A retrospective look at *Renibacterium salmoninarum*: research from the 20th century on relatives, pathogenicity and antibiotics

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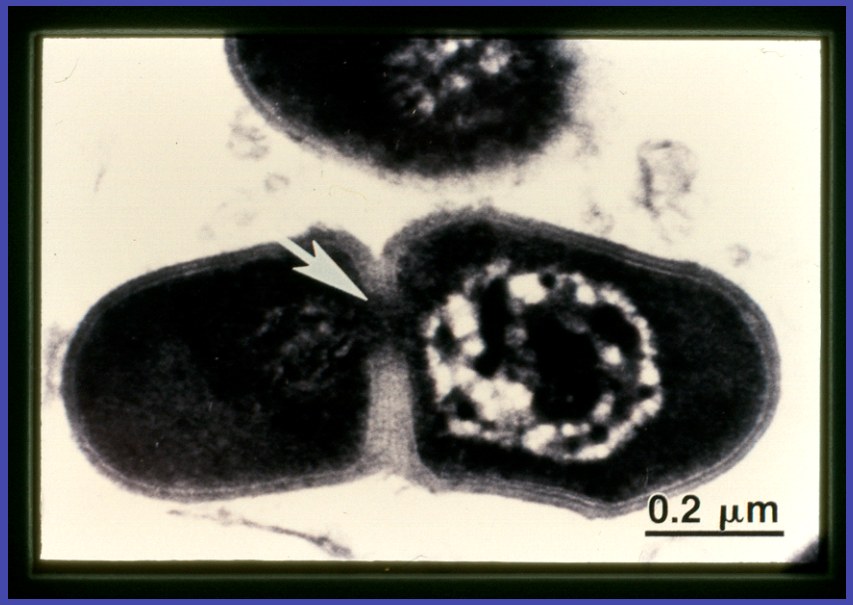
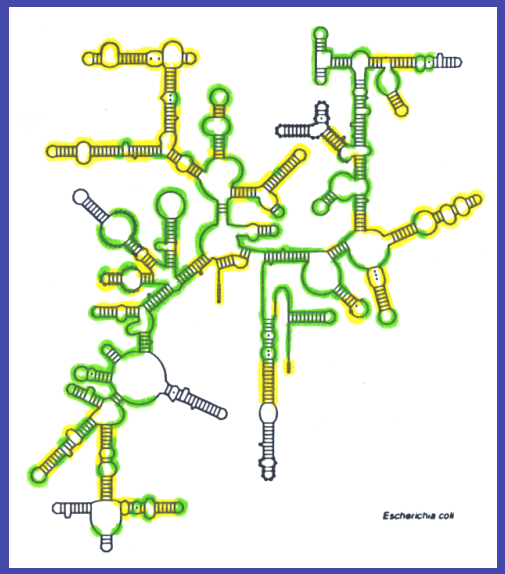
Characteristics of *R. salmoninarum,* a tough bug



1. Durable cell wall
2. p57
3. Slow replication

Phylogeny and Taxonomy of RS



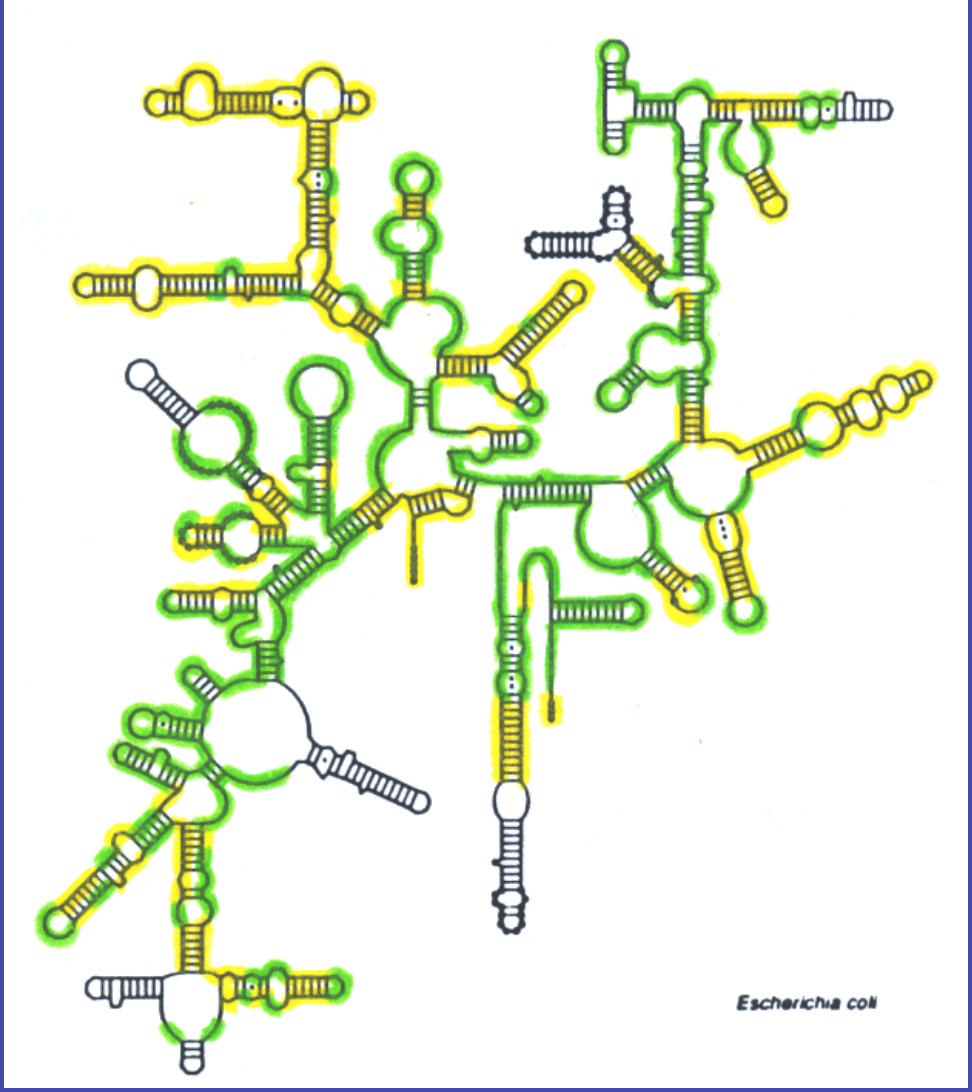
- Where did RS come from?

Ribosomal RNA

>Occurs in all organisms

>Molecular chronometer

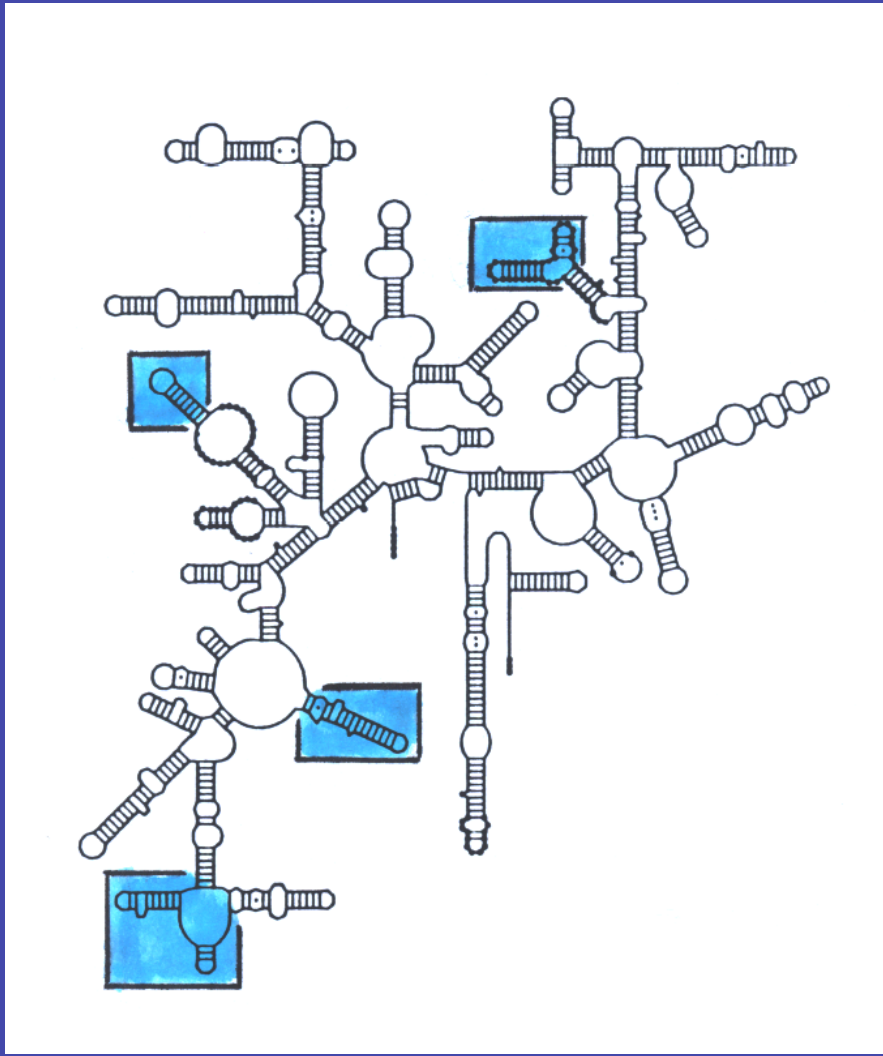
>16S rRNA has large conserved and hypervariable regions



Hypervariable regions on 16S rRNA of *R. salmoninarum*

>Hypervariable regions show relatedness of organisms

>Can be used as probes in PCR to identify an organism



16S rRNA sequence of *R. salmoninarum*

EMBL gene bank, no. X5160

97% complete, 1475 nucleotides

```
UUUUACGGAG  AGUUU-AUUC  UGGCUCAGGA  UGAACGCUGG  CGGCGUGCUU  AACACAUGCA  60
AGUCGAACGA  UGAAGCGGUC  GUUGCGCCGU  GGAUUAGUGG  CGAACGGGUG  AGUAAUACGU  120
GAGUAACCUG  CCCUUGACUU  CGGGAUAAGC  CUGGGAAACU  GGGUCUAAUA  CUGGAUACGA  180
CCUAUCACCG  CAUGGUGUGU  AGGUGGAAAG  UUUUUGCGGU  UUUGGAUGGA  CUCGCGGCCU  240
AUCAGCUUGU  UGGUGAGGUA  AUAGCUAACC  AAGGCGACGA  CGGGUAGCCG  GCCUGAGAGG  300
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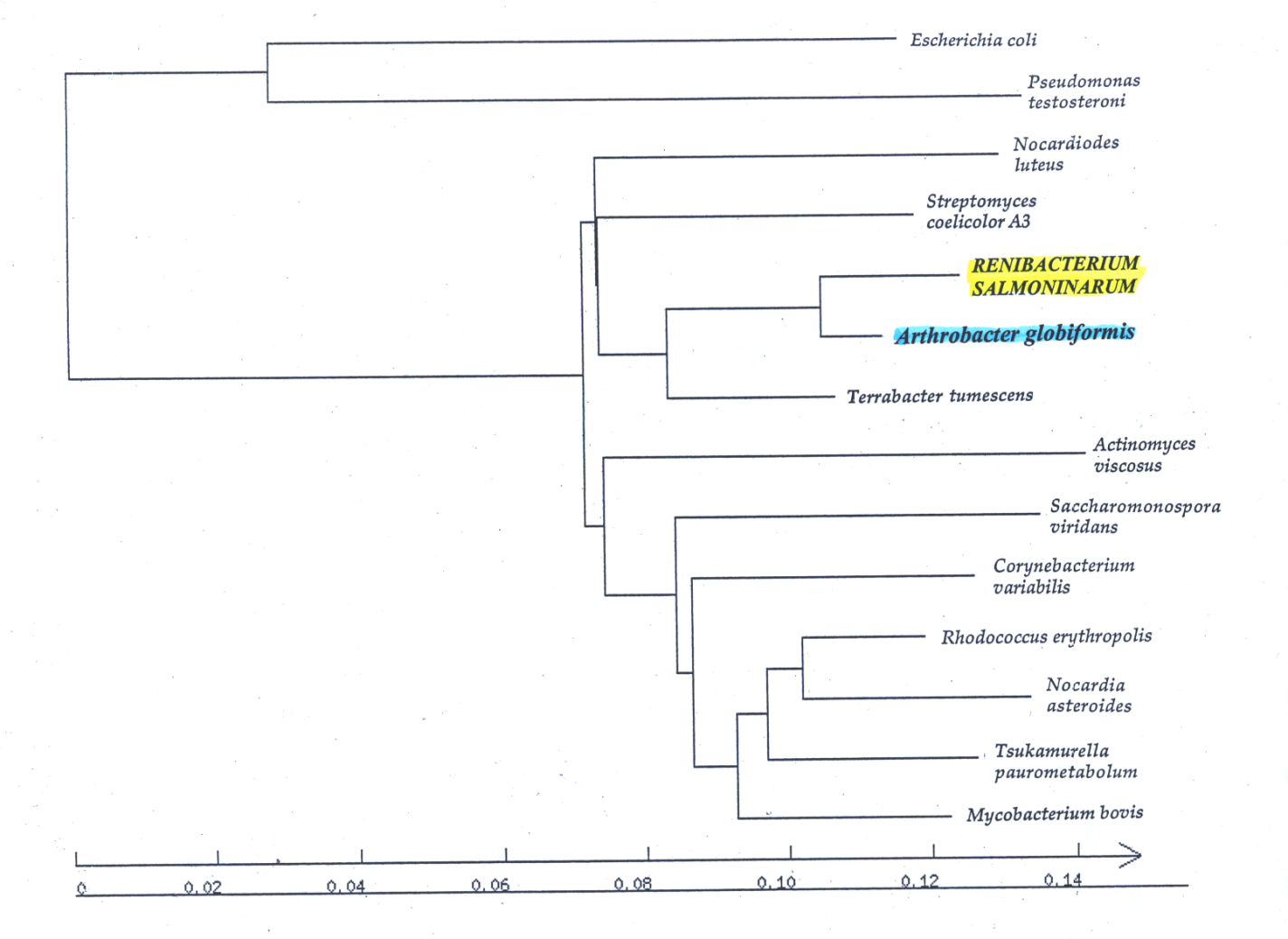
Comparison of bacterial 16S rRNAs



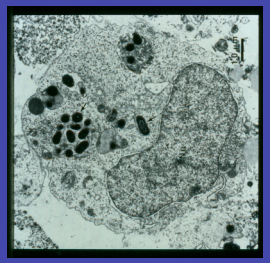
Phylogenetic tree based on comparison of 1218 nucleotides (111-182, 221-453, 480-838, 849-1005, 1042-1133, 1139-1449) from 16S rRNA sequence

Attributes of *Arthrobacter globiformis* that make it an interesting relative

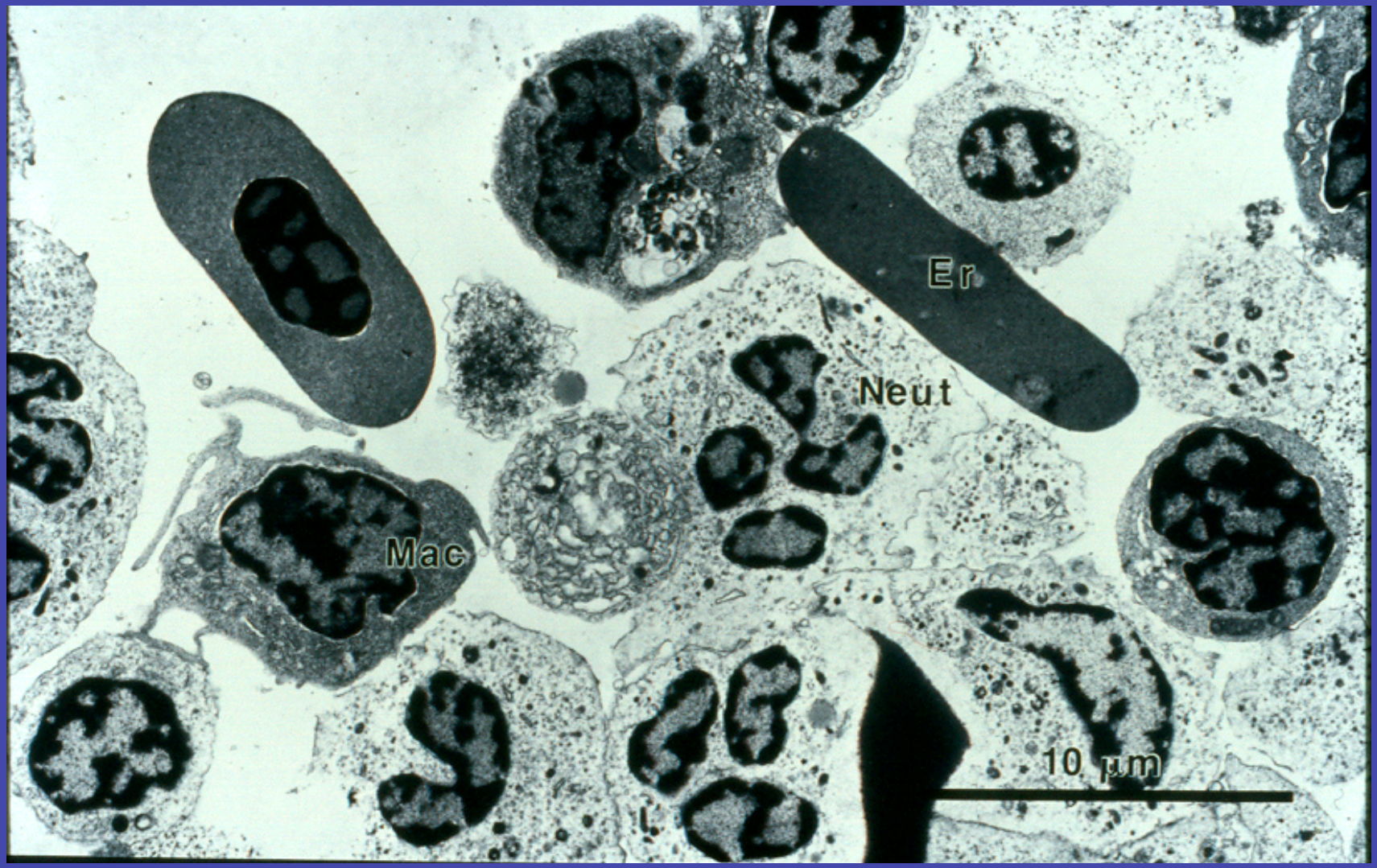
- Soil bacterium with cell wall containing the rare amino sugar, N-acetyl-fucosamine
- Renogen vaccine with *Arthrobacter davidanielli*, closely related to *A. globiformis*?
- Steroid conversion—is it possible that RS also uses this as a mechanism to streamline transmission?

The Mechanism for RS Pathogenicity

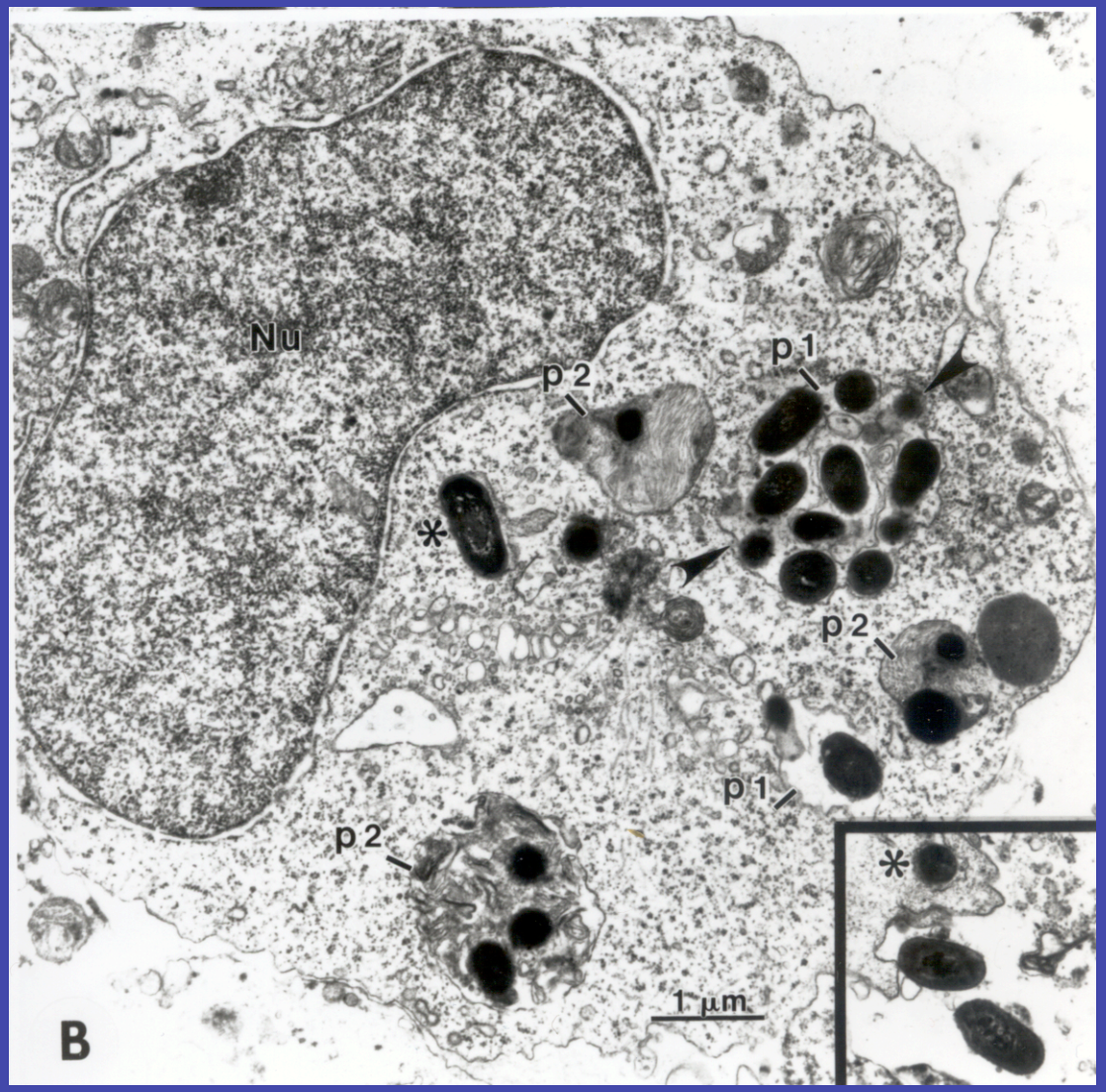
- Intracellular survival and replication in the mononuclear phagocytes
  - Avoids the immune system
  - Reduces susceptibility to antibiotics
  - Long-term survival



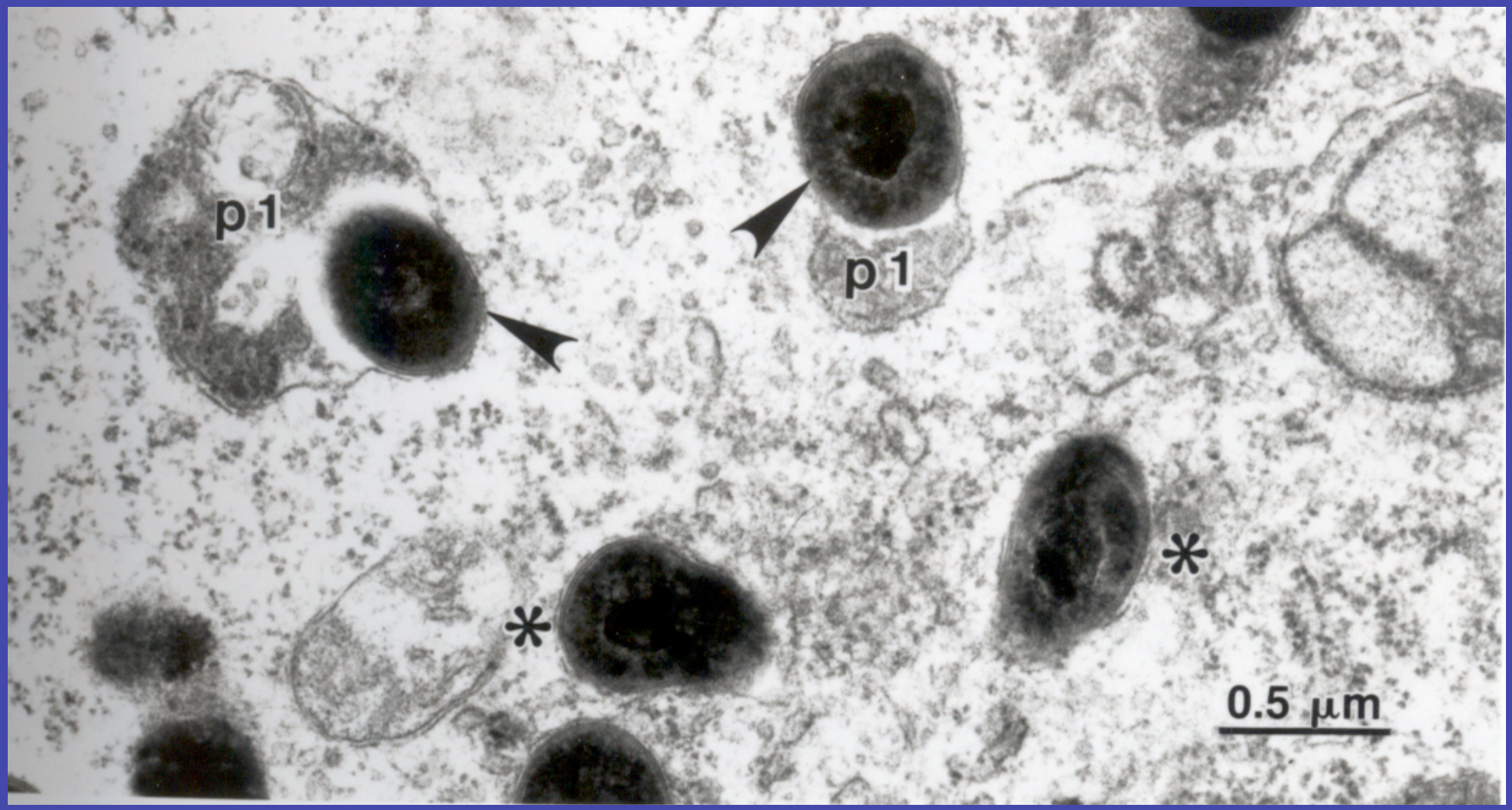
Trout cells homogenized into suspension cultures



RS in the macrophage

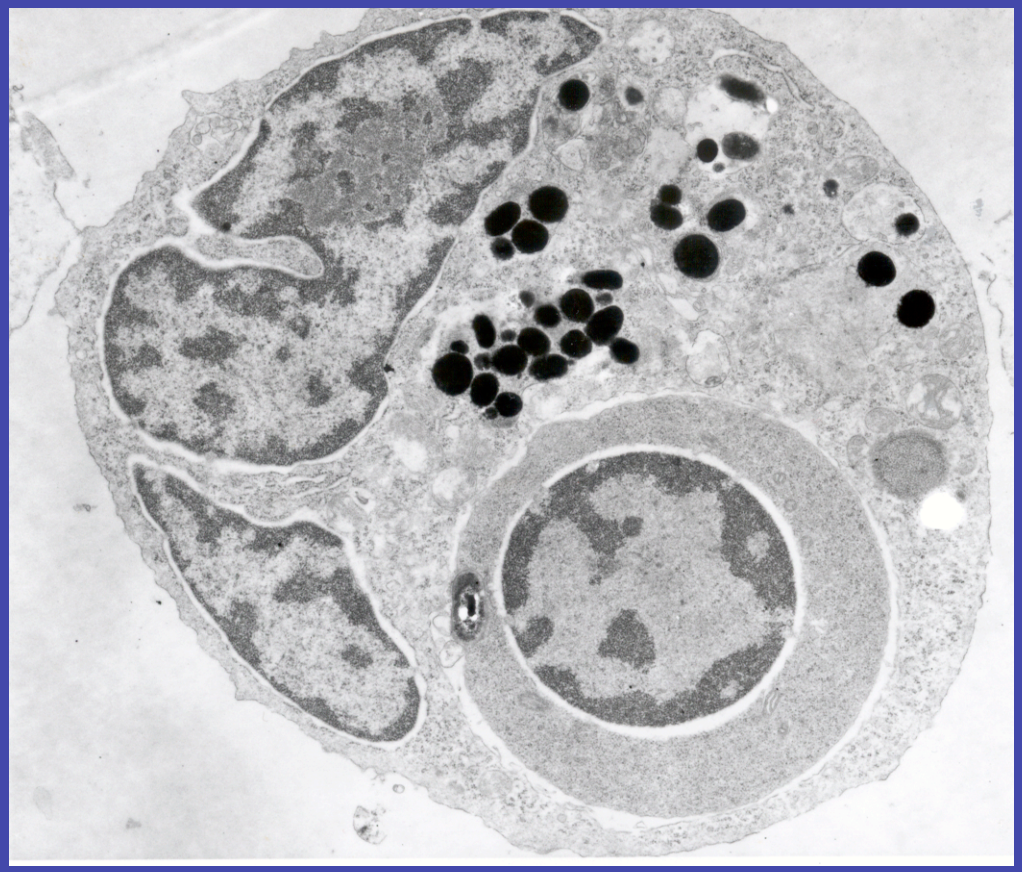


RS in phagolysosomes & cytoplasm of macrophage



RS in lymphocyte

Macrophage engulfs lymphocyte erythrocyte with embedded RS



Attributes that make RS a viable intracellular pathogen

- Extracellular protein (p57)
  - Enhances phagocytosis
  - Helps lyse the phagosome membrane to allow RS into safety of cytoplasm
- Durable cell wall
  - Formalin-killed RS was structurally intact through 240 hr
- Slow replication
  - Too many internalized RS kill the MP
  - Dormancy or slow growth reduces susceptibility to many antibiotics

The MPs aren't defenseless!

- Individual genetic inheritance also dictates intracellular killing by MP (activated versus non-activated)
- Differences between species/stocks.
- Susceptibility to RS may be related to the inherent ability of an individual's MP to respond quickly to bacterial pathogens.

Pathogenicity of RS

- An infection can begin with 1-2 bacteria
- A balance between the MP & internalized RS allows some animals to survive many years until
  - secondary infections, changing environment or reproductive stress
- Ability to survive in the cytoplasm, maintain slow or nonexistent replication that is un-inhibitory to the fish until conditions favor maximum re-infection.

100+experimental antibiotics & RS

Minimum inhibitory concentration of over 100 experimental antibiotics tested against 4 strains of RS

Table 2: Effects of antibiotics on four strains of *R. salmoniarum* (Based on 7 day readings)

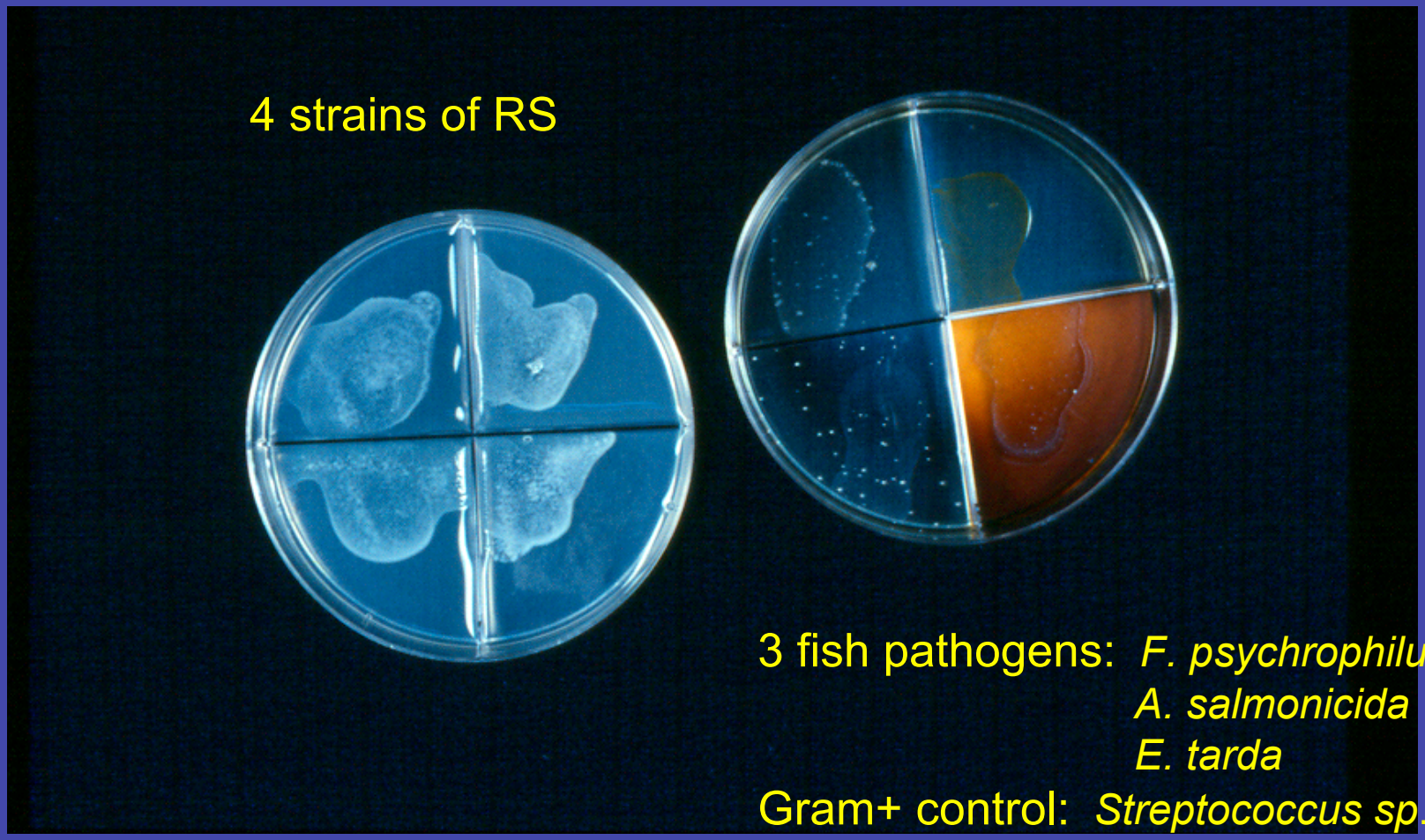
MIC mg/L

Drug #	33209	D-6	33139	932	Mode	Drug #	33209	D-6	33139	932	Mode
1	[35]	35	35	35	35	56	*1	*1	*1	*1	‘1
6	35					57	[1]	5	5	5	5
7	0.5	*0.5	*0.5	*0.5	0.5	58	(0.51	1	1	1	1
9	35				0	59	*35	*35	‘35	*35	+35
10	5	‘10	*10	‘10	10	62	[5]	5	5	5	5
11	35				0	63	[-]			35	
12	0.5	0.5	0.5	0.5	0.5	64	[10]	*10	*10	*10	10
13	5	*10	*10	*10	10	67	*0.5	*0.5	*0.5	*0.5	*0.5
14	<0.01	0.1	0.1	0.1	0.1	70	10	10	10	10	10
15	*35	*35	*35	*35	35	71	*0.5	*0.5	‘0.5	*0.5	*0.5
17	(-)			35		72	0.5	0.5	0.5	0.5	0.5
18	5	10	10	10	10	73	*5	*5	‘5	5	*5
19	10	35	35	35	35	78	1	1	1	1	1
20	35					79	35	35	35	35	35
21	5	35	35	35	35	82	35	35	35	35	35
22	35					87	5	1	5	5	5
23	10	35	35	35	35	88	‘1	*1	1	*1	*1
24		35	35	35	35	89	10	10	10	10	10
25	,335;	35	35	35	35	90	0.1	0.05	0.1	0.05	0.1/0.05
26	[35]					91	0.05	0.1	0.1	0.1	0.1
27	10					92	*1	*5	*5	*5	*5
28	35					93	0.05	0.1	0.1	0.1	0.1
29	35					94	1	1	1	1	1
30	35					95	35				
31	5	10	10	10	10	96	1	*1	*1	‘1	*1
32	35					97	*5	*10	‘10	*10	*10
33	35					98	0.05	0.1	0.1	0.1	0.1
34	[5]	5	5	5	5	99	‘0.05	*0.5	0.5	0.5	0.5
35	5	5	5	5	5	100	0.5	1	1	1	1
36	5	5	5	5	5	107	0.05	0.05	0.05	0.05	0.05
37	35					108	*35				
39	[35]					110	*35	*35	*35	‘35	‘35
41	(5]	5	5	5							
42	*5	*5	*5	‘5	5	Ampicillin	[5]	5	5	5	5
44	*35	*35			-/*35	Rifampicin	0.05	0.05	0.1	0.1	0.1
45	*35	‘35	*35	*35	‘35	Penicillin-G	1	1	1	1	1
46	[35]					Erthromycin	0.1	0.1	0.1	0.1	0.1
47	[10]	*5	*5	*5	5	Oxytetracycline	5	5/ 10	5.0/10	5/ 10	5.0/10

(-) = no effect by drug; * = change over time

[ ]= value taken from 15 or 30 day reading (no growth at 7 day). These values are not included in final evaluation of drug.

Bacteria tested against 100+ experimental antibiotics

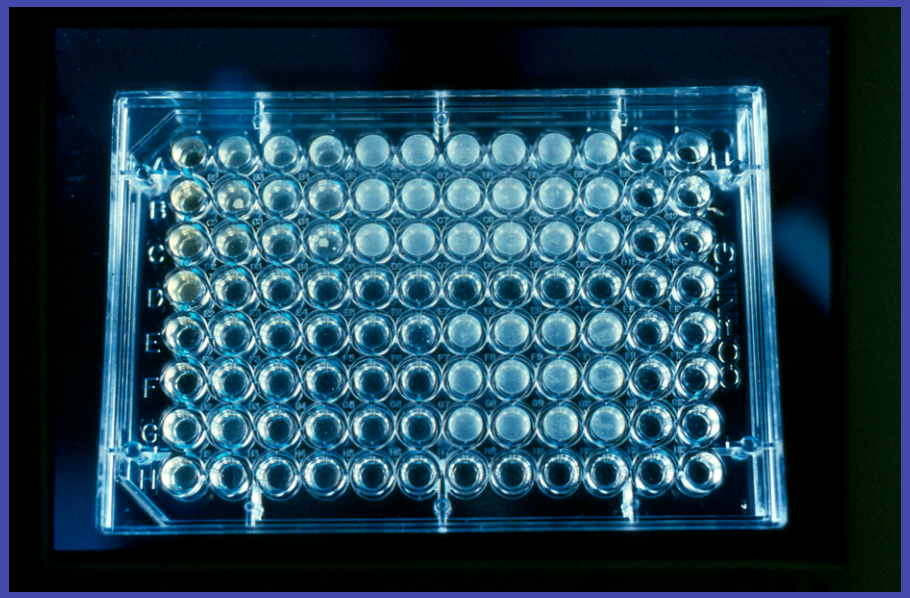


Four strains of RS on antibiotic media

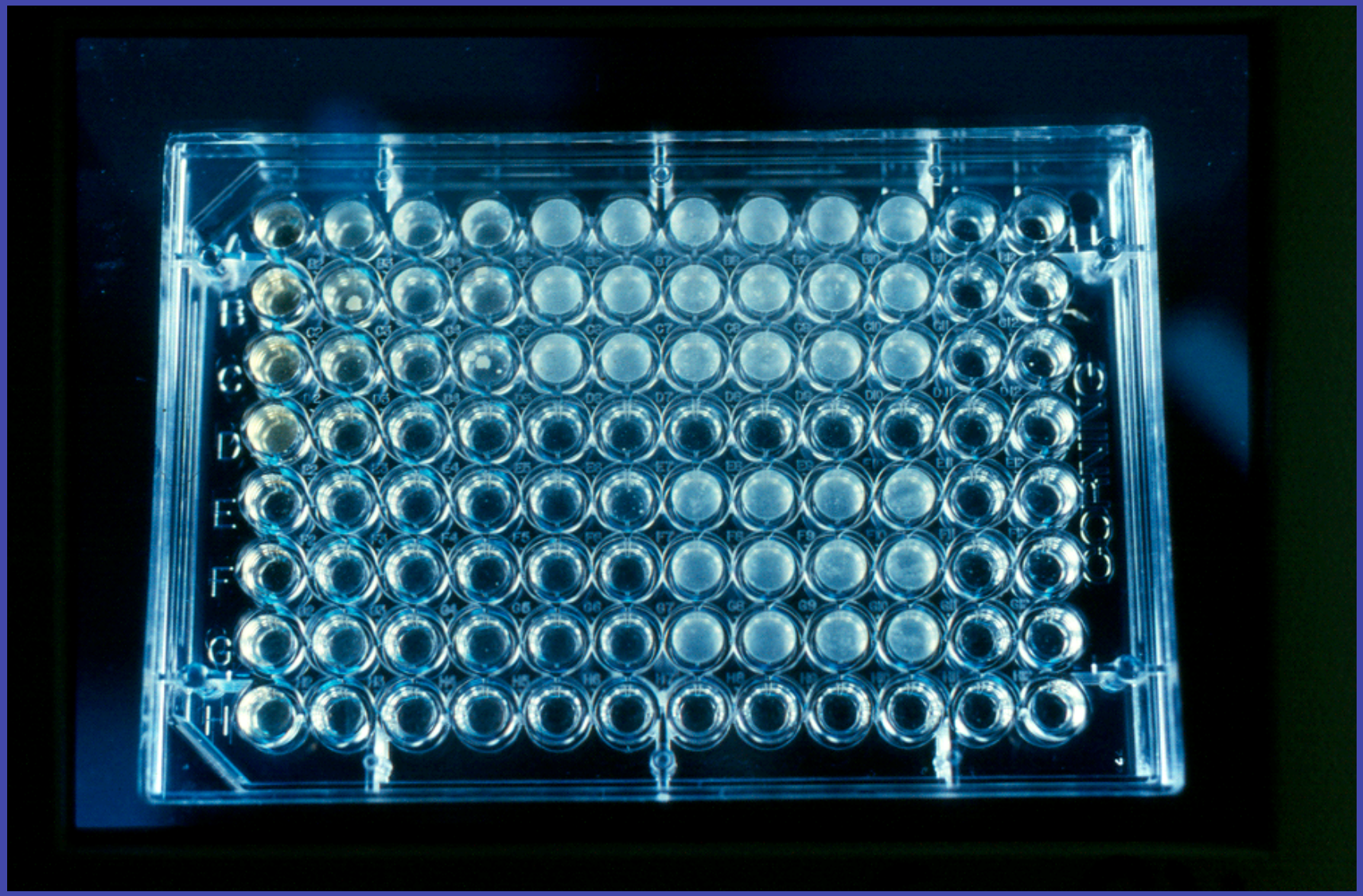
Results of antibiotic study (agar culture)

- **All four strains of RS reacted similarly to all drugs**
- **19 drugs & oxytetracycline = MIC of 5-10 mg/L**
- **7 antibiotics & penicillin G = MIC of 1 mg/L**
- **5 antibiotics, erythromycin, rifampicin = MIC of 0.1 mg/L**
- **# 107 = MIC of 0.05 mg/L**
- **Sensitivity to rifampicin and 20 other drugs decreased over 30 days (i.e., RS initially inhibited but then grew)**

Microtiter plates—an alternative culture method to test antibiotics



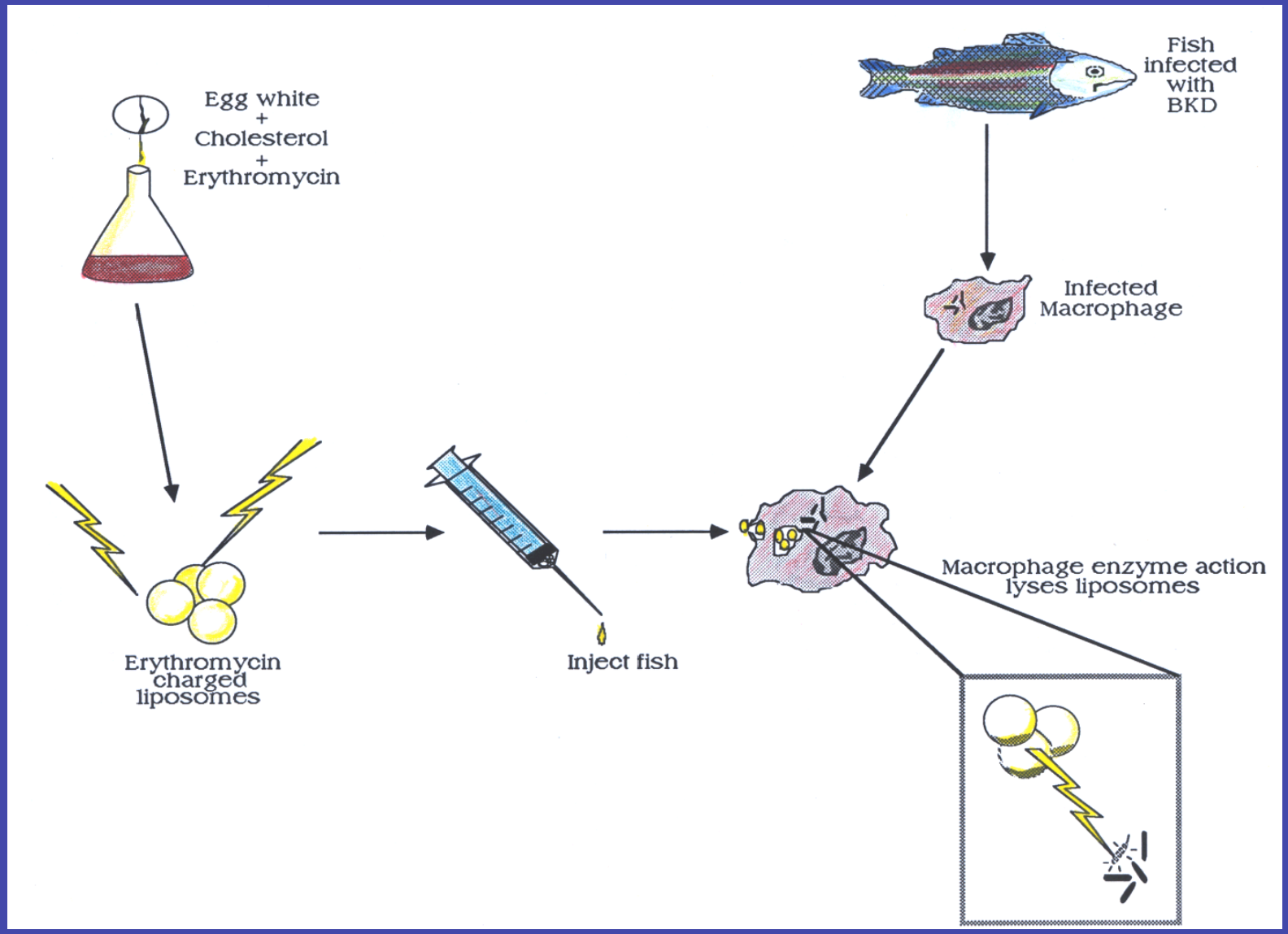
KDM2 broth + 8-9 dilutions of drug
2 to 8 strains of RS tested
Incubated at 16 C on a shaker
Read at 7, 15, 30 days

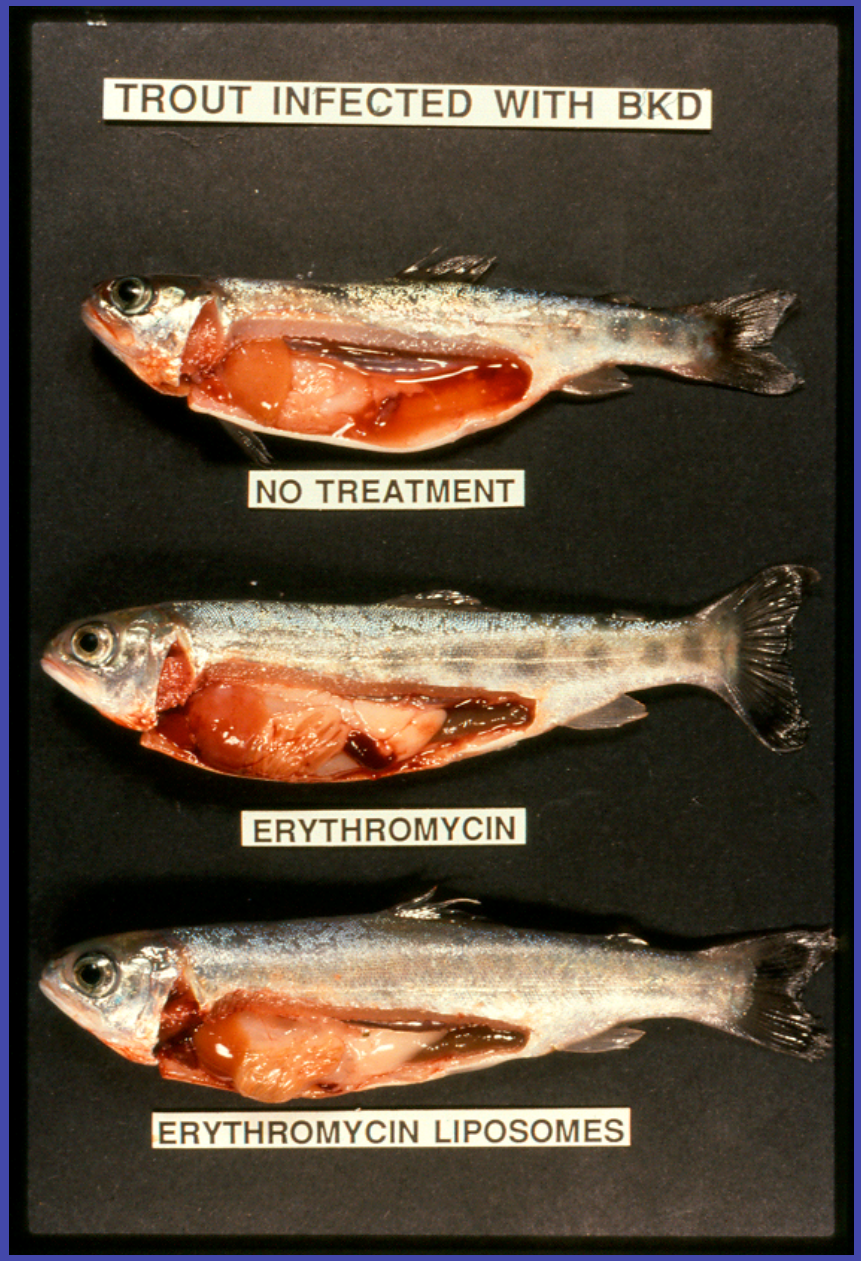

B
C
D
E
F
G
H

Results of antibiotic study (microtiter plate)

- MIC equivalent to agar
- Early reading at 7 days produced more false negatives; 15 & 30 day reading
- Development of resistance was apparent on agar but not seen in microtiter plates
- RS more sensitive to some drugs in microtiter plate (solubilizing agent)

Encapsulation of Erythromycin into Liposomes: Targeting Intracellular RS



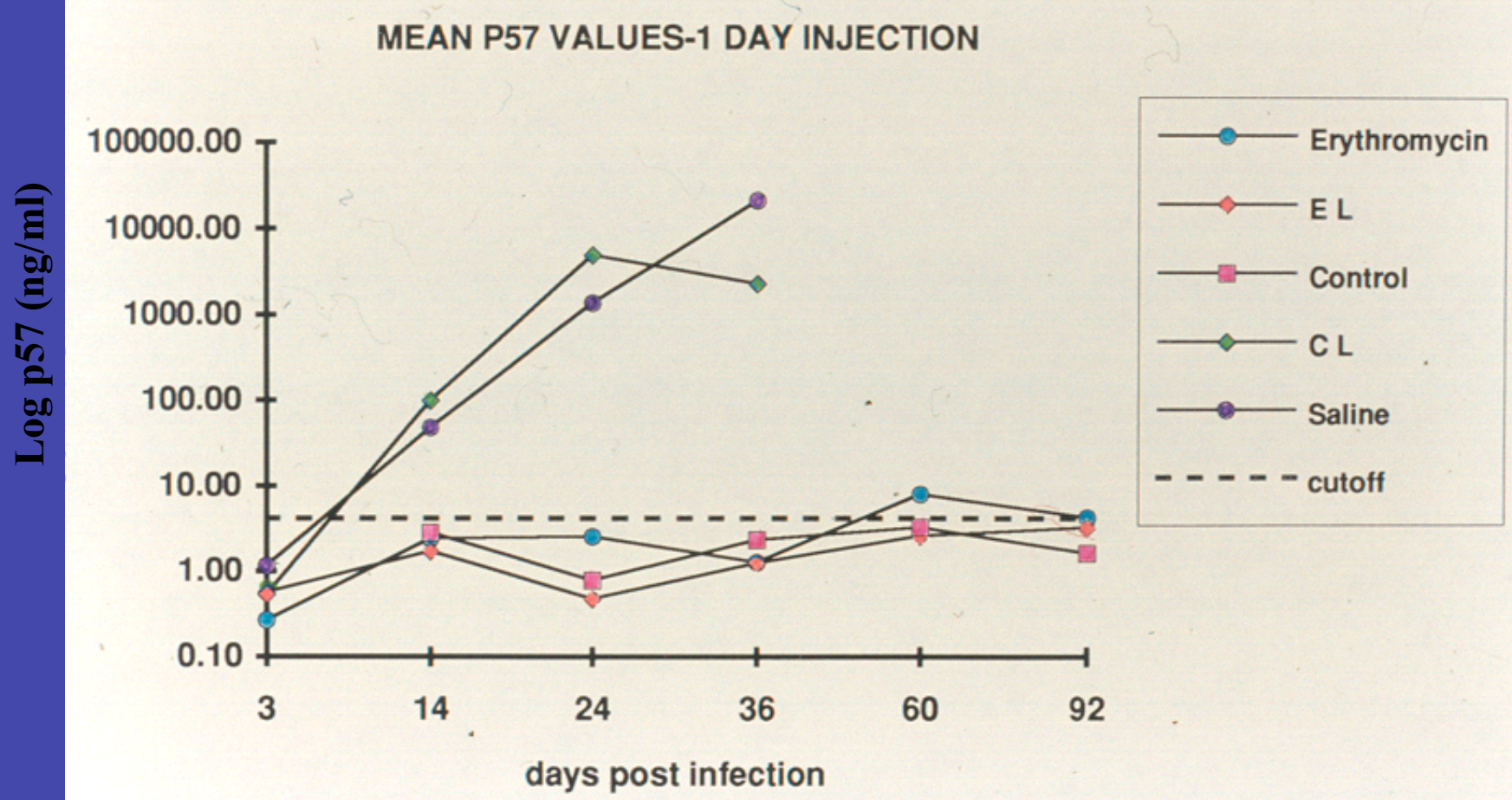


Liposome Study

1. IP infections with D-6 strain of RS
2. Injection of erythromycin liposomes, erythromycin, saline liposomes or saline at 1 or 7 days post infection

Experimental parameters measured:
Pathology
P57 levels (ELISA)
Mortality
Mean days to death

Levels of p57 in liposome study



Liposome Study

- Erythromycin liposomes equivalent to erythromycin
  - **Mortality**

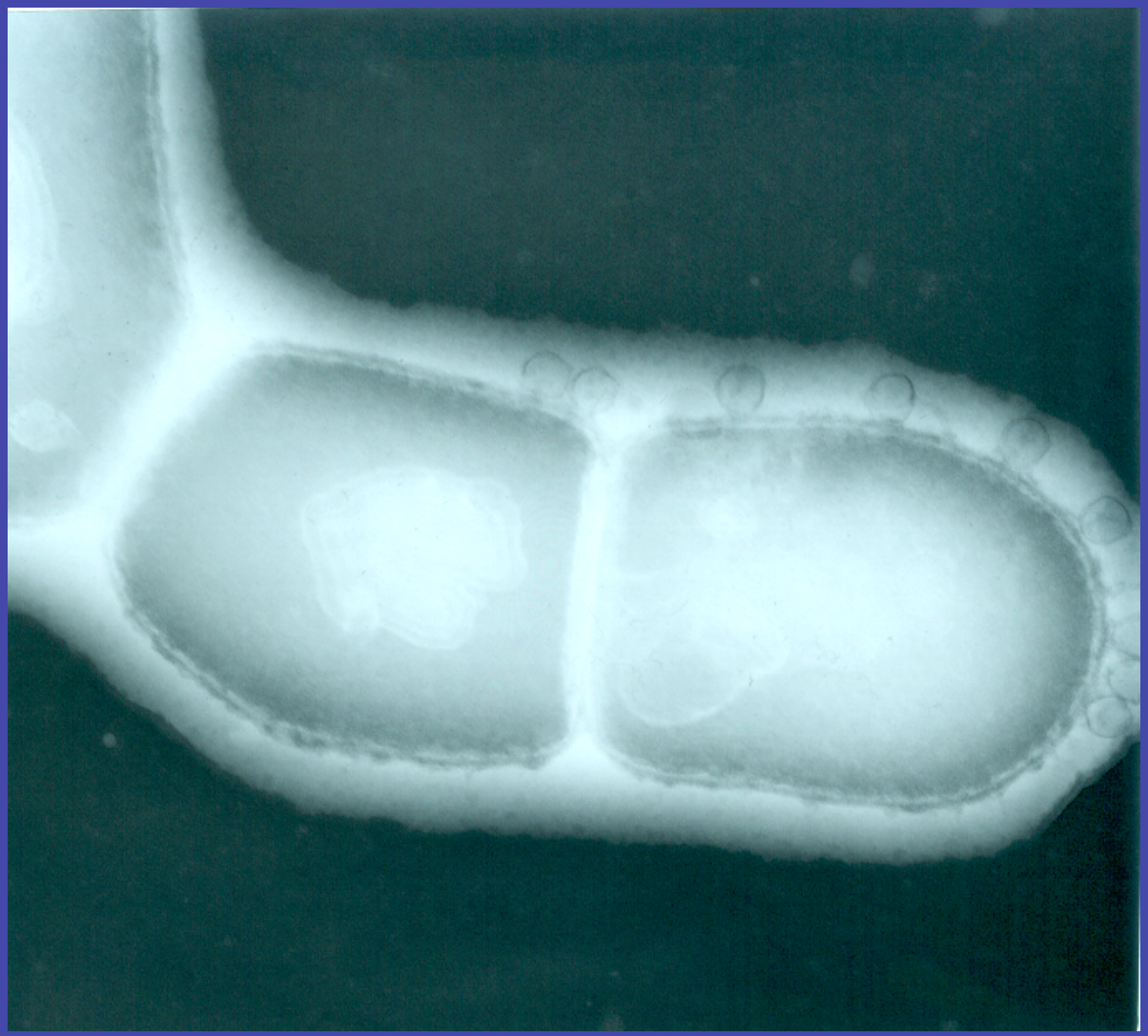
    1 day treatment --13-17%

    7 day treatment – 14-21%

    Control liposome, saline – 79-90%

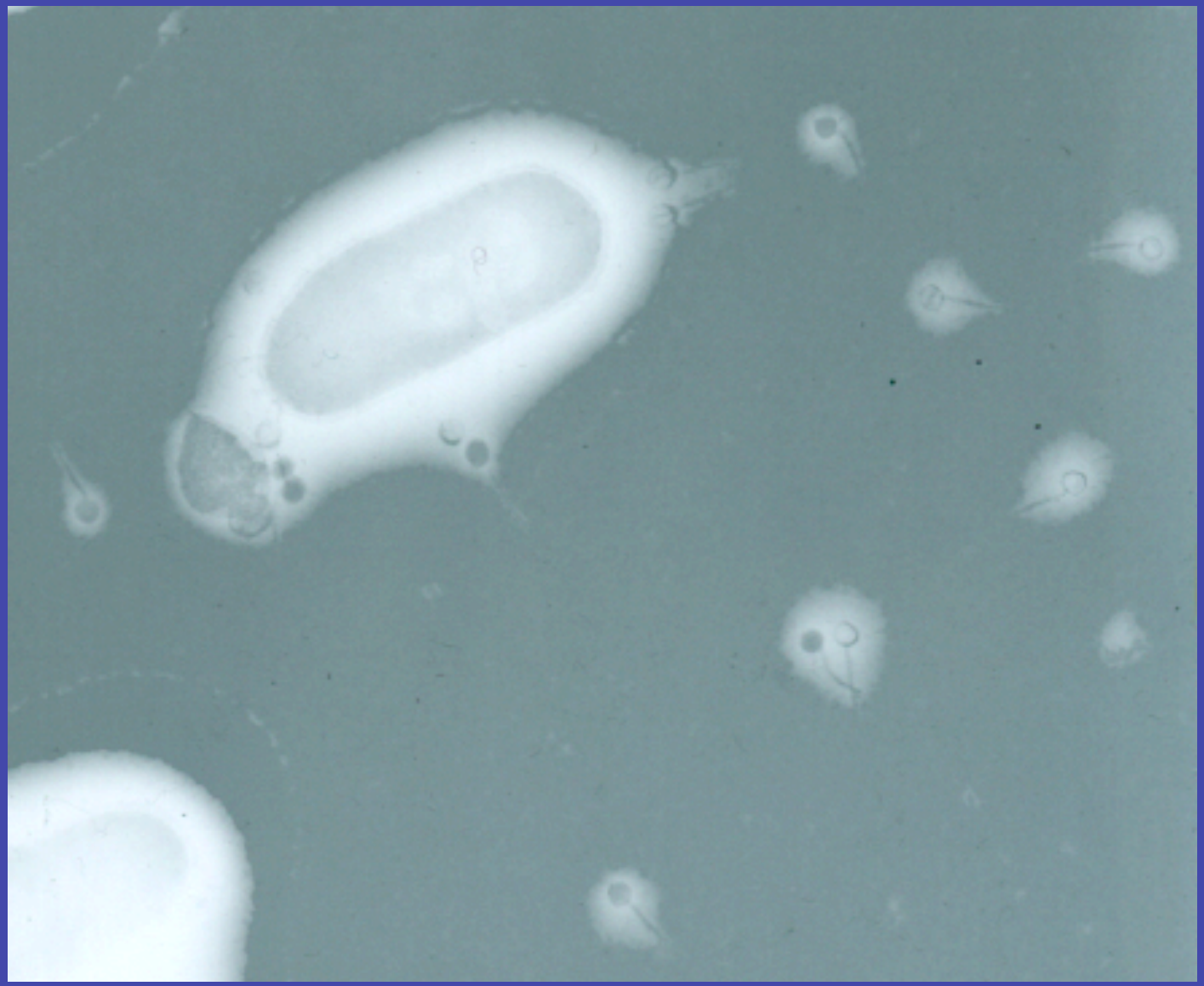
‘Can fatty acid components change the effectiveness of macrophages in controlling the bacterium?

Bacteriophage of RS



From centrifugation synchronization study, 10,000 rpm flask, D-6, pass 5, 9/17m90

Phage embedded in cell wall of RS

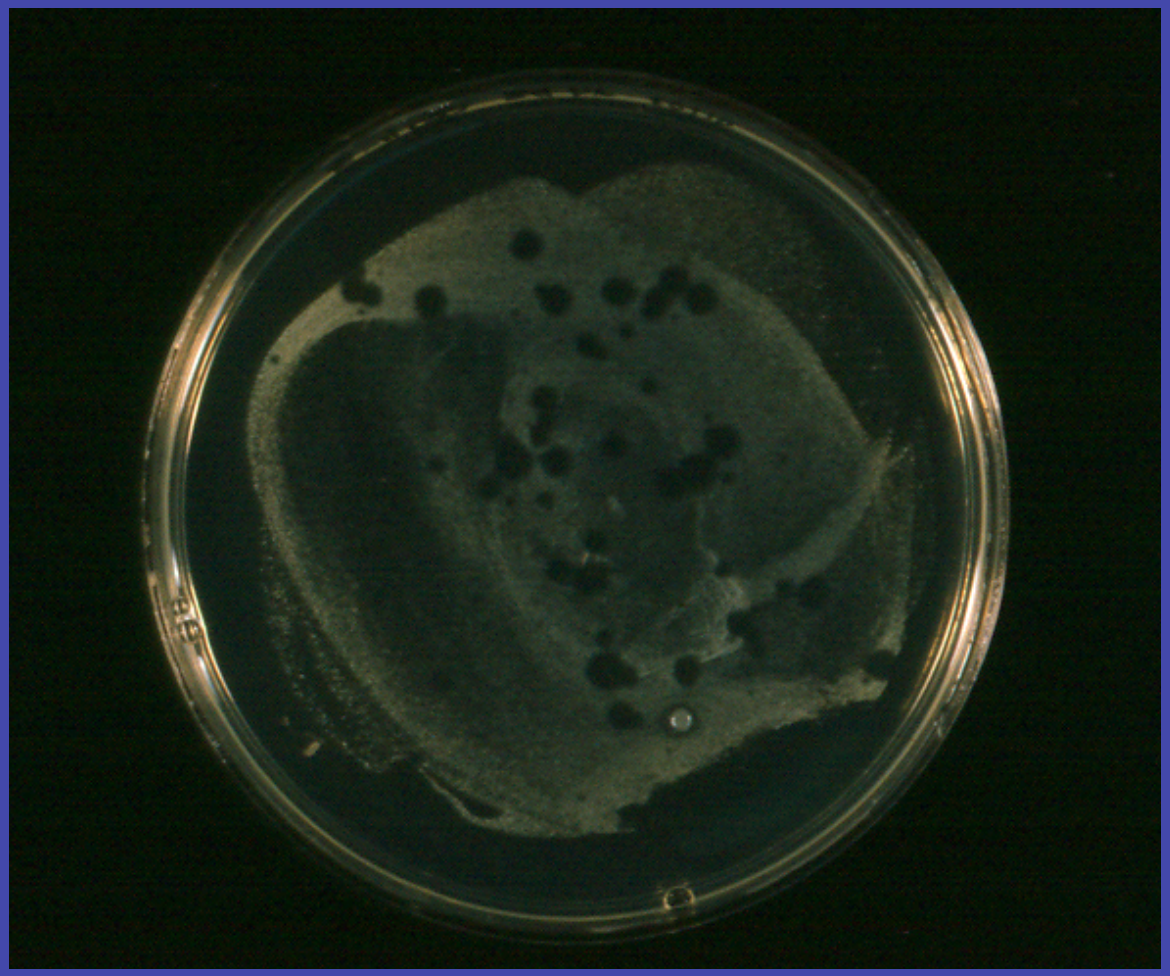


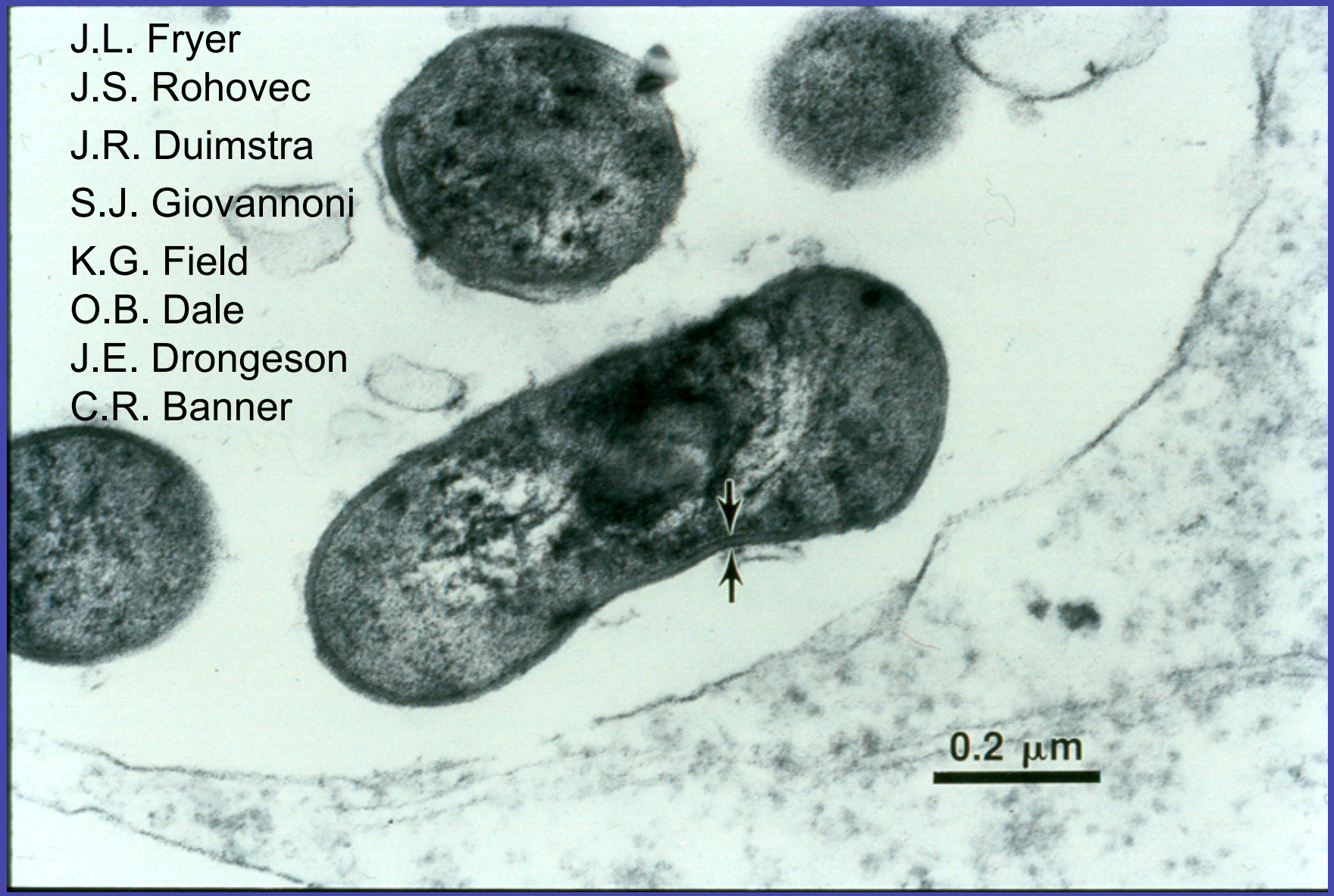
From centrifugation synchronization study, 10,000 rpm flask, D-6, pass 5, 9/17m90

From nalidixic acid synchronization study, D-6, passage 5

From nalidixic acid synchronization study, D-6, passage 5

A natural means to control RS? Bacteriophage discovery



Acknowledgements

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