THE BASIS OF VIRULENCE IN *PASTEURELLA PESTIS*: ATTEMPTS TO INDUCE MUTATION FROM AVIRULENCE TO VIRULENCE.

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OF organisms pathogenic for higher animals, *Pasteurella pestis* is unique in that its optimum temperature for growth on laboratory media is 28° (Sokhey and Habbu, 1943). This temperature is considerably lower than that of its mammalian hosts and probably closely approaches that of the arthropod alternative host in its normal habitat. In considering the evolution of the virulent plague organism the significance of this relatively low optimum temperature must be assessed, and it is pertinent to ask to what extent does a temperature similar to that required for maximal growth rate assist an organism in pathogenesis ? Presumably the answer to this question will largely depend on the mechanism by which the pathogen overcomes the defensive systems of the host. For some pathogens multiplication at a maximum rate may be of prime importance in establishing disease whilst for others this may be relatively unimportant.

The synthetic ability of *P. pestis* at 28° is known to be greater than at 37°, as evidenced by its simpler nutritional requirements at the lower temperature (Hills and Spurr, 1951), and we have confirmed this observation. On first thoughts one would expect that in the course of the evolution of plague in higher animals an organism would have evolved in which the most perfect balance of metabolic systems was attained at 37°. However, providing those growth factors which cannot be synthesised by the organism from simpler components are freely available at the higher temperature, no selective pressure may be exerted against it in favour of organisms capable of their synthesis. It need not necessarily appear incongruous, therefore, that a pathogen of warm-blooded animals should have an optimum temperature for growth and maximum synthetic ability at 28°. In fact, it may very well be that interference in the synthesis of particular metabolites by supra-optimal temperatures may contribute to the ability of the organism to survive in the mammalian host and to establish a disease process.

If it is assumed that every property retained during the evolution of an organism has a certain survival value, the question then arises as to the value conferred by retention of synthetic systems functional at 28° but inoperative at 37°. It is feasible to assume that animal pathogens originated from free-living ancestral types, with or without the intermediate establishment of a commensal relationship. In the evolution of virulent *P. pestis*, as we now know it, it is tempting to suggest that parasitism of the flea by an organism well equipped for survival and multiplication at 28° was an initial step in this process and by mutation, or other change, forms arose which were capable of infecting mammals.

If this is so, do existing avirulent laboratory strains represent reversion to the hypothetical flea commensal type from which virulent cells may be reproduced, and if so, what is the nature of the change resulting in virulence ?

Since the recognition of *P. pestis* as the causative agent of plague in rodents and man, several investigations of possible factors determining virulence in this organism have been made. Apart from the claim made by Rockenmacher (1949) that virulent strains show significantly greater catalase activity than do avirulent strains, so far as we are aware no investigator has established a criterion based on cultural, morphological, serological, nutritional or other demonstrable *in vitro* property whereby the two types, virulent and avirulent, may be differentiated with certainty.

The ability of a strain of *P. pestis* to elaborate a well-defined capsule or envelope is insufficient to confer virulence by itself, certain avirulent strains having been shown to produce abundant capsule material serologically identical with, and of comparable protective power to, that derived from fully virulent strains (Amies, 1951).

We have tried to induce mutation of an avirulent strain of this organism to the virulent form, partly because of the evolutionary and epidemiological interest of this possibility and partly to obtain virulent and avirulent strains, as genotypically identical as possible, for use in investigating the basis of virulence in *P. pestis*. The results of these attempts are recorded in this paper.

EXPERIMENTAL.

The classical strain "Tjiwidej" smooth described by Otten (1936) and extensively used as live vaccine in human plague prophylaxis was selected as the parent avirulent organism. This strain, which for convenience we have called TS throughout, was originally obtained from a plague rat at Tjiwidej in Netherlands East Indies. After rat passage followed by four months' storage in deep serum stab culture the strain was found to be avirulent.

The average lethal dose of TS for mice (Porton strain, 18–22 g.) within a 7-day observation period, following intraperitoneal injection of organisms suspended in 0·2 ml. phosphate buffer pH 7·4, was approximately 150 million cells. Under the same conditions fully virulent laboratory strains of *P. pestis* have an A.L.D. in the range of 5–100 cells. This large difference in the A.L.D. of typical virulent and avirulent strains theoretically should permit the selection of one virulent cell when accompanying *ca.* 10 million avirulent cells, using the mouse as a selective medium. Unfortunately for our purpose, however, a given small number of virulent cells (strain 337) was found to be less virulent when in the presence of large numbers (1×10^7) of avirulent organisms than when injected alone. This loss of virulence of accompanied virulent cells in terms of the A.L.D. was approximately 10-fold, lowering the theoretical efficiency of the selection method correspondingly.

The parent culture TS was repeatedly purified by single colony selection and maintained in the dry state. Suspensions prepared from 16-hr. growths on tryptic meat agar (T.M.A.) incubated at 37° and containing 1×10^9 avirulent parent cells per ml. in phosphate buffer were subjected to X- or ultraviolet radiation under the conditions described in a previous paper (Bacon, Burrows and Yates, 1950). Irradiated samples after storage for 24 hr. at 5° on T.M.A., were incubated for 16 hr. at 37° and the resulting organisms injected intraperitoneally into 20–50 mice (1×10^7 cells per mouse.) The animals were observed for a minimum period of 7 days and those dying examined for the presence of plague organisms by microscopical and cultural observation of spleen smears, peritoneal and thoracic fluids.

Where cultures of *P. pestis* were recovered from these animals, the virulence of the recovered strains was tested by intraperitoneal injection of 1×10^7 organisms into each of 20 mice. Failure to kill test animals at this level showed that the recovered organisms were not virulent mutants. From 70 experiments using TS as parent, 23 strains were recovered and tested in this manner. They were designated MP1 . . . MP23.

RESULTS.

Isolation of strain MP6.

With one exception the recovered strains MP1 to 23 were avirulent and presumably represented the residues of the injected suspensions. The exception was strain MP6. Its virulence was comparable with that of the virulent laboratory strain 337 and was retained unimpaired through 20 daily subcultures on T.M.A. at 37°. The question immediately arose whether this was in fact a virulent mutant or an accidental contamination with the virulent strain 337. Unfortunately, the identity of behaviour of the three strains TS, 337 and MP6 in all *in vitro* tests applied to them (with the exception of infrared absorption spectrometry referred to later) makes this question unanswerable. It is, however, difficult to visualise how accidental contamination with a virulent laboratory strain could occur particularly since the greatest care is taken when manipulating virulent plague bacilli to avoid any possibility of aerosol production or contamination of any materials or apparatus. The mistaken use of a virulent whole culture, wrongly labelled, would be a more likely, though still very remote possibility. In the experiment yielding MP6 this possibility was excluded by testing for virulence 40 isolated colonies derived by plating the final suspension used for infecting the 50 mice. All proved avirulent indicating the proportion of virulent to avirulent cells to have been less than 1 to 40 and that a virulent culture had not been employed. Reconstruction experiments recorded in Table I suggest that the probable ratio of virulent to avirulent cells in this suspension was in the range 1 : 1000 to 1 : 10,000, which is not dissimilar to the frequency with which the biochemical mutants listed in Table II occurred in similarly irradiated suspensions.

TABLE I.—*Reconstruction Experiment Suggesting Probable Ratio of Virulent to Avirulent Cells in X-irradiated Suspension Yielding Strain MP6*

	Mice injected.	Total intraperitoneal dose. MP6.	TS.	Total deaths on days after injection. 1.	2.	3.	4.	5.	6.	7.
Experiment yielding MP6	50	?	$+ 10^7$	0	0	14	28	37	44	46
Reconstruction experiment	50	10^3	$+ 10^7$	0	0	3	13	30	38	40
	50	10^4	$+ 10^7$	0	5	24	35	45	50	50

Isolation of biochemical mutants.

The desirability of labelling our parent strain with some property serving to differentiate it from strain 337 (the only virulent strain then being handled) became obvious. Accordingly biochemical mutants of strain TS and of the avirulent strain "Soemedang" were selected using a modified minimal supplement selection technique similar to that employed in the selection of biochemical mutants of *Salmonella typhi* (Bacon *et al.*, 1950). As modified for use with *P. pestis* the minimal agar employed had the same composition as used for *Salm. typhi*, plus haemin 40μM and phenylalanine, cystine, methionine, glycine, valine and isoleucine each to 100 μM final concentration. While not essential for growth at 28° the last three amino acids are very stimulatory and improve the reliability of this synthetic agar as a plating medium. (Since these mutants were selected

we have found that the addition of sodium bisulphite at a final concentration of 0·02 per cent greatly improves growth on minimal agar and yields parent colonies of more uniform size, aiding the recognition of small mutant colonies). X-rays were used as the mutagenic agent and the minimal supplemented plates were incubated for 4 days at 28° (the optimum temperature for growth of *P. pestis*), prior to selecting small nutritionally limited mutant colonies. The mutants obtained from 4 experiments are listed in Table II.

TABLE II.—*Nutritionally Deficient Mutants of Avirulent Strains of* P. pestis.

Parent.	Mutant.	Growth Factor.	Frequencies of mutant to parent colonies on minimal supplement agar.
TS	T1	Tryptophan	1 : 32,450
,,	T3	Adenine	1 : 6,000
,,	T4	Arginine	1 : 7,800
" Soemedang "	S1	,,	1 : 10,000
,,	S2	Nicotinamide	1 : 63,000

From previous studies with *Salm. typhi* (Bacon *et al.*, 1951) the adenine-requiring strain T3 was known to be unsuited as parent for further attempts at the selection of virulent mutants. From a total of 122 trials using the other nutritionally deficient mutants listed in Table II as parents, no virulent mutants were recovered.

Evidently if mutation from avirulence to virulence in *P. pestis* is possible it is a very rare event. The possibility was considered that two distinct mutations were involved which would result in such a low frequency and that the strains MP1–23, with the exception of MP6, may represent organisms in which one of the hypothetical double mutations had occurred facilitating their survival in the host. In 41 experiments using the strain MP22 as parent organism, no virulent mutants were recovered. Similarly the repeated irradiation of strain TS with intervening mouse passage between irradiation treatments (4 in all) also failed to yield a virulent form.

DISCUSSION.

In the absence of evidence which clearly indicates strain MP6 to have been derived from strain TS, no claim can be made that mutation from the avirulent to the virulent state in *P. pestis* has been observed. The failure of numerous attempts to recover virulent cells from irradiated avirulent strains carrying nutritional markers would make such a claim very questionable. This work has been recorded, however, to describe the origin of strain MP6 which we have used as the virulent representative in comparative experiments with the avirulent strain TS, in an attempt to identify factors determining virulence in this organism. Our results will be reported later.

Although we are not able to differentiate the strains TS, 337 and MP6 by the conventional laboratory criteria, the infrared absorption spectra of these three strains, using material grown and harvested under as nearly identical conditions as possible, are clearly distinguishable. MP6 is thus an addition to the list of *P. pestis* strains which can be differentiated by their characteristic spectra. As the technique of bacterial infrared spectrometry is still in its infancy, it is not

at present certain how much significance can be attached to such spectra in deciding the identity or otherwise of strains under examination. Nevertheless the evidence as it stands suggests that MP6 and 337 are not identical strains, which is indirect evidence for, but not proof of, the derivation of MP6 from the avirulent TS.

Whereas in this paper we have shown that mutation from avirulence to virulence occurs, if at all, with very low frequency, the reverse mutation can occur readily (Jawetz and Meyer, 1943; Burgess, 1930). It is unknown whether mutation from avirulence to virulence involves gain or loss of synthetic ability. From the work of Engelsberg (1952) it is apparent that *P. pestis* can mutate to give forms with increased synthetic powers, *e.g.*, becoming independent of exogenously supplied methionine. We have confirmed this observation with the strains TS, MP6 and 337, all of which appear to mutate to methionine independence at comparable rates. Similarly, these three strains mutate spontaneously to be independent of the stimulatory effects of glycine and valine + isoleucine, without which the parent organisms grow very slowly on synthetic agar at 28°. It is possible, however, that these mutations are not true gains but reflect the loss of synthetic systems whose products inhibit methionine or other syntheses.

SUMMARY.

The hypothesis that virulent plague organisms have evolved from avirulent strains by mutation, or other change, and that existing avirulent laboratory strains represent reverse mutation, was tested by attempting to bring about artificial mutation of an avirulent strain "Tjiwidej" to the virulent form, using X-rays as mutagenic agent and the mouse as a selective medium. From 237 trials one virulent strain was isolated, but cannot be proved beyond doubt to have arisen by mutation of the avirulent parent. Using nutritionally marked avirulent parents, no virulent organisms were recovered. The isolation of these biochemical mutants, with requirements for tryptophan, arginine, nicotinamide and adenine respectively, is described.

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