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

Richard L. Hodinka
The University of Montana

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

By

Richard L. Hodinka

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Approved by:

<u>ꓱn ꓢ. ꓠakamura</u>
Chairman, Board of Examiners

<u>ꓱenyr ꓒ ꓒaul</u>
Co-Chairman, Board of Examiners

<u>ꓣꓣ ꓟurray</u>
Dean, Graduate School

<u>8-1-79</u>
Date

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A Study of Conjugal Gene Transfer in <u>Yersinia</u> <u>enterocolitica</u> Strain W277 Harboring the F'lac Plasmid of <u>Escherichia</u> <u>coli</u>. (111 pp.)

Directors: Mitsuru J. Nakamura, George L. Card

    In as much as the pathogenic mechanism of <u>Yersinia</u> <u>enterocolitica</u> 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 <u>Y</u>. <u>enterocolitica</u> employing F'lac plasmids.

    Conjugal transfer of the F'lac plasmid from donor strains of <u>Escherichia</u> <u>coli</u> to recipient strains of <u>E</u>. <u>coli</u> and <u>Y</u>. <u>enterocolitica</u> and from donor to recipient strains of <u>Y</u>. <u>enterocolitica</u> 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 <u>Y</u>. <u>enterocolitica</u> 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 <u>E</u>. <u>coli</u> to <u>Y</u>. <u>enterocolitica</u> 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 <u>E</u>. <u>coli</u>. The transferred genetic elements were unstable in the <u>Y</u>. <u>enterocolitica</u> strain, but repeated plating on MacConkey agar permitted the selection of relatively stable lactose positive strains of this organism. The transconjugants of <u>Y</u>. <u>enterocolitica</u> were able to transfer the F'lac plasmid to a recipient strain of <u>Y</u>. <u>enterocolitica</u> 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 <u>Y</u>. <u>enterocolitica</u> transconjugants with acridine orange failed to eliminate the F'lac plasmid from the organisms. Two of the four F'lac plasmid donor strains of <u>Y</u>. <u>enterocolitica</u> 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 <u>Y</u>. <u>enterocolitica</u> 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 <u>Y</u>. <u>enterocolitica</u> using the systems employed.

ACKNOWLEDGMENTS

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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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ABBREVIATIONS

API	Analytab Products Incorporated
BHI	Brain Heart Infusion
C	temperature Celsius
CSH	Cold Spring Harbor
$CuSO_4$	Cupric sulfate
Cys	Cysteine
d	days
DNA	deoxyribonucleic acid
EMB	Eosin Methylene Blue
F	fertility factor or sex factor
g	grams
Hfr	High frequency recombinant
His	Histidine
KCN	potassium cyanide
l	liter
LF	lactose fermenting
LIA	Lysine Iron Agar
M	molar
Met	Methionine
mg	milligram
min	minute
ml	milliliter
mm	millimeter
MNNG	N-methyl-N'-nitro-N-Nitrosoguanidine
N	normal

Na_2CO_3	Sodium carbonate
Na-K tartrate	Sodium-potassium tartrate
NaOH	Sodium hydroxide
NLF	non-lactose fermenting
nm	nanometer
OD	optical density
ONP	o-nitrophenol
ONPG	o-nitrophenyl-β-D-galactosidase
Pro	Proline
R	drug resistant plasmid
SDS	Sodium dodecyl sulfate
SDW	sterile distilled water
SS	Salmonella-Shigella
TSI	Triple Sugar Iron
Trp	Tryptophan
μg	micrograms
μm	micrometer
YM	Yersinia-Mannitol

CHAPTER I

INTRODUCTION

HISTORY

Prevalence

In recent years, <u>Yersinia enterocolitica</u> has received increasing attention as a human pathogen. Since the first recognized case of human disease caused by <u>Y</u>. <u>enterocolitica</u> 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 <u>Y</u>. <u>enterocolitica</u> on deposit at the Pasteur Institute in Paris, France (13). Between the years 1966-1976 the majority of <u>Y</u>. <u>enterocolitica</u> 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, <u>Y</u>. <u>enterocolitica</u> 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 <u>Y</u>. <u>enterocolitica</u> from swine, dog, cow,

1

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 <u>Enterobacteriaceae</u> (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 <u>Yersinia</u> <u>(Pasteurella)</u> <u>pestis</u> in 1894. It has been proposed by some workers that <u>Y</u>. <u>enterocolitica</u> be subdivided into the species <u>Y</u>. <u>rhamnophilica</u> and <u>Y</u>. <u>enteritidis</u> due to the differences in their biochemical behavior from typical strains of <u>Y</u>. <u>enterocolitica</u> (15,28,84). To date, the proposal has not been universally accepted.

<u>Y</u>. <u>enterocolitica</u> meets the criteria established by Edwards and Ewing (45) for the Family <u>Enterobacteriaceae</u>. 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 <u>Bacterium</u> <u>enterocoliticum</u>, <u>Pasteurella</u> <u>pseudotuberculosis</u> type B, <u>Pasteurella</u> X, <u>Pasteurella</u> Y, <u>Pasteurella</u> <u>pseudotuberculosis</u>-like organisms, Germ X and Bacterium X (65,138). In 1964, Frederiksen (48) recognized the similarities between these organisms and <u>Bacterium</u> <u>enterocoliticum</u> described by Schleifstein and Coleman in 1939 (136) and proposed the name <u>Yersinia</u> <u>enterocolitica</u>.

Descriptive Characteristics

<u>Y</u>. <u>enterocolitica</u> 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 <u>Y</u>. <u>enterocolitica</u>, (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 Mac-Conkey 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°-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°-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-galacto-

sidase (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°-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
3.5×10^9 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

sulfamethorazole. The organisms are resistant to oleandomycin, novobiocin,

methicillin, fucidina, 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°C; these frequencies were increased to about 2.5×10^{-1} per donor cell at 37°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 5.0×10^{-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 7.7×10^{-6}. This frequency was increased when the recipient strain was heat-treated before mating. Heat exposure for optimum fertility was $50^{\circ}-52^{\circ}C$ 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, pGC1, 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 trans-

poson, Tn951, from the pGC1 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 inter-generic recombination through conjugation has been demonstrated repeatedly (21,42). For the purpose of discussion, the process of bacterial con-jugation, as described by Curtiss (42), can be divided into five stages. Upon mixing appropriate concentrations of male (donor) and female (recip-ient) 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) prepara-tion 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 pre-sence 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 4.5×10^7 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 pili, 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

F' FACTOR FORMATION
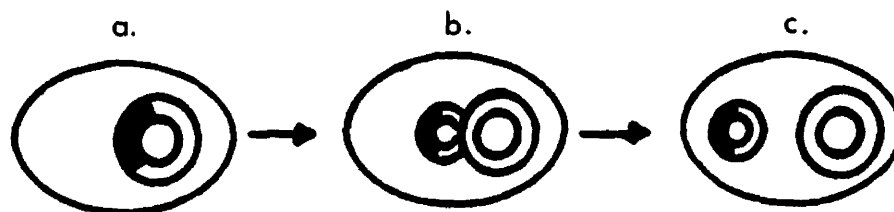
INTEGRATION AND

CHROMOSOME TRANSFER
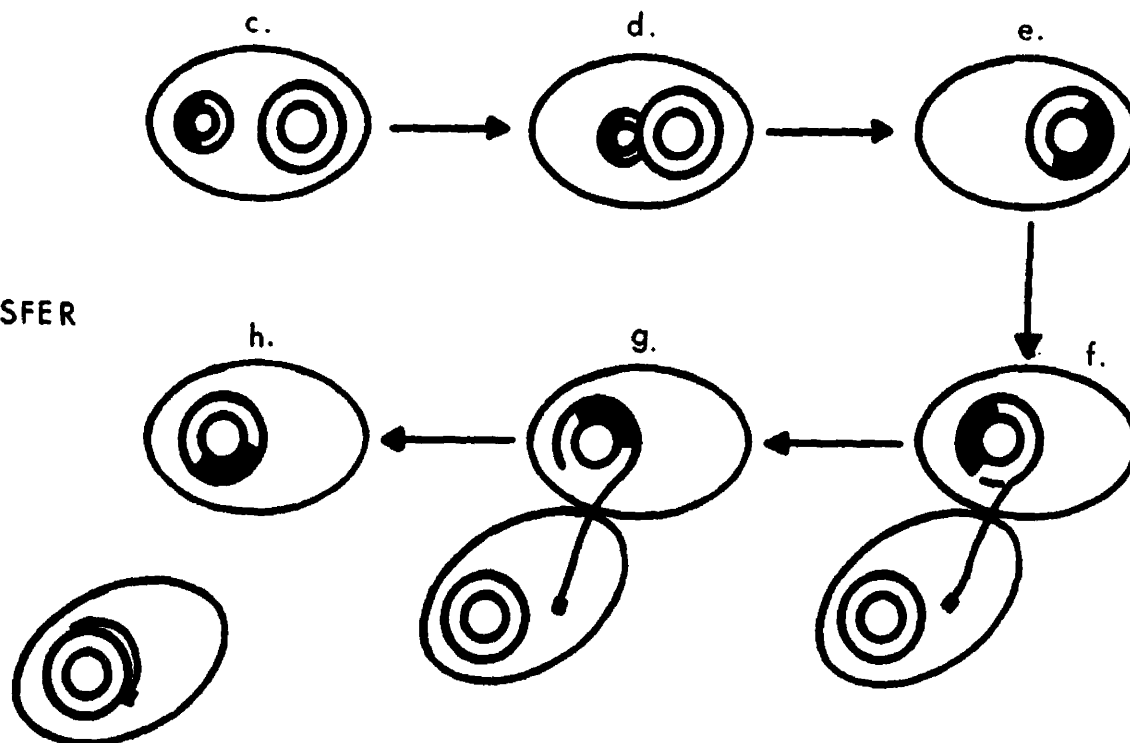
Figure 1. Formation of the F' state and transfer of the Hfr chromosome. The F-factor of an Hfr cell reverses the integration process, but in doing so, retains some bacterial DNA (a. - c.). A bacterial cell that has the F'-factor (c.) may be converted to an Hfr cell through the integration of the fertility 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⁻.

O

F FACTOR

BACTERIAL
CHROMOSOME

A   B
                C

CROSSOVER

A   B
                C

INTEGRATION

O

A   B
                C
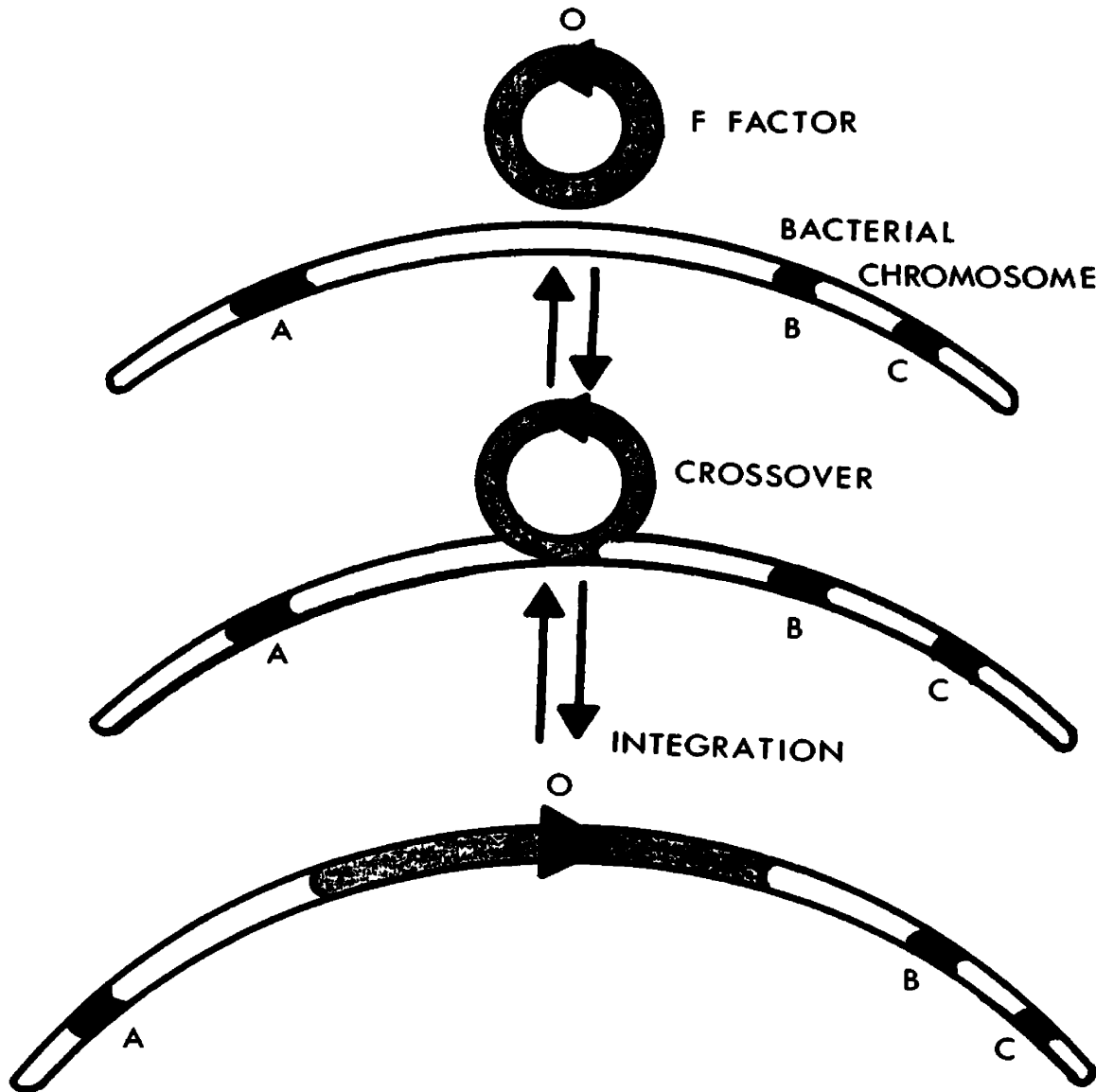
Figure 2. The conversion of an F^+ bacterium into the Hfr state. Circular F-factor DNA, bearing among its genes O, the origin of oriented chromosome transfer from donor to recipient cell, happens to pair with the DNA of the circular bacterial chromosome between genes A and B. A reciprocal crossover between 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 intial studies performed by Lederberg and Tatum (91,92), auxotrophic 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-N'-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.
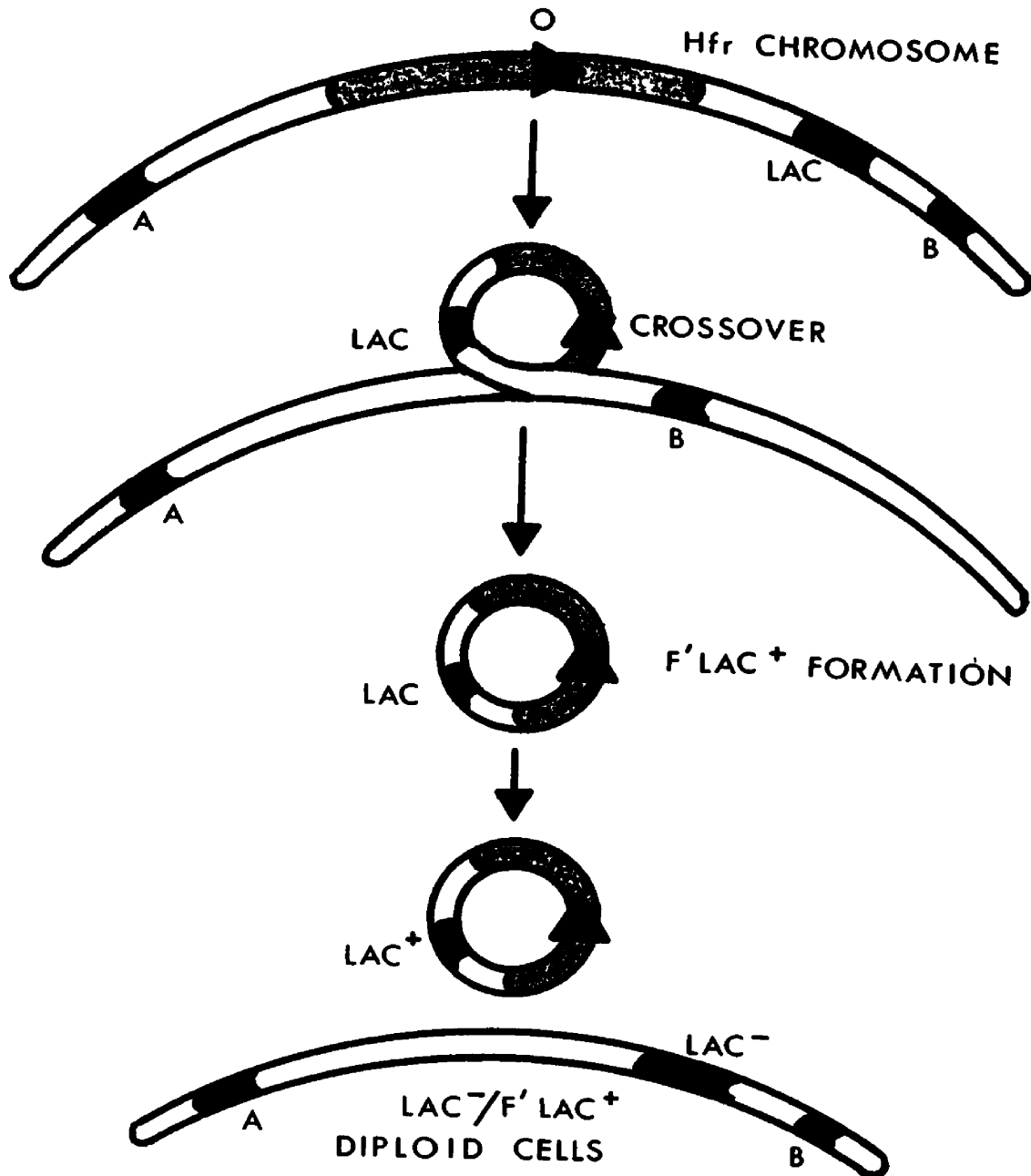
Figure 3. The production of an F' fertility factor, bearing the lac gene of the bacterial chromosome, or F'lac. The F-factor integrated into the Hfr chromosome between gene A and the lac gene and now loops out improperly, so that the chromosomal lac sector has been included in the looped-out portion 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 Y. enterocolitica employing plasmids. Specific aspects of the study include investigation into: (a) the transfer of an F'lac plasmid from E. coli to Y. enterocolitica, from donor to recipient strains of E. coli, and from donor to recipient strains of Y. enterocolitica, (b) the integration of the plasmid into the chromosome of 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 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 Micro-
biology, University of New Hampshire, Durham, New Hampshire. Dr. G.
Cornelis, Department of Microbiology, Universite Catholique de Louvain,
Brussels, Belgium, kindly supplied the needed strains of Yersinia entero-
colitica, 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 relavent 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 sub-
culture was made, points for optical density (OD) readings were taken

20

Table I. Strains of <u>Escherichia coli</u> and <u>Yersinia enterocolitica</u> used in this study.

ORGANISM	STRAIN NUMBER	SEX	GENOTYPE	IMPORTANT PROPERTIES
<u>Escherichia coli</u>				
	CSH 61	HfrC	trpR thi	-
	CSH 36	F'lacI proA$^+$,B$^+$	Δ(lac pro) supE thi	Constitutive for the lac enzyme (i$^-$)
	CSH 24	F'$_{ts}$lac	Δ(lac pro) supE thi	Carries temperature sensitive F'lac; Str^S Bl$^-$ Ara$^+$ Met$^-$ Str^S Ara$^+$
	CSH 23.10s	F'lac	met	
	CSH 23	F'lac proA$^+$,B$^+$	Δ(lac pro) supE thi	Bl$^-$ Str^S Ara$^+$
	CSH 28	F'lac proA$^+$,B$^+$	Δ(lac pro) supF trp pyrF his str thi	Trp$^-$ Ura$^-$ His$^-$ Str^r Bl$^-$ Ara$^+$
	CSH 50	F$^-$	Ara Δ(lac pro) strA thi	Ara$^-$ Lac$^-$ Pro$^-$ Str^r Bl$^-$
<u>Yersinia enterocolitica</u>				
	W277	wild type	lac	Lac$^-$
	W2771	wild type	str lac	Str^r Lac$^-$
	W2772	wild type	nal lac	Nal^r Str^S Lac$^-$
	W27723	F'lac proA$^+$,B$^+$	str	Str^r Nal^S
	W27724	F'$_{ts}$lac	str	Str^r Nal^S
	W27728	F'lac proA$^+$,B$^+$	str	Str^r Nal^S
	W27723.10s	F'lac	str	Str^r Nal^S
	W2773	wild type	his met pro nal lac	His$^-$ Met$^-$ Pro$^-$ Nal^r Str^S Lac$^-$

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 care-
fully monitored with a thermometer. The plates were observed for 7 days
before discarding. For the carbohydrate plates, any growth of Y. entero-
colitica was considered positive for the ability to utilize that carbo-
hydrate 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 waterbath at 37°C. The cells were harvested when the growth
reached the upper portion of the logrithmic 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 (Na_2CO_3), cupric sulfate ($CuSO_4$), 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
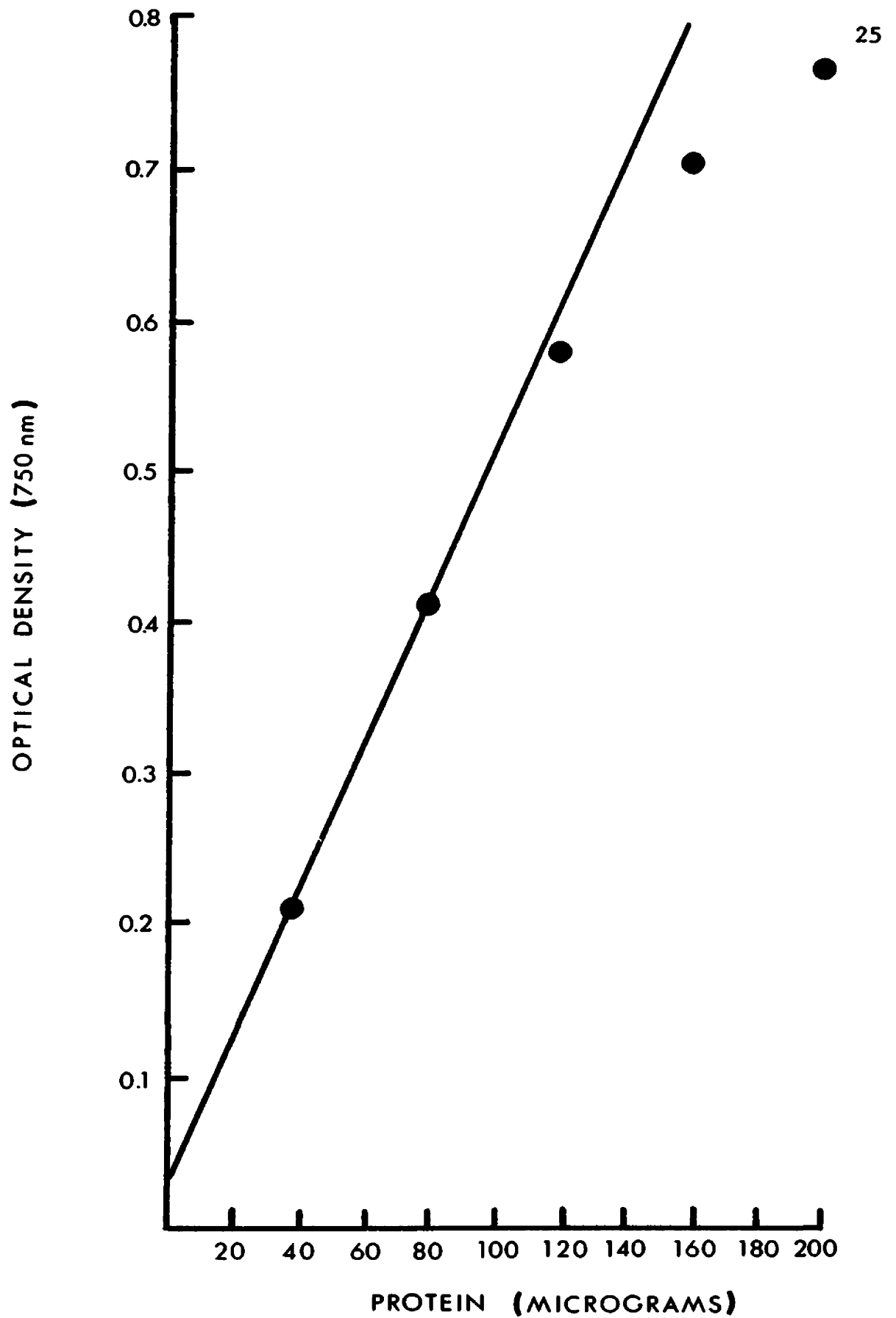
Figure 4. A protein standard curve showing the optical density readings for various concentrations of bovine serum albumin at 750 nm on a Coleman Junior II spectrophotometer.

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_2CO_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:

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

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 incu-

bated 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 $OD_{550 \ nm}$ 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 amd 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. entero-colitica 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. entero-colitica 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 pre-

heated 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 pre-

viously 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 over-

night 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 10^8

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 differ-

ent 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 $\underline{E}$. $\underline{coli}$ 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 $\underline{Y}$. $\underline{enterocolitica}$ 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 ($OD_{550 \text{ nm}}$ 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 poly-auxotroph 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.
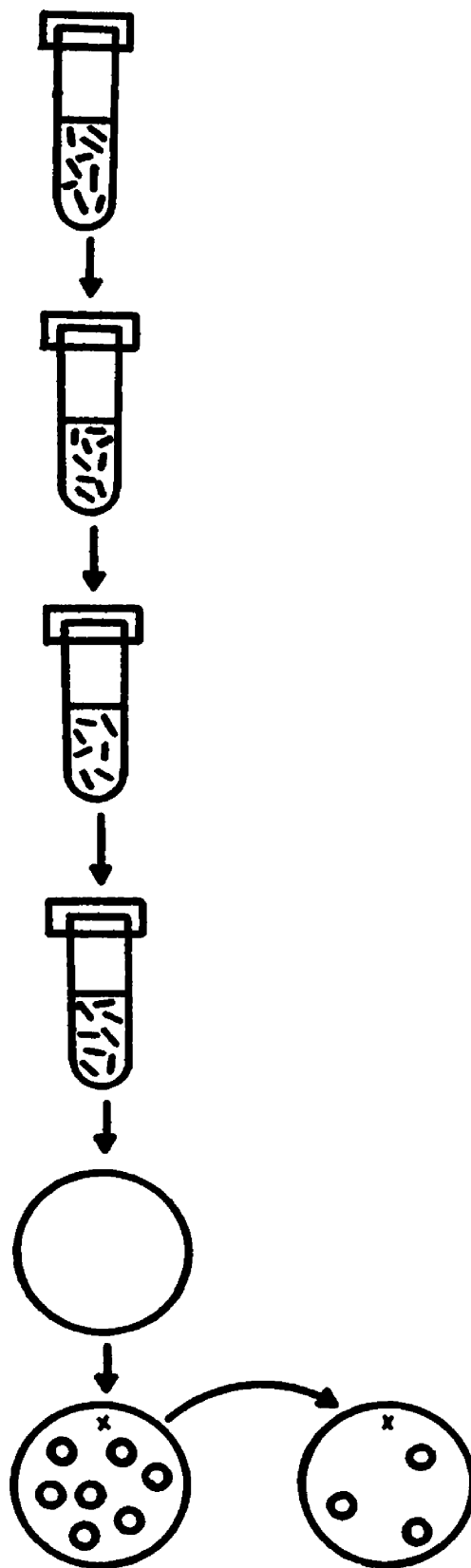
Figure 5.   Nalidixic acid treatment for the isolation of auxotrophic
            mutants.

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 recipient 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. entero-
colitica 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

39
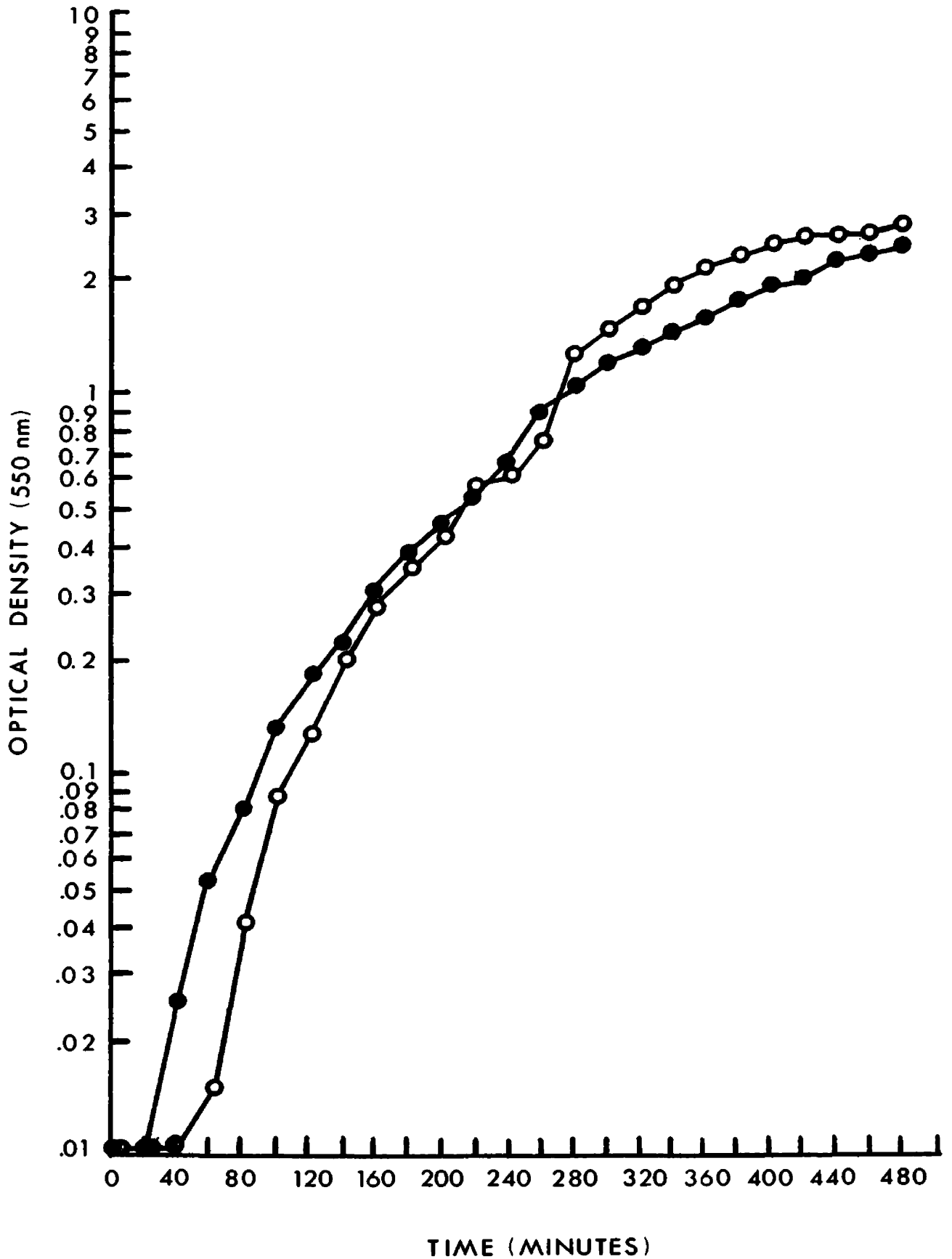
Figure 6. Growth curves of Y. enterocolitica strain W277 at 25°C and 37°C showing the optical density readings ($OD_{550\ nm}$) for various times of incubation. 25°C ●————●; 37°C ○————○.
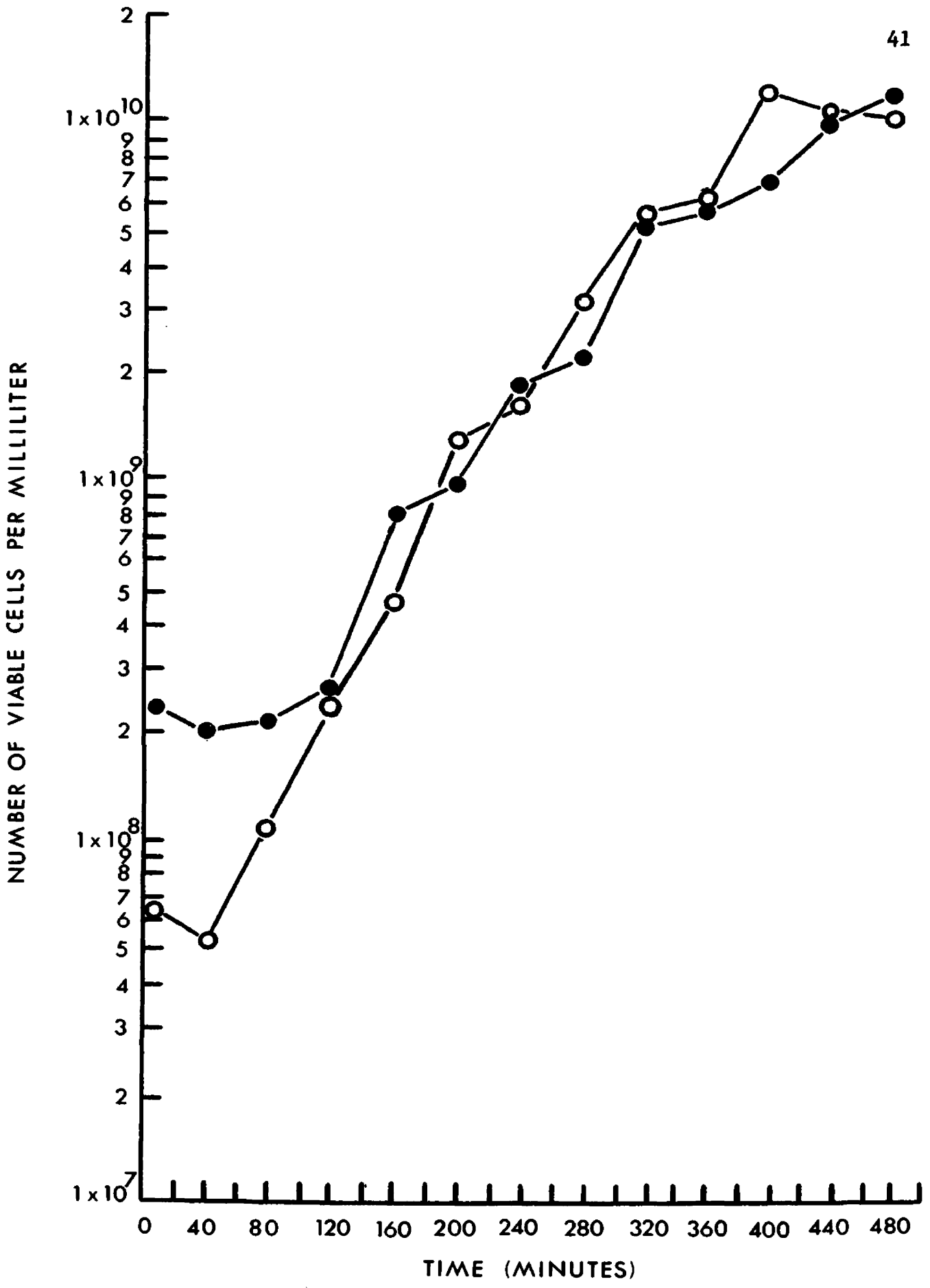
Figure 7. Growth of Y. enterocolitica strain W277 at 25°C and 37°C showing the number of viable cells per ml for various times of incubation.   25°C ●——●;   37°C O——O.
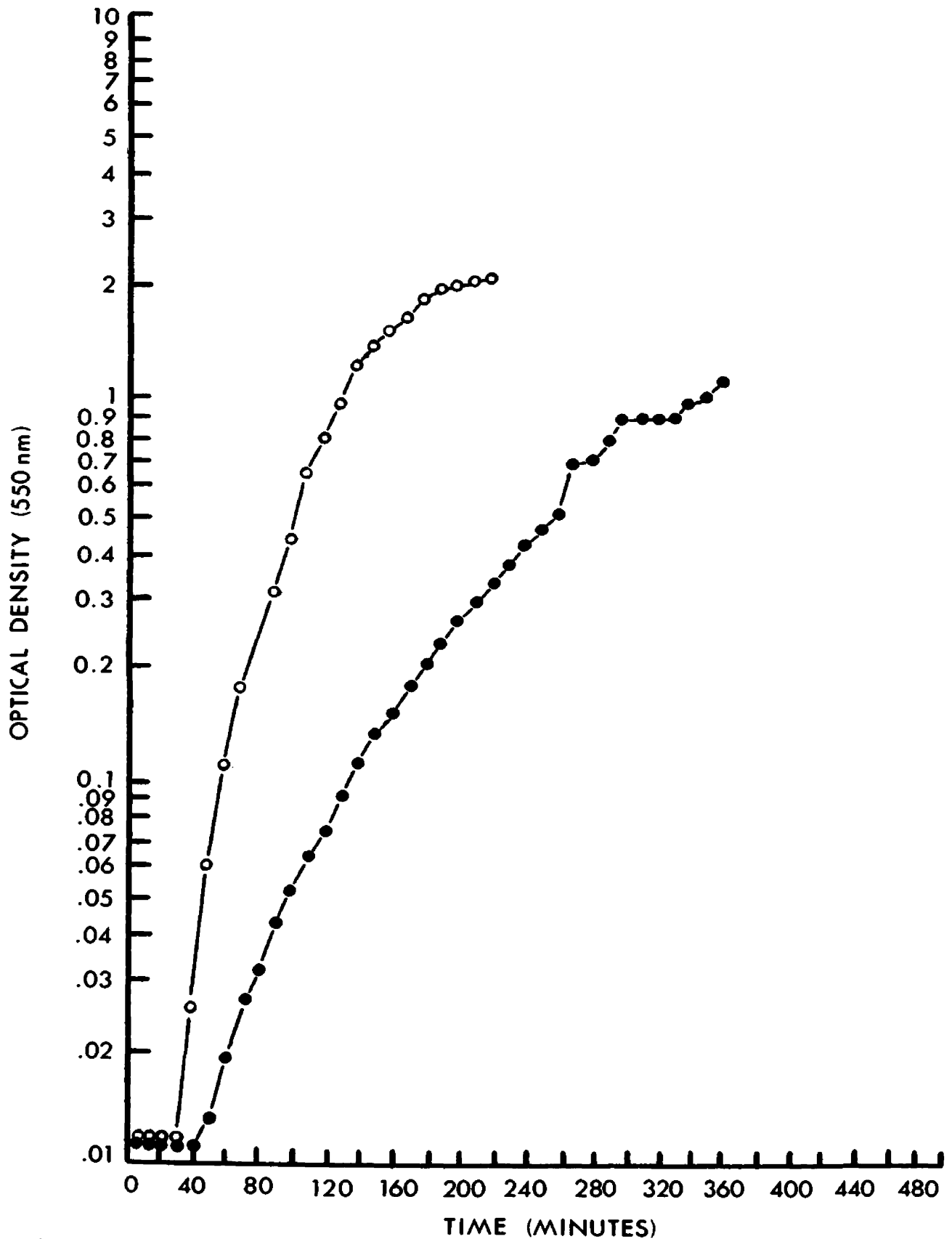
Figure 8. Growth curves of E. coli strain CSH 61 at 25°C and 37°C showing the optical density readings (OD_{550} nm) for various times of incubation. 25°C ●———●; 37°C o———o.

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.

INCUBATION TEMPERATURE	GLUCOSE MINIMAL MEDIUM[a]			CONTROLS[b]	
	CYSTEINE	METHIONINE	CYSTEINE AND METHIONINE	MINIMAL MEDIUM WITHOUT AMINO ACIDS	LB MEDIUM
25°C	+++[c] (2d)	+++ (2d)	+++ (2d)	+++ (2d)	+++ (1d)
37°C	+++ (2d)	+++ (2d)	+++ (2d)	+++ (2d)	+++ (1d)
40°C	+[d] (7d)	+ (7d)	+ (7d)	+ (7d)	+++ (1d)

[a]Organisms were inoculated by streaking for isolated colonies on minimal medium with and without the amino cysteine and methionine.

[b]Controls consisted of inoculating organisms onto minimal medium without the amino acids 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]+ = 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.

INCUBATION TEMPERATURE	MINIMAL MEDIUM[a]					CONTROLS[b]	
	GLUCOSE	GLUCOSE 0.1% CONC.	LACTOSE	GALACTOSE	GALACTOSE AND GLUCOSE	MINIMAL MEDIUM WITHOUT CARBOHYDRATE	LB MEDIUM
25°C	+++[c] (2d)	+++ (6d)	+ (4-7d)	+++ (2d)	+++ (2d)	– (7d)	+++ (1d)
37°C	+++ (2d)	+++ (7d)	– (7d)	+++ (3d)	+++ (2d)	– (7d)	+++ (1d)
40°C	++[d] (5d)	+[e] (7d)	–[f] (7d)	++ (7d)	++ (7d)	– (7d)	+++ (1d)

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

[b]The 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.

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supports growth of this organism at 25°C and 37°C. Minimal medium con-
taining 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-galacto-
sidase 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.

ORGANISMS	PROTEIN CONTENT		β-GALACTOSIDASE		VIABLE CELLS/ML[c]
	µg/ml[a]	%protein[b]	units/min ml	units/mg min	
Yersinia enterocolitica strain W277 (25°C)	2115	44	25	12	1.92 x 10^9
Yersinia enterocolitica strain W277 (37°C)	2076	47	9	4	1.33 x 10^9
Escherichia coli strain CSH 36 (25°C)	1713	43	2457	1434	1.72 x 10^9
Escherichia coli strain CSH 36 (37°C)	1129	49	3075	2723	1.52 x 10^9

[a] µg/ml = optical density readings (420 mu) 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.

colitica 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
1×10^{10} 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 V. Isolation of streptomycin resistant mutants of E. coli strains CSH 24 and 23.10s and Y. enterocolitica strain W277 at 25°C and 37°C temperatures.

ORGANISMS	CONCENTRATION OF STREPTOMYCIN $(\mu g/ml)^d$								
	10	50	100	200	400	600	800	1000	CONTROL
Yersinia enterocolitica strain W277 (25°C)	CG[a]	CG	CG	151[c]	165	119	92	111	CG
Yersinia enterocolitica strain W277 (37°C)	CG	CG	16	15	27	15	23	11	CG
Escherichia coli strain CSH 24 (25°C)	CG	2	2	NG[b]	NG	NG	NG	NG	CG
Escherichia coli strain 23.10s (37°C)	CG	CG	CG	1	2	NG	NG	NG	CG

[a]CG = CONFLUENT GROWTH

[b]NG = NO GROWTH

[c]Number of resistant colonies/~1 x 10^{10} cells/ml

[d]Organisms 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.

CONJUGATION		NUMBER OF	FREQUENCY OF TRANSFER OF[a]
DONOR	RECIPIENT	RECOMBINANTS/ML	F'LAC PLASMID
CSH 23	CSH 50	4.05×10^8	1.00 (ALL TRANSFERRED)
CSH 24	CSH 50	6.60×10^7	1.28×10^{-1}
CSH 28	CSH 50	2.16×10^8	9.04×10^{-1}
23.10s	CSH 50	1.80×10^7	4.80×10^{-2}

[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 - 4.05×10^8 cells/ml
CSH 24 - 5.15×10^8 cells/ml
CSH 28 - 2.85×10^8 cells/ml
23.10s - 3.75×10^8 cells/ml

transfer of the F'lac plasmid from four donor strains of $\underline{E}$. $\underline{coli}$ to

$\underline{E}$. $\underline{coli}$ 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 $\underline{E}$. $\underline{coli}$, 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 $\underline{E}$. $\underline{coli}$ to $\underline{Y}$. $\underline{enterocolitica}$ strain W2771 were successful, but the

frequency of transfer was much lower than that observed for matings

between strains of $\underline{E}$. $\underline{coli}$, occurring at a frequency of approximately

10^{-6} (Table VII).

In a separate experiment, $\underline{Y}$. $\underline{enterocolitica}$ strain W2771 was pre-

heated to 51°C for three minutes prior to mating with the four $\underline{E}$. $\underline{coli}$

donor strains. Table VIII indicates that heat treatment of the recipient

caused no increase in the frequencies of F'lac plasmid transfer from

$\underline{E}$. $\underline{coli}$ to $\underline{Y}$. $\underline{enterocolitica}$ 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 $\underline{E}$. $\underline{coli}$ CSH 50 or $\underline{Y}$. $\underline{enterocolitica}$ 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 $\underline{Y}$. $\underline{enterocolitica}$ strain. Upon

initial isolation, some segregation of lactose negative colonies was

observed but repeated plating on MacConkey agar permitted the selection

of relatively stable $\underline{Y}$. $\underline{enterocolitica}$ lactose positive organisms.

Table VII. Direct selection of F'lac plasmid transfers involving strains of $\underline{E}$. $\underline{coli}$ and $\underline{Y}$. $\underline{entero-}$ $\underline{colitica}$ strain W2771 in a 60 minute broth mating system.

CONJUGATION		NUMBER OF	FREQUENCY OF TRANSFER OF[a]
DONOR	RECIPIENT	RECOMBINANTS/ML	F'LAC PLASMID
CSH 23	W2771	2.00×10^3	5.48×10^{-6}
CSH 24	W2771	1.00×10^3	3.44×10^{-6}
CSH 28	W2771	1.50×10^3	5.52×10^{-6}
23.10s	W2771	2.00×10^3	5.26×10^{-6}

[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 - 3.65×10^8 cells/ml
CSH 24 - 2.91×10^8 cells/ml
CSH 28 - 2.72×10^8 cells/ml
23.10s - 3.80×10^8 cells/ml

Table VIII. Direct selection of F'lac plasmid transfers involving strains of $\underline{E}$. $\underline{coli}$ and a heat-treated recipient, $\underline{Y}$. $\underline{enterocolitica}$ strain W2771, in a 60 minute broth mating system.

CONJUGATION		NUMBER OF		FREQUENCY OF TRANSFER OF[a]
DONOR	RECIPIENT	RECOMBINANTS/ML		F'LAC PLASMID
CSH 23	W2771	WITH HEAT[b]	2.45×10^3	8.78×10^{-6}
		WITHOUT HEAT	1.95×10^3	6.99×10^{-6}
CSH 24	W2771	WITH HEAT	2.95×10^3	9.61×10^{-6}
		WITHOUT HEAT	1.50×10^3	4.89×10^{-6}
CSH 28	W2771	WITH HEAT	6.50×10^2	3.40×10^{-6}
		WITHOUT HEAT	8.50×10^3	4.45×10^{-5}
23.10s	W2771	WITH HEAT	2.10×10^3	9.09×10^{-6}
		WITHOUT HEAT	2.50×10^3	1.08×10^{-5}

[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 - 2.79×10^8 cells/ml
CSH 24 - 3.07×10^8 cells/ml
CSH 28 - 1.91×10^8 cells/ml
23.10s - 2.31×10^8 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 $\underline{Y}$. $\underline{enterocolitica}$. 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 ferment-
ing colonies of Y. enterocolitica strain W277 (wild
type) (right) on MacConkey agar. LF - Lactose Ferment-
ing, NLF - Non-Lactose Fermenting.

Figure 10. A mixture of lactose fermenting colonies of Y. entero-
colitica 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.
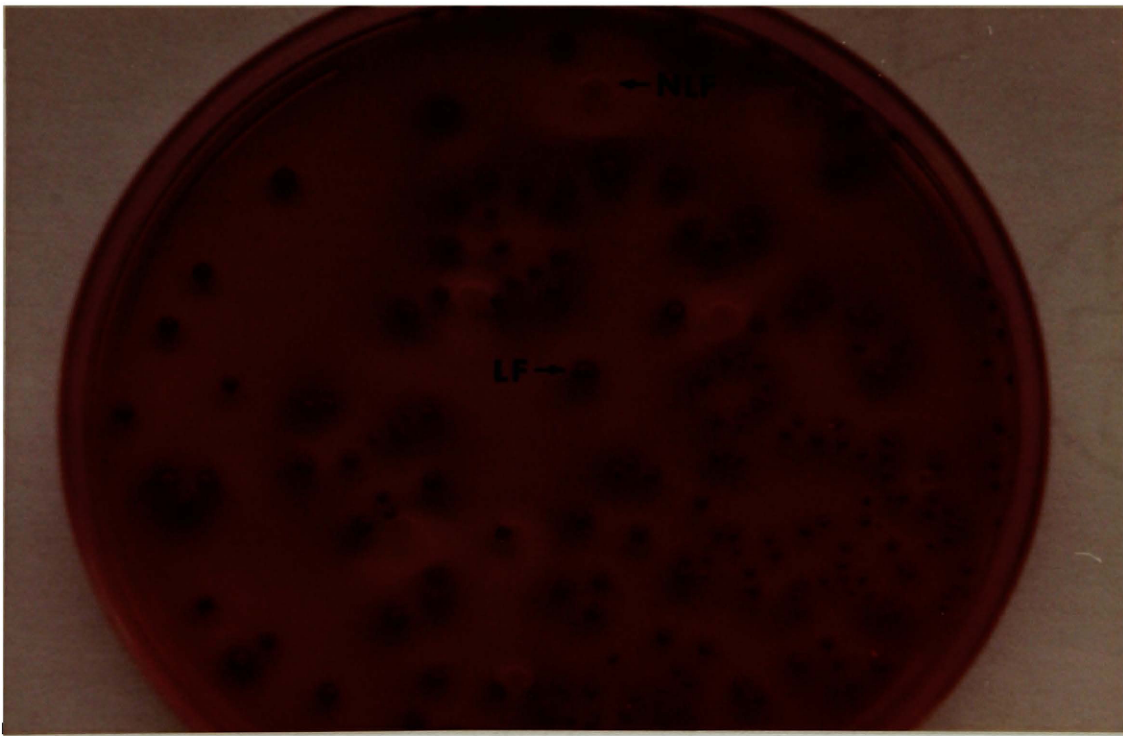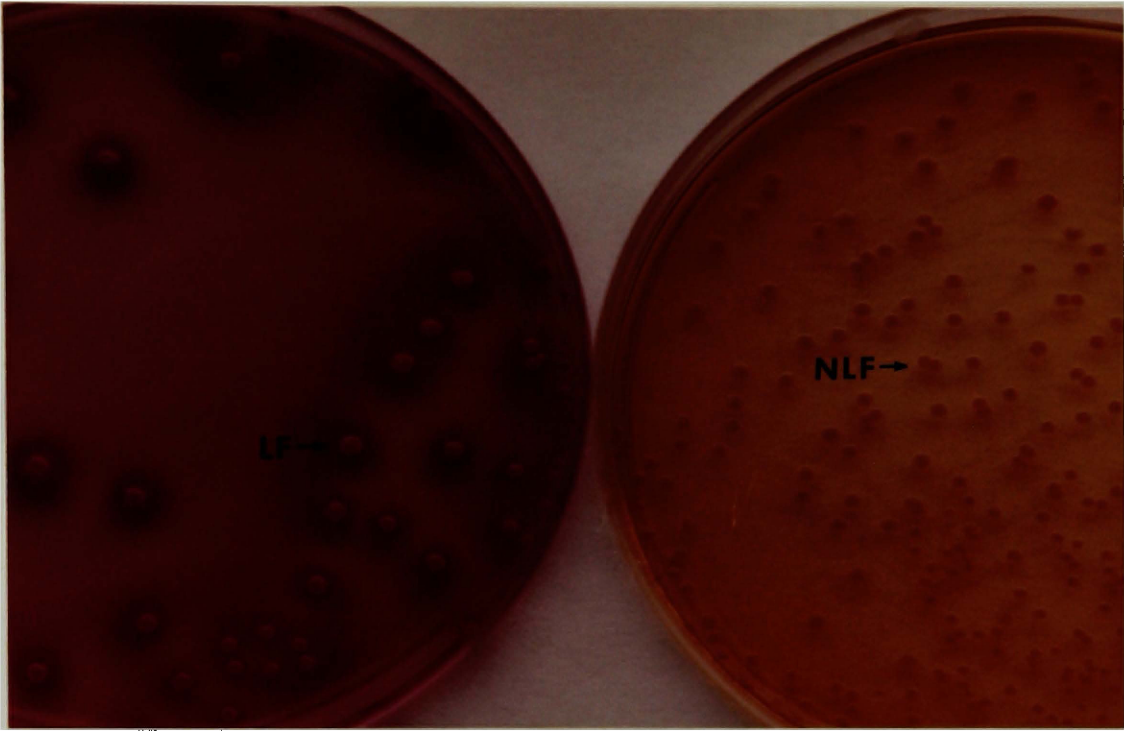
Figure 11. A comparison of lactose fermenting colonies of Y.
enterocolitica strain W27723 (left), suspected of
harboring the F'lac plasmid from E. coli CSH 23,
with Y. enterocolitica strain W27724 (right),
suspected of harboring the F'lac plasmid from
E. coli CSH 24, on MacConkey agar.

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.
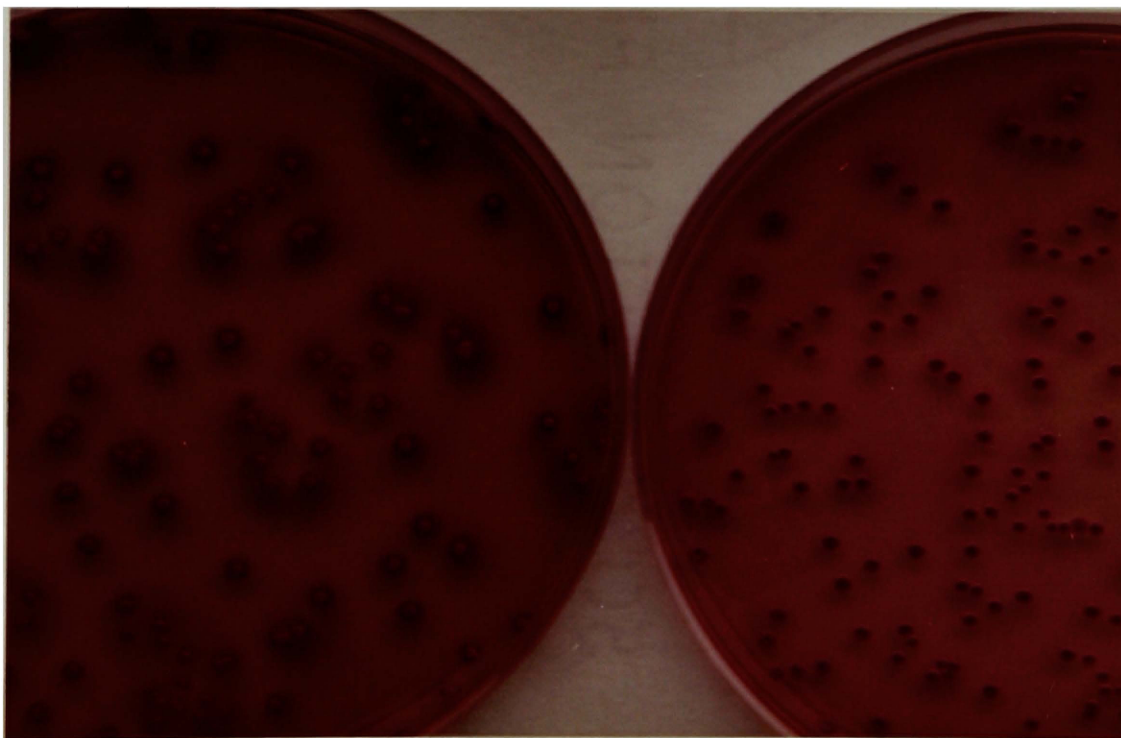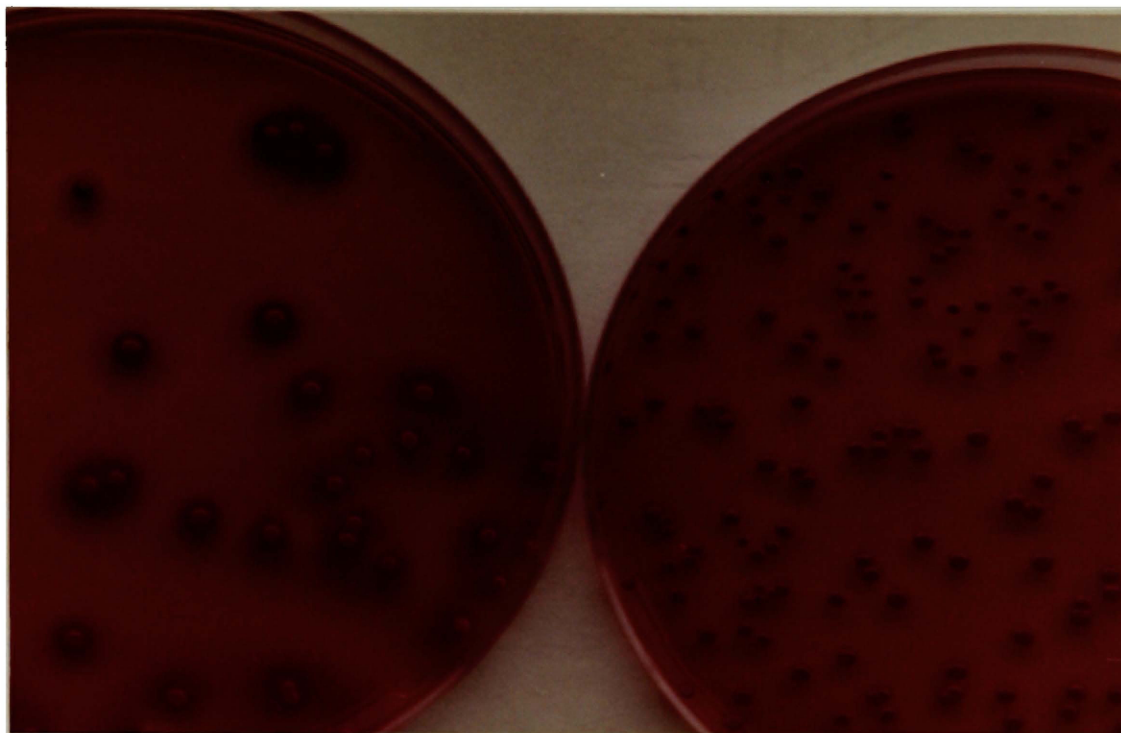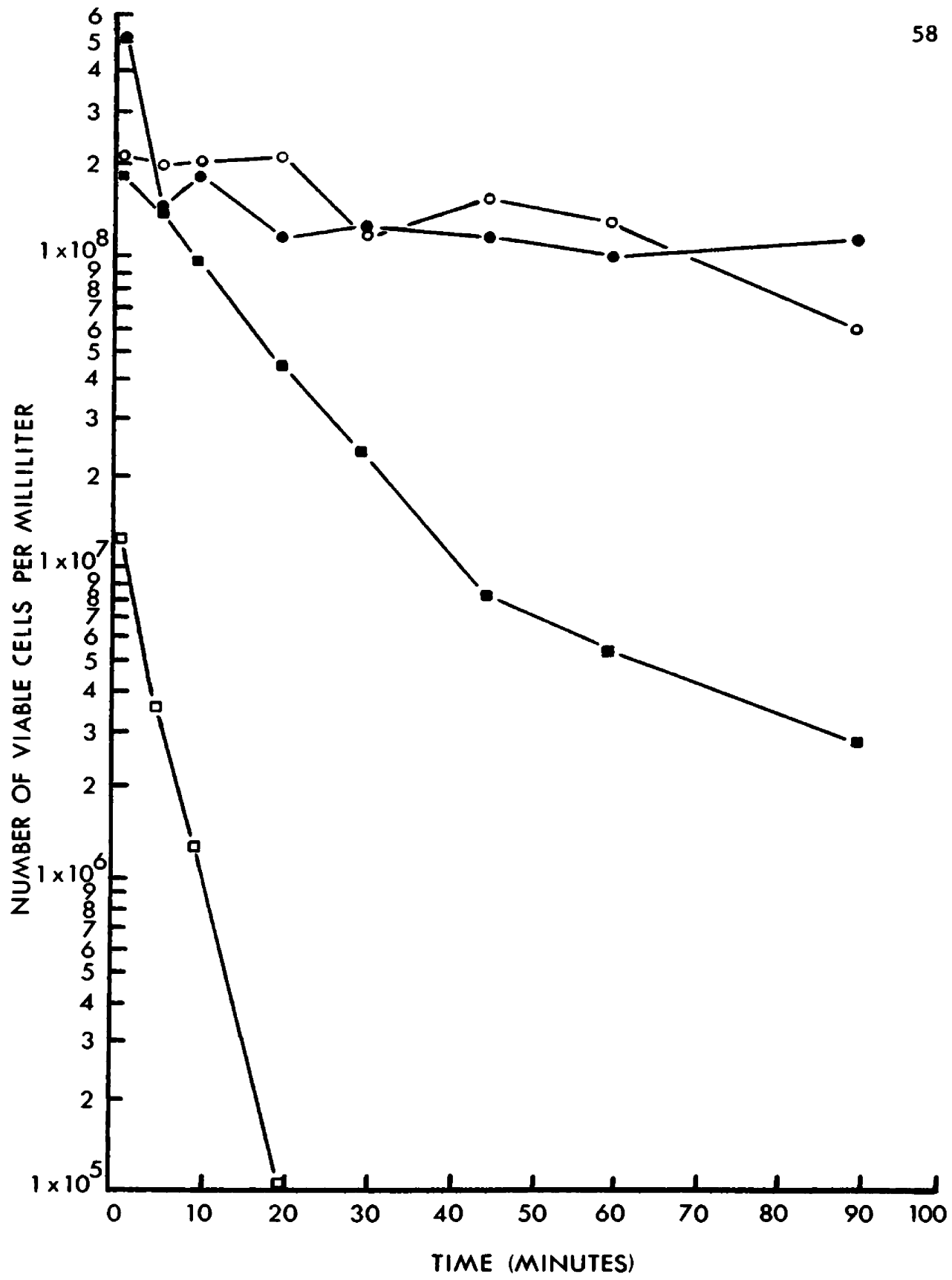
Figure 13. Survival curves of E. coli strain CSH 50 and Y. enterocolitica strain W277 mutagenized with 50 µg/ml of MNNG at 25°C and 37°C. E. coli strain CSH 50 25°C ●————●; E. coli strain CSH 50 37°C o————o; Y. enterocolitica strain W277 25°C ■————■; Y. enterocolitica strain W277 37°C ▫————▫.

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.

	VIABLE CELL COUNTS[a]			
DILUTION	MINIMAL GLUCOSE[b] PLUS HISTIDINE	MINIMAL GLUCOSE MINUS HISTIDINE	MINIMAL GLUCOSE MINUS HISTIDINE	PERCENT AUXOTROPHY
10^{-7}	976	975	975	0.1%
10^{-8}	198	198	198	0%

[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.

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.

	VIABLE CELL COUNTS[a]			
DILUTION	MINIMAL GLUCOSE[b] PLUS HISTIDINE	MINIMAL GLUCOSE MINUS HISTIDINE	MINIMAL GLUCOSE MINUS HISTIDINE	PERCENT AUXOTROPHY
10^{-7}	305	288	288	5.57%
10^{-8}	45	42	42	6.67%

[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.

Table XI. Isolation of methionine auxotrophic mutants from a histidine requiring mutant of $\underline{Y}$. $\underline{entero\text{-}colitica}$ strain W277 at 25°C after 18 hours incubation with 100 µg/ml of nalidixic acid.

	VIABLE CELL COUNTS[a]			
DILUTION	MINIMAL GLUCOSE[b] PLUS HIS MET	MINIMAL GLUCOSE PLUS HISTIDINE	MINIMAL GLUCOSE PLUS HISTIDINE	PERCENT AUXOTROPHY
10^{-7}	180	152	152	15.56%
10^{-8}	36	27	27	25.00%

[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.

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.

| DILUTION | VIABLE CELL COUNTS[a] | | | |
	MINIMAL GLUCOSE[b] PLUS HIS MET PRO	MINIMAL GLUCOSE PLUS HIS MET	MINIMAL GLUCOSE PLUS HIS MET	PERCENT AUXOTROPHY
10^{-7}	395	391	391	1.01%
10^{-8}	65	63	63	3.08%

[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.

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.

| | VIABLE CELL COUNTS[a] | | | |
DILUTION	MINIMAL GLUCOSE[b] PLUS HIS MET CYS	MINIMAL GLUCOSE PLUS HIS MET	MINIMAL GLUCOSE PLUS HIS MET	PERCENT AUXOTROPHY
10^{-7}	159	159	159	0%
10^{-8}	33	33	33	0%

[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.

Table XIV. Isolation of tryptophan 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.

	VIABLE CELL COUNTS[a]			
DILUTION	MINIMAL GLUCOSE[b] PLUS HIS MET TRP	MINIMAL GLUCOSE PLUS HIS MET	MINIMAL GLUCOSE PLUS HIS MET	PERCENT AUXOTROPHY
10^{-7}	153	153	153	0%
10^{-8}	17	17	17	0%

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

SELECTION OF F'$_{ts}$LAC PLASMID INTEGRATION

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

F'LAC PLASMID-MEDIATED CHROMOSOME TRANSFER

$\underline{Y}$. $\underline{enterocolitica}$ strains W27723, W27724, W27728, and W27723.10s were tested for their ability to behave as gene donors in crosses with the polyauxotroph, $\underline{Y}$. $\underline{enterocolitica}$ 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.

DILUTION	TOTAL NUMBER OF VIABLE CELLS	NUMBER OF LACTOSE NEGATIVE CELLS	NUMBER OF POSSIBLE F'_{ts}LAC INTEGRATIONS	PERCENTAGE OF POSSIBLE F'_{ts}LAC INTEGRATIONS
10^{-7}[a]	5555	5395	160[b]	2.88%
10^{-8}	1216	1194	22	1.81%

[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.

CONJUGATION		NUMBER OF RECOMBINANTS/ML[b]			FREQUENCY OF CHROMOSOME TRANSFER		
DONOR[a]	RECIPIENT	HIS$^+$	MET$^+$	PRO$^+$	HIS$^+$	MET$^+$	PRO$^+$
W27723	W2773	0	0	0	0	0	0
W27724	W2773	0	0	0	0	0	0
W27728	W2773	0	0	0	0	0	0
W27723.10s	W2773	0	0	0	0	0	0
W277	W2773	0	0	0	0	0	0

[a]Viable cell counts of the donors (donor input) are as follows:

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

[b]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.

CONJUGATION		NUMBER OF RECOMBINANTS/ML[b]			FREQUENCY OF CHROMOSOME TRANSFER		
DONOR[a]	RECIPIENT	HIS$^+$	MET$^+$	PRO$^+$	HIS$^+$	MET$^+$	PRO$^+$
W27723	W2773	0	0	0	0	0	0
W27724	W2773	0	0	0	0	0	0
W27728	W2773	0	0	0	0	0	0
W27723.10s	W2773	0	0	0	0	0	0
W277	W2773	0	0	0	0	0	0

[a]Viable cell counts of the donor (donor input) are as follows:

W27723 - 2.79 x 10^8 cells/ml
W27724 - 3.66 x 10^8 cells/ml
W27728 - 1.94 x 10^8 cells/ml
W27723.10s - 2.87 x 10^8 cells/ml
W277 - 4.30 x 10^8 cells/ml

[b]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.

CONJUGATION		NUMBER OF	FREQUENCY OF TRANSFER OF[a]
DONOR	RECIPIENT	RECOMBINANTS/ML	F'LAC PLASMID
W27723	W2772	0	0
W27724	W2772	0	0
W27728	W2772	0	0
W27723.10s	W2772	0	0

[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:

W27723 - 1.63×10^8 cells/ml
W27724 - 2.68×10^8 cells/ml
W27728 - 2.38×10^8 cells/ml
W27723.10s - 3.63×10^8 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.

CONJUGATION		NUMBER OF	FREQUENCY OF TRANSFER OF[a]
DONOR	RECIPIENT	RECOMBINANTS/ML	F'LAC PLASMID
W27723	W2772	1.42×10^4	1.73×10^{-5}
W27724	W2772	0	0
W27728	W2772	1.80×10^4	1.07×10^{-5}
W27723.10s	W2772	0	0

[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:

W27723 - 8.20×10^8 cells/ml
W27724 - 3.53×10^9 cells/ml
W27728 - 1.69×10^9 cells/ml
W27723.10s - 5.48×10^9 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.

CONJUGATION		NUMBER OF	FREQUENCY OF TRANSFER OF[a]
DONOR	RECIPIENT	RECOMBINANTS/ML	F'LAC PLASMID
W27723	W2772	1.37×10^5	7.33×10^{-4}
W27724	W2772	0	0
W27728	W2772	1.09×10^5	6.09×10^{-4}
W27723.10s	W2772	0	0

[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:

W27723 - 1.87×10^9 cells/ml
W27724 - 2.68×10^8 cells/ml
W27728 - 1.79×10^8 cells/ml
W27723.10s - 3.63×10^8 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 $\underline{Y}$. 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 $\underline{Y}$. 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 $\underline{Y}$. enterocolitica using acridine orange. Approximately 27% of the total cell population of $\underline{E}$. 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 $\underline{Y}$. 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 $\underline{Y}$. 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 $\underline{Y}$. 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.

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

[a]Concentration 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 Y. enterocolitica (W27724 and W27723.10s) previously

isolated, but not to Y. enterocolitica strains W27723 and W27728

(Figures 9 thru 12).

CHAPTER IV

DISCUSSION

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 pseudo-tuberculosis (88,90,103,116), as well as Escherichia coli (61). The system employed for Y. pestis was unsuccessfully extended to Y. entero-colitica in the early 1970's (90). The purpose of the present study, therefore, was to develop a genetic system that would enable the success-ful 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,

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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 concetration 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 5.0×10^{-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-

colitica was observed at a frequency of only 7.7×10^{-6}. This frequency was increased when the recipient strain was heat-treated before mating. The conditions for optimum fertility were 50°-$52^\circ C$ 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 5×10^6 donor cells increased with heat-treatment of the recipient. A self-transmissible lac plasmid, pGC1, 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 demostrated 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 necessay for conjugation were fuctional 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 $40^{\circ}-43^{\circ}C$. 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 $41^{\circ}C$. 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 $25^{\circ}C$ or $37^{\circ}C$. Y. enterocolitica strain W27723.10s was suspected of carrying an F'lac plasmid which was not temperature sensitive and would ferment lactose at $41^{\circ}C$. 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 $41^{\circ}C$. 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 E. coli F'lac plasmid in the four donor strains of Y. enterocolitica would be in order.

Attempts to cure the F'lac plasmid from 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 unsucessful in the two fertile donor strains of 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 Y. pseudo-tuberculosis 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 success-fully used this technique to isolate Hfr strains of Erwinia amylovora by selecting for clones resistant to curing by acridine orange. They found that, in the presence of 1.0 μg/ml of acridine orange, F'lac plasmid-containing strains of this organism demonstrated 89% segrega-tion to lactose negative colonies. Colonies resistant to curing were then screened for the ability to transfer chromosome markers and Hfr strains of E. amylovora were isolated. The need for future research in this area could prove benificial in isolating Hfr strains of Y. enterocolitica if an operative system could be established to differ-entiate integrated F'lac plasmid clones from those not possessing integrations.

It seems quite likely that 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. entero-

colitica 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 success-

fully 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-methyl-urea 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 accomadate 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, β-galactosidase activity, and isolation of various mutants of

this organism have been presented. F'lac plasmid transfer from strains of E. coli to 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 Y. enterocolitica. Functional mechanisms for F-pili production and pair formation were present for two of the four donor strains of Y. enterocolitica used in this study.

There is a need to investigate the conjugal genetics of a number of different strains and serotypes of 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 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 Enterobacteriaceae. At this stage, it would be premature to generalize that 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 Y. entero-
colitica strain W277 can accept the F'lac plasmid from E. coli and
transfer this plasmid to a recipient strain of Y. enterocolitica.
Transconjugants (which harbored the F'lac plasmid) of Y. enterocolitica
were not capable of transferring chromosome markers in crosses with a
polyauxotrophic mutant of this organism.

Studies relating to the growth, β-galactosidase activity, and isola-
tion of antibiotic resistant and auxotrophic mutants of this organism
have presented some interesting and unusual results. Y. enterocolitica
strain W277 was found to grow equally well at both 37°C and 25°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
β-galactosidase enzyme at both 25°C and 37°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 E. coli at both 25°C and 37°C. Auxotrophic
mutants of 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 µg/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)

K_2HPO_4..................................... 10.5 g

KH_2PO_4..................................... 4.5 g

$(NH_4)_2SO_4$................................. 1.0 g

Sodium citrate $\cdot$ $2H_2O$..................... 0.5 g

$MgSO_4$ $\cdot$ $7H_2O$	Add 1.0 ml from a stock solution of 20.0 g/100 ml after auto-claving.
B1 (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. Therfore 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 µg/ml of the D,L-amino acid (or 20 µg/ml of the L-amino acid) is sufficient. Vitamins are required in smaller amounts (1.0 µg/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.

Streptomycin concentration	ml of stock solution
10 μg/ml	0.1 ml/l
50 μg/ml	0.5 ml/l
100 μg/ml	1.0 ml/l
200 μg/ml	2.0 ml/l
400 μg/ml	4.0 ml/l
600 μg/ml	6.0 ml/l
800 μg/ml	8.0 ml/l
1000 μg/ml	10.0 ml/l

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. Sterlize 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 µg/ml = 0.4 g/1000 ml

1. Prepare a 400 µg/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 µg/ml)
    b. 0.4 ml (160 µg/ml) plus 0.1 ml of distilled water
    c. 0.3 ml (120 µg/ml) plus 0.2 ml of distilled water
    d. 0.2 ml (80 µg/ml) plus 0.3 ml of distilled water
    e. 0.1 ml (40 µg/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 tatrate (Dissolve 2.0 g Na-K tartrate in 100 ml of distilled water).

    c. 2.0% Na_2CO_3 (Dissolve 2.0 g Na_2CO_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_2CO_3 stock solution add 1.0 ml of the mixture prepared in step 2.

4. Make fresh each day.

<u>RESUME</u>

Richard Lee Hodinka

Birthplace: Brownsville, Pennsylvania
Birthdate: January 11, 1955
Citzenship: U.S.A.
Ancestry: Slovak-Russian
Height: 5'11"
Weight: 155 lbs.
Marital Status: Married
Permanent Address: 3121 Lodwick Dr., Warren, Ohio, 44485

Education

Marietta College, Marietta, Ohio, B.S. Biology, 1972-1976

University of Montana, Missoula, Montana, M.S. Microbiology, 1977-1979

Experience

Graduate Teaching Assistant, Department of Microbiology, University
of Montana, Missoula, Montana, 1977-1979.

Research Assistant on <u>Yersinia</u> Research Project, Department of
Microbiology, University of Montana, Missoula, Montana, 1978.

Membership in Professional Societies

Tri Beta Society for Biology, 1972-1976

American Society for Microbiology, 1977 to present

Montana Academy of Sciences, 1977 to present

Honors and Awards

Elected to Graduate Council, University of Montana, Missoula, Montana, 1979

President, Graduate Student Club, Department of Microbiology, University
of Montana, 1978-1979