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Influence of Na^+, Dicarboxylic Amino Acids, and pH in Modulating the Low-Calcium Response of *Yersinia pestis*

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The virulence of yersiniae is promoted in part by shared ≈70-kb plasmids (pCD in *Yersinia pestis* and pYV in enteropathogenic *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*) that mediate a low-calcium response. This phenotype is characterized at 37°C by either bacteriostasis in Ca^{2+}-deficient medium with expression of pCD/pYV-encoded virulence effectors (Yops and LcrV) or vegetative growth and repression of Yops and LcrV with ≥2.5 mM Ca^{2+} (Lcr^+). Regulation of Yops and LcrV is well defined but little is known about bacteriostasis other than that Na^+ plus L-glutamate promotes prompt restriction of *Y. pestis*. As shown here, L-aspartate substituted for L-glutamate in this context but only Na^+ exacerbated the nutritional requirement for Ca^{2+}. Bacteriostasis of *Y. pestis* (but not enteropathogenic yersiniae) was abrupt in Ca^{2+}-deficient medium at neutral to slightly alkaline pH (7.0 to 8.0), although increasing the pH to 8.5 or 9.0, especially with added Na^+ (but not L-glutamate), facilitated full-scale growth. Added L-glutamate (but not Na^+) favored Ca^{2+}-independent growth at acidic pH (5.0 to 6.5). Yops and LcrV were produced in Ca^{2+}-deficient media at pH 6.5 to 9.0 regardless of the presence of added Na^+ or L-glutamate, although their expression at alkaline pH was minimal. Resting Ca^{2+}-starved Lcr^+ cells of *Y. pestis* supplied with L-glutamate first excreted and then destroyed L-aspartate. These findings indicate that expression of Yops and LcrV is necessary but not sufficient for bacteriostasis of Ca^{2+}-starved yersiniae and suggest that abrupt restriction of *Y. pestis* requires Na^+ and the known absence of aspartate ammonia-lyase in this species.

The ≈70-kb plasmid termed pCD in *Yersinia pestis*, the causative agent of bubonic plague, and pYV in enteropathogenic *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* encodes a type III secretion system (TTSS) capable of injecting cytotoxic yersinial outer proteins (Yops) directly into the host cell cytoplasm and secreting the immunomodulator LcrV directly into supernatant fluids (5, 17). Expression of the TTSS is mediated at 37°C by melting (54) a structural gene present on pCD/pYV encoding a transcriptional activator designated VirF in *Y. enterocolitica* (40) and LcrF in *Y. pseudotuberculosis* (58) and *Y. pestis* (34). Carriage of pCD/pYV confers a low-calcium response (LCR) in vitro at 37°C, characterized as the ability to undergo either vegetative growth without expression of the TTSS upon addition of Ca^{2+} or bacteriostasis in its absence coupled with synthesis of VirF/LcrF-activated functions (Lcr^+) (10, 29, 53, 61). However, Ca^{2+} does not downregulate Yops in tissue culture, where their expression occurs following attachment of yersiniae to both professional and nonprofessional phagocytes (24, 27, 52). The mechanism accounting for the ability of host cells to upregulate the TTSS of docked yersiniae is not fully defined but may involve competition between Ca^{2+} and a host cell receptor for a bacterial outer membrane sensor (17).

Although *Y. pestis* and the enteropathogenic yersiniae share the pCD/pYV-encoded TTSS, the typically chronic diseases caused by *Y. pseudotuberculosis* and *Y. enterocolitica* are radically different from plague. This distinction is due in part to carriage by *Y. pestis* of a unique ≈10-kb plasmid termed pPCP (22) encoding a plasminogen activator (Pla) that facilitates bacterial metastasis (39), thereby accounting for prompt colonization of visceral organs following subcutaneous infection (8, 60). A second ≈100-kb plasmid termed pMT, encoding capsular antigen fraction 1 and murine toxin (38), is also specific to *Y. pestis* (22). However, these activities are not essential for virulence (12, 33, 64) and thus the primary role of pMT is to facilitate colonization of the flea (32). Transformation of pPCP into *Y. pseudotuberculosis* did not significantly enhance its dissemination in mice (37), suggesting that this enteropathogen possesses factors that curtail acute disease.

Despite the very recent evolution of *Y. pestis* from *Y. pseudotuberculosis* (1), comparison of the two species by DNA microarray analysis revealed the existence of 11 loci containing numerous *Y. pestis*-specific chromosomal genes (31). However, subsequent direct annotation of the two genomes using additional isolates of *Y. pseudotuberculosis* decreased the number of such plague-specific genes to 25, the bulk of which are unidentified open reading frames (16). Analysis of more strains of *Y. pseudotuberculosis* (especially the immediate progenitor of the plague bacillus) could further reduce this number, perhaps to zero. In this case, the remarkable lethality of *Y. pestis* would obviously entail an explanation other than the action of unique chromosomal gene products.

Since its emergence from *Y. pseudotuberculosis*, the chromosome of *Y. pestis* has undergone physical rearrangements, numerous small additions and deletions, and amplification of insertion sequence elements that eliminate about 10% of total genome function (16, 20, 50). This observation is consistent with the hypothesis that enhanced virulence of *Y. pestis* is a function of chromosomal gene loss in addition to lateral transfer of pPCP. Mutations that prevent utilization of nutrients

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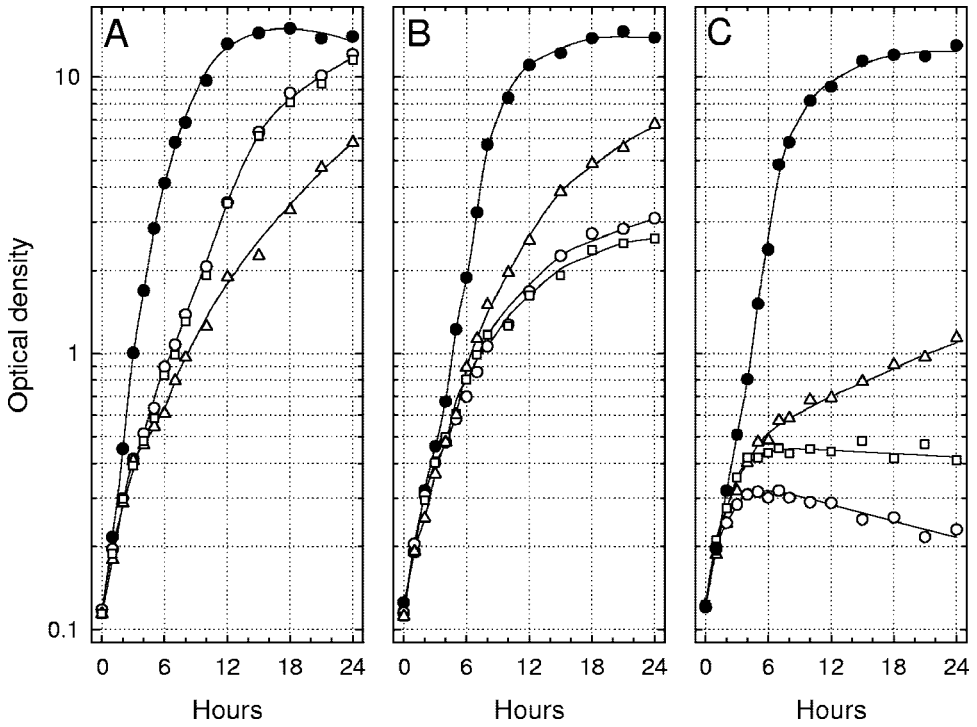

FIG. 1. Growth of *Yersinia enterocolitica* strain WA (A), *Yersinia pseudotuberculosis* strain PB1/+ (B), and *Yersinia pestis* strain KIM (C) at 37°C in chemically defined medium containing added L-glutamate (25 mM) either with (●) or without (○) added 4.0 mM Ca^{2+} after two prior subcultures at 26°C in the same medium lacking added Ca^{2+}. Parallel growth at 37°C of a third culture either lacking (△) or containing (□) L-glutamate (25 mM) is shown after prior subculture at 26°C for two transfers without added L-glutamate or Ca^{2+}. All cultures contained 100 mM Na^+ and possessed an initial pH of 7.0.

found only in natural environments or those accounting for specific biochemical requirements would not be expected to favor acute disease. However, plague bacilli lack the cell invasins and adhesins required by the enteropathogenic yersiniae to initiate chronic gastrointestinal disease (16, 20, 50), as would be expected if these structures hinder dissemination in tissue (5, 7). The LCR of *Y. pestis* is also unique in that the organisms undergo especially abrupt restriction of growth (15) and Pla is utilized to degrade undelivered Yops (37, 55, 59).

At 37°C, Ca^{2+}-starved yersiniae cease vegetative growth but remain viable and exhibit reduced adenylate energy charge, suggesting a lesion in energy metabolism (66). Bacteriostasis is enhanced by Mg^{2+} (10, 30, 65), Na^+ (4, 26), and L-glutamate (26) and relieved by exogenous nucleotides (65). Furthermore, upregulation of VirF/LcrF-activated functions is increased by Mg^{2+} (10, 41), L-glutamate (42), and fermentable carbohydrates (10). Plague bacilli, as opposed to the enteropathogenic yersiniae, lack detectable aspartate ammonia-lyase (aspartase) (21) and glucose 6-phosphate dehydrogenase (45, 46) activities and therefore possess potentially serious lesions in bioenergetics and efficient flow of metabolic carbon.

The purpose of this report is to integrate these variables by demonstrating that both of the naturally occurring dicarboxylic amino acids (L-glutamate and L-aspartate) as well as their porter (Na^+) favor bacteriostasis but not expression of VirF/LcrF-activated functions, the LCR is significantly modified by initial culture pH, and the abrupt onset of bacteriostasis in *Y. pestis* involves uptake, conversion, and excretion of L-glutamate in the form of L-aspartate. These observations provide important insights into the LCR and may relate directly to expression of acute disease.

MATERIALS AND METHODS

Bacteria. The *Y. pestis* isolate used throughout this study was an Lcr^+, nonpigmented mutant (35) of strain KIM-10 (25) known to have undergone high-frequency ≈100-kb chromosomal deletion (6, 23, 43) resulting in loss of the Hms locus and high-pathogenicity island encoding the ability to synthesize the siderophore yersiniabactin (11, 51). An isogenic Lcr^- mutant lacking pCD was isolated by selection on magnesium oxalate agar (30). Enteropathogenic *Y. pseudotuberculosis* strain PB1/+ (13), *Y. enterocolitica* WA (14), and their similarly selected Lcr^- mutants were used in comparative experiments. *Y. pseudotuberculosis* PB1/+ is of serotype O:1b and thus on the direct evolutionary line leading to *Y. pestis* (57).

Chemically defined medium. The basic chemically defined medium was modified from that reported previously (26) by inclusion of 40 mM potassium D-gluconate and omission, unless stated otherwise, of L-glutamate, L-aspartate, and Na^+. First and second transfers prepared to inoculate third cultures for use in experiments always contained *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) at a final concentration of 25 mM and pH 7.0. HEPES was replaced in the third culture by an equimolar concentration of another Good buffer depending upon the desired initial pH. The buffers used were morpholinethanesulfonic acid (MES) for a final pH of 5.0, 5.5, and 6.0, morpholinopropanesulfonic acid (MOPS) for a final pH of 6.5, 7.0, and 7.5, Tris for a final pH of 8.0 and 8.5, and 2-(cyclohexylamino)ethane-2-sulfonic acid (CHES) for a final pH of 9.0. Solid NaCl (100 mM) and L-glutamic acid (25 mM) were added when required, and when dissolved, the pH was adjusted precisely to the desired initial value by dropwise addition of 5 M HCl or 5 M KOH. After addition of all stock solutions (26) the fully constituted medium was sterilized by filtration and dispensed aseptically.

Cultivation. Storage of bacteria at −20°C in buffered glycerol and cultivation on solid medium for use in experiments were previously described (26). Rates of growth in chemically defined medium were determined in stoppered Erlenmeyer flasks (25 ml of medium per 250-ml flask) aerated at 200 rpm in a temperature-

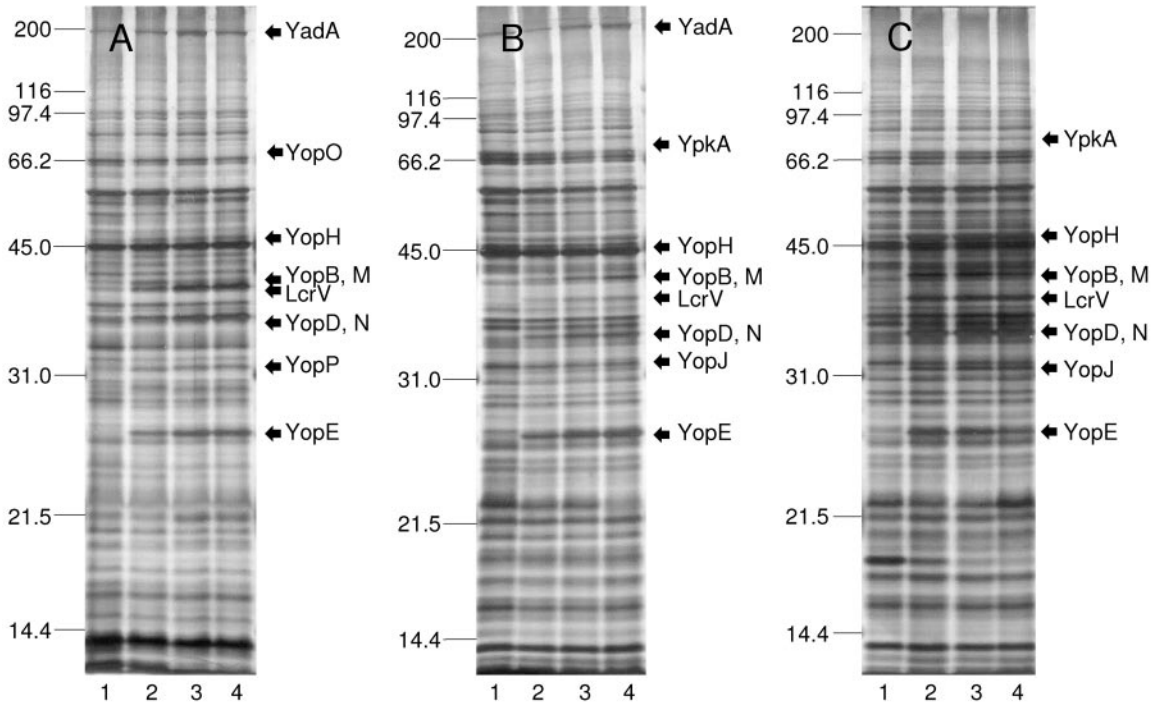

FIG. 2. Silver-stained preparations following SDS-PAGE of whole cultures of *Yersinia enterocolitica* strain WA (A), *Yersinia pseudotuberculosis* strain PB1/+ (B), and *Yersinia pestis* strain KIM (C) after subculture at 26°C in chemically defined medium containing added 25 mM L-glutamate without Ca^{2+} followed by cultivation at 37°C in the same medium either without (lane 4) or with (lane 1) 4.0 mM Ca^{2+}; comparable samples of cells previously subcultured twice at 26°C without Ca^{2+} or L-glutamate and then transferred to third Ca^{2+}-deficient cultures at 37°C either lacking (lane 2) or containing (lane 3) L-glutamate are shown. All cultures contained 100 mM Na^+ and exhibited an initial pH of 7.0. Expression of YopO/YpkA was too faint for reproduction.

controlled model G76 water bath gyrotory shaker (New Brunswick Scientific Co., Inc., New Brunswick, N.J.). Multiplication was compared in cultures containing Na^+ plus L-glutamate, Na^+ alone, L-glutamate alone, or neither nutrient. In these determinations, the organisms underwent two subcultures at 26°C in medium identical to that used for the third culture (with respect to Na^+ and L-glutamate) except, as noted above, a buffered-salt stock solution containing HEPES was used and the initial pH was always adjusted to 7.0.

The first and second cultures were inoculated at an optical density (OD) of about 0.1 and transferred during late phase at an OD of between 5 and 6. Third cultures composed of the Good buffers noted above (initial pH of 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, or 9.0) were then inoculated at an OD of about 0.125 and incubated at 37°C. Bacterial growth was determined at 620 nm with a Beckman DU spectrophotometer using 1-ml cuvettes and cultures suitably diluted in 0.033 M potassium phosphate buffer (pH 7.0) to yield an OD of between 0.05 and 0.7. The results of separate control experiments indicated that the chosen Good buffers controlled pH without significantly interacting with other ingredients of the media. The pH of individual cultures remained remarkably stable for at least 9 h of incubation.

Excretion of L-aspartate. Lcr^+ or Lcr^- cells of *Y. pestis* were grown for two transfers at 26°C in chemically defined medium (initial pH of 7.0 using HEPES with 100 mM Na^+ plus 25 mM L-glutamate) and then aerated as described above in the same medium (initial pH of 7.5 using MOPS) with or without added 4.0 mM Ca^{2+} (200 ml of medium per stoppered 2-liter Erlenmeyer flask). After 7 h of cultivation, the organisms were harvested by centrifugation (10,000 × *g* for 30 min), washed twice in excess cold potassium phosphate buffer (pH 7.0), and suspended to a concentration equivalent to 20 mg of protein per ml of distilled water. A portion (1.0 ml) of this suspension was immediately added to a 50-ml Erlenmeyer flask containing 2.0 μmol of $MgCl_2$ and 20 μmol of sodium L-glutamate in 1.0 ml of 0.066 M potassium buffer (pH 7.0). The flask was aerated at 37°C as described above, and samples of 0.1 ml were removed at intervals, sedimented for 1 min in a model 11 Microfuge (Beckman Coulter, Inc., Fullerton, Calif.), passed through a 4-mm Acrodisc HT Tuffryn low-protein-binding filter disk (Pall Life Sciences, Ann Arbor, Mich.), and frozen until analysis. After thawing, the samples were appropriately diluted in distilled water, applied (10 μl) to Whatman no. 1 filter paper, and chromatographed with phenol-water (4:1, wt/vol, in an HCN atmosphere). The sheets were then dried, sprayed with 0.25% (wt/vol) ninhydrin in water-saturated *n*-butanol and, after full development of color in a water-saturated environment at 26°C, scanned and evaluated against a standard curve using the SigmaGel software program (Systat Software, Inc., Point Richmond, Calif.).

Miscellaneous. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), silver staining, and immunoblotting were performed as previously described (9, 48). Positions on stained SDS-PAGE gels corresponding to individual Yops and LcrV were characterized previously (37).

RESULTS

Comparison of the LCR. Lcr^+ cells of *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis* were cultured twice at 26°C in chemically defined medium containing 100 mM Na^+ but lacking both added Ca^{2+} and L-glutamate, transferred to third cultures of the same medium either with or without Ca^{2+} (4.0 mM) and L-glutamate (25 mM), and aerated at 37°C. Increases in optical density were compared to those obtained in parallel third transfers of yersiniae previously grown with both Na^+ and L-glutamate (Fig. 1). L-Glutamate did not influence doubling times at 37°C in the presence of Ca^{2+} although the terminal population of *Y. pestis* was slightly reduced in its absence (data not shown). The nutritional requirement of Lcr^+ *Y. enterocolitica* for Ca^{2+} was minimal, and L-glutamate stimulated growth whether or not the organisms were previously adapted to this amino acid. In contrast, L-glutamate enhanced bacteriostasis of Ca^{2+}-starved *Y. pseudotuberculosis*; this effect

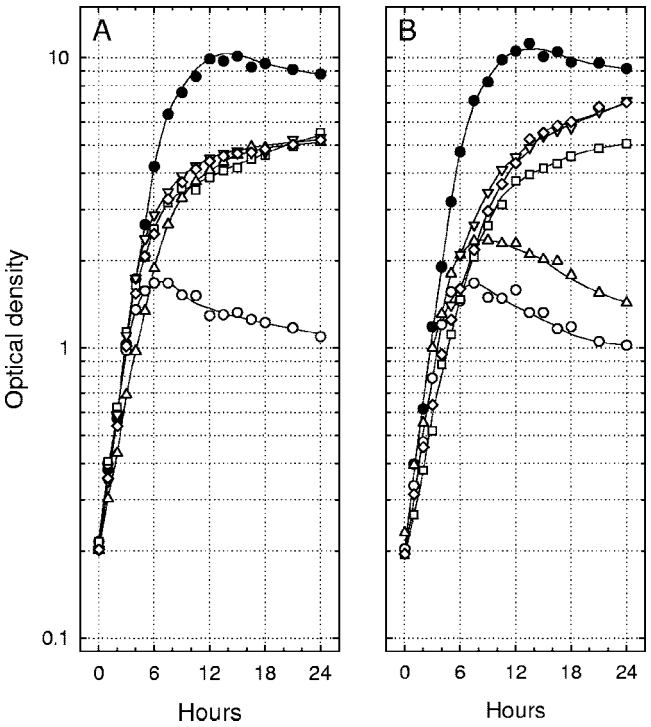

FIG. 3. Growth of *Yersinia pestis* strain KIM at 37°C in Ca^{2+}-deficient chemically defined medium containing (A) constant L-glutamate (25 mM) and 100 mM Na^+ (○), Li^+ (△), K^+ (□), or $Tris^+$ (▽); a parallel control culture containing Na^+ and Ca^{2+} (4.0 mM) is included (●). Also shown (B) is growth of *Y pestis* strain KIM at 37°C in Ca^{2+}-deficient chemically defined medium containing constant Na^+ (100 mM) and 25 mM L-glutamate (○), L-aspartate (△), L-glutamine (□), or L-asparagine (▽) or no added amino acid (◇); a parallel control culture containing Na^+ and Ca^{2+} (4.0 mM) is included (●). The initial pH of all cultures was 6.0.

was not dependent on prior adaptation. Cells of *Y. pestis* exhibited an even more abrupt pattern of restriction that was also favored by L-glutamate, especially if the latter was present during prior growth at 26°C.

Growth with Ca^{2+} always prevented expression of VirF/LcrF-activated functions except for YadA regardless of the presence or absence of Na^+ or L-glutamate (Fig. 1 and data not shown). Samples of the cultures illustrated in Fig. 1 were subjected to SDS-PAGE and silver staining after 7 h of incubation; full complements of Yops and LcrV were expressed during Ca^{2+} privation regardless of the presence or absence of added L-glutamate (Fig. 2). This finding indicates that L-glutamate enhances the nutritional requirement for Ca^{2+} as expressed by *Y. pseudotuberculosis* and *Y. pestis* but is not necessary for expression of LcrV and Yops, as reported for *Y. enterocolitica* (42).

Contribution of Na^+ and L-glutamate to the LCR. The results of previous studies showed that Na^+ is essential for abrupt bacteriostasis of *Y. pestis* (26). To determine the specificity of this process, the ability of 100 mM Na^+, K^+, Li^+, and $Tris^+$ to inhibit growth of Lcr^+ cells of *Y. pestis* in Ca^{2+}-deficient media was compared in the constant presence of 25 mM L-glutamate. As shown in Fig. 3A, only Na^+ promoted prompt bacteriostasis. These monovalent cations were not toxic to Lcr^+ cells cultivated with Ca^{2+} nor were they inhibitory to Lcr^- mutants grown with or without Ca^{2+} (data not shown). A similar experiment was undertaken to determine if L-amino acids metabolically related to L-glutamate could also promote abrupt bacteriostasis in Ca^{2+}-deficient medium containing constant 100 mM Na^+. Both L-glutamate and L-aspartate caused abrupt restriction, whereas the bacteriostasis observed with L-glutamine or L-asparagine was less pronounced (Fig. 3B). These amino acids were not inhibitory to either Lcr^+ cells grown with Ca^{2+} or Lcr^- mutants grown with or without this cation; a full component of VirF/LcrF-activated functions was observed in all media lacking added Ca^{2+} (data not shown).

Effect of pH on the LCR. The initial culture pH dramatically influenced the ability of Na^+ and L-glutamate to affect bacteriostasis after a shift of Lcr^+ cells of *Y. pestis* from 26°C to 37°C in Ca^{2+}-deficient medium. The significance of acidic pH was defined by measuring the growth of Lcr^+ yersiniae at 37°C with 100 mM Na^+ plus 25 mM L-glutamate, Na^+ alone, L-glutamate alone, or neither nutrient at initial pHs ranging from 5.0 to 7.0. Mild to moderate acidity (pH 5.5 to 6.5) favored multiplication in all four Ca^{2+}-deficient environments, although the extreme of pH 5.0 was often initially toxic (Fig. 4A). The favorable effect of moderate acidity was especially evident in the medium containing L-glutamate alone, where, at pH 6.0, the doubling time (≈70 min) approached that observed at pH 7.0 with added 4.0 mM Ca^{2+} (Fig. 4C). Addition of Na^+, either alone (Fig. 4B) or with L-glutamate (Fig. 4D), depressed growth except at the lowest pHs tested. The toxic effect of Na^+ was pronounced at pH 7.0 in the presence of L-glutamate (Fig. 4D), where the onset of bacteriostasis was especially abrupt.

Mild alkalinity (pH 7.5 and 8.0) inhibited multiplication in all four environments, although a higher initial pH of 8.5 and especially 9.0 promoted excellent growth provided Na^+ was present (Fig. 5). Indeed, the multiplication observed at pH 9.0 in Ca^{2+}-deficient medium containing Na^+ alone (Fig. 5B) was indistinguishable from that detected in a parallel control culture containing added Ca^{2+} (doubling time, ≈70 min). Addition of L-glutamate to alkaline media containing Na^+ significantly inhibited growth (Fig. 5D), as was observed in Fig. 4 for mildly acidic and neutral media. These results demonstrate that the temperature-dependent nutritional requirement of Lcr^+ cells of *Y. pestis* for physiological levels of Ca^{2+} is essentially eliminated in the chemically defined medium by incubation at pH 6.0 with added L-glutamate alone or at pH 9.0 with the sole addition of Na^+. The determinations further emphasize that the combination of Na^+ plus L-glutamate at neutral to mildly alkaline pH in Ca^{2+}-deficient medium promotes especially abrupt bacteriostasis of Lcr^+ *Y. pestis*.

Samples of the cultures illustrated in Fig. 4 and 5 were subjected to immunoblotting against mouse monoclonal anti-LcrV (Fig. 6) and SDS-PAGE and silver staining (Fig. 7) after 7 h of incubation. Full components of Yops and LcrV were expressed regardless of the presence or absence of added Na^+ or L-glutamate provided that the initial pH was 6.5 or above (Fig. 6); significant expression occurred even at pH 6.0 if only L-glutamate was added. This finding effectively uncouples activation of pCD/pYV-encoded genes by VirF/LcrF from the phenomenon of bacteriostasis as considered in the definition of the LCR provided above.

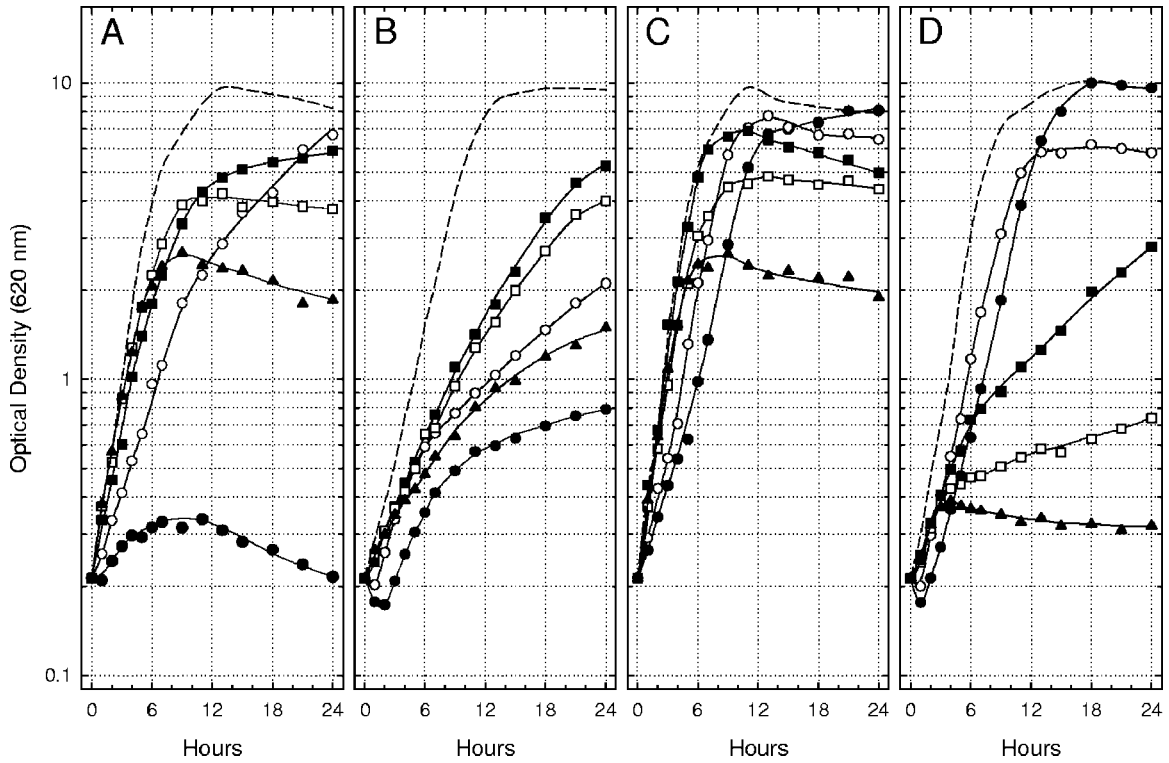

FIG. 4. Growth of Lcr^+ cells of *Yersinia pestis* strain KIM at 37°C in Ca^{2+}-deficient chemically defined medium lacking added Na^+ and L-glutamate (A), containing only 100 mM Na^+ (B), containing only 25 mM L-glutamate (C), and containing both Na^+ and L-glutamate (D) at an initial pH of 5.0 (●), 5.5 (○), 6.0 (■), 6.5 (□), and 7.0 (▲). Dashed lines indicate the extent of growth in control cultures containing 4.0 mM Ca^{2+} at an initial pH of 7.0.

LCR-mediated excretion of L-aspartate. Lcr^+ cells of *Y. pestis* were cultivated under conditions that optimized abrupt bacteriostasis (no added Ca^{2+}, 100 mM Na^+, and 25 mM L-glutamate at pH 7.0 and 37°C) and prepared for incubation in buffer containing exogenous L-glutamate. Growth in this environment permitted catabolism of L-glutamate with net accumulation of L-aspartate followed by destruction of the latter after L-glutamate had disappeared (Fig. 8B). Lcr^+ cells previously grown in the same medium with 4.0 mM Ca^{2+} (Fig. 8A) or Lcr^- cells grown either with (Fig. 8C) or without (Fig. 8D) Ca^{2+} did not release detectable L-aspartate. Similar export of L-aspartate at 26°C or by Ca^{2+}-starved cells of *Y. enterocolitica* or *Y. pseudotuberculosis* was not detected (data not shown).

DISCUSSION

Y. pestis KIM, *Y. pseudotuberculosis* PB1, and *Y. enterocolitica* WA are well-defined type species of highly pathogenic yersiniae known to exhibit distinct patterns of growth at 37°C in Ca^{2+}-deficient media (15). Fowler and Brubaker (26) used a modification of this environment to show that Na^+ and L-glutamate were necessary for abrupt bacteriostasis of Lcr^+ cells of *Y. pestis* and that, for full effectiveness, both metabolites had to be present in prior subcultures incubated at 26°C. In the present study, growth of Lcr^+ *Y. enterocolitica* in Ca^{+}-deficient medium containing Na^+ was enhanced by L-glutamate whether or not the cells were adapted to this amino acid. In contrast, L-glutamate was inhibitory in parallel cultures of Lcr^+ *Y. pseudotuberculosis* regardless of prior adaptation at 26°C and toxic to Lcr^+ *Y. pestis*, especially after previous adaptation. This difference suggests that *Y. pseudotuberculosis* and *Y. pestis* possess a common sensitivity to L-glutamate not shared by the distantly related *Y. enterocolitica* and that an additional metabolic lesion involving Na^+ that accounts for abrupt restriction of growth is superimposed on *Y. pestis*.

Lee et al. (42) reported that L-glutamate or metabolically related amino acids were necessary for expression of the TTSS by *Y. enterocolitica* in Ca^{2+}-deficient tissue culture medium. L-Glutamate was unnecessary in the Ca^{2+}-deficient chemically defined medium used here for production of LcrV and Yops by all three species of yersiniae. A likely explanation for this discrepancy is that the level of Mg^{2+} in the present study was increased to 20 mM. As noted previously, this concentration favors bacteriostasis in the absence of Ca^{2+} (30, 64) and ensures expression of LcrV (41). It is also equivalent to that present in mammalian cytoplasm (36) known to be released by invading yersiniae during rapid terminal growth within necrotic lesions of visceral organs (49, 63).

The observation that both Na^+ and L-glutamate are necessary for abrupt bacteriostasis (26) was refined in this report by demonstrating that the contribution made by Na^+ was specific (not due to increase of ionic strength or some allied effect), whereas the requirement for L-glutamate could also be met by L-aspartate (but not L-glutamine or L-asparagine). The literature regarding the role of Na^+ in prokaryote physiology and the metabolism of dicarboxylic amino acids is voluminous al-

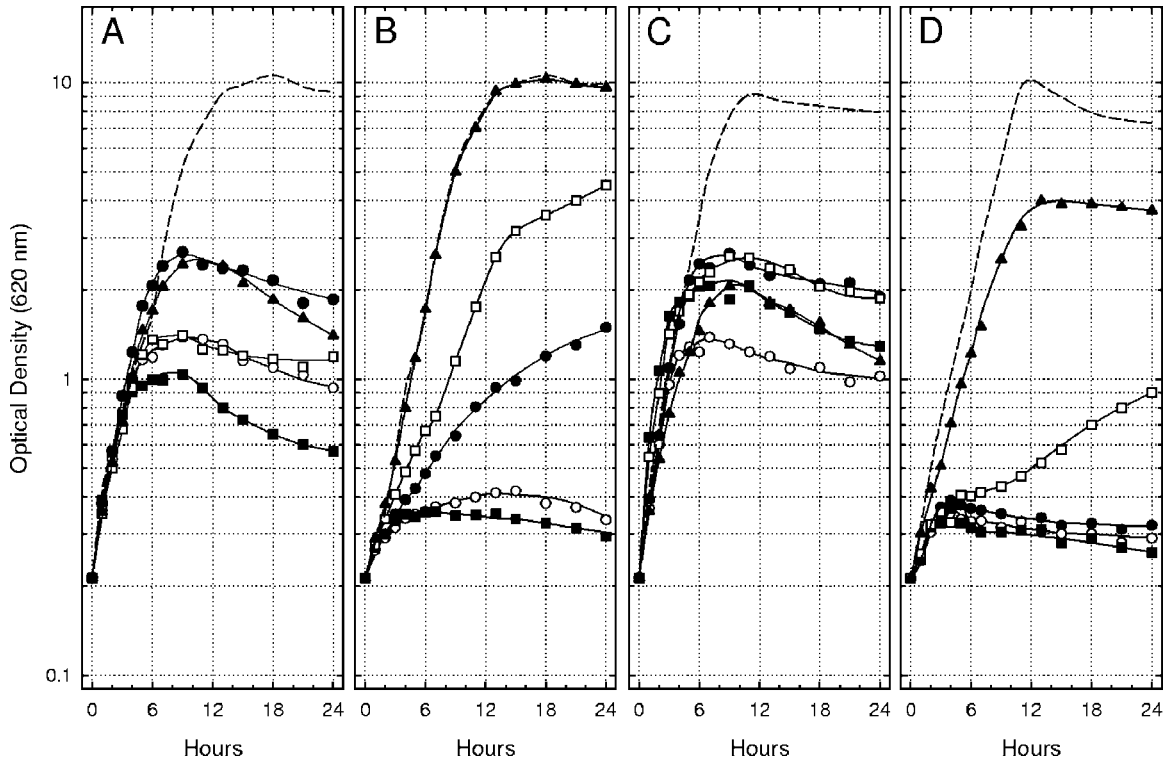

FIG. 5. Same as Fig. 4 except that the cultures possessed an initial pH of 7.0 (●), 7.5 (○), 8.0 (■), 8.5 (□), and 9.0 (▲).

though the overlapping set is limited to the observation that Na^+ serves as a porter for the high-energy transport of both L-glutamic acid and L-aspartic acid via the action of GltS (18, 44, 56). This translocase exists in yersiniae (16, 20, 50) as do alternative Na^+-independent L-glutamate transport systems mediated by GltJKL and GltP (2, 19, 62). As noted below, the possibility exists that GltS may modulate the LCR of *Y. pestis*, as judged by the release of L-aspartate by Ca^{2+}-starved organisms.

Duplication of the modest acidic pH of mammalian cytoplasm (pH 6.0) permitted essentially full-scale growth of Lcr^+ cells at 37°C in chemically defined medium lacking both added Ca^{2+} and Na^+, provided that L-glutamate was present. This environment also facilitated full synthesis of LcrV and Yops,

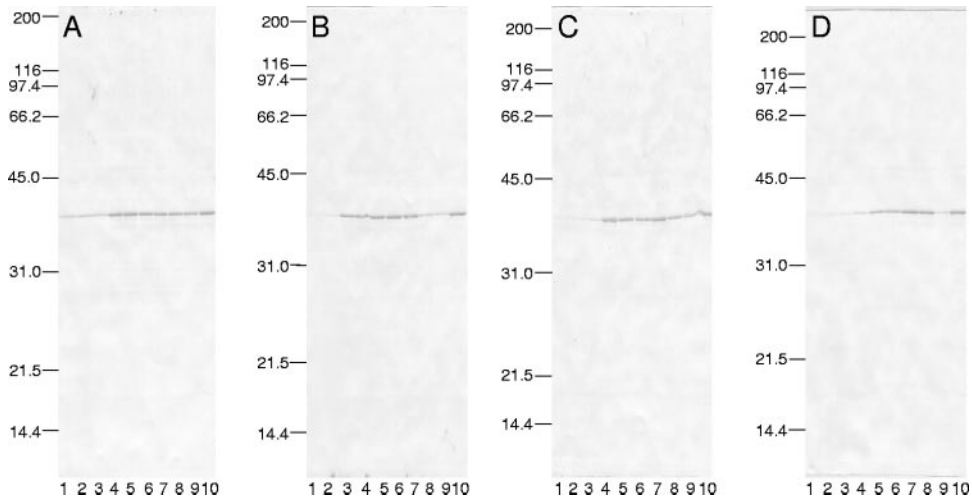

FIG. 6. Immunoblot using mouse monoclonal anti-LcrV following SDS-PAGE of whole cultures of *Yersinia pestis* strain KIM cultured in Ca^{2+}-deficient chemically defined medium lacking both Na^+ and L-glutamate (A), containing only 100 mM Na^+ (B), containing only 25 mM L-glutamate (C), and containing both Na^+ and L-glutamate (D) at an initial pH of 5.0 (lane 1), 5.5 (lane 2), 6.0 (lane 3), 6.5 (lane 4), 7.0 (lane 5), 7.5 (lane 6), 8.0 (lane 7), 8.5 (lane 8), and 9.0 (lane 9); a comparable positive control of a Ca^{2+}-deficient culture containing both Na^+ and L-glutamate is shown in lane 10.

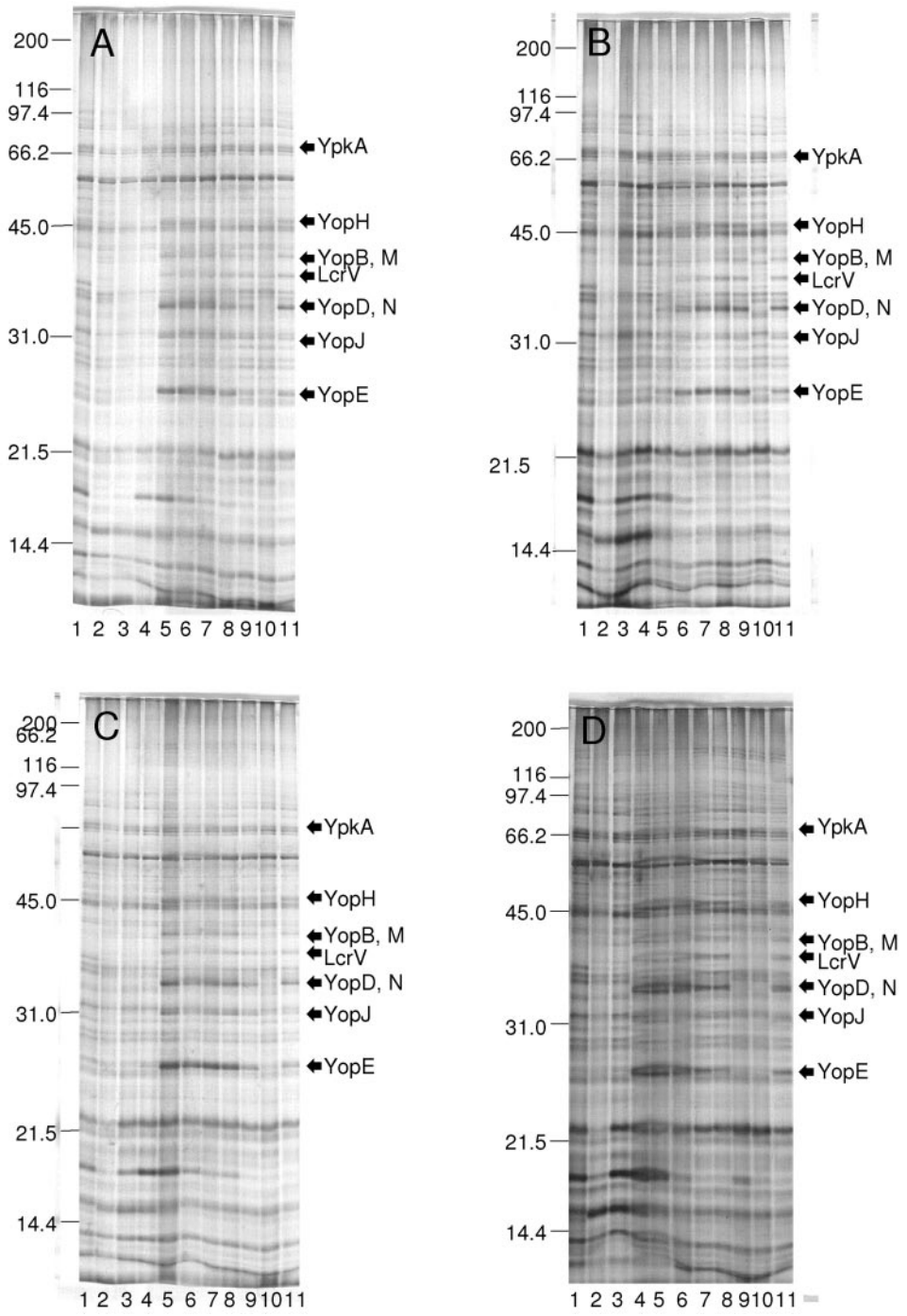

FIG. 7. Silver-stained preparations following SDS-PAGE of whole cultures of *Yersinia pestis* strain KIM cultured in Ca^{2+}-deficient chemically defined medium lacking both Na^+ and L-glutamate (A), containing only 100 mM Na^+ (B), containing only 25 mM L-glutamate (C), and containing both Na^+ and L-glutamate (D) at an initial pH of 5.0 (lane 2), 5.5 (lane 3), 6.0 (lane 4), 6.5 (lane 5), 7.0 (lane 6), 7.5 (lane 7), 8.0 (lane 8), 8.5 (lane 9), and 9.0 (lane 10); comparable negative (Lcr^- cells) and positive (Lcr^+ cells) controls of Ca^{2+}-deficient cultures containing both Na^+ and L-glutamate are shown in lanes 1 and 11, respectively.

demonstrating that the correlation in vitro between bacteriostasis and expression of VirF/LcrF-activated genes is not obligatory (as implied in the present definition of the LCR). Medium adjusted to pH 9.0 and containing added Na^+ but not L-glutamate also facilitated full-scale multiplication in the absence of added Ca^{2+}. In this case, synthesis of LcrV and Yops was less pronounced than at neutral to mildly acidic pH but still detectable. One possible explanation for sustained Na^+-dependent growth in this alkaline environment is the presence in *Y. pestis* of NADH:ubiquinone oxidoreductase (20, 50), an

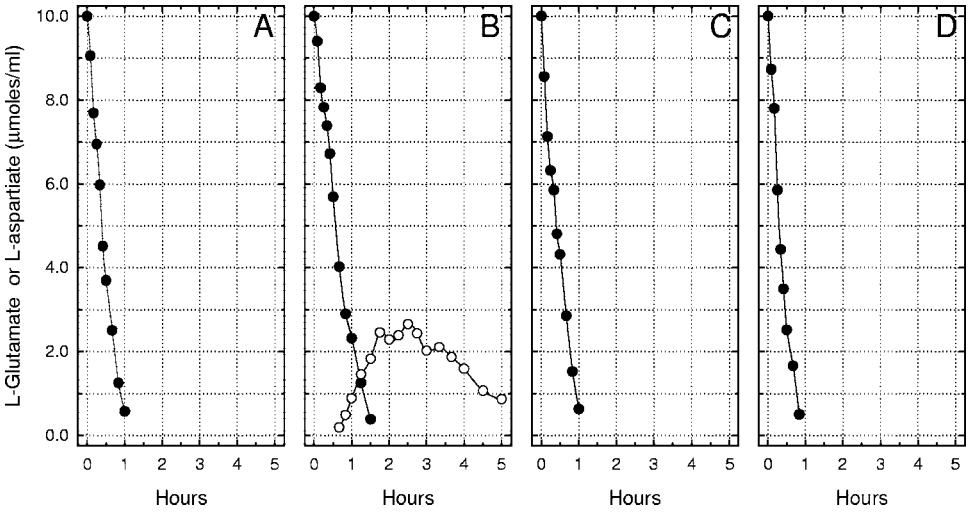

FIG. 8. Catabolic destruction of L-glutamate (●) with the appearance of metabolic L-aspartate (○) by resting cells of *Yersinia pestis* strain KIM previously grown at 37°C in synthetic medium containing 25 mM L-glutamate and 100 mM Na^+. (A) Lcr^+ cells grown with 4.0 mM Ca^{2+}, (B) Lcr^+ cells grown without added Ca^{2+}, (C) Lcr^- cells grown with 4.0 mM Ca^{2+}, and (D) Lcr^- cells grown without added Ca^{2+}.

enzyme capable of serving as a primary electrogenic sodium pump at alkaline pH (68). Further study will be necessary to define the mechanism that excludes Na^+ from the cytoplasm of yersiniae cultivated at neutral and acidic pHs and to determine if this process is inactivated during expression of LcrV and Yops. If so, then one or more VirF/LcrF-activated gene products may be involved in modulating bacteriostasis of Ca^{2+}-starved yersiniae by enabling Na^+ to access the cytoplasm.

Cells of *Escherichia coli* grown in enriched medium rapidly transaminate internalized L-glutamate (via aspartate aminotransferase) with a catalytic level of oxalacetate to yield 2-oxoglutarate and L-aspartate. 2-Oxoglutarate is then catabolized via enzymes of the tricarboxylic acid cycle to regenerate oxalacetate and accumulated L-aspartate is converted to fumarate via aspartase, thereby reentering the tricarboxylic acid cycle (28). Although the same process can function in the enteropathogenic yersiniae, the absence of aspartase activity in *Y. pestis* precludes use of this mechanism, limiting catabolism of L-aspartate to the formation of oxalacetate by transamination with some convenient amino group acceptor, especially 2-oxoglutarate (thereby generating L-glutamate). As a consequence, L-glutamate undergoes stoichiometric conversion to L-aspartate in resting plague bacilli (21). The observation provided here showing that secretion of L-aspartate occurs only in previously restricted Lcr^+ cells is new information and was unsuspected. Since both bacteriostasis and secretion are dependent upon prior cultivation with Na^+, the latter probably directly or indirectly promotes the export of L-aspartate. A likely candidate for this exit reaction would be GltS, where the normal flow of L-aspartate would become reversed by internalized Na^+.

Considered together, these findings suggest that upregulation of VirF/LcrF-activated functions by cultivation at 37°C in Ca^{2+}-deficient medium results in loss of the normal ability to exclude Na^+ from the cytoplasm. One generally recognized consequence of this lesion would be interference with normal bioenergetic processes involving the proton motive force at acidic pH. A second effect unique to *Y. pestis* would involve Na^+-facilitated export of L-aspartate, resulting in loss of metabolic carbon and consequent abrupt bacteriostasis; neither of these events could occur at alkaline pH due to the activity of NADH:ubiquinone oxidoreductase. This hypothesis can readily be subjected to experimental verification. Further study will also be necessary to determine what effect, if any, the ability to convert L-glutamate to L-aspartate has on expression of acute disease.

The results obtained by microarray analysis using essentially the same chemically defined medium described here showed that a shift of *Y. pestis* from 26°C to 37°C causes marked upregulation of oxidative metabolism with attendant profligate catabolism of nutrients readily converted to tricarboxylic cycle intermediates (47). This extravagant process, coupled with the potential of radically altering host L-glutamate and L-aspartate pools, may directly contribute to the acute symptoms of plague. An additional observation favoring this notion is the observation that the aspartase sequenced in attenuated *Y. pestis* strain 91001 (biovar *microtus*) (67) is similar to the active form found in *Y. pseudotuberculosis* (16). It may also be significant that L-glutamate undergoes stoichiometric conversion to L-aspartate in typhus rickettsiae (3) in a manner similar to that found for *Y. pestis*.

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