation of the F *repE* gene (Wada *et al.*, 1987). RepE is a replication protein that is required for activation of DNA replication in the F plasmid. In an *rpoH* mutant, F is unstable and is rapidly lost after growth in the absence of a selective medium (Wada *et al.*, 1986). A σ^H-consensus binding site has been identified in the *repE* promoter. Moreover, Penfold noticed that P_{traM}, particularly P_{M2}, has sequence homology similar to P_{repE}, which is subject to the control by the σ^H-RNA polymerase (Penfold, Ph.D. Thesis, 1995}. In this study, we have uncovered a novel and specific role of σ^H in F conjugation, which will be discussed in Chapter 5.

1.8 Summary and research objectives

Without doubt, the regulation of F conjugation is complex and strict. Within the *tra* region, promoters of key regulators P_M, P_J, and P_Y are subject to various controls from both chromosomal- and plasmid-encoded factors (Figure 1.7). By no means does the present study attempt to explore details at each individual control system; however the complexity of F *tra* regulation demonstrates that while any control system is being studied, other factors cannot be counted out. Previous studies also showed the hierarchy of control. For example, in an *hns*-deleted F^+ host, TraJ is not required to activate P_Y. This illustrates that a single regulator, in this case TraJ, does not function in isolation.

Figure 1.7 Regulation of F *tra* expression in a nutshell. The *traM*, *traJ*, and *traY* genes are preceded by their own promoters, P_M, P_J, and P_Y. TraM is autorepressed and activated by TraY. TraJ translation is inhibited by the FinOP repression system. Lrp and CRP activate TraJ by increasing its transcript levels. The global regulator H-NS represses transcription of all three promoters. TraJ and SfrA independently activate, or de-repress, transcription of the polycistronic *tra* operon preceded by P_Y. The CpxA/R two-component stress response system destabilizes TraJ protein in times of extracytoplasmic stress. The alternative sigma factor, σ^H, is proposed to increase transcription at P_M. The two question marks indicate the proposed research: The influence of σ^H and the mechanism of CpxAR control on TraJ.


CpxAR
cAMP-CRP
Lrp
SfrA
σH
H-NS
?
?
M
J
Y
P M
P J
P Y
traM
traJ
traY
P finP
FinP/FinO
complex

Instead of an activator, TraJ is now perceived as a de-repressor. The tight control of F is an excellent model to examine gene regulation in newly acquired or "xenogeneic DNA" (Navarre *et al.*, 2007) and type IV secretion system gene expression in particular.

The first objective of this study is to examine the regulation of F TraJ by the Cpx system in times of extracytoplasmic stress. As detailed above, TraJ is essential for transcription of the main *tra* operon in a wild-type F^+ host. When the Cpx system is activated, F conjugation is severely reduced as a result of decreased levels of TraJ. Since a transcriptional control is not supported, we attempted to examine the post-transcriptional control of TraJ by Cpx. The first goal was to determine the protease or chaperone that is up-regulated in a Cpx-activated cell that degrades TraJ. A host peptidase/chaperone pair was found to catalyze proteolysis of TraJ, which was subject to various conditions. The dynamics of TraJ during the growth cycle was further investigated.

The second objective of this study was to examine the mechanism of σ^H control on F regulators. In an *rpoH* mutant, F conjugation is abolished. Presently, TraM is the only known *tra* regulator that is possibly controlled by σ^H. Does σ^H control transcription initiation at P_J and P_Y? How does σ^H assert its function on the F regulator(s)? Is σ^H involved directly in transcriptional initiation, or does it activate another regulator to stimulate/de-repress transcription at P_M, P_J or P_Y? Chapter 5 shows experimental data that uncover the function of σ^H.

The ability of the F plasmid to inhibit RP4 conjugation is an interesting and specific operation. The third goal of this work was to determine the repressive mechanism of F PifC on RP4 conjugation. As illustrated in Subsection 1.2, results of PifC obtained from previous findings appear contradictory. PifC is believed to exert its

effect on one of the essential RP4 transfer gene products, $TraG_{RP4}$ ($TraD_F$). Chapter 6 reveals details of experiments and findings that support the theory of a PifC-$TraG_{RP4}$ interaction. It further explores the possible conformation of PifC used to inhibit RP4 conjugation.

Chapter 2: Materials and Methods

2.1 Bacterial strains, media, antibiotics and growth conditions.

The *Escherichia coli* strains used in this study are listed in Table 2.1. The genotypes and sources of each strain are listed in the table. Standard genetic techniques were employed to construct the strains (Silhavy *et al.*, 1984). In experiments involving *hslV*, *hslU*, *cpxA** or *hns*, mutants were constructed by P1 phage transduction of the *hslV* allele from SG12064, the *hslU* allele from SG12065, the *cpxA101** allele from TR189, or the *hns* allele from PD32 into the recipients using their antibiotic resistant cassettes as markers. The mutants were confirmed by polymerase chain reaction (PCR) to show the presence of the antibiotic resistance insertion and their growth on media containing designated antibiotics.

All cultures were grown and maintained in Luria-Bertani (LB; 1% (w/v) Difco Tryptone, 0.5% (w/v) Difco Yeast Extract, 1% (w/v) NaCl) broth or on agar plates at 30°C (for experiments involving *cpxA**, *rpoH*, *hslV* or *hns*) or 37°C. Antibiotics were used at the following concentrations in selective media: ampicillin, 100 μg/ml; kanamycin, 25 μg/ml; chloramphenicol, 20 μg/ml; tetracycycline, 10 μg/ml; streptomycin, 100 μg/ml; nalidixic acid, 20 μg/ml; and rifampicin 200μg/ml. *cpxA** strains were supplemented with 3 μg of amikacin/ml to prevent reversion (Raivio *et al.*, 1999). X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) was used at a final concentration of 100 μg/ml.

2.2 Plasmids and plasmid construction

Plasmids used in this study and the sources of each are listed in Table 2.1. Isolation of plasmid DNA was performed using QIAprep® Spin Miniprep Kit and according to the manufacturers' instructions (Qiagen). *E. coli* MC4100 genomic DNA was isolated using standard methods (Wilson, 1994) and used as the template for various

Table 2.1 Strains and plasmids used in this study.

Strain or plasmid	Genotype[a]	Source or reference
E. coli strains		
BTH101	F⁻ *cya-99 araD139 galE15 galK16 rpsL1* (Str^r) *hsdR2 mcrA1 mcrB1*	(Karimova *et al.*, 1998)
C600	*supE44 thi-1 thr-1 leuB6 lacY1 tonA21*	Laboratory Collection
IL1	SG12064 *cpxA101*	This study
IL2	SG12065 *cpxA101*	This study
IL5	MC4100 *hslV::cm*	This study
IL7	MC4100 *hflB::km*	This study
IL8	*cpxA101 hflB::km*	This study
IL9	C600 *cpxA101*	This study
IL26	MC4100 *hns::amp rpoH::km*	This study
KY1621	*rpoH::km*	(Klein *et al.*, 2003)
MC4100	F⁻ *araD139*Δ(*argF-lac*)*U169 rpsL150* (Str^r) *relA1 flb5301 deoC1 ptsF25 rbsR*	(Casadaban, 1976)
PD32	MC4100 *hns-206*::Amp^r	(Dersch *et al.*, 1993)
PhB767	JM105 *hflB::km*	(Herman *et al.*, 1997)
RFM475	*rpsL galK2* Δ*lac74 gyrB221 gyrB203* Δ*trpE* Δ(*topA cysB*)*204*	(Drolet *et al.*, 1995)
YT475H	RFM475 *hns*::Tn*5*	(Steward *et al.*, 2005)
SG12064	C600 *hslV::cm*	Susan Gottesman
SG12065	C600 *hslU::cm*	Susan Gottesman
TR20	MC4100 *cpxA101*	(Gubbins *et al.*, 2002)
TR51	MC4100 *cpxR::spc*	(Raivio *et al.*, 1999)
TR49	MC4100 λ*RS88*[*degP-lacZ*]	(Raivio and Silhavy, 1997)
TR189	MC4100 *cpxA101 zii*::Tn*10*λ*RS88*[*degP-lacZ*]	(Gubbins *et al.*, 2002)
VL584	F⁻ *thi ara*Δ(*lac pro*) Δ(*uxu fimD*) *rspL*	(Schandel *et al.*, 1992)
XK1200	F⁻ Nal^r Δ*lacU124* Δ(*nadA araG gal att(Wu et al., 1999)L*)	(Moore *et al.*, 1981)
Plasmids		
F*lac traJ90*	*traJ lac*$^+$ F derivative	(Achtman *et al.*, 1971)
pACYC184	Cm^r, Tc^r, general cloning vector	(Chang and Cohen, 1978)
pBAD18	Amp^r, general cloning vector	(Guzman *et al.*, 1995)
pBAD24	Amp^r, general cloning vector	(Guzman *et al.*, 1995)
pBAD33	Cm^r, general cloning vector	(Guzman *et al.*, 1995)
pBADTraJ	Amp^r, *traJ* cloned into pBAD24	(Gubbins *et al.*, 2002)
pBC SK	Cm^r, general cloning vector	Stratagene
pBHB1	Cm^r, *hflB* cloned into pBAD33	(Herman *et al.*, 1997)
pBR322	Amp^r, Tc^r general cloning vector	New England Biolabs

pED851	Amp^r, F *tra* region cloned into pBR322	(Johnson and Willetts, 1980)
pIL17	Cm^r, Amp^r, *hslV-lacZ* transcriptional fusion in pJLac101	This work
pIL18	Cm^r, *hslV-lacZ* into pACYC184	This work
pILS8	Amp^r, *sraF* cloned into pBAD24	This work
pILJ11	Cm^r, *traJ* fragment cloned into pBC SK	This work
pILJ12	Cm^r, *traJ* fragment cloned into pACYC184	This work
pIL13	Amp^r, *hslVU* cloned into pBR322	This work
pILJ14	Cm^r, *traJ* cloned into pBAD33	This work
pILJ15	Km^r, *traJ* cloned into pBAD33 with KIXX	This work
pILJ16	Cm^r, *his$_6$-traJ* cloned into pBAD33	This work
pIL21	Amp^r, Cmr, P_{traG}-*lacZ*	This work
pJLac101	pPR9tt-1-derived transcriptional fusion-based promoter assessment plasmid	(Will *et al.*, 2004)
pJLac105	Amp^r, Cmr, P_{lac}-*lacZ*	(Lu, Ph.D. Thesis, 2004)
pKT25	Km^r, BTH cloning vector	(Karimova *et al.*, 1998)
pLD404	Amp^r, *nlpE* cloned into pBR322	(Snyder *et al.*, 1995)
pLJ5-13	Ampr, *T7Φ10-finP* fusion in pUC19	(Jerome *et al.*, 1999)
pLF71	Ampr, *pifC* in pT7-7	Lab collection
pLF181	Ampr, *pifC* in pT7-7	Lab collection
pML100	Amp^r, RP4 *traF*$^+$ *traG*$^+$	(Lessl *et al.*, 1993)
pND18	Amp^r, P_{BAD}-*nlpE* cloned into pBAD18	(Danese *et al.*, 1995)
pOX38-Km	Km^r, F *tra* region, Rep FIA replicon	(Chandler and Galas, 1983)
pOX38-Tc	Tc^r, F *tra* region, Rep FIA replicon	(Chandler and Galas, 1983)
pOX38::*traR354*	Km^r, F *tra* region, *traR* mutant	(Maneewannakul and Ippen-Ihler, 1993)
pOX38::*traX482*	Km^r, F *tra* region, *traX* mutant	(Maneewannakul and Ippen-Ihler, 1993)
pPR9tt	Amp^r, Cm^r, RK2 replicon	(Santos *et al.*, 2001)
pPR9tt-1	Amp^r, Cm^r, BstBI site-disrupted pPR9tt	(Lu, Ph.D. Thesis, 2004)
pRS27	Tc^r 9-kb partial EcoRI F fragment in pSC101	(Skurray *et al.*, 1978)
pRWJ2	Amp^r, *his$_6$-traJ* cloned into pBAD24	This work
pRS2496	Km^r, *pif* operon in pACYC177	(Cram *et al.*, 1984)
pSK470	Amp^r, P_{tac}-*traG*$_{RP4}$	(Schroder *et al.*, 2002)
pUC-KIXX	Kmr, pUC4 containing Tn*5*	Amersham Pharmacia Biotech
pUT18	Amp^r, BTH cloning vector	(Karimova *et al.*, 1998)
pUT18C	Amp^r, BTH cloning vector	(Karimova *et al.*, 1998)
p25TraG-N	Km^r, N-terminal of *traG* fused to T25 in pKT25	This work
p25PifC-N	Km^r, N-terminal of *pifC* fused to T25 in	This work

	pKT25	
p18TraG-C	Amp^r, C-terminal of *traG* fused to T18 in pUT18	This work
p18TraG-N	Amp^r, N-terminal of *traG* fused to T18 in pUT18C	This work
p18PifC-C	Amp^r, C-terminal of *pifC* fused to T18 in pUT18	This work
p18PifC-N	Amp^r, N-terminal of *pifC* fused to T18 in pUT18C	This work
p25TraG$_{123-635}$-N	Km^r, N-terminal of *traG*$_{123-635}$ fused to T25 in pKT25	This work
p25TraG$_{245-635}$-N	Km^r, N-terminal of *traG*$_{245-635}$ fused to T25 in pKT25	This work
p25TraG$_{398-635}$-N	Km^r, N-terminal of *traG*$_{398-635}$ fused to T25 in pKT25	This work
p25TraG$_{123-196}$-N	Km^r, N-terminal of *traG*$_{123-196}$ fused to T25 in pKT25	This work
RP4	Km^r, Amp^r, IncPα plasmid	(Lanka *et al.*, 1983)
R751	Tp^r, IncPβ plasmid	(Thorsted *et al.*, 1998)

[a] Str^r, streptomycin resistant; Nal^r, nalidixic acid resistant; Km^r, kanamycin resistant; Tc^r, tetracycline resistant; Amp^r, ampicillin resistant; Cm^r, chloramphenicol resistant, Spc^r, spectinomycin resistant, Tp^r, trimethoprim resistant.

PCR reactions listed below. All clones constructed during the course of this work were sequenced using the DYEnamic ET fluorescent sequencing system (Amersham Pharmacia Biotech). Restriction enzymes and T4 DNA ligase used for DNA cloning were purchased from either Roche Diagnostics or Fermentas Life Sciences. Methods for restriction digestion, ligation, and standard PCR were performed using standard protocols as previously described (Sambrook, 1989)

pILJ11 was constructed by digesting pRS27 with SalI and PstI and ligating the *traJ*-containing fragment into SalI/PstI-digested pBC SK+ (Stratagene) using T4 DNA ligase. pILJ12 was constructed by digesting pILJ11 with SalI and XbaI and ligating the *traJ* fragment into SalI/XbaI-digested pACYC184, conferring Cm^R. In both ligations, the pre-digested fragments were purified from 1% agarose gels using QIAquick® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

E. coli MC4100 genomic DNA was used as the template for PCR amplification of the *hslVU* coding region for pIL13 and the *hslV* promoter region for pIL17. Primers ILA19 and ILA20 (Table 2.2) were used to amplify a 2386-bp PCR product with EcoRI and BamHI sites at the 5' and 3' ends, respectively, which was inserted directly into pCR4Blunt-TOPO® cloning vector according to the manufacturer's instructions (Invitrogen). The resulting plasmid was digested with EcoRI and BamHI and ligated to EcoRI/BamHI-digested pBR322 to construct pIL13. pIL17 was constructed using the upstream primer ILA49 and downstream primer ILA50 to amplify the *hslV* 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 (Will *et al.*, 2004). pIL18 was constructed by digesting pIL17 with BglII and XbaI and ligating the *hslV*::*lacZ* fragment into BamHI/XbaI-digested pACYC184 using T4 DNA

Table 2.2 Primers used in this study

Primers	Sequence	Remarks
ILA19	5'-*GGAATTC*CTGACGCGCCAAAACCG-3'	*hslV* forward
ILA20	5'-*CGGGATCCCG*ACGATAATTGCAGC-3'	*hslV* reverse
ILA27	5'-*GCTAGC*CATACTTTGTTACCTGCA-3'	*sraF* forward
ILA28	5'-*AAGCTT*CATAAAAAAACGGCCAACG-3'	*sraF* reverse
ILA29	5'-*AGATCT*GAAGAGGGCTAAAGCCCG-3'	*traGp* forward
ILA30	5'-*CCATGG*CCAAGAAGTAGATGAGG-3'	*traGp* reverse
ILA38	5'-*GGATCC*GATGCTAAGCCAGCT-3'	*pifC* forward
ILA39	5'-*GGTACC*AGATCTCCGTACAGG-3'	*pifC* reverse
ILA40	5'-*GGATCC*GATGAAGAACCGAAAC-3'	*traG* forward
ILA41	5'-*GGTACC*ATTATCGTGATCCCCTC-3'	*traG* reverse
ILA45	5'-*GGATCC*GGACAAGAAGGACATAC-3'	$traG_{123}$ forward
ILA46	5'-*GGATCC*GCTCGATGAAATCCGC-3'	$traG_{245}$ forward
ILA47	5'-*GGATCC*GCTGTTCATCGTGACG-3'	$traG_{398}$ forward
ILA48	5'-*GGTACC*ATTACCGGGTCGTCGT-3'	$traG_{396}$ reverse
ILA49	5'-*AGATCT*GACGCGCCAAAACCGACG-3'	P_{hslV} forward
ILA50	5'-*GGTACC*GAGCTGACCCCTTGGTTAC-3'	P_{hslV} reverse
RWI34	5'-CCATGGTA*CATCATCATCATCATCAT*ATGT ATCCGA TGGATCGTATTC-3'	his_6-*traJ* upstream
RWI35	5'-CTGCAGTTAACGCGTATTTATGATACACA TAGCC-3'	his_6-*traJ* downstream

ligase. Positive clones were sequenced using the DYEnamic ET fluorescent sequencing system to confirm that the *hslVU* genes and the *hslV* promoter were correctly cloned into the vectors.

E. coli MC4100 chromosome was used as the template for PCR amplification of the SraF small RNA. Primers ILA27 and ILA28 were used to amplify the 220-bp PCR product with an NheI site and a HindIII site at the 5' and 3' ends, respectively, which was inserted directly into pCR4Blunt-TOPO® cloning vector according to the manufacturer's instructions (Invitrogen). This resulting plasmid was digested with NheI and HindIII and ligated to NheI/HindIII-digested pBAD24 to construct pILS8.

pILJ14 was constructed by digesting pBADTraJ (Gubbins *et al.*, 2002) with ClaI and HindIII and ligating the *traJ*–containing fragment into pBAD33. pILJ15 was constructed by digesting pUC4-KIXX with SmaI and ligating the 1.4kb, Km^r fragment into PvuII-digested pILJ14. Positive clones of pILJ15 were selected based on their resistance to Km and Cm. pRWJ2 was constructed by Dr. R. Will using the upstream primer RWI34 and the downstream primer RWI35 to amplify the F *traJ* gene and to introduce an NcoI site 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 NcoI/PstI-digested pBAD24. pILJ16 was constructed by digesting pRWJ2 with ClaI and HindIII and ligating the his_6-*traJ* fragment into pBAD33. The *traJ* coding region on pILJ11, pILJ12, pILJ14, pILJ15, and pILJ16 was sequenced and the plasmids were tested in a complementation experiment using a F*lac traJ90* mutant to ensure they were functional *in vivo*.

pML100, a plasmid that contains $traG_{RP4}$, was used as a template for PCR amplification of the *traG* promoter (*traGp*). Primers ILA29 and ILA30 were used to amplify the 0.2-kb PCR product with BglII and KpnI sites at the 5' and 3' ends,

respectively. The *traGp* region was cloned into pJLac101 to become pIL21 (P_{traG}-*lacZ*). pLF181, a plasmid that contains *pifC*, was used as a template for PCR amplification of the *pifC* coding region. Primers ILA38 and ILA39 were used to amplify the 1.1-kb PCR product with BamHI site and KpnI site at the 5' and 3' ends, respectively. pML100, a plasmid that contains *traG*, was used as a template for PCR amplification of the *traG* coding region. Primers ILA40 and ILA 41 were used to amplify the 1.9-kb PCR product with BamHI and KpnI sites at the 5' and 3' ends, respectively. The PCR products were inserted directly into pCR4Blunt-TOPO® cloning vector according to the manufacturer's instructions (Invitrogen). These plasmids were digested with BamHI and KpnI, and fragments containing *pifC* or *traG* were gel purified and ligated to BamHI/KpnI-digested bacterial two-hybrid (BTH) vectors pKT25, pUT18, or pUT18C. Six plasmids were generated: p25TraG-N, p25PifC-N, p18TraG-C, p18TraG-N, p18PifC-C, p18PifC-N. The number in the name of the plasmid indicates the adenylate cyclase peptide, whereas the last letter indicates the terminus (C- or N-) of the test protein that is fused to the peptide. For example, p25TraG-N expresses the T25-TraG fusion protein in which the N-terminal end of *traG* is fused in frame to the C-terminal end of T25.

For truncated versions of *traG*, primers ILA45, ILA46, ILA47 were used in place of ILA39 in PCR amplifications with primer ILA40 to generate *traG* fragments that lack the N-terminal end at various lengths. PCR generated $traG_{123\text{-}635}$, $traG_{245\text{-}635}$, and $traG_{398\text{-}635}$, respectively, where the numbers in the subscripts indicate the amino acid number of $TraG_{RP4}$. Primers ILA45 and ILA48 were used to amplify a truncated *traG* fragment that lacks the N- and C-terminus, $traG_{123\text{-}396}$. The resulting PCR fragments were treated the same way as the full length *traG*, and cloned into the vector pKT25.

2.3 Microarray analysis

E. coli MC4100/pOX38-Km and TR189/pOX-38Km 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 of 1.0 (OD_{600}). 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 20µl 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 3µm with a GeneArray scanner. Data analysis was performed using Affymetrix Microarray Suite5.0 software (http://www.affymetrix.com/support/technical/ whitepapers.affx).

2.4 Bacterial Matings

Liquid matings were performed as previously described (Gubbins *et al.*, 2002). Donor cells containing F, or F derivatives, and recipient cells (XK1200) were grown to an OD_{600} of 0.5 to 1.0. One hundred µl of each donor and recipient was added to 800 µl of LB broth and the mating mixtures were incubated for 45 minutes at 30°C, followed by vigorous vortexing to disrupt mating pairs. The mating mixtures were serially diluted in cold 1X Saline-Sodium citrate (SSC, pH 7.0) and 10 µl of each dilution was spotted on appropriate antibiotic plates to select for donors and transconjugants. Mating efficiency was calculated as the ratio of transconjugants to 100 donors.

Solid matings were performed on MC4100 cells harbouring the IncPα RP4 or IncPβ R751 plasmid. One hundred µl of donors and recipients (XK1200) in mid-log

phase were filtered onto 55mm Whatman® filter papers, which were placed on LB agar plates and incubated (with lids, face-up) at 37°C to allow conjugation. After 1 hr, each filter paper was submerged in 1-ml LB broth and vortexed vigorously to resuspend the cells. Serial dilution was performed as described above and 10 µl of each dilution was spotted on appropriate antibiotic plates.

2.5 Immunoblot analysis

Volumes of cell pellets corresponding to 0.1 OD_{600} were collected from cultures that were grown to an OD_{600} of 0.5 to 1 for all immunoblot analyses. Samples were boiled in 10 µl of sodium dodecyl-sulphate (SDS) loading buffer (Laemmli, 1970) for 5 min, and were separated by SDS-12% Polyacrylamide Gel Electrophoresis (PAGE) using the Bio-Rad Protean Minigel system. Proteins were transferred to Immobilon-P membranes (Millipore) using Towbin buffer (25mM Tris, 192mM glycine, 20% methanol; Towbin *et al.*, 1979). Membranes were blocked for 2 hrs 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 horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G (1:10 000, 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 the 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 His_6-TraJ was normalized to time zero, which was arbitrarily assigned a value of 100.

2.6 Purification of His_6-TraJ and *in vitro* proteolysis of TraJ

The His_6-TraJ was purified from pRWJ2 in MC4100 (in the absence or presence of pBR322 or pLD404) as described by Folichon *et al.* (2003). Briefly, a three-ml culture was grown at 30°C or 37°C in LB broth with Amp and 0.4% glucose to approximate 0.5 OD_{600} and then centrifuged. Cell pellets were resuspended in 250 ml fresh LB with Amp and induced with 0.05% arabinose for 2 hours. Cultures were then pelleted, and the pellets were stored at -80°C until processing. Pellets were resuspended in 10 ml B-PER® Bacterial Protein Extraction Reagent (Pierce) and mixed with one tablet of EDTA-free protease inhibitor cocktail (Complete mini, Roche) for 10 minutes. Insoluble proteins were removed by centrifugation. One hundred-µl of 1M imidazole was added to the supernatant, which was incubated with 1ml of Ni^{2+}-NTA agarose with gentle agitation at 4°C for one hour. Following incubation, the slurry was applied to a column and washed with approximately 5ml buffer A [20 mM Tris-HCl (pH 7.8), 300 mM NaCl, and 20 mM imidazole] three times. Protein was eluted from the column with buffer containing 50 mM sodium phosphate, 300 mM NaCl, and 250 mM imidazole. Protein fractions containing TraJ were identified by SDS-PAGE and concentrated by dialysis using the Amicon® Ultra-4 Centrifugal Filter Device (Millipore). Pure TraJ was quantified using a standard curve of bovine serum albumin (BSA) via the Bicinchoninic acid (BCA, Pierce®) protein assay and stored at 4°C.

Pure HslV, HslU, and MBP-SulA (Maltose Binding protein fused to SulA) were generous gifts from Dr. Eunyong Park (Seoul National University, Korea).

The degradation of His_6-TraJ was assayed by incubating the 20-μl reaction mixture at 37°C for stated time periods. The reaction mixtures contained 3 μg of MBP-SulA or 3 μg of His_6-TraJ, 0.8 μg of HslV and 2 μg of HslU in 25 mM Tris-HCl buffer (pH 7.8), 10 mM $MgCl_2$, 100 mM NaCl, 1 mM Dithiothreitol (DTT), 1 mM EDTA, 10% glycerol and 2 mM ATP. After incubation, the reaction was stopped by adding 4 μl of 6× SDS-PAGE sample buffer (0.35M Tris, pH 6.8, 30% glycerol, 10% SDS, 0.26M DTT, 0.6% Bromophenol blue) and analysed on 12% SDS-PAGE. Proteins were quantified by the BCA protein assay using BSA as a standard.

2.7 Stability of TraJ *in vivo*

Cultures of *E. coli* containing the arabinose-inducible plasmids pILJ14 and pILJ15 were grown at 37°C with 0.4% glucose and appropriate antibiotics to an OD_{600} 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 was 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 out 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 rifampicin was added to prevent further expression from the arabinose promoter P_{BAD}. Samples were collected at 30, 60, 120, 180, 240 or/and 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 pILJ14 and 15.

2.8 β-galactosidase assay

E. coli strains containing various transcriptional/translational fusions with the *lacZ* gene were assayed for their activities. β-Galactosidase assays were performed as

described (Miller, 1972). Overnight cultures were diluted 1:50 into fresh LB broth containing appropriate antibiotics. One hundred to five hundred µl of each culture were added to Z-buffer [60mM $Na_2HPO_4 \cdot 7H_2O$, 40mM $NaH_2PO_4 \cdot H_2O$, 10mM KCl, 1mM $MgSO_4$, and 0.27% (v/v) β–mercaptoethanol] to bring the final volume to 1.0 ml. Two drops of 0.1% SDS and chloroform were added and the tubes were vortexed vigorously. The tubes were incubated at 28°C for 5 min, and the reactions were initiated by addition of 13.3mM ONPG. Reactions were stopped by adding 0.5ml of 1M $NaCO_3$. Activity in Miller Units (MU) is determined by using the formula MU= $(A_{420}*1000)/(tvOD_{600})$ where t = time (in minutes), v = volume (in ml) and A_{420} = absorbance at 420nm. Experiments were performed at least three times and the average and standard deviation of the results were determined.

2.9 Transmission electron microscopy (TEM)

E. coli C600, IL9 (C600 *cpxA101**), and IL1 (C600 *cpxA101*hslV*) cells were grown to 0.5 OD_{600} in LB broth. Two-ml of cells were washed and resuspended in 1X Phosphate buffered saline [PBS; 0.038M NaH_2PO_4, 0.162M Na_2HPO_4, 1.5M NaCl]. Approximately 40 µl of the sample was applied to formvar-carbon coated 300 mesh grids. The sample was allowed to dry for a few minutes and negatively stained with Phosphotungstic acid (PTA). The samples were examined with a FEI Morgagni Electron Microscope at 70 kV. Digital images were captured with a Megaview II camera of Soft Imaging Systems in the Advanced Microscopy Facility with assistance from Rakesh Bhatnagar.

2.10 Northern blot analysis

Northern blot analysis was performed as described previously (Will *et al.*, 2004). Briefly, cultures of MC4100 and KY1621 (in the presence or absence of pED851) were

grown at 30°C and collected at 1.0 OD_{600}. Total RNA was isolated using the hot phenol method (Jerome and Frost, 1999). Cell pellets were resuspended in 300µl of lysis buffer. Three hundred µl of phenol was added to the cells, followed by vigorous vortexing for 30 sec. The tube was incubated at 65°C for 15 min with regular vortexing every 10 sec. Following centrifugation, the aqueous phase was extracted twice with 300 µl of chloroform. RNA was precipitated by adding 30 µl of 3 M sodium acetate and 600 µl of 95% ethanol to the aqueous phase. The RNA pellet was washed twice with 70% ethanol and dissolved in 20 µl of DEPC–treated Milli-Q® water. RNAs were quantified using an Amersham Pharmacia Ultrospec 3000.

Samples containing 20µg of total RNA were resuspended in 2X RNA loading dye [50% deionized formamide, 5% formaldehyde, 1XMOPS buffer (20mM MOPS, pH 7.0, 50mM sodium acetate, 1mM EDTA), and 0.05% bromophenol blue] and incubated at 65°C for 10 min. Samples were separated by electrophoresis on a 1.5% agarose gel containing 5% formaldehyde in MOPS buffer. The RNA was transferred to a Zeta-Probe membrane (Bio-Rad) in 20XSSC for overnight. The membrane was washed in 2X SSC for 5 min, and allowed to dry. RNA on the membrane was then cross-linked using a Bio-Rad GS Gene-linker at 150mJoules. Membrane was then re-wetted in 2X SSC and stained in reversible Northern Blot Staining Solution (Sigma-Aldrich) for loading control visualization.

For probing, the membrane was destained in Milli-Q® water, dried, and pre-hybridized at 58°C for 4 hours in 30ml of hybridization buffer (50% formamide, 5X Denhardt's, 2.5X SSC, 1.5% SDS, 200µg/ml *E. coli* tRNA XX, and 200µg/ml sonicated calf thymus DNA. The blot was incubated at 58°C overnight in the presence of a ^{32}P-UTP labelled FinP RNA probe (see below) synthesized *in vitro*. The blot was washed at room

temperature for 5 min in 2X SSC, 10 min in 2X SSC and 0.1% SDS, 10min in 0.5X SSC and 0.1% SDS, and then at 55°C for 5 min in 0.1X SSC and 0.1% SDS. After washing, it was dried and exposed on a Molecular Dynamics Storage Phosphor Screen and visualized using a Molecular Dynamics Phosphorimager 445 SI.

For *in vitro* transcription of the FinP RNA probe, pLJ5-13, a plasmid that contains *finP* behind a T7 promoter, was used as the template (Jerome and Frost, 1999). Briefly, fresh pLJ5-13 was digested with BamHI and electrophoresed on a 1% agarose TBE gel. The bands were cut and purified with the Qiagen extraction Kit. Approximate 2 μg of linearized pLJ5-13 was used in a 20-μl transcription reaction. Transcription was performed for 3 hours at 37C with 0.5 mM CTP, ATP, GTP, 0.02 mM UTP and 50 μCi [α-^{32}P-UTP] (3000 Ci/mmol; Perkin-Elmer), in the presence of 20U of T7 RNA polymerase. Completed reactions were incubated at 37°C for 15min with DNaseI to remove any remaining template. The RNA was then electrophoresed on a denaturing 8% Tris/borate/EDTA (TBE)-polyacrylamide gel containing 8M urea in 1X TBE buffer [89mM Tris, 89mM boric acid, 2mM EDTA] and visualized with Kodak X-Omat film. The radioactive RNA probe was cut out of the gel and eluted in diethylpyrocarbonate (DEPC)-treated elution buffer [0.5M ammonium acetate, 1mM EDTA] at 37°C with rotation. The eluent was extracted with 1:1 phenol:chloroform, followed by chloroform (see above). The probe was precipitated with 1μl of glycogen, 30μl sodium acetate, and 600 μl 95% ethanol. The pellet was washed in 70% ethanol, dried, dissolved in water and frozen until the membrane was ready for hybridization.

2.11 Bacterial two-hybrid analysis

The principal of the bacterial two-hybrid system was described in Karimova *et al.* (1998). It takes advantage of the two fragments, T25 and T18, which constitute the

catalytic domain of adenylate cyclase from *Bordetella pertussis*. When these fragments are separated, the enzyme is not functional. However if each of the two interacting proteins is fused to T25 and T18, a functional enzyme is produced. In an adenylate cyclase deficient *E. coli* host (*cya*$^-$), interaction between the two test proteins will produce a functional adenylate cyclase that catalyzes the synthesis of cAMP. The cAMP/CAP complex activates catabolic genes, such as the *lac* operon, involved in lactose catabolism. Therefore, positive protein interactions are selected on X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) plates.

Each of the two test candidates, *pifC* and *traG*$_{RP4}$, was cloned into one of the BTH vectors, pKT25, pUT18, and pUT18C (Table 2.1). The pKT25 plasmid is a vector that encodes the T25 fragment of *B. pertussis* adenylate cyclase and expresses the kanamycin resistance selectable marker. A multiple cloning site (MCS) was inserted at the 3' end of T25 to allow construction of fusion proteins in frame at the C-terminal end of T25. The pUT18 and pUT18C plasmids are vectors that encode the T18 fragment of *B. pertussis* adenylate cyclase and express the ampicillin resistance marker. The MCS lies upstream of the T18 open reading frame in pUT18 and downstream of that in pUT18C, thus allowing constructions of fusion proteins in frame at the N-terminal end in pUT18 and C-terminal end in pUT18C.

Plasmids encoding fused proteins were co-transformed into competent *E. coli* BTH101 strain. The plasmids pKT25-*zip* and pUT18C-*zip* serve as positive controls for complementation, in which the leucine zipper of GCN4 is fused in frame to the T25 and T18 fragments. Interaction of the hybrid proteins will produce functional adenylate cyclase, which catalyses production of cAMP that complexes with CAP to activate expression of *lac* gene. For negative controls, the empty vectors or one of the empty

vectors and a vector containing the test gene were used. Transformations were plated on LB agar containing Amp, Km, and the chromogenic substrate X-Gal. Blue colonies were selected as positive clones after overnight incubation. β-galactosidase assays were performed to allow quantification of protein interaction *in vivo*.

2.12 Cross-linking and Immunoprecipitation of $TraG_{RP4}$ and PifC complex

E. coli cultures containing pML100 and pRS2496 (encode *traG* and *pifC*, respectively) were grown to mid-log phase. The volume of cells equivalent to 1 OD_{600} were collected, washed with 1 ml of cold 1X PBS and resuspended in 200 µl PBS. Cross-linking was performed by adding the chemical reagent BS^3 (Pierce) to a final concentration of 500 µM and incubated at room temperature for 30 min. Reactions were quenched with 12µl of 1M Tris-HCl (pH 7.5) at room temperature for 5 min. Cells were pelleted and washed with cold 1X PBS and frozen. Samples were prepared by resuspending the pellets in 50µl of 1X protein load dye, incubated at 95°C for 10 min, vortexed, and centrifuged for 10 sec at 14K. Six-µl of each sample was loaded on SDS-PAGE gel.

For immunoprecipitation, cells were collected, washed, and resuspended in cold 1X PBS similar to the above procedures for cross-linking. The non-cleavable cross-linking reagent Dithiobis(succinimidylpropionate) (DSP; dissolved in 100% anhydrous DMSO, Pierce) was used to treat cells at a final concentration of 1 mM for 30 min at room temperature. Reactions were quenched the same manner as stated above, and cells were washed and resuspended in 200 µl of PBS. Glass beads were added in a final concentration of 6g/l to lyse cells with vigorous vortexing for 10 min at 4°C. The clear lysate was diluted to 200µl with PBS. 500µl IMP buffer [50mM Tris-HCl, pH 7.5, 300mM NaCl, 1mM EDTA, 1mg/ml BSA, 2% Nonidet P40, 1X complete, EDTA-free

protease inhibitor (Roche)] and 6μg of anti-PifC or anti-TraG antibodies were added to the cells. The immunoprecipitation reaction mixtures were incubated at 4°C overnight with end-to-end mixing. Forty-μl of 50% protein A sepharose slurry in IMP buffer was added and continually incubated at 4°C for 6 hours with end-to-end-mixing. The resin was pelleted by centrifugation at 16,100 X g, 4°C for 2 min, washed 3 times with 1 ml IMP buffer, and 3 times with 1 ml IMP wash buffer (50mM Tris-HCl, pH 7.5, 300mM NaCl, 0.5% Nonidet P40, 0.1% SDS). The resin was resuspended in 20μl of 2X non-reducing SDS sample buffer [100mM Tris, pH 6.8, 4% SDS, 0.2% Bromophenol blue, 20% glycerol] and boiled at 95°C for 5 min. The supernatant was analyzed by 12% SDS-PAGE. PifC and $TraG_{RP4}$ immunoblot analyses were performed to allow visualization of any interaction.

Chapter 3: Activation of the Cpx envelope stress response destabilizes TraJ via the HslVU protease*

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3.1 Introduction

The *cpx* (Conjugative plasmid expression) locus was first identified by isolating chromosomal mutations (*cpx*) that reduce levels of F transfer efficiency by a reduced level of TraJ (Sambucetti *et al.*, 1982). The Cpx regulon was later found to be an extracytoplasmic stress response system that responds to the accumulation of misfolded proteins or overproduced cell envelope proteins (Raivio and Silhavy, 1997). The Cpx pathway has been shown to be involved in cell surface composition (Mileykovskaya and Dowhan, 1997), synthesis of adhesive pili (Hung *et al.*, 2001; Nevesinjac and Raivio, 2005), adhesion (Otto and Silhavy, 2002) and growth (De Wulf *et al.*, 1999; DiGiuseppe and Silhavy, 2003). 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 -R 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, autophosphorylates at a conserved histidine in the cytoplasmic domain and transfers the phosphate group to a conserved aspartate in the cytoplasmic response regulator CpxR (Raivio and Silhavy, 1997). Phosphorylated CpxR (CpxR-P) acts as a transcriptional regulator by binding to the promoters of target genes at the consensus sequence 5'-$GTA_3N_5GTA_3$-3' (Pogliano *et al.*, 1997; De Wulf *et al.*, 2002). Examples of known CpxR-P target genes are *cpxP* (Danese and Silhavy, 1998a), *degP* (Cosma *et al.*, 1995), *ppiA* and *dsbA* (Danese and Silhavy, 1997; Pogliano *et al.*, 1997), encoding chaperones, proteases and other enzymes that maintain 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 catalyze the dephosphorylation of CpxR-P, thereby down-regulating 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 P_Y 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 is a well-characterized mutation that involves a single amino acid change from threonine to proline at position 253 (Raivio and Silhavy, 1997), and results in strong, constitutive activation of the *cpx* regulon. Previous results showed that *cpxA101** 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 up-regulated when perceived stress is sensed in *cpxA**. In this study, microarray analysis was performed to identify candidate protease or chaperone genes that are up-regulated in the *cpxA101** strain. Chapter 8 presents the detailed transcription profile of genes that are regulated and influenced in *cpxA101**. Several candidates were tested and the HslVU heat shock protease-chaperone pair was found to be involved in TraJ degradation. We present data here to show that activation of the Cpx system by *cpxA101** or overexpression of the outer membrane protein NlpE leads to HslVU-mediated degradation of TraJ. Electron microscopy results are also presented to show that

the filamentous phenotype of *cpxA**, which is due to randomized FtsZ ring assembly (Pogliano *et al.*, 1998), is suppressed by an *hslV* mutation in a *cpxA101** *hslV* double mutant.

3.2 Results

3.2.1 Heat shock genes are upregulated in *cpxA101**

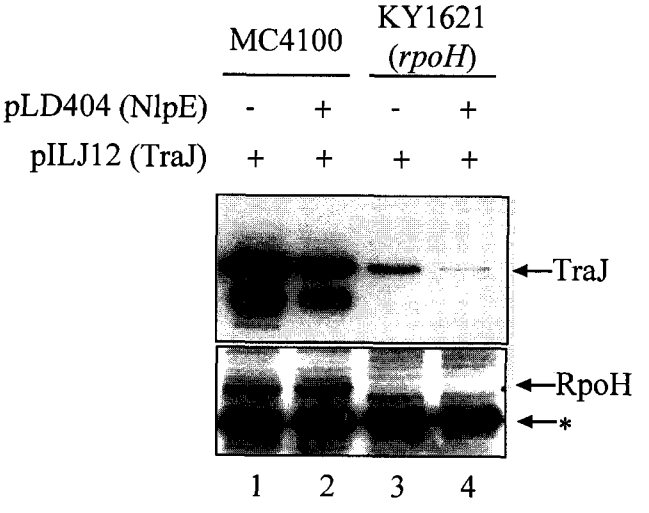
We hypothesized that a protease or a chaperone is upregulated in *cpxA** leading to the degradation of TraJ. Accordingly, microarray analyses were performed to compare the gene profile in *E. coli* MC4100 (wild-type) and TR189 (*cpxA101**), both containing pOX38-Km, an F derivative. Protease or chaperone genes that are activated by 2-fold or greater in *cpxA101** are listed in Table 3.1. The *cpxA101** mutation appeared to stimulate the heat shock regulon. However we discounted the heat shock regulator, σ^H, as a factor in TraJ degradation in Cpx-activated cells because mutations in *rpoH*, the gene encoding σ^H, did not restore TraJ levels (Figure 3.1). σ^H acts on the F plasmid at several levels: it is required for F plasmid vegetative replication (Wada *et al.*, 1987) and for efficient *traJ* transcription (Chapter 5). As shown in Figure 3.1, the level of TraJ expressed from the P_J promoter in pILJ12 is greatly reduced in an *rpoH* mutant (compare lanes 1 and 3). However, the level of TraJ is further reduced in the presence of pLD404, which overexpresses *nlpE* and induces the Cpx regulon (Snyder *et al.*, 1995), suggesting that σ^H is not required for TraJ degradation (Figure 3.1, compare lanes 3 and 4). A *cpxA101** *rpoH* double mutation was lethal and could not be tested.

Table 3.1 Protease, chaperone or heat shock genes with increased expression in a *cpxA101** background

Blattner no.	Gene	Gene description	Signal Log_2 Ratio[a]
b0015	*dnaJ*	heat shock protein	2.1
b0161	*degP*	periplasmic serine protease	2.5
b0439	*lon*	ATP-dependent protease La	2.1
b0473	*htpG*	chaperone Hsp90	3.5
b1829	*htpX*	heat shock membrane protein	2.5
b2592	*clpB*	heat shock protein	3.5
b2699	*recA*	DNA- and ATP- dependent coprotease	1.9
b3686	*ibpB*	heat shock protein	2.6
b3687	*ibpA*	heat shock protein	3.3
b3931	*hslU*	chaperone, HslVU proteosome	3.1
b3932	*hslV*	peptidase, HslVU proteosome	2
b4142	*mopB*	GroES, chaperone	1.9
b4143	*mopA*	GroEL, chaperone, Hsp60	2.2

[a]Signal log_2 ratio of transcript levels for TR189 (*cpxA101**) relative to the MC4100 (wild-type) strain. Both strains contain the F derivative, pOX38-Km.

Figure 3.1 σ^H is not required for TraJ proteolysis in times of extracytoplasmic stress. Immunoblot analysis was performed with polyclonal antisera directed against TraJ and RpoH. *E. coli* MC4100 wild type (lanes 1 and 2) and KY1621 *rpoH* (lanes 3 and 4) strains containing pILJ12 were grown in the presence (+) or absence (-) of envelope stress induced by the *nlpE*-containing plasmid, pLD404. Over-expression of NlpE was known to activate the Cpx system. The positions of TraJ and RpoH are indicated on the right with an arrow. The asterisk indicates a band that cross-reacts nonspecifically with the antiserum and serves as loading control. Immunoblots were performed as described in the Materials and Methods.


MC4100
KY1621
(rpoH)
pLD404 (NlpE) - + - +
pILJ12 (TraJ) + + + +
TraJ
RpoH
*
1 2 3 4

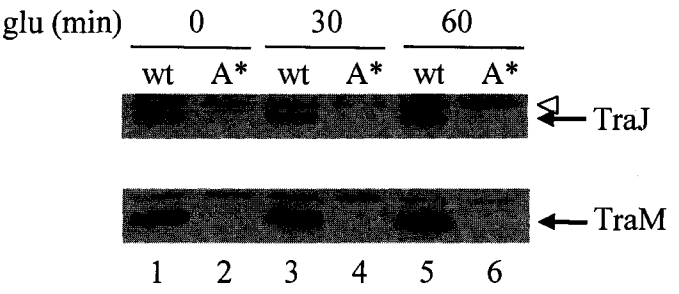
3.2.2 HflB and SraF are upregulated in *cpxA101** but not involved in the degradation of TraJ

Previously *degP*, *recA*, *clpP*, *lon* (Gubbins *et al.*, 2002) have been discounted as being important in the degradation of TraJ. HflB, an essential protease in *E. coli*, was tested for its ability to degrade TraJ in the presence of envelope stress. PhB767 (JM105 *hflB*::Km/pBHB1) was a generous gift from Dr. Philippe Bouloc (Université Paris-Sud, France). Since a mutation in *hflB* is toxic to the cell, PhB767 contains pBHB1 that expresses HflB from the arabinose-inducible promoter in pBAD33 (Herman *et al.*, 1997). IL7 (MC4100 *hflB::km*) and IL8 (*cpxA101* hflB::km*) containing pOX38-Tc and pBHB1 were grown in LB broth plus arabinose to an OD_{600} of 0.4 (0 min). Cells were washed and resuspended in LB broth plus glucose (HflB depletion) and grown for an additional 30, 60, 90 and 120 minutes. During this growth, the arabinose-inducible promoter is suppressed and HflB is depleted. TraJ and TraM proteins were assayed by immunoblot analyses and were found to be absent in the *cpxA101** strain with or without the HflB protease (Figure 3.2A, lanes 4 and 6). Furthermore, the levels of TraJ and TraM remained unchanged when HflB was over-expressed in the wild-type *E. coli* MC4100 containing pOX38-Tc and pBHB1 (Figure 3.2B). This indicates that *hflB*, although up-regulated in a *cpxA101** background, is not responsible for TraJ degradation in an F-containing *cpxA101** cell.

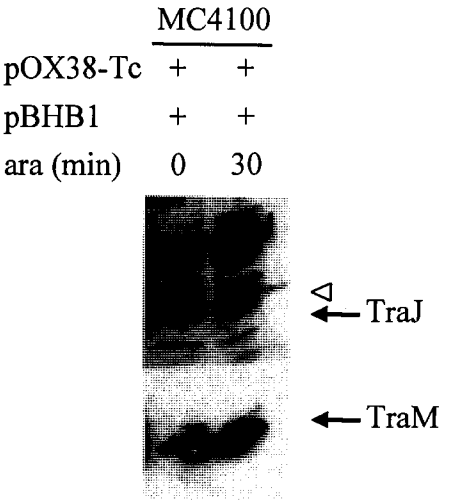
During the course of this study, Dr. Rahul Kulkarni from NEC laboratories (America; personal communication) suggested that translation of *traJ* may be affected in *cpxA** cells by a small RNA (sRNA). This sRNA, named SraF (Argaman *et al.*, 2001), is encoded in the intergenic region between chromosomal genes *ygjR* and *ygjT* and is partially complementary to the *traJ* 5'-UTR, and contains the CpxR-P consensus box at

Figure 3.2 The *hflB* mutation does not rescue TraJ expression in the *cpxA101 mutant.** Immunoblot analysis was performed with polyclonal antiserum directed against TraJ and TraM. **A.** *E. coli* IL7 (MC4100 *hflB*, lanes 1, 3 and 5) and IL8 (*cpxA** *hflB*, lanes 2, 4, and 6) harbouring pOX38-Tc and pBHB1 (P_{ara}-*hflB*) were assayed at 0 (lanes 1 and 2), 30 (lanes 3 and 4), and 60 (lanes 5 and 6) minutes after the addition of 0.4% glucose to suppress production of HflB from P_{ara} . Three bands are shown in the TraJ blot where the top band is nonspecific cross-reaction with the antiserum indicated by an open triangle, the middle band is TraJ indicated by an arrow, and the bottom band is possibly a degradation product of TraJ. **B.** *E. coli* MC4100/pOX38-Tc/pBHB1 was assayed for TraJ and TraM after 30 minutes of arabinose induction. Although the TraJ immunoblot appears cluttered, the TraM immunoblot shows that no degradation occurs when HflB is overexpressed.

A


glu (min)
0
30
60
wt
A*
wt
A*
wt
A*
TraJ
TraM
1
2
3
4
5
6

B


MC4100
pOX38-Tc
+
+
pBHB1
+
+
ara (min)
0
30
TraJ
TraM

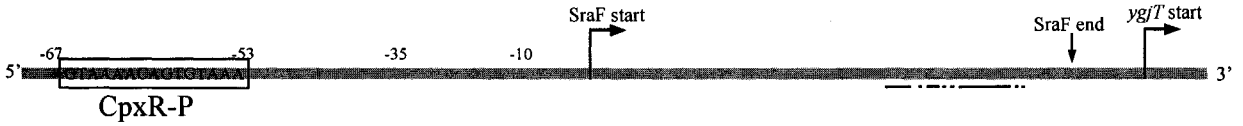
its promoter (Figure 3.3). Although the promoter of SraF contains the CpxR-binding box, the efficiency of conjugation was unchanged when SraF was overexpressed from pILS8 (Table 3.2), a pBAD24 based-plasmid in wild-type cells. Thus, this suggests that SraF is not involved in regulating conjugation.

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

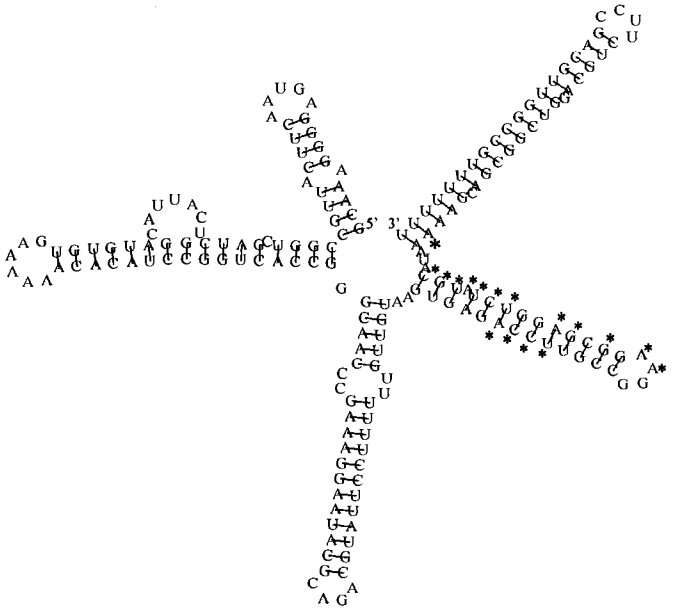
Among the other genes, *hslU* (8.6-fold increase) and *hslV* (4-fold increase) were considered strong candidates for affecting TraJ stability (Table 3.1). They encode the components of the HslVU chaperone/protease pair that are involved in the degradation of SulA (De Wulf *et al.*, 1999) and RcsA (Kuo *et al.*, 2004). SG12064 (*hslV*::Cm) and SG12065 (*hslU*::Cm) were generous gifts from Dr Susan Gottesman (National Institution of Health). The double mutants IL1 (*cpxA101** *hslV*) and IL2 (*cpxA101** *hslU*) were constructed by P1 transduction of the *cpxA101** allele into SG12064 and SG12065, which were otherwise isogenic to *E. coli* C600. pOX38-Km was mated into these strains and provided TraJ, which was detected by immunoblot analysis (Figure 3.4). All experiments were performed at 30°C because of the temperature-sensitive phenotype of *cpxA101** strains. TraY and TraM levels were also assayed by immunoblot to monitor the P_Y and P_M 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 (Figure 3.4A, compare between lanes 3 and 4) and IL2 (*cpxA101** *hslU*) /pOX38-Km (data not shown). Thus both the chaperone (HslU) and the protease (HslV) are required for TraJ level 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 only partially restored in the double mutants MC4100 *cpxA101** *hslV* (Figure 3.4B, compare

Figure 3.3 Structure of SraF, a sRNA that is activated in *cpxA101 and complementary to *traJ* mRNA.** A. The promoter of SraF contains a perfect match for the CpxR-P consensus sequence (boxed). SraF is encoded in the intergenic region between chromosomal genes *ygjR* and *ygjT* (Argaman *et al.*, 2001). B and C. Secondary structures of SraF and *traJ* mRNA. The proposed complementary sequences in both structures are asterisked. Structure are obtained from Kulkarni (2004, personal communication) and Gubbins (Ph.D. Thesis, 2002).

A SraF promoter



B SraF



C *traJ* mRNA

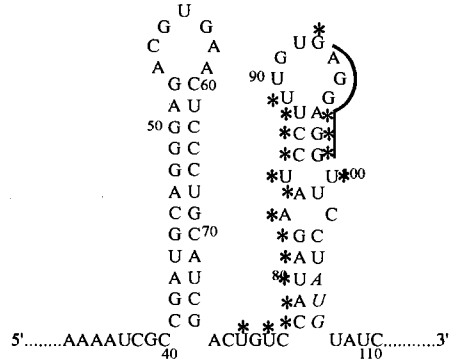
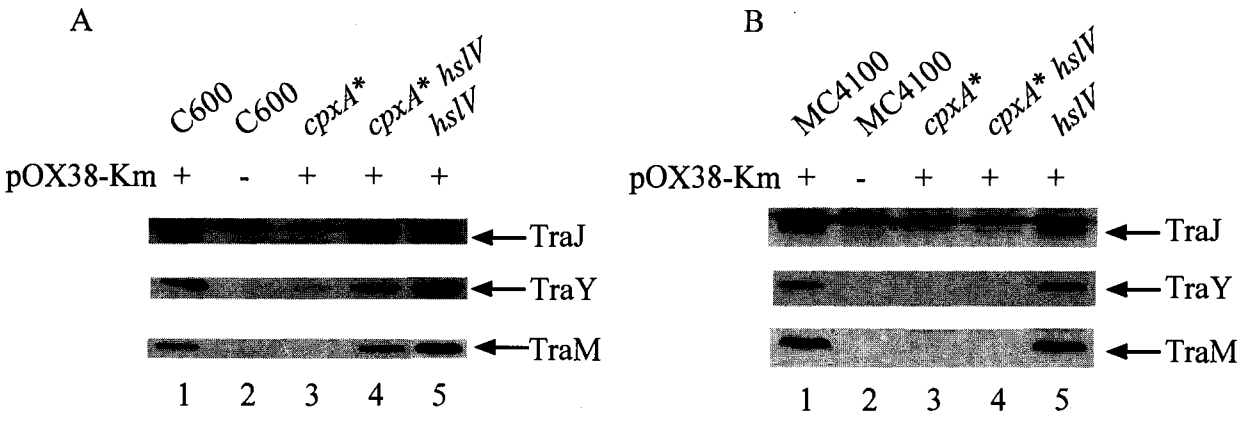


Table 3.2 Mating efficiencies in *E. coli* MC4100 harboring pILS8 (SraF)

Donors (pOX38-Km in[a])	Transconjugants/100 donors	% Mating efficiency
MC4100	50	100
MC4100/pBAD24	38	76
MC4100/pBAD24 (glu)	21	42
MC4100/pBAD24 (ara)	27	54
MC4100/pILS8	22	44
MC4100/pILS8 (glu)	52	104
MC4100/pILS8 (ara)	50	100

[a]*E. coli* XK1200 was used as the recipient in this mating assay

Figure 3.4 TraJ is rescued in the C600 double mutant *hslV cpxA, and partially rescued the MC4100 double mutant.** Immunoblot analyses with polyclonal antisera directed against TraJ, TraY and TraM. *E. coli* strains with (+) or without (-) pOX38-Km were grown to early log phase. The numbers below refer to the lanes in each immunoblot. The positions of TraJ, TraY and TraM are indicated on the right with arrows. The top band above TraJ cross-reacts nonspecifically with the antiserum and serves as a loading control. **A.** *E. coli* C600 strains wild-type (lanes 1 and 2), IL9 (*cpxA101**, lane 3), IL1 (*cpxA101* hslV*, lane 4), and SG12064 (*hslV*, lane 5). **B.** *E. coli* MC4100 strains, wild-type (lanes 1 and 2), TR20 (*cpxA101**, lane 3), IL3 (*cpxA101* hslV*, lane 4), and IL5 (*hslV*, lane 5). The levels of TraJ are insufficient to activate P_Y in IL3, as detected by TraY and TraM immunoblot analyses.


A
C600
C600
cpxA*
cpxA* hslV
hslV
pOX38-Km + - + + +
TraJ
TraY
TraM
1 2 3 4 5
B
MC4100
MC4100
cpxA*
cpxA* hslV
hslV
pOX38-Km + - + + +
TraJ
TraY
TraM
1 2 3 4 5

between lanes 3 and 4). This strain difference indicates that proteases other than HslVU can potentially degrade TraJ in MC4100, and deleting the *hslV* gene results in the substitution of its function by other proteases.

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 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 (Figure 3.5) as was TraY and TraM (data not shown). Supplying HslVU *in trans* in C600/pOX38-Km/pIL13 also resulted in the degradation of TraJ (Figure 3.5), indicating that TraJ or a protein required for TraJ stability was a substrate for HslVU *in vivo*.

3.2.4 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 in *cpxA* hslV*/pOX38-Km using *E. coli* XK1200 as the recipient strain (Table 3.3). The mating efficiency of IL9 (C600 *cpxA101**)/pOX38-Km decreased to 6% of wild-type levels whereas the mating efficiency of IL1 (C600 *cpxA101* hslV*)/ pOX38-Km was restored to 76% of wild-type. These results were consistent with the levels of TraJ, -Y, and -M detected by immunoblot analyses (Figure 3.4).

TraJ levels were reduced in wild-type cells (C600/pOX38-Km) expressing *hslVU* from pIL13 (Figure 3.4). This was also reflected in the reduced mating efficiency of these cells (0.5%). Mating efficiency remained low for IL1 (*cpxA101* hslV*)/pOX38-Km/pIL13 (0.9%). Because TraJ is essential for F *tra* operon activation, it appears to be an important substrate for HslVU.

Figure 3.5 TraJ levels are reduced when HslVU is overexpressed from a multicopy plasmid. Immunoblot analysis was performed with polyclonal antiserum directed against TraJ. *E. coli* C600 wild-type (lanes 1 and 2), IL1 *cpxA101*hslV* (lanes 3 and 4), and IL2 *cpxA101*hslU* (lanes 5 and 6) cells containing pOX38-Km and either pBR322 (lanes 1, 3, and 5) or pIL13 (lanes 2, 4, and 6) were assayed for TraJ abundance. The band that cross-reacted nonspecifically with the antiserum served as a loading control.

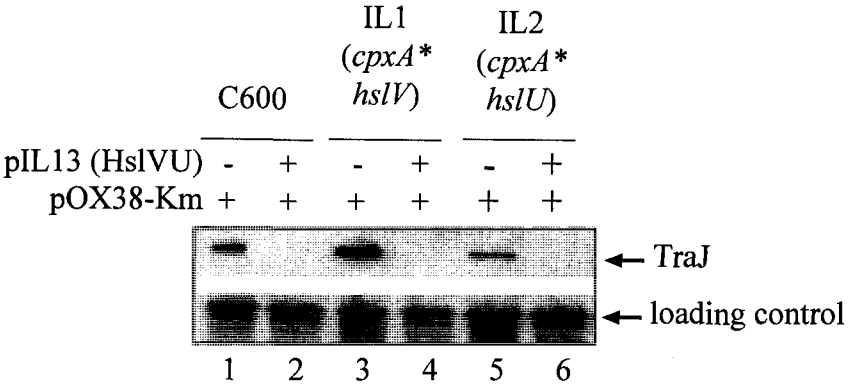

C600
IL1 (cpxA* hslV)
IL2 (cpxA* hslU)
pIL13 (HslVU) - + - + - +
pOX38-Km + + + + + +
TraJ
loading control
1 2 3 4 5 6

Table 3.3 Transfer efficiency of pOX38-Km from various donor strains[a]

Donors (pOX38-Km in)	Transconjugants/1000 donors[b]	% Mating efficiency[c] (versus wild type)
C600	23.8	100
IL9 (*cpxA101**)	1.3	6
IL1 (*cpxA101* hslV*)	18.1	76
MC4100	22.5	100
TR20 (*cpxA101**)	0.2	0.8
IL3 (*cpxA101* hslV*)	1.5	7
C600/pBR322	18.3	100
C600/pIL13	0.1	0.5
IL1/pBR322	14.2	77.3
IL1/pIL13	0.17	0.9
MC4100/pBR322	15.4	100
MC4100/pIL13	0.3	1.9
IL3/pBR322	0.3	3.5
IL3/pIL13	0.02	0.2

[a] Matings were performed at 30°C for 45 minutes

[b] Average number of transconjugants per 1000 donors from 3 mating results.

[c] Mating efficiency expressed as a percentage of the number of transconjugants per 1000 donors in each strain divided by the number of transconjugants per 1000 donors in a wild-type background.

Transfer ability of MC4100 *cpxA**/pOX38-Km decreased to 0.8% of wild-type levels. Whereas mating is restored by one log in IL3 (MC4100 *cpxA** *hslV*) to 7% of the wild-type level, restoration of TraJ is minimized as shown by immunoblot (Figure 3.4). Similar to C600 background, over-expressing HslVU protease from pILJ13 resulted in reduction of transfer efficiency.

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

Overproduction of the outer membrane lipoprotein NlpE activates the Cpx pathway (Snyder *et al.*, 1995) and 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 (Table 2.1) in C600/pOX38-Km and SG12064 (*hslV*)/pOX38-Km. The mating efficiency of wild-type cells decreased 5.7-fold when *nlpE* was overexpressed (Table 3.4) whereas the mating efficiency in SG12064/pLD404 was unaffected. The levels of TraJ and TraY reflected these results as shown by immunoblot analysis (Figure 3.6). Thus, HslVU was implicated in the reduction of TraJ levels and mating efficiency during induction of stress by NlpE overproduction.

3.2.6 CpxAR influences TraJ degradation in cells overexpressing NlpE.

Our results could be explained by the activation of another stress regulon that acts in parallel to the CpxAR response system. To test this possibility, the degradation of TraJ in *cpxA* and *cpxR* mutants was monitored in the presence and absence of pLD404 (NlpE). Whereas *cpxA* mutants carrying pOX38-Km and pLD404 had intermediate levels of TraJ (data not shown), the levels of TraJ and mating efficiency were restored to wild-type

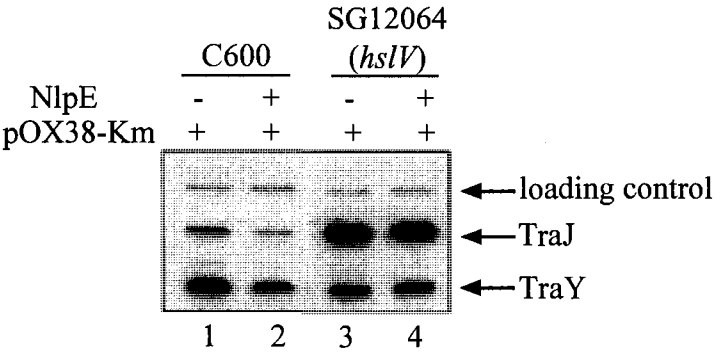
Table 3.4 Transfer efficiency of pOX38-Km from donors under envelope stress induced by NlpE overproduction

Donor (+pOX38-Km)	Transconjugants /1000donors[a]	% Mating efficiency	Fold decrease[b]
C600/pBR322	40	100	5.7
C600/pLD404	7	17.5	
SG12064(C600 *hslV*)/pBR322	30	100	1.1
SG12064(C600 *hslV*)/pLD404	28	93	

[a]Matings were performed at 30°C for 45 minutes and are the average of 3 independent mating results. See figure 3 for details.

[b]Fold decrease refers to the decrease in mating efficiency in each pair of strains.

Figure 3.6 Restoration of TraJ in *hslV* cells overexpressing NlpE. Immunoblot analysis was performed with polyclonal antisera directed against TraJ and TraY. *E. coli* C600 wild-type (lanes 1 and 2) and SG12064 *hslV* (lanes 3 and 4) strains containing pOX38-Km and either pBR322 (lanes 1 and 3) or pLD404 (lanes 2 and 4) expressing NlpE were subjected to immunoblot analyses. The band above TraJ that cross-reacted nonspecifically with the antiserum served as a loading control. The positions of TraJ and TraY are indicated with arrows on the right.


SG12064
C600
(hslV)
NlpE - + - +
pOX38-Km + + + +
loading control
TraJ
TraY
1 2 3 4

levels in a *cpxR* mutant background (Figure 3.7, compare lanes 2 and 4). These results suggest that envelope stress induced by NlpE leads to the degradation of cellular TraJ mainly through CpxR. The reduced level of TraJ in *cpxR* in the absence of stress may be due to activation of cellular protease such as HslVU (discussion).

3.2.7 The *hslVU* promoter is activated upon heat shock and envelope stress induction

To confirm the microarray results and to demonstrate that the *hslV* promoter is upregulated upon Cpx activation, cells harbouring pIL18, a plasmid carrying a *hslV-lacZ* transcriptional fusion, were assayed for β-galactosidase activity. Since *hslVU* is known to be activated in a heat shock response, *hslV* promoter activity was assayed in both heat shock (42°C) cells and Cpx-activated cells. When wild-type/pIL18 cells were grown at 42°C, the *hslV* promoter was up-regulated 2.7-fold. The *hslV* promoter was induced 4.3-fold in the presence of pLD404 in wild-type but not *cpxR* mutant cells (Figure 3.8). Figure 3.8 also reveals a perplexing phenomenon that *hslVU* is upregulated in a *cpxR* null mutant (compare lanes 1 and 3). To this end, the *hslV* promoter is not extensively studied. But it includes two heat shock-stimulated mRNA transcriptional start sites and two HS-repressed mRNA transcriptional start sites (Chuang *et al.*, 1993). This experiment suggests the notion that low, steady levels of CpxR (or CpxR-P) could act as a repressor of the *hslV* promoter. When Cpx is activated, for example through induction of NlpE, high levels of CpxR-P activate *hslVU*.

3.2.8 The filamentous phenotype of *cpxA** is suppressed by the *hslV* mutation

Our results indicate that HslVU, the host protease, is activated in *cpxA** or NlpE-overexpressing cells. As such, we asked whether the aberrant cell division and

Figure 3.7 CpxR is required for TraJ proteolysis in time of extracytoplasmic stress. Immunoblot analysis was performed with polyclonal antiserum against TraJ. MC4100 (lanes 1 and 2) and TR51 *cpxR* (lanes 3 and 4) carrying pOX38-Km in the presence (+) or absence (-) of pLD404 were subjected to TraJ immunoblot analysis.

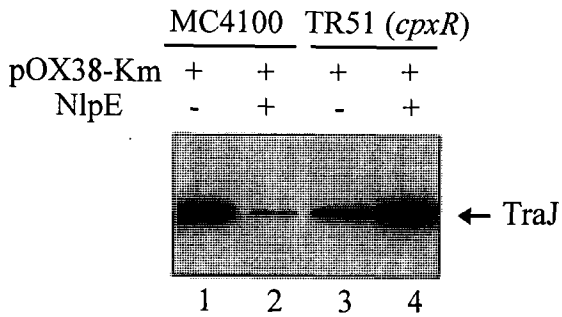
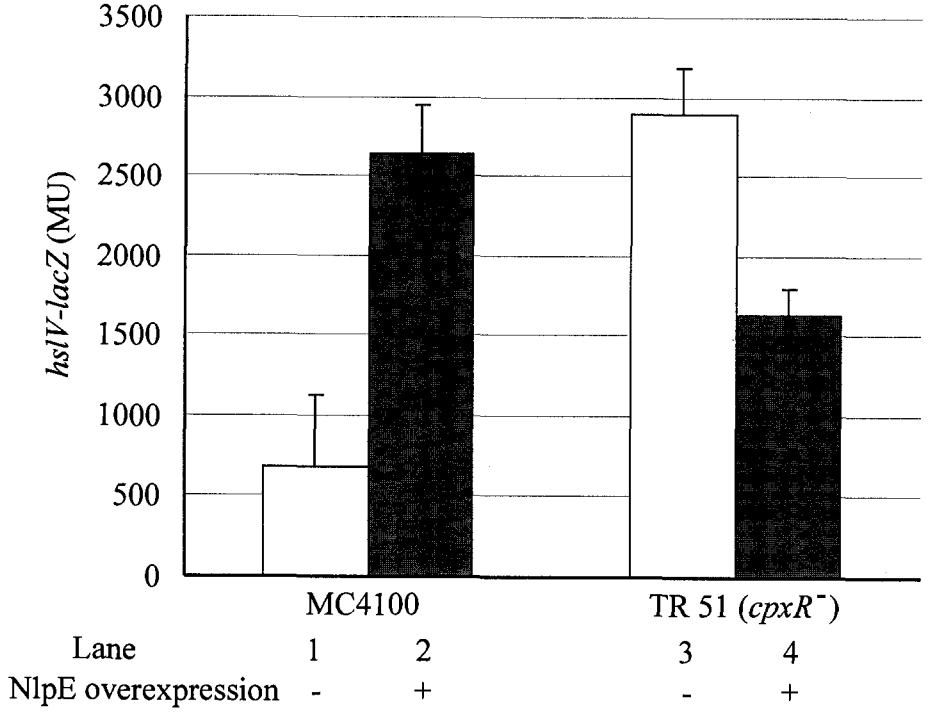

MC4100
TR51 (cpxR)
pOX38-Km + + + +
NlpE - + - +
← TraJ
1 2 3 4

Figure 3.8 The *hslVU* promoter is activated in cells expressing NlpE in a CpxR-P dependent fashion. Expression from the *hslVU* promoter was measured by monitoring β-galactosidase expression from pIL18 (*hslV-lacZ* fusion) harboured in MC4100 (lanes 1 and 2) and TR51 *cpxR* (lanes 3 and 4), which co-harbour either pBR322 (lanes 1 and 3) or pLD404 (lanes 2 and 4) expressing NlpE. Cells were grown to 0.5 OD_{600} at 37°C. All assays were performed in duplicate and repeated at least twice. The data shown represent the mean and standard deviation.

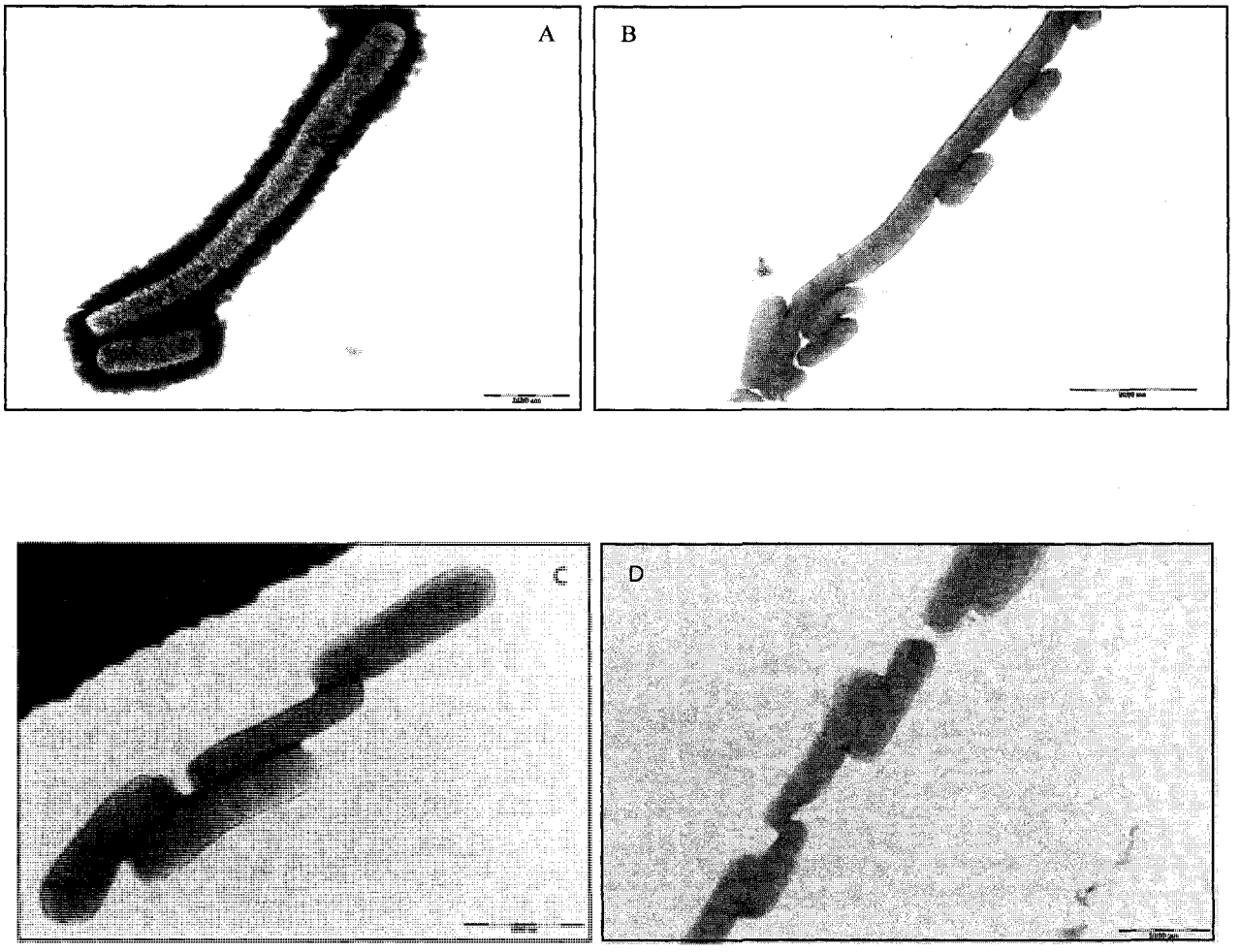

3500
3000
2500
2000
1500
1000
500
0
hslV-lacZ (MU)
MC4100
TR 51 (cpxR⁻)
Lane 1 2 3 4
NlpE overexpression - + - +

filamentous phenotype observed in *cpxA** cells by Pogliano *et al.* (1998) is due to rapid degradation of SulA. SulA is a cell division inhibitor that is encoded by the SOS-inducible *sulA* gene. The target of SulA is FtsZ, which forms a cytoskeletal Z ring at mid-cell position during cell division (Bi and Lutkenhaus, 1991). SulA prevents cell division occurring at incorrect positions. Randomized FtsZ ring assembly was observed in *cpxA** cells, which resulted in abnormal cell division (Pogliano *et al.*, 1998). Since SulA was found to be an *in vivo* substrate of HslVU (De Wulf *et al.*, 1999; Seong *et al.*, 1999), we tested whether mutation in *hslV* can suppress this phenomenon in *cpxA** cells. *E. coli* C600, IL9 (*cpxA101**) and IL1 (*cpxA101* hslV*) were grown in LB and mounted onto the grid as described in Materials and Methods. Images were recorded using a transmission electron microscope with CCD camera (Figure 3.9). Interestingly, the filamentous phenotype of *cpxA101** cells is suppressed by an *hslV* mutation in IL1 (*cpxA101* hslV*), suggesting that HslVU is involved in SulA degradation during cell division.

3.3 Discussion

Our results suggest that TraJ, the activator of F transfer operon transcription, is a substrate for the host protease HslVU during the envelope stress response mediated by the Cpx regulatory system. Microarray analysis of a *cpxA101** mutant revealed that the protease-chaperone pair *hslVU* was up-regulated and, based on its role in degrading other regulators, was considered the most promising candidate. Mutations in *hslVU* restored or increased TraJ levels in the presence of stress or in wild-type cells, respectively. The introduction of HslVU *in trans* complemented these mutations and also led to decreased TraJ levels and mating ability in wild-type cells. While these results seem straightforward, the intransigence of intracellular TraJ to degradation suggests a more complex story, which will be elucidated in the next chapter.

Figure 3.9 Transmission electron microscopic results of IL9 (*cpxA) and IL1 (*cpxA*hslV*).** A and B. Electron micrographs of IL9 (C600 *cpxA101**). The *cpxA** cells are characterized by filamentous morphology. C and D. Electron micrographs of IL1 (C600 *cpxA101** *hslV*). In the double mutant, the filamentous phenotype in *cpxA** is suppressed by an *hslV* mutation. Cells were grown to mid-log phase and washed and resuspended in PBS buffer. EM grids were prepared as described in Material and Methods. Scale bars represent 2μm (each black or white division = 0.4μm) for A, C, D and 5μm for B (each division = 1μm).


A
B
C
D

In this chapter, we explore TraJ degradation at two levels: translational or post-translational. The sRNA SraF that is complementary to *traJ* mRNA, while being activated in *cpxA101**, did not reduce transfer ability in the assays we used (Table 3.2). Recently, SraF was found to be involved in response to pH activation (Altuvia *et al.*, 2008). Four ATP-dependent protease families have been recognized in prokaryotes: Lon, ClpAP and ClpXP, HslVU (also termed ClpQY), and HflB (also termed FtsH; Wu *et al.*, 1999). Among these, Lon and ClpP were found to be not responsible for the degradation of TraJ in *cpxA101** (Gubbins *et al.*, 2002). HflB, the only essential protease in *E. coli*, was found also to not be involved in TraJ degradation (Figure 3.2).

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 (Gottesman, 2003; Rohrwild *et al.*, 1996). 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 co-regulated by the Lon protease. HslVU, along with other ATP-dependent proteases, is additionally responsible for the degradation of σ^H, the heat shock sigma factor (Kanemori *et al.*, 1999b), as part of a mechanism for maintaining σ^H homeostasis. 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 *hslVU* promoter, which contains the consensus sequence for promoters recognized by σ^H (RpoH), is regulated by this sigma factor (Chuang *et al.*, 1993). σ^H is also proposed to be regulated by the Cpx system at one ($rpoHp_1$) of its four promoters (Pogliano *et al.*, 1997; De Wulf *et al.*, 2002; Zahrl *et al.*, 2006), suggesting that CpxR-P

could potentially activate *hslVU* via σ^H . Our microarray results indicated that *rpoH* was up-regulated approximately 4-fold in a *cpxA101** mutant (Table 8.1). Immunoblot analysis also revealed that σ^H protein levels increased slightly in TR20 (*cpxA101**) and an *rpoH::lacZ* promoter fusion showed a 2-fold increase in activity in *cpxA101** strains (data not shown). In contrast, a deletion mutation in *rpoH* revealed that σ^H is not essential for TraJ degradation in cells overproducing NlpE (Figure 3.1). Therefore *hslVU* expression can be dependent on other factors in addition to σ^H, such as σ^{70}. We interpret these contradictory results as suggesting that *hslVU* transcriptional control is not the major factor causing the degradation of TraJ. Instead, TraJ is a substrate for HslVU under all conditions and that its susceptibility to degradation is affected by another factor to be discussed in the next chapter.

We noticed that HslVU-mediated TraJ degradation appears to be strain-specific, since a *hslV* mutation in C600 but not MC4100, resulted in complete restoration of TraJ and F conjugation in the presence of the *cpxA101** allele. Moreover, a more dramatic decrease in mating efficiency was noticed in MC4100 compared to C600 when stress was induced by either CpxA101* or overexpression of NlpE (Tables 3.3 and 3.4). This can be explained by two possibilities.

First, another protease, along with HslVU, could be involved in TraJ degradation in the MC4100 strain. This would not be extraordinary since other substrates of HslVU have been found to be targets for multiple proteases (Wu *et al.*, 1999; Kuo *et al.*, 2004). HslVU appears to have an overlapping role with Lon, a single component energy-dependent protease (Gottesman) that mediates the degradation of SulA and RcsA (Wu *et al.*, 1999; Kuo *et al.*, 2004). In Lon^+ *hlsVU* hosts, no SulA was detected and the half life

of overexpressed SulA was 2 minutes. In *lon* HslVU$^+$ hosts, SulA is stable (half-life 30 min), whereas in *lon hslVU* hosts, its stability increases (half-life 120 min; Wu *et al.*, 1999). The turnover rate of RcsA has the same features, in which the degradation of RcsA in a Lon$^+$ *hslVU* host did not result in maximal levels of RcsA. Thus, HslVU was only noticeably active in a *lon* background. Similarly, deleting *hslVU* in MC4100 may not result in the complete restoration of TraJ, if other proteases are involved.

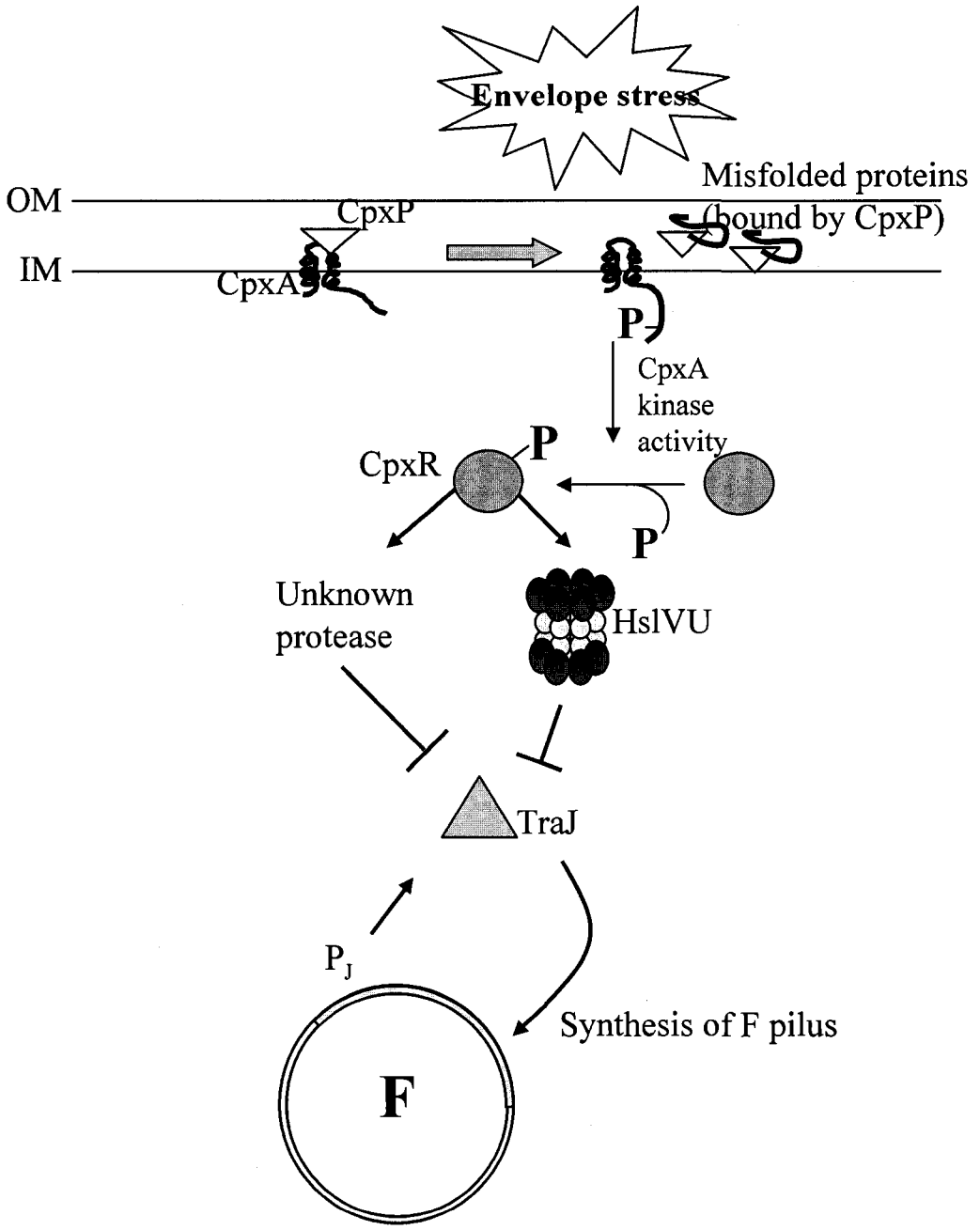
Secondly, it has been shown that the level of Lon increases appreciably in Δ*hslVU* mutants (Kanemori *et al.*, 1999a). Since σ^H is a substrate of HslVU, stabilization of σ^H in a Δ*hslVU* strain may result in increased transcription of other heat shock proteins (HSPs), which are mainly proteases and chaperones (Arsene *et al.*, 2000). Taken together, increased levels of other cellular proteases in a Δ*hslVU* host might contribute to the degradation of TraJ. In studying the degradation of RcsA by HslVU, Kuo *et al.* (2004) also encountered difference between strains, in which the levels of RcsA were rescued to a greater extent in the double *lon hslV* mutant (RecD$^+$ strain) than in the triple *lon hslV hslU* mutant (*recD* strain). Accordingly different levels of proteases expressed in different backgrounds can contribute to, and affect the degradation of the same substrate, as in the case of F TraJ.

Aberrant cell division and randomized FtsZ ring assembly has been observed in *cpxA** cells by Pogliano *et al.* (1998). It is speculated that rapid degradation of SulA, a substrate of HslVU, may have caused this phenomenon. Interestingly, the filamentous phenotype of *cpxA101** cells is suppressed by an *hslV* mutation in IL1 (*cpxA101* hslV*) as revealed by electron microscopy (Figure 3.9). Therefore, it is highly possible that

increased level of HslVU protease causes reduction of the cell division inhibitor, SulA, and leads to aberrant cell division and random placement of FtsZ in *cpxA** cells.

A model incorporating observations in this chapter is summarized in Figure 3.10. An inducing cue is sensed in the envelope to activate CpxA and -R yielding CpxR-P that activates the Cpx regulon, including *hslVU.* Unknown proteases may contribute to the degradation of TraJ in some *E. coli* strains, such as MC4100, but remain to be determined. In spite of this, one of the regulatory circuits in controlling F *tra* expression in the *cpx* mutants originally noted by Sambucetti *et al.* (1982) is uncovered in this chapter.

Figure 3.10 Schematic diagram summarizing findings in this chapter. Upon envelope stress signal detection, CpxA is freed from its inhibitor CpxP and phosphorylates CpxR. Increased amounts of CpxR-P leads to the accumulation of cellular HslVU, which degrades TraJ and thus impairs F pilus synthesis. Envelope stress can be induced by high pH, misfolding or aggregation of envelope proteins, overexpression of NlpE, adhesion to hydrophobic surfaces, and altered membrane composition etc. Unknown protease(s) are responsible for residual degradation of TraJ in certain *E. coli* backgrounds. OM, outer membrane; IM, inner membrane; P, phosphate group.


Envelope stress
Misfolded proteins
(bound by CpxP)
OM
IM
CpxP
CpxA
P
CpxA
kinase
activity
CpxR
P
P
Unknown
protease
HslVU
TraJ
P_J
Synthesis of F pilus
F

Chapter 4: Degradation of TraJ and accumulation of TraJ*†

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4.1 Introduction

From the previous chapter, we have shown that activation of the Cpx envelope stress response system leads to a reduction in F conjugation and reduced levels of the activator, TraJ, through activation of the host HslVU protease/chaperone pair. Deletion mutation of *hslV* in *cpxA101** harbouring pOX38-Km results in restoration of TraJ and the double mutant becomes transfer-proficient. While this circuit may appear straightforward (as illustrated in Figure 3.10), intracellular TraJ is intransigent to degradation in stationary phase (Frost and Manchak, 1998). This chapter explores the fate of TraJ once it is synthesized in the cytoplasm, when it encounters extracytoplasmic stress, or when cells enter stationary phase.

When F^+ cells enter stationary phase, conjugation ceases. This phenomenon, termed "F^- phenocopies" (Jacob and Wollman, 1961), is characterized by a decrease in the nicking at *oriT* and in transfer gene transcription. Whereas *tra* gene transcription decreases, the TraJ protein - the activator of P_Y, persists. These contradictory observations were elucidated when key promoters, P_M, P_J, P_Y, were found to be silenced by host H-NS in a growth phase-dependent manner (Will *et al.*, 2004; Will and Frost, 2006a). H-NS is a host nucleoid-associated protein that binds preferentially to AT-rich promoters and inhibits transcriptional initiation from these promoters upon environmental and nutritional cues (Williams and Rimsky, 1997). Whereas TraJ is an essential activator for transcriptional initiation at P_Y, TraJ is not necessary for plasmid transfer or P_Y transcription in an *hns* mutant host (Will and Frost, 2006a). Accordingly, the newly assigned role of TraJ is to counteract H-NS repression when growth resumes as cells are diluted into fresh medium or as glucose is added (Will and Frost, 2006a). As growth continues, however, TraJ is hypothesized to be post-translationally modified (Will, Ph.D.

Thesis, 2006). As such, it loses its ability to bind DNA or other regulators, and the promoters become accessible to H-NS.

In this chapter, we attempt to investigate the degradation of TraJ from various perspectives. Firstly, we asked whether inducing extracytoplasmic stress in F^+ exponential phase cells, where pili are fully synthesized and conjugation is proficient, would result in a decrease in the levels of TraJ or/and F conjugation. Our observations reveal that once pili are fully established and conjugation is possible, TraJ becomes inactive and resistant to degradation. Secondly, we observed whether fresh TraJ, which was induced at mid-exponential phase in cells experiencing extracytoplasmic stress or HslVU overexpression, was subject to degradation. To that end, we present evidence that only a portion of TraJ molecules, perhaps with modifications, are resistant to degradation. Possible modifiers of TraJ are suggested. Lastly, we present data on the *in vitro* degradation of TraJ to support the above hypothesis.

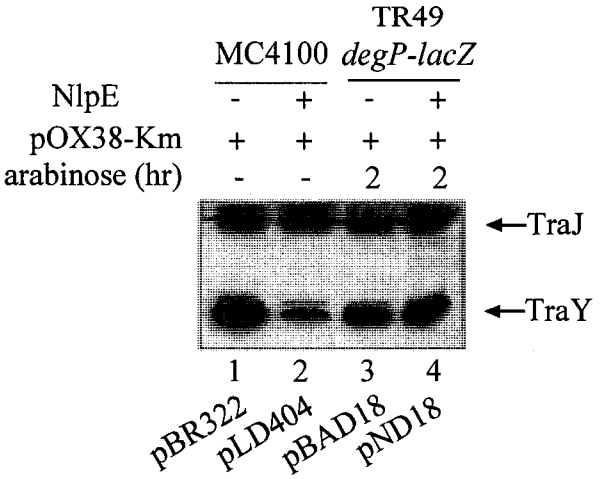
4.2 Results

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

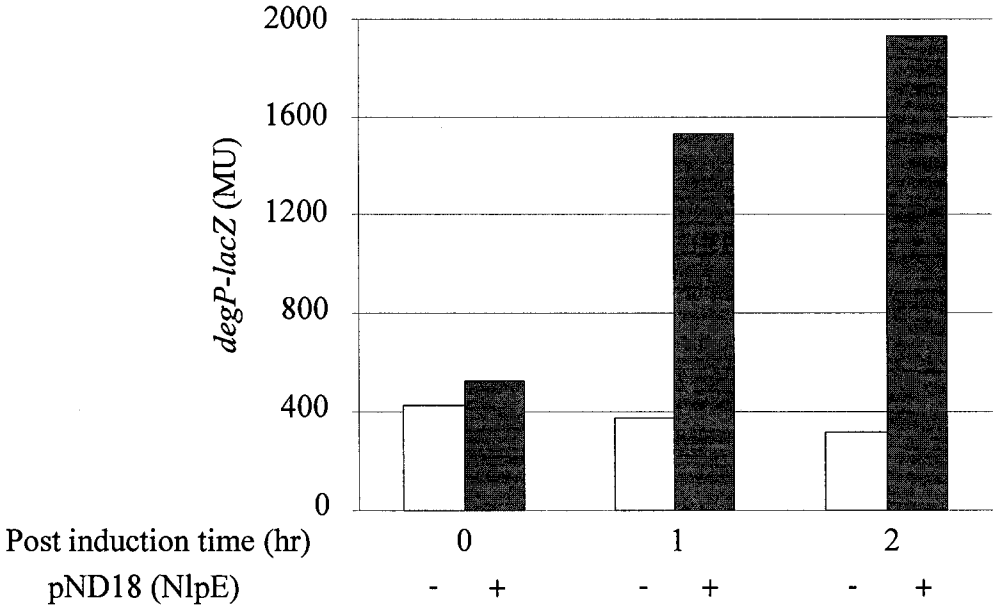
We asked whether TraJ levels were decreased when stress was induced during exponential growth by expressing *nlpE* from the arabinose-inducible promoter in pBAD18 (pND18; Table 2.1). TR49/pOX38-Km/pND18 was assayed for TraJ and TraY levels after 2 hours of induction with 0.05% arabinose. Cells were monitored for activation of the Cpx regulon by following the induction of a *degP::lacZ* transcriptional fusion (Raivio and Silhavy, 1997). Figure 4.1 shows that TraJ and TraY levels remained constant (compare between lanes 3 and 4). Similarly, the half-life of TraJ, as measured using the protocol in Gubbins *et al.* (2002), was greater than 8 hours (data not shown) indicating that previously synthesized TraJ was very stable. This is in agreement with the

Figure 4.1 TraJ is protected from proteolysis when extracytoplasmic stress is induced in mid-log phase. Immunoblot analyses were performed with polyclonal antisera directed against TraJ and TraY. **A.** *E. coli* MC4100 (lanes 1 and 2) and TR49 *degP*::*lacZ* strains (lanes 3 and 4) carrying pOX38-Km in the absence (-; lanes 1 and 3) or presence (+; lanes 2 and 4) of stress were subjected to immunoblot analyses. 0.05% arabinose was added (lanes 3 and 4) for 2 hours at mid-log phase to induce extracytoplasmic stress in TR49[*degP-lacZ*]/pND18/pOX38Km. Whereas constitutive Cpx-activation (lane 2) led to a reduction in the levels of TraJ and TraY, the transfer regulators were stable when envelope stress was induced in mid-log phase. **B.** The induction of NlpE was confirmed by activation of the *degP* promoter in TR49 carrying pND18 but not pBAD18. White bars, pBAD18, vector control; grey bars, pND18, NlpE-containing plasmid.

A.


TR49
MC4100 degP-lacZ
NlpE - + - +
pOX38-Km + + + +
arabinose (hr) - - 2 2
TraJ
TraY
1 2 3 4
pBR322
pLD404
pBAD18
pND18

B.


2000
1600
1200
800
400
0
degP-lacZ (MU)
Post induction time (hr) 0 1 2
pND18 (NlpE) - + - + - +

results of Frost and Manchak (1998), who showed that TraJ is stable in stationary phase. Mating efficiencies were also stable in these cells where the Cpx system was activated by induction of NlpE during exponential phase (Table 4.1). Whereas constitutive Cpx activation led to a reduction in the levels of TraJ (Chapter 3), TraJ was resistant to degradation when the Cpx system was activated by overexpression of NlpE in mid-log phase when pili and the transfer apparatus (TraD, -I, etc.) are already established.

We then asked whether TraJ, when synthesized by induction of a transcriptional fusion of *traJ* to the *araBAD* promoter in pBAD33 (pILJ14), was susceptible to degradation by HslVU. pILJ14 was introduced into MC4100/F*lac traJ90* cells containing pBR322, pLD404 or pIL13 (expressing *hslVU*). TraJ production was induced by the addition of 0.05% arabinose for 50 minutes followed by removal of the arabinose and addition of glucose and 0.2 mg/ml rifampin to halt further transcription. The levels of TraJ were monitored over four hours by immunoblot and were estimated using densitometry of the bands (Figure 4.2). TraJ was found to be stable in the presence of pBR322 (97%) and degraded partially in the presence of pIL13 (HslVU, 81%) and pLD404 (NlpE, 41%). Thus, TraJ appears to be stable in wild-type cells and is degraded in the presence of excess HslVU or stress.

4.2.2 Presence of the F plasmid stabilizes TraJ protein

The above experiment was performed in the presence of the F*lac traJ90* plasmid that carries an amber mutation in *traJ* (Achtman *et al.*, 1971). We suspected that the presence of F gene products (presumably Tra proteins) might stabilize TraJ. To examine the effect of the F plasmid on TraJ stability, the experiment was repeated in the absence of F*lac traJ90* (Figure 4.3A). Interestingly, over a period of 6 hours after the halt of *traJ* transcription, the stability of TraJ protein was maintained in the presence of F*lac traJ90.*

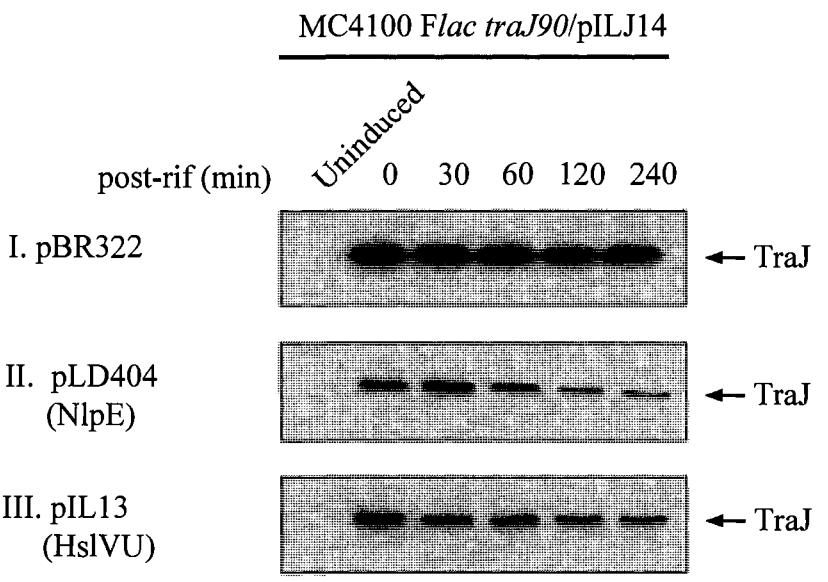
Table 4.1 Mating efficiency is not affected when NlpE is overproduced in mid-exponential phase.

Time (hours)	Mating efficiency with	
	pBAD18 (vector)	pND18 (NlpE)[a]
0	29	17
1	16	13
2	22	70
4	23	46
6	18	27
8	27	25

[a]NlpE was induced by the addition of 0.05% arabinose in mid-log phase ($0.4OD_{600}$) *E. coli* MC4100 harbouring pOX38-Km and either pBAD18 (vector) or pND18 (NlpE). Mating assays were performed in duplicate at each time point and mating efficiencies are reported as the average of the number of transconjugants per 100 donors.

Figure 4.2 Rifampicin-chase experiments reveal *in vivo* degradation of TraJ in cells experiencing envelope stress or expressing HslVU protease. A. TraJ was induced by the addition of 0.05% arabinose to MC4100/F*lac traJ90/* pILJ14 for 50 minutes. After washing, 0.4% glucose and 3μM rifampicin in fresh LB were added to prevent further rounds of *traJ* transcription. The amounts of remaining TraJ were detected by immunoblot analyses 0, 30, 60, 120, and 240 minutes after the addition of glucose and rifampicin. **A**. MC4100/F*lac traJ90*/pIL14 with pBR322 (vector control, panel I); pLD404 (expressing NlpE, panel II); or pIL13 (expressing HslVU, panel III). **B**. TraJ levels detected in A were quantified with AlphaEase software and a FluorChem IS-5500 imaging system as described in Material and Methods. The intensity of each band in A was normalized to the band corresponding to 0 minute in each strain and plotted versus post-induction time. The percentages of TraJ remaining in MC4100/F*lac traJ90*/pIL14 with pBR322 (vector, diamond); pLD404 (expressing NlpE, square); or pIL13 (expressing HslVU, triangle) are shown.

A


MC4100 F*lac traJ90*/pILJ14
Uninduced
post-rif (min) 0 30 60 120 240
I. pBR322
TraJ
II. pLD404
(NlpE)
TraJ
III. pIL13
(HslVU)
TraJ

B

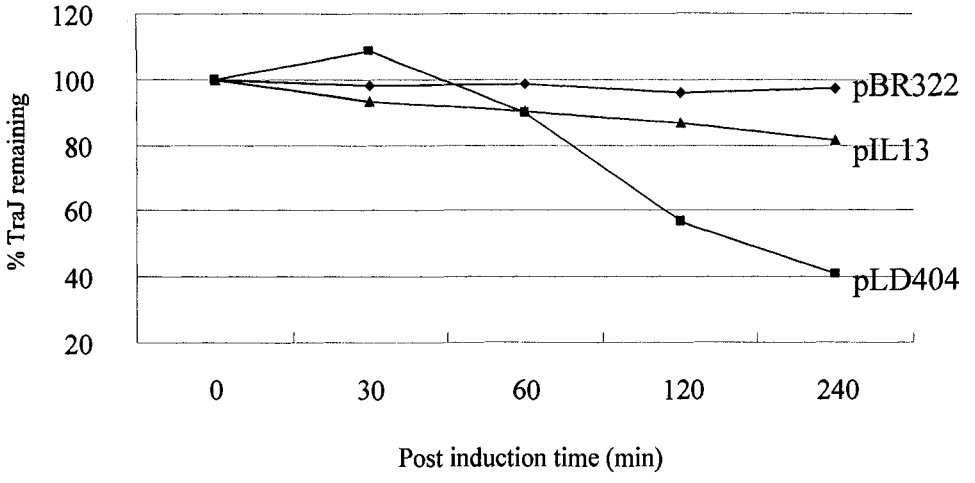
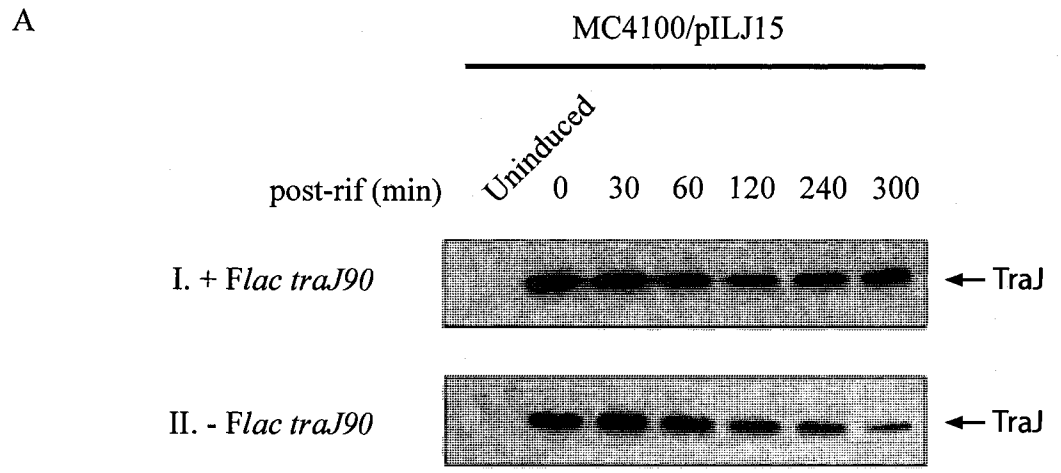
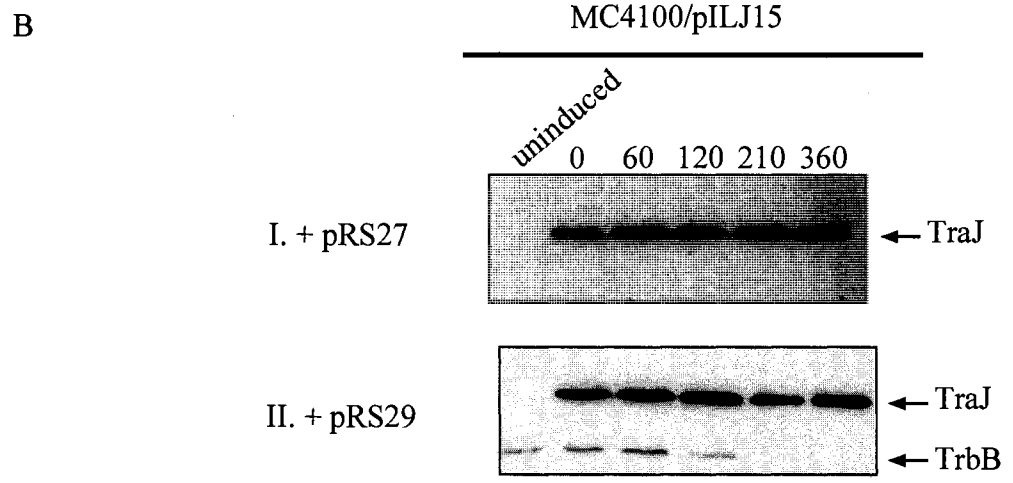

120
100
80
60
40
20
% TraJ remaining
0
30
60
120
240
Post induction time (min)
pBR322
pIL13
pLD404

Figure 4.3 The presence of F*lac* protects TraJ from degradation. A. The abundance of TraJ in MC4100/pIL15 in the presence (upper panel) or absence (lower panel) of F*lac traJ90* is shown. TraJ was induced with 0.05% arabinose for 50 minutes and its transcription was then halted at time 0 by the addition of rifampicin and glucose. The levels of TraJ were detected over 5 hours by immunoblot analysis. **B.** TraJ is stable in the presence of pRS27 or pRS29 (Skurray *et al.*, 1978). The abundance of TraJ in MC4100/pIL15 (Table 2.1) harbouring pRS27 or pRS29 over 6 hours after the addition of rifampicin is shown. **C.** pRS27 and pRS29 containing EcoRI fragments of the transfer region were cloned into pSC101 (Manning *et al.*, 1984). The overlapping region consists of *trbG*, *traR*, and *traV*, which are candidates for TraJ stabilization. Adapted from Frost *et al.* (1994).

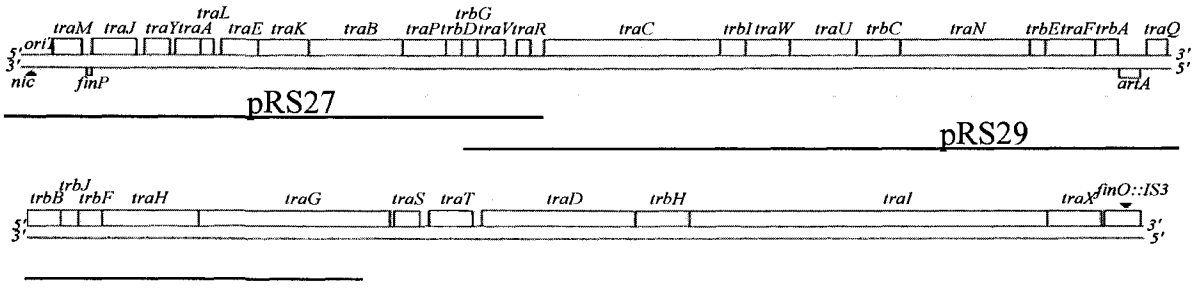
A


MC4100/pILJ15
Uninduced
post-rif (min)
0
30
60
120
240
300
I. + F*lac traJ90*
II. - F*lac traJ90*
TraJ
TraJ

B


MC4100/pILJ15
uninduced
0
60
120
210
360
I. + pRS27
II. + pRS29
TraJ
TraJ
TrbB

C


traL
trbG
traM traJ traY traA traE traK traB traP trbD traV traR traC trbI traW traU trbC traN trbE traF trbA traQ
oriT
nic
finP
artA
pRS27
pRS29
trbB trbJ trbF traH traG traS traT traD trbH traI traX finO::IS3

Next we investigated the element(s) on the F plasmid that cause this difference. The presence of pRS27 or pRS29 was found to stabilize TraJ over 6 hours after halt of transcription (Figure 4.3B). As a negative control, the TrbB protein was found to be unstable 2 hours after the halt of transcription. Taken together, the element that stabilizes TraJ protein was mapped to an EcoRI fragment containing *trbG, traR and traV* (Frost *et al.*, 1994; Figure 4.3C). These results agree with the observation that TraJ is stable over the growth cycle (Frost and Manchak, 1998) and suggest that TraJ exists in two forms, which we denote as TraJ and TraJ* (see Discussion), with TraJ* being resistant to HslVU degradation.

4.2.3. TraR, the candidate protein that modifies TraJ

We investigated possible TraJ modifier(s) in the overlap region of pRS27 and pRS29 (Achtman *et al.*, 1971). TraR appeared to be an excellent candidate since it is a homolog of DksA, a suppressor of defects in DnaK, an Hsp70 family member (Doran *et al.*, 1994). The stability of TraJ was observed in an *E. coli* VL584 strain containing pOX-Km::*traR354* (Maneewannakul and Ippen-Ihler, 1993). Over a 5-hour period, the levels of TraJ decreased dramatically, with a pattern resembling that of MC4100/pILJ15 in the absence of F*lac*. The majority of degradation occurred in the first 30-minutes after the halt of transcription initiation (Figure 4.4A). In a separate experiment, pOX38-Km::*traX* and pOX38-Km were used as controls. The levels of TraJ did not decrease as dramatically (Figure 4.4 B and C), suggesting that TraR appears to be protecting TraJ immediately following its production.

4.2.4 *In vitro* degradation of His_6-TraJ by purified HslV and HslU

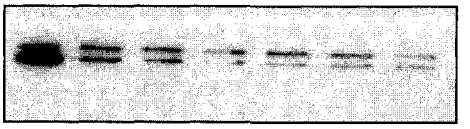
The previous results suggest that TraJ could be either a direct or indirect substrate for HslVU. To demonstrate direct proteolysis, TraJ was incubated with purified HslV and

Figure 4.4 TraJ stability decreases in *traR* mutants. Immunoblot analyses of *E. coli* VL584/pOX38::*traR354* (A) or pOX38::*traX482* (B) (Table 2.1; Maneewannakul and Ippen-Ihler, 1993) to detect the levels of TraJ after 0 to 5 hours after halting transcription by the addition of rifampicin and glucose. At each time point, an aliquot equivalent to 0.1 OD_{600} of cells was pelleted and frozen until ready to be run on SDS-PAGE. C. The stability of TraJ in MC4100/pOX38-Km was determined in the same manner with omission of the time point collected at 3 hours.

A

VL584 / pOX38-Km *traR*354

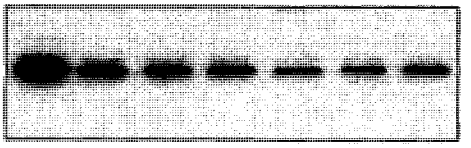
Time (hr) 0 0.5 1 2 3 4 5



B

VL584 / pOX38-Km *traX*482

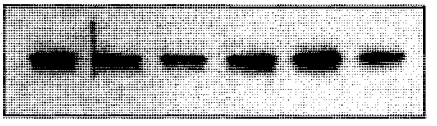
Time (hr) 0 0.5 1 2 3 4 5



C

MC4100 / pOX38-Km

Time (hr) 0 0.5 1 2 4 5

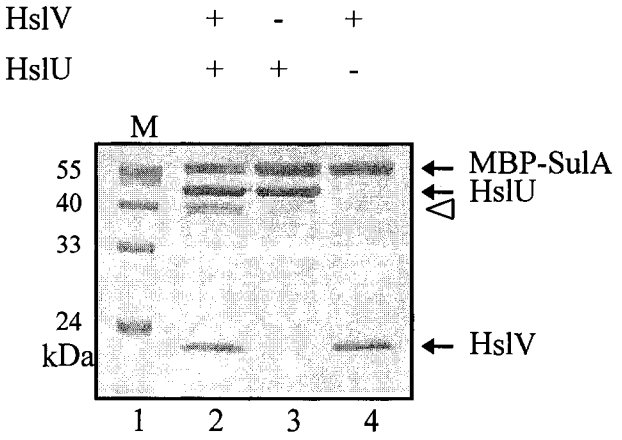


HslU (kindly provided by Dr. Eyoung Park, Seoul National University) in the presence of ATP and detected by immunoblot analysis. MBP-SulA, a known substrate of HslVU (also provided by Dr. Park), was used as a positive control. MBP-SulA was partially degraded when incubated with HslV and HslU for 2 hours (Figure 4.5A), which is in agreement with previous results (Seong *et al.*, 1999).

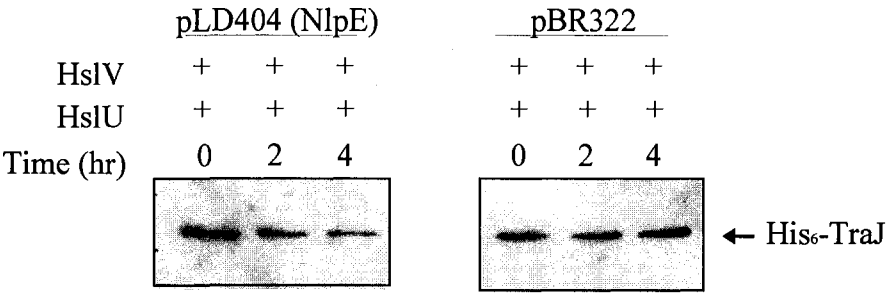
An arabinose-inducible plasmid encoding His_6-TraJ (pILJ16) was constructed and shown to be able to complement the *traJ* amber mutation in F*lac traJ90* (data not shown). pILJ16 was induced with 0.05% arabinose and purified by Ni-NTA agarose (Qiagen) chromatography. 1μg of His_6-TraJ was incubated with HslV and HslU at 37°C over four hours (Figure 4.5B). The amount of His_6-TraJ at time 0 was set as 100% and was stable over 4 hours when purified from wild-type cells. A control digestion was performed in the absence of HslV and HslU for 4 hours to ensure that His_6-TraJ was not degraded by contaminating proteases (Figure 4.5C). In contrast, His_6-TraJ was degraded to 62% of the original amount when it was purified from MC4100/pLD404/pILJ16 (Figure 4.5B, Stress). Thus the presence of stress induced by pLD404 (NlpE) appears to alter the susceptibility of TraJ to HslVU. Small proteins such as TraR (8.3 kDa) were not visible on stained SDS-gels of pure His_6-TraJ. However, a band near 60 kDa was consistently present in His_6-TraJ preparations. Recent studies hypothesized that TraJ complexes with GroEL (58 kDa) during heat shock (Zahrl *et al.*, 2007). Using GroEL antisera kindly provided by Dr. Gunther Koraimann (Karl-Franzens-Universität Graz, Austria), GroEL was detected in approximately equivalent amounts in His_6-TraJ purified from cells with or without stress (data not shown). We suspect that the presence of GroEL is a result of the overexpression of His_6-TraJ and may not be physiologically relevant.

Figure 4.4 TraJ stability decreases in *traR* mutants. Immunoblot analyses of *E. coli* VL584/pOX38::*traR354* (A) or pOX38::*traX482* (B) (Table 2.1; Maneewannakul and Ippen-Ihler, 1993) to detect the levels of TraJ after 0 to 5 hours after halting transcription by the addition of rifampicin and glucose. At each time point, an aliquot equivalent to 0.1 OD_{600} of cells was pelleted and frozen until ready to be run on SDS-PAGE. C. The stability of TraJ in MC4100/pOX38-Km was determined in the same manner with omission of the time point collected at 3 hours.

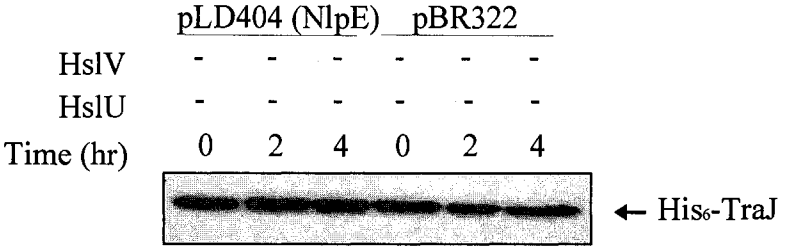
A


HslV + - +
HslU + + -
M
55
40
33
24
kDa
MBP-SulA
HslU
HslV
1 2 3 4

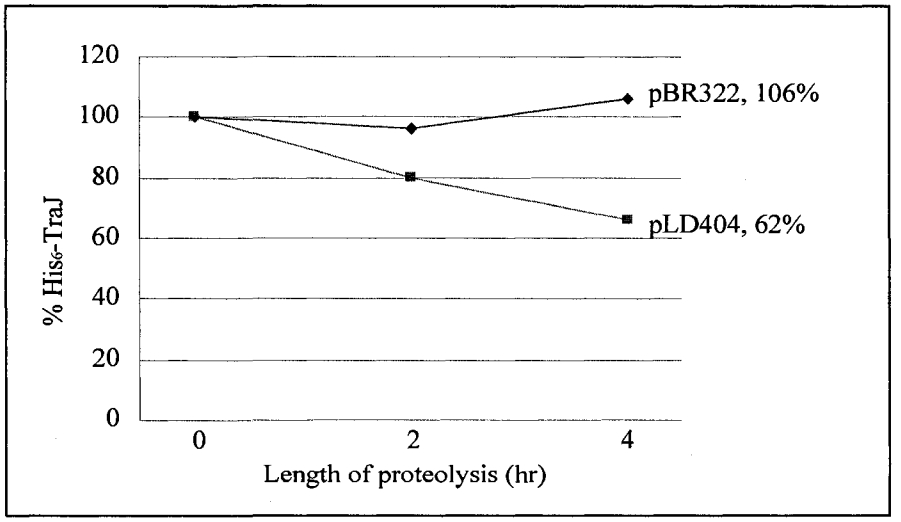
B


pLD404 (NlpE)
pBR322
HslV + + + + + +
HslU + + + + + +
Time (hr) 0 2 4 0 2 4
His6-TraJ

C


pLD404 (NlpE) pBR322
HslV - - - - - -
HslU - - - - - -
Time (hr) 0 2 4 0 2 4
His6-TraJ

D


120
100
80
60
40
20
0
% His6-TraJ
0 2 4
Length of proteolysis (hr)
pBR322, 106%
pLD404, 62%

4.3 Discussion

In this chapter, we investigated TraJ degradation under various conditions in *E. coli* cells, including the presence and absence of F, and under *in vitro* conditions (Figure 4.5). Our results suggest that: 1) TraJ appears to be present in two forms, only one of which is active (TraJ) and is susceptible to degradation by HslVU. From evidence presented in this chapter, which shows that TraJ is resistant to degradation in stationary phase, the active form of TraJ is present predominantly in early exponential phase; 2) A factor encoded on F modifies TraJ and makes it more stable and 3) TraJ is more susceptible to degradation when stress is induced, for instance by the overproduction of NlpE, in a CpxR-dependent manner. 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 resumption of growth, for instance by dilution of a stationary culture into fresh medium, existing TraJ* (inactive) is inferred to be activated by the reversal of modification. Simultaneously, fresh TraJ (active) can be produced by stimulation of the *traJ* promoter. If stress (NlpE) or perceived stress (*cpxA101**) is present during resumption of growth, the newly synthesized TraJ would be active but would be immediately degraded, leading to continued silencing by H-NS. This would be an efficient mechanism for controlling F transfer region gene expression at the first instance of renewed growth. The induction of stress in mid-exponential phase cells did not cause an appreciable change in the level of TraJ (Figure 4.1), suggesting that once transfer gene expression is activated and the transfer apparatus is synthesized, TraJ becomes

modified/stabilized by the F-encoded factor (and converted to TraJ*) and is no longer subject to Cpx-induced degradation.

Our data may explain why TraJ binding to DNA has been difficult to detect previously. Purified His_6-TraJ did not bind DNA (Will and Frost, unpublished observations) although Ohtsubo reported that "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 that are visible in the immunoblots or in pure TraJ preparations may be inactive (TraJ*). The small portion of TraJ that is synthesized to activate the transfer region at each cell division can be masked by this pool of inactive TraJ*. Why TraJ* accumulates in wild-type cells is unclear at this time.

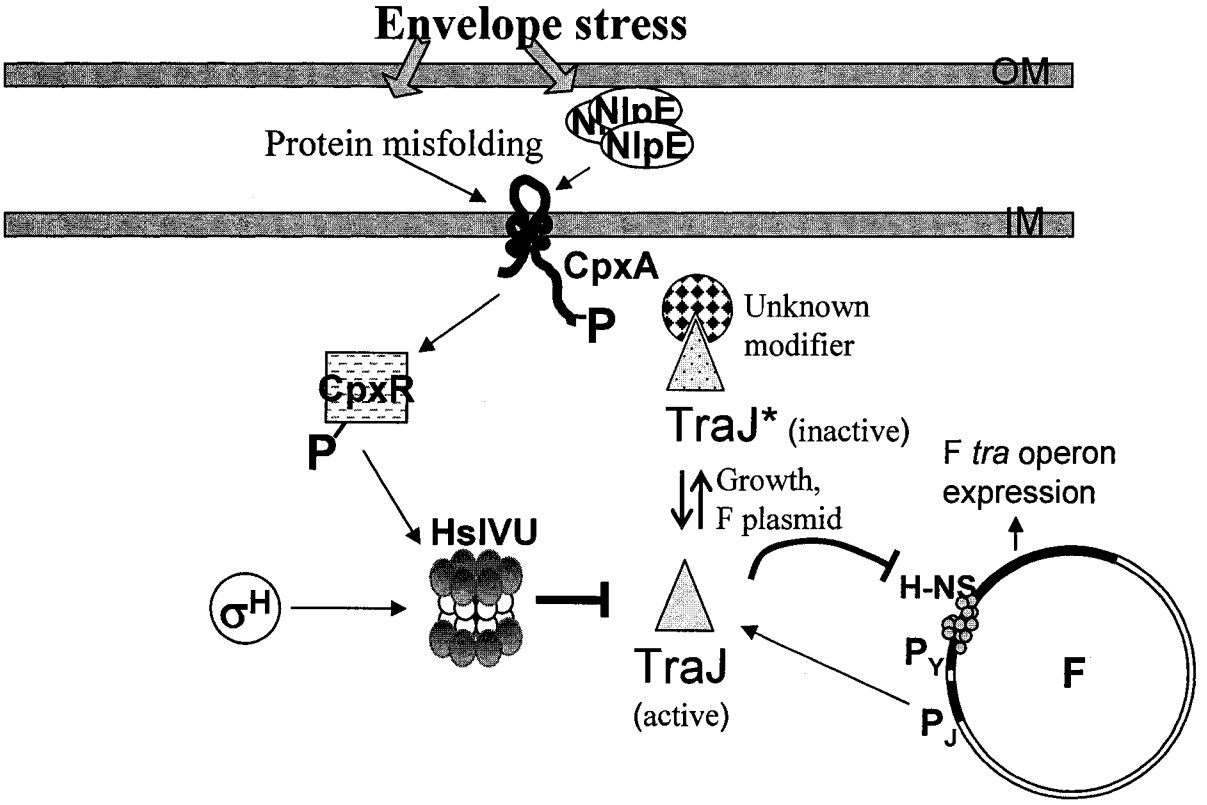
The presence of the F plasmid appeared to protect TraJ from degradation by HslVU *in vivo* in the absence of stress. Our results suggested that TraR might play such a role (Figure 4.4). Sequence homology search has revealed that TraR contains a thioredoxin (CXXC) motif and a zinc-finger motif that can potentially mediate protein binding (Villenueve and Frost, personal communication). The notion that TraR can bind and reduce TraJ that has been aged and oxidized is currently being examined (Beadle and Frost, unpublished results).

Recently GroEL, the chaperone protein in *E. coli*, has been proposed to interact with F-like TraJ and target it for degradation during the heat shock response (Zahrl *et al.*, 2007). Using anti-GroEL antibodies provided by Dr. Koraimann, we determined that GroEL was present in equivalent amounts in His_6-TraJ preparations purified from cells in the presence or absence of pLD404 (NlpE; data not shown) suggesting it did not directly affect TraJ susceptibility to HslVU *in vitro*. 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 conjugation.

A model incorporating our observations is summarized in Figure 4.6. An inducing cue is sensed in the envelope to activate CpxAR yielding CpxR-P that activates the Cpx regulon, including *hslVU.* We suspect that certain conditions must exist for TraJ to be degraded by HslVU and that only active TraJ is targeted. TraJ could be modified in some way, such as by TraR or by being bound to DNA, and is converted to TraJ* that is protected from degradation. These two possibilities are not mutually exclusive and could indicate that TraJ is degraded only when it is actively opposing H-NS silencing in an as yet unknown way. 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.

Figure 4.6 A model for F repression during the extracytoplasmic stress response. CpxA undergoes autophosphorylation and transfers the phosphate group (P) to the cytoplasmic response regulator CpxR in response to inducing cues (extracytoplasmic stress) such as protein misfolding or NlpE overproduction. CpxR-P directly or indirectly increases transcription of several protease and chaperone genes, one of them being *hslVU*. The newly synthesized F positive regulator, TraJ, is active in reversing the silencing of the F *tra* operon by H-NS and is a target for HslVU. As growth progresses, TraJ accumulates in a modified, apparently inactive form (TraJ*), that is resistant to degradation, a process which is dependent on the presence of the F plasmid. OM, outer membrane; IM, inner membrane.


Envelope stress
OM
NlpE
NlpE
Protein misfolding
IM
CpxA
P
Unknown
modifier
CpxR
P
TraJ* (inactive)
F tra operon
expression
Growth,
F plasmid
HslVU
σH
H-NS
TraJ
(active)
PY
F
PJ

Chapter 5: Regulation of TraJ by the heat shock sigma factor, σ^H

5.1 Introduction

In contrast to laboratory strains that are grown in rich media at 37°C with aeration, natural bacteria reside in diverse environments such as soil, plants, or animal digestive tracts. There are infinite challenges faced by these bacteria, including nutrient shortage and changes in pH, temperature, moisture, or oxygen availability. Therefore, bacteria have evolved ways to overcome these challenges. This chapter will review some of the consequences when bacteria are compelled to grow under heat stress. It will also present several findings that the same regulator for heat shock genes is indeed needed for activation of the F *tra* operon.

When bacteria are exposed to elevated temperatures, a group of proteins, called the heat shock proteins (HSPs), are rapidly induced. In *E. coli*, HSPs are synthesized by the alternative sigma factor, σ^H, the gene product of *rpoH*. Most of the HSPs are chaperones (DnaK, DnaJ, GroEL) or proteases (FtsH, Lon, HslVU) which serve to ensure correct folding of proteins, prevent aggregation, and degrade proteins that are recalcitrant to proper folding when cells are under heat stress (Arsene *et al.*, 2000; Hengge and Bukau, 2003). σ^H in turn, is regulated at the translational and post-translational levels. The *rpoH* mRNA itself is a built-in thermosensor in which the ribosome binding site (RBS) is obscured by a secondary structure at low temperature, limiting its translation. At high temperature, the mRNA is melted, exposing the Shine-Dalgarno sequence within the RBS for ribosomal access (Morita *et al.*, 1999). In addition, stability of σ^H is increased from 50 molecules per cell at 30°C to approximately 1000 molecules per cell immediately after they are placed under 42°C (Straus *et al.*, 1987). This transient increase of σ^H molecules is due to titration of the DnaK/DnaJ chaperones, along with other HS proteases, which are usually bound to σ^H and responsible for its degradation. These HSPs

are directed towards misfolded or aggregated proteins during the transient change of temperature from 30°C to 42°C (Herman *et al.*, 1995; Kanemori *et al.*, 1999b). Free σ^H combines with core RNA polymerase and directs transcription initiation of the heat shock regulon. When the amount of HSPs is sufficient to relieve cellular protein aggregation, during the adaptation period, excess HSPs bind to σ^H and reduce its intracellular level (Arsene *et al.*, 2000). Thus the heat shock response is rapid and only transiently induced in *E. coli.*

In addition to transcription of HS genes, σ^H was also found to be essential for F replication (Wada *et al.*, 1986). F cannot be stably maintained in *rpoH* mutants since transcription of F *repE*, encoding a replication initiator protein, is σ^H-dependent (Wada *et al.*, 1987). Interestingly, a subset of HSPs, DnaK, J, and GrpE, has been shown to activate the RepE protein (Ishiai *et al.*, 1992). Penfold noted some similarities between the F *traM* promoter (P_{M2}) and the *repE* promoter (Penfold, Ph.D. Thesis, 1995). Both promoters possess a σ^H recognition -35 box, and are regulated by autorepression. While RepE is involved in binding to *incC* for replication initiation, TraM is involved in binding to *oriT* for F DNA transfer initiation. However, a requirement of σ^H for *traM* transcription remains undetermined.

In the course of this study, another requirement for σ^H in conjugation was further revealed. We originally wanted to test whether an *rpoH* mutation could restore TraJ in a Cpx-activated *E. coli* strain background harboring pOX38-Km (Chapter 3). Recurrent failure to conjugate pOX38-Km into an *rpoH* strain prompted us to examine the requirement for σ^H by F. Using pED851, a pBR322-replicon-based plasmid containing the F *tra* region (Table 2.1; Johnson and Willetts, 1980), however, led us to believe that

σ^H is required by F for more than replication. This chapter presents interesting findings that an *rpoH* mutation results in decreased levels of F conjugation by reducing TraJ. When cells are entering stationary phase, H-NS down-regulates P_M, P_J, and P_Y (Will and Frost, 2006a). H-NS is a 15.4 kDa host nucleoid-associated protein that binds preferentially to a region of curved DNA and acts as a transcriptional silencer. EMSA and DNase I footprinting analysis indicated that H-NS binds extensively at the *traJ* promoter (Will *et al.*, 2004). As cells progress through the growth cycle, the affinity of H-NS for P_M, P_J and P_Y increases, either in response to altered curvature resulting from decreased supercoiling or in response to a decrease in bound competitor proteins. This binding of H-NS is believed to form an extensive nucleosomal complex, thus repressing the major transfer gene promoters P_M, P_J, and P_Y. TraJ was further found to have a specific role in opposing H-NS-mediated repression of P_Y (Will and Frost, 2006a). Detailed experiments in this chapter show that σ^H is not required for *traJ* transcription. σ^H is proposed to be involved in initiation of transcription of a cellular factor that antagonizes P_{traJ} repression by H-NS or directly involved in the transcription of a readthrough *traM* transcript initiated at P_{M2}.

5.2 Results

5.2.1 The levels of TraJ and mating ability are decreased in KY1621/pED851

KY1621, an *E. coli* MC4100 strain carrying a deletion mutation at the *rpoH* locus, was kindly provided by Dr. Raina (Centre Médical Universitair, Switzerland) in order to study the effect of *rpoH* on F (Missiakas *et al.*, 1993). Transfer of F*lac* from *E. coli* XK1200 into KY1621 was not possible, since there is a requirement for σ^H for F replication (data not shown). As a result, a chimera consisting of the large BamH1

fragment of F (containing the entire F *tra* operon) cloned into pBR322 was used (Johnson and Willetts, 1980). pED851 has a higher (20 per cell) copy number than F (1-2 per cell), contains the Amp^r gene and is stably maintained in *rpoH* mutants. Surprisingly, the transfer ability of KY1621 (*rpoH*)/pED851 was reduced (ME = 0.25% compared to wild-type). In addition, the levels of TraJ and TraM were decreased in the *rpoH* mutant (Figure 5.1). This is the first study showing that σ^H is involved in F conjugation and the synthesis of TraJ.

5.2.2 TraJ protein level is not influenced in an *rpoH* mutant containing pED851

Since P_J does not contain a consensus σ^H-binding sequence (-35 sequence: 5'-CCCTTGAA-3'; 13-15 bp separation; -10 sequence: 5'-CCCGATNT-3'; Lewin, 2000) , TraJ was initially thought to be controlled by σ^H in a post-transcriptional manner. To determine if TraJ stability is affected in an *rpoH* mutant, the *traJ* coding region was fused to the arabinose-inducible promoter in pBAD24. The resulting construct, pBADTraJ (Gubbins et al., 2002), was expressed in wild-type and *rpoH* mutant cells, and the stability of TraJ was determined by immunoblot analysis to be identical in both strains (Figure 5.2). Therefore the translation and stability of TraJ appear to be unaffected by the absence of σ^H. Moreover, the degradation pattern of TraJ is different in an *rpoH* mutant compared to the wild-type cells, possibly due to the absence of particular protease(s) that are σ^H-regulated.

5.2.3 Promoter strength of various transfer genes is reduced in *rpoH* cells

Chimeric plasmids of various F *tra* fragments linked to the *lacZ* reporter gene were constructed by Dr. Jun Lu (Ph.D. Thesis, 2004; Figure 5.3). These constructs were built using the vector pJLac101, which is an RK2 replicon-based plasmid that contains the RBS and the first 24 codons of *traM* fused to *lacZ*. They were used to determine

Figure 5.1 TraJ is undetectable in KY1621 (*rpoH*)/pED851. Immunoblot analysis was performed to detect the levels of TraJ and TraM in MC4100 or KY1621 (*rpoH*), containing pED851. The levels of TraJ and TraM correlated with the reduced mating efficiency (ME), which was 0.25% for KY1621/pED851 compared to wild-type. After SDS-PAGE, proteins were transferred to a nitrocellulose membrane, which was blocked and cut in half where indicated by the open triangle. The top and bottom portions were probed by anti-TraJ (1:40,000) anti-TraM (1:10,000) antisera, respectively.

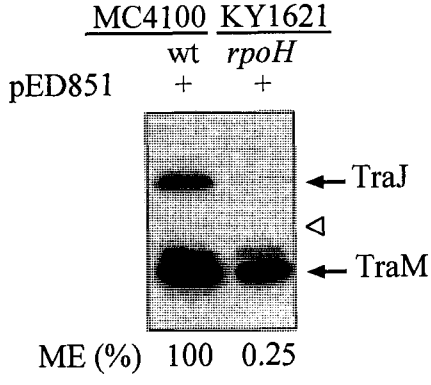

MC4100
KY1621
wt
rpoH
pED851
+
+
TraJ
TraM
ME (%)
100
0.25

Figure 5.2 σ^H regulates TraJ at the transcriptional level. TraJ expressed from pBAD-TraJ (Gubbins *et al.*, 2002) in MC4100 (lanes 1, 3 and 4) and KY1621 (*rpoH*, lanes 2, 5, and 6) was subjected to immunoblot analysis. (–) and (+) indicate the absence and presence of TraJ induction which was achieved by using 0.05% arabinose for 50 minutes. The position of TraJ is indicated by an arrow on the right. The bands below TraJ are possibly degradation products. Lanes 4 and 6 are duplicates of lanes 3 and 5, respectively.

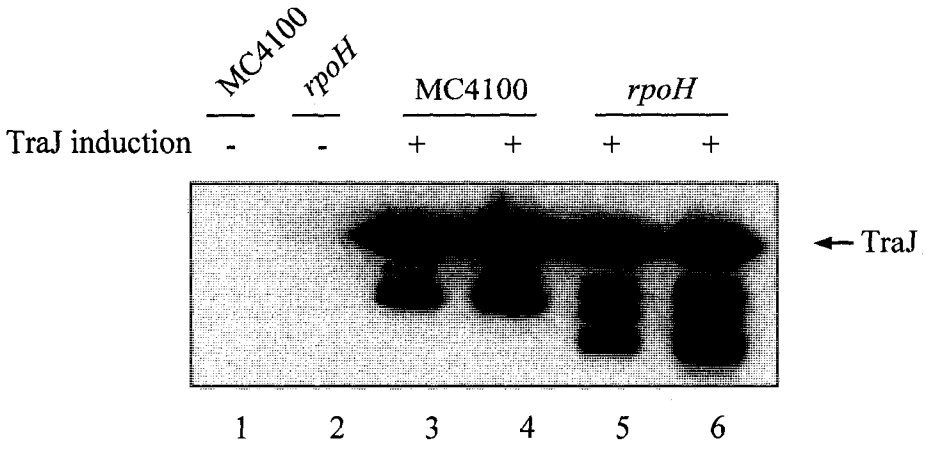

MC4100
rpoH
MC4100
rpoH
TraJ induction - - + + + +
TraJ
1 2 3 4 5 6

Figure 5.3 Promoter strengths of fragments in the F *tra* region in MC4100 and KY1621 (*rpoH*). β-galactosidase assays were employed to determine the activity of various F promoter fragments using the promoter assessment plasmid, pJLac101. Promoter activities were shown as Miller units (MU) in MC4100 (grey bars) and KY1621 (white bars). The F *tra* region from *oriT* to the beginning of *traY* is illustrated. The lines below represent different fragments from the *tra* region that are inserted in pJLac101. The open box represents incomplete *traY* with an arrow indicating its orientation. The grey boxes are indicative of the positions of *traM* and *traJ*. Angled arrows indicate the location and the direction of promoters. P_{M1} and P_{M2} are the two *traM* promoters (collectively called P_M). T_M denotes the terminator sequence of *traM*.

pJLac101, vector (RK2 replicon)

pJLac102, F P_M and *traM*

pJLac104, F P_M

pJLac106, F P_J (including *finP* and P_{finP})

pJLac107, F P_J *traJ* (no P_{finP})

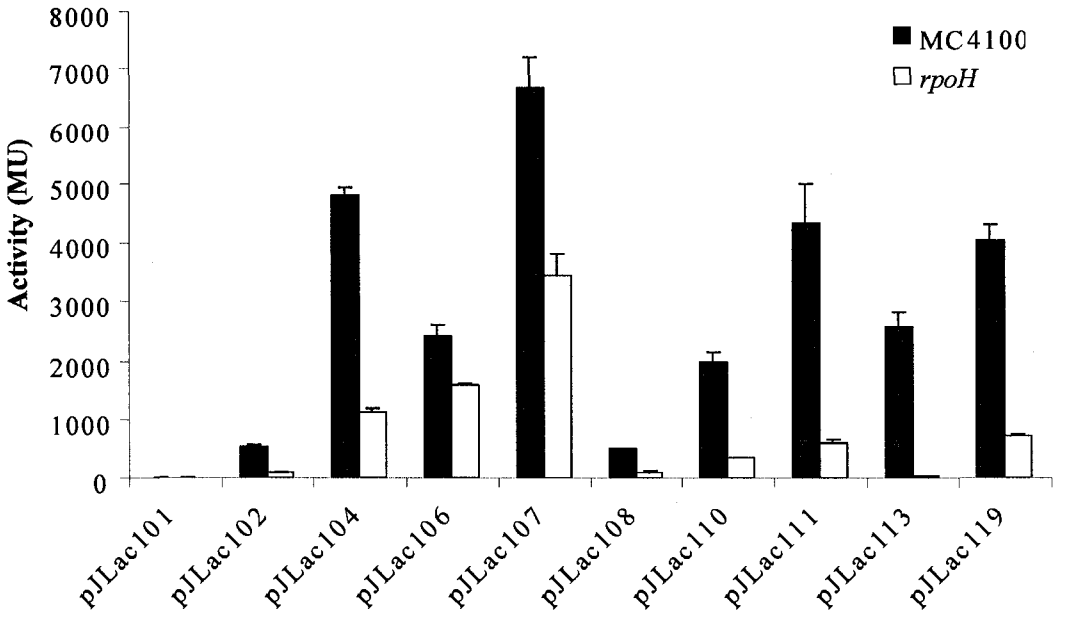
pJLac108, F P_{finP}

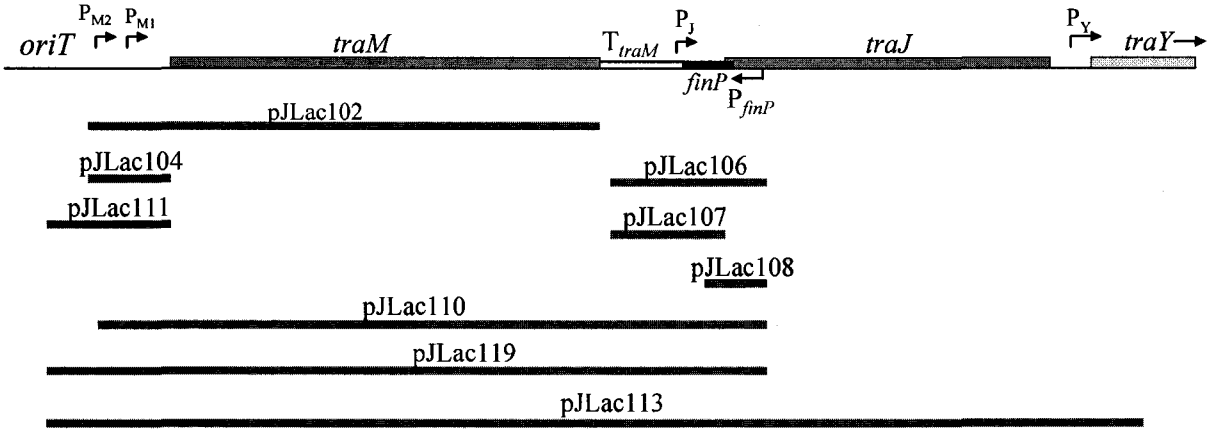
pJLac110, F P_M, *traM*, T_M, P_J (including *finP* and P_{finP})

pJLac111, F *oriT* and P_M

pJLac113, F *oriT* to P_Y

pJLac119, F *oriT*, P_M, *traM*, T_M, and P_J


8000
7000
6000
5000
4000
3000
2000
1000
0
Activity (MU)
MC4100
rpoH
pJLac101
pJLac102
pJLac104
pJLac106
pJLac107
pJLac108
pJLac110
pJLac111
pJLac113
pJLac119

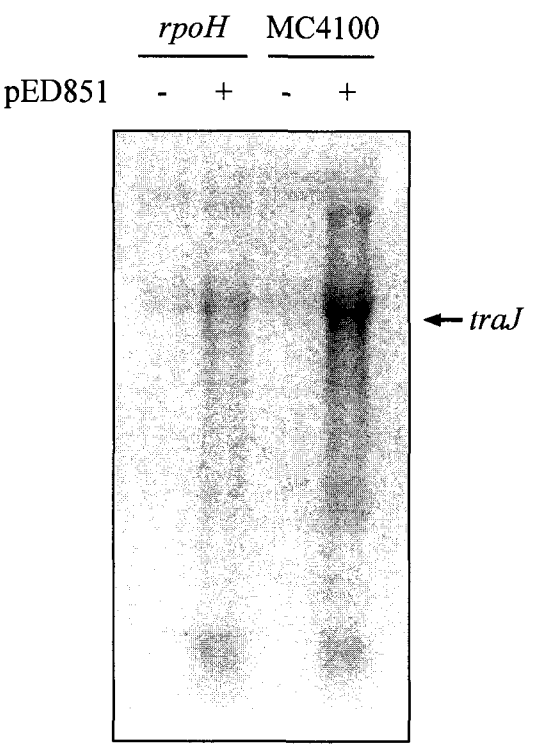

oriT
P M2
P M1
traM
T traM
P J
traJ
P Y
traY
finP
P finP
pJLac102
pJLac104
pJLac106
pJLac111
pJLac107
pJLac108
pJLac110
pJLac119
pJLac113

various P_{tra} activities in MC4100 and KY1621 (*rpoH*). Figure 5.3 shows that in general, all P_{tra} promoters tested were expressed to a lesser extent in KY1621 than MC4100. In wild-type cells, pJLac102, which contains P_M and the *traM* gene upstream of *lacZ*, has lower activity than pJLac104 due to autorepression by TraM. In pJLac107, the absence of an intact *finP* increased P_J activity when compared to pJLac106. Since Will noted that activities of P_{tra} are extremely context-dependent (Will, Ph.D. Thesis, 2006), long fragments were used when examining P_J promoter strength. pJLac110 and 119 show that the presence of *traM* did not affect P_J activity since the strength of these promoters were comparable to pJLac106, where *traM* is absent. Therefore in wild-type cells under the conditions employed, P_J is independent of P_M and the *traM* gene product. The present experiment, however, was not sufficient to detect the effect of *traM* read-through into *traJ*. In pJLac113, where P_Y is preceded by a long fragment starting from *oriT*, P_Y promoter activity was greatly diminished in KY1621. Therefore, it appears that P_Y is not activated in *rpoH*, either due to a lack of TraJ or other activators required at P_Y.

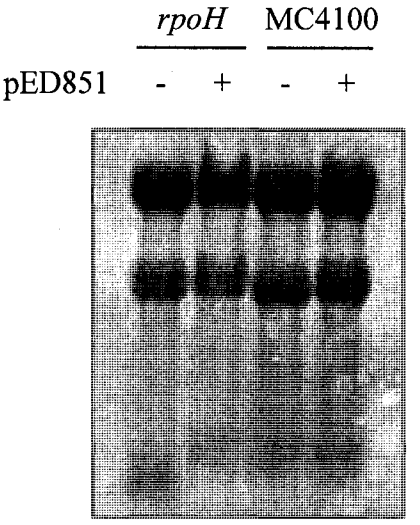
In order to examine the levels of *traJ* mRNA in an *rpoH* mutant, a Northern blot analysis was performed. RNA was extracted from MC4100 and KY1621 (*rpoH*). 20 μg of RNA was separated by electrophoresis on a 1.5% agarose gel containing 5% formaldehyde, transferred to a nylon membrane, and then probed with [^{32}P]-labelled *finP* RNA, synthesized *in vitro*, to detect *traJ* transcript (Materials and Methods). As shown in Figure 5.4, *traJ* transcript levels were decreased in KY1621/pED851. The control using RNA blot dye verified that this decrease is not attributable to loading errors. Thus, σ^H regulates TraJ at the transcriptional level, possibly via RNA polymerase or indirectly through activation of another transcriptional regulator.

Figure 5.4 Northern analysis of transcript levels of *traJ*. Samples of MC4100 and KY1621 (*rpoH*) in the absence (-) or presence (+) of pED851 were collected and total RNA was isolated and analyzed by Northern blotting. The blot was probed for *traJ* (**A**) and 16S rRNA (**B**) as a loading control. Twenty-μg of total RNA was loaded on each lane.

A



B



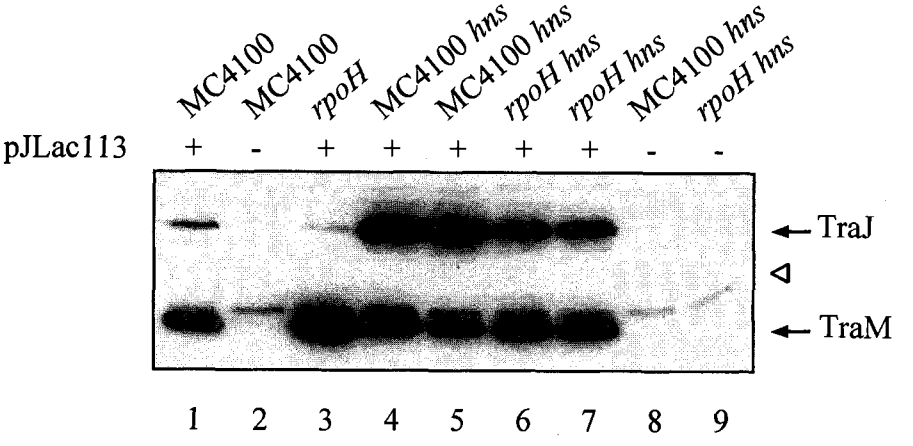
5.2.4 σ^H is required for transcription of a factor that antagonizes H-NS

To determine whether the control of σ^H on *traJ* transcription is direct, pJLac113 (Figure 5.3) was transformed into IL26, an *hns rpoH* double mutant. In PD32 (MC4100 *hns* mutant), transcription from P_J is de-repressed (Will *et al.*, 2004). We reasoned that if transcription initiation of P_J requires $E\sigma^H$, TraJ would not be detectable in IL26. Conversely, if the requirement for σ^H is not direct, TraJ will be rescued in IL26. As detected by immunoblot analysis, TraJ is restored in IL26 carrying pJLac113 (Figure 5.5, compare lanes 6 and 7 to lane 3). This indicates that the presence of σ^H is not essential for *traJ* transcription. Accordingly, once repression of H-NS on P_J is relieved, $E\sigma^{70}$ is able to initiate transcription efficiently. The decrease in the transcript level of P_J in KY1621/pED851 or KY1621/pJLac113 thus appears to be indirect. Accordingly, σ^H is expected to initiate transcription of a cellular or plasmid-encoded factor that counteracts H-NS repression at P_J. In the *rpoH* mutant containing the F *tra* operon, P_J cannot be relieved from H-NS repression due to the absence of this factor. As a result, P_J transcriptional initiation is blocked.

5.2.5 TopA, topoisomerase I, is not involved in releasing H-NS at P_J

During the course of this work, Tse-Dinh *et al* (Stewart *et al.*, 2005) published a study on the effect of *topA*, encoding topoisomerase I, on the acid resistant GAD (*gadA* and *gadBC*) system. In their study, TopA (topoisomerase I) is believed to bind and release H-NS from *gadA* and *gadBC* promoters. Of the four promoters for the transcriptional initiation of *topA*, the P1 promoter has been shown to be σ^H-dependent (Qi *et al.*, 1996). In addition, TopA is indeed activated in an σ^H-dependent manner as determined by microarray analysis (Zhao *et al.*, 2005). Consequently, the involvement of TopA in releasing H-NS from P_J was tested.

Figure 5.5 Immunoblot analyses of TraJ and TraM from pJLac113 in *rpoH*, *hns*, and *rpoH hns* double mutants. Early-log phase samples equivalent to 0.1 OD_{600} were collected and separated on SDS-PAGE followed by protein transfer. The membrane was blocked and cut where indicated by the open triangle. (-) and (+) indicate the absence and presence of pJLac113, which contains an F fragment from *oriT* to P_Y. The positions of TraJ and TraM are indicated with arrows on the right. Lanes 5 and 7 are duplicates of lanes 4 and 6, respectively.


MC4100
MC4100
rpoH
MC4100 hns
MC4100 hns
rpoH hns
rpoH hns
MC4100 hns
rpoH hns
pJLac113
+ - + + + + + - -
TraJ
TraM
1 2 3 4 5 6 7 8 9

pED851 was mated into *E. coli* RFM475 (*topA* mutant; Drolet *et al.*, 1995) and YT475H (*topA hns* double mutant; Stewart *et al.*, 2005), which were generous gifts from Dr. Tse-Dinh (New York Medical College). The resulting strains were tested for their abilities to transfer the conjugative plasmid pED851. We found that for RFM475/pED851, the absence of topoisomerase I had no effect on conjugation (data not shown). Similarly *topA hns* double mutations do not affect the transfer ability of cells. Therefore although one of the promoters of *topA* is σ^H-dependent and its product interacts with H-NS (Butland *et al.*, 2005), it does not appear to be involved in counteracting H-NS repression at P_J when cells enter exponential phase.

5.3 Discussion

Regulation of F transfer gene expression involves a complex network controlling P_M, P_J, and P_Y. In the classic model, TraJ, which contains a putative helix-loop-helix DNA binding domain (Frost *et al.*, 1994), along with cellular SfrA (ArcA; Strohmaier *et al.*, 1998) protein, binds to P_Y and activates its transcription. Although Taki *et al* (Taki *et al.*, 1998) have shown the *in vitro* binding of R100 TraJ to P_Y by EMSA, several attempts to characterize the binding of F TraJ to P_Y were unsuccessful (Will and Frost, unpublished results). Yet, as noted in Chapter 1, the positive regulatory effect of TraJ on P_Y is sequence context-dependent (Gaudin and Silverman). Will *et al.* (2004) were able to discover a role for TraJ using *hns* mutants. In stationary phase, "F^- phenocopies" are achieved through repression of P_M, P_J, and P_Y by H-NS silencing, thus rendering F^+ cells deficient in conjugation. TraJ was found to counter this H-NS repressive effect at P_Y when growth resumed (Will and Frost, 2006a). Therefore, instead of a classic transcriptional activator that binds to a specific DNA-binding sequence upstream of a promoter thereby recruiting RNA polymerase, TraJ can be viewed as a de-repressor that

serves to disrupt the nucleo-protein complex formed by H-NS, allowing transcription to begin. The precise understanding of the mechanism that relieves H-NS *in vivo*, however, remains elusive.

Many examples of counter-silencing by DNA-binding proteins have been described. The MarR family regulator SlyA counteracts H-NS silencing at sites upstream and downstream of the hemolysin gene *hlyE* transcriptional start site by competing with H-NS for binding (Lithgow *et al.*, 2007). The response regulator SsrB, upon phosphorylation in an acidic environment, activates genes within *Salmonella* pathogenicity island 2 (SPI-2) by binding to promoters of the apparatus and effector clusters (Walthers *et al.*, 2007). In the absence of H-NS, the requirement for SsrB in activating SPI-2 genes however is significantly reduced. RovA, a transcriptional activator in *Yersinia*, binds to sites in the promoter regions of the *inv* and *rovA* genes that superimpose the H-NS binding sites (Heroven *et al.*, 2004). Similarly, RovA is not essential for activation of the *inv* and *rovA* genes in the absence of H-NS. Interestingly, both SsrB in *Salmonella* and RovA in *Yersinia* are proposed to have dual functions: binding to the promoters to displace H-NS and activation by recruiting the RNA polymerase. In fact, a number of positive regulators that counteract H-NS also activate transcription by promoting direct interaction with the RNA polymerase, such that RNAP can interact more productively with the promoter. Examples include the ToxT protein of *Vibrio cholerae* and the CfaD protein in *E. coli* (Jordi *et al.*, 1992; Yu and DiRita, 2002). Therefore, TraJ may function similarly to one of the above de-repressors.

The requirement for σ^H and σ^H-regulated proteins (such as DnaK) in F plasmid maintenance have long been documented (Wada *et al.*, 1987; Ezaki *et al.*, 1989). The essentiality of σ^H for F plasmid transfer, however, was newly uncovered in this study.

With diminished levels of TraJ and reduced activity of P_Y, *rpoH* mutants are unable to conjugate. Whereas F *repE*, encoding the replication protein, requires σ^H for initiation of transcription, σ^H is not required for the transcription of *traJ* directly. In *hns rpoH* double mutants, P_J is de-repressed and TraJ expression is evident (Figure 5.5). Therefore, similar to TraJ (Will and Frost, 2006a), σ^H becomes dispensable in the activation of P_J when *hns* is absent.

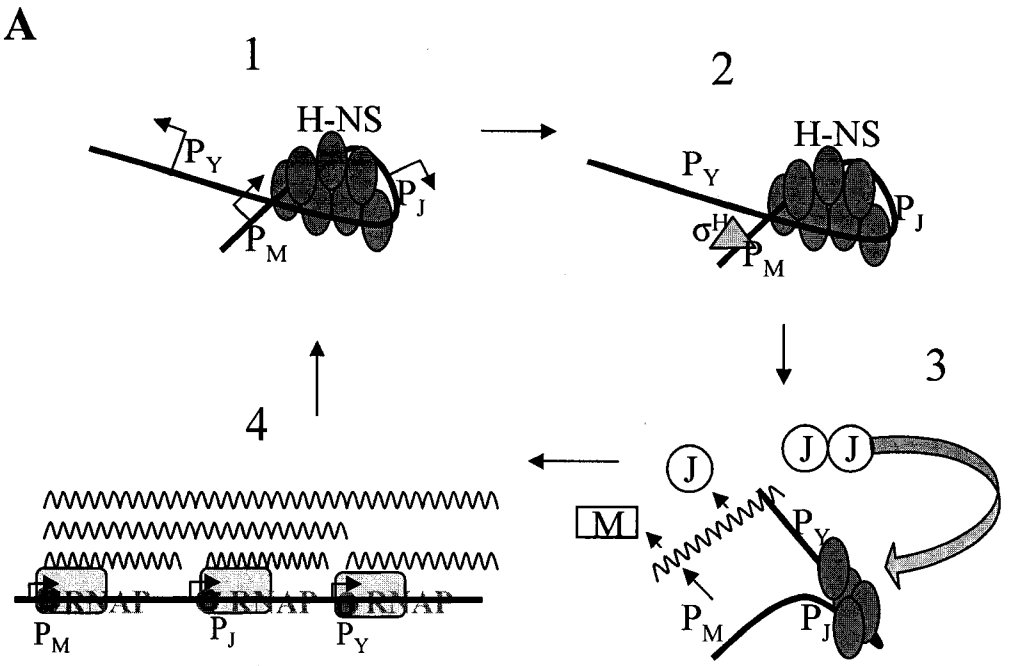
It is possible that TraJ, which undergoes post-translational modification when cells enter stationary phase (Will, Ph.D. Thesis, 2006), loses its ability to bind DNA. Preliminary data came from an isoelectric focusing gel electrophoresis experiment, which showed that the pI of TraJ after 3 hours of growth is different than the pI after 7 hours of growth (Will and Frost, unpublished results). As suggested in previous chapters, TraJ becomes an inactive form, TraJ*, that is unable to initiate transcription at P_Y and is resistant to degradation. In early exponential phase, TraJ (active TraJ) may bind directly to P_Y and counter H-NS repression by hindering its access to P_Y. The aforementioned protein that is proposed to be transcribed by σ^H, is possibly responsible for modifying TraJ. In the absence of this protein, the interaction of TraJ with DNA may be reduced in *rpoH* null cells containing pED851. Thus, transcription of P_Y is dependent on TraJ, SfrA (ArcA), and this unknown factor. TraR, which is proposed to be a modifier of TraJ, was recently shown to not be regulated by σ^H (Beadle, Villenueve and Frost, personal communication).

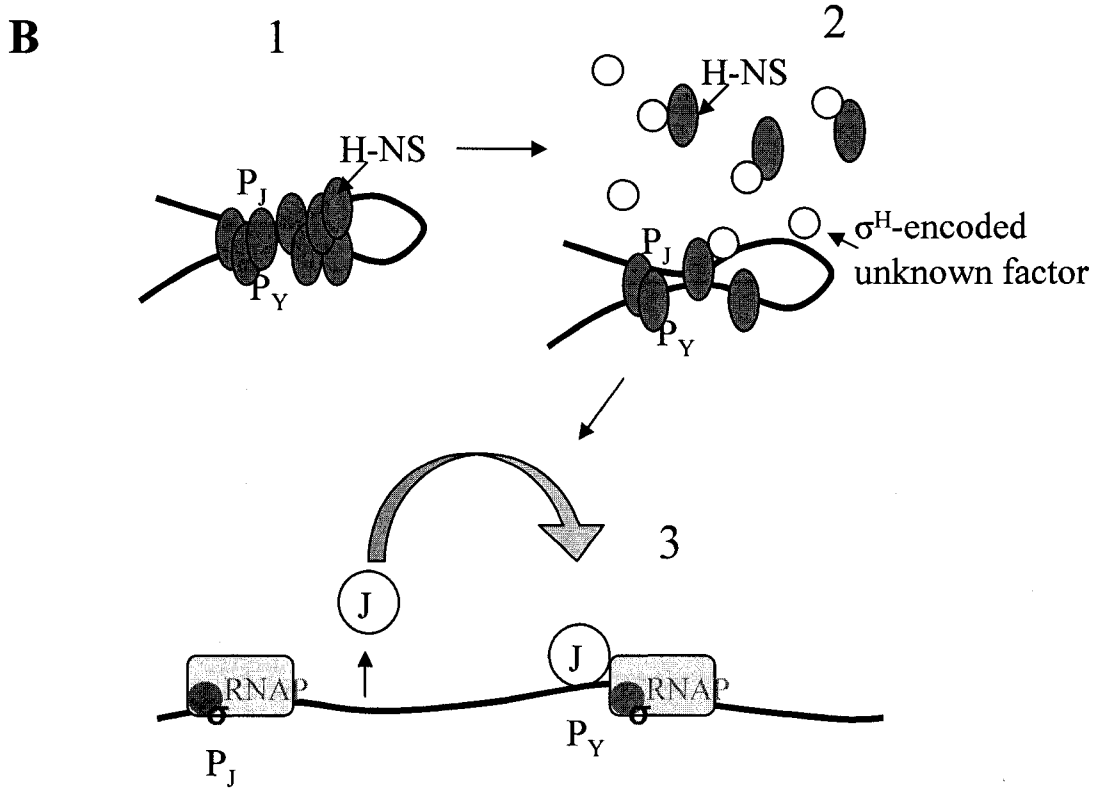
Alternatively, σ^H may be responsible for the transcription of an adaptor protein that mediates TraJ binding to P_Y. This is similar to the effect of *topA* on the acid resistant GAD system (Stewart *et al.*, 2005). The P1 promoter of *topA* is stimulated by σ^H. TopA binds to sites upstream of the *gad* genes and counteracts the H-NS repressive effect.

However, the involvement of TopA in relieving H-NS from P_Y has been discounted (See section 5.2.5). There have been other examples in which expression of genes that are repressed by H-NS can be activated by an alternative sigma factor or H-NS homologs (Fang and Rimsky, 2008). The sigma factor in stationary phase, σ^S, is required for expression of the *csgBA* and *hdeAB* loci of *E. coli* only in the presence of H-NS (Arnqvist *et al.*, 1994). In the absence of *hns*, both $E\sigma^{70}$ and $E\sigma^S$ can activate the expression of these genes. It has been inferred that H-NS forms nucleoprotein complexes with $E\sigma^{70}$ preferentially over other sigma factors in complexes with RNAP (Shin *et al.*, 2005). As such, this may account in part for the selectivity of alternative sigma factors. Our current data suggests that σ^H is not required for direct activation of *traJ* when *hns* is absent (Figure 5.5). This, however, does not refute the possibility that both $E\sigma^{70}$ and $E\sigma^H$ can initiate transcription at P_J or P_M.

A search for a σ^H-consensus binding site at the promoter regions of P_J and P_Y was unsuccessful. Although promoter activity assessment revealed that P_M, P_J, and P_Y are repressed in the absence of *rpoH* (Figure 5.3), characterization of σ^H binding to each of the promoters awaits further experimentation. At present, the binding of σ^H to P_M is the most likely candidate since this is the only promoter (of the three) that contains the σ^H-consensus binding site although the results that show the expression of TraM in the absence of *rpoH* oppose this hypothesis (Figure 5.5). Since P_M has two promoters, P_{M1} and P_{M2}, σ^H can be required for the first few rounds of transcription initiated at P_{M2} when supercoiling is at the appropriate level. During exponential phase, transcription initiation at P_{M1}, the predominant promoter, can be accomplished by $E\sigma^{70}$. In a parallel experiment, *traM* transcript levels were found to be reduced in an *rpoH* mutant containing the F *tra* regulatory region (Beadle and Frost, personal communication). Accordingly it remains

possible that σ^H first initiates transcription of *traM* by displacing H-NS at P_{M2}. TraJ can be translated from the read-through of the *traM-traJ* transcript and further activate the polycistronic *tra* operon by relieving the nucleoprotein complex of H-NS at P_J and P_Y. Alternatively, as illustrated in Figure 5.6, an unknown factor can be activated to relieve H-NS at P_J at the beginning of cell growth. Once de-repressed, $E\sigma^{70}$ initiates transcription of *traJ*. Subsequently, TraJ activates P_Y and allows expression of the F pilus. The identity of this H-NS antagonist, presumably under the control of σ^H, is presently under investigation. Although the unknown factor that requires σ^H for transcription awaits additional research, the roles of TraJ and σ^H as de-repressors for F *tra*, are further defined in this study.


A
1
H-NS
P_Y
P_J
P_M
2
H-NS
P_Y
σ^H
P_M
P_J
3
J
J
J
M
P_Y
P_M
P_J
4
RNAP
RNAP
RNAP
P_M
P_J
P_Y


B
1
H-NS
P_J
P_Y
2
H-NS
P_J
P_Y
σ^H-encoded
unknown factor
3
J
J
RNAP
RNAP
P_J
P_Y

Chapter 6: Inhibition of RP4 conjugation by F PifC

6.1 Introduction

Conjugative DNA transfer requires the synthesis of three protein complexes: the mating pair formation complex (the Mpf system), the DNA transfer and replication complex (the Dtr system) and the coupling protein. In the case of the broad-host-range IncP plasmids (both IncPα and IncPβ), the membrane-associated Mpf system involves P-pilus formation, which promotes intimate cell-cell contact for DNA export or adsorption of donor-specific phages. Twelve plasmid-encoded proteins of RP4, an IncPα plasmid, (TrbB-L of Tra2 and TraF of Tra1) are components of this complex (Lessl *et al.*, 1992). The Dtr proteins interact at *oriT* to form the relaxosome and initiate transfer of a single-strand of DNA. Three transfer proteins encoded by the Tra1 region (TraI, TraJ, and TraK) are components of this system. Encoded in Tra1, RP4 TraG (F TraD equivalent) is essential for conjugation (Waters *et al.*, 1992; Llosa *et al.*, 1994). $TraG_{RP4}$ is a 70kDa inner membrane protein that connects the relaxosome (the Dtr system) to the transfer machinery (the Mpf system), thus coordinating conjugative transfer (Hamilton *et al.*, 2000). Homologs of TraG occur in various conjugative systems and are essential both for transfer of the conjugative plasmid and for mobilization of non-conjugative plasmids (Cabezon *et al.*, 1997). As a result, TraG family proteins (for examples, $TraG_{RP4}$, $TrwB_{R388}$, $TraD_F$, $TraG_{Ti}$ and $VirD4_{Ti}$) are referred to as coupling proteins (Llosa *et al.*, 2002).

In a bacterium co-harboring both F and P plasmids, the transfer of the P plasmid is blocked by F (Tanimoto and Iino, 1983). Whereas the P plasmid has no effect on F transfer, F reduces RP4 transfer by approximately 500-fold (Tanimoto and Iino, 1983). The F *pifC* gene product located on an operon that spans 43.3 kb to 37.2 kb on F (reviewed in Chapter 1) has been implicated in this repression. PifC is a 40.7kDa protein

that is involved in the regulation of *pif* gene expression in addition to the initiation of F plasmid replication. It acts as an autorepressor by binding to *pifO2*, a *cis* acting element in the *pif* operon (Figure 1.2). In the presence of PifC, expression of *pif* is reduced 6- to 45-fold (Miller and Malamy, 1983). PifA has been shown to affect translation of bacteriophage T7 mRNA in a F^+ cell at late stages of infection by interacting with the T7 phage protein gp1-2, which is involved in viral replication (Schmitt and Molineux, 1991; Molineux *et al.*, 1989). PifB is responsible for causing membrane lesions in an F^+ host, leading to increased permeability (Willetts and Skurray, 1987). As such, the *pif* operon in F inhibits replication of bacteriophage, giving an advantage to the F^+ bacterium.

In early studies, Miller *et al.* (1985) suggested that PifC interferes with RP4 conjugation by repressing promoters of RP4 *tra* genes. This was based on the observations that the presence of RP4 *in trans* to F*lac* resulted in a decrease in T7 bacteriophage plating, which was attributed to increased PifA and PifB activity. Therefore, titration of PifC away from its operator *pifO* by RP4 DNA was proposed to be the mechanism of inhibition (Miller *et al.*, 1985). Subsequently, PifC has been postulated to inhibit RP4 conjugative transfer by sequestering $TraG_{RP4}$, the coupling protein that drives DNA transport during bacterial conjugation (Santini and Stanisich, 1998). Evidence demonstrating *traG* as the specific target of inhibition was obtained in an artificial system in which cloned *traG* was used to enhance RSF1010 mobilization via the N pilus system. Such enhancement did not occur in the presence of *pifC*. This chapter aims to study the mechanism of RP4 conjugative inhibition by F PifC, as well as to re-examine the implications of RP4 TraG inhibition. Using the bacterial two hybrid system, we show for the first time an *in vivo* F PifC and $TraG_{RP4}$ protein-protein interaction, which is further verified by cross-linking and co-immunoprecipitation experiments.

6.2 Results

6.2.1 RP4 conjugal transfer is reduced in cells overexpressing F PifC

E. coli wild-type cells harbouring P and F plasmids were tested for their ability to conjugate the P plasmids on solid and liquid media, into XK1200 recipient cells according to Materials and Methods. The presence of F decreases RP4 mating efficiencies by 3 logs on solid medium and 2 logs in liquid medium (Table 6.1). PifC was overexpressed from pLF71 (Amp^r, Table 2.1), a pT7-7 based plasmid encoding *pifC* under the pT7 $Ø_{10}$ promoter that is transcribed by an IPTG-inducible T7 polymerase in BL21. Since RP4 has multiple antibiotic resistant genes, an IncP β plasmid R751 (Tp^r) was used. When overexpressed, PifC reduced R751 transfer by over 5 logs on solid and over 2 logs in liquid media, indicating strong conjugation inhibition. This is in agreement with previous results (Santini and Stanisich, 1998), showing that 1) IncP plasmids transfer less efficiently on liquid than on solid medium and 2) the mating efficiencies are further reduced in the presence of F *pifC* product.

6.2.2 PifC reduces RP4 conjugative transfer through a post-transcriptional mechanism

Miller *et al* (1985) found that the F *pifC* gene product is required for the inhibition of RP4 transfer, and Santini and Stanisich (1998) found that the only inhibition target for PifC on RP4 is *traG*. Whereas the previous study favoured a transcriptional control, the latter suggested protein-protein interactions between TraG and PifC. An electrophoretic mobility shift assays (EMSA) was performed to test whether purified PifC binds to the $traG_{RP4}$ promoter, P_{traG}. A non-related promoter *fisYp* was used as a control. Purified PifC binds very weakly to P_{traG}. The PifC-P_{traG} complex first appears when PifC concentration reaches 200 nM (data not shown). To determine if PifC represses the RP4 *traG* promoter,

Table 6.1 Inhibition of IncP conjugative transfer by the F plasmid or PifC in pLF71.

P Plasmid	F		PifC	
	-	+	-	+
RP4 (solid[a])	$2.5X10^{-1}$	$6.3X10^{-4}$		
RP4 (liquid[b])	$7.5X10^{-4}$	$4.0X10^{-6}$		
R751 (solid[a])			$1.5X10^{-1}$	$<10^{-6}$
R751 (liquid[b])			$5.3X10^{-4}$	$<10^{-6}$

Mating assays were performed as described in Materials and Methods.
[a]For solid matings, donor and recipient cells were filtered and allowed to mate on LB agar plate.
[b]For liquid matings, cell cultures were pelleted and resuspended in LB broth. Matings were performed at 37°C for one hour. Mating efficiencies were reported as the number of transconjugates per donor cell. For $<10^{-6}$, no transconjugate appeared in the undiluted mating tubes.

the transcriptional fusion-based promoter assessment plasmid pPR9tt-1 (Santos *et al.*, 2001; Table 2.1) was used. The *traG* promoter (P_{traG}) followed by a ribosome binding site and an initiation codon was fused to the *lacZ* gene to give pIL21. IPTG was added to induce *pifC* from pLF71. P_{traG} is not repressed when *pifC* is induced since there is no difference in the expression levels of *lacZ* from P_{traG} (Figure 6.1). pJLac105 (P_{lac}–*lac*) was used as a control to show that PifC does not affect unrelated promoters. Therefore, even though PifC was shown to bind weakly to P_{traG}, *traG* promoter activity does not appear to be affected by PifC. This suggests PifC does not bind and repress P_{traG} in the same way as it does at the *pif* operator (Miller and Malamy, 1986).

6.2.3 $TraG_{RP4}$ level is not affected in cells overexpressing PifC

To determine if the protein level of $TraG_{RP4}$ decreases in the presence of overexpressed PifC, immunoblot analysis was performed with $TraG_{RP4}$ antiserum provided by Dr. Erich Lanka (Max-Planck_Institut fur Molekulare Genetik, Germany). In Figure 6.2, two different *traG* clones, pML100 expressing *traG* from its native promoter (P_{traG}-*traG*); and pSK470 expressing *traG* from a foreign promoter (P_{tac}-*traG*), along with the vector control pBR322 were tested. When PifC on pRS2496 was co-expressed, the levels of $TraG_{RP4}$ did not decrease (Figure 6.2, TraG levels in lanes 5 and 6 are comparable to that in lanes 2 and 3). Therefore, P_{traG} is not affected by F PifC. In addition, PifC does not inhibit RP4 conjugative transfer by reducing $TraG_{RP4}$ levels. Instead, it may bind to and sequester $TraG_{RP4}$ from the RP4 Mpf proteins or the relaxosome, thereby blocking conjugation.

6.2.4 PifC-$TraG_{RP4}$ protein interaction as shown by the bacterial two-hybrid system

In order to test for an interaction between PifC and $TraG_{RP4}$, a bacterial two-hybrid (BTH) system was employed. In the BTH assay, proteins being tested are fused to

Figure 6.1 Assessment of RP4 *traG* promoter activities. β-galactosidase assays were performed to assess the promoter activities of P_{traG} and P_{lac} in the absence and presence of PifC. *E. coli* MC4100 harbouring pIL21 (P_{traG}-*lacZ*) or pJLac105 (P_{lac}-*lacZ*) and pLF71 were grown to log phase. The cultures were divided into two, and IPTG was added to one of the cultures to induce *pifC*. Activities were obtained as the average of triplicate assays and reported as Miller units (MU).

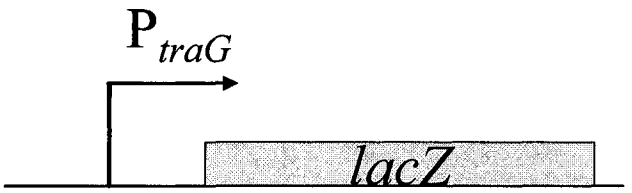
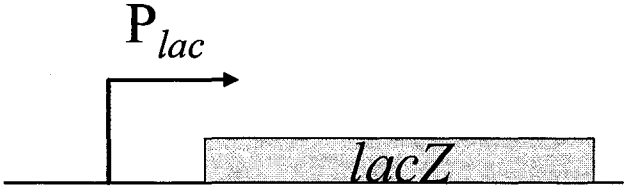
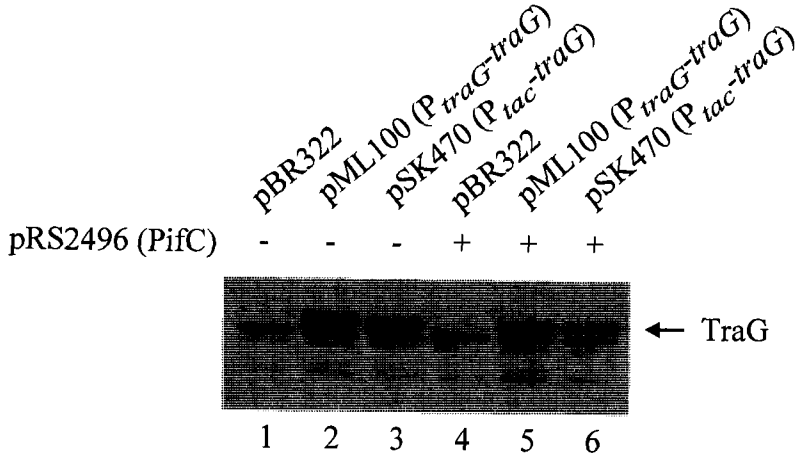
	β-galactosidase activities (X10^3 MU)	
	-PifC	**+PifC**
	2.6	2.5
	3.1	3.0

Figure 6.2 TraG is not degraded in *pifC*-overexpressing cells. Immunoblot analysis detecting the levels of $TraG_{RP4}$ in *E. coli* MC4100 harbouring pBR322 (vector control, lanes 1 and 4), pML100 (P_{traG}-*traG*, lanes 2 and 5), or pSK470 (P_{tac}-*traG*, lanes 3 and 6) in the presence (+) or absence (-) of pRS2496 (Cram *et al.*, 1984, Table 2.1). Mid-log phase cultures equivalent to 0.1 OD_{600} were pelleted and separated on a 10% SDS-PAGE. The band below TraG reacts non-specifically with the TraG-antiserum and serves as loading control. The level of TraG does not decrease in the presence of excess PifC.


pBR322
pML100 (P traG-traG)
pSK470 (P tac-traG)
pBR322
pML100 (P traG-traG)
pSK470 (P tac-traG)
pRS2496 (PifC) - - - + + +
TraG
1 2 3 4 5 6

one of two adenylate cyclase fragments (T18 or T25) that are not active when physically separated. Upon interaction of the test proteins, the fragments are brought into close proximity, synthesizing cAMP. cAMP binds to catabolite activator protein, CAP, and the cAMP-CAP complex activates catabolic genes including *lac*. In a cyclase-deficient strain of *E. coli* (BTH101), interacting clones will be identified on chromogenic plates such as LB-X-gal agar or in a β-galactosidase assay upon ONPG catabolism.

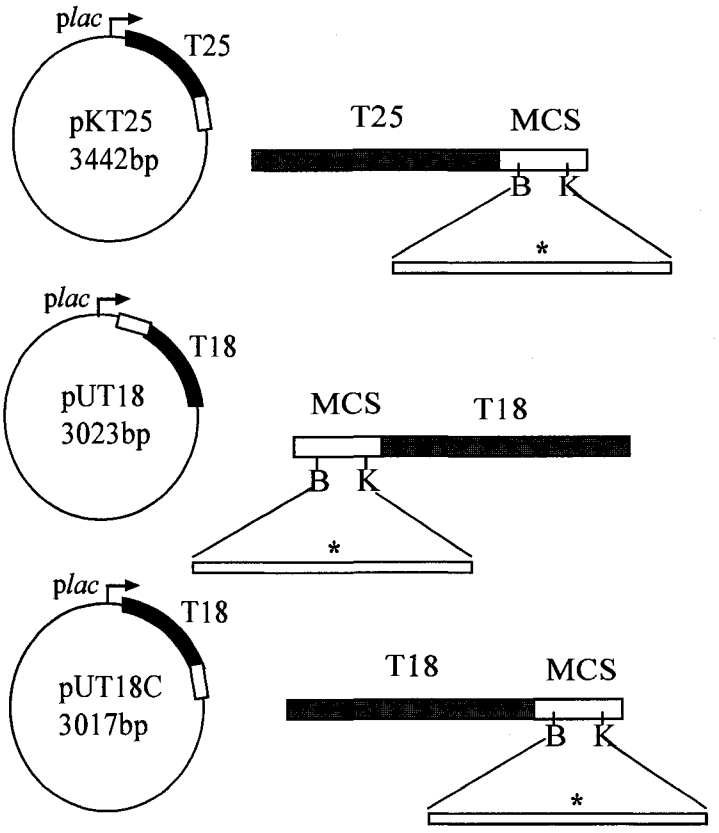
pifC and *traG* genes encoding the entire PifC (362 aa) and TraG (635 aa) in the absence of their stop codons were amplified by PCR with primers ILA38/39 and ILA40/41 respectively (Table 2.2). Each of these genes was fused in-frame to one of the adenylate cyclase fragments encoded by the BTH vectors pKT25, pUT18, pUT18C (Figure 6.3A) as described in Materials and Methods. In these clones, the number 25 or 18 denotes the adenylate cyclase fragments T25 or T18; and the letter N or C following the name of the protein indicates the terminus (N- or C-) that is fused to the adenylate cyclase peptide.

Two test plasmids, pKT25 and pUT18 or pUT18C were co-transformed into *E. coli* BTH101. Positive interactions were selected on X-gal plates. Two pairs (p25TraG-N/p18PifC-C and p25TraG-N/p18PifC-N) yielded dark blue colonies, and another two pairs (p25PifC-N/p18TraG-C and p25PifC-N/p18TraG-N) yielded pale blue colonies; whereas the positive control with two fragments of leucine zipper (pKT25-Zip/pUT18C-Zip) yielded blue colonies and the negative control (empty BTH vectors) showed white colonies on LB-X-gal agar plates (Table 6.2).

Quantification of β-galactosidase activity was performed using a standard assay (Miller). The two pairs that gave rise to pale blue colonies showed slight interaction of 93 and 84 Miller Units (MU, Figure 6.3B, lanes 3 and 4). Of the interacting pairs, one

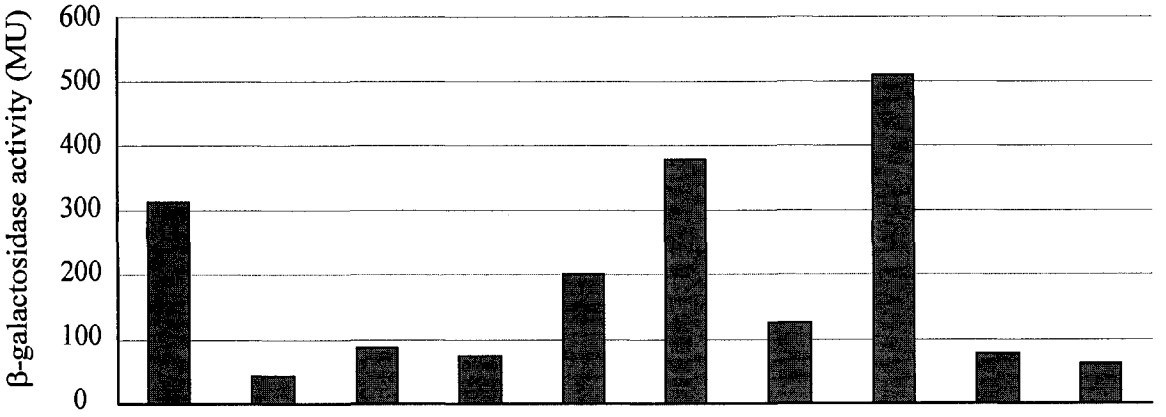
Figure 6.3 Bacterial Two-hybrid analysis of TraG-PifC and PifC-PifC interaction. A. Plasmid maps of BTH vectors used in this study: pKT25, pUT18, and pUT18C. T25 and T18 are two peptides that produce active adenylate cyclase when they interact physically. MCS: multiple cloning site. For simplicity, only the restriction enzymes used in this study: BamH1 (B) and KpnI (K) are indicated. The asterisks indicate genes encoding the test proteins and their lengths (in aa) that have been fused to the peptides in each vector. The name of the resulting plasmid is listed on the right. **B.** β-galactosidase activity of *E. coli* BTH101 (*cya*) harbouring various test plasmids. pKT25-Zip and pUT18C-Zip are control plasmids containing two leucine zipper peptides that are known to interact and synthesize functional adenylate cyclase (Karimova *et al.*, 1998). Lane 2 (-) denotes a negative control where only the vectors are co-transformed. Lanes 3 to 6 illustrate the interaction of PifC and TraG. Lanes 7-8 and 9-10 illustrate the level of PifC-PifC and TraG-TraG interactions respectively.

A



*Fusion peptide	Plasmid
PifC 1-362aa	p25PifC-N
TraG 1-635aa	p25TraG-N
TraG 123-635aa	p25TraG$_{123-635}$-N
TraG 245-635aa	p25TraG$_{245-635}$-N
TraG 398-635aa	p25TraG$_{398-635}$-N
TraG 123-396aa	p25TraG$_{123-396}$-N
PifC 1-362aa	p18PifC-C
TraG 1-635aa	p18TraG-C
PifC 1-362aa	p18PifC-N
TraG 1-635aa	p18TraG-N

B



pKT25	Zip	-	PifC	PifC	TraG	TraG	PifC	PifC	TraG	TraG
pUT18			TraG		PifC		PifC		TraG	
pUT18C	Zip	-		TraG		PifC		PifC		TraG
	1	2	3	4	5	6	7	8	9	10

Table 6.2 Phenotypes of *E. coli* BTH101 (*cya*$^-$) harbouring various clones.

pKT25	pUT18C	Phenotype (X-Gal plate)
Zip	Zip	Blue
-	-	White
PifC	TraG	Light blue
TraG	PifC	Dark blue
TraG	-	White
-	PifC	White
TraG C1	PifC	White
TraG C2	PifC	White
TraG C3	PifC	White
TraG I1	PifC	White
PifC	PifC	Dark blue
TraG	TraG	White

revealed strong interaction: p25TraG-N and p18PifC-N. Reversing the orientation of PifC such that its C-terminal end is fused to the adenyl cyclase peptide (p18PifC-C) was also assayed. Interaction of p25TraG-N and this plasmid was reduced approximately two-fold to 200MU (Figure 6.3B, lane 5 compare to lane 6). Accordingly, we hypothesize that TraG and PifC interact most strongly at the C-terminal domains of both proteins. This is the first demonstration of a molecular interaction between TraG and PifC (Figure 6.4).

We also tested PifC-PifC and TraG-TraG interactions using the BTH clones (Figure 6.3B). Self-interaction of PifC was observed most strongly when the C-termini were unhindered (512MU, Lane 8). TraG self-interaction could not be demonstrated using the BTH method, possibly due to orientation or folding of the protein in the fusion.

Figure 6.4 shows that PifC, a cytoplasmic protein, interacts most strongly with the C-terminal cytoplasmic tail of TraG. To delimit the domain on TraG that interacts with PifC, we constructed deletion mutants of TraG according to its proposed topology (Figure 6.5A; Schroder *et al.*, 2002). Each of the constructs, including N-terminal fusions of TraG ($TraG_{123-635}$, $TraG_{245-635}$, $TraG_{398-635}$, and $TraG_{123-396}$ respectively) to the T25 peptide in pKT25 was co-transformed with pUT18C-PifC. Unexpectedly, none of the truncated TraG fusions showed a positive interaction with PifC (Table 6.2), signifying that the N-terminal (1-122) part of the protein is required for interaction to occur.

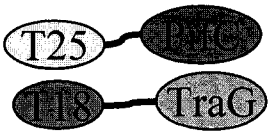
6.2.5 Cross-linking and Immunoprecipitation of TraG and PifC

To confirm the *in vivo* interaction between TraG and PifC, cross-linking experiment and immunoprecipitation analysis were performed. MC4100/pML100/ pRS2496 cell pellets were washed and treated with the chemical cross-linking agent bis(sulfosuccinimidyl) suberate (BS^3 , Pierce Chemical Co.). BS^3 cross-links proteins and results in protein complexes that are non-cleavable when boiled and separated on SDS-

Figure 6.4 Schematic diagram illustrating the orientation of PifC and TraG and their interactions as determined by the bacterial two-hybrid system. The *pifC* and *traG* genes were cloned into each of the various BTH vectors. The lines between the test proteins (PifC or TraG) and adenylate cyclase (Cya) peptides (corresponding to amino acids 1-224, named T25 or corresponding to amino acids 225-399, named T18) indicate the sites of fusion. Positive interaction (+ or ++) and negative interaction (–) are indicated.

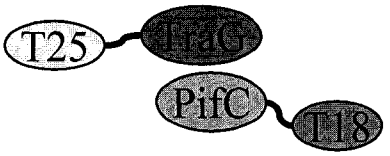
-

p25PifC-N
p18TraG-C



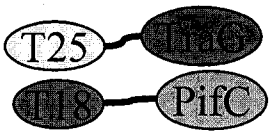
-

p25PifC-N
p18TraG-N



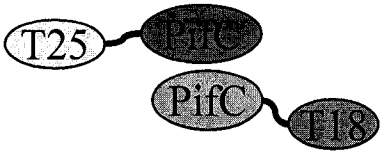
+

p25TraG-N
p18PifC-C



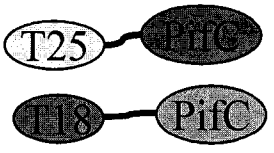
++

p25TraG-N
p18PifC-N



-

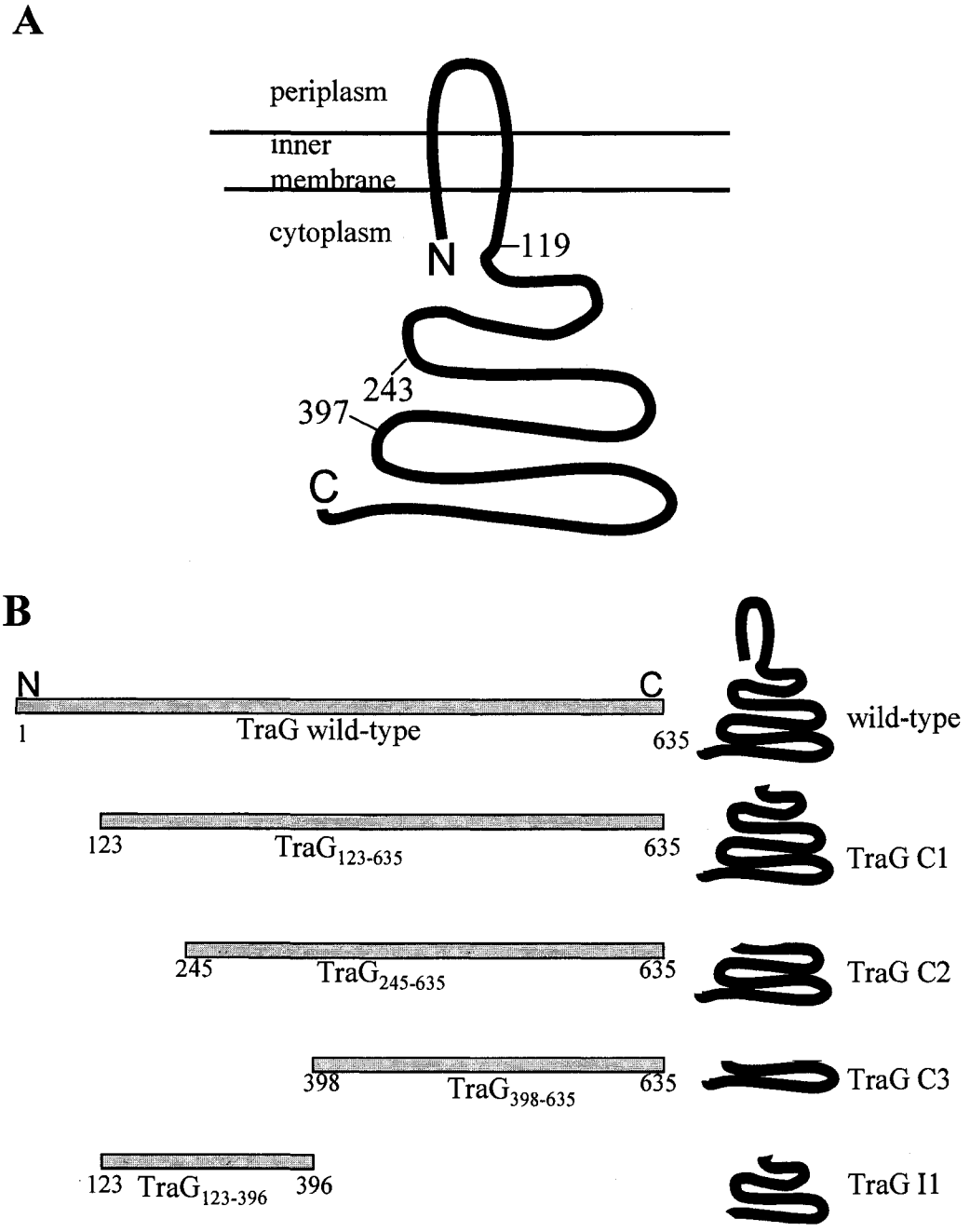
p25PifC-N
p18PifC-C



+++

p25PifC-N
p18PifC-N

Figure 6.5 RP4 TraG. A. Proposed topology of RP4 TraG by Schroder *et al.* (2002) using insertional mutations. Amino acids 119, 243, and 397 are indicated. Figure adapted from (Schroder *et al.*, 2002). **B.** Constructs of TraG deletion mutants. Each of the constructs was fused to the C-terminus of T25 peptide in pKT25. TraG C1 contains aa 123-635, C2 contains aa 245-635, C3 contains aa 398-635, and I1 contains aa 123-396. Plasmids were co-transformed with p18PifC-N into *E. coli* BTH101. Results on LB X-gal agar plates are listed in Table 6.2.


A
periplasm
inner
membrane
cytoplasm
N
119
243
397
C
B
N
C
1
TraG wild-type
635
wild-type
123
TraG123-635
635
TraG C1
245
TraG245-635
635
TraG C2
398
TraG398-635
635
TraG C3
123
TraG123-396
396
TraG I1

PAGE. A cross-linking experiment was performed according to the protocol described in Chapter 2. In Figure 6.6, a high-molecular-weight cross-linked complex was only observed in BS^3-treated cells that harbour *traG*- and *pifC*-encoding plasmids (lanes 4). As a control, cross-linking cells harbouring only *pifC* but not *traG* did not result in this high-molecular-weight band (lane 2). Interestingly, when *traG* is co-expressed in the cell, the level of PifC protein is elevated (compare between lanes 1 and 3). This agrees with the previous finding that overexpression of the RP4 *traG* gene titrates cellular PifC, and therefore potentially de-represses the *pif* promoter (Miller *et al.*, 1985).

Co-immunoprecipitation (Co-IP) analysis was also used to investigate the specific interaction of TraG and PifC. Cells containing *pifC* and *traG*-expressing plasmids, pML100 and pRS2496, were treated with membrane-permeable dithiobis[succinimidyl propionate] (DSP), a chemical cross-linking agent that results in protein complexes that are cleavable upon addition of a reducing agent (like β-mercaptoethanol). MC4100/pML100/pRS2496 cells were washed and crushed with glass beads and the cell-free extracts were incubated overnight with Sepharose A beads conjugated with anti-PifC antibody. The resin was resuspended in reducing SDS sample buffer and boiled. The supernatant was analyzed by SDS-PAGE and proteins were transferred to nitrocellulose membrane for immunoblot analysis. TraG was precipitated only in the presence of anti-PifC antibody conjugated to Sepharose A (Figure 6.7, lane 2). The thick band that appears at 50kDa is possibly the heavy chain fragment of anti-PifC antibody, which reacts with the secondary antibodies, because a reducing SDS-sample buffer was used. Accordingly, although other controls are needed to support the interaction between TraG and PifC, this preliminary Co-IP experiment reveals the specific *in vivo* interaction of TraG and PifC in their native states.

Figure 6.6 BS^3 cross-linking analysis of PifC and TraG interaction. *E. coli* MC4100 harbouring pRS2496 (*pifC*) and pBR322 (vector control) or pML100 (*traG*) were treated with BS^3 and separated by SDS-PAGE. The proteins were then transferred to nitrocellulose membrane and reacted with anti-PifC antiserum. Lanes 1 and 3 untreated cells; lanes 2 and 4, whole cells treated with 200μM BS^3. The position of PifC is indicated with an arrow on the right. The position for the $TraG_{RP4}$-PifC complex is indicated with an open triangle.

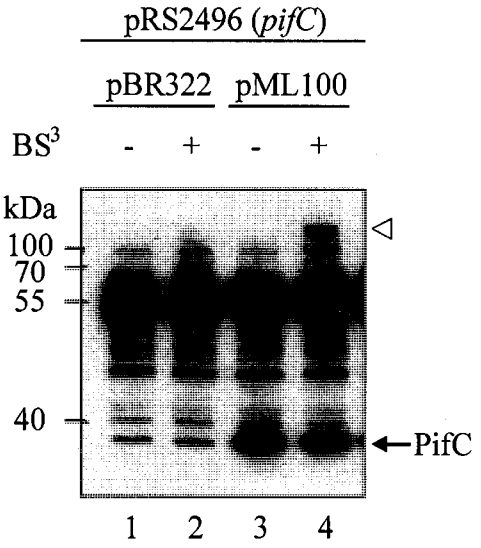
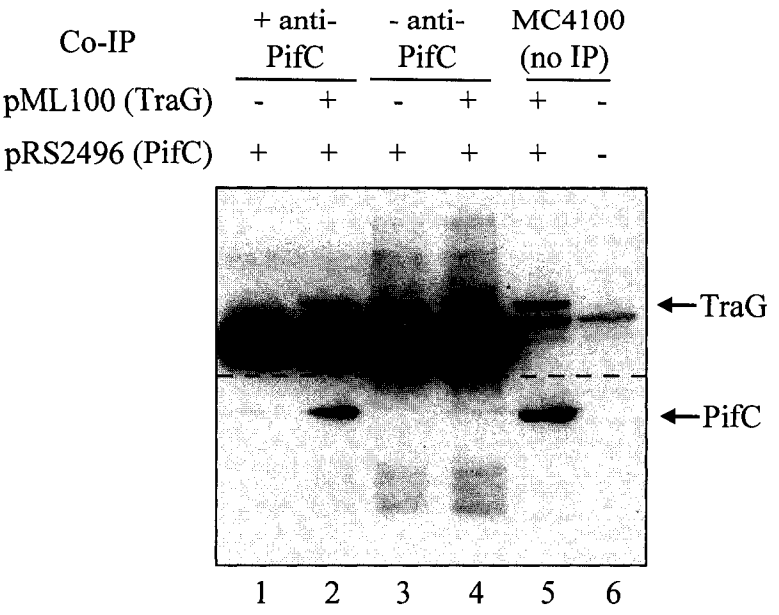

pRS2496 (*pifC*)
pBR322
pML100
BS3
-
+
-
+
kDa
100
70
55
40
PifC
1
2
3
4

Figure 6.7 Cross-linking and Co-Immunoprecipitation of $TraG_{RP4}$-PifC complex. *E. coli* MC4100 containing *traG*- and *pifC*-expressing plasmids (pML100 and pRS2496) were treated with DSP, washed and disrupted by using glass beads. Cell-free extract of cultures were incubated with (lanes 1 and 2) or without (lanes 3 and 4) anti-PifC antibody conjugated to Sepharose A beads slurry, washed, and resolved by SDS-PAGE. Proteins were transferred to nitrocellulose and the membrane was blocked and cut at the indicated dash line. The top and bottom portions were probed with anti-TraG and anti-PifC antisera, respectively. Cell pellets of MC4100/pML100/pRS2496 and MC4100 serve as positive and negative controls, respectively (lanes 5 and 6).


Co-IP
+ anti-PifC
- anti-PifC
MC4100 (no IP)
pML100 (TraG) - + - + + -
pRS2496 (PifC) + + + + + -
TraG
PifC
1 2 3 4 5 6

6.3 Discussion

The role of F PifC as a repressor for the conjugal transfer of the promiscuous IncP plasmids is investigated in this study. When F and RP4 plasmids are both present in a cell, RP4 conjugation is greatly reduced (Tanimoto and Iino, 1983; Table 6.1). The mechanism of this inhibition has been perplexing but is believed to involve RP4 TraG protein. Studying the promoter of RP4 *traG* allowed us to conclude that PifC does not inhibit *traG* transcription. In addition, the level of TraG is unaltered in the presence of F PifC. Interaction between RP4 TraG protein and F PifC is shown using a bacterial two-hybrid system, and further using cross-linking and co-immunoprecipitation analyses. This is the first study to reveal that TraG interacts with a regulator protein of a different Inc group.

F TraD, RP4 TraG, R388 TrwB are the best studied coupling proteins in conjugative systems (Schroder *et al.*, 2002; Gomis-Ruth and Coll, 2001). Since conjugation uses a type IV secretion system, these TraG-like proteins are also termed type IV coupling protein (T4CP; Tato *et al.*, 2007). The crystal structure of one T4CP, TrwB of the IncW plasmid R388, is known. The TrwB structure resembles that of ring helicases (Gomis-Ruth *et al.*, 2001). It forms a membrane-anchored hexamer, with the trans-membrane domain near the amino terminus and a long cytoplasmic tail. A central channel runs from the cytoplasmic pole to the membrane pole. The channel is 20 Å in diameter, which is enough for the transfer strand (T-strand) to pass through. The entrance of the channel is plugged by a ring of asparagine residues and restricted to 8Å in diameter. This narrow entrance is thought to be open when TrwB interacts with the relaxosome, allowing it to pass through (Llosa *et al.*, 2003). The hexamer is also involved in recognition of the substrate to be secreted in conjugation and the related type IV

secretion systems (Cabezon *et al.*, 1997). For example, the F TraD hexamer only recognizes and allows conjugation of the F relaxosome complex. F TraD cannot complement RP4 TraG in recognizing the RP4-relaxosome. Such specificity may also explain why F TraD is not blocked by PifC. Our BTH results suggest that PifC interacts with the C-terminal cytoplasmic tail of TraG and that PifC binds to the bottom of the TraG hexamer, preventing the gate from opening, thereby inhibiting RP4 conjugation. The TraG domain(s) with which PifC interacts could not be defined by truncations of *traG* fusion clones (Table 6.2 and Figure 6.6), possibly due to incorrect folding of the truncated proteins or importance of transmembrane domain in subunit interactions. Nevertheless this study provides the first evidence that F inhibits the promiscuous transfer of RP4 by interaction of the PifC protein and the coupling protein, $TraG_{RP4}$.

Chapter 7: General Discussion

7.1 Extracytoplasmic stress inhibits F conjugation

Chromosomal mutation at the *cpxA* locus was originally found to impede conjugative plasmid expression of F (Sambucetti *et al.*, 1982). Subsequently, upon discovery of the cognate regulator encoded by the *cpxR* gene (Dong *et al.*, 1993), Cpx was recognized as a two-component signal transduction system that alleviates envelope stress. In addition, Cpx was found to be essential for virulence gene expression in some pathogens, namely the *icm* and *dot* virulence genes in *Legionella pneumophila* (Gal-Mor and Segal, 2003), the *pap* genes in uropathogenic *E. coli* (Hung *et al.*, 2001), the *invE* gene and the type III secretion system in *Shigella sonnei* (Mitobe *et al.*, 2005), the lipase and colonization genes in *Xenorhabdus nematophila* (Herbert *et al.*, 2007), and *Yersinia enterocolitica* (Heusipp *et al.*, 2004). Over the past decade, studies on Cpx focused mainly on its downstream regulon and how their gene products assist in combating envelope stress. With better understanding of the Cpx system nowadays, this is the first study to revisit its effect on F conjugation since 1993.

It is interesting that Cpx, an envelope stress response regulon, contributes to the regulation of F conjugation through inactivation of its cytoplasmic regulator, TraJ. This is in contrast to the classical Cpx regulon where genes are regulated at the transcriptional level, involving activation or repression of promoters by CpxR-P. The control of other signaling pathways by Cpx through diverse mechanisms suggests that Cpx is an important factor in the hierarchy of regulatory networks underlying cell adaptations (Dorel *et al.*, 2006). In the case of F, activation of the Cpx system in times of extracytoplasmic stress causes posttranscriptional degradation of F TraJ by HslVU; this prevents the formation of F pili that otherwise span the envelope and establish contact with surrounding F^- cells.

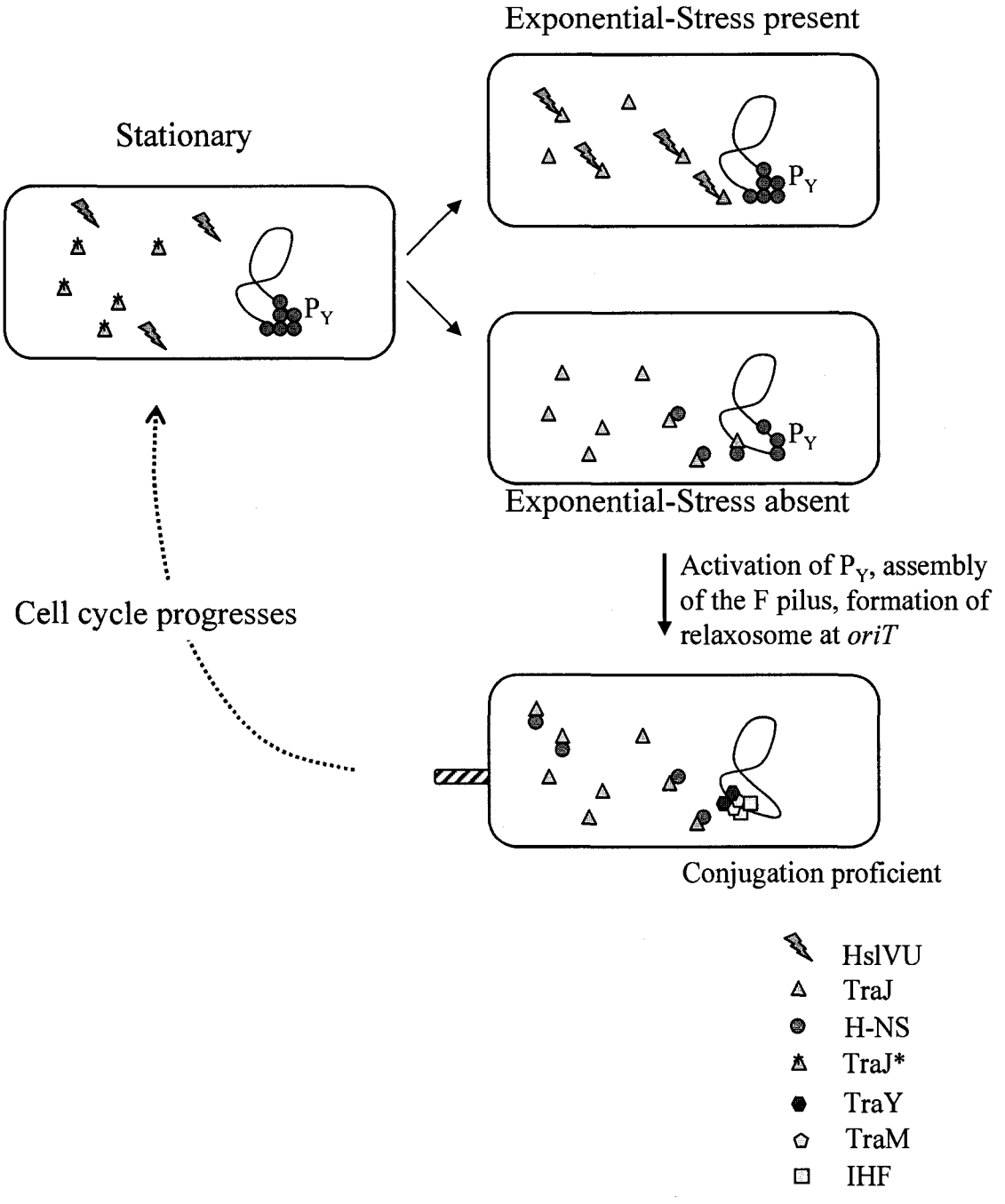
7.2 Degradation of TraJ in times of envelope stress

This study underlines the importance of the amount of active TraJ in F^+ cells for establishing the mating apparatus. Based on this and previous findings (Frost and Manchak, 1998), the majority of TraJ molecules present in stationary phase cells are potentially inactive. This pool of TraJ, which we refer to as TraJ*, is modified from TraJ as cells age and is proposed to be incapable of activating the major *tra* operon, as well as being resistant to degradation by cellular proteases. Currently, the type of modification TraJ undergoes is unclear. That only unmodified TraJ is capable of DNA binding may explain why *in vitro* binding of TraJ to P_Y has been difficult to demonstrate. If the appearance of active TraJ is only transient in early log phase, and once the mating apparatus is synthesized, TraJ is modified, then purified TraJ proteins may have undergone inactivation during the purification process by modifier(s) in the cell-free extract. As a result, it is difficult to obtain purified, active TraJ. A semi-conserved, putative helix-turn-helix motif has been suggested to be within TraJ C-terminal region (Takeda *et al.*, 1983; Frost *et al.*, 1994; Rodriguez-Maillard and Frost, personal communication). Modification of TraJ by cellular or plasmid-encoded factor(s) may obscure its DNA binding function by changing its conformation. Similarly, modification of TraJ may render it resistant to degradation by occluding its protease recognition site. Unfortunately, little is known about the substrate specificity of HslVU, since experimentations on the natural substrates of HslVU (SulA, σ^H, and RcsA) are hampered by the difficulty of expressing and solubilising these proteins at high levels and the lack of structural information to guide modification of the substrates (Kwon *et al.*, 2004). It has been shown that mutations on two amino acids, L57 and M89, on the exterior of SulA reduce its binding with the chaperone HslU (Lee *et al.*, 2003). The M89I substitution is

positioned in between a turn and an α-helix. Disruption of the amino acid residues in the TraJ helix-turn-helix region renders it conjugative-deficient (Rodriguez-Maillard and Frost, unpublished results). In accord with our hypothesis, modification of TraJ may lead to a conformational change that prevents it from being recognized by HslVU or bind to P_Y.

Our data shows that the levels of HslVU are increased when the Cpx envelope stress response system is activated. However, it was not merely an increase in the HslVU protease in F^+ *cpxA** cells that results in the reduction of TraJ levels, but also an increased susceptibility of TraJ to the protease in the presence of envelope stress. Parallel to the findings of Hernday *et al.*, (2004) that CpxR-P must be present immediately following DNA replication in order to bind to the *pap* operon and inhibit its expression in newly divided cells, our findings suggest that envelope stress must be present when bacterial growth resumes in order to degrade TraJ and inhibit *tra* expression. Chapter 4 shows that if the Cpx system is activated in mid-log phase when cells are already piliated, TraJ is not subject to degradation and conjugation is not affected. Accordingly, when growing cells experience envelope stress, nascent, unmodified TraJ can be degraded by HslVU protease as a way to shut off F conjugation (Figure 7.1, see below). This is especially important in new transconjugants, where F and F-like plasmids are transiently de-repressed during epidemic spread. De-repression will be limited in cells experiencing envelope stress through activation of the Cpx system that degrades the essential F activator, TraJ. Prevention of F piliation and conjugation is desirable for cells under envelope stress, since they require a considerable investment in energy and metabolic resources, and piliation leads to extensive alteration of the cell envelope (Nishiyama *et al.*, 2008). In addition, downregulation of F *tra* will avert infection by F-specific phage.

Figure 7.1 Regulation of F *tra* expression in stationary and exponential phases and in the presence of stress. During stationary phase, F TraJ is modified and inactive (TraJ*). H-NS silences P_M, P_J, and P_Y. Transcription of the *tra* operon is repressed. When growth resumes, or when new transconjugants express fresh TraJ, unmodified TraJ alleviates repression by H-NS, and together with cellular SfrA (ArcA), activates transcription of P_Y. Subsequently, assembly of the F pilus and formation of the relaxosomal complex at *oriT* prepare this cell to be conjugative-proficient. In another scenario, if envelope or cytoplasmic stress is enduring, degradation of TraJ by HslVU and presumably other proteases results in prolonged repression of P_Y. As a result, the F pilus is not assembled and the cell becomes conjugative-deficient. For simplicity, only the F plasmid is shown and the chromosomal DNA is not shown in the diagram.


Exponential-Stress present
Stationary
P_Y
P_Y
P_Y
Exponential-Stress absent
Activation of P_Y, assembly of the F pilus, formation of relaxosome at *oriT*
Cell cycle progresses
Conjugation proficient
HslVU
TraJ
H-NS
TraJ*
TraY
TraM
IHF

7.3 *rpoH* encodes a sigma factor that is important to the existence of F

The requirement of σ^H for F replication was first documented in 1986 (Wada *et al.*, 1986). In addition to the *repE* promoter, the *traM* promoter, P_{M2}, was also noted to contain σ^H-consensus binding sequences (Penfold, Ph.D. Thesis, 1995). This study reveals a novel role for σ^H in terms of the survival of F. Conjugation and transcription of *traJ* mRNA was found to be diminished in KY1621 (*rpoH*)/ pED851. Strikingly, the requirement of σ^H for *traJ* transcription is unnecessary in an *rpoH hns* double mutant harbouring pED851. Taken together, σ^H is hypothesized to initiate transcription of a cellular/plasmid factor that is able to relieve H-NS repression at P_M, P_J or P_Y. Our study shows that the *topA* gene product, the DNA topoisomerase omega fragment, is not responsible for such a de-silencing function at the *tra* promoters.

In searching for the proposed H-NS binding factor, we reasoned that it may possess chaperone activity. Alternatively, this factor could serve to modify or complex with TraJ in order to antagonize H-NS, since TraJ has been suggested to have a role in relieving H-NS repression (Will and Frost, 2006a). We tested the possibility of F TraR, a chaperone-like protein that is encoded in the long P_Y transcript, as being regulated by σ^H. Preliminary results suggest that a weak P_{traR} does exist, although it is not activated by σ^H (Villeneuve, Beadle and Frost, unpublished results). Accordingly, the role of TraR appears to be stabilizing TraJ or increasing the activity of TraJ since an absence of *traR* in a wild-type cell harboring pOX38::*traR354* mutant resulted in a delay in conjugation.

7.4 Regulation of F conjugation

The current study aims to explore the mechanism of TraJ activity further. When growth resumes, σ^H is hypothesized to initiate transcription of *traM*, since P_{M2} is proposed to be regulated by this alternative sigma factor (Penfold, Ph.D. Thesis, 1995).

The signal that activates σ^H is currently unknown, but is inferred to be due to an increased amount of free σ^H molecules in the cell as chaperones DnaK/J, GrpE and GroEL are occupied by nascent, unfolded polypeptides in exponential growth phase. The residual transcription of *traM* observed in the TraM immunoblot in KY1621 (*rpoH*) /pJLac113 (Figure 5.1) may reflect the fact that this promoter is subject to transcription initiation by both σ^{70} and σ^H. In the absence of σ^H, transcription is initiated by σ^{70}, a phenomenon that is common to promoters regulated by alternative sigma factors (Typas *et al.*, 2007; Li *et al.*, 2000). Using the galactose (*gal*) operon as an example, studies have suggested that the frequency of Rho-dependent transcription termination could vary between the two promoters, P1 and P2 (Lee *et al.*, 2008). One of the ways to couple transcription initiation to termination is that each RNA polymerase in an elongation complex "remembers" which promoter it has just left.

In F^+ wild-type cells, TraJ is translated from the readthrough of the *traM-traJ* transcript, which is initiated at P_{M2} by σ^H. Since the *traM* transcript initiated from P_{M1} is more abundant (Penfold *et al.*, 1996) and that *traM-traJ* transcripts are destabilized by host Hfq (Will and Frost, 2006b), the amount of TraJ produced by the readthrough transcript is relatively low. Currently, TraJ is proposed to be a dimer (Arutyunov, Rodriguez-Maillard and Frost, unpublished results) that undergoes modification by other factors, such as TraR, in order to relieve H-NS repression. Studies on H-NS antagonists have shown that they can be bound to H-NS directly, such as TopA (Butland *et al.*, 2005; Stewart *et al.*, 2005), and prevent its binding to DNA. Alternatively, they can compete with H-NS for binding to the promoter region as a way to remove H-NS from the promoter. Furthermore, the antagonist can perform dual functions by binding to the promoter and recruiting the RNAP, such as RovA in *Yersinia* (Heroven *et al.*, 2004).

Interestingly, the sequence and the charge of the amino acids of the TraJ, SlyA and RovA C-terminal regions are similar (Arutyunov, Rodriguez-Maillard and Frost, unpublished results). In addition, these regulators contain a helix-turn-helix motif that can bind to promoter DNA. Therefore, TraJ, initially synthesized in small amounts, binds to P_Y and activates transcription of the long *tra* operon. The first gene product, TraY, would then bind and further activate P_M and P_Y. Activation of P_M, P_J, and P_Y would result in the relief of H-NS repression by TraJ and TraY. If constitutive envelope stress is present at the initiation of de-silencing, such as in the presence of the *cpxA101** mutation, TraJ would be degraded by HslVU and activation of P_Y would be inhibited.

Alternatively, σ^H may activate transcription of another factor in addition to *traM*. This factor is hypothesized to bind H-NS and release it from P_J. Subsequently, *traJ* could be transcribed and translated, and newly synthesized TraJ could then activate P_Y. This model illustrates a more direct effect for σ^H, and is supported by previous findings that a single major transcript originating from P_J was detectable as early as after 3 hours of growth (Will *et al.*, 2004). To detect cellular factors that can potentially restore *traJ* transcription in KY1621/pJLac113, a gene library that overexpresses each of the *E. coli* open reading frames (Zolli-Juran *et al.*, 2003) was transformed, and positive clones were identified by LB X-gal plates (Beadle and Frost, unpublished results). Unfortunately, this preliminary experiment led to selection of a few false positive clones and identification of this σ^H-regulated cellular factor thus remains inconclusive.

7.5 F, the selfish plasmid, inhibits transfer of IncP plasmid by utilizing PifC

In comparison to the F plasmid, IncP plasmids are considered "promiscuous", or broad-host-range (Thomas and Smith, 1986). This is because expression of the P plasmid replication and transfer genes is autonomous, or independent of host proteins. As a result,

the RP4 plasmid is able to transfer and stably maintain itself in a wide variety of Gram-negative bacteria (Thomas and Smith, 1987). In the F system, however, host-encoded factors like SfrA (ArcA), IHF, and Fis proteins are required directly or indirectly to activate *tra* operon transcription as well as relaxosome formation (Silverman *et al.*, 1991, Tsai *et al.*, 1990; Will and Frost, unpublished). In order to compete with the broad-host-range RP4 plasmid, F has evolved *pifC*, which encodes an inhibitor that impedes transfer of RP4 when both plasmids coexist in the same host (Tanimoto and Iino, 1983).

The *pif* operon resides outside the *tra* region on the "dark side" of F (Gubbins *et al.*, 2005). The presence of Pif inhibits replication of the female-specific phage T7, a feature that is beneficial to F^+ hosts (Wang *et al.*, 1999). PifC is responsible for autorepression of the *pif* operon, thus maintaining the levels of PifA and PifB. In addition, PifC is known as RepC, a replication protein that is essential for initiation of replication at *oriV* (Wehlmann and Eichenlaub, 1980; Tanimoto and Iino, 1984). Therefore using this important protein to inhibit RP4 transfer seems to be a way to increase the competiveness of F during epidemic spread in *E. coli*.

The first study of the inhibition of RP4 transfer by F was documented in 1983 (Tanimoto and Iino, 1983). Subsequently, PifC was postulated as a DNA-binding protein that represses expression of RP4 transfer genes (Miller *et al.*, 1985). It was not until the study by Santini and Stanisich in 1998 that a mechanism of inhibition and the involvement of $TraG_{RP4}$ was reported (Santini and Stanisich, 1998). However, evidence of direct interaction between PifC and $TraG_{RP4}$ has not been previously demonstrated. With the bacterial two-hybrid system and co-immunoprecipitation experiments that make interaction of even membrane proteins possible (Karimova *et al.*, 1998), PifC was shown to interact with the cytoplasmic region of $TraG_{RP4}$. Thus, PifC performs two functions

including binding to DNA (the *pifO* operator or *oriV*) and binding to a protein (other replication proteins or $TraG_{RP4}$).

$TraG_{RP4}$ is a type IV coupling protein (T4CP) that serves as a gate in the inner membrane for DNA to pass through during RP4 conjugation (Tato *et al.*, 2007). TraG or TraG-like proteins have been demonstrated to interact with both the Mpf proteins ($TrhB_{R27}$; Gilmour *et al.*, 2003) and the relaxosome complex (Mob_{pBHR1}, Szpirer *et al.*, 2000; $MbpB_{pLV22a}$, Thomas and Hecht, 2007; and $TraM_F$, Lu and Frost, 2005). It is interesting to note that some mobilizable elements are only mobilized by RP4 and R388 but not F, due to the specificity of the F TraD coupling protein (Szpirer *et al.*, 2000; Thomas and Hecht, 2007). One of the relaxosome proteins, TraM, is able to sense environmental stress and convey this signal by changing its state of protonation (Lu *et al.*, 2006). Four protonated glutamic acid residues (Glu88) in the tetrameric domain of TraM increase TraM-TraD interaction (Lu and Frost, 2005). *In vivo* experimentation with $TraD_F$ showed that the N-terminal domain is essential for oligomerization, and moreover, the presence of F-encoded factor(s) is required for a stable TraD complex formed in the inner membrane (Haft *et al.*, 2007). The crystal structure of one of the T4CPs, $TrwB_{R388}$, has shown that the cytoplasmic domain has a hexameric pore-like structure (Hormaeche *et al.*, 2002; Moncalian *et al.*, 1999; Gomis-Ruth *et al.*, 2001). The N-transmembrane domain of $TrwB_{R388}$ was proposed to be required for binding specific nucleotides (Hormaeche *et al.*, 2006). Thus, in a cell harbouring RP4, the presence of RP4-encoded factor(s) causes the oligomerization of the $TraG_{RP4}$ coupling protein in the cell membrane, possibly through interaction between the RP4 transferosome and the N-terminal periplasmic domain of $TraG_{RP4}$. A positive conjugation signal is triggered via the C-terminal cytoplasmic domain of $TraG_{RP4}$, which interacts with the relaxosome

complex. $TraG_{RP4}$ then pumps the DNA through using energy from the hydrolysis of ATP (Gomis-Ruth *et al.*, 2001). In the presence of F, however, PifC interacts specifically with the C-terminal domain of $TraG_{RP4}$, preventing it from binding to the RP4 relaxosome. Accordingly, F can out-compete RP4 by having a high specificity F coupling protein, TraD (Thomas and Hecht, 2007), and by inhibiting the RP4 coupling protein, TraG. These strategies would ensure successful conjugation of F but not RP4. As observed from the high level of β-galactosidase activity in the BTH experiments, interaction between $TraG_{RP4}$ and PifC appears to be relatively strong, which suggests that the interaction is not transient.

7.6 Future perspectives

The mechanism by which the regulatory circuit of the Cpx pathway reduces F conjugation was shown to be unexpectedly complex. Whereas this study identifies HslVU as the protease responsible for TraJ degradation in Cpx-activated cells and in *cpxA101** cells experiencing perceived stress, proteolysis of TraJ is not limited to this protease pair. Chapter 3 presents evidence that eliminating the HslV protease did not result in full restoration of TraJ in *cpxA** *hslV* in the MC4100 background strain harbouring pOX38-Km. It has been reported that one regulator, for example σ^H or SulA, can be subjected to degradation by multiple proteases (Wu *et al.*, 1999; Kanemori *et al.*, 1999a). Therefore besides HslVU, other proteases exist that can degrade TraJ in times of envelope stress. Previous studies have shown that single mutations in *hflB*, *lon*, *clpP* or *recA* do not result in full restoration of TraJ in *cpxA**. Eliminating another protease encoded by *clpB*, *clpXP*, or *hflC*, or constructing a multiple protease-deficient strain might allow full restoration of TraJ in MC4100 *cpxA101**.

One important finding from this study is possible modification of TraJ. As cells enter stationary phase, TraJ could be modified to give TraJ* that appears to be resistant to degradation. When cells enter exponential growth again, modification of TraJ* to TraJ or synthesis of new TraJ could be an important mechanism for de-silencing H-NS repressed genes. Possible mechanisms of modification of TraJ are suggested but not limited to the phosphorylation, dimerization, or/and conformational change due to being bound to DNA. It is also possible that CpxR-P, a phosphatase that dephosphorylates non-cognate sensor kinases (Dorel *et al.*, 2006), may catalyze modification of F TraJ directly. Although F^+ *cpxR* mutants are able to conjugate as efficiently as wild-type cells (Gubbins *et al.*, 2002), a time-course mating assay and a promoter assessment study of P_Y could be performed to monitor the activity of TraJ over a 24-hour period in *cpxR* or other phosphatase- or kinase-deficient strains. It is also possible that a chaperone of TraJ, but not TraJ itself, is being modified and thus affecting the stability and activity of TraJ. As such, the phosphatase or kinase identified in the former experiment should be purified and incubated with TraJ to examine whether the modification is direct, using *in vitro* studies. The mechanism of TraJ modification awaits further research. Nonetheless this study uncovers the characteristics of the two forms of TraJ and their fates in times of stress.

Future research should also investigate involvement of σ^H for initiation of transcription at P_M. While the consensus binding site was found, true activation will be revealed through footprinting and primer extension experiments. Whether P_{M2} is silenced in KY1621 is another interesting area to explore. This is the answer to the question in our model that σ^H serves to initiate *traM* transcription at P_{M2} that potentially reads through into *traJ* when cell growth resumes. Future experiments can also utilize the *rpoB3595*

mutant (Li *et al.*, 2000), which synthesizes an RNA polymerase that terminates transcription with lower efficiency than the wild-type; or the *rho* partial mutant (Lee *et al.*, 2008), which has decreased Rho activity leading to impaired transcriptional termination. Such a mutation in combination with *rpoH* (KY1621) would rescue *traJ* expression if transcription initiation at P_{M1} can bypass termination and read into *traJ*. Performing mutational studies within the P_{M2} DNA sequence would also delimit whether σ^H is directly involved in *traM* and *traJ* transcription.

Several attempts to construct a gene library for this purpose were not successful due to low recombination frequency between the chromosomal fragments and the chosen vector pBC-SK. With a genomic library that over-expresses each *E. coli* ORF in comparable amounts (Zolli-Juran *et al.*, 2003) one can identify the gene(s) that is responsible for de-silencing H-NS by using a detection system that selects for activation of P_Y in *rpoH* null mutants. Care must be taken to consider both host- and plasmid-encoded factors in this screening method. Currently, over-expression of *dnaK*, *topA*, *yhdN*, and F *traR* (Beadle and Frost, unpublished results) have been proposed and have been discounted as candidates in suppressing the *rpoH* mutation in terms of P_Y activation.

Studies are also required to determine the amino acids on the cytoplasmic tail of $TraG_{RP4}$ that interact with F PifC. Such an understanding will extend our knowledge on the mechanism employed by F to inhibit transfer of RP4 or perhaps other conjugative plasmid transfer when both plasmids coexist. Thus far TrwB in R388 is the only coupling protein whose structure has been solved (Gomis-Ruth *et al.*, 2001). The comparison of the structures of $TraG_{RP4}$ and $TraD_F$ will certainly help deduce the difference between the coupling proteins and the domains on $TraG_{RP4}$ that interact with PifC. PifC, which

possesses both DNA- and protein-binding characteristics, is also an important F regulator to be explored.

Chapter 8: Appendix I- The transcriptional profile of *cpxA101**

8.1 Introduction

Bacteria reside in a dynamic environment that ranges from, for example, warm, nutritious intestinal tracts inside animals, to cool, nutrition-deficient water trough sediments (Hancock *et al.*, 2001). Being able to activate pathways that elicit a rapid response to cope with challenges is important. In *E. coli*, extracytoplasmic stress is sensed by both the Cpx two-component signal transduction system and the σ^E pathway that activates transcription of response genes via the alternative sigma factor (Raivio and Silhavy, 1999). Upon signal activation, CpxR, the response regulator, is phosphorylated through a phospohorelay signal that is initiated in the envelope. CpxR-P regulates genes that encode proteins, for example, the periplasmic protease DegP (Danese *et al.*, 1995) or the periplasmic disulfide oxidoreductase DsbA (Danese and Silhavy, 1997), to combat envelope stress. In the σ^E pathway, activation of the pathway is initiated by proteolysis of the anti-sigma factor RseA through the membrane anchored DegS and membrane embedded YaeL (Alba *et al.*, 2002). Released σ^E, along with RNAP, activates genes that are required to alleviate protein misfolding in the envelope (Ades, 2004). A third envelope stress response system, the BaeS/R signal transduction pathway, was found to be distinct of the Cpx and σ^E pathways (Raffa and Raivio, 2002).

In order to examine the cellular factor(s) that are up-regulated in response to activation of the Cpx pathway, microarray analysis was performed to obtain the transcriptional profile of *E. coli* using wild-type and *cpxA101** strains harbouring pOX38-Km. The *cpxA101** mutation locks the cell in a constitutively "Cpx-on" state (Raivio and Silhavy, 1997) due to high CpxR-P levels. In Chapter 3, genes encoding chaperones or proteases that can potentially degrade F TraJ were chosen for further study. In this appendix, complete list of the microarray data is presented.

8.2 Results and Discussion

Two strains, *E. coli* MC4100 and *cpxA101**, both harbouring pOX38-Km, were used in the microarray analysis (Affymetrix) as described in the Materials and Methods. The array was scanned at 570 nm with a GeneArray scanner (Affymetrix). Data analysis was performed by using the Affymetrix Microarray Suite 5.0 software. The software calculates change calls, change *p*-values, and signal log ratio. The intergenic regions and the genes in which no signal was detected in both strains were not analyzed further. The software uses statistical algorithms to calculate change *p*-values. Among the genes listed in Tables 8.1 and 8.2, the *p*-values were smaller than 0.005, suggesting that the changes in expression levels are significant. Since *cpxA101** is pleiotropic (De Wulf *et al.*, 1999), the current study cannot distinguish genes that are secondary to the control of the Cpx system. As a reference, genes whose promoters have been shown to bind CpxR-P and those that have been recognized by the CpxR-P matrix screening method (De Wulf *et al.*, 2002) are indicated in the tables.

Table 8.1 Genes that are up-regulated in the *cpxA101 mutant as detected by microarray analysis[a].**

Gene	Blattner no.	Gene description[b]	*In vitro* binding of CpxR-P[c]	Detected by CpxR-P screening[d]
		Amino Acid		
sdaA	b1814	L-serine deaminase	no	no
argS	b1849	arginine tRNA synthetase	no	no
leuZ	b1909	leucine tRNA4	no	no
cysT	b1910	cysteine tRNA	no	no
serU	b1975	serine tRNA2	no	no
proL	b2189	proline tRNA2	no	no
argW	b2348	arginine tRNA 5	no	no
gltX	b2400	glutamate tRNA synthetase, catalytic subunit	no	no
sseB	b2522	enhanced serine sensitivity	no	no
glyA	b2551	serine hydroxymethyltransferase	no	no
metZ	b2814	initiator methionine tRNA f1; triplicate gene	no	no
glyU	b2864	glycine tRNA1	no	no
ilex	b3069	ile tRNA2	no	no
metY	b3171	initiator methionine tRNA f2	no	no
leuU	b3174	leucine tRNA2	no	no
trpS	b3384	tryptophan tRNA synthetase	no	no
proK	b3545	proline tRNA 1	no	no
tdh	b3616	threonine dehydrogenase	no	no
kbl	b3617	2-amino-3-ketobutyrate CoA ligase (glycine acetyltransferase)	no	no
tyrU	b3977	tyrosine tRNA2	no	no
glyT	b3978	glycine tRNA2, UGA suppression	no	no
alr	b4053	alanine racemase I	no	no
lysU	b4129	lysine tRNA synthetase, inducible; heat shock protein	no	no
pheU	b4134	phenylalaline tRNA	no	no
leuX	b4270	leucine tRNA 5 (amber [UAG] suppressor)	no	no
		Cell Division		
sulA	b0958	FtsZ ring inhibitor	no	no
hflB	b3178	degrades sigma 32, integral membrane peptidase, cell division protein	no	yes
ftsJ	b3179	cell division protein	no	yes
mreB	b3251	regulator of FtsI, PBP3, septation	no	no

		function		
gidB	b3740	glucose-inhibited division; chromosome replication	no	no
gidA	b3741	glucose-inhibited division; chromosome replication	no	no
mioC	b3742	initiation of chromosome replication	no	no
		Damage/Resistance		
dinI	b1061	damage-inducible protein I	no	no
marA	b1531	multiple antibiotic resistance; transcriptional activator of defense systems	no	no
marB	b1532	multiple antibiotic resistant protein	no	no
bcp	b2480	bacterioferritin comigratory protein	no	no
yggT	b2952	putative resistance protein	no	no
		DNA		
add	b1623	adenosine deaminase	no	no
	b2496	putative DNA replication factor	no	no
guaA	b2507	GMP synthetase	no	no
grpE	b2614	phage lambda replication, host DNA synthesis, heat shock protein, protein repair	no	no
recN	b2616	protein used in recombination and DNA repair	no	no
recA	b2699	DNA strand exchange and renaturation, DNA-dependent ATPase, DNA- and ATP- dependent coprotease	no	no
thyA	b2827	thymidylate synthetase	no	no
gidB	b3740	glucose-inhibited division; chromosome replication	no	no
gidA	b3741	glucose-inhibited division; chromosome replication	no	no
uvrD	b3813	DNA-dependent ATPase I and helicase II	no	no
uvrA	b4058	excision nuclease subunit A	no	no
priB	b4201	primosomal replication protein N	no	no
		Envelope Protein		
secD	b0408	protein secretion	no	no
secF	b0409	protein secretion	no	no
ybeJ	b0655	putative periplasmic binding transport protein	no	no
pspA	b1304	phage shock protein (IM)	no	no
pspB	b1305	phage shock protein (IM)	no	no
spy	b1743	periplasmic protein related to	no	yes

		spheroplast formation		
htpX	b1829	heat shock protein, integral membrane protein	no	no
cvpA	b2313	membrane protein required for colicinV production	no	no
lepA	b2569	GTP-binding elongation factor, maybe IM protein	no	no
yiaD	b3552	putative OM protein	no	no
glmU	b3730	N-acetyl glucosamine-1-phosphate uridyltransferase	no	no
rfe	b3784	UDP-GlcNAc-undecaprenylphosphate GlcNAc-1-phosphate transferase; synthesis of enterobacterial common antigen (ECA)	no	no
cpxP	b3914	periplasmic protein	no	yes
murB	b3972	UDP-N-acetylenolpyruvoyl glucosamine reductase	no	no
lamB	b4036	phage lambda receptor protein; maltose high-affinity receptor	no	no
slt	b4392	soluble lytic murein transglycosylase	no	yes
		Kinase / Phosphatase		
ackA	b2296	acetate kinase	no	no
yrfG	b3399	putative phosphatase	no	no
pita	b3493	low-affinity phosphate transport	no	no
grnK	b3648	guanylate kinase	no	no
phoU	b3724	negative regulator for pho regulon and putative enzyme in phosphate metabolism	no	no
ppa	b4226	inorganic pyrophosphatase	no	no
		Protease / Chaperone		
dnaK	b0014	chaperone Hsp70	no	yes
dnaJ	b0015	heat shock protein	no	no
lspA	b0027	prolipoprotein signal peptidase	no	no
htrA (degP)	b0161	periplasmic serine protease; heat shock protein HtrA	yes (Pogliano *et al.*, 1997; Raivio and Silhavy, 1997)	yes
yajG	b0434	putative polymerase/proteinase	no	no
clpP	b0437	ATP-dependent proteolytic subunit of clpA-clpP serine protease; heat shock protein F21.5	no	no
lon	b0439	DNA-dependent specificity	no	no

		component of clpP serine protease, chaperone		
htpG	b0473	chaperone Hsp90, heat shock protein C 62.5	no	no
	b0955	putative ATP-dependent protease	no	no
	b1599	possible chaperone	no	no
	b1600	possible chaperone	no	no
htpX	b1829	heat shock protein, intergral membrane protein	no	no
pepB	b2523	putative peptidase	no	no
hscA	b2526	heat shock protein, chaperone, member of Hsp70 protein family	no	no
lepB	b2568	leader peptidase (signal peptidaseI)	no	no
clpB	b2592	heat shock protein	no	no
grpE	b2614	phage lambda replication, host DNA synthesis, heat shock protein, protein repair	no	no
recA	b2699	DNA strand exchange and renaturation, DNA-dependent ATPase, DNA- and ATP- dependent coprotease	no	no
ygiD	b3064	putative O- sialoglycoprotein endopeptidase	no	no
prlC	b3498	oligopeptidase A	no	no
ibpB	b3686	heat shock protein	no	no
ibpA	b3687	heat shock protein	no	no
dsbA	b3860	protein disulfide isomerase I, essential for cytochrome C synthesis and formate-dependent reduction	yes (Pogliano *et al.*, 1997)	yes
hslU	b3931	heat shock protein *hslVU*, ATPase subunit, homologous to chaperones	no	no
hslV	b3932	heat shock protein hslVU, elleted i-related peptidase subunit	no	no
lexA	b4043	regulator for SOS (*lexA*) regulon	no	no
yibK	b4046	putative regulator	no	no
lysU	b4129	lysine tRNA synthetase, inducible; heat shock protein	no	no
mopB	b4142	GroES, chaperone binds to Hsp60 in presence of Mg-ATP, suppressing its ATPase activity	no	no
mopA	b4143	GroEL, chaperone Hsp60, peptide-dependent ATPase, heat shock protein	no	no
efp	b4147	elongation factor P (EF-P)	no	no
		Protein Folding		
ppiB	b0525	peptidyl-prolyl *cis-trans* isomerase B	no	no

		(rotamaseB)		
tktA	b2935	transketolase/ isozyme	no	no
ygiC	b3038	putative synthetase/ amidase	no	no
ppiA	b3363	peptidyl-prolyl cis-trans isomerase A (rotamaseA)	yes (Danese and Silhavy, 1997)	yes
ppiC	b3775	peptidyl-prolyl *cis-trans* isomerase C (rotamaseC)	no	no
yibO	b3612	putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase	no	no
dsbA	b3860	protein disulfide isomerase I, essential for cytochrome c synthesis and formate-dependent reduction	no	no
sodA	b3908	superoxide dismutase, manganese	no	no
pgi	b4036	glucosephosphate isomerase	no	no
msrA	b4219	peptide methionine sulfoxide reductase	no	no
Regulator				
fur	b0683	negative regulator	no	no
relB	b1564	negative regulator of translation	no	no
lrhA	b2289	NADH dehydrogenase transcriptional regulator, LysR family	no	no
yfhF	b2528	putative regulator	no	no
yhdM	b3292	putative transcriptional regulator	no	no
cspA	b3556	cold shock protein 7.4, transcriptional activator of *hns*	no	no
phoU	b3724	negative regulator for pho regulon and putative enzyme in phosphate metabolism	no	no
birA	b3973	biotin-[acetyl CoA carboxylase] holoenzyme synthetase and biotin operon repressor	no	no
lexA	b4043	regulator for SOS (*lexA*) regulon	no	no
yibK	b4046	putative regulator	no	no
soxS	b4062	regulation of superoxide response regulon	no	no
hflX	b4173	GTP-binding subunit of protease specific for phage lambda cII repressor	no	no
hflK	b4174	protease specific for phage lambda cII repressor	no	no
hflC	b4175	protease specific for phage lambda cII repressor	no	no
Ribosome				

yggV	b2954	putative ribosomal protein	no	no
rpsU	b3065	30S ribosomal subunit protein S21	no	no
rpsG	b3341	30S ribosomal subunit protein S7, initiates assembly	no	no
rpmG	b3636	50S ribosomal subunit protein L33	no	no
rpmH	b3703	50S ribosomal subunit protein L34	no	no
rpmE	b3936	50S ribosomal subunit protein L31	no	no
rpsF	b4200	30S ribosomal subunit protein S6	no	no
		Sigma Factor		
yfiA	b2597	putative *yhbH* sigma 54 modulator	no	no
rpoD	b3067	RNA polymerase, sigma 70 factor, regulation of proteins induced at high temperature	no	no
rpoH	b3461	RNA polymerase, sigma 32 factor, regulation of proteins induced at high temperature	yes (De Wulf *et al.*, 1999)	yes
		Sugar Metabolism/Energy		
purK	b0522	phosphoribosylaminoimidazole carboxylase: AIR carboxylase, CO_2-fixing subunit	no	no
purE	b0523	phosphoribosylaminoimidazole carboxylase, catalytic subunit	no	no
ybiX	b0877	putative enzyme	no	no
prsA	b1207	phosphoribosylpyrophate synthetase	no	no
fabI	b1288	enoyl-[acyl-carrier-protein] reductase (NADH)	no	no
ydfG	b1539	putative oxidoreductase	no	no
purT	b1849	phosphoribosylglycinamide formyltransferase 2	no	no
rpiA	b2914	ribosephosphate isomerase	no	no
tktA	b2935	transketolase/ isozyme	no	no
prlC	b3498	OM protein induced after carbon starvation	no	no
gor	b3500	glutathione oxidoreductase	no	no
kbl	b3617	2-amino-3-ketobutyrate CoA ligase (glycine acetyltransferase)	no	no
ubiB	b3844	ferrisiderophore reductase; flavin reductase (NADPH: flavin oxidoreductase)	no	no
fpr	b3924	ferredoxin-NADP reductase	no	no
menG	b3929	menaquinone biosynthesis, unknown	no	no
menA	b3930	1,4-dihydroxy-2-naphthoate – dimethylmenaquinone	no	no

		Transcription		
nusB	b0416	transcription termination; L factor	no	no
rstA	b1608	response transcriptional regulatory protein (RstB sensor)	no	no
purR	b1658	transcriptional repressor for *pur* regulon, *glyA*, *glnB*, *prsA*, *speA*	no	yes
rnc	b2567	Rnase III, dsRNA	no	no
rpoD	b3067	RNA polymerase, σ^{70} factor, regulation of proteins induced at high temperature	no	no
yhdM	b3292	putative transcriptional regulator	no	no
cspA	b3556	cold shock protein 7.4, transcriptional activator of *hns*	no	no
rph	b3643	Rnase PH	no	no
spoU	b3651	putative RNA methylase	no	no
rhoL	b3782	*rho* operon leader peptide	no	no
rho	b3783	transcription termination factor Rho; polarity suppressor	no	no
		Translation		
prfB	b2891	peptide chain release factor RF-2	no	no
selC	b3658	selenocysteyl tRNA UCA (converted from serine tRNA)	no	no
efp	b4147	elongation factor P (EF-P)	no	no
miaA	b4171	delta (2)- isopentenylpyrophosphate tRNA- adenosine transferase	no	no
prfC	b4375	peptide chain release factor RF-3	no	no
		Transport / Transferase		
yla	b0459	putative transferase	no	no
msbA	b0914	ATP-binding transport protein; multicopy suppressor of *htrB*	no	no
chaA	b1216	Sodium-calcium/ proton antiporter	no	yes
pta	b2297	phosphotransacetylase	no	no
purF	b2312	amidophosphoribosyltransferase, PRPP amidotransferase	no	no
fabB	b2323	3-oxoacyl-[acyl-carrier-protein] synthase I	no	no
fadL	b2344	transport of long-chain fatty acid; sensitivity to phage T2	no	no
cysP	b2425	thiosulfate binding protein	no	no
yfhO	b2530	putative aminotransferase	no	no
glyA	b2551	serine hydroxymethyltransferase	no	no
yggB	b2924	putative transport protein	no	no
yhfC	b3364	putative transport	no	yes

pita	b3493	low-affinity phosphate transport	no	no
pyrE	b3642	orotate phosphoribosyltransferase	no	no
yicE	b3654	putative transport protein	no	no
pstB	b3725	ATP-binding component of high-affinity phosphate-specific transport system	no	no
glmU	b3730	N-acetyl glucosamine-1-phosphate uridyltransferase	no	no
pyrI	b4244	aspartate carbamoyltransferase	no	no
pyrB	b4245	aspartate carbamoyltransferase	no	no
rimI	b4373	acyltransferase for 30S ribosomal subunit protein S18; acetylation of N-terminal alanine	no	no
yjjK	b4391	putative ATP-binding component of a transport system	no	no

[a]Affymetrix microarray was performed as described in the Materials and Methods. Total mRNA was isolated from *E. coli* wild-type and *cpxA101** mutant, both containing pOX38-Km, and enriched prior to hybridization. The array was scanned at 570 nm with a resolution of 3 μm using a GeneArray scanner (Affymetrix). Data analysis was performed by using Affymetrix Microarray Suite 5.0 software.

[b]Gene descriptions are taken from the Affymetrix Expression Analysis Sequence Information Database.

[c]Promoters of genes that have been shown to bind CpxR-P

[d]Promoters recognized by the CpxR-P weight matrix, 5'-GTAAA$(N)_5$GTAAA-3' (De Wulf *et al.*, 2002).

Table 8.2 Genes that are down-regulated in the *cpxA101 mutant as detected by microarray analysis[a]**

Genes	Blattner no.	Gene Description[b]	*In vitro* binding of CpxR-P[c]	Detected by CpxR-P screening[d]
		Amino Acid		
trpB	b1261	tryptophan synthase, β protein	no	no
pheT	b1714	phenylalaline tRNA synthetase, α subunit	no	no
pheS	b1715	phenylalaline tRNA synthetase, β subunit	no	no
hisL	b2018	*his* operon leader peptide	no	no
hisG	b2019	ATP phosphoribosyltransferase	no	no
hisA	b2024	N-(5 –phospho-L-ribosyl-formimino)-5-amino-1-(5 – phosphoribosyl)-4-imidazolecarboxamide isomerase	no	no
hisF	b2025	imidazole glycerol phosphate synthase subunit in heterodimer with HisH	no	no
hisI	b2026	phosphoribosyl-amp cyclohydrolase; phosphoribosyl-ATP pyrophosphatase	no	no
proV	b2677	ATP-binding component of transport system for glycine, betaine, and proline	no	no
alaS	b2697	alanyl-tRNA synthetase	no	no
sdaC	b2796	serine transporter	no	no
sdaB	b2797	serine dehydratase (deaminase)	no	no
gcvP	b2903	glycine decarboxylase, P protein of glycine cleavage system	no	yes
gcvH	b2904	in glycine cleavage complex, carrier of aminomethyl moiety	no	yes
gcvT	b2905	aminomethyltransferase of glycine cleavage system	no	yes
speA	b2938	biosynthetic arginine decarboxylase	no	no
metK	b2942	methionine adenosyltransferase; methyl and pro	no	no
gltB	b3212	glutamate synthase, large subunit	no	no
gltD	b3213	glutamate synthase, small subunit	no	no
cysG	b3368	uroporphyrinogen III methylase; sirohaeme biosynthesis	no	no
asd	b3433	aspartate-semialdehyde	no	no

		dehydrogenase		
asnA	b3744	asparagine synthetase A	no	no
glnA	b3870	glutamine synthetase	no	no
aspA	b4139	aspartate ammonia-lyase (aspartase)	no	no
		Cell Division		
minE		cell division inhibitor	no	no
minD		cell division inhibitor	no	no
minC		cell division inhibitor	no	no
ynaF	b1376	putative filament protein	no	no
zipA	b2412	cell division protein involved in FtsZ ring	no	no
yfjN	b2630	putative cell division protein	no	no
fic	b3361	induced in stationary phase, recognized by *rpoS*, affects cell division	no	no
		Damage / Resistance		
	b1448	putative resistance protein	no	no
	b1840	putative resistance protein	no	no
yhjX	b3547	putative resistance protein	no	no
		DNA		
sbmC	b2009	SbmC protein (DNA gyrase inhibitor)	no	no
thiD	b2103	phosphomethylpyrimidine kinase	no	no
cdd	b2143	cytidine/ deoxycytidine deaminase	no	no
polA	b3863	DNA polymerase I, 3 → 5 polymerase, 5 → 3 and 3 → 5 exonuclease	no	no
		Envelope Protein		
acrB	b0462	acridine efflux pump	no	no
ompT	b0565	outermembrane protein, protease VII	no	no
oppA	b1243	oligopeptide transport; periplasmic binding protein	no	no
cls	b1249	cardiolipin synthase, a major membrane phospholipid, novobiocin sensitivity	no	no
lpp	b1677	murein lipoprotein	no	no
fliY	b1920	putative periplasmic binding transport protein	no	no
slp	b3506	OM protein induced after carbon starvation	no	no
atpF	b3736	membrane bound ATP synthase	no	no

atpE	b3737	membrane bound ATP synthase	no	no
atpB	b3738	membrane bound ATP synthase	no	no
malF	b4033	part of maltose permease, periplasmic	no	no
malE	b4034	periplasmic maltose-binding protein; substrate recognition for transport and chemotaxis	no	no
fecB	b4290	citrate-dependent iron transport, periplasmic protein	no	no
fecA	b4291	outer membrane receptor; citrate-dependent iron transport, outer membrane receptor	no	no
		Kinase / Phosphatase		
carA	b0032	carbamoyl-phosphate synthetase	no	no
carB	b0033	carbamoyl-phosphate synthetase	no	no
yaeD	b0200	putative phosphatase	no	no
psiF	b0384	induced by phosphate starvation	no	no
agp	b1002	periplasmic glucose-1-phosphatase	no	no
pdxY	b1636	pyridoxal kinase 2/ pyridoxine kinase	no	no
thiD	b2103	phosphomethylpyrimidine kinase	no	no
		Nitrogen Metabolism		
narK	b1223	nitrite extrusion protein	no	no
narG	b1224	nitrate reductase 1, alpha subunit	no	no
narH	b1225	nitrate reductase 1, beta subunit	no	no
narJ	b1226	nitrate reductase 1, delta subunit, assembly function	no	no
narI	b1227	nitrate reductase 1, cytochrome b, gamma subunit	no	no
fdnH	b1475	formate dehydrogenase –N, nitrate inducible	no	no
fdnI	b1476	formate dehydrogenase –N, nitrate inducible	no	no
nirB	b3365	nitrite reductase (NAD(P)H) subunit	no	no
nirD	b3366	nitrite reductase (NAD(P)H) subunit	no	no
nirC	b3367	nitrite reductase activity	no	no
		protease/chaperone		
pepD	b0237	aminoacyl-histidine dipeptidase (peptidase D)	no	no
prc	b1830	carboxy-terminal protease for penicillin-binding protein 3	no	no
secB	b3609	protein export; molecular chaperone; may bind to signal sequence	no	no

		Protein Folding		
acpD	b1412	acyl carrier protein phosphodiesterase	no	no
ydfG	b1539	putative oxidoreductase	no	no
hypD	b2729	pleotrophic effects on 3 hydrogenase isozymes	no	no
msrA	b4219	peptide methionine sulfoxide reductase	no	no
		Regulator		
hnr	b1235	Hnr protein, regulator response protein homolog	no	no
hns	b1237	DNA binding protein HLP-II (HU, BH2, HD, NS); pleiotropic regulator	no	no
cysB	b1275	positive transcriptional regulator for cysteine regulon	no	no
himA	b1712	integration host factor (IHF), alpha subunit, site specific recombination	no	no
uvrY	b1914	putative two-component transcriptional regulator	no	no
wzzB	b2027	regulator of length of *o*-antigen component of lipopolysaccharide chains	no	no
gatR	b2090	split galactitol utilization operon repressor, fragment 2	no	no
yeiE	b2157	putative transcriptional regulator LysR-type	no	no
yojN	b2216	putative two-component sensor protein	no	yes
rcsB	b2217	positive response regulator for capsule biosynthesis	no	no
yfeU	b2428	putative regulator	no	no
rseA	b2572	σ^E factor, negative regulatory protein	yes	yes
icc	b3032	regulator of *lacZ*	no	no
fis	b3261	site-specific DNA inversion stimulation factor; DNA-binding protein; a trans activator for transcription	no	no
yhhX	b3440	putative regulator	no	no
uspA	b3495	universal stress protein; broad regulatory function	no	no
yhiX	b3516	putative ARA C type regulatory protein	no	no
yjaE	b3995	putative transcriptional regulator	no	no

Ribosome				
rpsV	b1480	30S ribosomal subunit protein S22	no	no
rplT	b1716	50S ribosomal subunit protein L20 and regulator	no	no
rpmI	b1717	50S ribosomal subunit protein A	no	no
rrlG	b2589	23S rRNA of *rrnG* operon	no	no
rrsG	b2591	16S RNA of *rrnG* operon	no	no
rrlD	b3275	23S rRNA of *rrnD* operon	no	no
rplQ	b3294	50S ribosomal subunit protein L17	no	no
rpsD	b3296	30S ribosomal subunit protein S4	no	no
rpsK	b3297	30S ribosomal subunit protein S11	no	no
rpmJ	b3299	50S ribosomal subunit protein L36	no	no
rrlC	b3758	23S rRNA of *rrnC* operon	no	no
Sugar Metabolism/ Energy				
adhE	b1241	CoA-linked acetaldehyde dehydrogenase and iron-dependent alcohol dehydrogenase, pyruvate-formate-lyase deactivase	no	yes
nadE	b1740	NAD synthetase, prefers NH3 over glutamine	no	no
gdhA	b1761	NADP-specific glutamate dehydrogenase	no	no
manX	b1817	PTS enzyme IIAB, mannose specific	no	yes
manY	b1818	PTS enzyme IIC, mannose specific	no	yes
manZ	b1819	PTS enzyme IID, mannose specific	no	yes
yedO	b1919	putative 1-aminocyclopropane-1-carboxylate deaminase	no	no
wbbK	b2032	putative glucose transferase	no	no
wbbJ	b2033	putative o-acetyl transferase	no	no
wbbI	b2034	putative Galf transferase	no	no
wbbH	b2035	o-antigen polymerase	no	no
gatD	b2091	galactitol-1-phosphate dehydrogenase	no	no
gatC	b2092	PTS system galactitol-specific enzyme IIC	no	no
gatB	b2093	galactitol-specific enzyme IIB of phosphotransferase system	no	no
gatA	b2094	galactitol-specific enzyme IIA of phosphotransferase system	no	no
gatZ	b2095	putative tagatose 6-phosphate kinase 1	no	no
gatY	b2096	tagatose-bisphosphate aldolase 1	no	no
dld	b2133	D-lactate dehydrogenase, FAD	no	no

		protein, NADH independent		
nuoN-A	b2276-b2288	NADH dehydrogenase I	no	no
ptsH	b2415	PTS system protein HPr	no	no
ptsI	b2416	PEP-protein, glucose-specific IIA component	no	yes
hmpA	b2552	dehydropteridine reductase, ferrisiderophore reductase activity	no	no
hypD	b2729	effects on 3 hydrogenase isozymes	no	no
ribB	b3041	3,4 dihydroxy-2-butanone-4-phosphate synthase	no	no
crp	b3357	cyclic AMP receptor protein	no	no
malQ	b3416	4-alpha-glucanotransferase (amylomaltase)	no	no
malP	b3417	maltodextrin phosphorylase	no	no
malT	b3418	positive regulator of mal regulon	no	no
gpsA	b3608	glycerol-3-phosphate dehydrogenase (NAD^+)	no	no
lamB	b4036	phage lambda receptor protein; maltose high-affinity receptor	no	no
frdD	b4151	fumarate reductase, anaerobic, membrane anchor polypeptide	no	no
frdC	b4152	fumarate reductase, anaerobic, membrane anchor polypeptide	no	no
frdB	b4153	fumarate reductase, anaerobic, iron-sulfur protein subunit	no	no
Transcription				
rne	b1084	RNaseE, membrane attachment, mRNA turnover, maturation 5S RNA	no	no
rnb	b1286	Rnase II, mRNA degradation	no	no
rpoE	b2573	RNA polymerase, σ^E factor, heat shock and oxidative stress	yes (De Wulf *et al.*, 2002)	yes
rpoS	b2741	RNA polymerase, σ^S (σ^{38}) factor, synthesis of many growth phase related proteins	no	no
rpoA	b3295	RNA polymerase, alpha subunit	no	no
Translation				
gcvP	b2903	glycine decarboxylase, P protein of glycine cleavage system	no	no
infB	b3168	protein chain initiation factor IF-2	no	no
Transport / Transferase				

yliJ	b0838	putative transferase	no	no
	b0847	putative transport protein	no	no
oppA	b1243	oligopeptide transport; periplasmic binding protein	no	no
acpD	b1412	acyl carrier protein phosphodiesterase	no	no
ydgR	b1634	putative transport protein	no	no
yeeF	b2014	putative amino acid/ amine transport protein	no	no
yehY	b2130	putative transport system permease protein	no	no
	b2290	putative aminotransferase	no	no
sdaC	b2796	serine transporter	no	no
gcvT	b2905	aminomethyltransferase of glycine cleavage system	no	no
dctA	b3528	uptake of C4 dicarboxylic acid	no	no
yifK	b3795	putative amino acid/ amine transport protein	no	no
yiiP	b3915	putative transport system permease protein	no	no
malF	b4033	part of maltose permease, periplasmic	no	no
malE	b4034	periplasmic maltose-binding protein; substrate recognition for transport and chemotaxis	no	no
malK	b4035	ATP-binding component of transport system for maltose	no	no
lamB	b4036	phage lambda receptor protein; maltose high-affinity receptor	no	no
fecB	b4290	citrate-dependent iron transport, periplasmic protein	no	no
fecA	b4291	outer membrane receptor; citrate-dependent iron transport, outer membrane receptor	no	no

[a]Affymetrix microarray was performed as described in the Materials and Methods. Total mRNA was isolated from *E. coli* wild-type and *cpxA101** mutant, both containing pOX38-Km, and enriched prior to hybridization. The array was scanned at 570 nm with a resolution of 3 μm using a GeneArray scanner (Affymetrix). Data analysis was performed by using Affymetrix Microarray Suite 5.0 software.

[b]Gene descriptions are taken from the Affymetrix Expression Analysis Sequence Information Database.

[c]Promoters of genes that have been shown to bind CpxR-P

Chapter 9: References

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