Study of conjugal gene transfer in Yersinia enterocolitica strain W277 harboring the F'lac plasmid of Escherichia coli

Richard L. Hodinka

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A STUDY OF CONJUGAL GENE TRANSFER IN YERSINIA ENTEROCOLITICA STRAIN W277 HARBORING THE F'LAC PLASMID OF ESCHERICHIA COLI

By
Richard L. Hodinka
B.S., Marietta College, 1976

Presented in partial fulfillment of the requirements for the degree of

Master of Science

UNIVERSITY OF MONTANA

1979

Approved by:

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Chairman, Board of Examiners
Co-Chairman, Board of Examiners
Dean, Graduate School

Date 8-1-79
A Study of Conjugal Gene Transfer in *Yersinia enterocolitica* Strain W277 Harboring the F'lac Plasmid of *Escherichia coli*. (111 pp.)

Directors: Mitsuru J. Nakamura, George L. Card

In as much as the pathogenic mechanism of *Yersinia enterocolitica* is unknown, conjugal gene transfer studies would provide a basis for elucidating the genetic mechanism(s) controlling human and animal virulence in this organism and establish more clearly the taxonomic relatedness of this organism to other enterobacteria. The purpose of this study was to develop a genetic system that would enable the successful transfer of the bacterial chromosome from donor to recipient strains of *Y. enterocolitica* employing F'lac plasmids.

Conjugal transfer of the F'lac plasmid from donor strains of *Escherichia coli* to recipient strains of *E. coli* and *Y. enterocolitica* and from donor to recipient strains of *Y. enterocolitica* was achieved by using broth and membrane filter mating systems. These systems were, in turn, used to determine if transconjugants (which harbored the F'lac plasmid) of *Y. enterocolitica* had the capability of transferring chromosome markers in crosses with a polyauxotrophic mutant of this organism. The auxotrophic mutants were isolated following successive cycles of mutagenesis with N-methyl-N'-nitro-N-Nitroso-guanidine for 10 minutes and enrichment with 100 μg/ml of nalidixic acid for 18 hours at 25°C.

The F'lac plasmid was transferred from four donor strains of *E. coli* to *Y. enterocolitica* strain W277 at a frequency of $10^{-6}$ compared to a much higher frequency (complete transfer to $10^{-2}$) exhibited for crosses involving donor and recipient strains of *E. coli*. The transferred genetic elements were unstable in the *Y. enterocolitica* strain, but repeated plating on MacConkey agar permitted the selection of relatively stable lactose positive strains of this organism. The transconjugants of *Y. enterocolitica* were able to transfer the F'lac plasmid to a recipient strain of *Y. enterocolitica* at a frequency of $10^{-5}$ in an 18 hour broth mating system and at a frequency of $10^{-4}$ using a membrane filter mating system. Treatment of the *Y. enterocolitica* transconjugants with acridine orange failed to eliminate the F'lac plasmid from the organisms. Two of the four F'lac plasmid donor strains of *Y. enterocolitica* proved sterile in the ability to transfer the plasmid to a recipient strain of the same organism.

In crosses involving two fertile donor strains of *Y. enterocolitica* and the polyauxotrophic recipient of this organism, gene transfer was not observed. Therefore, this study offered a limited data base for establishment of a model for plasmid-mediated gene transfer in *Y. enterocolitica* using the systems employed.
ACKNOWLEDGMENTS

The author would like to express his thanks and heartfelt appreciation to Drs. Mitsuru J. Nakamura and George L. Card. Their encouragement, guidance and friendship persevered throughout the course of this study.

A special thanks is extended to Dr. John J. Taylor for relinquishing space in his laboratory for conducting research. It was pleasing to know that bacteria and fungus could live in such close contact without cross-contamination.

The author also appreciates the professional guidance and critical review by the other members of his thesis advisory committee: Dr. R. A. Faust and Dr. E. W. Pfeiffer.

To my wife, there is an expression of deep love, warmth, and happiness for enduring long hours and changing temperaments. Her technical assistance was beyond reproach.

With much respect, admiration, and love, the author would like to thank his parents: Mr. and Mrs. James Hodinka and Mr. and Mrs. Herbert Hall. Their timely visits and expressions of love made the last few years all worthwhile.
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F'LAC PLASMID TRANSFER

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Membrane Mating

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<tr>
<td>API</td>
<td>Analytab Products Incorporated</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain Heart Infusion</td>
</tr>
<tr>
<td>C</td>
<td>temperature Celsius</td>
</tr>
<tr>
<td>CSH</td>
<td>Cold Spring Harbor</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>Cupric sulfate</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>d</td>
<td>days</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EMB</td>
<td>Eosin Methylene Blue</td>
</tr>
<tr>
<td>F</td>
<td>fertility factor or sex factor</td>
</tr>
<tr>
<td>g</td>
<td>grams</td>
</tr>
<tr>
<td>Hfr</td>
<td>High frequency recombinant</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>KCN</td>
<td>potassium cyanide</td>
</tr>
<tr>
<td>l</td>
<td>liter</td>
</tr>
<tr>
<td>LF</td>
<td>lactose fermenting</td>
</tr>
<tr>
<td>LIA</td>
<td>Lysine Iron Agar</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>Met</td>
<td>Methionine</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>mm</td>
<td>millimeter</td>
</tr>
<tr>
<td>MNNG</td>
<td>N-methyl-N′-nitro-N-Nitrosoguanidine</td>
</tr>
<tr>
<td>N</td>
<td>normal</td>
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<tr>
<td>Symbol</td>
<td>Term</td>
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<td>--------</td>
<td>-------------------------------------------</td>
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<tr>
<td>Na₂CO₃</td>
<td>Sodium carbonate</td>
</tr>
<tr>
<td>Na-K tartrate</td>
<td>Sodium-potassium tartrate</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NLF</td>
<td>non-lactose fermenting</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
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<tr>
<td>ONP</td>
<td>o-nitrophenol</td>
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<tr>
<td>ONPG</td>
<td>o-nitrophenyl-β-D-galactosidase</td>
</tr>
<tr>
<td>Pro</td>
<td>Proline</td>
</tr>
<tr>
<td>R</td>
<td>drug resistant plasmid</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDW</td>
<td>sterile distilled water</td>
</tr>
<tr>
<td>SS</td>
<td>Salmonella-Shigella</td>
</tr>
<tr>
<td>TSI</td>
<td>Triple Sugar Iron</td>
</tr>
<tr>
<td>Trp</td>
<td>Tryptophan</td>
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<tr>
<td>µg</td>
<td>micrograms</td>
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<tr>
<td>µm</td>
<td>micrometer</td>
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<td>YM</td>
<td>Yersinia-Mannitol</td>
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HISTORY

Prevalence

In recent years, *Yersinia enterocolitica* has received increasing attention as a human pathogen. Since the first recognized case of human disease caused by *Y. enterocolitica* was described in the United States in 1939 by Schleifstein and Coleman (136), disease caused by this organism has been reported with increasing frequency, especially in north western European and Scandinavian countries. There were only 23 reported cases in the world in 1966 (111), but there were 642 in 1970 (110), more than 1,000 in 1972 (111), and more than 4,000 in 1974 (144). At present, there are well over 5,800 isolates of *Y. enterocolitica* on deposit at the Pasteur Institute in Paris, France (13). Between the years 1966-1976 the majority of *Y. enterocolitica* was isolated from epidemic outbreaks and survey studies in Belgium (152), France (111), Finland (4,5,56,143), Norway (87), and Sweden (159). Yersiniosis was also reported in Hungary (142), Canada (144,145), the United States (10,44,80), Japan (8,162,163), and South Africa (128).

Initially, *Y. enterocolitica* was considered to be primarily of veterinary importance causing epizootic diseases in chinchillas (6) and wild hares (109). However, the first 12 isolates of this organism studied in the New York State Health Department Laboratories from 1923-1947 were from human illness (49,136). Recently, isolates have been reported from a wide variety of animals as well as man. Workers in several European countries have isolated strains of *Y. enterocolitica* from swine, dog, cow,
chinchilla, horse, sheep, guinea pig, and monkey. Recently, rats, other small rodents, small mammals, and fish have been found to carry the organism, but swine has been implicated as the primary reservoir of *Y. enterocolitica* in European countries and Canada (77,78,79,124,146,164). In the United States and parts of Canada, isolates have been reported from frogs and snails (14), swine (68,146), wapiti (101), the cat, cow, goat, rabbit, sheep, squirrel (68), beaver, raccoon (55), North American deer (156), and birds, including the canary, dove, pigeon, robin, and goose (55,68).

The evidence presented above suggests that zoonosis may be an important mode of transmission of *Y. enterocolitica* and that animals are probably the reservoir of infection. Although this may be the case, other modes of transmission include ingestion of contaminated food (20,94), person-to-person transmission through hand-to-mouth contact (54), as well as contact with infected animals (54). The organism has also been isolated from a variety of diverse sources such as drinking water (58,63,73,86,87,125,133) and nonchlorinated well-water (63,133); foodstuffs such as milk (11,69,134,135), ice cream (112), cheese (135), mussels (140), oysters (144), samples of market meat, and vacuum-packed beef (57). The implication is that *Y. enterocolitica* is important not only as an epizootic disease in animals but may be a potential human health hazard from environmental sources. Due to widespread distribution of *Y. enterocolitica*, no correlation between disease and mode of transmission has been established at the present time.

**Current Nomenclature and Taxonomy**

The genus *Yersinia* is comprised of five species: *Y. pestis*, *Y. pseudotuberculosis*, *Y. ruckeri*, *Y. philomiragia*, and *Y. enterocolitica*
and is now classified as a member of the Family Enterobacteriaceae (46, 74, 114, 137). The genus name was proposed by Van Loghem (151) in 1944 in honor of the French bacteriologist A. J. E. Yersin (113), who first isolated the plague bacillus Yersinia (Pasteurella) pestis in 1894. It has been proposed by some workers that Y. enterocolitica be subdivided into the species Y. rhamnophilica and Y. enteritidis due to the differences in their biochemical behavior from typical strains of Y. enterocolitica (15, 28, 84). To date, the proposal has not been universally accepted.

Y. enterocolitica meets the criteria established by Edwards and Ewing (45) for the Family Enterobacteriaceae. The organism is a Gram-negative rod which is asporogenous and peritrichous. Glucose is fermented, nitrate is reduced to nitrite, and cytochrome oxidase is absent. During the 1960's the organism was variously described as Bacterium enterocoliticum, Pasteurella pseudotuberculosis type B, Pasteurella X, Pasteurella Y, Pasteurella pseudotuberculosis-like organisms, Germ X and Bacterium X (65, 138). In 1964, Frederiksen (48) recognized the similarities between these organisms and Bacterium enterocoliticum described by Schleifstein and Coleman in 1939 (136) and proposed the name Yersinia enterocolitica.

**Descriptive Characteristics**

Y. enterocolitica is a relatively large coccobacillary, ovoid or rod-shaped Gram-negative facultatively anaerobic bacteria. Cultures of the organism are motile at 25°C, but not at 37°C. Depending on both age and composition of the medium, the organism can be highly pleomorphic (13). Deoxyribonucleic Acid (DNA) homology studies by Brenner et al. (15) suggest that there are four relatedness groups within this species: (a) typical Y. enterocolitica, (b) rhamnose-positive strains,
(c) rhamnose-positive, melibiose-positive, alpha-methylglucoside-positive, and raffinose positive strains, as well as (d) sucrose negative strains of this organism.

Morphologically, *Y. enterocolitica* colonies are quite distinct and can best be recognized with the aid of a dissecting microscope. On MacConkey agar the colonies are a light peach color and are readily examined using oblique transmitted light. Highsmith et al. (64) have evaluated standard enteric media using *Y. enterocolitica* strains. The organism grows well at both 25°C and 37°C on Brain Heart Infusion (BHI) agar where the colonies are large, transparent, and often confluent. On M-Endo agar, distinct, dark red colonies are observed. MacConkey agar is equal to M-Endo agar in supporting growth of *Y. enterocolitica* at 25°C, but at 37°C colonies are very small and difficult to observe without prolonged incubation. Growth of this organism on Eosin Methylene Blue (EMB) agar is poor. Because *Y. enterocolitica* produces a metallic sheen on EMB agar, some strains would be confused with *Escherichia coli*. Salmonella-Shigella (SS) agar was found to be unreliable for supporting growth of all strains of *Y. enterocolitica* (65). Three other selective media have been proposed for the isolation and differentiation of *Y. enterocolitica* from other enterobacteria. MacConkey agar modified with Tween 80, DNAse agar containing Tween 80 and sorbitol, and *Yersinia*-Mannitol (Y-M) agar have been used with varied success (95,127). Clinically, cold temperature (4°C-7°C) enrichment procedures are valuable in the recovery of *Y. enterocolitica* from fecal samples and other mixed cultures where this organism does not compete well with other bacteria (4,53,120,144,157,164). It is known that this organism grows well at temperatures ranging from 4°C-41°C (120).
Typical strains of *Y. enterocolitica* display an acid slant/acid butt reaction with no gas in Triple Sugar Iron (TSI) agar slants and an alkaline slant/acid butt reaction on Lysine Iron Agar (LIA) slants. Oxidase, phenylalanine deaminase, gelatin hydrolysis, tryptophan deaminase, lysine decarboxylase, and arginine enzymes are absent. The esculin and citrate reactions are negative. The indole reaction is variable. Most human isolates in the United States produce a positive reaction, whereas, the majority of European and Canadian isolates are negative. The reactions for urease, catalase, nitrate reductase type B, methyl red, β-D-galactosidase (ONPG), and ornithine decarboxylase are positive. Carbohydrates are hydrolyzed fermentatively but no gas is produced except for small amounts when tested in Durham tubes (137). Glucose, xylose, mannitol, inositol, amygdalin, maltose, arabinose, galactose, levulose, sucrose, mannose, trehalose, sorbitol, and cellibiose are fermented within 48 hours. The reactions for lactose, salicin, esculin, and indole are strain variable, but should be included since some of these reactions determine the biotypes described by Nilehn (120) and Wauters (153). Lactose is rarely fermented within 48 hours and normally requires up to 30 days of incubation before a visible acid pH change in the indicator is observed (13). Rhamnose, raffinose, melibiose, dulcitol, and adonitol are not utilized but the first three should be tested since most water isolates in the United States have given positive reactions to these carbohydrates (65). The tests for β-D-galactosidase, acetylmethylcarbinol (Voges-Proskauer), growth in potassium cyanide (KCN), and motility are temperature dependent and are expressed at 22°C-25°C but are negative (or delayed) at 37°C (13,137).

*Y. enterocolitica* has 34 "O" and 19 "H" serotypes (120,154,160).
Some antigens of this bacterium are shared with other bacteria, e.g., *Y. enterocolitica* serotype 9 cross reacts with *Brucella* species (3,31,70, 71,102). Other cross-agglutination reactions can be observed with *Vibrio* and *Salmonella* species (31). Agglutination or hemagglutination tests are used for antigenic analysis. The most common European serotypes in man are types 3 and 9, while serotype 3 is most common in Japan and Canada. Most human isolates in the United States are of serotype 8 (155).

Because of the biochemical heterogeneity that exists among strains of *Y. enterocolitica*, Nilehn (120) has explained the arrangement of these strains into biochemical groups or biotypes which show a variation in certain test results with the incubation temperature or with the test method. Also, a phage typing system has been developed in Europe, but most of the isolates from the United States are insensitive to this typing scheme (85,118,119).

The mechanism of pathogenicity of *Y. enterocolitica* is not yet known. In one experiment involving a human volunteer subject, a dose of 3.5 x 10^9 organisms/ml was required to produce an infection (117). Recently, investigators have been successful in establishing experimental infection in mice (7,22,23,24,126), guinea pigs (142), rats (22,23,24), and rabbits (129). The pathogenicity of this organism may vary according to the method of cultivation, route of infection, and temperature of incubation of *Y. enterocolitica* (121,129). Lee et al. (96), Une et al. (147,148,149,150), and Maki et al. (99) simultaneously discovered that pathogenic strains of *Y. enterocolitica* have the ability to invade tissue culture cells and the epithelial linings of the intestinal mucous membranes of experimentally infected rabbits. This invasive property is dependent on both temperature of incubation and formation of flagella in some strains.
Y. enterocolitica isolates are highly susceptible in vitro to chloramphenicol, nitrofurantoin, sulfonamide, nalidixic acid, colistin, tetracycline, aminoglycosides, and to the combination of trimethoprim and sulfamethoxazole. The organisms are resistant to oleandomycin, novobiocin, methicillin, fucidin, penicillin, and partially resistant to ampicillin, carbenicillin, and cephalothin (120,13). The resistance to the penicillins has been attributed by Cornelis et al. (33) to the production of a beta-lactamase by this species. Two distinct beta-lactamases, designated A and B, have been discovered. Type A enzymatically degrades ampicillin, carbenicillin, penicillin, and cephalosporins, but type B only hydrolyzes cephalosporins and penicillin (34,35).

**Genetics**

Attempts to use Yersinia pestis, the causative agent of plague, as a model for investigating the genetics of virulence in this organism and other species of Yersinia have been hampered due to lack of a gene transfer system. Gene transfer experiments involving conjugation, transduction, or transformation with these organisms were unsuccessful (16,17). In 1962, Martin and Jacob (100) reported that an avirulent strain of Y. pestis accepted the F'lac plasmid from E. coli. Later, observations were made that strains of Y. pseudotuberculosis accepted R factors (88). These significant findings stimulated efforts to obtain plasmid-mediated gene transfer in the genus Yersinia. Although the ultimate interest of the above investigators was in the genetics of Y. pestis, the subject was approached through the very closely related but less pathogenic organism, Y. pseudotuberculosis, and the transfer of markers from an F'lac infected donor strain to different recipient strains of this latter species have
been studied (88,116). At the present time, there is evidence that the conjugal system employing plasmid-mediated chromosome gene transfer is useful in delineating various genetic mechanisms in a diverse group of organisms. The system has been extended to such organisms as Neisseria gonorrhoeae (130,139), Pseudomonas fluorescens (105), Proteus species (47), species of the genus Erwinia (25,26,27), as well as Escherichia coli (61).

Since 1967, it has been known that strains of Y. pseudotuberculosis can accept the F'lac plasmid from E. coli and behave as a gene donor in crosses with several different auxotrophs of Y. pseudotuberculosis. Some selected donor markers were transferred at frequencies of $10^{-4}$ to $10^{-5}$ per donor cell while others appeared not to be transferred (88). A strain of Y. pestis, harboring the F'Cm plasmid from E. coli, was able to donate its chromosome to auxotrophic recipient strains of Y. pestis. The frequency of gene transfer in this organism was approximately $10^{-6}$ per donor cell, 100 times less efficient than gene transfer in Y. pseudotuberculosis. An attempt to extend the conjugation system to different serotypes of Y. pseudotuberculosis and to Y. enterocolitica by Lawton et al. (90) did not succeed.

In 1971, a High frequency recombinant (Hfr) strain of Y. pseudotuberculosis Group A was isolated from a strain which had previously accepted an F'lac plasmid from E. coli. This donor transferred a variety of genetic markers to Y. pseudotuberculosis Group A recipients. Transfer frequencies of approximately $10^{-2}$ per donor cell were obtained for early markers from membrane matings at $28^\circ\text{C}$; these frequencies were increased to about $2.5 \times 10^{-1}$ per donor cell at $37^\circ\text{C}$. Recombinant analysis and the
gradient of transmission of the markers suggested that markers were transferred linearly (103).

By interrupted mating experiments (89,104), the sequential transfer of selected and unselected markers to recipient bacteria was followed in Y. pseudotuberculosis. Under the best mating conditions employed, the earliest marker was transferred within 13 minutes and the latest within 98 minutes of impinging mating mixtures on membranes. Markers from the Hfr donor were transferred in a clockwise direction from an origin corresponding to a point near 60 minutes on the E. coli map.

Genetically, relatively little is known about Y. enterocolitica compared to other Yersinia species related to this organism. In recent years, the interest in Y. enterocolitica and the ability of various organisms to transfer drug resistance through conjugative drug resistant (R) plasmids has lead to the discovery that this organism has the ability to accept and transfer these R factors at a relatively high frequency (9,32, 75,76,81,82,83,132). Cornelis et al. (36) reported that a derepressed R factor can be transferred at a frequency of $5.0 \times 10^{-3}$ between two strains of Y. enterocolitica mated on a membrane. Under the same conditions, transfer of this R factor from E. coli to Y. enterocolitica was observed at a frequency of only $7.7 \times 10^{-6}$. This frequency was increased when the recipient strain was heat-treated before mating. Heat exposure for optimum fertility was $50^\circ$-52°C for a period of 2-3 minutes. They found that an F'lac plasmid from E. coli can be transferred to Y. enterocolitica, increasing the frequency of transfer of this plasmid through the effect of recipient heat-treatment. A self-transmissible lac plasmid, pGCl, that originated in Y. enterocolitica, has also been described. This plasmid
is freely transmissible between strains of *Y. enterocolitica* and strains of *E. coli*. The plasmid is transferred between *E. coli* strains and back to *Y. enterocolitica*, although at a reduced frequency. A lactose transposon, Tn951, from the pGCl plasmid, has been transposed to plasmid RP1 and was examined by DNA heteroduplex and restriction endonuclease analysis. A coordinate system for the transposon was constructed and evidence was presented that Tn951 can transpose to at least eight different sites on plasmid RP1 (37,38,39,40). No mention was made concerning chromosome mobilization by this plasmid. This is the extent of the knowledge obtained on the genetics of *Y. enterocolitica* since the 1960's. The development of a genetic system that would enable the successful transfer of the bacterial chromosome from donor to recipient strains of *Y. enterocolitica* employing plasmid mediation could prove benificial in this respect. As previously mentioned, the mechanism for pathogenicity in *Y. enterocolitica* is not yet known so an adequate model to study the genetics of virulence would be valuable.

CONJUGAL GENE TRANSFER

**Conjugation**

Bacterial conjugation was discovered by Lederberg and Tatum in 1946 (91,92). The process was defined as a transfer of genetic material from one bacterial strain to another which required cellular contact between the two different genotypes. Even though mutants of *E. coli* K-12 were used extensively in these initial studies and in subsequent future research dealing with conjugation, exchange of genetic material by conjugation appears to be a widespread phenomenon among many families of bacteria,
such as Enterobacteriaceae (Escherichia coli, Salmonella, Shigella, Serratia, Yersinia, and Proteus), Vibrionaceae (Vibrio), Pseudomonadaceae (Pseudomonas), and Rhizobiaceae (Rhizobium). Interspecies and intergeneric recombination through conjugation has been demonstrated repeatedly (21,42). For the purpose of discussion, the process of bacterial conjugation, as described by Curtiss (42), can be divided into five stages. Upon mixing appropriate concentrations of male (donor) and female (recipient) cells, the following events ensue: (a) formation of donor:recipient cell unions which are stable during gentle dilution, (b) establishment of a cellular connection between donor and recipient cells, (c) preparation of the circular donor chromosome (or conjugal fertility factor) for linear sequential transfer, (d) chromosome or conjugal fertility factor transfer, and (e) integration of parts of the transferred donor chromosome into the chromosome of the recipient to produce genetic recombinants.

**Sexual States in Bacteria**

The ability to act as a donor cell in conjugation is due to the presence of an F factor. This sex factor (plasmid) is a small circular double-stranded DNA particle which can exist either as a free self-replicating genome or integrated within the host bacterial chromosome. In the extrachromosomal state the plasmid is stably inherited and is generally dispensable to the cell. The F factor has been found to have a molecular weight of $4.5 \times 10^7$ daltons which may code for about 40 genes. Some of the important functions of the F factor genome in a donor cell are: (a) independent replication and maintenance of the factor itself, (b) expression of sexuality in the donor bacterial cell, and (c) a role in the sequence of steps that occur during conjugation. The F factor also
prompts the donor bacterial cell to produce the F pilis, which acts as specific attachment organs during the formation of conjugal pairs with F− cells. This F pilus has also been implicated as the passageway for the transfer of DNA to the recipient bacteria (21,107). The genetics, nomenclature, integration, and mobilization of the bacterial chromosome by plasmids has been and currently is an important area of investigation (60,67,72,115,122,141,158).

Strains of E. coli have been divided into two main groups, F+ or male cells and F− or female cells, depending on pair formation and conjugal mating properties. F+ cells are able to transfer the F factor to F− cells at a high frequency after forming conjugal pairs, but these cells transfer host chromosomal markers at a very low frequency. Hfr mutants of an F+ population can transfer host chromosomal markers at a very high frequency when mated to an F− strain due to the integration of the F factor into the bacterial chromosome (19,107). Each Hfr transfers the chromosome in a linear fashion from a fixed origin which is mediated by the integrated F factor and begins at a point within the F factor (60,72). Figures 1 and 2 illustrate the formation of Hfr cells and the steps involved in chromosome transfer.

On occasion, the F factor has the ability to break out of its integrated state taking with it one or more of the bacterial Hfr chromosome markers. Adelberg et al. (1) described this condition as an F' (F-prime) factor in 1960. If an F' factor, such as that carrying the lactose operon, is transferred to a wild type bacterial strain, then a partial diploid (or merodiploid) for the lactose region is created. Because bacterial recombination enzymes promote recombination between regions of
Figure 1. Formation of the $F'$ state and transfer of the Hfr chromosome. The $F$-factor of an Hfr cell reverses the integration process, but in doing so, retains some bacterial DNA (a. - c.). A bacterial cell that has the $F'$-factor (c.) may be converted to an Hfr cell through the integration of the fertility plasmid into the cell chromosome (d. - e.). Contact with an $F^-$ cell (f.) initiates the linear transfer of one strand of the Hfr chromosome, starting at the integrated $F'$-factor (g. - h.). Replication of donor and recipient DNA may occur during transfer (only donor DNA replication is shown). At some random time following initiation of the transfer, conjugation may be interrupted, at which time the $F^-$ cell departs with all, or part of the Hfr chromosome, and about one-third of the $F'$ plasmid. Unless the $F^-$ cell receives the entire Hfr genome, it does not gain the entire $F'$-factor and thus remains $F^-$. 
Figure 2. The conversion of an F\(^+\) bacterium into the Hfr state. Circular F-factor DNA, bearing among its genes O, the origin of oriented chromosome transfer from donor to recipient cell, happens to pair with the DNA of the circular bacterial chromosome between genes A and B. A reciprocal crossover between F-factor and bacterial chromosomes results in the insertion of the F-factor into the continuity of the chromosome. This act has created an Hfr strain that transfers gene A as one of its first genes and gene B as one of its last genes upon conjugation with an F\(^-\) cell. This sequence of events is reversible.

Adopted from Stent (141).
identical sequence homology, the F'lac plasmid will integrate readily into
the host chromosome at these regions of homology. The integrated F'
factor can then transfer certain chromosomal markers at a moderate
frequency. The polarity of chromosomal transfer is dependent upon F'
factor orientation (2,107). Figure 3 illustrates the formation of the
F' factor.

Auxotrophs

In initial studies performed by Lederberg and Tatum (91,92), auxo­
trophic mutants played a significant role in the elucidation of conjugal
gene transfer. In an Hfr x F⁻ cross, transfer of the bacterial chromosome
and subsequent recombinant formation can be detected by using auxotrophic
recipients which are unable to produce specific growth factors that the
donor or prototrophic cell is capable of synthesizing and donating to this
recipient.

The isolation of spontaneously occurring auxotrophic mutants from a
prototrophic strain of bacteria is rare. Only a small percentage of the
total prototrophic population is auxotrophic for a given selected growth
factor. For this reason, the concurrent discovery by Davis (43) and
Lederberg and Zinder (93) of a selection technique which enriches for
auxotrophic mutants in the bacterial population became invaluable to the
genetics of conjugation. N-methyl-N¹-nitro-N-Nitrosoguanidine (MNNG) has
been used as a potent mutagen which induces a high frequency of mutations
at doses which result in little killing of the bacterial cell. After
mutagenesis with MNNG the cells are exposed to penicillin or its analog
ampicillin. Under the appropriate conditions, these bacteriocidal agents
kill only dividing cells, enriching for the auxotrophic population.
Figure 3. The production of an F' fertility factor, bearing the lac gene of the bacterial chromosome, or F'lac. The F-factor integrated into the Hfr chromosome between gene A and the lac gene and now loops out improperly, so that the chromosomal lac sector has been included in the looped-out portion bearing the fertility-factor genes. A reciprocal crossover generates a circular F-factor into which the lac gene has been incorporated and which can now be readily transferred from F' donor to F' recipient cell.

Adopted from Stent (141).
DNA Synthesis During Transfer and Genetic Recombination

Many models have been proposed to explain the mechanism of DNA synthesis and transfer during bacterial mating (12, 30, 41, 42, 59, 72, 123, 131, 161) but the rolling circle model for DNA replication proposed by Gilbert and Dressler (50) is widely accepted. In this model, replication is initiated in the male upon a closed double-stranded DNA circle such as the F factor or the bacterial chromosome. A sequence-recognizing endonuclease puts a nick into the positive strand converting it into a DNA rod with a 3'-OH terminus and a 5'-P terminus. The DNA polymerase enzyme adds nucleotides onto the 3' end of the open strand, displacing a tail. Continuous elongation of the open positive strand causes continuous displacement of the single-stranded tail. It is this tail which is transferred into the female cell during bacterial mating. Inside the female, complementary fragments begin to be synthesized on the elongated tail converting it to a double-stranded form. The nuclease mediated cutting out of unit length segments containing sticky 5' ends and pairing between these ends cause circulation of the DNA. The gaps in the DNA are closed by the DNA ligase repair enzyme. The formation of double-stranded DNA in the female is necessary for recombination to occur (107, 131).

The formation of recombinants following conjugal transfer of genetic material from donor to recipient requires: (a) joining between homologous segments of donor and recipient genomes, (b) formation of continuous or discontinuous regions of effective homologous pairing between synapsed homologues, (c) reassortment of donor and recipient genetic information to yield new combinations of genetic information, and (d) segregation of recombinant chromosomes from nonrecombinant chromosomes (42).
These concepts have been put forth in the copy-choice model and breakage and reunion model that have been proposed to explain the mechanism of recombination (21,91,106).

**Chromosome Mapping**

Upon transfer of the bacterial chromosome from a donor to a recipient cell through conjugation and establishment of a recombinational event, it is then possible to estimate the locations of the particular genes on the bacterial chromosome that are transferred by the donor to the recipient. The techniques employed include mapping by interrupted mating for a coarse structural map of the chromosome and transduction by a transducing phage. Transduction is a valuable tool for determining the distances between very closely spaced bacterial genes and establishing the exact order of genes on the chromosome (21).

**Statement of Problem**

Members of the Family Enterobacteriaceae such as Escherichia coli, Salmonella typhimurium, and Yersinia pseudotuberculosis have been shown to transfer chromosome markers in a linear, progressive fashion during conjugation and have been used extensively as models for mapping of the bacterial chromosome. In comparison, knowledge on the genetics of Y. enterocolitica is substantially lacking. In as much as the pathogenic mechanism of Y. enterocolitica is unknown, conjugal gene transfer studies would provide a basis for elucidating the genetic mechanism(s) controlling human and animal virulence in this organism and establish more clearly the taxonomic relatedness of this organism to other enterobacteria. It is the purpose of this study to develop a genetic system that would enable
the successful transfer of the bacterial chromosome from donor to recipient strains of \textit{Y. enterocolitica} employing plasmids. Specific aspects of the study include investigation into: (a) the transfer of an \textit{F}''\text{lac} plasmid from \textit{E. coli} to \textit{Y. enterocolitica}, from donor to recipient strains of \textit{E. coli}, and from donor to recipient strains of \textit{Y. enterocolitica}, (b) the integration of the plasmid into the chromosome of \textit{Y. enterocolitica}, (c) the transfer of the chromosome from Hfr donor cells to recipient auxotrophs of this organism, and (d) formation and analysis of recombinants. In doing so, there will exist a basis for future research in the area of gene structure and function in \textit{Y. enterocolitica}. 
CHAPTER II
MATERIALS AND METHODS

BACTERIAL STRAINS AND PLASMIDS

Strains of *Escherichia coli*, stored in 40% glycerol at -15°C, were obtained from Dr. G. L. Card, Department of Microbiology, University of Montana, Missoula, Montana and Dr. R. M. Zsigray, Department of Microbiology, University of New Hampshire, Durham, New Hampshire. Dr. G. Cornelis, Department of Microbiology, Université Catholique de Louvain, Brussels, Belgium, kindly supplied the needed strains of *Yersinia enterocolitica*, which were identified as human isolates of serotype 9. The organisms were maintained on Trypticase Soy agar (Difco) slants at 4°C. They were streaked to LB agar (Appendix) plates to obtain freshly growing cultures prior to experimental use. All strains of *E. coli* were incubated at 37°C for 24 hours. Strains of *Y. enterocolitica* were grown at 25°C for 48 hours. The phenotypes of all strains were experimentally confirmed and the biochemical properties of the organisms were monitored using the Analytab Products Incorporated (API) 20E System for the identification of members of the Family *Enterobacteriaceae*. Table I shows the organisms employed in this study and their relevant properties.

DETERMINATION OF VIABLE CELL COUNTS: GROWTH CURVE

An overnight culture of *Y. enterocolitica* strain W277 and *E. coli* strain CSH 61 incubated at 25°C and 37°C in LB broth (Appendix) was diluted 1:50 into 100 ml of LB broth in a 500 ml side arm flask and shaken vigorously (115 oscillations/minute) in a shaking waterbath (American Optical) at the two different temperatures. Thirty minutes after the subculture was made, points for optical density (OD) readings were taken.
Table I. Strains of *Escherichia coli* and *Yersinia enterocolitica* used in this study.

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>STRAIN NUMBER</th>
<th>SEX</th>
<th>GENOTYPE</th>
<th>IMPORTANT PROPERTIES</th>
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<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSH 61</td>
<td>HfrC</td>
<td></td>
<td>trpR thi</td>
<td>Constitutive for the lac enzyme (i&lt;sup&gt;-&lt;/sup&gt;)</td>
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<tr>
<td>CSH 36</td>
<td>F'&lt;lacI proA&lt;sup&gt;+&lt;/sup&gt;,B&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
<td>Δ(lac pro) supE thi</td>
<td></td>
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<tr>
<td>CSH 24</td>
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<td></td>
<td>Δ(lac pro) supE thi</td>
<td>Carries temperature sensitive F'&lt;lac; Str&lt;sup&gt;+&lt;/sup&gt; Bl&lt;sup&gt;-&lt;/sup&gt; Ara&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>CSH 23.10s</td>
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<td></td>
<td>met</td>
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<td>His&lt;sup&gt;-&lt;/sup&gt; Met&lt;sup&gt;-&lt;/sup&gt; Pro&lt;sup&gt;-&lt;/sup&gt; Nal&lt;sup&gt;r&lt;/sup&gt; Str&lt;sup&gt;s&lt;/sup&gt; Lac&lt;sup&gt;-&lt;/sup&gt;</td>
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every 20 minutes and time points for viable cell counts every 40 minutes until the OD readings leveled off. Every other OD sample was also diluted for plating. The OD was read at 550 nm in a spectrophotometer (Coleman Junior II). For viable cell counts, the samples were diluted in LB broth and the dilutions plated on LB agar. The plates were incubated at 25°C and 37°C. Growth curves for both organisms at both temperatures were constructed. Only OD readings were taken on cultures of *E. coli* strain CSH 61 at 25°C and 37°C. In this case, readings were made every 10 minutes.

GROWTH ON VARIOUS MINIMAL MEDIUM AT 25°C, 37°C, AND 40°C

A culture of *Y. enterocolitica* strain W277 was inoculated into 20 ml of LB broth and allowed to shake at 37°C for 18 hours. A 10 ml sample of this overnight culture was placed in a sterile 16 x 125 millimeter (mm) conical centrifuge tube and centrifuged for 10 minutes in an International Clinical centrifuge. The pellet was washed three times in sterile distilled water (SDW) and resuspended in 1.0 ml of SDW. The following plates were inoculated by streaking for isolated colonies: 3 lactose minimal agar plates, 3 glucose minimal agar plates, 3 glucose (1/10 of normal concentration) minimal agar plates, 3 galactose minimal agar plates, 3 galactose plus glucose minimal agar plates, 3 minimal agar plates without a carbohydrate (Appendix), and 3 LB agar plates. Also, 3 glucose minimal agar plates plus methionine, 3 glucose minimal agar plates plus cysteine, 3 glucose minimal agar plates with both amino acids, 3 glucose minimal agar plates without the amino acids, and 3 LB agar plates were inoculated to test the requirements of this organism for the two amino acids. The plates were incubated at 37°C. The procedure was also repeated and the selection
plates incubated at 25°C and 40°C. The incubator temperature was carefully monitored with a thermometer. The plates were observed for 7 days before discarding. For the carbohydrate plates, any growth of *Y. enterocolitica* was considered positive for the ability to utilize that carbohydrate as a sole source of carbon at the given temperature. Colonies were gram stained and checked biochemically using the API 20E System.

**DETERMINATION OF DRY WEIGHT AND PROTEIN**

An overnight culture of *Y. enterocolitica* strain W277 and *E. coli* strain CSH 36 incubated at 37°C in LB broth was diluted 1:50 into 100 ml of LB broth in a 500 ml side arm flask and the cells shaken vigorously in a shaking water bath at 37°C. The cells were harvested when the growth reached the upper portion of the logarithmic growth phase as measured in a spectrophotometer at 550 nm. The cultures were poured into plastic centrifuge bottles and centrifuged at 10,000 x g for 20 minutes. The cells were washed three times in 25 ml of SDW and resuspended to a final volume of 10 ml for analysis.

To determine the dry weight of the cell suspension, an aluminum foil weighing dish was used. The dish was placed in a 100°C drying oven (Fisher) for several minutes, cooled in a desiccator, and weighed to determine the tare weight. A 1.0 ml sample of the cell suspension was placed in the dish and dried in the drying oven overnight. The sample was then cooled in a desiccator and weighed. The dry weight was measured in milligrams (mg) per ml of sample.

Protein was determined by the method of Lowry et al. (98). The method can be applied to whole microorganisms if these are heated to 100°C in sodium hydroxide (NaOH), which extracts all of the cell proteins in soluble
form. In this procedure, 0.2 ml samples of a $10^{-1}$, $10^{-2}$, and $10^{-3}$ dilution of the cell suspension were pipetted into sterile 13 x 100 mm test tubes, 0.25 ml of 1.0 N NaOH was added to each tube, and the tubes were placed in a boiling waterbath for 10 minutes. The tubes were cooled. Then, 2.5 ml of a reagent containing sodium carbonate ($\text{Na}_2\text{CO}_3$), cupric sulfate ($\text{CuSO}_4$), and sodium-potassium tartrate (Na-K tartrate) (Appendix) was added and the tubes were allowed to stand for 10 minutes. Then, 0.25 ml of Folin-Ciocalteau (Phenol) reagent was rapidly added to each tube and mixed immediately. After standing for 30 minutes to allow full color development, the absorbance was measured against a reagent blank at 750 nm in a spectrophotometer. The reagent blank, containing 0.2 ml of distilled water, and a set of standard protein solutions (Appendix) were treated in the same manner as the cell suspensions. A standard curve was drawn by plotting absorbancy of the protein standards against micrograms (µg) of protein (Figure 4) and used to calculate the amount of protein in the cell samples. The percent protein, based on the dry weight of the cells, was calculated. The determinations of dry weight and protein content were made on cultures of *Y. enterocolitica* strain W277 and *E. coli* strain CSH 36 grown at 25°C as well.

**ASSAY FOR BETA–GALACTOSIDASE ACTIVITY**

The enzyme β-galactosidase (β-galactosidase hydrolyase) must be functional if an organism is to use lactose for a carbon and energy source. β-galactosidase can be measured with chromogenic substrates such as o-nitrophenyl-β-D-galactoside (ONPG). This compound is colorless, but in the presence of β-galactosidase it is converted to galactose and o-nitrophenol (ONP). The ONP is yellow and can be measured by its absorption at
Figure 4. A protein standard curve showing the optical density readings for various concentrations of bovine serum albumin at 750 nm on a Coleman Junior II spectrophotometer.
420 nm in a spectrophotometer. If the ONPG concentration is high enough, the amount of ONP produced is proportional to the amount of enzyme present and to the time the enzyme reacts with the ONPG.

An overnight culture of *Y. enterocolitica* strain W277 and *E. coli* strain CSH 36, incubated at 37°C in LB broth plus lactose (1% concentration), were inoculated into 500 ml side arm flasks containing 100 ml of the same medium and allowed to shake on a shaking waterbath at 37°C until the OD$_{550}$ nm reached 0.600. The cells were then harvested, washed, and resuspended in a final volume of 10 ml of SDW. The cell suspensions were assayed for dry weight, protein, and β-galactosidase. Viable cell counts were done at the time the OD reached 0.600.

In assaying for β-galactosidase activity, 0.5 ml of dilutions of the original cell suspension was added to 1.5 ml of 0.1 M phosphate buffer (pH 6.5) (Appendix) in a 13 x 100 mm test tube. Two drops of chloroform and 1 drop of 0.1% sodium dodecyl sulfate (SDS) solution were added to each ml of assay mix. The tubes were then vortexed for 10 seconds. The tubes were placed in a waterbath at 37°C and allowed to sit 5 minutes for the temperature to equilibrate. The reaction was started by the addition of 0.2 ml of 0.01 M ONPG and timed with a stop watch. After sufficient yellow color had developed (15 minutes to 6 hours), the reaction was stopped by removing the tubes from the waterbath and adding 1.5 ml of cold 0.25 M Na$_2$CO$_3$ solution. Before reading the OD$_{420}$ nm of the samples, the cell debris was removed by centrifugation using a tabletop centrifuge. The units of enzyme/ml of sample was calculated as:

$$\frac{\text{units of enzyme}}{\text{ml of sample}} = \frac{1000 \times \text{absorbance}_{420\,\text{nm}}}{\text{time ml of sample}} \times \frac{\text{units of enzyme}}{\text{minutes ml}}.$$  

To determine the enzyme activity per mg of cells, the units of enzyme
were divided by the protein content in mg contained in the aliquot of cells. The activity of β-galactosidase was recorded as the number of micromoles of o-nitrophenyl-β-D-galactoside hydrolyzed per unit time per mg of protein at 37°C. The assay for β-galactosidase activity was also done at 25°C.

ISOLATION OF STREPTOMYCIN RESISTANT MUTANTS

Six drops of fresh overnight cultures in 20 ml of LB broth of Y. enterocolitica strain W277 and E. coli strains CSH 24 and 23.10s incubated at 37°C and 25°C were inoculated and thoroughly spread with glass rods (hockey sticks) over the surface of 3 LB agar plates containing streptomycin (Sigma) in the following concentrations: 0 (Control), 10 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml, 400 µg/ml, 600 µg/ml, 800 µg/ml, and 1000 µg/ml. The plates were incubated at 37°C and 25°C. Viable cell counts of the overnight cultures were made to determine the number of mutants per total cell population for each temperature. After 24 hours, the plates were examined and colonies were repurified on streptomycin plates. Colonies were plated on both plates with and without streptomycin to determine streptomycin resistant versus streptomycin dependent mutants. If a mutant is resistant to streptomycin it will grow on both types of plates; if streptomycin dependent the mutant will grow only on medium containing streptomycin. Streptomycin is the antibiotic of choice for counterselection against the male bacterial cell in conjugal mating with a recipient cell. The recipient cell is streptomycin resistant but is selected for by requiring some growth factor that can be received from the donor during mating and prior to elimination of the donor by streptomycin. For convenience, streptomycin resistant mutants of Y. enterocolitica
strain W277 were given the strain number W2771. Several of these mutants, isolated and repurified on LB agar containing 1000 μg/ml of streptomycin, were maintained on Trypticase Soy agar slants at 4°C for further experimentation.

F’LAC PLASMID TRANSFER

Broth Mating

Overnight cultures of *E. coli* strains CSH 23, CSH 28, CSH 50, and 23.10s and *Y. enterocolitica* strain W2771 were subcultured by diluting the donors 1:40 and the recipients 1:20 in LB broth and grown at 37°C until the donor reached 2-3 x 10^8 cells/ml. The cell density was measured by reading the OD_{550} in a spectrophotometer and comparing it to the growth curves obtained for each genus of organisms. A mating mixture was prepared by mixing 0.5 ml of the donor and recipient together in a sterile 16 x 125 mm screw capped tube. The tubes were placed in a waterbath at 37°C and gently shaken (49 oscillations/minute) for 60 minutes. For *E. coli* strain CSH 24, which carries a temperature sensitive F’lac plasmid, growth and matings of this organism with recipients were carried out at 30°C. After mating, 0.1 ml of dilutions of the mating mixtures were plated onto lactose minimal agar plates with 100 μg/ml of streptomycin and incubated at 30°C. The donors and recipients alone were plated onto lactose minimal agar plates with streptomycin as controls. Viable cell counts of the donors were made to enable determination of the efficiency of transfer of the F’lac plasmid between *E. coli* donors and *E. coli* and *Y. enterocolitica* recipients. The number of diploids generated per ml was computed and the efficiency of F’lac plasmid transfer was determined. In matings between *E. coli* strains and *Y. enterocolitica*
strain W2771, the transconjugant colonies were checked for the lactose marker and the *Y. enterocolitica* strain W2771 background by plating on lactose minimal agar plates with streptomycin and MacConkey agar plates, and by checking the colonies biochemically with the API 20E System. In matings between *E. coli* donors and *E. coli* strain CSH 50, transconjugant colonies were checked for the lactose marker as for the previous matings. Verification of the *E. coli* strain CSH 50 background was made by testing for the arabinose marker using arabinose minimal agar plates. *E. coli* strain CSH 50 will not grow on these plates while all the *E. coli* donor strains will.

Once the F'lac plasmid was transferred from strains of *E. coli* to *Y. enterocolitica* strain W2771, this organism was tested for the ability to transfer the F'lac plasmid to recipient strains of *Y. enterocolitica* using the same procedure as above except that matings were accomplished at both 60 minute and 18 hour time periods. Donor strains of *Y. enterocolitica* strain W2771 carrying the F'lac plasmids from different strains of *E. coli* were given the strain numbers W27723, W27724, W27728, and W27723.10s. These were mated with the wild type *Y. enterocolitica* strain W277 at 30°C and the selection plates were incubated at 30°C. Since the donor strains of *Y. enterocolitica* were resistant to streptomycin, due to the use of this antibiotic in the previous crosses with strains of *E. coli*, nalidixic acid was used for counterselection of the donor strains. Resistant mutants of the recipient wild type *Y. enterocolitica* strain W277 to nalidixic acid were isolated and given the strain number W2772. To verify that the colonies growing on the selection plates were of the *Y. enterocolitica* strain W2772 background, colonies were plated on LB agar contain-
ing 100 μg/ml of streptomycin. *Y. enterocolitica* strain W2772 is sensitive to this antibiotic. The four donor strains were also plated on LB agar containing 100 μg/ml of streptomycin to verify their resistance to this antibiotic.

In a separate experiment, *Y. enterocolitica* strain W2771 was preheated to 51°C for three minutes prior to mating with the four *E. coli* donor strains to eliminate the restriction and modification system that is present in *Y. enterocolitica* (36). Two three ml samples of an 18 hour culture of *Y. enterocolitica* strain W2771 incubated at 28°C were centrifuged and the bacteria resuspended in 0.05 ml of sterile 0.85% saline. One sample was then heated at 51°C for three minutes before mating, while the other sample received no treatment. The cell suspensions were transferred to an ice bath, allowed to cool, and centrifuged. The pellets of bacteria were immediately resuspended in 0.5 ml of an exponentially growing culture of the donor and mated as described earlier. From this point on, the procedure remained as previously described.

**Membrane mating**

A membrane mating technique was used to facilitate the transfer of the F'lac plasmid from the four donor strains of *Y. enterocolitica* to the recipient *Y. enterocolitica* strain W2772. In this technique, fresh overnight cultures of both donors and recipient, grown in 20 ml of LB broth at 30°C, were subcultured by diluting the donors 1:40 and the recipient 1:20 in LB broth and grown at 30°C until the donors reached 2-3 x 10⁸ cells/ml. At this point, a mating mixture was prepared by mixing 0.5 ml of each donor with the same volume of recipient in a 16 x 125 mm sterile
screw capped tube. The mixtures were immediately filtered through 0.30 μm Millipore membrane filters (25 mm) and the filters placed onto the surface of LB agar plates. The plates were incubated at 30°C for 4 hours, the filters were then removed, the cells resuspended in 2.0 ml of sterile saline, and diluted. The dilutions were plated on lactose minimal agar containing 20 μg/ml of nalidixic acid. Dilutions of both donors and recipient alone were plated on lactose minimal agar with nalidixic acid as a control. Viable cell counts of the donors were made to enable the determination of the efficiency of F'lac plasmid transfer. The number of diploids generated per ml was computed and the efficiency of F'lac plasmid transfer was determined. Transconjugant colonies were checked for the lactose marker by plating them on lactose minimal agar with nalidixic acid and MacConkey agar plates. To verify that the colonies growing on the selection plates were of the Y. enterocolitica strain W2772 background, they were plated on LB agar containing 100 μg/ml of streptomycin. Y. enterocolitica strain W2772 is sensitive to this antibiotic. The four donor strains of Y. enterocolitica were also plated on LB agar containing 100 μg/ml of streptomycin to verify their resistance to this antibiotic. The colonies were also tested biochemically using the API 20E System.

ISOLATION OF NALIDIXIC ACID RESISTANT MUTANTS

Six drops of a fresh overnight culture of Y. enterocolitica strain W277 in 20 ml of LB broth was spread with a hockey stick over the surface of 9 LB agar plates containing 20 μg/ml of nalidixic acid. The plates were incubated at 25°C for 48 hours. Resistant colonies were purified on LB agar plates containing 20 μg/ml of nalidixic acid. Two gene loci are involved in conferring resistance of an organism to nalidixic acid.
Mutants resistant to high levels (10 μg to 100 μg/ml) map at nal A on an E. coli chromosome. Nal B confers low level resistance (1 μg to 10 μg/ml) (107). Resistant mutants of Y. enterocolitica strain W277 were replica plated onto LB plates containing 20 μg/ml of nalidixic acid and those plates containing 100 μg/ml of nalidixic acid to confirm that the higher level resistance was selected for. Colonies were checked biochemically using the API 20E System.

NITROSOGUANIDINE MUTAGENESIS: SURVIVAL CURVE

N-methyl-N'-nitro-N-Nitrosoguanidine (MNNG) is a potent mutagen that induces a high frequency of mutations at doses which result in little killing. Survival curves are done with a constant time of exposure and varying doses of MNNG or with a constant concentration of MNNG and different times of exposure. The aim is for 50% survival of the cell population, where the highest frequency of mutations would be obtained (107). In this method, an overnight culture of Y. enterocolitica strain W277 in 20 ml of LB broth was subcultured into six test tubes, each with 5.0 ml of LB broth, and aerated at 37°C until a density of 3-5 x 10^8 cells was reached. The cultures were pooled together and centrifuged. The pellet was washed twice in 5.0 ml of citrate buffer (pH 5.5) (Appendix) and resuspended in 32 ml of citrate buffer. A 4.0 ml sample was measured into each of eight test tubes and placed in a waterbath at 37°C. A 0.2 ml volume of a 1 mg/ml solution of MNNG in citrate buffer was added to each tube. The final concentration of MNNG was approximately 50 μg/ml in each tube. At different times a culture was removed from the waterbath, centrifuged, and washed once in phosphate buffer (pH 7.0) (Appendix). Each pellet was resuspended in 5 ml of phosphate buffer and immediately diluted and
plated on LB agar plates to determine viable cell counts. Points were taken at the following times: 0, 5, 10, 20, 30, 45, 60, and 90 minutes. A graph was constructed plotting the cell survival against the time of exposure to MNNG. The procedure was repeated on *E. coli* strain CSH 50 as a control and also accomplished at 25°C as well as 37°C for each organism.

**ISOLATION OF AUXOTROPHIC MUTANTS**

A culture of *Y. enterococolitica* strain W277 was mutagenized at 25°C with MNNG for 10 minutes following the previously described procedure. After washing the cells in phosphate buffer, they were grown overnight in glucose minimal medium containing 100 µg/ml of histidine. A 5 ml sample of the minimal overnight was centrifuged, washed twice in 5 ml of phosphate buffer and resuspended in 5 ml of phosphate buffer. A 10 ml volume of glucose minimal medium without histidine was inoculated with enough bacteria to give a final concentration of 1-2 x 10^7 cells/ml (OD550 nm of 0.04) and shaken at 25°C to starve for histidine. The cells were aerated until the OD increased 4 to 5-fold. At this point, nalidixic acid, at a final concentration of 100 µg/ml, was added and the cells aerated for 18 hours. The cells were then centrifuged, washed in phosphate buffer, resuspended in glucose minimal medium containing histidine, and grown overnight at 25°C. Dilutions of the sample were then plated on glucose minimal agar containing histidine and incubated at 25°C. When the colonies were at least 2 mm in diameter, they were replica plated in succession onto glucose minimal agar with histidine, then two plates of glucose minimal agar without histidine and incubated at 25°C. Colonies were retested for growth with and without histidine. Using a
mutant colony that now required histidine for growth, the procedure was repeated to isolate a mutant colony that required both histidine and methionine for growth. Using this mutant, a polyauxotroph requiring histidine, methionine, and proline was isolated and given the number **Y. enterocolitica** strain W2773. Each successive mutant was checked for its sensitivity to nalidixic acid after each enrichment procedure by plating the mutants on LB agar containing 100 μg/ml of nalidixic acid and LB agar plates without nalidixic acid. Each marker of the polyauxotroph was individually tested after each enrichment procedure using selection plates with and without the given amino acid being selected for. The mutants were also tested biochemically using the API 20E System after each successive enrichment procedure. Figure 4 shows the procedure used for isolation of auxotrophic mutants of **Y. enterocolitica** strain W277.

**SELECTION OF F'_ts LAC FACTOR INTEGRATION**

**Y. enterocolitica** strain W27724 contains a temperature sensitive F'lac plasmid that was received from **E. coli** strain CSH 24. This plasmid is unable to replicate at high temperatures (40°-43°C). Although the DNA replication system of the F' factor cannot function in the autonomous (non-integrated) state, it can replicate at high temperatures if the F' factor is integrated into the host chromosome (107). Therefore, among the revertants of **Y. enterocolitica** strain W27724 which can grow at high temperatures will be a class of organisms which have the F' factor integrated at various points along the host chromosome. These are now Hfr strains. These strains were selected for by plating dilutions of a fresh overnight culture of **Y. enterocolitica** strain W27724, grown in 20 ml
1. MUTAGEN TREATMENT.

2. STARVATION IN MINIMAL MEDIUM MINUS THE AMINO ACID.

3. ADD NALIDIXIC ACID AND INCUBATE AT 25°C FOR 18 HOURS.

4. CENTRIFUGE, WASH, AND RESUSPEND IN MINIMAL MEDIUM PLUS THE AMINO ACID. INCUBATE OVERNIGHT AT 25°C.

5. PLATE DILUTIONS ONTO MINIMAL MEDIUM PLUS THE AMINO ACID.

6. REPLICA PLATE IN SUCCESSION ONTO MINIMAL MEDIUM PLUS THE AMINO ACID, MINIMAL MEDIUM MINUS THE AMINO ACID, AND AGAIN MINIMAL MEDIUM MINUS THE AMINO ACID.

Figure 5. Nalidixic acid treatment for the isolation of auxotrophic mutants.
of LB broth at 25°C, onto lactose tetrazolium plates (Appendix) which were prewarmed at 41°C. Dilutions were selected that yielded approximately 500 to 1,000 colonies per plate and 10 plates were inoculated. The plates were incubated at 41°C for 48 hours. Lactose tetrazolium plates of the same dilutions were also incubated at 25°C as a control. The incubator was carefully monitored with a thermometer. After 48 hours, the lawn of colonies was observed and white colonies (lactose fermenters) were restreaked onto lactose tetrazolium plates and incubated at 41°C for another 48 hours. The lactose positive colonies on these plates are possible insertions. Stability of these colonies was monitored by continued streaking on lactose indicator plates at 41°C. Strains which segregated the least number of lactose negative colonies were saved for further experimentation.

CURING OF THE F' LAC PLASMID

The replication of plasmids can be selectively inhibited by acridine orange. Growth of strains carrying F' or F factors in the presence of acridine orange results in loss ("curing") of the sex factor. Fresh overnight cultures of Y. enterocolitica strains W27723, W27724, W27728, and W27723.10s and E. coli strain CSH 23 were diluted and 5 ml of LB broth (pH 7.6) containing different concentrations of acridine orange (25, 50, 75, 100, and 125 μg/ml) were inoculated with approximately 1,000 to 2,000 cells. The cells were grown overnight in the dark to saturation at 30°C. In a similar manner, a control culture without acridine orange was prepared. The overnight cultures with the highest concentration of acridine orange which still allowed growth of the organism were diluted and plated on lactose tetrazolium agar. The
plates were incubated at 30°C. The indicator plates were observed for
curing of the F'lac plasmid by comparing the number of lactose negative
colonies present in the culture grown in the presence of acridine orange
with respect to the control.

F'LAC PLASMID-MEDIATED CHROMOSOME TRANSFER

Broth Mating

A mating mixture of the multiply marked recipient, Y. enterocolitica
strain W2773, with each of the four donor strains (Y. enterocolitica
strains W27723, W27724, W27728, W27723.10s) was prepared. A 2 ml sample
of the recipient was mixed with 0.2 ml of each donor strain in a
16 x 125 mm sterile screw capped tube and placed on a gently shaking
waterbath at 25°C and 30°C. Both donor and recipient cultures were grown
in LB broth to a density of 2-3 x 10^8 cells/ml before mixing. After 60
minutes and 18 hour incubation times, dilutions of the matings were plated
onto glucose minimal medium containing: (a) proline, methionine, and
10 μg/ml of nalidixic acid, (b) proline, histidine, and 10 μg/ml of
nalidixic acid, and (c) histidine, methionine, and 10 μg/ml of nalidixic
acid to independently select for each marker that may be received by
the recipient. The plates were incubated at 25°C and 30°C. Donor and
recipient controls on each type of selection plate were done. Viable
cell counts of the donor population were accomplished so as to determine
the frequency of transfer of the bacterial chromosome from donor to re­
cipient for each time period of mating. Also, the recipient was mated
with the wild type Y. enterocolitica strain W277 to establish that the
recipient could not be equally cross-fed by a donor not carrying the
F'lac plasmid from E. coli. The selection plates were examined for 72
hours and a determination as to the markers inherited by *Y. enterocolitica* strain W2773 from the F'lac plasmid donors was made.

**Membrane Mating**

Fresh overnights of the above mentioned donor strains of *Y. enterocolitica* and the recipient, *Y. enterocolitica* strain W2773, were subcultured in LB broth by diluting them 1:40 and growing at 30°C until both donors and recipient were at 2-3 x 10^8 cells/ml. A mating mixture was prepared by mixing 2.0 ml of the recipient with 0.2 ml of each donor strain. Immediately, these mixtures were filtered onto 0.30 μm Millipore membrane filters (25 mm), the filters placed on the surface of an LB agar plate, and the plates incubated at 30°C for 4 hours. At this time, the filters were removed, the cells resuspended in 2.0 ml of sterile 0.85% saline and diluted. Dilutions were plated on the same selection plates as for the broth matings and the plates were incubated at 30°C. Both donor and recipient controls were also plated on the various selection plates. Dilutions of the donor strains were plated on LB agar to enable the determination of the efficiency of chromosome transfer. As for the broth matings, the recipient was mated with the wild type *Y. enterocolitica* strain W277 as a control. The selection plates were examined for 72 hours and a determination as to the markers inherited by *Y. enterocolitica* strain W2773 from the F'lac plasmid donors was made.
CHAPTER III
RESULTS

DETERMINATION OF VIABLE CELL COUNTS: GROWTH CURVE

The growth pattern of Y. enterocolitica strain W277 was compared with that of a strain of E. coli using the same experimental system at two different temperatures. The data accumulated was used in proceeding experiments. Figures 6 and 7 show an unusual and interesting feature of the growth of Y. enterocolitica strain W277. The results indicate that this organism grows equally well at both 25°C and 37°C as shown by the marked similarities of OD readings and viable cell counts of the organism at the two temperatures. Figure 8 shows typical growth curves of a strain of E. coli at 25°C and 37°C.

GROWTH ON VARIOUS MINIMAL MEDIUM AT 25°C, 37°C, AND 40°C

Burrows and Gillett (18) reported that all strains of Y. enterocolitica required thiamine and some strains required either cysteine or methionine as nutritional requirements for growth. They noted that as the temperature of incubation increased, additional requirements for growth existed. Table II reveals that Y. enterocolitica strains W277 showed no requirement for either cysteine or methionine at 25°C and 37°C. The organism grew equally well on glucose minimal medium with and without the amino acids. At 40°C, growth of the organism on glucose minimal medium was quite poor.

In a separate experiment, Y. enterocolitica strain W277 was examined for its growth on minimal medium with different carbohydrates as the sole source of carbon and energy. Table III shows that minimal medium containing glucose, galactose, and a combination of glucose and galactose readily
Figure 6. Growth curves of *Y. enterocolitica* strain W277 at 25°C and 37°C showing the optical density readings (OD 550 nm) for various times of incubation. 25°C •—•; 37°C •—•.
Figure 7. Growth of *Y. enterocolitica* strain W277 at 25°C and 37°C showing the number of viable cells per ml for various times of incubation. 25°C ● — ●; 37°C ○ — ○.
Figure 8. Growth curves of *E. coli* strain CSH 61 at 25°C and 37°C showing the optical density readings (OD550 nm) for various times of incubation. 25°C ■■■; 37°C ●●●.
Table II. Determination of the requirement for the amino acids cysteine and/or methionine by *Yersinia enterocolitica* strain W277 at 25°C, 37°C, and 40°C temperatures.

<table>
<thead>
<tr>
<th>INCUBATION TEMPERATURE</th>
<th>GLUCOSE MINIMAL MEDIUM&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CONTROLS&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>CYSTEINE</td>
<td>METHIONINE</td>
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<td>25°C</td>
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<td>(7d)</td>
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<sup>a</sup>Organisms were inoculated by streaking for isolated colonies on minimal medium with and without the amino cysteine and methionine.

<sup>b</sup>Controls consisted of inoculating organisms onto minimal medium without the amino acids and enriched LB agar medium.

<sup>c</sup>+++ = Good growth as determined by observation. The time of incubation necessary for observation of the given growth pattern is presented in number of days.

<sup>d</sup>+ = Poor growth as determined by observation.
Table III. Growth of *Y. enterocolitica* strain W277 on various minimal media at 25°C, 37°C, and 40°C temperatures.

<table>
<thead>
<tr>
<th>INCUBATION TEMPERATURE</th>
<th>MINIMAL MEDIUMª</th>
<th>CONTROLSᵇ</th>
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<tr>
<td></td>
<td>GLUCOSE 0.1% CONC.</td>
<td>LACTOSE GALACTOSE AND GLUCOSE</td>
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<td>40°C</td>
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</tbody>
</table>

ªOrganisms were inoculated by streaking for isolated colonies on minimal medium containing various carbohydrates as the sole source of carbon and energy.

bThe controls consisted of inoculating organisms onto minimal medium containing no carbohydrate and enriched LB agar medium.

c+++ = Good growth as determined by observation. The time of incubation necessary for observation of the given growth pattern is presented in number of days.

d++ = Moderate growth.

e+ = Poor growth. Observations of lactose minimal agar plates at 25°C revealed a few small colonies (0.5-1.0 mm in diameter) that were not present on the same medium incubated at 37°C and 40°C temperatures.

f- No growth observed after 7 days of incubation.
supports growth of this organism at 25°C and 37°C. Minimal medium containing glucose in 0.1% of the normal concentration also allows for good growth of the organism, but after an extended period of incubation. *Y. enterocolitica* strain W277 did not grow on lactose minimal medium at 37°C and 40°C and grew poorly at 25°C. Observations of the lactose minimal agar plates at 25°C revealed a few small colonies (0.5 to 1.0 mm in diameter) that were not present on the same medium incubated at 37°C and 40°C. At 40°C, growth of the organism on minimal medium was moderate to poor.

**ASSAY FOR PROTEIN CONTENT AND β-GALACTOSIDASE ACTIVITY**

The presence of a small number of colonies growing on lactose minimal medium at 25°C but not at 37°C or 40°C and the observation that *Y. enterocolitica* strain W277 can utilize glucose or galactose as a sole source of carbon and energy prompted the investigation into the activity of β-galactosidase in this organism. The protein content and β-galactosidase activity of *Y. enterocolitica* strain W277 and *E. coli* strain CSH 36 were compared at 25°C and 37°C. The results in Table IV illustrate that, given approximately the same amount of protein and number of viable cells per ml for the two organisms at the two temperatures, *E. coli* strain CSH 36 contains one hundred times more β-galactosidase activity than *Y. enterocolitica* strain W277 at 25°C and more than three hundred times more enzyme activity than the latter organism at 37°C. The specific activity of this enzyme is extremely low in *Y. enterocolitica* strain W277 compared with *E. coli* strain CSH 36 at the two temperatures. A difference can also be seen in comparing the units and specific activity of β-galactosidase of the same organism at both temperatures. *Y. entero-
Table IV. Determination of the protein content and β-galactosidase activity of *E. coli* strain CSH 36 and *Y. enterocolitica* strain W277 at 25°C and 37°C temperatures.

<table>
<thead>
<tr>
<th>ORGANISMS</th>
<th>PROTEIN CONTENT</th>
<th>β-GALACTOSIDASE</th>
<th>VIABLE CELLS/ML</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/ml</td>
<td>% protein</td>
<td>units/min ml</td>
</tr>
<tr>
<td><em>Yersinia enterocolitica</em> strain W277 (25°C)</td>
<td>2115</td>
<td>44</td>
<td>25</td>
</tr>
<tr>
<td><em>Y. enterocolitica</em> strain W277 (37°C)</td>
<td>2076</td>
<td>47</td>
<td>9</td>
</tr>
<tr>
<td><em>Escherichia coli</em> strain CSH 36 (25°C)</td>
<td>1713</td>
<td>43</td>
<td>2457</td>
</tr>
<tr>
<td><em>E. coli</em> strain CSH 36 (37°C)</td>
<td>1129</td>
<td>49</td>
<td>3075</td>
</tr>
</tbody>
</table>

*a* µg/ml = optical density readings (420 μm) x slope of protein standard curve x dilution factor

*b* % protein = µg of protein/µg dry weight x 100

*c* Viable cell counts were done on actively growing cell cultures which had an optical density reading of .600.
coli strain W277 exhibits an increase in units and specific activity of the enzyme at 25°C whereas E. coli strain CSH 36 shows the opposite effect.

**ISOLATION OF STREPTOMYCIN RESISTANT MUTANTS**

Streptomycin is the antibiotic of choice for counterselection against the donor cells in conjugal mating with recipient cells. The recipient cells are streptomycin resistant. This resistance is routinely selected for by plating the organism on enriched medium containing 100 µg/ml of the antibiotic. Cornelis et al. (36) used a concentration of 1000 µg/ml of streptomycin to isolate resistant colonies of Y. enterocolitica. To confirm these results and isolate resistant mutants of Y. enterocolitica strain W277, the organism was plated on LB agar containing concentrations of streptomycin ranging from 0 to 1000 µg/ml. Table V shows the isolation of streptomycin resistant mutants of Y. enterocolitica strain W277 and E. coli strains CSH 24 and 23.10s at 25°C and 37°C. Resistant mutants of Y. enterocolitica strain W277 were easily obtained at both temperatures at levels up to and including 1000 µg/ml of streptomycin. On the average, seven times more mutants were isolated at 25°C than at 37°C per 1 x 10¹⁰ viable cells per ml. For the strains of E. coli used, few mutants were obtained at either temperature and resistance was not observed above the level of 400 µg/ml of streptomycin.

**F′LAC PLASMID TRANSFER INVOLVING DONOR AND RECIPIENT STRAINS OF E. COLI AND DONOR STRAINS OF E. COLI WITH Y. ENTEROCOLITICA STRAIN W2771.**

Transfer of the F′lac plasmid from donor to recipient strains of E. coli and from donor strains of E. coli to Y. enterocolitica strain W2771 was accomplished using a broth mating system. Table VI shows the
<table>
<thead>
<tr>
<th>ORGANISMS</th>
<th>CONCENTRATION OF STREPTOMYCIN (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td><strong>Yersinia enterocolitica</strong></td>
<td></td>
</tr>
<tr>
<td>strain W277 (25°C)</td>
<td>CG^a</td>
</tr>
<tr>
<td><strong>Yersinia enterocolitica</strong></td>
<td></td>
</tr>
<tr>
<td>strain W277 (37°C)</td>
<td>CG</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
</tr>
<tr>
<td>strain CSH 24 (25°C)</td>
<td>CG</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
</tr>
<tr>
<td>strain 23.10s (37°C)</td>
<td>CG</td>
</tr>
</tbody>
</table>

^aCG = CONFLUENT GROWTH

^bNG = NO GROWTH

^cNumber of resistant colonies/-1 x 10^10 cells/ml

^dOrganisms were inoculated onto LB agar plates containing different concentrations of streptomycin (μg/ml)
Table VI. Direct selection of F'lac plasmid transfer involving strains of *E. coli* in a 60 minute broth mating system.

<table>
<thead>
<tr>
<th>CONJUGATION</th>
<th>NUMBER OF RECOMBINANTS/ML</th>
<th>FREQUENCY OF TRANSFER OF F'LAC PLASMID</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSH 23 CSH 50</td>
<td>4.05 x 10^8</td>
<td>1.00 (ALL TRANSFERRED)</td>
</tr>
<tr>
<td>CSH 24 CSH 50</td>
<td>6.60 x 10^7</td>
<td>1.28 x 10^-1</td>
</tr>
<tr>
<td>CSH 28 CSH 50</td>
<td>2.16 x 10^8</td>
<td>9.04 x 10^-1</td>
</tr>
<tr>
<td>23.10s CSH 50</td>
<td>1.80 x 10^7</td>
<td>4.80 x 10^-2</td>
</tr>
</tbody>
</table>

The frequencies of transfer of the F'lac plasmid were calculated in respect of donor input. Viable cell counts of the donors are as follows:

CSH 23 - 4.05 x 10^8 cells/ml
CSH 24 - 5.15 x 10^8 cells/ml
CSH 28 - 2.85 x 10^8 cells/ml
23.10s - 3.75 x 10^8 cells/ml
transfer of the F'lac plasmid from four donor strains of *E. coli* to *E. coli* strain CSH 50. The results revealed that: (a) the plasmids were present in the donor strains, (b) they were transmissible from donor to recipient strains of *E. coli*, and (c) the frequency of transfer, with respect to donor input, ranged from total transfer of the plasmid to a frequency of approximately $10^{-2}$. Attempts to transfer the F'lac plasmids from *E. coli* to *Y. enterocolitica* strain W2771 were successful, but the frequency of transfer was much lower than that observed for matings between strains of *E. coli*, occurring at a frequency of approximately $10^{-6}$ (Table VII).

In a separate experiment, *Y. enterocolitica* strain W2771 was preheated to 51°C for three minutes prior to mating with the four *E. coli* donor strains. Table VIII indicates that heat treatment of the recipient caused no increase in the frequencies of F'lac plasmid transfer from *E. coli* to *Y. enterocolitica* strain W2771. In two experiments, a slightly higher transfer was observed in matings where the recipient received no treatment.

In the above experiments, all suspected transconjugant colonies were experimentally tested for the lactose marker and verified that they were of the *E. coli* CSH 50 or *Y. enterocolitica* strain W2771 background as described in Materials and Methods before calculating the frequencies of F'lac plasmid transfer. The transferred genetic elements were relatively stable in the recipient *Y. enterocolitica* strain. Upon initial isolation, some segregation of lactose negative colonies was observed but repeated plating on MacConkey agar permitted the selection of relatively stable *Y. enterocolitica* lactose positive organisms.
Table VII. Direct selection of F’lac plasmid transfers involving strains of *E. coli* and *Y. enterocolitica* strain W2771 in a 60 minute broth mating system.

<table>
<thead>
<tr>
<th>CONJUGATION</th>
<th>NUMBER OF RECOMBINANTS/ML</th>
<th>FREQUENCY OF TRANSFER OF F’LAC PLASMID</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSH 23 W2771</td>
<td>$2.00 \times 10^3$</td>
<td>$5.48 \times 10^{-6}$</td>
</tr>
<tr>
<td>CSH 24 W2771</td>
<td>$1.00 \times 10^3$</td>
<td>$3.44 \times 10^{-6}$</td>
</tr>
<tr>
<td>CSH 28 W2771</td>
<td>$1.50 \times 10^3$</td>
<td>$5.52 \times 10^{-6}$</td>
</tr>
<tr>
<td>23.10s W2771</td>
<td>$2.00 \times 10^3$</td>
<td>$5.26 \times 10^{-6}$</td>
</tr>
</tbody>
</table>

*a* The frequencies of transfer of the F’lac plasmid were calculated in respect of donor input. Viable cell counts of the donors are as follows:

- CSH 23 - $3.65 \times 10^8$ cells/ml
- CSH 24 - $2.91 \times 10^8$ cells/ml
- CSH 28 - $2.72 \times 10^8$ cells/ml
- 23.10s - $3.80 \times 10^8$ cells/ml
Table VIII. Direct selection of F'lac plasmid transfers involving strains of _E. coli_ and a heat-treated recipient, _Y. enterocolitica_ strain W2771, in a 60 minute broth mating system.

<table>
<thead>
<tr>
<th>CONJUGATION</th>
<th>NUMBER OF RECOMBINANTS/ML</th>
<th>FREQUENCY OF TRANSFER OF F'LAG PLASMID</th>
</tr>
</thead>
<tbody>
<tr>
<td>DONOR</td>
<td>RECIPIENT</td>
<td>WITH HEAT</td>
</tr>
<tr>
<td>CSH 23 W2771</td>
<td></td>
<td>2.45 x 10^3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WITHOUT HEAT</td>
</tr>
<tr>
<td>CSH 24 W2771</td>
<td></td>
<td>2.95 x 10^3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WITHOUT HEAT</td>
</tr>
<tr>
<td>CSH 28 W2771</td>
<td></td>
<td>6.50 x 10^2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WITHOUT HEAT</td>
</tr>
<tr>
<td>23.10s W2771</td>
<td></td>
<td>2.10 x 10^3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WITHOUT HEAT</td>
</tr>
</tbody>
</table>

^a^ The frequencies of transfer of the F'lac plasmid were calculated in respect of donor input. Viable cell counts of the donors are as follows:

- CSH 23 - 2.79 x 10^8 cells/ml
- CSH 24 - 3.07 x 10^8 cells/ml
- CSH 28 - 1.91 x 10^8 cells/ml
- 23.10s - 2.31 x 10^8 cells/ml

^b^ The recipient was heated in a waterbath at 51°C for three minutes before mating with the donors to inhibit the restriction and modification system present in _Y. enterocolitica_. Unheated controls were also done.
Figures 9 and 10 show colonies of *Y. enterocolitica* strain W27723, suspected of harboring the F'lac plasmid from *E. coli* CSH 23, compared with typical non-lactose fermenting colonies of the wild type *Y. enterocolitica* strain W277. Figures 11 and 12 show that *Y. enterocolitica* strains W27723 and W27728, suspected of harboring the F'lac plasmid from either *E. coli* strains CSH 23 or CSH 28, have the same morphological appearance. On MacConkey agar, the colonies are flat, dull, undulated, dark pink in color, and have a large dark pink to purple halo surrounding each colony. This is quite similar to a typical lactose fermenting colony of *E. coli* on this medium. *Y. enterocolitica* strains W27724 and W27723.10s, suspected of harboring the F'lac plasmid from either *E. coli* strains CSH 24 or 23.10s, have the same morphological appearance, but are characteristically different from *Y. enterocolitica* strains W27723 and W27728. On MacConkey agar, the colonies are raised, smooth, glistening, entire, dark pink in color and have no halo surrounding each colony.

**NITROSOGUANIDINE MUTAGENESIS: SURVIVAL CURVE**

When isolating mutants of a given organism after treatment with MNNG, it is important to know the survival of the organism when exposed to this potent mutagen. The aim of a survival curve is for 50% survival of the cell population, where the highest frequency of mutations would be obtained (107). Figure 13 shows survival curves of *Y. enterocolitica* strain W277 and *E. coli* strain CSH 50 at 25°C and 37°C. The results show that mutagenesis with 50 µg/ml of MNNG for 10 minutes at 25°C and 2-3 minutes at 37°C causes 50% killing of *Y. enterocolitica* strain W277 compared to a time of 30 minutes exposure to the same concentration of MNNG for *E. coli* strain CSH 50.
Figure 9. Lactose fermenting colonies of *Y. enterocolitica* strain W27723 (left), suspected of harboring the F'lac plasmid from *E. coli* CSH 23, compared with non-lactose fermenting colonies of *Y. enterocolitica* strain W277 (wild type) (right) on MacConkey agar. LF - Lactose Fermenting, NLF - Non-Lactose Fermenting.

Figure 10. A mixture of lactose fermenting colonies of *Y. enterocolitica* strain W27723, suspected of harboring the F'lac plasmid from *E. coli* CSH 23, with non-lactose fermenting colonies of *Y. enterocolitica* strain W277 (wild type) on MacConkey agar. LF - Lactose Fermenting, NLF - Non-Lactose Fermenting.
Figure 11. A comparison of lactose fermenting colonies of *Y. enterocolitica* strain W27723 (left), suspected of harboring the F'lac plasmid from *E. coli* CSH 23, with *Y. enterocolitica* strain W27724 (right), suspected of harboring the F'lac plasmid from *E. coli* CSH 24, on MacConkey agar.

Figure 12. A comparison of lactose fermenting colonies of *Y. enterocolitica* strain W27728 (left), suspected of harboring the F'lac plasmid from *E. coli* CSH 28, with *Y. enterocolitica* strain W27723.10s (right), suspected of harboring the F'lac plasmid from *E. coli* strain 23.10s, on MacConkey agar.
Figure 13. Survival curves of E. coli strain CSH 50 and Y. enterocolitica strain W277 mutagenized with 50 μg/ml of MNNG at 25°C and 37°C. E. coli strain CSH 50 25°C ———○; E. coli strain CSH 50 37°C ———○; Y. enterocolitica strain W277 25°C ———●; Y. enterocolitica strain W277 37°C ———○.
ISOLATION OF AUXOTROPHIC MUTANTS

A polyauxotrophic mutant of *Y. enterocolitica* strain W277 was isolated after several cycles of MNNG mutagenesis and enrichment with 100 μg/ml of nalidixic acid. Tables IX and X show the isolation of histidine auxotrophic mutants from *Y. enterocolitica* strain W277 using 3 hours and 18 hours incubation of cells with nalidixic acid. For strains of *E. coli*, incubation of cells for 3 hours with this antibiotic enriches for a large number of auxotrophic mutants (66). Only 0.1% of the total population of cells of *Y. enterocolitica* strain W277 was auxotrophic for histidine after 3 hours incubation with nalidixic acid. Approximately 6.0% of the cell population was auxotrophic for histidine after enrichment for 18 hours. Tables XI and XII show the successive isolation of methionine auxotrophic mutants from a histidine requiring mutant of *Y. enterocolitica* strain W277 and the isolation of proline auxotrophs from the histidine and methionine requiring mutant of this organism. Attempts to isolate cysteine and tryptophan auxotrophic mutants from the histidine and methionine requiring mutant of *Y. enterocolitica* strain W277 were unsuccessful (Tables XIII and XIV). After each successive enrichment procedure, the mutants were tested for their sensitivity to nalidixic acid and for the auxotrophic stability of each marker as described in Materials and Methods. The mutants remained sensitive to 100 μg/ml of nalidixic acid through three successive enrichment procedures and the auxotrophic markers showed no reversion to the prototrophic type.
Table IX. Isolation of histidine auxotrophic mutants from *Y. enterocolitica* strain W277 at 25°C after 3 hours incubation with 100 µg/ml of nalidixic acid.

<table>
<thead>
<tr>
<th>DILUTION</th>
<th>MINIMAL GLUCOSE&lt;sup&gt;b&lt;/sup&gt; PLUS HISTIDINE</th>
<th>MINIMAL GLUCOSE MINUS HISTIDINE</th>
<th>MINIMAL GLUCOSE MINUS HISTIDINE</th>
<th>PERCENT AUXOTROPHY</th>
</tr>
</thead>
<tbody>
<tr>
<td>10&lt;sup&gt;-7&lt;/sup&gt;</td>
<td>976</td>
<td>975</td>
<td>975</td>
<td>0.1%</td>
</tr>
<tr>
<td>10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>198</td>
<td>198</td>
<td>198</td>
<td>0%</td>
</tr>
</tbody>
</table>

<sup>a</sup>The viable cell counts consist of the number of colonies tested from duplicate plates at the given dilution.

<sup>b</sup>The colonies were replica plated in succession onto the various selection plates. Auxotrophic colonies were retested for growth with and without the required amino acid.
Table X. Isolation of histidine auxotrophic mutants from *Y. enterocolitica* strain W277 at 25°C after 18 hours incubation with 100 μg/ml of nalidixic acid.

<table>
<thead>
<tr>
<th>DILUTION</th>
<th>MINIMAL GLUCOSE PLUS HISTIDINE</th>
<th>MINIMAL GLUCOSE MINUS HISTIDINE</th>
<th>MINIMAL GLUCOSE MINUS HISTIDINE</th>
<th>PERCENT AUXOTROPHY</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^-7</td>
<td>305</td>
<td>288</td>
<td>288</td>
<td>5.57%</td>
</tr>
<tr>
<td>10^-8</td>
<td>45</td>
<td>42</td>
<td>42</td>
<td>6.67%</td>
</tr>
</tbody>
</table>

^aThe viable cell counts consist of the number of colonies tested from duplicate plates at the given dilution.

^bThe colonies were replica plated in succession onto the various selection plates. Auxotrophic colonies were retested for growth with and without the required amino acid.
Table XI. Isolation of methionine auxotrophic mutants from a histidine requiring mutant of *Y. enterocolitica* strain W277 at 25°C after 18 hours incubation with 100 μg/ml of nalidixic acid.

<table>
<thead>
<tr>
<th>DILUTION</th>
<th>MINIMAL GLUCOSE&lt;sup&gt;b&lt;/sup&gt;</th>
<th>MINIMAL GLUCOSE</th>
<th>MINIMAL GLUCOSE</th>
<th>PERCENT AUXOTROPHY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PLUS HIS MET</td>
<td>PLUS HISTIDINE</td>
<td>PLUS HISTIDINE</td>
<td></td>
</tr>
<tr>
<td>10^-7</td>
<td>180</td>
<td>152</td>
<td>152</td>
<td>15.56%</td>
</tr>
<tr>
<td>10^-8</td>
<td>36</td>
<td>27</td>
<td>27</td>
<td>25.00%</td>
</tr>
</tbody>
</table>

<sup>a</sup>The viable cell counts consist of the number of colonies tested from duplicate plates at the given dilution.

<sup>b</sup>The colonies were replica plated in succession onto the various selection plates. Auxotrophic colonies were retested for growth with and without the required amino acid.
Table XII. Isolation of proline auxotrophic mutants from a histidine and methionine requiring mutant of *Y. enterocolitica* strain W277 at 25°C after 18 hours incubation with 100 μg/ml of nalidixic acid.

<table>
<thead>
<tr>
<th>DILUTION</th>
<th>VIALBE CELL COUNTS&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MINIMAL GLUCOSE&lt;sup&gt;b&lt;/sup&gt; PLUS HIS MET PRO</td>
</tr>
<tr>
<td>10&lt;sup&gt;-7&lt;/sup&gt;</td>
<td>395</td>
</tr>
<tr>
<td>10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>65</td>
</tr>
</tbody>
</table>

<sup>a</sup>The viable cell counts consist of the number of colonies tested from duplicate plates at the given dilution.

<sup>b</sup>The colonies were replica plated in succession onto the various selection plates. Auxotrophic colonies were retested for growth with and without the required amino acid.
Table XIII. Isolation of cysteine auxotrophic mutants from a histidine and methionine requiring mutant of *Y. enterocolitica* strain W277 at 25°C after 18 hours incubation with 100 μg/ml of nalidixic acid.

<table>
<thead>
<tr>
<th>DILUTION</th>
<th>MINIMAL GLUCOSE&lt;sup&gt;b&lt;/sup&gt; PLUS HIS MET CYS</th>
<th>MINIMAL GLUCOSE PLUS HIS MET</th>
<th>MINIMAL GLUCOSE PLUS HIS MET</th>
<th>PERCENT AUXOTROPY</th>
</tr>
</thead>
<tbody>
<tr>
<td>10&lt;sup&gt;-7&lt;/sup&gt;</td>
<td>159</td>
<td>159</td>
<td>159</td>
<td>0%</td>
</tr>
<tr>
<td>10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>33</td>
<td>33</td>
<td>33</td>
<td>0%</td>
</tr>
</tbody>
</table>

<sup>a</sup>The viable cell counts consist of the number of colonies tested from duplicate plates at the given dilution.

<sup>b</sup>The colonies were replica plated in succession onto the various selection plates. Auxotrophic colonies were retested for growth with and without the required amino acid.
Table XIV. Isolation of tryptophan auxotrophic mutants from a histidine and methionine requiring mutant of \textit{Y. enterocolitica} strain W277 at 25°C after 18 hours incubation with 100 μg/ml of nalidixic acid.

<table>
<thead>
<tr>
<th>DILUTION</th>
<th>MINIMAL GLUCOSE$^b$ PLUS HIS MET TRP</th>
<th>MINIMAL GLUCOSE PLUS HIS MET</th>
<th>MINIMAL GLUCOSE PLUS HIS MET</th>
<th>PERCENT AUXOTROPHY</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-7}$</td>
<td>153</td>
<td>153</td>
<td>153</td>
<td>0%</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>17</td>
<td>17</td>
<td>17</td>
<td>0%</td>
</tr>
</tbody>
</table>

$^a$The viable cell counts consist of the number of colonies tested from duplicate plates at the given dilution.

$^b$The colonies were replica plated in succession onto the various selection plates. Auxotrophic colonies were retested for growth with and without the required amino acid.
ISOLATION OF NALIDIXIC ACID RESISTANT MUTANTS

Nalidixic acid resistant mutants of the polyauxotrophic Y. enterocolitica strain W2773 were easily isolated. At a concentration of 20 \( \mu g/ml \) of nalidixic acid, an average of 251 resistant colonies were isolated per \( 7.0 \times 10^9 \) viable cells per ml. Because nalidixic acid resistance is known to afford an organism with additional requirements for growth (62, 108), each marker of Y. enterocolitica strain W2773 was retested as described in Materials and Methods. The organism required only histidine, methionine, and proline. All mutants resistant to 20 \( \mu g/ml \) of nalidixic acid were also resistant to 100 \( \mu g/ml \) of the antibiotic as well.

SELECTION OF F' \(_{ts}^{lac}\) PLASMID INTEGRATION

Table XV illustrates the selection of F' \(_{ts}^{lac}\) lac plasmid integrations in Y. enterocolitica strain W27724 at 41°C. Approximately 2.0% of the total cell population carried possible F' \(_{ts}^{lac}\) lac integrations. These colonies were retested as described in Materials and Methods and two were chosen for gene transfer studies with the polyauxotroph, Y. enterocolitica strain W2773.

F' LAC PLASMID-MEDIATED CHROMOSOME TRANSFER

Y. enterocolitica strains W27723, W27724, W27728, and W27723.10s were tested for their ability to behave as gene donors in crosses with the polyauxotroph, Y. enterocolitica strain W2773. An 18 hour broth mating system and a Millipore membrane filter mating system were employed to facilitate chromosome transfer. None of the markers selected for in this study (histidine, methionine, or proline) were transferred
Table XV. Selection of F'_{ts} lac plasmid integrations in Y. enterocolitica strain W27724 at 41°C.

<table>
<thead>
<tr>
<th>DILUTION</th>
<th>TOTAL NUMBER OF Viable CELLS</th>
<th>NUMBER OF LACTOSE NEGATIVE CELLS</th>
<th>NUMBER OF POSSIBLE F'_{ts} LAC INTEGRATIONS</th>
<th>PERCENTAGE OF POSSIBLE F'_{ts} LAC INTEGRATIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^{-7}^a</td>
<td>5555</td>
<td>5395</td>
<td>160^b</td>
<td>2.88%</td>
</tr>
<tr>
<td>10^{-8}</td>
<td>1216</td>
<td>1194</td>
<td>22</td>
<td>1.81%</td>
</tr>
</tbody>
</table>

^a Dilutions of a fresh overnight of Y. enterocolitica strain W27724 that yielded approximately 500 to 1,000 colonies per plate were plated onto lactose tetrazolium plates which were preheated to 41°C. The plates were incubated at 41°C for 48 hours. Control plates of the same dilutions were incubated at 25°C for 24 hours.

^b Colonies that were suspected of carrying F'_{ts} lac plasmid integrations were tested by continuously streaking them onto lactose tetrazolium agar plates and incubating at 41°C. Those colonies that segregated the least number of lactose negative colonies were chosen for further experimentation.
by the donors to the auxotrophic recipient. Matings between the wild
type *Y. enterocolitica* strain W277 and the polyauxotroph also proved
to be sterile using either system of mating (Tables XVI and XVII).

**F'lac Plasmid Transfer Involving Donor and Recipient Strains of *Y. enterocolitica*.

Due to the sterility of the gene transfer experiments, F'lac
plasmid transfer involving donor and recipient strains of *Y. enterocolitica* was examined to ascertain if the plasmids actually existed
within the donor strains. A 60 minute and 18 hour broth mating system
and a Millipore membrane filter mating system were employed. The 60
minute broth mating system proved to be sterile as no F'lac plasmid
transfer was observed (Table XVIII). Table XIX reveals that, by
allowing the mating mixtures to incubate for a period of 18 hours,
transfer of the F'lac plasmid was accomplished in two of the four mating
pairs. Two mating pairs proved to be sterile. The frequency of F'lac
plasmid transfer, with respect to donor input, was approximately $10^{-5}$.
A Millipore membrane filter mating system increased the frequency of
F'lac plasmid transfer to approximately $10^{-4}$ for two of the four mating
pairs. Again, the same two mating pairs proved to be sterile (Table
XX). All suspected transconjugant colonies were checked for the lactose
marker and verified that they were of the *Y. enterocolitica* strain
W2772 background as described in Materials and Methods before calculating
the frequencies of F'lac plasmid transfer.

Nalidixic acid, in a final concentration of 20 µg/ml, was used as
the counterselecting agent and resistant mutants of *Y. enterocolitica*
strain W2772 were isolated. Because nalidixic acid resistance can
Table XVI. F'lac plasmid-mediated chromosome transfer involving donor and recipient strains of *Y. enterocolitica* using an 18 hour broth mating system.

<table>
<thead>
<tr>
<th>CONJUGATION</th>
<th>NUMBER OF RECOMBINANTS/ML&lt;sup&gt;b&lt;/sup&gt;</th>
<th>FREQUENCY OF CHROMOSOME TRANSFER</th>
</tr>
</thead>
<tbody>
<tr>
<td>DONOR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>HIS&lt;sup&gt;+&lt;/sup&gt;</td>
<td>MET&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>W27723</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>W27724</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>W27728</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>W27723.10s</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>W277</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Viable cell counts of the donors (donor input) are as follows:

- W27723 - 1.13 x 10<sup>9</sup> cells/ml
- W27724 - 2.10 x 10<sup>9</sup> cells/ml
- W27728 - 1.65 x 10<sup>9</sup> cells/ml
- W27723.10s - 2.44 x 10<sup>9</sup> cells/ml
- W277 - 3.04 x 10<sup>9</sup> cells/ml

<sup>b</sup>Each marker (histidine, methionine, and proline) required by the recipient was independently selected for using glucose minimal agar minus the given amino acid.
Table XVII. F'lac plasmid-mediated chromosome transfer involving donor and recipient strains of *Y. enterocolitica* using a Millipore membrane filter mating system.

<table>
<thead>
<tr>
<th>CONJUGATION</th>
<th>NUMBER OF RECOMBINANTS/ML</th>
<th>FREQUENCY OF CHROMOSOME TRANSFER</th>
</tr>
</thead>
<tbody>
<tr>
<td>DONOR</td>
<td>RECIPIENT</td>
<td>HIS⁺</td>
</tr>
<tr>
<td>W27723</td>
<td>W2773</td>
<td>0</td>
</tr>
<tr>
<td>W27724</td>
<td>W2773</td>
<td>0</td>
</tr>
<tr>
<td>W27728</td>
<td>W2773</td>
<td>0</td>
</tr>
<tr>
<td>W27723.10s</td>
<td>W2773</td>
<td>0</td>
</tr>
<tr>
<td>W277</td>
<td>W2773</td>
<td>0</td>
</tr>
</tbody>
</table>

*Viable cell counts of the donor (donor input) are as follows:*

- W27723 - 2.79 x 10⁸ cells/ml
- W27724 - 3.66 x 10⁸ cells/ml
- W27728 - 1.94 x 10⁸ cells/ml
- W27723.10s - 2.87 x 10⁸ cells/ml
- W277 - 4.30 x 10⁸ cells/ml

*Each marker (histidine, methionine, and proline) required by the recipient was independently selected for using glucose minimal agar minus the given amino acid.*
Table XVIII. Direct selection of F'lac plasmid transfers involving donor and recipient strains of Y. enterocolitica using a 60 minute broth mating system.

<table>
<thead>
<tr>
<th>CONJUGATION</th>
<th>NUMBER OF RECOMBINANTS/ML</th>
<th>FREQUENCY OF TRANSFER OF F'LAC PLASMID</th>
</tr>
</thead>
<tbody>
<tr>
<td>DONOR</td>
<td>RECIPIENT</td>
<td></td>
</tr>
<tr>
<td>W27723</td>
<td>W2772</td>
<td>0</td>
</tr>
<tr>
<td>W27724</td>
<td>W2772</td>
<td>0</td>
</tr>
<tr>
<td>W27728</td>
<td>W2772</td>
<td>0</td>
</tr>
<tr>
<td>W27723.10s</td>
<td>W2772</td>
<td>0</td>
</tr>
</tbody>
</table>

*The frequencies of transfer of the F'lac plasmid were calculated in respect of donor input. Viable cell counts of the donors are as follows:

W27723 - 1.63 x 10^8 cells/ml
W27724 - 2.68 x 10^8 cells/ml
W27728 - 2.38 x 10^8 cells/ml
W27723.10s - 3.63 x 10^8 cells/ml
Table XIX. Direct selection of F'lac plasmid transfers involving donor and recipient strains of *Y. enterocolitica* using an 18 hour broth mating system.

<table>
<thead>
<tr>
<th>CONJUGATION</th>
<th>NUMBER OF RECOMBINANTS/ML</th>
<th>FREQUENCY OF TRANSFER OF* F'LAC PLASMID</th>
</tr>
</thead>
<tbody>
<tr>
<td>W27723, W2772</td>
<td>$1.42 \times 10^4$</td>
<td>$1.73 \times 10^{-5}$</td>
</tr>
<tr>
<td>W27724, W2772</td>
<td>$0$</td>
<td>$0$</td>
</tr>
<tr>
<td>W27728, W2772</td>
<td>$1.80 \times 10^4$</td>
<td>$1.07 \times 10^{-5}$</td>
</tr>
<tr>
<td>W27723.10s, W2772</td>
<td>$0$</td>
<td>$0$</td>
</tr>
</tbody>
</table>

*The frequencies of transfer of the F'lac plasmid were calculated in respect of donor input. Viable cell counts of the donors are as follows:

W27723 - $8.20 \times 10^8$ cells/ml
W27724 - $3.53 \times 10^9$ cells/ml
W27728 - $1.69 \times 10^9$ cells/ml
W27723.10s - $5.48 \times 10^9$ cells/ml
Table XX. Direct selection of F'lac plasmid transfers involving donor and recipient strains of Y. enterocolitica using a Millipore membrane filter mating system.

<table>
<thead>
<tr>
<th>CONJUGATION</th>
<th>NUMBER OF RECOMBINANTS/ML</th>
<th>FREQUENCY OF TRANSFER OF F'LAC PLASMID</th>
</tr>
</thead>
<tbody>
<tr>
<td>W27723</td>
<td>1.37 x 10^5</td>
<td>7.33 x 10^{-4}</td>
</tr>
<tr>
<td>W27724</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>W27728</td>
<td>1.09 x 10^5</td>
<td>6.09 x 10^{-4}</td>
</tr>
<tr>
<td>W27723.10s</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The frequencies of transfer of the F'lac plasmid were calculated in respect of donor input. Viable cell counts of the donors are as follows:

W27723 - 1.87 x 10^9 cells/ml
W27724 - 2.68 x 10^8 cells/ml
W27728 - 1.79 x 10^8 cells/ml
W27723.10s - 3.63 x 10^8 cells/ml
afford an organism with additional requirements for growth (62,108), the recipient was tested for growth on glucose minimal medium with and without the amino acid proline. It was suspected that lack of phenotypic expression of the F'lac plasmid in the recipient, after being transferred from Y. enterocolitica strains W27724 and W27723.10s, was due to the recipient requiring proline for growth. Phenotypic expression of the F'lac pro A⁺B⁺ plasmid was observed after being transferred from Y. enterocolitica strains W27723 and W27728 to the recipient strain of this organism. No requirement for proline was observed in the recipient strain.

CURING OF THE F'LAC PLASMID

Table XXI shows the results of curing of the F'lac plasmid from E. coli strain CSH 23 and the four donor strains of Y. enterocolitica using acridine orange. Approximately 27% of the total cell population of E. coli strain CSH 23 was cured of the plasmid at 30°C using 75 μg/ml of acridine orange. Only 1% of the control cells were lactose negative. All four donor strains of Y. enterocolitica showed very poor curing of the F'lac plasmid after 18 hours incubation in 125 μg/ml of acridine orange at 30°C. No significant increase in lactose negative colonies compared to control cultures was observed. The genes coding for lactose were relatively stable in the donor strains of Y. enterocolitica as the spontaneous loss of the ability to ferment lactose occurred at a low frequency. Segregation of lactose negative colonies in the control cultures of the four donor strains of Y. enterocolitica ranged from 0.0 to 7.7% of the total cell population, depending on the strain.
Table XXI. Curing of the F'lac plasmid from *E. coli* CSH 23 and four F'lac plasmid donor strains of *Y. enterocolitica* with acridine orange at 30°C.

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>TOTAL VIABLE CELLS/ML</th>
<th>NUMBER OF LACTOSE POSITIVE CELLS/ML</th>
<th>NUMBER OF LACTOSE NEGATIVE CELLS/ML</th>
<th>PERCENT LACTOSE NEGATIVE CELLS/ML</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSH 23 (Control)</td>
<td>$1.40 \times 10^{10}$</td>
<td>$1.38 \times 10^{10}$</td>
<td>$2.00 \times 10^{8}$</td>
<td>1.43%</td>
</tr>
<tr>
<td>CSH 23 (75 ug/ml)</td>
<td>$9.95 \times 10^{9}$</td>
<td>$7.30 \times 10^{9}$</td>
<td>$2.65 \times 10^{9}$</td>
<td>26.60%</td>
</tr>
<tr>
<td>W27723 (Control)</td>
<td>$8.45 \times 10^{9}$</td>
<td>$7.80 \times 10^{9}$</td>
<td>$6.50 \times 10^{8}$</td>
<td>7.69%</td>
</tr>
<tr>
<td>W27723 (125 ug/ml)</td>
<td>$2.56 \times 10^{9}$</td>
<td>$2.43 \times 10^{9}$</td>
<td>$1.40 \times 10^{8}$</td>
<td>5.47%</td>
</tr>
<tr>
<td>W27724 (Control)</td>
<td>$3.56 \times 10^{9}$</td>
<td>$3.56 \times 10^{9}$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>W27724 (125 ug/ml)</td>
<td>$2.60 \times 10^{9}$</td>
<td>$2.59 \times 10^{9}$</td>
<td>$1.00 \times 10^{7}$</td>
<td>0.38%</td>
</tr>
<tr>
<td>W27728 (Control)</td>
<td>$1.64 \times 10^{10}$</td>
<td>$1.64 \times 10^{10}$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>W27728 (125 ug/ml)</td>
<td>$4.35 \times 10^{9}$</td>
<td>$4.30 \times 10^{9}$</td>
<td>$5.00 \times 10^{7}$</td>
<td>1.15%</td>
</tr>
<tr>
<td>W27723.10s (Control)</td>
<td>$2.08 \times 10^{9}$</td>
<td>$2.07 \times 10^{9}$</td>
<td>$1.00 \times 10^{7}$</td>
<td>0.48%</td>
</tr>
<tr>
<td>W27723.10s (125 ug/ml)</td>
<td>$2.25 \times 10^{9}$</td>
<td>$2.22 \times 10^{9}$</td>
<td>$3.00 \times 10^{7}$</td>
<td>1.33%</td>
</tr>
</tbody>
</table>

*aConcentration of acridine orange that cells were exposed to.*
In preceding experiments, *Y. enterocolitica* strains W27724 and W27723.10s did not have the ability to transfer F'lac plasmids to a recipient strain of *Y. enterocolitica*. An exploratory study was conducted to examine the ability of these two strains to ferment lactose at 41°C. Dilutions of overnight cultures of the two strains were spread over the surface of duplicate sets of lactose tetrazolium plates and MacConkey agar plates. One set of plates was incubated at 41°C and the other at 25°C. It was found that both donor strains showed a weak fermentation of lactose at 41°C on both types of medium used. Also, lactose tetrazolium plates proved more sensitive in detecting the decreased ability of both strains to ferment lactose as the majority of all colonies were lactose negative on this medium. On MacConkey agar, many more colonies of both strains demonstrated the ability to ferment lactose, but the ability was diminished in comparison with the control plates at 25°C.

Another exploratory experiment was conducted to investigate the isolation of spontaneous lactose fermenting mutants from the wild type (lactose negative) *Y. enterocolitica* strain W277. In this study, 0.1 ml of a 10⁻¹ dilution of a fresh overnight of this organism was spread over the surface of six lactose minimal agar plates. After 5-7 days incubation at 25°C, 18 small colonies (0.5 to 1.0 mm in diameter) were isolated. These colonies were streaked on lactose minimal medium and MacConkey agar. Only two colonies showed any increased ability to ferment lactose, as they grew on lactose minimal medium and displayed pink colonies on MacConkey agar after 48 hours incubation at 25°C. These colonies showed marked similar-
ities in morphological appearance to the lactose fermenting donor strains of \textit{Y. enterocolitica} (W27724 and W27723.10s) previously isolated, but not to \textit{Y. enterocolitica} strains W27723 and W27728 (Figures 9 thru 12).
At the present time, there is well documented evidence that the conjugal system employing plasmid-mediated chromosome gene transfer is useful in delineating various genetic mechanisms in a diverse group of organisms. The system has been extended to such organisms as Neisseria gonorrhoeae (130,139), Pseudomonas fluorescens (105), Proteus species (47), species of the genus Erwinia (25,26,27), Yersinia pestis and pseudotuberculosis (88,90,103,116), as well as Escherichia coli (61). The system employed for Y. pestis was unsuccessfully extended to Y. enterocolitica in the early 1970's (90). The purpose of the present study, therefore, was to develop a genetic system that would enable the successful transfer of the bacterial chromosome from donor to recipient strains of Y. enterocolitica employing F'lac plasmids. In order to do this, standard techniques used in conjugal studies involving E. coli were modified and experimental conditions were refined so as to establish a workable system for Y. enterocolitica.

Initially, experiments were done to determine the growth of this organism in enriched LB medium at 25°C and 37°C. By using the growth curves obtained for Y. enterocolitica strain W277 and a strain of E. coli as a reference, the number of viable cells per ml in a given sample could be easily estimated for the two temperatures used. A peculiarity was observed in the growth of Y. enterocolitica strain W277. Marked similarities of OD readings and viable cell counts of the organism at 25°C and 37°C suggested that this organism is capable of growing equally well at both temperatures. This is in contrast to other members of the Family Enterobacteriaceae, such as E. coli,
which exhibit a slower rate of growth at temperatures below 37°C. Chester et al. (29) and Abbas et al. (unpublished data) have also observed this phenomenon. Clearly, more research into this matter would be in order.

The studies conducted on the growth of *Y. enterocolitica* strain W277 on various minimal medium were consistent with those of Burrows and Gillett (18), who have examined the nutritional requirements of the genus *Yersinia* on minimal agar medium at 28°C and 37°C. They found that all strains of *Y. enterocolitica* required thiamine at both temperatures with an additional requirement of cysteine and/or methionine for growth at 37°C. It was found that *Y. enterocolitica* strain W277 does not require additional growth factors beyond thiamine at either 25°C or 37°C, but additional requirements were necessary to support good growth at 40°C.

Nilehn (120) has investigated the growth of 330 strains of *Y. enterocolitica* on single carbohydrates in minimal medium at 25°C and 37°C. Differences in growth requirements at different temperatures appeared among the strains. Only 2 out of 330 strains grew in minimal glucose medium at 37°C while the majority of strains readily grew at 25°C in the same medium. The majority of the strains capable of utilizing glucose at 25°C did not grow in lactose minimal medium at this temperature or 37°C. Strains growing in lactose minimal medium usually gave visible turbidity after an extended period of incubation. The β-galactosidase activity of these strains was best demonstrable at 25°C where positive reactions were stronger and developed more rapidly than at 37°C. Upon investigating the growth of *Y. enterocolitica* strain W277
on minimal agar medium with different carbohydrates, it was evident that minimal medium containing glucose, galactose, and a combination of glucose and galactose readily supported the growth of this organism after 2 days incubation at 25°C and 37°C without additional growth factors. At 40°C, additional incubation time is required to support good growth of this organism. The organism did not grow on lactose minimal medium at 37°C and 40°C and grew poorly at 25°C.

When investigating the units and specific activities of β-galactosidase in *Y. enterocolitica* strain W277 at 25°C and 37°C, it was evident that functional β-galactosidase was present in this organism at both temperatures. Unlike *E. coli*, the units and activities of the enzyme were inversely related to the temperature. Observations of growth of this organism on lactose minimal medium at 25°C is not surprising, even though this organism is considered to be unable to utilize or ferment lactose. This would be of some concern to clinicians because occurrence of these lactose positive variants in nature from a normally lactose negative pathogenic bacteria would obscure routine identification of the pathogen. LeMinor (97) has demonstrated that 9 strains of *Y. enterocolitica* lacked a functional β-galactosidase enzyme in comparison with other genera of bacteria. In assaying for units of the enzyme in these strains, however, the study was accomplished at 37°C but not at 25°C so information concerning the ability of this organism to actually utilize lactose was incomplete. A review of the literature revealed a limited knowledge in this area of interest for *Y. enterocolitica*. Further investigation would be warranted.

Attempts were made to isolate streptomycin resistant mutants of *Y.*
enterocolitica strain W277. In these studies, an increase in the number of resistant mutants to this antibiotic was observed at 25°C compared with 37°C. Like numerous biochemical reactions tested in this organism (13,120), the isolation of increased numbers of streptomycin resistant mutants appeared to be related to the temperature of incubation.

If streptomycin sensitive cultures of E. coli are spread over the surface of an agar plate containing 100 μg/ml of streptomycin, only 1 in 10^9 cells can form colonies. These resistant colonies are placed in three different classes: (a) mutants with slight resistance that can withstand only the original concentration of 100 μg/ml of streptomycin, but are killed by higher concentrations, (b) mutants with intermediate resistance that can withstand up to 500 μg/ml of streptomycin, and (c) mutants with high resistance that can withstand even the highest streptomycin concentrations employed (141). Evidence in this study suggested that the frequency of resistant colonies isolated per 1 x 10^10 cells per ml was much greater for Y. enterocolitica strain W277 in comparison to strains of E. coli at both 25°C and 37°C. Also, the vast majority of resistant colonies of Y. enterocolitica strain W277 showed a high level resistance to streptomycin compared to slight and intermediate resistance observed for E. coli. A large number of nalidixic acid resistant colonies were also isolated for Y. enterocolitica strain W2773 at 25°C. At a concentration of 20 μg/ml of this antibiotic, an average of 251 resistant colonies were isolated per 7.0 x 10^9 viable cells per ml. These mutants were also resistant to high levels of nalidixic acid. Kimura (81,82,83) and Kanazawa (75,76) have shown that streptomycin resistance factors in some strains of Y. enterocolitica
and *Y. pseudotuberculosis* were carried by conjugative R plasmids. They observed that *Y. enterocolitica* could accept and transfer these plasmids at high frequencies, conferring a high level resistance to the antibiotic in cells that carried the R plasmid. The possibility exists that some of the streptomycin resistant mutants isolated from *Y. enterocolitica* strain W277 carry a conjugative R plasmid which confers a high level resistance to this antibiotic. The increase in number of resistant mutants isolated for *Y. enterocolitica* strain W277 compared with *E. coli* could be due, in part, to this drug resistant (R) plasmid, which would be transmissible between donor and recipient cells in a wild type population, such as *Y. enterocolitica* strain W277. However, this would not account for the temperature sensitivity in streptomycin resistance observed in this organism, unless the R plasmid was not capable of replicating at higher temperatures. Due to time limitations, R plasmid transfer studies and comparative studies on different temperatures and different organisms were not done. However, further investigation into the nature of streptomycin resistance and antibiotic resistance, in general, would be warranted in *Y. enterocolitica*.

As previously stated, several investigators have found that *Y. enterocolitica* has the ability to accept and transfer drug resistant plasmids at a relatively high frequency. Attempts to isolate and transfer naturally occurring R plasmids in this organism have been successful (9,32,75,76,81,82,83,132). Cornelis et al. (36) have shown a derepressed R factor to be transferred at a frequency of 5.0 x 10^{-3} between two strains of *Y. enterocolitica* mated on a membrane. Under the same conditions, transfer of this R plasmid from *E. coli* to *Y. entero-
coli was observed at a frequency of only $7.7 \times 10^{-6}$. This frequency was increased when the recipient strain was heat-treated before mating. The conditions for optimum fertility were $50^\circ-52^\circ$C for a period of 2-3 minutes. An F' lac plasmid from *E. coli* was also transferred to *Y. enterocolitica*, with the observation that the number of transfers per $5 \times 10^6$ donor cells increased with heat-treatment of the recipient. A self-transmissible lac plasmid, pGCl, that originated in *Y. enterocolitica*, has also been described (37). This plasmid is freely transmissible between strains of *Y. enterocolitica* at frequencies of $10^{-2}$ to $10^{-3}$ and to strains of *E. coli* at frequencies of $10^{-4}$ to $10^{-6}$. Chatterjee et al. (25) have shown the transfer of F' lac plasmids to recipients of *Erwinia* species to vary from $10^{-5}$ to $10^{-8}$. It was demonstrated by Falkow et al. (47) that donor strains of *E. coli* and *Salmonella typhosa* could transfer F' lac plasmids to recipient *Proteus* species at a frequency of $10^{-4}$ to $10^{-8}$. When mated with recipient strains of *E. coli* and *S. typhimurium*, *Proteus* donor strains transferred the F' lac plasmids at similar frequencies.

F' lac plasmids were transferred from strains of *E. coli* to *Y. enterocolitica* strain W2771 at a frequency of $10^{-6}$ which is consistent with current literature for intergeneric transfer of this plasmid. Contrary to previous findings, heat-treatment of the recipient prior to mating resulted in no increase in the frequency of F' lac plasmid transfer. Transfer of the F' lac plasmid between donor and recipient strains of *Y. enterocolitica* revealed a frequency of transfer of $10^{-5}$ in an 18 hour broth mating system and a frequency of $10^{-4}$ using a Millipore membrane filter mating system.
The F'lac plasmid could not be transferred from *Y. enterocolitica* donor strains W27724 and W27723.10s to the recipient strain of this organism. There are several possible explanations for this. First, the F'lac plasmid, harbored in the two donor strains, could have integrated into the bacterial chromosome during prolonged storage and when retested could not be transferred by itself. Mergeay and Geritis (105) have shown this to be the case for approximately 50% of *Pseudomonas fluorescens* strain 6.2 transconjugants that harbored F'lac plasmids from *E. coli*. They suggested the possibility of integration of parts of the plasmid into the genome of *P. fluorescens* but this was not experimentally examined. Secondly, evidence was presented in Table IV that there exists a functional β-galactosidase enzyme in the wild type *Y. enterocolitica* strain W277. Therefore, the possibility exists that spontaneous mutants, with an increased level of β-galactosidase and subsequent increased ability to ferment lactose, were isolated from the lactose negative recipient during the selection for F'lac plasmid transfer between *E. coli* and *Y. enterocolitica*. However, in this selection process, background growth of the recipient, *Y. enterocolitica* strain W2771, presented no problem. Lastly, there exists the possibility that the F'lac plasmid, harbored in *Y. enterocolitica* strains W27724 and W27723.10s, did not function properly resulting in no expression of sexuality in the bacterial cells, i.e., no pili formation or conjugal pair formation. This would result in an inhibition of conjugation and plasmid transfer. Transfer of the F'lac plasmid from *Y. enterocolitica* strains W27723 and W27728 to a recipient of the same organism suggested that the phenotypic surface alterations associated with F+ and F−
factors which are necessary for conjugation were functional in these two donor strains.

It was suspected that *Y. enterocolitica* strain W27724 carried a temperature-sensitive F'lac plasmid that could not replicate in the autonomous state at 40°C-43°C. If the plasmid were integrated within the host chromosome, it could replicate and lactose would be fermented. An experiment was conducted to select for these integrations at 41°C. A problem encountered in this study, was the fact that *Y. enterocolitica* strain W27724 showed moderate growth at this temperature and required prolonged incubation for good growth in comparison to growth at 25°C or 37°C. *Y. enterocolitica* strain W27723.10s was suspected of carrying an F'lac plasmid which was not temperature sensitive and would ferment lactose at 41°C. Evidence presented in this study suggested that both of the donor strains showed a sensitivity to high temperatures as segregation of a large number of lactose negative colonies from both strains was observed at 41°C. This observation implied that the F'lac plasmid, whether integrated or in the autonomous state, had an impaired function at the higher temperatures. Falkow et al. (47) studied the synthesis of β-galactosidase in *Proteus* species harboring the F'lac plasmid from *E. coli* to determine whether enzyme synthesis was affected by a diverse cellular environment and whether *Proteus* species could correctly interpret the information contained in DNA of different base composition. They found that the regulatory functions of *E. coli* lactose genes were impaired in *Proteus* species. They also showed an impairment in the function of the sex factor in this genus. *Proteus* species, harboring the F'lac plasmid from *E. coli*, were poor plasmid
donors and did not exhibit the changes in surface properties normally associated with the presence of an F\(^+\) or F'-factor. More research into the functional capabilities of the \textit{E. coli} F'lac plasmid in the four donor strains of \textit{Y. enterocolitica} would be in order.

Attempts to cure the F'lac plasmid from \textit{Y. enterocolitica} strains W27724 and W27723.10s using acridine orange produced no evidence for the existence of the plasmid in these organisms. Curing with acridine orange was also unsuccessful in the two fertile donor strains of \textit{Y. enterocolitica}. This does not seem unusual for this organism, however, as Lawton and Stull (89) have shown that curing of the F'lac plasmid from \textit{Y. pseudotuberculosis} was poor and that none of the lactose positive clones tested exhibited an increased ability to transfer chromosome markers. This was disappointing because Chatterjee and Starr (26) have successfully used this technique to isolate Hfr strains of \textit{Erwinia amylovora} by selecting for clones resistant to curing by acridine orange. They found that, in the presence of 1.0 \(\mu\)g/ml of acridine orange, F'lac plasmid-containing strains of this organism demonstrated 89% segregation to lactose negative colonies. Colonies resistant to curing were then screened for the ability to transfer chromosome markers and Hfr strains of \textit{E. amylovora} were isolated. The need for future research in this area could prove beneficial in isolating Hfr strains of \textit{Y. enterocolitica} if an operative system could be established to differentiate integrated F'lac plasmid clones from those not possessing integrations.

It seems quite likely that \textit{Y. enterocolitica} strains W27724 and W27723.10s were lactose fermenting mutants of the recipient strain used
in this study rather than transconjugants. Two mutants were obtained from the wild type *Y. enterocolitica* strain W277 that closely resembled the colonies illustrated in Figures 11 and 12 for *Y. enterocolitica* strains W27724 and W27723.10s. Also, experiments conducted on the β-galactosidase activity of *Y. enterocolitica* strain W277 (wild type) showed that the units and activities of the enzyme were inversely related to the temperature. Furthermore, *Y. enterocolitica* has many biochemical reactions that are temperature dependent; some of which are expressed at 25°C but not at 37°C (13,120). This could account for the impaired lactose fermentation observed at 41°C for *Y. enterocolitica* strains W27724 and W27723.10s. However, the other proposed explanations cannot be clearly eliminated with the evidence presented herein.

Characterization of DNA from the lactose positive strains of *Y. enterocolitica* using CsCl density-gradient centrifugation and chromatographic fractionation of DNA (47) may prove fruitful in demonstrating the existence of F'lac plasmids in these strains of *Y. enterocolitica*. Studies of this nature were beyond the scope of this study.

Auxotrophic mutants of *Y. enterocolitica* strain W277 were successfully isolated using a modification of the penicillin enrichment technique (43,93). Resistance of this organism to both penicillin and ampicillin necessitated the use of the antibiotic nalidixic acid, which works on the same basic principle of lysis of growing cells and enrichment of non-growing auxotrophic cells under appropriate conditions. Hoffman et al. (66) isolated auxotrophic mutants from a strain of *E. coli* using this antibiotic. After mutagenesis with MNNG for 30 minutes at 37°C, a cell population was treated with 100 µg/ml of nalidixic acid
for three hours to enhance the auxotrophic population. A modification of this procedure was used to isolated auxotrophic mutants of *Y. enterocolitica* strain W277 as the results obtained in this study revealed a decreased time of exposure to MNNG was necessary to stimulate effective mutagenesis and an increased time of exposure to 100 μg/ml of nalidixic acid was required for enrichment of the auxotrophic population. Overall, the modified enrichment procedure employed was an effective system for isolating auxotrophic mutants of *Y. enterocolitica* and would serve as a valuable tool for subsequent investigations.

Nalidixic acid has been shown to selectively and reversibly inhibit DNA synthesis with little effect on ribonucleic acid (RNA) or protein synthesis, but the molecular basis of inhibition is unknown (51,52). Helling et al. (62) and Mishan'kin et al. (108) have shown that nalidixic acid resistant variants of *E. coli*, *Y. pestis* and *Vibrio cholerae* exhibited new growth requiements for a number of amino acids and concluded that the auxotrophy and resistance to nalidixic acid were the result of concurrent mutations. It was found that auxotrophic mutants of *Y. pestis*, obtained after treatment with N-nitroso-N-methylurea without use of nalidixic acid, differed in their amino acid requirements from nalidixic acid resistant variants, which presupposed the presence of mutagenic properties in nalidixic acid itself. In isolating auxotrophic mutants of *Y. enterocolitica* strain W277, evidence was presented showing the sensitivity of this organism to nalidixic acid after each successive isolation of an auxotrophic mutant. After isolating a polyauxotrophic mutant of this organism, a nalidixic acid resistant variant of the polyauxotroph was then isolated.
This mutant developed no additional requirements for growth other than the markers selected for in the enrichment technique. This evidence suggested that the enrichment procedure employed was responsible for the isolation of auxotrophic mutants of Y. enterocolitica strain W277 and that the mutants were not the result of resistance to nalidixic acid.

In 1967, Lawton et al. (88) demonstrated that strains of Y. pseudotuberculosis could accept the F'lac plasmid from E. coli and behave as gene donors in crosses with several different auxotrophs of Y. pseudotuberculosis. With further investigation, a strain of Y. pestis, harboring the F'Cm plasmid from E. coli, was found to donate its chromosome to auxotrophic recipient strains of Y. pestis, but the frequency of gene transfer in this organism was 100 times less efficient than gene transfer in Y. pseudotuberculosis. An attempt to extend this plasmid-mediated gene transfer system to different serotypes of Y. pseudotuberculosis and Y. enterocolitica by Lawton et al. (90) was unsuccessful. With this in mind, it was conceivable that gene transfer in Y. enterocolitica could be demonstrated if experimental conditions were modified to accommodate this organism. However, evidence presented in the present study suggested that establishment of a model for plasmid-mediated gene transfer in Y. enterocolitica was unsuccessful. Since the F'lac plasmids were transferred at high frequencies from Y. enterocolitica donor strains W27723 and W27728 to the recipient Y. enterocolitica strain W2772, the necessary mechanisms of F pili production and pair formation were functioning. As mentioned earlier, this may not be the case for Y. enterocolitica donor strains W27724 and W27723.10s. Two explanations can be put forth for the failure to observe gene
transfer in Y. enterocolitica. First, the origin of F'lac plasmid integration within the bacterial chromosome could be at a substantial distance from the positions of the three auxotrophic markers selected for. Therefore, transfer of these gene markers did not take place or was not detectable by the systems employed. It was apparent that polyauxotrophic mutants carrying a variety of markers would be more conducive in detecting transfer of various genes. Secondly, it was possible that poor homology between the F'lac plasmids of E. coli and the chromosome of Y. enterocolitica strain W277 did not permit the necessary integration of the F' factor within the bacterial chromosome and subsequent chromosome mobilization. Either absence of homology in the structural sense or absence of functional homology due to the lack of appropriate recombinant enzymes could be responsible. Y. pseudotuberculosis and, to some extent, Y. pestis have enough homology between the bacterial chromosome and the F'lac plasmid of E. coli to allow integration of the plasmid and mobilization of the bacterial chromosome. Using DNA hybridization studies, Brenner et al. (15) have shown that Y. pseudotuberculosis is only 40 to 60% related to Y. enterocolitica and that both species showed low (20% related to E. coli K-12) relatedness to other members of the Family Enterobacteriaceae. Clearly, more research into this matter would be in order.

Evidence has been presented in this investigation which implied that donor strains of Y. enterocolitica strain W277 did not have the capability of transferring chromosomal markers in a plasmid-mediated gene transfer system. Interesting and unusual results concerning the growth, ß-galactosidase activity, and isolation of various mutants of
this organism have been presented. F'lac plasmid transfer from strains of \textit{E. coli} to \textit{Y. enterocolitica} strain W2771 was successful but the frequency of transfer was much lower than that observed for matings involving donor and recipient strains of \textit{Y. enterocolitica}. Functional mechanisms for F-pili production and pair formation were present for two of the four donor strains of \textit{Y. enterocolitica} used in this study.

There is a need to investigate the conjugal genetics of a number of different strains and serotypes of \textit{Y. enterocolitica} and to modify existing gene transfer systems to better facilitate genetic studies of this organism. In as much as the pathogenic mechanism of \textit{Y. enterocolitica} is unknown, conjugal gene transfer studies would provide a basis for elucidating the genetic mechanism(s) controlling human and animal virulence in this organism and establish more clearly the taxonomic relatedness of this organism to other members of the Family \textbf{Enterobacteriaceae}. At this stage, it would be premature to generalize that \textit{Y. enterocolitica} is not capable of plasmid-mediated conjugal gene transfer from donor to recipient based on the examination of one strain and the systems employed.
CHAPTER V
SUMMARY

This study has presented evidence which indicates that \textit{Y. enterocolitica} strain W277 can accept the F'\textit{lac} plasmid from \textit{E. coli} and transfer this plasmid to a recipient strain of \textit{Y. enterocolitica}. Transconjugants (which harbored the F'\textit{lac} plasmid) of \textit{Y. enterocolitica} were not capable of transferring chromosome markers in crosses with a polyauxotrophic mutant of this organism.

Studies relating to the growth, \(\beta\)-galactosidase activity, and isolation of antibiotic resistant and auxotrophic mutants of this organism have presented some interesting and unusual results. \textit{Y. enterocolitica} strain W277 was found to grow equally well at both \(37^\circ\text{C}\) and \(25^\circ\text{C}\) as suggested by marked similarities of OD readings and viable cell counts of the organism at the two temperatures. The organism possessed a functional \(\beta\)-galactosidase enzyme at both \(25^\circ\text{C}\) and \(37^\circ\text{C}\). The units and activities of this enzyme were shown to be inversely related to the temperature. Like numerous biochemical reactions for this organism, the isolation of increased numbers of streptomycin resistant mutants appeared to be inversely related to the temperature of incubation as well. Also, a significantly large number of mutants were obtained for this organism compared to strains of \textit{E. coli} at both \(25^\circ\text{C}\) and \(37^\circ\text{C}\). Auxotrophic mutants of \textit{Y. enterocolitica} strain W277 were isolated using a modified nalidixic acid enrichment technique. Results revealed a decreased time of exposure to MNNG was necessary to stimulate effective mutagenesis and an increased time of exposure to 100 \(\mu\text{g/ml}\) of nalidixic acid was required for enrichment of the auxotrophic population compared to
auxotrophic enrichment techniques for *E. coli*.

The frequency of transfer of the F'lac plasmid from four donor strains of *E. coli* to *Y. enterocolitica* was $10^{-6}$ compared to a frequency of complete transfer to $10^{-2}$ exhibited for crosses involving donor and recipient strains of *E. coli*. Stability of the F'lac plasmid in the *Y. enterocolitica* strains was obtained by repeated plating of the organisms on MacConkey agar. The F'lac plasmid was transferred from transconjugants of *Y. enterocolitica* to a recipient of this organism at a frequency of $10^{-5}$ in an 18 hour broth mating system and $10^{-4}$ using a membrane filter mating system. Treatment of the *Y. enterocolitica* transconjugants with acridine orange failed to eliminate the F'lac plasmid from the organisms. Two of the four F'lac plasmid donor strains of *Y. enterocolitica* proved sterile in the ability to transfer the plasmid to a recipient strain of the same organism.

In crosses involving two fertile donor strains of *Y. enterocolitica* and a polyauxotrophic recipient of this organism, conjugal gene transfer was not observed. It was apparent that several polyauxotrophic mutants carrying a variety of markers would be more conducive in detecting transfer of various genes on the bacterial chromosome. Research involving a number of different strains and serotypes of *Y. enterocolitica* could be beneficial in disproving that this organism is not capable of transferring chromosome markers from donor to recipient in a plasmid-mediated conjugal gene transfer system.
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APPENDIX

Culture Media Formulations

LB Medium

Bacto tryptone ................................... 10.0 g
Bacto yeast extract ............................... 5.0 g
NaCl ...................................................... 10.0 g
Distilled water .................................... 1000 ml

If used in plates, add 15.0 g of Difco agar per liter.

Minimal Medium

Per liter:

Difco minimal agar .................................. 15.0 g

Salts: (in this case 1 x A)

K₂HPO₄ ................................................. 10.5 g
KH₂PO₄ .................................................. 4.5 g
(NH₄)₂SO₄ .............................................. 1.0 g
Sodium citrate • 2H₂O .............................. 0.5 g

MgSO₄ • 7H₂O Add 1.0 ml from a stock solution of 20.0 g/100 ml after autoclaving.

Bl (thiamine hydrochloride) Add 0.5 ml from a 1% stock solution.

Sugar Add 10.0 ml from a 20% stock solution.

Amino acids (as required) Add 10.0 ml from a 4 mg/ml stock solution.
Minimal Medium (continued)

Antibiotics as required*  
Streptomycin  
Nalidixic acid

The salt solution and the agar should be prepared and autoclaved separately at 15 lbs./in.\(^2\) for 15 minutes. Therefore the salts are usually prepared in more concentrated form. It is convenient to autoclave 15.0 g of agar in 500 ml of distilled water and the salts (at 2 x normal strength) in 500 ml of distilled water. The Mg\(^{++}\) and nutrients are prepared and autoclaved separately. Normally, 40 \(\mu g/ml\) of the D,L-amino acid (or 20 \(\mu g/ml\) of the L-amino acid) is sufficient. Vitamins are required in smaller amounts (1.0 \(\mu g/ml\)). These are then added to the salts after autoclaving. The vitamins must be filter sterilized.

Lactose Tetrazolium Medium

These plates work best with Difco antibiotic medium #2. Dissolve 25.5 g of this medium in 950 ml of distilled water. Add 50 mg of 2,3,5-triphenyltetrazolium chloride and continue to heat until completely dissolved. Autoclave and then add 50 ml of a 20% solution of the desired sugar (in this case lactose). It is extremely important that the tetrazolium be added before autoclaving.

*Refer to Antibiotic Stock Solutions
Antibiotic Stock Solutions

Streptomycin Sulfate (Sigma)

100 mg/ml = 2.0 g/20.0 ml

1. Weigh 2.0 g of streptomycin and dissolve in 20.0 ml distilled water.

2. Filter sterilize using a 0.22 μm Millipore membrane filter.

3. Add appropriate ml of stock solution to medium for desired concentration of streptomycin.

<table>
<thead>
<tr>
<th>Streptomycin concentration</th>
<th>ml of stock solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 μg/ml</td>
<td>0.1 ml/l</td>
</tr>
<tr>
<td>50 μg/ml</td>
<td>0.5 ml/l</td>
</tr>
<tr>
<td>100 μg/ml</td>
<td>1.0 ml/l</td>
</tr>
<tr>
<td>200 μg/ml</td>
<td>2.0 ml/l</td>
</tr>
<tr>
<td>400 μg/ml</td>
<td>4.0 ml/l</td>
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<tr>
<td>600 μg/ml</td>
<td>6.0 ml/l</td>
</tr>
<tr>
<td>800 μg/ml</td>
<td>8.0 ml/l</td>
</tr>
<tr>
<td>1000 μg/ml</td>
<td>10.0 ml/l</td>
</tr>
</tbody>
</table>

Nalidixic Acid (Sigma)

10 mg/ml = 0.1 g/10 ml or 100 mg/ml = 1.0 g/10 ml

1. Weigh 0.1 g or 1.0 g of nalidixic acid and dissolve in 10.0 ml of 1 N NaOH.

2. Sterilize by autoclaving at 121°C, 15 psi for 15 minutes.

3. Add appropriate ml of stock solution to medium for desired concentration of nalidixic acid.

Citrate Buffer (pH 5.5)

Stock Solutions

1. A: 0.1 M solution of citric acid (Dissolve 21.01 g in 1000 ml of distilled water).

2. B: 0.1 M solution of sodium citrate (Dissolve 29.41 g of...
Citrate Buffer (continued)

\[ C_6H_6O_7Na_2 \cdot 2H_2O \] in 1000 ml of distilled water.

3. Add 16.0 ml of solution A with 34.0 ml of solution B, and dilute to a total of 100 ml.

4. Adjust pH with 1 N HCl or 1 N NaOH, if necessary.

Phosphate Buffer (pH 6.5 and 7.0)

Stock Solutions

1. A: 0.2 M solution of monobasic sodium phosphate (Dissolve 27.8 g in 1000 ml of distilled water).

2. B: 0.2 M solution of dibasic sodium phosphate (Dissolve 53.65 g of \( Na_2HPO_4 \cdot 7H_2O \) in 1000 ml of distilled water).

3. For a pH of 6.5, add 68.5 ml of solution A with 31.5 ml of solution B and dilute to a total of 200 ml. Add 39.0 ml of solution A and 61.0 ml of solution B and dilute to a total of 200 ml for a pH of 7.0.

4. Adjust the pH with 1 N HCl or 1 N NaOH, if necessary.

Standard Protein Solutions

Bovine Serum Albumin (Armour)

400 \( \mu g/ml \) = 0.4 g/1000 ml

1. Prepare a 400 \( \mu g/ml \) stock solution of bovine serum albumin by dissolving 0.4 g in 1000 ml of distilled water.

2. Make the following dilutions of the protein standard:

   a. 0.5 ml (200 \( \mu g/ml \))
   b. 0.4 ml (160 \( \mu g/ml \)) plus 0.1 ml of distilled water
   c. 0.3 ml (120 \( \mu g/ml \)) plus 0.2 ml of distilled water
   d. 0.2 ml (80 \( \mu g/ml \)) plus 0.3 ml of distilled water
   e. 0.1 ml (40 \( \mu g/ml \)) plus 0.4 ml of distilled water
Reagent for Protein Determination

1. Prepare stock solutions of:
   a. 1.0% CuSO$_4$ (Dissolve 1.0 g CuSO$_4$ in 100 ml of distilled water).
   b. 2.0% Na-K tartrate (Dissolve 2.0 g Na-K tartrate in 100 ml of distilled water).
   c. 2.0% Na$_2$CO$_3$ (Dissolve 2.0 g Na$_2$CO$_3$ in 100 ml of distilled water).

2. Mix together 1.0 ml of Na-K tartrate stock solution with 1.0 ml of CuSO$_4$ stock solution.

3. To 50.0 ml of the Na$_2$CO$_3$ stock solution add 1.0 ml of the mixture prepared in step 2.

4. Make fresh each day.
RESUME

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