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PROTEIN EXPRESSION DURING THE LOW CALCIUM RESPONSE IN YERSINIAE

By

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ABSTRACT

PROTEIN EXPRESSION DURING THE LOW CALCIUM RESPONSE IN YERSINAE

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The plasmid-mediated low calcium response (Lcr) occurs when pathogenic yersiniae are grown *in vitro* at 37°C in Ca^{2+}-deficient media and is characterized by a stepdown in vegetative growth accompanied by induction of Lcr-specific virulence factors. The latter include Lcr plasmid-encoded outer membrane peptides (Yops) that undergo hydrolysis in *Yersinia pestis* due to the plasminogen activator/coagulase (PAC) activity encoded on the pesticin plasmid which is not found in the enteropathogenic strains. In this report, protein expression was examined to determine the production and degradation of important Lcr-mediated peptides and also to identify the production of other virulence factors or auxiliary proteins during the Lcr. Using [^{35}S]-methionine in pulse-chase experiments, important Yops of *Y. pestis* were found to be maintained in the steady state or exist as stable degradation products. Using the above method, a *Yersinia pseudotuberculosis* strain carrying the pesticin plasmid exhibited a similar Yop degradation pattern as *Y. pestis*. Differences in this *Y. pseudotuberculosis* strain

include the presence of a major Yop that was only partially degraded and a species specific adhesion protein (Yop A) that exhibited a discrete, stepwise degradation pattern. Pulse-chase studies of Y. pestis revealed the production of two major heat-shock protein occurs before onset of Lcr. Radiolabeling of Y. pestis during the Lcr revealed the production of several major stable peptides. These cytoplasmic peptides were labeled and then fractionated on various columns for identification of the peptides produced during the Lcr. Correlation of the peptides produced during the Lcr with known products produced by Y. pestis was done mainly by immunoblotting. Products identified include the Lcr plasmid-encoded V antigen and an unknown 20 kilodalton peptide, the pesticin plasmid-encoded pesticin and a 35 kilodalton degradation product of the PAC, and the 110 kb cryptic plasmid-encoded capsular antigen (fraction 1) and the plague murine exotoxin. Chromosomally-encoded peptides of 70 and 56 kilodaltons were found to be a novel basic peptide with catalase activity and the yersiniae equivalent to the E. coli Gro EL heat shock peptide, respectively.

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INTRODUCTION

There are three Yersinia species pathogenic for humans; Yersinia pestis, the causative agent of bubonic plague, and the enteropathogenic yersinia, Y. enterocolitica and Y. pesudotuberculosis. These three gram-negative organisms are facultative intracellular parasites. An important virulence factor common to all three is the carriage of a approximate 70 kb plasmid that mediates the low calcium response (Lcr). The Lcr occurs when the cells are grown at 37°C in media lacking Ca^{2+}, whereby the organisms undergo a slow down or a restriction of growth and the induction of Lcr plasmid-mediated virulence factors. During this restriction of growth in Y.pestis, only the Lcr-mediated peptides are produced while most vegetative protein production is shutdown.

This plasmid encodes several virulence factors that are necessary for virulence of this organism, since loss of this plasmid results in avirulence of these pathogens. A subset of these Lcr-mediated virulence factors termed Yops (yersiniae outer membrane peptides) undergo hydrolysis only in Y. pestis due to the presence of a second plasmid, the

pesticin plasmid. This pesticin plasmid encodes a plasminogen activator/coagulase (PAC) that has fibronlytic activity and it degrades the Yops. Most of the research involving the low calcium response has been to identify the role of these Yops and the regulation of expression of the Lcr. This dissertation continues to examine the production and degradation of Yops in Y. pestis and also examines Yop degradation in a second strain of yersiniae, Y. pseudotuberculosis. In addition to the Lcr, Y. pestis has other discrete virulence factors the are necessary for virulence of the organism. Since most protein production ceases during the low calcium response, it was of interest to see what other kinds of proteins are produced during the Lcr. Major stable proteins of Y. pestis were examined and identified to see if they were virulence factors or if they were proteins necessary for survival of the organism.

CHAPTER 1

LITERATURE REVIEW

Yersinia pestis, the etiological agent of bubonic plague, is a gram negative bacterium that is capable of causing a fatal infection in mice when only about ten organisms are injected subcutanously (68). *Y. pestis* is a facultative intracellular parasite that is able to multiply within mammalian phagocytic cells, (24) usually macrophages. Alexander Yersin was the first person to correctly identify this bacterium as the causative agent of bubonic plague during an epidemic in Hong Kong in 1894 (76). This organism was assigned to a number of different genera until 1970 when it was assigned its own genus, *Yersinia*, to honor the discovery by Dr. Yersin and that the organism is unique to other known pathogens.

The primary transmission of the plague to man is through a flea bite. The fleas feed on an infected host which is carrying the organism in its blood. The blood coagulates in the flea and blocks its ability to ingest more blood. This distressed flea abandons its normal host and attempts to feed on other hosts. The flea regurgitates the bacteria into the bloodstream of the new host where they travel to the lymphatic system. They gather in lymph nodes and begin

to multiply intracellularly until the lymph nodes are tender and swollen. Eventually the bacteria swamp the lymph system and spill into the blood creating a septicemia. Death usually follows within a few days if the infection remains untreated due to the large number of bacteria present in the host. Another form of the disease is pneumonic plague which is caused by airborne bacteria that infect the lungs. This results in a rapid pulmonary infection that is usually fatal.

There are two other yersiniae species that are pathogenic for man; Yersinia enterocolitica and Yersinia pseudotuberculosis. Both of these species are closely related to Y. pestis but they are much less virulent (68). The normal route of infection of these organism is through contaminated food or water thus eliminating the need for the highly invasive properties found in Y. pestis. They cause prolonged enteric diseases with fever and diarrhea or an appendicitis-like syndrome.

Yersiniae are susceptible to a number of antibiotics, thus these types of infections can be controlled, but these organisms serve as an excellent model system. There have been many important discoveries made in bacterial pathogenesis using the yersiniae system. Plasmid borne invasive functions being genetically linked to bacteriocin production was first found in Y. pestis (12). Iron uptake by a non-siderophore mechanism and the ability to utilize

exogenous hemin as a sole source of iron were both initially reported in Y. pestis (47) as well as the importance of iron privation as a non-specific mechanism of host defense (6). Other important discoveries include; (i) plasmids can mediate bacterial invasiveness (11,30,42), (ii) mutations in purine biosynthesis but not other metabolites can cause avirulence (13,17), and (iii) the ability to absorb exogenous hemin or congo red serves a virulence function (38,62).

Yersinia pestis serves as an important model for facultative intracellular parasites. Avirulence can result from the loss of any one of several distinct virulence factors as shown in Table 1. The different virulence factors give different degrees of avirulence as measured by the lethal dose needed to kill half of the experimental animals (LD_{50} values). Each of these virulence factors is explained in more detail below.

PURINE BIOSYNTHESIS

The ability to synthesize purines (Pur^+) is a requirement of all pathogens. This virulence factor has been reported for many pathogens including Y. pestis (17). It is assumed that free purines are not available in the mammalian host cell and that they must be manufactured de novo by the pathogen in order to proliferate (1). Thus, the loss of the ability to produce them results in avirulence of

Table 1 Virulence factors of *Yersinia pestis* and their effect on the LD_{50} value for guinea pigs and mice

Virulence Factor[a]					LD_{50}[b]		
Lcr	Pst	Pgm	Fra	Pur	mouse	guinea pig	mouse + Fe^{3+}[c]
+	+	+	+	+	<10	<10	<10
O	+	+	+	+	$>10^7$	$>10^8$	$>10^7$
+	O	+	+	+	$\sim10^5$	$\sim10^6$	<10
+	+	O	+	+	$>10^7$	$>10^8$	<10
+	+	+	O	+	<10	$\sim10^4$	<10
+	+	+	+	O[d]	$\sim10^2$	$\sim10^4$	$\sim10^2$
+	+	+	+	O[e]	$>10^7$	$>10^8$	$>10^7$

[a]abbreviations for Virulence Factors

Lcr = Carriage of the Lcr Plasmid
Pst = Carriage of the Pesticin Plasmid
Pgm = ability to absorb exogenous hemin (pigmentation)
Fra = Fraction 1 (capsular antigen)
Pur = Purine Biosynthesis

[b]intraperitoneal injection

[c]Sufficient iron injected to saturate serum transferrin

[d]Mutation blocks Purine biosynthesis before Inosine monophosphate

[e]Mutation blocks Purine biosynthesis after Inosine monophosphate

the organism. Evidence to support this idea came from *in vitro* infection of cultured macrophages with *Y. pestis*. Purine auxotrophs were unable to survive or replicate unless hypoxanthine or guanosine were added to the media while Pur^+ strains were able to grow in the macrophage (63).

The location of the mutation in the purine biosynthetic pathway is important in the loss of virulence (Table 1). If the block occurs before the synthesis of inosine monophosphate (IMP), an early step in purine biosynthesis, then there is only a slight reduction in the pathogencity of the organism for mice. However, If the block occurs in the conversion of IMP to guanosine monophosphate, then the strain becomes completely avirulent in mice (13).

FRACTION 1

Fraction 1 (Fra^+) represents the highly immunogenic capsular antigen of *Y. pestis* which has been purified and described as a protein-carbohydrate complex (2,5). The subunits are approximately 15 kdal which consists of a protein bound to a small carbohydrate described as an oligomeric galactan (5). These subunits form structures up to 300 Kdal in size and can be dissociated into subunits with SDS and upon removal of SDS will spontaneously reform large structures (5). Its production is temperature dependent with maximum production at 37°C and only small

levels detected at lower temperatures (5). Mutation to Fra^- reportedly occurs at a high frequency both *in vivo* and *in vitro* (20,50) which may verify its reported existence on the 110 kb cryptic plasmid in *Y. pestis* (30,51). There is a second class of mutations designated $F1^{\pm}$ (21) which produces fraction 1 but fails to incorporate the antigen into an extracellular capsular structure.

The exact role of fraction 1 during an infection is unclear. When Fra^- strains of *Y. pestis* are injected intraperitoneally into guinea pigs there was reduced virulence (20) but the organism remained fully virulent for mice(14) (Table 1). Visible encapsulation of *Y. pestis* had no effect on resistance to ingestion by murine phagocytes *in vivo* (19). It has also been reported that Fra^- strains were able to cause chronic and sometimes lethal infections in laboratory rats(73,74). There has also been a $F1^{\pm}$ *Y. pestis* strain isolated from a plague patient (75). All of this evidence casts a doubt on whether or not fraction 1 is an important virulence factor in humans.

PIGMENTATION

Virulent strains of *Y. pestis* usually form pigmented colonies (Pgm^+) when grown on semi-solid media containing hemin (38) or Congo red(66). The importance of this observation was not known until nonpigmented isolates were

found to be avirulent by subcutaneous or intraperitoneal routes of injection (39,68) but were still fully virulent when injected intravenously (68). These nonpigmented strains could become fully virulent by peripheral routes if sufficient iron was injected to saturate serum transferrin of the host (39) (Table 1). Hence, pigmentation was then thought to play a role in iron acquisition in the mammalian host.

Iron is found in very minute amounts in the extracellular environment of a mammal, so for invading organisms to proliferate in this niche they must have some type of high affinity iron uptake system. Many organisms use excreted low molecular weight peptides, termed siderophores, that chelate iron and then are taken up by the pathogen. In iron limiting conditions, yersiniae do not produce siderophores and only the enteropathogenic strains are capable of using them as an iron source (49). Y. pestis is capable of growth with ferritin, hemin, and hemin-containing proteins but not transferrin or lactoferrin (57). When strains of Y. pestis are grown in iron limiting conditions, nonpigmented strains can only grow for a few generations while the pigmented strains grow for at least 16 generations (56). This evidence has led to the theory that yersiniae have some type of cell-bound high-affinity iron uptake system that is lost upon mutation to nonpigmented.

This mutation may be part of the uptake system or found in a iron storage function.

Recent work in this area has revealed that a deletion occurs in the chromosome (unpublished data) upon mutation to the nonpigmented phenotype which results in the loss of 5 outer membrane peptides (57). The peptides that are lost are only produced under iron privation and are repressed when sufficient quantities of iron is present (57). This would be expected if the peptides were needed for iron uptake under iron limiting conditions. A mutant strain that contains one of these peptides, but not the other four, has been correlated with the pigmentation phenotype, but this peptide alone does not confer growth in iron limiting conditions (57).

The enteropathogenic yersiniae were not found to be pigmented (66), on a modified Congo red medium except for some clinical isolates of Yersinia enterocolitica (52). This was a different type of pigmentation which correlated with the presence of the Lcr plasmid and was limited to only virulent isolates of Y. enterocolitica (52). Pigmentation has also been found in virulent strains of E. coli, Shigella species and Neisseria meningitidis and may play a similar role in these organisms (48).

PESTICIN PLASMID

The pesticin plasmid is a 9.5 kb plasmid named after the first product to be associated with the plasmid; the bacteriocin pesticin. Pesticin is a 44 kilodalton protein that has N-acetylglucosaminidase activity (29) and it has been shown to inhibit the *in vivo* incorporation of diaminopimelic acid into the peptidogylcan of *E. coli* Ø. It has been shown to promote the *in vitro* and *in vivo* release of diaminopimelic acid from peptidoglycan.

Strains of *Y. pestis* that produce pesticin also produce an inhibitor of pesticin termed immunity protein, which prevents self-destruction (9). Strains of *Y. pestis* lacking the pesticin plasmid may or may not be sensitive to pesticin. A majority of cells resistant to pesticin were also found to be nonpigmented. One of the outer membrane peptides that is lost upon mutation to nonpigmented apparently acts as the uptake protein for pesticin (57).

Coagulase and fibrinolysin activities were also linked to the production of pesticin (4,22) and eventually to the pesticin plasmid (62). These two activities were found to be accomplished by the same protein (59). This outer membrane peptide is found as a 37 kilodalton protein and a 35 kilodalton degradation product (59). It is not known if only one or both of these peptides has the activities or if the activity is different for each of the peptides. It has been shown that coagulase activity is predominate at

temperatures below 30°C while the fibrinolytic activity is greater at higher temperatures (45). This temperature dependence may cause the blockage in the flea whose body temperature is lower than that of mammals.

Y. pestis strains that lack the pesticin plasmid show a drop in virulence (Table 1), especially by the peripheral routes, which may indicate that the pesticin plasmid is responsible for the invasiveness of the organism (11,67). The LD_{50} for mice of a wild type strain of Y. pestis is less than ten organisms by any method of injection. An isogenic strain lacking the pesticin plasmid retains a similar LD_{50} (71 organisms) by the intravenous route of injection, an intermediate LD_{50} (3.8×10^5) by intraperitoneal injection and is completely avirulent by the subcuteneous route. The virulence of this mutant strain could be restored by the intraperitoneal route but not the subcutenous by injecting the mice with an excess of iron before the challenge (11). This effect of iron is similar to that reported for the non-pigmented mutation (39) but there are no iron regulated proteins found on the pesticin plasmid (57). The effect in this case may reflect suppression of non-specific mechanisms of host defense rather than a loss of iron acquisition ability (56,70,72). The loss of these mechanisms may give the pathogen enough time to find a suitable host cell in which to replicate. The only function specific to the pesticin plasmid that may be responsible for the

invasiveness is the fibrolytic activity. More work is needed to determine the exact function the pesticin plasmid plays in the mammalian host during infection.

LCR PLASMID

Early *in vitro* research on *Y. pestis* was hampered by an unusual switch to avirulence of the cultures when the organisms were grown at 37°C in standard culture media. The virulent organisms failed to replicate and lysed under these conditions and were quickly overgrown by strains lacking the Lcr plasmid. Addition of 22 mM Mg^{2+} could prevent lysis but it was eventually found that 2-4 mM Ca^{2+} would allow the virulent strains to grow at this temperature (37). What made this problem so baffling was both strains grew equally well at 26°C irregardless of the calcium concentration. It was later found that zinc and strontium also allowed the virulent cells to grow at 37°C. The severity of the growth restriction was also found to depend on the Mg^{2+} concentration. The lower the level of Mg^{2+} the lower the levels of Ca^{2+} needed to allow the cells to grow (10). Using this information, a medium was developed to mimic mammalian intracellular fluid with respect to divalent cations (20 mM Mg^{2+}, 0 mM Ca^{2+}). When virulent *Y. pestis* are grown in this medium at 37°C, they undergo restriction of vegetative growth and begin synthesizing a set of

virulence factors. This change is referred to as the low calcium response (LCR) (34).

The LCR is mediated by an approximate 75 kilobase plasmid (lcr^+) common to all pathogenic yersinae. When lcr^+ cells, growing logarithmically at 26°C in calcium-deficient media, are switched to 37°C the LCR is induced. The first known change in the cells is a termination of stable ribonucleic acid (rRNA) synthesis but not necessarily messenger RNA (25). There is also a reduction in adenylate energy charge and a blockage in DNA synthesis initiation (79). Addition of exogenous adenine triphosphate (ATP) could relax the restriction of growth but the ATP was not metabolized. It is believed that the ATP acts as a chelator of the magnesium thus lowering the stringency of the restriction (80). The LCR appears to be an ordered metabolic shutdown resulting from a block in stable RNA synthesis (25).

The exact mechanism of how this plasmid restricts growth and begins producing virulence factors is not known. There is a 17-kb region on the lcr plasmid which seems to control the regulation of temperature and calcium. Transposon insertions in this region result in Ca^{2+}-independent mutants which do not require Ca^{2+} for growth at 37°C (26,27,34,77). These mutants also lose their ability to produce Lcr^+-specific virulence factors and the resulting avirulent strains are similar to Lcr^- strains. There are several

different loci identified with this region termed lcrA to lcrF in Y. pestis (48) and virA to virF in Y. enterocolitica (26,27). The lcrA locus was further divided into lcrD and lcrE loci(78). There were mutants found within the lcrA locus that were termed Ca^{2+}-blind (78). This mutant showed a constitutive lcr phenotype and its growth is restricted at 37°C even in the presence of 2.5 mM Ca^{2+} (78). A 3.8-kb fragment of this locus was able to complement this mutation and was designated lcrE. In the lcrE locus the mutation was mapped to the same location as the structural gene for an lcr encoded protein termed Yop N (71). Upon further investigation it was found that Yop N (renamed LcrE for its role in regulation of the LCR) was the first gene in an operon of six genes (71). The other five genes have no known function but are able to produce proteins(71). The virF gene has been shown to be a transcriptional activator controlling the lcr virulence genes (28). The expression of this protein is increased at 37°C and is probably responsible for the production of Yops, while Ca^{2+} concentration has no effect on the expression of this gene(28). In addition to this regulatory area, at least one important gene in virulence, Yop E, has a regulatory protein located just upstream from its coding sequence (33). Also one of the Lcr proteins, encoded on the plasmid, appears to be needed for export of the outer membrane proteins that are

produced (53). Therefore, the regulation of the LCR is very complex and will take a long time to decipher.

In addition to the restriction of growth there is a set of virulence proteins that is only made under these restrictive conditions (37°C, No Ca^{2+}). These virulence factors are important, since loss of the plasmid results in complete avirulence of the organism (Table 1). V and W antigens were shown to be produced only by virulent strains of Y. pestis both in vitro and in infected laboratory animals (18). These proteins were eventually found to be produced only under the conditions needed to induce the LCR and production was elevated when high levels (>20mM) of magnesium were present (10,43). It was shown that antibodies specific for V antigen could passively protect mice from intraperitoneal challenge with a lethal dose of Y. pestis. However, this did not hold true for antibodies specific for W antigen(43). Both V and W antigens were shown to be produced by the enteropathogenic yersiniae during growth at 37°C in calcium deficient media (22,23).

The first protein to be characterized was V antigen. This protein is a 90 kdal protein that is found both in the bacterial cytoplasm and its culture fluid (15). V antigen has been shown to prevent granuloma formation at the foci of infection in liver tissue (69). Monoclonal or polyclonal antisera to V antigen injected along with the organisms restores granuloma formation and limits the invading

organisms (69). This lack of neutrophil and mononuclear cell response indicates that V antigen may act as a anti-chemotactic agent. V antigen has been purified but this protein undergoes rapid proteolysis in the purified form (15). There is not much information on W antigen but it has been reported to be a lipid-protein complex (22).

In addition to V and W antigens, the lcr plasmid also encodes a set of yersiniae outer membrane peptides termed Yops. These proteins are only produced during the low calcium response (6,61). There have been at least 13 different Yops described and they have been given the nomenclature of YopA through YopM (64). Yop A is an adhesion protein and is only found in the enteropathogenic yersiniae. It seems to have many roles including such things as autoagglutination (58), mannose-resistant haemagglutination of guinea-pig erythrocytes (40,41), and inhibition of the anti-invasive effect of interferon (16). In Y. enterocolitica Yop A has been associated with serum resistance (3), adherence and inhibition of internalization of the bacterium in HEp-2 cells (36), and expression of fibrils on the surface of the bacteria (41). This Yop is different from the other Yops in that it is induced by the higher temperature (37°C) and its production is not regulated by calcium (7,8,31). Yops G and I are only found in Y. pseudotuberculosis and their function remains unknown (65). Yops K, L and M are only found in Y. pestis (65).

Yop M has been shown to inhibit platelet aggregation and may be necessary for virulence (44).

The two most important Yops are Yop E and Yop H (32,64). These Yops are highly conserved between the three species and avirulence results upon mutation of these genes. Yop H is a protein tyrosine phosphatase (PTPase) related to the eukaryote PTPases such as CD45 and leukocyte antigen-related protein (LAR) (35). How it effects the host still remains unknown. Yop E is cytotoxic for HeLa cells but only if the bacteria are bound to them (53). It is also cytotoxic to mouse macrophages and it may also influence the ability of the pathogen to resist phagocytosis (53). Insertional mutation of this gene results in avirulence (32,64) but this can be restored by injection with iron (47).

Large quantities of Yops were originally only found on the enteropathogenic strains. Y. pestis did not appear to make Yops in vitro although immune sera from convalescent plague patients contained antibodies to Yops which recognized the Yops in the enteropathogenic strains (8,46) thus indicating Yop production in vivo. Transfer of the Lcr plasmid from Y. pestis to a Lcr^- strain of Y. pseudotuberculosis allowed full expression of Yops indicating that Y. pestis had the coding capacity for Yops (64). The controversy was resolved when Sample et. al. showed that curing the organism of the pesticin plasmid resulted in the full production of Yops (54). Pulse-chase

studies of protein expression later showed that the Yops were being produced but were then rapidly degraded (55). This proteolysis was later found to be the fibrinolytic activity that is encoded on the pesticin plasmid (60).

The low calcium response is an important virulence factor in all of the yersiniae strains but there is still a lot of information to be gathered to truly decipher the exact role in pathogenesis. In this study, the general protein expression during the LCR is investigated with most of the emphasis on Yersinia pestis.

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CHAPTER 2

Expression of the low calcium response in *Yersinia pestis*

by

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Abstract

Pathogenic yersiniae undergo an established low calcium response (LCR) at 37°C in Ca^{2+}-deficient media characterized by restricted growth with synthesis of Lcr plasmid-encoded virulence functions. The latter include outer membrane peptides (Yops) known to undergo Pst plasmid-mediated post-translational degradation in Yersinia pestis but not in the enteropathogenic yersiniae lacking this plasmid. Salient Yops of Y. pestis are shown here to be either maintained in the steady state or exist as a stable degradation product (p24 of YopE). Processing of plague plasminogen activator (p36 to p33), responsible for the hydrolysis of Yops, required 2 hours. Avirulence of mutants with inserted Mu dl1 (Ap^rlac) in yopE was verified and shown to occur independently of introduced fusion-dependent peptides. However, avirulence of such yopE mutants but not that of isolates lacking the Lcr plasmid was phenotypically suppressed in mice injected with iron. Appearance of 20,500 and 40,55 Da heat-shock peptides preceded onset of the LCR. Lcr plasmid mediated V antigen (p38) and p20, Pst plasmid-encoded p36, and chromosomally promoted p56 and p70 were synthesized throughout the LCR. Classical antigen 5 was equated with p70 which was shared by Yersinia pseudotuberculosis but not Yersinia enterocolitica.

INTRODUCTION

Yersinia pestis, the causative agent of bubonic plague, and the enteropathogenic yersiniae (Yersinia pseudotuberculosis and Yersinia enterocolitica) are generalized facultative intracellular parasites capable of growth in either Ca^{2+}-enriched blood and lymph or Ca^{2+}-deficient intraleukocytic fluids (11). However, at 37°C in typical culture media lacking Ca^{2+} these organisms undergo a unique metabolic stepdown, termed the low calcium response (LCR)(21), characterized by restriction of vegetative growth accompanied by selective synthesis of putative and established virulence factors (lcr^{+}). An approximate 70 kb shared Lcr plasmid mediates the LCR which does not occur at 26°C in Ca^{2+}-deficient media or at 37°C in the presence of at least 2.5 mM Ca^{2+}. Lcr^{-} mutants cured of this plasmid are avirulent and do not express the LCR regardless of temperature or concentration of Ca^{2+}(10,16,34)

Regulatory mechanisms of the LCR probably serve to identify the intracellular or extracellular environments occupied by invading yersiniae (6,30) thereby permitting induction of an appropriate attack against the distinct nonspecific mechanisms of host defense resident to these niches (11). An approximate 20 kb segment of the Lcr plasmid serves to promote the temperature-dependent response to Ca^{2+} (4,15,21,29,32,33,52). Mutations within this region (3,6,15,21,51) and a linked operon (29) typically permit

Ca^{2+}-independent growth with loss of ability to express Lcr^{+}-specific virulence functions, thereby conferring an avirulent phenotype similar to that resulting from cure of the Lcr plasmid. Products of genes within these regions were assigned diverse regulatory roles including an activator and repressor of virulence factor synthesis and possible sensors of Ca^{2+} and temperature (20,52).

Lcr^{+}-specific virulence functions shown to be induced during expression of the LCR and repressed by Ca^{2+} (3,4,6,15,19,20,29-33,37,51,52,54) include the soluble V and W antigens (13,26) and certain yersiniae outer membrane peptides (30,43) termed Yops (3). Structural genes for these determinants are usually located outside of the 20 kb regulatory region. Mutation in many such genes, especially yopE and yopH (3,5,36,43,45) and possibly that for V antigen (29), lead to avirulence. A full component of yops is only expressed in vitro by Lcr^{+} cells of nonpesticinogenic Y. pestis (37,38) lacking the 10 kb pesticin or Pst plasmid unique to this species (2,18). These structures do not accumulate in typical pesticinogenic (Pst^{+}) isolates (37,43) where they are synthesized but undergo rapid post-translational degradation (38) catalyzed by a Pst plasmid-mediated (7,8) outer membrane plasminogen activator/coagulase activity (PAC) (1,41,42,44). PAC exhibits a molecular weight of 38,000 Da but undergoes rapid processing to 37,000 Da during secretion followed by slow

conversion (in minicells) to 35,000 Da (41). It is an enigma that Yops are not expressed in Lcr^+, Pst^+ cells of Y. pestis since mutational loss of Yop E and H in this species results in avirulence (45).

One objective of this paper is to reconcile this discrepancy by showing that significant concentrations of salient Yops are maintained in vitro by Y. pestis in the steady state or exist as stable degradation products. We also demonstrate that avirulence caused by mutational loss of Yop E can be phenotypically suppressed in mice by injection of iron as opposed to that resulting from the cure of the Lcr plasmid. Finally, previously described (38) evident chromosomal and Pst plasmid-encoded peptides are defined which are regulated by a mechanism that abrogates the LCR.

MATERIAL AND METHODS

Bacteria

Origins of isolates used in this study have been described (18,37,38,48). The parent of all derivatives of Y. pestis was strain KIM (9). This Lcr^+, Pst^+ isolate was non-pigmented thus it and its mutants are avirulent in mice unless injected intravenously (46) or intraperitoneally with exogenous iron (24). Other yersiniae used possessed insertions of Mu dl1 (Ap^rlac) in the Lcr plasmid in yopE or yopJ; construction of these isolates has been described

(45). Lcr$^-$ and Pst$^-$ mutants, lacking the Lcr or Pst plasmid (18), were selected at 37°C on magnesium oxalate agar (22) or by cold cure (37). Y. pestis strain EV76, Y. pseudotuberculosis PB1, and Y. enterocolitica WA have been described (37,38,43,45). Where necessary, methionine meiotrophs of Y. pestis strain KIM were isolated as previously reported (38).

Media and Cultivation.

Bacteria were cultivated in chemically defined medium (54) lacking methionine as already described (38). All cultures contained 20 mM Mg^{2+} and no added Ca^{2+}. To assure balanced growth prior to pulse with ^{35}S-methionine, the organisms were subcultured twice at 26°C and then shifted to 37°C to induce the LCR (38).

Pulse-chase labeling of bacteria

Procedures used for pulse-chase experiments were identical to those reported earlier (38). A typical experiment involved pulsing methionine meiotrophs, starved for Ca^{2+} in methionine-free medium, with ^{35}S-methionine and then chasing with unlabeled methionine at great excess. Samples of whole cultures were removed at appropriate intervals, precipitated with cold 10% TCA and then prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The resulting gels were

stained with Coomassie Brilliant Blue G prior to autoradiography as previously described (38).

In experiments concerned with stable peptides expressed during the LCR, the organisms were pulsed and chased as already performed (38) to assure degradation of Yops and then centrifuged at 10,000 x g for 15 min at 4°C, washed twice in chilled 0.033 M potassium phosphate buffer, pH 7.0 (phosphate buffer) and then prepared for SDS-PAGE as described for TCA-precipitated samples. This process assured removal of radioactivity loosely associated with the outer membrane (primarily p24). SDS-PAGE and autoradiography were undertaken as defined previously (38).

Determination of virulence.

Organisms were injected either intraperitoneally or intravenously in 0.1 ml of phosphate buffer. Iron, if administered, was injected intraperitoneally as $FeCl_2$ (40 μg of iron per mouse) suspended in arachis oil (24). Doses of about 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 organisms were injected into seven mice apiece in determinations of LD_{50} (calculated by the method of Reed and Meunch) (35). Mice were maintained as already defined (47) for 2 weeks.

Immunoblotting

Immunoblots of antigen 5 were prepared as previously described (12). The primary antibody was the same monospecific polyclonal rabbit anti-E serum used as a temperature-dependent control in initially defining the LCR (6). The precipitin formed upon diffusion of this serum against cell-free extracts of yersiniae was previously shown (25) to be similar or identical to antigen 5 (17).

RESULTS

Peptides translated during the LCR

We have shown previously that Yops of LCR^+, Pst^+ *Y. pestis* undergo immediate post-translational proteolysis *in vitro* (38). To detect putative long-term degradation products and to further define the kinetics of Pst plasmid-dependent destruction of Yops, we pulsed restricted Lcr^+, Pst^+ yersiniae for 1 min with ^{35}S-methionine in a similar manner but extended the period of chase with unlabeled methionine for up to 6 h.

As shown in autoradiograms, previously defined (36) major stable peptides of 70,000 Da (p70), 56,000 Da (p56), 38,000 Da (p38 or V antigen), and 20,000 Da (p20) remained intact throughout this prolonged chase in cultures of both Lcr^+, Pst^+ (Fig. 1) and Lcr^+, Pst^- yersiniae (Fig. 2). Only Yops of Lcr^+, Pst^- mutants exhibited equivalent stability (Fig. 2). As anticipated, pulsed radioactive Yops of

Figure 1. Stained gel (A) and corresponding autoradiogram (B) of trichloroacetic acid-precipitated material from cultures of Lcr^+, Pst^+ cells of Yersinia pestis strain KIM after 6 h of cultivation at 37°C in Ca^{2+}-deficient medium (at which time vegetative growth had ceased). Bacteria were pulsed for 1 min with [^{35}S]-methionine and then chased with excess unlabeled methionine for 0 (lane 1), 5 min (lane 2), 15 min (lane 3), 30 min (lane 4), 1 h (lane 5), 2 h (lane 6), 3 h (lane 7), 4 h (lane 8), 5 h (lane 9), and 6 h (lane 10); molecular weight markers (kDa) are shown in lane M. Peptides were separated by 12.5% SDS-PAGE. Open arrowheads represent stable peptides known to be produced during the LCR and closed arrowheads indicate Yops; stable degradation products (p33 and p24) are shown by arrows.

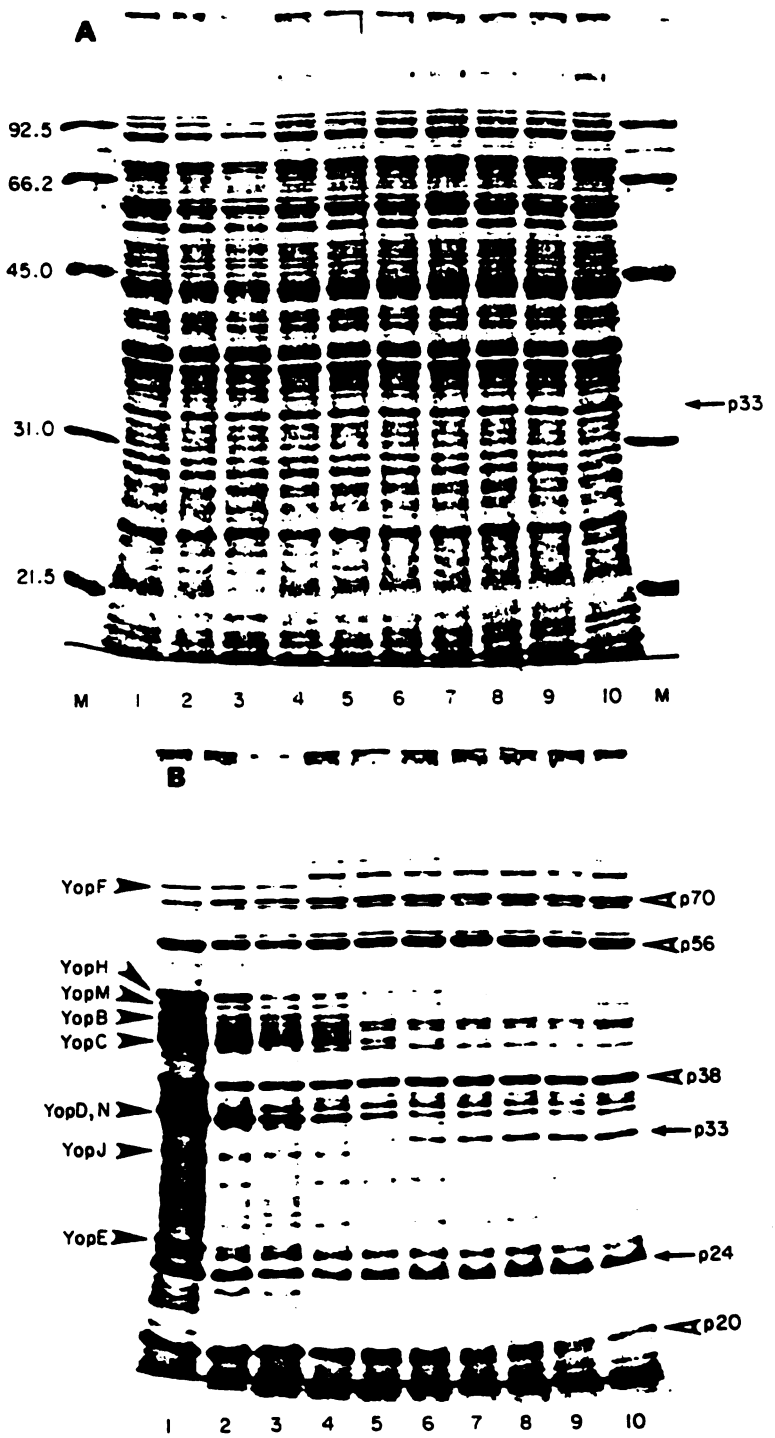
A
92.5
66.2
45.0
31.0
21.5
p33
M 1 2 3 4 5 6 7 8 9 10 M
B
YopF
YopH
YopM
YopB
YopC
YopD, N
YopJ
YopE
p70
p56
p38
p33
p24
p20
1 2 3 4 5 6 7 8 9 10

Figure 1.

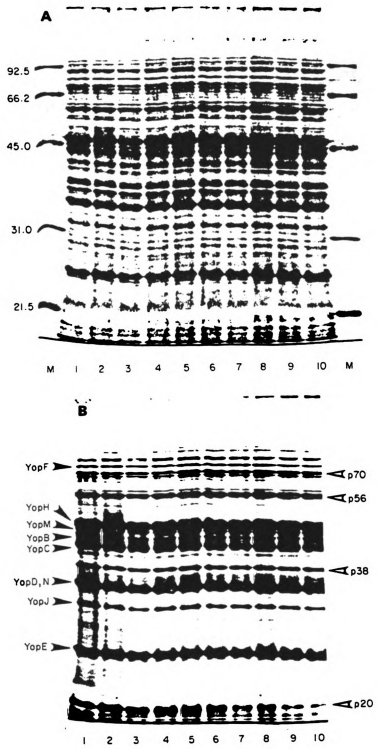

Figure 2. Same as Figure 1 except that an isogenic Lcr$^+$, Pst$^-$ isolate was used.

Lcr$^+$, Pst$^+$ organisms underwent rapid degradation (Fig. 1). Nevertheless, reduced but detectable levels of peptides with molecular weights corresponding to Yop H (47,000 Da), Yop B (44,000 Da), Yop C (42,000 Da) PAC or p36 (36,000 Da) (20, 35, 37) and Yop D (34,500 Da) occurred in all lanes of the corresponding stained gel of Lcr$^+$, Pst$^+$ yersiniae (Fig. 1). This finding suggests that diminished but nevertheless significant levels of at least these four Yops are maintained by Lcr$^+$, Pst$^+$ organisms in the steady state.

We have shown previously that a unique 24,000 Da peptide (p24) is generated during degradation of Yops in Lcr$^+$, Pst$^+$ yersiniae (38). As shown in Fig. 1, this hydrolytic product remained intact throughout the extended chase used in the present study. This observation, plus occurrence of significant concentrations of the peptide in all lanes of the stained gel (Fig. 1), demonstrates that p24 is a major stable intermediate.

A radioactive peptide of 33,000 Da (p33) accumulated in Lcr$^+$, Pst$^+$ (Fig. 1) but not Lcr$^+$, Pst$^-$ yersiniae (Fig. 2) after chase for 2 h with unlabeled methionine. Like p24, p36 and the four Yops already noted, this structure was present in all lanes of the corresponding stained gel (Fig. 1) indicating that, in the steady state, it exists as a major processed peptide. In view of prior work showing slow conversion of PAC to a smaller processed form (41), we

assume that p33 is identical to the smaller (33,000 Da) of two outer membrane peptides previously equated with this activity (37,42,44). The larger such peptide (p36 of 36,000 Da) could thus serve as the precursor of p33. A radioactive structure of this size was initially present (Fig. 1) which underwent degradation at a rate sufficient to account for generation of p33.

Yop E is the p24 precursor

The kinetics of p24 synthesis suggested that this structure arose as a degradation product of Yop E (38). To establish this relationship, we shortened the pulse of Lcr^+, Pst^+ organisms from 1 min to 15 s and also decreased the period of chase. This modification illustrated a transient increase in p24 accompanied by reduction in concentration of Yop E [Fig. 3(A)]. To prove that Yop E per se was the precursor of p24, the same determination was performed with a mutant of the Lcr^+, Pst^+ parent possessing a Mudl1(Ap^r lac) insertion in yopE (45). As shown in Fig. 3(B), neither Yop E nor p24 were evident thus demonstrating a precursor-product relationship. However, this isolate expressed at least two stable peptides (102,000 and 42,000 Da) not produced by the parent. To ascertain whether these structures represented either some unprocessed form of Yop E or, more likely Mudl1(Ap^rlac) dependent functions, we performed a similar determination with a yopJ mutant

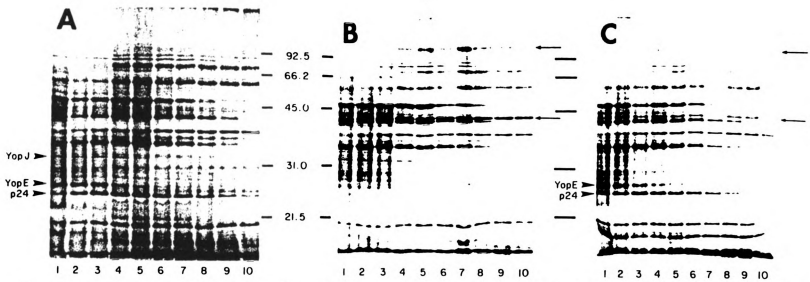

Fig. 3. Autoradiogram of trichloracetic acid-precipitated material from cultures of Lcr$^-$, Pst^r (A), Lcr^r (*yopE*), Pst^r (B), and Lcr^r (*yopJ*), Pst^r (C) cells of *Yersinia pestis* strain KIM after 6 h of cultivation at 37°C in Ca^{2+}-deficient medium at which time vegetative growth had ceased. Bacteria were pulsed for 15 s with ^{35}S-methionine and then chased with excess unlabeled methionine for 0 (lane 1), 15 s (lane 2), 30 s (lane 3), 1 min (lane 4), 2 min (lane 5), 4 min (lane 6), 8 min (lane 7), 15 min (lane 8), 30 min (lane 9), and 1 h (lane 10). Peptides were separated by 12.5% SDS-PAGE. Yops and p24 are indicated by closed arrowheads; Mu dl1 (Ap' *lac*) fusion-dependent peptides are shown by arrows. Molecular weights are shown in kDa.

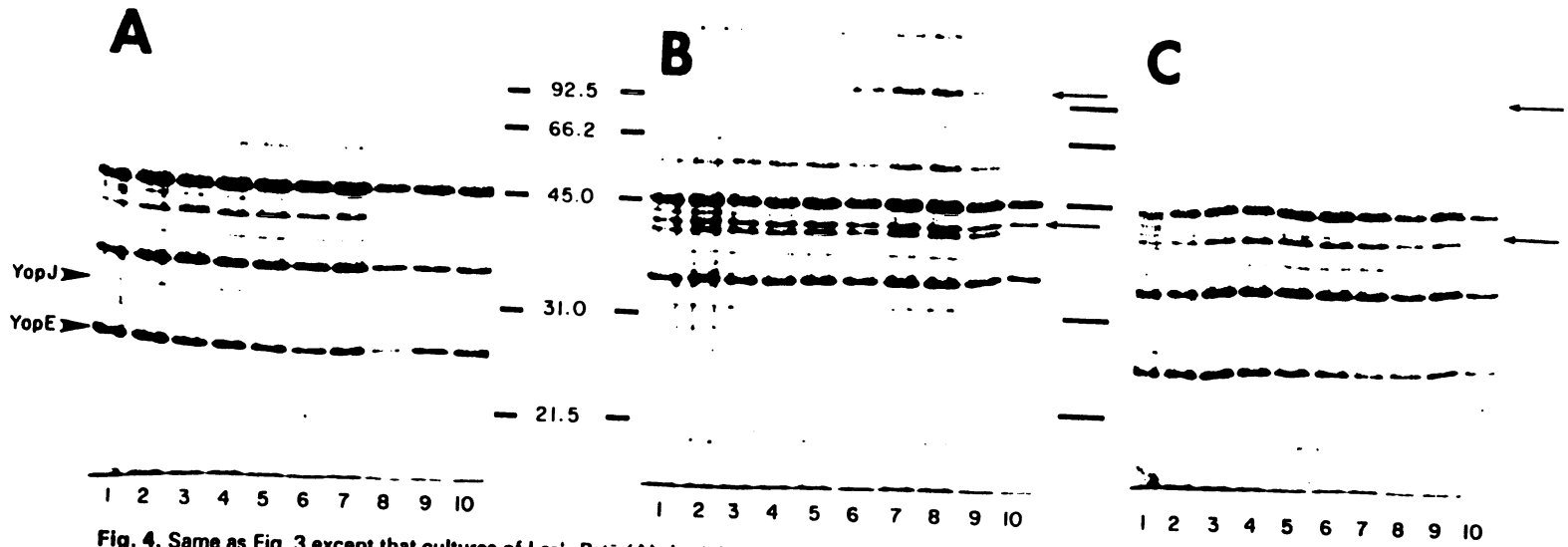

Fig. 4. Same as Fig. 3 except that cultures of Lcr', Pst⁻ (A), Lcr' (*yopE*), Pst⁻ (B), and Lcr' (*yopJ*), Pst⁻ (C) cells of *Yersinia pestis* strain KIM were used.

possessing an analogous Mudl1(Ap^{r}lac) insertion in the Lcr plasmid. Identical extra peptides were expressed in this isolate [Fig. 3(C)] indicating that they are fusion-dependent functions rather than specific Yop precursors. The kinetics of Yop E degradation with conversion to p24 in this mutant were similar to those of the parent.

To further clarify these relationships, this determination was repeated with the same strains after cure of the Pst plasmid. As shown in Fig. 4, both Yop E and Yop J remained stable in isolates capable of their production, p24 was never detected, and the extra fusion-dependent peptides were similarly expressed in the yopE and yopJ mutants.

Iron-dependent phenotypic suppression of avirulence in yopE mutants

Mice were injected either intravenously or intraperitoneally with Lcr$^+$, Pst$^+$ yersiniae or their yopE and yopJ mutants. As expected, these non-pigmented isolates were all avirulent via the intraperitoneal route (LD_{50} > 10^7) (23,24,46) but the parent (46) and yopJ (45) strains were fully lethal by intravenous injection (Table 1). The corresponding yopE mutant was essentially avirulent by this route thus verifying the results of Straley and Bowmer (45). This finding also demonstrated that the necessity to synthesize the extra fusion-dependent peptides described

Table 1 Interperitoneal and intravenous LD_{50} of Yop-deficient and non-pesticinogenic strains of *Yersinia pestis* strain KIM in normal and iron-treated mice[a]

Organism injected	Injected iron		No injected iron	
	Bacteria injected i.p.	Bacteria injected i.v.	Bacteria injected i.p.	Bacteria injected i.v.
Lcr^+, Pst^+	$2.4x10^1$	$4.3x10^1$	$>10^7$	$1.8x10^2$
Lcr^+ (*yopE*), Pst^+	$1.4x10^3$	$2.3x10^6$	$>10^7$	$3.5x10^6$
Lcr^+ (*yopJ*), Pst^+	$2.5x10^1$	$6.0x10^1$	$>10^7$	$6.5x10^1$
Lcr^+ Pst^-	$2.1x10^3$	$2.0x10^6$	$>10^7$	$7.8x10^5$
Lcr^+ (*yopE*), Pst^-	$1.7x10^6$	$3.7x10^6$	$>10^7$	$9.8x10^6$
Lcr^+ (*yopJ*), Pst^-	$5.4x10^1$	$2.2x10^4$	$>10^7$	$3.1x10^3$
Lcr^-Pst^+	$>10^7$	$>10^7$	$>10^7$	$>10^7$

[a]Iron was injected as $FeCl_2$ in arachis oil (40 μg/mouse). (37)

above in yopE mutants does not inhibit expression of disease because the same peptides were produced by the virulent yopJ isolate. However, virulence of the yopE mutant was essentially restored by intraperitoneal (LD_{50} = 1.4 x 10^3) but not by intravenous injection (LD_{50} = 2.3 x 10^6) in mice treated with exogenous iron (Table 1). This pattern is distinct from that of the parent and yopJ mutant where lethality could be enhanced by either use of the intravenous route (with or without injected iron) or by intraperitoneal injection in mice receiving injected iron. The Lcr^-, Pst^+ mutant (cured of the Lcr plasmid) was unable to kill either normal or iron-treated mice at sub-endotoxic doses (intravenous and intraperitoneal LD_{50} >10^7). Accordingly, exogenous iron can phenotypically suppress the yopE mutation to avirulence whereas this treatment is unable to restore virulence of mutants lacking the Lcr plasmid.

As anticipated, the Lcr^+, Pst^- isolate was attenuated under all tested conditions (Table 1) although virulence was significantly enhanced via intraperitoneal infection in mice receiving exogenous iron (LD_{50} = 2.1 x 10^3) (7). This route of injection did not significantly suppress avirulence of the Pst^- yopE mutant (LD_{50} = 1.7 x 10^6), an observation that would be expected if processing of Yop E to p24 is required for lethality. Curiously, the control Pst^- yopJ mutant was

somewhat more virulent under most conditions of assay than was its Lcr^+, Pst^- parent.

Expression of stable peptides during the low calcium response

Since the consequences of mutation to yopE but not avirulence resulting from cure of the Lcr plasmid, was phenotypically suppressed by injection of iron, we assumed that Lcr^+-dependent functions other than Yops fulfill essential roles in causing disease. To identify such candidates without interference by Yops and to define more fully regulatory events associated with onset of the LCR, we prepared a series of parallel cultures of Lcr^+, Pst^+ yersiniae in Ca^{2+}-deficient medium. The latter were aerated at 26°C until logarithmic growth was assured and then shifted to 37°C. At different times thereafter, each culture was pulsed for 1 min with ^{35}S-methionine and then chased for 1 h with unlabeled methionine to assure complete degradation of Yops.

Growth in these cultures (plus that of a control which was maintained at 26°C) and the times that each culture was pulsed is shown in Fig. 5(A). The autoradiogram of the corresponding samples prepared after incubation with excess unlabeled methionine for 1 h [Fig 5(B)] demonstrated that no significant difference occurred if the cultures were chased at 26°C or 37°C and that bulk protein, contributing to

Figure 5. (A) Optical density of parallel cultures of Lcr^+, Pst^+ cells of Yersinia pestis strain KIM versus time (h) showing points (arrows) where samples were pulsed for 1 minute with [^{35}S]-methionine prior to chase for 1 h with unlabeled methionine and preparation for autoradiography. Cultures (lacking Ca^{2+}) were shifted from 26°C to 37°C at an optical density of 0.25. Growth was then monitored during onset and expression of the LCR (O) and in a control culture maintained at 26°C (●). Protein synthesized during corresponding pulses were separated by 12.5% SDS-PAGE (B); 0 chased at 26°C(lane 1), 0 chased at 37°C(lane 2), 1 h(lane 3), 2 h(lane 4), 4 h(lane 5), 6 h (lane 6), 8 h(lane 7), 10 h(lane 8), and 12 h(lane 9); all chases after 0 h were performed at 37°C. Open arrowheads indicate Lcr plasmid-mediated peptides and closed arrowheads represent chromosomal (p70, p56) or Pst plasmid-mediated structures. Molecular weights are shown in kDa.

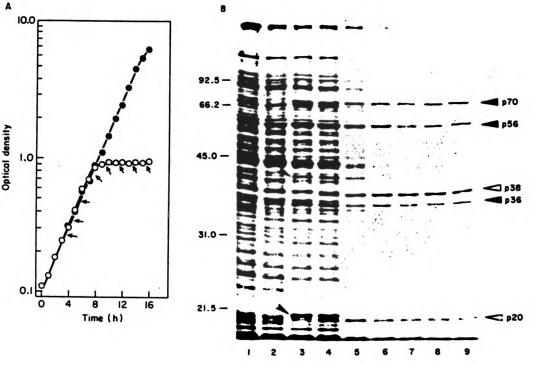
A
10.0
1.0
0.1
Optical density
0
4
8
12
16
Time (h)
B
92.5
66.2
45.0
31.0
21.5
p70
p56
p38
p36
p20
1
2
3
4
5
6
7
8
9

Figure 5.

vegetative growth, continued to be synthesized during the two anticipated (53) residual post-shift doublings. However, two evident heat-shock peptides of 20,500 and 40,500 Da were induced within 1 h after shift. Although appearance of these structures is the first known detectable event associated with onset of the LCR, their synthesis was not maintained after cell-division had ceased. At this time (6 h after shift to 37°C) and thereafter, the organisms continued to synthesize detectable levels of only p70, p56, p38, p36 and p20.

As already noted, p38 is V, p36 is a Pst plasmid-mediated precursor of p33 (either or both of which serve as the PAC) (41,42) and p20 is a major, but unknown, Lcr plasmid-mediated function (38,unpublished results). Both p70 and p56 were detected as major temperature-dependent peptides 30 min after shift from 26°C to 37°C in both Lcr^+ and Lcr^- organisms [Fig. 6(A)]. This observation suggests that these structures are chromosomally encoded but, like Lcr plasmid-mediated functions, are regulated by a mechanism that abrogates repression during the LCR. The identity of p56 has not been determined but p70 reacted in immunoblots with monospecific polyclonal rabbit antiserum (6) directed against antigen E [Fig. 6(B)], a structure probably identical (25) to the classical temperature-dependent antigen 5 of Crumpton and Davies (17). Antigen 5 was also

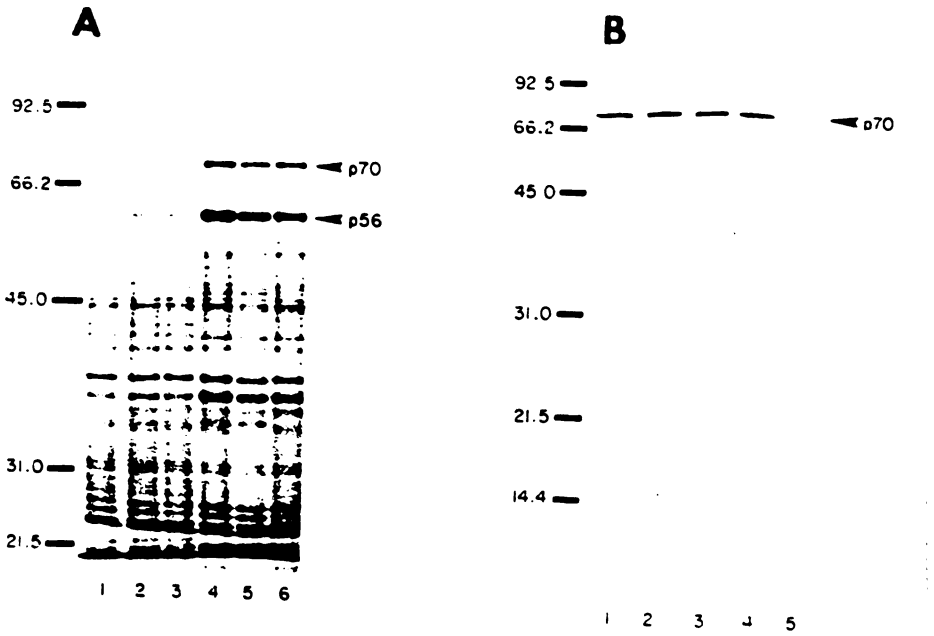

Figure 6. Autoradiograms (A) of trichloroacetic acid-precipitated material from culture of Lcr^+,Pst^+ (lane 1), Lcr^+,Pst^- (lane 2), and Lcr^-,Pst^+ (lane 3) cells of Yersinia pestis strain KIM growing at 26°C and from Lcr^+,Pst^+ (lane 4), Lcr^+,Pst^- (lane 5), and Lcr^-,Pst^+ (lane 6) cells from parallel cultures 30 min after shift to 37°C. Immunoblot (B) of similar preparation of cells of Lcr^+,Pst^+ Y. pestis strain KIM (lane 1), Lcr^-,Pst^+ Y. pestis strain KIM (lane 2), Lcr^+,Pst^+ Y. pestis strain EV76 (lane 3), Lcr^+ Y. pseudotuberculosis strain PB1 (lane 4), and Yersinia enterocolitica strain WA (lane 5) developed after growth for 6 h at 37°C with anti-E antigen (1:5000 dilution). Molecular weights are shown in kDa.

produced by Y. pseudotuberculosis strain PB1/+ but not by Y. enterocolitica WA [Fig. 6(B)].

DISCUSSION AND CONCLUSIONS

As previously noted, Yops are not expressed during the LCR by wild type cells of Y. pestis (33,37,38,43). Nevertheless, isolates blocked in synthesis of Yops E, H and K (as determined after transformation of mutant Lcr plasmids into Y. pseudotuberculosis) were found to be of reduced virulence (45). One approach towards resolving this enigma was to propose that the Yops of Y. pestis undergo full expression in vivo (34). Evidence purported to support this notion was the observation that antibodies directed against Yops are present in the sera of experimental animals and humans after recovery from the plague (28,51). However, a full spectrum of anti-Yops was similarly recovered after immunization with a sterile extract of Lr $^{+}$, Pst^{+} cells of Y. pestis harvested during expression of the LCR (37). This observation negated the proposal that the Yops of Y. pestis are only expressed in vivo and indicates that these structures or their degradation products are highly antigenic.

Results presented here verified that Yops undergo rapid destruction in Lcr^{+}, Pst^{+} organisms as judged by rapid chase of radioactivity into the trichloroacetic acid (TCA)-soluble

fraction. However, the stained gels used to make autoradiograms exhibited detectable levels of major Yops throughout the extended period of chase. This observation indicates that the rates of Yop synthesis and degradation in Lcr^+, Pst^+ organisms are equivalent thereby assuring maintenance of significant levels of these structures during steady state synthesis of protein by restricted organisms. This balanced production and hydrolysis of Yops probably only exists during expression of the LCR because their net accumulation was previously detected after step-up by shift to 26°C or by addition of Ca^{2+} at 37°C. Further study will be required to determine if these environmental changes promote reduced catalytic turnover of the PAC known to mediate hydrolysis of Yops (42).

Rapid but limited Pst plasmid-directed hydrolysis of Yop E yielded p24, a stable degradation product possessing over 90% of the primary structure of the parent molecule. This observation is in accord with the finding that Lcr^+ cells of Y. pestis express an antigen capable of interacting with antibody directed against Yop E (28). Accordingly, it is not improbable that any enzymatic activity of Yop E required for promoting disease would also be maintained in p24. These findings demonstrated that a transient but significant level of major Yops or their degradation products are maintained in vitro during the LCR. Accordingly, it is now unnecessary to invoke mechanisms of specific in vivo

induction of Yops in Y. pestis (or inhibition of PAC activity) to account for genetic evidence demonstrating that some of these structures serve as virulence factors. Nevertheless, it is curious that the Yops of Y. pestis undergo turnover whereas those of the enteropathogenic yersinae do not. It is established that Yops are exposed on the surface of the organism (27). Accordingly, an obvious explanation for degradation of Yops in Y. pestis, and one which we favor, is that this turnover serves to prevent lethality mediated by prolonged contact with molecules involved in host defense (e.g. antibodies, enzymes associated with non-oxidative mechanisms of killing, and possibly even $HClO_3^-$) (11).

An advantage of using Mu dl1(Ap^{r}lac) to induce mutations is that sites of insertion are easily identified thus providing assurance that a single fusion accounts for a given phenotype (39). However, this element necessarily introduces new information into the mutant genome which could modify its resulting phenotype. This possibility was of real concern during expression of the LCR where the necessity to synthesize extra fusion-specific peptides under known conditions of markedly reduced adenylate energy charge (53) might prevent full induction of virulence factors. At least two such fusion-specific peptides were discovered in the tested yopE mutant. To demonstrate that limited energy and metabolites required for synthesis of these extra

structures did not account for avirulence, we also tested a known virulent isolate possessing a similar insert in yopJ (45). Identical fusion-specific peptides were present in this mutant indicating that introduction of their genes did not modify virulence. Further study will be necessary to identify these extra peptides and to determine why their expression, like that of Yops, overrides Lcr plasmid-mediated restriction of transcription typical of bulk protein involved in vegetative growth.

Results of prior studies showed that avirulence of wild type Y. pestis, caused by mutation to non-pigmentation (23,24) (a lesion in iron transport,40) or Pst^- (7), could be phenotypically suppressed in mice by concomitant intraperitoneal injection of inorganic iron. As expected, avirulence of Lcr^+, Pst^+ isolates of Y. pestis blocked in synthesis of Yop E was similarly suppressed by exogenous iron. This treatment did not restore virulence of mutants cured of the Lcr plasmid indicating that one or more genes in addition to yopE are also required for promotion of disease. These determinations were repeated with isogenic mutants cured of the Pst plasmid which were thus unable to degrade Yops. The latter were less virulent than their Pst^+ parents. This finding could be construed as evidence suggesting that Pst plasmid-mediated processing of Yops is required for full expression of virulence in Y. pestis. Alternatively, increased lethality of Pst^+ organisms could

reflect the invasive action of PAC (1,7,8) which only incidentally degrades Yops.

It is established that administration of iron to the host not only serves to fulfill an essential nutritional requirement of invading organisms (49,50) but can also block non-specific mechanisms of host defense (e.g. synthesis of transferrin, chemotaxis of professional phagocytes, and both oxidative and nonoxidative mechanisms of killing (49). The parent Lcr^+, Pst^+ strain of Y. pestis used in this study was non-pigmented thus this isolate and its mutants are avirulent unless injected intraperitoneally with Fe^{3+} (24) or intravenously with or without the cation (46), a route that permits immediate access to iron-rich cytoplasm of cells of the reticuloendothelial system (46). Avirulence promoted by mutation to non-pigmentation probably reflects an established lesion in assimilation of iron (40). In contrast, intravenous injection of yopE mutants did not restore virulence. This observation would be expected if Yop E or, in Y. pestis, its degradation product p24 is not required for transport of iron. Instead, these structures may serve to neutralize one or more of the nonspecific mechanisms of host defense noted above. Regardless of the process involved, it is evident that avirulence caused by mutation to yopE can be phenotypically suppressed by iron whereas this treatment does not restore virulence to mutants cured of the Lcr plasmid.

Accordingly, additional Lcr-plasmid mediated functions are obviously required for expression of virulence in mice. In order to identify such possible virulence factors other than Yops, we pulsed and then chased the organisms for 1 h throughout onset and maintenance of the LCR. Shift to 37°C resulted in immediate production of two heat shock proteins which, like bulk protein, become repressed as the organisms ceased vegetative growth. Further study will be required to define the role of these proteins in yersinae and to determine if they are only expressed by Lcr^+ organisms. Observed major peptides known to be mediated by the Lcr plasmid were p38 or V (implicated in immunosuppression, 47,48) and p20 (38).

Other major peptides produced throughout the LCR were not encoded by the Lcr plasmid. These included p36 which, as noted above, required 2 h for processing to p33. Additional work will be required to determine if only one or both of these peptides exhibit PAC activity. Both p56 (data not shown) and p70 are chromosomally encoded. The latter was also expressed by Lcr^- cells of Y. pestis and Y. pseudotuberculosis but not by Y. enterocolitica. Because this peptide reacted against monospecific anti-E antigen (6), it is probably identical (25) to classical antigen 5 of Crumpton and Davies (41). These findings demonstrated that regulation of at least three major peptides not encoded by the Lcr plasmid occurs by a mechanism that abrogates the

typical restriction of transcription that exists during expression of the LCR.

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CHAPTER 3

Lcr Plasmid-mediated Protein Expression and Processing in a Pesticinogenic *Yersinia pesudotuberculosis* Strain

by

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Abstract

The low calcium response (Lcr) plasmid is an important virulence factor common to all pathogenic yersiniae. The Lcr is induced *in vitro* when cells are grown at 37°C in media lacking Ca^{2+}. The Lcr causes a slow down or stoppage of vegetative growth and induction of Lcr-specific virulence factors. The latter includes a group of yersiniae outer membrane peptides (Yops) encoded on the Lcr plasmid. These structures are stably maintained in the enteropathogenic *Yersinia pseudotuberculosis* but not in *Yersinia pestis*. In *Y. pestis*, the plasminogen activator/coagulase (PAC) encoded on the pesticin plasmid degrades the Yops. In this study, the pesticin plasmid was transformed into the highly related *Y. pseudotuberculosis* and Yop expression was examined. Silver stained gels of this Lcr^+, Pst^+ strain of *Y. pseudotuberculosis* did not indicate stable production of Yops. Immunoblotting the new *Y. pseudotuberculosis* strain revealed the presence of a stable 40 kilodalton Yop that results from a partial degradation of a unidentified Yop. Radiolabeling of the cells indicated nearly identical processing of all the Yops including the important Yops H and E. Yop E was processed into a stable 24 kilodalton product in the same way as that found in *Y. pestis*. A *Y. pseudotuberculosis* specific Yop (Yop A), was processed into discrete degradation fragments in the Pst^+ strain. This

report gives further evidence of the conserved nature of these peptides and that they probably serve a similar function for both species.

INTRODUCTION

Yersinia pestis, the causative agent of bubonic plague, and the enteropathogenic yersiniae, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* are all human pathogens. An important virulence factor common to all three organisms is an approximate 70-kb plasmid that mediates the low calcium response (LCR) (13,25). In addition to the low calcium response plasmid (Lcr), *Y. pestis* also contains a 9.5-kb pesticin plasmid and a 110-kb cryptic plasmid (21,43) neither of which are found in the enteropathogenic strains. The cryptic plasmid is reported to encode a murine specific exotoxin and a capsular antigen termed fraction I (43) and its role in pathogenesis of plague remains unclear. The pesticin plasmid promotes pesticinogency (Pst^+) and is known to encode the bacteriocin pesticin (4,20,28) and a plasminogen activator/coagulase (PAC) activity (3,52,55). The latter function is temperature dependent with most coagulase activity occurring at temperatures below 30°C, while the fibrinolytic activities are dominant above this temperature (37). The pesticin plasmid is also thought to be responsible for the

invasiness of Y. pestis allowing the organism to disseminate from peripheral routes of injection (11).

The LCR is a unique metabolic event in which normal vegetative growth is completely restricted in Y. pestis and a set of virulence factors are induced (Lcr^+). The LCR is induced at 37°C in media lacking Ca^{2+} and does not occur at this temperature if at least 2.5 mM Ca^{2+} is present or at temperatures lower than 30°C regardless of Ca^{2+} concentration (10,27). The LCR is similar in the enteropathogenic strains, but the metabolic stepdown is not as severe as Y. pestis and growth of the organisms continues at a reduced rate (18).

Virulence factors induced during the LCR include the soluble V and W antigens (17,34) and a set of yersiniae outer membrane peptides termed Yops (7,41,54). V antigen is a stable, cytoplasmic peptide found in all three species (15) but Yops can only be detected in the enteropathogenic yersiniae and the non-pesticinogenic Y. pestis (46). Yops are produced by the pesticinogenic Y. pestis but undergo rapid post-translational degradation by the fibrinolytic activity of the PAC encoded on the pesticin plasmid(46,47,53).

The Yops are a diverse group of 13 different proteins whose exact role in pathogenesis is now being elucidated. Each of the different yersiniae species has only 10 of the 13 total Yops. Yops K, L, and M are unique to Y. pestis

while Yops A, G, and I are found in Y. pseudotuberculosis serotype I (57). Yop A is common to all of the enteropathogenic yersiniae and is an adhesion protein regulated by temperature and not calcium (6,49). This protein has been implicated in agglutination reactions (31,32,48), serum resistance (2), and inhibition of the anti-invasive effect of interferon (16). Yops G and I do not have any known function (57). Yops E and H are common to all three yersiniae (8,24,56) and the Yop E gene has been sequenced in all three and was found to be highly conserved (9). Mutations in these two Yops leads to a decrease in virulence of the organism (8,23,24,56), but injection of iron can restore the virulence of Yop E (38). Yop H is a protein tyrosine phosphatase (PTPase) related to the eukaryote PTPases and how it functions in pathogenesis is still not known (26). Yop E is cytotoxic for HeLa cells and mouse macrophages and may also influence the ability of the pathogen to resist phagocytosis (45). The role of Yops between the enteropathogenic yersiniae and Y. pestis may seem to be different because of the degradation of Yops in Y. pestis. However, it has been shown that significant levels of these Yops are maintained in vitro in the steady state or as stable degradation products (38).

Y. pestis and Y. pseudotuberculosis share about 90% DNA homology and have nearly identical Lcr plasmids (5,9,39,42). However, Y. pestis causes a lethal acute disease while Y.

pseudotuberculosis causes a chronic enteropathogenic disease. However, they both share a important virulence factor in the Lcr plasmid, so it is of interest to see how another virulence factor, the pesticin plasmid, affects the expression of Lcr-mediated proteins during the LCR. We have found a remarkable similarity between these strains when they carry the pesticin plasmid. Important Yops such as Yop H and E undergo nearly identical processing by the PAC. In Y. pseudotuberculosis, significant levels of two Yops that were only partially degraded were noted.

MATERIALS AND METHODS

Bacteria

The isogenic isolates of Y. pestis were derived from strain KIM. This Lcr^+, Pst^+ isolate is non-pigmented (30), thus it is virulent in mice only by intravenous injection and not by peripheral routes of injection (10,14). The Y. pestis methionine meiotroph used in the pulse-chase experiment was isolated and described as previously reported (47). The Lcr^+, Pst^+ Y. pseudotuberculosis was constructed by M.M.S. Orkrovskaya and G. B Smirnov of the Gamaleya Research Institute of Epidemiology and Microbiology. USSR Academy of Medical Sciences. Moscow, USSR. The mapping of the Tn1 transposon was accomplished by multiple restriction enzyme digest of the plasmid p2PCP::Tn1 (36). The parental strain was a guanine, biotin auxotroph derived from a Y.

pseudotuberculosis PB1 which is a serotype I strain that is avirulent by any route of injection (12). Isogenic strains of both organisms lacking the Lcr plasmid or the pesticin plasmid were obtained as previously described (47). Loss of the plasmids by these derivatives was verified by alkaline lysis plasmid DNA mini-preps of each strain and electrophoresis on a 0.6% agarose gel(36).

Media and Cultivation.

Bacteria were grown in a chemically defined media (58) as previously reported (47). All media contained 20 mM Mg^{2+} and no added Ca^{2+} and the media used for radiolabeling the organisms also lacked methionine. Guanosine and hypoxanthine were added to the growth media to a final concentration of 0.5 mM to allow growth of the Y. pseudotuberculosis.

Immunoblotting.

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (33). Immunoblots were then performed as previously described (15) with antisera prepared against the Yops of Y. pestis (46).

Assays.

Pesticin was assayed by plating the organisms on tryptose blood agar (TBA) plates (Difco, Detroit, MI) and incubated for 1 to 2 days. The resulting colonies found on the plates were then chloroform killed and overlayed with 5 ml of 1/2 strength TBA containing the pesticin-sensitive Y. pseudotuberculosis strain (~$1x10^5$ CFU/ml). Pesticin producing strains produced zones of growth inhibition in the top agar strain. Coagulase was assayed by mixing a suspension of the organism with 0.5 ml of fresh plasma and incubating the tubes at 37°C for up to 12 hours, checking the tubes hourly for clotting. Fibrinolytic activity was measured by placing the organisms on fibrin plates (3) and looking for lysis of the fibrin film. To quantitate fibrinolytic activity, the cells were grown at 37°C without Ca^{2+} as described above. After 6 hours under these conditions, the cells were harvested by centrifigation, washed twice in ice-cold phosphate buffer, and resuspended in borate buffer to an optical density of 20 at 620 nm. The cells were briefly sonicated and then serially diluted in borate buffer. Equal quantities of the dilutions were spotted on fibrin plates and incubated at 37°C for 12 hours. The titer was estimated by the lowest dilution that still retained fibrinolytic activity. The amount of protein added to each plate was checked by the method of Lowery (35).

Pulse-chase Labeling of Bacteria.

The procedure used in the pulse-chase experiments was essentially the same as described by Sample et al (47). Briefly, the organisms were grown in chemically defined media lacking methionine and Ca^{2+} at 37°C. Bacteria were pulsed with [^{35}S]-methionine at a final concentration of 10 μCi/ml and were chased with excess unlabeled methionine (final concentration of 1.6 mM). Whole culture samples were removed at timed intervals and precipitated with an equal volume of cold 10% trichloroacetic acid. The samples were then prepared for SDS-PAGE. The gels were silver stained by the method of Morrissey (40) and were then dried and exposed to film as previously described (47).

RESULTS

Detection of Pesticin Plasmid Activities

The pesticin plasmid has the bacteriocin pesticin, coagulase and fibrinolytic activities encoded on the plasmid. This plasmid was "tagged" with a Tn1 transposon to facilitate selection of a Pst^+ strain by selection with ampicillin (Tn1 encodes a beta-lactamase). The location of the transposon was mapped to determine if its insertion had inactivated any of the known gene products of the plasmid (Figure 1). The first step in characterizing this Pst^+ Y. pseudotuberculosis was to verify that these same activities also exist in this strain. As seen in Table 1, these

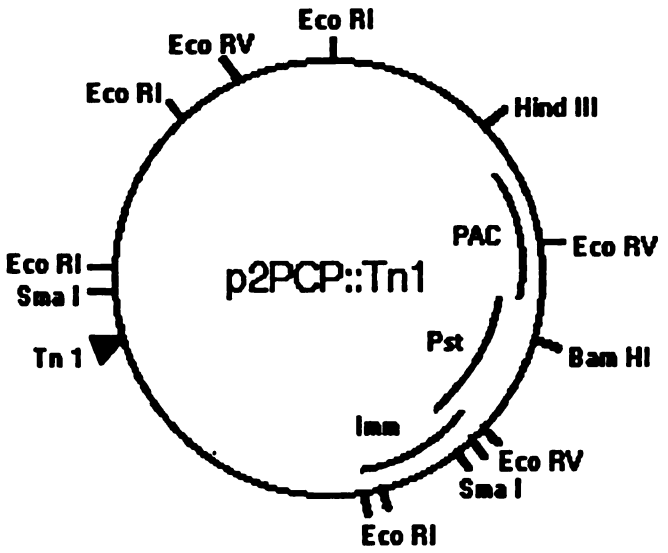

Figure 1. Plasmid map of the pesticin plasmid transformed into Y. pseudotuberculosis strain PB1. Solid triangle denotes location of Tn1 transposon location. Approximate location of known genes are shown on inside of plasmid; PAC=plasminogen activator/coagulase, Pst=bacteriocin pesticin, Imm=protein needed for immunity to pesticin.

Table 1. Assay for Pesticin Plasmid Activities

Strain	Pesticin	Coagulase	Fibrinolytic
Y. pestis Pst^+	+	+	+
Y. pestis Pst^-	O	O	O
Y. pseudotuberculosis Pst^+	+	+	+
Y. pseudotuberculosis Pst^-	O	O	O

+ = activity present
O = lack of activity

activities were present in the Pst$^+$ Y. pseudotuberculosis strain examined, therefore, the transposon did not effect expression of the pesticin plasmid-mediated genes. An isogenic strain lacking the Pst plasmid did not have these activities. Isogenic Pst$^+$ and Pst$^-$ Y. pestis control strains exhibited a similar pattern of these activities (Table 1).

Detection of Lcr-mediated Yersiniae Outer Membrane Peptides (Yops).

In order to identify proteins produced during the low calcium response, Lcr$^+$, Pst$^+$ cells were grown at 37°C in media lacking Ca^{2+} to induce production of Yops. These cells were then harvested, prepared for SDS-PAGE, and separated on a denaturing gel. In order to identify the Yops, isogenic strains lacking one or both plasmids were prepared in a similar manner and compared to the Lcr$^+$, Pst$^+$ cells. The Yops appear as distinct bands in the Lcr$^+$ Pst$^-$ strains as seen in lanes 2 and 6 of Figure 2. Only four Yops are visible with molecular weights of 44, 40, 34, and 26 kilodaltons. These molecular weights are that reported for Yops H, C, D, and E respectively. The Yops made in lower amounts are not distinctive in these lane gels nor can any Yops be seen in the Lcr$^+$, Pst$^+$ strains of either organism. There is a band at 38 kilodaltons (p38) that is

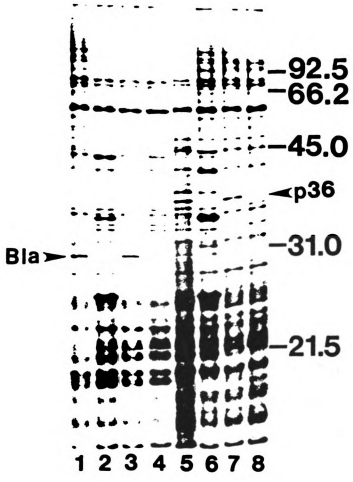

Figure 2. Silver-stained gel of whole cells of Y. pseudotuberculosis PB1 (lanes 1-4) and Y. pestis KIM (lanes 5-8) grown at 37°C in Ca^{2+}-deficient media. Peptides were separated by 12.5% SDS-PAGE. Each lane contains isogenic strains lacking one or both plasmids as follows: Lcr^+, Pst^+ (lanes 1 and 5), Lcr^+, Pst^- (lanes 2 and 6), Lcr^-, Pst^+ (lanes 3 and 7), Lcr^-, Pst^- (lanes 4 and 8). Bla= beta-lactamase and p36 is a Pst specific peptide.

specific for those strains carrying the Lcr plasmid (Figure 2, Lanes 1, 2, 5, and 6) and this is V antigen. In the Pst^+ *Y. pestis* strains (Figure 2. lanes 5 and 7) there is a band at about 36 kilodaltons that represents the plasminogen activator/coagulase factor encoded on this plasmid. This band cannot be seen in the Pst^+ *Y. pseudotuberculosis* strains as seen in Figure 2, lanes 1 and 3. However, there is a band at about 29 kilodaltons found in these lanes and this protein is the processed form of the beta-lactamase gene product.

Only some of the Yops could be detected on the silver-stained gels so a Western blot was done on these same samples using polyclonal antisera raised against Yops of *Y. pestis*. Again the Yops are most distinct in the Lcr^+, Pst^- strains as shown in Figure 3 lanes 2 and 6. In the Lcr^+, Pst^- *Y. pestis* strain only Yops of 45 and 34 kilodaltons are distinct but there are a few of the less prominent Yops visible which are clustered around 45 kilodaltons in size. In the Lcr^+, Pst^- *Y. pseudotuberculosis* only bands of 45, 43 and 34 kilodaltons are visible which probably represent Yops H, B, and D respectively. The surprising result is the appearance of a Yop at 40 kdal in the Lcr^+, Pst^+ *Y. pseudotuberculosis* strain. There are no detectable Yops in the Lcr^+, Pst^+ *Y. pestis* control and there is no Yop of that size reported in the literature (57). Since this Yop is

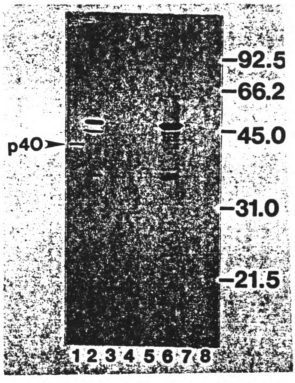

Figure 3. Immunoblot with antisera raised against Yops of Y. pestis KIM. The blot contains whole cell extracts of Y. pseudotuberculosis PB1 (lanes 1-4) and Y. pestis KIM (lanes 5-8) grown at 37°C in Ca^{2+}-deficient media. Peptides were separated by 12.5% SDS-PAGE. Each lane contains isogenic strains lacking one or both plasmids as follows: Lcr^+, Pst^+ (lanes 1 and 5), Lcr^+, Pst^- (lanes 2 and 6), Lcr^-, Pst^+ (lanes 3 and 7), Lcr^-, Pst^- (lanes 4 and 8).

only in the Lcr$^+$, Pst$^+$ Y. pseudotuberculosis strain it probably represents a stable degradation product of one of the higher molecular weight Yops.

Radiolabeling of the Lcr$^+$ Pst$^+$ Yersinia

Yop production could not be detected in any great quantity in the Lcr$^+$, Pst$^+$ Y. pseudotuberculosis strain. Therefore, to show that Yops are produced and subsequently hydrolyzed in this strain is was necessary to use a radiolabeled amino acid to follow protein production. The cells were grown at 37°C in Ca$^+$ deficient media to induce the production of Yops. They were then pulsed with [^{35}S]-methionine for 15 seconds and then chased with an excess of unlabeled methionine for one hour. Aliquots were taken at timed intervals, and then these protein samples were separated by SDS-PAGE and autoradiographed. From the autoradiogram in Figure 4, Yops are being produced with molecular weights of 76, 46, 44, 42, 34, and 25 Kdals. These correspond to Yops F, H, B, C, D, and E respectively. All of these Yops undergo hydrolysis and are not detectable after the one hour chase. A control experiment was performed with Lcr$^+$, Pst$^+$ Y. pestis (Figure 5) to compare the kinetics of degradation between the two species. A comparison of Figures 4 and 5 reveals an almost identical degradation pattern between the two organisms. The only

Figure 4. Stained gel (left) and corresponding autoradiogram (right) of trichloroacetic-acid precipitated material from cultures of Y. pseudotuberculosis PB1 grown at 37°C in Ca^{2+}-deficient medium. Bacteria were pulsed for 15 s with [^{35}S]-methionine and then chased with excess unlabeled methionine for 0 (lane 1), 15 s (lane 2), 30 s (lane 3), 1 min (lane 4), 2 min (lane 5), 4 min (lane 6), 8 min (lane 7), 15 min (lane 8), 30 min (lane 9), and 1 h (lane 10). Control lanes (pulsed for 15 s and then precipitated) contained the following isogenic derivatives of Y. pseudotuberculosis PB1 Lcr^+, Pst^- (lane 11), Lcr^-, Pst^+ (lane 12), Lcr^-, Pst^- (lane 13). Lane M contains molecular weight markers (kDa). Peptides were separated by 12.5% SDS-PAGE. Closed arrowheads indicate Yops and open arrowheads indicate Pst plasmid specific products. Arrows indicate stable degradation products (p24 and p40).

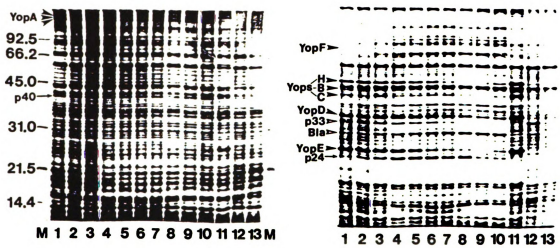
YopA
92.5
66.2
45.0
p40
31.0
21.5
14.4
M 1 2 3 4 5 6 7 8 9 10 11 12 13 M
YopF
YopE
H
B
A
C
YopD
p33
Bla
YopE
p24
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 4.

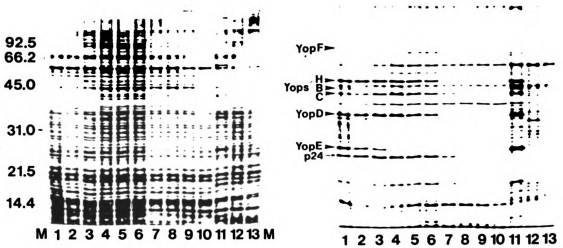

Figure 5. Essentially the same as Figure 4 except that a Lcr$^+$, Pst$^+$ Y. pestis strain KIM was used in the pulse-chase. Control lanes (11-13) contained isogenic derivatives of Y. pseudotuberculosis.

major difference is the presence of two distinct bands in Y. pseudotuberculosis. The smaller band is the 29 kdal form of the beta-lactamase (Bla) and 33 kdal band that may represent the larger form of the beta-lactamase or the smaller processed form of the PAC (38,52).

A comparison of individual Yops shows that there is not much difference in how the PAC degrades the Yops. Yop E is probably the most important Yop and, like Y. pestis, this Yop is degraded into a 24 kdal product. However, this hydrolysis is slower in Y. pseudotuberculosis in which it takes about 15 minutes for the labeled Yop E to be degraded, while it only takes about 1 minute in Y. pestis. The degradation of Yop D is also slower in Y. pseudotuberculosis (Figure 4) with detectable quantities throughout the one hour chase while it is lost after 8 minutes in Y. pestis (Figure 5). The hydrolysis of Yops H, B, C and F is nearly identical between the two organisms.

The stable degradation product in the Lcr^+, Pst^+ Y. pseudotuberculosis seen in the immunoblot (Figure 3) was not detectable in the autoradiogram. However, this band can be seen on the stained gel (Figure 4). Also, on the same gel 3 distinct degradation products from the Yop A protein that is unique to Y. pseudotuberculosis. This protein is not well labeled in the 15 second pulse, but these degradation products can be seen in the autoradiogram (Figure 4) from 1

minute into the chase until one hour. Yops G and I are also unique to Y. pseudotuberculosis but they cannot be detected in either the stained gel or the autoradiogram.

DISCUSSION

The construction of a pesticinogenic Y. pseudotuberculosis strain allowed us to compare the production and degradation of Yops between species. The products associated with the pesticin plasmid are produced in Y. pseudotuberculosis and there is no interference by the Tn1 transposon that was used as a marker for the plasmid because all of the products of the plasmid are produced at levels similar to that of Y. pestis (Table 1). This plasmid was stably maintained even without selection with ampicillin. However, this is to be expected since this Y. pseudotuberculosis strain is sensitive to pesticin. Hence, production of the bactericin would eliminate any organism that lost the plasmid.

The most interesting aspect of this comparison between the pesticinogenic strain and Y. pestis is the similarity in Yop production and processing. The Yops form obvious structures in the protein profile of Pst^- strains but they seem to disappear when the pesticin plasmid is present. The only visible differences between the pesticin plasmid containing species are 2 protein bands (Figure 2). The Pst^+ Y. pseudotuberculosis has a band at 29 kdal which represents

the Beta-Lactamase gene product from the transposon. The Pst$^+$ *Y. pestis* has a band at about 36 kdal which is one form of the PAC (38,52). There is no similar band in the Pst$^+$ *Y. pseudotuberculosis* strains but it has the same amount of fibrinolytic activity per gram of cells as *Y. pestis*.

Immunoblotting is a more sensitive method for detection of protein production. However, the antisera only identified a few of the Yop proteins. One of these was a novel stable degradation product of 40 kdal only found in the Lcr$^+$, Pst$^+$ *Y. pseudotuberculosis* strain. This product probably represents either Yop H or Yop B and probably represents a difference in primary structure in that particular Yop between the two species. This Yop has a structure that is only partially degraded by the PAC while its counterpart in *Y. pestis* is completely degraded.

The presence of this single Yop did not actually show that Yops in general are produced in this strain. Therefore, it was necessary to use a radioactively labeled amino acid to trace protein production. A possible problem with this approach is the fact that the enteropathogenic strains continue to replicate during the LCR while *Y. pestis* does not replicate (18). Therefore, the bulk cellular proteins were labeled in *Y. pseudotuberculosis* but not in *Y. pestis*. It was not know ahead of time whether or not Yops could be detected in *Y. pseudotuberculosis*. To help

alleviate this problem, isogenic strains lacking one or both plasmids were also labeled with the radioactive amino acid to help identify the origins of plasmid-specific proteins. Most of the Yops were easily distinguished in Y. pseudotuberculosis, with the exception of the Yops that are made in small amounts such as Yops J, G, and I (57). The short labeling time (15 s) also does not allow a lot of time for incorporation of the label into the bulk proteins, thus, cutting down on the background but not allowing these Yops to be well labeled.

The kinetics of degradation of Yops between these two species is very similar (Figures 4 and 5). This result is not surprising because the two plasmids found in these species are highly related (42). Sequence analysis of the Yop E gene between Y. pseudotuberculosis and Y. pestis EV76 show that only 2 out of 219 amino acids are different (24), while immunological similarity of Yop E between these species is also quite high (19). This similarity can be seen in the degradation pattern between these species (Figures 4 and 5). This Yop is quickly degraded in a 24 kdal protein in Y. pestis (38,47) (Figure 5) and this also occurs in Y. pseudotuberculosis (Figure 4). Therefore, this important protein may still function in the same manner as the Lcr^+, Pst^- strains (38). The other visible Yops also behave in a similar manner as the counterparts in Y. pestis including another important Yop, Yop H. This information

gives indirect evidence that these Yops are highly related between these two species and probably function in a similar manner.

Yops A, G and I are unique to Y. pseudotuberculosis PB1 (57) and it was of interest to see how they were affected by the fibrinolytic activity of the PAC. Yops G and I are minor Yops (57) and their production could not be detected due to the short pulse time as well as the effect of being masked by labeling of the bulk protein. Yop A, an adhesion protein, shows a distinctive degradation pattern. This protein has a normal molecular weight of between 200-240 kdal when run on SDS-PAGE (51). This protein can be dissociated into 45-52.5 kdal subunits if the sample is boiled for a prolonged time (15 min) or by addition of 8M urea in the gel (48,50,59). The size of these subunits varies between different serotypes of Y. pseudotuberculosis (50). This Yop can be clearly seen on the stained gel in the radiolabeling experiment (Figure 4) as a large band and also as a series of large degradation products. These large degradation products differ in size by about 40-50 kdal which may represent fragments removed from all of the subunits or the loss of an entire subunit. How this processing effects its role in pathogenesis is not known. However, other studies have indicated that loss of this protein and another adhesion protein needed for invasion of non-phagocytic cells (ivn^+) (22,29) actually increases the

virulence of this organism in mice (44). The ivn gene is chromosomally encoded but does not seem to be expressed in Y. pestis (44) or it can not function properly in Y. pestis due to production of the species specific capsular antigen (1). Y. pestis has an similar coding region for Yop A but it is not produced due to a 1 bp deletion (44). If production of Yop A is restored in Y. pestis, its virulence is decreased (44). Therefore, this protein, along with ivn protein, seems to be important in virulence of Yersinia and whether its causes a chronic or an acute disease.

It is interesting to speculate on how the addition of the pesticin plasmid affects the virulence of Y. pseudotuberculosis. In this study we used a purine auxotroph, thus this strain is avirulent by any route of injection (12). Studies are under way to see how the pesticin plasmid affects the virulence of Y. pseudotuberculosis in a wild type strain. The pesticin plasmid is thought to be responsible for the invasiveness of Y. pestis and the spread of the organism from peripheral sites (18). However, Y. pestis does not produce any adhesion proteins that are found in Y. pseudotuberculosis (44). These proteins may be needed for the enteropathogenic type of disease produced by Y. pseudotuberculosis and Y. enterocolitica. These same proteins seem to limit the virulence of the organisms since the loss of these proteins increases the virulence of the organisms (44). Therefore,

it seems that the pesticin plasmid may only contribute a small amount to the virulence of Y. pseudotuberculosis. However, the Lcr-mediated virulence factors appeared to be highly conserved and most likely play the same role in both organisms.

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CHAPTER 4

Major stable peptides of *Yersinia pestis* produced during the low calcium response

by

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(manuscript to be submitted to Infection and Immunity)

ABSTRACT

The ~70-kb Lcr plasmid of yersiniae contains 2 functionally distinct sets of genes known to mediate the low calcium response (Lcr^+). Those of the first set encode activities that promote restriction of cell division at 37°C in Ca^{2+}-deficient medium. Products of the second set are selectively synthesized within this environment and serve as virulence factors (soluble V antigen and outer membrane peptides termed Yops). Ability of restricted Lcr^+ cells of Yersinia pestis (incapable of synthesizing vegetative protein) to produce additional virulence functions was defined in this study. Bacteria were pulsed with [^{35}S]-methionine and then chased to assure ~10 kb pesticin plasmid-encoded plasminogen activator (PAC)-catalyzed degradation of Yops. The 14 remaining radioactive peptides were cytoplasmic and could be separated by conventional chromatography. They consisted of V antigen plus other Lcr-mediated peptides of about 20 and 15 kd, pesticin plasmid-encoded pesticin and a 35 kd degradation product of PAC, and ~110 kb cryptic plasmid-mediated fraction 1 (capsular antigen) plus 3 peptides comprising the murine exotoxin. Evident chromosome-encoded activities were the 15 kd antigen 4 (pH 6 antigen) and temperature-inducible peptides of about 70 and 56 kd tentatively identified as a unique catalase (antigen 5) and Gro EL, respectively. The p56 peptide has

also been equated with W antigen as a p56/V antigen complex. This is the first report of production of other virulence factors during the Lcr.

INTRODUCTION

Yersinia pestis, the causative agent of bubonic plague, and enteropathogenic Yersinia pseudotuberculosis are facultative intracellular parasites capable of growth within Ca^{2+}-deficient host cell cytoplasm and in Ca^{2+}-enriched extracellular fluids (8,40). Virulence of these species is dependent upon carriage of a common ~70 kb Lcr plasmid that mediates a unique temperature-dependent low calcium response (Lcr^{+}). This phenomenon is characterized by shutoff of vegetative growth under restrictive conditions (cultivation at 37°C in Ca^{2+}-deficient media) accompanied by selective synthesis of Lcr plasmid-encoded virulence functions (6,17,33,52). The latter include a series of yersiniae outer membrane peptides (33,43) termed Yops (5) and V antigen (10), a soluble peptide of 38 kd (9,43). Yops are synthesized but undergo immediate post-translational degradation in Y. pestis (28,37,38) catalyzed by plasminogen/prothrombin activator (PAC) activity (42) encoded on a species specific ~10 kb pesticin (Pst) plasmid (16). In contrast, Yops exhibit net accumulation in the enteropathogenic yersiniae (33,43) or nonpesticinogenic Y.

pestis (36). V antigen is stable and accumulates in all 3 yersiniae (7,38).

Onset of the low calcium response reflects an ordered nutritional stepdown as judged by typical reduction of adenylate energy charge (51) and shutoff of stable but not mRNA synthesis (12). Mutation to Lcr^- caused by cure of the plasmid results in loss of both the nutritional requirement for Ca^{2+} and the ability to express Yops and V antigen (7,34). Although a number of Lcr-plasmid-encoded genes regulate the low calcium response (17,46,49,50), the sequences whereby they or their products interact together, or with environmental sensors are not yet fully resolved. This process is evidently complex in that loss of only one of these regulatory functions often mimics cure of the Lcr plasmid in that significant growth occurs under restrictive conditions with greatly reduced synthesis of Yops and V antigen (17,46).

The assumption that Lcr^+-specific virulence functions are selectively induced following uptake by host cells (45) was directly substantiated (45). These functions, therefore, may contribute to survival within intracellular niches. However, the ability of yersiniae to synthesize distinct virulence factors encoded by other plasmids or the chromosome during residence within these or other Ca^{2+}-deficient environments is unknown. To resolve this question, we cultivated Lcr^+ cell of Y. pestis under

restrictive conditions and then, after growth had ceased, determined their ability to synthesize virulence factors other than V antigen and Yops. Results demonstrated that possibly all established virulence determinants of the species were produced during restriction even though these organisms are known to be incapable of synthesizing bulk vegetative protein (28). These factors included capsular or fraction 1 antigen (1), antigen 4 or "pH 6" antigen (3,25), plague murine exotoxin (29), PAC (2,41), and 2 small Lcr plasmid-mediated peptides in addition to V antigen. Other major peptides synthesized by restricted yersiniae were tentatively identified as a novel catalase (antigen 5,28) and the protein export function Gro EL (22,25). The Gro EL factor has also been correlated with W antigen believed to be a Gro EL/V antigen complex.

MATERIALS AND METHODS

Bacteria

A methionine-independent meiotrophic mutant (15) of nonpigmented (21) Y. pestis KIM, known to lack outer membrane peptides associated with assimilation of iron (39,44), was used in all experiments. This isolate possesses the 3 plasmids typical of wild type (16,35) and is virulent by intravenous (47) but not peripheral(47) routes of injection.

Cultivation and Radiolabeling

Bacteria, stored in liquid buffered glycerol at -20°C, were transferred to slopes of Tryptose blood agar (Difco Laboratories, Detroit, Michigan), incubated for 2 days at 26°C, and then transferred to flasks containing the chemically defined medium of Higuchi et al (19) as modified by Zahorchak and Brubaker (52), except that L-methionine was omitted (the concentration of Mg^{2+} was 20 mM and no Ca^{2+} was added). After two pregrowths that were at 26°C as previously defined (38), a subculture of the same medium was inoculated at an optical density (620 nm) of 0.1 (200 ml/ 2 L Erlenmeyer flask). This flask was aerated at 26°C until the optical density was 0.25 and then shifted to 37°C where, after further aeration for 6 hours, vegetative growth completely ceased (28,38,51).

The culture was then pulsed by addition of carrier-free [^{35}S]-methionine (new England Nuclear, Boston Mass.) to a final concentration of 10 μCi/ml. After 1 minute an excess of unlabeled L-methionine (final concentration=1.6 mM) was added to the culture to chase the radioactive label from the unstable Yops. After 1 hour of chase, the cells were centrifuged at 10,000 X g and washed twice in ice-cold phosphate buffer. The cells were resuspended to an optical density at 620 nm of ~20 and then sonicated for 2 minutes to disrupt the cells. The cell debris was removed by centrifugation at 10,000 X g and the supernatant was

filtered through a 0.22 micron membrane to remove any remaining cells. This radioactively labeled cytoplasmic extract was mixed with about 4 times as much unlabeled cytoplasmic extract grown and prepared in the exact same manner as described above. Aliquots of this cytoplasmic mixture were separated on the columns and used in the lane gels and immunoblots.

Radiolabeling of Lcr^+ and Lcr^- cells found in Figure 1 were prepared in a similar manner. Smaller quantities of cells (10 ml) were pregrown and then shifted to restrictive conditions as above. The cultures were then pulsed for one minute in 10 μCi/ml [^{35}S]-methionine and then chased for 1 hour with an excess unlabeled L-methionine. Aliquots of the cultures were removed at 0 and 1 hour into the chase and precipitated with an equal volume of cold 10% trichloroacetic acid. These samples were then prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Antisera and Immunoblots.

Monospecific polyclonal antisera to the p56 and p70 peptides was prepared by injecting partially purified peptides into rabbits with the RIBI conjugate (RIBI Immunochem research Inc. Hamilton, MT) according to the manufactures instructions. To make these antisera monospecific, anti-p70 sera was absorbed (9) with

lyophilized, disrupted, Lcr$^+$ Y. enterocolitica which does not produce p70 while anti-p56 was clarified against a lyophilized cytoplasmic protein mixture that did not contain p56. Immunoblots were performed as previously described (9).

W antigen purification and two-dimensional gel electrophoresis

An Lcr$^+$ Y. pestis cytoplasmic fraction was separated on a DEAE cellulose column. Fractions were assayed for the presence of W antigen by the double diffusion method of Ouchterloney (32) using monospecific anti-W obtained from Dr. William Lawton (23). The fractions that contained W antigen were combined and precipitated and then separated on a Sepharose S-200 sizing column. These fractions were again checked for W antigen and positive fractions were pooled. This is crude W antigen and a cytoplasmic sample were separated by two-dimensional gel electrophoresis as described by O'Farrell (31). The amount of protein loaded on each gel was determined by the method of Lowery et al (26).

p70 purification.

A Lcr$^-$, Pst$^-$ strain of Y. pestis KIM was grown at 37°C in large fermenter vessels, containing a complex NZ amine medium (9), to an optical density at 620 nm of ~8. The

cells were harvested, washed twice in ice-cold phosphate buffer, and resuspended in 50 mM Tris-HCl pH 8.0. The cells were then disrupted by passing ~80 g of cell paste through an ice-cold French pressure cell twice. The cell debris was removed by centrifugation at 17,000 X g for 20 minutes. This crude extract was loaded on a 225 ml (2.5 x 45 cm) DEAE cellulose (Whatman Biosystems. Maidstone, England) column equilibrated with 50 mM Tris-HCl pH 8.0 buffer. The p70 protein eluted in the wash as a brown pigmented protein. The first 32 ml containing p70 were combined and brought to 1 M $[NH_4]_2SO_4$. This sample was then loaded on a 50 ml (1.5 x 30 cm) Phenyl-sepharose CL-4B (Pharmacia. Uppsala, Sweden) column equilibrated with 50 mM Tris-HCl pH 7.0 + 1M $[NH_4]_2SO_4$. After the sample was loaded, it was washed with 50 ml of the equilibration buffer. The p70 peptide was eluted with a step gradient using 50 mM Tris-HCl pH 7.0 buffer, and the pigmented fractions were subsequently combined. This protein solution was precipitated by addition of solid $[NH_4]_2SO_4$ to 80% saturation. The precipitated protein was collected by centrifugation at 27,000 X g for 30 minutes. The pellet was resuspended in 1.5 ml of 50 mM CHES buffer pH 10 . This sample was concentrated to ~0.5 ml in a centricon-10 tube (Amicon. Danvers, MA). This extract was loaded on a 150 ml (1.5 x 170 cm) Sephacryl S-300 (Pharmacia. Uppsala, Sweden) sizing column equilibrated in 50 mM CHES pH 10.0 + 0.25 M

$[NH_4]_2SO_4$. About 150 ml was collected before 4 ml fractions were taken. The fractions with positive optical density at 280 nm were separated by SDS-PAGE and those fractions which were seen to contain pure p70 were combined. Detection of catalase activity was performed as described by the Worthington Biochemical Corporation (Freehold, NJ).

RESULTS

Radiolabeling Lcr restricted Y. pestis

To examine protein production during the low calcium response, growing cells were shifted to restrictive conditions (37°C, no Ca^{2+}) and pulsed for 1 minute with [^{35}S]-methionine. These cells were chased for 1 hour with an excess of unlabeled methionine. Aliquots taken at the beginning of the chase and 1 hour later show that peptides of 70, 56, 38, 20, and 15 kdal remain stable throughout the chase in the Lcr^+ cells (Figure 1). The other peptides in the Lcr^+ cells that are labeled at the beginning of the chase but are subsequently lost 1 hour later represent the unstable Yops which are degraded (28,38) (Figure 1).

An isogenic strain of Y. pestis lacking the Lcr plasmid was labeled in a similar manner. Both the p70 and p56 peptides can be seen in this strain, while p38, p20, and p15 are missing. The p38 peptide is V antigen and it is encoded on the Lcr plasmid (16). The p20 and p15 peptides are unknown and may be encoded on the Lcr plasmid or require the

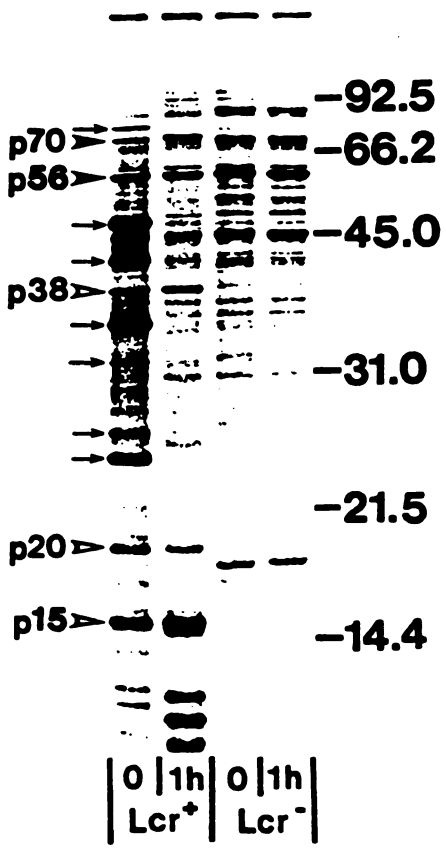

Figure 1. Autoradiogram of protein profiles of Lcr^+ and Lcr^- cells of Y. pestis KIM grown at 37°C in Ca^{2+}-deficient medium. Cells were pulsed for one minute with [^{35}S]-methionine and then chased with an excess of unlabeled methionine. Aliquots of the culture were precipitated with trichloroacetic acid at the beginning of the chase and one hour later. Peptides were separated by 12.5% SDS-PAGE. Small arrows represent unstable Yops, open arrowheads represent Lcr-mediated stable peptides and closed arrowheads represent chromosomally encoded stable products. Molecular weight markers are in kilodaltons.

Lcr plasmid for expression. The p70 and p56 peptides are found in large amounts in the Lcr^- strain and represent chromosomally encoded peptides (38).

Chromatography of the labeled cytoplasm

Most of the stable peptides were found to be located in the cytoplasm (38, unpublished results), so to further characterize and identify these peptides, we labeled Lcr^+ cells with [^{35}S]-methionine and extracted the cytoplasm. Aliquots of the labeled cytoplasm were fractionated on a Sepharose A-1.5 sizing column,a DEAE cellulose column, and a calcium hydroxyapatite column. The protein profile and radioactivity were determined for each fraction from each of the columns and plotted as shown in Figure 2. The protein profiles are typical for a Lcr^+ Y. pestis cytoplasmic extract (unpublished results).

In each of the columns there are distinct peaks of radioactivity which indicates the presence of one or more stable peptides. To correlate these radioactive peaks with specific stable peptides, aliquots of the column fractions were precipitated with 10% trichloroacetic acid and then separated on a denaturing gels. To identify the exact location of the labeled peptides the gels were dried and

Figure 2. Fractionation of radioactively labeled Y. pestis KIM cytoplasm extracts of (A) Sepharose A-1.5, (B) DEAE cellulose, and (C) calcium hydroyapatite. Eluted macromolecules were monitored by absorbance at 280 nm (●) and cpm/ml (O). Buffers used were 0.05 M Tris-HCl pH 8.0 (A), 0.05 M Tris-HCl pH 8.0 with introduction at arrow of gradient (dashed line) of NaCl (0 to 0.5 M) in same buffer (B), and 0.5 M Tris-HCl pH 8.0 with introduction at arrow of gradient (dashed line) of phosphate (0 to 0.5 M). Fraction sizes were 6.0 ml (A) and 4.0 ml (B and C).

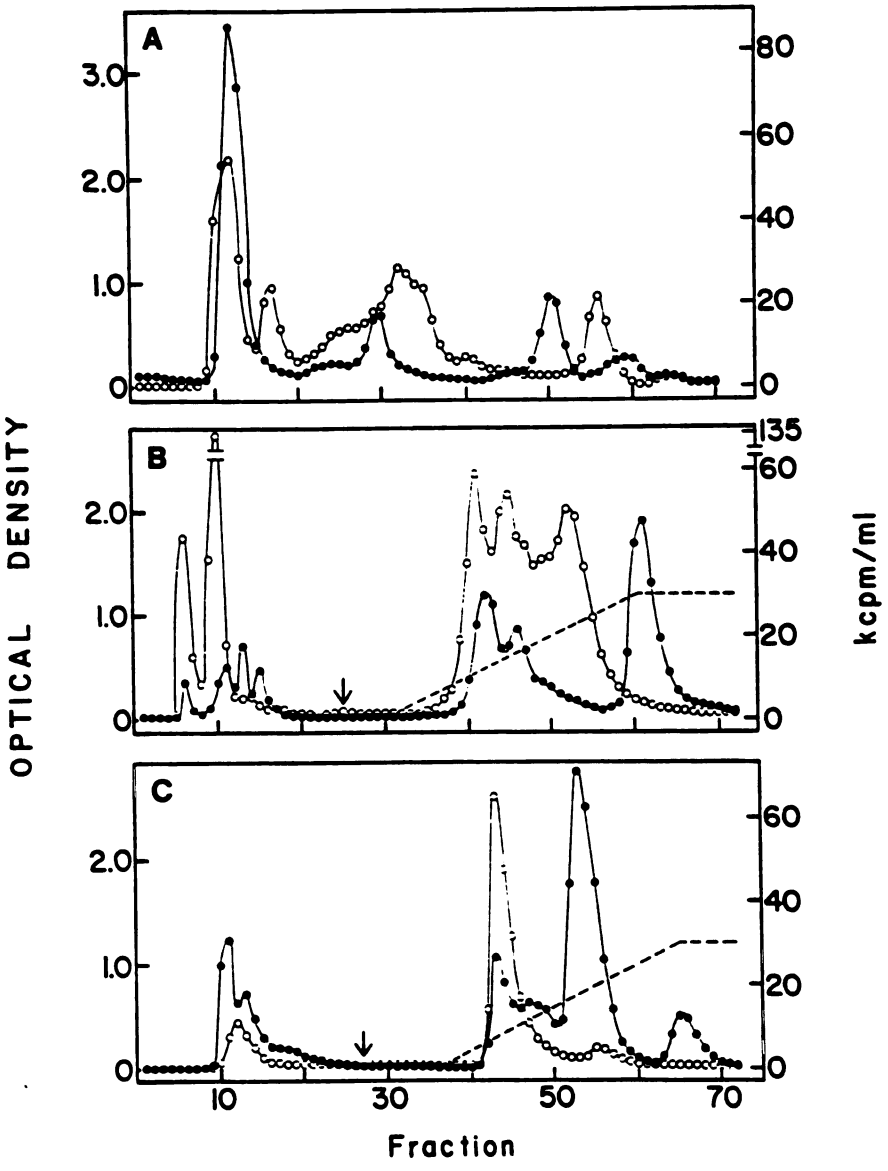
A
B
C
OPTICAL DENSITY
kcpm/ml
Fraction
3.0
2.0
1.0
0
80
60
40
20
135
10
30
50
70

Figure 2.

exposed to film. The results of this analysis are shown in Figures 3-5. An unexpected result of this analysis was the appearance of other radioactively labeled peptides in the autoradiograms of the gels. These new peptides were labeled F1, Toxin, p35, and Pst (Figures 3-5).

Identification of the stable peptides

It was of interest at this stage to see if any of these stable products could be correlated with any known virulence factor. To accomplish this task, a series of immunoblots were performed of the radioactive cytoplasm using antisera to pH 6 antigen (antigen 4), V antigen, the bacteriocin pesticin, the plague murine exotoxin, and to monospecific polyclonal sera prepared against the p70 and p56 peptides. In addition to these antisera, a comparison was made to purified capsular antigen (fraction 1) and pH 6 antigen. These immunoblots were compared to a stained gel of the labeled cytoplasm and its corresponding autoradiogram shown in Figure 6.

The antisera to pH 6 antigen, kindly provided by Dr. Susan Stratley, recognized a 15 kdal peptide. This antisera also reacted with all of the column fractions that contained the radioactive p15 peptide (unpublished data), thus indicating that the major p15 peptide is the pH 6 antigen.

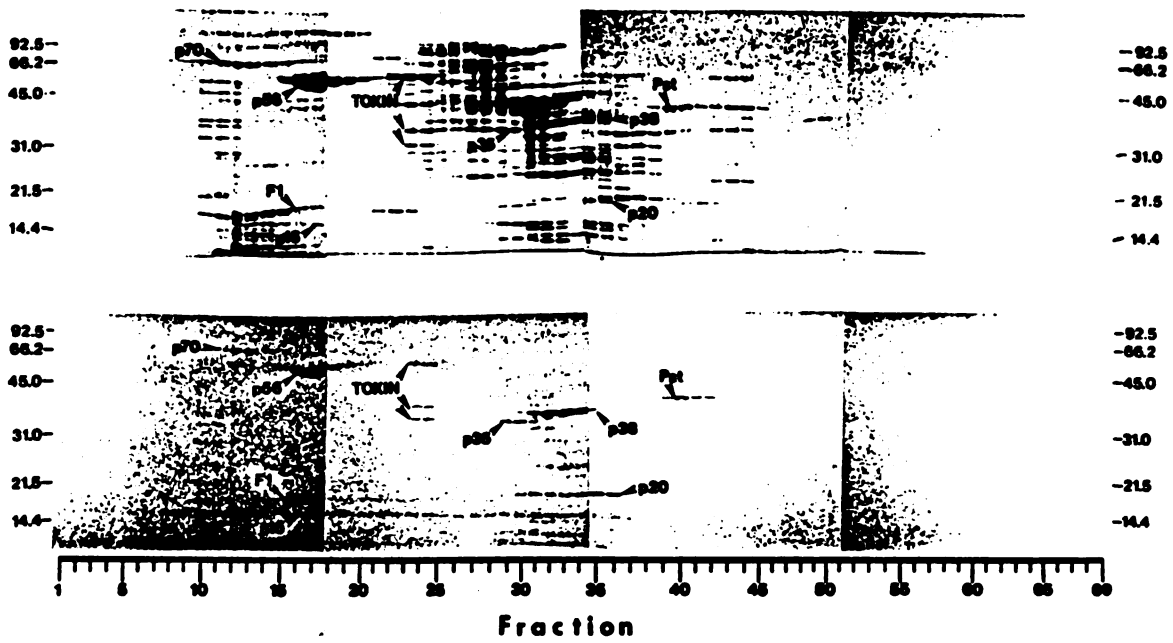

Figure 3. Coomassie blue stained gels (top) and corresponding autoradiograms (bottom) of trichloroacetic acid precipitated proteins of the column fractions of the Sepharose A-1.5 sizing column. Molecular weight markers are in kilodaltons. Labeled proteins are discussed in text.

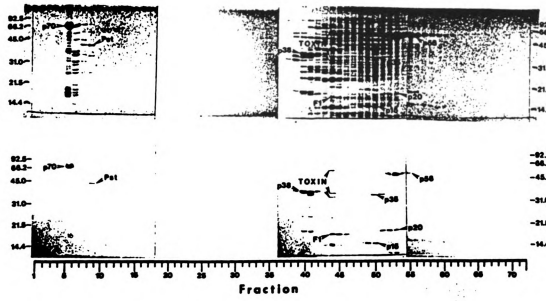

Figure 4. Coomassie blue stained gels (top) and corresponding autoradiograms (bottom) of trichloroacetic acid precipitated proteins of the column fractions of the DEAE cellulose column fractionation of the labeled cytoplasmic proteins of Y. pestis. Molecular weight markers are in kilodaltons. Labeled proteins are discussed in text.

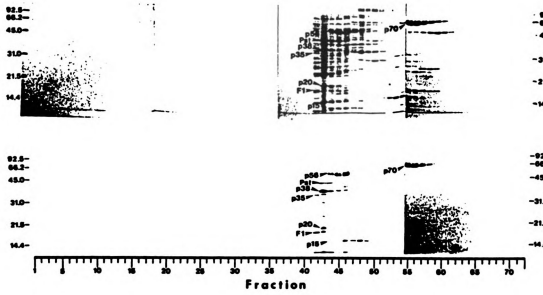

Figure 5. Coomassie blue stained gels (top) and corresponding autoradiograms (bottom) of trichloroacetic acid precipitated proteins of the column fractions of the calcium hydroxyapatite column fractionation of the labeled cytoplasmic proteins of Y. pestis. Molecular weight markers are in kilodaltons. Labeled proteins are discussed in text.

Purified fraction 1, the capsular antigen, has a molecular weight of about 18 kdal and has the same apparent molecular mass as the radioactive peptide labeled F1 in each of the columns. This peptide is found in nearly all of the fractions in the sizing column fractionation (Figure 3) which would indicate a diverse native molecular weight that would be expected of this capsular antigen (4).

The anti-V and anti-pesticin antibodies recognized peptides of 38 and 45 kdal, respectively. These correspond to the p38 and Pst radioactive peptide bands. These results were verified by immunoblots of the column fractions which contain the respective peptides using antisera to them (unpublished data).

The murine exotoxin (toxin) is a distinct 3 subunit polypeptide in which the largest band is about 58 kdal (unpublished data, Figure 3). In Western blots prepared against whole cytoplasm only the largest subunit is identified (Figure 6). This toxin was clearly visible in the stained gels of the Sepharose A-1.5 column and the DEAE cellulose column (Figures 3 and 4). However, it was not labeled well with the radioactive amino acid which indicates that this peptide is only expressed at low levels during the low calcium response.

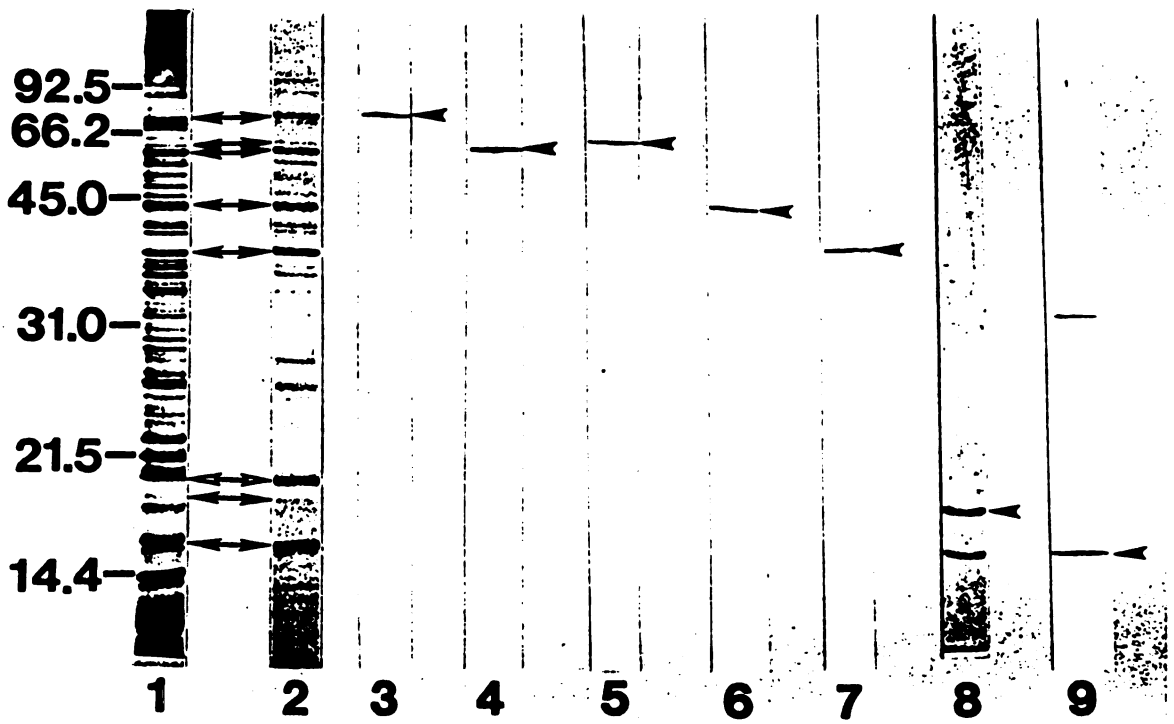

Figure 6. Stained gel (lane 1) and corresponding autoradiogram (lane 2) of radioactively labeled Y. pestis KIM cytoplasm as described in materials and methods. Immunoblots prepared against this cytoplasm used monospecific polyclonal rabbit antisera raised against; p70 (lane 3), p56 (lane 4), murine exotoxin (lane 5), pesticin (lane 6), and V antigen (lane 7). Antisera to pH 6 antigen (lane 9) was kindly provided by Dr. Susan Straley. Lane 8 contains purified fraction 1 (capsular) antigen. Molecular weight markers are in kilodaltons.

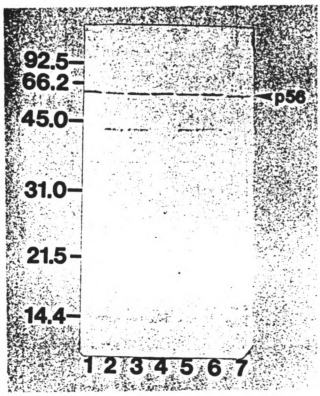

Figure 7. Immunoblots of whole cell extracts of S typhimurium LT2 (lanes 1 and 4), E. coli K-12 (lanes 2 and 5), E. coli B (lanes 3 and 6), and Y. pestis KIM (lane 7) developed with monospecific polyclonal rabbit antisera to the p56 peptide. Cells were grown at 26°C (lanes 1-3) or 37°C (lanes 4-7) in medium that lacked Ca^{2+}. Peptides were separated on 12.5% SDS-PAGE and molecular weight markers are in kilodaltons.

The p20 and p35 peptides could not be correlated with any known virulence factor. However, the p35 peptide may represent a processed form of the plasminogen activator/coagulase (PAC) encoded on the pesticin plasmid (28,41).

p56 and W antigen

The p56 product was found in all strains of yersiniae in our laboratory and did not appear to be correlated with any of the plasmids (unpublished data). Monospecific polyclonal antisera to the p56 peptide cross-reacted with a 58 kdal peptide in S. typhimurium and E. coli (Figure 7). This is the same molecular weight as the Gro EL peptide in E. coli (18). The p56 peptide purifies in the same manner as Gro EL (unpublished results) and is probably serves the equivalent function in yersiniae. The p56 peptide also migrated to the same point in two-dimensional gels as partially purified W antigen (Figure 8). The p56 peptide is also the major cytoplasmic protein (Figure 8).

p70 is a catalase

The p70 peptide was purified as described in the materials and methods. SDS-PAGE analysis of the proteins during this purification can be seen in Figure 9. This

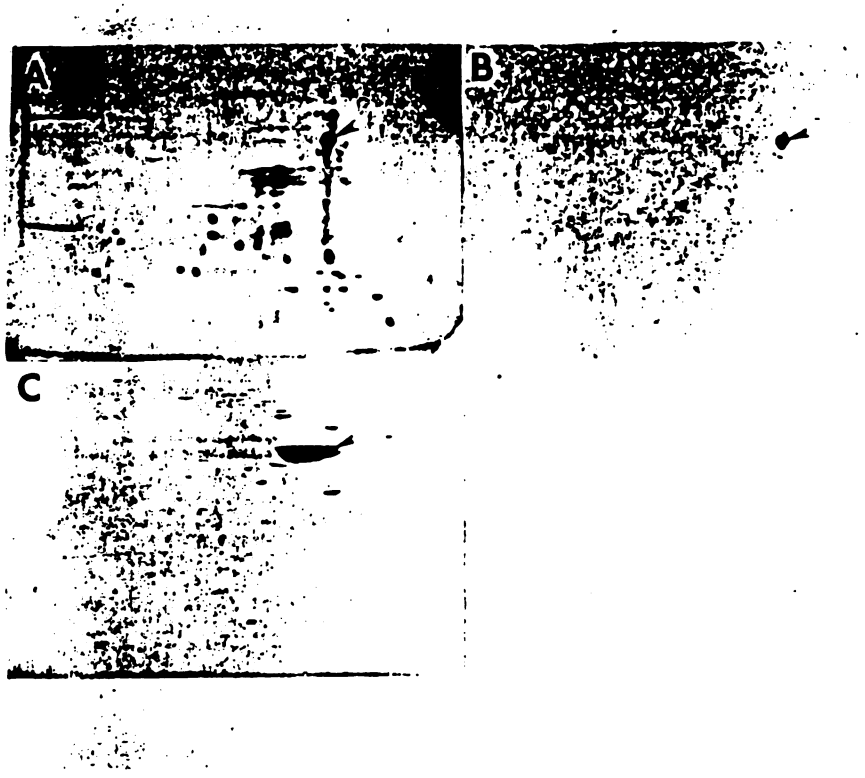

Figure 8. Stained two-dimensional gels of Y. pestis KIM cytoplasmic peptides of cells grown at 37°C (A) and of purified W antigen (C). Immunoblot of Y. pestis KIM cytoplasmic peptides was developed with monospecific polyclonal antisera to the p56 peptide. The isoelectric focusing of the peptides is in the horizontal direction with the acidic peptides to the right side. The second dimension was a 6-15% exponential gradient SDS-PAGE. Arrowhead indicates location of p56 peptide.

brown pigmented peptide had a sorêt band at 405 nm associated with a heme-containing enzyme and also stained with a hemoprotein specific stain (unpublished results). This protein exhibited moderate catalase activity, but no peroxidase activity. Purification of this catalase resulted in only about 5% of the total catalase activity as shown in Table 1. A majority of the catalase activity comes from a second catalase distinct from the p70 peptide (Figure 10). These are distinct enzymes since the p70 peptide is eluted in the wash of a DEAE column (Figure 4) while the second activity is very acidic because it is eluted at the very end of the DEAE column (unpublished results). Overall, Y. pestis has an enormous catalase activity as compared to E. coli (13,14).

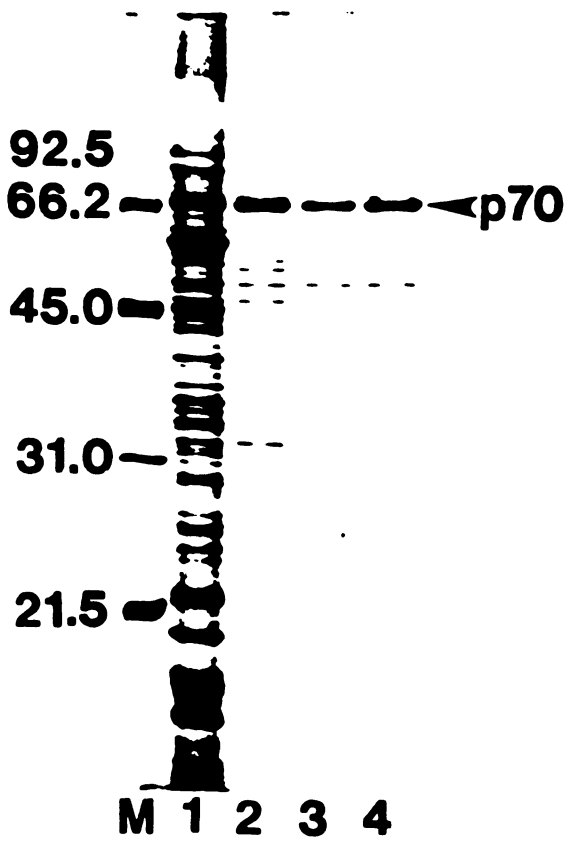

Figure 9. Silver stained 12.5% gel of the purification of the p70 peptide (as described in the materials and methods) showing crude extract (lane 1), after the DEAE cellulose (lane 2), after the phenyl-sepharose CL-4B (lane 3), and after the Sephacryl S-300 (lane 4). The molecular weight markers can be seen in lane M and they are in kilodaltons.

Table 1. Purification of the p70 protein

Step	Volume (ml)	[protein] (mg/ml)	total protein (mg)	specific activity (U/mg)	total units	yield (%)	Purification
Crude extract	26	63.8	1658.8	468	776,318	100	1.0
DEAE	32	2.6	83.2	658	54,746	7.0	1.4
Phenyl-Sepharose	63	0.88	55.4	704	44,352	5.7	1.5
S-300	15.6	0.94	14.7	2459	36,147	4.7	5.3

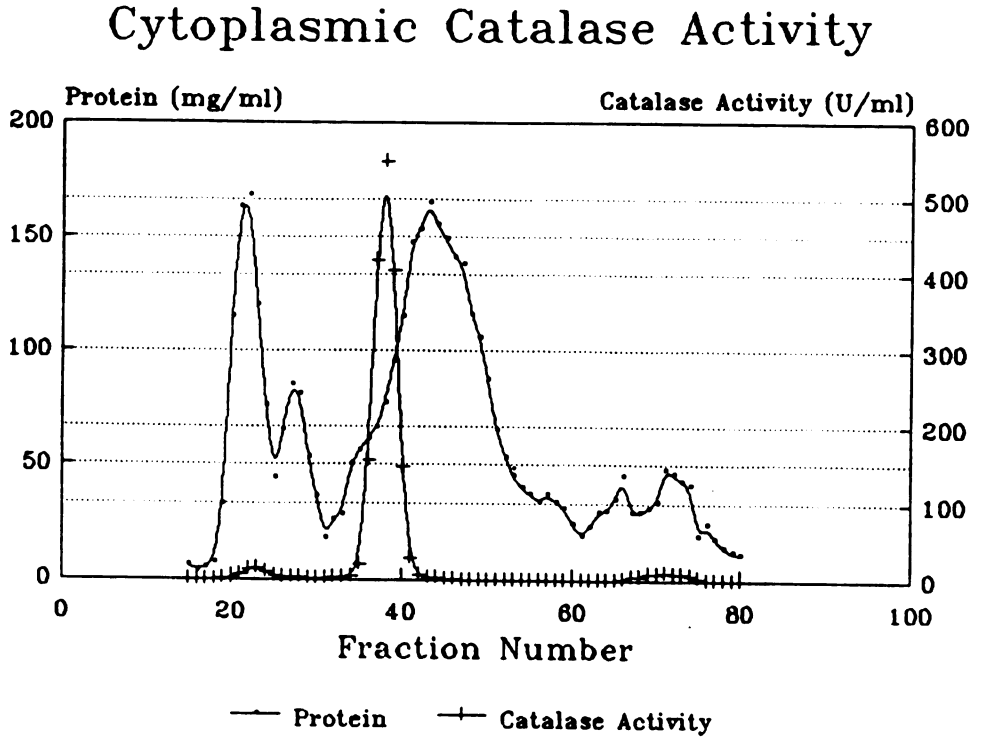

Figure 10. A Lcr⁻, Pst⁻ Y. pestis strain KIM cytoplasm was separated on a Sepharose A-1.5 column in 0.05 M Tris-HCl and fractions were assayed for protein by the method of Lowery (26) and for catalase activity. As verified by stained gels and immunoblots (Figure 3,unpublished data), the peak of the p70 peptide elutes at fraction 24 with small amounts found in fractions 70-75. No p70 can be detected in fraction 35-40 where the peak of catalase activity is found.

DISCUSSION

Much of the research done on the low calcium response has focused on the Yops. While these structures are stable in Y. pseudotuberculosis and Y. enterocolitica they undergo degradation in Y. pestis (38,42). This degradation in Y. pestis has allowed us a unique opportunity to examine how the LCR effects other virulence factors. The restriction of growth in Y. pestis also allows us to distinguish proteins that may be important in virulence without the interference of protein production needed for vegetative growth.

Labeling protein production during restriction is a sensitive method for determining what proteins are produced during the LCR, but lane gels may obscure minor but important proteins. In Figure 1, 5 major stable peptides could be visualized. The p38, p20 and p15 peptides are specific for the Lcr plasmid and cannot be detected upon loss of this plasmid. The p70 and p56 peptides are chromosomally encoded (28, unpublished results) and they are able to bypass the restriction normally found on chromosomal peptides during the low calcium response.

In addition to p70 and p56, the pH 6 antigen is another chromosomally encoded product (25) that circumvents the restriction during the LCR. This protein is a homopolymer with a base subunit size of 15 kdal (25). This can be verified by its presence in multiple fractions in the sizing column (Figure 3). This peptide is normally found on the

exterior of the cell but is a soluble peptide and can be found in the cytoplasmic fraction when the cells are disrupted by sonication. This peptide was designated a virulence factor because Y. pestis strains that produce it kill mice faster than those that don't and the purified protein was found to be cytotoxic to cultured macrophages (25). This peptide was named pH 6 antigen because it was only made in the greatest amounts at pH values below 6.7, and at host body temperatures (37°C) (3). These conditions would be expected in a macrophage phagolysosome. This same niche would also induce the LCR, therefore, this result is not that surprising.

Two other peptides produced during the LCR are the murine exotoxin and the capsular antigen termed fraction 1. Both of these peptides are believed to be encoded on the 110 kb cryptic plasmid (16,35). Their production during the LCR is very low at best, but it is detectable above backround. Fraction 1 production is greater than the production of toxin. The toxin protein reported here is much different that reported in the literature (29) and work is continuing to further characterize this protein. Fraction 1 is a glycoprotein homopolymer with a subunit size of 18 kdal with native complexes ranging up to 300 kdal (4). This size diversity can be seen in the sizing column fractionation where this peptide is found in almost all of the fractions (Figure 3). Purification of this peptide also resulted in

the copurification of the pH 6 antigen. These two proteins may interact in some manner or possibly act in a synergistic way.

Another protein that is produced during the LCR is the bacteriocin pesticin. This peptide is encoded on a third plasmid named after it, the pesticin plasmid (16). This plasmid also encodes the PAC which has both fibrinolytic and coagulase activities (27,41). This protein is already known to be encoded during the LCR because it is responsible for degradation of the Yops (28,38,41). The protein has a 37 and 35 kdal forms (41) and the latter form probably represents the p35 peptide seen in the autoradiograms (Figures 3-5). This is normally an outer membrane peptide (41,44) but small quantities of this protein remain in solution upon disruption of cells by sonication.

The p20 peptide is a minor peptide and could not be purified to any great degree. It is Lcr specific and it may represent a regulatory peptide, but at this time its identity and function remain unknown.

The p56 peptide is the major cytoplasmic peptides made during the LCR (Figure 8). This peptide represents the yersiniae equivalent to the Gro EL peptide in E. coli or the common antigen (20). This protein has been identified in many pathogens the Chlamydia species Gro EL is cross reactive to N. gonorrhoeae, R. rickettsii B. burgdorferi, and M. tuberculosis (30). The Gro EL protein is a large

acidic peptide that is believed to "chaperone" proteins for export (22,24). This "chaperone" protein holds newly produced proteins in an "open" conformation so that they can be exported or be assembled into large complexes. It seems reasonable to assume that this peptide is necessary during the LCR because there is a large number of proteins being exported at this time. Most of these proteins are Yops, but they only seem to need the LcrK gene for export (36). Therefore, Gro EL is may not needed for export of Yops.

An important virulence factor of yersiniae is the Lcr-mediated V antigen which has been shown to prevent granuloma formation during infection (48). This protein has a subunit molecular weight of 38 kdal (9) and can be seen in the figures as the p38 peptide. This peptide is mainly found in the cytoplasm but significant quantities, of the intact protein, can be found in the culture supernatant (9). Since the Gro EL peptide (p56) has been shown to be involved in the export of some proteins, we believe that the p56 peptide is responsible for the export of V antigen.

At the same time V antigen was identified as a virulence factor, there was also another peptide thought to be important in virulence termed W antigen (11). This antigen was found to be chromosomally encoded and, until recently, seems to have been forgotten. This protein has been purified in using the original anti-W antisera (23). A crude purification revealed a peptide of 56 kdal. Two-

dimensional gel electrophoresis of this crude W antigen preparation and a cytoplasmic extract revealed that W antigen is the same as the p56 peptide (Gro EL). Therefore, we propose that W antigen is actually a V antigen/p56 complex in which the V antigen is in the process of being exported. V antigen could not be detected in this complex due to its instability (9) during the purification of the W antigen. Purified V antigen is not stable in the purified form unless special measures are undertaken (9). However, anti-W antigen sera cross reacted with V antigen although W antigen was found to be much larger (90 kdal to 140 kdal) and have a more acidic isoelectric point (23).

The second chromosomally encoded peptide, p70, was found to be a catalase. Since this organism can survive intracellularly, it not unreasonable to assume that this type of enzyme would be necessary to help protect the organism from oxygen radicals. However, this peptide only makes up between 7-20% of the total catalase activity of the cytoplasm (Figure 10). Therefore, its role remains unclear inside the cell. The purified form of the enzyme has a specific activity level comparable to that of the hydroperoxidases of *E. coli* (13,14). However, this is the first reported catalase with an isoelectric point that is highly basic and it is very different than that of any other reported catalase.

The restriction of cells during the Lcr in *Y. pestis* results in the shut down of protein production except for Lcr-plasmid encoded protein which are induced. In this report, we have shown that non-Lcr virulence factors also continue to be produced under these energy limiting conditions (51). More work will have to be done to determine if there is some type of coordinate regulate of these virulence factors. We have also identified 2 chromosomally important peptides that may be required for the Lcr-mediated virulence factors to be effective during infection by *Y. pestis*.

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SUMMARY AND CONCLUSIONS

The low calcium response (Lcr) is a very complex virulence factor in yersiniae. The Lcr induces several distinct peptides that are necessary for virulence. Among these is a group of outer membrane peptides termed Yops. These Yops undergo a pesticin plasmid-mediated degradation in Y. pestis, that does not occur in the enteropathogenic strains. In this thesis, I have shown that despite this degradation there are sufficient levels of important Yops maintained. This Yop degradation can also occur in Y. pseudotuberculosis upon transformation of the pesticin plasmid. The resulting degradation is very similar to Y. pestis indicating that these two species have a similar virulence factor but yet cause two distinct types of disease.

This report has also identified the production of numerous other peptides during the LCR in Y. pestis when most vegetative protein production has ceased. Among these were several other virulence factors not associated with the LCR and some new products. Two of these virulence factors (fraction 1 and murine exotoxin) were encoded on the large cryptic plasmid while the plasminogen activator/coagulase and the bacteriocin pesticin were encoded on the pesticin plasmid. The pH 6 antigen was also produced and it is a chromosomal peptide. During the onset of the LCR, 2 heat-shock proteins were produced. Another heat-shock protein,

Gro EL, was identified as a major cytoplasmic peptide that may be necessary for the export of a known virulence factor (V antigen). A second chromosomally encoded product was identified as a novel catalase whose role is as yet unclear. Therefore, the LCR allows expression of other proteins that are important besides the Lcr-encoded products.