THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

THE IDENTIFICATION OF A CELL WALL INHIBITOR OF CONJUGATION IN *ESCHERICHIA COLI* K-12

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

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Norman, Oklahoma

1971

THE IDENTIFICATION OF A CELL WALL INHIBITOR OF CONJUGATION IN *ESCHERICHIA COLI* K-12

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ACKNOWLEDGEMENT

I am indebted to Dr. John H. Lancaster for his invaluable direction, advice, and interest during this study. I also wish to thank all of the faculty who have assisted with suggestions and criticisms of this work.

DEDICATION

"To Jo Ann and Amy"

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CHAPTER I

INTRODUCTION

Bacterial conjugation is a mode of genetic transmission requiring cell to cell contact (1). Two distinct cell types exist since genetic transfer via conjugation is always unidirectional (2). One cell type is called the recipient or female cell. This cell must survive if recombinants are to be formed because it alone forms the zygote. The other cell type, the male or donor, is the donor of genetic information.

The genetic determinant which characterizes the male cell is called the *F* particle (3). *F* is an episome capable of existing either as an independently replicating cytoplasmic element or integrated into the chromosome. When *F* is in the cytoplasmic state, the male is called an F^+. F^+ cells transfer *F* with a probability approaching one. Chromosomal markers are transferred only at low frequency by F^+ cells. When *F* is in the integrated state, chromosomal markers are

transferred with high efficiency. These cells, termed Hfr, transfer F only infrequently, if at all. Kinetic studies with various Hfr types indicate that F attachment to or integration into the chromosome occurred at different sites on the chromosome (4). Variants of Hfr cells are found which transfer F with high efficiency along with a small portion of the chromosome. These male cells are termed F' (5).

The female cells most often used in conjugation studies are derived from E. coli K-12. However, conjugation can also occur between E. coli K-12 donors and recipients from E. coli strains C and B. In addition, intergeneric crosses between K-12 donors and recipients from other genera occur. A partial list includes Shigella, Salmonella, Serratia, Vibrio, and Pseudomonas (6). Frequencies of recombination are always low in these intergeneric crosses. The reasons for low frequencies are differences in genetic homology, which prevents chromosome pairing prior to recombination, and restriction, which destroys deoxyribonucleic acid (DNA) before pairing can occur.

Conjugation can be divided into a number of steps (7). The initial steps are pair formation and stabilization. The later steps include genetic transfer and recombination. The purpose of this paper is to investigate further the initial steps of conjugation.

The initial events in conjugation must involve phenotypic surface differences between mating cell types. Male

cells differ from female cells in a number of ways which indicate surface differences. Those include differences in affinities for certain dyes and the pH at which the cells autoagglutinate (8, 9). An antigen termed F^+ is located only on male cells (10). The F^+ antigen is probably identical to F pili (11). F pili are synthesized under the control of the F particle and are probably the receptors for male specific phage (12, 13). More extensive information on male specific phage is the subject of a recent book and review (14, 15).

Phages specific or quasi-specific for female cells also have been reported (16). Most of the female specific phage adsorb equally well to male and female cells. These phage inject their nucleic acid and kill the male cells without yielding new phage progeny. The rare phage liberated from male cells are not modified because they still exhibit a low efficiency of plating on male cells. Curtiss (7) has pointed out that these features are similar to superinfection immunity. Another female specific phage, Ø1, adsorbs at a higher efficiency to female cells than male cells (16). It is unknown whether a specific surface substance on the female allows adsorption or whether a substance on the male prevents adsorption.

Another reported property of male cells is surface exclusion (18). Surface exclusion refers to an F specific substance which prevents male homosexuality. The nature of the surface substance is unknown but it appears to be

distinct from F pili. Evidence that the substance is distinct from F pili is that F' DNA is transferred to DNA-less minicells derived from female cells. However, F' DNA is not transferred to minicells derived from male cells, despite the absence of F pili or F DNA (19). Periodate destroys surface exclusion and male fertility simultaneously (20). This property indicates that a periodate sensitive component may be responsible for male fertility and surface exclusion. Periodate sensitivity indicates that the surface component possibly is carbohydrate in nature.

The evidence cited above indicate that the presence of F does indeed alter the surface characteristics of cells.

Kern (21) and Yura (22) were first to demonstrate that isolated cell walls from both fertile cells inhibited conjugation at an early step. Lancaster, et al. (23) confirmed their work and developed an assay for cell wall preparations. In addition, they showed that the aqueous phase from phenol-water extracts of male and female cells showed similar, though reduced inhibition levels. The purpose of this paper is to present evidence for the identity of the cell wall inhibitory component.

CHAPTER II

MATERIALS AND METHODS

Cultures

Escherichia coli K-12[1]

Hfr G6 *his*$^-$

F$^-$464 *ileu*$^-$

ERW F' *his*$^+$/*his*$^-$, Sm^S

B380 F$^-$*his*$^-$, Sm^R

Hfr G6 *his*$^-$ and F$^-$464 *ileu*$^-$ were supplied by Dr. John H. Lancaster. ERW F' *his*$^+$/*his*$^-$, Sm^S and B380 are isogenic. The only differences between the two are that ERW carries the F' *his*$^+$ episome, while B380 carries the chromosomal Sm^R characteristic. ERW and B380 are non-piliated, although ERW possesses F pili.

Culture medium

Stock cultures were routinely maintained on nutrient agar slants.

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[1]The abbreviations used are: *his*$^-$= histidineless, *his*$^+$= capacity to synthesize histidine, *ileu*$^-$= isoleucineless, Sm^R= resistance to streptomycin, Sm^S= sensitivity to streptomycin.

Broth cultures for mating experiments were grown on Difco Pennassay broth medium. The media used to carry out mating experiments varied, and will be described with the different experiments.

Vogel's minimal salts glucose agar (24) was used to select recombinants in Hfr G6 X F$^-$464 matings. Vogel's minimal salts glucose agar plus 0.4 milligrams per milliliter (mg/ml) of streptomycin was used to select recombinants in ERW F' *his*$^+$/*his*$^-$,Sm^S X B380 F$^-$*his*$^-$, Sm^R matings.

Nutrient broth was used for cultivation of large quantities of cells for extraction.

Hot phenol extraction of cells

The procedure used was that of Westphal as described in Kabat and Mayer (25). Five grams of washed cells were suspended in 175 ml of distilled water preheated to 65°C. An equal volume of 90% phenol was added and stirred with a teflon magnetic stirrer for twenty minutes. The temperature was maintained at 65°-68°C during this period. The suspension was cooled and centrifuged at low speed to separate the layers. The phenol layer was re-extracted with water and the combined aqueous layers were dialysed overnight to remove excess phenol. After dialysis, the aqueous layer was concentrated at 80°C under vacuum. The concentrate was washed twice with ether to remove residual phenol. The concentrate was then precipitated with 0.025M $MgCl_2$ (26) at 4°C. The

precipitate was dialyzed overnight to remove $MgCl_2$ and then lyophilized.

Assay procedure for conjugation factors (CF)

The assay procedure except for slight modifications is that of Lancaster, et al. (23). Logarithmic phase cultures were grown in a New Brunswick gyrotary shaker incubator. When the absorbance at 420 nm reached 0.4, the cultures were harvested by centrifugation. The cultures were washed in either double strength Vogel's broth plus 0.2% glucose or in double strength Pennassay broth, resuspended in the wash medium, and standardized to an absorbance of 0.7 at 420 nm.

The material to be assayed was either weighed out and diluted to a known concentration or assayed by volume and the concentration chemically determined.

Serial two fold dilutions of the material to be assayed were then prepared in sterile distilled water. The final volume of each dilution was 0.5 ml.

One-fourth ml of each culture was then added to the assay tubes as well as to a number of tubes containing 0.5 ml of distilled water, which served as a control.

The tubes were incubated in a 37°C stationary water bath for one hour to allow for maximal yield of recombinants. Recombinants were detected by plating on Vogel's minimal salts glucose agar or Vogel's minimal salts glucose agar plus 0.4 mg/ml of streptomycin.

The activity of the assayed material, expressed as per cent inhibition, was calculated relative to the number of recombinants observed in the control tubes, which were taken as zero per cent inhibition.

Recombination in cation chloride solutions

Two-tenths per cent inocula into fresh Pennassay broth were made from overnight broth cultures of Hfr G6 and F⁻464. The cell density of the cultures was standardized at 420 nm with a Bausch and Lomb spectronic 20. After reaching a cell density corresponding to an absorbance reading of 0.40, both cultures were harvested by centrifugation. The cultures were washed twice in deionized distilled water and resuspended in the same. One-fourth ml each of Hfr G6 and F⁻464 were added to 0.5 ml of the cation chloride solution in distilled deionized water. Controls were prepared in a similar manner with the omission of cation chlorides. The mating mixture was incubated at 37°C in a stationary water bath for one hour for maximal yield of recombinants. After the incubation period, appropriate dilutions were prepared and plated on Vogel's glucose minimal agar plates.

Recombinant colonies were counted after 36-48 hours. Measurements were expressed as the ratio of the number of recombinants in cation chloride to the number of recombinants in distilled water.

Recombination in cation chlorides, buffer, and glucose

Inocula (0.2%) into fresh Pennassay broth were made from overnight broth cultures of Hfr G6 and F⁻464. The fresh cultures were standardized at 420 nm with a Bausch and Lomb spectronic 20. The cultures were washed separately in 0.05M tris (hydroxymethyl) amino methane (Tris) buffer, pH 8.3, plus 0.2% glucose. The cultures were resuspended in the wash medium and the cell concentrations adjusted to give an Hfr G6 : F⁻464 ratio of one to ten. To 0.5 ml of cation chloride solutions (prepared in 0.05M Tris, pH 8.3, plus 0.2% glucose), were added 0.25 ml of Hfr G6 cells and F⁻464 cells in a one to ten ratio. Controls were prepared in a similar manner, except for the omission of cation chloride. The final volume of each tube was one milliliter.

The mating tubes were incubated at 37°C in a stationary water bath for one hour. After the incubation period, appropriate dilutions of the mating mixture were made and plated on Vogel's minimal salts agar. The plates were incubated at 37°C for 36-48 hours, at which time the recombinant colonies were counted.

Effect of cation chlorides on the activity of conjugation factors (CF)

Conjugation factors were prepared as described by Lancaster, et al. (23) from Hfr G6 and F⁻464. Hfr G6 and F⁻464 conjugation factors at 1.0 mg/ml were treated with 0.1M

ethylenediaminetetraacetic acid, disodium salt (EDTA) for two hours at 4°C. The EDTA was removed by prolonged dialysis against distilled deionized water at 4°C.

For the assay of the above treated conjugation factors, logarithmic cultures of Hfr G6 and F⁻464 were grown at 37°C in a New Brunswick gyrotary shaker. The cultures were harvested by centrifugation and washed twice in 0.1M Tris buffer, pH 8.3, plus 0.4% glucose. The cultures were resuspended to an absorbance of 0.7 at 420 nm in the wash solution plus the cation chloride. To half milliliter volumes of CF in distilled water were added 0.25 ml of each culture. Controls were prepared in a similar manner using half milliliter volumes of distilled deionized water. The tubes were incubated at 37°C in a stationary water bath for one hour. After the mating period, samples were withdrawn, diluted, and plated on Vogel's minimal salts agar. The plates were incubated at 37°C for 36-48 hours and the recombinant colonies were counted.

Separate but similar assays were performed on EDTA treated Hfr G6 CF and F⁻464 CF in buffer plus 0.2% glucose.

Purification of phenol water extracts

Purification of phenol water extracts was accomplished by the method of Yuasa, et al. (27). The concentrated, ether washed extracts were centrifuged at 105,000 x g for ninety minutes to pellet the lipopolysaccharide (LPS). The nucleic

acid was removed from the supernatant by precipitation with $HClO_4$ added to a final concentration of 5%. The $HCLO_4$ was neutralized with KOH. The supernatant was then dialyzed overnight, and lyophilized. This fraction contained the soluble polysaccharide fraction (sPF) (28). The LPS pellet was resuspended in 0.025M Tris buffer, pH 7.5, containing 0.05 mg/ml of pancreatic ribonuclease. This suspension was incubated at 56°C for two hours. After two hours, the suspension was repeatedly pelleted in distilled water at 105,000 x g until the absorbance at 260 nm became negligible. The LPS pellet was resuspended in distilled water and lyophilized.

This procedure was modified when only purified LPS was required. The modification was that ribonuclease was added prior to the first centrifugation (105,000 x g). The pellet (LPS) was then washed repeatedly in distilled water at 105,000 x g until the absorbance at 260 nm became negligible. The LPS pellet was then lyophilized as before.

A flow sheet of the extraction and modification of the purification scheme is shown in Figure 1.

Acid hydrolysis of purified LPS

LPS was hydrolyzed with dilute acetic acid (29). Twenty milligrams of LPS were suspended in 4.0 ml of 0.2N acetic acid and heated for thirty minutes in a boiling water bath. The precipitate (Lipid A) was collected by low speed centrifugation, washed twice with water, and suspended in 2.0 ml of distilled water. This fraction (Lipid A) was

stored at minus twenty degrees centrigrade for assay. The supernatant fraction (degraded polysaccharide) was neutralized with 0.15M NH_4OH and also stored at minus twenty degrees centigrade for assay.

Deacylation of purified LPS

LPS from F' ERW and F⁻B380 were deacylated according to Niwa, et al. (30). For alcoholic deacylation, 30 mg samples were suspended in 3.8 ml of absolute ethanol. Two-tenths ml of 2N NaOH was added and the tubes were incubated at 30°C for one hour. After one hour, the samples were neutralized with acetic acid in 95% ethanol and rapidly chilled in an ice bath. Five volumes of cold absolute ethanol was added and the samples were centrifuged at 10,000 x g for twenty minutes. The pellets were washed twice more with cold absolute ethanol and then dissolved in a few milliliters of water. After exhaustive dialysis at 4°C, the water-soluble, deacylated LPS preparations were stored at minus twenty degrees centigrade for assay.

For dimethylsulfoxide (DMSO) treatment, 30 mg of purified LPS was suspended in 3.2 ml of DMSO. Eight-tenths ml of aqueous 0.5N NaOH was added and the tubes incubated at 30°C for one hour. The remainder of the procedure was exactly as described for alcoholic deacylation except that 0.1M sodium acetate in 95% ethanol was used to facilitate precipitation of the LPS.

Separation of core and side-chain polysaccharide of purified LPS

The method used for core and side-chain polysaccharide isolation was obtained through a personal communication from Dr. Petar Alaupovic, Oklahoma Medical Research Foundation. LPS samples were hydrolyzed by refluxing at 90°C in 1% acetic acid for four hours. After cooling, chloroform was added to extract the lipid. The phases were separated and the aqueous phase dialyzed against distilled water (1:60) for 12 hours. The dialysis water was saved (outer dialysate). The dialysis was repeated three more times and the outer dialysates (O.D.) were saved. The combined outer dialysates were flash evaporated at 80°C under vacuum to about ten milliliters and stored at minus twenty degrees centigrade. The inner dialysate (I.D.) was also stored at minus twenty degrees centigrade.

Chemical determinations

Standard curves were prepared for all determinations. The phenol sulfuric acid reagent of Dubois, et al. (31) was used for estimating hexose. D-glucose (Sigma) was used as a standard. Heptose was measured by the cysteine sulfuric acid method of Dische as modified by Osborn (26). Glucoheptose (Sigma) was used as a standard. Glucose and galactose were determined with "Glucostat" and "Galactostat" reagent kits respectively. Both were obtained from Worthington Biochemicals. D-glucose and D-galactose from Sigma were used as

standards. Rhamnose was measured by the cysteine sulfuric acid method of Dische, et al. (32) with a ten minute heating period. L-rhamnose (Sigma) was used as a standard. Phosphate was determined by the method of Ames (59). KDO was measured by the thiobarbituric acid method of Weissbach and Hurwitz as modified by Osborn (26). An authentic sample of KDO was purchased from Pfanstiehl Laboratories, Waukegan, Illinois. All of the colorimetric measurements were determined with a Gilford model 2000 recording spectrophotometer, as were the spectra of fractions from purifications of LPS.

Viability controls

Viability controls were performed on all fractions showing inhibitory activity. These controls consisted of incubating the male and female cultures separately in the material used for the assay. The number of cells, time of incubation, and temperature of incubation were identical to those used in the assay.

Effect of (lipo) polysaccharides on mating pair formation

Mating pair formation was measured by the standard technique of deHann and Gross (37). F' ERW and F$^-$B380 cells were cultured as described in the assay, washed twice and resuspended in double strength Vogel's broth plus 0.4% glucose. One-half milliliter volumes of deacylated (DMSO) LPS from F' ERW and F$^-$B380 were prepared. Each preparation

contained sufficient hexose to give more than 99% inhibition of recombination. Control tubes consisted of 0.5 ml of saline and deacylated (alcoholic) LPS from Serratia marcescens. To each of the tubes at 37°C were added 0.25 ml each of F⁻B380 and F' ERW cells. Beginning at three minutes after the addition of cells, and at two minute intervals thereafter, samples were removed from the tubes and "gently diluted" 1:1000 by using tissue culture pipettes and allowing the sample to flow onto the side of a 500 ml flask into pre-warmed (37°C) Vogel's broth plus 0.2% glucose. The flask was then swirled to dilute the suspension. The flasks were left stationary in a 37°C water bath for one hour. All operations were performed using 37°C water baths. After one hour, plating were made on Vogel's minimal agar (0.2% glucose) plus 0.40 mg/ml of streptomycin. After 36-48 hours at 37°C, the recombinant colonies were counted.

CHAPTER III

RESULTS

Effects of cation chlorides on conjugation

Certain environmental optima have been reported for conjugation in *E. coli* K-12. Hayes (33) found the optimal temperature to be 37°C. Czerwinska (34) showed a pH range optima between 6.5 and 7.5. Czerwinska (34) also reported that potassium ion addition to previously deionized broth resulted in a stimulation of recombination. It was not determined at which stage in conjugation the ion functioned.

Because of an interest in the complementary surface components which permit mating pair formation, the role of cations was reinvestigated. Calcium has been reported as a requirement for male specific phage adsorption to male cells (35). Therefore, it was not considered illogical to look for a cation which might increase the sensitivity of the assay procedure for conjugation.

The results of measuring recombination in a strictly ionic environment are shown in Figures 2 to 5. A characteristic of all four of these figures is the appearance of two maxima each.

The first maximum for the monovalent cations, KCl and NaCl, is exactly at 10^{-2}M. At this concentration, NaCl shows a greater amount of recombination than does KCl. The second maximum for the monovalent cations is at 10^{-7} M for KCl and at 10^{-6}M for NaCl.

The first maximum for the divalent cations, $CaCl_2$ and $MgCl_2$, occurs at 10^{-2}M and 10^{-3}M respectively. The second maximum for the divalent cations is at 10^{-5}M for $MgCl_2$ and $CaCl_2$. At all concentrations tested, the divalent cations show much lower levels of recombination when compared with the monovalent cations.

From the previous results, it is easy to generalize and believe that the first maxima represents a surface effect and that the second maxima represents an internal effect. However, the viability of both male and female is quite low in these experiments. At some ionic concentrations the viability is as low as 30%. There is also a problem in that low numbers of recombinants were observed, which requires that.large numbers of cells be plated out per plate. When large numbers of cells are on the plate, mating may occur on the plate and not in the ionic solution.

Fisher (36) found that phosphate buffer and a carbon source functioned almost as well as a complex medium for conjugation. To eliminate the problems involved in strictly ionic matings, Tris buffer (to eliminate phosphate) and glucose were added to the cation chlorides.

Recombination levels in Tris buffer, cation chlorides and glucose are shown in Table 1. Recombination is approximately ten fold higher in this medium when compared to ionic medium. Therefore, less cells have to be plated out and the problem of matings on the plate is eliminated. In these experiments the female cell concentration is ten times that of the male cell concentration.

Two ionic concentrations, 2.5×10^{-2}M and 2.5×10^{-6}M, were used. These concentrations correspond, on the average, to the maxima observed in ionic media. Controls are measured in the absence of the cation chlorides only. Of the [illegible] concentrations used in these experiments, only 2.5×10^{-6}M $CaCl_2$ clearly stimulates recombination when compared to the control. NaCl at 0.025M stimulates recombination, whereas 2.5×10^{-6}M NaCl is inhibitory. KCl at 0.025M is inhibitory compared to the control. 2.5×10^{-6}M KCl shows recombination levels very close to the control.

Effect of cation chlorides in Tris buffer plus 0.2% glucose on the activity of conjugation factors

The effect of cation chlorides in this medium on the ability of conjugation factors (CF) to inhibit recombination was then assayed. The same Tris buffer plus 0.2% glucose was used. The ratio of male to female cells was one to one. This alteration in relative cell numbers was made in order to standardize the relative number of cells available for

interaction with CF. In addition, the concentration of conjugation factors was sufficient to give maximal inhibition in Pennassay broth. Conjugation factors were treated with EDTA to remove divalent cations. The treated CF of both male and female cells were then dialyzed exhaustively to remove the EDTA.

EDTA treated CF from F^-464 cells has no inhibitory activity as shown in Figure 6. When certain ions are added, however, the activity is increased. $CaCl_2$ and $MgCl_2$, at 2.5×10^{-6}M, increase the activity by only 10% and 14% respectively. KCl at 0.025M increases the inhibition of F^-464 CF to 58%. This value approaches the inhibition of F^-464 CF observed in Pennassay broth. All other cation chlorides tested show no inhibition of recombination in the presence of EDTA treated F^-464 CF.

EDTA treated CF from Hfr G6 cells shows between 10-20% inhibition in Tris buffer plus 0.2% glucose. This value, along with the effect of other cations on Hfr G6 CF, is shown in Figure 7. The activity of G6 CF in 2.5×10^{-6}M NaCl is increased by 21%. However, as is similar to the case of 464 CF, 0.025M KCl gives a 49% increase in the activity of G6 CF. This value also is very close to the value observed in Pennassay broth for G6 CF. These are the only cations showing any activity.

Assays of conjugation factors in Vogel's minimal salts broth versus Pennassay broth

deHann and Gross (37) have reported that recombination in a minimal salts medium is greater than recombination in nutrient broth. When amino acids were added to the minimal salts medium, the levels of recombination reverted back to those levels in nutrient broth. deHann and Gross (37) hypothesized that amino acids caused mating pair instability. The assay of conjugation factors described by Lancaster, et al. (23) was in Pennassay broth.

Vogel's minimal salts medium differs primarily from Pennassay broth in that the latter contains amino acids and peptides. Both media contain glucose and potassium phosphates as well as other inorganic factors. If amino acids do cause mating pair instability, as suggested by deHann and Gross, amino acids may also have an effect on the activity of isolated conjugation factors.

When conjugation factors of either type are assayed in Pennassay broth, the inhibition curve plateaus at about 40-60%. However, when the assay is carried out in Vogel's minimal salts medium, the inhibition curve plateaus above 90%. The results of such an experiment for isogenic F' ERW CF is shown in Figure 8. Results similar to this occur for the non-isogenic Hfr G6 CF and the non-isogenic $F^{-}464$ CF.

In view of these results, all subsequent assays were carried out in Vogel's minimal salts medium plus 0.2% glucose instead of Pennassay broth.

Purification of conjugation factors from isogenic strains

Isogenic strains were used exclusively in the purification of conjugation factors in order to minimize chemical differences in cell walls due to non sex-related genetic inhomology.

The aqueous phase of phenol water extracts yields lipopolysaccharide (LPS), polysaccharides of various types, nucleic acid, and fragments of lipid. Even with these many components, however, the extract is well defined chemically. LPS is an amphipathic molecule and forms aggregates (of up to 70S) in aqueous solution. Therefore, LPS is readily sedimented in the ultra centrifuge, leaving other components in the supernatant. Fragments of lipid are removed from the supernatant by ether washes, leaving only nucleic acid and the soluble polysaccharide fraction (sPF). Nucleic acid is removed by perchlorate precipitation, leaving the polysaccharide fraction. The purification scheme of F' ERW CF and F⁻B380 CF is shown in Figure 1. The sPF is still contaminated with residual nucleic acid. However, the LPS fraction is substantially free of nucleic acid after two to three washes in distilled water at 105,000 x g. A spectrum of LPS and sPF is shown in Figure 9.

The assay of nucleic acid contaminated sPF from F' ERW and F⁻B380 is shown in Table 2. No significant inhibition is observed at any of the concentrations tested.

The assay of purified LPS from F' ERW and F⁻B380 is shown in Figure 10. The inhibition curves for both plateaus above 90%. It is noted here that after purification and lyophilization of LPS, sonication is required to break up aggregates for the assay.

Effects of acid and base treatment of LPS on its ability to inhibit conjugation

Dilute acid treatment of LPS leads to the release of Lipid A and the lipid free degraded polysaccharide. Osborn (26) found that 2-keto,3-deoxyoctonoate (KDO) is resistant to borohydride reduction and suggested that the reducing terminus of the polysaccharide is probably KDO. Therefore, KDO probably provides the covalent linkage between the polysaccharide and Lipid A.

The action of base on LPS results in a rapid disaggregation in particle size as well as a release of fatty acids (30). The amount of deacylation by base depends on the conditions used, such as normality, temperature, and organic solvent. Niwa, et al. (30) have found that 95% ethanol and 80% DMSO (dimethylsulfoxide) are excellent solvents for deacylation of LPS. Niwa, et al. (30) have also shown that there is no release of certain monosaccharides under the

conditions they used indicating minimal polysaccharide degradation. They also found that with 80% DMSO as a solvent no fatty acid esters were detectable even with 0.03N NaOH.

The acid and base degraded LPS from B380 and ERW were assayed to determine what components were required for inhibition of recombination. The results are shown in Figures 11 and 12. The acid degraded polysaccharide from B380 is completely inactive (Figure 11). However, deacylation of B380 LPS with base results in a pronounced increase in activity of the polysaccharide fraction. As little as 40 micrograms per milliliter of hexose from deacylated B380 LPS gives more than 99% inhibition of recombination. It is noted that base deacylation of LPS gives a completely soluble preparation. The greater solubility of deacylated LPS over untreated LPS may contribute to the enhanced activity of the deacylated preparations. Figure 12 shows the assay of acid and base treated ERW LPS. At high hexose concentrations of 70 micrograms per milliliter, ERW degraded polysaccharide shows 40% inhibition of recombination. Deacylated ERW LPS shows an enhanced activity compared to untreated ERW LPS. Thirty micrograms of hexose per milliliter of deacylated ERW LPS gives more than 99% inhibition.

Because acid and base treatment of LPS may produce toxic products, viability controls were performed under conditions similar to those used in the assay. B380 cells and ERW cells were tested separately and in combination with the

deacylated LPS from B380 and ERW. In addition, cells of both B380 and ERW were tested with Lipid A fractions and polysaccharide fractions of each.

The viability of B380 and ERW cells in the presence of an excess of deacylated LPS from each is shown in Table 4. Even with 80 micrograms of hexose per milliliter, no decrease in cell numbers is observed when cells are exposed to deacylated LPS. In some cases, the presence of deacylated LPS appears to show an increase in cell number. Cell viability apparently is not a factor in the inhibition of recombination demonstrated by alcoholic base deacylated LPS.

The assays of ERW and B380 Lipid A preparations are shown in Table 3. The assays were by volume only. However, the upper concentrations (0.5 ml) correspond to much more than 0.5 mg of Lipid A. ERW Lipid A at 0.5 ml shows 50% inhibition of recombinations. However, no inhibition of B380 Lipid A occurs at any other concentration. Chang and Hager (29) report that Lipid A has bactericidal activity. It is possible then that the inhibition observed with B380 Lipid A is due to the death of cells.

The viability of B380 and ERW cells in the presence of Lipid A and degraded polysaccharide is shown in Table 5. B380 cells in the presence of Lipid A from ERW show about a 15% decrease in viability. ERW cells in the presence of Lipid A from B380 show a 20% decrease in viability. However, mixtures of equal amounts of the two cells remained stable or

slightly higher than the control. At any rate, a 15-20% decrease does not account for the 50% inhibition observed with B380 Lipid A. The inhibition observed with ERW degraded polysaccharide is also not explained by the viability controls.

Niwa, et al. (30) have reported that, under the conditions used (0.1N NaOH in 95% ethanol), over 90% of the fatty acids were removed from E. coli O 111:B4. However, they also reported that DMSO was a better solvent than 95% ethanol. For E. coli O 111:B4, no fatty acid esters were detectable after twenty minutes in 0.03N NaOH in 80% DMSO. In addition, the particle size of LPS was shown to drop much more rapidly than when 95% ethanol was used as a solvent (30). Because of the reportedly more efficient removal of fatty acids in base plus 80% DMSO, this solvent was also used to deacylate ERW LPS and B380 LPS.

The results of the assay of deacylated ERW LPS and B380 LPS using 80% DMSO as a solvent are shown in Figures 13 and 14. Deacylated LPS in 80% DMSO is as good or better than deacylated LPS using 95% ethanol as a solvent. For the assay, these results suggested that the lipid is of no importance. However, no measurements of fatty acid esters were made in this laboratory.

There is no difference in cell viability when cells are incubated in deacylated (DMSO) LPS from B380 and ERW

(Table 6). These results indicate that cell viability is not related to the inhibition observed (Figures 13, 14).

Assays of LPS from other genera

Three commercial LPS preparations (Difco) were obtained to test for specificity of the inhibition reaction. It was possible that the interaction or association of LPS with the cell surface, observed by these assays, is a general property of LPS. All three LPS preparations (Salmonella typhimurium, Serratia marcescens, and E. coli 0) formed a very fine suspension without sonication. They were assayed as received, without further purification.

The results of the assay of the commercial LPS preparations is shown in Table 7. A control of B380 LPS is also shown. Rather than observing any low degree of inhibition, only stimulation of recombination occurs with all three. The control B380 LPS shows 88% inhibition of recombination. A complication of these results is that all three commercial preparations contain large "0" specific side chains. The only commercial LPS preparations available are from cells which contain side-chain polysaccharide. Side chains may prevent the core polysaccharide from interacting with a complementary site on the male or female cells.

The chemical linkage which attaches side-chain polysaccharide to core polysaccharide is acid labile (38). Therefore, this linkage is split early during hydrolysis. This generality is true for all "0" strains tested. After

hydrolysis, the two polysaccharides are easily separated by dialysis. Core polysaccharide is no larger than 7000 Daltons, whereas side-chain polysaccharide has a molecular weight between 20,000-40,000 Daltons.

The procedure for the separation of core and side-chain polysaccharides is from Dr. Petar Alaupovic, Oklahoma Medical Research Foundation, who routinely uses it for S. marcescens.

The assay of core and side-chain polysaccharide from the three commercial LPS preparations is shown in Table 9. Forty-three micrograms per milliliter of E. coli 0 core polysaccharide shows 31% inhibition of recombination. Forty-eight micrograms per milliliter of S. typhimurium core polysaccharide shows 40% inhibition of recombination. S. marcescens gives no inhibition. ERW LPS, when hydrolyzed in the same manner as a control, shows no inhibition of recombination. B380 LPS, when treated likewise, shows only 14% inhibition of recombination. These results may mean that the conditions for the separation of polysaccharides are too rigorous. That is, the sugars required for inhibition may be acid sensitive and are destroyed by the treatment. Side-chain polysaccharide from all three show no inhibition of recombination.

The assays of the deacylated commercial LPS (0.1N NaOH in 95% ethanol) are shown in Table 8. At hexose concentrations greater than 200 micrograms per milliliter, S.

marcescens shows 10% inhibition of recombination. S. typhimurium also, at more than 200 micrograms per milliliter, shows 21% inhibition of recombination.

Effect of (lipo) polysaccharide on mating pair formation

deHann and Gross (37) have reported that gentle dilution (1:1000) of mating pairs after five minutes of incubation effectively separates pair formation from chromosome transfer. However, a small percentage of mating pairs begin chromosome transfer prior to five minutes while other mating pairs take up to fifteen minutes. Table 11 indicates that at three minutes, the majority of mating pairs are formed. Because isogenic parents are used, this finding is not unusual. The results are taken to indicate that (lipo) polysaccharide prevents pair formation because in the presence of deacylated LPS from F' ERW and F⁻B380, the number of mating pairs drops over one hundred fold (Table 11). The presence of deacylated LPS from S. marcescens shows a slight decrease from the control.

Rapin and Mayer (28) have examined some of the constituents of E. coli K-12 polysaccharides. F' ERW and F⁻B380 contain all of the constituents of what Rapin and Mayer consider "wild type" E. coli K-12 (Table 10).

Under the hydrolysis conditions of Alaupovic, et al. (56), rhamnose was present in ERW LPS but not detectable in B380 LPS. These conditions included refluxing LPS in 2N HCl

at 100°C for four hours. When the hydrolysis was carried out in sealed evacuated tubes (57), rhamnose was found in both LPS (Table 10). It appears that qualitatively, the presence of F does not lead to the total loss of these sugars or phosphate.

CHAPTER IV

DISCUSSION

The extent of recombination in distilled water supplemented with cation chlorides depends upon not only the type of cation but also the concentration of cation. There is a maximal yield of recombinants at 10^{-2}M NaCl and a minimal number (excluding absence of cation chlorides) at various other ion concentrations. Part of the explanation for these effects include a lowered cell viability. The maximal viability is about 70%. However, maximal viability does not correlate directly with maximal recombination. For example, the viability of F$^-$464 and Hfr G6 cells at 10^{-3}M NaCl is the same as at 10^{-2}M NaCl. The number of recombinants is about eight times greater at 10^{-2}M NaCl than at 10^{-3}M NaCl. Recombination via conjugation is dependent upon the availability of energy sources for both male and female cells (17). The stimulation of recombination observed in the various cation chlorides may reflect a metabolic activation or the possibility that cations are involved in cell surface interactions (39). However, because of low viability and low numbers of

recombinants, no more mating experiments were attempted in this medium.

In an attempt to increase the number of recombinants for worthwhile measurements, cation chlorides were supplemented with Tris buffer and glucose. Fisher (36) had reported that recombination was as high in the presence of phosphate buffer and a carbon source as it was in complete media. For our studies phosphate buffer was not used because of the insolubility of metallic ion phosphates and the necessity of adding either sodium or potassium ions in conjunction with the phosphates. In addition, Lancaster and Gallup (39) had already investigated the effect on mating of dilute ionic solutions in phosphate buffer. Glucose (0.2%) was used because Curtiss, et al. (17) have found that both parents require an energy source.

In Tris buffer and glucose only 2.5×10^{-6}M $CaCl_2$ clearly stimulates recombination above the level obtained in the control (Table 1). $CaCl_2$ is a cofactor for male specific phage attachment (12). It is possible that $CaCl_2$ is affecting F pili or F pili associated structures important in conjugation. Cations may still have two effects (internal and surface) in which an internal negative effect obscures a positive surface effect. The converse is also possible.

It is possible to estimate part of the surface effect of cations by using conjugation factors (CF) as indicators of surface interaction. Conjugation factors are

extracts of fertile cells which, when added prior to mixing of compatible fertile cells, inhibit recombination (23).

To assess part of the surface role cations play in conjugation, F⁻464 CF and Hfr G6 CF were treated with EDTA and assayed in Tris buffer, glucose, and cation chloride. The EDTA treated CF from Hfr G6 always retained activity, whereas the activity of 464 CF was lost in the absence of cation chlorides. This difference in the activity of the two CF preparations may indicate that the two extracts are different in some way. This is speculative because EDTA was presumed to have been removed completely and it is known that EDTA disrupts the envelope of gram negative bacteria (40). Both EDTA-treated CF preparations inhibit recombination best when 0.025M KCl is added to Tris plus glucose. It is interesting that EDTA-treated 464 CF, which shows no inhibition in the absence of ions, does give 10% and 14% inhibition, respectively, in $CaCl_2$ and $MgCl_2$. EDTA chelates both of these metallic ions. It is tempting to speculate that metallic ions are removed more efficiently from 464 CF by EDTA than they are from G6 CF. Ten and fourteen percent inhibition is very low however, and approaches plate count error.

Activity of conjugation factors were assayed in Vogel's minimal salts medium because of the high potassium phosphate concentration (10 g per liter) and the absence of amino acids which are reported to cause mating pair instability (37). Assays in Pennassay broth were compared to

assays in Vogel's broth because Pennassay broth was originally used for the assay (23). Recombination via conjugation is 10-15% greater in Vogel's broth than in Pennassay broth as measured in our laboratory. In Vogel's broth, inhibition of recombination is above 90% compared to about 45% in Pennassay for ERW conjugation factors (Figure 8). As expected, Hfr G6 CF and 464 CF show similar levels of inhibition. Both Vogel's broth and Pennassay broth contain glucose, potassium phosphates, and other inorganic factors. However, Pennassay broth contains both peptides and amino acids whereas Vogel's does not. Lipopolysaccharides or endotoxin bind peptides or proteins with subsequent inactivation (41). In the presence of Pennassay broth the conjugation factors may complex with peptides or amino acids and not interact with the complementary cells. Vogel's broth also contains approximately twice as much potassium phosphate as does Pennassay broth. It is possible that the extra potassium ion in Vogel's broth may account for all or part of the increase in activity of CF. The addition of amino acids or peptides to Vogel's may distinguish whether excess potassium ion activates CF or whether organic constituents inhibit CF. The possibility that the effects are on the cells rather than CF also exists.

The discovery that LPS is responsible for inhibition of conjugation in E. coli K-12 is not surprising (Figure 10). Bacteriophage T-3, T-4, and T-7 are inactivated by LPS (43). Resistance to certain antibiotics (52) and colicin adsorption

(42) are also thought to involve LPS in the cell envelope. Nucleic acid as an inhibitor of conjugation is eliminated by the results of the assay of the soluble polysaccharide fraction (sPF) which contains appreciable nucleic acid (Figure 9). The involvement of LPS in conjugation is suggested by the work of Ørskov, et al. (44), who found that a relationship apparently exists between female fertility in the Salmonella and the chemotype or sugar composition. Fertility appears to be negatively related to more complex chemotypes.

In general, glycosidic linkages are acid labile and base stable. Acid degraded polysaccharide from F⁻B380 LPS shows no activity (Figure 11). F' ERW degraded polysaccharide gives up to 40% inhibition (Figure 12). The same normality of acid, amount of LPS, time of hydrolysis, and temperature was used for both LPS preparations. The reason for this difference is unknown. There is the possibility that the presence of F in F' ERW may alter the structure of the polysaccharide. F has been likened to a prophage, and temperate phage, at least in Salmonella, are active in LPS modifications, as has been indicated through the studies of Robbins and his associates (45). Phage epsilon 15, which represses transacetylase (O acetylation), inhibits alpha specific cellular polymerase, and causes the appearance of a new beta polymerase (45).

F has a molecular weight of 45 million Daltons (46) and could code for between forty to sixty proteins, some of

which are involved in F pili synthesis. Other proteins are responsible for the expression of F, be it negative or positive. Surface exclusion (18) may account for another protein. The surprising thing, according to Hayes (6), is that more sex factor functions have not yet become apparent.

The base stability of LPS, as revealed by the assay, strongly suggests that the polysaccharide is important in the inhibition. The reasons for this conclusion is the known resistance of these polysaccharides to base degradation and the lability in base of the long chain acyl esters which constitute the bulk of the lipid (30). In addition, the enhanced activity after deacylation of LPS certainly indicates that the lipid is of little importance to the inhibitory activity (Figures 11, 12). The use of 80% DMSO as a solvent for deacylation gives a preparation with activity as high or higher than preparations obtained using 95% ethanol as a solvent (Figures 13, 14). Fatty acid esters are reportedly not detectable under conditions of saponification less strenuous than those used in this work (30). However, no measurements were made on the quantity of fatty acid esters remaining from these treatments. It is certainly possible that some few acyl linkages are left intact. Heath, et al. (47) have found that beta hydroxymyristate is resistant to both acid and base hydrolysis. 3N HCl and 100°C for 2 hours or 2.5N aqueous NaOH at 80°C for sixteen hours is required to liberate all of this fatty acid. The effect of the solvents (80% DMSO

and 95% ethanol) used in these studies on the saponification of beta hydroxymyristate has not been reported.

Toxicity to mating cells could account for apparent inhibition of recombination in these assays (Figures 11, 12, 13, 14). LPS from E. coli K and W has no toxicity for the homologous cells (23, 29). In the presence of acid degraded polysaccharides from B380 and ERW, the cells show little difference in viability compared to control saline (Table 5). Saponification or deacylation results in the release of salts of fatty acids which are removed by ethanol washes and dialysis. Residual salts of fatty acids if present, could damage the cells and result in inhibition of recombination.

The results of viability control studies (Tables 4, 6) indicate that cell viability is not influenced by base treated LPS (DMSO or ethanol). These fractions also show the greatest activity (more than 99% inhibition).

Lipid A is reported to be toxic for E. coli W (29). Lipid A from B380 shows 50% inhibition of recombination, which cannot be accounted for by decrease in viability (Table 5). It is possible that some molecules of LPS in this preparation escaped hydrolysis, or that inhibition from B380 Lipid A is more stable to dilute acid (pH 3) than is ERW Lipid A.

The frequency of genetic transfer between F' ERW and F^-B380 is about one male cell in five completing transfer. Stanier, et al. (48), using the data of Baron, list the frequencies of F' lac transfer between the enterics. The

frequencies vary between 10^{-4} for *E. coli* X *Salmonella*, to 10^{-7} or 10^{-8} for *Salmonella* X *Serratia*. The assay of *S. typhimurium* LPS, *S. marcescens* LPS, and *E. coli* O 111B:4 LPS indicates no inhibition of recombination (Table 7). This data is in accord with the low frequency of genetic transfer between *E. coli* K-12 and other genera as mentioned earlier.

A characteristic of the LPS from *S. typhimurium*, *S. marcescens*, and *E. coli* O 111B:4 is the presence of side-chain polysaccharide. These side-chains are large (20,000 - 30,000 Daltons) and, at least in the *Salmonella*, are polymerized onto the end of the core polysaccharide. (See Westphal and Lüderitz (51) for a more comprehensive review of the chemistry of LPS.) *E. coli* K-12 is "rough" (28). That is, it lacks side-chain polysaccharide and possesses only a core polysaccharide. The bulky side-chains, which extend out from the core, of smooth organisms such as *S. typhimurium*, *E. coli* O 111B:4, and *S. marcescens*, may sterically prevent core polysaccharide of other genera from interacting with the surface of *E. coli* K-12. The core region of *S. marcescens*, *S. typhimurium*, and *E. coli* O may be compatible with *E. coli* K-12, if the side-chains were removed. The exposed core then may be more amenable for interaction with *E. coli* K-12 cells. It has recently been suggested by Watanabe, *et al.* (49) that mutants of *S. typhimurium* lacking side-chains were more fertile than wild types.

If the possession of side-chains on the LPS of a fertile cell do prevent potential mating cells from pairing, two general methods are possibly available for that study. Chemically, side-chain polysaccharides can be separated from core polysaccharides and then assayed for their ability to inhibit conjugation. Secondly, "O" specific or side-chain specific virulent bacteriophage, which are now known (50), could be used to enrich a population for "rough" mutants. Any correlation between fertility and inhibitory activity of LPS could then be developed. The latter is preferred because of the heterogeneous nature of chemically separated cores and side-chains. The chemical separation of core and side-chain polysaccharide is used in this work because virulent "O" specific phage were not available to us.

The assay of separated core and side-chain polysaccharide indicates that side-chain polysaccharide is not functional in inhibition of mating (Table 9). The core preparations from E. coli O 111B:4 and S. typhimurium apparently show 36% and 40% inhibition. However, B380·LPS, when treated as a control for core separations, gives only 14% inhibition. The loss of activity with B380 and ERW LPS is not so surprising. The loss of activity of "core" polysaccharide from B380 LPS and ERW LPS is seen also with degraded polysaccharides (Figures 11, 12). The methods used for preparing core polysaccharide and degraded polysaccharide are similar with respect to the acetic acid normality, but differ with

respect to time and temperature (see methods). The exact degraded products from both hydrolysates is unknown. KDO is one of the most acid labile sugars in LPS (51). Free KDO is released during dilute acid hydrolysis more rapidly than is the polysaccharide from LPS (26).

Kalkar (53) has examined galactose negative E. coli K-12 mutants with respect to fertility and found no correlation between the presence or absence of galactose in the LPS. He has suggested that perhaps the backbone region of the core is of primary importance for fertility. The backbone of LPS consists primarily of KDO and heptose (51). In E. coli K-12, rhamnose is reported to be attached to KDO (54). Rhamnosyl-KDO is detectable only under mild acid hydrolysis. It may be that the configuration of the sugars in the backbone of E. coli K-12 (KDO, heptose, rhamnose) is important for cell-cell interaction during conjugation. Mutants of E. coli K-12, resistant to bacteriophage lambda, T-3, T-4, and T-7 are believed to possess only the backbone sugars (43). It would be of interest to compare these mutants with regard to male and female capabilities and the activity of their LPS in the assay. The inhibition observed with core polysaccharide from S. typhimurium, and E. coli O 111B:4 may be a measurement of potential mating pair compatability between S. typhimurium and E. coli O and E. coli K-12. However, the core polysaccharides should be purified and cell viability determined before any such conclusion is reached.

Further work on core polysaccharide was terminated in the expectation that "O" specific phage would eventually be available. Fertility correlations would then be available also.

Of the deacylated LPS from E. coli O, S. marcescens, and S. typhimurium, only S. marcescens shows any activity (10%), although the level of activity obtained may not be significant. As mentioned earlier, base has no effect on side-chain polysaccharide.

The results of the qualitative analysis of B380 and ERW LPS indicate that the usual sugars and phosphate found in E. coli K-12 strains are present in both. This does not rule out the possibility that F in the male may add a particular sugar or alter chemical linkages. Various types of chromatography should reveal whether a new sugar is added in the male or whether one is being overlooked. If the possible alteration of the polysaccharide by F is in the structure of the polysaccharide, the techniques of polysaccharide structural analysis are available. These would include partial acid hydrolysis and periodate oxidation. Because the fine structure of E. coli K-12 polysaccharide is unknown, it may be advantageous to examine males and females which are resistant to certain phage (58). These mutants are believed to have deficient backbone structures (52).

The process of pair formation in conjugation is poorly understood. Curtiss (17) has defined specific pair

as a union stable to dilution. He defines effective pair formation as a union ready to initiate chromosome transfer. An effective pair would presumably have an adequate "conjugation bridge."

There is little doubt that both specific and effective pairs are being measured when pairs are diluted at timed intervals (Table 11). However, no recombination would have occurred prior to dilution of the mating pairs (three to seven minutes after mixing). Only a small fraction of the mating pairs would have begun chromosome transfer. Upon dilution of the cells, inhibiting (lipo)polysaccharide is also diluted out to the point that it can no longer cause inhibition in the assay (0.05 micrograms per milliliter). Prevention of pair formation prevents any of the successive steps in conjugation.

At least three substances on mating cells have been inferred to be involved in pair formation. Two of these, F pili and an unidentified substance, are on the male (19). The identity of the substance on the female cell is also unreported, although it is believed to be chromosomally specified. The unknown substance on the male is F specific and also functions to prevent male homosexuality (19). It is recalled also that this unknown component is destroyed by periodate. F pili are F specific and are required for pair formation as measured by blending spectra and visual reappearance (55).

It is likely that (lipo)polysaccharide is the component on the female required for pair formation. Almost every mating pair is inhibited by (lipo)polysaccharide and the inhibition correlates well with the high fertility. Twenty percent of the males conjugate with females. The LPS from S. marcescens, S. typhimurium, and E. coli O 111B:4 may be considered as female LPS. The inability of the LPS from these three genera to inhibit conjugation correlates with their lack of fertility. This finding is negative, however, and fertile "mutant" cells devoid of side-chains may provide a positive correlation with "mutant" LPS. The recent results of Watanabe, et al. (49) with S. typhimurium mutants blocked in the synthesis of side-chain polysaccharide support that approach. Mutant female cells of S. typhimurium lacking side-chains are more fertile than those cells with side-chains.

The male (lipo)polysaccharide is as active as the female in preventing pair formation. It may be that male (lipo)polysaccharide is the unknown component responsible for both pair formation and surface exclusion.

Attempts to adsorb (lipo)polysaccharide from B380 and ERW to the respective and complementary cells were unsuccessful. A centrifugal wash was used to remove residual polysaccharide and the wash may have removed the polysaccharides from the cells. It is not known if the assay works with a membrane mating system. However, if a membrane assay

procedure is functional, the two (lipo)polysaccharides may be adsorbed to cells and the specificity determined.

It is possible that (lipo)polysaccharide, extracted from male cells, is partially [or completely] female material. If this is so, the polysaccharides, which are normally exposed to the aqueous environment, must be covered in some way to account for surface exclusion. Alternatively, the structure of the polysaccharide of the male may be altered by the presence of F to prevent male-male matings (surface exclusion).

In conclusion, the lipopolysaccharide from fertile E. coli K-12 cells inhibit conjugation. The (lipo)polysaccharide probably inhibits some step important in pair formation.

CHAPTER V

SUMMARY

Recombination measurements in cation chlorides and distilled water suggested a role for cations in conjugation. When Tris buffer and glucose were substituted for distilled water, of those tested, only $CaCl_2$ at 2.5×10^{-6}M clearly stimulated recombination. Conjugation factors from the aqueous phase of phenol-water extracts were treated with EDTA and tested with cations in Tris buffer and glucose. EDTA-treated conjugation factors from F$^-$464 and Hfr G6 showed 58% and 49% inhibition, respectively, in the presence of 0.025M KCl. Untreated conjugation factors showed more than 90% inhibition in Vogel's salts broth medium compared to only 40-60% in Pennassay broth.

Conjugation factors from isogenic males and females of E. coli K-12 were extracted and purified by centrifugation at 105,000 x g. The supernatant was made 5% in $HClO_4$ to precipitate the nucleic acid. The nucleic acid was removed by centrifugation and the resulting supernatant, which contains soluble polysaccharides (sPF), was neutralized and lyophilized for assay. The pellet from centrifugation at

105,000 x g contained lipopolysaccharide (LPS) and was further purified from nucleic acid by ribonuclease treatment and repeated washings at 105,000 x g. The purified LPS was lyophilized for assay.

Assays in Vogel's broth of purified LPS from isogenic ERW (males) and B380 (females) showed that only LPS retained activity (85%-95% inhibition). At all concentrations tested, the soluble polysaccharide fractions (sPF) showed no activity.

Purified LPS from isogenic males and females was treated with dilute acetic acid (0.2N) to separate Lipid A and degraded polysaccharide for assay. Degraded polysaccharide from B380 showed no inhibition whereas 40% inhibition was obtained with degraded polysaccharide from ERW.

Alcoholic base deacylation of LPS from males and females resulted in an enhanced inhibition by the polysaccharide-rich fraction. Thirty to forty micrograms per milliliter of hexose from fractions obtained from either male or female gave more than 99% inhibition of recombination in the assay.

Assays of LPS from Salmonella typhimurium, Serratia marcescens, and Escherichia coli $O111B_4$ were assayed for the ability to inhibit conjugation. All three were inactive. Assays of alcoholic base deacylated LPS from the above three were also inactive. Assays of separated core and side-chain polysaccharide from LPS suggested that the side chains were not functional in inhibition of conjugation.

It was indicated that (lipo)polysaccharide is important in conjugal fertility for the bacteria. In addition, it was suggested that the (lipo)polysaccharide of other genera may play an initial limiting role in intergeneric bacterial crosses.

Figure 1.--A flow sheet illustrating the extraction procedure of phenol water extracts and the modification of the purification scheme.

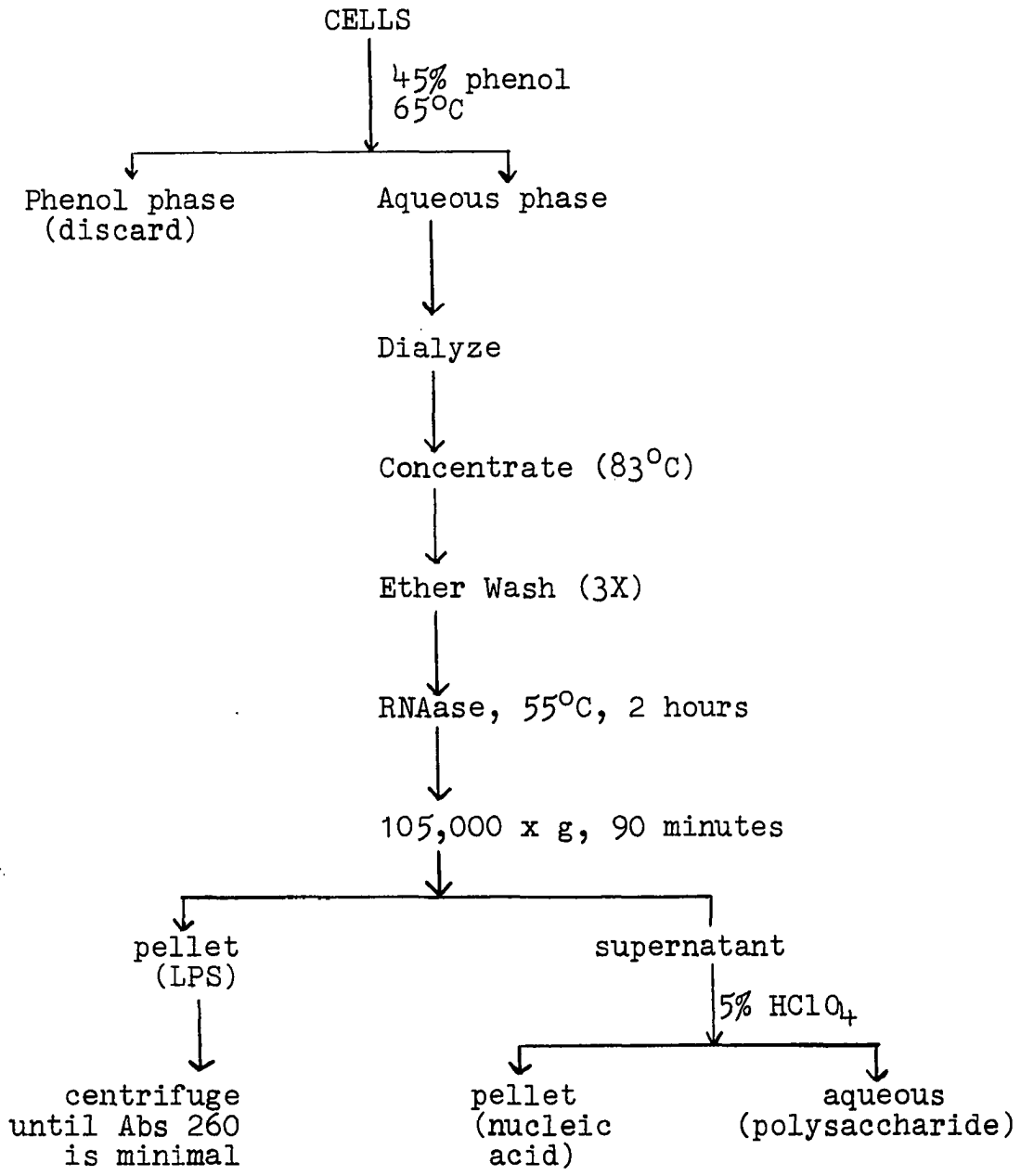

CELLS
45% phenol
65°C
Phenol phase
(discard)
Aqueous phase
Dialyze
Concentrate (83°C)
Ether Wash (3X)
RNAase, 55°C, 2 hours
105,000 x g, 90 minutes
pellet
(LPS)
supernatant
5% HClO4
centrifuge
until Abs 260
is minimal
pellet
(nucleic
acid)
aqueous
(polysaccharide)

Figure 2.--Recombination measured in the presence of various concentrations of magnesium chloride by crossing $F^{-}464$ with Hfr G6.

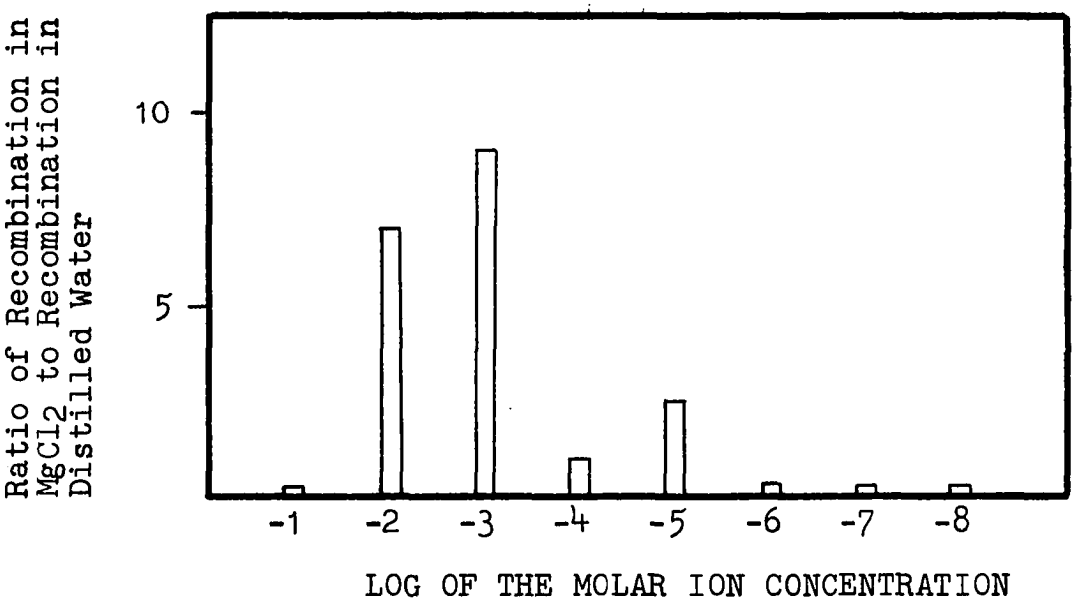
The relative number of recombinants are expressed as the ratio of the number of recombinants in $MgCl_2$ to the number of recombinants in distilled water.

The number of recombinants in distilled, deionized water is 260.

Figure 3.--Recombination measured in the presence of various concentrations of calcium chloride by crossing $F^{-}464$ with Hfr G6.

The relative number of recombinants are expressed as the ratio of the number of recombinants in calcium chloride to the number of recombinants in distilled water.

The number of recombinants in distilled, deionized water is 300.


Ratio of Recombination in $MgCl_2$ to Recombination in Distilled Water
10
5
-1
-2
-3
-4
-5
-6
-7
-8
LOG OF THE MOLAR ION CONCENTRATION

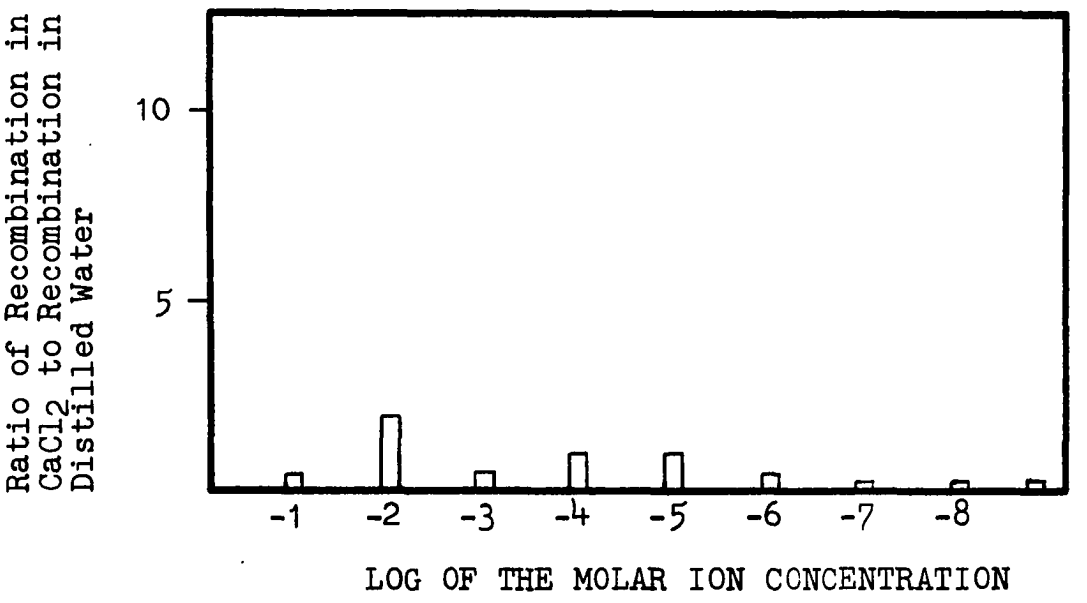

Ratio of Recombination in $CaCl_2$ to Recombination in Distilled Water
10
5
-1
-2
-3
-4
-5
-6
-7
-8
LOG OF THE MOLAR ION CONCENTRATION

Figure 4.--Recombination measured in the presence of various concentrations of potassium chloride by crossing F-464 with Hfr G6.

The relative number of recombinants are expressed as the ratio of the number of recombinants in KCl to the number of recombinants in distilled water.

The number of recombinants in distilled, deionized water is 320.

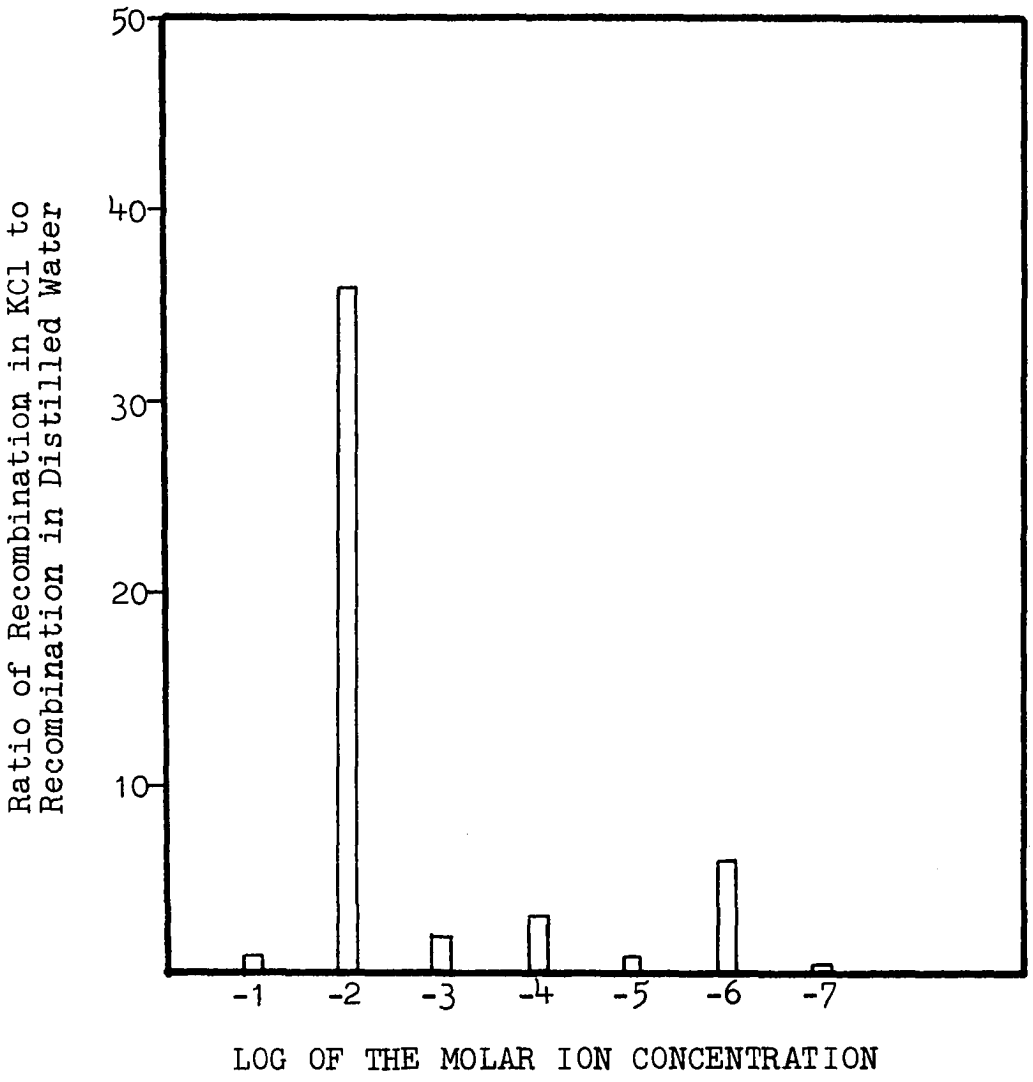

Ratio of Recombination in KCl to Recombination in Distilled Water
50
40
30
20
10
-1
-2
-3
-4
-5
-6
-7
LOG OF THE MOLAR ION CONCENTRATION

Figure 5.--Recombination measured in the presence of various concentrations of sodium chloride by crossing $F^{-}464$ with Hfr G6. The relative number of recombinants are expressed as the ratio of the number of recombinants in NaCl to the number of recombinants in distilled water.

The number of recombinants in distilled, deionized water is 310.

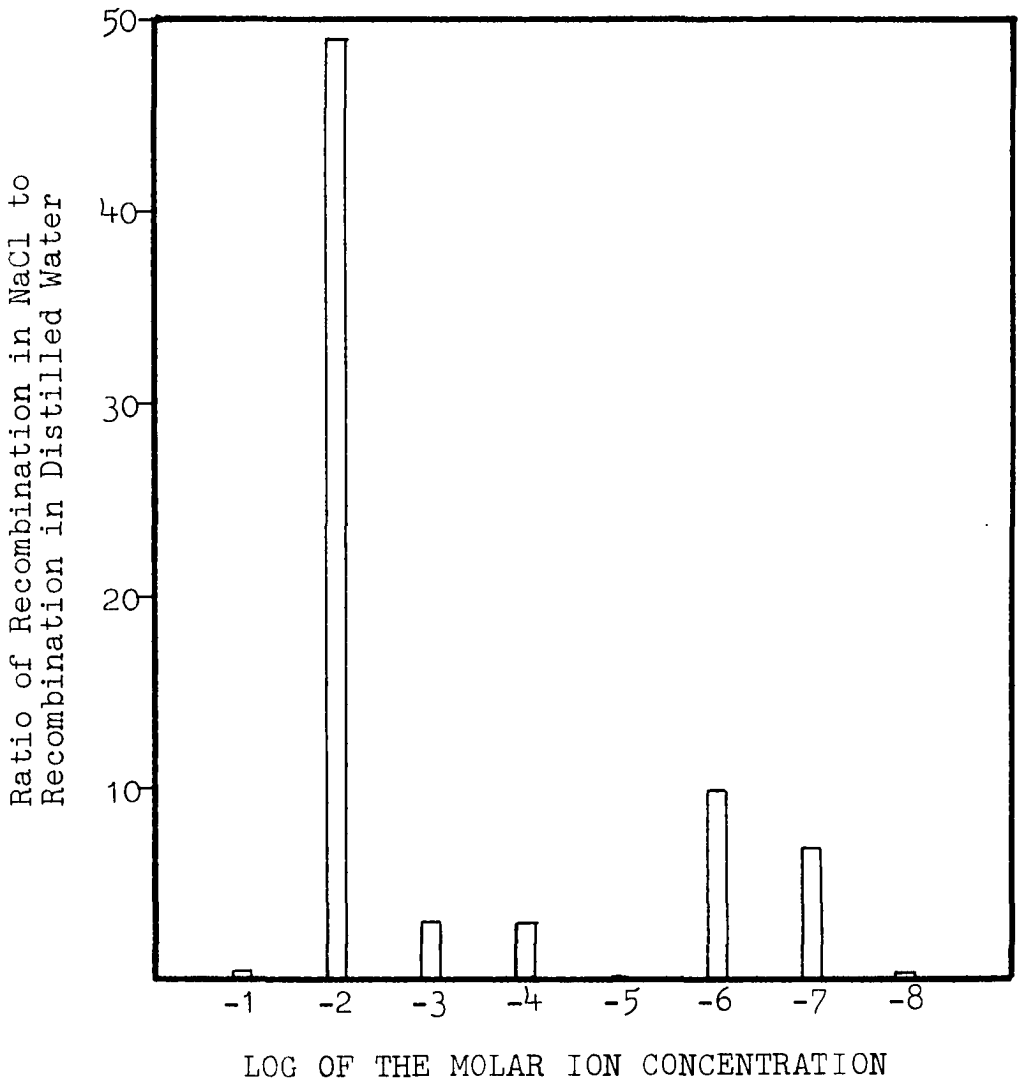

Ratio of Recombination in NaCl to Recombination in Distilled Water
50
40
30
20
10
-1
-2
-3
-4
-5
-6
-7
-8
LOG OF THE MOLAR ION CONCENTRATION

TABLE 1

RECOMBINATION IN 0.05M TRIS BUFFER (pH 8.3), 0.2% GLUCOSE, AND IN CATION CHLORIDES

	Number of Recombinants in:			
Molarity	KCl	NaCl	$MgCl_2$	$CaCl_2$
2.5×10^{-2}	1.5×10^3	7.0×10^3	0.3×10^3	---
2.5×10^{-6}	4.0×10^3	3.2×10^3	1.5×10^3	30×10^3
0	4.2×10^3	4.2×10^3	4.2×10^3	4.2×10^3

Figure 6.--The ability of EDTA treated F^-464 conjugation factors (CF) to inhibit recombination in the presence of 0.05M Tris buffer (pH 8.3), plus 0.2% glucose. To half milliliter volumes of EDTA treated F^-464 CF was added 0.25 ml volumes of each culture. Mating was for one hour followed by plating on Vogel's minimal agar plus 0.2% glucose.

Concentrations:

KCl - .025M.

$CaCl_2$ - 2.5×10^{-6}M

$MgCl_2$ - 2.5×10^{-6}M

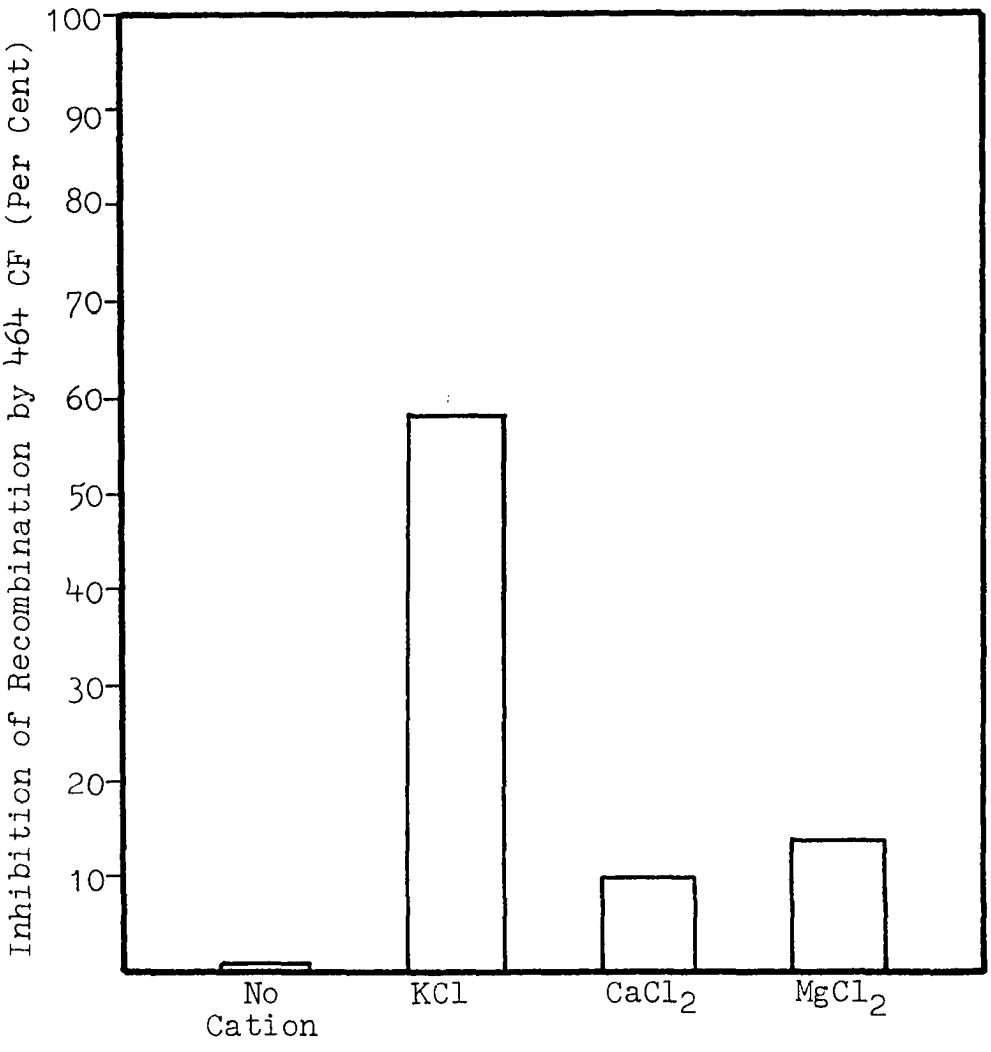

Inhibition of Recombination by 464 CF (Per Cent)
100
90
80
70
60
50
40
30
20
10
No Cation
KCl
$CaCl_2$
$MgCl_2$

Figure 7.--The ability of EDTA treated Hfr G6 conjugation factors (CF) to inhibit recombination in the presence of 0.05M Tris buffer (pH 8.3), plus 0.2% glucose. To half milliliter volumes of EDTA treated Hfr G6 CF was added 0.25 ml volumes of each culture. Mating was for one hour followed by plating on Vogel's minimal agar plus 0.2% glucose.

Concentrations:

KCl - .025M

NaCl - 2.5×10^{-6}M

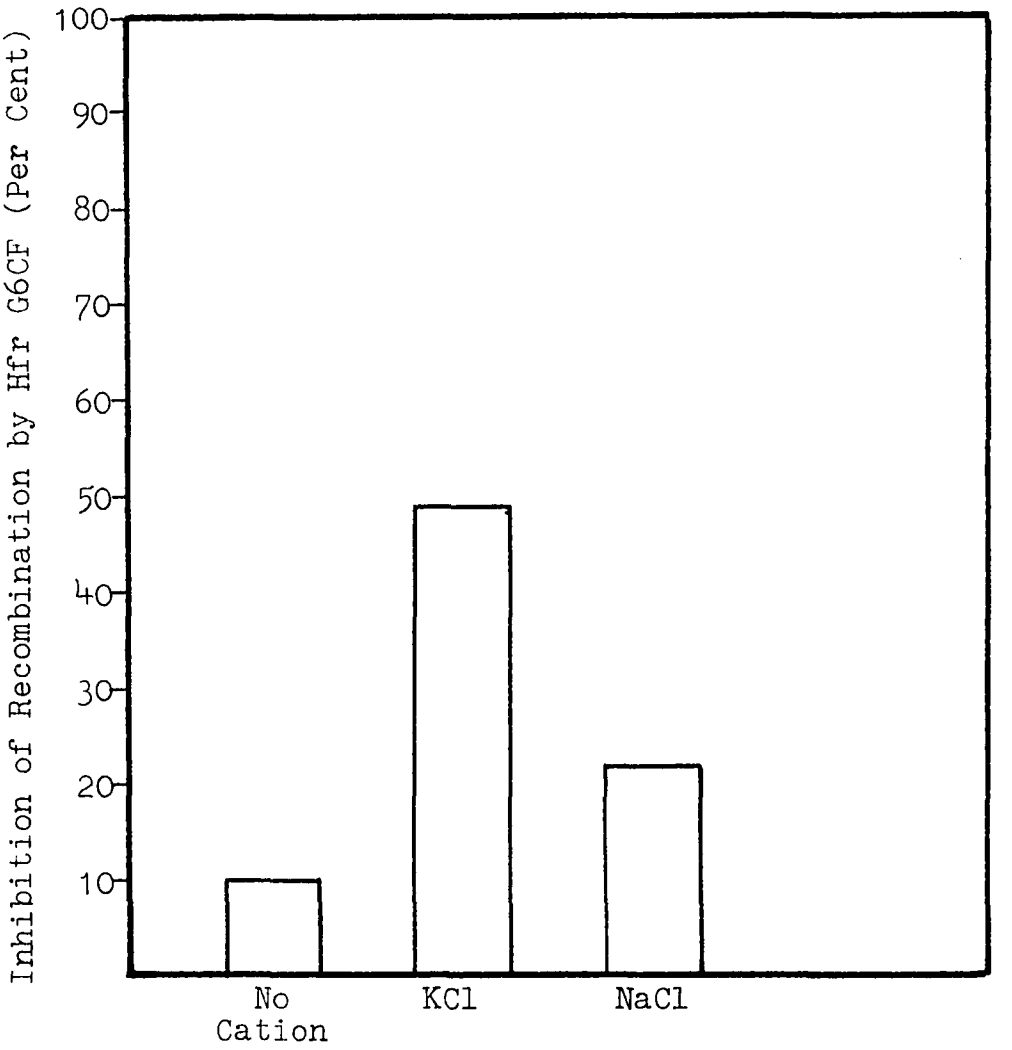

Inhibition of Recombination by Hfr G6CF (Per Cent)
100
90
80
70
60
50
40
30
20
10
No Cation
KCl
NaCl

Figure 8.--The comparison of the activity of F' ERW CF in Vogel's minimal salts broth and Pennassay broth. The assay procedure is described in the methods section.

o = Conjugation factors of isogenic F' ERW CF in Pennassay broth

● = Conjugation factors of isogenic F' ERW CF in Vogel's minimal salts medium.

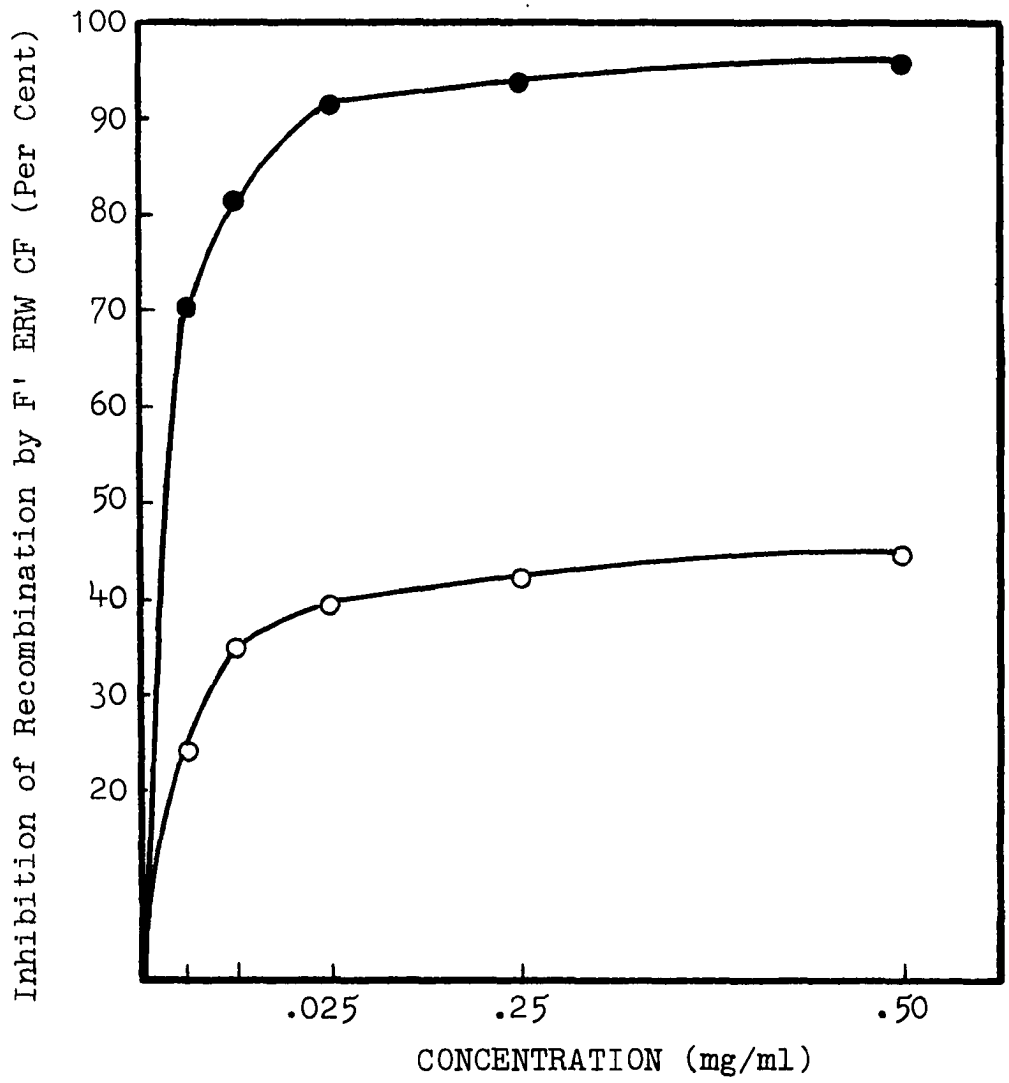

Inhibition of Recombination by F' ERW CF (Per Cent)
100
90
80
70
60
50
40
30
20
.025
.25
.50
CONCENTRATION (mg/ml)

Figure 9.--Ultraviolet spectra of soluble polysaccharide fraction (sPF) and lipopolysaccharide fractions (LPS) from F' ERW.

o = sPF

△ = LPS

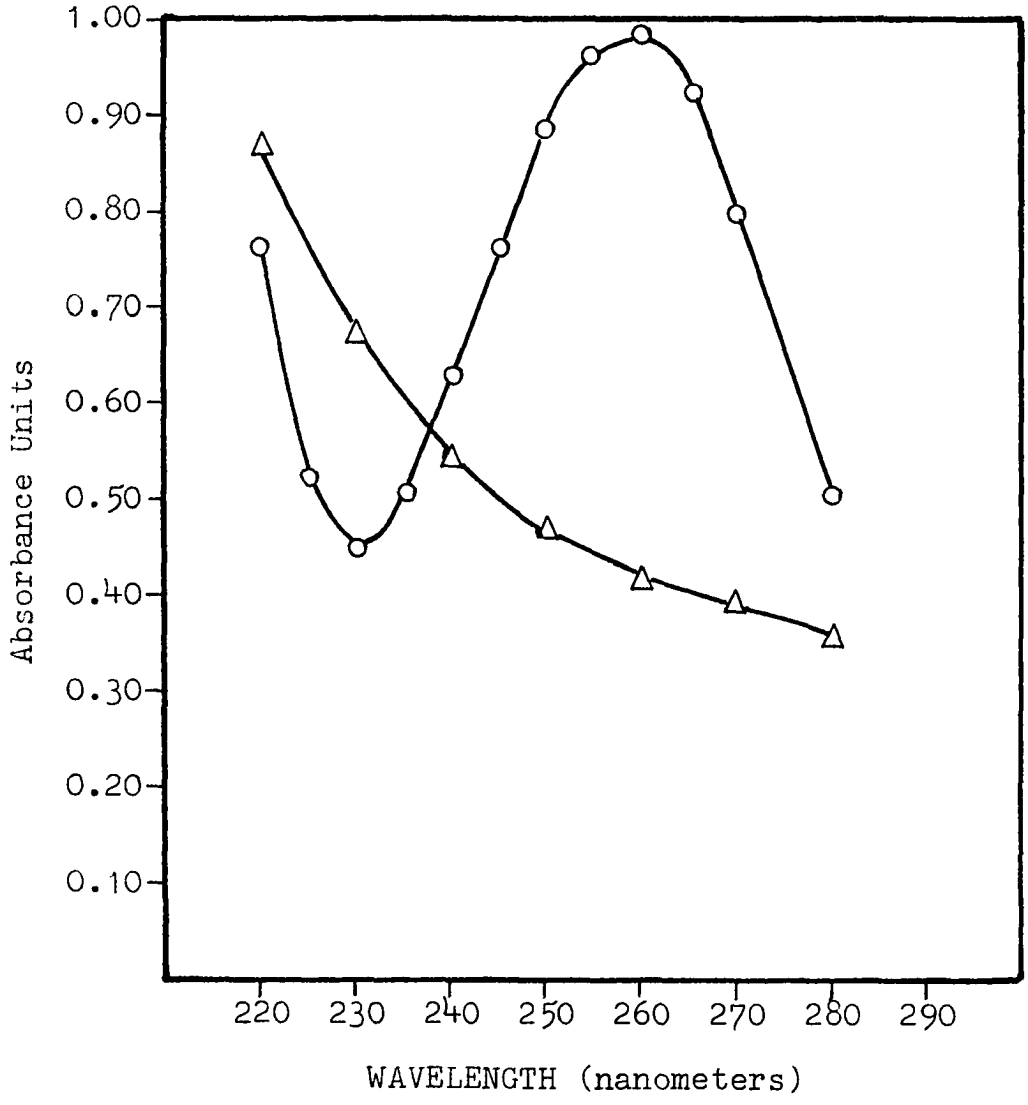

1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
Absorbance Units
220
230
240
250
260
270
280
290
WAVELENGTH (nanometers)

TABLE 2

ASSAY OF SOLUBLE POLYSACCHARIDE FRACTIONS FROM F^- B380 AND F' ERW

ERW (male) sPF		B380 (female) sPF	
Fraction Weight	Number of Recombinants	Fraction Weight	Number of Recombinants
0.5 mg	137×10^5	0.5 mg	126×10^5
0.25 mg	133×10^5	0.25 mg	94×10^5
0 mg	124×10^5	0 mg	124×10^5

Figure 10.--The assay of purified LPS fractions from F' ERW and F⁻B380 in vogel's. The assay procedure is described in the materials section. The assay cells are F' ERW and F⁻B380.

o = purified LPS from F' ERW

● = purified LPS from F⁻B380

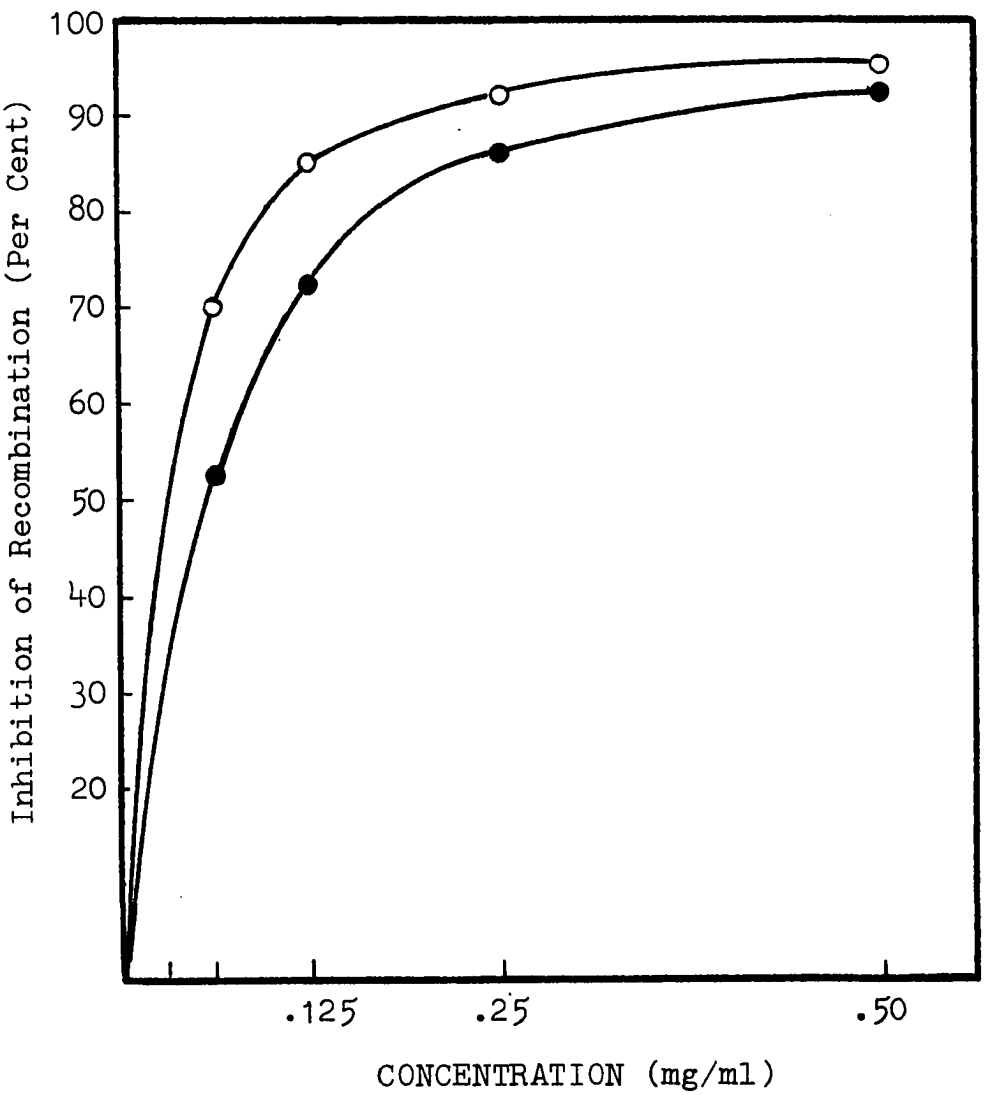

100
90
80
70
60
50
40
30
20
Inhibition of Recombination (Per Cent)
.125
.25
.50
CONCENTRATION (mg/ml)

Figure 11.--The effect on the assay of the polysaccharide-rich fractions obtained from 0.2N Acetic Acid and 0.1N NaOH in 95% alcohol treatment of F⁻B380 Lipopolysaccharide. The assay procedure is described in the materials section and the assay cells are F' ERW and F⁻B380. The treatment of LPS with acid and base is also described in the materials section.

o = B380 deacylated LPS

● = B380 LPS

Δ = B380 degraded polysaccharide

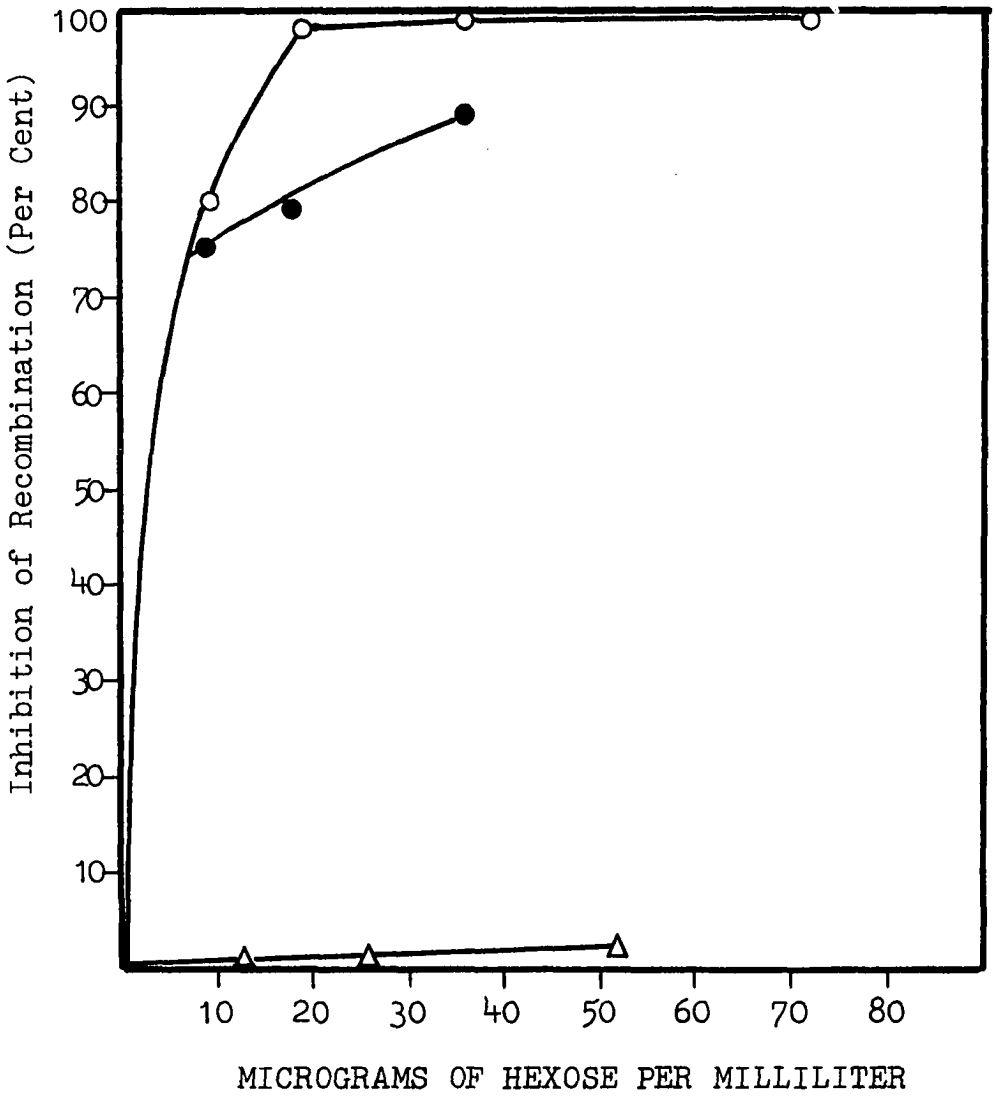

Inhibition of Recombination (Per Cent)
100
90
80
70
60
50
40
30
20
10
10
20
30
40
50
60
70
80
MICROGRAMS OF HEXOSE PER MILLILITER

Figure 12.--The effect on the assay of the polysaccharide-rich fractions obtained from 0.2N acetic acid and 0.1N NaOH in 95% alcohol treatment of F' ERW lipopolysaccharide. The assay procedure is described in the materials section and the assay cells are F' ERW and F⁻B380. The treatment of LPS with acid and base is also described in the materials section.

o = ERW deacylated LPS

● = ERW LPS

Δ = ERW degraded polysaccharide

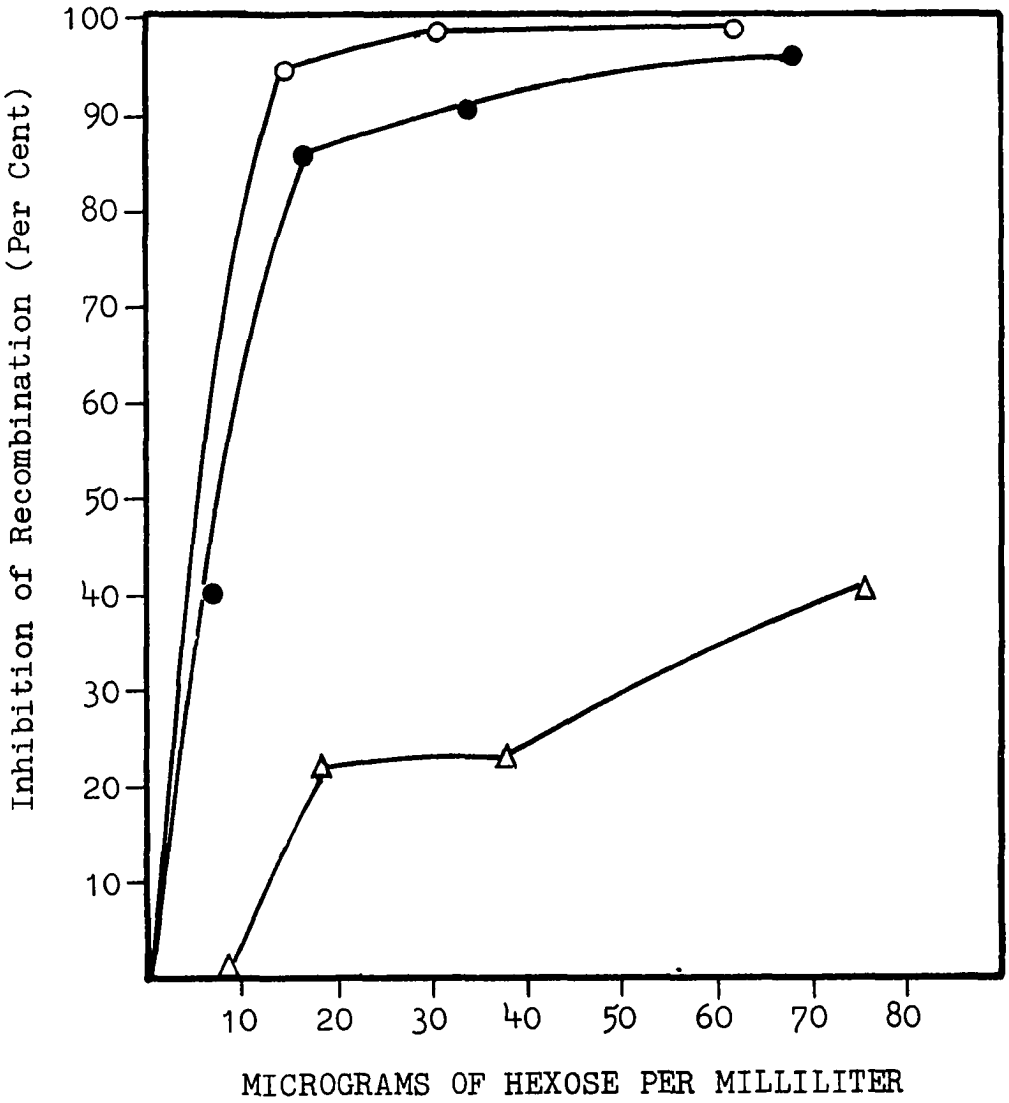

Inhibition of Recombination (Per Cent)
100
90
80
70
60
50
40
30
20
10
10
20
30
40
50
60
70
80
MICROGRAMS OF HEXOSE PER MILLILITER

TABLE 3

ASSAY OF LIPID A FRACTIONS FROM F⁻B380 AND F' ERW

ERW		B380	
Volume	Inhibition of Recombination	Volume	Inhibition of Recombination
0.5	0%	0.5	50%
0.25	0%	0.25	0%
0.125	0%	0.125	0%

TABLE 4

THE EFFECT OF ALCOHOLIC BASE DEACYLATED ERW LPS (80 MICROGRAMS PER MILLILITER) AND B380 LPS (70 MICROGRAMS PER MILLILITER) ON CELL VIABILITY

	Cell Count		
Component	B380	ERW	Both
Deacylated ERW LPS	22.7×10^7	12.1×10^7	19×10^7
Deacylated B380 LPS	18.2×10^7	13.2×10^7	14×10^7
Saline	15.4×10^7	11.3×10^7	15×10^7

TABLE 5

THE EFFECT OF LIPID A AND DEGRADED POLYSACCHARIDE FRACTIONS FROM B380 LPS AND ERW LPS ON CELL VIABILITY. THE CONCENTRATIONS OF THE COMPONENTS ARE THE HIGHEST USED FOR THE ASSAY

Component	Cell Count		
	B380	ERW	Both
Saline	1.0×10^8	2.2×10^8	1.27×10^8
Lipid A (ERW)	0.85×10^8	1.92×10^8	1.35×10^8
Lipid A (B380)	0.88×10^8	1.85×10^8	1.30×10^8
Degraded (ERW) Polysaccharide	1.44×10^8	2.35×10^8	1.33×10^8
Degraded (B380) Polysaccharide	0.95×10^8	1.95×10^8	1.33×10^8

Figure 13.--A comparison of the activities of F' ERW polysaccharide-rich fractions obtained from the treatment of F' ERW lipopolysaccharide with 0.1N NaOH in 95% alcohol and in 80% dimethylsulfoxide (DMSO). The cells for the assay were F' ERW and F⁻B380.

o = F' ERW LPS Treated with 0.1N NaOH in 95% ethanol

● = F' ERW LPS Treated with 0.1N NaOH in 80% DMSO

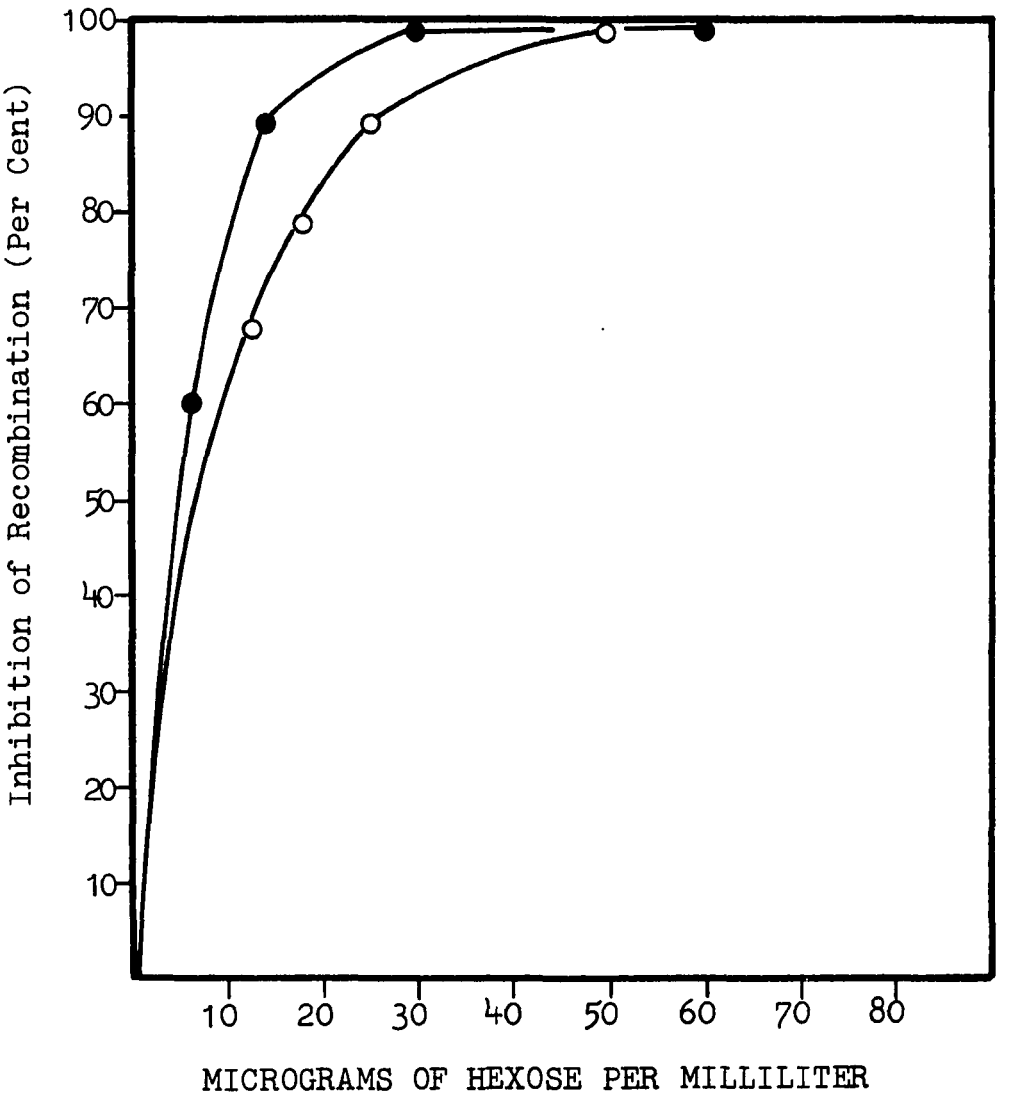

Inhibition of Recombination (Per Cent)
100
90
80
70
60
50
40
30
20
10
10
20
30
40
50
60
70
80
MICROGRAMS OF HEXOSE PER MILLILITER

Figure 14.--A comparison of the activities of F⁻B380 polysaccharide-rich fractions obtained from treatment of F⁻B380 lipopolysaccharide with 0.1N NaOH in 95% alcohol and in 80% Dimethylsulfoxide (DMSO). The cells for the assay were F' ERW and F⁻B380.

o = F⁻B380 LPS treated with 0.1N NaOH in 95% ethanol

Δ = F⁻B380 LPS treated with 0.1N NaOH in 80% DMSO

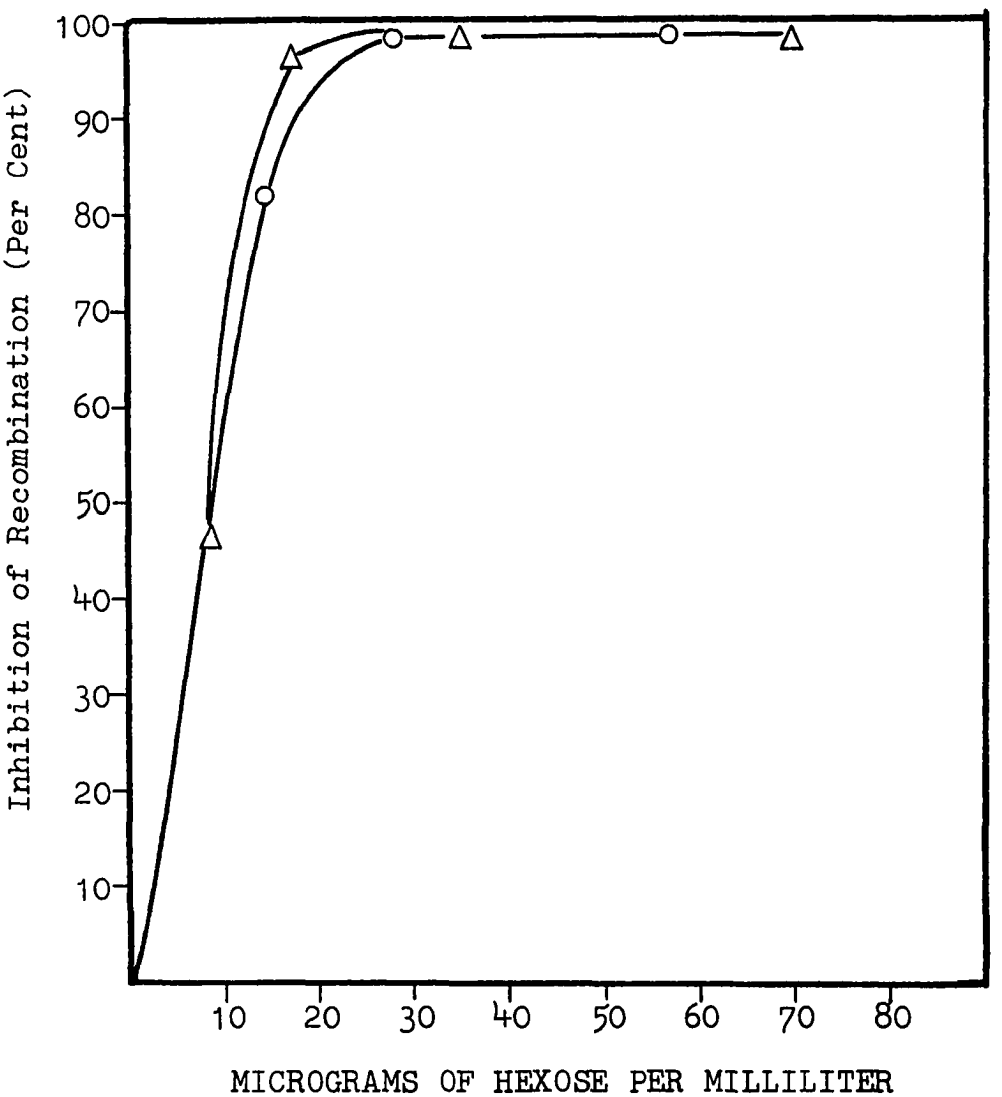

Inhibition of Recombination (Per Cent)
100
90
80
70
60
50
40
30
20
10
10
20
30
40
50
60
70
80
MICROGRAMS OF HEXOSE PER MILLILITER

TABLE 6

EFFECT OF DEACYLATED (DMSO) B380 LPS AND ERW LPS ON CELL VIABILITY

Component	Cell Count		
	B380	ERW	Both
B380 (DMSO) LPS	22 X 10^7	27 X 10^7	18 X 10^7
ERW	21 X 10^7	24 X 10^7	21 X 10^7
Saline	22 X 10^7	24 X 10^7	16 X 10^7

TABLE 7

CONJUGATION INHIBITION ASSAYS OF LPS FROM S. TYPHIMURIUM, E. COLI 0, AND S. MARCESCENS

LPS	Concentration (mg/ml)	% Inhibition of Recombination
E. coli 0	0.5	-23
	0.25	- 8
S. marcescens	0.5	-11
	0.25	+ 2
S. typhimurium	0.5	-11
	0.25	- 2
F⁻B380	0.5	88

TABLE 8

ASSAY OF DEACYLATED (95% ALCOHOLI) LPS FROM E. COLI 0, S. MARCESCENS, AND S. TYPHIMURIUM

Concentration (micrograms per milliliter)	Fraction	Source of LPS	% Inhibition of Recombination
greater than 200	deacylated	E. coli 0	0
greater than 200	deacylated	S. typhimurium	21
greater than 200	deacylated	S. marcescens	10

TABLE 9

ASSAY OF CORE AND SIDE-CHAIN POLYSACCHARIDE FROM LPS OF E. COLI 0, S. MARCESCENS, AND S. TYPHIMURIUM

Concentration (micrograms per milliliter)	Fraction	Source of LPS	% Inhibition of Recombination
43	core	E. coli 0	36
48	core	S. typhimurium	40
31	core	S. marcescens	0
22	core	B380	14
26	core	ERW	0
greater than 150	side-chain	E. coli 0	0
greater than 150	side-chain	S. typhimurium	0
greater than 150	side-chain	S. marcescens	0

TABLE 10

QUALITATIVE CHEMICAL COMPARISON OF THE COMPONENTS OF F' ERW & F⁻B380 LPS

LPS	% Lipid	KDO	Heptose	Glucose	Galactose	Rhamnose	Phosphate
B380	51	+	+	+	+	±	+
F'ERW	50	+	+	+	+	+	+

Note: ± refers to qualitative determination under two conditions of hydrolysis. In one case rhamnose was present while under other conditions rhamnose was absent. All the other sugars and phosphate were present under both conditions of hydrolysis.

TABLE 11

THE EFFECT OF POLYSACCHARIDE-RICH FRACTIONS FROM BASE TREATED LPS OF F' ERW, F⁻B380, AND S. MARCESCENS ON MATING PAIR FORMATION

Time of Dilution (minutes)	Type of (lipo)polysaccharide	Number of Recombinants
3	saline	2.5×10^7
5	ERW	1.9×10^5
7	B380	1.5×10^5
9	saline	3.2×10^7
11	ERW	2.0×10^5
13	B380	1.4×10^5
15	saline	4.5×10^7
17	ERW	2.5×10^5
18	S. marcescens	3.0×10^7

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