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TECHNICAL MANUSCRIPT 515

CHROMOSOME MAPPING OF PASTEURELLA PSEUDOTUBERCULOSIS BY INTERRUPTED MEMBRANE MATING

William D. Lawton
Harold B. Stull

JUNE 1969

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

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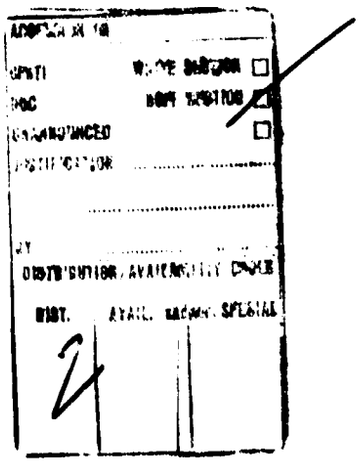

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CHROMOSOME MAPPING OF PASTEURELLA PSEUDOTUBERCULOSIS BY INTERRUPTED MEMBRANE MATING

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June 1969

ABSTRACT

Pasteurella pseudotuberculosis F'*lac*, after growth in brain heart infusion to obtain maximum F piliation, mated on membranes with three different multiple auxotrophs of *P. pseudotuberculosis* F⁻. Samples of mating pairs were periodically separated by vigorous mixing and plated on selective media containing nalidixic acid to counterselect against the donor strain. The transfer of six markers was observed within 65 minutes after mating, resulting in a partial chromosome map as follows:

Origin
of entry – – – – *arg* *his* *pro* – – *thr* – – – *tyr* – – *met* – – –

Assignment of precise entry times for each marker was confused by the fact that all of the three recipient strains showed slightly different entry times for their common markers, *arg* and *his*.

I. INTRODUCTION*

Gene transfer by conjugation between auxotrophs of Pasteurella pseudotuberculosis has been demonstrated by Lawton, Morris, and Burrows.[1] Their data were obtained by mixing donor (F'lac) and recipient cells on selective plates and permitting conjugation, transfer, and recombinant colony formation on the plate. Alternative explanations for their results such as syntrophy or reversion were not substantiated by experiments showing that (i) replacement of the donor by an F⁻lac⁻ derivative, which would be expected to cross-feed the recipient equally well, resulted in infertility; (ii) separation of parents by a membrane essentially eliminated fertility; and (iii) a large number of recombinants showed unselected donor markers. The extension of the initial observations to classical interrupted mating experiments was hindered by the apparent lack of gene transfer in broth, the difficulty of preventing the donor strain from remating on the selective plate, and—most critically—the apparent need for the donor and recipient to be together for at least 16 hours before gene transfer.

We have solved some of these problems and report here our method of interrupted membrane mating and the resulting preliminary chromosome map for P. pseudotuberculosis.

II. MATERIALS AND METHODS

A. ORGANISMS

All the bacterial strains used were derived from P. pseudotuberculosis strain 32IV obtained from Professor E. Thal, Stockholm. The initial auxotrophs were obtained at the Microbiological Research Establishment (MRE) and are described in the publication of Lawton, Morris, and Burrows.[1] The donor strain used in this study, which was obtained from strain MRE 2027 by isolating a single colony resistant to 1 mg/ml streptomycin sulfate, was designated YsD-20 (cys-5, pth-2, str-50; F'lac). The recipient strains, derived from strain MRE 2205 (met-5, arg-8) after treatment with nitrosoguanidine,[2] were designated YsD-16 (met-5, arg-8, his-50, thr-50, nal-51), YsD-17 (met-5, arg-8, his-50, pro-50, nal-52), and YsD-19 (met-5, arg-8, his-50, tyr-50, nal-54).

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B. ABBREVIATIONS

The designation of genotype and phenotype essentially follows the recommendations of Demerec et al.[3] with the additional symbols: pth, double requirement for any purine + thiamine; nal, nalidixic acid.

C. MEDIA

In attempting to improve the initial mating system, we explored empirically a variety of media for mating and for selection of recombinants. A dramatic increase in recombinants occurred on the SD medium used by Landman and Haile[4] for maintaining protoplasts. We modified their SD medium to arrive at an optimum mating medium, which was made by combining the following ingredients (grams per liter) in the order listed: KH_2PO_4 (1.5); $MgCl_2 \cdot 6H_2O$ (1.0); NH_4NO_3 (1.0); K_2HPO_4 (1.5); sodium succinate (13.5); Bacto agar (9.0); and gelatin (10.0). After autoclaving, 10 ml per liter of a 20% filtered glucose solution were added aseptically.

Although the mating agar could be used as a selective medium for some markers, the presence of 1% gelatin made it impossible to select for certain markers and permitted a disturbing amount of background growth of the parental strains. We finally modified this medium to obtain rapid growth of recombinants but no background growth by replacing the gelatin with glutamic acid. Our final selective agar was made as follows (per liter): K_2HPO_4 (1.5 g); L-glutamic acid, CalBiochem, (0.5 g); 12 N HCl (0.55 ml); $MgCl_2 \cdot 6H_2O$ (1.0 g); NH_4NO_3(1.0 g); bring volume to 1,000 ml, pH should be 6.65; add Bacto agar (9.0 g); after autoclaving, aseptically add stock solutions to obtain final concentrations of 0.2% glucose (20% stock solution, filtered), 3 μg/ml nalidixic acid (50 mg/ml alkaline stock solution, filtered), and 50 μg/ml of required amino acids (10 mg/ml stock solutions).

Viable counts were made on Difco purple broth base supplemented with 1.5% agar, 1.0% lactose, and 125 μg/ml triphenyltetrazolium chloride. This medium permitted us to distinguish lac^+ and lac^- colonies and consistently produced slightly higher viable counts than other complete media.

D. PREPARATION OF CULTURES FOR MATING

Donor cells were shaken in Difco brain heart infusion broth plus 0.1% lactose (BHI + lac) at 26 C for 19 hours (50 ml per 250-ml flask). Three-milliliter samples were exposed, with gentle agitation in a petri plate, to a GE 15-watt ultraviolet lamp at a distance of 24 inches for 60 seconds. One milliliter of the treated sample was added to 1 ml of a 50:50 mixture of BHI + lac and 0.03 M potassium phosphate buffer pH 7.2, and the diluted sample was shaken very gently at 37 C for 5 hours. The initial viability of approximately 8×10^8 cells/ml was reduced approximately 50% after ultraviolet treatment and remained at that number after the subsequent 5-hour incubation.

Recipient cells were grown on a Difco blood agar base slant at 26 C for 21 hours, and the cells were suspended in 9 ml of a 10^{-2} dilution of the minimal selective agar. The viable count was approximately 3 x 10^{9} cells/ml.

E. MEMBRANE MATING PROCEDURE

One milliliter of recipient cells plus 1 ml of donor cells were impinged with suction on each of several membranes (Millipore, 0.22μ, sterilized by autoclaving for 10 minutes). The cells were washed with 3 ml of a 10^{-2} dilution of minimal broth, and the membranes were immediately transferred to mating agar at 34 C. At various times, a membrane was transferred to a flask containing 5 ml of minimal diluent plus 3 μg/ml nalidixic acid, the cells were scrubbed off and violently agitated on a Vortex mixer for 1 minute to break mating pairs, and appropriate dilutions were plated on selective media. Recombinant colonies were counted after incubation at 26 C for 3 days.

III. RESULTS

The original medium used to culture the donor strain was a tryptic digest of meat broth prepared at the Microbiological Research Establishment, U.K. We obtained the same results using Difco heart infusion broth (HIB), confirming the apparent need for at least 16 hours of incubation of the donor and recipient before transfer began. We reasoned that the unusually long period of time to obtain transfer might be due to a lack of F pili formation under the conditions used to grow the donor strain; after 16 hours on the selective plate, the donor might produce F pili and begin to conjugate. This hypothesis was supported by the finding that the donor strain, when grown in HIB, did not adsorb the male-specific phage MS2, but after incubation for several hours on minimal agar or on Difco blood agar base, the donor cells did adsorb MS2 phage. Dorothy Molnar in our laboratory demonstrated, by growing the donor cells in several different media, that Difco brain heart infusion (BHI) was optimum for the production of cells able to adsorb MS2 phage.

To test the donor strain grown in BHI for rate of gene transfer, it was necessary to find a better method of eliminating all remating on the selective plate. The elimination of the donor by the classical methods of streptomycin or phage was unsatisfactory; we therefore used nalidixic acid to prevent conjugational transfer on the selective plates.[5] We isolated spontaneous mutants resistant to 20 μg/ml nalidixic acid from each of our three auxotrophic recipient strains, and, by interrupted membrane conjugation of these mutants with our donor strain, established entry times for six different markers (Fig. 1-3). Figures 1 to 3 represent a typical

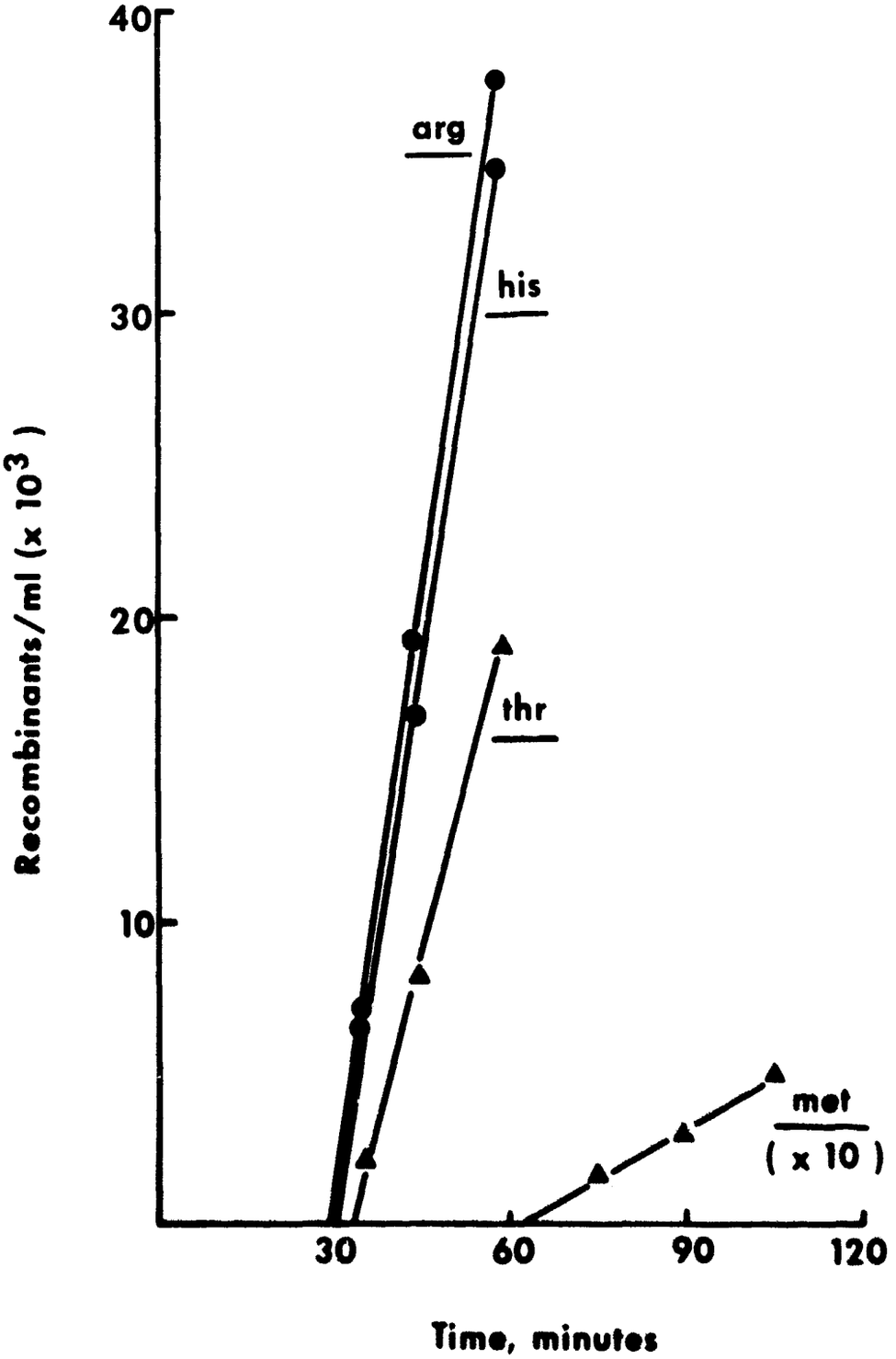

FIGURE 1. Interrupted Membrane Mating Between P. pseudotuberculosis F'lac (YsD-20) and P. pseudotuberculosis F⁻ (YsD-16).

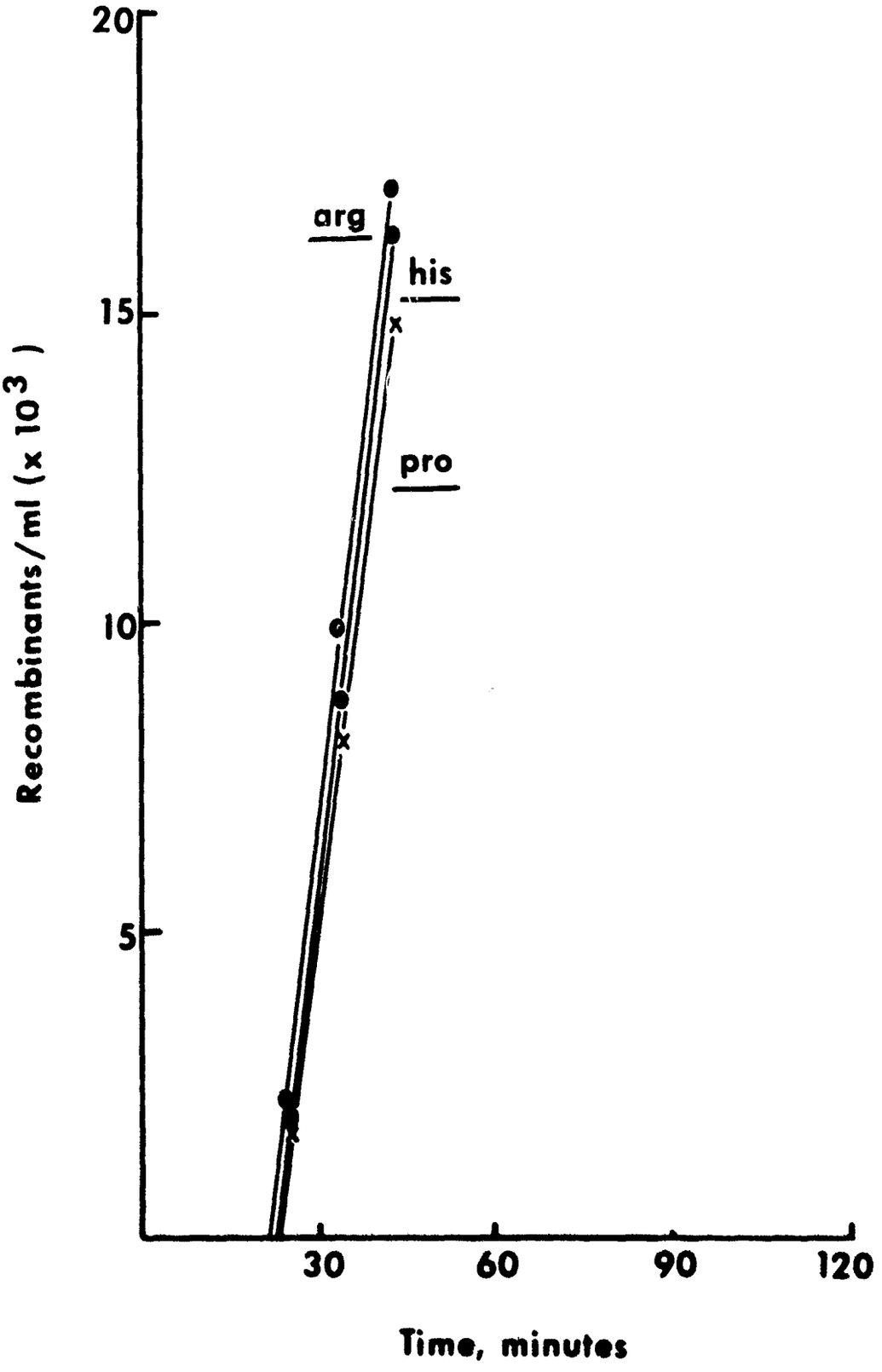

FIGURE 2. Interrupted Membrane Mating Between P. pseudotuberculosis F'lac (YsD-20) and P. pseudotuberculosis F^- (YsD-17).

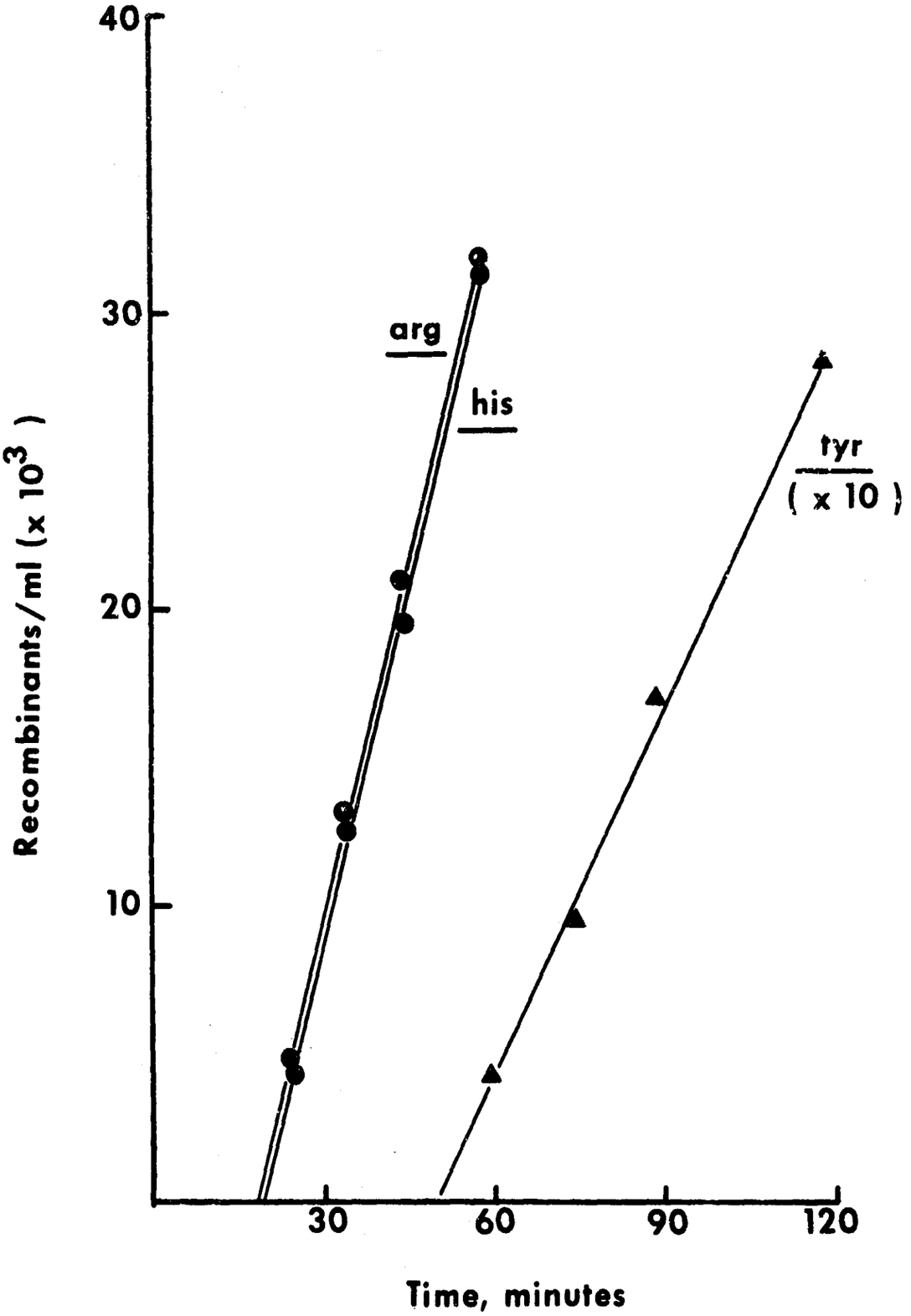

FIGURE 3. Interrupted Membrane Mating Between P. pseudotuberculosis F'lac (YsD-20) and P. pseudotuberculosis F⁻ (YsD-19).

experiment with each of the three recipient strains. The results of additional experiments (Table 1) indicate that reproducible entry times can be obtained with each recipient, but that the three recipients differ from each other in the entry times of their common markers, arg and his. During these experiments, we isolated from the donor strain MRE 2027 a mutant resistant to 1 mg/ml streptomycin sulfate. This mutant (YsD-20) segregates lac$^-$ clones at a lower frequency than the parent (approximately 1% versus 10%) and produces two to three times more recombinants than the parent when mated with recipient strains. The results in Table 1 show that the entry times of various markers are the same using either strain. Other mutants of MRE 2027 picked for their resistance to 1 mg/ml streptomycin sulfate were equal to or poorer than the parent in ability to donate chromosomal genes. Our efforts to transfer high level streptomycin resistance have not succeeded.

TABLE 1. EXTRAPOLATED ENTRY TIME FOR VARIOUS CHROMOSOMAL MARKERS IN P. PSEUDOTUBERCULOSIS

Recipient Strain	Entry Time, minutes					
	arg	his	pro	thr	tyr	met
YsD-16						
Exp 1	29	29		33		63
Exp 2	29	27		33		62 to 65
YsD-17						
Exp 1[a/]	23	24	24			
Exp 2	22	23	24			
Exp 3	23	24	27			
YsD-19						
Exp 1[a/]	20	21			55	
Exp 2	20	20			52	
Exp 3	19	20			51	

a. Experiment performed with donor strain MRE 2027 instead of the usual strain YsD-20.

IV. DISCUSSION

Our results demonstrate unequivocally that chromosome transfer occurs in *P. pseudotuberculosis*. The chromosome map obtained is not yet extensive enough to permit a meaningful comparison with the established chromosome maps of *Escherichia coli* or *Salmonella typhimurium*, but the methods are now available to extend our knowledge of the chromosome map of *Pasteurella* and to make such a comparison.

The observation that the markers *arg* and *his* have very close entry times supports the original finding of Lawton et al.[1] that 85% of the recombinants selected as *arg*$^+$ were also *his*$^+$. Morris and Burrows[6] have recently published a partial chromosome map of *P. pseudotuberculosis* based on a frequency analysis of multiple unselected markers. Our entry times support their conclusion that *arg*-8 and *his*-8 enter early and *met*-5 enters late. Since the other three markers we used were different from the other three markers used by Morris and Burrows, no other comparisons are possible.

The most unexpected finding was that three mutants all derived from the same parent strain each showed a different entry time for the common markers *arg* and *his* (Table 1). One possible explanation is that each recipient may differ slightly in the rate of entry of the donor DNA.[7] Other alternatives such as different rates of attaching to the donor strain or different times of permitting initial entry of donor chromosome are also possible, but untested. The fact that the common markers have different entry times makes it impossible to draw a precise composite chromosome map. We can write the gene order as follows:

Origin of entry ___ *arg* *his* *pro* ___ *thr* _____ *tyr* ___ *met* ___ Terminus unknown

As we obtain additional mutants that have common useful markers toward the terminus, we should be able to determine if the entry of the entire chromosome shifts slightly when comparing two recipients.

We used a membrane mating technique because all past attempts to observe mating in broth had failed. The membrane technique, however, had certain disadvantages. Each time sample required a separate membrane, and although we could usually extrapolate a straight line through three or more points, we sometimes observed a sample to be very high or very low. On the basis of our experience during 23 separate experiments, we concluded that the variability due to the technique itself (e.g., differences in suction when depositing the cells on membranes or in washing them off) was greater than we desired. For that reason and because of the greater efficiency of effort, we are currently attempting to obtain chromosome transfer in broth.

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Origin
of entry ____ arg his pro __ thr ___ tyr __ met ___

Assignment of precise entry times for each marker was confused by the fact that all of the three recipient strains showed slightly different entry times for their common markers, arg and his.

14. Key Words

*Pasteurella pseudotuberculosis
*Chromosomes
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