GROWTH OF *YERSINIA PSEUDOTUBERCULOSIS* IN MOUSE SPLEEN DESPITE LOSS OF A VIRULENCE PLASMID OF MOL. WT 47×10^6

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SUMMARY. A highly virulent strain of *Yersinia pseudotuberculosis* (LD₅₀ c. 10^2 bacteria/mouse) harboured two plasmids with mol. wt of 47×10^6 and 61×10^6 . The role of these plasmids in virulence was studied in mice with derived strains cured of plasmids. It was confirmed that the plasmid of mol. wt 47×10^6 played a major function in virulence. This was shown both by the increase of the LD₅₀ and the lower rate of multiplication in the spleen obtained with strains cured of the plasmid of mol. wt 47×10^6 . The plasmid of mol. wt 61×10^6 did not play any role in virulence. This work also demonstrates that the strain cured of the plasmid of mol. wt 47×10^6 and the plasmid-free strain were able to multiply in the spleens of infected mice during a 7-day period. This suggests that virulence factors not associated with plasmids are also responsible for the bacterial growth in tissues *in vivo*.

INTRODUCTION

Yersinia pseudotuberculosis causes epizootic outbreaks with various patterns of disease in birds and in domestic and wild mammals. It may also cause disease in man (Mollaret, 1965). Several authors have reported that plasmids with mol. wt between 42×10^6 and 47×10^6 are associated with the virulence of the various species of *Yersinia*, i.e., *Y. enterocolitica*, *Y. pestis* and *Y. pseudotuberculosis* (Gemski, Lazere and Casey, 1980a; Gemski *et al.*, 1980b; Ben-Gurion and Shafferman, 1981; Portnoy and Falkow, 1981). It has been shown that the presence of these plasmids is related to the synthesis of V and W antigens which appear to be essential for the virulence of the genus *Yersinia* (Brubaker, 1972). Pathogenicity of *Y. pseudotuberculosis* has been studied by inducing keratoconjunctivitis in the guinea pig (Gemski *et al.*, 1980b; Mäki *et al.*, 1983) and by assessing mortality in the mouse (Ben-Gurion and Shafferman, 1981; Bölin, Norlander and Wolf-Watz, 1982). The purpose of this work was to study the virulence of *Y. pseudotuberculosis* strains with or without a plasmid of mol. wt 47×10^6 by following the kinetics of bacterial growth in the spleens and survival of infected mice.

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MATERIALS AND METHODS

Bacterial strains. The strain of *Y. pseudotuberculosis* used in these experiments was isolated from a blood culture from a patient with fatal septicaemia (Simonet, Berche and Mazigh, 1983); it was *Y. pst.* 2637 (Collection of the Centre National des Yersinia, Institut Pasteur, Paris). Bacteria were grown in trypto-casein soy broth (TCSB) or agar (TCSA). Stock cultures were kept at -70°C until required.

A strain of *Y. pestis* (*Y.p.* EV 76) harbouring three plasmids of mol. wt 6×10^6 , 47×10^6 and 61×10^6 (Portnoy and Falkow, 1981), and a strain of *Y. enterocolitica* 09 (*Y.e.* IP 383) harbouring a plasmid of mol. wt 47×10^6 (Mazigh *et al.*, 1983) were used as controls.

Screening and cure of plasmids. Plasmid DNA was isolated from bacteria grown at 25°C in peptone water and analysed by electrophoresis in 0.7% agarose gels according to the method of Portnoy, Moseley and Falkow (1981). The gel was stained by ethidium bromide and DNA was visualised in UV light. The mol. wt of plasmids extracted from *Y. pst.* 2637 and its derivative *Y. pst.* 2637a were estimated by comparison of their distances of migration with those of plasmids of known mol. wt extracted from reference strains *Y.p.* EV76 and *Y.e.* IP383. Loss of plasmids was obtained by five subcultures of *Y. pseudotuberculosis* strains at 37°C or 43°C in TCSB, according to classical methods for temperature-induced cures (Cuzin and Jacob, 1967; Vesikari *et al.*, 1981).

Infection of mice. Specific pathogen-free ICR female Swiss mice (Iffa Credo, France), 6 weeks old, were given intravenous (IV) injections of 0.5 ml of various dilutions of an overnight bacterial culture grown at 25°C in TCSB. The LD50 was determined in groups of five mice by the probit method. Growth of bacteria in the spleen was followed in relation to time after the IV injection. Groups of four animals were killed by cervical dislocation and the organs were removed aseptically and homogenised separately in 9 ml of sterile saline; 0.1-ml volumes of serial ten-fold dilutions in saline were spread on TCSA and colonies were counted after incubation for 48 h at 25°C .

RESULTS

Strain *Y. pst.* 2637 was subcultured five times in TCSB at 37°C and a derivative, designated *Y. pst.* 2637a, was obtained after screening five colonies for the presence of plasmids. This derivative was then subcultured five times at 43°C , which allowed us to isolate, after the same screening, a second derivative designated *Y. pst.* 2637b. The agarose gel electrophoretic profiles of plasmid DNA extracted from the parent *Y. pst.* 2637 strain and the two temperature-induced derivatives are illustrated in fig. 1. The parent strain harboured two plasmids which migrated like the plasmid DNA of the reference strains used as controls, *Y.p.* EV76 (6×10^6 , 47×10^6 and 61×10^6 mol. wt) and *Y.e.* IP383 (47×10^6 mol. wt). The derivative *Y. pst.* 2637a harboured only the plasmid of mol. wt 61×10^6 , whereas the derivative *Y. pst.* 2637b had no plasmid detectable by this electrophoretic method (fig. 1).

The virulence of the parent strain and its two derivatives was studied by determining their LD50. The parent strain (*Y. pst.* 2637) was highly virulent in mice; the LD50 after IV injection was *c.* 10^2 bacteria/mouse. Loss of the plasmid of mol. wt 47×10^6 resulted in a 10^4 increase of the LD50. The LD50 of the strains *Y. pst.* 2637a and 2637b were assessed to be *c.* $1-2 \times 10^6$ bacteria/mouse.

The bacterial growth in the spleens of mice was measured in mice infected by the parent strain and its two derivatives (dose 5×10^3 bacteria IV). The data obtained during a 14-day period are shown in fig. 2. The parent strain rapidly proliferated in the spleen during the first 3 days with early formation of abscesses. After the third day, mice began to die and no mice survived after day 6. In contrast, after inoculation of either

FIG. 1.—Agarose gel electrophoresis of plasmids extracted from various strains of *Yersinia*. The tracks contain DNA from strains *Y.p.* EV 76 (A), *Y. pst.* 2637 (B), *Y. pst.* 2637a (C), *Y. pst.* 2637b (D), *Y.e.* IP 383 (E).

strain *Y. pst.* 2637a or strain *Y. pst.* 2637b, bacteria grew more slowly in the spleen than did those of the parent strain. This growth reached a peak by day 7 and then the number of bacteria steadily declined until they were almost completely eliminated by day 21 (data not shown). Electrophoretic analysis of lysates of 10 colonies isolated from the spleen on day 7 of infection with *Y. pst.* 2637a and *Y. pst.* 2637b, showed the absence of the plasmid of mol. wt 47×10^6 in these two derivatives, whereas *Y. pst.* 2637a still harboured the plasmid of mol. wt 61×10^6 .

DISCUSSION

The parent strain of *Y. pseudotuberculosis* used in this study harbours two plasmids (fig. 1): (i) a plasmid of mol. wt 47×10^6 as previously described in virulent strains of *Y. pseudotuberculosis* (Gemski *et al.*, 1980b; Ben-Gurion and Shafferman, 1981); and (ii) a plasmid of mol. wt 61×10^6 , so far never described in *Y. pseudotuberculosis*, which migrated similarly to the cryptic plasmid of mol. wt 61×10^6 of *Y. pestis* (Ferber and Brubaker, 1981; Portnoy and Falkow, 1981). This strain was highly virulent in mice ($LD_{50} = c. 10^2$ bacteria/mouse) and quickly invaded the spleen (fig. 2).

The loss of the plasmid of mol. wt 47×10^6 in the two derived strains of *Y. pseudotuberculosis* resulted in loss of virulence ($LD_{50} = (1-2) \times 10^6$ bacteria/mouse). This confirms the major role of the plasmid of mol. wt 47×10^6 in virulence of

FIG. 2.—Bacterial growth of *Y. pseudotuberculosis* in the spleens of mice given IV injections of 5×10^3 bacteria prepared from the parent strain *Y. pst.* 2637 (▲—▲) or its two derivatives *Y. pst.* 2637a (■ · · · · ■) and *Y. pst.* 2637b (●-----●). Each point is the mean value of groups of 4 mice $\pm$ SD.

yersiniae (Gemski *et al.*, 1980*a* and *b*; Ben-Gurion and Shafferman, 1981; Portnoy and Falkow, 1981). However, it is shown in fig. 2 that the two derived strains, harbouring or not the plasmid of mol. wt 61×10^6 , were still capable of growing in the spleen during a 7-day period. These data suggest that the plasmid of mol. wt 61×10^6 is probably not involved in virulence and its role remains unknown. Moreover, some strains of *Y. enterocolitica* that lack the virulence-associated plasmid are rapidly eliminated from organs of infected mice without any growth (Berche and Carter, 1982; Mazigh *et al.*, 1983) and our data with *Y. pst.* 2637 and its derivatives suggest that factors not associated with plasmids are responsible for the bacterial growth in organs observed when the plasmid of mol. wt 47×10^6 was absent.

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