INFECTION AND IMMUNITY, Oct. 1988, p. 2749–2752
0019-9567/88/102749-04$02.00/0

Vol. 56, No. 10

NOTES

Plasminogen Activator/Coagulase Gene of *Yersinia pestis* Is Responsible for Degradation of Plasmid-Encoded Outer Membrane Proteins

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Received 5 April 1988/Accepted 23 June 1988

The related family of virulence plasmids found in the three major pathogens of the genus *Yersinia* all have the ability to encode a set of outer membrane proteins. In *Y. enterocolitica* and *Y. pseudotuberculosis*, these proteins are major constituents of the outer membrane when their synthesis is fully induced. In contrast, they have been difficult to detect in *Y. pestis*. It has recently been established that *Y. pestis* does synthesize these proteins, but that they are rapidly degraded due to some activity determined by the 9.5-kilobase plasmid commonly found in *Y. pestis* strains. We show that mutations in the *pla* gene of this plasmid, which encodes both the plasminogen activator and coagulase activities, blocked this degradation. A cloned 1.4-kilobase DNA fragment carrying *pla* was also sufficient to cause degradation in the absence of the 9.5-kilobase plasmid.

Plasmids approximately 70 kilobases (kb) in size that are required for expression of virulence are found in the three major pathogens of the genus *Yersinia*: *Yersinia pestis* (the causative agent of plaque), *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica* (3, 10, 11, 27). These plasmids share large regions of homology which contain three recognized elements: a set of regulatory genes that control expression of other plasmid genes in response to temperature and Ca^{2+} concentration; a gene encoding a cytoplasmic protein known as V antigen; and a set of genes encoding outer membrane proteins, at least some of which are serologically related across the three species (see reference 9 for a recent review). Although it is unclear how these elements contribute to virulence, loss of the entire plasmid and mutations in the regulatory genes and in some outer membrane protein genes result in a dramatic increase in 50% lethal dose (LD_{50}) values in mouse infection experiments (9). Mutants with specific defects in the V antigen gene have not yet been examined.

Although the *Yersinia* outer membrane proteins (YOPs [5]) encoded by this plasmid family are abundant in membrane preparations from *Y. enterocolitica* and *Y. pseudotuberculosis*, they are not seen in similar preparations from wild-type *Y. pestis* strains (19, 24). Nonetheless, antibodies against these proteins are developed in animals infected with *Y. pestis* (5). These observations have led to some doubt about the importance of the YOPS in the virulence of *Y. pestis* and also to suggestions that a unique regulatory mechanism allows their expression in vivo but not in vitro (4). More recently, it has been established that the YOPs are synthesized by *Y. pestis* in vitro but are then rapidly degraded (19, 25). Moreover, degradation does not occur in strains lacking the 9.5-kb plasmid found in wild-type *Y. pestis* (19). This plasmid is not found in the other yersiniae (3).

The 9.5-kb plasmid also plays a role in plaque pathogenesis, since strains which lack it have increased LD_{50} values when used to infect mice by subcutaneous or intraperitoneal injection (6). It determines four known biochemical activities: a bacteriocin called pesticin, immunity to pesticin, plasminogen activator activity, and coagulase activity (1, 2, 8, 15). The plasminogen activator and coagulase activities are both determined by a single gene, which as a result of posttranslational processing produces two distinct outer membrane protein species (22). It has not been determined how plasminogen activator and coagulase activities are distributed between these species. In this report, we show that the plasminogen activator/coagulase gene is also responsible for degradation of the YOPs.

All *Y. pestis* strains used in our experiments were derived from a nonpigmented isolate of strain KIM. Like most natural isolates of this species (10), KIM contains three plasmids of 110, 75, and 9.5 kb. The 75-kb plasmid in this strain is designated pCD1, and the 9.5-kb plasmid is designated pPCP1. All strains used retained the 110-kb plasmid, but pPCP1 and pCD1 were removed, altered, or replaced in particular experiments as indicated. Spontaneous segregants lacking pCD1 were isolated by selection on magnesium oxalate agar (14), and segregants lacking pPCP1 were isolated after growth in the cold (19). Construction of transposon Tn*5* mutants of pPCP1 has been described elsewhere (22). Construction of pOS30 was accomplished by standard methods (16). m-Tn*3*(*lacZ*) mutants of pOS30 were constructed by the method of Seiffert el al. (20) in *Escherichia coli* K-12 and transferred to $pPCP1^-$ KIM via P1 transduction (12).

E. coli K-12 strain LE392 has the genotype F^- *hsdR514 supE44 supF58* Δ(*lacIZY*)*6 galK2 galT22 metB1 trpR55* λ^- (16).

Plasminogen activator activity of the mutants was scored by the fibrinolytic assay of Beesley et al. (1). To achieve

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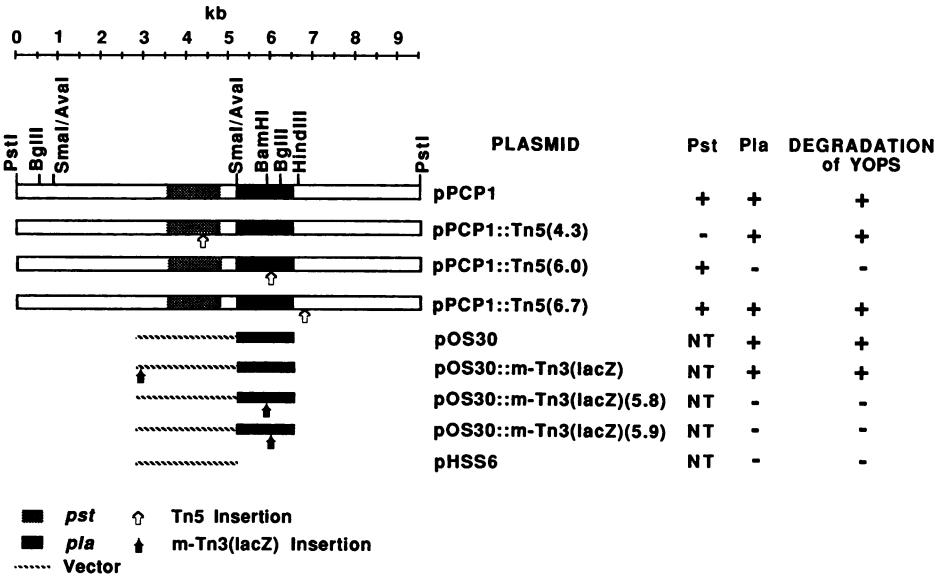

FIG. 1. Map of pPCP1 and derivative plasmids. Numbers following designations for plasmids containing transposon insertions indicate the position of the insertion on the pPCP1 map (in kilobases). *pst* and *pla* indicate the pesticin and plasminogen activator genes, respectively. Pst and Pla indicate the pesticin and plasminogen activator phenotypes conferred by the plasmids. NT, Not tested. A more detailed map of pPCP1 is given in reference 22. The vector pHSS6 is described in reference 20.

induction of YOP synthesis, *Y. pestis* strains were grown in Ca^{2+} deficient defined medium at 26°C and then shifted to 37°C (26). Outer membranes were prepared by a method previously developed for use with *Y. pestis* (24). Silver-stained sodium dodecyl sulfate-polyacrylamide gels were prepared as described before (17, 24), as were immunoblots of such gels (7). YOP-specific rabbit antiserum prepared with *Y. pseudotuberculosis* was used in staining the immunoblots. Preparation of this antiserum has been detailed elsewhere (19).

To determine which of the pPCP1 genes was required for degradation of the YOPs, we began by examining the outer membrane proteins produced by *Y. pestis* strains carrying Tn*5* insertion mutants of pPCP1. Of the three mutants tested (Fig. 1), one with the transposon inserted in the plasminogen activator/coagulase gene (*pla*) was found to have several prominent proteins with molecular masses corresponding to those reported for the YOPs and that were not seen in strains with either wild-type pPCP1 or the other pPCP1 mutants (Fig. 2). As expected, this strain also lost the two outer membrane proteins derived from *pla*, α-Pla and β-Pla (22). To confirm that the presence of an active *pla* gene was sufficient for degradation of the YOPs, a 1.4-kb fragment of pPCP1 previously shown to contain this gene (22) was cloned into the vector pHSS6 to form plasmid pOS30 (Fig. 1). A. *Y. pestis* strain carrying pOS30 but cured of pPCP1 had an outer membrane protein profile identical to that of strains with wild-type pPCP1, lacking the YOPs but containing α- and β-Pla (Fig. 2). Two mutant plasmids in which the transposon m-Tn*3*(*lacZ*) (20) was inserted into this cloned fragment, inactivating the *pla* gene, were constructed (Fig. 1). The YOPs were again present in membrane preparations from these strains (Fig. 2), as they were in a strain carrying pHSS6 with no inserted pPCP1 DNA. A strain carrying pOS30 with m-Tn*3*(*lacZ*) inserted outside of the pPCP1 fragment (Fig. 1 and 2) lacked the YOPs but contained the Pla proteins. Thus, in all of the strains tested, there was a perfect correlation between the presence of the intact *pla* gene, the Pla proteins, and lack of the YOPs.

To provide additional evidence that the protein species lost in the presence of *pla* were indeed the YOPs encoded by the 75-kb plasmid, we performed two additional experiments. First, an immunoblot was prepared from a gel identical to that shown in Fig. 2 and stained with antisera prepared against the YOPs of *Y. pseudotuberculosis* (Fig. 3). This antiserum clearly detected several of the YOP species, but only in those strains which lacked an intact *pla* gene. Second, we compared outer membrane proteins from iso-

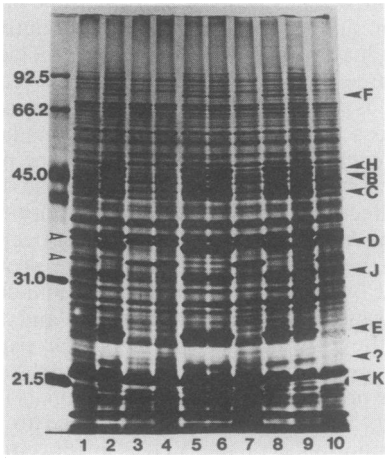

FIG. 2. Outer membrane proteins of Pla^+ and Pla^- *Y. pestis* strains. Letters indicate the various YOP species as designated by Straley and Bowmer (23). The two open arrowheads at left indicate α- and β-Pla (22); α-Pla is the larger of the two species. All strains contained the 75-kb plasmid pCD1 and differed only in their pPCP1 derivatives as follows: lane 1, pPCP1; lane 2, none; lane 3, pPCP1::Tn*5*(4.3) (Pst^- Pla^+); lane 4, pPCP1::Tn*5*(6.7) (Pst^+ Pla^+); lane 5, pPCP1::Tn*5*(6.0) (Pst^+ Pla^-); lane 6, pHSS6; lane 7, pOS30 (Pla^+); lane 8, pOS30::m-Tn*3*(*lacZ*)(5.8) (Pla^-); lane 9, pOS30::m-Tn*3*(*lacZ*)(5.9) (Pla^-); lane 10, pOS30::m-Tn*3*(*lacZ*) (Pla^+). These plasmids are diagrammed in Fig. 1. Molecular mass markers are shown, with masses indicated in kilodaltons.

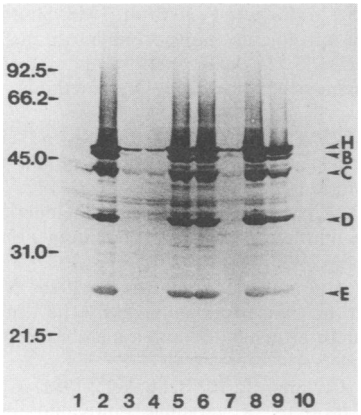

FIG. 3. Immunoblot stained with anti-YOP antisera. This blot was prepared from a gel identical to that shown in Fig. 2. Letters indicate YOP species. See text and Fig. 2 legend for additional details.

genic pPCP1$^+$ and pPCP1$^-$ strains, both of which lacked pCD1 (Fig. 4). As expected, these differed only in that the Pla proteins were present in the pPCP1$^+$ strain; no additional species appeared when pPCP1, and thus the *pla* gene, was removed. Combined with the results presented above, this experiment shows that the appearance of the YOPs in the absence of *pla* occurs only if the 75-kb plasmid is present.

Figure 4 also shows the effect of pPCP1 on the outer membranes of *E. coli* K-12 strain LE392: the two Pla proteins appeared, but no other significant changes were observed. Both plasminogen activator and coagulase activities were expressed in this *E. coli* strain. This result, along with comparisons of membranes from *Y. pestis* strains, illustrates the specificity of the *pla*-determined degradation; few proteins other than the YOPs were severely affected.

Why do *Y. pestis* strains both synthesize and degrade this set of proteins? One possible explanation is that the degradation observed in these experiments is an artifact of in vitro culture. If the same active center of the Pla protein(s) that is responsible for proteolysis of the YOPs also catalyzes plasminogen activation, the former reaction may well be competitively inhibited in vivo by high plasminogen concentrations. This is a reasonable possibility, since both degradation of the YOPs and plasminogen activation are proteolytic events. It is also supported by the observation that intact YOPs can be detected by immunoblotting in *Y. pestis* harvested from culture chambers implanted in guinea pigs (21). If this hypothesis proves correct, the primary role of *pla*-mediated YOP degradation in *Y. pestis* physiology has been to confuse researchers investigating the function of the 75-kb plasmid.

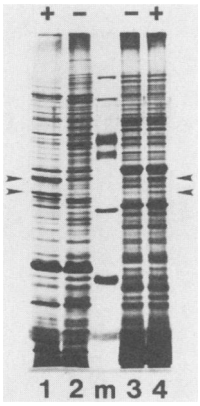

FIG. 4. Effect of *pla* on outer membranes from *Y. pestis* and *E. coli* strains lacking pCD1. Lane 1, *Y. pestis* KIM; lane 2, *Y. pestis* KIM cured of pPCP1; lane m, Molecular mass markers (as in Fig. 2); lane 3, *E. coli* LE392(pOS30); lane 4, LE392. Arrows indicate α- and β-Pla (see Fig. 2 legend).

Alternatively, it is conceivable that the release of YOP fragments from *Y. pestis* as a result of degradation plays an important role in pathogenesis. Although we favor the former hypothesis, two observations lend some support to this notion. The first is the apparent specificity of *pla*-mediated degradation for the YOPs as opposed to other outer membrane proteins. Such specificity suggests adaptation via natural selection. The second observation is that at least some of the YOPs are readily released from both *Y. enterocolitica* and *Y. pseudotuberculosis* in culture, although in intact rather than degraded form (4, 13). Proteolytic degradation could be an alternative form of release if some of the resulting fragments retain the required biological activity.

Finally, it is possible that the *Y. pestis* YOPs are degraded both in vitro and in vivo and have no role in pathogenesis, but there is strong evidence against this hypothesis. Mutations in several YOP genes of *Y. pestis* have been shown to dramatically increase LD_{50} values in mouse infection experiments (23). Because the mutations in the strains tested were transposon insertions, which are strongly polar, it is possible that more than a single gene was inactivated in each, complicating interpretation of these experiments. However, unless inadvertent inactivation of unknown virulence genes occurred in every case—an unlikely possibility—at least one of the YOPs plays an important role in the virulence of *Y. pestis*.

This work was supported by Public Health Service grants AI 22176 (to J.D.G.) and AI 19353 and AI 13590 (to R.R.B.) from the National Institutes of Health.

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