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Birrer, Teresa Sandelin, Ph.D.

University of New Hampshire, 1993

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ANALYSIS OF CHROMOSOMAL INVOLVEMENT IN *YERSINIA PESTIS* VIRULENCE PLASMID EXPRESSION

BY

TERESA SANDELIN BIRRER

BA Mount Holyoke College, 1986

DISSERTATION

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

Doctor of Philosophy

in

Microbiology

May, 1993

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DEDICATION

To my parents.

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ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Zsigray for his guidance and support as well as the other members of my committee, Dr. Chesbro, Dr. Green, Dr. Rodgers and Dr. Margolin for all of their help. I would also like to thank Dr. Pistole for his encouraging words throughout my career at UNH. Thank you to UNH and the Graduate School for several years of support in the form of CURF grants, Summer Fellowships and a Dissertation Fellowship.

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TABLE OF CONTENTS

DEDICATION.....iii

ACKNOWLEDGEMENTS.....iv

LIST OF TABLES.....vii

LIST OF FIGURES.....viii

ABSTRACT.....x

CHAPTER PAGE

INTRODUCTION.....1

I. LITERATURE REVIEW.....3

The Genus *Yersinia*.....3

Plasmid Expression in the *Yersinia*.....5

Mechanisms of Plasmid Regulation.....25

Rational for the Present Study.....28

II. MATERIALS AND METHODS.....30

Bacterial Strains and Growth Conditions.....30

Nucleic Acid Manipulations.....30

Gene Transfer Methods.....36

Analysis of Gene Function.....37

Non-aerobic Growth.....39

III. RESULTS.....41

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Isolation of Calcium Independent Strains of *Y. pestis*.....41

Conjugation of F'Cm to *Y. pestis* O19CI2.....41

Construction of F'133::Tn*10* and F'105::Tn*10* and

their Expression in *Y. pestis* O19CI2.....43

Expression of Cloned *rfa*H, *cpx*A, and *arc*A Genes in

Y. pestis Strains O19 and O19CI2.....47

Hybridization of *Y. pestis* DNA with Plasmids

pRA310 and pKZ17.....50

Anaerobiosis and Virulence Plasmid Expression.....52

IV. DISCUSSION.....70

E. coli F Expression Genes in *Y. pestis*.....70

Virulence Plasmid Expression in Anaerobically

Grown *Y. pestis*.....76

Summary.....80

LITERATURE CITED.....82

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LIST OF TABLES

1. *Yersinia* Plasmid and Chromosome Encoded Genes Involved in Virulence....10
2. Bacterial Strains and Sources....31
3. Bacteriophages and Sources....32
4. Frequencies of Conjugation....44
5. Calcium Requirement and MS2 Sensitivity of *Y. pestis* O19 Harboring Different *E. coli* F Expression Genes....45
6. Expression of Characteristics Associated with *E. coli* F Regulatory Genes in *Y. pestis* O19....49
7. Expression of the Lcr Trait under Different Atmospheric Conditions....60
8. Alleviation of the Lcr of *Y. pestis* by the addition of 10 mM $NaHCO_3$ to magnesium-oxalate medium....69

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LIST OF FIGURES

1. *Y. pestis* Virulence Plasmid Map....9
2. Current Theory of Virulence Regulation in *Y. pestis*....14
3. Agarose Gel of *Y. pestis* O19 Plasmidless Isolates and *Y. pestis* F'Cm Transconjugants....42
4. Construction of F'133::Tn*10* and F'105::Tn*10*....46
5. Agarose Gels and Corresponding Southern Hybridizations of *Yersinia* and *E. coli* Genomic DNA Probed with pRA310 and pKZ17 plasmid DNA....51
6. Bar Graph of Lcr Expression During Growth under Different Atmospheric Conditions....53
7. Plasmid Profiles of *Y. pestis* K25 isolates Grown at 37°C on MgOx Medium under Aerobic or Anaerobic Conditions....55
8. Bar Graph of Aerobic Lcr Expression after Growth on MgOx Plates Incubated under Different Atmospheric Conditions....57
9. Graph of β-galactosidase Activity of *Y. pestis* Kim and its Isogenic MuD1 Insertion Mutants....58
10. Graph of *Y. pestis* K25 grown aerobically and anaerobically at 37°C in HIB over a 4 h period....61
11. Graph of *Y. pestis* K25 grown aerobically and anaerobically

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at 37°C in HIB over a period of 42.5 h.....62

12. Graph of *Y. pestis* K25 grown in HIB at 37°C under 2 different anaerobic environments.....64

13. Graph of *Y. pestis* K25 grown in BHI at 37°C under 2 different anaerobic environments.....65

14. Growth of *Y. pestis* K25 grown anaerobically at 37°C in BHI with the addition of either sodium acetate or sodium bicarbonate.....67

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ABSTRACT

ANALYSIS OF CHROMOSOMAL INVOLVEMENT IN *YERSINIA PESTIS* VIRULENCE PLASMID EXPRESSION

by

Teresa Sandelin Birrer

University of New Hampshire, May, 1993

Virulence plasmid expression in the *Yersinia* has been shown to vary depending upon which species harbors the virulence plasmid suggesting that chromosomal regulation may in some way be involved. In this study, fertility plasmid expression in *Y. pestis* was investigated as a means of evaluating mechanisms of virulence plasmid regulation in this bacterium. The F plasmid was found to be minimally expressed in *Y. pestis* transferring at a rate of $8.8 \times 10^{-5}\%$. In addition, *Y. pestis* failed to express F pili as determined by infection with the male specific phage MS2. When F plasmids containing at least 2 *E. coli* F regulatory genes were transferred to *Y. pestis* and monitored for MS2 sensitivity, they were found to confer MS2 sensitivity to *Y. pestis* .

In an attempt to determine if these known plasmid regulatory genes could influence virulence plasmid expression, three clones *arc*A, *rfa*H, and *cpx*A were observed in *Y. pestis*. All of these genes were found to increase

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the frequency of F transfer from *Y. pestis*, but none were able to alter virulence plasmid expression. The effects of anaerobic growth on plasmid expression were investigated. While anaerobicity was observed to have no effect on F plasmid expression, surprisingly virulence plasmid expression was found to be repressed in strains with and without the *arc*A gene.

Further analysis of two ß-galactosidase insertion mutants of *Y. pestis* revealed that both *yop*J and *yop*E genes failed to be induced by the normal stimuli when the mutants were grown anaerobically. However, it was noted that when these strains were returned to an aerobic environment plasmid expression returned to normal. Varying gas mixtures were studied for their ability to repress plasmid expression, but only the original mix of 85%N_2, 5%CO_2, and 10%H_2 was able to repress plasmid expression. $NaHCO_3$ at a final concentration of 10mM was found to relieve growth restriction aerobically suggesting that the anaerobic repression was due to the formation of HCO_3- in the presence of CO_2 and H_2.

These results represent a new means of relieving *Yersinia* plasmid-mediated growth restriction *in vitro* which may be related to the physiological environment encountered *in vivo* by this bacterium.

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INTRODUCTION

As the causative agent of the bubonic plague, *Yersinia pestis* has been intensively studied since it was first isolated by Yersin in 1894. Although a vaccine and several effective antibiotics are available, delayed diagnosis (three to four days) of infected individuals has resulted in a high fatality rate from this disease. Because of its extreme pathogenicity and the curious ability of this organism to survive within the immune cells whose function is to destroy foreign bacteria, *Y. pestis* represents a model for studying virulence mechanisms in other intracellular pathogens as well. An early discovery toward understanding the pathogenesis of *Y. pestis* was that it contained a 72 kilobase plasmid encoding several virulence associated characteristics (Ferber and Brubaker, 1981; Portnoy and Falkow, 1981). Without the plasmid the organism is avirulent (Portnoy and Falkow, 1981). Since the identification of the virulence plasmid, numerous studies have been done to characterize its proteins and to determine how they influence pathogenicity. Relatively little work has been done on how bacterial physiology is involved in plasmid expression or regulation (Cornelis *et al.*, 1991; Delwiche *et al.*, 1959; Harakeh and Matin, 1989)

In this study, the fertility plasmid F was used as a model to try and

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understand the host factors involved in virulence plasmid regulation in *Y. pestis.* Accordingly, several *Escherichia coli* genes known to influence F expression were analyzed for function in *Y. pestis.* In addition, anaerobiosis, known to regulate plasmid expression in *E. coli* and other organisms, was studied in *Y. pestis* as a possible means of virulence plasmid regulation.

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I. REVIEW OF THE LITERATURE

The Genus *Yersinia*

The *Yersiniae* are characterized as Gram-negative, bipolarly staining, coccobacilli to rods. All species are non-motile at 37°C, and all but *Y. pestis* are motile by peritrichous flagella at 30°C. They are facultative anaerobes having the capacity for both respiratory and fermentative metabolism. Growth is rather slow compared to other members of the *Enterobacteriaceae*. In addition, the three species pathogenic for humans are auxotrophic at 37°C requiring at least thiamin and biotin for growth (Burrows and Gillet, 1972). *Y. pestis* is also auxotrophic at 26°C requiring L-methionine and L-phenylalanine. This species has the additional growth requirements of L-isoleucine, L-valine, glycine, L-threonine, and cysteine for growth at 37°C (Brownlow and Wessman, 1959). The optimum temperature and pH for growth are 27°C to 28°C and 7.2-7.6 respectively, but growth will occur at temperatures between 5°C and 45°C and within the pH range of 5.0-9.6 (Sokhey and Habbu, 1943a; 1943b).

Y. pestis carries out the Embden-Meyeroff-Parnas pathway for dissimilation of glucose, but it cannot utilize the hexose monophosphate

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shunt due to a lack of glucose-6-phosphate dehydrogenase (Santer and Ajl, 1955; Mortlock and Brubaker, 1962). *Y. pestis* also lacks formic hydrogenlyase activity; therefore, no appreciable gas is produced from growth on glucose (Santer and Ajl, 1955). Studies of electron carriers in *Y. pestis* have shown the absence of cytochrome C oxidase in both aerobically and anaerobically grown cells (Englesberg *et al.*, 1954). Another oddity of *Y. pestis* intermediary metabolism is the presence of a constitutive isocitrate lyase at 26°C in addition to an acetate inducible isozyme at both 26°C and 37°C (Hillier and Charnetzky, 1981). Other species of the genus appear to behave as the rest of the members of the Enterobacteriaceae with respect to metabolism.

Three members of the Yersiniae are of significant pathogenicity to humans. These are *Y. enterocolitica*, *Y. pestis*, and *Y. pseudotuberculosis*. *Y. enterocolitica* causes adenitis and ileitis in children and arthritis, septicemia, and erythema nodosum in adults (Schaechter *et al.*, 1989). *Y. pseudotuberculosis* results in epizootics in most mammals causing mesenteric adenitis and diarrhea (Schaechter *et al.*, 1989). This infection, if not self-limiting, may lead to septicemia in humans. The third species, *Y. pestis*, is the etiological agent of the bubonic plague (Schaechter *et al.*, 1989). Although plague is mostly associated with rodents, it may be transmitted to humans via the flea vector. Colonization in this manner results in enlarged lymph nodes known as buboes. Left untreated this form of disease can lead to pneumonic

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plague which when spread via aerosols results in death within four days. Although occasional resistant strains develop, most *Yersinia* are susceptible to tetracycline, chloramphenicol, streptomycin, and sulfonamides (Swartz, 1973; Schaechter *et al.*, 1989). Although not routine, vaccination with formalin treated organisms is available for protection against bubonic but not pneumonic plague (Swartz, 1973).

While the outcome of infection from these organisms is varied, they are genetically very similar. The closest relation is shared by *Y. pestis* and *Y. pseudotuberculosis* which are 90% homologous (Bercovier *et al.*, 1980). In addition, all three species maintain a 60-75 kilobase virulence plasmid required for pathogenicity. *Y. pestis* exhibits four additional virulence factors not related to this plasmid. These are the ability to produce F1 antigen (provides antiphagocytic protection) and pesticin I (functions as an antibacterial as well as a post-translational modifier for virulence plasmid-encoded proteins), to synthesize purines *de novo* (since *Y. pestis* apparently cannot obtain these from its environment) and to bind hemin resulting in pigmentation (necessary for iron acquisition) (Surgalla *et al.*, 1964; Brubaker, 1972). It is the virulence plasmid, however, that has received the most attention in the past ten years.

Plasmid Expression in the *Yersiniae*

The Virulence Plasmid

The virulence or Vwa plasmid of the *Yersiniae* is large (60-75 kb), single copy number, and highly conserved. It encodes a set of temperature

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and calcium sensitive proteins referred to as Yops for *Yersinia* outer membrane proteins. It is now known that the Yops are membrane bound only in *Y. enterocolitica* and *Y. pseudotuberculosis* which lack a 9 kb plasmid unique to *Y. pestis*. The 9 kb plasmid encodes pesticin I as well as plasminogen activator which is involved in cleavage and secretion of the Yops of *Y. pestis in vitro* (Sodeinde *et al.*, 1988; Mehigh *et al.*, 1989). For this reason, it was only recently shown that *Y. pestis* does indeed produce Yops, and that they are secreted into the environment.

Although several significant contributions have been made toward the understanding of plasmid expression in *Yersinia*, the regulation of Yop production remains unclear. It is known that transcription of the virulence plasmid is enhanced in response to an increase in temperature from 26°C to 37°C and a decrease in calcium concentration to $\leq$ 2.5mM (Straley and Bowmer, 1986). Expression is even greater in the presence of at least 2.5mM magnesium (Brubaker and Surgalla, 1964; Zahorchak *et al.*, 1979). These cations apparently mimic the environment seen by the organism during an infection (Straley and Brubaker, 1981).

Increased plasmid transcription and subsequent Yops production lead to an ordered metabolic stepdown referred to as growth restriction. This is characterized by a failure of the cells to undergo more than one or two rounds of DNA replication, a reduction in cell mass and protein production, as well as an overall reduction in accumulation of RNA due to a shift in RNA

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species (Zahorchak *et al.*, 1979); mRNA continues to increase comprising 95% of the total cell RNA after 2 h under restrictive conditions (Charnetzky and Brubaker, 1982). Zahorchak *et al.* (1979) also found that while *Y. pestis* does undergo a typical stringent response during amino acid starvation, only trace amounts of guanosine tetraphosphate and no guanosine pentaphosphate were detected during plasmid mediated growth restriction. After 10 to 12 h of restriction, cells can be rescued by shifting to 26°C whereas the addition of calcium after this amount of time is virtually ineffective (Hall *et al.*, 1974; Goguen *et al.*, 1984; Zahorchak *et al.*, 1979). In addition to calcium, several other factors have been identified which relieve growth restriction at 37°C. These include the addition of ATP (Zahorchak *et al.*, 1979; Zahorchak and Brubaker, 1982), GTP (Zahorchak and Brubaker, 1982), Zn^{2+} (Higuchi *et al.*, 1959), Sr^{2+} (Higuchi *et al.*, 1959), and bicarbonate ions (increased alkalinity) (Delwiche *et al.*, 1959; Surgalla *et al.*, 1964; Zahorchak and Brubaker, 1982). Only Ca^{2+} and ATP have been studied to any extent revealing that neither is bound nor taken up by the cell during relief from restriction (Perry and Brubaker, 1987; Zahorchak and Brubaker, 1982). Zahorchak and Brubaker (1982) have suggested that the nucleotides may alleviate restriction by chelating the magnesium. Brubaker (1991) has also suggested that the removal of Na^{+} will alleviate restriction.

This plasmid associated growth restriction, or low calcium response (Lcr), has been exploited by the development of magnesium oxalate (MgOx)

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medium (Higuchi and Smith, 1961). Virulent strains of *Yersinia* are able to grow on this medium only at 26°C. Colonies arise at 37°C at a frequency of 1 in 10^4 (Higuchi and Smith, 1961; Portnoy and Falkow, 1981; Portnoy *et al.*, 1984). Goguen *et al.* (1984) found approximately 30% of calcium independent colonies to be missing the virulence plasmid or to have a mutation in it. The remaining 70% of his isolates appear to have arisen due to chromosomal mutations involved in plasmid expression. Straley and Bowmer (1986) also reported a high percentage of uncharacterized chromosomal mutations that altered plasmid expression. These results strongly suggest that some of the genes governing normal cell behavior are also involved in virulence plasmid regulation.

Based upon growth characterisitics on MgOx medium, many of the virulence plasmid genes responsible for producing the Lcr and Yops have been identified. Figure 1 shows the locations of the genes that have been identified to date. The cross-hatched area represents the low calcium response (Lcr) region. The genes located in this region have been found to be essential virulence determinants required for the bacterial response to temperature and calcium (Portnoy *et al.*, 1983; Goguen *et al.*, 1984)). Table 1 lists the known plasmid and chromosomal genes related to virulence and their functions where known.

Mutations which alter growth on MgOx medium have been induced directly by ethyl-methane-sulfonate (EMS) (Yother and Goguen, 1985) and

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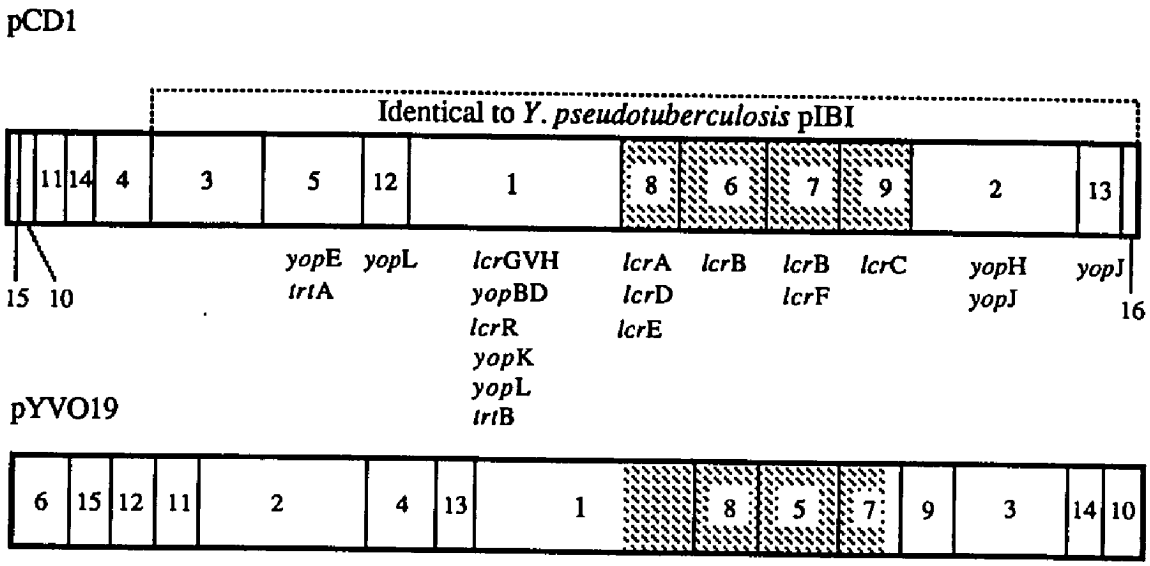

FIG. 1. *Bam*HI restriction pattern of pCD1 from *Y. pestis* KIM (Yother and Goguen, 1985) andpYVO19 from *Y. pestis* O19 (Portnoy and Falkow, 1984). The stippled area represents the Lcr region.

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TABLE 1. *Yersinia* plasmid and chromosome encoded genes involved in virulence.

Gene	Description	Reference
Plasmid-encoded		
*lcr*A	Transcription increases due to a shift in temperature to 37°C in the absence of calcium. Mutants are calcium blind and are transcribed at 37°C regardless of calcium concentration. (*vir*A in *Y. enterocolitica*)	Goguen *et al.*, 1984
*lcr*B	Behaves the same as *lcr*A to a lesser degree (*vir*B in *Y. enterocolitica*)	Goguen *et al.*, 1984
*lcr*C	Transcription is similar to *lcr*A and lcrB in *Y. pestis*. In *Y. enterocolitica* (*vir*C) it has been found to comprise a large single operon containing 13 *ysc* genes. Transcription of the operon is dependent upon VirF.	Goguen *et al*, 1984 Michiels and Cornelis, 1991 Michiels *et al.*, 1991
*lcr*D	Membrane-bound regulator with homology to the *Caulobacter crescentus flb*F protein. Mutants do not undergo growth restriction, do express V antigen or LcrG protein, and show an increased expression of YopH and YopM.	Ramakrishan *et al.*, 1991 Plano *et al*, 1991
*lcr*E	Encodes the YopN which is involved in repression of the Lcr in the presence of calcium. Mutants are calcium blind and exhibit an Lcr regardless of calcium concentration.	Yother and Goguen, 1985 Forsberg *et al.*, 1991
*lcr*F	DNA binding protein responsible for regulating the response to increased temperature. Specifically activates transcription of *trt*A and B. The product VirF in *Y. enterocolitica* shares homology with AraC, RhaS, RhaR and the regulator of CS pili.	Cornelis *et al.*, 1989 Lambert de Rouvroit *et al*, 1992
*lcr*G	Possibly the promoter of the lcrGVH operon.	Bergman *et al.*, 1991

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TABLE 1 cont.

*lcr*H	Lcr repressor that is inactivated by another protein when calcium is low. It is necessary for Lcr relief due to ATP or calcium and production of YopJ. Mutants are avirulent but still produce V antigen.	Bergman et al., 1991 Price and Straley, 1989
*lcr*Q	Mutant is temperature sensitive for growth at 37°C regardless of calcium.	Rimpilainen *et al.*, 1992
*lcr*R	Represses V antigen expression in the presence of calcium. It is not responsive to ATP. It is transcriptionally regulated by temperature not calcium although it regulates other proteins in response to calcium.	Barve and Straley, 1990
*lcr*V	Encodes the V antigen which is necessary for the Lcr. It counteracts the repression of the Lcr by *lcr*H.	Bergman *et al.*, 1991 Perry *et al.*, 1986
*trt*A	Activated by *lcr*F at 37°C.	Yother *et al.*, 1986
*trt*B	Activated by *lcr*F at 37°C. Mutants do not produce V antigen.	Yother *et al.*, 1986
*yer*A	Regulates expression of *yop*E product.	Forsberg and Wolf-watz,1990
*ylp*A	*Y. enterocolitica* lipoprotein related to *tra*T of F.	China *et al.*, 1990
*ypk*A	*Y. pseudotuberculosis* protein kinase.	Galyov *et al.*, 1993
*yop*E	Obstructs antibacterial activities of professional phagocytes. Has cytotoxic activity when attached to host cells and complexed with the product of *yop*H. *In vitro* it is repressed by calcium and activated by elevated temperature.	Straley and Bowmer, 1986 Straley and Cibull, 1989 Forsberg and Wolf-watz,1988 Rosqvist *et al.*, 1990
*yop*K	Strongly transcribed in a low calcium environment. Unique to *Y. pestis*.	Straley and Bowmer, 1986 Straley and Cibull, 1989
*yop*L	Unique to *Y. pestis* and not necessary for virulence in the mouse model.	Straley and Cibull, 1989

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TABLE 1 cont.

*yop*H	Essential for resistance to phagocytosis, interacts with *yop*E product to have cytotoxic activity, and acts as a tyrosine phosphatase.	Straley and Bowmer, 1986
*yop*J	Partially regulated by *lcr*H, not necessary for virulence in the mouse.	Straley and Bowmer, 1986
Chromosome-encoded		
*ymo*A	Encodes a histone-like protein which modulates thermoregulation of *vir*F in *Y. enterocolitica*. Mutants allow expression of yops at 28°C on MacConkey agar. It is also involved in *yst* regulation.	Cornelis *et al.*, 1991
yst	Encodes the heat stable enterotoxin of *Y. enterocolitica*. Expressed at 28°C not 37°C.	Michiels and Cornelis, 1991 Michiels *et al*, 1991
*ysc*D	Required for export of yops.	Michiels and Cornelis, 1991 Michiels *et al.*, 1991
*ysc*J	Same as above	
*ysc*L	Same as above	
*ysc*C	Same as above	
*ysc*M	No viable mutants have been obtained. Similar to central portion of *yop*H.	Michiels *et al.*, 1991

Mu dI1 (Apr *lac*) insertions (Goguen *et al.*, 1984; Perry *et al.*, 1986; Straley and Bowmer, 1986) and indirectly by transposon mutagenesis with Tn*5* in *E. coli* harboring the *Yersinia* virulence plasmid (Portnoy *et al.*, 1984). Two major divisions of mutants have been defined. These are calcium independent mutants which grow at 37°C and calcium blind mutants which are unable to grow at 37°C, regardless of the calcium concentration (Portnoy *et al.*, 1984; Yother and Goguen, 1985; Yother *et al.*, 1986). There are also reports of an ATP blind mutant which does not respond to ATP at 37°C but is still responsive to calcium (Price and Straley, 1989), and several mutants identified by alterations in transcription that have typical MgOx growth patterns but are avirulent (Forsberg and Wolf-watz, 1988; Straley and Bowmer, 1986).

Bergman *et al.* (1991) and others (Forsberg *et al.*, 1991; Price *et al.*, 1991) have attempted to describe an overall scheme for regulation of the *Yersinia* virulence plasmid. The current outline of regulation is diagrammed in Figure 2. It is postulated that a temperature inducible activator protein is responsible for the positive control of the Lcr and that calcium is the effector responsible for repression of the Lcr (Forsberg and Wolf-watz, 1988). The major candidate for the positive control gene, *lcr*F (*vir*F in Y. *enterocolitica*), has been found to encode a DNA binding protein that acts as a diffusable activator of the Lcr at 37°C (Cornelis *et al.*, 1989; Yother *et al.*, 1986).

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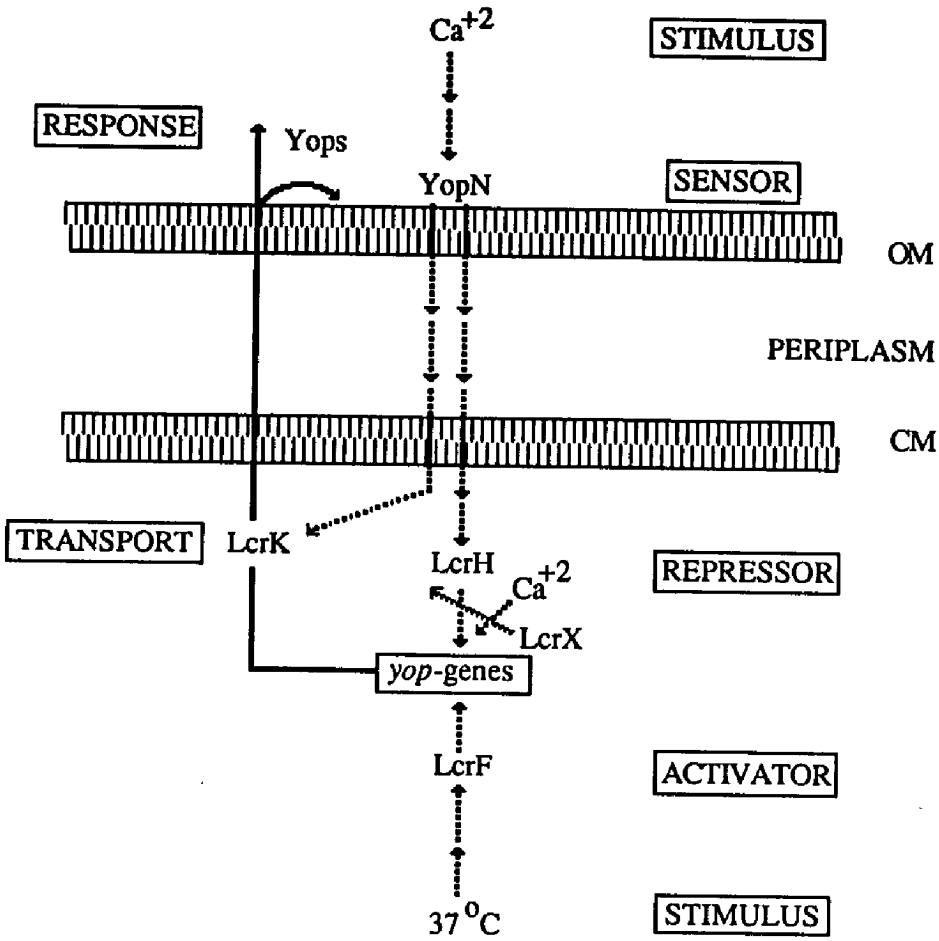

FIG. 2. Outline of *Yersinia* regulation according to Bergman *et al.*, 1991.

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Yother *et al.* (1986) found expression of this protein to be independent of temperature in *Y. pestis*, while Cornelis *et al.* (1989) and Bergman *et al.* (1991) have shown temperature inducible transcription in *Y. enterocolitica*, *Y. pseudotuberculosis*, and to a lesser degree in *E. coli*. Both groups agree that expression of *lcr*F is autoregulated or chromosomally regulated since it is transcribed in the absence of any other plasmid genes (Cornelis *et al.*, 1989).

This model also includes a repressor (Bergman *et al.*, 1991; Forsberg and Wolf-watz, 1988) which is transcribed at 37°C in response to high levels of calcium and in turn depresses transcription of Yops. LcrH has been shown to act with another unknown protein to regulate Yop production at 37°C in the presence of calcium (Bergman *et al.*, 1991). Bergman *et al.* (1991) have suggested that the unknown protein is active at low calcium concentrations and interferes with the repressor activity of LcrH alone. Others (Price *et al.*, 1991) have suggested that LcrV is the protein that interferes with LcrH function.

LcrH has also been shown to vary in only one amino acid (threonine to proline) between the three plasmids (Bergman *et al.*, 1991) suggesting that it is a conserved regulator of virulence plasmid expression. Price and Straley (1989) showed that an *lcr*H insertion mutant expressed an increased level of *lcr*V product at 37°C in the presence of ATP or calcium. The expression of *lcr*V was shown to be higher in the presence of ATP than in the presence of calcium indicating that LcrH is more responsive to ATP levels than to

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calcium concentration. They also found that the *lcr*H mutant showed depressed levels of *yop*J transcription, in contrast to higher levels of *lcr*V, suggesting that LcrH regulates *lcr*V and *yop*J expression differently.

Another protein found to have repressor activity is LcrR (Barve and Straley, 1990). LcrR apparently represses the *lcr*GVH operon in the presence of calcium; however, this product is also necessary for LcrG activity in the absence of calcium (Barve and Straley, 1990). This group has also shown that LcrR behaves in the opposite manner of LcrH in that it is more responsive to calcium than to ATP. It has been shown that the negative regulation of *lcr*GVH in the presence of calcium is at the transcriptional level and that the positive regulation of LcrG in the absence of calcium is at the mRNA or protein level (Barve and Straley, 1990). The possibility exists that the activation of *lcr*G is actually potentiated by the product of *lcr*D which has recently been located immediately upstream of *lcr*R and characterized as an activator of virulence operons (Plano *et al.*, 1991).

In addition to activator and repressor proteins, Bergman *et al.* (1991) have found regulation of the virulence plasmid to involve post-translational location. It has been shown that *lcr*H, *lcr*V, *yop*B, and *yop*D mutants all exhibit transcription of *yop* genes at 37°C but that the Yops are only secreted in the absence of calcium (Bergman *et al.*, 1991; Cornelis *et al.*, 1989). Secretion of Yops has only recently been investigated, but reports have shown that the Yop proteins do not require any signal sequences or

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recognition sequences for export (Michiels and Cornelis, 1991). Studies on protein export done in *Y. enterocolitica* have shown that the *vir*C (*lcr*C) locus is a large operon containing 13 *ysc* genes (Michiels *et al.*, 1991). They have shown that three of the 13 genes are required for protein export. Transcription of the *vir*C operon was found to be regulated by *vir*F (*lcr*F) much in the same way the Yops are regulated (Michiels *et al.*, 1991).

The functions of most of the Yop proteins remains unknown; however, a few significant Yops have been characterized. *yop*E was found by Forsberg and Wolf-watz (1988) to encode a cytotoxic protein that is active only when attached to eukaryotic cells indicating that it functions extracellularly at relatively high calcium concentrations. Forsberg *et al.* (1991) have shown the *yop*N encoded protein to be surface located and involved in calcium signal transduction. YopM, which is unique to *Y. pestis*, has been found to inhibit platelet aggregation (Leung *et al.*, 1990). Lastly, the most exciting discoveries of Yop function to date are that of the *yop*H and *ypk*A encoded proteins which have been shown to be a tyrosine phosphatase active on eukaryotic cells (Guan and Dixon, 1990) and a protein kinase (Galyov *et al.*, 1993) respectively.

The Fertility Plasmid

The fertility plasmid, F, associated with *E. coli* can be transferred and expressed in *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis* (Bakour *et al.*, 1983; McMahon, 1971; Zsigray *et al.*, 1983 and1985). Because of

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homologous *inc*D regions, F and the *Yersinia* virulence plasmid are incompatible and unable to be maintained together as autonomously replicating cytoplasmic forms (Bakour *et al.*, 1983). F and the *Y. enterocolitica* virulence plasmid also share a structurally and functionally similar replicon, RepFIIA, which has been found on the virulence plasmids of *Salmonella typhimurium*, *Shigella* spp., and *E. coli* as well (Vanooteghem and Cornelis, 1990). As with the virulence plasmid, F is large (90-100kb) and maintains a single copy number. Due to the strict regulation of copy number, F is tightly linked to the process of cell division through its *ccd* genes (Tam and Kline, 1989). When F is lost , CcdB is produced and acts as a cytotoxin to kill the F- segregants (Tam and Kline, 1989). When F is stably maintained, the product of *ccd*A combines with that of *ccd*B to relieve the cytotoxicity (Tam and Kline, 1989). Oddly, F is dependent upon the highly conserved host replication machinery for its own replication and several other ill-defined host proteins for expression of many of its conjugal properties. Specifically, the F encoded properties of pilus formation and surface exclusion are highly host dependent (Beutin and Achtman, 1979; Beutin *et al.*, 1981).

The group of plasmid genes responsible for pilus formation and surface exclusion is termed the *tra* operon (Beutin and Achtman, 1979). It comprises a 33kb uninterrupted region of the F plasmid which is made up of at least 19 genes referred to as *tra*Y-Z preceded by a positive regulatory gene *tra*J (Beutin *et al.*, 1981; Gaffney *et al.*, 1983). Several groups have arrived at

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nine chromosomal genes whose products exhibit some influence over F plasmid expression in *E. coli* primarily through their action on *tra*J or the ability of TraJ to positively regulate the *tra* operon. The genes, as they have been most recently labelled, are *arc*A, *arc*B, *cpx*A, *cpx*B, *rfa*H, *dna*B, *fex*A, *him*A, and *hip* (Bachman, 1990).

<u>*arc*A</u>. The most well-defined of the chromosomal F regulatory genes is the *E. coli arc*A locus. The name *arc* refers to its involvement in the normal aerobic respiratory control of *E. coli* (Iuchi and Lin, 1988). Others have identified this locus as *dye*, *msp*, *seg*, *fex*A, *cpx*C, and *sfr*A according the various mutant phenotypes (Beutin and Achtman, 1979; Lerner and Zinder, 1982; McEwen and Silverman, 1980b; Willetts, 1977). This pleiotropic gene has been mapped to 0/100 min on the current *E.coli* linkage map (Bachman, 1990). Its gene product is a 238 amino acid, soluble, cytplasmic protein whose primary function appears to be to inhibit aerobic respiratory enzymes during anaerobic growth (Iuchi and Lin, 1988). As noted above several secondary characteristics have been associated with this locus including the ability to grow aerobically in the presence of redox dyes such as toluidine blue (Buxton and Drury, 1983). This characteristic seems to be a visual representation of the same system that senses aerobic and anaerobic conditions for enzyme control. Thus, it is probably indicative of the cell's ability to alter its respiratory pathways (Iuchi and Lin, 1988). The unusual aspect of *arc*A regulation is that the enzymes involved belong to a variety of

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regulons (tricarboxylic acid cycle, glyoxylate shunt, and fatty acid degradation enzymes, flavoprotein dehydrogenases, and the cytochrome c oxidase complex) suggesting that the *arc*A locus encodes a conserved regulatory protein able to interact with several types of genes (Iuchi and Lin, 1988; Iuchi *et al.*, 1989b; Fu *et al.*, 1991).

In addition to influencing aerobic respiratory enzyme expression, the *arc*A product stimulates expression of the F plasmid presumably by enhancing transcription from *tra*J, *tra*Y or both (Beutin *et al.*, 1981; Iuchi *et al.*, 1989a and 1989b; Silverman *et al.*, 1991b). It appears that ArcA acts in both a positive and a negative manner to repress one set of genes while stimulating another. Apparently, these functions can be genetically separated (Silverman *et al.*, 1991a). How such diverse regulation is accomplished will be discussed later.

<u>*arc*B</u>. The *arc*B gene was identified shortly after *arc*A as another gene involved in mediating aerobic respiratory control (Iuchi *et al.*, 1989a). *arc*B is located at 69.5 min on the *E. coli* chromosome (Bachman, 1990) and has been determined to be a transmembrane protein (Iuchi *et al.*, 1989b). Its role is presumed to be as a sensor of the cell oxidation and reduction state(Iuchi *et al.*, 1989b; Stock *et al.*, 1989). Recently, Iuchi *et al.* (1989b) examined *arc*B mutants grown both aerobically and anaerobically. They determined that F pili were elaborated normally in the presence of oxygen while they were produced in levels greater than wild type in the absence of oxygen. One

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explanation for how oxygen might influence F pilus production in *arc*B mutants will be discussed later.

*cpx*A. Another fairly well-characterized gene involved in F conjugal activity is *cpx*A (conjugative plasmid expression) which is located at 87.5 min on the *E. coli* map (Bachman, 1990). It codes for a 52,000 molecular weight inner membrane protein (Weber and Silverman, 1988). It has been suggested that the *cpx*A gene product is a sensor of the sexual status (the presence or absence of F) of the cell and transmits this signal to another protein to activate transcription of *tra*J (Stock *et al.*, 1989; Weber and Silverman, 1988).

The normal role of CpxA to the bacterial cell involves the biosynthesis of the amino acids isoleucine and valine. Wild type *E. coli* produce two isozymes (I and III) of acetohydroxyacid synthase, an enzyme required for biosynthesis of these amino acids (McEwen and Silverman, 1980c; Sutton *et al.*, 1982). When leucine is present in the growth medium only isozyme I is functional; it has been shown that *cpx*A mutants are auxotrophic for isoleucine and valine under these conditions due to a missing membrane protein required for enzyme activity (McEwen and Silverman, 1980c; Sutton *et al.*, 1982). It has not been shown whether the activation of isozyme I and the activation of *tra*J transcription are through the same process.

*cpx*B. Another gene implicated in expression of F transfer genes is cpxB which has been mapped at 40.5 min on the *E. coli* chromosome (Bachman, 1990). Very little is known about this locus. Its product has not

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been determined; therefore neither its cellular location nor its function have been defined. What has been studied is the effect of *cpx*B mutations on F pilus expression in *cpx*A and *cpx*A+ backgrounds (McEwen and Silverman, 1980a and 1980b; Silverman *et al.*, 1980). Single mutant *cpx*B/*cpx*A+ cells were found to elaborate normal levels of F pili while double mutants (*cpx*B/*cpx*A) were found to be even more impaired than *cpx*A mutants (McEwen and Silverman, 1980b). No other work has been done toward characterizing *cpx*B.

*rfa*H. The *rfa*H locus was initially identified as a transcriptional control element of the F *tra* operon (Beutin and Achtman, 1979; Beutin *et al.*, 1981). It is located at 86 min on the *E. coli* chromosome and encodes an 18,000 molecular weight protein (Rehemtulla *et al.*, 1986). Early studies on the effects of *rfa*H mutations on F expression found that *tra* operon transcripts were prematurely terminated in the absence of RfaH (Beutin *et al.*, 1981). More recently, Farewell *et al.* (1991) have established that the *rfa*H product is an antiterminator at rho-dependent transcriptional termination sites.

It was later observed that cells with *rfa*H mutations expressed altered bacteriophage and antibiotic sensitivities as well as non-functional flagella (Rehemtulla *et al.*, 1986; Willetts, 1977). Shortly after the discovery that the *rfa*H product was involved in membrane alteration, it was found that the *rfa*H gene of *Salmonella typhimurium* was homologous in function to *rfa*H in *E. coli* (Rehemtulla *et al.*, 1986). Mutations in the *S. typhimurium* gene were

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known to affect lipopolysaccharide core synthesis due to two inactive glycosyltransferases enzymes (Rehemtulla *et al.*, 1986; Sanderson and Stocker, 1981). A secondary effect of the altered LPS was the change in phage and antibiotic sensitivities. It has yet to be shown whether premature termination of transcripts is responsible for the defective glycoslytransferases.

*fex*B. The *fex*B locus has not been well studied. The gene is located at 85 min on the *E.coli* linkage map (Bachman, 1990). Nothing is known of its gene product except that at the time of its identification it was thought to function interdependently with the *fex*A (*arc*A) gene product (Lerner and Zinder, 1982). At this time no subsequent reports have appeared on the nature of the *fex*B product or its function.

*him*A and *hip*. Studies of the function of the DNA binding protein integration host factor (IHF) have shown it to be required for F plasmid transfer (Dempsey, 1987; Friedman, 1988; Gamas *et al.*, 1987). IHF is a 20,000 molecular weight heterodimer whose subunits are encoded by the *him*A gene at 37 min and the *hip* gene at 20 min on the *E. coli* chromosome (Friedman, 1988; Mendelson *et al.*, 1991). Mutations in either gene have been shown to reduce F transfer by as much as 500 fold; however, this effect can be overcome if F is present as a high copy number plasmid fusion (Gamas *et al.*, 1987). It has also been found that IHF does not exhibit its effects by altering expression of TraJ (Gamas *et al.*, 1987). It has been speculated that IHF could act indirectly by influencing expression or function

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of any other chromosomal genes necessary for F plasmid transfer (Gamas *et al.*, 1987). Friedman (1988), however, reports the existence of binding sites specific for IHF on the F plasmid. This evidence could support the theory of Gamas *et al.* (1987) if IHF binding to F DNA is required for subsequent interactions by proteins such as ArcA or FexB. One of the IHF binding sites has been located in the promoter for the *pif* operon whose products are required for replication of mini F and inhibition of phage T_7 growth (Friedman, 1988). It is not known if IHF regulation of the *pif* operon is related to its control of plasmid transfer.

<u>*dna*B</u>. The *dna*B gene is located at 91 min on the *E. coli* map (Bachman, 1990) and appears to influence the F plasmid property of surface exclusion (Iyer *et al.*, 1974; Palchoudhury and Iyer, 1971). Few studies have been done on the effects of *dna*B mutations on F expression, but what has been reported indicates some involvment with the cell membrane. Palchoudhury and Iyer (1971) found that surface exclusion of donor cells was reduced in *dna*B mutants such that two F plasmids could coexist in the same cell. Iyer *et al.* (1974) later discovered that the *dna*B-induced reduction in pilus production and surface exclusion could be reversed by addition of 0.15M KCl to the growth medium. Both Palchoudhury and Iyer (1971) and Iyer *et al.* (1974) conducted their experiments with temperature sensitive *dna*B mutants that were thought to encode heat labile proteins which would interfere with DNA replication at the non-permissive temperature (42°C).

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Oddly, the F related phenotypes observed with these mutants were seen at the permissive temperature (37°C) as well. No new studies on the influence of DnaB on F expression have been forthcoming. Possibly it was found that the strains used for the previous studies carried a mutation in one of the other genes required for F plasmid transfer.

Mechanisms of Plasmid Regulation

Two-component Signal Transduction

The *arc*A and the *cpx*A loci have been shown to have complex and sometimes incongruous regulatory patterns that have only recently been explained by the process of two-component signal transduction. This system requires the presence of at least two proteins referred to as the sensor and the response regulator (RR) (Stock *et al.*, 1989). The sensor is usually a membrane associated protein kinase which becomes phosphorylated in response to an environmental signal (Stock *et al.*, 1989). The phosphorylated sensor then tranduces a signal to the RR by transferring the phosphoryl group (Stock *et al.*, 1989). The activated RR can in turn modulate the cell's response to the environmental stimulus. Such regulatory systems have been described for several stimuli such as osmolarity (Forst *et al.*, 1990), nitrogen utilization (Weglenski *et al.*, 1989), and sporulation (Stock *et al.*, 1989). Most of the response regulators have been shown to be DNA binding proteins required for activation or inhibition of transcription (Stock *et al.*, 1989).

ArcA has been identified as a response regulator for two systems:

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aerobic respiratory control and F conjugal expression (Iuchi *et al.*, 1989a; Stock *et al.*, 1989). Silverman *et al.* (1991a) have shown that the site of phosphorylation of ArcA determines which system it will act on. The two speculative kinases associated with ArcA are the products of the *arc*B and the *cpx*A genes (Stock *et al.*, 1989). According to Iuchi *et al.* (1989a), ArcB is the sensor of redox state and phosphorylates ArcA when oxygen becomes depleted. The phosphorylated ArcA then inhibits transcription from the genes for enzymes involved in aerobic respiratory pathways. CpxA has been shown to be a sensor of cell sexual state (as well as other uncharacterized states) (Iuchi *et al.*, 1989b; Silverman, 1985). It phosphorylates at a different site from ArcB causing ArcA to act as a transcriptional activator of the F *tra* operon (Silverman *et al.*, 1991a). It has been shown that cross talk can occur between the sensors of one system and the response regulators of another to the consternation of most researchers.

DNA Supercoiling

It has been known for a long time that the degree of DNA supercoiling varies within the cell over time, but only recently have researchers realized that this variation is a very controlled process of gene regulation (Pruss and Drlica, 1989; Dorman, 1991). Both increased supercoiling and relaxation of DNA have been found to stimulate or repress transcription of different genes (Pruss and Drlica, 1989; Menzel and Gellert, 1987). Interestingly, transcription from strong promoters has been shown to alter superhelicity as

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well (Figueroa and Bossi, 1988). In particular, supercoiling has been shown to be involved in the expression of sets of genes which respond to significant environmental stimuli. Several examples have been characterized.

Transcription of the osmotic stress locus, *pro*U, of both *E. coli* and *S. typhimurium* has been found to be activated by increased negative supercoiling induced by high osmolarity (Higgins *et al.*, 1988). Because of this, topoisomerase I mutants which reduce supercoiling result in osmotically sensitive cells that fail to induce *pro*U (Higgins *et al.*, 1988). The *omp*F and *omp*C porin genes are also regulated by changes in DNA supercoiling due to osmotic stress (Higgins *et al.*, 1988).

Another condition known to influence gene expression through altered DNA topology is anaerobicity. Several genes are speculated to belong to this group, but the *ton*B and colicin genes of *E.coli* and *S. typhimurium* have been shown to respond to anaerobically induced changes in supercoiling (Dorman *et al.*, 1988; Malkhosyan *et al.*, 1991). Dorman *et al.* (1988) have shown that TonB is expressed aerobically and repressed anaerobically when negative supercoiling is increased. They have also shown that *ton*B expression increases with growth phase as DNA becomes more relaxed. The opposite has been shown to be true for colicin gene expression which is enhanced by the increased negative supercoiling associated with anaerobic growth (Malkhosyan *et al.*, 1991). Anaerobicity has also been found to induce the production of at least two *Neisseria gonorrhoea* proteins normally

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expressed during the infectious process (Clark *et al.*, 1987 and 1988). While regulation of these proteins has not been directly linked to changes in supercoiling, it has been suggested (Clark *et al.*, 1987).

Several pathogenic bacteria are known to alter gene expression in response to temperature shifts (*Bordetella pertussis*, *S. typhimurium*, *Shigella flexneri*, and *Yersinia* spp.). Recently it has been shown that DNA supercoiling may be responsible for this as well (Dorman *et al.*, 1990; Cornelis *et al.*, 1991). In *S. flexneri*, the temperature regulated *vir*R gene appears to be induced in response to DNA relaxation (Dorman *et al.*, 1990). Conversly, one of the *S. typhimurium* temperature dependent genes required for entry into host cells, *inv*A, has been found to be stimulated by increased superhelicity (Galan and Curtiss, 1990). Working with *inv*A, Galan and Curtiss (1990) also found that the specific degree of negative supercoiling was critical to gene expression. It was possible to repress *inv*A if the supercoiling density was too high as in topoisomerase I mutants.

Rational for the Present Study

The pathogenesis of *Y. pestis* has been extensively studied for the past thirty years, yet a clear explanation of how this organism causes such a devastating disease as the plague remains elusive. Since the genes of the *Yersinia* virulence plasmid have been identified and are continuing to be characterized and their relationships to one another defined by other laboratories, it was the goal of this study to look at how chromosomal genes

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and normal cell behavior might fit into virulence plasmid expression. The expression of F in *Yersinia* was chosen as a starting point since so much is known about chromosomal regulation of this plasmid. Growth anaerobically was considered as an example of altered cell physiology that might possibly be a significant factor in the *in vivo* infection process.

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II. MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

All bacterial strains used are listed in Table 2. *Escherichia coli* cultures were grown in nutrient broth (Difco Laboratories, Detroit, Mich.) or LB broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.0) with aeration, or on plates of the same medium containing 1.5% agar. Growth was at 37°C unless otherwise noted. *Yersinia* cultures were grown in Heart Infusion Broth (HIB) (Difco Laboratories, Detroit, Mich.) or on Heart Infusion Agar (HIA) plates (Difco Laboratories, Detroit, Mich.) at either 26°C or 37°C. All strains were stored frozen at -70°C in 8% dimethylsulfoxide.

Bacteriophages used are listed in Table 3. MS2 and T4 phage stocks were maintained in broth at 4°C to which a drop of chloroform had been added. U3 and C21 phages were stored in 1.0 ml aliqouts at -70°C.

Nucleic Acid Manipulations

Isolation of Plasmid DNA

Plasmid DNA of all sizes was isolated by the alkaline lysis method of Birnboim and Doly (1979). Minipreparations were performed using 1.5 ml of a broth culture or 1 loopful of cells from a plate. For volumes up to 250 ml, the procedure was scaled up by a factor of 10. All plasmid DNA

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TABLE 2. Bacterial strains and sources.

Strain	Plasmid/Genotype	Source
E. coli K25	F'Cm	W. Lawton
E. coli TB1	R-, Streptomycin^r	J. Hopper
E. coli KL706	F'133(*rfa*H, *cpx*A)	B. Bachman
E. coli KL724	F'105(*rfa*H, *cpx*A)	B. Bachman
E. coli NF859	*met*B, *arg*A	J. Gallant
E. coli NF161		
E. coli WIA-2	Naladixic acid^r	P. Guyre
E. coli SAB2791	pKZ17 (*rfa*H)	
E. coli SAB2796	pKZ23(*rfa*H and Tn*5*)	K. Sanderson
E. coli AE2136	pRA310(*cpx*A)	P. Silverman
E. coli 11560	pMW2(*arc*A)	B. Wanner
E. coli SP314	*arc*A	B. Bachman
Y. pestis O19	72kb plasmid	S. Straley
Y. pestis O19CI.2	Plasmidless(Lcr-)	This study
Y. pestis O19CI.3	F'Cm	This study
Y. pestis O19CI.4	F'Cm, pKZ23	This study
Y. pestis O19.4	72kb, pKZ23	This study
Y. pestis O19CI.5	F'Cm, pRA310	This study
Y. pestis O19.5	72kb, pRA310	This study
Y. pestis O19CI.6	F'Cm, pMW2	This study
Y. pestis O19.6	72kb, pMW2	This study
Y. pestis O19CI.7	F'133::Tn*10*	This study
Y. pestis O19CI.8	F'105::Tn*10*	This study
Y. pestis K25	gua-	R. Brubaker
Y. pestis K25	gua-, Lcr-	R. Brubaker
Y. pestis K25	gua-, pst-	R. Brubaker
Y. pestis KIM	*yop*E::Mu dI1(Ap *lac*)	S.Straley
Y. pestis KIM	*yop*J::Mu dI1(Ap *lac*)	S. Straley
Y. pestis 51F	91.5kb, 72kb, 9kb	R. Brubaker
Y. enterocolitica MY844	72kb	Dr. Yu
Y. enterocolitica	F'Lac	S. Torosian
Y. pseudo-tuberculosis	Wild Type	R. Brubaker
Y. pseudo-tuberculosis	F'Lac	S. Torosian

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TABLE 3. Bacteriophages and sources.

Phage	Host	Source
C21	*E. coli rfa*H *S. typhimurium rfa*H	K. Sanderson
MS2	*E. coli* expressing F pili	Lab stock
T_4	*E. coli*	Lab stock
U3	*E. coli* WT *S. typhimurium* WT	K. Sanderson

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was stored under ethanol at -20°C or 0°C.

Isolation of Chromosomal DNA

E. coli and *Yersinia* chromosomal DNA was isolated by the method of Marmur (1961). DNA was treated with 10 ug/ml of ribonuclease A [E.C. 3.1.27.5, Sigma Chemical Co., St. Louis, MO] before storing under ethanol at 0°C or in sterile glass distilled water with chloroform at 4°C.

Isolation of mRNA

RNA was isolated from 10-25 ml cultures of *Y. pestis* by the hot phenol method described by Emory and Belasco (1991). Clean, DNase treated RNA was stored at -20°C in 20 mM Na phosphate (pH 6.5), 1 mM EDTA (pH 8.0).

Enzymatic Digestion and Recovery of DNA

Plasmid and chromosomal DNA samples were treated with restriction endonucleases as described in the manufacturer's instructions (Bethesda Research Laboratories, Gaithersburg, MD). Restriction fragments to be purified after digestion were separated on 0.8% low melting temperature agarose at 4°C in Tris acetate buffer (0.04 M Tris, 0.02 M Na acetate, 2 mM disodium EDTA, pH 7.8). Fragments were then sliced from the gel and purified by the method described by Burns and Beacham (1983).

Agarose Gel Electrophoresis

Both plasmid and chromosomal DNA were observed in 0.8% agarose

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gels run in Tris acetate buffer (0.04 M Tris, 0.02 M Na acetate, 2 mM disodium EDTA, pH 7.8). Vertical and horizontal gels were run at a constant voltage of 40-50 V. Gels were stained with ethidium bromide and viewed under UV light. Documentation was with Polaroid type 55 black and white negative film exposed for approximately 2 min at F8 with a yellow filter.

RNA samples were run in 1% agarose, 2.2 M formaldehyde gels as described by Sambrook *et al.* (1989). Samples were run at a constant voltage of 30-40 V for 3.5 h. After 1-2 h, the buffer was removed and recirculated to prevent gradient formation. Gels were observed and documented as described for DNA gels.

Southern Transfers

Plasmid and chromosomal DNA was transferred from agarose gels to nitrocellulose (BA85, Schleicher and Schuell, Keene, NH) by the method of Southern (1979). Nitrocellulose containing DNA was stored under vacuum until used.

Northerns Blots

RNA was transferred to nitrocellulose or Nytran™ nylon membranes (Schleicher and Schuell, Keene, NH) by a combination of the methods described by Sambrook *et al.* (1989) and Thomas (1980). Gels were soaked in several changes of water for 30 min to remove the formaldehyde. They were then soaked in 20 X SSC for 45 min. The gels were then placed on a filter paper wick soaked in 20 X SSC (the transfer buffer). A piece of

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nitrocellulose soaked in 20 X SSC, and 2 pieces of filter paper soaked in 2 X SSC were placed on top of each gel. Finally, a stack of paper towels about 5 cm high and a 500 g weight were placed on the nitrocellulose sandwich to facilitate transfer. After about 18 h, the gels were removed, air dried, and baked at 80°C in vacuo. Unlike the DNA containing filters, these filters were not soaked in a lower salt buffer before being dried. Filters were stored under vacuum. Nitrocellulose filters were stained with methylene blue (Sambrook *et al.*, 1989) to visualize RNA transfer.

Labelling of DNA Probes

DNA samples to be used as probes were labelled with ^{32}P, according to the manufacturer's instructions, using nick translation kits from Bethesda Research Laboratories (Gaithersburg, MD) or by random priming with digoxigenin labelled dUTP using the Genius™ system (Boehringer Mannheim Corporation, Indianapolis, IN).

Hybridizations

Southerns were hybridized according to the method described by Sambrook *et al.* (1989). The entire contents of nick translated DNA probe was generally incorporated into the hybridization solution. Hybridization was carried out at 68°C in 5 X Denhardt's, 0.5% SDS, 6 X SSC, 100 ug/ml denatured salmon sperm DNA. Autoradiography was performed with Kodak XAR-5 film at -70°C for 15 min to 2 w. Film development was in GBX developer for 3 min.

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Northerns were hybridized according to the directions accompanying the Genius™ probe kit (Boehringer Mannheim Corporation, Indianapolis, IN). Hybridization was done in 50% formamide at 42°C.

Gene Transfer Methods

Conjugation

Transfer of F plasmids from *E. coli* to *Y. pestis* was carried out in liquid matings similar to those described by Miller (1972). Briefly, the donor culture was diluted 1:50 and allowed to grow approximately 3 h to reach log phase. Equal volumes of the diluted donor and an over night culture of the recipient were mixed gently and incubated with slow shaking for 30 min to 1 h at 37°C. At the end of this period a drop of T_4 phage (3.4×10^{11} PFU/ml) was added for 15 to 20 min to eliminate the *E. coli.* Selection was on HIA containing 40 ug/ml phosphomycin. Transfer of F from *Y. pestis* to *E. coli* was done in a similar manner but without the T_4 phage and the phosphomycin.

Electroporation

Plasmids other than F were transferred to *Yersinia* species and *E. coli* by electroporation with a BioRad Gene Pulsar apparatus. Following the manufacturer's instructions, 10 to 100 ml of log phase cells were washed and resuspended in 1/20 of the original volume in ice cold electroporation buffer (272 mM sucrose, 1 mM $MgCl_2$, and 7 mM Hepes, pH 7.2, filter sterilized).

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The cells and appropriate plasmid DNA (also resuspended in electroporation buffer) were mixed and left on ice for 5 to 15 min. The sample (0.8 ml) was then subjected to an electrical pulse in a 0.4 cm cuvette at 2.5 Kv and 25 μF. The pulsed sample was diluted in 8 volumes of HIB and incubated 1 h at 37°C, before being diluted further and plated on media containing the appropriate selective antibiotics.

Transposon Mutagenesis

λ89::Tn*10* was used to transfer tetracycline resistance to plasmids F'133 and F'105. A stock of λ89 was grown in *E. coli* CSH23, a *sup*E mutant. Cultures were grown and infected as described by Miller (1972).

Analysis of Gene Function

Determination of the LCR

The activity of the low calcium response (Lcr) region of the *Yersinia* virulence plasmid was monitored by growth on magnesium oxalate (MgOx) medium (Higuchi and Smith, 1961) or EGTA medium. MgOx medium was made by adding Na oxalate and $MgCl_2$ to HIA or HIB each at a final concentration of 0.02 M. EGTA medium was made by adding 10-100 mM EGTA (made as a 10-100 X stock solution and filter sterilized) to HIA or HIB. Cell counts were determined on these media at both 26°C and 37°C. Cells exhibiting a functional Lcr region grew poorly at 37°C due to their requirement for calcium. Cells having lost the virulence plasmid or having an

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altered Lcr region grew well at both temperatures.

Fertility Plasmid Expression

Efficient expression of F plasmids was determined by sensitivity to the male specific phage MS2. A 0.2 ml sample of a log phase culture was plated in soft agar. After the agar had solidified, the plates were spotted with 20 to 50 μl of concentrated phage stock (1.5×10^{11} PFU/ml). After 8 to 24 h at 37°C, the plates were checked for zones of lysis indicating sensitivity to the phage and thus expression of the F pilus.

*sfr*B Gene Expression

Phages C21 and U3 were used as indicators of the *srf*B gene product UDP- galactosyl-transferase. Phage U3 infects cells having a functional product while C21 infects the mutants. Sensitivity tests were done as described for MS2.

*arc*A Gene Expression

Expression of the global regulatory gene *arc*A was determined by plating cells on HIA containing 0.2% toluidine blue O (Sigma Chemical Company, St. Louis, MO). Cells deficient in ArcA product are unable to grow in the presence of this redox dye (Iuchi and Lin, 1989).

*cpx*A Gene Expression

E. coli cultures were tested for *cpx*A activity at 41°C on Vogel-Bonner minimal medium (Vogel and Bonner, 1956) lacking the amino acids isoleucine and valine. Cells lacking a functional *cpx*A gene were unable to grow under

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these conditions (McEwen and Silverman, 1980c).

Chlorate Sensitivity

Cultures were diluted and plated on 0.4% filter sterilized potassium chlorate. Sets of plates were incubated at both 26°C and 37°C for 48 h before colonies were counted.

Yops Expression

ß-galactosidase activity was measured to determine if Mu dII (Apr *lac*) fusions in the *Yop*J and *Yop*E genes of *Y. pestis* were being expressed. ß-galactosidase was assayed as described by Miller (1972) using chloroform and SDS to lyse the cells. All samples were frozen at -20°C until the assay was performed. All volumes were scaled up by a factor of three. Absorbances at 420 nm and 550 nm were read on a Bausch and Lomb Spectronic 20.

Non-Aerobic Growth

General Growth Conditions

Anaerobic growth conditions were maintained in either an anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, Mich.) containing a mixture of 85% N_2 (or 85% Ar), 10% H_2 and 5% CO_2 (Northeast Airgas, Inc., Manchester, NH) or in BBL (Becton Dickinson and Co., Cockeysville, MD) Gas Pack jars using BBL anaerobic system envelopes. Elevated CO_2 levels were achieved with a continuous flow CO_2 incubator or a BBL CO_2 Gas Pack system. A nitrogen environment was maintained in a Torbal jar

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(Torsian Balance Co., Clifton, NJ) vacuumed and filled five times with 99.98% N_2 gas (Northeast Airgas, Inc., Manchester, NH). 90% N_2 and 10% H_2 was maintained by displacing 10% of the N_2 from a filled Torbal jar with 99.999% H_2 (Northeast Airgas, Inc., Manchester, NH). 95% N_2 and 5% CO_2 was maintained as described above except that the N_2 was displaced with 99.9% CO_2 (Northeast Airgas, Inc., Manchester, NH). Microaerobic conditions were obtained using a BBL microaerobic Gas Pack system. In all cases other than aerobic, *Yersinia* strains were grown in the presence of 0.1% glucose or 0.2% xylose.

Specific Growth Studies

Y. pestis K25 Lcr+ and Lcr- strains were grown aerobically at 26°C with shaking in either HIB or BHI (Brain Heart Infusion) broth (Difco Laboratories, Detroit, MI) until cultures reached approximately 2-4 x 10^8 cells/ml. After the addition of 0.2% xylose, the flasks were transferred to an anaerobic chamber at 37°C for 24 h. Four side-arm flasks (250 ml) containing 25 ml of the same medium were inoculated from these cultures. One flask of each strain was tightly stoppered before all flasks were removed from the chamber to be incubated at 37°C New Brunswick G-10 gyrotory shaker (250 rpm). The optical densities of all cultures were monitored at 670 nm with a Bausch and Lomb Spectronic 20. In some cases the anaerobic head space consisting of N_2, H_2, and CO_2 was replaced by 2 parts H_2 to 1 part CO_2.

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III. RESULTS

Isolation of Calcium Independent Strains of *Y. pestis*

Because the virulence plasmid of *Y. pestis* was known to be incompatible with F, it was necessary to obtain plasmidless forms of *Y. pestis*. Consequently,*Y. pestis* strain O19 harboring only the 72kb virulence plasmid was plated on MgOx medium at 37°C in order to obtain isolates cured of this plasmid. Figure 3A shows the plasmid profiles of 6 calcium independent isolates and the control calcium dependent strain. A deletion in the 72kb plasmid (lane 1) was noted while lanes 2, 4, and 5 show apparently unaltered plasmids. Only lanes 3 and 6 represent isolates having lost the virulence plasmid. Isolate 6 (lane 6) grew the best and was designated *Y. pestis* O19CI.2.

Conjugation of F'Cm to *Y. pestis* O19CI.2

Figure 3B shows the plasmid profiles of transconjugants of *Y. pestis* O19CI.2 harboring the 92kb F'Cm plasmid from *E. coli* K57. Lanes 1 and 3(9) show the normal plasmid content of strains O19 and O19CI.2 respectively. Lane 5 contains plasmid size standards from *Y. pestis* 51F. The remaining lanes are from chloramphenicol resistant transconjugants. Lanes 2, 4, 6, 8, and 10 show the presence of the F plasmid. One isolate carrying the F'Cm plasmid was designated *Y. pestis* O19CI.3 and was used

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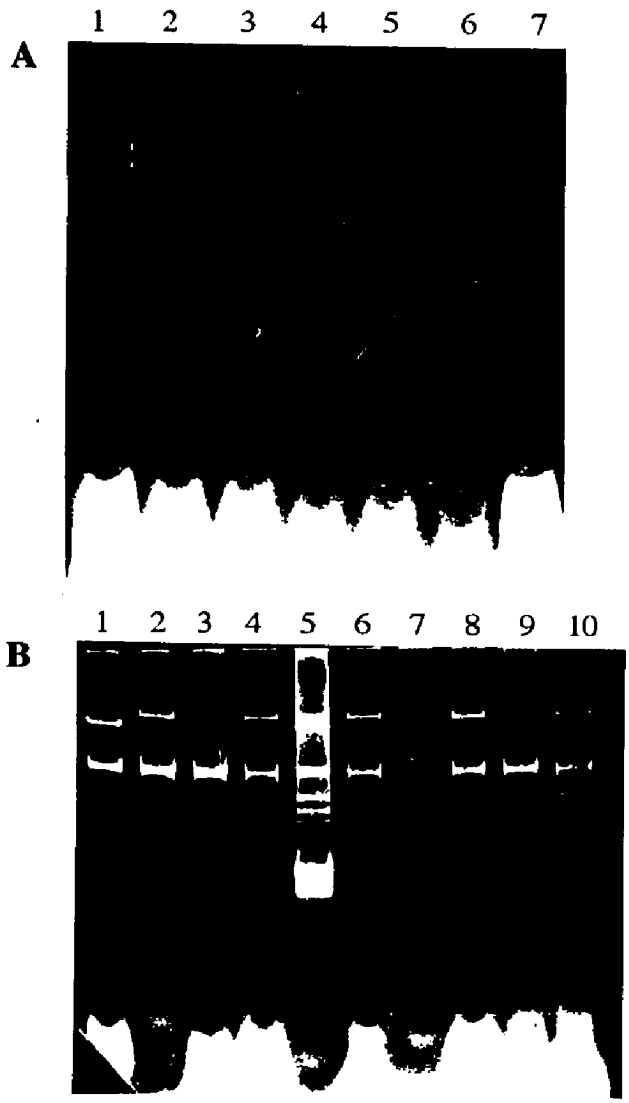

FIG. 3. (A) Plasmid profiles of *Y. pestis* O19 Lcr- isolates. Lane 1, Lcr- due to a deletion in the 72kb plasmid; lanes 2,4, and 5, Lcr- isolates retaining the 72kb plasmid; lanes 3 and 6, plasmidless Lcr- isolates; lane 7, 72kb plasmid from control *Y. pestis* grown at 26°C. (B) *Y. pestis* O19.CI2 F'Cm transconjugants. Lane 1, *Y. pestis* O19 control; lanes 3 and 9, *Y. pestis* O19.CI2; lanes 2,4,6,8, and 10, *Y. pestis* O19.CI2 F'Cm transconjugants; lane 5, plasmid MW markers from *Y. pestis* 51F (91.5 and 72kb markers are indicated); lane 7, *E. coli* K57 F'Cm donor.

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to determine phage MS2 sensitivity and conjugation frequency from *Y. pestis*. *Y. pestis* O19CI.3 was found to transfer F to *E. coli* TB1 at a frequency of 8.8 X 10^{-5} % (Table 4) and to be resistant to the male specific phage MS2 (Table 5). This transfer frequency was found to be significantly lower than those frequencies observed for *E. coli* K57 and two other species of *Yersinia* tested (Table 4).

Construction of F'133::Tn*10* and F'105::Tn*10* and Their Expression in *Y. pestis* O19CI.2

Since it was possible that the low donor activity of *Y. pestis* was due to a deficiency in F pilus formation as shown by the lack of MS2 sensitivity, the effects of at least two *E. coli* genes required for efficient F expression in that bacterium were observed in *Y. pestis*. Plasmids F'133 and F'105 carrying genes from min 84.5 to 90 and 86.5 to 90 of the *E. coli* chromosome were mutagenized with the tetracycline resistance transposon Tn*10* as depicted in Figure 4. After it was shown that the plasmids were still able to transfer between *E. coli* strains, the tetracycline resistant plasmids were conjugated to *Y. pestis* O19CI.2 resulting in strains designated O19CI.7 (F'133::Tn*10*) and O19CI.8 (F'105::Tn*10*). Both F'133 and F'105 contain the genes *rfa*H and *cpx*A which are known to influence F plasmid expression in *E. coli*. When these plasmids were transferred to *Y. pestis*, they were found to confer MS2 sensitivity by the appearance of turbid plaques (unlike the clear plaques obtained with *E. coli*). Due to the extreme instability of F'133::Tn*10* and

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TABLE 4. Frequencies of conjugation of *Y. pestis* harboring F'Cm along with known *E. coli* F expression genes compared with frequencies for *E. coli* and other pathogenic *Yersinia* species.

Strain	Plasmid(s)	Frequency of Conjugation(%)[a]
E. coli K57	F'Cm	4.7×10^{0}
Y. enterocolitica	F'Lac	2.5×10^{1}
Y. pseudo-tuberculosis	F'Lac	1.2×10^{1}
Y. pestis O19CI.3	F'Cm	8.8×10^{-5}
Y. pestis O19CI.4	F'Cm, pKZ23	7.8×10^{-4}
Y. pestis O19CI.5	F'Cm, pRA310	2.3×10^{-2}
Y. pestis O19CI.6	F'Cm, pMW2	2.6×10^{-4}
Y. pestis O19CI.7	F'133::Tn*10*	[b]
Y. pestis O19CI.8	F'105::Tn*10*	[b]

[a] Recipient in all cases was *E. coli* TB1. All frequencies were determined after 1h.
[b] Not determined due to instability of F plasmids.

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TABLE 5. Calcium requirement and MS2 sensitivity of *Y. pestis* O19 harboring different *E. coli* F expression genes.

Strain	Plasmid(s)	Ca^{2+}	MS2
Y. pestis O19	72kb	+	-
Y. pestis O19CI.2	none	-	-
Y. pestis O19CI.3	F'Cm	-	-
Y. pestis O19.4	72kb, pKZ23	+	-
Y. pestis O19CI.4	F'Cm, pKZ23	-	-
Y. pestis O19.5	72kb, pRA310	+	-
Y. pestis O19CI.5	F'Cm, pRA310	-	-
Y. pestis O19.6	72kb, pMW2	+	-
Y. pestis O19CI.6	F'Cm, pMW2	-	-
Y. pestis O19CI.7	F'133::Tn*10*	-	+
Y. pestis O19CI.8	F'105::Tn*10*	-	+

\+ Calcium dependent growth, MS2 sensitivity
\- Calcium independent growth, MS2 resistant

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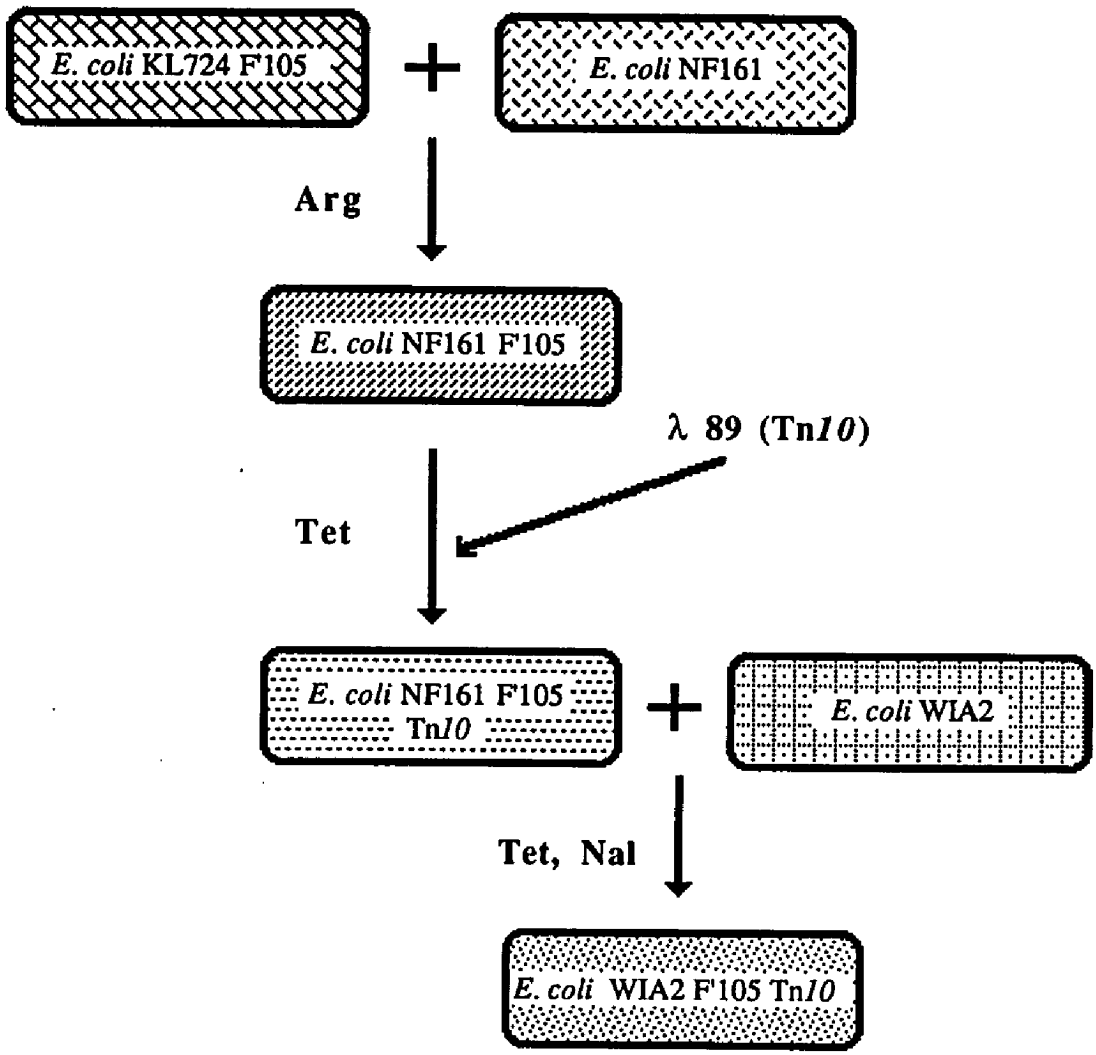

FIG. 4. Construction of F'133::Tn*10* and F'105::Tn*10*.

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F'105::Tn*10*, the frequencies of conjugation could not be tested, and since the transposon could have inserted in any of the non-essential F genes, it was not known if either of the two genes of interest were interrupted. Furthermore, due to the incompatibility of the *Yersinia* virulence plasmid and any F plasmids, it was not possible to test the effects of the *rfa*H and *cpx*A genes on calcium dependency using this system.

Expression of Cloned *rfa*H, *cpx*A, and *arc*A Genes in *Y. pestis* O19 and *Y. pestis* O19CI.3

Fertility and Virulence Plasmid Expression

To avoid working with the large, unstable F plasmids and to better determine which of the genes carried by the F plasmids was responsible for MS2 sensitivity of *Y. pestis* strains O19CI.7 and O19CI.8, clones of the genes of interest were obtained. Plasmids pKZ17 (*rfa*H), pKZ23(Tn*5* outside *rfa*H gene), pRA310 (*cpx*A) and pMW2(*arc*A) were obtained from the sources indicated in Table 2. After several unsuccessful attempts to transfer the cloned genes by P1 transduction, each of these plasmids was transferred by electroporation to *Y. pestis* O19 and *Y. pestis* O19CI.3. Tables 4 and 5 show the conjugation frequencies and MS2 sensitivities obtained with each of the electroporated strains. In addition, Table 5 shows the effects of the *E. coli* genes on expression of the 72kb virulence plasmid. The presence of any of the three genes *cpx*A, *rfa*H, or *arc*A appeared to increase the frequency of conjugation to some extent as shown in Table 4. Plasmid pRA310 (*cpx*A)

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was found to increase the rate of F transfer by a factor of about 250 which was still at least 100 times lower than that observed with *Y. enterocolitica* and *Y. pseudotuberculosis* (Table 4). None of these genes alone were found to confer MS2 sensitivity to *Y. pestis* (Table 5) as seen when plasmids F'133::Tn*10* and F'105::Tn*10* were tested. Table 5 also shows that all of the *Y. pestis* strains harboring the virulence plasmid remained calcium dependent in the presence of the *E. coli* genes while those strains previously lacking the 72kb plasmid remained calcium independent.

Sensitivity to Bacteriophages C21 and U3

The expression in *Y. pestis* of other characteristics associated with the cloned *E. coli* genes is shown in Table 6. *Y. pestis* strain O19 harboring only the 72kb virulence plasmid was found to be sensitive to the bacteriophage C21 and resistant to phage U3. Transfer of the *E. coli rfa*H gene (which is involved in C21 and U3 receptor synthesis) into this strain did not reverse the phage sensitivities (Table 6) as would be expected in an *rfa*H wild type organism. The presence of both F'133::Tn*10* and F'105::Tn*10* in *Y. pestis* resulted in sensitivity to phage U3, but only F'133::Tn*10* was able to confer resistance to phage C21. This was unexpected since the receptor for U3 is an external galactose residue which blocks the more internal C21 receptor (Rehemtulla *et al.*, 1986; Sanderson and Stocker, 1981). Sensitivity to these phages was not determined with *Y. pestis* containing pMW2 since the *arc*A gene is not known to influence expression of the LPS sugars which act

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TABLE 6. Expression of characteristics associated with *E. coli* F regulatory genes in *Y. pestis* O19.

Strain	Plasmid(s)/ Genotype	C21	U3	TBO(37°C)	TBO(26°C)
Y. pestis O19	72kb	s	r	s	r
Y. pestis O19.4	72kb pKZ23	s	r	-	-
Y. pestis O19.6	72kb pMW2	-	-	s	r
Y. pestis O19CI.7	F'133::Tn*10*	r	s	-	-
Y. pestis O19CI.8	F'105::Tn*10*	s	s	-	-
E. coli TB1	none	r	s	r	r
E. coli SAB2796	*rfa*H	s	r	r	r
E. coli SP314	*arc*A	r	s	s	s

Sensitive (s), Resistant (r), Not determined (-)

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as receptors for U3 and C21.

Dye Sensitivity

Growth in the presence of 0.2% of the redox dye toluidine blue O (TBO) was used as a possible means to determine *arc*A expression. Table 6 shows that *Y. pestis* O19 was found to be sensitive to this concentration of TBO at 37°C as has been shown for *arc*A mutants of *E. coli* (Iuchi and Lin, 1988); however, at 26°C *Y. pestis* was found to be resistant to TBO (Table 6). A similar pattern was observed when *Y. pestis* was grown in the presence of 0.02% methyl green, 0.02% metronidozole, and 0.02 % rose bengal which do not all have the same mode of action.

Hybridization of *Y. pestis* DNA with plasmids pRA310 and pKZ17

Since the *rfa*H genes from *E. coli* and from *Salmonella typhimurium* are able to complement each other in *rfa*H mutants of both species, it seemed possible that the closely related *Yersinia* chromosome might also have a similar gene. In order to determine if the *Yersinia* chromosome carried copies (possibly non-functional) of genes homologous to the *E. coli cpx*A and *rfa*H genes, whole cell DNA from *Y. pestis* strains O19CI.2, 51F and K25 (pesticin negative) along with DNA from *Y. pseudotuberculosis* WT and *E. coli* 12435 was digested with *Eco*RI, separated on a 0.8% agarose gel, transferred by capillary action to nitrocellulose and hybridized with ^{32}P-labelled plasmid DNA probes. Figure 5 shows the results of hybridization with plasmid probes pRA310 and pKZ17. In addition to the expected

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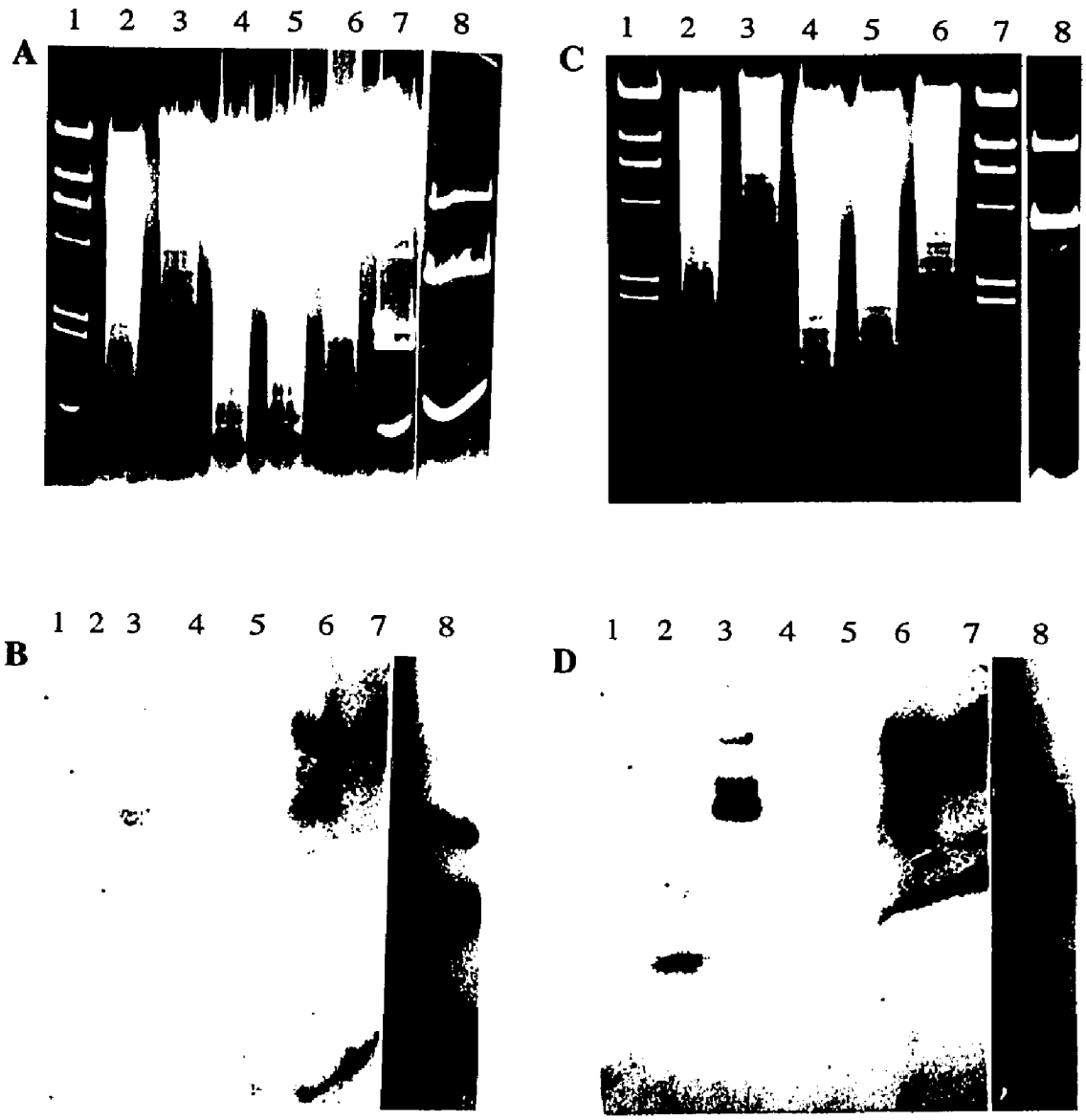

FIG.5. Photographs of agaraose gels and corresponding Southern hybridizations of *Yersinia* and *E. coli* genomic DNA *Eco*R1 digests probed with pRA310 (*cpx*A) and pKZ17 (*rfa*H) plasmid DNA. (A -D) Lanes 1 and 7, λ *Hin*dIII digests (sizes in kilobases: 23.7, 9.4, 6.7, 4.3, 2.3, 1.9); lane 2, *Y. pseudotuberculosis* WT ; lane 3, *Y. pestis* 51F; lane 4, *Y. pestis* O19.Cl2; lane 5, *Y. pestis* K25 pst-; lane 6, *E. coli* 12435; lane 8, (A and B) pRA310, (C and D) pKZ17. (B) Hybridization is to *Y. pestis* 51F, *E. coli* 12435 and the control plasmid DNA. (D) Hybridization is as above including the top band of the λ digest.

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hybridization of the plasmids to themselves, homology was detected between both probes and the DNA obtained from *E. coli* (lane 6 B and D) and from *Y. pestis* 51F (lane 3 B and D). Subsequent hybridization experiments (data not shown) confirmed that the hybridization of pRA310 and pKZ17 to *Y. pestis* 51F was due to homology between the common cloning vector pBR322 (from which pRA310 and pKZ17 are derived) and the 9kb pesticin plasmid found in *Y. pestis* strain 51F. No hybridization was due to homology between the *E.. coli cpx*A and *rfa*H genes and the *Yersinia* chromosme.

Anaerobiosis and Virulence Plasmid Expression

Anaerobic Growth on Magnesium Oxalate Medium

The *arc*A gene carried on plasmid pMW2 has been shown to repress aerobic metabolic pathways under anaerobic growth conditions (Iuchi and Lin, 1988). For this reason the low calcium response of *Y. pestis* harboring both the 72kb virulence plasmid and the *arc*A containing pMW2 plasmid was tested under anaerobic conditions represented by 85% N_2, 10% H_2, and 5% CO_2. As a control *Y. pestis* without pMW2 was tested anaerobically as well. Suprisingly, both *Y. pestis* with and without the *arc*A containing plasmid were found to be calcium independent when grown in the anaerobic chamber. Apparently the overproduction of ArcA product did not have an effect on the virulence plasmid but anaerobic growth did influence plasmid expression.

Figure 6 shows the percentage of calcium independent cells that arose

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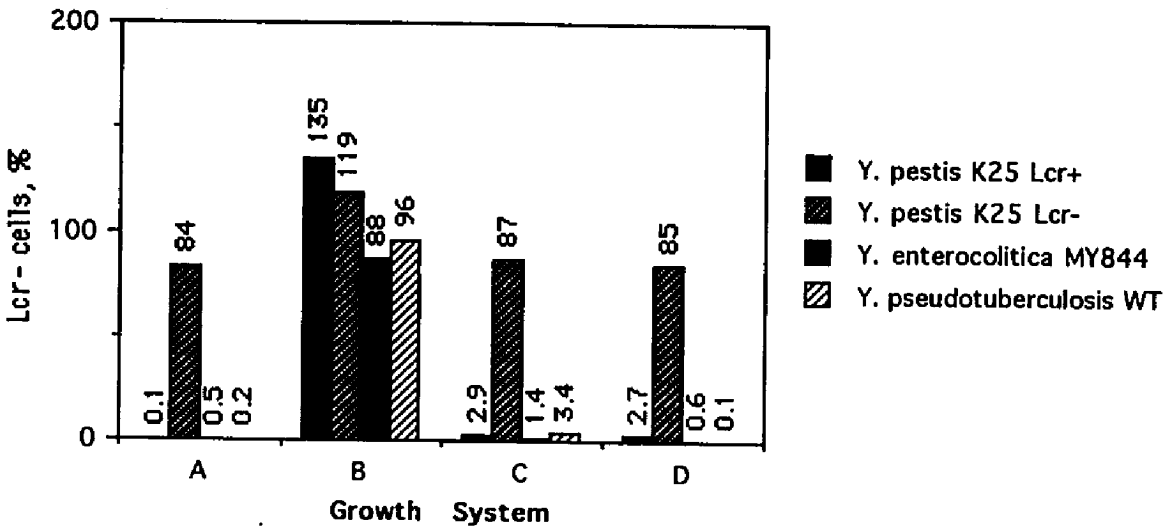

FIG. 6. Expression of the Lcr trait under different atmospheric conditions. Atmospheric Condition: A=aerobic, B=anaerobic (85% N_2, 10% H_2, 5% CO_2), C=microaerobic, and D=5% CO_2 incubator. Each bar represents the average value obtained from three trials.

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when *Y. pestis* K25 (with and without the 72kb plasmid), *Y. enterocolitica* MY844, and *Y. pseudotuberculosis* WT were plated on magnesium oxalate (MgOx) medium at 37°C under different atmospheric conditions. As expected *Y. pestis* without the virulence plasmid was found to be calcium independent under every condition tested. The other three strains were found to be calcium dependent when grown aerobically, microaerobically, or in a 5% CO_2 incubator. However, when grown in an anaerobic chamber containing 85% N_2, 10% H_2, and 5% CO_2, these strains were all found to be calcium independent. Similar results were seen when cultures were incubated under anaerobic conditions consisting of 85% Ar, 10% H_2, and 5% CO_2 (data not shown). Figure 6 also shows that an elevated level of CO_2 or a microaerobic atmosphere allowed more cells to grow on MgOx medium than were able to grow aerobically; however, these conditions did not allow 100% growth as seen with the anaerobic mix. This was most apparent for *Y. pestis* which exhibited an almost 30 fold increase in cell number under these conditions when compared to growth aerobically at 37°C.

Determination of Plasmid Alteration

Since it was possible that anaerobic growth was able to cause a higher frequency of plasmid mutation resulting in more calcium independent colonies, the plasmid contents of several aerobic and anaerobic calcium independent isolates were analyzed. Figure 7 shows the plasmid profiles of 9

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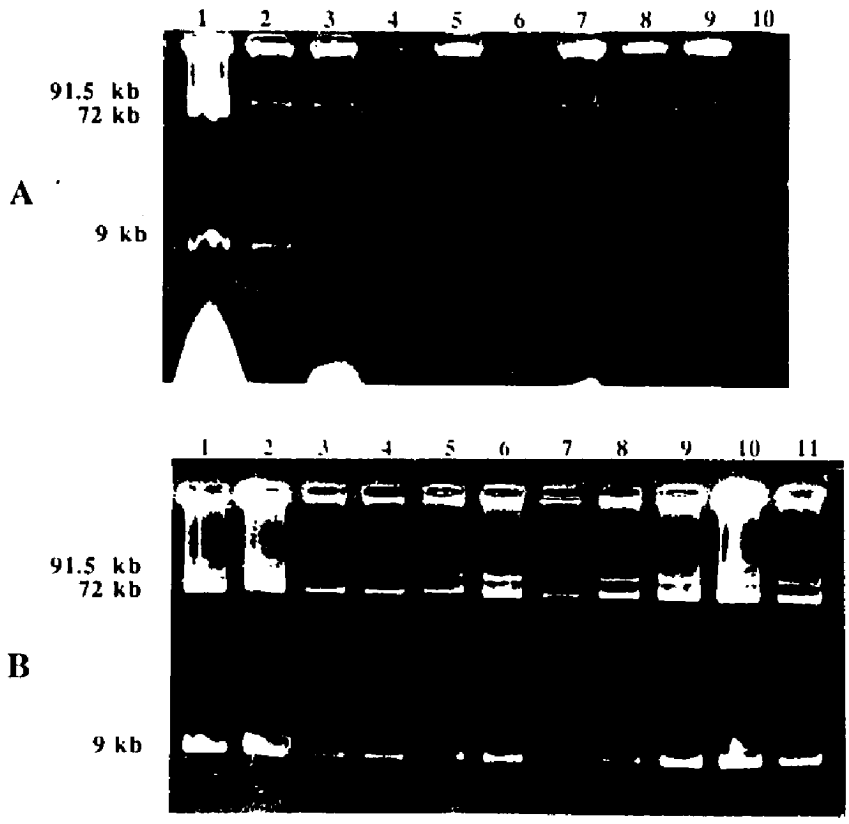

FIG. 7. Plasmid profiles of *Y. pestis* K25 isolates grown at 37°C on magnesium oxalate plates under aerobic or anaerobic (85% N_2, 10% H_2, 5% CO_2) conditions. Lanes (A) 2-10, aerobically grown cells; lanes (B) 2-11, anaerobically grown cells; lanes (A) 1 and (B) 1, *Y. pestis* K25 control grown at 26°C.

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aerobic and 10 anaerobic isolates. These results confirmed the data reported by Portnoy and Falkow (1981) that colonies arising aerobically under restrictive conditions undergo a loss or alteration of the virulence plasmid which confers an Lcr- phenotype. Anaerobically no changes were seen in the 72kb plasmid. Plasmid profiles from cells grown under elevated CO_2 or microaerophilic conditions showed a pattern of plasmid alteration similar to that of the aerobically grown cells (data not shown).

Each of the isolates used for plasmid analysis was further tested for plasmid alteration by repeating the test for growth restriction aerobically. Figure 8 illustrates that cells arising on MgOx medium under all conditions except anaerobiosis (85% N_2, 10% H_2, and 5% CO_2) became permanently Lcr- while those arising under anaerobic conditions retained the ability to express the Lcr trait aerobically. These results confirmed that the virulence plasmid was not altered in the anaerobically grown cells.

ß-galactosidase Determination

To determine if the virulence plasmid was being expressed in the absence of growth restriction, *Y. pestis* constructs containing Mu d1 gene fusions were analyzed for ß-galactosidase activity aerobically and anaerobically. Figure 9 shows the level of enzyme expressed from the *Y. pestis yop*E and *yop*J genes in cultures grown aerobically at 26°C and 37°C and anaerobically at 37°C. Only the cultures grown aerobically at 37°C were

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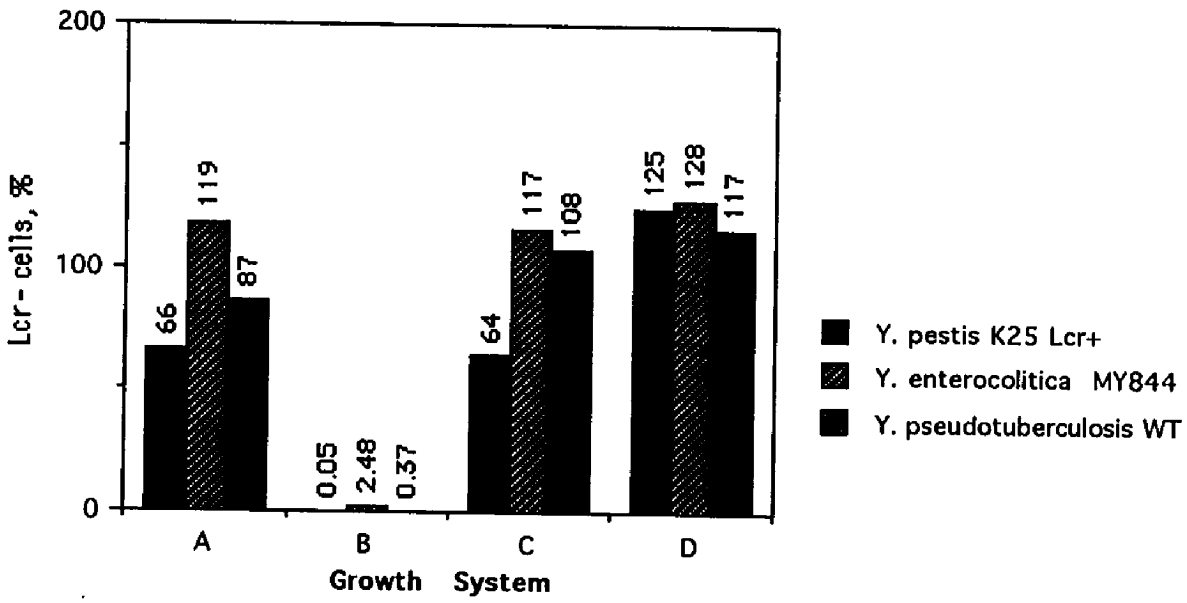

FIG. 8. Expression of the Lcr trait after isolation from MgOx plates incubated under different atmospheric conditions. Atmospheric Conditions are defined as in Figure 1. Each bar represents the average value obtained from three trials.

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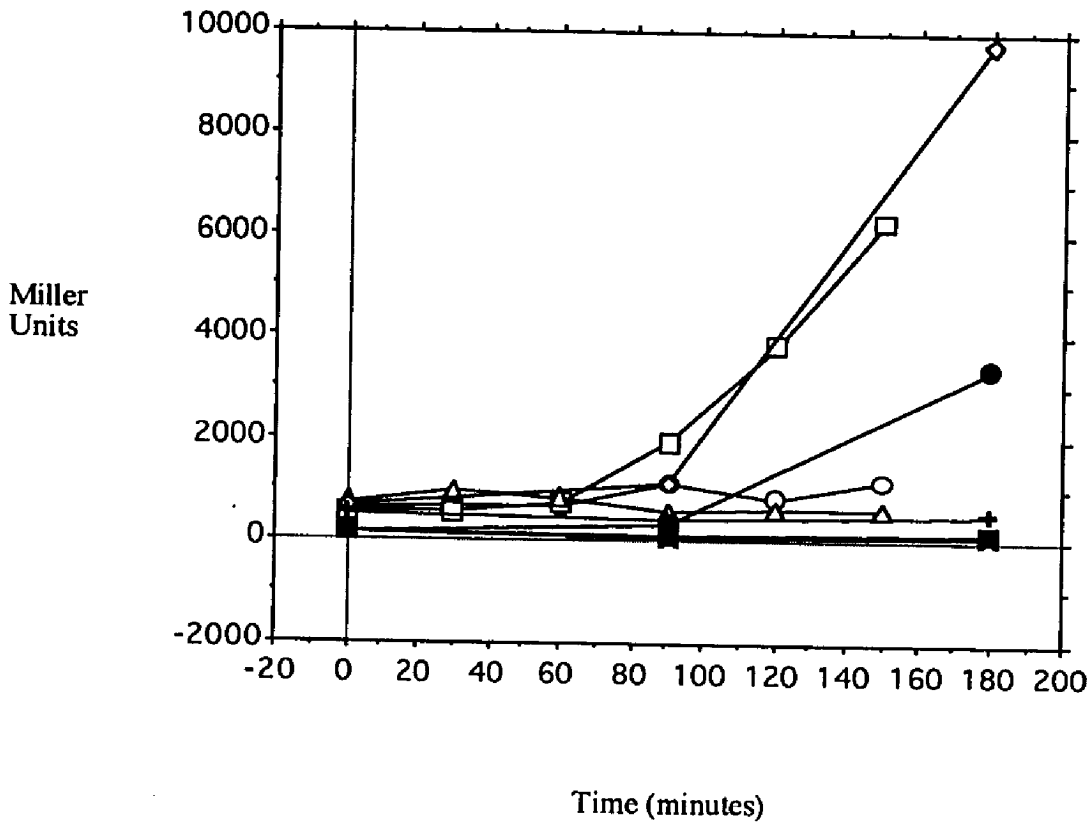

FIG. 9. β-galactosidase activity of *Y. pestis* strain KIM and its isogenic Mu d1 insertion mutants. Activity is given in Miller Units as described in the text. O =*Y. pestis* KIM, 37°C, aerobic; ■ =*Y. pestis* yopE, 26°C, aerobic; □ =*Y. pestis* yopE, 37°C, aerobic, trial 1; ◊ =*Y. pestis* yopE, 37°C, aerobic, trial 2; Δ =*Y. pestis* yopE, 37°C, anaerobic; ✚ =*Y. pestis* yopJ, 26°C, aerobic; ✖ =*Y. pestis* yopJ, 37°C, anaerobic; ● =*Y. pestis* yopJ, 37°C, aerobic.

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found to induce the two plasmid encoded genes in a manner similar to that reported by Straley and Bowmer (1986). The anaerobically grown cultures were found to maintain the same levels of ß-galactosidase detected in the uninduced cultures grown aerobically at 26°C.

Oxygen, Nitrate Reductase, and Formate Hydrogen Lyase

In order to determine if the absence of oxygen was the major factor in Lcr repression, *Y. pestis* was grown on MgOx medium under varying anaerobic conditions. Table 7 shows that anaerobic conditions consisting of 100% N_2 or a mix of 90% N_2 and 10% H_2 or 95% N_2 and 5% CO_2 were not able to relieve growth restriction in the manner observed with the N_2, H_2, and CO_2 gas mix (Figure 6). Although the condition representative of 20% O_2, 10% H_2, 5% CO_2 and 65% N_2 could not be tested due to the explosive nature of O_2, the environment of the microaerobic chamber (consisting of the same 4 gases but in different concentrations) was not found to relieve restriction (Figure 6).

In order to determine the effects of anaerobiosis on growth of Lcr+ and Lcr- cells under typically permissive conditions, *Y. pestis* K25 cultures were grown aerobically or anaerobically in HIB at 37°C. Figure 10 shows that over a 4 h period the Lcr- cells grew at a faster rate than the Lcr+ cells under aerobic conditions while both strains grew at approximately the same rate under anaerobic conditions. In a separate study (Figure 11), the same pattern

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TABLE 7. Expression of the Lcr trait under different atmospheric growth conditions.[a]

Organism	Growth Condition		
	N_2	N_2+H_2	N_2+CO_2
Y. pestis K25	0.2± 0.15[b]	0.1± 0.06	0.14± 0.02
Y. pestis K25 (Lcr-)	83± 4	84± 8	-

[a] Lcr trait was determined on MgOx medium at 37°C.
[b] Values are percentages of Lcr- cells determined by using growth at 26°C as the 100% baseline. Each value is the average of three trials.

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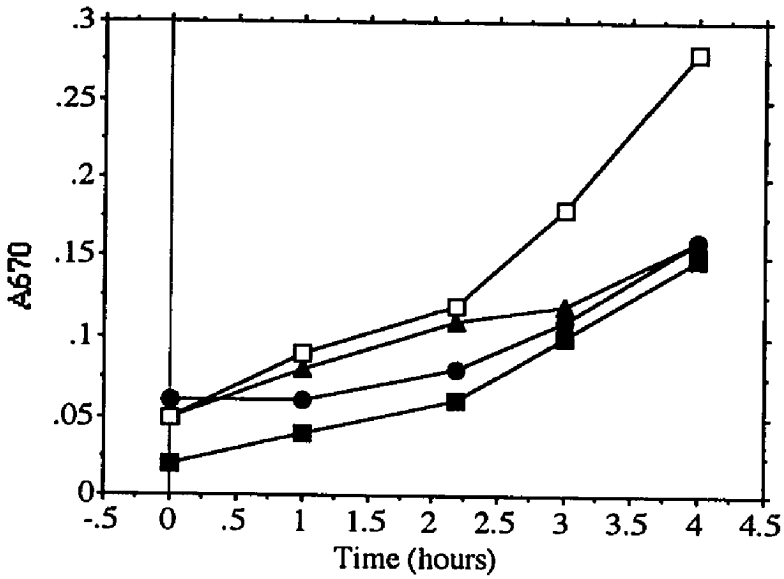

FIG. 10. *Y. pestis* K25 Lcr+ and Lcr- strains grown at 37°C in HIB under aerobic or anaerobic conditions. □=Lcr-, aerobic; ▲=Lcr-, anaerobic; ■=Lcr+, aerobic; ●=Lcr+, anaerobic.

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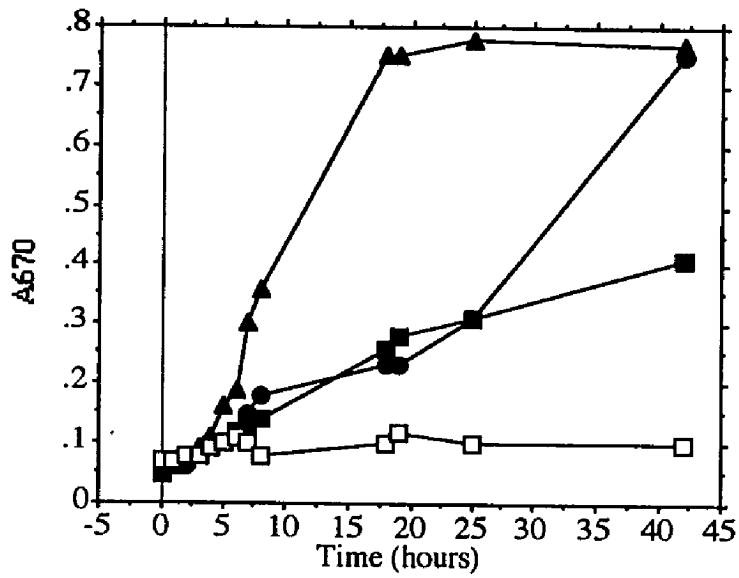

FIG. 11. *Y. pestis* K25 Lcr+ and Lcr- strains grown at 37°C in HIB aerobically or anaerobically. ▲=Lcr-, aerobic; □=Lcr-,anaerobic; ●=Lcr+, aerobic; ■=Lcr+, anaerobic.

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was seen during the first 4 h of incubation; however, as the incubation time increased, the anaerobic Lcr- cells reached a plateau at approximately 7 h while the Lcr+ cells continued to increase over the duration of the experiment. Aerobically, the Lcr+ cells were found to grow at a faster rate after approximately 20 h. This is consistent with the finding that Lcr+ populations are overtaken by faster growing Lcr- cells over time(Fukui *et al.*, 1957).

When Lcr+ and Lcr- cells were grown on either HIB (Figure 12) or BHI (Figure 13) at 37°C anaerobically (85% N_2, 10% H_2, 5% CO_2) or with the head space replaced with H_2 (2 parts) and CO_2 (1 part), the Lcr- cells were again seen to level off between 2 and 7 h while the Lcr+ cells continued to increase until at least 12 h independent of growth medium. Oddly, the increased concentration of H_2 and CO_2 appeared to inhibit both Lcr+ and Lcr- cells grown in HIB but not BHI.

Since the 72kb plasmid appeared to be repressed anaerobically at 37°C as it is aerobically at 26°C, it seemed possible that the same mechanism of repression might be functioning in both cases. Potassium chlorate (0.4%) was used to determine if *Y. pestis* had a functional nitrate reductase and if it was expressed aerobically as well as anaerobically. The enzyme nitrate reductase is known to reduce chlorate to the toxic compound chlorite which prevents growth (Stewart, 1988). When *Y. pestis* was grown in the presence of

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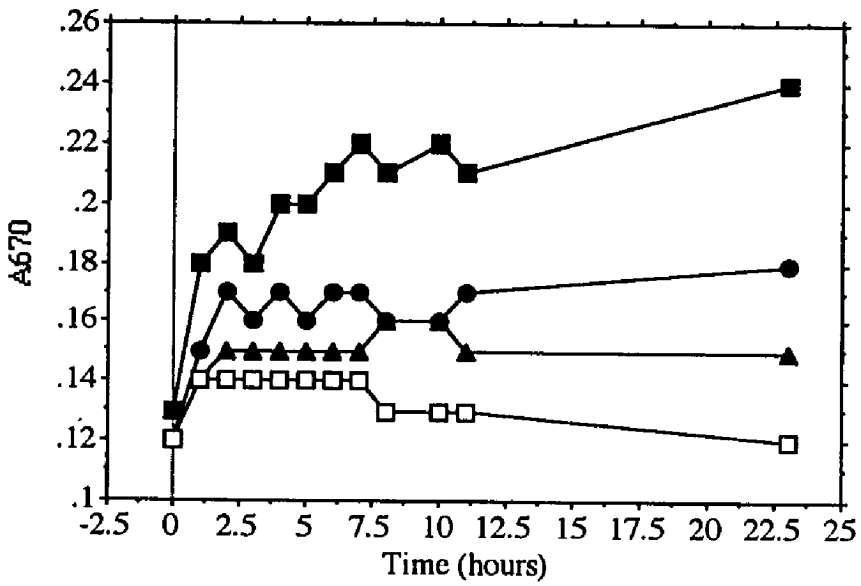

FIG. 12. *Y. pestis* K25 Lcr+ and Lcr- strains grown at 37°C in HIB with shaking under varying anaerobic environments. ■=Lcr+, $N_2 + H_2 + CO_2$; ●=Lcr+, $H_2 + CO_2$; □=Lcr-, $N_2 + H_2 + CO_2$; ▲=Lcr-, $H_2 + CO_2$.

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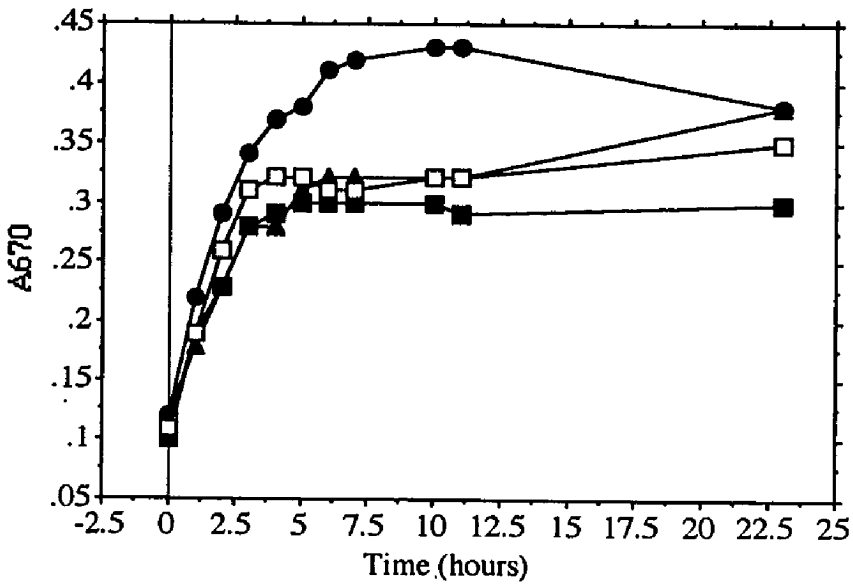

FIG. 13. *Y. pestis* K25 Lcr+ and Lcr- strains grown in BHI at 37°C with shaking under varying anaerobic environments. ●=Lcr+, $H_2 + CO_2$; ■=Lcr+, N_2 + H2 + CO_2; ▲=Lcr-, $H_2 + CO_2$; □=Lcr-, $N_2 + H_2 + CO_2$.

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potassium chlorate it was found that anaerobically the cells were inhibited while aerobically the cells grew normally at 37°C but were inhibited at 26°C. After 3 to 4 d the expected number of colonies was visible at 26°C aerobically while only an occassional mutant colony was ever isolated anaerobically. Results were the same when done in broth cultures as well.

In addition to nitrate reductase, formate hydrogen lyase is an enzyme produced anaerobically that has the potential to be involved in plasmid repression. It has been shown that the presence of 1% nitrate will repress the production of formate hydrogen lyase (Stewart, 1988). When *Y. pestis* was grown anaerobically on MgOx medium in the presence of 1% potassium nitrate, it was found that the Lcr trait was still repressed indicating that this enzyme was not required for relief of plasmid restriction.

Another possible explanation for the mechanism of Lcr repression by CO_2 and H_2 is that these gases allow the cells to undergo acetogenesis to produce acetate or that bicarbonate is formed which has previously been shown to maintain virulent populations of cells (Delwiche *et al.*, 1959). Figure 14 shows the results of growing *Y. pestis* Lcr+ and Lcr- cultures in BHI supplemented with 10 mM sodium acetate or sodium bicarbonate at 37°C anaerobically (85% N_2, 10% H_2, 5% CO_2). Compared with unsupplemented BHI cultures, the sodium bicarbonate cultures appeared to be stimulated while the sodium acetate flasks were inhibited.

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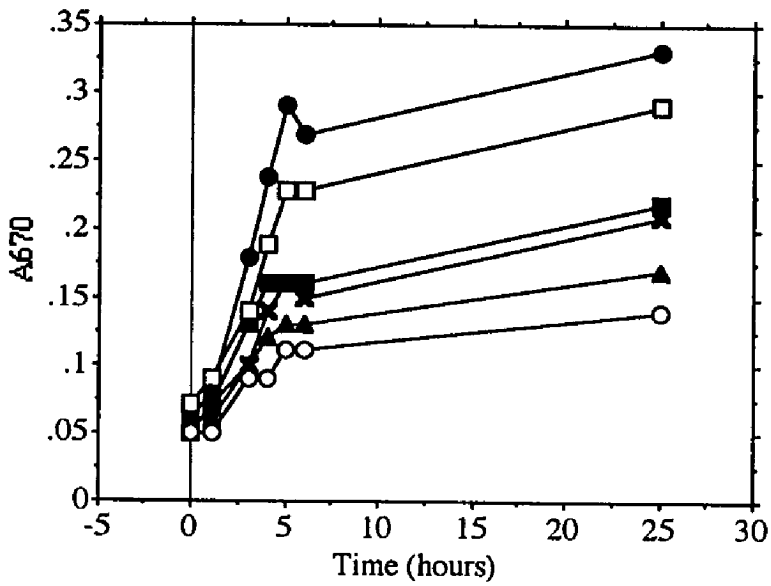

FIG. 14. *Y. pestis* K25 Lcr+ and Lcr- strains grown anaerobically at 37°C in BHI containing 10 mM sodium acetate or 10 mM sodium bicarbonate. ●=Lcr-, bicarbonate; ▲=Lcr-, acetate; ■=Lcr-, unsupplemented; □=Lcr+, bicarbonate; O=Lcr+, acetate; ✖=Lcr+, unsupplemented.

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Since the presence of bicarbonate was found to enhance the growth of *Y. pestis*, MgOx medium was supplemented with 10 mM sodium bicarbonate to determine its effect on the Lcr of *Y. pestis* K25. Table 8 shows that this addition was able to relieve growth restriction. To detemine whether this effect was due to the presence of bicarbonate or the resulting pH (8.0), the same supplemented medium and MgOx medium with the pH adjusted to 8.0 were used to determine the Lcr of *Y. pestis* EV76 51F. In all cases the bicarbonate supplemented medium was able to relieve restriction while the pH adjusted medium was unable to do so or allowed only pinpoint colonies in comparison with the colonies on the bicarbonate plates suggesting that the presence of bicarbonate was more significant in repressing the Lcr than the change in pH.

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TABLE 8. Alleviation of the Lcr of *Y. pestis* by the addition of 10mM $NaHCO_3$ to magnesium-oxalate medium.

Strain	Temperature(°C)	Colony Forming Units/ml on MgOx	Colony Forming Units/ml on MgOx + $NaHCO_3$
K25 Lcr+	26	-	1.0 (±.08) x 10^9
	37	3.3 (±1.4) x 10^5	1.2 (±.01) x 10^9
K25 Lcr-	26	-	1.7 (±.03) x 10^9
	37	1.5 (±0.34) x 10^9	1.4 (±.11) x 10^9

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IV. DISCUSSION

The human pathogens of the genus *Yersinia* all harbor a 60-75 kb plasmid which encodes several virulence related proteins and their regulatory elements (Portnoy and Falkow, 1981). Expression of the majority of plasmid encoded genes appears to be activated by elevated temperature and reduced calcium concentration (Brubaker, 1986; Straley and Brubaker, 1981). In recent years, several laboratories have used the relatively standard conditions of growth at 37°C in the presence of high magnesium and low calcium to determine the regulatory patterns of numerous plasmid encoded genes (Rosqvist *et al.*, 1990; Skurnik, 1985; Straley and Bowmer, 1986). Under these conditions, plasmid-bearing *Yersinia* cultures become restricted for growth after approximately 2 h (Zahorchak *et al.*, 1979). This Low Calcium Response trait or Lcr has not been shown to occur under these conditions *in vivo* although plasmid encoded proteins have been readily detected (Pollack *et al.*, 1986). Although numerous studies have been done to characterize the genes of the virulence plasmid and their possible functions, relatively little has been done towards understanding how the plasmid is regulated. In this study the influence of chromosomal genes on plasmid expression was explored.

E. coli F Expression Genes in *Yersinia*

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The *E. coli* associated F plasmid and the *Yersinia* virulence plasmid are similar in that they are both large, low copy number and able to restrict growth of their hosts under specific conditions (Tam and Kline, 1989; Zahorchak *et al.*, 1979). In addition, these plasmids belong to the same incompatibility group due to homology between their *inc*D regions (Bakour *et al.*, 1983). Because of these similarities and the well characterized nature of F expression, F was studied as a possible model of virulence plasmid regulation in *Yersinia pestis*. Chromosomal genes are known to be required for F plasmid expression in *E. coli* (Beutin and Achtman, 1979; Beutin *et al.*, 1981), and it has been hypothesized that chromosome encoded genes in *Yersinia* are necessary for virulence plasmid expression (Cornelis *et al.*, 1987 and 1991; Emody *et al.*, 1989). Differences in expression of both F and the virulence plasmid in three species of *Yersinia* (Lawton and Stull, 1972; McMahon, 1971 and 1973; Wolf-watz *et al.*, 1985) suggested that there might be some common regulatory gene involved in expression of both plasmids in the *Yersinia*. Early studies have shown that F can be transferred to and from *Y. enterocolitica* and *Y. pseudotuberculosis* with relative ease while tranfer from *Y. pestis* is virtually non-existent (Lawton and Stull, 1972; McMahon, 1971 and 1973). Furthermore, the sex pili by which F transfers itself from cell to cell are not detectable on *Y. pestis* by the traditional phage (MS2) sensitivity method.

Plasmids F'133 and F'105 which carry regions of the *E. coli*

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chromosome containing genes known to be involved in F plasmid expression were mutagenized with the transposon Tn*10* to ensure a selectable marker for transfer to *Y. pestis*. Genes *cpx*A and *rfa*H are reported to be carried on these plasmids (Bachman, 1990; Low, 1972). The *cpx*A gene product has been shown to be a membrane protein involved in the pathway for isoleucine and valine synthesis and in F plasmid expression (McEwen and Silverman, 1980a, b, and c; Silverman *et al.*, 1980) while *rfa*H has been found to be an antiterminator of rho-dependent transcriptional termination for the genes involved in sugar transport for LPS synthesis and transfer in F expression.

Both F'133::Tn*10* and F'105::Tn*10* were conjugated to the plasmidless *Y.pestis* strain O19CI2. This strain was generated by curing *Y. pestis* O19 of its virulence plasmid thus enabling it to harbor the normally incompatible F plasmids. It was found that both F plasmids were able to confer MS2 sensitivity to *Y. pestis* indicating the presence of F pili. These plasmids were also observed to change the sensitivity of *Y. pestis* to the LPS specific phages C21 (from sensitive to resistant) and U3 (from resistant to sensitive) indicating that the *E. coli rfa*H gene was functioning to alter the sugar residues of the LPS. No direct test was available for detecting expression of the *cpx*A gene.

While preliminary analysis indicated that these *E. coli* genes were able to influence F plasmid expression in *Yersinia*, it can not be ruled out that some other genes carried on the plasmids were responsible for the observed effects. In addition, the location of Tn*10* was not determined; therefore, it is

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possible that a mutation within the normal F transfer genes resulted in an over-expression of F pili able to be detected with MS2 and having nothing to do with the presence of cpxA or *rfa*H.

Since the *rfa*H gene has been shown to be required for efficient F expression in *E. coli*, it was expected that a similar gene should be present in the *Yersinia* although possibly not functional in *Y. pestis* since it does not efficiently express F. Such a gene seemed a likely candidate to encode a virulence plasmid regulatory protein as well. In order to determine if there was DNA homology between the *E. coli* F regulatory genes and the *Yersinia* chromosome, whole cell DNA from *Y.enterocolitica, Y. pseudotuberculosis, Y. pestis*, and *E. coli* was isolated, restricted with *Eco*RI, separated by agarose gel electrophoresis, blotted to nitrocellulose, and hybridized with ^{32}P-labelled pKZ17 (*rfa*H) or pRA310 (*cpx*A) plasmid DNA. Autoradiographs indicated that there was some homology between these plasmids and the *Y. pestis* DNA; however, it was later shown that this homology was due to sequences from the pBR322 derived cloning vectors and the 9kb plasmid associated with *Y. pestis*. Although no DNA sequence homology was detected between the *E. coli cpx*A and *rfa*H genes and the *Yersinia* chromosome, it is still likely that homologous proteins exist between these two species since F can be expressed in both.

Since no genes were identified on the *Yersinia* chromosome that might be involved in regulation of the 72kb virulence plasmid, clones of *E. coli*

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genes were transferred to *Y. pestis* to observe their effects on virulence expression. Unlike the F'133 and F'105 plasmids, the cloned genes could be stably maintained in plasmid bearing strains of *Y. pestis*. The three genes tested were *cpx*A (pRA310), *rfa*H (pKZ23) and *arc*A (pMW2)which have all been shown to be critical to F expression in *E. coli* (Beutin and Achtman, 1979; Beutin *et al.*, 1981; Gaffney *et al.*, 1983; Silverman, 1985). Each of the cloned genes was transferred to *Y. pestis* by electroporation after several unsuccessful attempts at transduction. Electroporation by the method described for *E. coli* (Bio-Rad Laboratories, Inc., Richmand, CA) was found to be a very efficient and reproduceable method of gene transfer in the *Yersinia* and was subsequently used for most genetic manipulations in this species.

Analysis of the three *E. coli* genes in *Y. pestis* revealed that each, individually, was able to increase the frequency of F transfer out of *Y. pestis* although none was able to confer MS2 sensitivity to F containing strains. Each plasmid was also observed for a possible influence on expression of the virulence plasmid as determined by the ability to grow on the typically restrictive magnesium-oxalate (MgOx) medium at 37°C. A fully expressed virulence plasmid would be expected to result in cessation of cell growth under these conditions. None of the *Y. pestis* strains harboring the *E. coli* genes along with the virulence plasmid were found to exhibit altered growth patterns on MgOx medium. Since it was possible that the genes were not

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being expressed in *Y. pestis*, characteristics specific to each gene were tested for in the *Yersinia* strains. The trait normally associated with the *cpx*A gene was not tested for in *Y. pestis* since there was no direct method. In *E. coli* the *cpx*A gene product is required for growth at 41°C in the absence of isoleucine and valine (McEwen and Silverman, 1980c).

As previously mentioned the *rfa*H product is necessary for synthesis of LPS containing galactose as a terminal sugar residue (Rehemtulla *et al.*, 1986; Sanderson and Stocker, 1981). The galactose can be detected by sensitivity to the phage U3 which takes advantage of this sugar as a receptor (Watson and Paigen, 1971; Rehemtulla *et al.*, 1986). *E. coli* cells unable to incorporate galactose into their LPS are resistant to U3 and sensitive to C21 which attaches to the glucose residue normally masked by the presence of galactose (Rapin *et al.*, 1968). When F'133 and F'105 were transferred to *Y. pestis* these phage patterns were altered, but when the cloned *rfa*H gene was transferred to the same strain it was not found to effect the phage sensitivities. This suggests that some other gene or genes carried on plasmids F'133 and F'105 acted in concert to alter the sensitivity of *Y. pestis* to these phages.

In addition to its function as a transcriptional activator for F, the *arc*A gene product has also been shown to act as a repressor of aerobic respiratory pathways in *E. coli* grown anaerobically (Iuchi and Lin, 1988). An indicator of this second function is the ability of *E. coli* to grow in the presence of the redox dye toluidine blue (Buxton and Drury, 1983; Iuchi and Lin, 1988)

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which explains the original designation of this locus as *dye*. When *Y. pestis* with and without the *E. coli arc*A gene was grown in the presence of toluidine blue (TBO), no difference was seen in growth pattern. However, an unexpected result was observed with both strains when growth at 26°C was compared with growth at 37°C. While both strains were unable to grow in the presence of TBO at 37°C (characteristic of *E. coli arc*A mutants), both were able to grow on the same medium at 26°C (indicative of *arc*A wildtype). Since ArcA in *E. coli* is known to be regulated by its phosphorylation state, it was assumed that the phosphorylation state of an ArcA homolog in *Y. pestis* varied with temperature. Analysis of growth on several other dye containing media including methyl green, rose bengal, violet red bile, eosin methylene blue and Salmonella-Shigella agar indicated that growth at 26°C was most likely due to dye exclusion caused by changes in LPS composition. Temperature sensitive synthesis of LPS has been reported in *Y. pestis* (Osasawara *et al.*, 1985), and it seems logical that the LPS would be more resistant to penetration at 26°C when the bacterium would most likely encounter harsher conditions (the flea gut).

Virulence Plasmid Expression in Anaerobically Grown *Y. pestis*

In *E. coli* it is possible for ArcA to be phosphorylated to its active form for F regulation without being in the active form for aerobic respiratory control (Iuchi *et al.*, 1989b; Silverman *et al.*, 1991). If this were the case in

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Yersinia, ArcA might be activated at 26°C but not at 37°C under aerobic growth conditions by a mechanism similar to that associated with F. However, anaerobically one would expect ArcA to be regulated by a different signal as is the case in *E. coli*. For this reason *Y. pestis* strains with and without the pMW2 plasmid were grown anaerobically to test for F and virulence plasmid expression. No difference was seen in either strain as to MS2 sensitivity; both strains remained resistant. Suprisingly, an effect on virulence plasmid expression was noted. Both the control *Y. pestis* and the *arc*A containing strain were found to grow as well on MgOx medium anaerobically as they did on heart infusion agar aerobically.

This observation was exciting since many factors have been identified, including the divalent cations Zn^{2+} and Sr^{2+} (Higuchi *et al.*, 1959) and the nucleotides ATP and GTP (Zahorchak and Brubaker, 1982), that alleviate growth restriction *in vitro* in addition to repressing expression of other plasmid related activities. However, no condition has been identified that will allow growth *in vitro* without also inhibiting production of plasmid encoded proteins. It seemed possible that anaerobic growth might represent such a condition since anaerobiosis has recently been shown to effect virulence related characteristics in other pathogens (Clark *et al.*, 1987; Clark *et al.*, 1988; Schiemann and Shope, 1991). In addition, the virulence plasmid containing species of *Yersinia* are all facultative anaerobes suggesting that anaerobiosis is a condition likely to be encountered during infection.

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To confirm or disprove this theory, varying "anaerobic" environments were tested for the ability to relieve growth restriction without repressing virulence protein production. These studies were done with a *Y. pestis* K25 guanine mutant strain which most closely resembles the wild type organism (Straley and Brubaker, 1982). Microaerobicity and elevated CO_2 were not found to alleviate the plasmid-encoded Lcr *in vitro*. In addition, anaerobic repression of the Lcr was found to be reversible indicating that anaerobiosis does not represent a selective pressure on the cells to mutate at a higher frequency than they would under aerobic conditions. Unfortunately, activation of the specific virulence plasmid-encoded *Y. pestis* genes *yop*E and *yop*J was also found to be repressed by anaerobic growth supporting an early report by Lawton *et al.* (1963) that the plasmid-encoded V antigen is not produced during anaerobic growth.

Although growth under microaerobic or elevated CO_2 conditions was not found to alleviate the Lcr trait, these conditions were observed to increase the percentage of the population mutated to an Lcr- phenotype. Previous studies on the ability of CO_2 to maintain virulent populations were done using non-restrictive media and at a time prior to the discovery of the virulence plasmid (Delwiche *et al.*, 1959; Surgalla *et al.*, 1964). It has been suggested that the effect of CO_2 is to increase the growth rate of the virulent population to that of the avirulent population thus preventing the loss of virulent cells

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through over growth of avirulent cells (Delwiche *et al.*, 1959; Surgalla *et al.*, 1964). Presumably, the situation differs here in that the virulent, plasmid-bearing population is restricted for growth on MgOx medium no matter what growth rate it could achieve in a non-restrictive medium; therefore, these results are not directly comparable to those from earlier studies.

It seemed likely from these initial results that the absence of oxygen was the signal for plasmid repression, but this does not appear to be the case. Experiments using anaerobic gas combinations of 100% N_2, 90% N_2 and 10% H_2, or 95% N_2 and 5% CO_2 revealed that only the original mix of 85% N_2 (or 85% Ar), 10% H_2 and 5% CO_2 was able to alleviate the Lcr. These results conflict somewhat with those of Lawton *et al.* (1963) who reported that the V antigen is not produced under anaerobic conditions consisting of 95% N_2 and 5% CO_2. In the present study cells grown under these conditions were found to exhibit a normal Lcr indicating the presence of V since it has been shown to be required for growth restriction (Price *et al.*, 1991).

One explanation for the alleviation of plasmid-mediated growth restriction anaerobically is that bicarbonate, which was found to relieve the Lcr aerobically, is formed from H_2 and CO_2. The mechanism by which bicarbonate relieves growth restriction aerobically is still unknown. Another possible explanation is that the numerous membrane associated proteins

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required for anaerobic growth in the presence of H_2 and CO_2 may act as barriers to the signal transduction pathway postulated to act in plasmid regulation (Bergman *et al.*, 1991). Alternatively, the degree of supercoiling could be significantly altered resulting in plasmid repression. Supercoiling has been shown to be effected by anaerobiosis in other organisms (Dorman *et al.*, 1988; Malkhosyan *et al.*, 1991), and as a regulatory mechanism for plasmid expression it has been implicated in the response of *Y. enterocolitica* to temperature (Cornelis *et al.*, 1991). Considering that the four gas mixtures discussed above all represent conditions requiring anaerobic growth, it seems unlikely that plasmid supercoiling is the best explanation of these results. However, it cannot be ruled out that the degree of supercoiling varies with the anaerobic environment.

Summary

The results of this study have identified a new condition which permits growth of pathogenic *Yersinia* under conditions typically thought of as restrictive. Other methods of alleviating Lcr-mediated growth restriction involve incorporation into the growth medium of some component that can block the cell's ability to sense either calcium or magnesium concentration (Higuchi *et al.*, 1959; Zahorchak and Brubaker, 1982). While a similar phenomenon could be occurring during growth under the specific anaerobic conditions described here, the interference is likely to be due to some metabolic change within the cell rather than to some component in the

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medium.

These results may be physiologically significant in two respects. First, both *Y. enterocolitica* and *Y. pseudotuberculosis* invade the anaerobic intestine as part of their infectious cycle. It may be that during this stage anaerobiosis provides a signal to repress plasmid expression until the cells reach a location where plasmid-encoded proteins are required. Secondly, *Y. pestis* spends part of its life cycle in the likely anaerobic gut of the rat flea. Bacteria isolated from the flea gut have been shown to lack the capsular antigen as well as the specific plasmid-encoded proteins V and W (Swartz, 1973). These proteins are only produced after the bacteria have entered the host lymphatic system and been engulfed by macrophages where they replicate free from contact with PMNs. It is in the macrophage that the *Y. pestis* cells produce these plasmid-encoded proteins that provide protection from further phagocytosis. Perhaps *Y. pestis* has adapted to the anaerobic environment of the flea as a mechanism of repressing virulence plasmid expression under conditions where it would not only be wasteful but also possible harmful to its future existence in a mammalian host.

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