# *Yersinia pseudotuberculosis* disseminates directly from a replicating bacterial pool in the intestine

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**Dissemination of *Yersinia pseudotuberculosis* within mice after oral inoculation was analyzed. *Y. pseudotuberculosis* translocated to organs such as the liver and spleen shortly after oral inoculation, but was quickly cleared. In contrast, a second temporally distinct bacterial translocation event resulted in successful hepatosplenic replication of the bacteria. Replicating pools of bacteria could be established in these organs in mouse mutants that lacked Peyer's patches. These animals frequently had sterile mesenteric lymph nodes, a finding consistent with translocation taking place independently of regional lymph node colonization. In further contradiction to accepted models for dissemination of enteropathogens, clonal analysis revealed that bacteria causing disease in the spleen and liver of C57BL/6J mice were derived from populations located outside the intestinal lymph nodes. Replication of bacteria in the intestine before translocation appeared critical for dissemination, as transient selective suppression by streptomycin of bacterial growth in the intestine delayed dissemination of *Y. pseudotuberculosis*. These results collectively indicate that hepatosplenic colonization appears intimately connected with the ability of *Y. pseudotuberculosis* to successfully establish replication in the intestinal lumen and does not result from ordered spread leading from the intestine to regional lymph nodes before dissemination.**

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Abbreviations used: MLN, mesenteric LNs; PP, Peyer's patches.

The *Yersinia* genus has three members that are pathogenic for humans (1, 2). *Y. pestis* infection, usually a zoonosis from fleas, causes the fatal systemic disease plague (3). In contrast, oral ingestion of the highly similar microorganism *Yersinia pseudotuberculosis* or the related *Y. enterocolitica* most often cause self-limiting enteritis and mesenteric adenitis, primarily as a consequence of eating contaminated food products (4, 5, 6). In patients with hemochromatosis, however, enteric *Yersinia* disseminate systemically with a case fatality of 70% (7). In addition to enteritis, oral inoculation of mice with enteropathogenic *Yersinia* results in replication of the bacteria in the liver and spleen, resulting in death of the animal (8). Murine enteropathogenic *Yersinia* disease is very similar to the typhoid-like syndrome observed after inoculation of mice by other enteric pathogens such as *Salmonella enterica* serovar typhimurium (9). Therefore, *Y. pseudotuberculosis* pathogenesis in mice provides insight into the pathways and virulence mechanisms of enteropathogens that cause enteritis and systemic infections (4, 10).

Intestinal epithelial cells mainly function to facilitate nutrient absorption and most are enterocytes that have secretory and endocrine functions. Two properties of intestinal epithelial cells, however, function to protect the intestine from pathogen invasion and disease. First, tight junctions between the epithelial cells help maintain the barrier between luminal contents and underlying mucosal tissue (11). Second, specialized epithelial cells, called M cells, overlay the lymphoid Peyer's patches (PP) (12) and facilitate mucosal immune responses by transporting antigens from the intestinal lumen to deep pockets in the basal surface of the cell (13). Within the pockets are dendritic cells and macrophages, which are ideally placed to phagocytose antigens,

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transport them to the regional lymph nodes, and initiate a local immune response (14, 15).

*Y. pseudotuberculosis* appears to take advantage of M cell function (16). Invasin, an outer membrane protein of enteropathogenic *Yersinia*, binds to $\beta_1$ integrins on the luminal surface of M cells (16, 17), facilitating translocation of bacteria across the M cell into the underlying PP tissue (18–21). Within 24 h after oral inoculation, enteropathogenic *Yersinia* are found in the mesenteric lymph nodes (MLN), then appear in the liver and spleen 48–72 h after inoculation (22, 23). These observations have led to a model in which bacterial colonization in the local lymphatic tissues leads to dissemination of enteropathogenic *Yersinia* into the blood stream (via lymph drainage to the thoracic duct), with subsequent infection of the liver and spleen (19, 22, 24).

Although the ordered spread from the lumen of the intestine into the PP and MLN followed by dissemination to the liver and spleen is the presumed model of enteropathogen dissemination, there is sparse and often contradictory evidence that this is the dissemination route. Previous work has shown that enteropathogenic *Yersinia* mutants lacking functional invasin are able to spread to the liver and spleen within 6 d, although shortly after inoculation these mutants fail to bind M cells and colonize PP with delayed kinetics (24). This suggests a pathway of intestinal translocation by enteropathogenic *Yersinia* that bypasses the PP. Similarly, a *Salmonella* mutant that is unable to colonize the PP (25) is detected in the blood stream of mice minutes after oral inoculation (26), probably after direct transport across the epithelial layer by a phagocytic cell. It has been proposed that this very early *Salmonella* bacteremia initiates the systemic terminal disease in mice (26). Collectively, these observations suggest that the conventionally accepted model for extraintestinal dissemination of enteropathogens may be incorrect.

This study used genetically modified mice and clonal analysis of *Y. pseudotuberculosis* to examine extraintestinal dissemination of enteropathogens to the liver and spleen. Our results indicate that systemic disease results from bacteria that spread from some site other than the intestinal lymphatic tissue. Furthermore, prolonged replication within the intestines before entry into the spleen and liver appears to be a prerequisite for growth within these tissues and thus systemic infection.

## RESULTS

### Dynamics of *Y. pseudotuberculosis* infection after oral inoculation

The goal of these studies was to determine if systemic disease resulting from oral inoculation of *Y. pseudotuberculosis* is a consequence of ordered dissemination from local lymph nodes to distal tissues such as the liver and spleen. To characterize the kinetics of enteropathogen dissemination, orogastric inoculation of $5 \times 10^8$ CFU of *Y. pseudotuberculosis* into C57BL/6J mice was performed. Organ colonization assays revealed that the PP and MLN of most mice were populated with bacteria 7 d after infection (Fig. 1 A). The bacterial load within these tissues varied markedly (Fig. 1, A–C). Increasing the dose to enhance hepatosplenic infection occasionally disrupted the intestinal barrier, permitting dissemination of normal bowel flora into the liver and spleen (unpublished data); therefore, $5 \times 10^8$ CFU was used as the standard orogastric dose. This lower dose also facilitated tracking individual clones of bacteria in clonal distribution studies described later.

The kinetics of the initial appearance of bacteria in each tissue site was followed (Fig. 1, B and C). Surprisingly, 30 min and 5 h after oral inoculation of *Y. pseudotuberculosis*, bacteria were cultured from the liver and spleen. The colony counts recovered were close to the limits of detection, therefore a broth recovery technique was used to

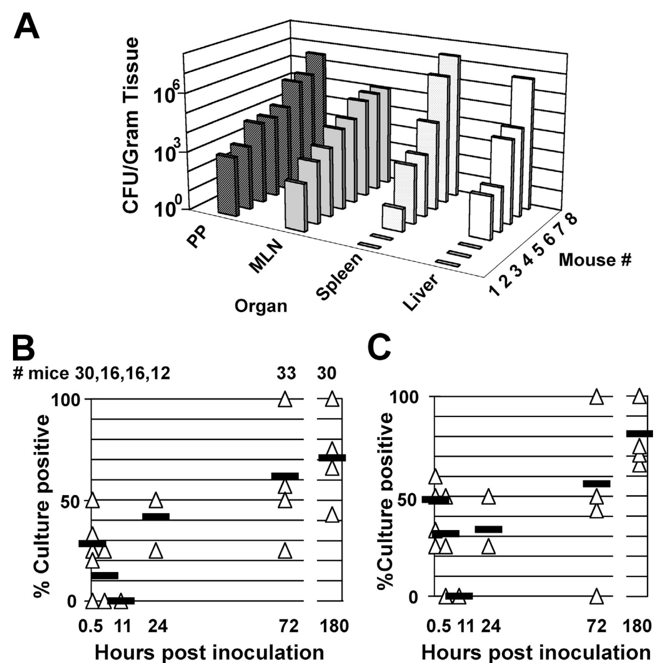


**Figure 1. Typical infection of C57BL/6J mice 7 d after orogastric inoculation of *Y. pseudotuberculosis* and temporal kinetics of hepatosplenic colonization.** C57BL/6J mice were orogastrically inoculated with $5 \times 10^8$ CFU of *Y. pseudotuberculosis*. 7 d after infection (A), the mice were killed and the organs were weighed and cultured for bacterial colony counts on MacConkey lactose plates (see Materials and methods). Similar results were obtained with BALB/c mice (see Fig. 2 A). PP, Peyer's patches; MLN, mesenteric lymph nodes. After oral inoculation, at 0.5, 5, 11, 24, 72, and 180 h after inoculation, groups of mice were killed and the spleens (B) and livers (C) were cultured for the presence of any bacteria using the broth recovery technique (see Materials and methods). The results, compiled from a series of experiments and displayed above the graph in B, are the total number of mice examined at each time point. Each triangle represents the percentage of mice with bacteria cultured from spleen or liver in one experiment at each time point. Bars are the mean percentages of organs containing bacteria. At some time points, the same proportion was obtained in different experiments (see 11 h); consequently, the triangles are superimposed. The total number of experiments performed at each time point were as follows: 0.5 h, seven experiments; 5 h, four experiments; 11 h, four experiments; 24 h, three experiments; 72 h, seven experiments; and 180 h, seven experiments.

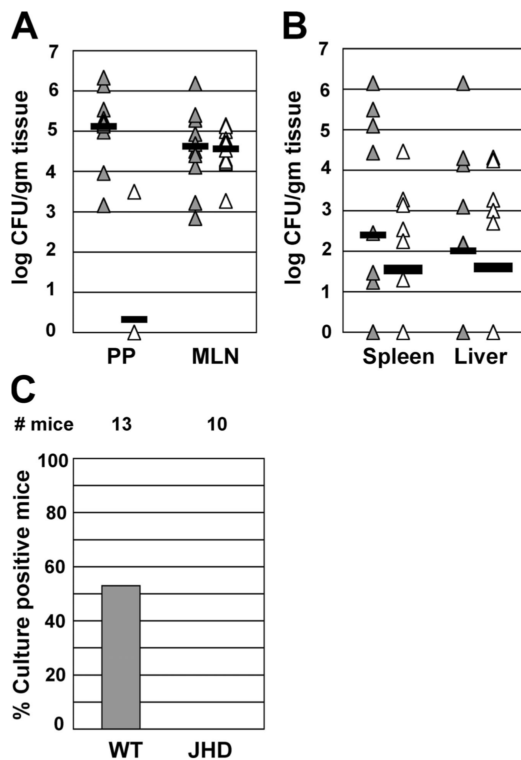


**Figure 2. Oral infection by *Y. pseudotuberculosis* in B cell–deficient mice indicates two mechanistically different waves of spread from the intestine.** 6 d after oral inoculation with 5 × $10^8$ CFU of *Y. pseudotuberculosis* (see Materials and methods), BALB/c mice and BALB/c-$J_H$D mice (B cell deficient) were killed and (A) PP and MLN or (B) spleen and liver were cultured for colony counts (see Materials and methods). Each triangle represents the CFU recovered from the organ of a mouse: (closed triangles) BALB/c mice and (open triangles) BALB/c-$J_H$D mice. At some time points, similar colony counts were obtained in different mice and the triangles are superimposed. Bars are the mean number of bacteria found in the tissues from all experiments. The results were accumulated from three separate experiments. In total, 10 wild-type and 11 BALB/c-$J_H$D mice were analyzed. There was no statistical difference in CFU recovered between the two mouse strains from the liver, spleen, or MLN. Only three BALB/c-$J_H$D mice contained identifiable PP and bacteria were cultured from the PP of only one of these mice. (C) B cell function is required for early dissemination of *Y. pseudotuberculosis* from the intestine to spleen and liver. BALB/c and BALB/c-$J_H$D mice were orogastrically inoculated with 5 × $10^8$ CFU of *Y. pseudotuberculosis*. The mice were killed between 30 min and 2 h after infection and the liver and spleens were cultured for the presence of bacteria, using the broth recovery technique. The results are pooled from three separate experiments. # mice, mean percentage of mice from three separate experiments that had culturable bacteria from the liver or spleen in the noted mouse strain.

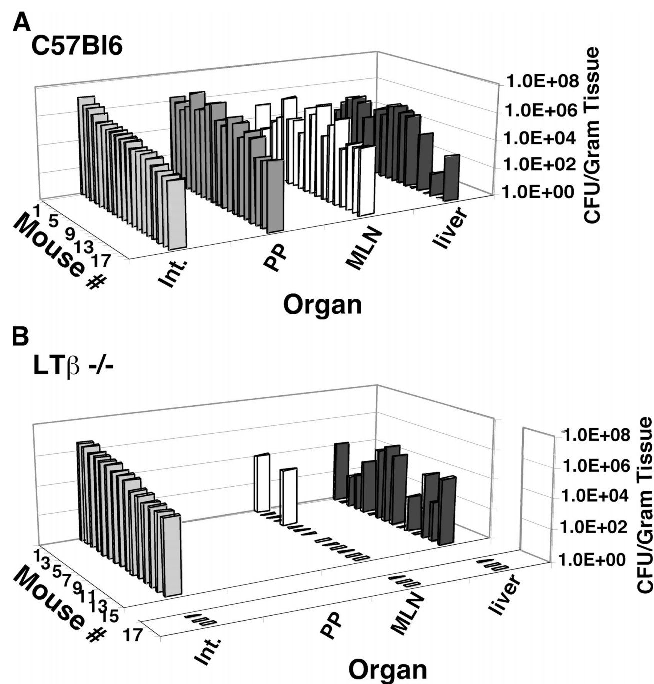


**Figure 3. *Y. pseudotuberculosis* colonizes the livers of the LT $\beta^{-/-}$ mouse.** Mice were orally inoculated with 5 × $10^8$ *Y. pseudotuberculosis*. 6 d after inoculation, they were killed and colony counts were obtained from noted tissues. Shown are individual animals in which intestinal colonization was established. Shown are three experiments from (A) C57BL/6J mice and (B) LT $\beta^{-/-}$ mice. The LT $\beta^{-/-}$ mice are displayed as separate graphs, with the three mice showing no intestinal bacteria separated from the other data. No PPs were found in the LT $\beta^{-/-}$ mouse, whereas only two mice had detectable colonization of the MLN in the KO strain.

enhance assay sensitivity (see Materials and methods). 30 min after oral inoculation, *Y. pseudotuberculosis* was cultured from an average of 48% of the livers of C57BL/6J mice (Fig. 1 C) and 28% of spleens (Fig. 1 B). At 5 h, there was a decrease in the frequency of liver (31%) and spleen (12%) infection, although the difference was not significant (Fig. 1, C and B) ($P = 0.122$ and $P = 0.150$, respectively). Unexpectedly, no *Y. pseudotuberculosis* could be cultured from liver or spleen 11 h after inoculation. This loss of bacteria in these tissues was remarkably reproducible over multiple experiments (Fig. 1).

After the sterile period, we next detected *Y. pseudotuberculosis* in the liver and spleen at 24 h after inoculation, with an average of 33% of livers and 42% of spleens having culturable bacteria (Fig. 1). By the 72-h time point after inoculation, replication appeared to be well established in most mice, with *Y. pseudotuberculosis* cultured from the liver and spleen in ∼60% of the mice (Fig. 1). After the onset of the second systemic infection, bacteria remained detectable by culture in the liver and spleen in >50% the mice 6–7 d after infection, when many animals were moribund (Fig. 1). A similar time course of dissemination was observed in BALB/c mice (Fig. 2 and not depicted). Therefore, there appears to be two temporally distinct processes of bacterial dissemination.

### B cell function and lymphotoxin β–dependent lymph nodes are not required for hepatosplenic infection caused by *Y. pseudotuberculosis*

To test the hypothesis that there may be different routes of spread into liver and spleen dependent on the time point at which spread occurs, we analyzed bacterial dissemination in

two mouse strains that are deficient for specific cells of the host immune system. First analyzed were BALB/c-$J_H$D mice, which produce no B cells, have reduced numbers of PP, and produce M cells with altered morphology and function (27, 28). As the presence of intact PP has been hypothesized to be critical for systemic disease caused by enteropathogens (27), it is predicted that such mutants should be severely depressed for large scale invasion into systemic infection sites. Consistent with published reports (27), between zero and two PP were detectable by eye in the BALB/cJ-$J_H$D mice, whereas from seven to nine PP were found in the parental mouse strain (unpublished data). The presence of dysfunctional PP in the mutant mouse strain is further supported by the fact that *Y. pseudotuberculosis* was only cultured from the PP of one of the few BALB/cJ-$J_H$D mice in which any PP were identified (Fig. 2 A).

Despite the absence of functional PP in BALB/cJ-$J_H$D mice, 7 d after orogastric inoculation, the infection of the liver and spleen by *Y. pseudotuberculosis* was similar in both the BALB/c-$J_H$D and the BALB/c mice (Fig. 2 B). Furthermore, the MLN were colonized as efficiently as wild-type mice (Fig. 2 A). In contrast, when early time points between 30 min and 2 h after orogastric inoculation of BALB/c-$J_H$D were investigated, *Y. pseudotuberculosis* was never cultured from the livers and spleens of BALB/c-$J_H$D, whereas >50% of wild-type mice had hepatosplenic infection (Fig. 2 C). These results indicate that although B cells or organs such as PP that are altered by lack of B cells play important roles in the early dissemination of *Y. pseudotuberculosis* to the liver and spleen, no such requirement exists for the later repopulation that leads to lethal disease. This result also argues against the hypothesis that the early bacteremia results from either mechanical disruption of the upper gastrointestinal tract or pulmonary aspiration during orogastric inoculation. Therefore, the presence of the bacteria in liver and spleen at early and late times after inoculation in wild-type mice likely results from mechanistically distinct processes.

To further investigate the possibility that colonization of PP is not a prerequisite for hepatosplenic infection, a second mouse strain lacking PP was challenged orogastrically with *Y. pseudotuberculosis*. Previous work indicates that lymphotoxin $\beta^{-/-}$ mice (LT$\beta^{-/-}$) lack PP, peripheral lymph nodes, splenic germinal centers, and follicular dendritic cells, but possess B cells and mesenteric lymph nodes (29). In our studies, we found that none of 18 LT$\beta^{-/-}$ mice dissected had PP, whereas 16 out of 18 had detectable MLN (Fig. 3 B). We analyzed colonization levels from three experiments after oral inoculation of either C57BL/6J mice (Fig. 3 A) or the congenic LT$\beta^{-/-}$ strain (Fig. 3 B). For ease of analysis, LT$\beta^{-/-}$ will be considered in two groups: those mice that were found to have bacteria in the lumen of the intestine and those in which *Y. pseudotuberculosis* failed to establish replication even within this site (Fig. 3 B).

For most of the LT$\beta^{-/-}$ mice that had detectable bacterial populations in the intestinal lumen, bacteria were also found within the livers, in spite of a lack of colonization of MLN (Fig. 3 B; only 2/15 LT$\beta^{-/-}$ mice had bacteria in MLN). For these animals, the frequency at which liver colonization was detected in the LT$\beta^{-/-}$ mutant (11/15 mice) was not significantly different from the C57BL/6J mouse (16/19 mice; Fig. 3 A; $P > 0.05$). There was, however, a significant depression in the number of bacteria in the liver and spleen in the LT$\beta^{-/-}$ mutant ($P < 0.05$; see Table I A for range and median colonization values). In addition to these animals, three of the LT$\beta^{-/-}$ mutants showed no evidence of bacteria in the intestine or in any other tissue (Fig. 3 B), perhaps because they lacked a pool of bacteria from which to initiate systemic disease. It is apparent from these studies that replication of *Y. pseudotuberculosis* in liver and spleen does not require colonization of the PP or MLN.

**Table I.** Analysis of *Y. pseudotuberculosis* infection in murine tissues

**A. Effect of LT$\beta^{-/-}$ mutation on bacterial yields in liver and spleen**

| | Spleen | | Liver | |
|---|---|---|---|---|
| Organ | C57BL/6 | LT$\beta^{-/-}$ | C57BL/6 | LT$\beta^{-/-}$ |
| Range CFU/g tissue | $10^2$–$2 \times 0^5$ | $10^1$–$3 \times 10^5$ | $10^1$–$10^5$ | $10^1$–$2 \times 10^5$ |
| Median CFU/g tissue | $1.5 \times 0^5$ | $1.8 \times 10^2$ | $4.4 \times 10^4$ | $4.2 \times 10^2$ |
| p-value | 0.002 | | 0.04 | |

**B. *Y. pseudotuberculosis* clone and CFU distributions in C57BL/6J mice**

| Hours after inoculation | 0.5 | 5 | 11 | 24 | 72 | 180 |
|---|---|---|---|---|---|---|
| No. of clones in intestine | | | | | | |
| median | 33.0 | 32.0 | 32.0 | 13.5 | 14.0 | 8.5 |
| range | all at 33 | all at 32 | all at 32 | 4–29 | 7–30 | 4–19 |
| No. of clones in liver | | | | | | |
| median | 0.0 | 0.0 | 0.0 | 0.0 | 0.5 | 1.0 |
| range | 0–11 | 0–4 | all at 0 | 0–3 | 0–16 | 0–8 |
| CFU in intestine | | | | | | |
| median | $8 \times 10^7$ | $10^7$ | ND | $2 \times 10^4$ | $6.5 \times 10^5$ | $5 \times 10^4$ |
| range | $7 \times 10^5$–$9 \times 10^7$ | $6 \times 10^6$–$3 \times 10^7$ | ND | $2 \times 10^3$–$10^5$ | $10^4$–$10^7$ | $9 \times 10^2$–$10^5$ |
| CFU in liver | | | | | | |
| median | $0.4 \times 10^1$ | 0.0 | 0.0 | ND | $1.1 \times 10^2$ | $5 \times 10^3$ |
| range | $0$–$2 \times 10^1$ | $0$–$2 \times 10^1$ | 0 | ND | $0$–$1 \times 10^5$ | $0$–$7 \times 10^4$ |

The absence of liver infection in those animals lacking populations in the intestine, however, raises the possibility that systemic infection may require the establishment of bacterial replication in the lumen of the intestine. Therefore, the connection between bacterial populations in sites associated with the intestine and the liver and spleen was studied further.

### Bacteria causing late stage hepatosplenic infection are not derived from local lymph nodes

To determine if bacteria recovered from the spleen and liver were derived from the intestinal lumen rather than regional lymph nodes, the clonal dissemination of *Y. pseudotuberculosis* from various tissues was examined beginning at different times after oral inoculation (Fig. 4). 33 uniquely tagged strains of wild-type *Y. pseudotuberculosis* were constructed (Fig. 4; see Materials and methods) and the presence of each clone in various tissues of C57BL/6J mice after orogastric inoculation of $5 \times 10^8$ CFU of bacteria ($1.5 \times 10^7$ of each clone) was detected using Southern hybridization (Fig. 4). 30 min after inoculation, an average of two clones were found in the liver (Fig. 5 B and Table I B). The population of clones in liver and spleen were also found in the lumen of the intestines, as expected (Table II; 30 min, liver vs. intestine, spleen vs. intestine). Clones in the liver, however, were not necessarily found in the MLN (Table II), indicating that bacteria did not appear to replicate in these glands before entry into distal tissue sites at early time points.

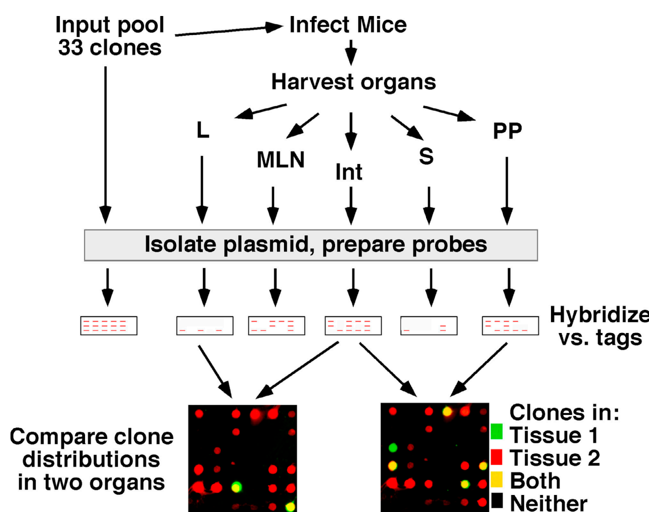


**Figure 4. Strategy for identification of *Y. pseudotuberculosis* clones present in different tissue sites.** 33 *Y. pseudotuberculosis* strains, each differing by only a unique oligonucleotide tag, were grown in broth and pooled immediately before oral inoculation into C57BL/6J mice (see Materials and methods). At various times after inoculation, the animals were killed, noted glands were removed, and bacteria were isolated by plating tissues on bacteriologic media (see Materials and methods). The colonies arising were pooled, DNA was isolated, and probes were constructed by PCR amplification of the unique oligonucleotide tags. The clones found in each tissue site were determined, and compared with the results obtained from other tissue sites.

11 h after inoculation, no bacteria were detectable in the spleens or livers of the mice (Figs. 1 and 5 A), but a second wave of entry became apparent by 72 h after inoculation (Figs. 1 and 5, A and B). At the later time point, clones in distal tissues were a subpopulation of the remaining clones in the intestines (Table II; 72 h, liver vs. intestine, spleen vs. intestine). The clones in the livers and spleens, however, were not consistently found in the intestinal lymphatic tissues, indicating that the hepatosplenic infection did not require replication in the PP and MLN before entry into distal tissue sites (Table II).

The total number of clones present in spleen or liver was consistently small, indicating the existence of a severe bottleneck that limited bacterial dissemination from the intestines into distal tissue sites (Fig. 5 B liver; spleen, not depicted). Between 72 h and 6 d after inoculation, the colony counts in the liver rose significantly (Fig. 5 A and Table I B), although the number of clones in this organ remained unchanged (Fig. 5 B and Table I B). Between 72 h and 6 d after inoculation, the proportion of mice with any bacteria detected in the liver was also unchanged (Fig. 1; $P > 0.1$). Collectively, these results are consistent with the model that a few clones reached distal organ sites within 72 h after inoculation and replicated after arrival. Further support for this model was obtained by pooling the data from all the animals and determining the total number of clones found at each time point after oral inoculation. When the data were normalized to the total number of clones found in the original inocula, the disappearance and reappearance of clones in the spleen and liver was readily observed (Fig. 5 E). Based on the clonal dissemination data, these clones were not siblings of bacteria from the regional lymph nodes (Table II). Therefore, bacteria seeding the liver and spleen were probably derived from a reservoir other than the PP or MLN.

**Table II.** Association of clones in tissues

| | 30 min | 72 h |
|---|---|---|
| Liver vs. spleen | 23% | **98%**[a] |
| Liver vs. MLN | 52% | 3% |
| Liver vs. PP | ND | 25% |
| Liver vs. intestine | **100%**[a] | **87%**[a] |
| Spleen vs. liver | 56% | **93%**[a] |
| Spleen vs. MLN | ND | 3% |
| Spleen vs. PP | ND | 30% |
| Spleen vs. intestine | **100%**[a] | **79%**[a] |
| Intestine vs. liver | 38% | 32% |
| Intestine vs. spleen | 9% | 28% |

C57BL/6J mice were inoculated orogastrically with *Y. pseudotuberculosis* labeled with 33 individual DNA tags. At times after inoculation, the mice were killed and harvested organs were examined for the presence of the individual clones using the technique described in Materials and methods. Percent association is expressed as the mean frequency that a clone present in one organ (organ x) was also present in the second organ of the same mouse (organ x vs. organ y). The association of the clones within the two organs was analyzed using the chi-squared test and significance for association ($P < 0.05$) is denoted by percentages in bold.
[a]ND, not determined.

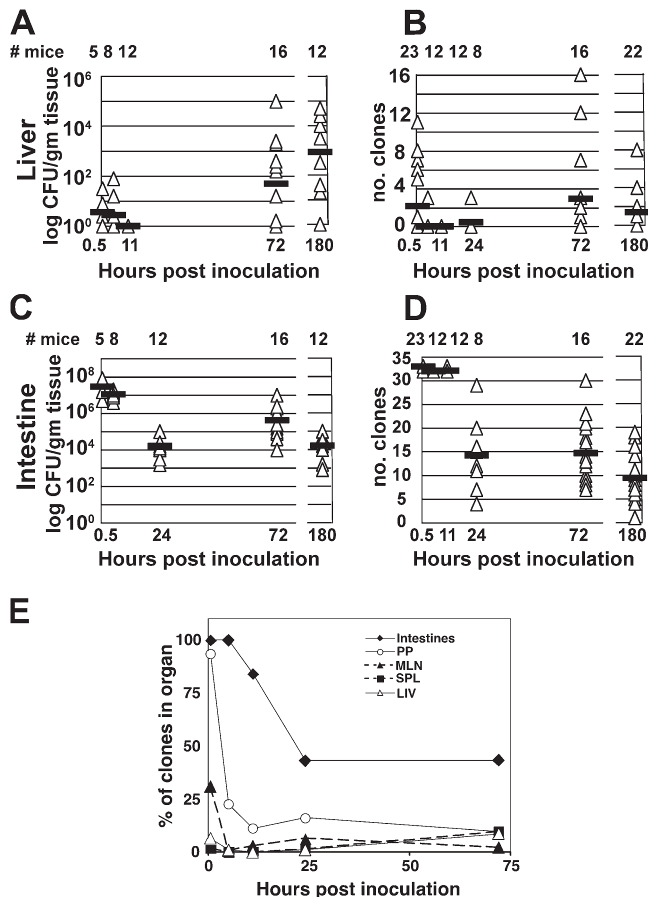


**Figure 5. Bacterial load and clonal dissemination of *Y. pseudotuberculosis*.** C57BL/6J mice were orally inoculated with $5 \times 10^8$ CFU of a pool of 33 uniquely tagged wild-type *Y. pseudotuberculosis* clones. At noted times after inoculation, the mice were killed and the liver (A and B) or intestines (C and D) (see Materials and methods for "intestines" definition) were assayed for the bacterial CFU (A and C) or the number of individual clones (B and D) (see Materials and methods). The results are pooled from separate experiments. Displayed above the graphs are the total number of mice examined at each time point and the number of experiments performed for each time point. Each triangle represents the CFU or number of clones isolated from the liver or intestines of one mouse in one experiment at each time point. Dark bars are the mean CFU or clones isolated from the livers of all mice at that time point. At some time points, the same CFU or number of clones was obtained in different mice; consequently, some triangles are superimposed. (E) Clonal distribution in infected organs over time. To determine the percentage of total clones found in each tissue, the total number of clones found in each tissue site was determined for all the animals killed at the noted time point. This number was then divided by the total number of clones that had been inoculated in all the animals analyzed at the noted time point to obtain the percentage.

### The second episode of hepatosplenic infection follows bacterial replication in the intestines

The kinetics of intestinal infection was analyzed next in the same mice used for liver and spleen clonal analysis (Fig. 5, C and D). All the clones inoculated were present in the intestines through 11 h after inoculation (Fig. 5 D and Table I B), but by 24 h there was a significant reduction in the number of clones to an average of 13 clones ($P < 0.01$; Fig. 5 D), accompanied by a fall to $5 \times 10^4$ CFU/g intestinal contents (Fig. 5 C). Between 24 and 72 h, replication in the intestines became apparent (Fig. 5 C) with a stable number of clones present in the intestine (Fig. 5, D and E). The appearance of bacteria in the liver at 72 h (Fig. 5 B) occurred after there was an expansion of the bacterial load in the intestines (Fig. 5 C and Table I B), consistent with the model that the pool of bacteria found in the intestinal lumen, rather than the lymph tissue, acts as the primary reservoir for the bacteria that cause late systemic disease. In support of this hypothesis, the liver and spleen populations of clones were usually a subpopulation of the intestinal clones found 72 h after oral inoculation (Table II; liver vs. intestine, spleen vs. intestine). Therefore, the clones that cause systemic disease appear to be derived directly from the bacteria present in the lumen of the intestines at this later time point, rather than from those present in local lymph nodes.

### Antibiotic-induced reduction of *Y. pseudotuberculosis* in the intestines lowers late stage systemic infection

To test the model that the clones that give rise to hepatosplenic infection were derived from bacteria that first replicated in the intestines, we used the antibiotic streptomycin to determine the effect of reduced intestinal bacterial load on extraintestinal dissemination. Streptomycin is reported to be poorly absorbed from the intestine (30), so oral administration is predicted to reduce intestinal but not spleen or liver bacterial replication. Mice were orally inoculated with *Y. pseudotuberculosis*, the initial dissemination events were allowed to progress, and then 1 mg of streptomycin was administered orogastrically at 5, 12, and 24 h after inoculation. The intestinal *Y. pseudotuberculosis* load was significantly reduced in streptomycin-treated mice at 24 and 72 h after inoculation, as compared with the PBS controls, demonstrating the efficacy of streptomycin killing of intestinal *Y. pseudotuberculosis* (Fig. 6 A). Despite early dissemination of *Y. pseudotuberculosis,* 72 h after oral inoculation of the bacteria, there was a significant reduction ($P < 0.05$) in the colony counts of *Y. pseudotuberculosis* in the livers of mice that had received oral streptomycin, compared with those that had received PBS (Fig. 6 B). Streptomycin did not leak into the bloodstream during intestinal *Y. pseudotuberculosis* infection, as serum streptomycin levels found in these animals were comparable to both uninfected, streptomycin-gavaged mice as well as to mice having no streptomycin treatment (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20060905/DC1). Additionally, if mice were inoculated intraperitoneally with *Y. pseudotuberculosis*, subsequent oral streptomycin administration produced no significant decrease in CFU in the liver (Fig. 6 C), confirming that systemic absorption of oral streptomycin was inefficient and its antimicrobial action was limited to bacteria within the intestines. Therefore, successful *Y. pseudotuberculosis* replication in the liver and spleen required an adequate intestinal bacterial load in addition to a period of intestinal replication.

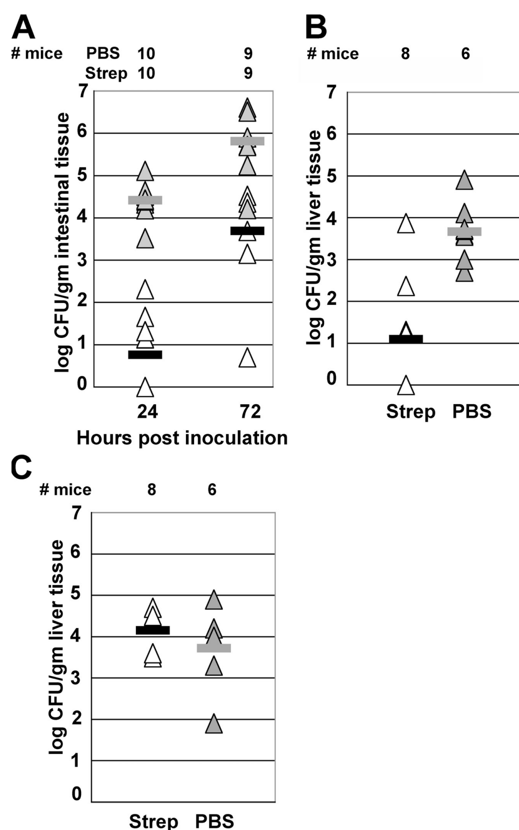


**Figure 6. Oral streptomycin reduces intestinal bacterial load and subsequent systemic infection after oral but not intraperitoneal inoculation.** C57BL/6J mice were orally inoculated (A and B) with $5 \times 10^8$ CFU of *Y. pseudotuberculosis* or inoculated i.p. (C) with $10^4$ CFU of *Y. pseudotuberculosis*. At 5, 12, and 24 h after inoculation, each mouse was orogastrically inoculated either with streptomycin (white triangles and black bars) or with PBS (gray triangles and bars). 24 and 72 h after inoculation, the intestines (see Materials and methods) were examined for CFU (A). The livers of the orally inoculated mice were also examined for colony counts 72 h after infection (B). The mice inoculated i.p. with *Y. pseudotuberculosis* were killed 72 h after inoculation and examined for CFU (C). The results are pooled from three separate experiments. Displayed above the graph is the total number of mice examined at each time point. Each triangle represents the CFU of a mouse. Bars are the mean number of bacteria found in the tissues from all experiments. At some time points, similar colony counts were obtained in different mice and the triangles are superimposed.

## DISCUSSION

There are two striking results from this study. First, *Y. pseudotuberculosis* did not efficiently initiate replication in systemic infection immediately after oral inoculation, as replication in the intestines appeared necessary before successful hepatosplenic infection. Second, liver and spleen colonization was independent of preliminary replication in the PP and MLN. These results conflict with models that propose systemic infections by enteropathogens proceeding in ordered pathways in which translocation across M cells and intestinal lymph tissue infection are prerequisites for systemic disease (27). The data do support, however, studies indicating that systemic disease can be initiated with bacterial mutants that are defective for colonization of local lymph nodes (9, 23). Although a route may exist through the intestinal lymphatic system that leads to systemic disease, the only evidence consistent with PP entry being essential for colonization of more distal tissues was the inability of PP-defective mice lacking functional B cells to support dissemination of bacteria shortly after oral inoculation. There is no evidence, however, that this early dissemination was necessary or sufficient for growth of bacteria in the liver and spleen. These results are consistent with the observation made from signature tagged mutagenesis studies of *Y. pseudotuberculosis*, in which bacterial clones could be isolated from liver and spleen that were not found in localized lymph nodes (22). These results also support prior findings of bottlenecks limiting dissemination from local to distal infection sites, as observed with enteropathogens (32, 33) and viruses (34).

The combined results of the clonal and kinetic analyses of spleen and liver colonization indicate that there is little connection between dissemination events that occur immediately after oral inoculation and systemic infection (Fig. 5; Table II). During the 24-h period after oral inoculation that witnessed loss of culturable bacteria from spleen and liver, clones were concurrently depleted from the intestinal pool. At 72 h after inoculation, the bacteria found in distal tissue sites were derived from the intestinal pool that remained after this depletion, arguing that bacteria in these organs are derived from clones that have first replicated in the intestine. The observed increase in intestinal bacterial load that occurred simultaneously with the second systemic dissemination is consistent with this intestinal population being the source of the disseminated bacteria, rather than the parent being the initial bolus that appeared in spleen and liver immediately after inoculation. This model was further supported when intestinal *Y. pseudotuberculosis* load was reduced by administration of the locally acting antibiotic, streptomycin, which in turn reduced late hepatosplenic infection. Therefore, it appears that the bacterial pool that translocates into spleen and liver is derived from a population that has been established in the intestinal lumen.

Infection of the PP-deficient BALB/c-$J_H$D mouse revealed no early bacterial translocation into spleen and liver, despite wild-type levels of colonization in these organs 6 d after inoculation. The absence of early bacterial dissemination in these mutant mice is consistent with the model that bacteria causing systemic disease are not derived from the population that crosses the epithelium shortly after oral inoculation. Either B cells or, more likely, B cell–dependent processes such as PP organogenesis, are important for early but not late stage extraintestinal dissemination. As we showed that tissues associated with immune sampling are important for the early dissemination events, it seems likely that the appearance of bacteria shortly after oral inoculation is advantageous to the host, because it allows early immune surveillance, defending against eventual bacterial replication in these sites.

During the second phase of infection, most of the bacterial clones observed in the liver and spleen were not derived from clones growing in regional lymph nodes (Table II), results consistent with prior observations that both *Salmonella* (35) and *Y. enterocolitica* (36) colonize the spleen and liver of lymphotoxin mutant mice after oral infection. Given that these other enteropathogens are both subject to bottlenecks limiting extraintestinal dissemination and use PP-independent routes of invasion, it seems likely that our results are not solely representative for *Y. pseudotuberculosis*. Collectively, these results suggest that undetermined routes may exist that lead the bacteria across the intestine before establishing systemic disease. There are at least three possible translocation mechanisms that bypass the PP, initiated from bacterial pools found within either the small intestine or colon (reference 37 and unpublished data). First, host or bacterial process may cause local micro-damage in the intestinal epithelium, providing sites for translocation. For instance, effectors from the *Yersinia* type III secretion system may break down the tight junctions between intestinal epithelial cells (38, 39), as could natural apoptotic processes occurring at the top of intestinal villi (40), permitting translocation of bacteria. Second, villous-associated M cells have recently been identified (41) that allow sampling of intestinal contents and are hypothetically portals across the epithelium. In fact, introduction of *Y. pseudotuberculosis* into intestinal ligated loops isolated from mutant mice lacking PP results in bacterial association with these newly identified cells (41), although no evidence has been provided that infection of regional lymph nodes or distal organs such as liver or spleen results from such a relationship, Finally, phagocytic or dendritic cells interdigitated within the intestinal epithelium have been demonstrated to sample luminal bacteria (42–44), and evidence exists for phagocytic routing of *S. typhimurium* across the intestine. Recent work indicates that transepithelial extensions of dendritic cells facilitate sampling of intestinal bacteria, dependent on the presence of chemokine receptor CX3CR1 (42), and these could be major sites of attachment by intestinal pathogens. The processes that lead to translocation across the intestine may not be particularly efficient, as large loads of bacteria in systemic infection sites can be derived from as few as one intestinal clone. Furthermore, the routes that establish systemic disease may not be identical in every animal.

Two pathways may route *Y. pseudotuberculosis* to distal infection sites once the organisms penetrate the intestinal epithelial layer as either free or host cell-associated bacteria. Blood-borne bacteria can travel vial the portal vein system, which transports intestinal blood directly to the liver. This allows unfiltered bacteria to transit to other downstream blood-filtering organs such as the spleen. Alternatively, bacteria entering via the lymphatics may first route to the mesenteric lymph nodes, drain into the thoracic duct and ultimately flow into the bloodstream. In either case, the colonization of organs such as the liver or spleen indicates that bacteria are present in the bloodstream at some point. Although it is possible that the initial conduit for disseminating bacteria is via intestinal lymph fluid with subsequent entry into the mesenteric lymph nodes, our data indicate that this would probably occur without colonizing this organ. Whatever the route chosen, it is likely that either free or host cell–associated enteropathogenic bacteria are provided with multiple strategies for initiating dissemination. Supporting this hypothesis is a recent study of mutant mice lacking either $CX3CR1^+$ or $CD11c^+$ dendritic cells in the intestinal lamina propria; invasion of a model enteropathogen across the intestinal epithelium can still occur in these mutant mice (45).

The results presented here raise the possibility that *Y. pseudotuberculosis* populations found in distal tissue sites are never derived from organisms growing in the regional lymph nodes. We believe, however, that exit from the regional nodes can still occur in our mouse model, even though there is severe attenuation of movement from the PP and MLN into the liver and spleen during systemic disease. In support of some movement out of the regional lymph nodes is the observation that the levels of bacteria found in the livers of the LT $\beta^{-/-}$ mouse lacking PP were not as high as in C57BL/6J (Table IA), even though systemic disease still occurred in the mutant. An explanation for this result is that loss of PP in the LT$\beta^{-/-}$ mouse results in loss of one of several possible translocation routes across the intestine, causing a reduction, but not elimination, of bacteria translocating to the liver. Furthermore, although the clonal analysis showed that none of the disseminated bacteria appear derived from colonies found in the regional lymph nodes of some of the animals, in other animals occasional clones were identified in the distal tissue sites that may have been derived from the lymph node population (Table II). As systemic disease requires the occurrence of only a few productive translocation events, small numbers of bacteria could certainly translocate across various sites in the intestine, including the regional lymph nodes. Single clones from any of these sites would then be sufficient to give rise to a large population of bacteria in systemic infection sites (Fig. 5).

Broth-grown *Y. pseudotuberculosis* appears to be poorly prepared to initiate replication in systemic infection sites after oral inoculation. The strong connection between intestinal growth of bacteria and successful systemic disease argues that bacterial proteins facilitating hepatosplenic infection are upregulated relative to broth culture during prolonged growth in the host. There are large numbers of virulence-associated proteins encoded by *Yersinia* species that are candidates for being activated within such a niche, because both the type III protein translocation system and the translocated Yop substrates of this system are selectively expressed at physiological temperature (10). As only a subset of Yops is required for intestinal survival (37), perhaps the intestine provides a permissive niche for the microorganism to express the full armory of virulence factors required for replication in the more restrictive tissues of liver and spleen. The Yop translocated substrates are involved in several biochemical processes that impair host immune cell function, and Yop expression is critical for successful systemic disease (10). In humans,

enteropathogenic *Yersinia* infection is usually a self-limiting illness, with no systemic spread. Perhaps, unlike in the mouse, replication in the human intestine does not induce the appropriate virulence factors required for the microorganism to deal with the highly restrictive nature of distal tissue sites in humans, or the intestinal innate immune response is sufficient to restrict *Y. pseudotuberculosis* to the human intestine.

The work described here demonstrates the complexity of systemic disease initiated by oral inoculation of an enteropathogen into a mammal. Although it may be attractive to describe the route taken by a pathogen as if it were a large-scale military invasion from a single beachhead, we do not believe that this accurately describes what occurs before establishing lethal disease. Rather, it appears that *Y. pseudotuberculosis* colonizes the intestinal niche, which possesses several entry portals for dissemination to systemic infection sites. The microorganism may even take advantage of delaying entry into the spleen and liver, as immediate entry into these organs does not result in productive replication. During establishment of intestinal colonization, the bacteria seem to armor themselves to allow replication in these sites and to guard themselves against host assault. This facilitates the eventual small-scale breaches of intestinal defenses. The relationship of the microorganism with the regional lymph nodes is similarly complex, as the low efficiency of movement out of these glands may be a reflection of the fact that the organism is inducing host defenses to ensure a localized disease and subsequent transmission to other hosts. Further work will focus on this tension between establishing a local and systemic disease, defining changes in *Y. pseudotuberculosis* that allow the organism to move from an intestinal site to extraintestinal organs, and identifying the many portals that allow movement out of the intestine.

## MATERIALS AND METHODS

**Bacterial strains, growth conditions, and clonal analysis of *Y. pseudotuberculosis*.** All bacterial strains are derived from YPIII($P^+$), a *Y. pseudotuberculosis* serogroup III strain (46). YPIII($P^+$) was passaged in mice and isolated from liver to obtain the strain M31. The strain PB1000 refers to M31 harboring pACYC184 derivatives having unique oligonucleotide tags.

Unique genetic sequences (tags) used to label clones of wild-type *Y. pseudotuberculosis* were inserted into the plasmid pACYC184 in *Escherichia coli* (47). pACYC184-tag plasmids were electroporated into *Y. pseudotuberculosis*. The pACYC184-tag plasmids were constructed as follows. The plasmid carrying oligonucleotide tags, (pUTminiTn5Kn2$^+$tags; references 22, 31) and pACYC184 were electroporated into *E. coli* strains SM10λpir and HB101, respectively. Transpositions of miniTn5Kn2$^+$ into pACYC184 were isolated after mating, selecting for kanamycin (30 μg/ml), and tetracycline (5 μg/ml) resistance. Loss of pUTminiTn5Kn2 transposition vector and insertions into the pACYC184 Cam$^R$ gene were determined by ampicillin and chloramphenicol sensitivity, respectively.

For probing of pools of oligonucleotide-tagged strains, the DNA-tags were amplified by PCR using the P2 and P4 primers described previously (22, 31). To separate the tag from the primer arms, the PCR product was digested with HindIII overnight at 37°C and the 40-bp tag was separated from the 20-bp primer arms by electrophoresis on an 8% polyacrylamide gel and eluted into TE at 4°C overnight. 20 μl of each tag-elute was placed onto nitrocellulose membranes (Schleider & Schuell Nytran) in a grid pattern using a 96-well vacuum dot-blotter (Bio-Rad Laboratories). The DNA was denatured with 0.5M NaOH and after neutralization and crossed linked to the membrane with ultraviolet light (Stratagene). After use, membranes were stripped according to the manufacturer's instructions (Roche) and reused multiple times.

For labeling of the DNA probes, each plasmid preparation was amplified by PCR using the P2 and P4 primers and incorporating digoxigenin dNTP (Roche) according to the manufacturer's instructions, except the ratio of labeled dNTP to unlabeled dNTP was 5:1. The probe was hybridized at 42°C overnight to the target membranes in hybridization buffer. Plasmids were identified that showed no cross-hybridization to other tags, and each tag was placed at a defined address on membranes in subsequent experiments. These membranes were stripped and reused multiple times and stored in PBS at 4°C.

**Preparation of *Y. pseudotuberculosis* for oral inoculation into mice.** One colony of *Y. pseudotuberculosis* freshly streaked on LB plates was grown in either LB (Figs. 1 and 6) or brain heart infusion (BHI) broth supplemented with 30 μg/ml kanamycin and 5 μg/ml of tetracycline (when needed) at 26°C to an $A_{600} = 4.4$. 1 ml of bacteria from this culture was washed twice with PBS and resuspended in 2 ml of PBS. 200 μl of this bacterial suspension contained $5 \times 10^8$ CFU, and was orogastrically inoculated into the mice.

**Mouse strains and husbandry.** BALB/cJ-$J_H$D mice, defective in Ig heavy chain switching (27, 48), and the lymphotoxin β-deficient C57BL/6J (LTβ$^{-/-}$) (29) mice were bred in house. BALB/c and C57BL/6J mice were obtained from The Jackson Laboratory at 5 wk of age and allowed to acclimatize to the new environment for 1 wk before the experiments. All mice used were 6-wk-old females.

**Orogastric mouse infections.** Mice were starved of food for 16 h before inoculations, but were provided water during this period. 200 μl of the suspension of *Y. pseudotuberculosis* was orogastrically inoculated into mice using a 21-gauge stainless steel ball-tipped feeding needle (Harvard Apparatus Inc.). If aspiration of inocula occurred during infection (visual detection of fluid after nasal respiration), mice were immediately killed and excluded from the experiment. The mice were starved of food for a further 90 min after infection and permitted food and water ab libatum. At various time points after inoculation, the mice were killed by $CO_2$ asphyxiation. If required, blood was obtained by a direct cardiac puncture immediately postmortem. All PP, MLN, liver, and spleen tissue that could be identified in the time course were retrieved. Intestinal tissue retrieved was the distal inch of terminal ileum (after the PP had been removed), cecum (minus PP), and proximal inch of the large intestines. The intestinal luminal contents were included in this sample.

Tissue for bacterial colony counts was placed in 200 μl of PBS immediately after dissection and stored on ice until samples were weighed. 800 μl of PBS was added to the vial and the tissue homogenized using a TissueTearor (Biospec). The total homogenates of all the organs, as well as serial dilutions of the homogenates were inoculated onto MacConkey-lactose agar plates. Colonies were counted after 48 h incubation at 28°C.

Tissue for the broth recovery method was diced into small pieces (∼5 mm × 5 mm) and inoculated into 3 ml of BHI supplemented with 30 μg/ml kanamycin and 5 μg/ml of tetracycline. To eradicate DNA cross-contamination between each organ and each mouse, a separate set of dissection instruments was used for removal of each organ from each mouse. In addition, instead of autoclaving, immediately after use, the instruments were placed in bleach, 1 ppm, scrubbed with soap and water, soaked for a further 20 min in bleach, rinsed, and air dried under ultraviolet light.

After dicing of the organs, the BHI/tissue suspension was placed in a shaker overnight at 28°C, and 250 μl of the BHI/tissue suspension was inoculated onto MacConkey and BHI plates both supplemented with kanamycin and tetracycline. After 48-h incubation at 28°C, plasmid preparations were made from the pool of arising colonies. The tags were amplified and digoxigenin labeled using PCR and detected by chemiluminescence (Roche).

All animal experiments used protocols approved by the Tufts University School of Medicine Division of Laboratory Animal Medicine.

**Mouse inoculations in the presence of streptomycin.** 5, 12, and 24 h after oral or i.p. inoculation of bacteria, the animals were orogastrically reinoculated with 1 mg of streptomycin suspended in 200 μl of PBS, or 200 μl of PBS-only control. 24 or 72 h after the bacterial inoculation, the mice were killed and the intestines, livers, and spleens homogenized for colony counts as described in the preceding section.

**Statistical analysis of results.** The variability of *Y. pseudotuberculosis* spleen and liver infections rendered the resulting data nonparametric. Therefore, the difference between the colony counts in two groups of tissues examined was compared using the Mann-Whitney U-test. The frequency of infection in two populations of mice, assayed using the broth recovery technique, was compared using two-sided Fisher's exact test mid-P. The association between clones in tissues was analyzed using the chi-squared test. Significance for all tests was defined by a p-value of $\leq 0.05$.

**Determination of streptomycin concentrations in bloodstream of infected mice.** Groups of mice were either mock inoculated with PBS or inoculated with $5 \times 10^8$ CFU of *Y. pseudotuberculosis*, followed by oral gavage with 1 mg of streptomycin at 5, 12, and 24 h after infection. Blood was obtained by cardiac puncture at 24 h after infection into serum separator tubes (Becton Dickinson) and centrifuged and resulting serum was pooled from individual mice to yield a minimum of 0.5 mL serum per condition. Controls included serum from uninfected mice (normal mouse serum), serum from mice injected intravenously with 200 μg of streptomycin, and normal mouse serum spiked with streptomycin to give 100 μg/ml final concentration. Serum samples were stored frozen at −70°C until assayed for streptomycin levels by an HPLC-based assay (Focus Diagnostics). The minimal inhibitory concentration of streptomycin upon *Y. pseudotuberculosis* growth was determined to be 12.5 μg/ml.

**Online supplemental material.** Experiment that measures the concentration of streptomycin in the mouse bloodstream after oral *Y. pseudotuberculosis* inoculation is found online as Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20060905/DC1.

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