AN UNKNOWN REGULATOR AFFECTS CELL DIVISION AND THE TIMING OF ENTRY INTO STATIONARY PHASE IN *Escherichia coli*

A Thesis

by

SHERRIE VALARIE BAIN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2005

Major Subject: Microbiology

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Approved as to style and content:

Deborah A. Siegele (Chair of Committee)	Luis Rene Garcia (Member)
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Vincent Cassone (Head of Department)	

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ABSTRACT

An Unknown Regulator Affects Cell Division and the Timing of Entry into Stationary Phase in *Escherichia coli.*

(May 2005)

Sherrie Valarie Bain, B.S., University of Tampa

Chair of Advisory Committee: Dr. Deborah A. Siegele

When an essential nutrient is depleted from the medium, cultures of wild-type *E. coli* cells enter a period called stationary phase. The transition into stationary phase is marked by distinct changes in cell physiology, gene expression, and morphology. Prüß and Matsumura (18) found a mutant strain of *E. coli* that was able to continue growing exponentially at a time when wild-type cells had stopped growing and entered stationary phase. They concluded that FlhD, a transcriptional activator of flagellar genes, was responsible for this growth phenotype and that it is a regulator of cell division (17, 18). Contrary to the findings of Prüß and Matsumura, research in our lab has shown that the mutant growth phenotype observed in the strain used by Matsumura and Prüß is *flhD* independent. This study sought to identify the second mutation, which we call *cdr* (cell division regulator) in the strain used by Matsumura and Prüß. We used Hfr mapping and P1 transduction to localize the mutation to a specific region of the chromosome. We also sought to determine if this growth phenotype was due to loss of function or gain of function and whether the

mutation in the *cdr* gene was sufficient to cause the observed growth phenotype in other strain backgrounds. In addition the growth phenotype of these two strains was compared to that of other wild-type and standard laboratory *E. coli* strains. Our results indicate that the *cdr* mutation is located in the 88.5' region of the chromosome and is due to loss of Cdr function. We also discovered that the growth phenotype assigned to the mutant strain more closely reflects that of other wild-type laboratory strains as did the morphology of cells in stationary phase. This evidence suggests that the actual mutant strain might be the one that was designated as the wild-type strain by Matsumura and Prüß and both strains may contain mutations that actually cause a decrease in cell number instead of an increase as previously reported.

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CHAPTER I

INTRODUCTION

Non-sporulating bacteria such as *Escherichia coli* exhibit four distinct stages of growth in batch culture (6). These stages are the lag phase, exponential phase, stationary phase, and eventually a death and decline phase. Initially when cells are inoculated into fresh medium, they do not increase in number. During this lag phase, the cells are synthesizing components such as ATP, ribosomes, and enzymes needed to optimize growth in the medium. The lag period also allows cells to adjust to other environmental changes such as temperature, pH, and osmolarity.

The length of the lag period can vary depending on the growth stage of the cells prior to being inoculated into the fresh medium, the type of medium the cells were growing in previously, as well as the type of media into which cells are transferred (12). If cells are growing in rich medium, where there is an abundant supply of nutrients, and then transferred to a minimal medium there will be a longer lag phase than if the cells were growing in a nutrient poor environment and then transferred into rich medium.

Cells diluted into fresh medium of the same type have different lag periods depending on the growth phase of the cells prior to the switch. Cells that are in the exponential growth phase have little or no lag, when compared to

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cells that had already entered stationary phase before the transfer occurred (6). This is because the cells that were in exponential phase are already adapted to that environment and have an adequate supply of ribosomes, proteins, and other factors necessary to maximize growth and therefore they do not need a lag time to produce those components unlike the cells that had already entered stationary phase.

Once the cells have produced the appropriate machinery, the lag phase ends, and the culture enters a period of exponential growth. In exponential phase, cells divide at the maximum rate possible for a given environment. The size of the population continues to increase proportional to the number of cells present at any previous time-point. Eventually, environmental perturbation results in conditions that are no longer support exponential growth. The culture might exhaust one or more key nutrients required for growth, or the metabolic waste products that are produced and excreted into the medium might be detrimental to the cells. Therefore, the culture enters a period of stasis, generally referred to as stationary phase.

During entry into stationary phase, the rate of cell division decreases and the density of the culture remain constant once the growth rate becomes zero. If conditions remain unfavorable, the culture will eventually enter a death phase and there is a decline in viable cell numbers (12).

Gene expression and cell physiology during stationary phase

Transition into stationary phase is marked by distinct changes in gene expression. Changes in gene expression are induced by physiological changes that signal a need to make this transition. For example, increases in the level of guanosine (penta)tetraphosphate [(p)ppGpp] during entry into stationary phase has been shown to decrease the affinity of RNA polymerase core enzyme for sigma70 (σ^{70}), thus allowing the stationary-phase and stress-induced sigma factor, RpoS (σ^{38}) to have greater access to the core enzyme (4). This in turn leads to induction of many stationary-phase specific genes, which are regulated by RpoS. The up-regulation of stationary-phase genes allows cells to cope with unfavorable conditions, including lack of nutrients, decreased oxygen availability, and accumulation of metabolic waste products (12).

During stationary phase, there is a decrease in the abundance of monounsaturated fatty acids in the inner membrane, the peptidoglycan thickens to approximately 5 layers instead of the two layers present in exponential phase, and the overall diversity of the protein composition decreases (2, 3, and 6). Cells that have entered stationary phase are more resistant to stresses such as high temperature, increased osmolarity, and increased acidity (3).

Specific signaling molecules that regulate the expression of target genes at high cell densities have been identified in other species of bacteria, such as *Vibrio harveyi* (22). In *E. coli*, a number of different metabolites, including acetate, glutathione, and 1,5-anhydroglucitol, accumulate during entry into

stationary phase (3). It is not known whether they serve as signals that a culture has reached its critical sustainable mass.

Growth phenotype of *cdr* strain

A mutant strain that continues to divide at the exponential rate when wild-type cells have reduced their cell division rate and entered stationary phase, has been reported (Fig. 1). This phenotype was previously assigned to a mutation in the *flhD* gene (18). FlhD, together with FlhC, forms a heterotetrameric transcription factor that serves as the master regulator of flagellar synthesis in *E. coli* (7). Prüß and Matsumura observed that cultures of *flhD* cells grow to approx. 10^9 cells per ml before entering stationary phase, while cultures of the *flhD*$^+$ parent strain only grow to 10^8 cells per ml, and concluded that FlhD regulates cell division (17). However, research in our lab has shown that the mutant growth phenotype observed in the strain used by Prüß and Matsumura is *flh*-independent.

Using phage P1, transduction of YK410 *flhD* cells to *flhD*$^+$ (strain DS522) did not rescue the mutant phenotype, and when the *flhD* mutation from YK4131 was transduced into the *flhD*$^+$ parental strain (to produce strain DS513), the cells did not demonstrate the mutant growth phenotype (Fig. 2). The growth curves were performed three times. A one-tailed Student t-test was used to compare the average CFU/ml for strains DS511 and DS522 to those for DS513 and DS514. The difference in the mean CFU/ml at 240 min for these two pairs of

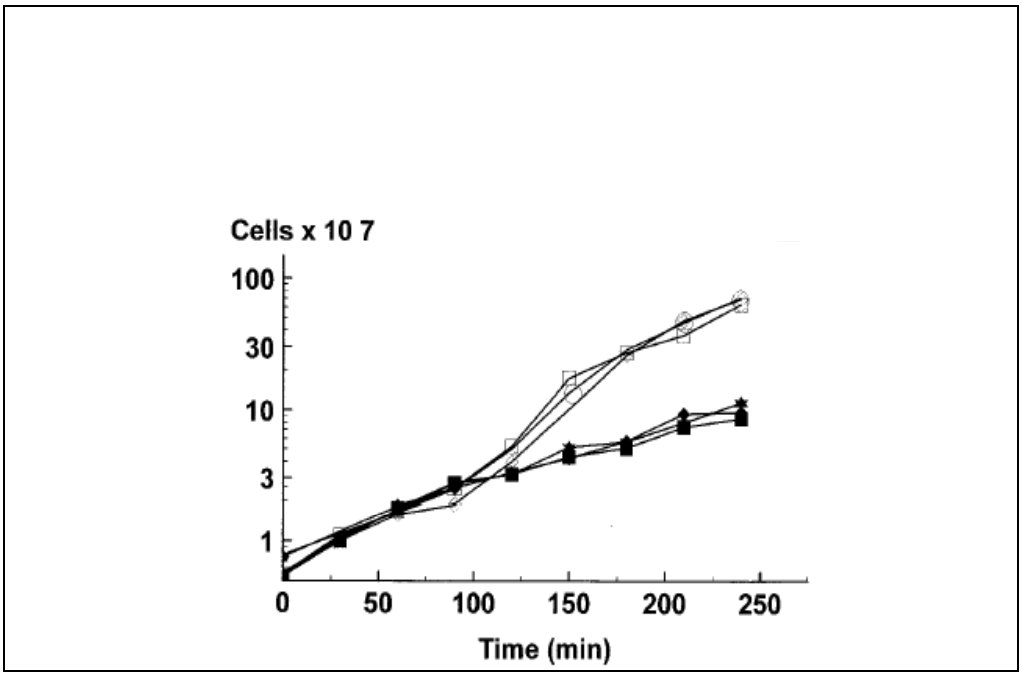

FIG. 1. Growth phenotype of wild-type, *flhD*, and *flhC* mutants in TB medium. Closed symbols indicate strains that show the same phenotype as the parental strain, YK410. YK410, closed squares; the *flhC* mutant strain YK4136 (YK410 *flhC*) closed stars; and a *fliA* mutant strain YK4104 (YK410 *fliA*), closed diamonds. The *fliA* gene encodes a sigma factor that is required for expression of late flagella genes (6). Open symbols indicate *flhD* strains that show the mutant phenotype. YK4131 (YK410 *flhD*), open squares; BP67 (YK410 *flhD::kan*), open circles; and YK4519 (MC1000 *flhD::kan*), open diamonds. The figure is from Prüß and Matsumura (18).

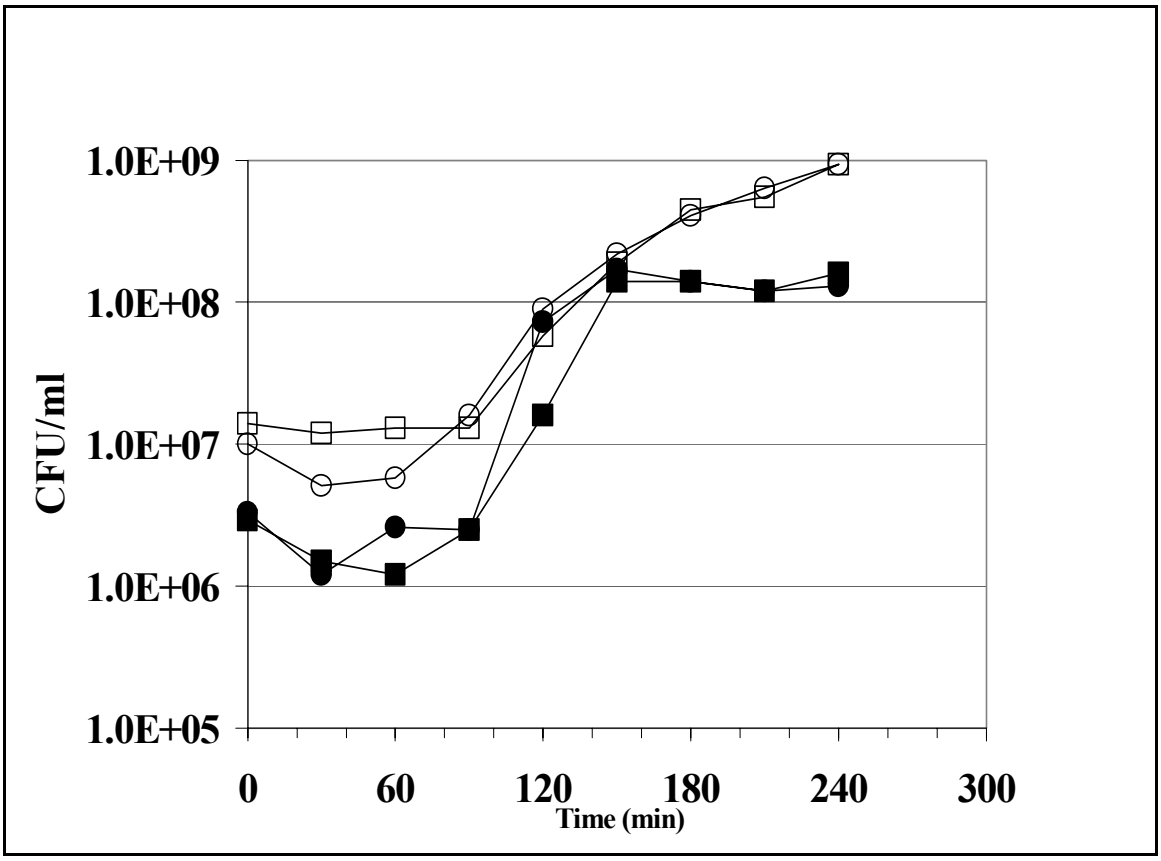

FIG. 2. FlhD is not responsible for the mutant growth phenotype (8). Growth conditions are the same as those used by Prüß and Matsumura (18). Open symbols represent strains that display the mutant growth phenotype. Strain DS511, open circles, the original *flhD4131* mutant strain. Strain DS522, open squares, the *flhD4131* allele was replaced by *flhD*$^+$. Strain DS514, closed squares, indicate *flhD*$^+$ parent strain. Strain DS513, closed circles, the *flhD4131* allele was transferred to DS514. This experiment was performed three times, a representative graph is shown.

strains is significant at the 95% confidence level ($p = 0.000004$). These results show that the phenotype is not caused by defects in *flhD*.

Prüß and Matsumura found that even though mutant cells grew to higher numbers than wild-type cells, the optical density of the wild-type and mutant cultures was comparable (18). My data (Fig. 3) are consistent with this finding. The unknown mutation will be referred to as *cdr* (cell division regulator).

The observed growth phenotype may be due to disruption of a signaling pathway that allows cultures to sense the onset of stationary phase and the need to reduce their cell division rate. The results of this disruption might be manifested in irregular division of mutant cells leading to increased cell numbers relative to the wild-type strain, which stops dividing. Alternatively, the ability of mutant cells to continue growing exponentially might be indicative of their ability to utilize an alternative energy source present in the growth medium that the wild-type cells cannot use.

It is not known whether the *cdr* mutants are also delayed in developing the increased resistance to environmental stress displayed by wild-type *E. coli* cells in stationary phase. When exposed to external stresses such as pH, high temperature and increased osmolarity, wild-type stationary phase cells survive better than their wild-type counterparts that are in exponential phase (6). Since the cells that exhibit the *cdr* growth phenotype appear to be locked in a prolonged period of exponential growth, they might also require a longer time to

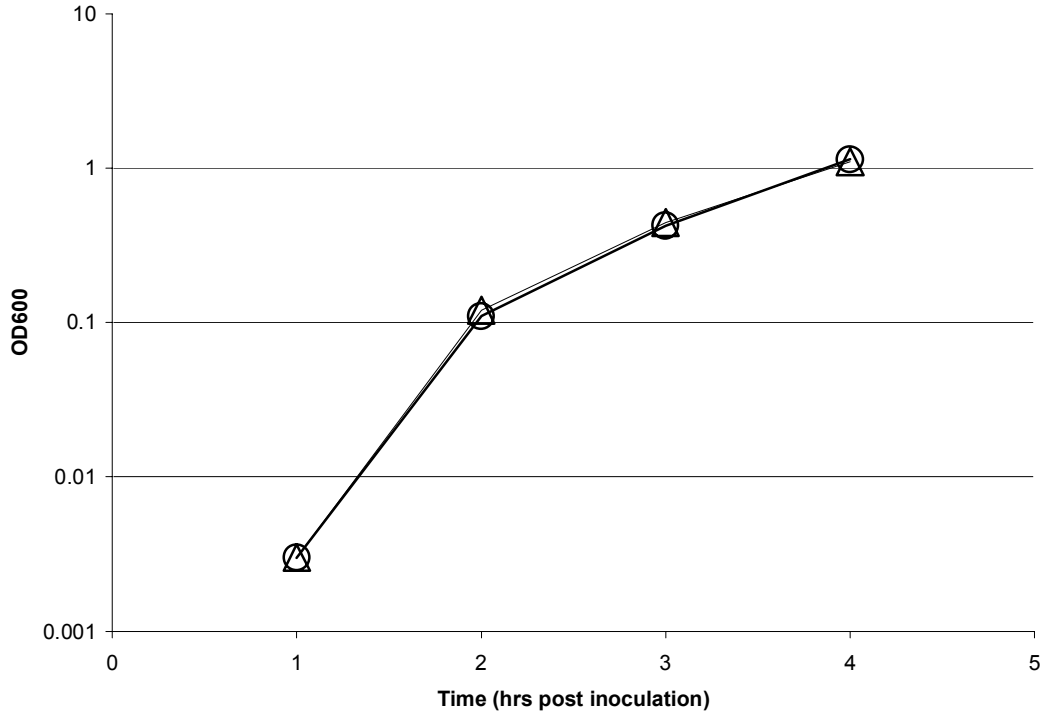

FIG. 3. The growth of the wild-type and *cdr* mutant strains. DS380, open circles, DS381, open triangles. Growth was monitored by measuring the optical density at 600 nm. The average of two independent cultures for each strain is plotted for each time-point. There was less than an eight percent difference in the readings at each time-point for the two experiments.

implement the protective measures that would allow them to survive as well under the same stress conditions as the wild-type strain.

Identification of the *cdr* mutation or mutations is important in order to be able to address the question of how and why this strain is able to overcome the stringent parameters that normally dictate the onset of stationary phase in wild-type cells.

CHAPTER II

MATERIALS AND METHODS

Bacterial strains

All strains used in this study are derivatives of *E. coli* K-12 and are listed in Table 1. Strains YK410 and YK4131 were obtained from P. Matsumura (University of Illinois–Chicago Circle). DS380 is a derivative of YK410 that is lysogenized with λRS45::P*mcb-lacZ*. This strain exhibits the wild-type growth phenotype. DS381 is a derivative of YK4131 (YK410 *flhD*) with the same λRS45::P*mcb-lacZ* prophage.

Media and growth conditions

All powder-based media were purchased from Difco Laboratory (Becton, Dickinson, & Co. Sparks, MD). Tryptone Broth (TB) containing 1% tryptone and 1% NaCl and supplemented with thiamine and thymidine, each at a concentration of 20μg/ml (1) was used to assay the growth phenotype. Luria-Bertani (LB) broth was used for the Hfr mating assays. For solid media, LB containing 2% Difco granulated agar and supplemented with thymidine (20 μg/ml) was used. MacConkey agar base with the appropriate sugar at a

Table 1. *E. coli* strains used in this study

Strain	Relevant genotype	Reference or source
CAG7041	C600 *galK recA56 srl*::Tn*10 rpoD800* pK01	Lab stock
CAG12185[a]	MG1655 *argE86*::Tn*10*	21
CAG18431[a]	MG1655 *ilv-500*::Tn*10*	21
CAG18499[a]	MG1655 *zid-501*::Tn*10*	21
CAG18450[a]	MG1655 *zhf-50*::Tn*10*	21
CAG18452	MG1655 *zhe-3085*::Tn*10*	21
CAG18477a	MG1655 *zii-510*::Tn*10 metF15*9	21
CAG18491a	MG1655 *metE3079*::Tn*10*	21
CAG18492a	MG1655 *zic-4901*::Tn*10*	21
CAG18495a	MG1655 *zih-35*::Tn*10*	21
CAG18496a	MG1655 *fadAB101*::Tn*10*	21
CAG18501a	MG1655 *zie-296*::Tn*10*	21
CAG18615a	MG1655 *zjb-3179*::Tn*10*Kan	21
CAG12200b	KL16 *zed-2120*::Tn*10*Kan	21
CAG12202b	KL96 *trpB3193*::Tn*10*Kan	21
CAG12203b	KL208 *zbc-3105*::Tn*10*Kan	21

Table 1. Continued

Strain	Relevant genotype	Reference or source
CAG12204[b]	KL 227 *btuB3192::*Tn*10kan*	21
CAG12205[b]	KL228 *zgh-3159*::Tn*10kan*	21
CAG12206[b]	HfrH *nadA3032*::Tn*10kan*	21
DS380	YK410 (λRS45::P*mcb-lacZ)*	Lab stock
DS381	YK4131 (λRS45::P*mcb-lacZ*)	Lab stock
DS631	MG1655 = *E. coli* K-12 λ^- F^- *rfb-50 rph-1*	C. Gross
DS473	MC4100 = *E. coli* K-12 λ^- F^- Δ(*argF-lac*)*U169* *araD139 rpsL150 ptsF25 flbB5301 rpsR* *deoC relA1*	W. Boos
DS511	DS 381 *uvrC*::Tn*10*	Lab stock
DS513	DS 380 *flhD4131 uvrC279*::Tn*10*	Lab stock
DS514	DS380 *uvrC*::Tn*10*	Lab stock
DS522	DS381 *flhD*$^+$ *uvrC279*::Tn*10*	Lab stock
EA1004[b]	KL16 *zeb*1::Tn*10*	21
EA1005[b]	KL228 *srl*::Tn*10*	21
EA1006[b]	KL14 *zje*::Tn*10*	21
KH51	MG1655 Δ*glpK202 zii-510::*Tn*10*	D. Pettigrew

Table 1. Continued

Strain	Relevant genotype	Reference or source
KL728	λ⁻ *leuB6(Am) fhuA2 lacY1 glnV44 gal-6*	
	hisG1 recA1 argG6 rpsL104 malT1 xylA7	
	mtlA2 metB1 F'111	CGSC[c] #4258
SB2	DS381 *zih-35*::Tn*10*	This Study
SB10	SO441 F'111	This Study
SB11	SO441 F'111 *zjb*-379::Tn*10*kan	This Study
SO441	λ⁻ F⁻ *ccd-5 upp-11 relA1 rpsL254 metB1*	CGSC[c] #5939
YK410	F⁻ *araD*139 (*argF-lac*)*U169 strA thy*	
	pyrC46 nalA thyA his	P. Matsumura
YK4131	YK410 *flhD4131*	P. Matsumura

[a] Mapping strains used in P1 transductions.

[b] Mating strains used in Hfr mating assays.

[c] Coli Genetic Stock Center

concentration of 1% was used for sugar utilization assays. M63 minimal medium (10) supplemented with glycerol, casamino acids, uridine (4 μg/ml), thymidine (20 μg/ml), and thiamine (20 μg/ml) was used to select glycerol-utilizing transductants. Where indicated the media were also supplemented with tetracycline (12.5 μg/ml), kanamycin (50 μg/ml), nalidixic acid (30 μg/ml), streptomycin (100 μg/ml), amikacin (12 μg/ml), and X-Gal (20 μg/ml). Unless otherwise indicated strains were grown at 37°C.

Mapping

Hfr matings were performed as described by Singer et al. (21). Donor strains were streaked onto LB plates supplemented with thymidine and tetracycline. Recipient strains were streaked onto LB plates supplemented with thymidine and either kanamycin or streptomycin. Single colonies were used to inoculate overnight cultures of donor and recipient cells in LB broth supplemented with thymidine and the appropriate antibiotic. Overnight cultures were diluted 1:50 into LB plus thymidine and grown at 37˚C in a shaking water bath at 25 rpm for approximately three hours. Control samples of donor and recipient cells were plated on LB agar supplemented with thymidine, X-Gal, and the appropriate antibiotics. Mating mixes of 0.5 ml donor and 9.8 ml recipient cells were incubated for 35 minutes without shaking and then diluted 1:10 into LB. The mating aggregates were disrupted by vortexing vigorously for 2 min. When Kan^R exconjugants selected, cells were incubated at 30°C with shaking for 1 hour after disruption of the mating pairs, and then for an additional 3 hours

at room temperature on a rocker. This was necessary to allow the kanamycin-resistance gene to be expressed before the cells were plated on selective media. Plates were incubated overnight and 16 to 20 exconjugants were colony-purified on selective media before growth assays were performed.

Transductions with phage $P1_{vir}$ were performed as described by Miller (10). A $P1_{vir}$ lysate was grown on each of the P1 mapping strains listed in Table 1. Transductants were colony-purified on selective media at least once before growth assays.

Growth phenotype assay

Cultures were grown overnight in TB medium supplemented with the appropriate antibiotics where indicated and diluted 1:100 into 10 ml of the same medium in a 125 ml Erlenmeyer flask and grown for 3 to 5 hr. In some experiments, the optical density at 600 nm was measured using a DU-65 spectrophotometer. At various time-points samples were serially diluted into 1XM63 salts and aliquots plated on LB medium supplemented with thymidine. Viable cell numbers were determined after overnight incubation.

Strain construction for complementation assays

F'111 was mated from donor strain KL728 into strain SO441. The resulting strain was transduced to Kan^R using a $P1_{vir}$ lysate grown on CAG12185 (*zjb-3179*::Tn*10kan*). To determine which transductants had the Kan^R marker on the F', the Kan^R transductants were used as donors and mated with DS381.

Exconjugants resistant to kanamycin and nalidixic acid were selected. One of the SO441 F'111 donors that was able to transfer the Kan^R marker (SB11) was used as the source of the F' plasmid in the complementation assays.

To prevent marker rescue from occurring during the complementation assays, Rec$^-$ derivatives of DS380 and DS381 were made using phage $P1_{vir}$ to introduce the *recA56* mutation by cotransduction with *srl*::Tn*10*.

Complementation assays

Once F'111 *zjb-3178*::Tn*10*kan (SB11) was constructed, it was mated into DS380 and DS381. Matings were performed as described by Miller (10). Single colonies were used to inoculate overnight cultures of donor and recipient cells in LB broth supplemented with thymidine and the appropriate antibiotic. Overnight cultures were diluted 1:50 into LB plus thymidine and grown at 37˚C in a shaking water bath at 25 rpm for approximately three hours. Control samples of donor and recipient cells were plated on LB agar supplemented with thymidine, and the appropriate antibiotics. Mating mixes of 0.5 ml donor and 9.8 ml recipient cells were incubated for 35 minutes without shaking and then diluted 1:10 into LB. Exconjugants were selected by plating on LB medium supplemented with kanamycin and nalidixic acid. Exconjugants were colony purified once on selective medium and then the growth phenotype of four individual exconjugants was assayed as described earlier. The wild-type phenotype was restored to DS381 and the growth phenotype of the *cdr*$^{+}$ parental strain with the F' was the same as the wild-type strain without the F'.

Amplification and sequencing of the *cpxA* gene

The *cpxA* gene from DS380 and DS381 was amplified using standard polymerase chain reaction (PCR) methodology. Chromosomal DNA was isolated using a quick-boiling preparation method. A 1 ml aliquot of an overnight culture was spun down in a microcentrifuge and the pellet was washed twice in sterile deionized water. After the final wash the pellet was resuspended in the same volume of water and boiled for five minutes. In order to remove cellular

debris, the lysate was spun for ten minutes in a microcentrifuge at 12,000 x g and the supernatant, containing the genomic DNA**,** was collected and transferred to a different Eppendorf tube.

The *cpxA* gene was amplified in two fragments. The primers used in this study are listed in Table 2. All oligonucleotides used for PCR amplification were purchased from Sigma-Genosys (The Woodlands, TX). Primers were complementary to sequences 53 nt upstream of the *cpxA* start codon, the middle of the coding sequence, and 96 nt downstream of the stop codon. The first fragment was amplified using primers cpxA_Fwd1 and cpxA_Rev1 and the second fragment was amplified using primers cpxA_Fwd2 and cpxA_Rev2. A graphical representation of the PCR amplification is shown in Figure 4. Additionally, the full-length *cpxA* gene was amplified using cpxA_Fwd1 and cpxA_Rev2.

PCR products were purified using a Wizard® PCR purification kit (Promega, Madison, WI) and visualized on a 1% agarose gel. This was done to determine if the PCR product was the anticipated size. Control PCR reactions were performed to ensure that the primers were not amplifying nonspecific sequences. The absorbance at 260 nm ($OD_{260)}$ was measured to determine the concentration of each sample. Primers for sequencing were purchased from New England BioLabs (Beverly, MA) and the Gene Technology Laboratory (Texas A&M University, College Station, TX). Sequencing reactions were performed with Big Dye Reaction Mix (ABI, Foster City, CA). Excess dye-

terminators were removed using Micro Bio-Spin P-30 spin columns (BioRad, Hercules, CA).

Table 2. Primers for amplification and sequencing

	Name	Description	Sequence
(a)	CpxA_Fwd1	*cpxA* 5' primer	5'gccagggttttcccagtcacgacggtcac ccgtggtttaaaaccttgc-3'
	CpxA_Rev1	*cpxA* 3' primer	5'agcggataacaatttcacacaggaggtgaca atcagcagtaataagcgg-3'
	CpxA_Fwd2	*cpxA* 5' primer	5'-cgccagggttttcccagtcacgacctgttgtgg ttgcctggagtct- 3'
	CpxA_Rev2	*cpxA* 3' primer	5'-agcggataacaatttcacacaggagag tgtaggcctgataagacgctatca -3'
(b)	M13Fwd	5' primer 1	5'-cgccagggttttcccagtcacgac-3'
	M13Rev	3' primer 1	5'-agcggataacaatttcacacagga-3'
	SeqFwd 2	5' primer 2	5'-gttccgggcgattgataagt-3'
	SeqRev 2	3'primer 2	5'-agatcgttgatcatgctgtc-3'

(a) Primers used to amplify *cpxA*. The underlined portions are identical to the M13 sequencing primers listed. The rest of the primer is complementary to the *cpxA* sequence.

(b) Primers used to sequence the PCR products generated using the primers listed in (a).

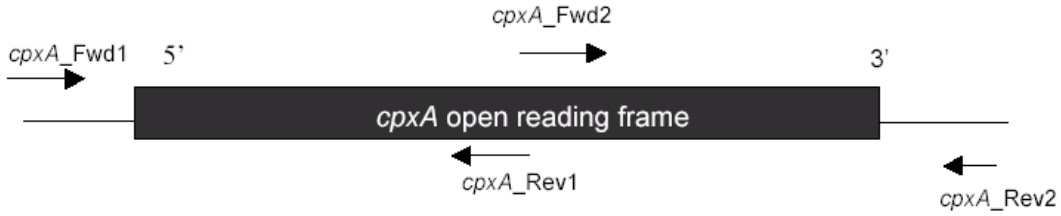

FIG. 4. Amplification of *cpxA*. Diagram depicting the *cpxA* open reading frame and relative positions of primers used to amplify the *cpxA* gene.

DIC microscopy imaging

Wild-type and mutant cultures were grown as described for the growth phenotype assay. Exponential phase samples were taken at an OD_{600} of 0.4 and stationary phase samples were taken at five hours. This was at the time when the growth phenotype was most distinct and coincides with an OD_{600} of 1.0-1.2. For the purpose of determining cell size, a 100 µl sample was spotted onto a poly-lysine coated glass cover slip and allowed to sit for five minutes to ensure adhesion of cells. The cover slip was then rinsed with sterile deionized water to remove any excess cells. The cover slip was mounted on a glass microscope slide and three sides of the cover slip were sealed with gel seal (Pharmacia) to prevent the sample from drying out. Slides were visualized using a Zeiss® Axioplan2 confocal microscope. Pictures were taken using a Hamamatsu® color chilled 3CCD camera.

CHAPTER III

RESULTS

Hfr mapping

The *cdr* mutation was localized to a specific region of the chromosome using a collection of Hfr mapping strains (21). Each Hfr has an origin of transfer (*oriT*) at a different location on the *E. coli* chromosome and contains a Tn*10* insertion approximately twenty minutes from *oriT* (Fig. 5). Matings between the mutant recipient strain DS381 and each of the Hfr donor strains were performed as described in Materials and Methods. The frequency of recombinants with an unselected marker located between *oriT* and the selected marker ranges between 15% and 50% depending on the position of the unselected marker relative to the *oriT* and the selected marker (1, 21). Therefore, the growth phenotype of at least 16 exconjugants from each Hfr mating was assayed to determine whether any of them had received the wild-type *cdr* allele from the donor strain or if they still retained the mutant *cdr* phenotype.

The Hfr mapping results indicated that the mutation was located between 67 min and 84 min on the chromosome (Table 3). The average number of CFU/ml at 300 min post-inoculation from three independent cultures of DS380 and DS381 is shown in Fig. 6A. Matings between DS381 and the Hfr strains EA1005 (Fig. 6B) and EA1006 (data not shown) gave rise both to exconjugants with a wild-type phenotype and exconjugants with a mutant phenotype, while

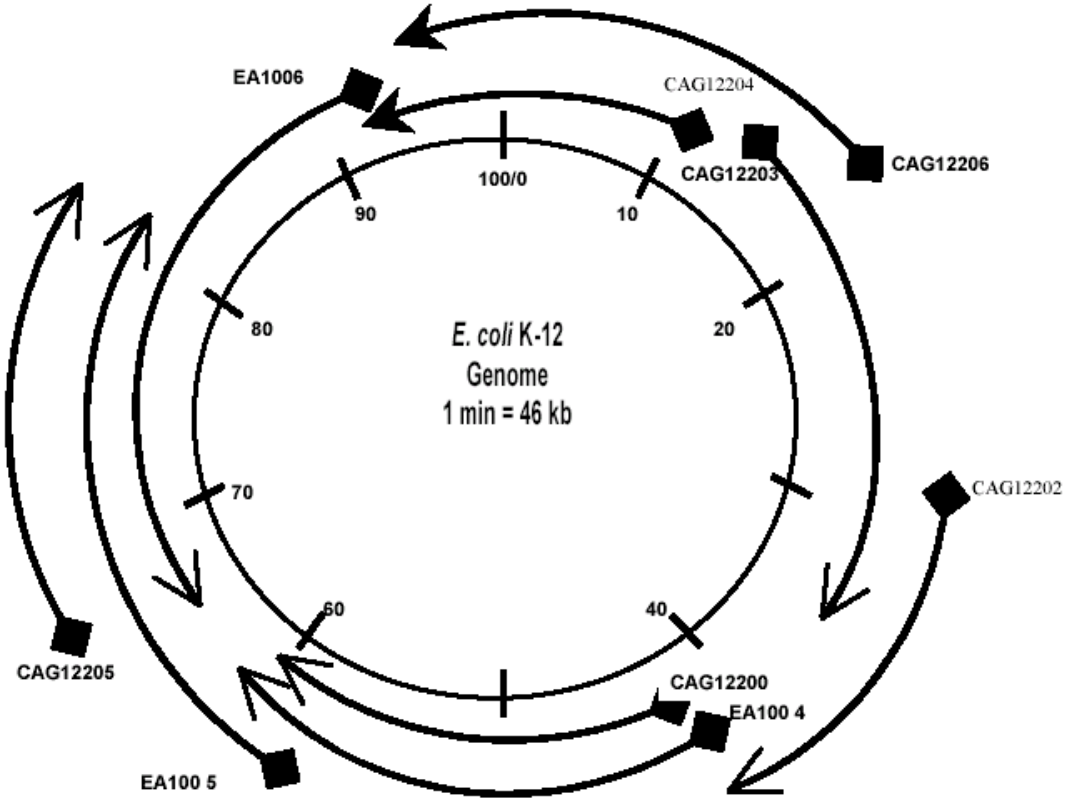

FIG. 5. Initial mapping of the *cdr* mutation. Diagram depicting the *E. coli* chromosome and the Hfr strains used for mapping. Arrows indicate the location of *oriT* and the closed diamonds represent the position of the Tn*10* insertion.

Table 3. Hfr mapping strains and results

Hfr donor	Position of *oriT*	Position of Tn10	Results [a]
CAG12200	61 min	43 min	0/16
CAG12202	44 min	28 min	0/16
CAG12203	36 min	12 min	0/16
CAG12204	89.7 min	6 min	0/16
CAG12205	84.5 min	67 min	0/16
CAG12206	95.4 min	16.8 min	0/16
EA1004	62 min	41 min	0/20
EA1005	84.5 min	58 min	4/8
EA1006	67 min	94 min	1/8

[a] Number of exconjugants showing the wild-type growth phenotype out of the total number tested.

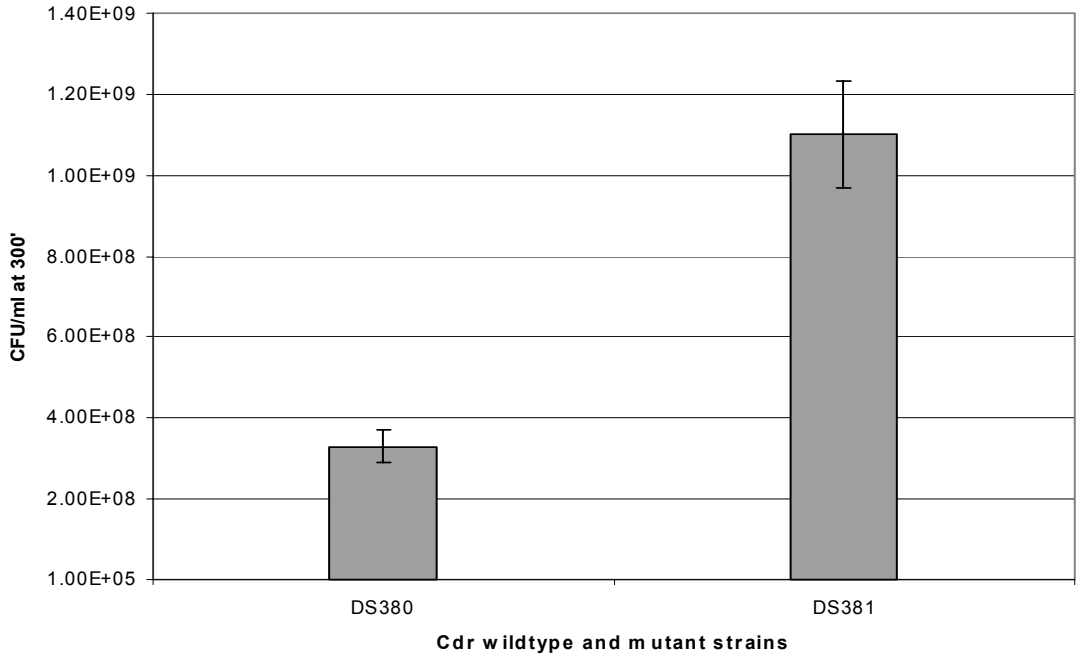

FIG. 6 (a). Growth phenotypes of control strains and exconjugants. The graph shows the average CFU/ml at 300 min from three independent experiments for the control strains. Error bars indicate the amount of variation between each experiment.

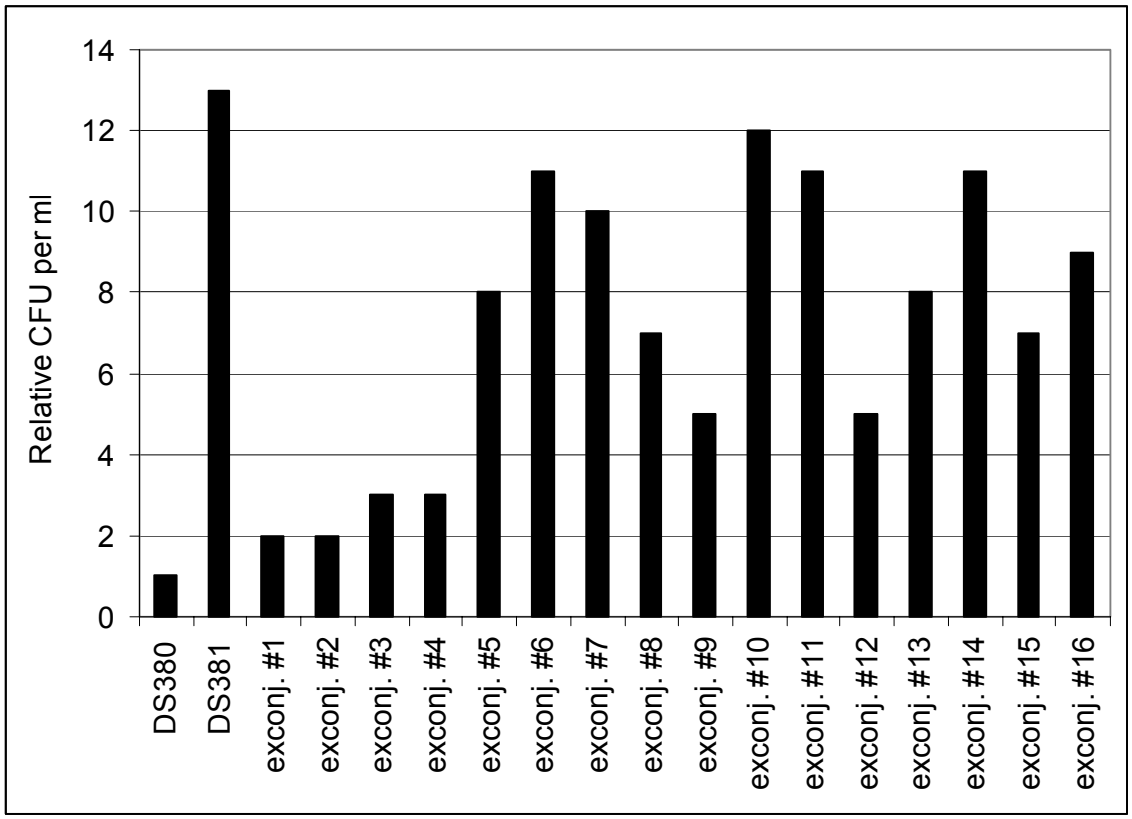

FIG. 6(b). Exconjugants from the mating between DS381 and EA1005 were tested. The number of CFU/ml at 240 min is expressed relative to the CFU/ml at 240 min for the wild-type control strain DS380 (1.0×10^8).

crosses with the remaining Hfr donor strains yielded only exconjugants that retained the mutant growth phenotype.

P1 mapping

A collection of ten P1 mapping strains, each with a Tn*10* transposon insertion at a position between 77.4' and 89.3' of the *E. coli* chromosome, was used to further localize the *cdr* mutation. $P1_{vir}$ was grown on each of the mapping strains and the lysate used to infect the *cdr* mutant strain DS381. Transductants were selected on the basis of Tet^R and then screened for the recovery of the wild-type growth phenotype. Initial screening of 8 transductants from each cross indicated that cdr^+ was linked to a Tn*10* insertion at 87.6 min (*zih-35*::Tn*10*) (Table 4). Eighteen percent (5/28) of the *zih-35*::Tn*10* transductants had the wild-type growth phenotype. None (0/8) of the Tet^R transductants from the other nine donor strains had the wild-type phenotype.

The distance between the *zih-35*::Tn*10* insertion and the *cdr* locus was determined using the Wu formula (23), which is used to convert cotransduction frequency (fraction of transductants with the unselected donor marker) to physical distance in minutes. The distance between *zih-35*::Tn*10* and the *cdr* mutation was determined to be 0.9 minutes (approximately 42 kb). This result would place the mutation at either 88.5' or 86.7'. Two of the donor strains had Tn*10* located at 86.8' and 89.3', respectively. If the mutation is at 86.7', I should

Table 4. P1 mapping strains and results

P1 donor strain	Position of Tn10	Results [a]
CAG18450	77.4	0/8
CAG18492	82.5	0/8
CAG18452	82.7	0/8
CAG18499	83.5	0/8
CAG18501	84.7	0/8
CAG18431	85.2	0/8
CAG18491	86.4	0/8
CAG18496	86.8	0/8
CAG18495	87.6	5/28
CAG18477	89.3	0/8

[a] The numbers indicate the number of transductants with the wild-type growth phenotype and the total number of transductants tested for each donor strain.

have seen linkage of the wild-type *cdr* allele to the Tn*10* insertion at 86.8', because the predicted cotransduction frequency is 86%. I was also able to use the *zih-35*::Tn*10* marker to transduce the *cdr* mutation out of DS381 back into the parental strain (DS380). The linkage between *zih-35*::Tn*10* and the *cdr* phenotype was 13% (4/30).

The question of whether the *cdr* mutation was sufficient to cause the observed growth phenotype in other strain backgrounds also was addressed. DS380 was transduced to Tet^R using a $P1_{vir}$ lysate grown on CAG18495 (*zih-35*::Tn*10)* and the growth phenotype of at least 14 transductants was scored. All of the transductants retained the wild-type phenotype and one of these (SB37) was used as a P1 donor and crossed with MG1655 (DS631), a wild-type *E. coli* K-12 strain. Eighteen Tet^R transductants of MG1655 were assayed and none had the wild-type growth phenotype. The growth phenotype of MG1655 was assayed and it had the same growth phenotype as the *cdr* mutant strain DS381. Another standard lab strain, MC1400 (DS473), also had a phenotype that was similar to the DS381 "mutant" strain (Fig. 7).

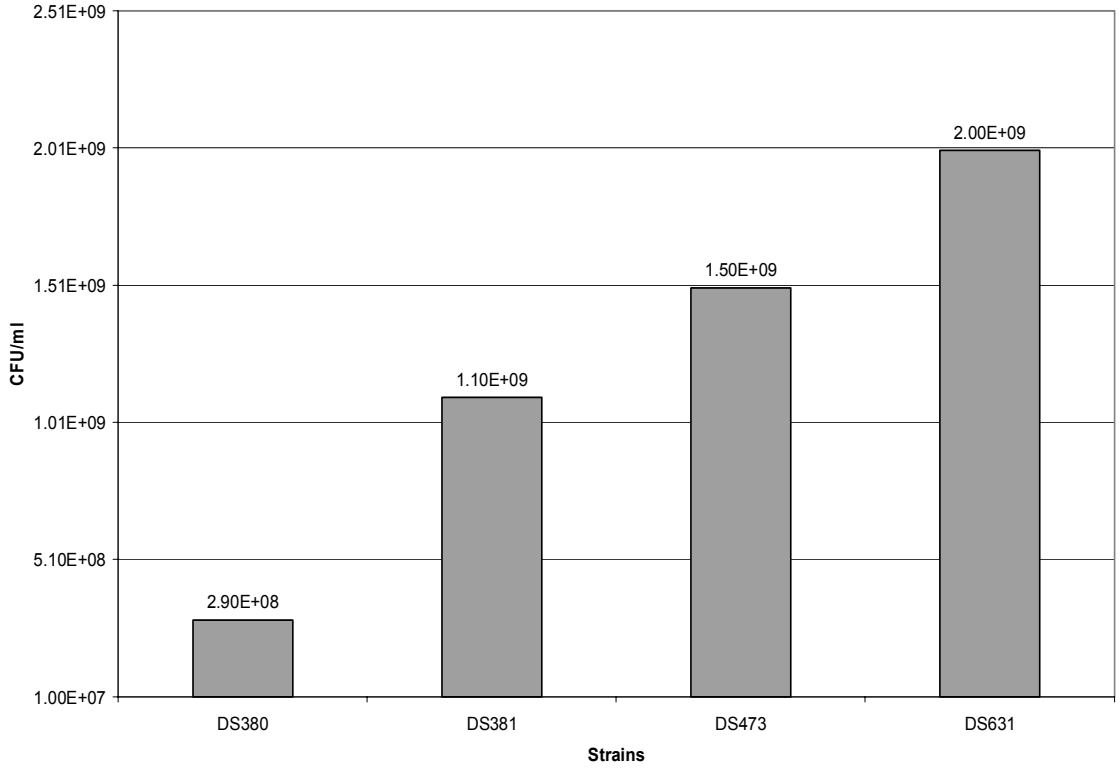

FIG. 7. Comparison of growth phenotypes. The growth phenotype of DS380 and DS381 were compared to that of other wild-type laboratory strains. The graph shows the CFU/ml after five hours of growth.

Complementation tests with F'111

F'111 carries the region of the *E. coli* chromosome from approximately 82.2 to 91.5 min. This F' plasmid was used to determine whether the *cdr* mutation is dominant or recessive. Before the actual complementation experiments were performed, it was necessary to transfer a drug-resistance marker onto the F'. This was necessary to select for exconjugants during the complementation assay and also to maintain selection for the F' plasmid once it was transferred into the recipient strain. F'111 *zjb-3178*::Tn*10kan* was constructed as described in Materials and Methods. The growth phenotype of four individual exconjugants from each mating was tested. The results indicate that the low CFU/ml phenotype was restored to DS381 after it was complemented with F'111 and the growth phenotype of DS380 remained the same in the presence and absence of F'111 (Fig. 8)

Marker rescue can occur when an F' plasmid is introduced into a strain, therefore *recA* mutant derivatives of DS380 and DS381 were constructed for the complementation tests. Introduction of the *recA56* mutation affected the growth of both DS380 and DS381 (Fig. 9). The Rec$^-$ derivatives of both strains grew more slowly then their wild-type counterparts but DS380 *recA* still grew to a lower cfu/ml than DS381 *recA*. When F'111 *zjb-3179::Tn10kan* was introduced into DS381 *recA*, the exconjugants entered stationary phase at a lower cell number than the strain without the F' plasmid (Fig. 10). .

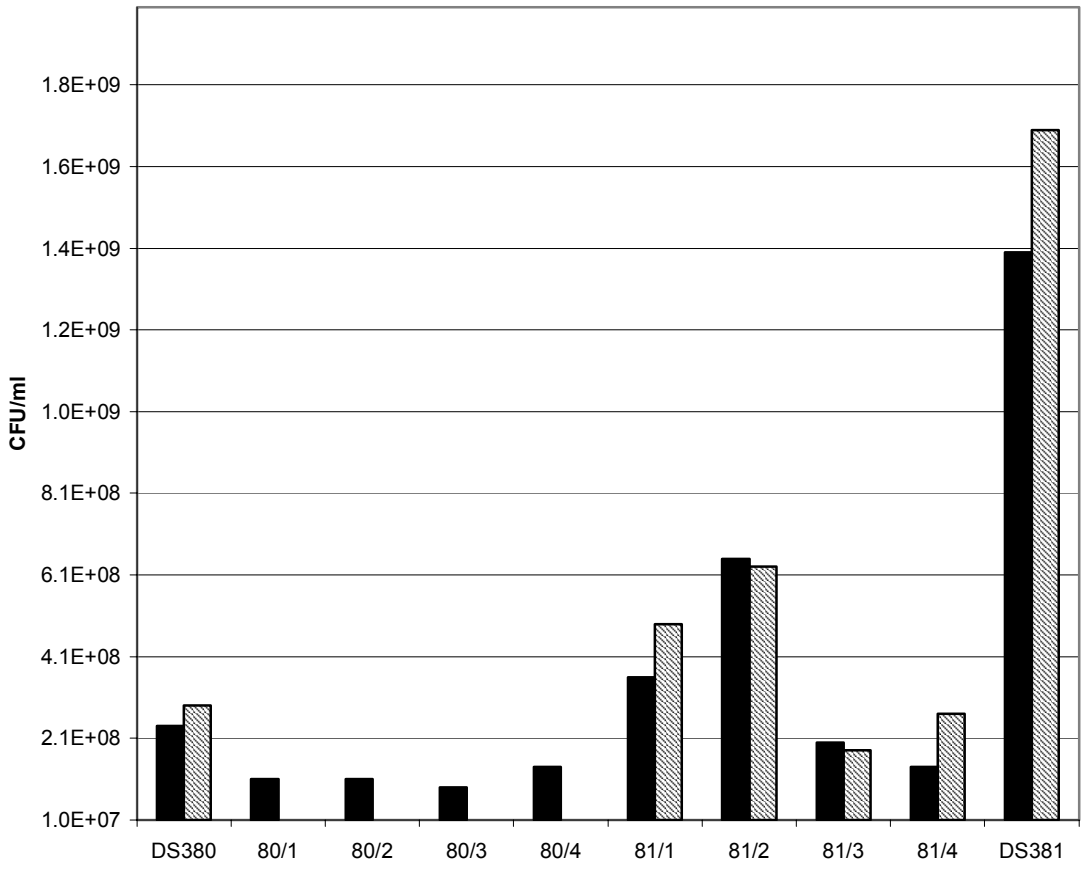

FIG. 8. Complementation of DS380 and DS381. Strains DS380 and DS381 were complemented using F'111 *zjb-3178*::Tn*10Kan*. Controls were grown in TB supplemented with thiamine and thymidine and exconjugants were grown in the same medium supplemented with kanamycin. The exconjugants from the DS381 x F'111 *zjb-3178::Tn10kan* cross were assayed twice on consecutive days; day one, solid bars, day two, hatch bars.

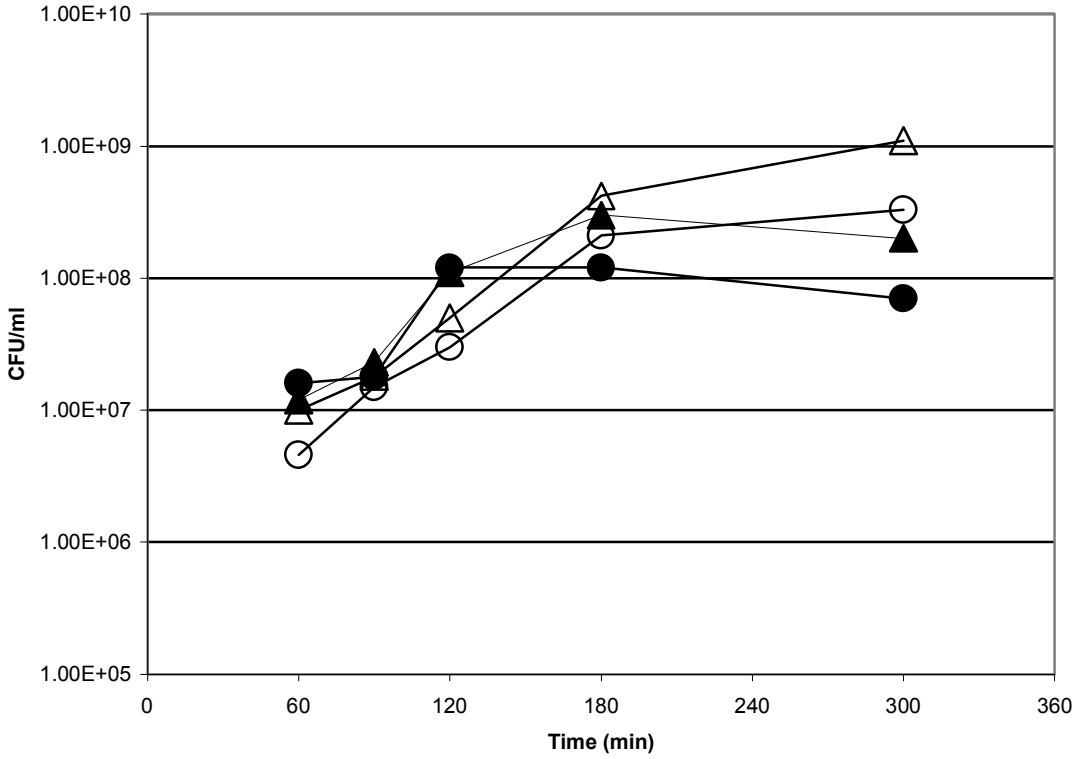

FIG. 9. Growth of Rec⁻ strains. Growth curves of DS380 and DS381 and their Rec⁻ derivatives. DS380, open circles; DS380 *recA*, closed circles; DS381, open triangles; DS381 *recA*, closed triangles.

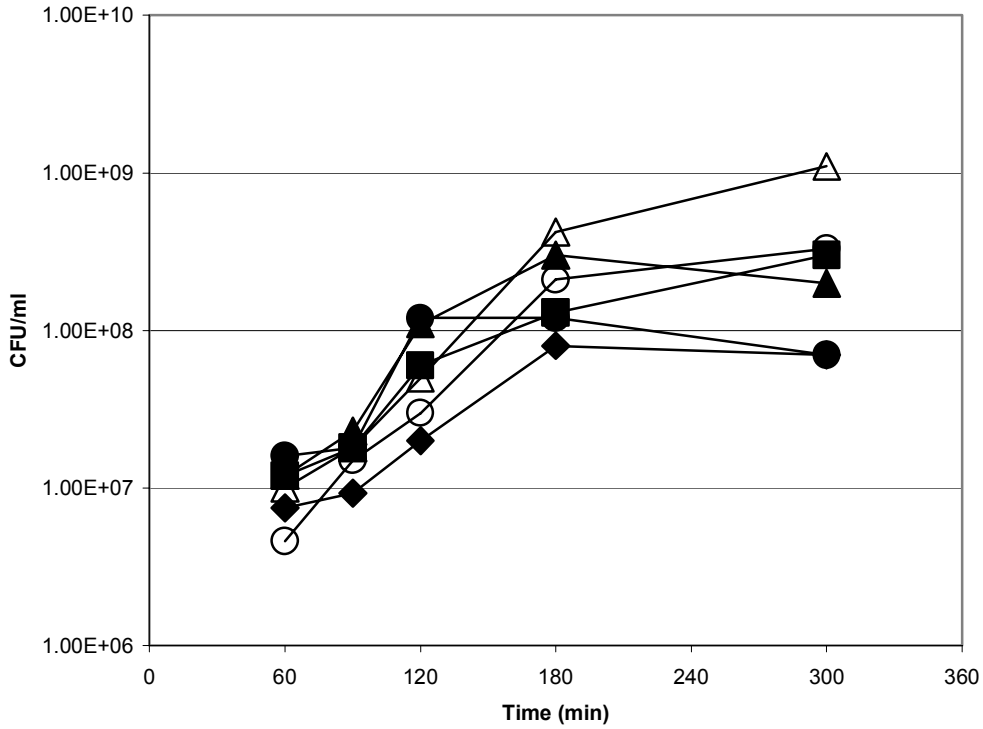

FIG. 10. Growth curves of the F'-complemented Rec$^-$ strains. DS380, open circles; DS380 *recA*, closed circles; DS380 *recA* F'111, closed squares; DS381, open triangles; DS381 *recA*, closed triangles; DS381 *recA* F'111, closed diamonds.

There was a four-fold difference in the final number of CFU per ml between the two strains. This would suggest that the mutant *cdr* allele is recessive, although the difference in final cell number is smaller than between DS381 *recA* and DS380 *recA*. However, when F'111 zjb-*3179::Tn10kan* was mated into DS380 *recA* the exconjugants had three times more CFU per ml than the parent strain after entry into stationary phase (Fig. 10). Because of these puzzling results, the complementation tests were also performed in DS380 and DS381. F'111 *zjb-3178*::Tn*10kan* was mated into DS380 and DS381 and the growth phenotype of four individual exconjugants from each mating was tested. The results are shown in Fig. 8. The wild-type phenotype was restored to DS381 and the growth phenotype of the *cdr*$^+$ parental strain with the F' was the same as the wild-type strain without the F'. These results indicate the *cdr* mutation is recessive. However, because DS381 is Rec$^+$, it would be necessary to determine whether the mutant *cdr* allele is still present in one or more of the 4 exconjugants.

D380 and DS381 are both *rec*$^+$ strains. During the F' complementation assay, recombination between the F' plasmid and the chromosome could occur, resulting in marker rescue instead of complementation of the *cdr* allele. To reduce this possibility the F' complementation tests were also performed with derivatives of DS380 and DS381 that had been transduced to *recA56* (Fig. 9 and 10.

Sequencing of the *cpxA* gene from DS380 and DS381

Prüß and Matsumura found that cells with the *cdr* mutant growth phenotype were smaller than wild-type cells (17). They also found a correlation between depletion of serine from the medium and the reduction in growth rate of wild-type cells. These phenotypes and the map location of the *cdr* locus near 88.5 min pointed to *cpxA* as a candidate for the gene affected by the *cdr* mutations.

The *cpxA* gene is located at 88.4' region of the *E. coli* chromosome, and its gene product is required for the regulation of the envelope stress response in *E. coli* (9, 11, 16, 20). CpxA is the transmembrane sensor kinase in the two-component CpxAR response regulatory system (16). Point mutations in *cpxA* have pleiotropic effects and show many different phenotypes including random localization of the FtsZ ring leading to aberrant cell division (14, 15), and increased levels of L-serine deaminase, which alters the ability of *E. coli* K-12 strains to utilize serine as a carbon source (13).

To test this hypothesis, the *cpxA* gene from both DS380 and DS381 was amplified using the polymerase chain reaction (PCR) and sequenced. The *cpxA* sequences from DS380 and DS381 were compared to one another and to the wild-type MG1655 reference sequence (GenBank Accession number U00096). The region sequenced included 53 base pairs upstream of the *cpxA* start codon to 160 base pairs downstream of the stop codon. In this interval the sequences of DS380 and DS381 were identical to one another and to MG1655.

Morphology of cells in exponential and stationary phase

The growth phenotype of MG1655 was similar to that of DS381. Another question addressed was whether or not these results reflected similarities in cell morphology between MG1655 and DS381 as well, and what differences in cell morphology might exist between these two strains and the DS380 *cdr*$^+$ strain. Samples were collected as described in Materials and Methods and DIC images of exponential and stationary phase cells were obtained. Representative photomicrographs are shown in Fig. 11 and 12.

Cell length was determined by measuring the photomicrographs and the length of the stage micrometer was used to calibrate the measurements and convert them to microns. 50 cells of each strain in each growth phase were measured, with the exception of the DS380 stationary phase sample, for which only 36 cells were measured (Fig. 13 and Fig. 14). DS381 and MG1655 have similar cell sizes in both exponential and stationary phase. In exponential phase, the cells sizes of DS380 and DS381 are comparable. However, in stationary phase, DS380 cells are larger than cells of DS380 and DS631.

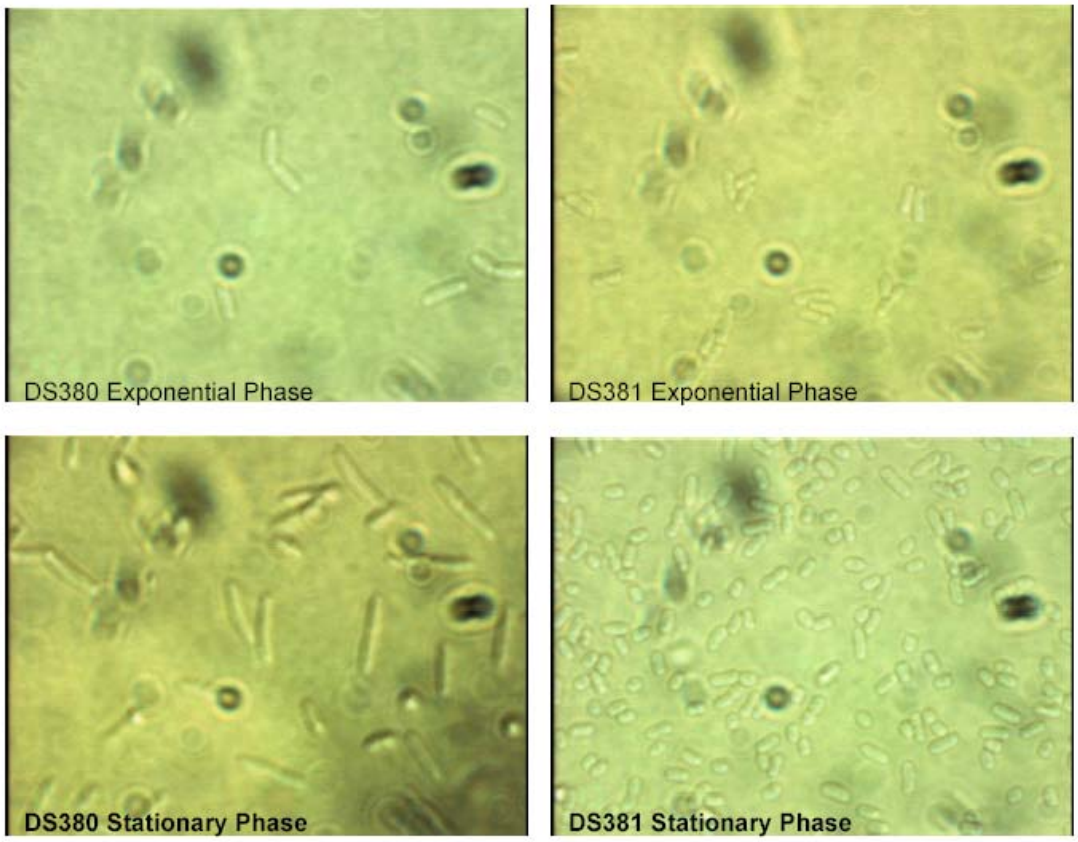

FIG.11. Morphology of exponential and stationary phase cells. DIC images of DS380 and DS381 cells in exponential and stationary phase. The images were taken at a magnification of 63X and a body magnification of 2X under oil immersion.

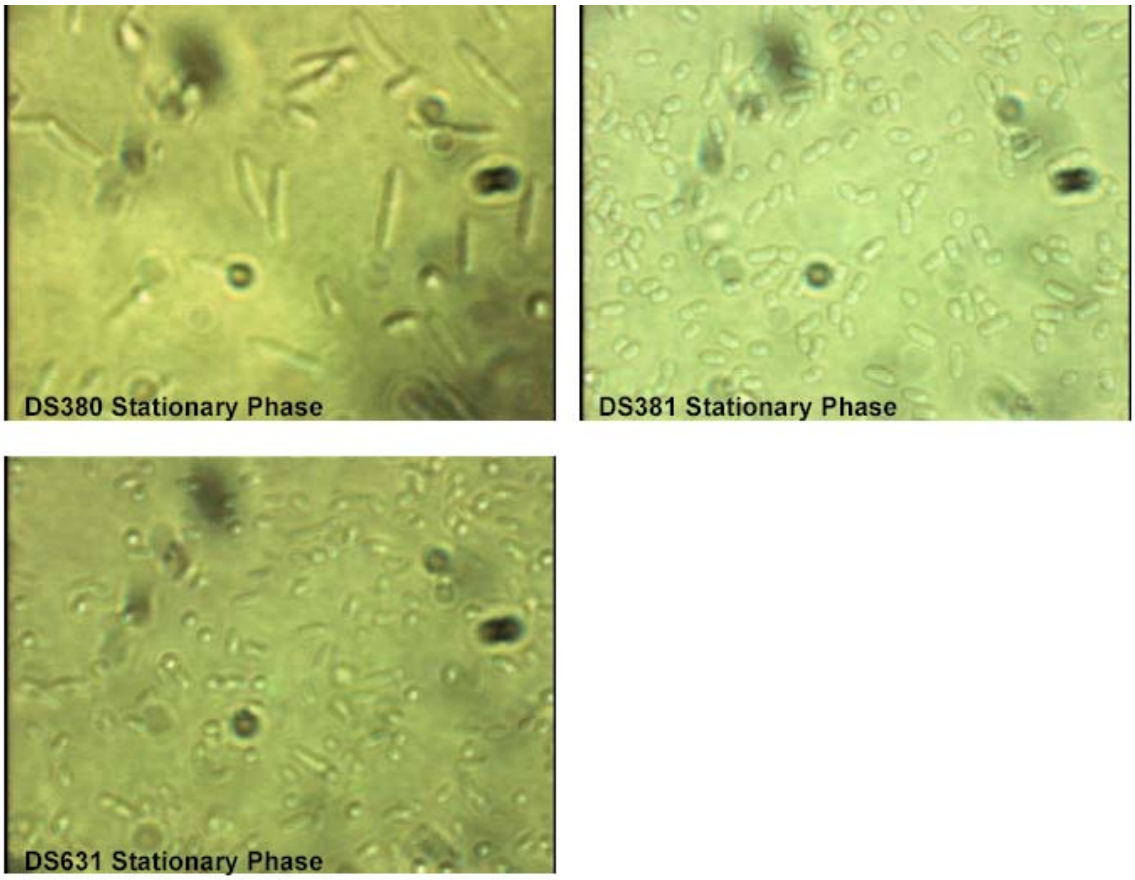

FIG. 12. Comparison of cell morphology in stationary phase. DIC images of DS380, DS381, and DS631 cells in stationary phase. The images were taken at a magnification of 63X and a body magnification of 2X under oil immersion.

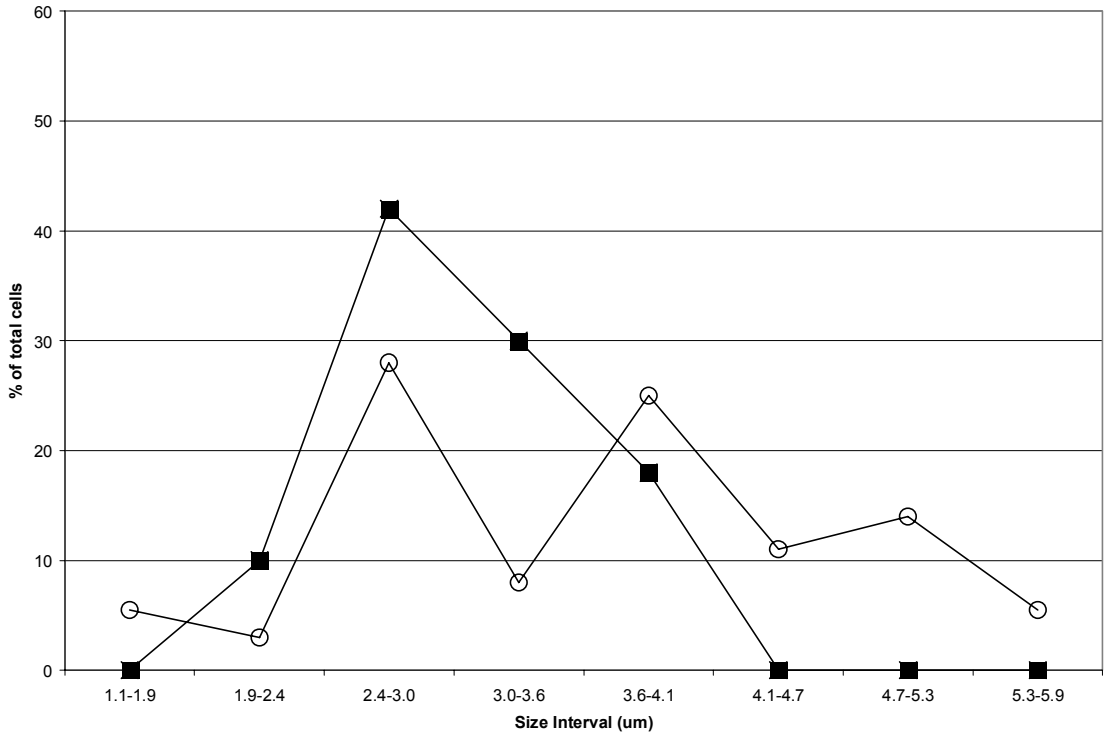

FIG. 13. Polygon of cell sizes in exponential phase. The data shown represent the percentage of cells in each size interval. DS380, open circles (N= 36 cells), DS381, closed squares (N = 50 cells).

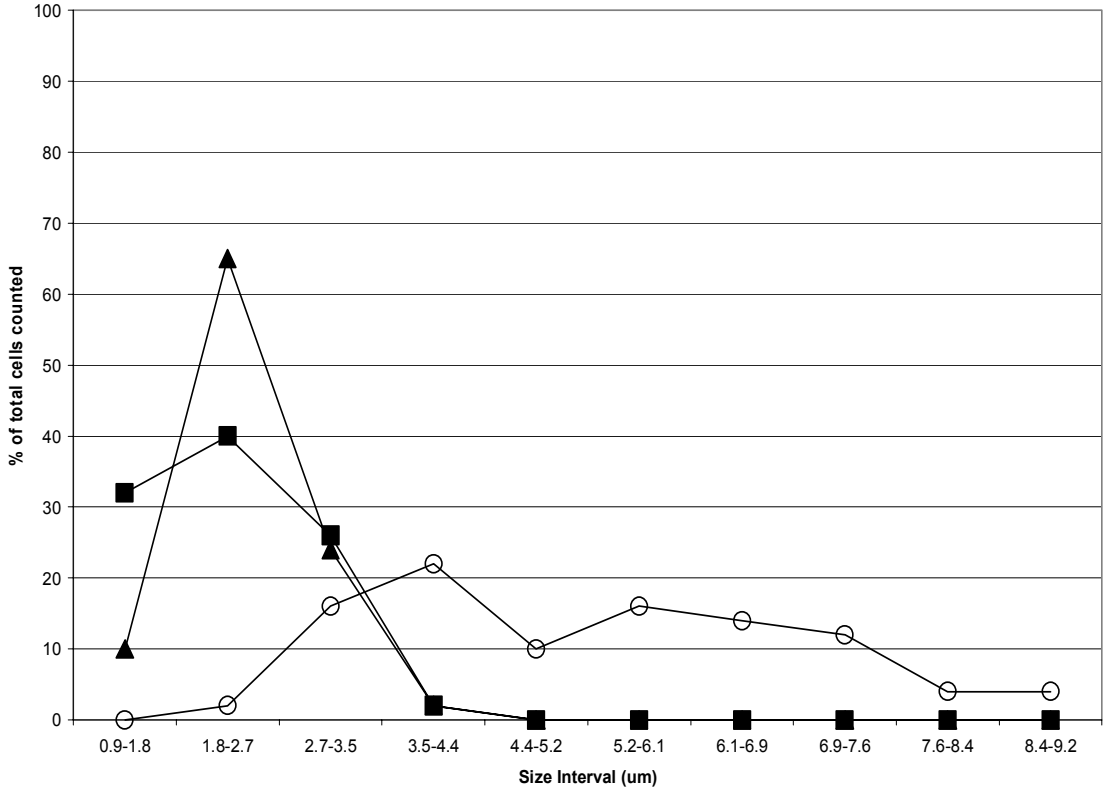

FIG. 14. Polygon of cell sizes in stationary phase. DS380, open circles, DS381, closed circles, DS631, closed triangles. Cells from a total of three independent cultures of each strain were examined and the cell size distribution for each strain was identical by visible inspection. The data shown are from a single culture of each strain. Fifty cells were measured for each sample.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

The Hfr mapping results localized the *cdr* mutation to the interval between 67' and 84' on the *E. coli* chromosome. However, there was a discrepancy in the results obtained with Hfr strains CAG12205 and EA1005, which have the same origin of transfer and transfer the region between 84.5 min and 67 min and 84.5 and 58 min, respectively (Table 3). The mating between DS381 and the EA1005 donor strain resulted in exconjugants that received the wild-type cdr^+ allele. However, none of the exconjugants from the mating between donor strain CAG12205 and DS381 received the wild-type allele. I should have recovered Cdr+ exconjugants from both crosses. CAG12205 has never been used in mating experiments in our laboratory and it is possible that it is not the Hfr strain that it is supposed to be. Because the results of the matings with all of the other Hfr strains are consistent with one another, I did not investigate this further.

The Hfr mapping and the mapping with phage P1 gave different results. Mapping with P1 indicated that the mutation is located near 88.5 min, rather than between 67 min and 84 min as indicated by the Hfr mapping results. The difference is probably due to the fact that the location of the Tn*10* insertions is known more precisely than the location of *oriT* in the Hfr strains. The location of *oriT* in the Hfr strains was determined genetically based on how long it took for known markers to be transferred from the donor to the recipient strain. In

contrast, the sites of the Tn*10* insertions used for mapping were determined by DNA sequencing.

My results indicate that DS380, the strain that was originally designated as the "wild-type" strain by Prüß and Matsumura, is in actuality a mutant strain. Analysis of the standard lab strains MG1655 (DS631) and MC4100 (DS473) showed that their growth phenotype is more akin to that of DS381 than DS380. The morphology of MG1655 cells in stationary phase is also more similar to that of DS381 than DS380.

If indeed DS380 is the actual mutant strain, the results from this study suggest that the DS381 strain may have picked up additional mutation(s) in the 88.5' region that suppress the original mutant phenotype, i.e. lower CFU/ml during entry into stationary phase. In order to map the original mutation in the DS380 strain, it would be necessary to carrying out the Hfr mating experiments using DS380 as the recipient strain and screen exconjugants for the DS381 growth phenotype.

To determine whether the "*cdr*$^{+}$" locus from DS380 was sufficient to cause the Cdr+ growth phenotype (lower CFU per ml during entry to stationary phase) in other strain backgrounds, this region was transduced into MG1655 using the linked *zih-35*::Tn*10* insertion as the selectable marker. Eighteen transductants were assayed and all retained the Cdr^{-} phenotype of MG1655, indicating that the *cdr*$^{+}$ allele does not cause a Cdr^{+} phenotype in the MG1655 background.

The results of the complementation test with F'111 showed that the *cdr* mutation in DS381 is recessive. This indicates that the mutant growth phenotype observed in the DS381 strain is due to loss of Cdr function. The complementation experiments were performed in a Rec^+ strain there is still a question of whether or not the results are due to marker rescue instead of complementation. To answer this question, I could use $P1_{vir}$ grown on the DS381 F'111 *zjb-3178::Tn10kan* exconjugants and infect SB42, which is DS380 with a Δ*glpK* mutation. The *glpK* gene, which is required for growth on minimal media with glycerol as the sole carbon source, is located at 88.6 min, and so is linked to the *cdr* locus. Transductants of SB42 that are able to grow on minimal glycerol medium can then be selected and the growth phenotypes tested. If transductants are found with the mutant phenotype, it will indicate that the mutant *cdr* allele is still present in the exconjugants of DS381 and that the *cdr* mutation is recessive. Alternatively, the F'111 can be cured from the *cdr* mutant strain by mating in another F plasmid with a selectable marker and then selecting for the loss of F'111. Since the two plasmids belong to the same incompatibility group they would not be maintained stably in the same cell. If selection is for the F plasmid that does not carry the *cdr* region of the chromosome, that plasmid would be present at a higher frequency than the F'111 plasmid. The growth phenotype of cells that have lost F'111 can be assayed to see if these cells retain the original mutant allele

The Kohara Phage collection can be used to further localize the *cdr* mutation to within a 5 kb region of the chromosome (5). Each member of this

collection of lambda phages carries a 20 kb portion of *E. coli* DNA. The collection is ordered and overlapping. We would have to make lysogens of the *cdr* mutant strain and score for recovery of the wild-type phenotype. This procedure will allow us to eliminate genes that are unlikely to be responsible for the *cdr* phenotype. Once the number of candidate genes is decreased to four or five genes, we can then sequence these genes from the mutant and wild-type strains.

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