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Major Stable Peptides of *Yersinia pestis* Synthesized during the Low-Calcium Response

RICHARD J. MEHIGH AND ROBERT R. BRUBAKER*

Department of Microbiology, Michigan State University, East Lansing, Michigan 48824-1101

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It is established that the medically significant yersiniae require the presence of physiological levels of Ca^{2+} (ca. 2.5 mM) for sustained growth at 37°C and that this nutritional requirement is mediated by a shared ca. 70-kb Lcr plasmid. The latter also encodes virulence factors (*Yersinia* outer membrane proteins [Yops] and V antigen) known to be selectively synthesized in vitro at 37°C in Ca^{2+}-deficient medium. In this study, cells of *Yersinia pestis* KIM were first starved for Ca^{2+} at 37°C to prevent synthesis of bulk vegetative protein and then, after cell division had ceased, pulsed with [^{35}S]methionine. After sufficient chase to ensure plasminogen activator-mediated degradation of Yops, the remaining major radioactive peptides were separated by conventional chromatographic methods and identified as Lcr plasmid-encoded V antigen and LcrH (and possibly LcrG), ca. 10-kb Pst plasmid-encoded pesticin and plasminogen activator, ca. 100-kb Tox plasmid-encoded fraction 1 (capsular) antigen and murine exotoxin, and chromosomally encoded antigen 4 (pH 6 antigen) and antigen 5 (a novel hemin-rich peptide possessing modest catalase activity but not superoxide dismutase activity). Also produced at high concentration was a chromosome-encoded GroEL-like chaperone protein. Accordingly, the transcriptional block preventing synthesis of bulk vegetative protein at 37°C in Ca^{2+}-deficient medium may not apply to genes encoding virulence factors or to highly conserved GroEL (known in other species to utilize a secondary stress-induced sigma factor).

The medically significant yersiniae consist of *Yersinia pestis*, the causative agent of bubonic plague, and the closely related enteropathogenic species *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*. Wild-type cells of these three species share an approximately 70-kb Lcr (low-calcium response) plasmid encoding a set of regulatory genes that mediate shutoff of cell division at 37°C in Ca^{2+}-deficient media (Lcr^+ phenotype) (4, 8, 14, 46, 59). Little is known about the mechanism responsible for this unique form of restriction other than that Ca^{2+}-starved yersiniae remain viable while undergoing an ordered metabolic stepdown resulting in concomitant reduction of adenylate energy charge and shutoff of stable RNA synthesis (12, 66). As an indirect consequence of these events, the organisms become progressively blocked in synthesis of the bulk cellular protein needed for vegetative growth (12, 34). It is therefore significant that this same Ca^{2+}-deficient environment promotes selective expression of most Lcr plasmid-encoded virulence factors (4, 8, 14, 46, 59).

These virulence factors consist of a series of released proteins termed *Yersinia* outer membrane proteins (Yops) (6) and a secreted ca. 38-kDa peptide designated LcrV (43) or V antigen (11). This peptide possesses the potential to express an internal secretion sequence (47) that may account for its observed exit from all three species of yersiniae without significant detectable accumulation at the cell surface (52, 59, 60). Evidence derived from genetic (4, 46, 47) and immunological (53, 61, 62) studies indicates that V antigen functions as both a regulator of restriction and a virulence factor. The peptide is encoded in the *lcrGVH-yopBD* operon (4, 43, 46); LcrH (4, 48) and possibly LcrG (2) are necessary to avoid constitutive restriction of growth at 37°C.

Most newly synthesized Yops in enteropathogenic yersiniae undergo a distinct exit reaction that is probably mediated by the Lcr plasmid (35, 36, 50) that facilitates their accumulation at the outer membrane (17, 45, 60) and release into culture supernatant fluid (21, 35, 36). Nevertheless, net synthesis of most Yops does not occur in wild-type cells of *Y. pestis* because the Yops are degraded immediately after translation (34, 51); the degradation is catalyzed by a plague plasminogen activator (57) encoded on a species-specific ca. 10-kb pesticin or Pst plasmid. This ca. 35-kDa outer membrane protease (56) requires about 2 h to accumulate as a smaller processed derivative in restricted yersiniae (34). The rationale for plasminogen activator-mediated degradation of Yops is not yet resolved, although this process is probably modified in vivo (8), and does not negate essential roles as virulence factors in *Y. pestis* for some if not most of these peptides (8, 14, 59).

Restricted yersiniae were pulsed with radioactive methionine and chased; after newly synthesized Yops had undergone degradation, the yersiniae were found to contain a series of labeled stable (i.e., undegraded) peptides termed p70, p56, p38, p36, and p20 (34). Of these, p70, p38, and p36 were identified as temperature-dependent antigen 5 (15) or antigen E (10, 28), V antigen, and unprocessed plasminogen activator, respectively (34). Additional major stable peptides synthesized during restriction are identified in the present study. Our results indicated that p56 is a GroEL-like protein and that p20 is LcrH. In addition, the organisms synthesized *Y. pestis*-specific ca. 100-kb Tox plasmid-encoded (49) fraction 1 (capsular) antigen (1) and plague murine exotoxin (37), Pst plasmid-encoded pesticin (24, 25), and chromosome-encoded antigen 4 (15) or pH 6 antigen (5, 30). Additional findings suggested that p70 functions as a hemin storage protein. These findings illustrate that virulence functions encoded on all known genetic elements of *Y. pestis* escape the Lcr plasmid-mediated restriction imposed on synthesis of bulk vegetative protein at 37°C in Ca^{2+}-deficient medium.

* Corresponding author.

MATERIALS AND METHODS

Bacteria. A methionine-independent meiotrophic mutant (34) of nonpigmented *Y. pestis* KIM (substrain D29) was used in most experiments. This isolate possesses the Lcr, Pst, and Tox plasmids present in the wild type but lacks the deletable ca. 100-kb chromosomal segment (32) known to encode iron transport functions required for expression of virulence after infection by peripheral but not intravenous routes of injection (8). A congenic *lcrH* mutant (48) was received from Susan Straley.

Cultivation and radiolabeling. Bacteria previously stored in liquid buffered glycerol at −20°C were transferred to slopes of tryptose blood agar base (Difco Laboratories, Detroit, Mich.), incubated for 2 days at 26°C, suspended in 1 ml of chemically defined medium (66) containing 20 mM Mg^{2+} but no added Ca^{2+} or L-methionine, and diluted into 25 ml of the same medium (at an optical density at 620 nm of 0.1) in a 250-ml Erlenmeyer flask. After aeration at 200 rpm overnight at 26°C in a model G76 water bath shaker (New Brunswick Scientific Co., Inc., New Brunswick, N.J.), the organisms were transferred again and similarly incubated until an optical density of about 4 was obtained. At this time, a third subculture of the same medium (200 ml per 2-liter flask) was prepared at an optical density of 0.1, incubated at 26°C until the optical density was 0.25, and then shifted to 37°C. Vegetative growth ceased after further incubation for 6 h at this temperature (34, 65, 66).

The resulting fully restricted bacteria were first pulsed by adding carrier-free L-[^{35}S]methionine (New England Nuclear, Boston, Mass.) at a concentration of 10 μCi/ml to the culture and then chased after 1 min by adding unlabeled methionine in great excess (1.6 μmol/ml). After further incubation for 1 h, a period sufficient to ensure complete degradation of labeled Yops (34, 52), the organisms were centrifuged at 10,000 × *g* for 15 min, washed twice in cold 0.033 M potassium phosphate buffer (pH 7.0) (phosphate buffer), and suspended in 0.05 M Tris-HCl buffer (pH 8.0) to an optical density of ca. 20. This preparation was treated for 2 min with an ultrasonic probe (MSE Ltd., London, England), and cellular debris was eliminated by centrifugation at 10,000 × *g* for 30 min. The resulting clarified cytoplasm was removed by decanting and passed through a 0.22-μm-pore-size, low-protein-binding membrane filter (Millipore Corp., Bedford, Mass.) to remove particulate matter and effect sterilization. This radioactive preparation was mixed with 4 volumes of unlabeled carrier cytoplasm prepared exactly as described above but without [^{35}S]methionine. Samples were then stored at −20°C and thawed before use in column chromatography, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting.

The radiolabeled Lcr^+ and Lcr^- cells used in the initial studies (Fig. 1) were prepared in a similar manner, except that smaller volumes of culture (10 ml of medium per 125-ml Erlenmeyer flask) were subcultured and then shifted to restrictive conditions as described above. The cultures were then pulsed with [^{35}S]methionine (10 μCi/ml) for 1 min and chased for 1 h with unlabeled L-methionine. Samples were removed immediately after the pulse and after 1 h into the chase, precipitated with an equal volume of cold 10% trichloroacetic acid, and prepared for SDS-PAGE.

Antisera and immunoblotting. Most stable radioactive peptides were identified by immunoblotting with rabbit polyclonal antisera raised against nearly homogeneous samples of the proteins in question. p70 was prepared as outlined below; after immunization, antibodies directed against minor contaminating peptides were eliminated by absorption with disrupted and lyophilized Lcr^+ cells of *Y. enterocolitica* (10 mg/ml). The method of Hendrix (22) for purification for GroEL was used to isolate p56. After immunization, antibodies directed against minor contaminating antigens were removed by absorption with disrupted lyophilized Lcr^+ cells of *Y. pestis* KIM grown at 10°C, a temperature at which production of GroEL was minimal. Murine exotoxin was isolated by a minor modification of an established method (37) and then subjected to high-pressure liquid chromatography (HPLC) with Mono Q HR 5/5 resin (Pharmacia LKB Biotechnology, Piscataway, N.J.) to remove remaining contaminating peptides; antiserum raised with this product was monospecific as judged by immunoblotting. Antiserum to pesticin was prepared by immunization with material purified to homogeneity as previously described (24) and found to be monospecific in immunoblots. The preparation of monospecific polyclonal anti-V antigen was as described previously (9, 61). An antiserum raised against antigen 4 (30) was a gift from Susan Straley. This reagent also reacted with a higher-molecular-mass component, which did not interfere with its ability to determine the ca. 15-kDa subunit of antigen 4. Fraction 1 antigen was purified by the method of Baker et al. (1), and immunoblotting was performed as previously described (52).

Purification of p70. p70 was purified by growing Lcr^- Pst^- cells to an optical density of ca. 8 in a fermentor with the same medium as that described for preparation of V antigen (9). The organisms were harvested by centrifugation (10,000 × *g* for 30 min), washed twice in cold phosphate buffer, suspended in 50 ml of 0.05 M Tris-HCl (pH 8.0) (ca. 80 g [wet weight]), and disrupted by two passages through a French pressure cell (500 lb/in^2). Cellular debris was removed by centrifugation (17,000 × *g* for 20 min). The resulting crude extract was sterilized by saturation with $CHCl_3$, gently aerated to remove residual $CHCl_3$, and chromatographed on a column (2.5 by 45 cm) containing DEAE cellulose; p70 eluted early in the wash (0.05 M Tris-HCl [pH 8.0]) as a brown pigment. The pigment was brought to 1.0 M $(NH_4)_2SO_4$, applied to a column (1.5 by 30 cm) containing Phenyl-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) in 0.05 M Tris-HCl (pH 7.0) in 1.0 M $(NH_4)_2SO_4$, equilibrated in the same buffer, and then eluted by application of a step gradient consisting of 0.05 M Tris-HCl alone. The protein was precipitated by adding solid $(NH_4)_2SO_4$ (80% saturation), collected by centrifugation (27,000 × *g* for 30 min), and dissolved in 1.5 ml of 0.05 M 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES) buffer (pH 10). After further concentration to ca. 0.5 ml in a Centricon-10 tube (Amicon, Danvers, Mass.), this sample was applied to a column (1.5 by 170 cm) of Sephacryl S-300 (Pharmacia) equilibrated in 0.05 M CHES buffer (pH 10) containing 0.25 M $(NH_4)_2SO_4$. Eluted p70 was dialyzed against 0.05 M CHES buffer (pH 10) before use in experiments.

Miscellaneous. Catalase (E.C. 1.11.1.6) and superoxide dismutase (E.C. 1.15.1.1) were directly assayed in chromatographic column eluents by the methods of Beers and Sizer (3) and Winterbourn et al. (64), respectively. Pesticin was assayed with cells of *Y. pseudotuberculosis* PB1/0 as previously described (25). In addition to immunoblotting, murine exotoxin was assayed by determining the ability of 0.1-ml samples of intraperitoneally injected column eluents to kill mice within 24 h. Protein was determined by the method of Lowry et al. (31), and two-dimensional gel electrophoresis was performed by the method of O'Farrell et al.

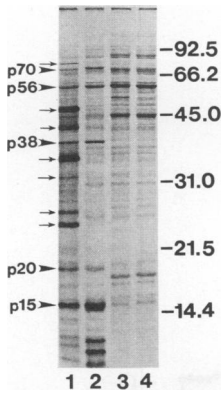

FIG. 1. Autoradiogram of SDS-PAGE gel (12.5% polyacrylamide) of trichloroacetic acid-precipitated samples of whole cultures of *Y. pestis* KIM cultivated at 37°C in Ca^{2+}-deficient medium after a pulse for 1 min with L-[^{35}S]methionine. Lanes: 1, restricted Lcr^+ cells without a chase; 2, restricted Lcr^+ cells after a chase for 1 h with excess unlabeled methionine; 3, growing Lcr^- cells without a chase; 4, growing Lcr^- cells after a chase for 1 h with excess unlabeled methionine. Small arrows represent unstable Yops, and large arrowheads indicate major stable peptides synthesized in Ca^{2+}-starved Lcr^+ yersiniae. The molecular masses of markers are given in kilodaltons.

(40) for analyses of LcrG. Protein in gels was determined by silver staining or use of Coomassie brilliant blue G as previously reported (34, 51). Autoradiographic procedures were described previously (51).

RESULTS

Lcr^+ and Lcr^- yersiniae were inoculated into Ca^{2+}-deficient medium and aerated at 26°C until logarithmic growth commenced. The cultures were then shifted to 37°C, at which a doubling time of 2 h was maintained by Lcr^- cells and vegetative growth of Lcr^+ organisms ceased entirely by 6 h. At this time the bacteria were pulsed with radioactive methionine and chased after 1 min by adding unlabeled methionine in excess. Samples were removed at the start of the chase and after 1 h of chase and subjected to SDS-PAGE and autoradiography for qualitative determination of the major peptides synthesized during restriction of growth.

Unstable peptides. Autoradiograms of pulsed Lcr^+ cells exhibited peptides recognized as Yops (Fig. 1, lane 1) as judged by their disappearance by 1 h with radioactivity undergoing chase into low-molecular-weight material (lane 2) and by their absence as intact peptides (lane 3) or degradation products (lane 4) in Lcr^- organisms. The seven bands of unstable peptides (lane 1) corresponded in molecular weight to YopF (76 kDa); YopB, -H, and -M (44 to 45 kDa); YopC (42 kDa); YopD and -N (34 kDa); YopJ (31 kDa); YopE (25 kDa); and YopK (22 kDa).

Stable peptides. Elimination of radioactive Yops by chasing with unlabeled methionine permitted identification of major stable proteins expressed by restricted Lcr^+ cells. These were present at both the beginning (Fig. 1, lane 1) and end (lane 2) of the chase and consisted of peptides of 70, 56, 38, 20, and 15 kDa. Of these, only p70 and p56 were shared by Lcr^- organisms, although a faint band probably corresponding to p15 was present in the latter (lanes 3 and 4). Bands appearing to be specific for Lcr^- cells represent major newly synthesized bulk or vegetative proteins and were also produced by Lcr^+ organisms during growth in Ca^{2+}-sufficient medium (data not shown).

Separation of radioactive peptides. Cytoplasm was prepared from fully restricted Lcr^+ cells after a similar pulse and chase as described above. Separate samples of this preparation were subjected to chromatography on columns containing Sepharose A-1.5 (Fig. 2A), DEAE cellulose (Fig. 2B), and calcium hydroxyapatite (Fig. 2C) to more fully resolve the stable peptides synthesized during starvation for Ca^{2+}; radioactivity and optical density were monitored as a function of volume eluted. Initial analyses showed that label often occurred in small peptides not present in similar extracts of Lcr^- organisms, consistent with their existence as degradation products of Yops. These small peptides were largely eliminated by precipitating a portion of each eluted sample with an equal volume of 10% trichloroacetic acid. Sedimented peptides were then suspended in SDS-PAGE buffer, resolved by SDS-PAGE, and stained; after drying, the gels were subjected to autoradiography.

Separation by molecular sizing resolved over 100 major peptides as judged by direct staining (Fig. 3, 4, 5); however, only 9 of these components contained sufficient radioactivity to register significant intensity on the corresponding autoradiograms. These newly synthesized proteins included the five stable peptides shown in Fig. 1 (lane 2) plus four additional peptides termed Pst, toxin, p35, and F1 (shown below to be pesticin, murine exotoxin, plasminogen activator, and fraction 1, respectively). In these experiments, the murine exotoxin always eluted as a 57-kDa peptide accompanied by two smaller peptides (Fig. 3 and 4; the smaller peptides are obscured in Fig. 5).

Chromatography on Sepharose A-1.5 showed that p70, F1, p56, and p15 were sized as high-molecular-weight components, indicating that they exist in native form as subunit complexes or in association with other macromolecules (Fig. 3). The earliest substance to elute on Sephadex A-1.5 was a brown pigment later equated with p70. Separation on the basis of net charge with DEAE cellulose provided a different pattern of unlabeled vegetative proteins and again showed early elution of p70 as a very basic brown pigment (Fig. 4). In contrast, p15, p35, p20, and p56 eluted late and thus possessed acidic isoelectric points. A distinct third pattern of elution was obtained after chromatography on calcium hydroxyapatite (Fig. 5), reflecting the dissimilar and not yet fully resolved mechanism of separation of this method (18). Although unidentified UV-absorbing material and radioactivity were detected in initial fractions (Fig. 2C), eluted peptides were not observed until the $NaHPO_4^-$ gradient was applied. A brown pigment, later identified as hemin, became irreversibly bound to calcium hydroxyapatite, resulting in late elution of p70 as a colorless peptide.

To verify that major radioactive peptides assigned the same name were identical, equal volumes recovered by the three processes were mixed and subjected to SDS-PAGE. As shown by subsequent radiography, the peptide in question always migrated as a compact single band (data not shown), thus indicating homogeneity among the pooled fractions. The three distinct processes of fractionation never resolved more than the nine major radioactive peptides already noted, although minor components of <15 kDa were also observed. One such example, considered below, is the small basic peptide eluted during chromatography on DEAE cellulose in fractions 6 and 7 (Fig. 4).

Identification of stable radioactive peptides. The identities of major peptides synthesized during restriction could usually be determined directly by comparison of immunoblots

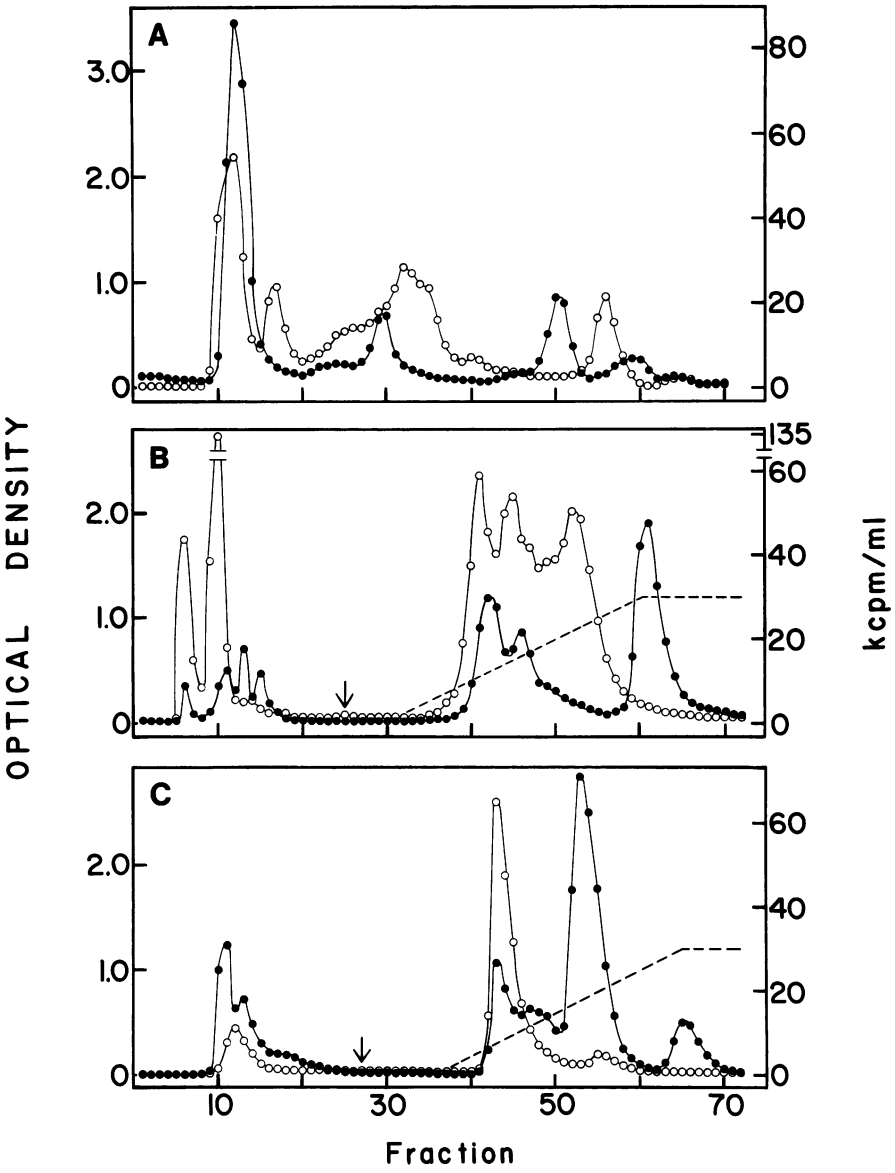

FIG. 2. Fractionation of cytoplasm from Ca^{2+}-starved Lcr^+ cells of *Y. pestis* KIM after a pulse for 1 min with L-[^{35}S]methionine: separation of (A) 40 mg of protein on Sepharose A-1.5 (column size, 1.5 by 100 cm) in 0.05 M Tris-HCl buffer (pH 8.0) in fractions of 6 ml, (B) 40 mg of protein on DEAE cellulose (column size, 2.5 by 46 cm) in 0.05 M Tris-HCl buffer (pH 7.8) with introduction (arrow) of gradient 0 to 0.5 M NaCl (- - -) in the same buffer in fractions of 4 ml, and (C) 40 mg of protein on calcium hybroxyapatite (column size, 2.5 by 45 cm) in 0.05 M Tris-HCl buffer (pH 7.8) with introduction (arrow) of gradient 0 to 0.5 M $NaHPO_4^-$ (- - -) in same buffer in fractions of 4 ml. Symbols: ●, optical density at 280 nm; ○, radioactivity.

with those of unfractionated cytoplasm (shown after staining and autoradiography in Fig. 6, lanes 1 and 2, respectively). For example, the molecular mass of the peptide termed toxin in Fig. 3 and 4 (57 kDa) was identical to that of known murine exotoxin (37), as judged by the reaction with its monospecific antiserum (Fig. 6, lane 5). All fractions recovered from chromatographic columns containing detectable concentrations of this peptide were lethal to mice, and toxicity in unfractionated cytoplasm was neutralized in vitro by addition of monospecific antiserum in excess followed by removal of the immune precipitate by centrifugation at 10,000 × *g* for 30 min (data not shown). Similarly, the peptide termed Pst in Fig. 3, 4, and 5 comigrated with homogenous β-pesticin (data not shown) and also reacted with monospecific antipesticin in immunoblots (Fig. 6, lane 6). Only the eluted column fractions containing this peptide possessed antibacterial activity directed against indicator cells of *Y. pseudotuberculosis*. The 15-kDa radioactive peptide was completely removed from unfractionated cytoplasm and chromatographic samples by precipitation with an excess of antiserum raised against antigen 4. This antiserum also reacted with p15 in immunoblots (Fig. 6, lane 9).

Fraction 1 was purified from whole cells by salt elution (1) and found to migrate during SDS-PAGE as a ca. 18-kDa monomer; unexpectedly, this process was also found to release antigen 4 (Fig. 6, lane 8). The stable radioactive peptide termed F1 in column eluents was also 18 kDa and appeared in fractions that were anticipated to contain this

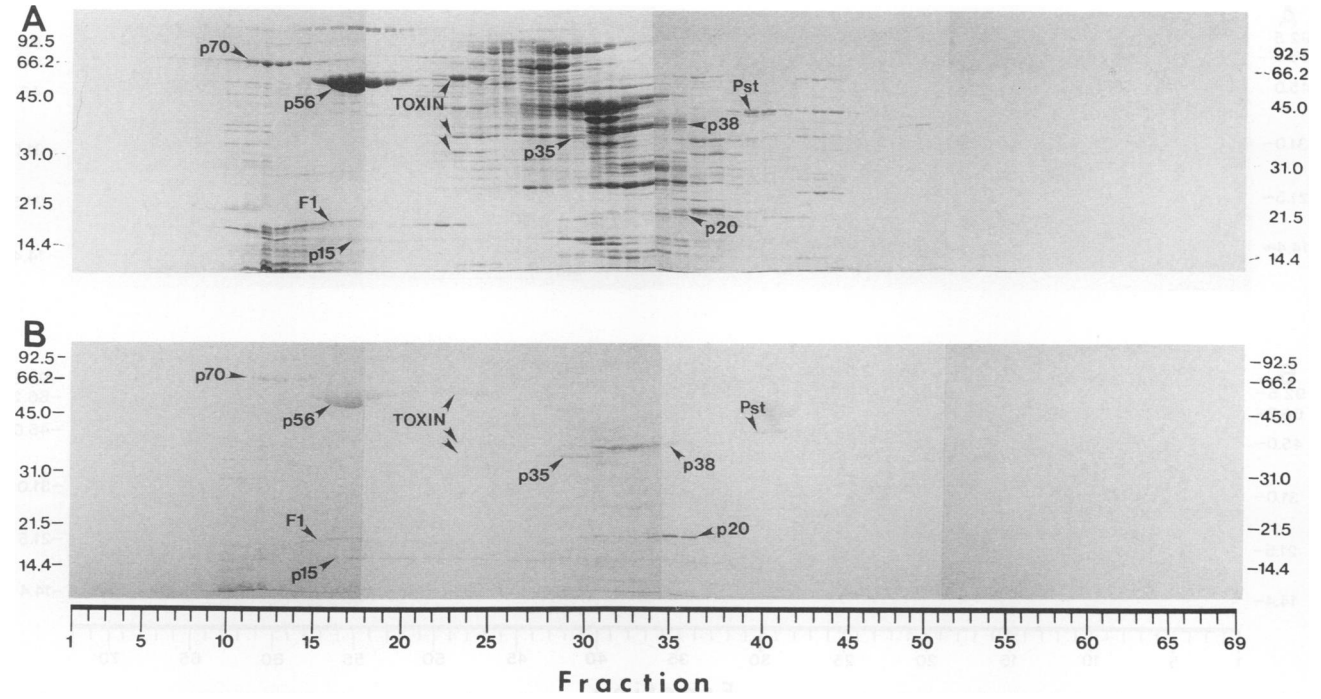

FIG. 3. Coomassie blue-stained gels (A) and corresponding autoradiograms (B) of trichloroacetic acid-precipitated proteins of the eluted fractions of the Sepharose A-1.5 column (Fig. 2A); molecular masses of markers are given in kilodaltons. Labeled proteins are discussed in the text.

capsular antigen as judged by its tendency to form aggregates and thus exhibit diverse molecular weights upon sieving (Fig. 3). Plasminogen activator had previously been equated with p35 primarily on the basis of corresponding molecular weights and its absence in isolates lacking the Pst plasmid (34). In the present work, the existence of newly synthesized p35 was initially obscured by radioactive undegraded comigrating YopD and N (Fig. 1, lane 1), although after the chase the peptide became detectable in both whole cells (Fig. 1, lane 2) and cytoplasm (Fig. 6, lane 2), where it

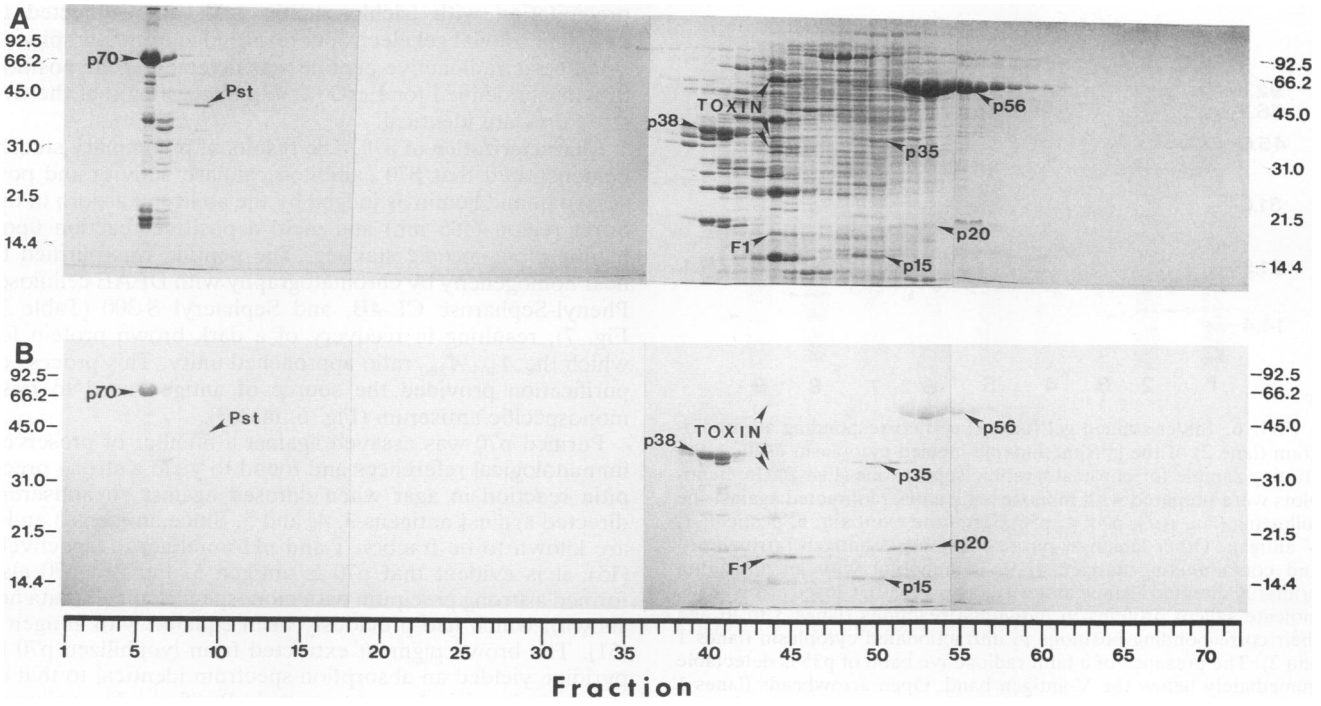

FIG. 4. Proteins eluted from the DEAE cellulose column (Fig. 2B) and processed as described in the legend to Fig. 3.

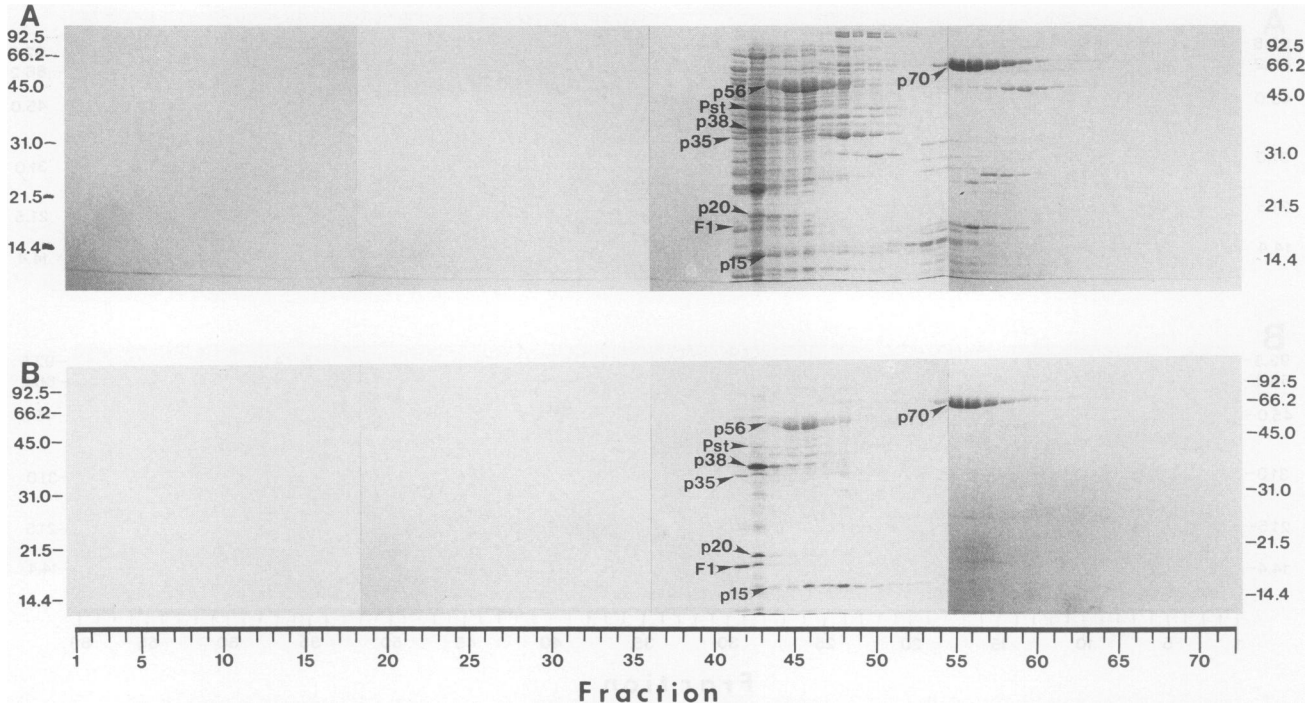

FIG. 5. Proteins eluted from the calcium hydroxyapatite column (Fig. 2C) and processed as described in the legend to Fig. 3.

could be fractionated by standard chromatographic procedures (Fig. 3, 4, and 5).

The observed Lcr^+ specificity, molecular weight, and net negative charge of p20 suggested an identity with LcrH. Attempts to demonstrate the presence of p20 in whole restricted *lcrH* cells or their cytoplasm were not successful (data not shown), indicating that the two activities are identical. V antigen was identical to p38 as judged by elution in only the chromatographic fractions retained during the process of purification (9) and by the ability to react in immunoblots against monospecific antiserum (Fig. 6, lane 7). The very basic low-molecular-weight radioactive peptide eluted in fractions 6 and 7 upon chromatography on DEAE cellulose (Fig. 4) was pooled, prepared for analysis by precipitation with trichloroacetic acid, and subjected to two-dimensional gel electrophoresis and autoradiography. A small basic radioactive peptide was detected at the position previously defined for LcrG (2, 47), suggesting that the two structures are identical.

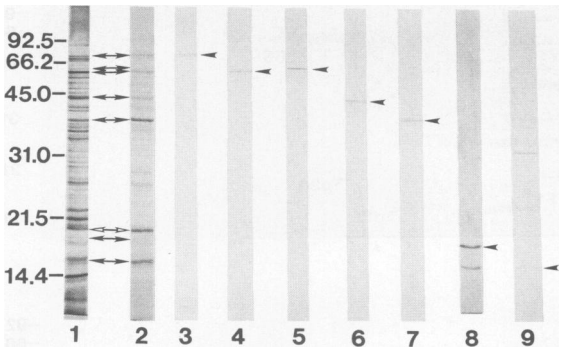

FIG. 6. Silver-stained gel (lane 1) and corresponding autoradiogram (lane 2) of the [^{35}S]methionine-labeled cytoplasm used as the starting sample for chromatographic separations (Fig. 2). Immunoblots were prepared with monospecific antisera directed against the following (lanes): 3, p70; 4, p56; 5, murine exotoxin; 6, pesticin; 7, V antigen. Other lanes: 8, purified fraction 1 antigen (arrowhead) and contaminating antigen 4; 9, immunoblot with an antiserum primarily directed against antigen 4 (arrowhead). Closed arrowheads indicate salient proteins in individual reactions (lanes 3 to 9) and their corresponding positions in unfractionated cytoplasm (lanes 1 and 2). The presence of a faint radioactive band of p35 is detectable immediately below the V-antigen band. Open arrowheads (lanes 1 and 2) indicate LcrH. Molecular masses of markers are given in kilodaltons.

Characterization of p70. The results of preliminary studies demonstrated that p70 exhibited catalase activity and possessed bound hemin as judged by the ability to absorb in the Soret region (405 nm) and yield a positive reaction upon hemoprotein-specific staining. The peptide was purified to near homogeneity by chromatography with DEAE cellulose, Phenyl-Sepharose CL-4B, and Sephacryl S-300 (Table 1, Fig. 7), resulting in recovery of a dark brown protein for which the A_{405}/A_{280} ratio approached unity. This process of purification provided the source of antigen used to raise monospecific antiserum (Fig. 6, lane 3).

Purified p70 was assayed against a number of preserved immunological references and found to yield a strong precipitin reaction in agar when diffused against an antiserum directed against antigens 3, 4, and 5. Since antigens 3 and 4 are known to be fraction 1 and pH 6 antigen, respectively (15), it is evident that p70 is antigen 5. Purified p70 also formed a strong precipitin with monospecific anti-E (data not shown), which had previously been equated with antigen 5 (51). The brown pigment extracted from lyophilized p70 in pyridine yielded an absorption spectrum identical to that of authentic hemin (data not shown). Purified p70 possessed modest but significant catalase activity (Table 1) but no

TABLE 1. Purification of p70

Prepn	Vol (ml)	Protein content		Catalase content		Sp act (U/mg of protein)	Yield (%)	Fold purification
		mg/ml	Total (mg)	U/ml	Total (U)			
Crude extract	26	63.8	1,658.8	29,858	776,308	468	100	1.0
DEAE cellulose	32	2.6	83.2	1,710	54,720	658	7.0	1.4
Phenyl-Sepharose CL-4B	63	0.88	55.4	704	44,352	801	5.7	1.7
Sephacryl S-300	15.6	0.94	14.7	2,317	36,145	2,459	4.7	5.3

detectable peroxidase activity. Loss of over 90% of the total catalase present in crude extract upon the first step of fractionation suggested that p70 accounts for only a minor portion of the activity present within yersiniae. This assumption was verified by molecular sizing of cytoplasm obtained from Lcr$^-$ organisms grown at 37°C, in which the majority of catalase activity arose from a distinct enzyme eluting after native p70 (Fig. 8A). Only p70 itself and a minor component eluting with low-molecular-weight material at the end of the determination reacted in immunoblots with anti-p70. No p70 was detected in the intermediate fractions containing the major catalase. The same eluted samples were tested to determine whether p70 corresponded to superoxide dismutase. Although two such activities, which may represent distinct enzymes, were eluted, neither was present in fractions containing p70 (Fig. 8B).

Characterization of p56. The molecular weight, net negative charge, abundance, and temperature dependence of p56 suggested identity with GroEL. This similarity was emphasized by the finding that the use of methods designed for purification of GroEL from *Escherichia coli* (22) resulted in isolation of nearly homogenous p56 from Lcr$^-$ cells of *Y. pestis*. Immunoblotting of polyclonal antiserum raised against the resulting product identified a single 56-kDa peptide (Fig. 6, lane 4) that was shared by Lcr$^-$ cells of *Y. pseudotuberculosis* PB1 and *Y. enterocolitica* WA as well as additional enteric bacteria including *E. coli*, salmonellae, and shigellae (34) (data not shown). These findings indicate that p56 is a GroEL-like protein.

DISCUSSION

The enigma of the low-calcium response is that the Ca^{2+}-deficient environment required for maximum induction in vitro of Lcr plasmid-encoded virulence factors is inconsistent with sustained bacterial multiplication. Conversely, these determinants are repressed in vitro in the presence of sufficient added Ca^{2+} to ensure continued vegetative growth (4, 8, 14, 46, 59). An initial interpretation of these relationships was that the virulence factors in question are probably not expressed in vivo by yersiniae growing in Ca^{2+}-sufficient vascular fluid but might be induced after phagocytosis and emergence into Ca^{2+}-deficient intraleukocytic fluid (10). Although later work with macrophages showed that this was the case (44), it is now recognized that yersiniae primarily proliferate as extracellular parasites within necrotic foci or abscesses (8). Nothing is known about the composition of the fluid contained within these lesions, although its origin from necrosed tissue suggests a reduced content of Ca^{2+} like that in mammalian intracellular fluid (26). In this case, extracellular yersiniae present within necrotic foci and ab-

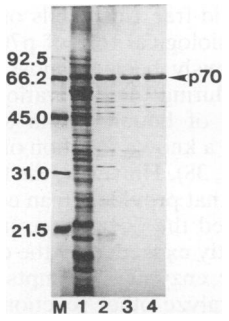

FIG. 7. Presence of p70 in crude extract (lane 1) and after chromatography by DEAE cellulose (lane 2), Phenyl-Sepharose CL-4B (lane 3), and Sephacryl S-300 (lane 4) as shown by silver staining; sample concentrations were adjusted to constant A_{405}. Molecular masses of markers (lane M) are given in kilodaltons.

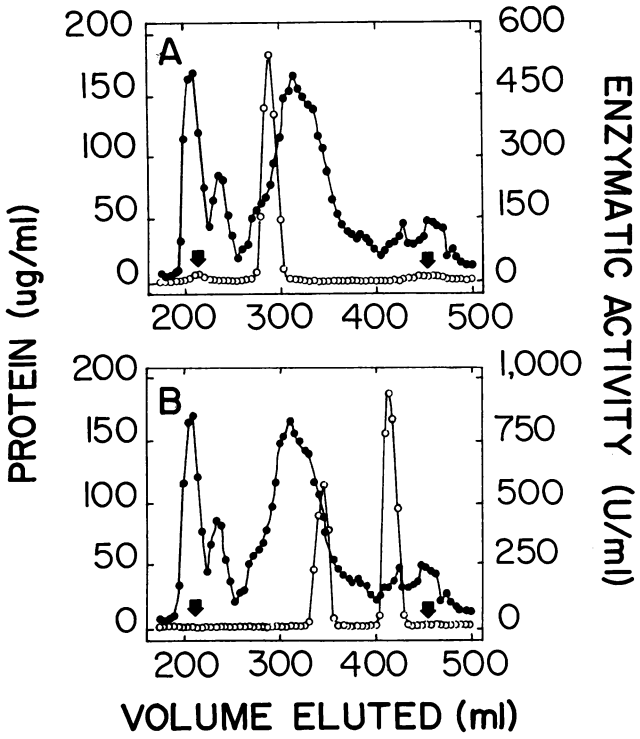

FIG. 8. Distribution of enzyme activities in the cytoplasm of Lcr$^-$ Pst$^-$ cells of *Y. pestis* KIM grown at 37°C in Ca^{2+}-deficient medium after molecular sieving on a column (1.5 by 100 cm) of Sepharose A-1.5 in 0.05 M Tris-HCl buffer (pH 8.0). Protein (●) and catalytic activity (○) are shown for catalase (A) and superoxide dismutase (B). Arrows indicate major peaks (at ca. 220 ml) and minor peaks (at ca. 450 ml) of eluted protein that reacted in immunoblots with anti-p70.

scesses would be expected to synthesize significant levels of Yops and V antigen.

There is no Ca^{2+}-deficient in vivo environment known to promote restriction of vegetative growth in a manner similar to that observed to occur in vitro. The nutritional requirement of Lcr^+ yersiniae for the cation may therefore be an artifact, as judged by reports of substantial growth at 37°C in Ca^{2+}-deficient medium with reduced Na^+ (7) or Mg^{2+} (65) or with added nucleotides (2, 48, 65). Further study may show that these modifications correct some in vitro deficiency or toxicity not encountered in Ca^{2+}-deficient fluids in vivo. In any event, the typical in vitro response of Lcr^+ yersiniae to Ca^{2+} privation has provided the basis of an excellent system for analysis of mutants blocked in the regulation of Yops and V antigen (4, 14, 46, 50).

Some caution, however, is necessary to avoid potential errors in determining these virulence factors in growing versus restricted yersiniae. For example, synthesis of Yops and V antigen in fully restricted cells of *Y. pestis* occurs as a zero-order reaction as illustrated by the present study. In contrast, expression of these virulence factors by wild-type organisms grown in Ca^{2+}-deficient but otherwise permissive environments (e.g., those containing nucleotides or reduced Na^+ or Mg^{2+}) takes place as a first-order reaction in which specific activities are reduced because of the contribution of newly synthesized vegetative protein. Similar kinetics describe Yop and V antigen synthesis in regulatory *lcr* mutants capable of growth under Ca^{2+}-deficient conditions that are known to promote restriction of wild-type yersiniae. Failure to distinguish between these physiological states could result in appreciable error in determining the rates of synthesis of Yops and V antigen or their concentrations in the steady state. Potential errors in estimating net synthesis were minimized in this work by use of a short pulse of [^{35}S]methionine. Restriction of yersiniae caused by Ca^{2+} privation is known, however, to deplete the internal pools of small molecules serving as nucleic acid and protein precursors (12, 65); thus, an exogenously supplied amino acid would be expected to undergo more extensive incorporation into macromolecules of Ca^{2+}-starved Lcr^+ cells than into those of growing Lcr^- mutants. This phenomenon probably accounts for the evident reduced incorporation of [^{35}S]methionine into growing Lcr^- mutants as compared with that observed in Lcr^+ organisms (Fig. 1).

These precautions only apply to executing precise quantitative determinations of Yops and V antigen; they do not question the generally accepted perception that these virulence factors are induced upon cultivation at 37°C in Ca^{2+}-deficient medium. However, it is possible that activation of the regulatory cascade leading to synthesis of Yops and V antigen reflects the existence of physiological stress arising as an indirect consequence of Ca^{2+} privation. Evidence favoring this concept was the finding that Ca^{2+} was not metabolized by yersiniae but excluded (42) as described previously for related enteric bacteria (55). Furthermore, first-order analysis demonstrated the occurrence of a burst of V-antigen synthesis in Lcr^+ yersiniae entering the stationary phase after full-scale growth at 37°C in Ca^{2+}-sufficient medium (65). This phenomenon would be expected if cells in the normal stationary phase and those starved for Ca^{2+} undergo some common physiological stress that, in itself, directly mediates induction of Yops and V antigen. A number of activities known to prevent cellular damage or prolong survival are induced in response to heat shock, nutritional privation, or other forms of stress (23, 39, 54, 58). Central to these activities in *E. coli* are stress-induced secondary sigma factors that mediate transcription of a variety of proteins required for survival in unfavorable environments (19, 41).

We formulated a working hypothesis that one or more secondary sigma factors in yersiniae recognize promoters of structural genes encoding both stress-inducible functions and Lcr plasmid-encoded virulence factors but not bulk protein amassed during vegetative growth. To test this possibility, we pulsed fully restricted Lcr^+ organisms (no longer capable of synthesizing vegetative protein) for 1 min with radioactive methionine and then chased for 1 h with unlabeled methionine to eliminate unwanted Yops. After SDS-PAGE, the organisms were shown by autoradiography to contain the expected high levels of newly synthesized V antigen and LcrH plus a GroEL-like protein. GroEL is a highly conserved heat shock protein required for a variety of fundamental processes (20, 27, 29, 33), all of which may reflect its ability to maintain other peptides in proper conformation (16, 63). The presence of a GroEL-like protein as a major radioactive component of Ca^{2+}-starved yersiniae is consistent with the notion that the low-calcium response is modulated by sigma factor conversion.

Cytoplasm of restricted organisms undergoing a similar pulse and chase was fractionated to retrieve and identify additional newly synthesized peptides. These were recognized as pesticin plus all Lcr plasmid-independent virulence factors so far recognized in the species (except for the Fur protein-regulated peptides required for assimilation of iron [8]). Constitutive expression of pesticin was not unexpected; this bacteriocin is inducible by UV light and thus normally subject to synthesis by a constant but statistically small fraction of the bacterial population (25). Production of significant concentrations of fraction 1 antigen, murine exotoxin, antigen 4, and plasminogen activator during restriction was not anticipated because these peptides are also synthesized during vegetative growth. With hindsight, however, it seems logical that these virulence factors would also be expressed within the same in vivo niche as are Yops and V antigen. A number of mechanisms could account for transcription of their structural genes in both restrictive and permissive environments. One possibility is reliance upon multiple promoters, including one utilized for synthesis of bulk protein during vegetative growth and another used during Ca^{2+} starvation for production of Yops and V antigen.

Another major peptide produced during restriction was hemin-rich p70 (i.e., antigen E or antigen 5), which was assumed to be chromosomally encoded as judged by its expression in plasmid-free Lcr^- cells of *Y. pseudotuberculosis* (34). The physiological role of p70 remains uncertain but was taken seriously because most of the other major peptides produced during Ca^{2+} privation are virulence factors. The presence of bound hemin suggested a role in oxygen metabolism, a known function of one class of stress-induced proteins (13, 38). However, the low catalase activity of p70 (matched by that provided in an equivalent amount of free hemin) suggested the occurrence of nonenzymatic catalysis and was greatly exceeded by the catalase activity of a distinct cytoplasmic enzyme. Attempts to demonstrate an ability of p70 to catalyze other reactions involving oxygen including superoxide dismutase were not successful. We are presently investigating the possibility that p70 functions as an internal storage depot for hemin, known to serve as a sole source of iron for yersiniae, in a manner analogous to that of bacterioferritin in storage of Fe^{3+} (8).

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REFERENCES

1. **Baker, E. E., H. Sommer, L. E. Foster, E. Meyer, and K. F. Meyer.** 1952. Studies on immunization against plague. I. The isolation and characterization of the soluble antigen of *Pasteurella pestis*. J. Immunol. **68:**131–145.
2. **Barve, S. S., and S. C. Straley.** 1990. *lcrR*, a low-Ca^{2+}-response locus with dual Ca^{2+}-dependent functions in *Yersinia pestis*. J. Bacteriol. **172:**4661–4671.
3. **Beers, R. F., Jr., and I. W. Sizer.** 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. **195:**133–140.
4. **Bergman, T., S. Hakansson, A. Forsberg, L. Norlander, A. Macellaro, A. Backman, I. Bolin, and H. Wolf-Watz.** 1991. Analysis of the V antigen *lcrGVH-yopBD* operon of *Yersinia pseudotuberculosis*: evidence for a regulatory role of LcrH and LcrV. J. Bacteriol. **173:**1607–1616.
5. **Bichowsky-Slomnicki, L., and S. Ben-Efraim.** 1963. Biological activities in extracts of *Pasteurella pestis* and their relation to the "pH 6 antigen." J. Bacteriol. **86:**101–111.
6. **Bolin, I., D. A. Portnoy, and H. Wolf-Watz.** 1985. Expression of the temperature-inducible outer membrane proteins of yersiniae. Infect. Immun. **48:**234–240.
7. **Brubaker, R. R.** 1967. Growth of *Pasteurella pseudotuberculosis* in simulated intracellular and extracellular environments. J. Infect. Dis. **117:**403–417.
8. **Brubaker, R. R.** 1991. Factors promoting acute and chronic diseases caused by yersiniae. Clin. Microbiol. Rev. **4:**309–324.
9. **Brubaker, R. R., A. K. Sample, D.-Z. Yu, R. J. Zahorchak, P. C. Hu, and J. M. Fowler.** 1987. Proteolysis of V antigen from *Yersinia pestis*. Microb. Pathog. **2:**49–62.
10. **Brubaker, R. R., and M. J. Surgalla.** 1964. The effect of Ca^{++} and Mg^{++} on lysis, growth, and production of virulence antigens by *Pasteurella pestis*. J. Infect. Dis. **114:**13–25.
11. **Burrows, T. W., and G. A. Bacon.** 1956. The basis of virulence in *Pasteurella pestis*: an antigen determining virulence. Br. J. Exp. Pathol. **37:**481–493.
12. **Charnetzky, W. T., and R. R. Brubaker.** 1982. RNA synthesis in *Yersinia pestis* during growth restriction in calcium deficient medium. J. Bacteriol. **149:**1089–1095.
13. **Christman, M. F., R. W. Morgan, F. S. Jacobson, and B. N. Ames.** 1985. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. Cell **41:**753–762.
14. **Cornelis, G., Y. Laroche, G. Balligand, M.-P. Sory, and G. Wauters.** 1987. *Yersinia enterocolitica*, a primary model for bacterial invasiveness. Rev. Infect. Dis. **9:**64–87.
15. **Crumpton, M. Y., and D. A. L. Davies.** 1956. An antigenic analysis of *Pasteurella pestis* by diffusion of antigens and antibodies in agar. Proc. R. Soc. Lond. Ser. B **145:**109–134.
16. **Ellis, R. J.** 1987. Proteins as molecular chaperones. Nature (London) **328:**378–379.
17. **Forsberg, A., I. Bolin, L. Norlander, and H. Wolf-Watz.** 1987. Molecular cloning and expression of calcium-regulated, plasmid-coded proteins of *Y. pseudotuberculosis*. Microb. Pathog. **2:**123–137.
18. **Gorbunoff, M. J., and S. N. Timasheff.** 1984. The interaction of proteins with hydroxyapatite. III. Mechanisms. Anal. Biochem. **136:**440–445.
19. **Grossman, A. D., J. W. Erickson, and C. A. Gross.** 1984. The *htpR* gene product of *E. coli* is a sigma factor for heat-shock promoters. Cell **38:**383–390.
20. **Grossman, A. D., W. E. Taylor, Z. F. Burton, R. R. Burgess, and C. A. Gross.** 1985. Stringent response in *Escherichia coli* induces expression of heat shock proteins. J. Mol. Biol. **186:**357–365.
21. **Heesemann, J., U. Gross, N. Schmidt, and R. Laufs.** 1986. Immunochemical analysis of plasmid-encoded proteins released by enteropathogenic *Yersinia* sp. grown in calcium-deficient media. Infect. Immun. **54:**561–567.
22. **Hendrix, R. W.** 1979. Purification and properties of GroE, a host protein involved in bacteriophage assembly. J. Mol. Biol. **129:**375–392.
23. **Hightower, L. E.** 1991. Heat shock, stress proteins, chaperones, and proteotoxicity. Cell **66:**191–197.
24. **Hu, P. C., and R. R. Brubaker.** 1974. Characterization of pesticin: separation of antibacterial activities. J. Biol. Chem. **249:**4749–4753.
25. **Hu, P. C., G. C. H. Yang, and R. R. Brubaker.** 1972. Specificity, induction, and absorption of pesticin. J. Bacteriol. **112:**212–219.
26. **Kugelmass, N. I.** 1959. Biochemistry of blood in health and disease. Charles C Thomas, Publisher, Springfield, Ill.
27. **Kumamoto, C. A.** 1991. Molecular chaperons and protein translocation across the *Escherichia coli* inner membrane. Mol. Microbiol. **5:**19–22.
28. **Lawton, W. D., G. M. Fukui, and M. J. Surgalla.** 1960. Studies on the antigens of *Pasteurella pestis* and *Pasteurella pseudotuberculosis*. J. Immunol. **84:**475–479.
29. **Lecker, S., R. Lili, T. Ziegelhoffer, C. Georgopoulos, P. J. Bassford, C. A. Kumamoto, and W. Wickner.** 1989. Three pure chaperonine proteins of *Escherichia coli*—SecB, trigger factor, and GroEL—form soluble complexes with precursor proteins *in vitro*. EMBO J. **8:**2703–2709.
30. **Lindler, L. E., M. S. Klempner, and S. C. Straley.** 1990. *Yersinia pestis* pH 6 antigen: genetic, biochemical, and virulence characterization of a protein involved in the pathogenesis of bubonic plague. Infect. Immun. **58:**2569–2577.
31. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
32. **Lucier, T. S., and R. R. Brubaker.** 1992. Determination of genome size, macrorestriction pattern polymorphism, and nonpigmentation-specific deletion in *Yersinia pestis* by pulsed-field gel electrophoresis. J. Bacteriol. **174:**2078–2086.
33. **Martin, A.** 1991. The molecular basis of carbon-starvation-induced general resistance in *Escherichia coli*. Mol. Microbiol. **5:**3–10.
34. **Mehigh, R. J., A. K. Sample, and R. R. Brubaker.** 1989. Expression of the low-calcium response in *Yersinia pestis*. Microb. Pathog. **6:**203–217.
35. **Michiels, T., and G. R. Cornelis.** 1991. Secretion of hybrid proteins by the *Yersinia* Yop export system. J. Bacteriol. **173:**1677–1685.
36. **Michiels, T., P. Wattiau, R. Brasseur, J.-M. Ruysschaert, and G. Cornelis.** 1990. Secretion of Yop proteins by yersiniae. Infect. Immun. **58:**2840–2849.
37. **Montie, T. C., and D. B. Montie.** 1971. Protein toxins of *Pasteurella pestis*. Subunit composition and acid binding. Biochemistry **10:**2094–2100.
38. **Morgan, R. W., M. F. Christman, F. S. Jacobson, G. Storz, and B. N. Ames.** 1986. Hydrogen peroxide-inducible proteins in *Salmonella typhimurium* overlap with heat shock and other stress proteins. Proc. Natl. Acad. Sci. USA **83:**8059–8063.
39. **Neidhardt, F. C., R. A. Van Bogelen, and V. Vaughn.** 1984. The genetics and regulation of heat shock proteins. Annu. Rev. Genet. **18:**295–329.
40. **O'Farrell, P. Z., H. M. Goodman, and P. H. O'Farrell.** 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell **12:**1133–1142.
41. **Ozaki, M., A. Wada, N. Fujita, and A. Ishihama.** 1991. Growth phase-dependent modification of RNA polymerase in *Escherichia coli*. Mol. Gen. Genet. **230:**17–23.
42. **Perry, R. D., and R. R. Brubaker.** 1987. Transport of Ca^{2+} by *Yersinia pestis*. J. Bacteriol. **160:**4861–4864.
43. **Perry, R. D., P. A. Harmon, W. S. Bowmer, and S. C. Straley.** 1986. A low-Ca^{2+} response operon encodes the V antigen of *Yersinia pestis*. Infect. Immun. **54:**428–434.
44. **Pollack, C., S. C. Straley, and M. S. Klempner.** 1986. Probing the phagolysosomal environment of human macrophages with a Ca^{2+}-responsive operon fusion in *Yersinia pestis*. Nature (London) **322:**834–837.
45. **Portnoy, D. A., S. L. Moseley, and S. Falkow.** 1981. Character-

ization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. Infect. Immun. **31:**775–782.

46. **Price, S. B., C. Cowan, R. D. Perry, and S. C. Straley.** 1991. The *Yersinia pestis* V antigen is a regulator protein necessary for Ca^{2+}-dependent growth and the maximal expression of low-Ca^{2+} response virulence genes. J. Bacteriol. **173:**2649–2657.
47. **Price, S. B., K. Y. Leung, S. S. Barve, and S. C. Straley.** 1989. Molecular analysis of *lcrGVH*, the V operon of *Yersinia pestis*. J. Bacteriol. **171:**5646–5653.
48. **Price, S. B., and S. C. Straley.** 1989. *lcrH*, a gene necessary for virulence of *Yersinia pestis* and for the normal response of *Y. pestis* to ATP and calcium. Infect. Immun. **57:**1491–1498.
49. **Protsenko, O. A., P. I. Anisimov, O. T. Mosarov, N. P. Donnov, Y. A. Popov, and A. M. Kokushkin.** 1983. Detection and characterization of *Yersinia pestis* plasmids determining pesticin I, fraction I antigen and mouse toxin synthesis. Genetika **19:**1081–1090.
50. **Rosqvist, R., A. Forsberg, M. Rempelainen, T. Bergman, and H. Wolf-Watz.** 1990. The cytotoxic Yop E of *Yersinia* obstructs the primary host defense. Mol. Microbiol. **4:**657.
51. **Sample, A. K., and R. R. Brubaker.** 1987. Posttranslational regulation of Lcr plasmid-mediated peptides in pesticinogenic *Yersinia pestis*. Microb. Pathog. **3:**239–248.
52. **Sample, A. K., J. M. Fowler, and R. R. Brubaker.** 1987. Modulation of the low calcium response in *Yersinia pestis* by plasmid-plasmid interaction. Microb. Pathog. **2:**443–453.
53. **Sato, K., R. Nakajima, F. Hara, T. Une, and Y. Osada.** 1991. Preparation of monoclonal antibody to V antigen from *Yersinia pestis*. Contrib. Microbiol. Immunol. **12:**225–229.
54. **Siegele, D. A., and R. Kolter.** 1992. Life after lag. J. Bacteriol. **174:**345–348.
55. **Silver, S., K. Toth, and H. Scribner.** 1975. Facilitated transport of calcium by cells and subcellular membranes of *Bacillus subtilis* and *Escherichia coli*. J. Bacteriol. **122:**880–885.
56. **Sodeinde, O. A., and J. D. Goguen.** 1988. Genetic analysis of the 9.5-kilobase virulence plasmid of *Yersinia pestis*. Infect. Immun. **56:**2743–2748.
57. **Sodeinde, O. A., A. K. Sample, R. R. Brubaker, and J. D. Goguen.** 1988. Plasminogen activator/coagulase gene of *Yersinia pestis* is responsible for degradation of plasmid-encoded outer membrane proteins. Infect. Immun. **56:**2749–2752.
58. **Storz, G., L. A. Tartaglia, and B. N. Ames.** 1990. Transcriptional regulator of oxidative stress-induced genes: direct activation by oxygen. Science **248:**189–194.
59. **Straley, S. C.** 1988. The plasmid-encoded outer-membrane proteins of *Yersinia pestis*. Rev. Infect. Dis. **10:**5323–5326.
60. **Straley, S. C., and R. R. Brubaker.** 1981. Cytoplasmic and membrane proteins of yersiniae cultivated under conditions simulating mammalian intracellular environment. Proc. Natl. Acad. Sci. USA **78:**1224–1228.
61. **Une, T., and R. R. Brubaker.** 1984. Roles of V antigen in promoting virulence and immunity in yersiniae. J. Immunol. **133:**2226–2230.
62. **Une, T., R. Nakajima, and R. R. Brubaker.** 1986. Roles of V antigen in promoting virulence in *Yersinia*. Contrib. Microbiol. Immunol. **9:**179–185.
63. **Van Dyk, T. K., A. A. Gatenby, and R. A. LaRossa.** 1989. Demonstration by genetic suppression of interaction of GroE products with many proteins. Nature (London) **342:**451–453.
64. **Winterbourn, C. C., R. E. Hawkins, M. Brian, and R. W. Carrell.** 1975. The estimation of red cell superoxide dismutase activity. J. Lab. Clin. Med. **85:**337–341.
65. **Zahorchak, R. J., and R. R. Brubaker.** 1982. Effect of exogenous nucleotides on Ca^{2+} dependence and V antigen synthesis in *Yersinia pestis*. Infect. Immun. **38:**953–959.
66. **Zahorchak, R. J., W. T. Charnetzky, R. V. Little, and R. R. Brubaker.** 1979. Consequences of Ca^{2+} deficiency on macromolecular synthesis and adenylate energy charge in *Yersinia pestis*. J. Bacteriol. **139:**792–799.