Br. J. exp. Path. (1974) **55**, 363.

VACCINE POTENCIES OF THE LIVE VACCINE STRAIN OF *FRANCISELLA TULARENSIS* AND ISOLATED BACTERIAL COMPONENTS

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Received for publication April 3, 1974

Summary.—The live vaccine strain of *Francisella tularensis* rapidly lost virulence (to mice) when grown under controlled conditions in the Porton mobile enclosed chemostat but protective activity was lost much less rapidly. In contrast, bacteria maintained their virulence and vaccine potency during batch culture in shaken flasks. Attempts to identify virulence and protective factors of *F.tularensis* were unsuccessful. The protective activities in mice of killed suspensions of *F.tularensis*, bacterial fractions and extracts of live bacteria were compared with the activity of live bacteria; all the non-living potential vaccines were ineffective.

MAN and experimental animals can be immunized against tularaemia with the viable live vaccine strain (LVS) of *Francisella tularensis* (Eigelsbach and Downs, 1961; Eigelsbach, Hornick and Tullis, 1967). Several non-living potential vaccines have been described (Foshay, 1940; Foshay, Ruchman and Nicholes, 1947; Shepard, Ribi and Larson, 1954; Ormsbee, Bell and Larson, 1955) and these may protect against moderately virulent strains (Bell *et al.*, 1952; Larson, Bell and Owen, 1954) but no non-living vaccine so far described protects against infection with a highly virulent strain such as Schu S4 (Eigelsbach *et al.*, 1951). Non-living vaccines may confer a limited immunity to experimental animals but this amounts to only a slight extension of survival time; substantial immunity is afforded only by a viable vaccine (Eigelsbach and Downs, 1961; Eigelsbach *et al.*, 1967; Claflin and Larson, 1972). Of the strains of *F. tularensis* so far tested for vaccine purposes, the most effective is that derived by Eigelsbach and Downs (1961) from a live tularaemia vaccine obtained from the Gamaleia Institute, USSR.

The objectives of the present work were to discover whether continuous culture techniques could be used to produce bacteria for vaccine purposes and whether killed bacteria, or fractions of them, conferred significant protection to mice against infection with highly virulent *Francisella tularensis*.

MATERIALS AND METHODS

Microorganism.—*Francisella tularensis* LVS (Eigelsbach and Downs, 1961; Tigertt, 1962; Sharer, Klein and Lincoln, 1968) and virulent Schu S4 strain (Eigelsbach *et al.*, 1951) were used.

Growth conditions.—(a) *Batch culture.* Bacteria were grown at 37° for 20 hours in shaken flasks (1·5 l) containing 100 ml of medium; a heavy inoculum (equivalent to about 10% of the final population) of a fully grown culture in the same medium was used. The media used were one similar to those described by Sharer *et al.* (1968), the plasma-catalase medium of Hood (1961), the medium of Tresselt and Ward (1964) and a medium similar to medium "B"

of Traub, Mager and Grossowicz (1955) to which peptic sheep blood (1%, v/v) was added. In some experiments the glycerol in the medium of Sharer *et al.* (1968) was replaced by glucose (0·5%, w/v).

Bacteria were also grown in batch culture with their growth rate limited by the rate of diffusion of glucose into the medium from a " diffusion capsule " (Pirt, 1971; L. H. Engineering Co Ltd, Stoke Poges, Bucks). Glucose solution (12·5%, 1·2 ml) was placed in a capsule which was autoclaved (15 psi, 15 min), then aseptically introduced into a conical flask (1·5 l) containing the medium of Sharer *et al.* (1968) minus glucose (165 ml); the flasks were inoculated and incubated as described above. (b) *Continuous culture*: *Francisella tularensis* LVS was grown in a Porton mobile enclosed chemostat (POMEC; Harris-Smith and Evans, 1969; Evans and Harris-Smith, 1970) containing the medium of Sharer *et al.* (1968). The culture vessel (2 l) was seeded with a batch grown culture (50 ml equivalent to about 0·8 mg bacterial dry wt/ml), the culture was forcibly aerated and the temperature (37° ± 0·5) and pH (6·6 ± 0·05) were maintained automatically. The system was operated as a controlled batch process for 48 hours, to allow the culture density to increase to about 1·5–2 mg equiv. bacterial dry wt/ml; thereafter it was operated as a continuous culture for over 700 hours at a constant or periodically changed growth rate.

Bacteria from batch and continuous culture were harvested and washed (×2) with 1% (w/v) peptone – 0·1% (w/v) cysteine HCl (cysteine broth) or saline phosphate buffer (pH 6·5, Strange, Dark and Ness, 1961) by centrifuging (10 000 *g*; 15 min) and resuspended in saline phosphate buffer (pH 6·5) or cysteine broth.

Determination of bacterial numbers.—Total bacterial numbers were determined with a Thoma counting chamber. Viable bacterial numbers were determined as described by Strange *et al.* (1972).

Determination of bacterial dry weight.—The method of Strange *et al.* (1961) was used.

Killed bacterial suspensions.—Suspensions of live *Francisella tularensis* (10^{10} organisms/ml) were killed with heat (60°, 2 hours), formaldehyde (1% w/v; 16 hours), ether (2·5 vol, 16 hours, room temperature) or phenol (50% w/v; 60°, 30 min) washed (×2) with saline buffer (pH 6·5) by centrifuging (10,000 *g*, 15 min) and finally suspended in the same buffer; in some instances bacteria killed by heat or formaldehyde were not washed. Bacteria killed with chemicals were freed from these by dialysis against saline phosphate buffer. Bacteria were also killed by suspending a bacterial paste in acetone (2·5 vol. 16 hours, −20°), the bacteria were separated and washed (×2) with saline buffer (pH 6·5) by centrifuging (10,000 *g*, 15 min) and finally resuspended in the same buffer; the suspension was dialysed against several changes of saline buffer (16 hours, 4°) to remove remaining acetone.

Bacterial extracts and fractions.—Ether–water, phenol–water and hot water (60°) extracts of *Francisella tularensis* LVS were prepared as described by Nutter (1971). Ether–water extracts were treated with $(NH_4)_2SO_4$ (final concentration 3·3 mol/l), the precipitate dissolved in saline phosphate buffer (pH 6·5) and dialysed against the same buffer (16 hours, 4°) to remove ammonium salt. Also bacteria (250 mg wet weight) harvested from a shaken flask culture by centrifuging (10,000 *g*, 10 min) were resuspended in saline buffer (pH 6·5, 20 ml), incubated at room temperature for 2 hours, the bacteria removed by centrifuging (10,000 *g*, 10 min) and the supernatant fluid concentrated by pressure dialysis through a cellophane membrane. All extracts were sterilized by filtration through Millipore membranes (GSWP; 0·22 μm mean pore size).

Francisella tularensis walls were prepared using a method similar to that described by Shepard *et al.* (1954) and sterilized by treatment with acetone or formaldehyde as for the preparation of killed bacterial suspensions.

A ribosomal fraction of *Francisella tularensis* LVS was prepared by shaking a suspension of the bacteria (5×10^{10}/ml) in 10 mmol/l tris HCl, 10 mmol/l magnesium acetate and 100 mmol/l KCl (pH 7·4) with ballotini (grade 14) in a Mickle shaker (12 min, 4°; H. Mickle, Hampton, Middlesex). After filtration through a glass sinter, bacterial debris and unbroken bacteria were removed by centrifuging (10,000 *g*, 30 min) and the preparation sterilized by filtration through a Millipore membrane (GSWP; 0·22 μm pore size). The filtrate was centrifuged (100,000 *g*, 16 hours) in sterile tubes and the pellet (ribosomal fraction) resuspended with a glass tissue homogenizer in saline phosphate buffer (pH 7·4) containing magnesium acetate (5 mmol/l).

Spent medium.—Bacteria were removed from a 20 hour shaken flask culture by centrifuging and the medium sterilized by filtration through a Millipore membrane (GSWP; 0·22 μm pore size). The spent medium was dialysed against saline buffer (pH 6·5, 16 hours,

4°) and concentrated by pressure dialysis through a cellophane membrane. Filtered spent medium was also treated with $(NH_4)_2SO_4$ (final concentration 3·3 mol/l) and the precipitate dissolved in saline buffer (pH 6·5), dialysed against the same buffer (16 hours, 4°) and concentrated by pressure dialysis.

Sterility checks.—Samples (0·1–0·25 ml) of killed bacterial suspensions, extracts, walls, ribosomal fractions and spent medium were spread on blood agar plates (Downs *et al.*, 1947) and incubated at 37°; preparations were considered to be sterile if no colonies appeared after 120 hours.

Determination of virulence and vaccine potency.—The virulence of *Francisella tularensis* LVS was tested by inoculating batches of 80 Porton mice intraperitoneally (i.p.) in groups of 10 with 1–10^6 viable bacteria and vaccine potency by challenging mice surviving after 3 weeks with about 10^3 lethal doses of the virulent Schu S4 strain (10^3 viable bacteria); LD_{50} and PD_{50} (dose to protect 50% of mice) values were calculated with a computer programme based on the method of Finney (1952).

The vaccine potency of killed bacterial suspensions, extracts, walls, ribosomal fractions and spent medium was tested by inoculating batches of 10 mice i.p. or subcutaneously (s.c.) over a period of 7 days with a single or up to 5 doses (0·1 ml/dose) of various amounts of the preparations; control animals received equivalent injections of saline phosphate buffer (pH 7·4). In some experiments ether–water extracts were administered with incomplete Freund's adjuvant (Bayol F, 85 ml; Arlacel A, 15 ml) or alum (potassium aluminium sulphate was added to the extract in saline phosphate buffer, pH 6·0, to a final concentration of 0·1% and left overnight at 4°). Two weeks after administration of the first dose mice were challenged with 10^3 lethal doses of the Schu S4 strain; deaths were recorded for 3 weeks post challenge.

Electrophoresis.—Components of bacterial extracts were separated by electrophoresis on Cellogel strips (90 min, 5·0 μA/cm, 18 V/cm; Chemetron, Milan, Italy) or polyacrylamide gels (90 min, 2mA/gel) with the method of Davis and Ornstein (1961). Extracts were also treated with sodium dodecyl sulphate (SDS; 1%, w/v), mercaptoethanol (1%, v/v) and glycerol (10%, v/v) before application to gels made in 10 mmol/l sodium phosphate buffer (pH 7·2). Bacterial walls were solubilized and the components separated on polyacrylamide gels as described by Robinson and Tempest (1973).

Protein bands on Cellogel strips were stained with Ponceau S, followed by destaining of the gel strip (Cellogel Instruction Sheet, Chemetron, Milan) and on polyacrylamide gels with naphthalene black (Sargent, 1969); carbohydrate reacting material was stained with Schiff's periodic acid reagent (Sargent, 1969). In some cases duplicate polyacrylamide gels were cut horizontally into 1 mm slices which were tested for immunological activity.

Immunological tests.—Immunological activity of bacterial extracts were tested by the agar-gel double diffusion technique (Ouchterlony, 1953) and that of the components separated on polyacrylamide gels by placing gel slices in the wells of agar diffusion plates with a few drops of saline phosphate buffer (pH 6·5); preparations were allowed to diffuse against rabbit anti-*Francisella tularensis* Schu S4 serum.

Estimation of RNA and protein.—RNA was estimated by the method of Herbert, Phipps and Strange (1972) and protein by the method of Lowry *et al.* (1951).

Materials.—Analar grade chemicals were used whenever possible and with acrylamide, NN′-methylene-bis-acrylamide and NNN′N′-tetramethylenediamine obtained from BDH Chemicals Ltd, Poole, Dorset.

RESULTS

Virulence and vaccine potency of Francisella tularensis (LVS) *grown in POMEC and shaken flask cultures*

Bacteria grown in POMEC for 48 hours under batch culture conditions, followed by up to 700 hours at a constant or periodically changed growth rate, rapidly lost virulence; the vaccine potency of the bacteria also decreased but more slowly and to a lesser extent. During the period of batch culture, LD_{50} values increased from 50–150 to 4×10^3–7×10^5 and PD_{50} values from about 10 to 150 bacteria; both values continued to increase during continuous culture (Fig. *a*, *b*).

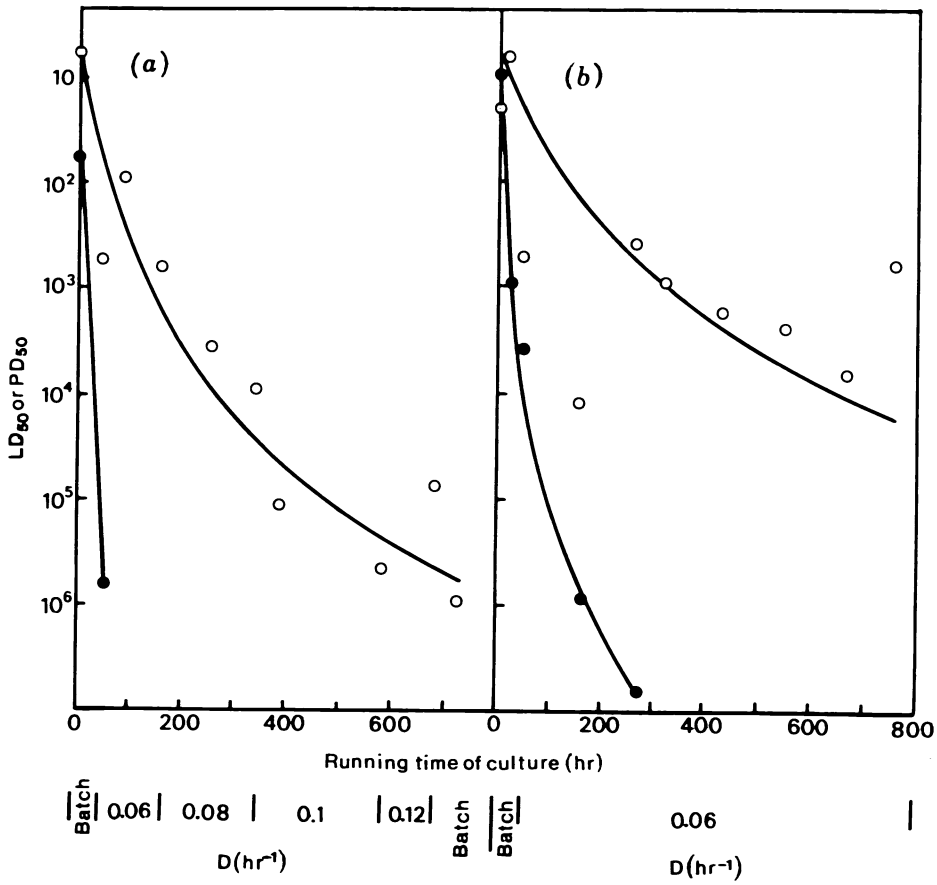

FIG.—Effect of batch and continuous growth in the POMEC on the virulence and vaccine potency of *Francisella tularensis* LVS. (a) Bacteria grown batchwise for 48 hours and then continuously at a periodically changed growth rate (D, 0·06–0·12 h^{-1}). (b) Bacteria grown batchwise for 48 hours and then at a constant growth rate (D, 0·06 h^{-1}). Virulence (LD_{50}, ●) and vaccine potency (PD_{50}, ○) in mice were determined as described in the text.

In contrast, the LD_{50} values of bacteria passaged 4 times over a period of 96 hours in shaken flask cultures with the same medium as in the POMEC increased only slightly from 5–40 to about 150 viable organisms.

Francisella tularensis grows well in shaken flasks of certain complex media (Sharer *et al.*, 1968; Hood, 1961; Mills *et al.*, 1949) and reports indicate that growth will occur in certain chemically defined media (Tresselt and Ward, 1954; Traub *et al.*, 1955; Chamberlain, 1965). In our hands, however, these chemically defined media supported only poor growth and on passaging in them the bacteria failed to grow at all; Sharer *et al.* (1968) obtained similar results with chemically defined media. Different LD_{50} values were obtained for bacteria grown in batch culture in shaken flasks for 20 hours in several different media. The same medium as used in POMEC (Sharer *et al.*, 1968) gave the most virulent bacteria (LD_{50}, 5–40 organisms) but populations from all the other media (Tresselt and Ward, 1954; Traub *et al.*, 1955; Hood, 1961) were more virulent (Table I, LD_{50} values of

390–2400 viable bacteria) than those recovered after growth in POMEC (Fig. *a*, *b*). Bacteria grown in the complex medium of Sharer *et al.* (1968) with glycerol replaced by glucose (0·5% w/v) had higher LD_{50} values (25–50) compared with organisms grown in the glycerol medium (LD_{50}, 5–40) but PD_{50} values were unchanged (Table I). Bacteria grown in complex medium in the presence of limiting amounts of glucose provided by diffusion capsules were of slightly decreased virulence (Table I).

TABLE I.—*The Virulence and Vaccine Potency to Mice of* Francisella tularensis *LVS grown in Shaken Flask Cultures* (20 *hours*, 37°) *with Different Growth Media*

Growth medium reference	LD_{50} (viable bacteria)	PD_{50} (total bacteria)
Sharer *et al.* (1968)	5–40	9
Sharer *et al.* (1968) with glycerol replaced by glucose (0·5%)	25–50	7–10
Sharer *et al.* (1968) with glycerol omitted and glucose (12·5%, w/v, 1·2 ml) in a diffusion capsule	130	7
Hood (1961)	390	ND
Traub *et al.* (1955) with peptic sheep blood (1%, v/v) added	1310	ND
Tresselt and Ward (1964)	2380	ND

Virulence (LD_{50}) was determined by inoculating groups of 10 mice with 1 to 10^6 viable bacteria and vaccine potency (PD_{50}) by challenging mice surviving after 3 weeks with 10^3 lethal doses of virulent Schu S4 strain.
ND Not determined.

Vaccine potency of killed Francisella tularensis *LVS, bacterial fractions and extracts of live bacteria*

Compared with living *Francisella tularensis* LVS, none of the materials tested was an effective vaccine although some significantly extended the survival time of mice subsequently challenged with the virulent Schu S4 strain; none of the preparations was lethal to mice.

Killed bacterial suspensions.—Suspensions of *Francisella tularensis* LVS killed with heat, formaldehyde, acetone, ether or phenol and acetone killed Schu S4 strain all slightly protected mice against a challenge of live virulent Schu S4 (Table II).

Extracts of live bacteria.—Nutter (1971) reported that ether–water extracts of *Francisella tularensis* were more immunogenic than either hot water or phenol–water extracts and Procházka and Dubanská (1972*a*,*b*) found that ether–water extracts contain a number of different protein species. The protein content of recovered material in bacterial ether–water extracts, prepared as described by Nutter (1971), accounted for 10–15% of the equivalent bacterial dry weight. A considerable amount of protein reacting material was also extracted from *F. tularensis* LVS with hot water but the bacteria-free aqueous phase after phenol treatment contained relatively little protein. The numbers of protein reacting components detected after electrophoresis of ether–water, hot water and phenol–water extracts of *F. tularensis* LVS on cellulose acetate strips were at least 13, 9 and 2 respectively; at least 2 carbohydrate reacting components were also present

TABLE II.—*Vaccine Potency of Killed* Francisella tularensis (*LVS*)

Preparation	Doses	Amount/dose	Range of survival time of mice challenged with virulent Schu S4 strain (days)
Saline phosphate buffer (pH 7·4)—controls	2	0·1 ml	3–4
Formalin killed LVS	2	10^9 bacteria	4–6
Formalin killed LVS (unwashed)	2	10^9 bacteria	6–9
Heat killed LVS	2	10^9 bacteria	4–10*
Heat killed LVS (unwashed)	2	10^9 bacteria	4–8
Ether killed LVS	2	10^9 bacteria	3–14*
Acetone killed LVS	2	10^9 bacteria	4–7
Phenol killed LVS	2	10^9 bacteria	4–6
Acetone killed Schu S4	2	10^9 bacteria	5–7

Mice in batches of 10 were inoculated (i.p.) with suspensions (0·1 ml) of virulent (in mice) *F. tularensis* (LVS) killed in various ways, and subsequently challenged with 10^3 lethal doses of virulent Schu S4 strain. Deaths were recorded for 3 weeks post challenge.

* Only 1 mouse survived beyond Day 6.

in each extract. Electrophoresis of ammonium sulphate precipitated and soluble fractions of ether–water extracts showed that the former contained most of the protein components in the original extract but the latter contained a fast moving component probably identical to the major component in phenol–water extracts. The ammonium sulphate precipitated fraction gave the same number and intensity of precipitation lines as whole ether–water extract with homologous anti-Schu S4 serum in double diffusion plates. No obvious differences were detected between the precipitation line patterns given by ether–water extracts of virulent (to mice) and non-virulent bacteria with anti-Schu S4 serum.

The separation of components in ether–water extracts of *Francisella tularensis* LVS was improved when samples were treated with sodium dodecyl sulphate (SDS) and mercaptoethanol before electrophoresis on polyacrylamide gels at pH 7·2. Under these conditions, about 27 protein staining components were detected, of which at least 6 precipitated with homologous anti-serum. When components in ether–water extracts of virulent (to mice) and non-virulent *F. tularensis* LVS separated on polyacrylamide gels were compared, the only difference detected was the apparent presence in the latter of an additional protein staining component. Since SDS decreased the number and intensity of precipitation lines given by whole ether–water extracts, electrophoresis was also conducted on untreated material in tris-glycine buffer, pH 8·9; here about 19 protein staining components were separated, of which at least 7 were immunologically active.

Ether–water extracts of virulent (to mice) but not non-virulent *Francisella tularensis* LVS slightly protected mice against challenge with virulent Schu S4 (Table III) but regardless of the amount tested, route of inoculation or presence of adjuvants, the protective activity was minimal compared with that of live bacteria although ether–water extracts and bacteria gave similar precipitation lines in double diffusion plates with homologous anti-serum. The slight protective activity of ether–water extracts was associated with the ammonium sulphate precipitated but not the soluble fraction (Table III).

Bacterial walls.—Walls of *Francisella tularensis* LVS gave precipitation lines against homologous anti-serum similar to those given by whole ether–water extracts. Components in solubilized isolated walls of virulent (to mice) bacteria were separated by polyacrylamide gel electrophoresis and stained for comparison

TABLE III.—*Vaccine Potency of Extracts of* Francisella Tularensis (LVS)

Preparation	Inoculation route	Doses	Amount/dose	Range of survival time of mice challenged with virulent Schu S4 strain (days)
Saline phosphate buffer (pH 7·4)-controls	i.p. or s.c.	1–5	0·1 ml	3–4
Whole other–water extract of non-virulent bacteria	i.p.	1	250 μg of protein	3–4
Whole other–water extract	i.p. or s.c.	1	250 μg of protein	4–5
$(NH_4)_2SO_4$-soluble fraction of other–water extract	i.p.	1	64 μg of protein	3–4
$(NH_4)_2SO_4$-precipitated fraction of other–water extract	i.p.	1–5	50–250 μg of protein	4–9
Incomplete Freund's adjuvant—control	i.p.	1	0·1 ml	3–5
$(NH_4)_2SO_4$-precipitated fraction of other–water extract with incomplete Freund's adjuvant	i.p.	1	250 μg of protein	3–5
Alum suspension-control	i.p. or s.c.	1–2	0·1 ml	3–4
$(NH_4)_2SO_4$-precipitated fraction of other–water extract + alum	i.p. or s.c.	1–2	125–380 μg of protein	4–14*
Hot water extract	i.p.	3	80 μg of protein	4–5
Phenol–water extract	i.p.	3	13·5 μg of protein	3–5

Mice in batches of 10 were inoculated with extracts (0·1 ml) of *F. tularensis* (LVS) and treated as in Table II. Unless otherwise stated, the extracts were derived from virulent (in mice) bacteria.

* Only 1 mouse survived beyond Day 6.

TABLE IV.—*Vaccine Potency of Walls and Ribosomal Fraction of Virulent (in Mice)* Francisella tularensis (LVS)

Preparation	Doses	Amount/dose	Range of survival time of mice challenged with virulent Schu S4 strain (days)
Saline phosphate buffer (pH 7·4)—control	3	0·1 ml	3–4
Formalin treated bacterial walls	3	100 μg equiv. dry wt of walls	5–9*
Acetone treated bacterial walls	3	150 μg equiv. dry wt of walls	3–9*
Acetone treated bacterial walls	3	750 μg equiv. dry wt of walls	4–9*
Saline phosphate buffer (pH 7·4) + 5 mmol/l magnesium acetate	3	0·1 ml	3–4
Ribosomal fraction	3	48–67 μg of RNA	5–21**

Mice in batches of 10 were inoculated with the preparations (0·1 ml) and treated as in Table II.

* Only 1 mouse survived beyond Day 6.

** 3 mice survived beyond Day 6 and 2 beyond Day 14.

with those in walls of non-virulent bacteria and similarly treated extracts of whole bacteria. About 21 protein staining components were detected in solubilized walls, the only difference between those in walls of virulent and non-virulent bacteria being the presence in the latter of an additional component that possibly was identical to the extra component found in ether–water extracts of non-virulent bacteria (see above). Several components in solubilized walls appeared identical with those in ether–water extracts but differences were apparent between the respective smaller molecular weight components.

Walls isolated from *Francisella tularensis* LVS and sterilized with formaldehyde or acetone slightly protected mice against a challenge with Schu S4 (Table IV) but relatively large amounts were necessary to give protection similar to that given by ether–water extracts.

Ribosomal fraction.—Of the non-living preparations tested, a crude ribosomal fraction of *Francisella tularensis* LVS gave the best protection to mice against the virulent Schu S4 strain. In a batch of 10 mice immunized with the fraction 8 survived for 6–14 days post-challenge while 2 survived completely (Table IV).

Extracellular material.—The possibility that *Francisella tularensis* (like *Bacillus anthracis*; Gladstone, 1946, 1948; Wright, Hedberg and Feinberg, 1951; Belton and Strange, 1954) produces an extracellular protective antigen and/or virulence factor was examined by injecting mice with sterile concentrated bacteria-free filtrate or material precipitated with saturated ammonium sulphate from 20 hours shaken flask cultures; neither material was significantly protective or lethal in mice challenged with Schu S4.

Effect of washing.—The possibility that protective antigen(s) was removed by washing was examined by injecting mice with unwashed suspensions of heat or formaldehyde killed bacteria or concentrated sterile bacteria-free filtrate from a live bacterial suspension. The killed bacteria gave slight protection (Table II) but the bacteria-free filtrate gave no significant protection to mice challenged with Schu S4; none of the preparations was lethal to mice.

Reaction of virulent and non-virulent bacteria with homologous anti-serum.—The precipitation lines in double diffusion plates given by freeze–thawed virulent and non-virulent *Francisella tularensis* LVS against hyperimmune anti-Schu S4 serum were identical. When anti-Schu S4 serum was absorbed with freeze-thawed non-virulent LVS and the absorbed serum tested in double diffusion plates against virulent and non-virulent LVS a similar weak precipitation line was produced in each case, apparently indicating the absence of an extra antigen in virulent bacteria.

DISCUSSION

The present findings show that continuous culture of the live vaccine strain of *Francisella tularensis* in a chemostat rapidly decreases the virulence and, to a lesser extent, decreases the vaccine potency of the bacteria. The results suggest that the factors concerned with virulence and protection are not related. Although long-term continuous culture may be unsuitable for vaccine production, bacteria of relatively high vaccine potency and extremely low virulence in mice were produced in short-term cultures. It is not clear why growth in the POMEC, in contrast to growth in batch culture, caused such rapid and significant changes in virulence and vaccine potency of *F. tularensis* LVS. Perhaps vigorous agitation in the culture vessel due to forced aeration and/or the controlled pH value of the

culture in the POMEC caused these changes. Bacteria grown in batch culture in shaken flasks of the same growth medium as used in the POMEC, but without pH control, maintained their virulence and vaccine potency during several passages.

Virulence and protection inducing factors in *Francisella tularensis* have not been identified, nor is the protective effect of the LVS strain understood. Several different antigens have been detected but it has not been demonstrated convincingly that all or any of them function as protective antigens (Alexander, 1950; Shepard *et al.*, 1954; Larson, Bell and Owen, 1954; Ormsbee *et al.*, 1955; Nutter, 1971). Although Procházka and Dubanská (1972*a*, *b*) fractionated and partially purified ether–water extractable antigens, they did not investigate the virulence or protective activity of the isolated components.

With the aim of developing an effective non-living vaccine, attempts were made to identify virulence and protective factors in *Francisella tularensis* but these were unsuccessful. Thus, relatively large amounts of killed bacteria, isolated walls, various bacterial extracts and crude ribosome preparations with and without adjuvants and using different routes of inoculation, all failed to kill mice or significantly protect them against tularaemia. All the preparations contained detectable antigens but evidently these did not confer significant immunity. The poor protection afforded by killed bacteria and isolated fractions of them could be due to the protective antigen(s) being extremely labile and destroyed by the various treatments, or it is possible that the good protection given by the live vaccine strain is due to the continued stimulus to the host antibody production apparatus during bacterial growth and division *in vivo* (Downs and Moody, 1956; Moody and Downs, 1956) although the protective antigen(s) is present in extremely small amounts. A third possibility also discussed by Downs and Moody (1956) is that during growth and division of *Francisella tularensis* in the host, but not in the culture media used, an antigen is produced that induces immunity. Non-living vaccines said to be effective against tularaemia were reported by Foshay *et al.* (1942) and Foshay *et al.* (1947), and later others that invoked antibody production and conferred some level of immunity to laboratory animals were described by Bell *et al.* (1952), Larson *et al.* (1954), Shepard *et al.* (1954), Ormsbee *et al.* (1955), Ormsbee and Larson (1955) and Nutter (1971). However, Eigelsbach and Downs (1961), Eigelsbach *et al.* (1967) and Claflin and Larson (1972) consider that, compared with living vaccines, present non-living vaccines are relatively ineffective and our results confirm this.

We thank Dr A. Robinson for polyacrylamide gel electrophoresis analyses, Mr R. Harris-Smith for providing continuously grown bacteria, Mr B. Maidment for statistical computations and Mrs S. M. Marriott for technical assistance.

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