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Growth in Mouse Peritoneal Macrophages of *Yersinia pestis* Lacking Established Virulence Determinants

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Cultured mouse resident peritoneal macrophages were challenged with strains of *Yersinia pestis* differing only with respect to the absence of one or more of the virulence determinants established for the species. Guanine-auxotrophic (Pur^-) yersiniae were unable to survive within the macrophages; exogenous hypoxanthine and guanosine permitted intracellular growth. This finding supports the idea that Pur^- yersiniae are avirulent due to inability to obtain sufficient free purines in host tissues for growth and maintenance and indicates that net biosynthesis is necessary to counteract the intracellular microbicidal environment of macrophages. Yersiniae unable to pigment in medium containing the dye Congo red (Pgm^-) or lacking either of the plasmids associated with the pesticin or calcium dependence virulence determinants (Pst^- and Vwa^-, respectively) were taken up as efficiently into macrophages and grew as well within these cells as did bacteria having all invasive virulence determinants intact. Opsonization with 100% homologous normal serum before infection of macrophages did not affect the ability of Pgm^- or Vwa^- Pgm^- yersiniae to grow within macrophages. Accordingly, attributes independent of these virulence determinants mediate the survival and growth of yersiniae in serum and within resident macrophages, and components of the mammalian environment other than serum and macrophages must interact with Pgm^-, Pst^-, and Vwa^- yersiniae to cause their avirulence in vivo.

Five virulence determinants have been established for the facultative intracellular parasite *Yersinia pestis* (5, 8, 10, 14, 25). (i) The ability to elaborate capsular material is associated with resistance to phagocytosis (9, 26). (ii) Roles in intracellular events have been proposed (27) for the plasmid-mediated (16) abilities to show a calcium or nucleotide requirement for growth at 37°C and to synthesize two proteins called the plague virulence antigens V and W (Vwa^+). In vivo, this virulence attribute is correlated with the ability to proliferate, causing septicemia (39). (iii) Expression of the plasmid-encoded (16, 28) bacteriocin pesticin and coagulase and fibrinolysin activities (Pst^+) is thought to contribute to the high invasiveness of *Y. pestis*, presumably due to the coagulase and fibrinolysin activities (7). In vivo, this property is associated with the retention of yersiniae in organs (39). (iv) The ability to form pigmented colonies due to adsorption from the medium of basic, planar compounds such as hemin and the dye Congo red (Pgm^+) is thought to play a role in iron acquisition for yersiniae in vivo (25). As with the expression of fibrinolysin and coagulase, the pigmentation virulence determinant is thought to play a role in extracellular survival. (v) The ability to synthesize purines de novo (Pur^+) may be necessary for full virulence of *Y. pestis*, because host tissues do not contain enough free purines to support maintenance and growth sufficient to overcome antibacterial elements of the mammalian environment (8, 9).

With the exception of expression of the capsular antigen, no systematic study has been made to assess whether these virulence attributes play a role in the interaction of *Y. pestis* with its target host cell, the macrophage. For the Pgm and Pst properties, no tests have been reported in the literature. For Pur, one study examined the effect of purine auxotrophy on the development of resistance to phagocytosis (9). Conflicting data are available concerning the role of the Vwa determinant in growth within phagocytes. Cavanaugh and Randall (12) found that fully virulent *Y. pestis* grew within cultured guinea pig elicited peritoneal macrophages but that the avirulent strain A1122, which now is known to be Pgm^- and Vwa^- (lacking the "calcium dependence plasmid" pCD associated with this latter property), was killed by cultured mouse-elicited peritoneal macrophages. In contrast, *Yersinia pseudotuberculosis*, which possesses a Vwa virulence determinant similar to that of *Y. pestis* (6, 11), grew equally well within dispersed rabbit spleen cells, whether the bacteria were Vwa^+ or Vwa^- (lacking pCD) (33).

This paper reports our study of the growth within cultured mouse resident peritoneal macrophages of congenic strains of *Y. pestis* differing in the presence of one or more of the Pur, Pst, Vwa, and Pgm virulence determinants. We show that, in keeping with their postulated extracellular roles, the Pgm and Pst properties are not required for growth of yersiniae within resident peritoneal macrophages. Contrary to the speculation about a role for the Vwa determinant in intracellular survival and growth, we found that Vwa^+ and Vwa^- yersiniae grew equally well in the macrophages even when opsonized with normal serum. However, Pur^- bacteria were unable to grow, and many were killed, in macrophages not supplemented with purines in their surrounding medium, indicating that net biosynthesis is necessary to counteract macrophage microbicidal properties.

MATERIALS AND METHODS

Bacterial strains and bacteriophage. *Escherichia coli* B and *E. coli* K-12 χ289 (F^- *supE42* $T3^r$) (13) were obtained from Roy Curtiss III, Washington University. All *Yersinia* strains

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were obtained from R. R. Brubaker, Michigan State University. They included Vwa^+ and Vwa^- strains of *Y. pestis* KIM (35), which is a Pgm^- derivative of the virulent parent strain *Y. pestis* KIM-10. The congenic series of strains isolated from the Pur^- derivative of *Y. pestis* KIM-10 (strain K25 [4]) is described elsewhere (36). Bacteriophage $T6h^{YP}$ is a host-range mutant of bacteriophage T6 that will lyse both *Y. pestis* and *E. coli* "from without" when present at a multiplicity of infection (MOI) greater than 100 (34). Its propagation on *E. coli* B, UV inactivation, and use in infectivity assays have been described previously (34). The presence of the appropriate plasmid profiles was confirmed for all of the strains of *Y. pestis* used in this study. Strains that were Vwa^- lacked the Vwa plasmid pCD, and Pst^- bacteria lacked the smaller plasmid associated with expression of pesticin, fibrinolysin, and coagulase.

Culture of bacteria. Bacterial stocks were stored at −20°C in 80% glycerol. Growth was initiated by streaking from these stocks onto tryptose blood agar base (Difco Laboratories, Detroit, Mich.) and incubating for 2 days at room temperature (*Y. pestis*) or 1 day at 37°C (*E. coli*). Cells from these cultures were suspended in 33 mM sodium phosphate buffer (pH 7.0) and used to inoculate heart infusion broth (Difco) supplemented with 0.2% xylose and 1 mM $MgCl_2$ (3) and, for the culture of Pur^- strains, 0.1 mM each of hypoxanthine and guanosine (36). This medium was employed because it yielded a homogeneous suspension of single and septating pairs of bacteria upon subsequent washing. In addition, growth in heart infusion broth at 26°C has been previously shown to result in strong expression of pesticin, fibrinolysin, and coagulase and modest expression of hemin binding (the pigmentation reaction) (36). The bacteria were maintained in exponential phase of growth for 10 generations at 26°C with shaking at 200 rpm (New Brunswick G-76 water bath) and with a ratio of flask volume to culture volume of 10:1. They were washed once by centrifugation with phosphate-saline buffer (solution "a" of Dulbecco phosphate-buffered saline [15]), resuspended at room temperature in phosphate-saline buffer at a density of 6×10^6 cells per ml unless otherwise specified, and immediately used to infect macrophages.

Opsonization with normal mouse serum. Normal mouse serum was obtained from peripheral blood of 9- to 12-week-old female CD-1 Swiss outbred mice [Crl:CD-1$^{(R)}$ (1CR) BR from Charles River Breeding Laboratories, Inc., Portage, Mich.]. Serum was exposed only to glass surfaces during its handling to minimize activation of complement. It was stored at −20°C for 1 week or less and was thawed just before use. Bacteria were suspended at a density of ca. 9×10^9 cells per ml in phosphate-saline buffer, diluted 1/100 in 100% serum or phosphate-saline buffer, and incubated for 10 min with gentle shaking. They were then diluted 1/5 with phosphate-saline buffer and immediately used to overlayer monolayers of macrophages. Thus, the concentration of normal mouse serum present during the 15-min infection of macrophages was 20% for the serum-treated bacteria. Throughout the handling of bacteria and macrophages, buffer and serum were maintained at 37°C. Determinations of numbers of viable bacteria made before, and immediately after, incubation in 100% serum showed that this treatment resulted in negligible decreases in viable numbers, consistent with the findings of Perry and Brubaker with 10% human serum (31).

Macrophage cultures. We used the procedures of Shaw and Griffin (33) with minor modifications to obtain and culture mouse peritoneal macrophages from 6- to 9-week-old female CD-1 Swiss outbred mice. All incubations of macrophages were at 37°C with a gas mixture of 95% air and 5% CO_2. All buffers and media added to macrophage cultures were warmed to 37°C before use. The macrophages, present at a nominal 10^6 per 16-mm well, were cultured for 20 to 42 h with 1 ml of medium 199 plus 10% heat-inactivated fetal bovine serum (FBS) (GIBCO Laboratories, Grand Island, N.Y.) per well. In one experiment, the time allowed for adherence was reduced from 2 to 1 h, and no further incubation preceded the challenge of the macrophages with yersiniae. In experiments employing Pur^- *Y. pestis*, the macrophage culture medium was supplemented with 0.1 mM each of hypoxanthine and guanosine. No antibiotics were employed in this work.

Infection of macrophages with bacteria. The macrophages were washed once with Hanks balanced salt solution (HBSS; GIBCO), overlayered with 0.25 ml of bacterial suspension (containing 1.4×10^6 bacteria for a nominal MOI of 1.4 unless otherwise specified), and incubated for 15 min with occasional gentle rocking. This protocol resulted in an average of 9.3 ± 1.1% of the macrophages being infected with an average of 1.6 ± 0.3 bacteria associated with each infected macrophage (combined data from five experiments; counts made from specimens stained with acridine orange). These numbers were essentially identical for all strains of *Y. pestis* employed. The number of CFU obtained per well was always significantly lower than that predicted by microscopic counts coupled with the input number of macrophages, chiefly because the vigorous washings of the macrophages removed many macrophages; also, many daughter bacterial cells, counted as two cells, probably grew as single colonies on plates.

After the infection, the macrophages were washed three times with HBSS (with brief agitation accompanying each wash), overlayered with HBSS containing 5% heat-inactivated FBS, and incubated. This incubation medium does not permit growth of *Y. pestis* even in the presence of macrophages (37); accordingly, any noninternalized bacteria remaining after the infection contributed a small, constant background number of CFU in our subsequent analyses. Estimates made from specimens stained with Giemsa stain and viewed both with phase-contrast and Nomarski differential interference contrast optics indicated that extracellular bacteria represented no more than 10% of the total after the infection procedure.

In one experiment, macrophages were exposed to *E. coli* K-12 χ289 at a nominal MOI of ca. 400 (4.0×10^8 bacteria per well). After infection and washing, each culture well received 0.2 ml containing 8×10^9 PFU of UV-inactivated $T6h^{YP}$ (titered on *E. coli* B before UV inactivation [33]) and was incubated for 5 min. The macrophages were washed once with HBSS, overlayered with 0.25 ml of medium 199, and incubated. We have previously shown that this bacteriophage treatment affects neither internalized bacteria nor phagocytosis or superoxide anion generation by the macrophages (34).

Determination of bacterial viable numbers in infected macrophages. The contents of duplicate wells were collected at various times and diluted into ice-cold water to lyse any macrophages present. Water (0.5 ml per well) was added, and the dish was placed on ice for 5 min. Macrophages remaining in the wells were lysed by vigorous pipetting. These lysates were combined with the original harvested liquid from the wells and vortexed vigorously (10 s at full speed of a Vortex Genie) to complete lysis and dissociate bacteria from cellular debris. Quantitative plating of these

mixtures on tryptose blood agar base yielded numbers of CFU present in each well. It was important to harvest the entire contents of the wells, since the infected macrophages increasingly tended to detach from the wells or cover slips, and bacteria were released from lysing macrophages as the incubation progressed. Maintenance of Pgm^+ and Vwa^+ phenotypes during the experiments was confirmed by plating samples of the cultures before infection of macrophages and after ca. 12 h of growth within macrophages on Congo red medium (38) and on calcium-deficient magnesium oxalate agar (22).

Light microscopic characterization of mixed cultures of *Yersinia* strains and macrophages. Infected macrophages attached to cover slips were stained with acridine orange (2) and used to determine the initial percentage of macrophages infected, the initial number of macrophage-associated bacteria, and the viability of the bacteria during the course of the experiment. Bacterial viability can be assessed this way because actively growing bacteria contain large amounts of RNA which gives off intense orange fluorescence, masking the green emission from DNA. Nongrowing bacteria, in which much RNA has been degraded, are green. At least 500 macrophages were scored to determine the percent infected; the bacteria associated with at least 100 infected macrophages were counted to determine the number of macrophage-associated bacteria. Replicate cover slips fixed and stained with Giemsa stain were used to obtain supplementary assessments of the events taking place in the course of the experiments. Additional replicate cover slips were used to determine the percentage of macrophages that were viable as judged by trypan blue exclusion (30). At least 100 macrophages were scored; these included all within at least nine fields evenly distributed over the cover slip and chosen without preview.

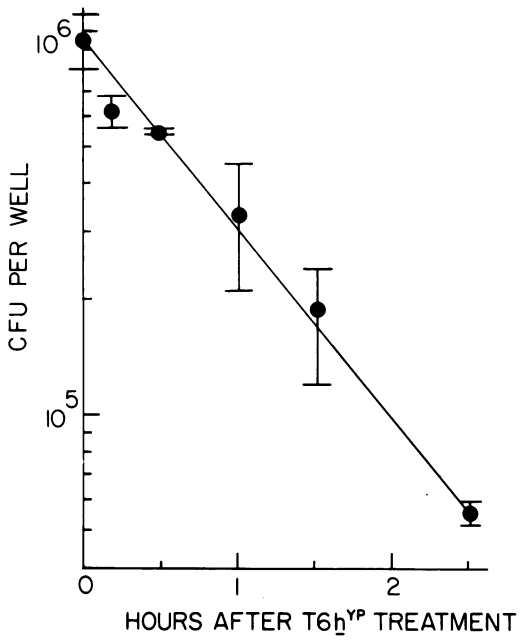

FIG. 1. Killing of *E. coli* K-12 by cultured resident mouse peritoneal macrophages. Macrophages cultured for 42 h were exposed for 15 min to *E. coli* K-12 χ289 at a nominal MOI of 400. Extracellular bacteria remaining after washing the infected macrophages were reduced in numbers by treatment for 5 min with 8×10^9 PFU of UV-inactivated bacteriophage T6h^{YP}. After the macrophages were washed with HBSS and overlayered with medium 199, they were incubated for 150 min. At various times thereafter, duplicate cultures were quantitatively harvested, the macrophages were lysed, and the bacteria were serially diluted and plated to determine viable numbers per culture well. The symbols represent the averages of the duplicate determinations, and the error bars represent the individual determinations.

RESULTS

This study was undertaken to determine whether the Pur, Pst, Pgm, and Vwa virulence determinants of *Y. pestis* play a role in the survival and growth of the bacteria within macrophages, their mammalian target cell. We employed strains of *Y. pestis* differing only with respect to one or more of these virulence attributes. Our system models initial events in infection by yersiniae, since the bacteria were cultured at 26°C, a temperature that might be found in the gut of a flea, the alternate host of these bacteria.

Bactericidal capacity of macrophages. We made preliminary tests to establish that our cultured resident peritoneal macrophages were capable of bactericidal activity. Macrophages cultured for 42 h were challenged with *E. coli* K-12, known to be readily engulfed and destroyed by macrophages. Our macrophages readily killed these bacteria, even though they were challenged with bacterial numbers greater than those to be used in experiments with *Y. pestis* (Fig. 1). Accordingly, it is unlikely that in experiments employing a much lower MOI of yersiniae, macrophage microbicidal properties might be nonspecifically titrated or neutralized by the challenge dose of bacteria.

Growth of Pst^-, Pgm^-, and Vwa^- *Y. pestis* within macrophages. Figure 2 shows the growth in purine-supplemented macrophages of purine-auxotrophic *Y. pestis* KIM K25 and derivatives of this strain that were Pst^-, Pgm^-, or Vwa^-. All *Yersinia* strains were engulfed to equal extents by the macrophages and subsequently grew equally well. The lag seen in this experiment with the Pst^- strain was not observed in other experiments. During experiments like that of Fig. 2, the growing yersiniae filled and burst the macrophages (Fig. 3) and initiated additional rounds of infection, ultimately involving most of the macrophages present. The fact that the net growth rate measured as CFU was the same for the four strains during the last third of the incubation period, when secondary rounds of infection were taking place (Fig. 2), suggests that even after growth at 37°C within their target host cell, these virulence attributes do not play a role in subsequent infection and growth of *Y. pestis* within macrophages.

The results of 14 experiments showed that immediately after infection, 85 ± 10% of the macrophages were viable as measured by trypan blue exclusion. This number remained constant for up to 9 h and then became an unreliable indicator, due to appreciable detachment of macrophages from the dishes and lysing of the macrophages. Before this stage, there was no correlation between trypan blue permeability and the presence or absence of bacteria within the cells. Thus, the intracellular development of yersiniae within macrophages did not kill these cells until they were packed with bacteria.

These plating experiments did not yield a meaningful value for the generation time for intracellular growth of *Y. pestis*, due to the formation of chains by the bacteria (e.g., Fig. 3) and to asynchrony in the culture, giving rise to staggered release of bacteria into the non-growth supportive surrounding medium. However, it is significant that the doubling time

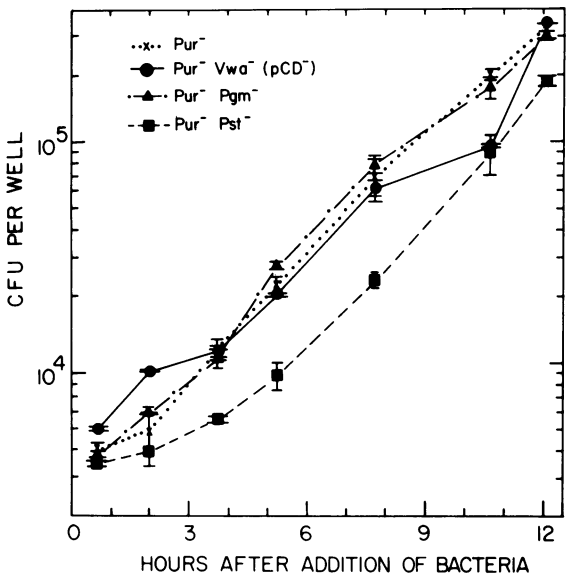

FIG. 2. Growth of *Y. pestis* KIM K25 (Pur⁻) congenic mutants in cultured mouse resident peritoneal macrophages. Macrophages cultured for 20 h in medium containing 0.1 mM each of hypoxanthine and guanosine were exposed for 15 min to *Y. pestis* (Pur⁻) strains missing one or more of the virulence determinants established for the species; each strain was present at an MOI of 1.4. The macrophages were then washed twice with HBSS, overlayered with HBSS containing 0.1 mM each of hypoxanthine and guanosine and 5% heat-inactivated FBS, and incubated. At various times thereafter, duplicate cultures were quantitatively harvested, the macrophages were lysed, and the bacteria were serially diluted and plated to determine viable numbers per culture well. The *Y. pestis* mutants were the purine-auxotrophic parent Pur⁻, Pur⁻ Vwa⁻ (lacking the calcium dependence plasmid pCD), Pur⁻ Pgm⁻, and Pur⁻ Pst⁻ (lacking the plasmid that confers expression of pesticin, fibrinolysin, and coagulase). The symbols represent the averages of the duplicate determinations, and the error bars represent the individual determinations.

of 1 h 49 min ± 24 min measured as CFU was comparable with that obtained in rich media in which chaining is not so extensive (determinations made during the first 6 h of 17 experiments). Accordingly, the bacteria probably were growing more rapidly than is seen in liquid media.

Culturing the macrophages for 20 to 42 h did not cause them to lose a property necessary for killing or inhibiting the growth of *Yersinia* strains. This was shown by culturing macrophages for only 1 h to allow adherence before challenge with Vwa⁺ and Vwa⁻ *Y. pestis* KIM (Pgm⁻); the results obtained were essentially the same as those shown in Fig. 2.

Failure of opsonization to affect growth of Vwa⁺ and Vwa⁻ yersiniae in macrophages. We considered the possibility that the fate of yersiniae within macrophages might be affected by opsonization; components of normal serum might interact with yersiniae that lacked invasive virulence determinants, eliciting uptake of bacteria into a more microbicidal environment than otherwise would be the case. This would be analogous to the ability of macrophages to kill only rickettsiae isolates that have been opsonized with immune serum (19). Both Vwa⁺ and Vwa⁻ *Y. pestis* KIM (Pgm⁻) grew with kinetics similar to those in previous experiments, whether the bacteria were opsonized or sham opsonized with phosphate-saline buffer. Since the bacteria were Pgm⁻ as well as Vwa⁺ or Vwa⁻, this indicates that serum is not sufficient to mediate the different fates of either Pgm⁺ and Pgm⁻ or Vwa⁺ and Vwa⁻ yersiniae in vivo.

Morphology and integrity of monolayers of macrophages infected with Vwa⁺ and Vwa⁻ *Y. pestis*. We noted no systematic differences in appearance of macrophage monolayers infected with Vwa⁺ and Vwa⁻ *Y. pestis*. We also tested for fragility of the infected cells in the monolayers, using an assay in which cultures of infected macrophages were vibrated for 10 min at >300 rpm (setting no. 6 on a Bellco Mini Orbital shaker). This resulted in rounding up of the macrophages and detachment of some, but there was no difference in tendency of the macrophages to detach or lyse when they were infected with Vwa⁺ or Vwa⁻ *Y. pestis*.

Inability of Pur⁻ *Y. pestis* to grow in macrophages without purine supplement. We tested whether purine-auxotrophic *Y. pestis* is able to grow within macrophages cultured in the presence of only those low (micromolar) concentrations of purines present in medium 199 and 10% FBS. Macrophages were cultured for 20 h with and without supplements of 0.1 mM each of hypoxanthine and guanosine. *Y. pestis* KIM K25 (Pur⁻) was grown in medium containing the purine supplements, washed with medium lacking the supplements, and then incubated for 2 h in this purine-deficient medium. The bacteria were then handled as usual, but the salts-FBS solution bathing the infected macrophages contained the purine supplements only for macrophages originally cultured in their presence. In the absence of the purine supplements, the Pur⁻ *Y. pestis* not only failed to grow but showed a fourfold decline in viable numbers during the experiment (Fig. 4). Staining of infected macrophages with acridine orange during the experiment showed a progressive change in color of fluorescence from orange to green for the bacteria in cultures lacking purines and a constant brilliant orange fluorescence by the bacteria in cultures containing purines.

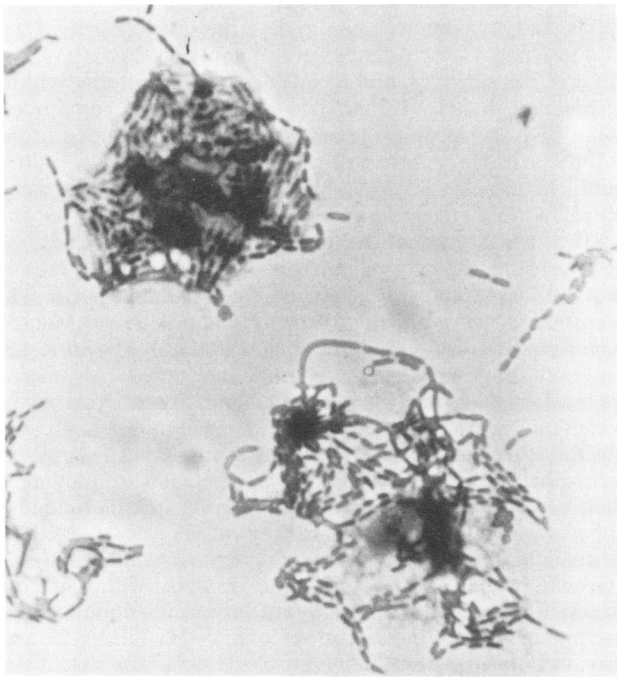

FIG. 3. *Y. pestis* KIM K25 (Pur⁻) completing a cycle of growth within cultured mouse peritoneal macrophages. Phase-contrast light photomicrograph of Giemsa-stained specimen. Magnification, ×1,466.

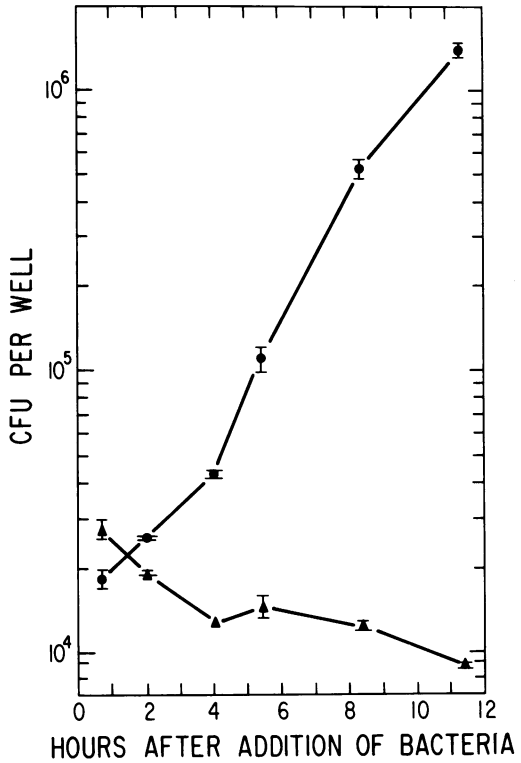

FIG. 4. Fate of purine-auxotrophic *Y. pestis* KIM K25 (Pur$^-$) in mouse peritoneal macrophages cultured with or without purine supplements. Macrophages cultured for 20 h were infected with *Y. pestis* KIM K25 (Pur$^-$), and numbers of CFU per culture well were determined as described in the legend to Fig. 2. For one set of macrophages (●), 0.1 mM each of hypoxanthine and guanosine were present during both the culture of the macrophages and the growth of the bacteria; for the other (▲), no purines were present either during macrophage culture or during bacterial growth within the macrophages. The symbols represent the averages of the duplicate determinations, and the error bars represent the individual determinations.

DISCUSSION

This study tested for a role of the Pur, Pst, Pgm, and Vwa virulence determinants in the survival and growth of *Y. pestis* in resident mouse peritoneal macrophages. We found that the inability to convert xanthine monophosphate to guanosine monophosphate (Pur$^-$) (4) resulted in failure of yersiniae both to grow and to survive in macrophages and that this lesion can be compensated in our Pur$^-$ strain of *Y. pestis* by the presence of hypoxanthine and guanosine in the mixed cultures. The purine-unsupplemented cultures were the only ones in this study in which yersiniae actually decreased in viable numbers. This decrease began without noticeable lag, indicating that the mechanism used by *Y. pestis* to counteract the bactericidal properties of macrophages involves bacterial growth or net biosynthesis. In an accompanying paper (37), we show that *Y. pestis* within macrophages is located in phagolysosomes. Our data with the Pur$^-$ strain suggest that the ability to withstand this environment is an active process and does not result simply from shielding by outer layers present at the time of phagocytosis. In our experiments with yersiniae grown at 26°C, a component of this active process may have been the biosynthesis of capsular material, which is expressed weakly at 26°C and strongly at 37°C (17). This would be consistent with the observations of Burrows and Bacon (9) that a purine auxotroph of *Y. pestis* not supplemented with purines was unable in vivo to develop full resistance to phagocytosis.

The ability to carry out the terminal steps of guanine or adenine biosynthesis has been demonstrated to be necessary for the virulence of *Bacillus anthracis* (23, 24), *Klebsiella pneumoniae* (20), *Salmonella typhimurium* (18, 21), *Salmonella typhi* (1), and *Pseudomonas pseudomallei* (29), three of which are extracellular pathogens, and probably necessary for the virulence of all bacterial parasites lacking the ability to obtain and utilize host nucleotides. Our data provide support for the hypothesis that avirulence of *Y. pestis* due to purine auxotrophy results from inavailability in host tissues and serum of sufficient adenine, guanine, or their nucleosides to support growth. It remains to be shown for this and other pathogens whether purine biosynthesis provides precursors for specific virulence attributes or whether deprivation of any essential compound is sufficient to shift the balance between mammalian antibacterial mechanisms and the maintenance processes of bacteria.

We found that the invasive Pst and Pgm properties do not play a role in survival and growth of *Y. pestis* within macrophages and also have little if any effect on efficiency of uptake of yersiniae. In addition, the absence of the pigmentation property did not render the bacteria more susceptible to the bactericidal effect of macrophages after exposure to normal serum. These observations are consistent with the idea that these two virulence determinants play their roles in promoting extracellular rather than intracellular survival of yersiniae. Further, our data imply that none of the multiple outer membrane protein species lost due to the Pgm$^-$ lesion (36) nor any product encoded by the plasmid associated with expression of the Pst property plays a significant role in mediating intracellular functions of *Y. pestis*.

Likewise, our data show that none of the products of the much larger (75-kilobase-pair) calcium dependence plasmid pCD that mediates expression of the Vwa property in *Y. pestis* plays essential roles in events within resident macrophages. Our Vwa$^-$ Pgm$^-$ derivatives of *Y. pestis* KIM or KIM K25 (Pur$^-$) grew well within macrophages, in apparent conflict with the previous finding of Cavanaugh and Randall that *Y. pestis* A1122 (Vwa$^-$ Pgm$^-$) was killed by elicited mouse macrophages (12). Our model system differed significantly from theirs in that we used unstimulated macrophages, whereas their cells had been elicited with the oil Bayol F and represented inflammatory macrophages. Even so, when we tested for growth of *Y. pestis* A1122 in our macrophages, we obtained only a 3-fold increase in viable numbers during 9 h of incubation, in contrast with 20-fold increases generally seen with other strains. Strain A1122 originally was attenuated by incubation in broth for 7 weeks at 32°C (27) and evidently acquired a lesion that affects intracellular growth. Our findings are consistent with those of Richardson and Harkness (32) with *Y. pseudotuberculosis* and suggest that the Vwa properties of the requirement of calcium for growth at 37°C and expression of V and W antigens do not mediate survival and growth within macrophages. In vivo, Vwa$^-$ yersiniae may grow within unstimulated tissue macrophages but may be eliminated through interactions with neutrophils and, ultimately, activated macrophages. The inflammatory macrophage also may play an important role early in a plague infection. These interactions presently are under investigation.

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