J. gen. Microbiol. (1968), **52**, 25–34
Printed in Great Britain

Gene Transfer in Strains of *Pasteurella pseudotuberculosis*

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(*Accepted for publication* 15 *November* 1967)

SUMMARY

Pasteurella pseudotuberculosis strain 32IV accepted the F'*lac* episome from *Escherichia coli* strain 23.10S and behaved as a gene donor in crosses with several different auxotrophs of *P. pseudotuberculosis*. Some selected donor markers were transferred at frequencies of 10^{-4}–10^{-5} per donor cell while others appeared not to be transferred. Up to 40% of recombinants were Lac+. Selected recombinants showed differing unselected marker frequencies with differing selected markers; those obtained by using double marker selection showed increased unselected marker frequencies. Some alternative explanations for the origin of recombinants (syntrophic growth, mixed clones, multiple recipient reversions) were not supported by experiment.

INTRODUCTION

Previous attempts to discover a gene transfer system in *Pasteurella pestis* involving conjugation, transduction or transformation were unsuccessful (Burrows, 1962). The report by Martin & Jacob (1962) that an avirulent strain of *P. pestis* could accept the F'*lac* episome from *Escherichia coli* and observations at the Microbiological Research Establishment that strains of *P. pseudotuberculosis* could accept R-factors (J. Boyle, unpublished) stimulated efforts to obtain episome-mediated gene transfer in Pasteurella. Although our ultimate interest is in the genetics of *P. pestis* we are approaching this subject through the very closely related but more easily handled organism *P. pseudotuberculosis* and have studied the transfer of markers from an F'*lac* infected donor strain to different recipient strains of this latter species. To avoid tedious repetition of non-committal terms such as 'presumed donor', 'presumed recombinant' in this first report, we use the terminology currently employed by others in their descriptions of the firmly established fertility system of *E. coli* and assume its applicability to the system here described for Pasteurella. In this paper we present evidence from which we deduce the occurrence of gene transfer and show that other explanations for the development of 'recombinant' strains are less acceptable.

METHODS

Strains. Escherichia coli K12 strain 23.10S Met-(F'*lac*) was kindly supplied by Dr R. C. Clowes. *Pasteurella pseudotuberculosis* strain 32IV was obtained from Professor E. Thal, Stockholm, in 1958 and held since at the Microbiological Research Establishment under the number MRE259. Auxotrophic derivatives of strain MRE259

were derived by presumed single-step mutations following treatment with nitrous acic (Kaudewitz, 1959). Derivatives resistant to *P.pseudotuberculosis* phage IV were isolated as colonies arising on lawns of sensitive bacteria lysed by phage. Streptomycin resistant strains were isolated from sensitive parent bacteria by plating on complete medium supplemented with streptomycin to 100 μg./ml. Strains were stored *in vacuo* at 2° as dried gelatin pellets (Stamp, 1947). Abbreviations designating genotypes and phenotypes of strains follow the recommendations of Demerec, Adelberg, Clark & Hartman (1966) with the additional symbols.

p4s = locus determining sensitivity to phage IV (phenotype symbols P4S and P4R for sensitivity and resistance respectively).

pth = Locus determining the presumed single-step mutation leading to a double requirement for any purine+thiamine (phenotype symbols Pth+ and Pth− for independence and dependence on these factors respectively).

The relevant genotypes of the strains used are shown in the tables.

Media. Tryptic digest of meat broth (TMB) or agar (TMA) and blood agar base (BAB) no. 2 (Oxo Limited) were used as complete media. Minimal agar medium (MA) was prepared as follows. Solution A (g./l. distilled water): Agar (Oxoid no. 3), 30; sodium citrate $2H_2O$, 1; autoclaved 115° for 15 min.; solution B (g./l. distilled water) K_2HPO_4, 21; KH_2PO_4, 9; $(NH_4)_2SO_4$, 2; sodium citrate $2H_2O$, 1; $MgSO_4.7H_2O$, 0·1; adjusted to pH 7·2 and autoclaved similarly. The final medium contained equal volumes of solutions A and B+glucose (sterilized by filtration) to give 0·2% (w/v).

Minimal salts solution (MS) was solution B+an equal volume of distilled water. Selective media were MA variously supplemented, as required, with growth factors to give the concentrations (mM): arginine, 0·5; cysteine, 0·4; glycine, 1; histidine, 0·2; isoleucine, 0·5; methionine, 0·2; phenylalanine, 0·5; serine, 1; threonine, 1; tryptophan, 0·1; tyrosine, 0·5; purine, 0·05; thiamine, 0·001.

Lactose indicator medium was BAB medium+lactose 1% (w/v), bromothymol blue 0·0025% (w/v) and triphenyltetrazolium chloride 0·005% (w/v). On this medium Lac+ colonies were yellow and Lac− colonies reddish purple.

Crossing procedure. The strain MRE2027 was used as donor throughout. It had the relevant genotype *cys-5 pth-2 p4s str-11* (F′*lac*). Donor bacteria were grown in 10 ml. TMB medium+lactose (0·1%, w/v) at 28° with rotation. After 18 hr incubation 5 ml. of the culture was added to 5 ml. of fresh medium and incubated, with rotation, for a further 2 hr. The exponentially growing bacteria were centrifuged and resuspended in 10 ml. of MS. Five ml. of this suspension (containing about 1×10^9 organisms/ml.) were exposed, with gentle agitation in a Petri dish, to a 15 W. ultraviolet lamp (Hanovia Limited) at a distance of 36 cm. for 30 sec. and then diluted 10-fold in MS to provide the donor bacteria suspension.

The various recipient strains were grown on TMA at 28° for 20 hr. They were then suspended in TMB to give concentrations of about 5×10^9 organisms/ml. to provide the recipient bacteria suspensions.

One-tenth ml. of recipient suspension was spread over the appropriate selective media plates followed by the same volume of donor suspension spread over the recipient lawns. Control plates spread with recipient suspension alone and with donor suspension alone were included in every experiment. All plates were incubated at 28° and scored for recombinants after 4–6 days.

Purification and analysis of recombinants. Colonies appearing on the different selective media were streaked on plates of the same media on which they had arisen and single colonies from these streaks were restreaked as before. Single colonies, one from each of the final growths, were inoculated to marked positions on plates of the same medium to provide master plates from which colonies could be replicated to variously supplemented media (by using a 76-needle replicator) to score for unselected markers. The purified recombinants always were replicated to donor selective medium to ensure their freedom from contaminating donor bacteria whose presence would have made unselected marker analysis suspect. In later experiments it was found adequate to replicate recombinant colonies 3 times on selective agar (without streaking and picking single colonies) to ensure their freedom from contamination by parent bacteria.

Recombinants were scored for phage sensitivity by replicating to BAB overlayered with 3 ml. soft agar containing 1×10^8 p.f.u. of phage IV per ml. and for streptomycin resistance by replicating to BAB+streptomycin, 100 μg./ml.

RESULTS

Isolation of donor strain MRE2027

From the *Pasteurella pseudotuberculosis* strain MRE259 we derived the Cys− strain MRE312 and from this the SmR strain MRE2077. This was grown in broth culture with *Escherichia coli* 23.10S and plated on minimal agar containing cysteine, lactose (in place of glucose) and streptomycin. Colonies arising on this medium were indistinguishable from strain MRE2077 except for being Lac+. Representative colonies transferred *lac* to other strains of *P. pseudotuberculosis*, to *P. pestis* and to *E. coli*, and were presumed therefore to have acquired the transmissible element F′*lac*. One such representative, MRE2007, was used as parent strain for the production of a series of auxotrophs, one of which had the double requirement for purine+thiamine and appeared to be the most efficient gene donor of a number tested in crosses with other, Lac−, auxotrophs. This Pth− derivative was chosen for further study and numbered MRE2027. Donor suspensions prepared for use in crosses usually contained about 2% Lac− bacteria.

Gene transfer

The crossing technique described under Methods was developed after considerable experience. Crosses made under conditions effective for *Escherichia coli*, i.e. mating in broth at 37° followed by dilution and plating on selective media, were sterile. It seemed that the growth conditions and treatment of the donor suspension were particularly critical. Ultraviolet irradiation of donor bacteria decreased viability to about 50% but increased the yield of recombinants 5- to 10-fold (i.e. a 10- to 20-fold increase per viable donor bacterium) above that of un-irradiated cells. When following ultra-violet irradiation the donor bacteria were incubated for 1 hr in TMB at 28° before crossing (Hayes, 1953) a further increase (about 1·5-fold) in yield sometimes occurred. This additional treatment, however, seemed to be an unwarranted modification of the technique for routine use.

Minimal enrichment of the selective media on which crosses were performed was necessary for fertility. Thus, when donor and recipient mixtures were incubated in TMB or in MS for 5 hr at 28°, the bacteria deposited, washed, resuspended in MS and plated on selective medium, no recombinants were obtained. The same washed

suspensions plated on selective medium enriched with 0·1 ml. TMB per plate yielded recombinants. Enrichment with 0·1 ml. TMB appeared to be optimal for maximum fertility with minimum background growth and was conveniently made by using recipient cells suspended in TMB.

Table 1. *Fertility of an F′ lac donor and infertility of its Lac− segregant in crosses with different recipient strains of Pasteurella pseudotuberculosis*

Donor: MRE 2027: *cys-5 pth-2 str-11* (F′ *lac*).

Recipient strain			Numbers of colonies on selective media*				
			Crosses		Controls		
No.	genotype	Selected phenotype	F′ *lac* donor	Lac− donor	Recipient alone	F′ *lac* donor alone	Lac− donor alone
MRE 2024	*leu-1 gly-2 ser-1*	Leu +	335	0	0	0	0
MRE 2056	*tyr-1 his-13*	Tyr +	200	5	5	0	0
		His +	20	0	0	0	0
MRE 2118	*met-5 arg-8*	Met+	0	0	0	0	0
		Arg +	112	4	8	0	0
MRE 2117	*met-5 leu-3*	Met+	0	0	0	0	0
		Leu +	320	0	1	0	0

* The different selective media were MA plus all growth factors of the particular recipient strain except that for which selection for independence was being made.

Table 2. *Occurrence of unselected donor markers in recombinants from crosses between strains of Pasteurella pseudotuberculosis*

Donor: MRE 2027: *cys-5 pth-2-str-11* (F′*lac*)

Recipient					Unselected donor markers* (%)			
Strain no.	Relevant genotype	Expt no.	Selected phenotype	Number analysed	*lac*	*p4s*	*met*	*Others*
MRE 2056	*tyr-1 his-13*	1	Tyr+	98	25	.	.	*his* 0
		2	His+	27	4	.	.	*tyr* 0
MRE 2117	*met-5 leu-3*	3	Leu+	69	20	.	0	.
MRE 2205	*met-5 arg-8 p4 -2*	4	Arg+	49	18	37	0	.
MRE 2258	*met-5 arg-8 ile-3 p4r-2*	5	Arg+	67	24	36	0	*ile* 5
			Ile +	68	22	6	0	*arg* 16
		6	Arg+	225	17	25	0	*ile* 2
			Ile +	304	18	4	0	*arg* 3
MRE 2263	*met-5 arg-8 aro-2 p4r-2*	7	Arg+	376	14	36	1	*aro* 3
			Aro+	300	25	9	18	*arg* 14
MRE 2256	*met-5 arg-8 his-8 p4r-2*	8	Arg+	138	28	37	0	*his* 81
		9	Arg+	304	22	35	0	*his* 85
MRE 2291	*met-5 arg-8 his-8 trp-3 p4r-2*	10	Arg+	225	20	32	0	*his* 85 *trp* 4
			Trp+	225	29	47	1	*his* 43 *arg* 36
			Arg+ Trp+	225	40	76	2	*his* 95

* No recombinants showed the donor phenotype SmR.

Table 1 summarizes the results of crosses involving four differently marked recipient strains. The recovery of many colonies per plate in the crosses in contrast to the small numbers on control plates indicated that the donor markers *arg*, *leu*, *his* and *tyr* were transferred to the different recipients at frequencies ranging from about 10^{-4} to 10^{-5} per donor bacterium. There was no indication of fertility when the donor was crossed with auxotrophs having requirements for methionine, nicotinamide, thiamin or uracil, nor when the donor strain was replaced by one of its Lac− spontaneous segregants.

From the Met− Arg− strain MRE2118 (Table 1) additionally marked strains were derived to test double marker selection and to permit unselected marker analysis of recombinants. As shown in Table 2 the markers *arg*, *ile*, *aro* and *trp* were transferred both as selected and as unselected markers and *his*, *p4s* and *met* as unselected markers. The donor marker *str* was not observed in recombinants in these experiments. Recombinants were obtained with double selection for the markers *arg* and *trp*; these showed increased unselected marker frequencies for *his* and for *p4s* when compared with recombinants obtained with single selection for *arg* or for *trp*.

Alternatives to gene transfer

Mechanisms other than gene transfer seemed unlikely to have been responsible for the development of colonies on selective media and for these colonies to have possessed unselected donor properties. Nevertheless, some other possibilities were examined. To explain the development of larger numbers of colonies from mixed parent platings than from parent control platings it could be argued that syntrophism, between the parental strains, permitted larger populations of recipient cells and consequently larger numbers of reversions. If this explanation held one would expect that following the plating of an Arg− recipient strain on *arg* selective media to which was added increasing concentrations of arginine, increasing numbers of revertants would arise to equal, or exceed, the numbers of recombinants in crosses with this recipient. The recipient strain MRE2205 (Table 2) tested in this way showed a maximum of 14 colonies from reversions of the *arg-8* locus (Table 3) in contrast to more than 100 recombinants/plate in crosses. A visual comparison of the background growth from mixed parent platings with those on arginine supplemented medium in these experiments indicated that the former did not exceed $1{\cdot}2 \times 10^9$ bacteria per plate. A total of 76 Arg+ revertants derived from these experiments were tested for unselected markers as if they had been Arg+ recombinants. None scored P4S or Lac+ in contrast to recombinants selected for Arg+, of which about 30% were P4S and about 20% were Lac+. The donor strain plated on minimal medium with increasing specific growth factor additions showed no double reversions. With this strain full supplementation with purine + thiamine allowed the detection of rare Cys+ revertants but no reversions to Pth+ were seen on plates fully supplemented with cysteine.

As a further test to exclude syntrophism a membrane filter was interposed between donor and recipient bacteria on selective media. These experiments resembled those of Dushman (1963) who showed that syntrophism could account for the apparent fertility of auxotrophic strains of *Serratia marcescens*. Our results (Table 4), in contrast to those reported with Serratia, showed that the number of colonies arising when parent bacteria were separated was less than 5% of that obtained when free contact of parent bacteria was permitted. Colonies arising on areas covered by membranes mostly

were restricted to the margins of the membranes where separation of parental bacteria may not have been complete. Further (Table 4), when parent bacteria were applied to selective media plates in separate, thin (0·3 mm.) agar layers, virtually no colonies developed whether or not the agar layers were separated by a membrane. However, when parent suspensions were mixed before adding to melted ag..r so that both parents were contained within the same agar layer, many colonies developed.

In an additional test of syntrophism confluent lawns of donor and of recipient cells (MRE2256) grown on separate BAB plates were replicated with the same 76-point replicator to another BAB plate which was incubated at 28° for 18 hr. This master plate, known to carry mixed clones, was replicated to the differently supplemented

Table 3. *Populations and numbers of spontaneous Arg+ revertants of Pasteurella pseudotuberculosis strain MRE2205 plated on Arg+ selective medium increasingly supplemented with arginine*

Strain MRE2205, Met− Arg− P4R, was grown and suspended in TMB as for use as a recipient in crosses and 0·1 ml. volumes (*c.* 5×10^8 organisms) spread on MA plus methionine plates containing increasing additions of arginine. One-tenth ml. of MS was then spread on the plates (to simulate application of donor cells) and the plates incubated for 5 days at 28°. Macroscopically obvious colonies were counted, removed from the agar, the remaining background population suspended in MS and measured turbidimetrically.

		Arginine addition (μM final concentrations)								
		0	4	8	16	32	64	125	250	500
Expt 1	Revertants per plate*	0·5	1·5	1	3·5	9	12	5·5	?	?
	Population per plate* ($\times 10^{-9}$)	0·93	1·23	1·59	1·44	1·83	9·9	9·0	17·7	20·7
Expt 2	Revertants per plate†	0	0·2	1	3	2·4	13·4	10·6	?	?
	Population per plate* ($\times 10^{-9}$)	0·6	0·63	0·87	0·84	1·25	2·7	8·1	18·7	19·3

? = Revertants not recognizable against the dense background growth.
* Means of duplicates.
† Means of five plates.

minimal media used in the analyses of recombinants. Two parallel series of plates were replicated with the donor alone and with the recipient alone and all plates incubated at 28° for 5 days. All separate donor and recipient replicates scored correctly. Mixed replicates grew both on minimal medium selective for the donor and on that selective for the recipient and all scored P4R and SmR on complete medium. On lactose indicator medium they produced brownish colonies. No growths were visible on the differently supplemented minimal media which would have been selective for syntrophic growths, reversions or for recombinants. The same results were obtained with the recipient strains MRE2258 and MRE2263. From the above experiments we obtained no support for the possibility that syntrophism followed by reversions could account for the fertility we had inferred. Further, the experiments excluded the possibility that recombinants falsely showed donor markers through contamination with donor cells since, unlike mixed clones, no recombinants grew on donor specific medium, none were SmR and several were P4S.

Table 4. *Reduction of the fertility of Pasteurella pseudotuberculosis crosses by interposing a membrane between parent bacteria or by separating them in different agar layers*

Suspensions of parent bacteria were prepared as for use in crosses. Donor, or recipient, bacteria were first applied to selective agar plates either by spreading 0·1 ml. of suspension or by adding this to 2 ml. of selective agar at 45° and overlayering the mixture. A semicircular membrane filter (standard grade, Oxo Limited, London) was then applied to cover one half of the agar surface, followed by the addition of recipient, or donor, bacteria as previously. The plates were incubated at 28° for 5 days and the colonies counted. The donor strain MRE 2027 was used throughout.

			Recipient strains				
			MRE 2267	MRE 2258		MRE 2291	
			Selection for				
Parent bacteria applied to plates as	Sequence of applications to plates	Separated (+) or not separated (−) by membrane	Arg+	Arg+	Ile+	Arg+	Trp+
			Numbers of colonies*				
Suspension (0·1 ml.) spread	†1. Recipient	+	0	18	0	4	1
	2. Membrane						
	3. Donor	−	1000	1000	430	570	22
Suspension (0·1 ml.) added to 2 ml. agar and over-layered	Donor alone	.	0	0	0	0	0
	Recipient alone	.	0	6	0	1	1
	1. Donor	+	1	2	0	0	0
	2. Membrane						
	3. Recipient	−	2	4	0	2	2
	1. Recipient	+	0	0	0	0	0
	2. Membrane						
	3. Donor	−	10	6	0	12	1
Mixed suspension (0·2 ml.) added to 2 ml. agar and over-layered	Donor and recipient simultaneously	.	400	233	26	128	1

* Sums of colonies on duplicated half-plate areas. On membrane-covered half-plate areas colonies growing under and on the membranes are summed; †they were stained deep red, to permit counting, by allowing 1 ml. triphenyltetrazolium chloride solution (0·25 %, w/v) to be absorbed by the membrane, followed by 20 min. incubation at 28°.

† Similar results were obtained with the reverse sequence. Membranes located above or below parental mixtures in normal crosses did not interfere with fertility.

Preliminary observations on the kinetics of marker transfer

Male specific phage $\mu 2$ (Dettori, Maccacaro & Piccinin, 1961) formed plaques on lawns of the donor strain but not on those of recipient strains, suggesting that it could be used selectively to eliminate the donor parent from mixtures with recipients. When parental mixtures were incubated in TMB for periods of 10 min. to 7 hr and then treated with phage before plating on selective media no recombinants were obtained. This result suggested either that gene transfer had not occurred in broth during the period (as earlier concluded) or that recombinants were sensitive to phage and were eliminated. Because of the failure to obtain gene transfer in broth, attempts were made to time the entry of the marker *arg* in crosses conducted on membranes (Matney & Achenbach, 1962). Donor and recipient (MRE 2205) suspensions prepared as for use in crosses were applied to membranes placed on selective medium plates and incubated at 28°. At intervals thereafter membranes were removed, the attached

organisms suspended in broth, the suspensions violently agitated using a vibrating mixer and divided into two. One half of the vibrated suspension was treated with phage for 20 min at 37°, the other untreated and both replated on selective medium. Recombinants appeared on untreated suspensions with all samples; they first appeared in phage treated samples after 16 hr had elapsed from the time of applying the mixtures to membranes. After 24 hr treated and untreated samples yielded similar numbers of recombinants. It would seem therefore that many recombinants could survive phage treatment and that *arg* transfer and expression required a minimum of 16 hr contact of parent strains under the conditions of the experiment.

DISCUSSION

We conclude from the experiments reported here that gene transfer occurred between strains of *Pasteurella pseudotuberculosis*. Some alternative explanations for the development of colonies on selective media, which we regard as recombinants, were not substantiated by experiment. It seemed most unlikely that syntrophism of parental mixtures leading to multiple reversions had given a false impression of fertility. First, cross plates did not show the heavy background growth indicative of syntrophism; secondly, permitting increased recipient populations by providing the growth factor for which selection was made, did not result in numbers of revertants approaching the numbers of recombinants obtained in crosses; thirdly, replacement of the donor by an F-Lac− derivative, which would be expected to cross-feed the recipient equally well, resulted in infertility; fourthly, separation of parents by a membrane greatly reduced fertility; and lastly, a large number of recombinants showed unselected donor markers.

Multiple reversions in the recipient strain to give the false impression that donor markers had been acquired seemed equally improbable. Spontaneous revertants subjected to the purification and replication treatment applied to recombinants showed no alteration in other properties for which they were tested. The relative ease with which recombinants appeared with double marker selection for *arg trp* and the fact that 95% of these were His+ is not explicable on a spontaneous mutation basis. The spontaneous mutation frequencies of the *arg-8* and *trp-3* loci were both about 1×10^{-8} and that for *his-8* considerably lower (none have yet been observed). The probability of obtaining triple reversions spontaneously would thus be less than 1×10^{-24}. Similarly recombinants could not have been revertants of the donor strain which carried the non-revertable *pth-1* locus, nor mixed clones of donor and recipient since no recombinants showed the donor marker *str-11*.

The apparent need for firm contact between parent bacteria for the production of recombinants and the inheritance of several donor markers indicate that gene transfer in *Pasteurella pseudotuberculosis* occurs by a process analogous to that mediated by F′*lac* in *Escherichia coli* and involving cell conjugation (Jacob & Adelberg, 1959). While there is at present no reason to doubt that the mechanisms of gene transfer would be similar in principle in the two genera we would expect to find differences in detail and to meet a number of puzzling situations at this early stage of studies with *P. pseudotuberculosis*. Thus, we failed to obtain recombinants in crosses with Met−, Nic−, Ura− or Thi− recipients. Possibly this means that the loci involved are terminal markers (as *str*-11 appears to be) and rarely transferred by our donor or excluded by

lethal zygosis, or they may be closely linked to contraselected donor markers, or *P. pseudotuberculosis* may have more than one linkage group. Again, we have not yet succeeded in obtaining transfer in broth cultures. Possibly this indicates that unions between mating pairs are more fragile than those of *E. coli* or do not form except under conditions where movement of bacteria is restricted, as when held on agar surfaces or membranes. It is puzzling that gene transfer takes some 16 hr under our conditions; does this mean that it is a slow process or do parent bacteria require this time to become competent to mate? Probably with further experience and improvements in technique these and other peculiarities of our system will be resolved. Meanwhile, however, departure from the behaviour shown by fertile systems in *E. coli* cannot be held as evidence against the occurrence of gene transfer in *P. pseudotuberculosis*.

A variable number of recombinants accepted *lac* in addition to the selected marker. Lac+ recombinants varied in the stability of this property and spontaneously produced Lac− sectors, or clones, with different frequencies. The Lac− segregants however showed no loss of their other selected and unselected donor markers which thus were stably inherited (Morris & Burrows, in preparation). We infer that Lac+ recombinants carry an integrated fragment of the donor chromosome and an autonomous F′*lac* plasmid.

The base composition of DNA extracted from our donor strain of *Pasteurella pseudotuberculosis* (determined by W.D.L. using the method of Marmur & Doty, 1962) was 45·6% G+C (T_m 88·0), and that from *Escherichia coli* strain 23.10S, determined in the same experiment, was 50% G+C (T_m 89.8). This 4·4% difference is noteworthy since donor chromosome mobilization by the F factor had not been observed in bacteria that differed in base composition from *E. coli* by more than 1% G+C (S. Falkow, personal communication). However, we know from gel-diffusion analyses (unpublished) that Pasteurella and Escherichia show at least three common antigens, they have some common phage sensitivities (Stocker, 1955; Smith & Burrows, 1962; Hertman, 1964) and show close taxonomic relationships in classifications based on Adansonian principles (Sneath & Cowan, 1958). The availability of a fertility system in *P. pseudotuberculosis* should eventually permit the construction of a chromosome map for this species and allow comparison with those of *E. coli* (Taylor & Thoman, 1964) and *Salmonella typhimurium* (Sanderson & Demerec, 1965).

Two other interesting problems would now seem to be amenable to investigation. Genetic studies on bacterial virulence have been initiated in strains of *Shigella flexneri* (Falkow, Schneider, Baron & Formal, 1963) and *Salmonella typhimurium* (Krishnapillai & Baron, 1964). The accumulated knowledge of factors determining virulence in Pasteurella, (Burrows, 1963) coupled with the ability to promote gene transfer in this genus should provide an excellent system for fruitful studies of the important property of virulence. Secondly, genetic studies should permit an assessment of the validity of claims for the inter-conversion of *Pasteurella pestis* and *P. pseudotuberculosis*, made by several investigators but never using genetically marked strains, nor in an entirely convincing manner (see discussion by Brubaker, Surgalla & Beesley, 1965). In recent experiments we have shown that crosses between *P. pseudotuberculosis* and *P. pestis* are fertile; there would therefore appear to be no impediment to a future decision on the interconvertibility of the two species.

It is a pleasure to acknowledge the contribution to our early studies by Mr J. Boyle and the excellent technical assistance of Mr P. E. Stone throughout this work. This study was mainly conducted during a one-year visit of W. D. L. to the Microbiological Research Establishment.

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