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In Vitro Persistence of Salmonella Typhimurium in a Dually Inoculated[1] Medium. I. with Proteus Morganii

By

Joseph L. Knuckles

The real potential of non-biting flies as biological vectors of certain enteric pathogens is incomprehensible due to an insufficiency of knowledge of environmental factors which may be related to the availability of these bacteria to flies both qualitatively and quantitatively, and those which may prevent the survival of pathogens within them.

That pH and temperature may influence bacterial growth, and a number of additional factors influence the persistence and/or multiplication of enteric pathogens have been reported. An isolated bacteriophage from *Musca domestica* found active against *salmonella typhosa* (cited as *Bacillus paratyphi* Type I) (8) and an unidentified agent thought to have been a bacteriophage, and found bacteriocidal for *Salmonella typhimurium* but not for *Salmonella schottmuelleri* in the digestive tract of *Phormia regina* (4) have been reported. The acidity in the digestive tract of *Blaberus craniifer* inhibited or destroyed *S. typhosa* but not *Salmonella enteridits,* (5) nor were several species of *Salmonella* destroyed by an acid pH in the developmental stages of *P. regina* (4). The survival of enteric pathogens in axenically and nonaxenically reared larvae and pupae (1) and in adult *Musca* (2) has been demonstrated experimentally. Yet, no correlation in fly activity with the prevalence of salmonellosis was observed (6,9) in field studies. This suggests the destruction of *Salmonella* by a bacteriophage or antagonistic action by bacteria normally present in the fly's gut or elsewhere in nature. Threshold numbers of several enteric pathogens required for their subsequent establishment and multiplication within *Musca* and *Phormia* are cited in (2) and (4), respectively,

This paper describes *in vitro* studies on the persistence of *S. typhimurium* in the presence of *Proteus morganii*

Methods and Materials

Modified S-F broth (MSFB) in a series of flasks (8-69) was inoculated with varied ratios of *S. typhimurium* and *P. morganii* / cc medium. Controls were established by inoculating S-F broth in flasks (1-7) with the pathogen only. The inoculated number of pathogen or nonpathogen employed / cc medium and the length of incubation prior to examination are shown in Table 1. Selenite-F broth and MSFB were prepared essentially as (7) except that acid selenite was omitted from the latter.

[1]Supported by a grant from the North Carolina Academy of Science.

Standardized bacterial suspensions were prepared by washing the growth from separate 18-24 hour-old agar slant cultures with distilled water, and then diluting it to photometrically match a Nephlometer standard no. 0.5 at a wave length of 600 mmr. Each of these suspensions was equivalent to 150 x 10^6 bacteria / cc, but was diluted further for inoculating purposes.

The number of bacteria in an inoculum was calculated by multiplying the fractional cc used x the concn of bacteria / cc suspension.

The number of *S. typhimurium* detected in a flask culture post-incubation was based on the highest serial dilution found positive for the species. One tenth cubic centimeter of a flask culture was transferred to the first of 10 dilution tubes, each of which contained 1.9 cc of distilled water, and serially diluted out. Then 1 cc from each dilution tube was transferred to a separate tube which contained 4 cc of MSFB, and incubated at 37 C for 24 hours. An inoculm of 0.05 cc was transferred from each tube culture and streaked on separate Brilliant Green agar and SS agar plates.

Salmonella typhimurium no. 63 and *P. morganii* no. 121 used in this work were initially obtained from the Department of Bacteriology, University of Connecticut. The pathogen was isolated from human excrement and had been attenuated in the laboratory for about seven years prior to this work. Specific antiserum was obtained from Case Laboratories, Chicago. The identification of S. *typhimurium* in randomly selected colonies from SS agar or Brilliant Green agar plates was based on (a) positive triple-sugar iron agar tests (an alkaline slant, an acid butt and the production of H_2S), (b) positive phenol red tartrate agar tests (an acid reaction with a distinct color change in the lower 80-95% portion of the medium), (c) colony appearance and (d) positive macroscopic slide agglutination tests. No special effort was made to identify *P. morganii*, but periodic checks were run on the tube cultures from serial dilutions with Urea broth for the hydrolysis of urea.

The tubed and plated media used were sterilized as prescribed on their original containers except that S-F broth and MSFB were sterilized in flowing steam for 20-30 minutes. Empty flasks, pipettes, Petri dishes, etc. were sterilized according to standard bacteriological methodology.

Results

The results of this study are presented in Table 1. *Salmonella typhimurium* based on the differences in the number inoculated and the numbers detected in MSFB post-incubation multiplied extensively in the presence of *P. morganii* during the first day of incubation. A high-level count of viable *S. typhimurium* persisted throughout the 19 day experimental period. The growth and persistence of this species was evident irrespective of the pathogennonpathogen inoculum ratios of 1:1, 1:4, 1:7, 1:90, and 1:300 / cc medium. No difference was reflected by the 10 serial dilutions employed in the growth cited

and persistence of *S. typhimurium* in MSFB irrespective of the pathogen-nonpathogen ratio employed. Spot checks with urea broth on MSFB cultures indicated the presence of *P. morganii* throughout the experiment in each of the 10 serial dilutions.

Discussion

A study of the multiplication of *S. typhimurium* in the presence of *P. morganii* was not of prime interest in this work. However, a difference of approximately 51.2 x 10^9 in the number of the former inoculated and the number detected / cc MSFB irrespective of the inoculum pathogen-nonpathogen ratio used and following a 24 hour incubation does indicate that a decided multiplication of *S. typhimurium* occured during this period. The multiplication indicated represents a minimum for the species since the number of viable cells in the highest serial dilution used was sufficient to give higher counts had the dilution range been extended. A generation time of 54.7 minutes for *S. typhimurium* as computed by $g = t$ is thought to be high. Its number of generations (n=35) during the first day of incubation as determined by $n = log\ p^1\ log^2\ p^3$ is considered to be low. Certain closely related bacterial species (e.g., *Escherichia coli*, g = 20 minutes) are known to show a greater number of generations and shorter generation periods than those obtained in this work for S. *typhimurium*. *In vitro* studies on the microbial competition relative to enteric pathogens and nonpathogens bacteria are generally lacking, but *S. typhimurium* did not multiply or survive in *Phormia* pupae and adults though their prepupae and adults had fed on this pathogen and *P. morganii* (4). The multiplication of *S. typhimurium* in this study and its inability to multiply or survive in *P. regina* fed *P. morganii* suggest that *P. morganii* does not significantly impair the multiplication of *S. typhimurium* in the external environment but when coupled with unknown ecological factors in the gut of certain flies may cause the extinction of *typhimurium*.

Proteus morganii demonstrated no significant inhibitory action on the survival of *S. typhimurium* as was evident by the persistence of a high-level count of at least 51.2 x 10^9 *typhimurium* / cc medium throughout a 19 day period. An extension of the serial dilution range in two cases indicated the presence of a greater population of the pathogen / cc medium than cited above. This persistence indicates that *P. morganii* would unlikely cause the extinction of *typhimurium* in nature even though the former tends toward the production of an acid pH in a confined medium. Results of work in progress show that *typhimurium* can tolerate a pH of less than 4.8. The survival of *Typhimurium* in this study, its persistence in pupae and multiplication in adult *Phormia* whose aseptically reared larvae and adults had fed on *typhimurium* only, and its complete absence in blowfly pupae and adults whose larvae and adults had fed on *typhimurium* and *P. morganii* and/or *Aerobacter* sp. (4) reemphasize the existence of some ecological factor(s) which act in conjunction with *P. morganii in vivo,* resulting in the destruction of *typhimurium* in the pupae and adults of *P. regina.*

TABLE 1

In Vitro Persistence of Salmonella Typhimurium in the Presence of Proteus Morganii

Flask number	Number of pathogen inoculated / cc medium	Number of nonpathogen inoculated / cc medium	Incubation period (days)	Number of viable *S. typhimurium* detected in medium / cc by serial dilution
1	750	0	1	512×10^8
2	750	0	3	512×10^8
3	750	0	5	512×10^8
4	750	0	8	512×10^8
5	750	0	10	512×10^8
6	750	0	13	512×10^8
7	750	0	19	512×10^8
8	750	7.50×10^2	1	512×10^8
9	750	7.50×10^2	3	512×10^8
10	750	7.50×10^2	5	512×10^8
11	750	7.50×10^2	8	512×10^8
12	750	7.50×10^2	10	512×10^8
13	750	7.50×10^2	13	512×10^8
14	750	7.50×10^2	19	512×10^8

15	750	3.00×10^3	1	512×10^8
16	750	3.00×10^3	3	512×10^8
17	750	3.00×10^3	5	512×10^8
18	750	3.00×10^3	8	512×10^8
19	750	3.00×10^3	10	512×10^8
20	750	3.00×10^3	13	512×10^8
21	750	3.00×10^3	19	512×10^8
22	750	5.25×10^2	1	512×10^8
23	750	5.25×10^2	1	512×10^8
24	750	5.25×10^2	3	512×10^8
25	750	5.25×10^2	3	512×10^8
26	750	5.25×10^2	5	512×10^8
27	750	5.25×10^2	5	512×10^8
28	750	5.25×10^2	8	512×10^8
29	750	5.25×10^2	10	512×10^8
30	750	5.25×10^2	10	512×10^8
31	750	5.25×10^2	13	512×10^8
32	750	5.25×10^2	13	512×10^8
33	750	5.25×10^2	16	512×10^8
34	750	5.25×10^2	17	512×10^8
35	750	5.25×10^2	19	512×10^8
36	750	5.25×10^2	19	512×10^8
37	750	6.75×10^3	1	512×10^8

38	750	6.75×10^3	1	512×10^8
39	750	6.75×10^3	3	512×10^8
40	750	6.75×10^3	3	512×10^8
41	750	6.75×10^3	5	512×10^8
42	750	6.75×10^3	5	512×10^8
43	750	6.75×10^3	5	512×10^8
44	750	6.75×10^3	8	512×10^8
45	750	6.75×10^3	8	512×10^8
46	750	6.75×10^3	10	512×10^8
47	750	6.75×10^3	10	512×10^8
48	750	6.75×10^3	13	512×10^8
49	750	6.75×10^3	13	512×10^8
50	750	6.75×10^3	16	512×10^8
51	750	6.75×10^3	17	512×10^8
52	750	6.75×10^3	18	512×10^8
53	750	6.75×10^3	19	512×10^8
54	750	2.25×10^5	1	512×10^8
55	750	2.25×10^5	1	512×10^8
56	750	2.25×10^5	3	512×10^8
57	750	2.25×10^5	3	512×10^8
58	750	2.25×10^5	5	512×10^8
59	750	2.25×10^5	5	512×10^8
60	750	2.25×10^5	8	512×10^8

61	750	2.25×10^5	8	512×10^8
62	750	2.25×10^5	10	512×10^8
63	750	2.25×10^5	10	512×10^8
64	750	2.25×10^5	13	512×10^8
65	750	2.25×10^5	13	512×10^8
66	750	2.25×10^5	16	512×10^8
67	750	2.25×10^5	16	512×10^8
68	750	2.25×10^5	19	512×10^8
69	750	2.25×10^5	19	512×10^8

Conclusions

1. *Salmonella typhimurium* persisted at a high level in MSFB for 19 days when this medium was inoculated with this species and *P. morganii* in ratios of 1:1-1:300 / cc.

2. The presence of *P. morganii* in a mixed culture with *S. typhimurium* had no significant inhibitory action on the persistence of the latter.

3 *Salmonella typhimurium* underwent a 35-plus fold multiplication during the first experimental day in MSFB inoculated with this species and *P. morganii* in ratios of 1:1-1:300 / cc. Neither of the bacterial species appeared to have had a significant inhibitory effect on the growth of the other. The persistence and/or multiplication of *S. typhimurium* in the presence of *P. morganii* appear to be limited primarily by the availability of nutrients for the total bacterial population or by products formed during their metabolism.

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In Vitro Persistence of Salmonella Typhimurium in a Dually Inoculated Medium. II. with Aerobacter Cloacae

By

Joseph L. Knuckles

In a previous paper, results of *in vitro* studies on the persistence of *Salmonella typhimurium* in the presence of varied numbers of *Proteus morganii* were described (4). The present paper deals with an extension of these studies to *Aerobacter cloacae.*

Materials and Methods

Modified S-F broth (MSFB) in a series of flasks was innoculated with varied ratios of *S. typhimurium* and *A. cloacae* / cc medium. Most of the materials and methods used throughout this study are presented in a previous paper (4) and will not be repeated in detail here. In addition, EMB agar streak plates were employed in the isolation and inumeration of *Aerobacter. A. cloacae* was obtained from the Carolina Biological Supply Company, Burlington, North Carolina. Its identity was based on: (a) positive triple-sugar iron agar tests (an acid slant, an acid butt, and the production of gas exclusive of H_2S); (b) positive phenol red tartrate agar tests (no color change in medium); (c) negative urea broth tests (lack of the hydrolysis of urea as indicated by no color change in the broth); (d) negative macroscopic slide aggultination tests with a polyvalent *Salmonella* antiserum. Again a serial dilution range of from 1:20 to 1:1024 x 10^{10} was used in the inumeration of bacteria found in MSFB post-incubation.

Results

The results of this study are presented in Table 1. *Salmonella typhimurium* based on differences in the numbers inoculated and the numbers detected in MSFB post-incubation multiplied extensively in the presence of *A. cloacae* during the first day of incubation. *A. cloacae* also underwent multiplication during this period. A high-level count of viable *typhimurium* and *cloacae* persisted throughout the 38 day study. The growth and persistence of *typhimurium* was evident irrespective of the pathogennonpathogen inoculum ratios of 1:8, 1:50, 1:200, 1:800, 1:1.8 x 10^6, and 1:7.2 x 10^6 / cc medium. No difference was reflected by the 10 serial dilutions used in the growth and persistence of *typhimurium* in MSFB irrespective of the pathogen-nonpathogen ratio employed.

Discussion

Aerobacter cloacae, as was true of *Proteus morganii* (4), demonstrated no significant inhibitory action on the survival of *S. typhimurium* as was evident by the persistence of a high-level count of at least 51.2 x 10^9 *typhimurium* / cc medium throughout a 38 day period. The same applies with respect to the influence of *typhimurium* on the survival of *A. cloacae.* These findings do not agree with the observation by Bowling and Wynne (1) that several strains of *Aerobacter* produced complete inhibition of *Salmonella paratyphi* on staled agar. The probable inability of *S. paratyphi* to feel on the dehydrated agar, the presence of a bacteriophage or an x-factor would have been a more feasible explanation for the inhibition observed by them rather than the presence of *Aerobacter.*

Identical findings in the number of *typhimurium* and *cloacae* in MSFB inoculated with these species in varied ratios (Table 1) suggest minimum populations and that more viable cells were present in each flask than detected by the highest serial dilution used. The results of this study indicate that *cloacae* alone would not likely prevent the survival of *typhimurium* in the insect's external environment. Jung and Shaffer (2) showed that *typhimurium* and *Salmonella montevido* could survive in human feces for at least 14 days and that the initial concentration of these organisms appeared to have no influence on the duration of their survival. *Aerobacter* sp. are normally associated with plants and may well have been a part of the microflora of the feces observed by Jung and Shaffer.

An approximate difference of 52.2 x 10^9 in the number of *S. typhimurium* inoculated and the number detected / cc MSFB which contained inoculum pathogen ratios up to 1:7.2 x 10^7, and following a 24-hour incubation period indicates that extensive multiplication of the pathogen occurred during this period. This was not unexpected since *typhimurium* underwent a decided multiplication in the presence of *Proteus morganii* (4). The survival and multiplication of *typhimurium* in this study, its inability to multiply or survive in polycontaminated *P. regina* and its ability to persist and multiply in monocontaminated blowflies (3) point to the importance of one or more *in vivo* ecological factors in the regulation of a fly's microflora.

TABLE 1

In Vitro Persistence of Salmonella Typhimurium In the Presence of Aerobacter Cloacae

Flask number	Number of pathogen inoculated / cc medium	Number of nonpathogen inoculated / cc medium	Incubation period (days)	Number of viable bacteria detected / cc medium by serial dilution	
				S. typhimurium / *A. cloacae*	
1	750	0	1	512×10^8	0
2	750	0	3	512×10^8	0
3	750	0	6	512×10^8	0
4	750	0	8	512×10^8	0
5	750	0	10	512×10^8	0
6	750	0	13	512×10^8	0
7	750	0	15	512×10^8	0
8	750	0	17	512×10^8	0
9	750	0	38	512×10^8	0
10	750	6.0×10^3	1	512×10^8	512×10^8
11	750	6.0×10^3	3	512×10^8	512×10^8

12	750	6.0×10^3	6	512×10^8	512×10^8
13	750	6.0×10^3	8	512×10^8	512×10^8
14	750	6.0×10^3	10	512×10^8	512×10^8
15	750	6.0×10^3	13	512×10^8	512×10^8
16	750	6.0×10^3	15	512×10^8	512×10^8
17	750	6.0×10^3	17	512×10^8	512×10^8
18	750	6.0×10^3	38	512×10^8	512×10^8
19	750	37.5×10^3	1	512×10^8	512×10^8
20	750	37.5×10^3	3	512×10^8	512×10^8
21	750	37.5×10^3	6	512×10^8	512×10^8
22	750	37.5×10^3	8	512×10^8	512×10^8
23	750	37.5×10^3	10	512×10^8	512×10^8
24	750	37.5×10^3	13	512×10^8	512×10^8
25	750	37.5×10^3	15	512×10^8	512×10^8
26	750	37.5×10^3	17	512×10^8	512×10^8
27	750	37.5×10^3	38	512×10^8	512×10^8
28	750	1.5×10^5	1	512×10^8	512×10^8
29	750	1.5×10^5	3	512×10^8	512×10^8
30	750	1.5×10^5	6	512×10^8	512×10^8
31	750	1.5×10^5	8	512×10^8	512×10^8
32	750	1.5×10^5	10	512×10^8	512×10^8
33	750	1.5×10^5	13	512×10^8	512×10^8
34	750	1.5×10^5	15	512×10^8	512×10^8

35	750	1.5×10^5	17	512×10^8	512×10^8
36	750	1.5×10^5	38	512×10^8	512×10^8
37	750	6.0×10^5	1	512×10^8	512×10^8
38	750	6.0×10^5	3	512×10^8	512×10^8
39	750	6.0×10^5	6	512×10^8	512×10^8
40	750	6.0×10^5	8	512×10^8	512×10^8
41	750	6.0×10^5	10	512×10^8	512×10^8
42	750	6.0×10^5	13	512×10^8	512×10^8
43	750	6.0×10^5	15	512×10^8	512×10^8
44	750	6.0×10^5	17	512×10^8	512×10^8
45	750	6.0×10^5	38	512×10^8	512×10^8
46	25	4.5×10^7	1	512×10^8	512×10^8
47	25	4.5×10^7	3	512×10^8	512×10^8
48	25	4.5×10^7	6	512×10^8	512×10^8
49	25	4.5×10^7	8	512×10^8	512×10^8
50	25	4.5×10^7	10	512×10^8	512×10^8
51	16	11.5×10^7	1	512×10^8	512×10^8
52	16	11.5×10^7	3	512×10^8	512×10^8
53	16	11.5×10^7	8	512×10^8	512×10^8
54	16	11.5×10^7	10	512×10^8	512×10^8

55	16	11.5×10^7	13	512×10^8	512×10^8
56	16	11.5×10^7	13	512×10^8	512×10^8
57	16	11.5×10^7	15	512×10^8	512×10^8
58	16	11.5×10^7	17	512×10^8	512×10^8
59	16	11.5×10^7	38	512×10^8	512×10^8

Conclusions

1. *Salmonella typhimurium* persisted at a high level in MSFB for 38 days when this medium was inoculated with this species and *A. cloacae* in ratios of 1:8 - 1:72 x 10^5 / cc.

2. *Salmonella typhimurium* underwent a 36-plus fold multiplication during the first experimental day in MSFB inoculated with this species and *A. cloacae* in ratios of 1:8 - 1:72 x 10^5 / cc. Neither of the species appeared to have had a significant inhibitory effect on the growth of the other. The persistence and/or multiplication of *S. typhimurium* in mixed cultures with *cloacae* appear to be limited primarily by the availability of nutrients for the total bacterial population or by products formed during their metabolism.

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Experimental Transmission of Enteric Pathogens from Fly to Fly by Aseptically Reared Phormia Regina (Meigen)

By

Joseph L. Knuckles

Eighty-two adult, aseptically reared *Phormia regina* were forced indirectly to feed on feces deposited by *Salmonella schottmuelleri* and/ or *Salmonella typhimurium* fed mounted flies. Several test flies were examined internally and control flies examined externally at intervals during a 21 day period. Bacteriological and serological tests indicated that: (a) all flies which had ingested feces deposited by flies previously fed *S. schottmuelleri* or *typhimurium* were positive for the test microorganism; (b) all flies which had fed on excreta deposited by flies which had not been fed bacteria but which had previously ingested feces from mounted flies fed both bacterial species were positive for both test microbes; (c) male and female black blowflies did not differ in their abilities to house *S. schottmuelleri* and *typhimurium,* and to pass them in feces; (d) all control flies were negative for the microbes employed. An apparatus for rearing bacteriafree fly larvae and pupae, and one for studying the passage of microorganisms from fly to fly via feces are reported.

*An abstract of a portion of dissertation studies in the Departments of Bacteriology, Zoology and Entomology, University of Connecticut.