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Plasmid-Determined Cytotoxicity in *Yersinia pestis* and *Yersinia pseudotuberculosis*

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***Yersina pestis* KIM5 was found to be cytotoxic for the IC21 and P388D1 mouse macrophage cell lines, as well as for resident peritoneal macrophages from C57BL/6 mice. Affected cells phagocytosed KIM5 inefficiently, became spherical, detached readily from culture dishes, and retained ^{51}Cr poorly. The cytotoxic effect was dependent on the presence of the 75-kilobase plasmid pCD1. Because this plasmid also encodes the low calcium response (LCR), three Mu d1 insertion mutants previously shown to be LCR$^-$ and of reduced virulence in mice were examined for cytotoxicity; all were found to be atoxic. The insertions in these mutants lie within three distinct LCR loci (*lcrB*, *C*, and *D*). Like LCR, cytotoxicity was expressed only at 37°C. Unlike LCR, it was not influenced by Ca^{2+} concentration, indicating that the V and W antigens are probably not involved. *Yersinia pseudotuberculosis* was found to have a similar plasmid-dependent cytotoxicity. Thus, biological activity observed as cytotoxicity in vitro may well be a common feature contributing to virulence of the yersiniae.**

A family of closely related plasmids provides genes required for expression of virulence in all three *Yersinia* species pathogenic for man (4–6). In strain KIM5 of *Yersinia pestis*, this family is represented by a 75-kilobase plasmid designated pCD1. We have previously shown (7, 21b) that this plasmid carries a cluster of at least three distinct genetic loci (*lcrB*, *C*, and *D*) that are essential for expression of both virulence in mice and an unusual phenotype which we call the low calcium response (LCR). Similar results have been obtained in another *Y. pestis* strain (11) and in *Y. pseudotuberculosis* (21). These results constitute strong evidence that there is some functional relationship between the LCR and virulence.

In vitro, LCR is observed as growth restriction and concomitant production of two proteins known as the V and W antigens when Ca^{2+}-free cultures are shifted from 26 to 37°C (2, 22). This temperature shift does not affect growth or induce V and W production if 2.5 mM Ca^{2+} is included in the medium. Although intriguing, this unusual behavior provides few clues regarding the mechanism by which LCR contributes to virulence. No in vitro biological activity has yet been associated with the V and W antigens. However, Une and Brubaker (18) have presented immunological evidence which indicates a direct contribution of V to the ability of *Y. pestis* to proliferate in mice.

We have recently found that both *Y. pestis* and *Y. pseudotuberculosis* grown under appropriate conditions are cytotoxic for mouse macrophages. Mutations which affect LCR expression also affect expression of cytotoxicity, although Ca^{2+} concentration in the growth medium, a factor strongly influencing expression of V and W antigens, does not. These results are described below.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains are listed in Table 1. LE392(pCD1) was constructed by simultaneous transformation of LE392 with pCD1 and pBR322, followed by curing of pBR322 (1) after transformants carrying both plasmids were identified by screening plasmid DNA from individual colonies (10). YPIII(pCD1::Mu d1 28.4) was constructed by transduction of the pCD1 derivative from *Escherichia coli* to YPIII by phage P1. Before transformation, the recipient strain was heated to inactivate restriction enzymes (21). References detailing the construction of other strains are given in the table.

Bacterial cultures. The Ca^{2+}-supplemented liquid medium used to grow bacteria for use in cytotoxicity experiments was heart infusion broth (HIB) supplemented with 2.5 mM $CaCl_2$. The Ca^{2+}-deficient liquid medium used for this purpose was prepared by treating HIB with 20 mM sodium oxalate to remove Ca^{2+} and supplementing the oxalated medium with 20 mM MgCl to improve expression of the LCR (8). LCR is expressed in the Ca^{2+}-deficient but not in the Ca^{2+}-supplemented medium at 37°C. HIB was used in these experiments rather than chemically defined medium to avoid complications due to the clumping of cells which occurs in defined medium (15).

Bacteria from exponential phase cultures were used in all infection experiments. Cultures grown at 26°C were prepared by inoculating the appropriate medium with 0.5 volume of a fresh overnight culture grown in HIB at 26°C and incubating the new culture at 26°C for 6 h on an orbital shaker. Cultures grown at 37°C were prepared similarly, except that after 2 h of incubation at 26°C they were shifted to 37°C and incubation was continued for an additional 4 h. This period of incubation at 37°C is just sufficient to achieve growth restriction of KIM5 in Ca^{2+}-deficient medium. Unless otherwise specified, bacteria used in cytotoxicity assays were grown under the 37°C regime in Ca^{2+}-supplemented medium.

Tryptose blood agar base was routinely used as a solid medium for bacterial culture. Magnesium-oxalate agar and defined liquid medium were used to confirm LCR phenotypes (7).

Cell cultures. Cultures of macrophage cell lines IC21 and P388D1 (19) were maintained in RPMI 1640 (GIBCO, Grand Island, N.Y.) supplemented with 7.5% heat-inactivated fetal

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TABLE 1. Bacterial strains

Strain	Species	Relevant properties	Reference
KIM5	*Y. pestis*	pCD1$^+$ LCR$^+$	7
KIM6	*Y. pestis*	pCD1$^-$ LCR$^-$	7
KIM7	*Y. pestis*	pCD1$^-$, pGW600$^+$, LCR$^-$, stabilizes Mu d1	7
KIM7(pCD1::Mu d1 28.4)	*Y. pestis*	LCR$^+$	7
KIM7(pCD1::Mu d1 44.8)	*Y. pestis*	*lcrD*::Mu d1, LCR$^-$	7
KIM7(pCD1::Mu d1 54.4)	*Y. pestis*	*lcrB*::Mu d1, LCR$^-$	7
KIM7(pCD1::Mu d1 60.9)	*Y. pestis*	*lcrC*::Mu d1, LCR$^-$	7
YPIII(pIB1)	*Y. pseudotuberculosis*	LCR$^+$	12
YPIII	*Y. pseudotuberculosis*	pIB1$^-$, LCR$^-$	12
YPIII(pCD1::Mu d1 28.4)	*Y. pseudotuberculosis*	LCR$^+$	This study
LE392	*E. coli* K-12	Transforms well	17
LE392(pCD1)	*E. coli* K-12	LCR$^-$	This study

calf serum. Resident peritoneal macrophages were obtained from C57BL/6 mice by peritoneal lavage (14) and maintained in RPMI 1640 plus fetal calf serum.

Infection of macrophages. Bacteria cultured under the appropriate conditions were washed once in RPMI 1640 plus fetal calf serum and suspended in this same medium at a density of 2×10^8 cells per ml as determined by optical density measurements. Where indicated, chloramphenicol (100 μg/ml) was added to the suspension medium to inhibit protein synthesis. A volume of the suspension sufficient to provide the desired multiplicity of infection (MOI) was then added to the macrophage culture. These cultures were maintained in 24-well culture dishes containing 0.5 ml of medium and approximately 2×10^5 macrophages per well. Thus, to provide an MOI of 100, 100 μl of the bacterial suspension was required. Chloramphenicol (100 μg/ml) also was added to the macrophage cultures 1 h before infection when inhibition of bacterial protein synthesis was required.

Immediately after infection, cultures were incubated at 37°C for 30 min, washed three times with 0.5 ml of fresh medium to remove extracellular bacteria, incubated at 37°C for the times indicated, and then washed, fixed, stained, and examined. Except in experiments involving chloramphenicol, no additional steps were taken to prevent growth of extracellular bacteria. *Y. pestis* grows in the tissue culture medium with a doubling time of about 2 h.

Electron microscopy. Cultures of the IC21 macrophage cell line were grown attached to glass cover slips within the culture dishes. Infection and a 4-h postinfection incubation were carried out as described above with an MOI of 100. The cover slips were gently rinsed with Dulbecco phosphate-buffered saline and immediately fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer. After postinfection incubation in 1% osmium tetroxide and dehydration in a graded series of ethanol solutions, the specimens were infiltrated with Spurr low viscosity imbedding medium directly on the cover slips, and the imbedding medium was polymerized at 60°C. The imbedded samples then were detached from the glass, sectioned, and stained with uranyl acetate and lead citrate. This procedure was designed to minimize operations that might detach cells affected by cytotoxicity from the cover slips, although some cells were lost in processing.

Monolayer detachment assay. IC21 cells were infected as described above by using an MOI of 100 unless otherwise specified. After a 5-h postinfection incubation at 37°C, the culture dishes were agitated for 2 min at 200 rpm on a Bellco Mini Orbital Shaker, washed with phosphate-buffered saline (pH 7), fixed in methanol, and stained with Giemsa. Most of the cells affected by cytotoxicity were detached during the agitation. After staining, wells containing toxic bacteria were essentially colorless, while those containing atoxic strains were blue due to staining of the adherent macrophages.

Toxicity of culture supernatants and sonic extracts. A KIM5 culture in Ca^{2+}-supplemented HIB was shifted from 26 to 37°C as described above. After 4 h at 37°C, the optical density of the culture was determined, and a 0.5-ml sample was centrifuged at 13,000 × *g* for 2 min in a microfuge. A portion of the supernatant fluid large enough to have contained 10^8 cells before centrifugation (0.25 ml) was then added to a macrophage culture in lieu of bacterial suspension, and the monolayer detachment assay was conducted as described above. A second 0.5-ml sample from the same KIM5 culture was prepared as described for infection of macrophage cultures but was subjected to sonication immediately before addition to the macrophages. Sonication was performed in a cup horn transducer for 5 min (setting 7, model W185 sonicator; Heat Systems-Ultrasonics, Plainview, N.Y.) with a continuous flow of cooling water from an ice bath. No significant heating of the sample occurred under these conditions. After sonication, the cell detachment assay was conducted as described above. A third sample from the same KIM5 culture was assayed for cytotoxicity at an MOI of 50 to confirm the cytotoxic effect of intact bacteria.

^{51}Cr release. The method used has been described in detail elsewhere (20). Briefly, macrophages preloaded with ^{51}Cr were infected at an MOI of 50 with the bacteria to be tested and washed as described above. At the times indicated, postinfection incubation was interrupted and the medium was carefully removed. Adherent cells were then lysed with sodium dodecyl sulfate (0.1%) and Triton X100 (1%). The ^{51}Cr content of the lysis fluid and of half of the medium removed from the culture was determined directly. The other half of the culture medium was centrifuged to remove detached cells before determination of ^{51}Cr. The proportion of the isotope contained in attached cells, in detached cells, and free in the medium was calculated from these measurements.

RESULTS

Basic observations. The micrographs in Fig. 1 illustrate our primary observation; pCD1$^+$ *Y. pestis* KIM5 was cytotoxic to the IC21 mouse macrophage cell line while its pCD1$^-$ derivative KIM6 was not. We have obtained similar results as judged by light microscopy with another mouse macrophage cell line, P388D1, and with resident peritoneal macrophages obtained from C57BL/6 mice. When an MOI of 100 was used, cultures infected with KIM5 could often be distinguished from those infected with KIM6 as early as 1 h postinfection by phase-contrast microscopy; cells infected with KIM5 began to change shape and acquired a granular

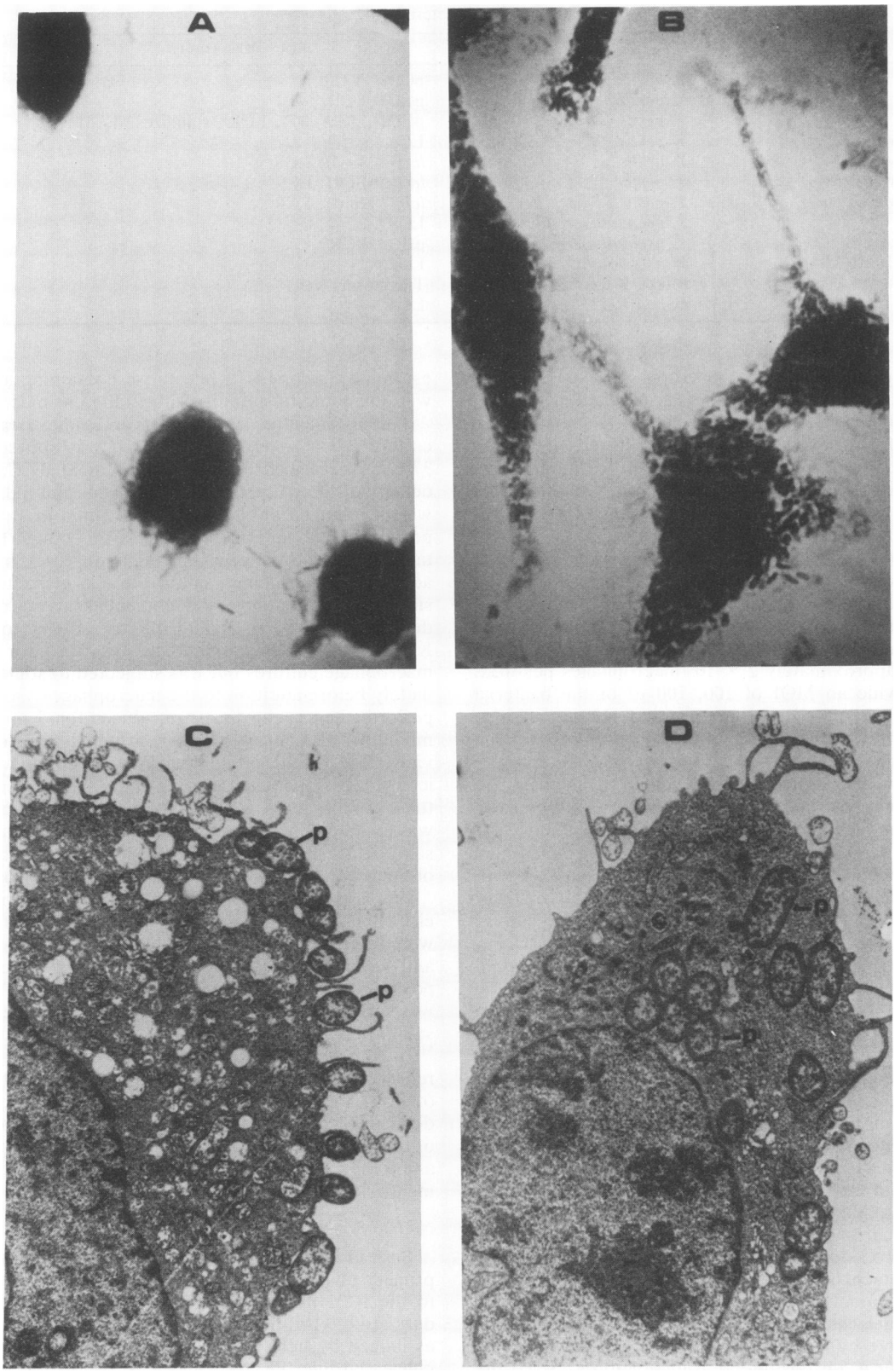

FIG. 1. Interaction of *Y. pestis* strains KIM5 (panels A and C) and KIM6 (panels B and D) with the IC21 mouse macrophage cell line. Both light micrographs (A and B) and transmission electron micrographs (C and D) are shown. Note the difference in shape between the KIM5-infected and KIM6-infected cells apparent in the light micrographs and that many KIM6 cells are visible within the macrophages in panel B. The electron micrographs show that KIM5 is only partially phagocytosed, while KIM6 is readily internalized. The KIM5-infected cells also contain numerous electron-translucent inclusions that are not present in the cells infected with KIM6. The label (p) indicates selected bacteria. Magnification of the electron micrographs, ×8,500.

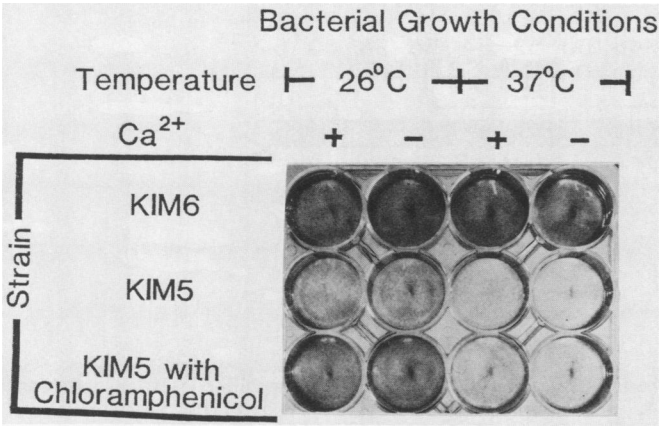

FIG. 2. Ca^{2+}, temperature, and cytotoxicity. The photograph shows part of a Giemsa-stained 24-well culture plate in which the monolayer detachment assay was conducted by using KIM5 and KIM6 grown under the conditions indicated. The monolayers are IC21 cells. See text for additional discussion.

appearance, whereas those infected with KIM6 retained their usual shape.

We also found that cells infected with KIM5 were easily detached from the culture dish or glass cover slips on which they were grown. This observation provided the basis for a convenient qualitative assay of cytotoxicity in which detachment of a cell monolayer is scored by macroscopic observation after staining (see above).

At MOIs below 25, destruction of an IC21 monolayer by KIM5 was not reliably observed when a 5-h postinfection incubation was used. Results at such low MOIs were improved by longer incubation periods (8 to 10 h), probably due to extracellular growth of the bacteria not eliminated by washing. Longer incubation periods also were required to obtain complete clearing of the monolayer when bacteria were grown at 26°C.

When IC21 cells were exposed to supernatant fluid or sonicated bacteria from KIM5 cultures, no cytotoxic effect was observed.

Effect of growth temperature and Ca^{2+} concentration on cytotoxicity. Because both temperature and Ca^{2+} concentration affect expression of LCR, the monolayer detachment assay was used to determine whether these factors also affect expression of cytotoxicity (Fig. 2). Chloramphenicol (100 μg/ml) was present during the postinfection incubation at 37°C in one series of infections to determine whether protein synthesis is required for expression of the cytotoxic effect. Chloramphenicol has been demonstrated to be concentrated two or more fold by macrophages (9), and therefore, should inhibit protein synthesis by both intracellular and extracellular yersiniae. Although Ca^{2+} concentration had no detectable effect on clearing of the monolayer, the temperature at which the bacteria were grown had a pronounced effect. KIM5 cells were cytotoxic both in the presence and absence of chloramphenicol when grown at 37°C, but were cytotoxic only when assayed in the absence of chloramphenicol when grown at 26°C. Compared to the cytotoxic effect of the cells grown at 37°C, the effect of the cells grown at 26°C was reduced and required a longer postinfection incubation to develop. These results suggest that KIM5 cells grown at 37°C are inherently cytotoxic, while cells grown at 26°C require incubation at 37°C under conditions which permit protein synthesis to develop cytotoxic properties.

^{51}Cr-release. These experiments were undertaken to determine whether KIM5 damaged the cytoplasmic membrane of infected cells. Results obtained with the P388D1 cell line are shown in Fig. 3. Substantial ^{51}Cr was lost from cells infected with KIM5 but not from those infected with KIM6. Similar results were obtained with both the IC21 cell line and with resident peritoneal macrophages from C57BL/6 mice. Expressed as ^{51}Cr retained in KIM5-infected monolayer/^{51}Cr retained in KIM6-infected monolayer at 6 h, the values obtained with these later cell types were: IC21, 63% (62 to 65%), C57BL/6, 54% (50 to 60%). Means and ranges obtained in triplicate experiments are given.

Effect of mutations in *lcr* loci on cytotoxicity. To determine whether the pCD1-dependent cytotoxicity of KIM5 was functionally related to the low Ca^{+} response, we tested three previously characterized LCR^{-} mutants for cytotoxicity by using IC21 cells in the monolayer detachment assay. Each of these mutants carries a lesion in a genetically distinct *lcr* locus and each has previously been shown to have reduced virulence for mice (7). Because the lesions carried by these strains result from insertions of Mu dl(Ap *lac*), we also tested a strain carrying this transposon inserted in pCD1 at a location which affects neither LCR nor virulence for mice.

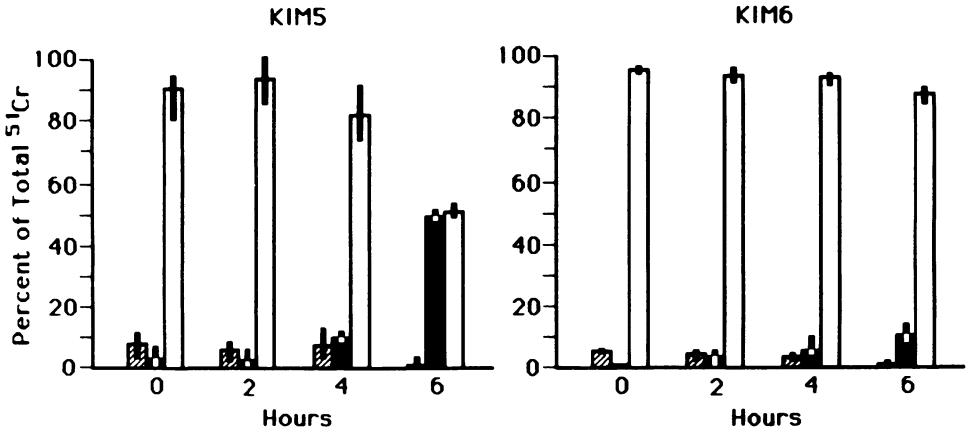

FIG. 3. Release of ^{51}Cr from the P388D1 macrophage cell line infected with KIM5 and KIM6. Means of triplicate experiments are shown. The error bars indicate the range of the values obtained. Symbols: ▨, in detached cells; ■, free in medium; □, in adherent cells.

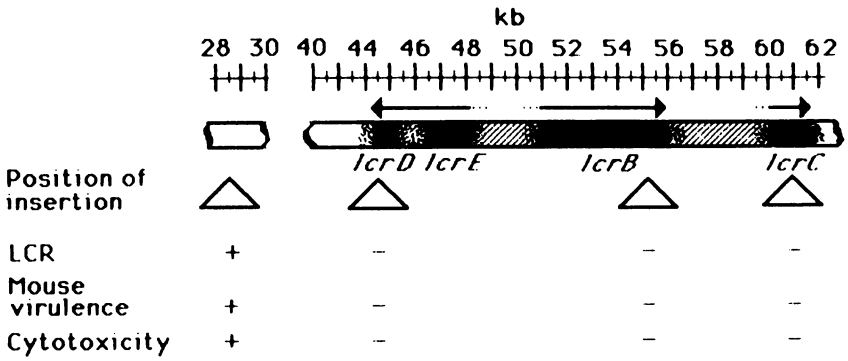

FIG. 4. Effect of Mu dl(Ap *lac*) insertions in pCD1 on cytotoxicity, the LCR, and mouse virulence. Mapping of these insertions (positions 28.4, 44.8, 54.4, and 60.9) and testing of their LCR and virulence phenotypes has been described previously, as has the genetic structure of the LCR region (●). *lcrD* and *lcrE* are derived from the original *lcrA* locus (J. Yother, and J. D. Goguen, submitted for publication.). Arrows indicate direction of transcription. The pCD1::Mu dl derivatives were carried by strain KIM7 in these experiments.

Results of these experiments demonstrated that expression of cytotoxicity, LCR, and virulence involves at least three common pCD1 genes (Fig. 4).

Plasmid-dependent cytotoxicity in other species. It has been demonstrated that the LCR-encoding plasmids of *Y. pestis* and *Y. pseudotuberculosis* have a high degree of homology (13) and that introduction of an LCR-encoding plasmid taken from *Y. pestis* can restore both expression of LCR and virulence to an otherwise plasmid-free derivative of *Y. pseudotuberculosis* YPIII (21). In contrast, introduction of LCR-encoding plasmids from *Y. pestis* into *E. coli* K-12 does not result in expression of either LCR or virulence for mice (11). Based on these findings and on the relationship between LCR, cytotoxicity, and virulence demonstrated above, it is reasonable to predict that: (i) *Y. pseudotuberculosis* strains carrying either a native LCR-encoding plasmid or an LCR-encoding plasmid from *Y. pestis* will be cytotoxic, while LCR^- plasmid segregants will be atoxic, and (ii) *E. coli* K-12 strains carrying LCR-encoding plasmids from *Y. pestis* will be atoxic.

To test these predictions, appropriate strains were examined for cytotoxicity by the monolayer detachment assay. Results of these experiments were as predicted (Table 2).

DISCUSSION

The results presented above show that the LCR-encoding plasmid pCD1 of *Y. pestis* specifies the synthesis of a cytotoxic component or components which at high MOIs cause gross damage to two mouse macrophage cell lines and to resident peritoneal macrophages obtained directly from mice. Cytotoxic bacteria bound to and were partially ingested by the affected cells, but they were not phagocytosed. Chloramphenicol failed to protect macrophages from *Y. pestis* grown at 37°C, demonstrating that postinfection bacterial growth is not required for the toxic reaction to occur. Because neither supernatant fluid from cultures of toxic *Y. pestis* nor sonic lysates of *Y. pestis* cells were cytotoxic, the toxic species appears to be cell bound and to require intact bacteria to be effective. This latter result suggests that the bacterium-macrophage membrane contact which occurs during phagocytosis may be important for effective presentation of a toxin, or that bioenergetically active bacteria are required to cause the cytotoxic effect. No specific cytotoxic component has been identified as yet.

TABLE 2. Cytotoxicity in *Y. pseudotuberculosis* and *E. coli*

Strain	Species	Toxicity
YPIII	*Y. pseudotuberculosis*	-
YPIII(pIB1)	*Y. pseudotuberculosis*	+
YPIII(pCD1::Mu dl 28.4)	*Y. pseudotuberculosis*	+
LE392	*E. coli*	-
LE392(pCD1)	*E. coli*	-

Expression of cytotoxicity, LCR, and virulence require genes within the same three pCD1 loci. These three characteristics are thus related at a functional level, although at present the details of this relationship remain obscure. A simple model consistent with most of our data is one in which either the V or W antigen is the cytotoxin and contributes directly to virulence in mice. Unfortunately, this model is inconsistent with the observation that Ca^{2+} concentration, which controls the LCR and thus synthesis of V and W (2, 22), does not affect expression of cytotoxicity. Although all atoxic mutants tested are reduced in virulence for mice, we cannot conclude without reservation that the activity observed as cytotoxicity in vitro contributes to virulence in vivo. All of these mutants are also LCR^- and hence synthesize markedly reduced amounts of V (and probably W) antigen (7). Thus, we are unable to distinguish between the effects of loss of cytotoxicity and loss of V on virulence. Resolution of this problem will require the isolation of V^+ Tox^- or V^- Tox^+ mutants.

Temperature seems to be a reasonable signal by which pathogenic bacteria might regulate expression of genes whose products are required only during infection of a mammalian host. It is clearly an important environmental signal controlling the synthesis of cytotoxic components since KIM5 cells grown at 26°C required de novo protein synthesis at 37°C for expression of cytotoxicity. Like cytotoxicity, LCR also is expressed at 37 but not 26°C. The data presently available suggest that regulation of both cytotoxicity and LCR in response to temperature probably is accomplished by regulation of transcription. Transcription is thermally regulated in the expected pattern at two of the three loci (*lcrB* and *lcrC*) known to be involved in expression of toxicity, LCR, and virulence (7). Moreover, we have recently found that pCD1 contains a complex thermally controlled regulon involved in LCR expression that contains at least three additional genes (21a). Preliminary data indi-

cate that mutations at some of these new loci also affect expression of cytotoxicity.

How might the activity observed as cytotoxicity in vitro contribute to virulence of *Y. pestis*? Although direct killing of phagocytic cells in vivo might occur, particularly at foci of infection where bacteria are numerous, the high MOIs required to achieve destruction of monolayers in vitro suggest that the effect of the toxic components in vivo may be more subtle. For example, at low MOIs, the toxin may poison the phagocytic process locally and prevent ingestion of individual bacteria without causing gross cell damage. This hypothesis is consistent with the recent observations of Charnetzky and Shuford (3). These authors found that *Y. pestis* grown at 37°C was poorly phagocytosed and grew more rapidly in the mouse peritoneum over a 1-h period immediately after infection than did *Y. pestis* grown at 26°C. This result was plasmid-dependent and occurred with small inocula (10^4 bacteria per mouse) that likely would exclude interactions of the phagocytes with large numbers of bacteria.

Straley and Harmon (15, 16) have recently reported that *Y. pestis* strains closely related to KIM5 grow within the phagolysosomes of resident mouse peritoneal macrophages and that the absence of pCD1 does not prevent such growth. Although MOIs as high as 3,000 were employed in their experiments, these workers did not observe the cytotoxic effect we report here. Nonetheless, their results are not inconsistent with ours. In all of their experiments the bacteria used were pregrown at 26°C, a condition we have shown to prevent expression of cytotoxicity. In addition, the medium used for postinfection incubation in their experiments (Hanks balanced salt solution supplemented with 5% fetal calf serum) did not support growth of *Y. pestis* and thus prevented the accumulation of toxic extracellular bacteria during the 37°C postincubation. If cytotoxic components were synthesized by the intracellular bacteria proliferating in the phagolysosomes of infected cells, their effects may have not been observed due to deterioration and detachment of these cells caused by bacterial growth, or because the cytotoxic components were ineffective when presented to the cell from within the phagolysosome.

In addition to demonstrating cytotoxicity in *Y. pestis*, we also found that *Y. pseudotuberculosis* YPIII expressed similar activity encoded by either its native LCR-encoding plasmid (pIB1) or by pCD1 from *Y. pestis*. Cytotoxicity determined by an LCR-encoding plasmid has also been reported in *Y. enterocolitica* (12). Although it has not been shown that this activity is directly linked to the LCR, it is temperature dependent and is observed only with intact bacteria. Thus, like the LCR, expression of cytotoxicity probably is universal among the yersiniae virulent for man. Because it is the first LCR-related phenotype that involves an in vitro biological activity that may relate directly to virulence, analysis of cytotoxicity is an attractive approach to dissecting this complex plasmid-borne virulence determinant.

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ADDENDUM IN PROOF

R. Rosqvist and H. Wolf-Watz (Microb. Pathogen., in press) have recently reported plasmid-dependent cytotoxicity of *Y. pseudotuberculosis* for HeLa cells.

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