

**The effects of *cpx* genes on the
regulation of L-serine metabolism in
Escherichia coli K-12**

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Abstract

The effects of *cpx* genes on the regulation of L-serine metabolism in *Escherichia coli* K-12

Anne Monette

The *E. coli* *ssd* mutant is so “sick” that it accumulates suppressor mutations that only partially restore the parental strain phenotype. Based on map location and phenotypic similarities, the *ssd* was annotated as a *cpxA* mutation despite unique phenotypes of its own. Here, the *ssd* mutation and one suppressor mutation are shown located within the *cpx* regulon, and each strain carrying a *cpxR*, *cpxP* or *cpxA* deletion demonstrates part of the *ssd* phenotype. The *cpxA** gain-of-function and *cpxA* deletion mutants are shown to have similar phenotypes likely because they both lack the phosphatase responsible for inactivating CpxR. Many deletion mutants are screened from the glycolytic, serine metabolism and Cpx pathways to reveal previously unknown gene products regulating growth on serine. A mucoid phenotype can arise in *ssd*, *cpxR* and *cpxP* mutants, whereas the *cpxA* mutants alone can have an effect on L-serine deaminase activity—indicating that the Cpx proteins have roles outside of the Cpx two-component regulatory system. RNA expression analysis reveals that the *cpxP* transcript is increased in *ssd* mutants, constitutive in the parent strain, and differentially regulated in *cpxR* and *cpxA* deletion mutants. Fluorescence microscopy techniques show that *ssd* mutant filaments do not have a damaged membrane despite a lack of proper DNA segregation, and merodiploid parental and *ssd* strains having an extra copy of ZipA have improper DNA segregation in cells that make filaments and lyse extensively.

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Introduction

In 1980, McEwen and Silverman described two *Escherichia coli* K-12 *cpx* genes—*cpxA* and *cpxB*—by mutations which produced a temperature-sensitive growth phenotype that could be remedied by adding isoleucine and valine. Three other laboratories had isolated mutations in genes mapping to the same chromosomal location between *pkfA* and *sodA*. Each research group described a different variety of phenotypic effects, based on experiments reflecting the interests of each group. The list of phenotypes for the *eup*, *ecf*, and *ssd* mutants includes amikacin and colicin K resistance, high temperature sensitivity, auxotrophies for isoleucine and valine, problems incorporating membrane proteins, the ability use L-serine as a carbon source, increased L-serine deaminase (L-SD) activity and inefficient energy utilization (Plate, 1976; Thorbjarnardottir, 1978; Morris and Newman, 1980). The Newman laboratory group concentrated on L-serine metabolism and isolated the *ssd* mutant by its ability to grow on serine and showed it had high L-SD activity (Morris and Newman, 1980). Despite the evidence indicating that the *ssd* and *cpxA* mutant strains had different phenotypes, the *eup*, *ecf*, and *ssd* mutants were annotated as alleles of *cpxA* (Rainwater and Silverman, 1990).

After almost 15 years, there still remained doubt that the annotation in many databases (e.g., www.ecocyc.org) of *ssd* as an allele of *cpxA* was correct. This thesis resolves what mutation causes the *ssd* phenotype by characterizing *ssd* and *cpx* mutant strains using a battery of phenotypic tests, including growth on serine, high L-SD activity

and UV-sensitivity. The *cpx* regulon of various *ssd* isolates was sequenced, and the sequencing results led to the use of an RNA expression analysis technique. This technique was used to quantify the *cpxP* transcript from *ssd* transductants and from strains carrying deletions of *cpxR* and *cpxA*. This technique was also applied to see which of the three L-SD genes are transcribed in glucose minimal medium. Microscopic examination of the *cpx* and *ssd* mutant strains revealed that some mutant strains grew as filaments. In addition to phase contrast microscopy, three other fluorescence techniques were used to further study the filaments. One was used in an attempt to visualize the localization of an essential cell division protein; another was used to determine whether DNA is properly segregated along filaments; and a third was used to test membrane fragility in order to resolve whether there are circumstances in which the *ssd* phenotype is lethal to *Escherichia coli* cells.

Part 1: The Cpx two-component regulatory system.

At the time the *ssd* mutation was annotated as a *cpxA* mutant, only two *cpx* genes (*cpxA* and *cpxB*) were known to exist (McEwen and Silverman, 1980). It has since been revealed that the *cpx* regulon comprises three genes—*cpxP*, *cpxR* and *cpxA*—separated by a 144bp intergenic dual promoter region. The *cpxP* gene is transcribed from one promoter, and the *cpxR* and *cpxA* genes are transcribed in the opposite direction from another promoter within this intergenic region (Figure 1; De Wulf *et al.*, 1999). The gram-negative bacterial envelope consists of two lipid bilayers called the cytoplasmic

membrane and outer membrane. These have distinct compositions and are separated by the soluble periplasmic space (Kadner, 1996; Nikaido, 1996; Raetz and Dowhan, 1990). CpxA, the sensor histidine kinase of the Cpx two-component signal transduction system, spans the cytoplasmic membrane and the CpxR response regulator is located in the cytoplasm (Figure 2).

The Cpx two-component signal transduction system is believed to be activated in response to various stress signals emerging from the cell envelope and the periplasm (Raivio *et al.*, 1999). CpxA activity is increased in response to envelope stresses predicted to result in protein misfolding. These include alkaline pH (Nakayama and Watanabe, 1995), the overexpression of the outer-membrane lipoprotein NlpE (Snyder *et al.*, 1995), misfolded or unassembled P-pilus subunits (Jones *et al.*, 1997) and alterations in membrane composition (Mileykovskaya and Dowhan, 1997). Although the mechanism of induction is not completely understood, activation of CpxA likely involves the release of its inhibition by the proteolysis of the periplasmic protein CpxP (Figure 2). CpxP is thought to inhibit CpxA activity through a direct protein-protein interaction (Buelow and Raivio, 2005).

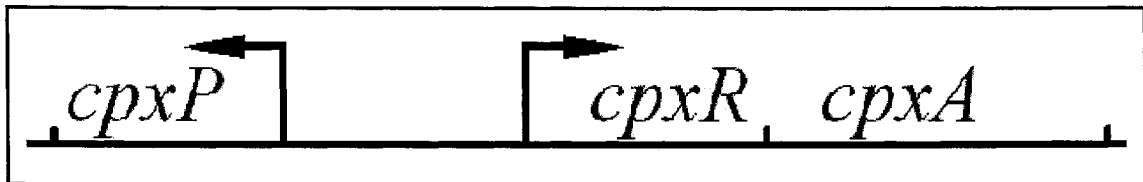


Figure 1: The *cpx* regulon has an intergenic region including divergent promoters for the transcription of *cpxP* and *cpxRA* (modified from De Wulf *et al.*, 1999).

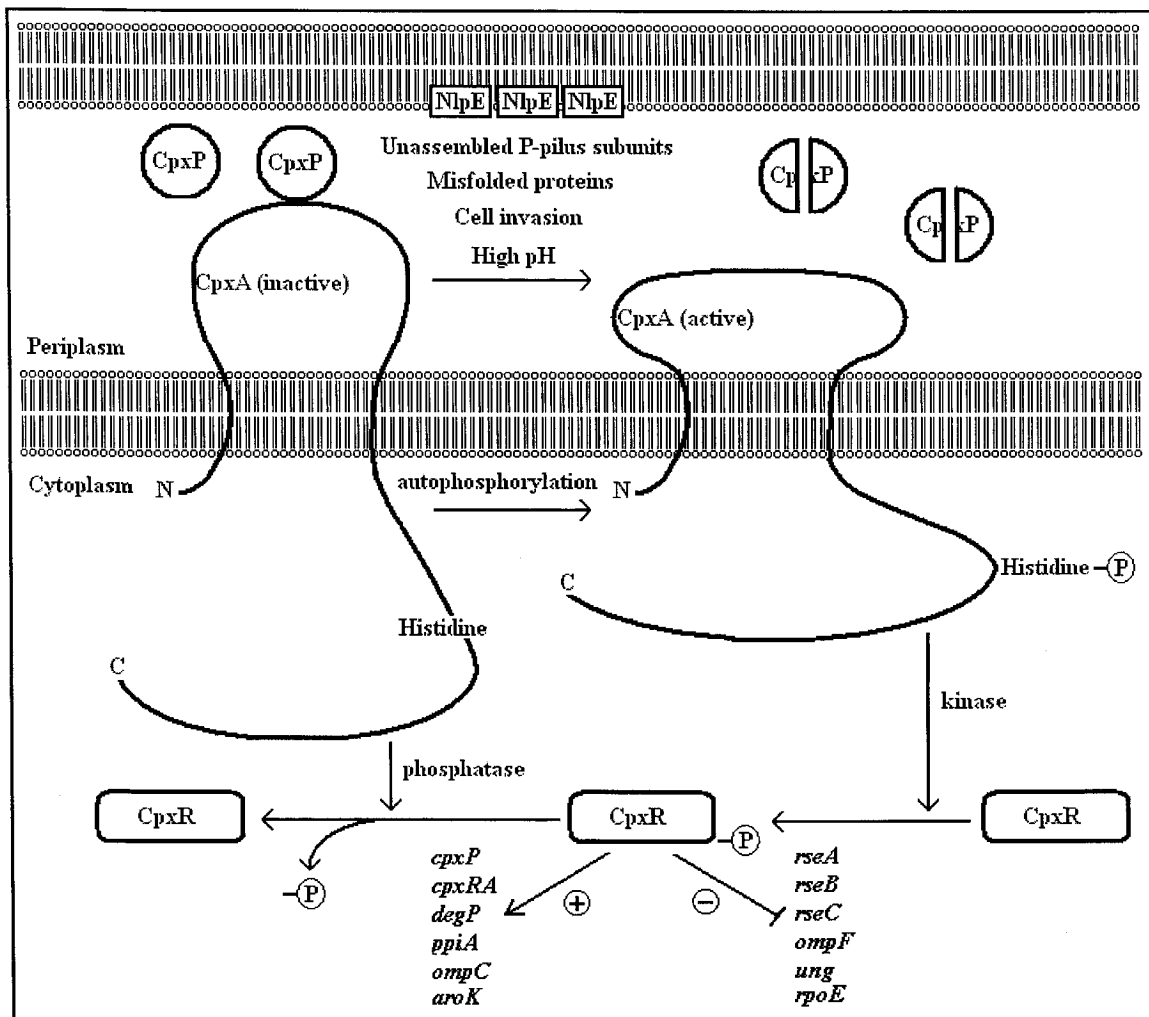


Figure 2: The Cpx two-component regulatory pathway (modified from Raivio and Silhavy, 1997 and 2001; Raivio *et al.*, 1999; De Wulf *et al.*, 2002).

Figure 2 shows that once CpxP is degraded, the CpxA becomes activated through autophosphorylation, and can then transfer its phosphate group to the CpxR response regulator. This leads to an increase of cytoplasmic phosphorylated CpxR (CpxR-P) (Raivio and Silhavy, 1997). CpxR-P then functions as a transcription factor to activate and repress the transcription of a number of target genes (Raivio and Silhavy, 2001). CpxR-P positively regulates genes encoding cell envelope protein folding and degrading factors, such as the periplasmic protease/chaperone DegP/HtrA (Danese *et al.*, 1995), the major disulphide oxidase DsbA (Danese and Silhavy, 1997), two peptidyl-prolyl-isomerases PpiA and PpiD (Pogliano *et al.*, 1997; Dartigalongue and Raina, 1998) and also the outer membrane porin OmpC (Batchelor *et al.*, 2005). CpxR-P regulates the Cpx-system in two ways: by increasing *cpxRA* transcription, leading to prolonged response of stress signals (Raivio *et al.*, 1999), and by increasing the transcription of *cpxP*, which inactivates the Cpx two-component signal transduction system, ending the response to stress (Diguissepe and Silhavy, 2003). CpxR-P is also a negative regulator of the outer membrane porin OmpF (Batchelor *et al.*, 2005), a DNA repair uracil glycosylase Ung (Ogasawara *et al.*, 2004) and the *ropErseABC* operon (De Wulf *et al.*, 2002).

E. coli has at least two other stress response systems that respond to protein misfolding upon envelope stress signals. These are the σ^E (RpoE) and BaeSR pathways, which have considerable overlap with the Cpx-system (Duguay and Silhavy, 2004; Raffa and Raivio, 2002; Ravio, 2005). The Cpx two-component signal transduction system may have the greatest metabolic effect of the three. De Wulf derived a matrix for CpxR-

P target DNA recognition sequences and defined 100 operons as probably being under the direct control of CpxR-P. Twenty five of the target genes coded for products associated with the periplasm. However, the other 75 gene products (many of which have been verified by secondary assays) were not associated with the periplasm or with any role previously defined for the Cpx two-component signal transduction system (De Wulf *et al.*, 2002). This multitude of newly discovered genes under transcriptional control by the Cpx-system is evidence that this system has a much broader ranging influence than previously suspected and is by no means limited to cell envelope related stress responses.

The invasive gram-negative bacteria *Salmonella typhimurium* and *Shigella flexneri* contain the highly conserved proteins CpxP, CpxR, CpxA, RpoE, RseA and RseB belonging to the Cpx- and σ^E -systems. These are found in the same genetic order on the chromosome seen in *E. coli* (De Wulf *et al.*, 2000). Experimental evidence strongly suggests that the corresponding signal transduction pathways in *S. typhimurium* and *S. flexneri* function like those of *E. coli* to sense and respond to bacterial envelope stress (Humphreys *et al.*, 2004; Mitobe *et al.*, 2005). The Cpx two-component signal transduction system has been implicated in the virulence of *S. typhimurium* and *S. flexneri*, and of other gram-negative bacteria that possess protein homologues of the Cpx and σ^E stress responses. These include *Yersinia enterocolitica* (Heusipp *et al.*, 2004), *Vibrio cholerae* (Kovacikova and Skorupski, 2002), *Legionella pneumophila* (Gal-Mor and Segal, 2003) and *Pseudomonas aeruginosa* (Yu *et al.*, 1995). Given the seemingly ubiquitous nature of the Cpx-system in these pathogenic gram-negative bacteria, a logical

connection to its importance for infection can be drawn. Despite the Cpx and σ^E systems overlap, studies suggest that in these bacteria the Cpx-response is involved in the regulation of genes essential to pathogenesis, while the σ^E -response is involved in post-invasion survival (Raivio, 2005).

Mutations in *cpxA* have a variety of phenotypic effects. These include: impaired donor conjugative ability (McEwen and Silverman, 1980); a deficiency in murein lipoprotein and OmpF in the cell envelope (McEwen and Silverman, 1982); anomalous positioning of the FtsZ ring during cell division (Pogliano *et al.*, 1998); decreased swarming ability (De Wulf *et al.*, 1999); partial auxotrophies for isoleucine and valine (McEwen and Silverman, 1980a); sensitivity to high temperature (McEwen and Silverman, 1980b) and sodium dodecyl sulfate (Cosma *et al.*, 1995); and enhanced tolerance to high pH (Danese and Silhavy, 1998), CuCl₂ (De Wulf *et al.*, 1999), amikacin, and kanamycin (Rainwater and Silverman, 1990).

Other phenotypes are ascribed to *cpxA* mutants based on the partially substantiated annotation that *ecfB*, *ssd*, and *eup* are mutations of *cpxA* (Rainwater and Silverman, 1990). Some of these phenotypes include the impaired ability to grow on L-lactose (Plate *et al.*, 1986) and L-proline (Plate and Suit, 1981); an acquired ability to utilize L-serine as the sole carbon source (Morris and Newman, 1980); and an enhanced tolerance to colicins A and K (Plate 1976).

Part 2: The *ssd* mutant.

The *ssd* mutant was isolated after exposure to ultraviolet (UV) light by virtue of its growth on serine, and was mapped to a chromosomal location between *rha* and *metB* (Morris and Newman, 1980). It was named *ssd* for succinate-nonutilizing [*s*] high serine [*s*] deaminase [*d*]. The *ssd* mutation was particularly interesting because it resulted in a decreased yield per glucose as compared to the parent strain, and was extremely fragile (Newman *et al.*, 1982a). Other *E. coli* strains with high L-SD activity do not show such fragility, and it was supposed that the *ssd* mutation could reveal a new role for L-SD. The *ssd* mutation caused the strain to grow slowly at 37°C and to grow filaments at 42°C. It was considered so “sick” that it quickly accumulated suppressor mutations and revertants that caused a partial restoration of the parental phenotype (e.g., lowered L-SD activity levels) (Newman *et al.*, 1982a). When the mutation of a gene causes serious metabolic changes to occur, suppressor mutations accumulate to alleviate the physiological difficulties caused by the first mutation. Intragenic suppressor mutations accumulate within the gene affected by the first mutation while extragenic mutations accumulate in other genes, where the gene product of the latter often interacts directly with the gene product of the former (Alberts *et al.*, 1994).

The *ssd* mutant shows some characteristic *cpxA* mutant phenotypes such as the growth on serine and high L-SD activity. These similar phenotypes, and the fact that they map to the same area of the chromosome was considered enough evidence to annotate the *ssd* as a *cpxA* mutant (e.g., www.ecocyc.org). However, it is difficult to

reconcile its extreme phenotype with that of *cpxA* mutants, since neither *cpxA** gain-of-function mutants nor *cpxA* deletion mutants have the exact *ssd* phenotype (De Wulf and Lin, 2000; this study). Notably, some *ssd* mutant strain phenotypes such as extreme UV-sensitivity, fragility and filamentous growth are found in *ssd* mutants and not in *cpxA* mutants.

When parental strains of *E. coli* are exposed to low doses of UV light at 265nm cell division is inhibited. Cell growth does continue, resulting in the formation of filaments (Deering, 1958; Witkin, 1947). DNA, RNA and protein synthesis are not altered by the UV light (Hanawalt and Setlow, 1960; Swenson and Setlow, 1966) and most of the filaments are capable of forming colonies on defined media, since they eventually recover their ability to divide (Deering, 1959). Conversely, *E. coli* mutants, like the *ssd* mutant, that tend to produce filaments prior to UV exposure are often much more UV-sensitive and cannot recover after UV exposure (Kantor and Deering, 1966).

Part 3. L-serine synthesis and degradation.

The synthesis of L-serine begins with a glycolytic intermediate, 3-phosphoglycerate, which is converted to L-serine by the enzymes SerA, SerB and SerC (Figure 3). The L-serine synthesized by the serine biosynthetic enzymes can be further metabolized into glycine and C1 units (Pizer, 1965), L-tryptophan (Yanofsky, 1960), cysteine (Kredich and Tomikins, 1966) and methionine (Tran et al., 1983). L-serine can

also be degraded to pyruvate and ammonia by L-serine deaminase A (SdaA)—a constitutively expressed gene in *E. coli* K-12 (Pardee and Prestidge, 1955). Pyruvate derived from L-serine, like any other pyruvate, can be used as a carbon source, but this is energetically wasteful in cells provided with glucose as carbon source (Ramotar and Newman, 1986). This may be an indication that SdaA is involved in other cellular roles (Lan, 1997). SdaA activity is regulated by lowering the concentration of its substrate L-serine. This occurs as a negative feedback loop, because the glycine synthesized from L-serine negatively regulates SerA activity, thereby lowering the amount of L-serine synthesized (Pizer, 1963). The gene products of *lrp* and *ssd* have also been shown to negatively regulate the transcription of *serA* (Tuan *et al.*, 1990).

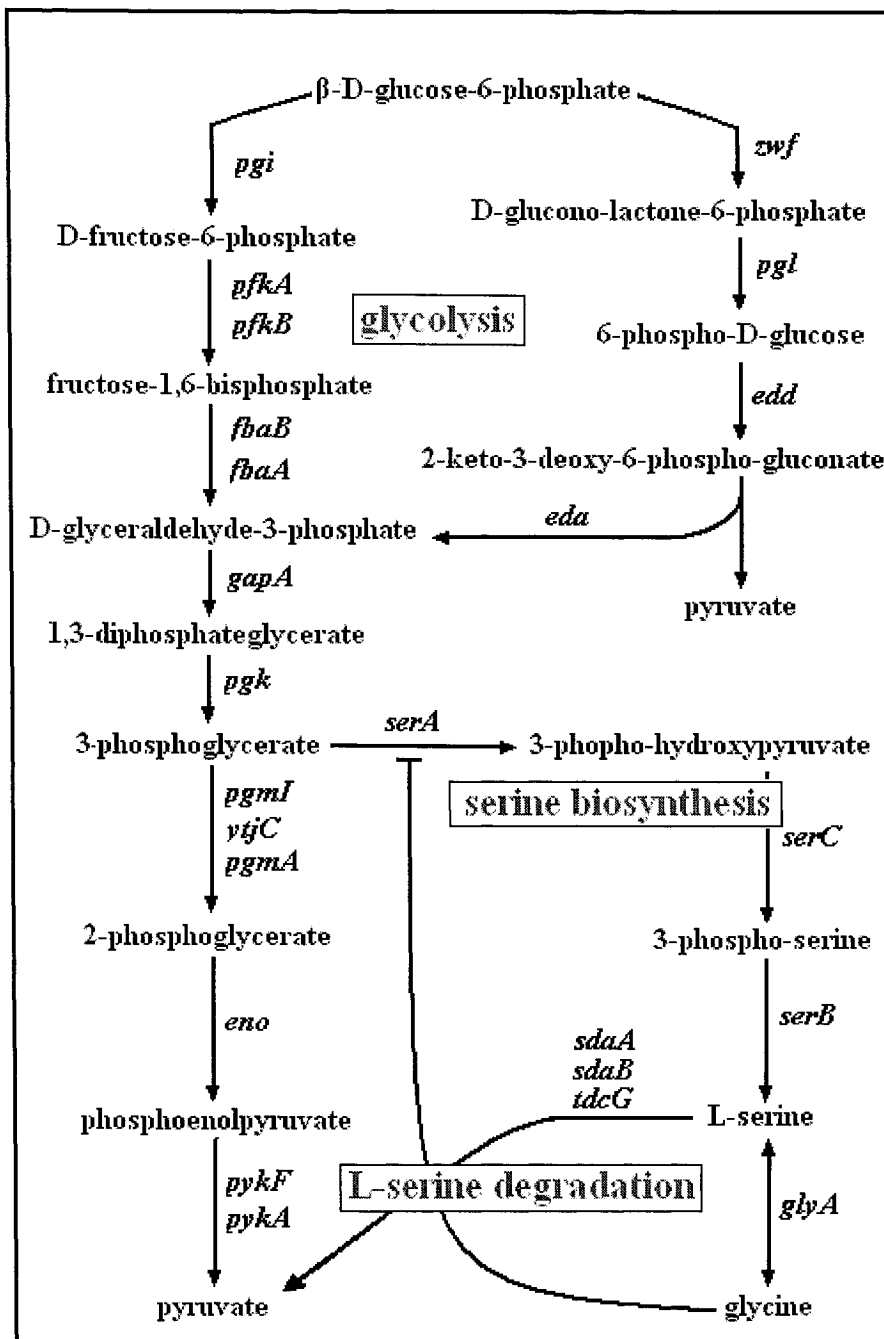


Figure 3: Glycolysis and serine metabolism (modified from EcoCyc: The Encyclopedia of *Escherichia coli* K-12 Genes and Metabolism).

There exist two other L-SD enzymes that degrade L-serine to pyruvate and ammonia. These are SdaB and TdcG. However, the only L-serine deaminase enzyme thought to be active in glucose minimal medium is the SdaA (Su and Newman, 1991) since a strain deficient in SdaA has no L-SD activity when grown in glucose minimal medium. This is because the *sdaB* and *tdcG* promoters are under catabolite repression—and therefore neither of the two genes should be transcribed when cells are grown in glucose minimal medium (Shao *et al.*, 1994; Hesslinger *et al.*, 1998). It is important to point this out because the high L-SD activity observed in the *ssd* mutant strain grown in glucose minimal medium should result from SdaA activity alone. However, studies revealed that a point mutation in the *sdaB* Shine-Dalgarno sequence caused this enzyme to be active in glucose minimal medium (Shao, 1993). As a consequence, it was speculated that the regulation of *sdaB* was one of post-transcriptional modification, rather than transcription repression. While the role of the SdaA in metabolism is not clear, the fact that three L-serine deaminases have been conserved in *E. coli* suggests that these play an important role in the cell.

Campylobacter jejuni, a gram-negative bacterium, is a leading cause of human enteric disease worldwide (Friedman *et al.*, 2000). *C. jejuni* has the *sdaA* and *sdaC* (L-serine symporter (Shao *et al.*, 1994) genes coding for proteins homologous to those found in *E. coli*. The *C. jejuni* SdaA has been demonstrated to be active in deamination and specific for L-serine. Although *sdaA* expression was not necessary for growth in complex media in vitro, SdaA was found to be essential for *C. jejuni* to grow on or utilize L-serine in defined media, as well as for the colonization of chickens. This suggests a

specific requirement for L-serine catabolism for pathogenesis by gram-negative bacteria (Velayudhan *et al.*, 2004).

Part 4. Cell division in *E. coli*.

The *ssd* mutant strain has been observed to grow into filaments at 42°C. This implies that part of its phenotype may be a result of a defect in cell division. *E. coli* cell division begins with the polymerization of the FtsZ protein at mid-cell, resulting in the formation of the “FtsZ-ring” (Figure 4). Other proteins are subsequently joined to the FtsZ-ring in a stepwise process with defined order. These proteins are FtsA, ZipA, FtsEX, FtsK, FtsQ, FtsL/FtsB, FtsW, FtsI, FtsN and AmiC (Figure 4). Cell division is completed by the constriction of the FtsZ-ring, and two new daughter cells are produced from the original (Weiss, 2004). FtsZ is thought to function as the scaffold for septum formation and to cause the contraction of the ring during the invagination process (Romberg and Levin, 2003). The other cell division proteins have been functionally grouped as follows: modulators of the assembly state of FtsZ by anchoring it to the membrane (FtsA and ZipA); proteins that synthesize cell wall (FtsI and FtsW); one that coordinates division with chromosomal segregation (FtsK); and one that hydrolyzes the cell wall to separate the daughter cells (AmiC) (Weiss, 2004). The formation of the “FtsZ-ring” is regulated by two mechanisms: nucleoid occlusion and the Min system. Nucleoid occlusion is the inhibition of the FtsZ-ring formation when the nucleoid is present at mid-cell. The Min system proteins MinC and MinD oscillate from pole to pole and can prevent FtsZ

polymerization. A third protein, MinE, is a positive regulator of polymerization (Weiss, 2004).

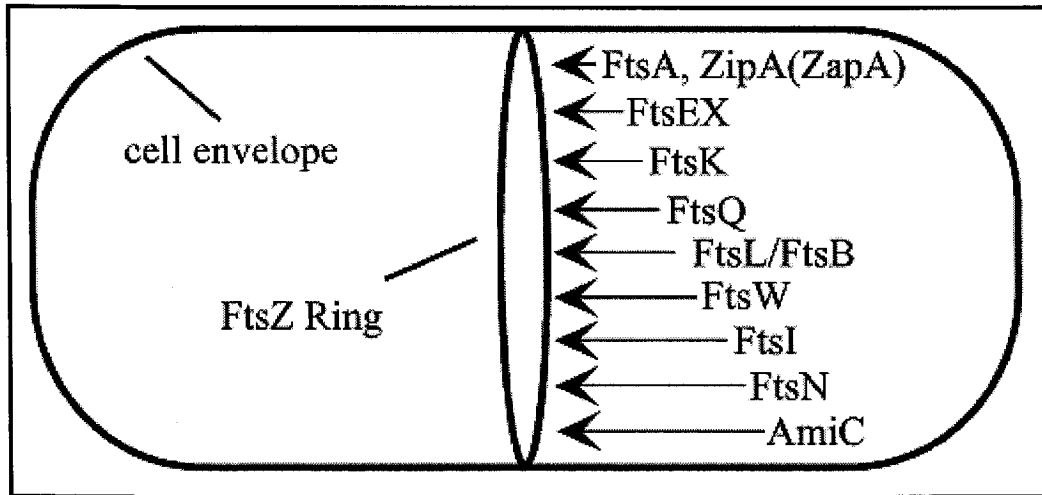


Figure 4. Step-wise incorporation of cell division proteins into the septum (Weiss, 2004).

Materials and Methods

A. Bacterial strains and plasmids

All bacterial strains used in this study were derivatives of *E.coli* K-12, and are listed in Table 1. All plasmids used are listed in Table 2.

B. Media and solutions

B.1. Luria-Bertani medium (LB or rich medium)

Ten grams of Bacto-peptone, 5 g of yeast extract and 5 g (85.5M) of NaCl were dissolved in 1 Litre of distilled water and autoclaved for 30 minutes at 15 lb/sq inch on liquid cycle, then stored at room temperature.

For solid media, 20 grams per Litre Bacto-agar was added to the medium before autoclaving.

B.2. Minimal medium (NIV)

Two grams (15.4M) of $(\text{NH}_4)_2\text{SO}_4$, 15 g (11.5M) of K_2HPO_4 and 5.25 g (110.2M) of KH_2PO_4 were dissolved in 1 Litre of distilled water (pH7.0) and autoclaved for 30 minutes at 15 lb/sq inch on liquid cycle, then stored at room temperature.

For solid media, 2X NIV was prepared and sterilized. Separately, 4 % v/w of Bacto-agar in water was prepared and sterilized. After autoclaving for 30 minutes at 15 lb/sq inch on liquid cycle, equal volumes of the 2X NIV and the 4 % Bacto-agar were mixed.

Since MEW1 ($\Delta ilvA$) and all of its derivatives require isoleucine and valine for growth, both isoleucine and valine were added to a final concentration of 50 $\mu\text{g/ml}$. NIV refers to liquid minimal medium with no added carbon source.

Prior to use, sterile CaCl_2 (1 %, w/v) and MgSO_4 (20 %, w/v) were added to autoclaved NIV medium to a final concentration of 0.004 % and 0.08 %, respectively.

B.3. Carbon sources for NIV minimal medium

B.3.1. D-glucose

D-glucose was 0.2 μm filter sterilized separately before being added to the NIV. The final concentration was 0.2 % (w/v).

B.3.2. L-serine

L-Serine was 0.2 μm filter sterilized separately before being added to the NIV. The final concentration was 0.2 % (w/v).

B.4. Antibiotics and supplements

The final concentrations used for the antibiotics were:

ampicillin (Ap) 100 $\mu\text{g/ml}$

chloramphenicol (Cm) 30 $\mu\text{g/ml}$

kanamycin (Km) 40 $\mu\text{g/ml}$

amikacin (Amik) 12 $\mu\text{g/ml}$

rifampicin (Rif) 50 mg/ml

methionine supplement was used at 50 µg/ml in NIV minimal medium for the *metB* derivative of CU1008.

All antibiotics and supplements were 0.2 µm filter sterilized.

Table 1. Strains used in this study

Strain	Relevant Genotype	Source
CU1008	<i>E. coli</i> K-12 <i>ilvA</i>	L.S. Williams
WES1	<i>metB</i> derivative of CU1008	Newman <i>et al.</i> , 1981
CU1008ssdL9-3	Ssd transductant	Newman
CU1008ssdL9-6	Ssd transductant	Newman
CU1008ssd01	Ssd transductant	Newman
CU1008ssd03	Ssd transductant from lysate CU1008ssd01	This study
CU1008ssd08UVS	Ssd transductant from lysate CU1008ssd01	This study
CU1008ssd11	Ssd transductant from lysate CU1008ssd01	This study
CU1008ssd17L	Ssd transductant from lysate CU1008ssd01	This study
CU1008ssd17SUVS	Ssd transductant from lysate CU1008ssd01	This study
TR 86	MC4100 <i>zii::Tn10</i>	Silhavy
TR 144	MC4100 <i>zii::Tn10</i> <i>cpxA24</i> RS88 [degP-lacZ]	Silhavy
TR 144UVS	MC4100 <i>zii::Tn10</i> <i>cpxA24</i> RS88 [degP-lacZ]	Silhavy
TR 188	MC4100 <i>zii::Tn10</i> <i>cpxA104</i> RS88 [degP-lacZ]	Silhavy
TR 189	MC4100 <i>zii::Tn10</i> <i>cpxA101</i> RS88 [degP-lacZ]	Silhavy
TR 198	MC4100 <i>zii::Tn10</i> <i>cpxA106</i> RS88 [degP-lacZ]	Silhavy
TR 199	MC4100 <i>zii::Tn10</i> <i>cpxA711</i> RS88 [degP-lacZ]	Silhavy

JWK3882_1	BW25113 $\Delta cpxA::kan^r$	H. Mori
JWK3883_1	BW25113 $\Delta cpxR::kan^r$	H. Mori
JWK3885_1	BW25113 $\Delta cpxP::kan^r$	H. Mori
CU1008 $\Delta cpxA$	WES1 $\Delta cpxA::kan^r$	This study
CU1008 $\Delta cpxR$	WES1 $\Delta cpxR::kan^r$	This study
CU1008 $\Delta cpxP$	WES1 $\Delta cpxP::kan^r$	This study
EC450	MC4100 $\Delta (\lambda attL-lom)::bla lacI^q P_{208-} zipA-gfp$	D. Weiss
CU1008zipA-gfp	CU1008 $\Delta (\lambda attL-lom)::bla lacI^q P_{208-} zipA-gfp$	Wang <i>et al.</i> , 2005
CU1008ssd17UVS zipA-gfp	CU1008ssd17UVS $\Delta (\lambda attL-lom)::bla lacI^q P_{208-}$ <i>zipA-gfp</i>	This study

All other strains carrying gene deletions (used in this study) are from H. Mori, and are derivatives of the *E. coli* BW25113 parental strain background.

Table 2. Plasmids used in this study

Plasmid	Description	Source
pcpxA	pCA24N derivative without GFP carrying <i>cpxA</i> gene, Cm ^r	H. Mori
pcpxR	pCA24N derivative without GFP carrying <i>cpxR</i> gene, Cm ^r	H. Mori
pcpxP	Overexpression plasmid ptrc99A containing <i>cpxP</i> , Amp ^r	Silhavy <i>et al.</i> , 1999

B.5. R-top agar for P1 phage transduction

Ten grams of Bacto-tryptone, 1 g of Yeast extract, 8 g (136.9M) of NaCl and 8 g of Bacto-agar were dissolved in 1 Litre of distilled water. Sterile 1M CaCl₂ and D-glucose (20%, W/V) were added to the medium to a final concentration of 2 mM and 0.1 %, respectively after autoclaving.

B.6. SOC medium for electro-transformation cell recovery

Twenty grams of Bacto-tryptone, 5 g of Yeast extract, 0.58 g (9.9M) of NaCl, 0.185 g (2.5M) of KCl, 2.03 g (21.3M) of MgCl₂ and 3.6 g of D-glucose were dissolved in 1 Litre of distilled water, which was then autoclaved.

C. Buffers

C.1. SM buffer

SM buffer was used for storage and dilution of P1 bacteriophage λ stocks.

Two grams (47.3M) of MgSO₄·7H₂O, 5.8 g (99.2M) of NaCl, 50 ml of 1M Tris·Cl (pH 7.5) and 5 ml of 2 % gelatin solution were dissolved in 1 Litre of distilled water, which was then sterilized by autoclaving for 20 minutes at 15 lb/sq inch on liquid cycle. The SM buffer was stored at room temperature.

C.2. Phosphate-buffered saline (PBS) buffer

Eight grams (136.9M) of NaCl, 0.2 g (2.7M) of KCl, 1.44 g (10.3M) of Na₂HPO₄ and 0.24 g (1.8M) of KH₂PO₄ were dissolved in 1 Litre of distilled water. The pH was

adjusted to 7.4 with HCl. The buffer was sterilized by autoclaving for 20 minutes at 15 lb/sq inch on liquid cycle and then stored at room temperature.

C.3. 10X Hepes buffer

119.2 grams (500.2M) of HEPES (238.31 x 0.5 x 0.5) was dissolved in 1 litre of distilled water, and the pH was adjusted to 7.8 using 10 % NaOH.

C.4. 2,4-Dinitrophenylhydrazine (DNPH) solution

Two hundred milligrams (1M) of DNPH and 27.6 ml HCl were dissolved in 1 Litre of distilled water and stored at 4°C in the dark.

C.5. 50X TAE buffer

10.5M EDTA, 242 g of Tris and 57.1 ml of glacial acetic acid were dissolved in 1 Litre of distilled water. The pH was adjusted to 7.8 with HCl.

C.6. RNA gel loading buffer

Nine hundred and fifty microlitres (21.1M) of Formamide, 25 µl of a 0.4 mg/ml (0.001M) Ethidium Bromide solution and 25 µl of a 0.3 % (w/v) Bromophenol Blue solution were premixed 10 minutes before use.

C.7. Sodium phosphate buffer (pH7.4)

77.4 ml of 1M Na₂HPO₄ and 22.6 ml of 1M NaH₂PO₄ were dissolved into 1 Litre of distilled water.

D. Fix solution for microscope

One hundred microlitres of 16 % paraformaldehyde (DHM Co., EM grade) and 0.2 µl of 50 % glutaraldehyde (Sigma Co.) were mixed prior to use.

E. DAPI (4'-6-Diamidino-2-phenylindole), DNA staining reagent

DAPI powder (Sigma Co.) was dissolved in PBS buffer to a final concentration of 20 mg/ml (0.08M). Aliquots of the stock were kept in the dark at -20°C until use.

F. Enzymes, primers, DNA ladder and DNA and RNA kits

TAQ polymerase and GeneRuler™ 1 kb DNA Ladder (#SM0311) were purchased from MBI Fermentas (Montreal, Canada). Primers were designed using Primer3 software and were purchased from BioCorp (Montreal, Canada). QIAprep Spin Miniprep Kit and GenElute Plasmid Miniprep Kit were purchased from QIAGEN (Montreal, Canada) or SIGMA (USA) respectively. DNA mini Kit, QIAEX II Gel Extraction Kit and QIAquick PCR Purification Kit were purchased from QIAGEN (Montreal, Canada). RNeasy Mini Kit, RNAprotect Bacteria Reagent and Sensiscript RT Kit were purchased from QIAGEN (Montreal, Canada).

G. Enzyme assays

G.1. Whole cell L-serine deaminase (L-SD) assay

The *in vivo* assay for L-SD was conducted as follows, which was the method of Isenberg and Newman (Isenberg *et al.*, 1974). Overnight cell cultures in minimal media plus glucose were subcultured and chilled on ice after reaching log-phase. Cells were

then resuspended in HEPES buffer to a density of 100 Klett units. The 0.05 ml resuspended cell sample, 0.3 ml HEPES buffer, 20 μ l L-serine (100 mg/ml) and 20 μ l toluene were added to a clean glass test tube. The reaction was stopped after 35 minutes of incubation in a 37°C water bath by adding 0.9 ml of 2,4-dinitrophenylhydrazine to the reaction mixture. After being allowed to stand at room temperature for 20 minutes, 1.7 ml of 10% NaOH was added to the reaction mixture. L-SD levels were determined by measuring keto acid formation using a spectrophotometer set to read at OD = 540nm. One unit of L-SD as measured in the whole cell assay is defined as the amount of enzyme which catalyzed the formation of 1 mol of pyruvate in 35 minutes in the whole-cell assay conditions.

H. Growth and viability experiments

H.1. UV-sensitivity experiment

Cells were grown overnight and subcultured to log phase, chilled and diluted to 10^{-2} in the appropriate medium. Thirty five microLitres of the dilution was drop-pipetted in a straight line from one end to the other of a square petri dish (Sarstedt 100 x 100 x 20 mm) containing the appropriate LB agar medium. A sterile loop head was run over the drops several times to smooth them out, creating a uniform line of 0.5mm width. Once all strains of interest were streaked out in this fashion, plates were irradiated (253.7nm) at a distance of 18 cm, covered and incubated in the dark at 37°C overnight. Extremely UV-sensitive is a term used to describe strains incapable of recovering from more than 5 seconds of 253.7nm UV-irradiation. Mildly UV-sensitive strains are able to sustain at least 40 seconds of the same UV-dose.

H.2. Amikacin-sensitivity experiment

Cells were grown overnight and subcultured to log phase, chilled and diluted to 10^{-2} in the appropriate medium. A square petri dish (100 x 100 x 20 mm) was prepared by placing them at a 5° elevation at one end, and pouring the 20 ml of 12 ug/ml amikacin LB agar medium. Once the medium had solidified, the plates were laid flat and an additional 25 ml LB agar was poured onto the plate so as to create an amikacin gradient. 35 µl of the cell dilutions was drop-pipetted in a straight line from one end to the other of the plate. A sterile loop was run over the drops several times to smooth them out, creating a uniform line of 0.5mm width. Once all strains of interest were streaked out side by side in this fashion, plates were incubated at 37°C overnight.

I. P1 phage transduction

P1 phage-mediated transduction was performed as described (Miller, 1972).

I.1. P1 phage lysate preparation

A single colony of donor strain was incubated in LB plus 0.05 % CaCl₂ overnight at 37°C with no shaking. 0.1 ml P1 phage (10^{5-6} phage/ml) was then added to 1 ml of the overnight culture and the mixture was incubated at 37°C for 15-30 minutes so as to allow the phage to infect the donor strain. 3 ml LB plus 0.05 % CaCl₂ and 3 ml melted R-top agar were then added, and the whole mixture was poured onto a freshly poured LB plate. After the plate had been incubating for 8 hours without inversion, the top layer was gently swirled in LB plus 0.05 % CaCl₂ and chloroform in a 37°C water bath, spun down, and the supernatant containing phage lysate was stored in a test tube in presence of chloroform at 4°C.

I.2. P1 phage-mediated transduction

An overnight culture of the recipient strain was subcultured and harvested for resuspension in 1/10 its volume of LB plus 0.05 % CaCl₂ until reaching late log phase (OD₆₀₀ = 0.7-1.0). 0.1 ml of the resuspended culture was mixed with 0.1 ml of the P1 phage-lysate containing the desired gene. The mixture was incubated at 37 °C for 15 minutes, after which the cells were centrifuged and resuspended in 1 ml of SM phage buffer. 0.2 ml aliquots were plated onto appropriate selection plates.

J. Plasmid extraction

Plasmids were extracted using QIAprep Spin Miniprep Kit (QIAGEN) or GenElute Plasmid Miniprep Kit (SIGMA) according to the manufacturers' instructions.

K. Restriction enzyme digestion

Plasmid DNA samples were digested by restriction enzymes from MBI fermentas following manufacturer's instruction in order to ascertain plasmid insert identity.

L. Transformation

L.1. Competent cells preparation

Overnight cultures were subcultured and grown to log phase, chilled on ice, centrifuged and washed three times with cold, sterile distilled water.

If cells were to be used right away, they were resuspended in 0.3 ml water and kept on ice until used.

If cells were to be used at later date, they are washed once with 10 % (w/v) glycerol and resuspended in 300 μ l 10 % (w/v) glycerol, then divided to 40 μ l aliquots for freezing at -86°C.

L.2. Heat transformation

Heat transformation was performed according to Maniatis (Maniatis *et al*, 1982).

L.3. Electro-transformation

Electro-transformation was performed using Gene Pulser (Bio-Rad) according to the manufacturer's instructions.

M. Genomic DNA extraction

Genomic DNA was extracted using DNA Mini Kit (QIAGEN) according to the manufacturer's instructions.

N. DNA gel electrophoresis analysis

DNA agarose gel electrophoresis analysis was performed as described elsewhere (Sambrook *et al.*, 1989). The final concentration was 1.0 % (w/v) agarose.

O. PCR product purification

PCR product purification was performed using QIAEX II Gel Extraction Kit Buffer QX1 along with PCR QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's instructions.

P. Sequencing

All DNA samples for sequencing were resuspended in HPLC water. Samples were sequenced by the McGill University & Genome Quebec Innovation centre using an ABI PRISM 3730XL DNA Analyzer system.

Q. RNA expression quantification

Q.1. Total RNA extraction

Cells were grown overnight and subcultured to log or early stationary phase, spun down and resuspended in RNA protect bacterial reagent (QIAGEN) for storage at -20°C. RNeasy Mini Kit (QIAGEN) was used for total RNA extraction according to the manufacturer's instructions. RNase-Free DNase Set (QIAGEN) was used during the RNA extraction procedure according to the manufacturer's instructions.

All equipment used for RNA experiments were treated with a nuclease removal agent (BioShop, Canada). All solutions were made using RNase free HPLC water.

Q.2. RNA gel electrophoresis and quantification

Q.2.1. RNA gel electrophoresis

RNA agarose gel electrophoresis analysis was performed using 1.0 % (w/v) agarose. Five microLitres was mixed with 10 µl RNA gel loading buffer and heated at 70°C for 5 minutes before loading into gel. This step allowed the visualization of the two distinct 16S and 23S ribosomal RNA bands that ensure the RNA has not been degraded.

Q.2.2. RNA quantification

For RNA quantification, 5 μ l of RNA was added to 995 μ l RNase free water, and spectrophotometer readings were taken from a quartz cuvette at both OD = 260nm and OD = 280nm to measure RNA concentration and sample purity, respectively.

Q.3. cDNA synthesis

cDNA synthesis was performed using random hexamer primers (BioCorp) and Sensiscript RT Kit (QIAGEN) according to the manufacturer's instructions.

Q.4. RNA (cDNA) expression quantification

RNA expression quantification was performed by PCR using primers internal to the genes of interest, under reaction conditions optimized for each individual set of primers.

R. Fixing cells

Cells were grown overnight and subcultured to log phase, from which 500 μ l of culture was mixed with 100 μ l fix solution plus 20 μ l sodium phosphate buffer. The mixture was left at room temperature for 15 minutes, placed on ice for another 15 minutes, washed three times with PBS buffer. Finally, the pellet was resuspended in 500 μ l of PBS buffer.

S. DAPI staining

One ml PBS buffer plus 10 μ l of a 20 μ g/ml DAPI solution were added to 500 μ l of fixed cells, incubated at room temperature for 10 minutes in the dark, then washed 3 times with PBS buffer to reduce DAPI background before resuspending cells in 500 μ l PBS buffer. Cells were kept in the dark until being visualized.

T. Live/Dead microscopy

Cells were grown overnight and subcultured to log phase, from which 1 ml of culture was harvested by washing two times with a 0.85 % (w/v) NaCl sterile “saline” solution. The cells were resuspended in 1 ml saline solution, and 3 μ l of pre-mixed equal amounts of the two Live/Dead Backlight L7007 (Molecular Probes) dyes diluted 1:2 in the saline solution. The mix was incubated in the dark until visualization.

U. Strain construction

U.1. *ssd* transduction

To create strains *CU1008ssd03*, *CU1008ssd08*, *CU1008ssd11* and *CU1008ssd17SUVS*, the stored *CU1008ssd01* was used as a donor to transduce to the WES1 recipient—a *metB* auxotroph of the parent strain. Transductants were first selected for methionine independence. Approximately 1/3 of the methionine-independent transductants were able use L-serine as a single carbon source; the percentage expected from the linkage of *ssd* or *cpx* to *metB*. Those transductants that could grow on L-serine were screened for high L-serine deaminase.

U.2. *cpx* deletion mutants

The *cpx* has, like the *ssd*, been mapped within the 2 minute chromosomal cluster surrounding *metB*. P1 phage-lysate was prepared for deletion strains JWK3882_1 ($\Delta cpxA::kan^r$), JWK3883_1 ($\Delta cpxR::kan^r$) or JWK3885_1 ($\Delta cpxPspeD::kan^r$) kindly provided by Dr. Mori. The phage-lysate was then used to infect WES1, the *metB* auxotroph of the parent strain. Transductants were first selected for methionine independence. 1/3 of the non-methionine requiring transductants were screened for growth in presence of kanamycin. PCR was used to verify the transductions.

U.3. Merodiploid ZipA-gfp strains

CU1008zipA-gfp and *CU1008ssd17UVSzipA-gfp* were created by transducing donor EC450—a merodiploid strain carrying a *zipA* gene fused to *gfp* inserted at the λ attachment site (Weiss *et al.*, 1999)—into our parent strain *CU1008* and into *CU1008ssd17SUVS*, and selecting for transductants on LB plates containing 100 μ g/ml ampicillin.

U.4. *CU1008* derivative strains transformed with *cpx* plasmids

Plasmids were extracted from JW3882 (*pcpxA*) and JW3883 (*pcpxR*) kindly provided by Dr. Mori, and analyzed by digestion with the restriction enzyme SfiI, whose recognition sites flank the gene insert. The plasmids were transformed into the appropriate strains and by electroporation and selected in LB with 30 μ g/ml chloramphenicol.

V. Primer list

Sequencing primers:

<i>cpxP</i>	5'gcattaagcagcaggcaaat	5'tgtttgcagcctgaatcgta
<i>cpx</i> promoter	5'gtactgcgctgcgtaagttct	5'aatcaatgctgtcgtccaga
<i>cpxR</i>	5'tgtcgcgattcaacgataga	5'gctatccagaagctcgggtca
<i>cpxA</i>	5'agatggtcaccctgggtta	5'ccggagtgtaggcctgataa
	5'gtttaccctgctctatttgctggc	5'ttcaatacgtccagttccttgc

RNA expression analysis primers:

<i>cpxP</i>	5'gtcacgcaactgctccattc	5'cgctggcagtcagttcatta
<i>cpxR</i>	5'agatggaaggcttcaacgtg	5'taaaccacgggtgaccatct
<i>cpxA</i>	5'tggtttgatgttacccaagc	5'gaatggcggttcaacaatc
	5'tgagctggtggcacgtattc	5'ctgtcaacgacttgcccatt
		5'gttgcgcttcggtttcaata
		5'tttccctgggcaacttcate
		5'tcggtggtcaccaataaaca
		5'agccagaagatggcgaagat
<i>sdaA</i>	5'cgtttcataacggcaacct	5'gaagtgggtgtgctgttc
<i>sdaB</i>	5'caaacggtcagcatgaagtg	5'cgatttctggtgctgaagtg
<i>sdaC</i>	5'tagcgtggcaatcaccaata	5'ccttctcggctactacctg
<i>tdcG</i>	5'tcttccatcccgaatgttg	5'taactgaactactgggcggc

Results

An *Escherichia coli* K-12 strain carrying a mutation in the *ssd* gene is very fragile and differs from its parent in many ways (Morris and Newman, 1980). This pleiotropic mutation has such a dramatic effect on the *E. coli* phenotype, and makes the strain so “sick,” that it quickly accumulated revertants and suppressors and even these could only partially restore the wild type phenotype (Newman *et al.*, 1982). The *ssd* mutation was mapped to a chromosomal area between *rha* and *metB* (Morris and Newman, 1980) – an area where the *cpx* regulon is now known to map. Though similarities between the *ssd* and *cpxA* mutant phenotypes exist, some phenotypes (e.g., UV-sensitivity) are unique to the *ssd* mutant. Nevertheless, a decade later the *ssd*, along with other *E. coli* mutants *ecfB* (Thorbjarnardottir *et al.*, 1978) and *eup* (Plate and Suit, 1981), was annotated as a *cpxA* mutant (Rainwater and Silverman, 1990). In this results section, we present evidence that the annotation is incorrect. Moreover, since Rainwater and Silverman made the suggestion that *ssd* is an allele of *cpxA*, the chromosomal locus thought to consist solely of *cpxA* has been shown to be a regulon comprised of three genes by the discovery of *cpxR* (Dong *et al.*, 1993) and *cpxP* (Danese and Silhavy, 1998). A decade has passed since mention of *ssd* mutant has been published (Ambartsoumian *et al.*, 1994). Since then much more has been revealed about the role of the Cpx two-component regulatory system (De Wulf *et al.*, 2002). This better understanding of the Cpx-system allows us to gain new insights on the *ssd* mutant.

This thesis sets out to resolve what mutation causes the *ssd* phenotype by using a battery of phenotypic tests, compensating *cpx* plasmids and by sequencing the *ssd* and *cpx* mutants. I also attempted to use RNA expression analysis to measure changes in *cpxP* expression resulting from different mutations. Considerable changes were found in the microscopically determined morphology of *ssd* and *cpx* mutants, and I attempted to visualize the localization of an essential cell division protein: ZipA. DAPI staining showed that the DNA is not always properly segregated in *ssd* mutants that grow into filaments. Finally, I employed a live/dead microscopic technique that allowed the visualization of the structural integrity of the *ssd* mutant cell membrane in order to determine if the *ssd* phenotype kills *E. coli* cells.

Part 1. The phenotypic effects of *cpxA*, *cpxR* and *cpxP* deletion mutants.

The phenotype of *cpxA* mutants is not identical to the original *ssd* mutant phenotype, though it is very similar. The original *ssd* and *cpxA* mutants are both grow on serine and have elevated L-SD activity. In addition, the *ssd* mutant is extremely UV-sensitive, but the *cpxA* mutant is not.

I transduced *cpx* deletions from strains (kindly given to our laboratory by Hirotada Mori from the Genome Analysis Project in Japan) with P1 phage into a *metB* derivative of our laboratory parent strain *CU1008* in order to produce *cpx* deletion

mutants with the same genetic background as the *ssd* mutant. Transductants were selected for methionine independence and screened for kanamycin resistance.

Table 3: Phenotypes of strains carrying deletions of individual *cpx* genes

Phenotype	Strain	<i>CU1008</i> (ps)	<i>CU1008</i> (ps)	<i>CU1008</i> $\Delta cpxA$	<i>CU1008</i> $\Delta cpxR$	<i>CU1008</i> $\Delta cpxP$	<i>CU1008</i> $\Delta cpxA$	<i>CU1008</i> $\Delta cpxR$	<i>CU1008</i> $\Delta cpxP$
	plasmid		<i>pcpxP</i>				<i>pcpxA</i>	<i>pcpxR</i>	<i>pcpxP</i>
L-SD ^a (nmol pyruvate)		7.876	23.931	233.551	7.876	13.328	22.719	4.847	9.088
Growth on serine ^b		-	-	+	-	+	-	-	-
Effect of UV ^c		ps	ps	ps	ps	>>ps	ps	ps	ps
Effect of Amikacin ^d		ps	ps	<ps	>ps	ps	ps	ps	ps
Cell shape ^e		ps	ps	curly	wt	filament	ND	ND	ND

Cells were grown to mid-log phase in glucose minimal medium, and tested as follows: (a) L-SD activity was measured by a whole cell assay, where the nmol of pyruvate formed in 30 minutes in a 50µl suspension of cells measuring 100 Klett units was measured at OD = 540nm. Cells were (b) plated on minimal medium with serine as sole carbon source, diluted further into glucose minimal medium and lightly (c) streaked on rich media plates and irradiated with UV for 0, 20, 40 and 60 seconds, then grown overnight at 37°C. Diluted cells were also (d) lightly streaked on amikacin rich medium gradient plates and grown for 24 hours at 37°C. Cells were (e) grown to late stationary phase before being observed using phase-contrast microscopy. The “+” indicates growth, “-” indicates no growth, “ps” indicates parental strain and “ND” indicates not determined.

The *CU1008ΔcpxA* strain was able to grow with serine as sole carbon source, had high L-SD activity and was amikacin resistant (Table 3, column 5). In late stationary phase, cells had an increased curvature, relative to the parent strain that is usually rectangular in shape (Figure 5a). I named the morphology observed for the *CU1008ΔcpxA* “curly” (Figure 5b). The growth on serine, amikacin resistance (De Wulf and Lin 2000) and high L-SD activity (Rainwater and Silverman, 1990) of *cpxA* mutants has previously been reported. It is clear that the *ssd* and *CU1008ΔcpxA* mutations have similar effects, but the *CU1008ΔcpxA* mutation does not confer extreme UV-sensitivity.

The *CU1008ΔcpxR* strain was slightly more amikacin-sensitive than the parent strain. All *CU1008ΔcpxR* transductants became highly mucoid after colonies grown on glucose minimal or rich medium plates at 37°C were subsequently left at room temperature (from 20°C to 23°C) for as little as 16 hours (Figure 6b). Parental strain colonies (Figure 6a) can survive on media plates placed in the refrigerator at 4°C for four months but the mucoid *CU1008ΔcpxR* mutant colonies cannot survive past ten days in the same conditions. The *CU1008ΔcpxR* mutant was no different from the parental strain for all other phenotypes tested (Table 3, column 6), and under the microscope its morphology was indistinguishable from the parental strain (Figure 5c).

About 20% of *CU1008ΔcpxP* transductants, isolated in the same fashion as the earlier transductants, had the mucoid appearance and fragility described for the *CU1008ΔcpxR* transductants and all could grow on serine and were very UV-sensitive (Table 3, column 7). Mucoid *CU1008ΔcpxP* colonies were extremely UV-sensitive.

Under the microscope $\Delta cpxP$ cells were 85% wild type in appearance and the remaining 15% were 4 to 10 times longer (Figure 5d). The extreme UV-sensitivity seen in some *CUI008* $\Delta cpxP$ mutants is particularly noteworthy since this is the sole *ssd* phenotypic change which could not be ascribed to a *cpxA* mutation.

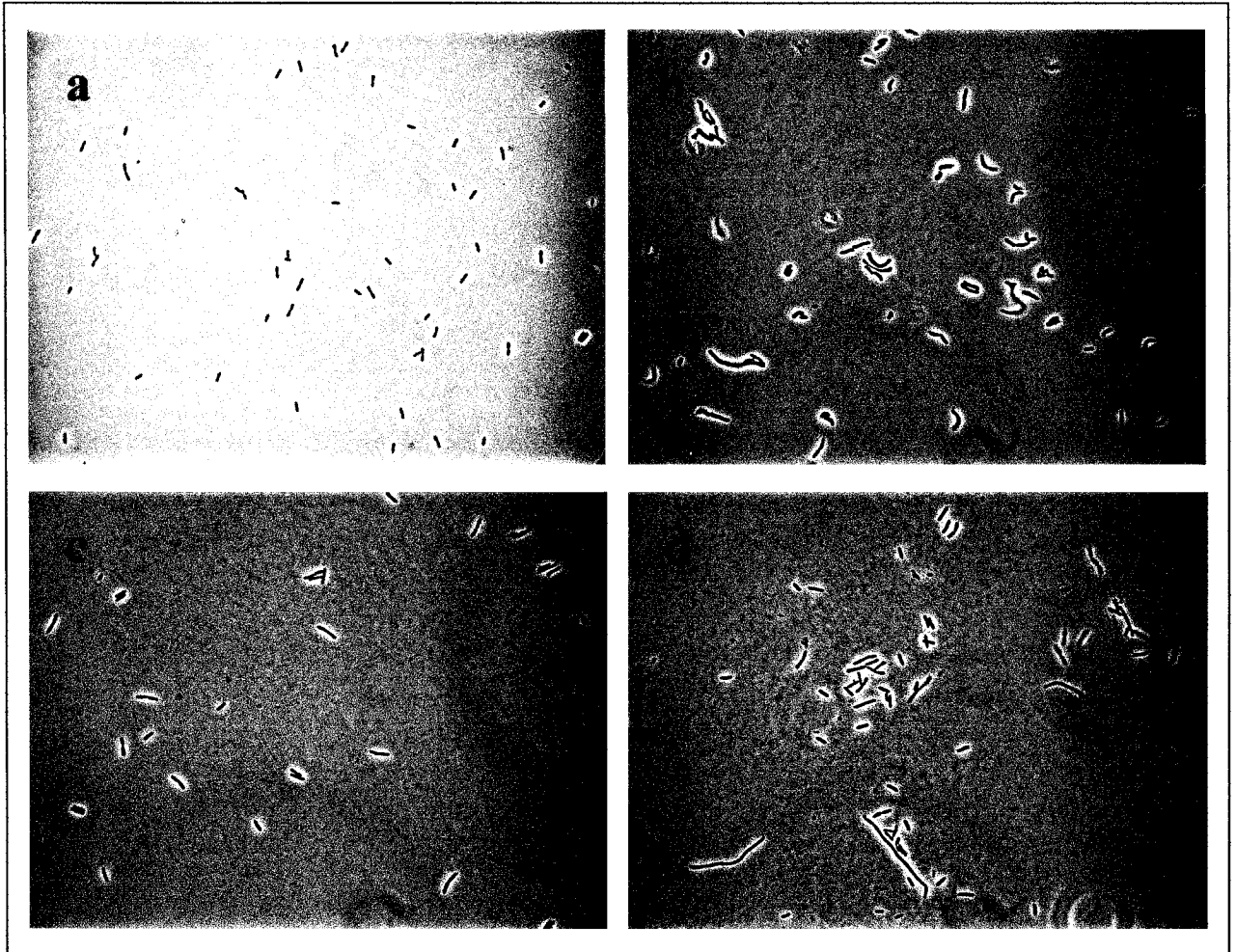


Figure 5: Morphology of parental and *cpx* deletion mutants. The parental (a) and *cpx* deletion mutant strains *CU1008ΔcpxA* (b), *CU1008ΔcpxR* (c) and *CU1008ΔcpxP* (d) were grown to late stationary phase in glucose minimal medium at 37°C, chilled and diluted in glucose minimal medium prior to visualization with phase-contrast microscopy.

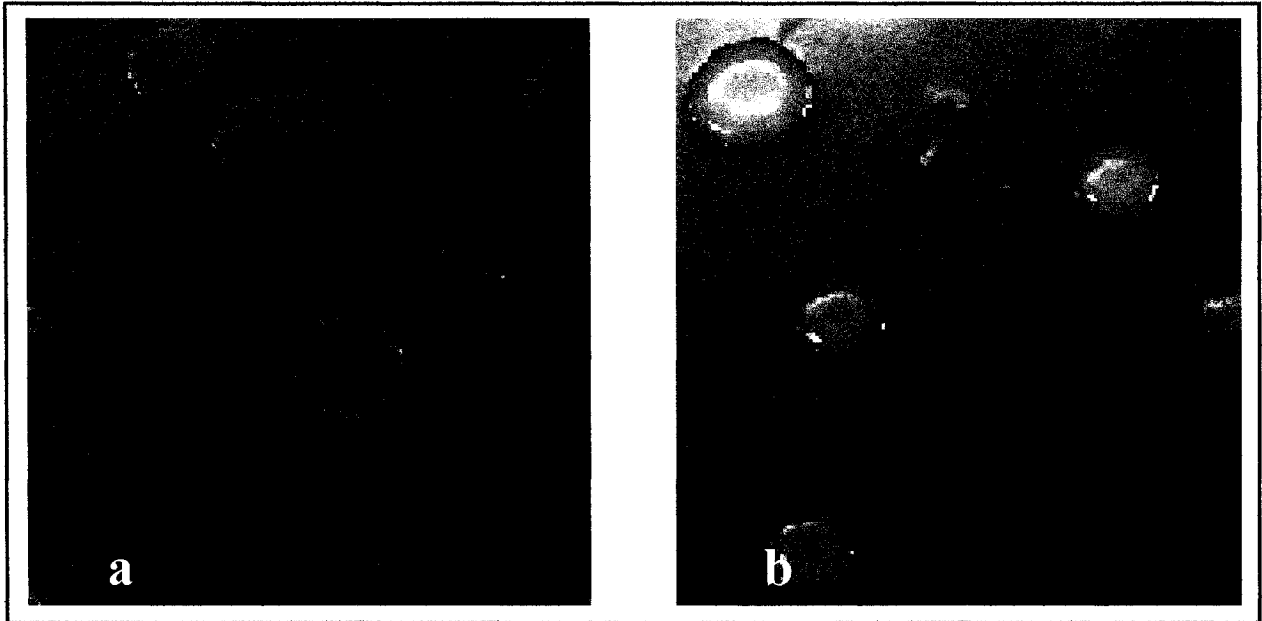


Figure 6: Appearance of wild type and mucoidal cells. The parental (a) and *CU1008ΔcpxR* (b) strains were grown to log phase in rich medium at 37°C, chilled and diluted in rich medium prior to being plated onto rich medium agar. Plates were incubated overnight at 37°C and then left on the bench top at room temperature (20°C to 23°C) for 24 hours prior to being photographed.

Part 2. Effects of *cpx* plasmids on the *cpx* deletion mutants and on the parent strain.

The *ssd* strain was shown to accumulate suppressor mutations (Newman *et al.*, 1982). If deletions of the *cpx* genes could also cause suppressor mutations to accumulate, the presence of the intact gene on a plasmid might not restore the original phenotype. To test this, I transformed each of the *CU1008Δcpx* mutants with the corresponding plasmid. In each case the parental strain phenotype was restored even without the addition of an inducer. The low level expression from the leaky *lac* promoters of *pcpxA* and *pcpxR* (H. Mori) or *tac* promoter of *pcpxP* (T.L. Raivio 1999) was enough to restore the parental phenotype (Table 3, columns 8-10).

When the three *cpx* plasmids were transformed into the parent strain, only the *cpxP* plasmid had a noticeable effect on its phenotypes. Even without the addition of an inducer, the *pcpxP* caused the parental strain to have elevated L-SD activity (Table 3, column 4). This indicates that *cpxA* is not the only gene which affects L-SD activity.

Part 3. The *cpxA gain of function mutant phenotypes.**

In 1998, a student (R. Wargachuk) from the Newman laboratory was interested in how L-SD activity was affected in different *cpxA** gain-of-function mutants. The *cpxA** gain-of-function mutants cause the Cpx system to be constitutively activated because the CpxA phosphatase is inactive, and cannot inactivate the CpxR-P response regulator (T.L.

Raivio and T.J. Silhavy, 1997). T.J. Silhavy kindly donated several *cpxA** mutants—TR188 (R33C), TR144 (*cpxA*Δ193-124), TR199 (P178S), TR198 (R192L) and TR189 (T253P), along with the parent strain TR86. Their L-SD activity was assayed by Wargachuk. Some *cpxA** mutants possessed exceptionally high L-SD activity (i.e. 20-fold higher than the parent strain – TR86) whereas others had levels only twofold more than the parent strain. Figure 7 shows the localization of the amino acids changed in these mutants along the CpxA protein as determined by Raivio and Silhavy. I recovered the TR strains from -86°C storage, and determined the phenotype for those that had higher L-SD activity than the parent strain (Table 4). The *cpxA** mutants were all amikacin resistant, had higher than parental strain L-SD activity and all but one (TR198) could grow on serine (Table 4, column 8)

One *cpxA** strain (TR144) was found stored as two variants. These were labelled TR144 and TR144UVS. The phenotypic tests showed that the TR144UVS variant was more UV-sensitive than the parent strain and had the 20-fold higher L-SD activity levels than the parent strain, like the TR144 assayed by Wargachuk. The TR144 variant had lower L-SD activity levels—about threefold higher than the parent strain (Table 4, column 6). Some morphological differences were seen when these two TR144 variants were viewed under the microscope. The TR144 had parent strain cell morphology (Figure 8a), whereas the TR144UVS was “curly” – much like the *CU1008*Δ*cpxA* mutant (Figure 8b). Therefore, gain-of-function and loss-of-function *cpxA* mutants showed the same change in morphology.

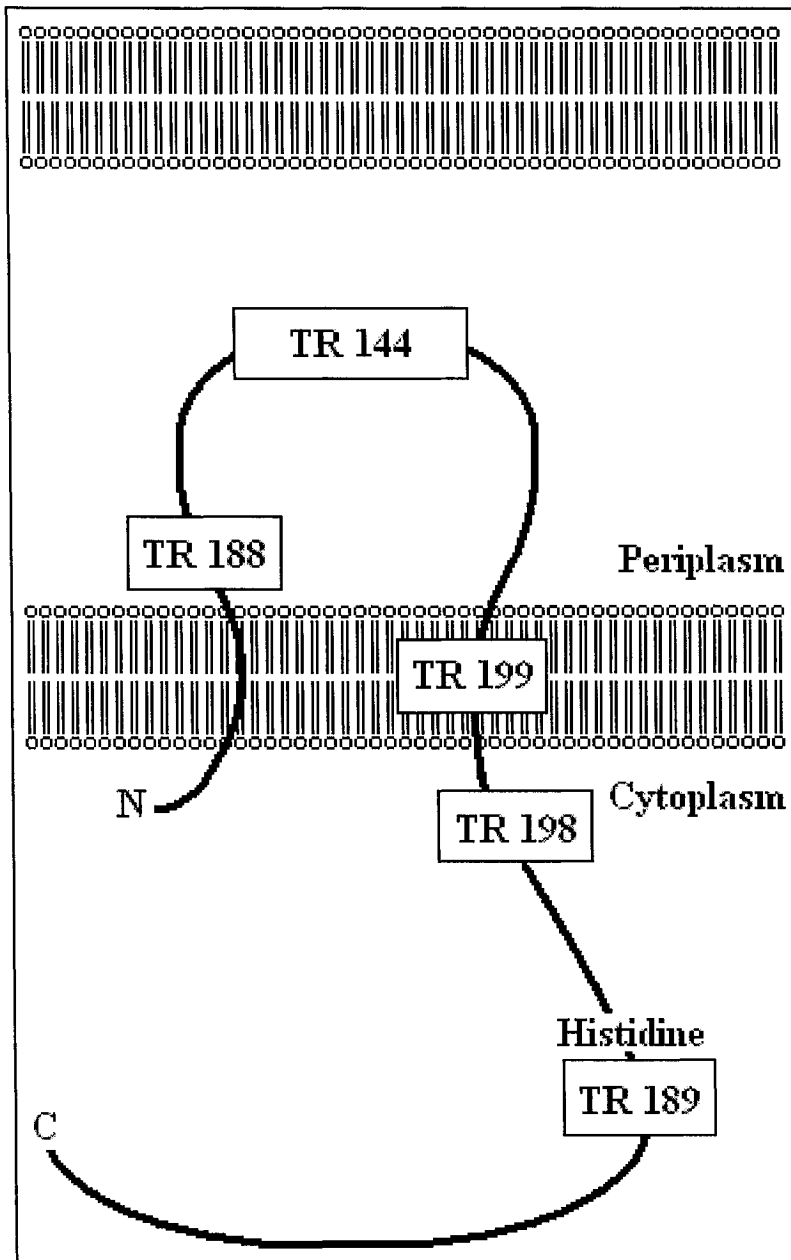


Figure 7: Location of amino acids altered in CpxA* gain of function mutants (modified from Raivio and Silhavy, 1997). All *cpxA** (TR) mutants have a single bp substitution except the TR144, which has a large *cpxA* deletion spanning bases 93-to-124 (i.e., a 32 codon deletion).

Table 4: Phenotypes of parental TR86 and *cpxA gain-of-function mutants.**

Phenotype	Strain	TR86 (ps)	TR144	TR144 UVS	TR188	TR189	TR198	TR199
L-SD ^a (nmol pyruvate)		10.723	56.949	449.988	206.289	70.430	25.142	43.015
Growth on serine ^b		-	+	+	+	+	-	+
Effect of UV ^c		ps	ps	>ps	ps	ps	ps	ps
Effect of Amikacin ^d		ps	<ps	<ps	<ps	<ps	<ps	<ps
Cell shape ^e		ps	ps	curly	ND	ND	ND	ND

These experiments were conducted as described in Table 3. The “+” indicates growth, “-” indicates no growth, “ps” indicates parental strain and “ND” indicates not determined.

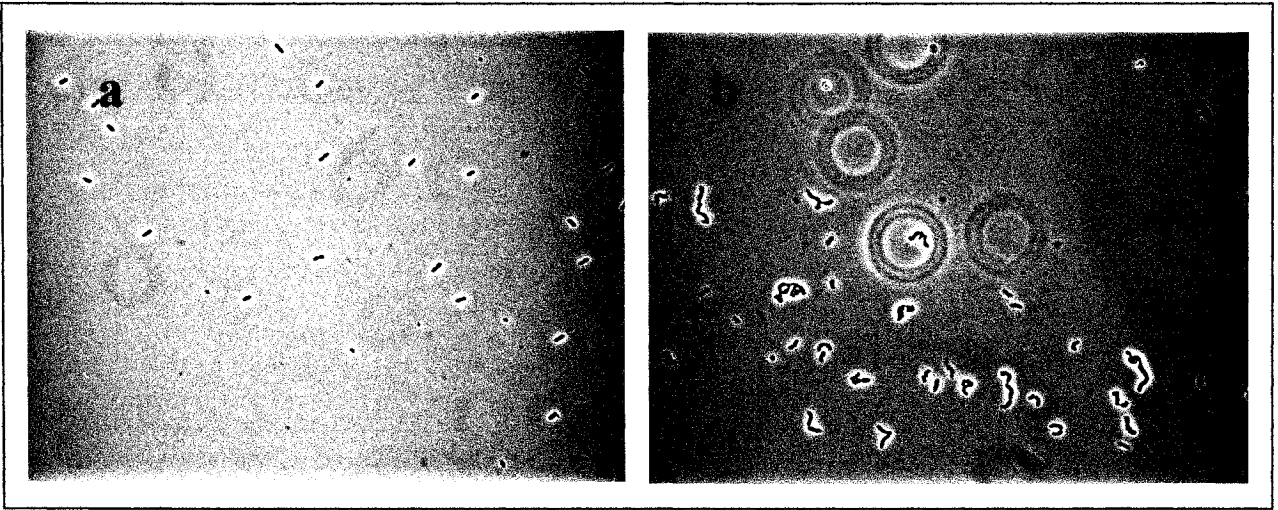


Figure 8: Morphology of *cpxA mutants TR144 (a) and TR144UVS (b).** Cells were treated as described in Figure 5.

Part 4. Transducing the *ssd* mutation to eliminate suppressor mutations.

The original *ssd* mutant had high L-SD activity, could grow on serine and was extremely UV-sensitive. It was also considered very “sick” and was subject to linked and unlinked suppressor mutations causing, for example, lower L-SD activity (Newman *et al.*, 1982). At that time, the *ssd* mutation was stored on a transducing phage (long since lost) and at least part of the phenotype was checked for every culture used during experimentation. In this study the growth on serine phenotype was checked for each experiment.

In an attempt to obtain a strain with the *ssd* mutation free from unlinked suppressor mutations that might exist in the -86°C stock, a P1 phage lysate was prepared from an old -86°C stored, *CU1008ssd01* mutant that could grow on serine and had higher than parental strain L-SD activity levels. This *ssd* isolate was not fragile and was only mildly UV-sensitive. The phage lysate was used to transduce the *ssd* mutation into the *metB* derivative of the parental strain. Transductants were selected for their growth on serine and then screened for high L-SD activity.

Several new *ssd* transductants were produced from two transductions and a few were characterized in detail. Figure 9 shows that the *CU1008ssd03* mutant comes from one transduction, and *CU1008ssd08UVS*, *CU1008ssd11* and *CU1008ssd17UVS* transductants come from a second transduction. These, like the older *CU1008ssd01*, all possessed amikacin resistance, were able to grow on serine and had the high L-SD

activity levels seen for the *CUI1008ssd01* (Table 5). However, only the *CUI1008ssd17UVS* and *CUI1008ssd08UVS* transductants were extremely UV-sensitive (like *CUI1008ΔcpxP* mutants) and mucoid in appearance (like the *CUI1008ΔcpxP* and *CUI1008ΔcpxR* mutants) (Table 5, column 7). Though the *CUI1008ΔcpxR* is always mucoid, it was never UV-sensitive. The *ssd* transductants showed characteristics of all three *cpx* deletion strains and the mucoid and extreme UV-sensitivity phenotypes did not have a single cause.

Under the microscope, the *ssd* transductants that were mildly UV-sensitive resembled the parental strain. The extremely UV-sensitive and mucoidal strains had more variety in cell lengths (85% short like the parent strain and the remaining 15% were filaments ranging from 4 to 70 cells in length) (Figure 10). This result is similar to the findings for the *CUI1008ΔcpxP* morphology (Figure 5d).

Months after its initial isolation, strain *CUI1008Ssd17UVS* exhibited two different colony types on either glucose minimal or rich media plates after it was recovered from -86°C storage (Figure 9). The majority (90%) of the colonies were mucoid and the remaining 10% had the parent strain appearance. Since colonies were stored after three successive single colony purifications, the possibility that stored colonies were contaminated was considered remote. Both colony types — *CUI1008ssd17UVS* (mucoid) and *CUI1008ssd17L* (the name given to the new nonmucoid colony type) — were retested for all phenotypes of interest. As seen in Table 5, the *CUI1008ssd17L* phenotype was identical to that of the *ssd* transductants that did not display extreme UV-sensitivity.

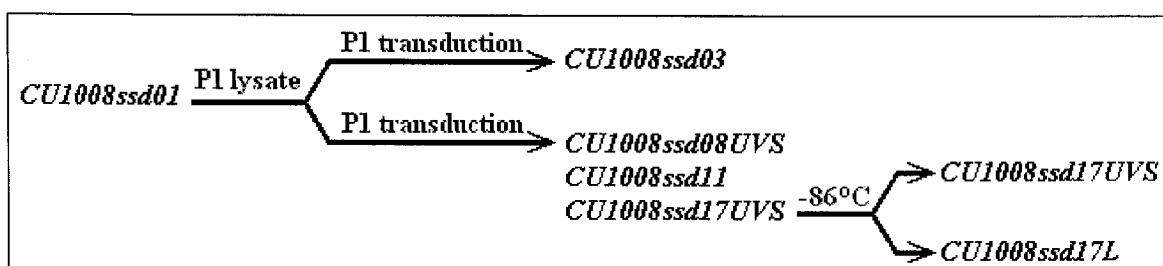


Figure 9: Two P1 transductions experiments using *CU1008ssd01* phage lysate preparation. Two P1 phage transductant experiments using *CU1008ssd01* P1 lysate produce *CU1008ssd03* in the first P1 transduction; and *CU1008ssd08UVS*, *CU1008ssd11* and *CU1008ssd17UVS* in the second P1 transduction. The *CU1008ssd17L* originates from the recovery of *CU1008ssd17UVS* from -86°C storage.

Table 5: Phenotypes of *ssd* transductants and effects of compensating *cpx* plasmids.

Phenotype	<i>CU1008</i> (ps)	<i>CU1008ssd</i> <i>L9-3, 1, 3,</i> <i>11 and 17L</i>	<i>CU1008ssd</i> <i>L9-3, 1, 3,</i> <i>11 and 17L</i>	<i>CU1008ssd</i> <i>L9-3, 1, 3,</i> <i>11 and 17L</i>	<i>CU1008ssd</i> <i>L9-3, 1, 3,</i> <i>11 and 17L</i>	<i>CU1008ssd</i> <i>8UVS and</i> <i>17SUVS</i>	<i>CU1008ssd</i> <i>8UVS and</i> <i>17SUVS</i>	<i>CU1008ssd</i> <i>8UVS and</i> <i>17SUVS</i>	<i>CU1008ssd</i> <i>8UVS and</i> <i>17SUVS</i>
plasmid			<i>pcpxA</i>	<i>pcpxR</i>	<i>pcpxP</i>		<i>pcpxA</i>	<i>pcpxR</i>	<i>pcpxP</i>
L-SD ^a (nmol pyruvate)	7.876	45.438	8.118	42.712	88.150	46.044	5.149	46.957	100.266
Growth on serine ^b	-	+	-	-	+	+	-	-	+
Effect of UV ^c	ps	>ps	>ps	>ps	ps	>>ps	>>ps	>>ps	>>ps
Effect of Amikacin ^d	ps	<ps	ps	<ps	<ps	<ps	ps	<ps	<ps
Cell shape ^e	ps	ps	ND	ND	ND	filament	ND	ND	ND

These experiments were conducted as described in Table 3. The “+” indicates growth, “-” indicates no growth, “ps” indicates parental strain and “ND” indicates not determined.

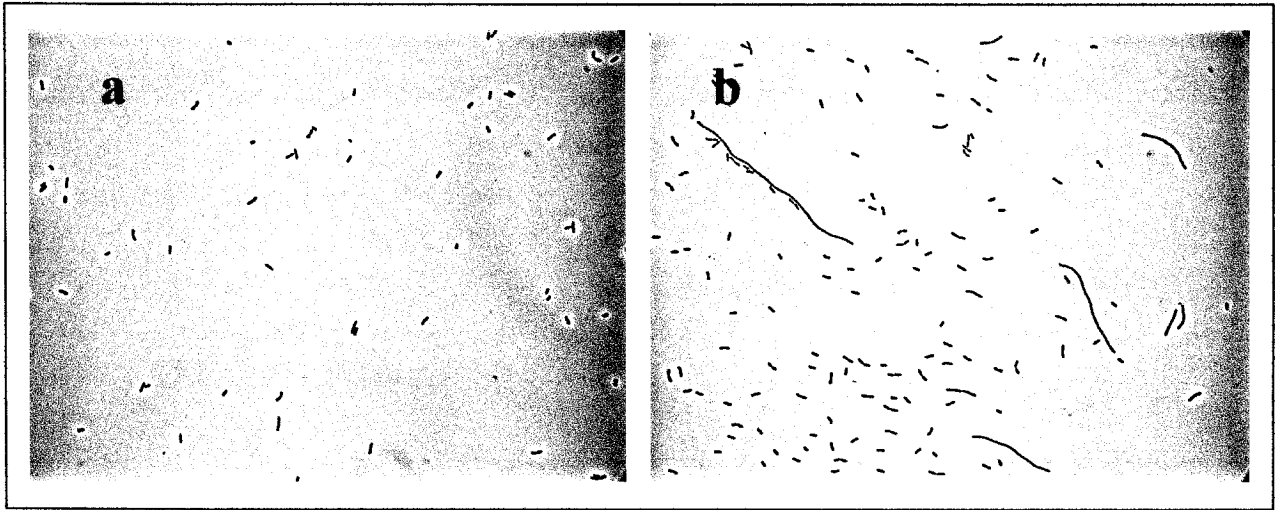


Figure 10: Mildly and extremely UV-sensitive *ssd* transductant morphology.
Mildly UV-sensitive strain *CU1008ssd11* (a) and extremely UV-sensitive *CU1008ssd17UVS* (b) were treated as described in Figure 5.

Part 5. Effects of *cpx* plasmids on the *ssd* transductant phenotypes.

If the *ssd* gene were an allele of any of the *cpx* genes, one of the plasmids carrying a *cpx* gene should complement it. I therefore transformed the various *ssd* transductants with the same set of *cpx* plasmids used earlier to test the strains carrying *cpx* deletions. The plasmid carrying *cpxA* decreased the L-SD activity, abolished amikacin resistance and the ability to grow on serine (Table 5) – just as it had when transformed into *CU1008ΔcpxA* (Table 3, column 8). The plasmid carrying *cpxR* did not decrease the L-SD activity, but did abolish the ability to grow on serine. The plasmid carrying *cpxP* decreased UV-sensitivity in the mildly UV-sensitive *ssd* only, and further increased all *ssd* transductant L-SD activity. These results suggest that the *ssd* mutation causes a phenotype affected by all three *cpx* gene products. However, since the extreme UV-sensitivity cannot be restored by *cpx* plasmids, there must exist at least one additional suppressor mutation outside of the *cpx* regulon.

Part 6. Glycolytic and Cpx pathway deletion mutants that grow on serine.

I obtained many deletion mutants implicated in glycolysis, in serine metabolism and in the Cpx two-component regulatory system, and am very grateful to Dr. Mori and the Genome Analysis Project in Japan for sending these strains to our laboratory. These were used in an attempt to discover how the Cpx protein products affect growth on serine. I tested all of these deletion mutants for their ability to grow on serine, by first

streaking on glucose minimal medium, and then streaking to serine minimal medium. These were all tested in the original Japanese strain without transducing to strain *CU1008*.

Figures 11 and 12 depict the deletion mutants tested in their corresponding pathways, and Table 6 describes their individual roles. All of those that could grow on serine ($\Delta fbaB$, $\Delta gpmI$, $\Delta gpmB$, $\Delta pykF$, $\Delta serA$, $\Delta serB$, $\Delta cutF$ and $\Delta ppiA$) were tested for L-SD activity, though none had higher levels than the parental strain (printed in red in Table 6 and Figures 11 and 12). I also tested L-SD activity in a strain carrying a deletion of *ung* (negatively regulated by CpxR; Ogasawara *et al.*, 2004) and one carrying a deletion of *degP* (positively regulated by CpxR; Danese *et al.*, 1995). However, these two strains did not have elevated L-SD activity. The deletion mutants were not studied further. However, it is clear that deletions of several genes from the glycolytic and serine biosynthetic pathways and the Cpx-system enable growth on serine.

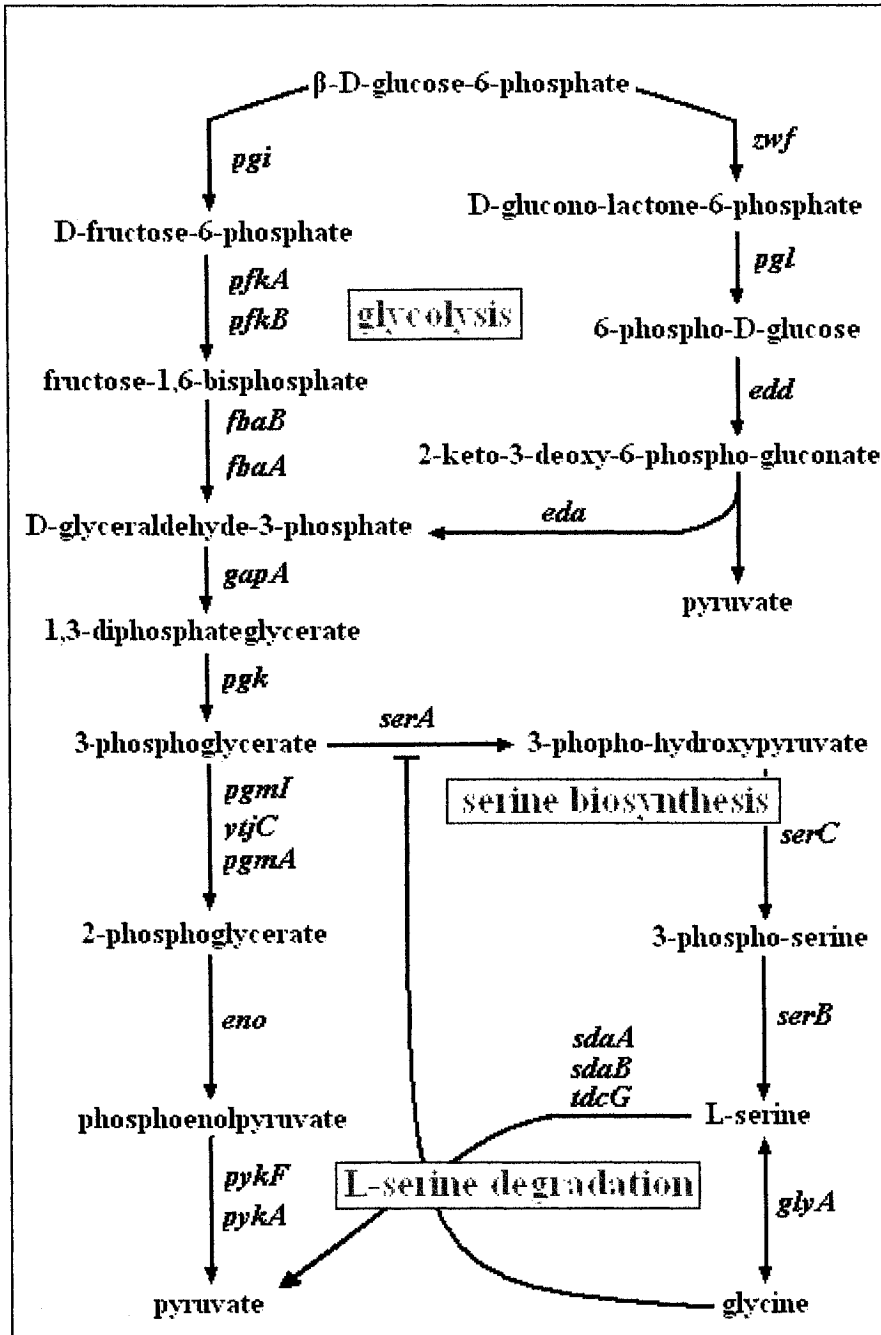


Figure 11: Glycolysis and serine metabolism (modified from EcoCyc: The Encyclopedia of *Escherichia coli* K-12 Genes and Metabolism). The deletion mutants that could grow on serine are printed in red.

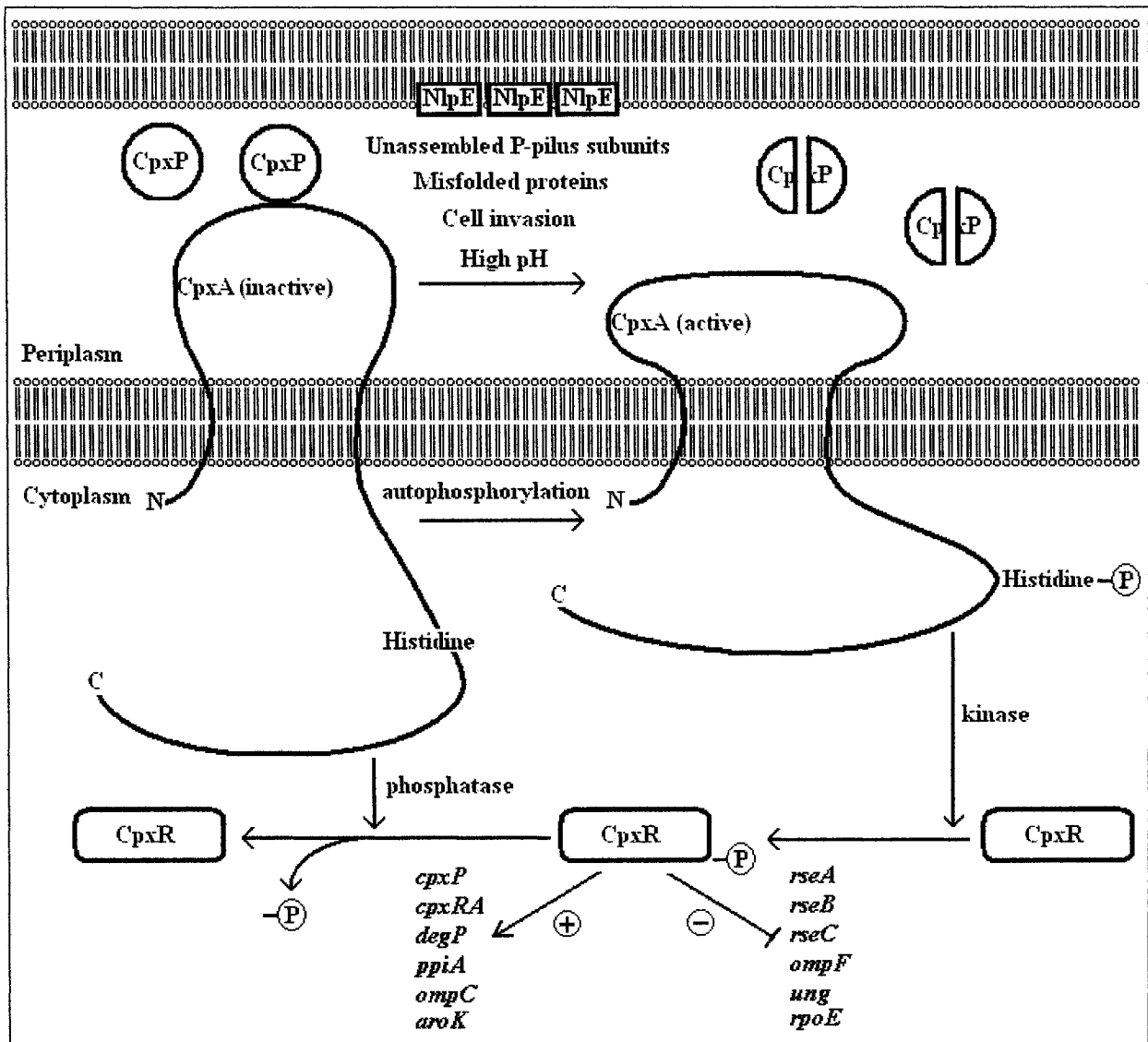


Figure 12: The Cpx two-component regulatory pathway (modified from Raivio and Silhavy, 1997 and 2001; Raivio *et al.*, 1999; De Wulf *et al.*, 2002). The deletion mutants that could grow on serine are printed in red.

Table 6: Cellular roles associated with gene products of deletion mutants tested for growth on serine and L-SD activity (modified from Colibri, Institut Pasteur, 1999-2001). The *E. coli* deletion mutants that could grow on serine are printed in red.

Pathway	Whole gene deletion mutant strain (plus synonyms)	Function	Growth on serine
Glycolysis	<i>pgi</i>	Glucosephosphate isomerase	-
	<i>pfkA</i>	6-Phosphofructokinase I	-
	<i>fbaB/dhmA</i>	Fructose 1,6-bisphosphate aldolase	+
	<i>fbaA/fba/fda</i>	Fructose-bisphosphate aldolase	-
	<i>pgmI/gpmI/yibO</i>	Phosphoglycerate mutase	+
	<i>pykF</i>	Pyruvate kinase I, fructose-stimulated	+
	<i>pykA</i>	Pyruvate kinase A (II)	-
	<i>eda/hga/kdgA</i>	2-keto-3-deoxygluconate 6-phosphate aldolase; 2-keto-4-hydroxyglutarate aldolase	-
Serine biosynthesis	<i>serA</i>	Phosphoglycerate dehydrogenase	+
	<i>serC/pdxF</i>	Phosphoserine aminotransferase	-
	<i>serB</i>	Phosphoserine phosphatase	+
Serine degradation	<i>sdaA</i>	L-Serine deaminase	-
	<i>sdaB</i>	L-Serine deaminase, L-SD2	-
	<i>sdaC/dcrA</i>	Regulator of L-SD2; putative serine transporter	-
	<i>tdcG/yhaP/yhaQ</i>	Anaerobic pathway, L-serine deaminase, L-serine dehydratase	-
Cpx	<i>rseA/mclA</i>	Membrane protein, negative regulator of sigma E	-
	<i>rseB</i>	Binds rseA, negative regulation of sigma E	-
	<i>rseC</i>	Deletion does not affect sigma E activity	-
	<i>atpA/uncA/papA</i>	Membrane-bound ATP synthase, F1 sector, alpha-subunit	-
	<i>atpB/uncB/papD</i>	Membrane-bound ATP synthase, F0 sector, subunit a	-
	<i>atpC/uncC/papG</i>	Membrane-bound ATP synthase, F1 sector, epsilon-subunit	-
	<i>atpD/uncD/papB</i>	Membrane-bound ATP synthase, F1 sector, beta-subunit	-
	<i>atpE/uncE/papH</i>	Membrane-bound ATP synthase, F0 sector, subunit c; DCCD-binding	-
	<i>atpF/uncF/papF</i>	Membrane-bound ATP synthase, F0 sector, subunit b	-
	<i>atpG/uncG/papC</i>	Membrane-bound ATP synthase, F1 sector, gamma-subunit	-
	<i>atpH/uncH/papE</i>	Membrane-bound ATP synthase, F1 sector, delta-subunit	-
	<i>degP/htrA/ptd</i>	DegP periplasmic serine endoprotease, protease Do, required for high-temperature growth; sigma E promoter	-
	<i>nlpE/cutF</i>	Copper sensitivity	+
	<i>clpP</i>	ClpP ATP-dependent protease proteolytic subunit	-
	<i>aroK</i>	Shikimate kinase I	-
	<i>ppiA/rotA/rot</i>	Rotamase; peptidylprolyl-cis-trans-isomerase A	+
	<i>ompF/tolF/cmiB/cry</i>	Outer membrane protein 1a (Ia;b;F)	-
	<i>ompC/meoA/par</i>	Outer membrane protein 1b (Ib, c)	-
	<i>hnr/rssB/sprE/mviA</i>	Two-component response regulator, affecting sigma S-dependent proteins	-
<i>hns/hnsA/pilC/topS</i>	Two-component response regulator, affecting sigma S-dependent proteins	-	
DNA repair	<i>ung</i>	Uracil-DNA-glycosylase	-
DNA methylase	<i>dam</i>	DNA adenine methylase	-
Global regulator	<i>crp</i>	cAMP receptor protein	-

Part 7. Sequencing the *cpx* gene deletion mutants.

I sequenced the entire *cpx* regulon from each strain carrying a *cpx* deletion to be certain that the strains which were thought to carry *cpx* deletions in fact did so, and that these deletions did not cause suppressor mutations in the remaining *cpx* genes. The sequencing results confirmed that all three *cpx* deletion mutants had the expected deletion and no other mutation. This ensured that neither the gene replacement strategy nor the transduction upset the transcription of these genes by altering the 144bp bidirectional promoter located between the *cpxP* and *cpxRA* RNA messages (Figure 1).

Part 8. Sequencing the *ssd* transductants reveals suppressor mutations inside and outside of the *cpx* regulon.

It is clear that the *ssd* transductants have similarities to both the *CU1008ΔcpxA* and *CU1008ΔcpxP* mutant strains. The entire *cpx* regulon of each *ssd* transductant used in this study was therefore sequenced. This region was also sequenced in two older *ssd* isolates from -86°C storage (*CU1008ssdL9-3* and *CU1008ssdL9-6*)—both with the same phenotype as *CU1008ssd01*.

The sequencing results in Table 7 and Figure 13 show that all of the *ssd* transductants — old and new — have a single C/A -to- G/T transversion mutation 35 bp upstream from the first *cpxP* codon (i.e., 1 bp outside of the putative -10 promoter

element for the transcription of *cpxP*; De Wulf *et al.*, 1999). This was the only mutation found in the *CU1008ssd01* strain used as the donor for the P1 lysate used for all transductions.

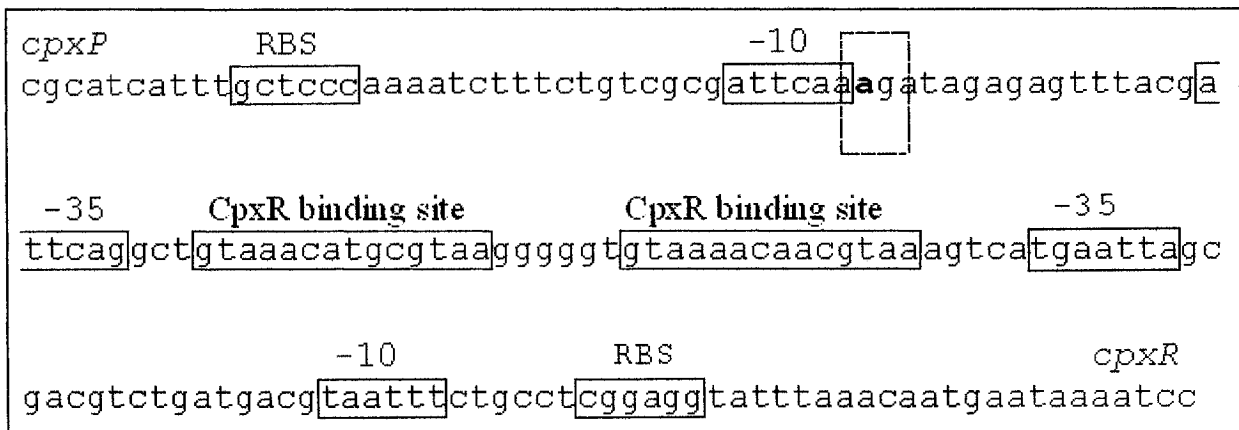


Figure 13: The location of the *cpxP* promoter mutation in the *ssd* transductants (modified from De Wulf *et al.*, 1999). The intergenic promoter region (black) separating the *cpxP* coding sequence region (pink) from the *cpxR* coding sequence (blue) is depicted. The *cpxP* promoter mutation found in all of the *ssd* transductants is the bold red cytosine to adenine (boxed in green) transversion mutation.

All *ssd* transductants created in this study have the same *cpxP* promoter region mutation observed in *CUI1008ssd01*, but this is not the only mutation observed in the *ssd* transductants produced in this study. Sequencing reveals that the *CUI1008ssd03* transductant (produced in the first transduction) has a G/C -to- A/T transition mutation located at +46 of *cpxA* coding sequence. The *CUI1008ssd8UVS*, *CUI1008ssd11*, *CUI1008ssd17UVS* and *CUI1008ssd17L* transductants (produced from the second transduction), along with the older *CUI1008ssdL9-3* and *CUI1008ssdL9-6* transductants, have a two-codon deletion spanning *cpxA* bases +46 to +51. This mutation is located in the predicted N-terminal inner membrane-spanning domain of CpxA (Figure 14; Weber and Silverman, 1988; Raivio and Silhavy, 1997). Apart from these demonstrated mutations, I presumed that other extragenic suppressor mutations outside the *cpx* regulon must account for the difference between the mildly and extremely UV-sensitive *ssd* transductants since these have identical *cpx* regulon sequences.

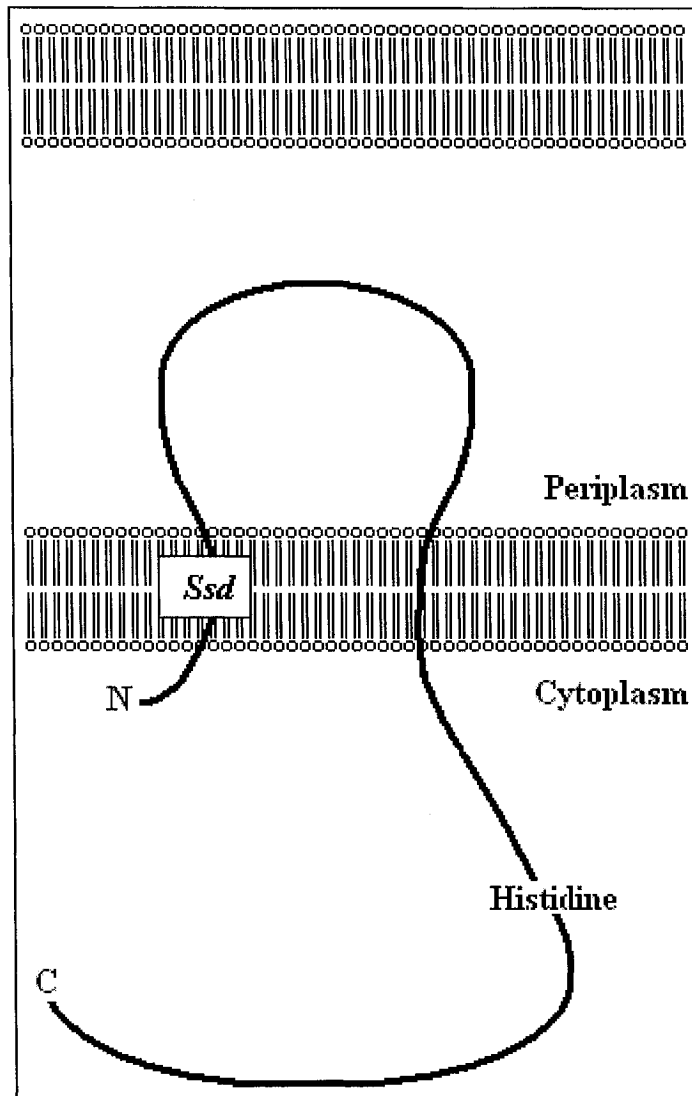


Figure 14: Location of CpxA mutations in the *ssd* transductants (modified from Raivio and Silhavy, 1997). The boxed *Ssd* represents the location of CpxA N-terminal membrane-spanning domain mutations found in the *ssd* transductants.

Part 9. Sequencing *cpxA mutants reveals a suppressor mutation.**

The fact that one mutation in the *cpx* regulon could result in the accumulation of other suppressor mutations suggested that strains which originally were isolated as *cpxA** mutants might also have accumulated suppressor mutations. I therefore sequenced the intergenic promoter region of *cpxA** mutants — TR188 and TR189 — but no suppressor mutations were found (data not shown).

I also compared the sequence of the entire *cpx* regulon of the two TR144 variant strains. Both strains sequences showed the expected *cpxA*Δ93-124 deletion encompassing 32 amino acids in the central region of the predicted *cpxA* periplasmic domain. However the TR144 variant with lower L-SD activity carries a second mutation. This is a G/C-to-A/T transition mutation at position -49 upstream from the *cpxR* coding sequence. This mutation is at the center of the putative -35 promoter element for the transcription of *cpxR* (Figure 15; De Wulf *et al.*, 1999). All mutations found in the sequences of the *ssd* and TR144 isolates are outlined in Table 7.

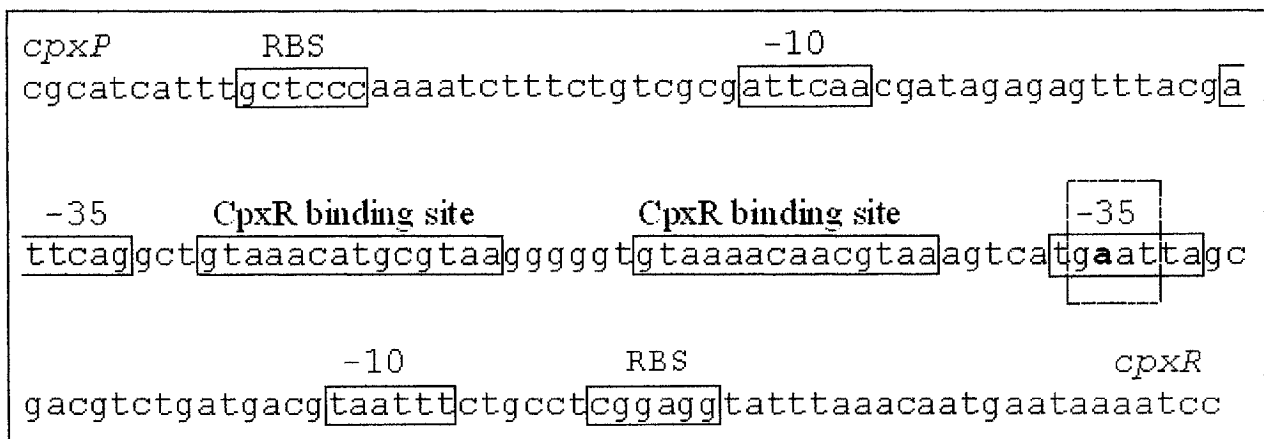


Figure 15: The location of the TR144 *cpxR* promoter mutation (modified from De Wulf *et al.*, 1999). The intergenic promoter region (black) separating the *cpxP* coding sequence region (pink) from the *cpxR* coding sequence (blue) is depicted. The mutation found in the TR144 *cpxR* promoter is the bold red guanine to adenine (boxed in green) transition mutation.

Table 7: Locations of mutations in *ssd* transductants and in *cpxA gain-of-function TR144 variants.**

Location of Mutations in <i>cpx</i> and <i>ssd</i> strains			
Strain	<i>cpxA</i> coding	<i>cpxP</i> promoter	<i>cpxR</i> promoter
<i>CU1008ssd01</i>		-30 (-10)	
<i>CU1008ssd03</i>	46	-30 (-10)	
<i>CU1008ssd08UVS</i>	Δ 46-51	-30 (-10)	
<i>CU1008ssd11</i>	Δ 46-51	-30 (-10)	
<i>CU1008ssd17UVS</i>	Δ 46-51	-30 (-10)	
<i>CU1008ssd17L</i>	Δ 46-51	-30 (-10)	
<i>CU1008ssdL9-3</i>	Δ 46-51	-30 (-10)	
<i>CU1008ssdL9-6</i>	Δ 46-51	-30 (-10)	
TR144	Δ 93-124		-49 (-35)
TR144UVS	Δ 93-124		

Part 10. Live/Dead fluorescence staining of *CU1008*, *CU1008ssd17UVS* and TR144 variants.

The *ssd* transductants that were mucoid and grew filaments were also extremely UV-sensitive. Since cells filament when they cannot divide, I wondered whether the basic defect in the *ssd* strains might be a failure in cell division. If so, this in turn might cause the cells that were not dividing to become fragile to environmental conditions such as UV irradiation. The Live/Dead fluorescence technique involves staining cells with dyes that fluoresce green if the cell membrane is intact, and fluoresce red if the membrane is damaged. This is based on the ability of intact membranes to exclude the red fluorescing dye. Lysed cells do not take up any dye, and these can only be seen using phase-contrast microscopy.

When the parental *CU1008* and the *CU1008ssd17UVS* strains were grown to early stationary phase in glucose minimal medium, both displayed 95% green fluorescence (Figure 16). This indicates that both long and short cells observed in *CU1008ssd17UVS* had intact membranes. Strain *CU1008* had the same green appearance when grown in either glucose rich medium or rich medium (Table 8). However, *CU1008ssd17UVS* became extremely fragile when grown in rich media— 60% of the cells lysed; 30% stained red, and only 10% could exclude the red dye and appeared green, like the parent strain. Furthermore, the filaments of the *CU1008ssd17UVS* observed in glucose minimal medium were far reduced in length when it was grown in rich media.

Filaments observed for *CU1008ssd17UVS* were a maximum of 12 cells long in rich medium and five cells long in glucose rich medium.

When the two *cpxA** variants – TR144 and TR144UVS – were grown in glucose minimal medium to early stationary phase, a proportion of 55% had intact cell membranes (green), 15% were lysed and 30% had damaged membranes that could not exclude the red dye (Figure 17).

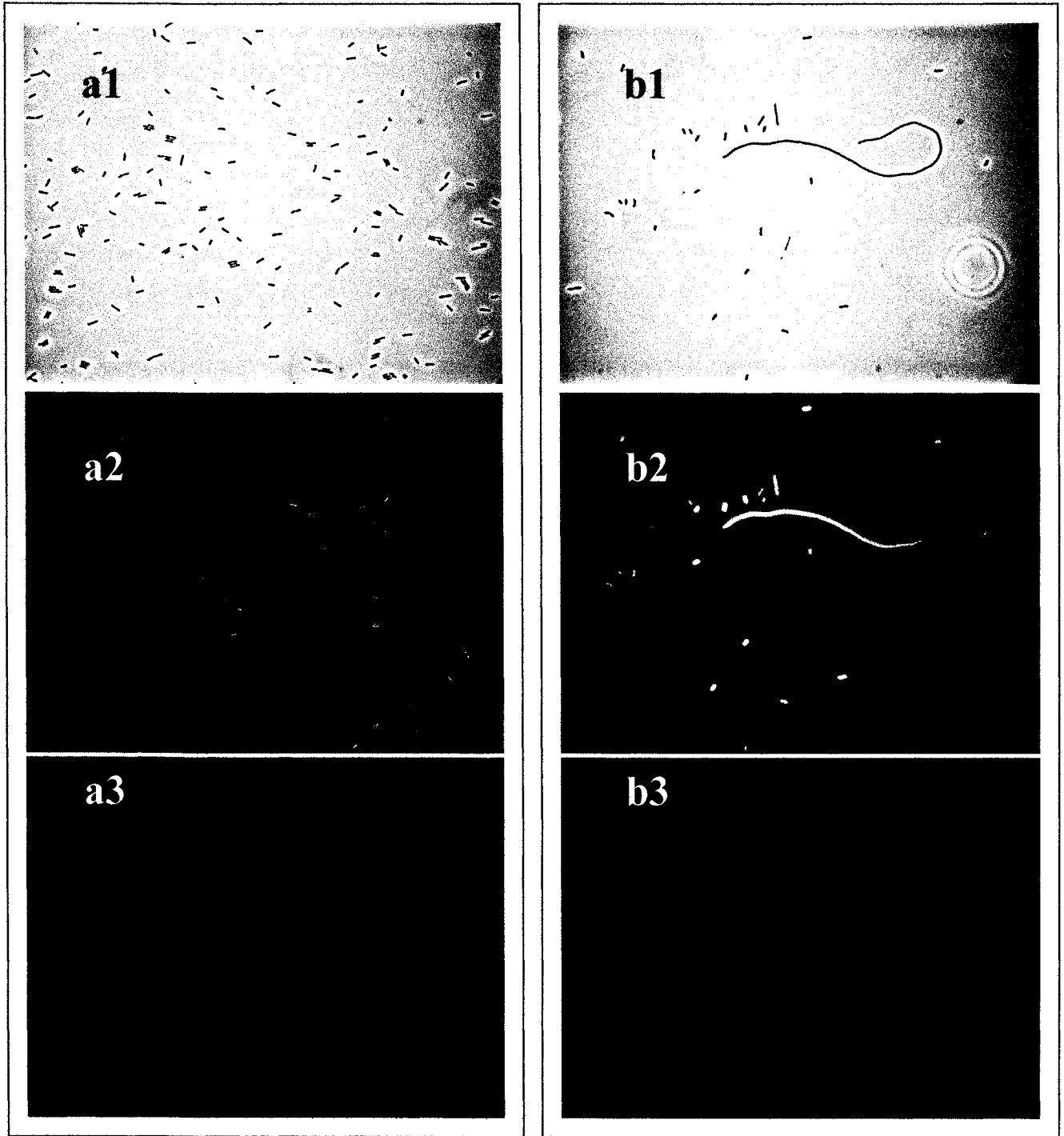


Figure 16: Properties of strains *CU1008* (a) and *CU1008ssd17UVS* (b) grown to log phase in glucose minimal medium. Panels represent: (1) phase-contrast microscopy, (2) green emission and (3) red emission spectra of live/dead stained cells.

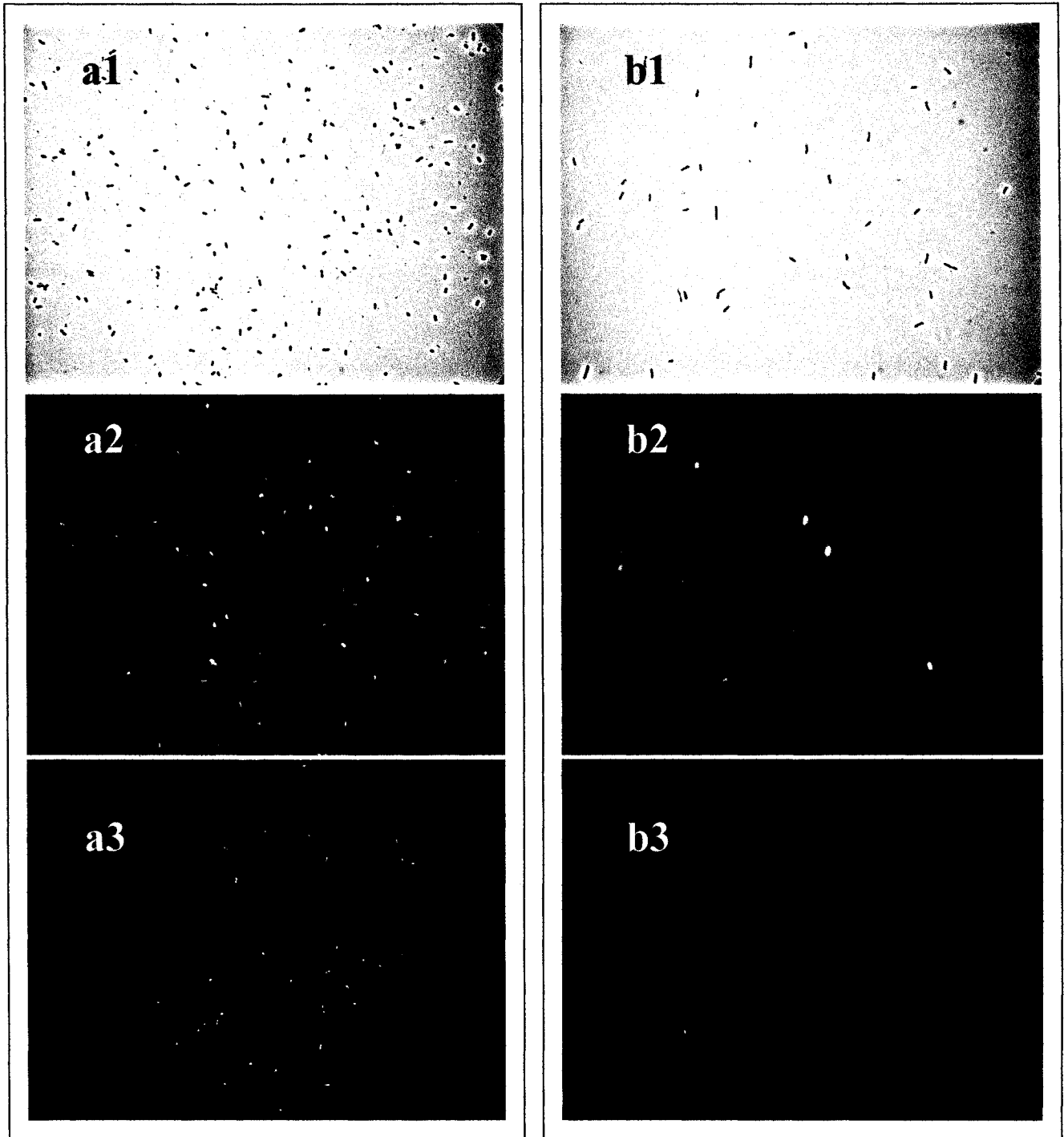


Figure 17: Properties of *cpxA variant strains TR144 (a) and TR144UVS (b) grown to early stationary phase in glucose minimal medium. Panel representation is as described in Figure 16.**

Part 11. The effect of overproduction of ZipA-Gfp on strains *CU1008* and *CU1008ssd17UVS*.

To determine whether the filaments observed for *CU1008ssd17UVS* contained whole or partial septa, derivatives of parental *CU1008* and the *CU1008ssd17UVS* transductant were constructed by transducing in a *zipA-gfp* fusion that recombines into the lambda attachment site to create merodiploid strains (Wang and Newman 2005). ZipA is a protein involved in early stages of septum formation during cell division. The green fluorescent protein (Gfp) is a green fluorophore fused, in this case, to the ZipA protein. The cellular localization of the Gfp, and by inference, of ZipA, can be detected using fluorescence microscopy. In this construct, *zipA-gfp* is under the control of the IPTG-inducible *trc* promoter and so, due to catabolite repression, its expression should be low in cells grown in the presence of glucose.

Both *CU1008zipA-gfp* and *CU1008ssd17UVSzipA-gfp* strains made filaments at early stationary phase in glucose minimal medium, even at the low expression level expected from the absence of the inducer. Strain *CU1008zipA-gfp* made filaments of 2-12 cell length; of these 85% were green in the live/dead test, 5% were red and about 10% were lysed. The filaments of the *CU1008ssd17UVSzipA-gfp* strain were longer (2-50 cells in length) with more red cells (50%), the same 10% lysis (no color) and the remaining 40% were green (Figure 18, Table 8).

When these two strains were grown in glucose rich medium, the *CU1008zipA-gfp* formed normal single cells with a considerable amount of lysis (70% green, 10% red, and 20% lysed cells). The *CU1008ssd17UVSzipA-gfp* filaments were somewhat shorter in this medium than in glucose minimal medium, ranging from 2-to-40 cells in length. Lysis was extensive at approximately 60% with only 25% green, and 15% red. The lysed cell ratio is the same as was observed for the *CU1008ssd17UVS* transductant. In rich medium, the *CU1008ssd17UVSzipA-gfp* made shorter filaments (2-12 cells long). The green/red fluorescence and lysed cell ratios were the same as was seen when this strain was grown in glucose rich medium (i.e., 60% lysed, 25% green and 15% red).

In an attempt to determine whether filaments could influence L-SD activity, the *CU1008zipA-gfp* and the *CU100817UVSzipA-gfp* were tested using the whole cell L-SD assay as described. However, neither of these two strains had higher than parental L-SD activity.

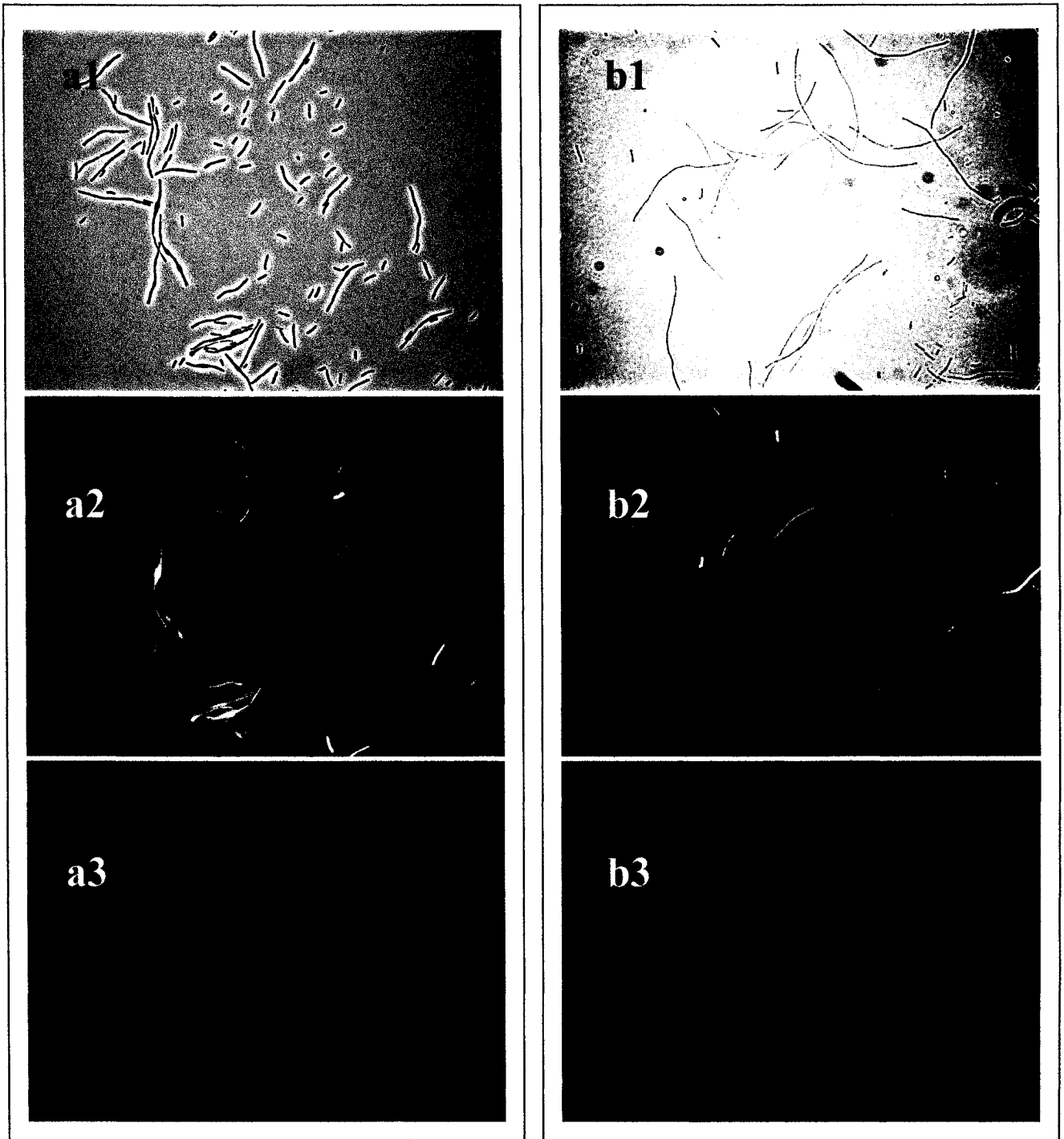


Figure 18: Properties of strains *CUI008zipA-gfp* (a) and *CUI008ssd17UVSzipA-gfp* (b) grown to early stationary phase in glucose minimal medium. Panel representation is as described in Figure 16.

Table 8: Summary of results for live/dead and DAPI staining *CU1008*, *CU1008ssd17UVS*, *CU1008zipA-gfp* and *CU1008ssd17UVSzipA-gfp*.

Strain (early stationary phase)	Medium	Phenotype				
		morphology	live/dead or lysis (%)			DAPI
		parental or length of filament (cells)	green	red	lysed	DNA segregation
<i>CU1008</i> (parental)	min glu	parental	95	4	1	+
<i>CU1008zipA-gfp</i>	min glu	2 to 12	85	5	10	-
<i>CU1008ssd17UVS</i>	min glu	2 to 40	95	0	5	ND
<i>CU1008ssd17UVSzipA-gfp</i>	min glu	2 to 50	40	50	10	-
<i>CU1008</i> (parental)	rich glu	parental	90	10	0	+
<i>CU1008zipA-gfp</i>	rich glu	parental	70	10	20	ND
<i>CU1008ssd17UVS</i>	rich glu	2 to 12	10	30	60	-
<i>CU1008ssd17UVSzipA-gfp</i>	rich glu	2 to 40	25	15	60	-
<i>CU1008</i> (parental)	rich	parental	90	10	0	+
<i>CU1008zipA-gfp</i>	rich	2 to 5	70	10	20	-
<i>CU1008ssd17UVS</i>	rich	2 to 5	10	30	60	-
<i>CU1008ssd17UVSzipA-gfp</i>	rich	2 to 12	25	15	60	-

Cells were grown to early stationary phase in glucose minimal (min glu), glucose rich (rich glu) and rich media at 37°C, chilled, washed and stained prior to being visualized using phase-contrast and fluorescence microscopy. The “+” indicates growth, “-” indicates no growth and “ND” indicates not determined.

Part 12. Determination of the distribution of DNA in strains *CU1008*, *CU1008ssd17UVS*, *CU1008zipA-gfp* and *CU1008ssd17UVSzipA-gfp*.

DNA can be visualized in cells using 4'-6-Diamidino-2-phenylindole (DAPI), a staining agent that complexes with DNA and emits blue fluorescence when illuminated with the proper excitation wavelength (R.P. Haugland, 1996). In filaments, if the DNA is properly segregated, blue emission by DAPI appears as a series of blue dots over the length of the filament whether or not the septum is formed (Newman *et al.*, 1998). In filaments where DNA that is not properly segregated an undifferentiated blue emission spread over the entire length of the filament is observed (Akihiro *et al.*, 2004).

When grown in glucose minimal medium, rich glucose medium or rich medium, DAPI-stained parental strain *CU1008* shows properly segregated DNA. However, the DNA in *CU1008ssd17UVS*, *CU1008zipA-gfp* and *CU1008ssd17UVSzipA-gfp* filaments was not properly segregated in any growth medium (Figure 19 and 20, Table 8).

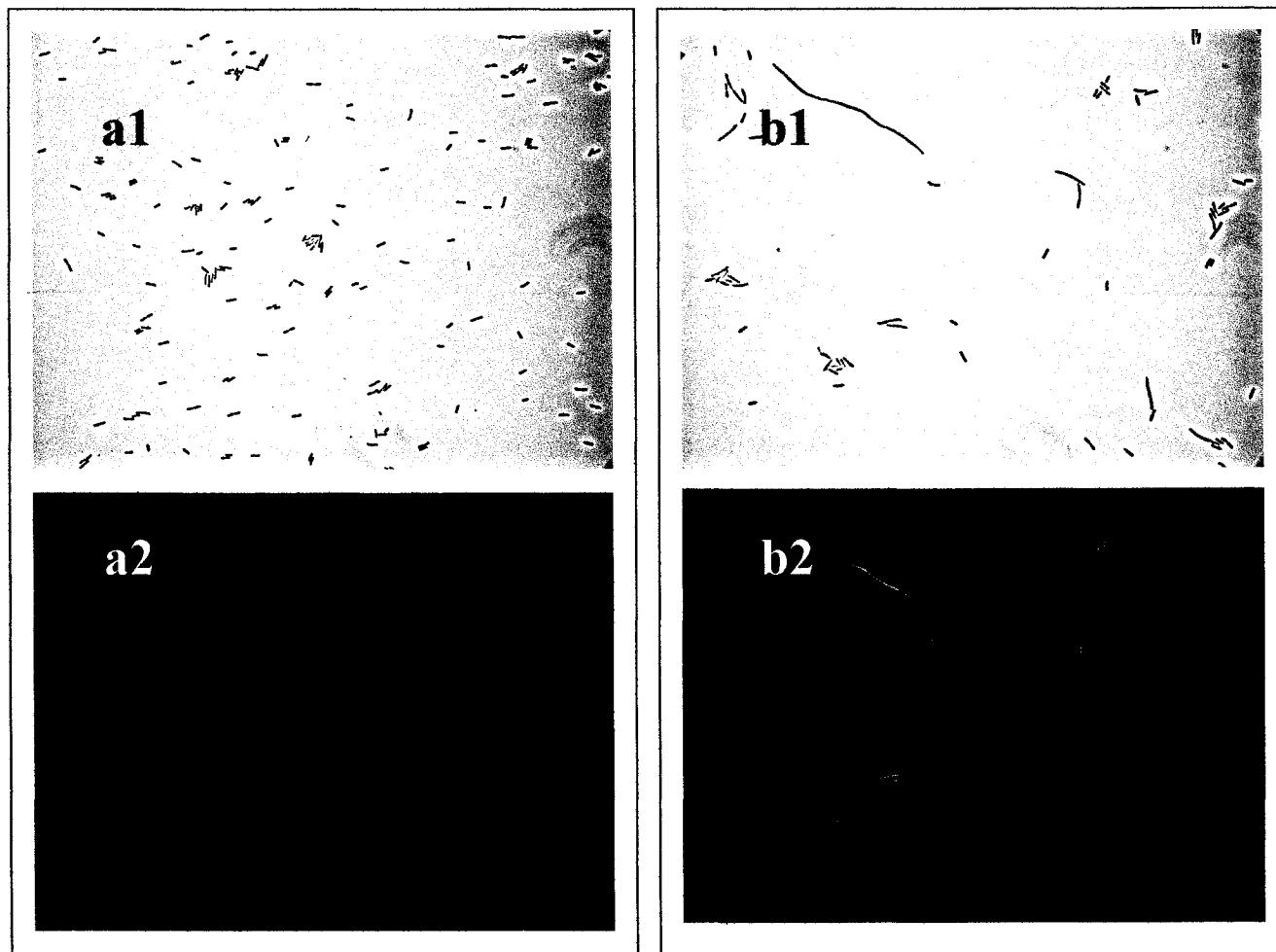


Figure 19: Localization of DNA by DAPI-staining strains *CU1008* (a) and *CU1008ssd17UVS* (b). Cells were grown to early stationary phase in glucose minimal medium at 37°C, chilled, washed and stained with DAPI prior to being visualized using phase-contrast (1) and fluorescence (2) microscopy. DNA does not properly segregate in *CU1008ssd17UVS* filaments.

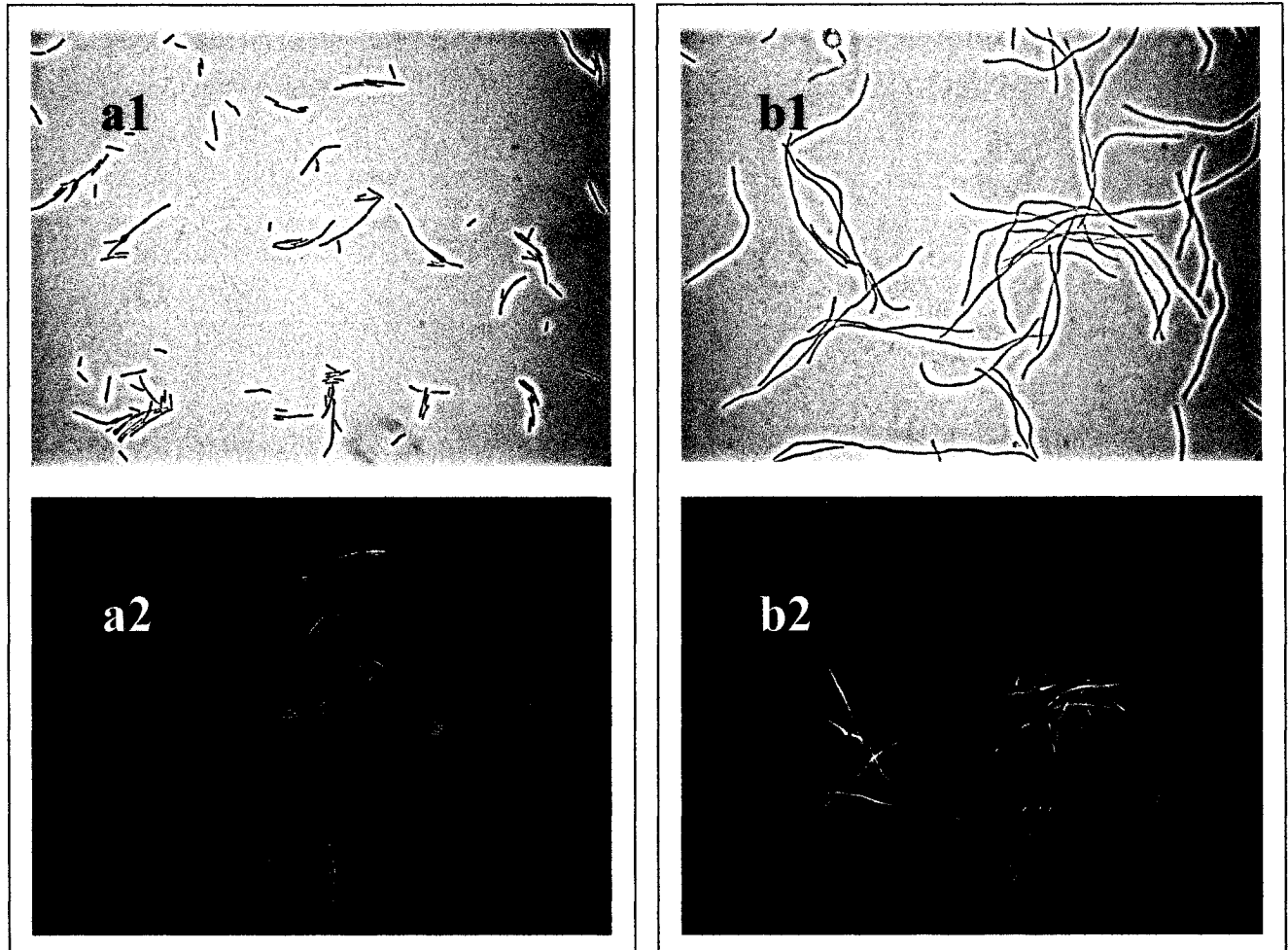


Figure 20: Localization of DNA by DAPI-staining strains *CU1008zipA-gfp* (a) and *CU1008ssd17UVSzipA-gfp* (b). Treatment of cells and panel representation is the same as described in Figure 19. DNA does not properly segregate in either of these two *zipA-gfp* merodiploid strains.

Part 13. *Ssd* transductants have increased *cpxP* transcription.

The mutation in the *cpxP* promoter region of the *ssd* mutant was suspected to cause an altered *cpxP* transcription. I therefore was interested in quantifying *cpxP* RNA expression in the parent strain and *ssd* transductants. The method I used involves isolating total RNA, reverse transcribing it into cDNA and amplifying the cDNA by PCR using specific primers designed to amplify an internal portion of the gene of interest. The results presented here are taken from experiments done in triplicate, with total RNA isolated from cultures grown in glucose minimal medium. Each extraction product was reverse transcribed, and each cDNA was PCR amplified at least once.

When grown to log phase in glucose minimal medium, RNA expression analysis showed that *cpxP* was transcribed in approximately equal amounts in the parental *CU1008* and in *ssd* transductants *CU1008ssd17UVS*, *CU1008ssdL9-3* and *CU1008ssd03* strains (Figure 21, panel 1). However, when these strains were grown to stationary phase *cpxP* was transcribed approximately 40-fold higher in all three *ssd* transductants relative to the parent strain (Figure 21, panel 2).

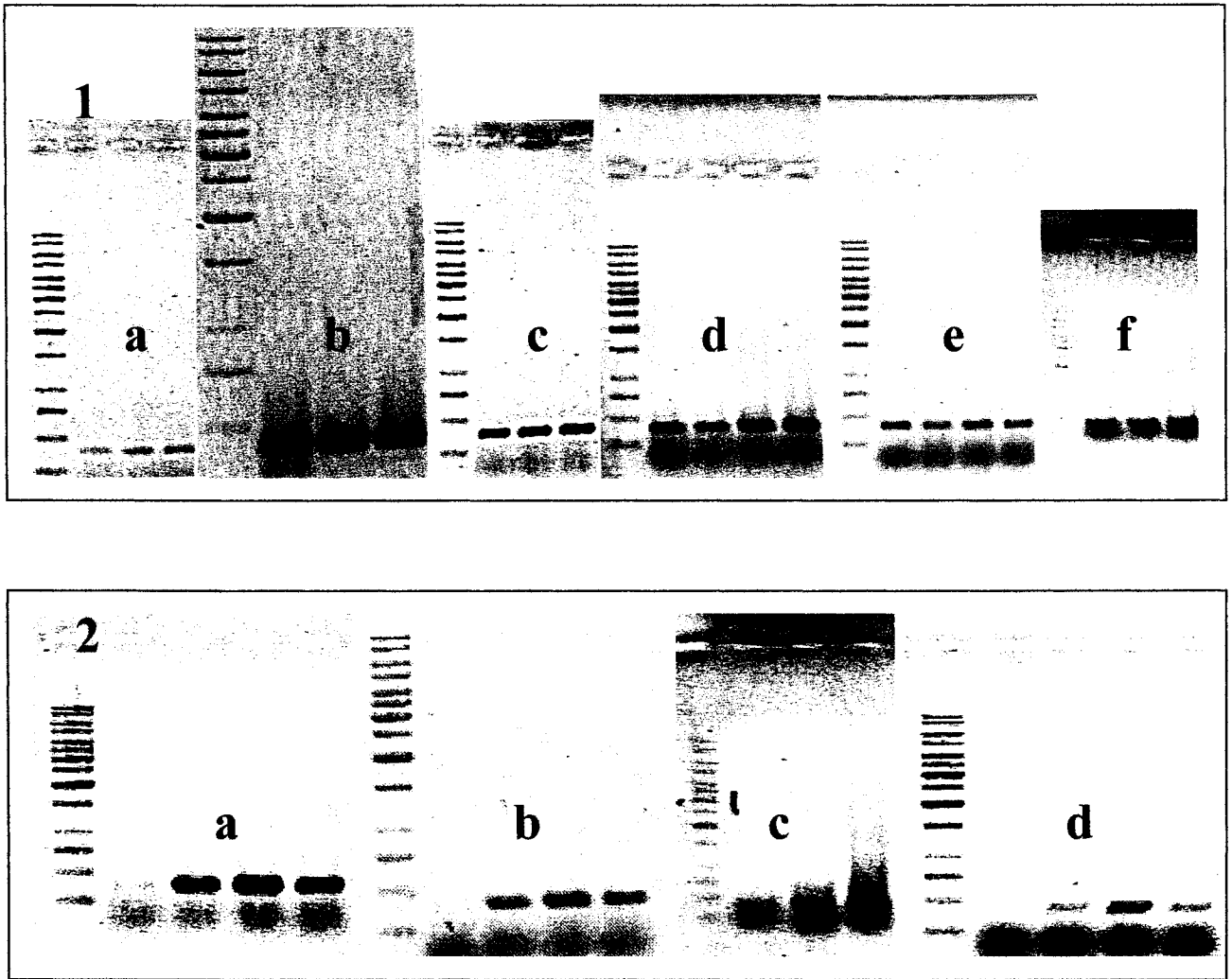


Figure 21: Parent strain *CU1008* and *ssd* transductant *cpxP* transcription at log (1) and stationary (2) phase in glucose minimal medium. At log phase (1), *cpxP* transcription levels are equivalent in parental *CU1008*, and *ssd* transductants *CU1008ssd17UVS*, *CU1008ssdL9-3* and *CU1008ssd03*. However, *cpxP* transcription is much higher in the *ssd* transductant at stationary phase (2). Sample order in panels 1 (a-to-f) and 2(a-to-d) is *CU1008*, *CU1008ssd17UVS*, and *CU1008ssdL9-3*. The fourth sample in shown in some panels is *CU1008ssd03*.

Part 14. The *cpxRA* transcript of *CU1008ssd17UVS* is degraded.

The 144bp intergenic promoter region between the *cpxP* and *cpxRA* RNA messages (containing divergent promoter elements for both transcripts) is crowded because the two -35 divergent promoter elements are separated by only 48bp. Since the CpxR is a positive regulator for *cpxP* transcription and its binding boxes are also within these 48bp, I felt it necessary to verify that elevated *cpxP* transcription observed in the *CU1008ssd* strains was not due to an elevated *cpxR* transcription leading to an increase in CpxR response regulator.

RNA expression analysis experiments showed the *cpxR* and *cpxA* transcripts were present in *CU1008*, *CU1008ssdL9-3* and *CU1008ssd03* strains (grown to log or early stationary phase in glucose minimal medium), but were no higher in the *ssd* transductants than in the parental strain (Figure 22, panels 1 and 2). Surprisingly, the *cpxR* and *cpxA* transcripts were faint (at log phase) or absent (at stationary phase) in the *CU1008ssd17UVS* strain grown in glucose minimal medium (Figure 22, panels 1 and 2). To check if the transcript was actually present but degraded, I designed two new sets of primers to PCR amplify at positions further into the *cpxRA* transcript. These new primer sets successfully PCR amplified the *CU1008ssd17UVS cpxRA* transcript (Figure 23). This indicated that the *CU1008ssd17UVS cpxRA* transcript may be degraded from both ends.

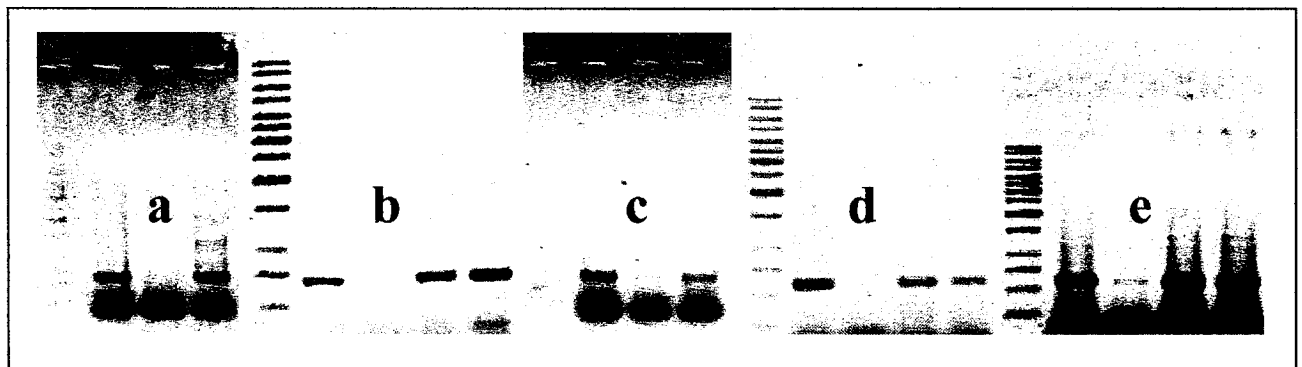
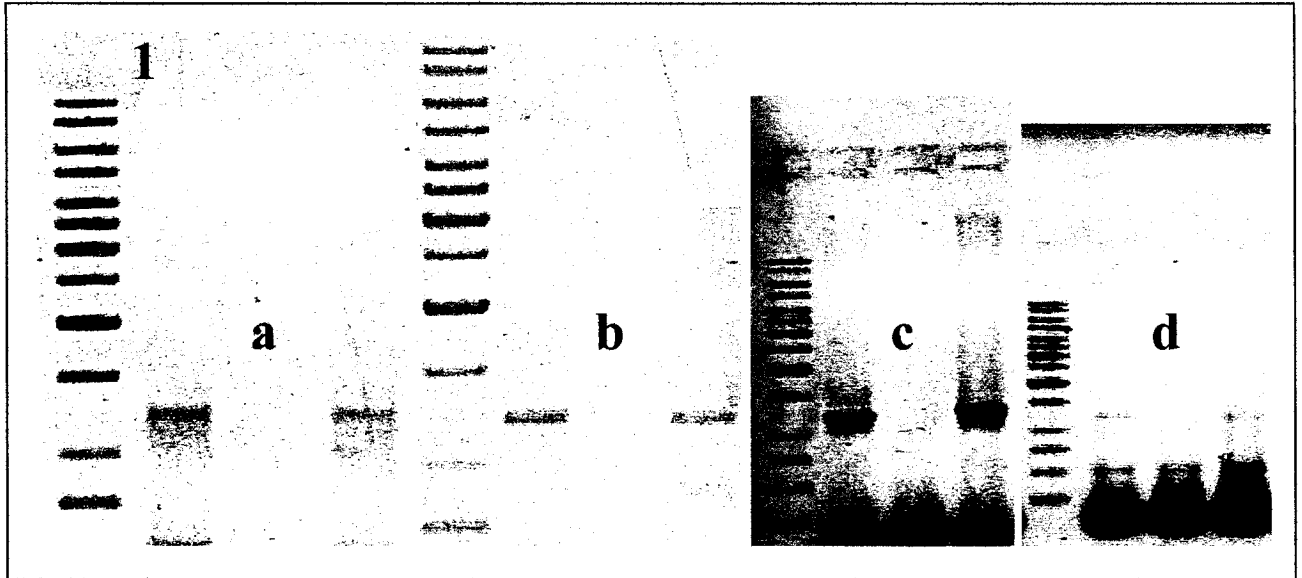


Figure 22: The *cpxR* (1) and *cpxA* (2) transcripts are faint or absent in *CU1008ssd17UVS* grown in glucose minimal medium. Sample order for *cpxR* transcript 1 (a-to-d) and *cpxA* transcript 2 (a-to-e) is the same as described in Figure 21.

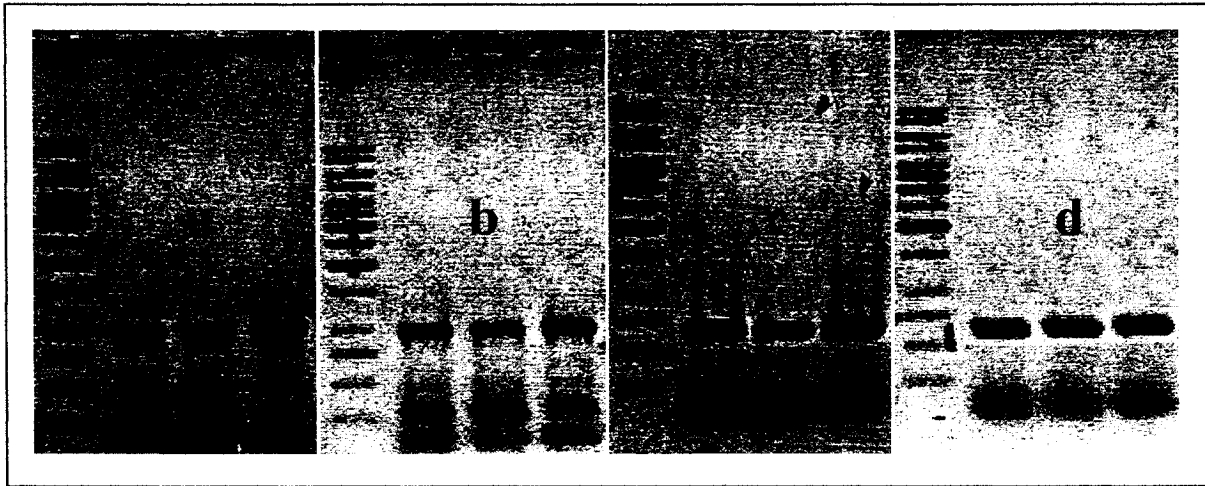


Figure 23: New internal *cpxR* and *cpxA* specific primer sets prove the *CU1008ssd17UVS cpxRA* message is transcribed. Sample order in a, b, c and d is *CU1008*, *CU1008ssd17UVS* and *CU1008ssdL9-3*.

Part 15. *cpxP* transcription in strains *CU1008ΔcpxR* and *CU1008ΔcpxA*.

The parental and *cpx* deletion strains were tested for variability of *cpxP* transcription. RNA expression analysis revealed that the *CU1008ΔcpxR* had approximately 25% *cpxP* expression levels relative to the parental strain. This was expected since CpxR positively regulates *cpxP* transcription, and a strain carrying a deletion in *cpxR* should diminish *cpxP* transcription (Figure 24; DiGiuseppe and Silhavy, 2003).

The *CU1008ΔcpxA* deletion mutant had approximately threefold higher *cpxP* transcription than that observed for the parental strain (Figure 24). This can be accounted for by the lack of the CpxA phosphatase responsible for inactivating CpxR-P, resulting in a constitutively activated CpxR. Since the CpxA phosphatase responsible for phosphorylating CpxR is absent in strains carrying a *cpxA* deletion, this suggests that another phosphate donor may phosphorylate CpxR.

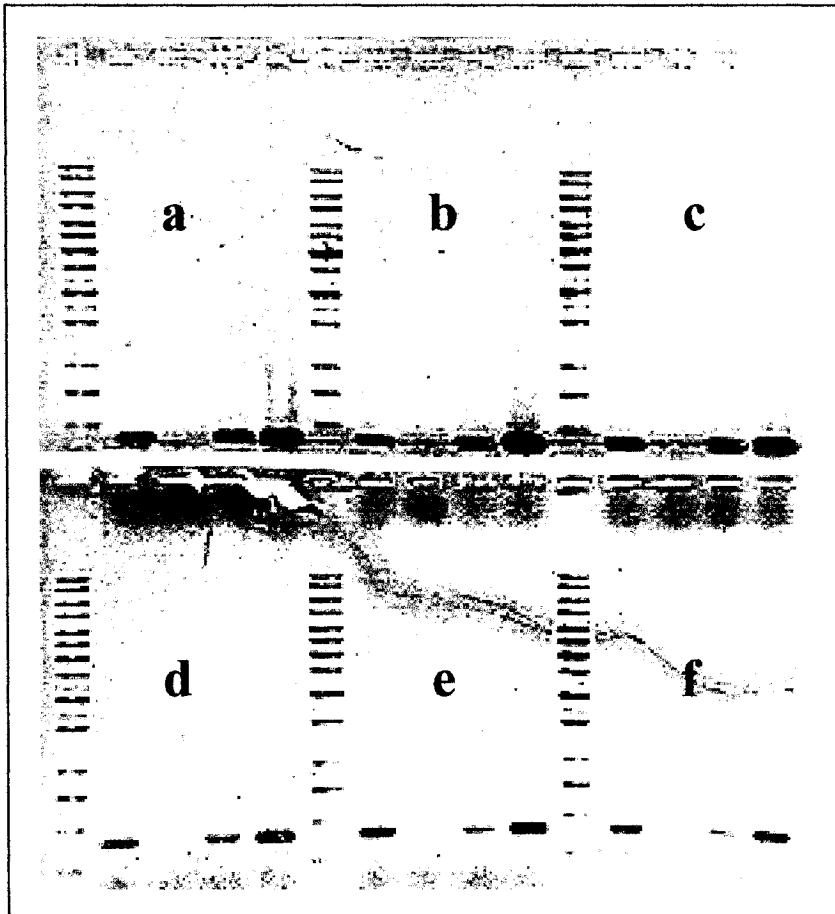


Figure 24: *CU1008* Δ *cpxR* and *CU1008* Δ *cpxA* deletion mutants have effects on *cpxP* transcription. The sample order in a to f is *CU1008*, *CU1008* Δ *cpxP*, *CU1008* Δ *cpxR* and *CU1008* Δ *cpxA*.

Part 16. L-SD activity in the *cpxA sdaA* double mutant strain.

A deletion of the *cpxA* gene results in an increase in L-SD activity (Table 3). This may be due to an increase in synthesis of *sdaA*, but there are two other serine deaminases – *sdaB* and *tdcG* – which could be involved. I investigated the effect of the *cpxA* deletion mutant on *sdaA* transcription. To do this, I constructed a *CUI1008ΔcpxA sdaA::lacZ* double mutant by transducing the *sdaA::lacZ* from a *CUI1008 sdaA::lacZ* lysate into the *CUI1008ΔcpxA* deletion strain.

A strain carrying the *sdaA::lacZ* fusion makes no L-SD in glucose minimal medium. Therefore, the *CUI1008ΔcpxA sdaA::lacZ* strain is not expected to have L-SD activity in glucose minimal medium. However, it does. The *CUI1008ΔcpxA sdaA::lacZ* strain has half the L-SD activity levels observed for the *CUI1008ΔcpxA* (Table 9). This indicates that the *sdaA* must be one of the serine deaminases affected by the *cpxA* deletion, but that *sdaB* or *tdcG* transcription or some other serine deaminase may also be affected by a *cpxA* deletion.

Table 9: L-SD activity levels of the *CU1008*, *CU1008sdaA::lacZ*, *CU1008ΔcpxA* and the *CU1008ΔcpxAsdaA::lacZ* double mutant.

Strain	L-SD activity (nmol pyruvate)
<i>CU1008</i>	7.876
<i>CU1008sdaA::lacZ</i>	NDA
<i>CU1008ΔcpxA</i>	233.248
<i>CU1008ΔcpxAsdaA::lacZ</i>	112.080

The whole cell L-SD assay was conducted as described in Table 3. “NDA” represents no detectable activity.

Part 17. The *sdaB*, *sdaC*, and *tdcG* genes are transcribed in glucose minimal medium.

I was curious to know which of the serine deaminase genes, or if the serine symporter (*sdaC*) was transcribed in *ssd* transductants and *cpxA* deletion mutant strains grown in glucose minimal medium. Using the RNA expression analysis technique, cDNA samples of these strains were amplified using PCR primers specific to internal portions of *sdaA*, *sdaB*, *sdaC* and *tdcG*. All four genes are expressed in the parental, *ssd* transductants and *cpx* deletion strains in glucose minimal medium. Figure 25 shows that the *sdaB*, *sdaC* and *tdcG* RNA messages are present in the parental and the three *cpx* deletion mutant strains. It has previously been shown that *sdaA* is transcribed in glucose minimal medium. The results presented here contradicts the idea that the *sdaB*, *sdaC* and *tdcG* genes are not transcribed in glucose minimal medium due to catabolite repression (Shao *et al.*, 1994; Hesslinger *et al.*, 1998), and suggests that a post-transcriptional regulation mechanism is responsible for the inactive-state of these L-serine deaminases in glucose grown cells.

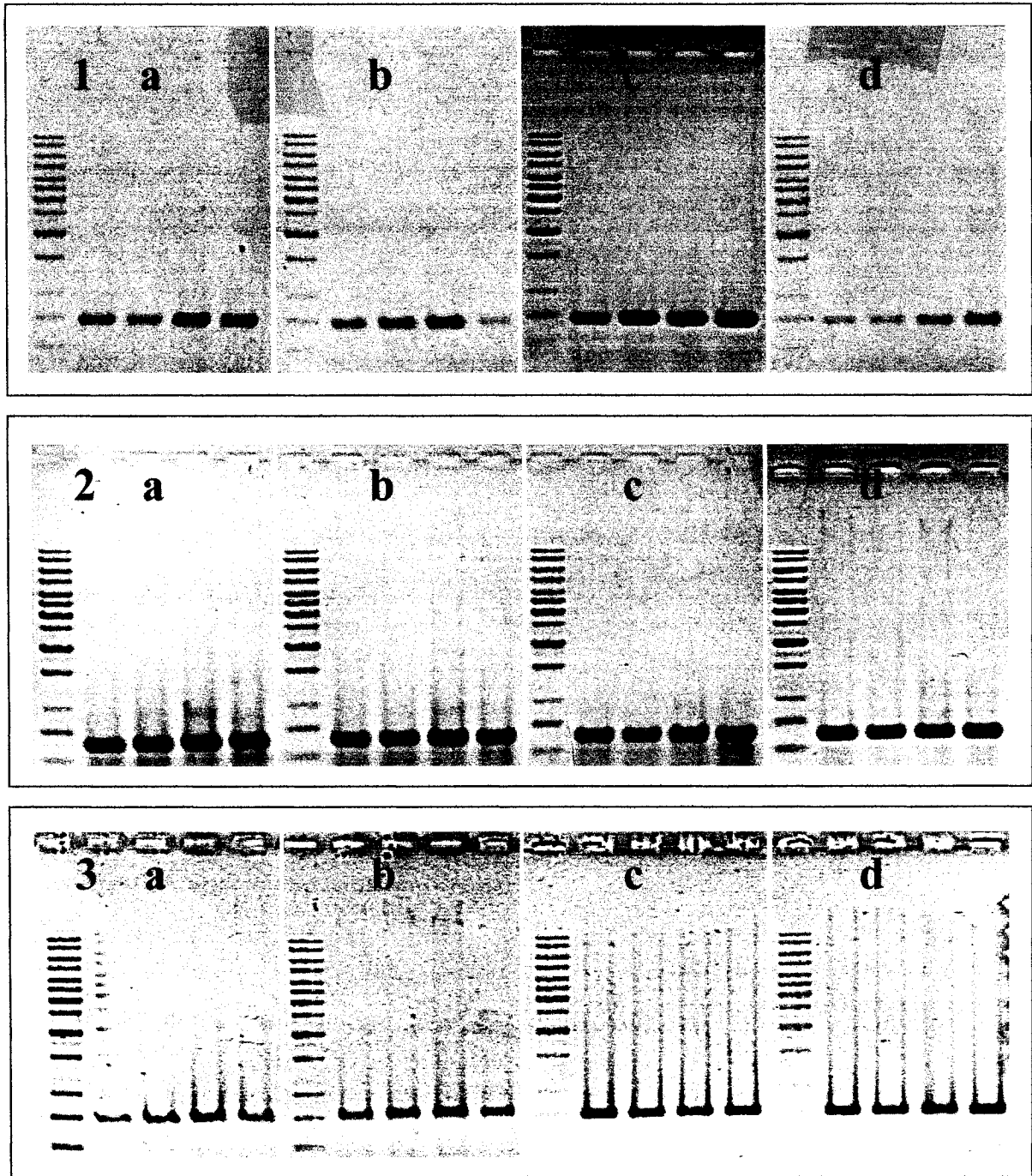


Figure 25: The *sdaB* (1), *sdaC* (2) and *tdcG* (3) genes are transcribed in the parental and *cpx* deletion mutants. Panels a, b, c and d represent repeats of sample order *CU1008*, *CU1008ΔcpxP*, *CU1008ΔcpxR* and *CU1008ΔcpxA*.

Discussion

In this work, I have shown that the *ssd* mutation lies within the *cpx* regulon but affects much more than *cpxA*. The *ssd* mutation, a *cpxA* deletion mutant and a variety of *cpxA** gain-of-function mutant all had high levels of L-SD activity, growth on serine and resistance to amikacin. However the strains carrying these mutations are not stable and tend to collect suppressor mutations which moderate the phenotype and probably increase their chances of survival.

Part 1. The occurrence of extragenic suppressor mutations in “sick” *ssd* transductants and in *cpxA gain-of-function mutants.**

The original mutation causing the *ssd* phenotype had such a dramatic effect on the *E. coli* phenotype, and made the strain so “sick” that it quickly accumulated revertants and suppressors that could partially restore the wild type phenotype. This was evidenced by lowered L-SD activity in *ssd* revertants, relative to the original *ssd* mutant (Newman *et al.*, 1982a). The *ssd* mutation was mapped to a chromosomal area between *rha* and *metB* (Morris and Newman, 1980). Linked and unlinked suppressor mutations were described at that time but not further characterized.

The chromosomal interval between *rha* and *metB* also contained a locus then known as *cpxA*. This is now better understood as the *cpx* regulon and is comprised of

three genes: *cpxP*, *cpxR* and *cpxA*. The *ssd* mutation has been widely annotated in databases as an allele of *cpxA* according to the suggestion of Rainwater and Silverman in 1990. Some parts of its pleiotropic phenotype are observed in *cpxA* mutants. However the sequencing results presented here show that the *ssd* phenotype is much more complex.

In this study, an *ssd* mutant isolated some 25 years ago was taken from -86°C storage and used to produce two new sets of *ssd* transductants. It was clear, even prior to sequencing, that these *ssd* transductants must carry suppressors since they were much less physiologically fragile than the mutants originally described. In spite of being able to grow on serine, having high L-SD, and being amikacin-resistant, only the mildly UV-sensitive *ssd* transductants (*CU1008ssd01*, *CU1008ssd03* and *CU1008ssd11*) possessed the same colony morphology as the parent strain when grown on plates (Figure 6a). Conversely, *ssd* transductants that were extremely UV-sensitive (*CU1008ssd17UVS* and *CU1008ssd08UVS*) were highly mucoid in appearance (Figure 6b). The mucoid *ssd* transductants were fragile and could not survive for more than 10 days at 4°C. Under the same conditions, the parental strain can survive for many months. Suppressor mutation accumulation was also suspected when the *CU1008ssd17UVS* transductant recovered from storage emerged as two colony types possessing different phenotypes, while having identical *cpx* regulon sequences. I conclude that the *ssd* strain originally described was similar to strain *CU1008ssd17UVS* and that other mildly UV-sensitive *ssd* transductants carry suppressors. These suppressors may have preexisted in strain *CU1008ssd01*, in which case they must be closely linked. Otherwise, the suppressor mutations could have

arisen following purification of the *ssd* transductants and thus could be anywhere in the genome.

I showed that all the *ssd* transductants carry a mutation in the *cpxP* promoter region mutation. This is in fact the only *cpx* regulon mutation in the donor *CU1008ssd01* used to produce the new *ssd* transductants in this study. All of the new *ssd* transductants had this mutation along with one of two possible mutations in *cpxA*. The *cpxA* mutation was either a point mutation or a two codon deletion affecting its N-terminal transmembrane domain (Table 7).

Since all strains had the same *cpxP* promoter mutation, and the donor *CU1008ssd01* had this mutation only, it was tempting to conclude that this was the original *ssd* mutation. In this case, both the single base pair change, and the two-codon deletion in *cpxA* would have been selected as a compensation for physiological difficulties resulting from the *cpxP* promoter mutation. However, it cannot be said with high confidence which of the two was the original *ssd* mutation and which was the second—extragenic suppressor mutation.

I also showed that the phenotype of the *ssd* mutant is strongly affected by mutations outside of the *cpx* regulon, as originally suggested by the isolation of unlinked suppressors (Newman et al., 1982a). Here I showed that *ssd* transductants having identical *cpx* regulon sequences may have very different phenotypes. They can be mildly UV-sensitive and nonmucoid, with a microscopically determined parental morphology,

or can be extremely UV-sensitive and mucoid, with a tendency to make filaments. Since these have the same *cpx* regulon sequence, it is fair to assume that they differ at some point outside the *cpx* regulon.

All *cpxA** gain-of-function mutants are amikacin-resistant and have high L-SD activity. They can all grow on serine except strain TR198 (Table 4). The two TR144 variants originating from a single strain from Silhavy exhibited different phenotypes. The TR144 cells look like the parent strain cells while the TR144UVS looks “curly” (Figure 8). TR144 has much lower L-SD activity than TR144UVS. TR144UVS had only one mutation in the *cpx* regulon (i.e., the expected *cpxA*Δ93-124 periplasmic space domain deletion; Figure 7), while the TR144 variant had this mutation and a second *cpx* regulon mutation in the *cpxRA* promoter (Figure 15). This presumably causes a change in CpxR and CpxA protein concentrations, which reduces UV-sensitivity and L-SD activity levels. Mutations destabilizing CpxA activity were postulated to be subject to extragenic suppressor mutations that result in a partial restoration of the parent strain phenotype (McEwen and Silverman, 1980). In the *ssd* transductant example, suppressors (inside and outside of the *cpx* regulon) cause lowered L-SD activity and UV-sensitivity. This is likely the result also brought about by the TR144 *cpxRA* promoter suppressor. However, the immediate effects of this suppressor mutation may be different. The long deletion in the *cpxA* gene may hinder CpxA function. The *cpxRA* promoter suppressor mutation might cause an increase in the transcription of *cpxRA*, so that higher cellular concentrations of CpxA and CpxR would then be present for activation through

phosphorylation events. The observation that the *cpxP* deletion mutant is UV-sensitive supports the hypothesis that the TR144UVS may have hampered *cpxP* transcription.

Whatever else the suppressor in TR144 does, it does not alter the fragility of the strain. The live/dead fluorescence technique showed that both TR144 variants show cell membrane fragility and lyse when grown to early stationary phase in glucose minimal medium (Figure 17).

Part 2. The *ssd* phenotype could be a result of the *cpxA* mutation or the *cpxP* promoter region mutation (or both).

Some *ssd* phenotypes—high L-SD activity, amikacin-resistance and the ability to grow on serine—are also seen in strains carrying the *cpxA* deletion (Tables 3 and 5). The growth on serine and other characteristics of the phenotype—UV-sensitivity, mucoid colonies and formation of filaments—are seen in strains carrying the *cpxP* deletion. Since we cannot know which was the original *ssd* mutation, two different interpretations can be made about the order of occurrence of the *cpxP* promoter (Figure 13) and *cpxA* (Figure 14) mutations and what effects these have on the *ssd* phenotype.

The first *ssd* mutation may have occurred in the CpxA N-terminal periplasmic membrane spanning domain, and destabilized the CpxA function by interfering with its localization to the membrane. Either the single point mutation or the two-codon deletion

might result in destabilizing the N-terminal region anchored to the periplasmic membrane. It is known that the *cpxA** gain-of-function mutant TR199 has a single point mutation in the CpxA C-terminal periplasmic membrane-spanning region and that this mutation alone causes the strain to grow on serine, gain amikacin-resistance and have high L-SD activity (Figure 7, Table 4).

Mutations altering the function of CpxA have a direct impact on the function of the response regulator CpxR. CpxR is responsible for regulating the transcription of many genes and a pleiotropic phenotype can be expected if its activation is altered. In this view, the *cpxP* promoter suppressor mutation may have arisen and caused an increase in *cpxP* transcription (as indicated by an increased RNA expression; Figure 21). The ensuing increase in the cellular pool of CpxP might have caused the altered CpxA to be more firmly secured into the membrane, resulting in a partial restoration of the parental phenotype. However, since this suppressor mutation can only partially restore the phenotype, other mutations outside of the *cpx* regulon also may occur, causing, for example, the extremely UV-sensitive *ssd* transductants to become only mildly UV-sensitive.

The second interpretation is that the first *ssd* mutation occurs in the *cpxP* promoter region mutation and causes an increase in the cellular pool of CpxP. This overabundance of CpxP proteins interacts with the CpxA and keeps it so highly negatively regulated that it cannot phosphorylate the response regulator CpxR. This results in a highly pleiotropic phenotype as was observed for the *cpxA* deletion mutant. A

suppressor mutation arises in the CpxA N-terminal periplasmic-spanning domain and causes this CpxA domain to be loosened from the periplasmic membrane. This can partially restore the parental strain phenotype by modifying the protein-protein interaction between CpxA and CpxP (Ruiz and Silhavy, 2005). The partial restoration of parental phenotype is most evident from the lowered activity levels of L-SD—which is the phenotype that can be best quantified in this and other *ssd* mutant studies (Newman *et al.*, 1982). This interpretation is further reinforced by the fact that the *pcpxP* plasmid transformed into the parent strain causes an increase in L-SD activity even without the presence of an inducer. If the inducer had been added the periplasmic CpxP pool would have been even larger, most likely leading to even higher levels of L-SD activity in the *CU1008pcpxP* (Table 3). This is what was also observed from transforming the *ssd* transductants with *pcpxP* (Table 5). Transforming the *ssd* transductants with this plasmid increased their L-SD activity to a level closer to what was observed in the original *ssd* mutant (E.B. Newman, personal communication). In this scenario, the suppressor mutation in *cpxA* causes additional phenotypes commonly observed in the *cpxA** and *cpxA* deletion mutant strains to be selected.

Part 3. The *cpxA gain-of-function and *cpxA* deletion mutants have similar phenotypes.**

Ssd transductants, *cpxA** gain-of-function mutants and *cpxA* deletion mutants all grow on serine, have high L-SD activity, are amikacin-resistant and have the same

“curly” morphology. The *cpxA** mutants lack the CpxA phosphatase activity responsible for dephosphorylating CpxR-P, and as a consequence the CpxR remains constitutively activated as the response regulator (De Wulf and Lin, 2000). It seemed odd that *cpxA** gain-of-function and *cpxA* deletion mutants could have similar phenotypes. If these phenotypes are a result of constitutively activated CpxR-P, then some other phosphate donor must be responsible for CpxR phosphorylation in the *cpxA* deletion mutant strain background. CpxR phosphorylation by an alternate phosphate donor (acetyl phosphate) has been demonstrated when a *cpxA* null mutant strain is grown in glucose minimal medium (Danese *et al.*, 1995). In such strains there may be lower concentrations of activated CpxR-P molecules. However the CpxR has been shown to tightly retain its phosphate group so that a phosphatase is necessary for its inactivation (Yamamoto *et al.*, 2005). Since no CpxA phosphatase activity exists in either the *cpxA** or *cpxA* deletion mutants, this is a likely explanation for how these two mutants demonstrate the same phenotype. Similarly, the *ssd* transductant *cpxA* mutation may result in a diminished CpxA phosphatase activity.

Part 4. The *ssd* transductants ability to grow on serine is the under the control of the CpxR response regulator.

The results from this study indicate that transforming the *ssd* transductants and *cpxA* deletion mutant strains with a *cpxA* plasmid abolishes growth on serine and high L-SD activity phenotypes (Tables 3 and 5). However, transforming a *cpxR* plasmid into the

ssd transductant can only abolish growth on serine, while L-SD activity remains high (Table 5). As the *ssd* transductant has elevated levels of *cpxP* expression, the CpxA must be highly inhibited—so much so that the *ssd* has very low CpxA kinase activity. The presence of *pcpxR* in the *ssd* transductant generates a higher concentration of CpxR available for phosphorylation by an alternative phosphate donor. Also, the lack of CpxA phosphatase activity in the *ssd* transductant would result in a perpetually activated CpxR-P response regulator. The fact that the *cpxP* deletion mutant grows on serine further supports this possibility because in this strain, CpxA cannot attain the phosphatase conformation that inactivates CpxR-P. Therefore, it is hypothesized that the loss of negative regulation of CpxA by CpxP, or increase in the pool of CpxR, will both have the effect of increasing the number of CpxR phosphorylation events. This, in turn, somehow allows such strains to grow on serine. Transforming the *ssd* transductants with *pcpxR* abolishes growth on serine, but has no effect on L-SD activity (Table 5). It is possible that the L-SD is not regulated by the Cpx two-component signal transduction systems CpxR response regulator.

Part 5. Several deletion mutants of glycolysis, serine biosynthesis and Cpx pathways can grow on serine.

The number of known promoters under the control of the CpxR response regulator is quickly growing (De Wulf et al., 2002). From the results presented in this thesis, CpxR appears to be responsible for inhibiting growth on serine in the parental

strain. Therefore I decided to test a variety of strains carrying deletions (H. Mori) in genes involved in the glycolytic and serine metabolic pathways (Figure 11), and in the Cpx two-component regulatory system (Figure 12) to see whether any could enable growth on serine. Of the 38 deletion mutants tested, three from glycolysis ($\Delta fbaB$, $\Delta pgmI$ and $\Delta pykF$), two from serine biosynthesis ($\Delta serA$ and $\Delta serB$) and two from the Cpx system ($\Delta nlpE$ and $\Delta ppiA$) could grow on serine (see Table 6).

Glycolysis involves the conversion of β -D-glucose-6-phosphate into pyruvate (Figure 11). The L-serine metabolic pathway can also produce pyruvate using glycolysis intermediate 3-phosphoglycerate (Ravnikar and Somerville, 1987). The *fbaB* gene encodes a fructose-1,6-bisphosphate aldolase which converts fructose to glyceraldehyde. This deletion mutant was not expected to grow on serine because its deletion interrupts the glycolytic pathway prior to the synthesis of the 3-phosphoglycerate substrate required for serine metabolism (Irani and Maitra, 1977). Growth on serine was a much more anticipated scenario for the *pgmI* deletion mutant. The *pgmI* gene encodes the phosphoglycerate mutase enzyme responsible for converting 3-phosphoglycerate into 2-phosphoglycerate, and its deletion is expected to cause increased 3-phosphoglycerate concentration for use by the serine metabolic pathway. Finally, the *pykF* gene encodes the last glycolytic step which converts phosphoenolpyruvate into pyruvate. It is, like the *pgmI* deletion strain, a much more likely target for the growth on serine phenotype because all enzymatic reactions leading to the production of phosphoenolpyruvate from 3-phosphoglycerate are reversible.

The serine biosynthetic pathway converts 3-phosphoglycerate into L-serine (Pizer, 1964 and 1963). It was tremendously surprising that deleting two of the three genes involved in serine biosynthesis could cause growth on serine to occur. When serine is transported from outside the cell, the pool of serine in the cell is much higher than when it is made *de novo*. In the parent strain, endogenous serine concentrations are limited by some compensatory mechanism. However, in these biosynthetic mutants that mechanism may be overridden because the mutants would otherwise not have enough serine to survive.

Strains carrying *cpxA* mutations are known to gain the ability to use serine as sole carbon source (De Wulf and Lin, 2000). The *ppiA* gene encodes a peptidylprolyl-cis-trans-isomerase-A that is positively regulated by the CpxR response regulator (Pogliano *et al.*, 1997). The fact that a strain carrying a *ppiA* deletion grows on serine further supports the idea that this phenotype is under the control of the Cpx two-component system, and suggests that the PpiA is directly involved in the negative regulation of growth on serine (Figure 12). Other results from this thesis showed that when *pcpxR* was transformed into an *ssd* transductant it caused growth on serine to be abolished (Table 5). It is therefore possible that growth on serine results from a non-phosphorylated, inactivated form of CpxR that cannot positively regulate *ppiA* transcription. In addition, the fact that a strain carrying a deleted *nlpE* gene was also able to use serine as a sole carbon source further supports the conclusion that the Cpx-system is responsible for inhibiting growth on serine in the parental strain. This is because the overproduction of

the NlpE outer membrane lipoprotein activates the Cpx-system (Snyder *et al.*, 1995), and its absence should therefore cause diminished concentrations of activated CpxR-P.

Not one of the deletion mutants that could grow on L-serine had detectable levels of L-SD activity using the standard whole-cell assay. This result further supports the hypothesis of separate schemes of regulation for growth on serine and L-SD activity phenotypes. This also suggests that growth on serine can result from mechanisms of serine metabolism that do not involve the three known L-serine deaminases.

Part 6. L-serine deaminase activity regulation is dependent upon a functional CpxA protein rather than on the CpxR response regulator.

The highest levels of L-SD activity are observed in the *cpxA** gain-of-function mutant TR144UVS and the *cpxA* deletion mutant. Strain TR144UVS has a large deletion in a portion of the CpxA periplasmic loop believed to make contact with CpxP, which normally keeps the CpxA kinase in an inactive state (Raivio *et al.*, 1999). In fact, this large deletion may cause the CpxA protein to lose the ability to fold into its proper conformation, resulting in a phenotype identical to that of a *cpxA* deletion mutant strain. Since both mutants have extremely high L-SD activity, what the TR144UVS and *cpxA* deletion mutants are lacking is likely an inhibitor of L-SD transcription or L-SD activity. Transforming the *ssd* transductants with *pcpxA* lowered their L-SD activity. However, transforming them with *pcpxR* had no effect on L-SD activity levels (Table 5). My

hypothesis is that L-SD regulation is controlled by a CpxA mechanism independent of the Cpx two-component regulatory system.

In 2003 Nakayama *et al.* conducted a study that demonstrated that CpxA—but not CpxR—was required for the transcription activation of bacterium *Salmonella enterica* serovar *Typhimurium* virulence gene *hilA* (Humphreys *et al.*, 2004). The mechanisms underlying the Cpx two-component regulatory system of *S. Typhimurium* closely resemble those in *E. coli*, and the Cpx system is essential for *in vivo* pathogenesis by gram-negative bacteria (Raivio, 2005). Humphreys *et al.*, also showed that a *cpxR* mutant had no effect on *S. Typhimurium* macrophage colonization, but that a *cpxA* mutant caused a 10% increase in colonization.

Given that the CpxA has functions which work independently of the Cpx two-component regulatory system, it is possible that the negative regulation of L-SD activity is under the control of a fully functional CpxA protein, and that this regulation does not involve the CpxR response regulator. Three results from this thesis suggest the possibility of an independent role for CpxA. First, neither the addition of *pcpxR* to the *ssd* transductant, nor the *cpxR* deletion mutant, has any effect on L-SD activity levels (Tables 3 and 5). Secondly, the *cpxA** gain-of-function mutant TR198 has high L-SD activity but cannot grow on serine (Table 4). The point mutation in the TR198 CpxA C-terminal cytoplasmic domain may have an effect on the CpxA tertiary structure needed for L-SD activity regulation (Figure 7), but no significant effect on that needed for CpxR activation. Finally, when *pcpxP* is transformed into the parental strain or the *ssd*

transductants, it causes an increase of L-SD activity levels (Tables 3 and 5). Furthermore, *cpxP* deletion mutants have no effect on L-SD since these strains have functional CpxA proteins free to negatively regulate L-SD activity by some unknown mechanism (Table 3).

Part 7. The high L-SD activity of *cpxA* mutant strains is from a combination of L-serine deaminases.

In this study, I constructed a *cpxA* and *sdaA::lacZ* double mutant with the expectation that it would have parental L-SD activity when grown in glucose minimal medium. This was because *sdaA* is considered to be the only L-serine deaminase gene expressed in this growth medium (Su *et al.*, 1989), so one would not expect to find any L-SD activity in the double mutant. At least two other L-serine deaminases (*sdaB* and *tdcG*) are known to exist and are active in rich medium, but these were not expected to be transcribed in glucose minimal medium because their transcription is controlled by catabolite repression (Shao *et al.*, 1994; Hesslinger *et al.*, 1998). However, the *cpxA-sdaA* double mutant had about half the L-SD activity levels observed for the *cpxA* deletion mutant possessing a functional *sdaA* gene (Table 9). This suggested that *sdaB*, *tdcG* or another gene coding for an unknown L-serine deaminase may be activated in strains carrying a *cpxA* deletion. One possibility is that CpxA may act (directly or indirectly) as a negative regulator for the transcription of *sdaCB* or *tdcG* or some other unknown serine deaminase gene. It is also possible that L-SD regulation by CpxA may

involve a protein-protein interaction mechanism since SdaA, SdaB and TdcG share amino acid sequence homology (Shao and Newman, 1993; Hesslinger et al., 1998), which indicates they are likely to have similar tertiary structure.

The RNA expression analysis technique (with primers specific for *sdaB*, *sdaC* and *tdcG* genes) was used to investigate whether these genes could be transcribed in glucose minimal medium. The *sdaB* and *sdaC* (serine symporter) genes are transcribed as a single RNA message. Therefore it was expected that both cDNA products would be present if transcription occurred at this promoter. After the parent strain (*CU1008*) and three *cpx* deletion mutants (*CU1008ΔcpxP*, *CU1008ΔcpxR* and *CU1008ΔcpxA*) were grown in glucose minimal medium, all strains revealed the expected PCR amplification product for the three genes tested (Figure 25). Owing to the high degree of sequence homology between these three genes, primer specificity was verified by using strains carrying deletions for these three genes. Indeed, the regulation of these two additional L-SD enzymes must be more complicated than previously anticipated. The results presented in this thesis suggest that the regulation of these L-serine deaminases (or possibly some other uncharacterized serine deaminase) involves the participation of a functional CpxA. I conclude that one or both of the other two L-SD enzymes may have increased activity in a *cpxA* deletion mutant, and that all three L-SD genes are transcribed in glucose minimal medium in the parent strain *CU1008*.

Part 8. Extreme UV-sensitive *ssd* transductants or strains carrying *cpxR* or *cpxP* gene deletions can become mucoids.

Strains carrying a *cpxR* deletion make mucoid colonies (Figure 6). Some *ssd* transductants are also mucoid as are some strains carrying a *cpxP* deletion. However, none of the *cpxA* deletion mutants make mucoid colonies. Also, mucoid *ssd* transductants and mucoid *cpxP* deletion mutant transductants were both extremely sensitive to UV irradiation—suggesting that the extreme UV-sensitivity and mucoid phenotypes have the same cause. UV-sensitivity has been previously reported for mucoid *E. coli* strains (Hamelin and Chung, 1975). However, the fact that strains carrying the *cpxR* deletion were always mucoid but never UV sensitive, suggest that mucoidy and UV-sensitivity are from different causes. The only other phenotypic effect of the *cpxR* deletion was an increased amikacin-sensitivity relative to the parental strain (Table 3). This suggests that the CpxR response regulator is responsible for the control of a basal parental strain aminoglycoside-resistance—which is highly increased in *cpxA* mutants. Unfortunately the basis of this resistance is not known.

Mucoidy is understood to result from a change in capsule formation (Beiser and Davis, 1957). A direct relation between the mucoid phenotype and *cpx* mutants has not been reported. However, one study revealed that the *E. coli* RpoE was capable of restoring the mucoid phenotype to a nonmucoid *Pseudomonas aeruginosa algU* mutant (Yu *et al.*, 1995). The mucoid phenotype is linked to the persistence of bacterial infection because mucoid isolates of *P. aeruginosa* (Doggett *et al.*, 1969) and *E. coli*

(Linker and Evans, 1968) are found in the lungs of cystic fibrosis patients. The *rpoE* gene is negatively regulated by CpxR, an inhibition missing in a strain carrying a *cpxR* deletion.

The extremely UV-sensitive *ssd* transductants are also mucoid. Apart from their mucoidy, they are very similar to the *cpxA** gain-of-function mutants. These are deficient in CpxA phosphatase phenotype, and have an abundance of CpxP so that the *ssd* transductants are likely to have relatively low concentrations of CpxR-P. If this is the case, the UV-sensitive *ssd* transductants should be unable to regulate the *rpoE* transcription and these result in a mucoid phenotype. Those *ssd* transductants that are not mucoid in appearance (e.g., the *CU1008ssd17L*) are expected to have additional extragenic suppressor mutations outside of the *cpx* regulon.

Only twenty percent of the *cpxP* deletion mutant transductants were mucoid. Mucoidy thus could be due to a linked suppressor mutation. The transcription of *cpxP* is strongly increased in response to all CpxR-activating signals (Danese and Silhavy, 1998). Based on RNA expression analysis and the literature (Isaac *et al.*, 2005), it seems that *cpxP* is constitutively expressed even in non-induced cells (Figure 24). Besides its role in the Cpx pathway, the CpxP has also been found to be a periplasmic chaperone (DiGiuseppe & Silhavy, 2003; Duguay & Silhavy, 2004). It is therefore likely that mutations causing imbalances in *cpxP* expression would cause a broad change in phenotype, and thus a good background from which suppressor mutations might arise. In

the case of the *cpxP* deletion mutant, CpxR is constitutively activated and *rpoE* should not be transcribed.

Part 9. Regulation of *cpxP* transcription by CpxR and CpxA.

It has been previously reported that induction of *cpxP* transcription is dependent on CpxR, but independent of CpxA, which suggested that activation of the Cpx-system occurs without the presence of activated CpxA (DiGiuseppe and Silhavy, 2003). The results of RNA expression analysis experiments conducted in this study for the transcription of *cpxP* support the idea that *cpxP* transcription is dependent on CpxR since the *cpxP* transcript is faint in a *cpxR* deletion mutant background (Figure 24). However, these results also show that *cpxP* transcription is also dependent upon a CpxA. The *cpxP* transcript was observed to be three times higher in a *cpxA* deletion mutant than it was in the parental strain. This is very interesting because it suggests that when no CpxA is present, there exist no negative regulation on *cpxP* transcription.

CpxR is known as the Cpx-system response regulator. It is able to both positively and negatively regulate gene expression (Rowley et al., 2006). It has been shown that the CpxR must be phosphorylated by the CpxA or by another phosphate donor such as acetyl phosphate to act as a negative regulator (Ogasawara et al., 2004). In a study of *Legionella pneumophila* genes positively induced by CpxR, mobility shift assays showed that CpxR binds to its DNA recognition sequence in the presence or absence of a

phosphate donor (Gal-Mor and Segal, 2003). Therefore, it is possible that a misconception of CpxR functioning exists. The CpxR may bind to its DNA element in two different conformations (one in presence of the phosphate and another in its absence). This may explain how the CpxR can both positively and negatively regulate gene transcription.

In the absence of CpxA, the CpxR may only be phosphorylated at low levels by other phosphate donors, and may act as a constitutive positive regulator for the transcription of *cpxP*—thereby explaining the RNA expression analysis results. In a *cpxA* deletion strain, there is no CpxA phosphatase activity to render the CpxR inactive. In this view, the prime function for CpxA may not be to activate CpxR, but rather to inactivate it.

Part 10. The extremely UV-sensitive *ssd* transductant makes long filaments and survives in glucose minimal medium despite improper DNA segregation.

Fluorescent live/dead and DAPI staining techniques were used on the parental strain (*CU1008*) and on the extremely UV-sensitive mucoid *ssd* transductant (*CU1008ssd17UVS*). These strains were grown in glucose minimal medium, glucose rich medium and in rich medium. The parental strain grew well, with intact membranes (i.e., fluoresced mostly green) and segregated its DNA properly in all three growth media (Figure 16). The *CU1008ssd17UVS* transductant, however, grew into long filaments in

glucose minimal medium, though these had functional membranes as seen by green fluorescence. These filaments did not show normal DNA segregation in any medium in which they appeared (Figure 19). Despite not exhibiting extensive filaments when grown in rich media, the *CUI008ssd17UVS* cells suffered a high (60%) proportion of lysed cells.

E. coli makes filaments under a number of conditions. Filaments results from mutations made to the early cell division proteins responsible for forming the septum as the site of division (Taschner *et al.*, 1988). Cell division requires the assembly of at least 10 currently known proteins at mid-cell, and studies have revealed these have a well defined order of recruitment of to the septum (Vicente and Rico, 2006). The filamentous and extremely UV-sensitive *ssd* transductants and *cpxP* deletion mutant may share a common transcription modification of one of these genes. In *E. coli*, the early cell division protein FtsK serves to sort chromosomal DNA during cell division (Lesterlin *et al.*, 2004). Since the DNA is not properly segregated in the filaments of the *CUI008ssd17UVS* transductant, the problem with cell division may be an effect of an alteration in FtsZ transcription or activity, or with its interaction with other proteins. It has been reported that *cpxA** gain-of-function mutants possessed random FtsZ-ring positioning causing both aberrant cell division and the formation of mini cells devoid of DNA (Pogliano *et al.*, 1998). The *ssd* transductant may have filamentous growth due to an altered *cpxP* transcription, and poor DNA segregation due to the *cpxA* mutation.

Part 11. ZipA-Gfp merodiploids show improper DNA segregation and make extensive filaments in glucose minimal medium.

In an effort to determine if the *CU1008ssd17UVS* transductant showed the aberrant FtsZ-ring positioning reported for the *cpxA** mutants, I created merodiploid strains by transducing in a second *zipA* gene (this one fused to *gfp*) into *CU1008ssd17UVS*, and into the parental *CU1008* strain with the intent to visualize the cellular localization of this protein fusion. The ZipA cell membrane protein stabilizes the FtsZ-ring at the site of division through a protein-protein interaction that anchors the FtsZ to the membrane (Liu *et al.*, 1999).

Surprisingly, the phenotypes of both merodiploid strains were affected when they were grown in glucose minimal medium even without the addition of inducer. Both strains made filaments in glucose minimal medium and in rich medium (with or without glucose), but the filaments were longest when the strains were grown in glucose minimal medium (Figure 18). DAPI staining showed that the DNA was not be properly segregated in the filaments grown in any medium (Figure 20). The proportions of lysed cells for the *CU1008ssd17UVSzipA-gfp* merodiploid strain was the same as that observed for the *CU1008ssd17UVS* transductant, but the proportion of damaged (red fluorescing) cells was far increased in the *CU1008ssd17UVSzipA-gfp*. However, cell lysis was also increased in *CU1008zipA-gpf* merodiploid strain, suggesting that the extra copy of *zipA* causes a problem to both the parental strain and *ssd* transductant. More experiments will be needed to determine how a second copy of *zipA* affects these strains.

Part 12. Summary

The Cpx two-component regulatory system is a highly intricate system comprised of the CpxA, CpxR and CpxP proteins that act together to regulate the transcription of multiple genes whose protein products are involved in an array of cellular roles. The *ssd* mutation has been annotated as a *cpxA* mutation, but the results in this thesis show that the *ssd* mutant is much more complex. This study provided an opportunity to use many mutant strains of the Cpx-system, glycolysis and serine metabolism to attempt to elucidate the causes behind the pleiotropic phenotype of the *ssd* mutation.

The *ssd* mutation accumulates extragenic suppressor mutations. Its phenotype does not result from a single mutation in the *cpxA*. It may rather be the result of two mutations, one in *cpxA* and another in the *cpxP* promoter. Both mutations can destabilize the strain causing suppressor mutations inside and outside of the *cpx* regulon to be selected.

The *cpxA** gain-of-function mutants have phenotypes similar to that observed for the *ssd* transductants. Mutations in *cpxA* are physiologically destabilizing and cause suppressor mutations to be selected. Therefore, one cannot know the true genotype of these strains because the characteristics attributed to *cpx* or *ssd* mutants may be the result of secondary, suppressor mutations inside or outside of the *cpx* regulon. The *cpxA** gain-of-function and *cpxA* deletion mutants show similar phenotypes because these strains are lacking a functional CpxA that is not able to inactivate the CpxR-P response regulator.

Some *ssd* transductant phenotypes (growth on serine and amikacin-resistance) can be attributed to a malfunctioning Cpx two-component regulatory system, whereas at least one other *ssd* phenotype (high L-SD activity) can only be accounted for by a non-functional CpxA. Other *ssd* transductant phenotypes (UV-sensitivity and filaments) can only be accounted for by altered physiological concentrations of CpxP.

L-SD activity is essential for the pathogenesis at least one other bacterium (*C. jejuni*). Therefore, the regulation of L-SD by CpxA may be part of *E. coli* pathogenesis. This thesis shows that the CpxA negatively regulates more than one L-serine deaminase because a *cpxA sdaA* double mutant strain still has high L-SD activity relative to the parental strain. High L-SD activity could be the result of a combination of more than one of the three known L-serine deaminases (*sdaA*, *sdaB* and *tdcG*) because all three are shown to be expressed in glucose minimal medium.

Mucoid *E. coli* and *P. aeruginosa* cells are often isolated from the lungs of cystic fibrosis patients. Mucoidy is observed in strains carrying deletions in *cpxR* and *cpxP*, and in the extremely UV-sensitive *ssd*, but is not a phenotype observed in strains carrying a *cpxA* deletion.

The RNA expression analysis technique corroborates the previously described result that the *cpxP* gene is transcribed in non-induced parental strain cells, and that its transcription is decreased in strains carrying a *cpxR* deletion. However, the results presented also demonstrate that CpxA regulates *cpxP* transcription and this supports the

hypothesis that the role of CpxA in the Cpx two-component signal transduction system is primarily as a phosphatase that inactivates the CpxR response regulator.

Live/dead and DAPI fluorescence staining techniques showed that the extremely UV-sensitive *ssd* transductant makes intact filaments in glucose minimal medium despite improper DNA segregation. However, this strain lyses extensively when grown in rich media. Fluorescence staining also shows that ZipA merodiploid strains cause improper DNA segregation and make long filaments in both parent stain and extremely UV-sensitive *ssd* transductant genetic backgrounds when grown in glucose minimal medium. Therefore, part of the *ssd* transductants phenotype may be a defect in an early cell division protein. Because the cell division protein FtsZ is involved in nucleoid segregation, the *ssd* mutation may be directly or indirectly affecting its activity, and this is lethal to *ssd* filaments in rich media.

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