INFECTION AND IMMUNITY, Jan. 1997, p. 327–330 Vol. 65, No. 1
0019-9567/97/$04.00+0

Reevaluation of the Virulence Phenotype of the *inv yadA* Double Mutants of *Yersinia pseudotuberculosis*

YIPING W. HAN[1] AND VIRGINIA L. MILLER[1,2]*

Department of Microbiology and Molecular Genetics[1] and Molecular Biology Institute,[2] University of California—Los Angeles, Los Angeles, California 90095

Received 15 July 1996/Returned for modification 13 September 1996/Accepted 16 October 1996

***Yersinia pseudotuberculosis* and *Yersinia enterocolitica* are closely related human pathogens causing gastroenteritis. Invasin and YadA are two of the most extensively studied virulence factors of the *Yersinia* genus. Invasin is the primary invasion factor encoded by the *inv* gene on the chromosome and is required for the penetration of the epithelial cells. YadA is encoded by the *yadA* gene on the 70-kb virulence plasmid and has multiple functions. Previous studies indicate that an *inv yadA* double mutant of *Y. enterocolitica* is avirulent while an *inv yadA* mutant of *Y. pseudotuberculosis* is hypervirulent. In this study, we investigated this unexpected difference. New constructs of the *inv yadA* mutants of *Y. pseudotuberculosis* were made and tested in mice. These new constructs were not hypervirulent; rather, they maintained the same virulence as the wild-type strain. Further examination of the *inv* mutant used for the previous study revealed that it carries an aberrant *inv* phenotype and has an altered outer membrane profile and an altered colony morphology. Therefore, the mutants used previously were not isogenic to the parental wild-type strain, which may in part account for the difference in the results obtained.**

Yersinia pseudotuberculosis and *Yersinia enterocolitica* are closely related human pathogens that cause gastroenteritis (for a recent review, see reference 4). When ingested orally, they can arrive at the terminal ileum of the small intestine, penetrate the epithelial layer, and reach their primary infection site, the Peyer's patches (7). In a mouse model and occasionally in humans, the bacteria can spread to deeper tissues, such as the liver and the spleen, to cause systemic infections (for a review, see reference 5). Virulence factors conserved between the two species have been identified on both the chromosome and the 70-kb virulence plasmid (1, 9, 15, 17, 21). The primary invasion factor, invasin, is encoded on the chromosome. The invasin of *Y. pseudotuberculosis* is an outer membrane protein of 103 kDa (9–11), while that of *Y. enterocolitica* is 92 kDa (15, 18). It has been shown for *Y. enterocolitica* that invasin allows the bacteria to penetrate the intestinal epithelium and reach the Peyer's patches (19). YadA is encoded on the virulence plasmid and has multiple functions, including the attachment and invasion of cultured mammalian cells, serum resistance, autoagglutination, and formation of fibrils (2, 8, 12, 14, 20, 25–27). In vivo, it has been shown that YadA is required for the persistent survival of *Y. enterocolitica* in the Peyer's patches (20). According to previous observations, the *inv yadA* double mutants of *Y. enterocolitica* and *Y. pseudotuberculosis* exhibit contrasting phenotypes in mice. For *Y. enterocolitica*, such a mutant was avirulent, with a 50% lethal dose (LD_{50}) 10,000-fold higher than that of the wild type (20). In contrast, the *inv yadA* double mutant of *Y. pseudotuberculosis* became hypervirulent, with LD_{50}s 100- to 1,000-fold lower than that of the wild type (23). This difference in the phenotypes of *inv yadA* mutations in *Y. enterocolitica* and *Y. pseudotuberculosis* suggests either that *Y. pseudotuberculosis* has virulence factors that are unmasked in the double mutant or that invasin and YadA perform different functions in the two species. To investigate this difference further, we first sought to reproduce the results obtained by Rosqvist et al. (23) for *Y. pseudotuberculosis*. This was done by testing newly constructed *inv* and *yadA* mutants along with those used by Rosqvist et al. (23).

Construction of the *inv yadA* double mutants. Summarized in Table 1 are the strains and plasmids used in this study. Throughout this paper, the strains that were obtained from the laboratory of H. Wolf-Watz or that were identical to the ones used by Rosqvist et al. (23) are marked with asterisks (*) in order to distinguish them from those constructed for this study.

(i) *inv* mutants. SF104 was obtained from R. Isberg and has a kanamycin resistance marker inserted in the *Cla*I site in the 5′ end of the gene (11). YP100* was obtained from H. Wolf-Watz and has a 2.2-kb Tn*5*::*kan* element inserted in the *Xho*I site in the 3′ end of the gene (23).

(ii) *yadA* mutants. Three different virulence plasmids carrying a *yadA* mutation were used, i.e., pIB103*, pYH4, and pYH7. pIB103* was obtained from H. Wolf-Watz and has a Tn*5*-132 (*tet*) element inserted in the 5′ end of the gene (6). pYH4 and -7 were constructed as follows. The *yadA*-containing plasmid pAY04 (27) was digested with *Nde*I, followed by (i) a religation to generate pYWH202 so that the amino acids 21 to 376 of YadA were deleted in frame or (ii) a replacement of the *Nde*I fragment of *yadA* with the *tet*-containing *Ssp*I-*Pvu*II fragment from pBR322 to generate pYWH204, resulting in a *yadA* mutant with a tetracycline resistance marker. Subsequently, both pYWH202 and -204 were digested with *Bst*BI and *Xba*I, and the *yadA*-containing fragments were isolated and cloned into the *Cla*I-*Xba*I sites of the suicide vector pEP185.2 (13) to generate pYWH210 and pYWH205, respectively. The latter two plasmids were then conjugated into appropriate *Y. pseudotuberculosis* strains by filter mating as described previously (13). Chloramphenicol-resistant exconjugants were selected and purified, followed by cycloserine enrichments for clones that resolved the plasmids yet still retained the mutations as previously described (20). The constructs were all verified by Southern blotting as described previously (16) (data not shown). The virulence plasmids containing different *yadA* mutations were introduced into identical chromosomal back-

* Corresponding author. Present address: Department of Molecular Microbiology, Campus Box 8230, Washington University School of Medicine, St. Louis, MO 63110-1093. Phone: (314) 747-2132. Fax: (314) 747-2135.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid[a]	Description and/or relevant genotype[b]	Source or reference
Strains		
E. coli S17-1λ*pir*	RP4-2-*tet*::Mu *kan*::Tn7 Tp Sm Pro$^-$ res$^-$ mod$^+$	24
Y. pseudotuberculosis		
YPIII*	Wild type	23
YPIII	Wild type	Lab strain
YPIIc	Cured of plasmid	This study
SF104	YP202 *inv*::*kan*	11
YP100*	*inv*::*kan*	23
SF104c	*inv*::*kan*, cured of virulence plasmid	This study
YP100*c	*inv*::*kan*, cured of virulence plasmid	This study
YPIII/pYH4	*yadA*::*tet*	This study
YPIII/pIB103*	*yadA*::Tn*5*-132 (*tet*)	This study
YPIII*/pIB103*	*yadA*::Tn*5*-132 (*tet*)	23
YP100*/pIB103*	*yadA*::Tn*5*-132 *inv*::*kan*	This study
YP100*/pYH4	*yadA*::*tet inv*::*kan*	This study
SF104/pYH4	*yadA*::*tet inv*::*kan*	This study
SF104/pYH7	Δ*yadA* (in frame), *inv*::*kan*	This study
Plasmids		
pAY04	Wild-type *yadA* clone	27
pEP185.2	Suicide plasmid vector	13
pYWH202	Δ*yadA* (in frame) clone	This study
pYWH204	*yadA*::*tet* clone	This study
pYWH205	*yadA*::*tet* in suicide vector pEP185.2	This study
pYWH210	Δ*yadA* (in frame) in suicide vector pEP185.2	This study

[a] *, obtained from the laboratory of H. Wolf-Watz.
[b] *tet*, tetracycline; *kan*, kanamycin; Sm, streptomycin; Tp, trimethoprim; res$^-$ mod$^+$, restriction negative and modification positive.

grounds with the wild-type or mutant *inv* locus by electroporation as described previously (20). The resulting recombinant strains were verified again by Southern blot analysis (data not shown) and are listed in Table 1.

Tissue culture invasion assays. The various *Y. pseudotuberculosis* constructs listed in Table 1 were tested for their ability to invade CHO cells as described previously (15) (Table 2).

TABLE 2. Tissue culture invasion and LD_{50} assays of wild-type and mutant *Y. pseudotuberculosis* strains

Strain[a]	% Invasion[b]	LD_{50}[c]
YPIII*/pIB1* (WT)	114	4.9×10^5
YPIII/pIB1 (WT)	89	5.4×10^4
YPIII*/pIB103* (*yadA*)	49	2.5×10^6
YPIII/pYH4 (*yadA*)	14	9.5×10^5
YP100*/pIB1* (*inv*)	1.7	4.4×10^5
SF104/pIB1 (*inv*)	3.6	3.0×10^5
YP100*/pIB103* (*inv yadA*)	0.1	8.2×10^4
YP100*/pYH4 (*inv yadA*)	0.3	3.7×10^4
SF104/pYH4 (*inv yadA*)	0.2	5.3×10^5
SF104/pYH7 (*inv yadA*)	0.5	8.8×10^6

[a] *, obtained from the laboratory of H. Wolf-Watz. WT, wild type.
[b] Bacteria (grown at 26°C overnight) were added to monolayers of CHO cells and allowed to incubate at 37°C for 45 min to 1 h. Extracellular bacteria were washed away with phosphate-buffered saline, and attached bacteria were killed by the addition of gentamicin for 30 min, followed by additional washes. The CHO cells were then lysed with Triton X-100 to release intracellular bacteria, which were then diluted and spread on selective plates. The invasion activity was expressed as percent intracellular bacteria with respect to the total number of bacteria added to the monolayer. The results presented are representative of three experiments and are the averages for duplicate samples. The range was <10%.
[c] Seven- to 8-week-old female BALB/c mice were infected intraperitoneally (five or six mice per group, four groups) as described previously (20). The LD_{50}s were calculated as described by Reed and Meunch (22). LD_{50}, bacterial dose required to kill 50% of the mice in 14 days.

The two *yadA* mutants (YPIII*/pIB103* and YPIII/pYH4) were slightly defective in invasion, the two *inv* mutants (YP100* and SF104) were severely defective in invasion, and the *inv yadA* double mutants were essentially noninvasive (Table 2). This is consistent with the previous findings that invasin is the primary invasion factor and that YadA plays a secondary role (27). Furthermore, the strains from different sources all behaved similarly in this assay.

LD_{50} assays. The same strains were then tested to determine the LD_{50} for mice as a measure of their virulence potentials. The LD_{50}s were determined by intraperitoneal injections into 7- to 8-week old female, virus-free BALB/c mice; this is the strain of mice used for the *Y. enterocolitica* study (19, 20). Shown in Table 2 are representative results of four experiments. Our results indicate that the disruptions of either *inv* or *yadA*, or both, do not have much effect on the virulence in mice as measured by LD_{50}s. Although during some experiments, two of the *inv yadA* double mutants, i.e., YP100*/pIB103* and YP100*/pYH4, both with a chromosomal background from YP100*c, seemed to have a slight trend of being more virulent, the difference was never more than 10-fold from the wild-type value. This is in contrast to what was reported by Rosqvist et al. (23). One explanation for the difference in the results obtained is the strain of mice used for the assay. Rosqvist et al. (23) used Swiss Albino mice from the Swiss Defense Research Establishment, which are no longer available. We tested this possibility by using Swiss Albino mice (from Charles River) in parallel experiments, but results similar to those found with BALB/c mice were obtained (data not shown).

Evaluation of YP100*c. YP100* has a distinctive colony morphology on Luria-Bertani agar plates when compared to SF104 or the two wild-type strains, YPIII and YPIII*. The colonies of YP100* were round and shiny as opposed to the flat, dull colony morphology of all other strains, suggesting that YP100* may have a different outer membrane protein profile.

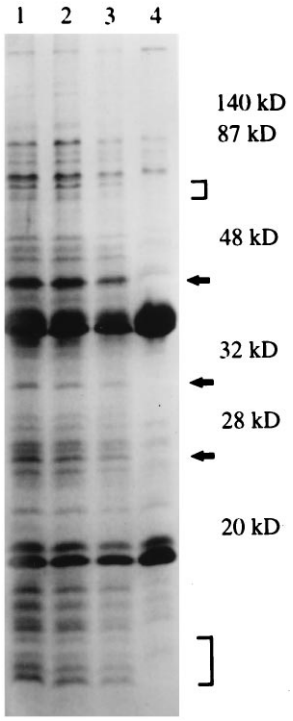

FIG. 1. Outer membrane profiles of wild-type and mutant *Y. pseudotuberculosis* strains. Bacteria were grown at 26°C in Luria-Bertani broth to stationary phase and then subcultured into minimal M9 medium and grown at 37°C overnight. The outer membrane samples were prepared with a French press as described previously (18). The samples were subjected to SDS–12.5% PAGE. The cultures attained equivalent cell densities, and equal volumes of each sample were loaded on the gel. After electrophoresis, the gel was stained with Coomassie blue. Lane 1, YPIIIc; lane 2, YPIII*c; lane 3, SF104c; lane 4, YP100*c. The arrows and brackets indicate the bands that differ between YP100*c and the other strains.

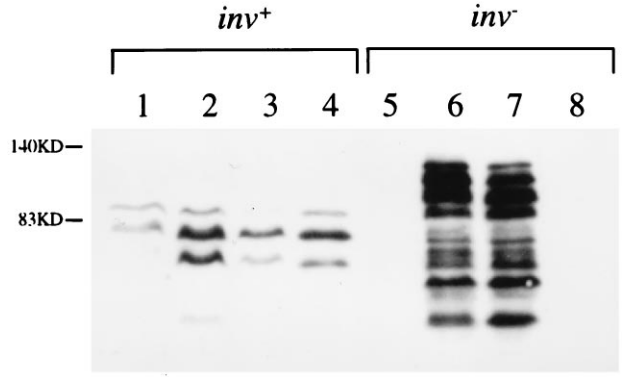

FIG. 2. Western blotting of the mutants. Bacteria were grown at 26°C to stationary phase. Equal counts of whole cells were subjected to SDS–12.5% PAGE. After electrophoresis, the gel was transferred to a nitrocellulose membrane and blotted with monoclonal anti-Inv 2A9-1 and an appropriate secondary antibody, followed by an enhanced chemiluminescence reaction, all as described by Pepe and Miller (18). Lane 1, YPIII; lane 2, YPIII*; lane 3, YPIIIc/pYH4; lane 4, YPIII*c/pIB103*; lane 5, SF104c/pYH4; lane 6, YP100*c/pYH4; lane 7, YP100*c/pIB103*; lane 8, SF104c/pYH7.

The distinctive colony morphology of YP100* remained even after the virulence plasmid was cured; thus, whatever causes the morphology change is probably chromosomally encoded. To test this hypothesis, outer membrane fractions of strains YPIIIc, YPIII*c, SF104c, and YP100*c were prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). YP100*c exhibited an outer membrane profile distinct from that of SF104c or either YPIIIc or its parental strain YPIII*c (Fig. 1). Since YP100* was constructed as an *inv* mutant, to verify the *inv* mutation we performed Southern (data not shown) and Western (Fig. 2) analyses with the monoclonal anti-invasin antibody 2A9-1 (kindly provided by R. Isberg), which recognizes the middle portion of invasin. It was noted before that invasin is unstable, and degradation products are often observed (11, 18). SF104c, as expected, showed no detectable invasin. YP100*c, however, gave rise to a variant invasin with slower mobility than wild-type invasin. There are two possible causes for this phenomenon: either the Tn*5* insertion in the *inv* gene created a large fusion protein or the truncated invasin formed multimers stable in the presence of SDS and heat. The aberrant outer membrane profile could be the effect of the accumulation of the aberrant form of invasin on the expression of other outer membrane proteins, or, alternatively, it could be the result of additional mutations introduced during strain construction. Together, these data suggest that YP100* is not isogenic to the parental strain.

Invasin and YadA are two of the most extensively studied virulence factors of the *Yersinia* genus. For *Y. enterocolitica*, a previous report showed that the *inv yadA* double mutant was avirulent (20). For *Y. pseudotuberculosis*, however, it was reported that such a mutant was unexpectedly hypervirulent (23). *Y. enterocolitica* and *Y. pseudotuberculosis* are closely related; thus, this difference in phenotype was unexpected, and we decided to investigate it further. Various constructs of the *inv yadA* mutants of *Y. pseudotuberculosis* were made and tested in mice. Our results indicate that these mutants are not hypervirulent in commercially available BALB/c and Swiss Albino mice, and yet, they are not avirulent like the equivalent mutants of *Y. enterocolitica*. Instead, their virulence remained almost the same as that of the wild-type strain in our mouse model. We believe that the *inv* and *yadA* mutants constructed for the present study are isogenic to the parental strain YPIII. However, it appears that the *inv* mutant used by Rosqvist et al. (23) (YP100*) is not isogenic to its parental strain. Closer examination of YP100* revealed that this strain actually produces either multimers of invasin or a fusion protein larger than the wild-type invasin. Additional mutations may also exist in this strain, resulting in an altered outer membrane protein profile and altered colony morphology; these mutations may have occurred during strain construction. Any one, or a combination, of these changes in YP100* could account for the slight increase of virulence observed during some LD_{50} experiments.

It remains interesting that for *Y. pseudotuberculosis* and *Y. enterocolitica*, a knockout of the *yadA* gene has different effects on virulence in mice. One possible explanation is that *Y. pseudotuberculosis* encodes an additional virulence factor(s) that compensates for the loss of the function of YadA. At present, we are testing this hypothesis in an effort to identify a new virulence factor(s). Alternatively, it is also possible that *Y. pseudotuberculosis* has an outer membrane layer very different from that of *Y. enterocolitica*, such that the function of YadA is masked or altered and is undetected in the current mouse model.

We thank Hans Wolf-Watz for sending the *Y. pseudotuberculosis* strains (YPIII*, YPIII/pIB103*, and YP100*) and Ralph Isberg for the monoclonal invasin antibody.

This work was support by National Institute of Health grants AI 27342 and AI 01230 to V.L.M. and AI 09268 to Y.W.H.

REFERENCES

1. **Ben-Gurion, R., and A. Shafferman.** 1981. Essential virulence determinants of different *Yersinia* species are carried on a common plasmid. Plasmid **5:**183–187.
2. **Bliska, J. B., M. C. Copass, and S. Falkow.** 1993. The *Yersinia pseudotuberculosis* adhesin YadA mediates intimate bacterial attachment to and entry into HEp-2 cells. Infect. Immun. **61:**3914–3921.
3. **Bölin, I., and H. Wolf-Watz.** 1984. Molecular cloning of the temperature-inducible outer membrane protein 1 of *Yersinia pseudotuberculosis*. Infect. Immun. **43:**72–78.
4. **Cornelis, G. R.** 1992. *Yersiniae*, finely tuned pathogens, p. 231–265. *In* C. E. Hormacche, C. W. Penn, and C. J. Smyth (ed.), Molecular biology of bacterial infection. Cambridge University Press, Cambridge, United Kingdom.
5. **Cover, T. L., and R. C. Arber.** 1989. *Yersinia enterocolitica*. N. Engl. J. Med. **321:**16–24.
6. **Forsberg, A., and H. Wolf-Watz.** 1988. The virulence protein Yop5 of *Yersinia pseudotuberculosis* is regulated at the transcriptional level by plasmid-pIB1-encoded transacting elements controlled by temperature and calcium. Mol. Microbiol. **2:**121–133.
7. **Hanski, C., M. Naunmann, H. Hahn, and E. O. Riecken.** 1989. Determinants of invasion and survival of *Yersinia enterocolitica* in intestinal tissue an *in vivo* study. Med. Microbiol. Immunol. **178:**289–296.
8. **Heesemann, J., and L. Grüter.** 1987. Genetic evidence that the outer membrane protein YOP1 of *Yersinia enterocolitica* mediates adherence and phagocytosis resistance to human epithelial cells. FEMS Microbiol. Lett. **40:**37–41.
9. **Isberg, R. R., and S. Falkow.** 1985. A single genetic locus encoded by *Yersinia pseudotuberculosis* permits invasion of cultured animal cells by *Escherichia coli* K12. Nature **317:**262–264.
10. **Isberg, R. R., and J. M. Leong.** 1988. Cultured mammalian cells attach to the invasin protein of *Yersinia pseudotuberculosis*. Proc. Natl. Acad. Sci. USA **85:**6682–6686.
11. **Isberg, R. R., D. L. Voorhis, and S. Falkow.** 1987. Identification of invasin: a protein that allows enteric bacteria to penetrate cultured mammalian cells. Cell **50:**769–778.
12. **Kapperud, G., E. Namork, M. Skurnik, and T. Nesbaken.** 1987. Plasmid-mediated surface fibrillae of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*: relationship to the outer membrane protein YOP1 and possible importance for pathogenesis. Infect. Immun. **55:**2247–2254.
13. **Kinder, S. A., J. L. Badger, G. O. Bryant, J. C. Pepe, and V. L. Miller.** 1993. Cloning of the *Yen*I restriction endonuclease and methyltransferase from *Yersinia enterocolitica* serotype O8 and construction of a transformable R^- M^+ mutant. Gene **136:**271–275.
14. **Martinez, R. J.** 1989. Thermoregulation-dependent expression of *Yersinia enterocolitica* protein 1 imparts serum resistance to *Escherichia coli* K-12. J. Bacteriol. **171:**3732–3739.
15. **Miller, V. L., and S. Falkow.** 1988. Evidence for two genetic loci in *Yersinia enterocolitica* that can promote invasion of epithelial cells. Infect. Immun. **56:**1242–1248.
16. **Miller, V. L., J. J. Farmer III, W. E. Hill, and S. Falkow.** 1989. The *ail* locus is found uniquely in *Yersinia enterocolitica* serotypes commonly associated with disease. Infect. Immun. **57:**121–131.
17. **Pai, C. H., and V. Mors.** 1978. Production of enterotoxin by *Yersinia enterocolitica*. Infect. Immun. **19:**908–911.
18. **Pepe, J. C., and V. L. Miller.** 1990. The *Yersinia enterocolitica inv* gene product is an outer membrane protein that shares epitopes with *Yersinia pseudotuberculosis* invasin. J. Bacteriol. **172:**3780–3789.
19. **Pepe, J. C., and V. L. Miller.** 1993. *Yersinia enterocolitica* invasin: a primary role in the initiation of infection. Proc. Natl. Acad. Sci. USA **90:**6473–6477.
20. **Pepe, J. C., M. Wachtel, E. Wager, and V. L. Miller.** 1995. Pathogenesis of defined invasive mutants of *Yersinia enterocolitica* in a BALB/c mouse model of infection. Infect. Immun. **63:**4837–4848.
21. **Portnoy, D. A., H. Wolf-Watz, I. Bölin, A. B. Beeder, and S. Falkow.** 1984. Characterization of common virulent plasmids in *Yersinia* species and their role in the expression of outer membrane proteins. Infect. Immun. **43:**108–114.
22. **Reed, L. J., and H. Meunch.** 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27:**493–497.
23. **Rosqvist, R., M. Skurnik, and H. Wolf-Watz.** 1988. Increased virulence of *Yersinia pseudotuberculosis* by two independent mutations. Nature **334:**522–525.
24. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784–791.
25. **Skurnik, M., and H. Wolf-Watz.** 1989. Analysis of the *yopA* gene encoding the Yop1 virulence determinants of *Yersinia* spp. Mol. Microbiol. **3:**517–529.
26. **Skurnik, M., I. Bölin, H. Heikkinen, S. Piha, and H. Wolf-Watz.** 1984. Virulence plasmid-associated autoagglutination in *Yersinia* spp. J. Bacteriol. **158:**1033–1036.
27. **Yang, Y., and R. R. Isberg.** 1993. Cellular internalization in the absence of invasin expression is promoted by the *Yersinia pseudotuberculosis yadA* product. Infect. Immun. **61:**3907–3913.

Editor: P. E. Orndorff