ELECTRON MICROSCOPIC EVIDENCE OF CONJUGATION BETWEEN *ESCHERICHIA COLI* AB785 AND *SALMONELLA PULLORUM* 35

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ABSTRACT

ELECTRON MICROSCOPIC EVIDENCE OF CONJUGATION BETWEEN *ESCHERICHIA COLI* AB785 AND *SALMONELLA PULLORUM* 35

by Otis W. Godfrey

The F'lac factor was transferred from *Escherichia coli* AB785 to *Salmonella pullorum* 35. Several micrographs of *E. coli* AB785 joined to *S. pullorum* 35 by a cellular tube were obtained with the aid of the electron microscope. Genetic analysis conducted upon completion of each mating experiment demonstrated that 2-99% of the recipient population (*S. pullorum* 35) acquired the ability to ferment lactose. When these lactose positive *S. pullorum* 35 cells were infected with low numbers of male specific bacteriophage (MS2) a 10^7-fold increase in MS2 number occurred within eight hours of incubation. Thus, it may be stated that the cellular tubes observed joining *E. coli* AB785 to *S. pullorum* 35 are in fact conjugal tubes.

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INTRODUCTION

Intrastrain conjugation of *Escherichia coli* (Lederberg and Tatum, 1946) and of *Salmonella typhimurium* is an established fact (Miyake and Demerec, 1959). Similarly intergenera conjugation of *E. coli* and various species of *Shigella* has been reported by Luria and Burrows (1957) and of *E. coli* and various species of *Salmonella* has been reported by Baron, Carey, and Spilman (1959) and Makela, Lederberg and Lederberg (1962). However conjugation of *E. coli* and *Salmonella pullorum* has been reported only by Robinson and Schoenhard (1964) and Robinson (1964). In the latter reference electron-microscopic evidence was included, but this aspect was not investigated in detail and was used only to corroborate the genetic analysis.

Conjugation is a process by which chromosomal material is transferred from one bacterial cell to another by direct cell to cell contact (Adelberg and Pittard, 1965). Morphological studies of mating bacterial pairs by electron-microscopy show the presence of a tube or bridge which unites the cells and through which, presumably, the genetic material passes (Hayes, 1964). Shadow-casting, autoradiography, the critical point method, and thin sectioning are the techniques which have been employed with the electron microscope to study conjugating bacteria (Jacob and Wollman, 1961).

In this study mating pairs were negatively stained with phosphotungstic acid and immediately examined with the electron microscope. This technique, employing negative staining, was chosen in preference to other techniques primarily for two reasons: (1) increased contrast and (2) preservation of cellular morphology (Anderson, 1962).

The mating pairs consisted of E. coli AB785 carrying the F'lac episome and S. pullorum, an F^- organism. Episomal transfer was studied in preference to chromosomal transfer for the following reasons: (1) rapidity of transfer (2) high percentage of transfer and (3) ease of genetic analysis.

The following study was undertaken to obtain cytological evidence of conjugation between E. coli and S. pullorum. This cytological evidence was substantiated further by genetic analysis and by an increase in titer of male specific bacteriophage when they were mixed with S. pullorum containing the F-genote derived from E. coli AB785.

HISTORICAL

Genetic Evidence of Conjugation

Lederberg and Tatum in 1946 discovered the transfer of chromosomal material by direct cell to cell contact (conjugation). Cells possessing a genetic element termed the male fertility factor, F, behaved as donors and those without it as recipients, F^-, (Lederberg and Cavalli, 1952). In rare instances a male fertility factor incorporates genetic information from a locus adjacent to its attachment site on the chromosome. These sex factors are called F-genotes (Adelberg and Pittard, 1965) and the bacteria bearing them are designated F'. The F-genote must contain a unique segment of DNA which includes the genetic determinants for the mating reaction and for the transfer of the sex factor itself; in addition, it must contain one or more segments of DNA which are homologous with regions of the host chromosome (Adelberg and Pittard, 1965).

A characteristic of sexuality in bacteria unique to them is that generally only a fraction of the male genome is transferred to the recipient cell. The cell thus becomes a zygote which is incompletely diploid. This resulting zygote is termed a merozygote (Hayes, 1964).

Frequency of collisions, thermal energy, aeration, media, and time of effective contact all play a significant role in merozygote formation. In order to obtain consistently high recombinant frequencies each of the preceding variables has to be strictly regulated. The role of each will be briefly reviewed since this information was taken into consideration in all the conjugation experiments conducted during this research.

Frequency of collisions.-Mating cells apparently collide in a random fashion as evidenced by the inability to demonstrate specific factors responsible for causing attraction between cells of opposite mating type (Clark, 1962). The frequency at which collisions occur is, within limits, a function of the concentration of bacteria and reaches a maximum value at approximately 1 x 10^9 cells per ml (Nelson, 1951).

Thermal energy.- Since the production of utilizable energy and possibly protein synthesis is a necessary prerequisite for the formation of the conjugal tube the role of thermal energy appears obvious. The temperature is kept constant at 37 C (Jacob and Wollman, 1961).

Aeration.-Since energy is a requirement for zygote formation, the cultures are supplied with oxygen by placing 10 ml of the mating mixture in a 250 ml erlenmeyer flask. The cultures are not aerated prior to mating primarily for the following reasons: (1) aerated cultures would use up glycogen, the energy of conjugation (Clark, 1962); (2) donors phenocopy as recipients under conditions of aeration in the stationary phase for several hours (Lederberg, Cavalli, and Lederberg, 1952).

Media.-The mating cells must be placed in a proper chemical environment if recombination is to occur. Both the hydrogen ion concentration and the overall ionic strength of the medium must be carefully adjusted so that cellular union can take place (Fisher, 1957). Since it is believed that energy is required for the formation of the conjugal tube, the medium must contain either a readily available source of energy or must enhance the cells capacity to utilize its stored energy (Fisher, 1957). De Haan and Gross in 1962 stated that the presence of a complete mixture of amino acids in the medium affects the cells of certain donor strains in such a way as to reduce the stability of mating pairs.

Time of effective contact.-Robinson in 1964 reported that 70% of the recipient population (S. pullorum) received the F'lac episome from E. coli AB785 within 17 min after the onset of mating.

Robinson (1964) reported a conjugation system in S. pullorum. She introduced F^{+} and F'lac sex factors into S. pullorum 35W from E. coli AB785 and subsequently was able to isolate Hfr mutants of these cultures. These Hfr cells were then employed to conduct chromosomal markers into recipient S. pullorum cultures.

Microscopic Evidence of Conjugation

Lederberg in 1954 observed conjugating bacteria in wet mounts employing the phase contrast microscope and subsequently was able to isolate individual conjugal pairs (Lederberg, 1954). Anderson (1957) using the critical point method in conjunction with the electron microscope noted that there was a breakdown in a small area where the mating cells are in juxtaposition and that a tube (100-300 mμ in diameter) connected the two cells. He noted also that there was no particular area of the bacterial surface that was characteristic of the site of the conjugal tube and that even in the smallest tubes there was no change in electron intensity from that of the cellular cytoplasm. These astute observations not only lent evidence for the requirement of cell contact in conjugation but also generated a great deal of interest concerning the kinetics of merozygote formation (Jacob and Wollman, 1961).

Mating Types as Evidence of Conjugation

Three criteria that may be employed to distinguish a male from a female cell are: (1) the permeability of a male cell to the dye eosin (Zinder, 1960), (2) the susceptibility of bacterial strains harboring the F-genote, derived from *E. coli* K-12, to male specific RNA containing bacteriophages (Loeb, 1960; Loeb and Zinder, 1961; Horiuchi and Adelberg, 1965), (3) the altered antigenicity of a donor cell (Ørskov, I. and F. Ørskov, 1966).

The staining reaction.-Robinson (1964) demonstrated that within certain limits of time and temperature male strains of *S. pullorum* took up the eosin dye more rapidly from the media than female strains of *S. pullorum*. The medium employed was eosin methylene blue agar without a fermentable sugar and enriched with casamino acids.

The susceptibility to male specific bacteriophages.-Loeb in 1960 reported the isolation of a number of bacteriophages that would grow only on *E. coli* K-12 donor strains. The inability of these phage to grow on F^- bacteria was attributed to their lack of adsorption (Loeb and Zinder, 1961). These phage were subsequently categorized into the following three groups:

f(1) .... which contains a single representative.
f(2)3........ consisted of many representatives.
f(2) .... possessed similar reactions to f(2).

The phage in group f(2) were chosen for further study (Loeb and Zinder, 1961). Zinder, in 1963, found that as much as 10-20% of the f(2) would elute in viable form, even when the infected bacteria were diluted through antiphage serum. He also found that no more than 85% of the bacteria were killed regardless of the multiplicity of infection, m.o.i., employed.

Horiuchi and Adelberg in 1965 demonstrated an increase in titer of MS2 (male specific bacteriophage) when they were used to infect Proteus mirabilis strains harboring the F-genote derived from E. coli K-12, but were unable to demonstrate plaque formation on agar plates.

Crawford and Gesteland in 1964 noted that a male-specific bacteriophage, R-17, adsorbed to fimbriae of an Hfr and an F^+ strain of E. coli, but not to fimbriae of an F^- strain. Valentine and Strand in 1965 were able to study f(2) adsorption to "F" fimbriae in vitro. Using the electron microscope, they were able to demonstrate "F" fimbriae f(2) complexes.

Fimbriae, first referred to as 'pili' (Brinton, 1959), are filamentous appendages associated with the bacterial cell surface. They differ in their appearance from flagella in that they are generally more rigid and show no evidence of forming patterns with wavelength or amplitude. Two types of fimbriae have been reported (Thornly and Horne, 1962). They are as follows: (1) structural fimbriae are composed of protein sub-units polymerized into right handed helices of 70Å in diameter with an axial hole 20-25Å in diameter. These fimbriae are 2-10 x 10^4Å in length with upwards of 400 on one cell (Brinton and Gemski, 1964), (2) "F" fimbriae are cytologically indistinguishable from structural fimbriae and are genetically controlled by the fertility factor of E. coli K-12. There are approximately one to four "F" fimbriae per cell. These fimbriae are recognized by the selective adsorption of male specific bacteriophage (Valentine and Strand, 1965). Electrophoretic studies suggest that fimbriae bear a weaker negative charge than the normal bacillary wall.

Brinton, Gemski and Carnahan in 1964 stated that the synthesis of "F" fimbriae was determined by the "F" factor and that structual fimbriae were genetically determined by the chromosome. They based this statement on the correlation of "F" fimbriae with the presence of the "F" factor in derivatives of E. coli K-12. That is, they noted the disappearance of "F" fimbriae when the "F" factor was removed and the subsequent appearance of "F" fimbriae when the "F" factor was added to the respective cells.

It is thought that "F" fimbriae may serve as hollow tubes through which the DNA molecules are transferred, On the basis of this hypothesis, Duguid, 1959, suggested that the conjugal tubes previously described may be an artifact.

Altered antigenicity of a donor cell.-Ørskov and Ørskov in 1960 demonstrated an antigen, termed f^+, in F^+ cultures. According to the features of the antigen, it was not identical to the O, K or H antigens. By the ordinary slide or tube agglutination method smooth cultures displayed an indisputable, but often weak and elusive reaction in sera produced with F^+

and Hfr cultures.

Staining Cells for Electron Microscopy

Scattering rather than absorption or reflection is the physical process responsible for a good electron microscope image. Contrast arises almost entirely from differences in density and thickness of the object (Cosslett, 1958).

Since the specific gravity of most biological specimens is approximately one it would be very difficult to observe some object less than 100 Å in thickness without enhancement of contrast (Valentine and Horne, 1962). Both positive and negative staining may be employed to increase the density of a specimen thus increasing the contrast.

Positive staining.-By combining the specimen with some element of high atomic weight (atomic weight of tungsten is 183.86) it is possible to increase the weight density of the specimen and thus enhance its contrast. Since it is hardly possible to more than double the specimen's weight without serious distortion it would seem that objects smaller than 50°A would not have sufficient contrast to be clearly visible (Valentine and Horne, 1962).

Negative staining.-By embedding the cells in a pool of electron dense material such as PTA contrast was enhanced (Brenner and Horne, 1959). Since the stain is not combining directly with the cell it is possible to achieve a three-fold increase in density and thus net a subsequent increase in resolution over conventional methods of positive staining (Valentine and Horne, 1962). As the specimen dries, theoretically PTA replaces all the water in the interstices of the specimen. If the embedding film is thicker than the object, the object escapes exposure to surface tension forces (Anderson, 1962). The best negative stain of a cell is dependent upon (a) the pH, as the pH is decreased the stain spreads more evenly (Horne, 1964), (b) extracellular protein, excessive protein results in poor contrast and uneven spreading (Parson, 1964) (c) wetting, bovine serum albumin is added to wett the cells (Valentine and Horne, 1962) (d) oil, oil from the diffusion pump must be removed since it will interfere with even spreading of the stain (Bradley, 1962).

Phosphotungstic acid.-Phosphotungstic acid (PTA) has weak or little reaction with nucleic acids, phospholipids, fats, but a strong reaction with protein. Twenty-four atoms of tungsten are in each molecule of PTA (Pease, 1964).

Sources of Artifact

Air drying causes a marked flattening of the cells due to surface tension forces associated with the evaporating liquid. The resultant flattened or collapsed cells may give rise to errors both in size and in morphology (Valentine and Horne, 1962). Changes in pH occur while the liquid drop (unbuffered) is evaporating. It is also possible for PTA to enter certain areas and mask various structures (Bradley, 1962).

MATERIALS AND METHODS

Cultures

S. pullorum 35W and five mutants of *E. coli* were employed in this study. The pertinent characteristics of each are listed in Table 1. Bacteriophage MS2 is a male specific one similar to f(2) (Davis, Strauss, and Sinsheimer, 1961).

Media

Penassay Broth, Levine's EMB Agar (supplemented with .8% casamino acids) and Nutrient Agar, all products of Difco, were used as indicated.

M-9 broth (Vaughan, 1962) was employed as the mating medium.

Eosin-methylene blue agar (Lederberg, 1950) was used as the medium for the staining reaction.

Tryptone broth (Cooper and Zinder, 1962) was used for routine culture and assay of MS2 phage.

Bacto-Tryptone	10.0g
Yeast extract	1.0g
Glucose	1.0g
NaCl	8.0g
CaCl	0.22g
Deionized Water	1000.0ml

Agar plates were prepared by supplementing the tryptone broth with 15 g of agar per liter. Top agar contained 7 g of agar per liter. All dilutions of MS2 were made in T2 buffer (Hershey and Chase, 1952).

Reagents

Indole production was detected in SIM agar stab cultures by overlaying the cultures with 0.5 ml of chloroform followed immediately by 0.5 ml of Kovac's reagent. A deep red color

Table 1. Characteristics of bacterial strains

Strain	Mating type	Auxotrophic characters*	Lactose utilization*	Indole*	Surface* appendages
Salmonella pullorum 35W	F^-	$cys^- leu^-$	-	-	None
Escherichia coli AB785	F'Lac	thi^-	+	+	Flagella-fimbriae
W6	F^+	met^-	+	+	N.D.
AB113	F^-	his^-	-	+	N.D.
AB312	Hfr	N.D.	+	+	N.D.
1895	Hfr	N.D.	+	+	N.D.

*Abbreviations and symbols: cys = cysteine; leu = leucine; met = methionine; his = histidine; thi = thiamine; + = utilized or produced; - = not utilized or produced; N.D. = not determined.

formed in the chloroform layer when indole was present.

Stains

Phosphotungstic acid (PTA) was used for negative staining.

Sucrose	3.0 mg
Bovine Serum Albumin	5.0 mg
Phosphotungstic acid	2.0 g
Deionized Water	100.0 ml

The pH was adjusted to 6.5 with 5N KOH a total of three times with a 15 min interval between each adjustment (Dr. T. F. Anderson, personal communication). The solution was passed through a millipore filter and stored at 5 C for not longer than a month.

Preparation of Support Films

A glass slide (precleaned with toluene) was immersed in a 0.17% formvar solution consisting of 125 mg of formvar per 75 ml of diethylene chloride. The slide was promptly removed and air dried. The formvar was removed from the slide by breathing on the slide and immediately immersing the slide in distilled water. The grids (300 mesh) were deposited on the film and the entire film, including the grids, was removed from the water with another glass slide. The film was blotted and allowed to dry for two hours at 37 C. A Kinney high vacuum evaporator was used to carbon coat the grids (Pease, 1964).

Immediately prior to staining, the grids were immersed in redistilled chloroform and allowed to air dry. This step was essential because it removed any traces of diffusion oil from the Kinney evaporator (Bradley, 1962).

Mating Procedure for Electron Microscopic Preparations

One ml of overnight cultures of *S. pullorum* 35W and *E. coli* AB785 were inoculated separately into 9 ml of penassay broth and grown to the exponential phase without aeration. The cultures were centrifuged (12,000 x g for 10 min. at 4C) and resuspended in M-9 broth. S. pullorum 35W was diluted to a concentration of 6 x 10^8 cells per ml (0.26 O.D. at 420 mμ). E. coli AB785 was diluted to a concentration of 2 x 10^9 cells per ml (0.90 O.D. at 420 mμ). Five ml of each culture were pipetted into a prewarmed 250 ml Erlenmeyer flask. The mating mixture was gently agitated for five minutes and then left undisturbed for an additional 10 minutes in a water bath at 37 C. At the end of this time period the conjugal pairs were stained for electron microscopy and samples were plated on Levine's EMB Agar containing 200 μg per ml of streptomycin sulfate. The cells were incubated for 48 hours and then were observed for genetic evidence of F-lac transfer.

Negative Staining of Conjugal Pairs

One ml of the conjugating cells was mixed with one ml of PTA. The resultant suspension was placed dropwise onto carbon coated grids and the conjugal pairs were allowed to settle for five minutes. The grid was then tilted and the excess stain was removed by touching the edge to a piece of filter paper. The phase microscope was employed then as a final check on the quality of the support film.

Shadow-casting

One ml of an overnight culture was pipetted into 9 ml of M-9 broth. After three hours of incubation at 37 C the cells were fixed for one hour in a 0.25% (w/v) solution of formaldehyde (Thornly and Horne, 1962). Drops of fixed cells were applied to grids and they were allowed to settle for five minutes. The grids were blotted dry; they were shadowcast using platinum for 5 seconds at an angle of 15^{o} (Siegel, 1964).

Male Specific Bacteriophage MS2

Method for phage assays.-The phage to be assayed were serially diluted into T2 buffer. One tenth ml of the phage was added to one tenth ml of an exponential culture of the indicator strain (*E. coli* 312) and allowed to adsorb for 10 minutes at 37 C. To this phage-bacterial mixture was added 2.5 ml of melted tryptone soft agar (0.7%) and one tenth ml of a suspension of *E. coli* washed from a nutrient agar slant with 5 ml of saline. The tubes were gently agitated and then poured onto tryptone agar plates. The poured plates were rocked gently to spread the soft agar over the surface of the plate. These plates were incubated for 5 hours at 37 C.

Phage adsorption determination.-One ml of a prewarmed suspension of MS2 phage (1×10^{7} particles per ml) was added to one ml of an exponential culture of *E. coli* and *S. pullorum*, both 2×10^{8} cells per ml. The mixture was incubated at 37 C for 15 min and then 0.2 ml of chloroform were added. The mixture was incubated for an additional five minutes and then it was assayed to score for free phage (Horiuchi and Adelberg, 1965).

Increase in phage titer.-One tenth ml of a prewarmed suspension of MS2 (500 phage per ml) was added to one ml of a log phase culture of bacteria (5×10^{8} cells per ml) in broth at 37 C. After 10 hours of incubation at 37 C the phage were assayed.

RESULTS

Electron Microscopy of E. coli AB785 and S. pullorum 35 Str^r

Many electron micrographs were made of *E. coli* and *S. pullorum* in pure culture to determine any morphological differences between the two species. This information was subsequently used in the analysis of conjugal pairs.

Morphology of S. pullorum 35.-Figures 1 and 2 show *S. pullorum* cells that are positive-stained. They clearly demonstrate the absence of both fimbriae and flagella. Figure 2 demonstrates an *S. pullorum* cell dividing by the "pinching off" process that seems to mediate division of gram negative cells.

Morphology of E. coli AB785.-The presence of both flagella and fimbriae on *E. coli* are evident in figures 3 and 4. The cells in figure 3 were shadow-cast whereas those of figure 4 were positive-stained. Both figures demonstrate the characteristic "blimp-like" shape of *E. coli*. Figure 3 lends observable evidence of the tremendous pressures associated with air drying. These pressures result in the surface appendages being firmly plastered to the surface of the film. Figure 4 demonstrates the differences both in number and size of fimbriae as compared to flagella.

The cells in figures 5 and 6 are negative-stained *E. coli* cells. The PTA appears dark because of its high electron density in comparison to biological material. Figure 5 illustrates two *E. coli* cells in proximity and figure 6 demonstrates two *E. coli* cells fused at the point of contact. Both figures

5 and 6 demonstrate the separation of the plasma membrane from the cell wall and also the presence of thin bridges joining the plasma membrane to the cell wall (Glauert, 1962).

Microscopic evidence of conjugating cells.-Figure 7 demonstrates two E. coli cells fused to one S. pullorum cell. S. pullorum is the middle cell as evidenced by the absence of flagella and fimbriae. Since the cells are both fused and positive-stained it is impossible to determine if these cells are conjugating.

In an attempt to investigate in greater detail both the area of contact and the conjugal bridge, mating pairs were negative stained by "embedding" them in pools of PTA. This technique not only enhanced contrast, but the conjugal bridge was partially protected against air drying if it was embedded in a pool of PTA.

Figures 8 and 9 illustrate E. coli cells that are joined by a cellular tube and are negative stained. This tube is approximately 100 - 300 mμ in diameter and at the point of junction the cell wall has disappeared.

Figure 10 shows two negative stained cells that are joined by a cellular tube. There seems to be a great deal of cellular material surrounding the tube, which may indicate that this is not a conjugal tube but rather a last stage of cellular division.

Figure 11 demonstrates an E. coli - S. pullorum conjugal pair (arrow). E. coli (the upper cell) is joined by a conjugal bridge to S. pullorum. The fimbriae on E. coli are just barely visible.

Figure 12 demonstrates a mating pair that is positive-stained and connected by a conjugal bridge. S. pullorum is the upper cell and E. coli is the lower one. Genetic analysis of cells from the same mating mixture used to prepare the electron micrographs of figure 13 was made and more than 99% of the S. pullorum cells observed was able to ferment lactose. Thirteen hundred colonies were observed.

Genetic Evidence of Conjugation

Immediately prior to staining 0.1 ml of the mating mixture was added to 9.9 ml of cold saline and agitated with a Vortex Jr. mixer. The interrupted mating mixture was plated onto Levine's E.M.B. Agar, supplemented with 200 μg per ml of streptomycin sulfate. After 48 hours incubation at 37 C the lactose positive colonies were inoculated into tubes of SIM Medium to test for motility and indole production. The percent of S. pullorum 35W receiving the F-genote from E. coli AB785 generally varied from 4 to 99% depending upon cell concentration and the ratio of donor to recipient cells in the mating mixture. Results from a Davis U-Tube experiment further substantiated the fact that cellular contact was a requirement for conjugation.

Tests for Infection of S. pullorum F'Lac with Male Specific Bacteriophage MS2.

Sensitivity to MS2 as evidenced by plaque formation.-A loopful of MS2, male specific phage, was spotted onto a lawn of various strains of E. coli and S. pullorum. After incubation at 37 C for 10-12 hours the spots were scored for lysis. The results, shown in Table 2, indicate no sensitivity of S. pullorum F'lac to MS2 phage.

Adsorption of MS2 to strains of E. coli and S. pullorum.- Bacteriophage-MS2 was added to strains of *S. pullorum* and *E. coli*. The phage were allowed to adsorb for 10 minutes, then chloroform was added to lyse the cells. The results, shown in Table 4, indicate that the adsorption of MS2 to *S. pullorum* F'lac was below the limits of resolution of this experiment.

Reproduction of MS2 in S. pullorum 35 F'lac.-Several strains of both *E. coli* and *S. pullorum* were tested for their sensitivity to MS2 phage as determined by an increase in the titer of MS2. The results, shown in Table 3, indicate a 10^7-fold increase in titer of MS2 when used to infect *S. pullorum* F'lac.

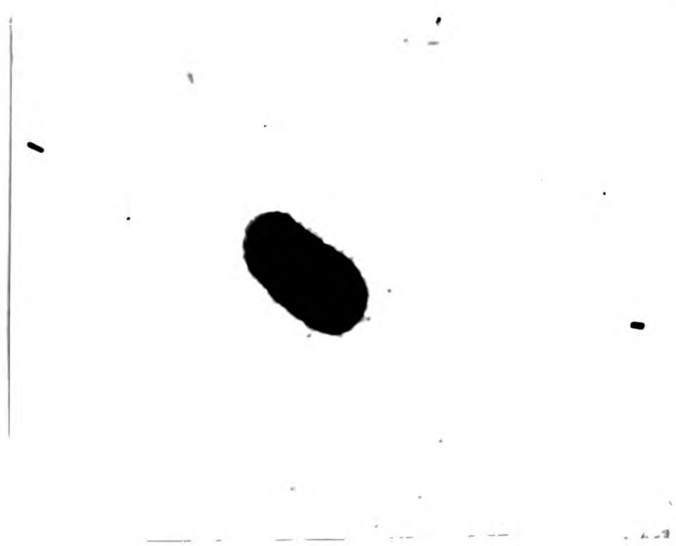

Figure 1. A positive stain of a S. pullorum cell. Note the absence of both flagella and fimbriae. X 10,000

Figure 2. A S. pullorum cell dividing. X 19,000.

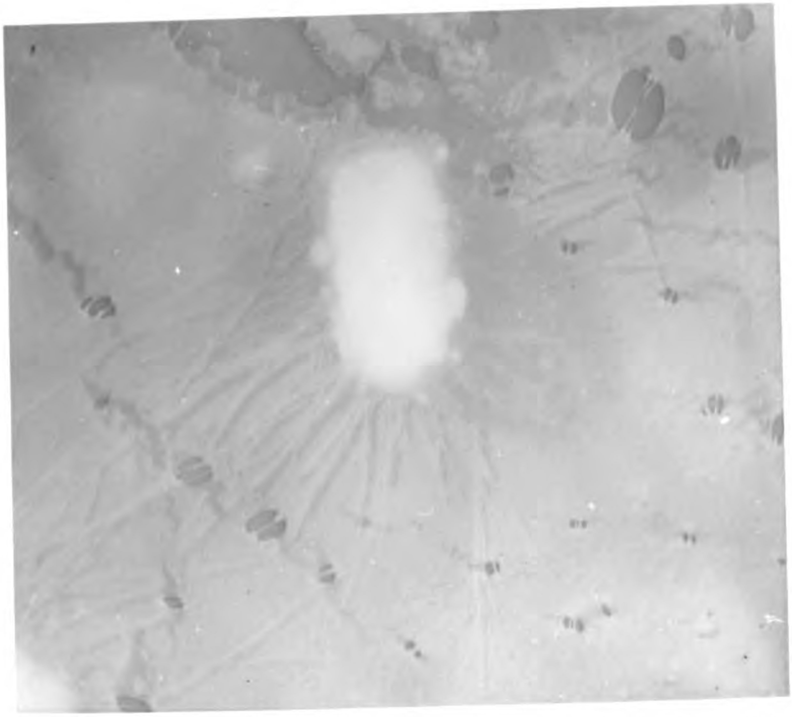

Figure 3. A shadow-cast preparation of <u>E. coli</u>. X 14,000

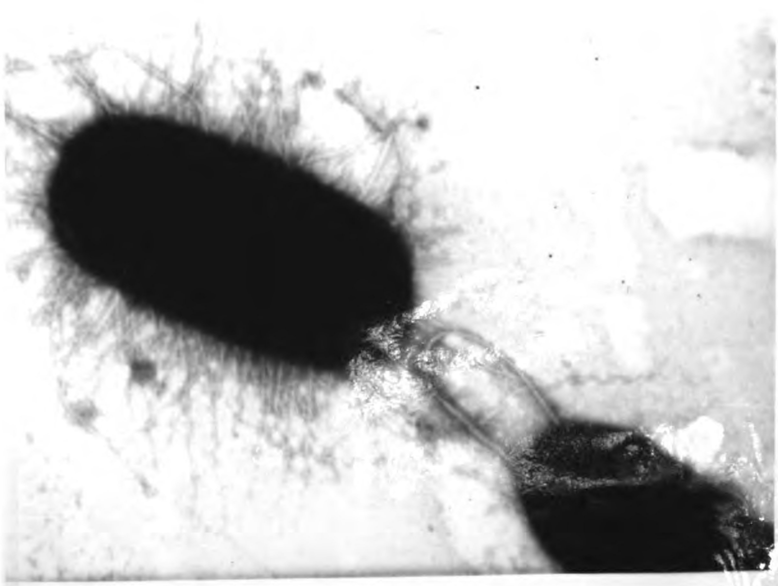

Figure 4. Two positive-stained <u>E. coli</u> cells demonstrating the presence of both fimbriae and flagella. X 22,000.

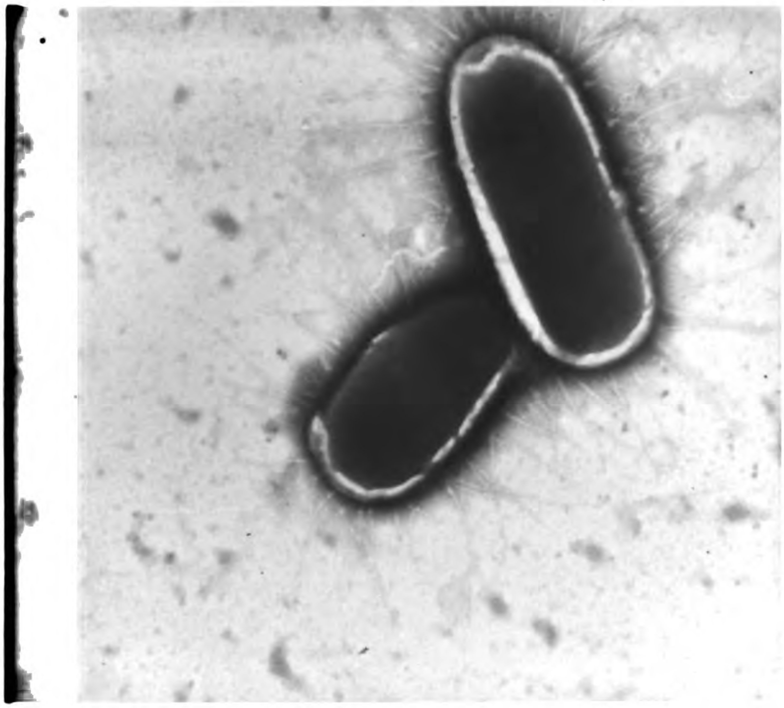

Figure 5. Contact between two E. coli cells. X 21,000

Figure 6. Fusion of two E. coli cells. X 18,000

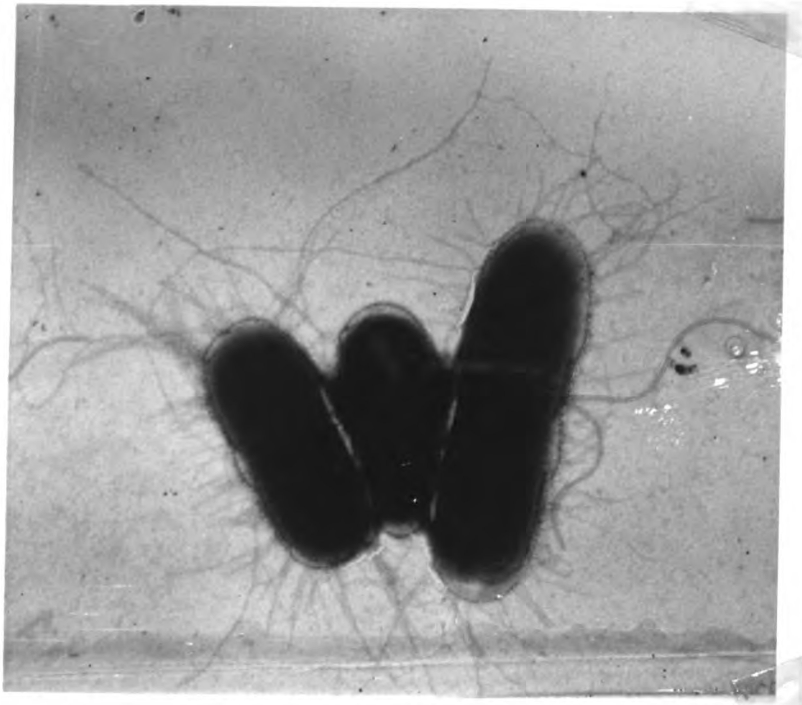

Figure 7. Demonstration of two E. coli in contact with S. pullorum. Note that S. pullorum is the middle cell. X 14,000

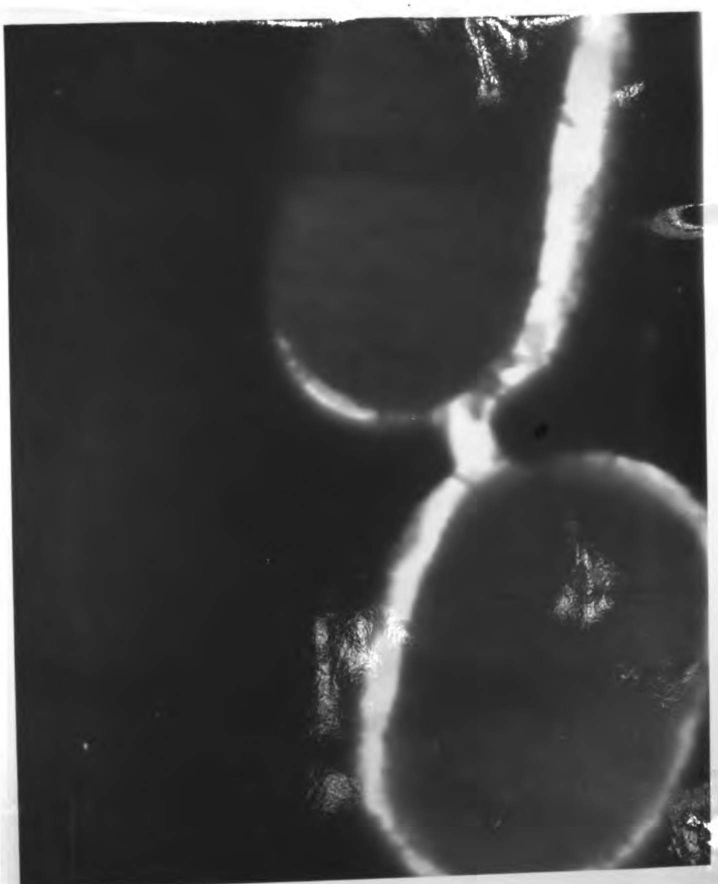

Figure 8. Demonstration of a bridge connecting two E. coli cells. X 43,000

Figure 9. Demonstration of a bridge connecting two E. coli cells. X 10,000

Figure 10. Demonstration of a bridge joining two cells. X 17,000

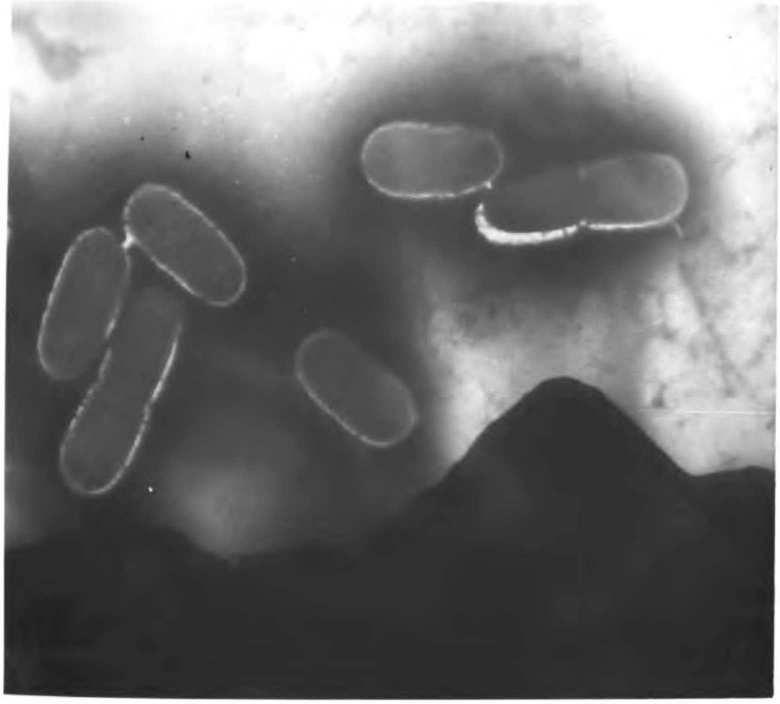

Figure 11. Demonstration of a conjugal bridge connecting members of an E. coli - S. pullorum conjugal pair. X 9,000

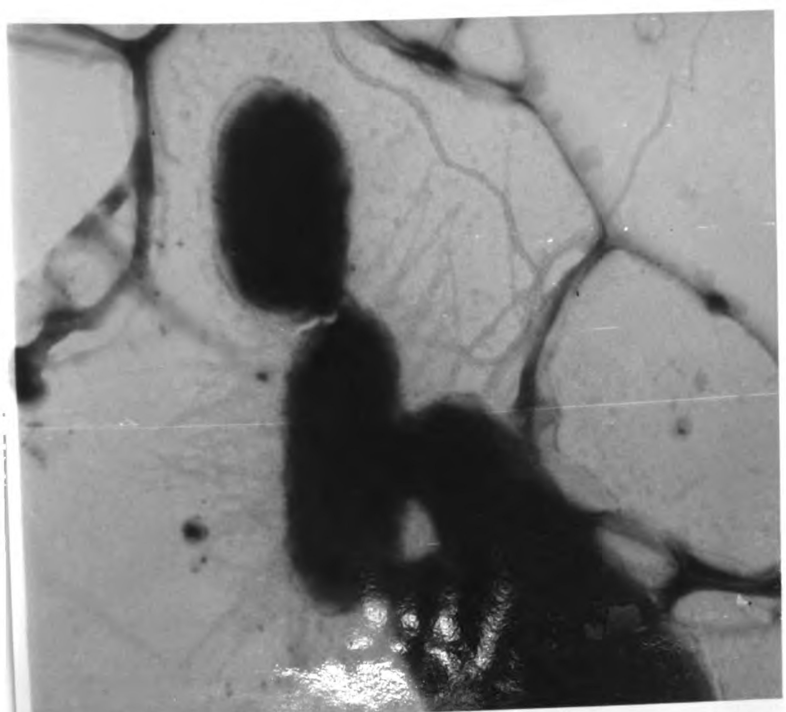

Figure 12. Demonstration of a conjugal bridge connecting members of an E. coli - S. pullorum conjugal pair. X 15,000

Table 2. Sensitivity of lactose fermenting *S. pullorum* to MS2

Strain		Mating-type	Response to MS2
Escherichia coli	W6	F^+	Lysis
	AB785	F'Lac	Lysis
	AB113	F^-	No lysis
Salmonella pullorum	35W	F^-	No lysis
	35	F'Lac	No lysis

Table 3. Reproduction of MS2 in lactose fermenting *S. pullorum*

Strain		Mating-type	Final number of MS2/ml
Salmonella pullorum	35W	F^-	30
	6	F^+	70
	35	F'Lac	5.8×10^8
Escherichia coli	AB785	F'Lac	1.3×10^{10}
	AB113	F^-	less than 10

Table 4. Adsorption of MS2

Strain		Mating-type	Concentration of bacteria/ml	Initial number of MS2/ml	M.O.I.	Final number of MS2/ml	% adsorption
Salmonella pullorum	6	F^+	3.8×10^8	7.2×10^6	.02	7.7×10^6	BLR
	35W	F^-	3.6×10^8	7.2×10^6	.02	7.2×10^6	BLR
	35	F'Lac	8.1×10^7	7.2×10^6	.09	8.1×10^6	BLR
Escherichia coli	AB312	Hfr	4.9×10^7	7.2×10^6	.15	3.3×10^5	95%
	AB785	F'Lac	1.8×10^8	7.2×10^6	.04	1.7×10^6	77%
	AB113	F^-	6.3×10^7	7.2×10^6	.11	6.3×10^6	12%

BLR = Below limits of resolution

$$\% \text{ adsorption} = \frac{\text{original phage titer} - \text{final phage titer}}{\text{original phage titer}} \times 100$$

DISCUSSION

The same procedure for negative staining was employed for all the cells photographed in this report except those that were shadow-cast. Many of the cells were not "embedded" in pools of PTA and thus left high and dry. These cells appeared to be positive-stained and were so labeled.

Cellular tubes joining male _E. coli_ cells have also been demonstrated (figures 8 and 9). It is of interest to note that these tubes were only evident when the cells were "embedded" in pools of PTA and that they were quite abundant. Five possibilities arise concerning the presence of these tubes joining male strains to each other: (1) The observed tubes merely represent a last stage in the division cycle of _E. coli_ that is preserved when "embedded" in PTA. (2) These tubes could be the result of a portion of the male population phenocopying as recipients. (3) They could be true conjugal tubes. (4) The tubes are artifact of electron microscope preparation and not directly related to chromosomal transfer. (5) The possibility also arises that a portion of the male population (_E. coli_ AB785) could spontaneously lose their F-genote and thus revert to F^-. This does not appear to be possible due to the current inability to cure _E. coli_ AB785. It would seem unlikely that a large number of the male population would phenocopy as recipients particularly since the cultures were not aerated prior to mating. The question of artifact will continually arise concerning these bridges, but since these bridges have been cytologically observed or at least indicated by many different preparative techniques (shadow-casting, negative staining, positive staining, dark field microscopy etc.) it would seem that the possibility of artifact may be eliminated. The possibility of the tubes representing a final stage in the division cycle can only be eliminated by a thorough study of cellular division with E. coli AB785, which was not conducted in this research. It is possible that these tubes could represent true conjugal tubes; thus, indicating that the male strains possess suitable receptor sites for attachment. I believe that the introduction of the F-genote into a cell produces a change in the electrostatic configuration on the surface of the cell as evidenced by F fimbriae and F^+ antigen, but there is no reason to believe that the receptor sites for conjugal bridge formation have been altered to a considerable extent.

Cytological evidence was accumulated which demonstrates the presence of a cellular tube joining E. coli AB785 to S. pullorum 35 (figures 13 and 14). This bridge resembles the conjugal bridge previously described by Anderson in 1957. The identification of the respective genera is based almost entirely on the presence or absence of surface appendages. Of some use is the fact that E. coli has a characteristic "blimp-like" shape and is somewhat larger than S. pullorum.

The electron microscope cannot be employed to directly determine the dynamics of the formation of the conjugal bridge and its function. This is due to the fact that specimens are dehydrated prior to being photographed. Genetic studies were undertaken to corroborate the electron micrographic observations.

Genetic analysis of F'lac transfer was done to determine the percent of the recipient population that received the F- genote. The percentage generally varied from a low of 3% to a high of 99% with an average of approximately 15%. In the initial experiments conducted in this research this information was used to determine both the cell concentration and donor recipient ratios to use. Once S. pullorum has received the male fertility factor from E. coli it should behave as a typical male strain (S. pullorum F'lac). Following the staining procedure as outlined by Robinson in 1964, I observed that S. pullorum F'lac produced a typical male reaction when compared to other S. pullorum strains. In addition, the male specific phage MS2 was used to infect a broth culture of S. pullorum F'lac and within 12 hours a 10^7-fold increase in titer occurred. Whereas, no increase in titer was evident when MS2 phage (under identical conditions) were used to infect S. pullorum F^- cells. Briefly, it may be stated that by introduction of the male fertility factor from E. coli AB785 into S. pullorum it has been converted to a receptive host for MS2.

As one studies the literature on bacterial conjugation, there seems to be the central theme of cellular union followed by the subsequent transfer of genetic material. Union via a cellular tube, conjugal bridge, seems currently in vogue; although Duguid (in 1959) stated that "F" fimbriae may serve as hollow tubes through which DNA may be transferred, thus, inferring that the conjugal bridges may simply be artifact.

When cells possessing the male fertility factor (F^+) are mated with homogenous recipient cells (F^-), it is noted that the male fertility factor is rapidly and efficiently transferred to the recipient population. To explain this phenomenon one would expect both a mechanism similar to positive chemotaxis and a large number of episomes per cell--neither of which have been demonstrated. But collisions are of a random nature and there are seldom more than four episomes per cell (Jacob and Brenner, 1963).

Jacob and Brenner (1963) proposed a model that would explain this paradox. They envisioned the F factor to be attached by its replicator to a point on the cell membrane at the site at which contact is formed with the recipient cell by means of the specific f^+ antigen. It would seem that a thorough study of the relation of the f^+ antigen, MS2 phage, and F fimbriae could contribute significant knowledge to this paradox.

S. pullorum F'lac could be used in future studies to determine the possible relationships of the f^+ antigen, "F" fimbriae, and MS2. The reason being that S. pullorum has neither structural nor "F" fimbriae and is a homogenous recipient population. During this research S. pullorum F'lac has been shown to be a male strain. If "F" fimbriae have an active role in bacterial conjugation then their presence should be demonstrated on S. pullorum F'lac. S. pullorum F'lac cells have been shadow-cast in various growth stages and to date no "F" fimbriae have been found. Due to the fact that "F" fimbriae are extremely small and few in number it is not possible at this time to positively claim their absence and thus negate the concept of conjugation via "F" fimbriae in a hybrid mating.

SUMMARY

Cytological evidence was presented demonstrating conjugation between *E. coli* AB785 and *S. pullorum* 35. The conjugal bridges connecting the mating pairs resembled descriptions cited previously. The transfer of the F'lac factor from *E. coli* to *S. pullorum* was also demonstrated by fermentation of lactose by *S. pullorum* 35 F'lac. Male specific bacteriophage (MS2) was employed to further substantiate the maleness of *S. pullorum* F'lac. *S. pullorum* F'lac cells were cytologically examined for the presence of "F" fimbriae, but none could be found.

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