# FACTORS INFLUENCING THE LOSS OF VIRULENCE IN *PASTEURELLA PESTIS*

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It was found in a previous investigation that various virulent strains of *Pasteurella pestis* showed a reduction in virulence in aerated broth at 37 C whenever several generations of growth occurred either from small inocula ($10^4$ cells/ml) or after repeated passage using larger inocula (Fukui, *et al.*, 1957*b*). This attenuation seemed to be caused by growth conditions favoring the selection of avirulent mutants present in the inoculum or arising during growth.

This report presents additional evidence that the loss of virulence in aerated broth cultures is due to selective conditions favoring the growth of avirulent mutants and, further, that this selection can be prevented either by addition of certain bacterial culture filtrates, or simply by adjustment of the pH to 7.8 to 8. The selection of avirulent mutants can also be reduced or prevented if the broth culture is incubated under reduced oxygen tension or in a cellophane sac placed in the peritoneal cavity of a guinea pig.

## MATERIALS AND METHODS

The strains of *P. pestis* used were (1) the progeny of a virulent single colony isolate of strain Alexander (Fukui *et al.*, 1957*b*) which had a mouse intraperitoneal $LD_{50}$ of less than 10 cells, and (2) an avirulent mutant of strain Alexander, designated 1A, which produced no deaths in mice at a dose of $1 \times 10^6$ viable cells. Both strains produced "smooth" colonies on tryptose-glucose agar. Stock cultures of the original strains and single colony isolates therefrom were maintained at 4 C on Difco blood agar base slants supplemented with 0.1 per cent glucose and 0.04 per cent sodium sulfite.

The liquid medium routinely used for the cultivation of *P. pestis* was heart infusion broth (Difco) supplemented (after sterilization) with 0.25 per cent D-xylose and 0.06 per cent $MgSO_4 \cdot 7H_2O$. Except where stated, 25 ml of this broth were placed in a 500 ml Erlenmeyer flask. Aeration was accomplished by placing the flasks on a reciprocating shaker operating at a rate of 100 cpm with a 3 in stroke.

The inoculum used for the broth cultures was prepared by suspending in M/15 phosphate buffer (pH 7.4) the growth from a blood agar base slant which had been incubated at 37 C for 24 hr. The suspensions were standardized with a nephelometer (Coleman) and dilutions were made in the sterile buffer to obtain the desired concentration of cells.

The number of viable cells in an inoculum or culture was estimated by plating appropriate dilutions on blood agar base and enumerating the visible colonies after 48 hr of incubation at 37 C. The approximate number of generations in a culture was calculated from determinations of the number of viable cells present immediately after inoculation and after incubation. The purity of cultures was ascertained by observation of the Gram reaction, fermentative reactions, and colonial morphology.

The $LD_{50}$ was determined by injecting intraperitoneally 0.2 ml doses of 10-fold serial dilutions of a culture into 5 Swiss-Webster mice per dilution. The $LD_{50}$ calculations were made according to the procedure of Litchfield and Wilcoxon (1949) and by the Reed and Muench method (1938).

## RESULTS

The observation of Fukui *et al.* (1957*b*) that, in aerated heart infusion broth cultures at 37 C, growth conditions are more favorable for avirulent cells than for virulent cells was confirmed by experiments utilizing inocula containing various known ratios of virulent to avirulent cells (table 1). The inocula were adjusted so that each flask contained a total of $10^8$ cells/ml of broth. The mouse $LD_{50}$ was determined at 0 hr and after 40 hr incubation. In the cultures incubated at 37 C, the growth of avirulent cells was favored, as shown by the increase in $LD_{50}$, whereas the

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TABLE 1

*Virulence of Pasteurella pestis cultures grown at 26 and 37 C from mixed inocula*

| Ratio of Virulent to Avirulent Cells | $LD_{50}$ at | | |
|---|---|---|---|
| | 0 hr | 40 hr, 26 C | 40 hr, 37 C |
| 9:1 | 10.0 | 8.8 | 118 |
| 5:5 | 16.8 | 8.8 | >55 |
| 1:9 | 86.0 | 72.0 | 3000 |

TABLE 2

*Effect of number of cell generations on virulence of Pasteurella pestis grown in aerated broth at 37 C*

| Time of Sampling | Viable Cell Count/ml | Approx no. of Generations Calculated from Viable Count at | | $LD_{50}$ (Cells) |
|---|---|---|---|---|
| | | 0 hr | 6 hr | |
| *hr* | | | | |
| 0 | $6.7 \times 10^3$ | — | — | 2 |
| 6 | $4.5 \times 10^2$ | — | — | 1 |
| 24 | $5.0 \times 10^3$ | — | 3–4 | 24 |
| 30 | $1.23 \times 10^5$ | 4 | 8 | 1,285 |
| 48 | $2.3 \times 10^8$ | 15 | 19 | >43,000 |
| 52 | $5.35 \times 10^8$ | 16 | 20 | >100,000 |
| 72 | $4.66 \times 10^9$ | 19 | 23 | >687,000 |

equilibrium between virulent and avirulent cells was unaltered in the cultures incubated at 26 C, as shown by the essentially unchanged $LD_{50}$.

Virulence tests performed at intervals during growth of a small inoculum ($10^4$/ml) of virulent cells indicated that with each new generation of cells the proportion of avirulent organisms increased in the population (table 2). At 6 hr, a reduction in viable count was noted but the $LD_{50}$ remained unchanged; as the viable count increased thereafter, the $LD_{50}$ also increased.

The virulence of cultures initiated from large inocula ($10^8$ cells/ml) was found to decrease after prolonged incubation at 37 C. One culture gave $LD_{50}$ values of 13, 29, and 48 at 40, 68, and 92 hr, respectively; another gave $LD_{50}$ values of 23, 303, and >446 at 68, 116, and 142 hr, respectively. Subcultures in broth or on agar did not regain virulence. Apparently the conditions in cultures at 37 C which favor selection of avirulent cells continue to exist upon aging.

Virulent cultures contained in cellophane dialysis tubing implanted in the peritoneal cavity of the guinea pig grew well and remained virulent. The culture technique was designed to test the possibility that the loss of virulence observed in flasks was due to adaptive mechanisms which might be reversed by growth *in vivo*. It was hoped that this culture environment might simulate *in vivo* conditions (Fukui *et al.*, 1957*a*) excluding the cellular defense mechanisms and nondialyzable host solutes. The method has some advantages over the collodion sac techniques as used by Harris (1939) and Harris and Miller (1940), since the tubing is commercially available, sturdy, and easily sterilized. Two types of growth chamber were used: one was a simple sac made from tubing of 8-in length and 1⅛-in diameter; the other (figure 1) permitted withdrawal or addition of fluid without removal of the sac from the animals. The sacs were implanted in 500 to 600 g guinea pigs under nembutal anaesthesia and the incision was closed by suture with 00 plain catgut. When aspiration tubes were used, the incision was sutured around the rubber tube and the external portion of tubing taped to the side and back of the animal. While broth cultures aerated *in vitro* at 37 C lost virulence, similar cultures (10 ml volume) incubated *in vivo* remained virulent (table 3).

Mixed inocula consisting of various ratios of

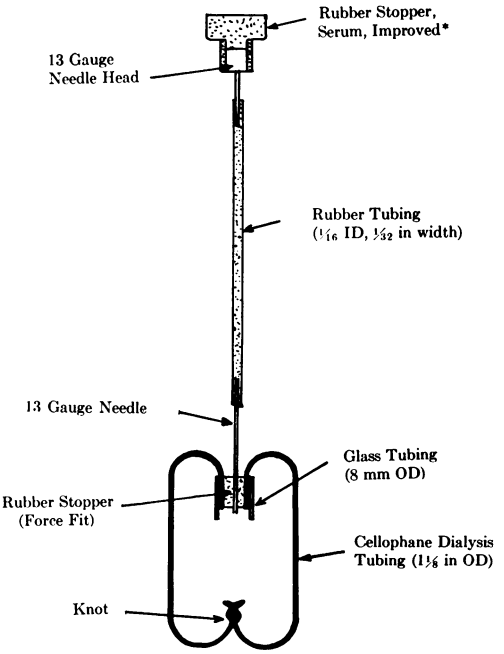


*Figure 1.* Cellophane growth chamber.

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TABLE 3

*Virulence of Pasteurella pestis cultures initiated from small inocula and grown in vivo and in vitro*

| Growth Conditions | Inoculum Size (Viable Cells/ml) | Final (40 hr) Viable Cell Count/ml | Approx no. Generations | $LD_{50}$ (Cells) |
|---|---|---|---|---|
| *in vitro* (37 C aerated) | $1.6 \times 10^4$ | $4.1 \times 10^8$ | 14 | >92,000 |
| | $1.6 \times 10^4$ | $1.9 \times 10^9$ | 16 | >56,000 |
| *in vivo* (cellophane sac) | $1.7 \times 10^3$ | $7.2 \times 10^8$ | 18 | 4 |
| | $9.1 \times 10^3$ | $8.6 \times 10^7$ | 13 | 4 |
| | $9.1 \times 10^3$ | $5.9 \times 10^7$ | 12 | 5 |

TABLE 4

*Virulence of Pasteurella pestis cultures grown in cellophane sacs in vivo from mixed inocula*

| Ratio of Virulent to Avirulent Cells | Inoculum Size (Viable Cells/ml) | Final (40 hr) Viable Cell Count | Approx No. Generations | $LD_{50}$ | |
|---|---|---|---|---|---|
| | | | | Initial (cells) | Final (cells) |
| 25:1 | $1.9 \times 10^6$ | $2.9 \times 10^9$ | 10 | 6 | 7 |
| 17:1 | $1.3 \times 10^6$ | $1.5 \times 10^9$ | 10 | 5 | 6 |
| 3:1 | $1.5 \times 10^6$ | $4.7 \times 10^9$ | 11–12 | 8 | 11 |
| 1:3 | $1.0 \times 10^6$ | $3.7 \times 10^9$ | 11–12 | 31 | 81 |
| 1:5 | $1.6 \times 10^6$ | $1.2 \times 10^9$ | 10 | 70 | 90 |

virulent to avirulent cells grew well in cellophane sacs *in vivo*, and gave no indication of selection of either the virulent or avirulent organisms (table 4).

Avirulent cultures in cellophane sacs *in vivo* remained avirulent even after 14 to 15 generations of growth, thus providing additional evidence that the cellular changes involved in loss of virulence are of a permanent nature.

Preliminary experiments indicated that lowered oxygen tension reduced the rate of virulence loss in heart infusion broth at 37 C. Twenty ml of heart infusion broth were placed in Thunberg tubes, giving a column of liquid about 75 mm in depth. The tubes were then inoculated with $10^4$ virulent (strain Alexander) cells/ml and incubated at 37 C for 44 hr under the conditions indicated in table 5. In the experiment in which cells were grown under a modified atmosphere, the tubes were first evacuated to 10 lb psi negative pressure and the partial vacuum replaced with the indicated gas. Agitation or stirring of the cultures was accomplished with a magnetic stirrer. Samples of the culture after incubation were tested for virulence in mice and the number of viable cells enumerated. Addition of 0.2 per cent sodium thioglycolate to the growth medium seemed to prevent population changes provided the cultures were incubated under static conditions. However, agitation of the cultures containing sodium thioglycolate resulted in a decrease in virulence, perhaps as a result of the oxidation of this reducing agent. Growth in the presence of increased carbon dioxide or nitrogen had no pronounced advantage over the control incubated under static conditions. This apparent effect of lowered oxygen tension in reducing the rate of virulence loss merits further investigation.

It was observed during the studies on the loss of virulence in aerated broth cultures at 37 C that addition of heart infusion broth culture filtrates from avirulent strain 1A to the heart infusion broth medium prevented or reduced the attenuation of virulent *P. pestis* cultures started from small inocula. Heart infusion broth was inoculated with the avirulent strain 1A and incubated with shaking at 37 C for 40 hr. The cells in the culture were then sedimented by centrifugation and the supernatant fluid filtered through a sterile fritted-glass filter. Three parts of avirulent culture filtrate were added to one part of heart infusion broth. Flasks containing this medium, along with controls containing only heart infusion broth, were inoculated with various sizes of inoculum of the virulent Alexander strain. These cultures were then incubated with aeration for 40 hr at 37 C and assayed for virulence. Table 6 summarizes the results of a typical experiment. There is a marked difference in virulence observed in *P. pestis* cultures grown with and without the addition of culture filtrate to the growth medium. The culture started with approximately $10^4$ cells/ml and incubated in the broth containing avirulent culture filtrate maintained its virulence, while the control culture grown in heart infusion broth showed a marked reduction in virulence. Such experimental results indicated that the broth culture filtrate of strain 1A contained an unknown factor or brought about an environmental condition which did not favor the selection of avirulent mutants in aerated broth cultures at 37 C.

Since the initial pH of the medium containing 3 parts of filtrate to 1 part of heart infusion broth (hereafter referred to as the 3:1 medium) was approximately 7.8, hydrogen ion concentration

TABLE 5

*Effect of oxygen tension on virulence of Pasteurella pestis at 37 C*

| Culture Conditions | Inoculum Size (Cells/ml) | Final (44 hr) Viable Cell Count | Approx No. Generations | $LD_{50}$ (Cells) |
|---|---|---|---|---|
| Control (static) | $1.25 \times 10^4$ | $1.00 \times 10^8$ | 13 | 590 |
| Control (agitated) | $1.41 \times 10^4$ | $6.25 \times 10^8$ | 15–16 | 14,500 |
| $CO_2$ (static) | $1.25 \times 10^4$ | $5.60 \times 10^7$ | 12 | 420 |
| $N_2$ (static) | $1.25 \times 10^4$ | $1.14 \times 10^8$ | 13 | 395 |
| 0.2% Sodium thioglycolate (static) #1 | $1.25 \times 10^4$ | $2.50 \times 10^7$ | 11 | 13 |
| 0.2% Socium thioglycolate (static) #2 | $2.35 \times 10^4$ | $3.70 \times 10^7$ | 11 | 12 |
| 0.2% Sodium thioglycolate (agitated) | $1.40 \times 10^4$ | $4.14 \times 10^8$ | 15 | 1,600 |

alone could have accounted for the maintanance of virulence. Therefore, the effect of pH as well as variations in the ratio of avirulent culture filtrate to heart infusion broth was tested. It was found that when the initial pH of heart infusion broth was adjusted with 0.1 N NaOH to approximately the same pH as the 3:1 medium, the virulence of the culture grown in such broth was maintained (refer to bottom line of table 7). However, the ability of 1A culture filtrate to prevent the selection of avirulent mutants was not necessarily due to the alkaline pH. The culture grown in the 3:1 medium, the initial pH of which was adjusted with 0.1 N HCl to 7 (the same pH as the normal control) also remained virulent (table 7). Even the culture grown in 1 part of filtrate to 1 part of heart infusion broth with an initial pH the same as the unaltered control gave a low $LD_{50}$.

The data from these experiments indicated that there might be two separate mechanisms for maintaining the equilibrium between virulent and avirulent cells in aerated broth cultures which were started from small inocula and incubated at 37 C. First, virulent cells apparently grew as rapidly as the avirulent cells at pH 7 if supplied with an unknown factor present in avirulent culture filtrate. The unknown factor had to be present in a definite concentration to exert its full effect. For example, the culture grown in 1 part filtrate to 1 part heart infusion broth had a lower $LD_{50}$ than the culture grown in 1 part filtrate to 3 parts heart infusion broth (table 7). Second, at an alkaline pH (7.8 to 8), virulent cells were able to multiply as rapidly (perhaps by being able to synthesize a particular growth factor) as the avirulent cells. This mechanism was shown more clearly in an experiment in which a range of pH from 7 to 7.8 was tested. The results of this experiment (table 8)

TABLE 6

*Effect of filtrates from avirulent cultures on the virulence of Pasteurella pestis strain Alexander*

| Ratio of Filtrate to Heart Infusion Broth | Inoculum Size (Viable Cells/ml) | Final (40 hr) Viable Cell Count) | Approx No. Generations | $LD_{50}$* (Cells) |
|---|---|---|---|---|
| 0:1 | $3.10 \times 10^8$ | $5.75 \times 10^8$ | 1 | <21 |
| 3:1 | $3.10 \times 10^8$ | $4.65 \times 10^9$ | 4 | 20 |
| 3:1 | $1.69 \times 10^7$ | $3.35 \times 10^9$ | 7–8 | 6 |
| 3:1 | $1.58 \times 10^4$ | $1.53 \times 10^8$ | 13 | 38 |
| 0:1 | $1.58 \times 10^4$ | $4.15 \times 10^8$ | 14–15 | >92,000 |

* Inoculum $LD_{50}$ = <10 cells.

showed that as little as a 0.2 difference in initial pH could markedly influence the $LD_{50}$ of the culture.

Further, it was found that treatment of 1A broth cultures with sonic vibrations apparently increased the concentration of the unknown factor in the growth medium. Strain 1A was grown in heart infusion broth for 48 hr at 37 C. The culture was divided into two parts. One part was treated with sonic oscillation (Raytheon sonic oscillator, 250 W, 10 KC, model DF 101) for 15 min and the cell debris removed by centrifugation. The supernatant was filtered through a fritted-glass filter. The cells from the untreated portion of the culture were removed by centrifugation and filtration. Various ratios of material obtained by sonic vibration and regular filtrate to heart infusion broth were prepared and inoculated with cells of the virulent strain Alexander. After 44 hr incubation with shaking at 37 C, the cultures were tested for virulence. The results indicated that disruption or lysis of cells by sonic vibration liberated more active material into the medium; the culture grown in a

TABLE 7

*Effect of pH and avirulent culture filtrate concentration on the virulence of Pasteurella pestis strain Alexander*

| Ratio of Filtrate to Heart Infusion Broth | Initial pH | Final pH | Final (40 hr) Viable Cell Count/ml | Approx No. Generations | $LD_{50}$ for Mice (Cells) |
|---|---|---|---|---|---|
| 3:1 | 7.8–8.0 | 8.0 | $2.85 \times 10^8$ | 14–15 | 12 |
| 2:1 | 7.2 | 7.2–7.4 | $2.37 \times 10^7$ | 11 | 7 |
| 1:1 | 7.0 | 7.2–7.4 | $1.78 \times 10^8$ | 14 | 14 |
| 1:2 | 7.0 | 7.0 | $3.15 \times 10^8$ | 14–15 | 960 |
| 1:3 | 7.0 | 7.0 | $4.70 \times 10^8$ | 15 | 10,000 |
| 0:1 (control) | 7.0 | 7.0 | $1.88 \times 10^8$ | 14 | >55,000 |
| 3:1 initial pH adjusted to | 7.0 | 7.2–7.4 | $3.10 \times 10^8$ | 14–15 | 15 |
| 0:1 initial pH adjusted to | 7.8–8.0 | 7.8–8.0 | $1.90 \times 10^8$ | 13–14 | 9 |

medium consisting of 1 part oscillated material to 2 parts heart infusion broth gave an $LD_{50}$ of 17 while the culture grown in 1 part filtrate to 2 parts heart infusion broth had an $LD_{50}$ of 144.

Heart infusion broth culture filtrates from *Pasteurella pseudotuberculosis*, *Escherichia coli* strain B, and *Shigella sonnei* also possessed a factor which prevented the selection of avirulent mutants in aerated broth cultures of virulent *P. pestis* started from small inocula. These factors may be similar to the one present in the culture filtrate from the avirulent *P. pestis* strain used in this study.

No evidence was found to show that addition of culture filtrates to heart infusion broth was merely dilution of an inhibitor which prevented the growth of virulent cells in an inoculum. For example, addition of culture filtrates could reduce the concentration of xylose, $MgSO_4$, and the normal constituents in heart infusion broth. Cultures started from small inocula and incubated in broth containing reduced amounts of the heart infusion ingredients mentioned above showed as great a reduction in virulence as the unaltered controls.

In general, it was found that the unknown factor in spent medium which prevents loss of virulence was relatively heat-stable. Autoclaving 1A culture filtrate for 15 min at 120 C inactivated the factor, while heating at 80 C for 15 min had no apparent effect on its activity. However, in another experiment, using a different lot of filtrate, autoclaving for 15 min seemed to reduce the activity. This variation in the effect of heat could have been due to the differences in concentration of the active principle noted from one lot of filtrate to another.

TABLE 8

*Effect of initial pH on the virulence of Pasteurella pestis grown in aerated broth at 37 C*

| Initial pH of Medium | Inoculum Size (Viable Cells/ml) | Final Viable Cell Count/ml | Approx No. Generations | $LD_{50}$ for Mice (Cells) |
|---|---|---|---|---|
| 7.0–7.1 | $1.62 \times 10^4$ | $5.13 \times 10^8$ | 15 | >76,000 |
| 7.4 | $1.62 \times 10^4$ | $1.70 \times 10^8$ | 13–14 | >34,000 |
| 7.6 | $1.62 \times 10^4$ | $4.93 \times 10^8$ | 15 | 22,900 |
| 7.8 | $1.62 \times 10^4$ | $1.27 \times 10^8$ | 13 | 6 |

In the course of these investigations, it was found that the rate of virulence loss varied with different lots of heart infusion broth. This observation was not surprising, since the composition of such a medium varied from lot to lot.

## DISCUSSION

Experimental evidence has been provided in this paper and in a previous publication (Fukui *et al.*, 1957*b*) which shows that reduction of virulence of *P. pestis* in aerated heart infusion broth incubated at 37 C is a result of the selective conditions favoring the growth of avirulent cells. This selection of avirulent mutants may occur because of their greater synthesizing abilities, or because of the inability of virulent cells to synthesize as rapidly the necessary growth factor or factors required at host temperatures. It is also possible that the avirulent cells are capable of coping more efficiently with the highly oxidative growth conditions at 37 C whereas the virulent cells may require, at least initially, a more reduced environmental condition. Reduced conditions, such as those seemingly required for the

multiplication of the virulent cells, may be necessary for the activation of certain essential enzymes. Thus, under high oxygen tension, i.e., aeration of the broth medium, the virulent cells seem unable to initiate growth as rapidly, if at all, and the population equilibrium shifts in favor of the avirulent cells; a reduction in the virulence of the whole culture results.

A similar situation with respect to oxygen tension was observed by Webster (1925) in the growth of *Pasteurella multocida*. He noted that meat extract broth was adequate for the growth of a G form (low virulence type) but not the D type (highly virulent strain). Reduction of oxygen tension favored the growth of the highly virulent form and retarded the appearance of the G type. Blood added to the growth medium gave the same result.

It is well known that some microorganisms require the addition of accessory growth factors as the temperature of incubation is increased. A similar phenomenon might obtain in the case of virulent cells of *P. pestis* grown in aerated heart infusion broth incubated at 37 C. Supplementation of the broth with filtrate from avirulent cultures, or with spent medium from either *E. coli*, *P. pseudotuberculosis*, or *S. sonnei*, may furnish a growth factor or may bring about an environmental change which allows the virulent cells to multiply. In this connection, mention should be made of the work of Schütze and Hassanein (1929) and Herbert (1949). The former found that addition to an agar medium of blood or a small amount of broth culture filtrate of *P. pestis*, which had previously been sterilized by heating at 60 C for 1 hr, augmented the growth of isolated cells of *P. pestis* and *P. multocida*. They attributed their results to a thermolabile substance present in the filtrate which lowered the oxygen tension and suggested that it was of an enzymatic nature. They implied that the enzyme was catalase since other bacteria which produced the augmenting substance were catalase-positive while the majority of nonproducers were catalase-negative. Herbert identified the factor in blood as hemin and hypothesized that it promoted the synthesis of catalase which decomposed the hydrogen peroxide formed under aerobic growth conditions. The factor (or factors) in culture filtrates which reduces or prevents the selection of avirulent *P. pestis* mutants in aerated broth at 37 C may be similar to the augmenter substance of Schütze and Hassanein; however, we have no experimental evidence to implicate catalase.

The effect of pH on the virulence of *P. pestis* is not surprising since the pH of the medium can affect the phenotypic expression of various genes. Stokes *et al.* (1943) described a pyridoxin-requiring mutant of Neurospora which requires pyridoxin below pH 5.8 but which can synthesize pyridoxin at higher pH levels. Srb and Horowitz (1944) also described a pH-dependent mutant of *Neurospora crassa* which was able to grow on a minimal medium at pH 6.7 to 8 but not at a pH of 5.5. In the case of *P. pestis* in aerated heart infusion broth at 37 C, the virulent cells may be able to obtain the necessary growth requirements at pH 7.8, perhaps by a compensating increase of a certain protective enzyme, but not at the lower pH values of 7 to 7.4. Production of additional catalase or another respiratory enzyme by virulent cells at the higher pH may provide the mechanism of inactivation of peroxide under highly oxidative conditions. The virulent cells could then multiply as rapidly as any avirulent cells resulting in no detectable loss in virulence of the culture. It could very well be that the substance synthesized at the higher pH is the same as that present in the culture filtrate from avirulent *P. pestis* and other bacteria.

The rapidity with which a culture of *P. pestis* grown in aerated heart infusion broth at 37 C from a small inoculum becomes attenuated suggests that cytoplasmic factors, i.e., cytoplasmic particles, may be involved. However, this particular aspect has not been investigated here.

## SUMMARY

Results of studies with small virulent inocula and with mixed (virulent and avirulent) inocula support the conclusion that aeration of broth medium at 37 C favors the growth of avirulent mutants of *Pasteurella pestis* over the virulent cells.

Addition of filtrates from avirulent broth cultures of *P. pestis* and three other genera of bacteria to the heart infusion medium eliminated or reduced the selective conditions which favor the growth of avirulent mutants in populations of *P. pestis* grown in aerated broth at 37 C.

Loss of virulence in broth cultures at 37 C did not occur when the initial hydrogen ion concentration of the medium was adjusted to pH 7.8.

Preliminary evidence suggests that reduced oxygen tension may play a role in the maintenance of virulence of *P. pestis* grown in broth at 37 C. Cultures started from small inocula (approximately $10^4$ cells/ml) and incubated in a medium containing the reducing agent, sodium thioglycolate, showed no reduction in virulence as long as they remained under static (nonagitated) conditions.

Selection of avirulent mutants did not occur in virulent cultures grown from small inocula in a cellophane sac in the peritoneal cavity of the guinea pig.

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