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GRANT NO: DAMD17-91-Z-1033

TITLE: SCIENTIFIC EXPERT IN METAGENESIS OF FRANCISELLA TULARENSIS (VACCINE STRAIN) TO PRODUCE MARKED STRAINS

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REPORT DATE: December 13, 1991

TYPE OF REPORT: Final Report

PREPARED FOR: U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
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13 December 1991 Final Report (5/15/91 - 12/15/91)

Scientific Expert in Metagenesis of Francisella Tularensis (Vaccine Strain) to Produce Marked Strains

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Final Report on Specific Tasks No. 1-5 of Contract (restated, for reference).

1. Independently plan and perform procedures to select for spontaneous mutations in the 10^{-6} to 10^{-10} frequency range, that render F. tularensis live vaccine strain resistant to one or more antibiotics or metabolic poisons (e.g., nalidixic acid, rifamycin, streptolydigin, azide) that can easily be incorporated into bacteriological media.

2. Independently plan and perform procedures to purify and store the mutants for further characterization.

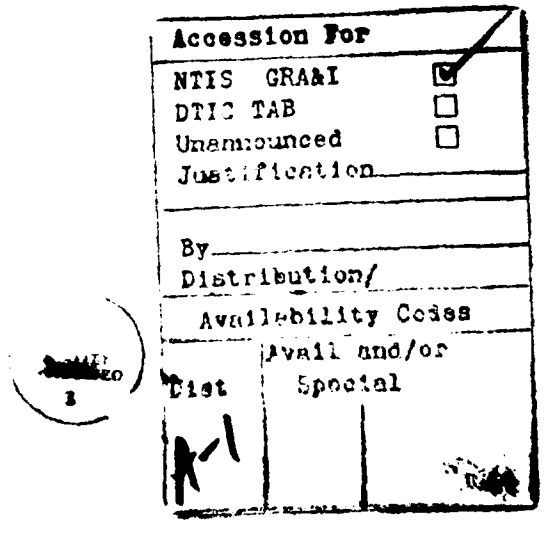
3. Independently plan and perform procedures to characterize the growth rates of the mutants both in the presence and absence of the antibiotic to which resistance has been obtained.

Vaccines

13. Abstract (continued)

4. Independently plan and perform procedures to determine the plasmid DNA composition of mutants having growth rates identical to that of the parent F. tularensis LVS.

5. Independently plan and perform procedures to measure the intradermal and intraperitoneal LD_{50} in C3H/HeN mice of selected mutants that have growth rates and plasmid profiles identical to those of the parent F. tularensis LVS.



Summary

Three strains marked with different antibiotic resistances were isolated and characterized as described above. In the course of the work, other isolates having the same antibiotic resistances and the same or different growth characteristics were isolated and stored in the event that they might be useful in future work. These latter were not characterized as thoroughly as the first three.

1. **Nalidixic acid-resistant (Nal^r) *F. tularensis* LVS.** This strain (called Nal^r 3) can be grown in the presence of 5 µg/ml nalidixic acid, a concentration which inhibits growth of the parent *F. tularensis* LVS. It has all of the properties described above for a versatile marked strain.

2. **Spectinomycin-resistant (Spc^r) *F. tularensis* LVS.** This strain (called Spc^r m1) can be grown in the presence of 80 µg/ml spectinomycin, a concentration which inhibits growth of the parent *F. tularensis* LVS. It can be used in experiments requiring two marked *F. tularensis*.

3. **Rifampicin-resistant (Rif^r) *F. tularensis* LVS.** This strain (called Rif^r 7) can be grown in the presence of 1 µg/ml rifampicin, a concentration which inhibits growth of the parent *F. tularensis* LVS. This strain is strongly decreased in virulence and cannot be used as a marked strain otherwise identical in properties to the parent *F. tularensis* LVS. However, it may be useful as a vaccine strain, because it can be immunize mice from either the intraperitoneal or intravenous route. This strain also may be useful in studies aimed at elucidating the virulence properties of *F. tularensis*. A second, independently derived Rif^r mutant (called Rif^r 5-2) was partially characterized. It differs from Rif^r 7 in retaining a low level of virulence. This strain also may be useful as a vaccine strain and in studies of the virulence properties of *F. tularensis*.

Technical Description of Strains Resulting From This Contract

Method of Obtaining Mutants.

Screening was carried out for isolates naturally resistant to antibiotics not useful for treatment of *F. tularensis* infections. These occur (and revert) at frequencies of natural mutations in genes – i.e., 10^{-6} - 10^{-10}. This approach has been used successfully for other facultative intracellular pathogens (e.g., *Salmonella, Yersinia*).

The antibiotics chosen were ones with a single, well-defined target: nalidixic acid, spectinomycin, and rifampicin. These were used at the highest concentration that permitted the isolation of mutants (determined empirically). The aim was to avoid mutations in respiration or transport, which can give low-level resistance and which are likely to cause alterations in growth. It also was crucial that the concentration of

antibiotic completely prevent the formation of colonies by the parent *F. tularensis* LVS even when present in the large numbers that might occur in some applications (e.g., 10^7).

The antibiotics were incorporated into the bacterial plating medium (after autoclaving), and a high density (10^7 to 10^{10} CFU) of bacteria was spread to allow colony-formation by bacteria making up 10^{-6} - 10^{-9} of the population.

Colonies were streak-purified three times, of which one streaking was made on nonselective medium to reveal (and subsequently avoid) any nongrowing bugs not killed by the antibiotic but carried along in the background during streaking and to avoid mutants that are dependent on the antibiotic for their growth. For each antibiotic, several colonies were picked for characterization. It was understood that colonies obtained from the same pool of bacteria might have arisen from daughter cells with the same mutation.

Properties of the Antibiotics:

Nalidixic acid (Nal). Target: the A subunit of DNA gyrase. DNA gyrase is necessary for the introduction and maintenance of supercoils in DNA and as such is necessary for the proper regulation of expression of DNA, for normal DNA replication (elongation reaction) and for normal decatenation of the chromosome during segregation into daughter cells.

The lethal action of nalidixic acid probably stems from the formation of drug-gyrase complexes covalently bound to DNA, resulting in inhibition of DNA replication.

Nalidixic acid-resistant (Nal^r) gyrase molecules do not bind the drug.

Rifampicin (Rif). Target: the β subunit of RNA polymerase. This subunit contains the catalytic site for RNA polymerization. Rifampicin prevents initiation of RNA chains but not their subsequent elongation.

Rifampicin-resistant (Rif^r) polymerase molecules do not bind Rif.

Spectinomycin (Spc). Target: ribosomal protein S5. Its action inhibits the function of the 30S ribosomal subunit and blocks the movement of the ribosome after initiation. It is bacteriostatic (i.e., the bacteria are inhibited in their growth but not killed).

Properties of the Mutant *F. tularensis* strains

1. Nalidixic acid-resistant *F. tularensis*

General Information

- *F. tularensis* LVS colony formation is inhibited by Nal concentrations as low as 1 μg/ml. When 2 x 10^7 CFU are spread, ca. 8 colonies arise on plates with 2 μg/ml Nal, 2 colonies with 5 μg/ml Nal, and 1 colony on plates containing 10 μg/ml Nal. These probably are Nal^r mutants in the LVS population and represent a negligible fraction of the total number plated.

- Nal stock: 5 or 10 mg/ml in 0.1 N NaOH (kept refrigerated). Used at 5 μg/ml. Added after autoclaving and tempering plating medium. It is recommended that stock solutions not be used longer than two weeks.

Parent strain: Kle 10/3/90 frozen stock subcultured to SCS 2/12/91 stock frozen in DMEM + 5% FBS + 1% proteose peptone.

Six isolates were saved: No. 1,3,4,5,7, and 8.

Nal^r 3

a.) Grows at ca. the same rate as *F. tularensis* LVS in Fran broth.
b.) Growth rate in Fran broth unaffected by 2 or 5 μg/ml Nal. Grows with 100% plating efficiency on plates with Nal concentrations up to 10 μg/ml (highest tested was 10 μg/ml), but rate of colony formation was slowed at Nal concentrations of $\geq$ 3.5 μg/ml.
c.) Same "plasmid" profile as parent *F. tularensis* LVS.
d.) Fully virulent in C3H/HeN male mice.

LD_{50}:

Exp. 1 **IP = 5ID = 3 x 10^4**
Exp. 2 **IP < 1ID = 8 x 10^4**
Exp. 3 **ID = 2 x 10^5**

This should be a useful marked strain.

Other Nal^r isolates were not characterized (with the exception of the growth of Nal^r 4 in Fran broth: ca. same rate as *F. tularensis* LVS) and could have the same mutation as Nal^{r}3.

2. Spectinomycin-resistant *F. tularensis*

General Information

- *F. tularensis* LVS inoculated at 2 x 10^7 CFU was completely inhibited in colony formation by Spc concentrations $\geq$ 50 µg/ml.

- Stock is 50 mg/ml in H_2O (stored refrigerated). Used at 80 µg/ml. It is added after tempering the plating medium. This antibiotic is very stable, and stock solutions probably can be used for several months.

Parent strain: Kle 10/3/90 frozen stock subcultured to SCS 2/12/91 stock frozen in DMEM + 5% FBS + 1% proteose peptone.

Three rounds of screening were made, resulting in isolates No. 1 through 8, No. 2p and 3p, and No. m1, m2, m3, and m4.

Spc^r m1

a.) Grows a little slower in Fran broth than *F. tularensis* LVS (e.g., in the O.D. range of ca. 0.1, LVS shows a generation time of ca. 2 h, whereas Spc^r m1 has a 2 h 25 min generation time).
b.) Growth in Fran broth is unaffected by Spc at 80 µg/ml. The plating efficiency is 100%, and the rate of colony formation is normal in the presence of Spc at concentrations up to 80 µg/ml (N.B.: determined with Cystine Heart Agar).
c.) Same "plasmid" profile as *F. tularensis* LVS.
d.) Similar virulence to *F.tularensis* LVS in C3H/HeN male mice (may have slightly higher LD_{50}, but many mice will be needed for a conclusive determination).

LD_{50}:

Exp. 1: IP $\leq$ 1 ID = 9 x 10^5
Exp. 2: IP = 5

This strain may be useful in experiments requiring two marked *Francisella.*

Other Spc^r isolates

No. m2 and m3.

Spc^r m2 and m3 were obtained in the same screening as Spc^r m1. Spc^r m2 shows the same growth rate in Fran broth as m1; both m2 and m3 could contain a mutation identical to that in Spc^r m1.

No. 1-8.

All grew more slowly than *F. tularensis* LVS in DMEM + 5% FBS + 1% proteose peptone. No. 2 and 3, which grew the fastest of this group of strains also grew slower than *F. tularensis* LVS in Fran broth (the generation time of *F. tularensis* LVS in old Fran broth was 3 1/4 h; Spc^r Nos. 2 and 3 showed 4 h 5 min).

No. 2 and 3 showed 100% plating efficiency on medium containing up to 10 µg/ml Spc (the plating efficiency and rate of colony formation were decreased on 20 and 50 µg/ml Spc).

Spc^r 2 at an IP dose of 2×10^2 killed all 5 C3H/HeN male mice.

These isolates are not useful as marked strains for experiments in which large numbers of *F. tularensis* LVS will be present simultaneously, because *F. tularensis* LVS shows a density-dependent tolerance of Spc. When $\geq 10^7$ CFU of LVS are inoculated, patches of confluent growth appear on plates containing $\leq$ 50 µg/ml Spc. Note, however, that *F. tularensis* LVS inoculated at low density (e.g., ca. 100 CFU) does not form colonies on plates with a Spc concentration as low as 10 µg/ml and has ca. 10% plating efficiency on 2 µg/ml.

No. 2p and 3p.

These were derived from No. 2 and 3 as papillae able to tolerate a higher concentration of Spc. They probably are derivatives with a secondary mutation.

They grow a little slower than the *F. tularensis* LVS parent in Fran broth, and their growth rate in Fran broth is unaffected (2p) or only slightly affected (3p) by 50 µg/ml Spc. Their growth on plates is density-dependent in the presence of 100 µg/ml Spc. Their tolerance for Spc probably is between that for strains No. 1-8 and No. m1.

3. Rifampicin-resistant *F. tularensis*

General Information

- *F. tularensis* LVS was prevented from forming any colonies by concentrations of Rif as low as 1 µg/ml -- even when 10^7 CFUs were applied to the plate. Ca. 2 colonies/plate are obtained in such tests. These probably are Rif^r mutants in the *F. tularensis* LVS population; their number is negligible compared to the total numbers inoculated on the plate.

- Rif stock: 10 to 30 mg/ml in 100% MeOH (Al foil; refrigerated). Final concentration in medium: 1 to 30 µg/ml, with 1 µg/ml being recommended. I recommend using 1 µg/ml. Rif is added after autoclaving and tempering the plating medium. Rif plates

should be protected from exposure to light (Al foil or black bag). The stock solution probably should be remade every two weeks.

Two rounds of screening were carried out.

Round 1

Parent: LVS 6/14-0 lab stock

4 isolates were saved; No. 1,2,5,7

Rif^r No. 7

a.) Same growth rate as the parent *F. tularensis* LVS in DMEM + 10% FBS + 1% proteose peptone pH 6.5; slightly slower rate than the parent in Fran broth.
b.) Growth in liquid medium unaffected by 1 to 30 µg/ml Rif; growth on plates with 1 µg/ml Rif: variously 62% to 100% of the number of CFU obtained without Rif.
c.) Same "plasmid" profile as parent.
d.) Binds FRAN-4 (FA assay).
e.) Decreased in virulence in both C3H/HeN male and C57Bl/6J female mice.

LD_{50} in C3H/HeN male mice:

ID > 2×10^7 IP = 2×10^6

f.) Immunizes against *F. tularensis* LVS in both C3H/HeN male and C57Bl/6J female mice. Two tests made with C3H/HeN:

1. 5 Mice were inoculated ID with 10^3 Rif^r 7; 35 d later, they received an IP inoculation of 2×10^3 LVS (Kle stock)

2. 5 Mice were inoculated IP with 2×10^3 Rif^r 7; 31 d later, they received an IP inoculation of 6×10^3 LVS (SCS frozen stock):

Both groups of mice survived.

g.) Elicits anti-*F. tularensis* LVS antibody in both C3H/HeN male and C57Bl/6J female mice, but the isotype distribution was not identical to that elicited by the parent *F. tularensis* LVS.

Rif^r No. 5

Not fully characterized; the antibody response elicited in mice is the only property that distinguishes this isolate from No. 7. Experiment f.) needs to be repeated to confirm this difference.

a.) Slightly slower growth rate than the parent *F. tularensis* in Fran broth (same rate as Rif^r 7).
b.) Growth in liquid medium unaffected by Rif (1 μg/ml); growth on plates with 1 μg/ml Rif: 59% to 100% of the number of CFU obtained without Rif.
c.) Binds FRAN-4 (FA assay).
d.) Decreased virulence in both C3H/HeN male and C57Bl/6J female mice.
e.) Immunizes against IP challenge by *F. tularensis* LVS (tested in C57Bl/6J females following IP immunization with nominal 10^3 CFU).
f.) The amounts of anti-*F. tularensis* LVS antibody elicited in C57Bl/6J female mice given a nominal 10^3 CFU IP were very low. Nonetheless, these mice were protected against 10^3 CFU of *F. tularensis* LVS given IP.

Rif^r 1 and 2

Not well characterized. Both No. 1 and No. 2 have growth rates in Fran broth similar to those of other Rif^r isolates -- slightly slower than *F. tularensis* LVS. No. 1 has the same growth rate as *F. tularensis* LVS in DMEM + 10% FBS + 1% proteose peptone, pH 6.5, whether or not 30 μg/ml Rif is present. No. 1 has the same "plasmid" profile as LVS. These isolates could have the same mutation as Rif^r 7.

Round 2.

Parent: LVS, Kle 10/3/90 frozen stock.

2 isolates were saved: No. 5-2 and 7-2.

Rif^r No. 5-2

a.) Grows slightly slower than *F. tularensis* LVS in Fran broth (similar growth rate to Rif^r 7 and 5).
b.) Growth in liquid medium is unaffected by Rif (1 μg/ml); grows on Rif plates with 100% plating efficiency.
c.) Same "plasmid" profile as *F. tularensis* LVS.
d.) Binds FRAN-4 (FA assay).
e.) Decreased virulence in both C3H/HeN male and C57Bl/6 female mice.

LD_{50} in C3H/HeN male mice:

IP = 1×10^5

f.) Immunizes against IP challenge by *F. tularensis* LVS (tested in C57Bl/6J female mice immunized IP with a nominal 10^3 CFU).
g.) Elicits anti-*F. tularensis* LVS antibody (tested in C57Bl/6J female mice after IP immunization with 10^3 CFU).

Rif^r No. 7-2

Not fully characterized.

a.) Grows slightly slower than *F. tularensis* LVS in Fran broth and in DMEM + 5% FBS + 1% proteose peptone, pH 6.5 (D+P).
b.) Growth in D+P unaffected by Rif (30 µg/ml); 100% plating efficiency on medium with 1 µg/ml Rif (but slower colony formation than other Rif^r strains on Rif plates).
c.) Same "plasmid" profile as *F. tularensis* LVS.