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Physiological Basis of the Low Calcium Response in *Yersinia pestis*

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It is established that duplication in vitro of that amount of Ca^{2+} (2.5 mM) and Mg^{2+} (1.5 mM) present in blood permits vegetative growth of *Yersinia pestis* with repression of virulence factors encoded by the Lcr plasmid (Lcr^+); similar simulation of intracellular fluid (no Ca^{2+} and 20 mM Mg^{2+}) promotes bacteriostasis with induction of these virulence determinants. However, proliferation of yersiniae in mice occurs primarily within necrotic focal lesions (supplied by Ca^{2+}-deficient host cell cytoplasm) within visceral organs rather than in Ca^{2+}-sufficient blood. The present study addressed this enigma by defining conditions necessary for achieving vegetative growth of Lcr^+ yersiniae at 37°C in simulated intracellular fluid. Maximum optical densities were increased by substitution of K^+ for Na^+ and elimination of Cl^-; the combination of Na^+ plus L-glutamate was selectively toxic to Lcr^+ cells. This phenomenon was attributed in part to the absence of aspartase in *Y. pestis* (a lesion known to facilitate massive accumulation of L-aspartate via transamination of the oxalacetate pool by L-glutamate). Replacement of L-glutamate by exogenous L-aspartate or α-ketoglutarate reversed this toxicity by favoring retention of oxalacetate. Proliferation of Lcr^+ cells in a medium containing K^+ and L-aspartate but lacking added Ca^{2+} and Na^+ was markedly enhanced by increasing the concentration of fermentable carbohydrate. Accordingly, in the worst-case scenario (i.e., added Na^+, Cl^-, and L-glutamate), Lcr^+ yersiniae underwent restriction of growth after one doubling, and in the best-case scenario (i.e., added K^+ and L-aspartate), the organisms completed more than five doublings, thereby achieving full-scale growth. Both of these Ca^{2+}-deficient media promoted maximum induction of Mg^{2+}-induced V antigen, a virulence factor encoded by the Lcr plasmid.

Early workers concerned with the nutrition of wild-type *Yersinia pestis* (the causative agent of bubonic plague) described the occurrence of a population shift resulting in avirulence during continuous cultivation at 37°C in undefined media prepared from natural sources (19, 23). The mutants selected by this process typically failed to produce the plague V antigen (LcrV) (10, 11) and were thus unable to express a unique "low calcium response" (25) now known to be mediated by a ca. 70-kb Lcr plasmid shared by enteropathogenic *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* (11, 16, 49). This shift in population could be postponed or prevented by the addition to culture media of Ca^{2+} (32), CO_2 (18), and products of CO_2 fixation in equilibrium with oxalacetate (10) or by replacement of D-glucose with D-xylose (54) or of Na^+ with K^+ (8). Addition of 20 to 40 mM Mg^{2+} to Ca^{2+}-deficient medium at 37°C favored bacteriostasis of wild-type cells (8, 13, 28, 29, 55, 56) and promoted maximum induction of V antigen (13, 33, 56). The latter was repressed by 2.5 mM Ca^{2+} (13), a concentration required for full-scale vegetative growth at 37°C regardless of the amount of added Mg^{2+} (9, 11, 28, 55, 56).

The Lcr plasmid encodes structural genes for major virulence factors, including excreted V antigen (11, 49) and certain released proteins termed Yops (16, 50) (Lcr^+). Mutational loss of these major determinants results in outright avirulence in the mouse model (increase in 50% lethal dose to $>10^7$ bacteria) (7, 24, 40, 42). Regulatory genes that mediate the low calcium response also reside on the Lcr plasmid because either its cure (Lcr^-) or introduction of certain point mutations causes loss of the temperature-dependent nutritional requirement for Ca^{2+} (16, 49). Studies of the physiological basis of the Ca^{2+} requirement showed that deprived yersiniae undergo a normal metabolic step-down characterized by reduction of adenylate energy charge (57), shutoff of stable RNA transcription (15), inhibition of vegetative protein synthesis (37), and evident σ-factor conversion of RNA polymerase (36). V antigen was implicated as a regulator of the low calcium response primarily because vegetative growth of a nonpolar *lcrV* mutant at 37°C occurred without addition of Ca^{2+} (40).

It is probably significant that addition to culture media of that amount of Ca^{2+} (2.5 mM) and Mg^{2+} (ca. 1.5 mM) reported (31) to exist in blood promotes vegetative growth of *Y. pestis* with repression of V antigen and Yops, whereas simulation of leukocyte cytoplasm with respect to Ca^{2+} (not added) and Mg^{2+} (20 mM) (31) causes bacteriostasis with induction of these virulence factors. The initial interpretation that this relationship indicated that V antigen is expressed only in vivo during residence within circulating phagocytes (13) was fallacious because later findings amply demonstrated that yersiniae proliferate primarily within isolated necrotic lesions of visceral organs and do not significantly spill into the vascular system until late in the course of disease (1, 38, 48, 51–53). Examination by electron microscopy of lesions of this type caused by Lcr^+ cells of *Y. pseudotuberculosis* revealed that the resident bacteria were extracellular (44). Results of recent work clearly indicate that bacterial adhesion is a requisite for polarized transfer of YopE into host cells (43). However, these studies were performed in tissue culture medium containing 20 mM Mg^{2+} plus only 1 mM Ca^{2+} and thereby provided no new information regarding regulation of Lcr plasmid-mediated virulence factors.

It thus remains a conundrum that Lcr^+ yersiniae undergo

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bacteriostasis in vitro in Ca^{2+}-deficient environments that favor synthesis of V antigen but fail to produce this virulence factor in media that promote cell division. Upon discovery that 2.5 mM Ca^{2+} ensured maximum yields of Lcr^+ cells at 37°C in vitro, further attempts to define alternative processes of avoiding temperature-dependent population shifts to avirulence were largely abandoned. The purpose of the present investigation was to reevaluate the variables of oxalacetate, Na^+, and carbohydrate. We demonstrate here that Lcr^+ cells of *Y. pestis* are capable of full-scale growth with maximum synthesis of V antigen at 37°C in Ca^{2+}-deficient medium containing 20 mM Mg^{2+} provided that toxic extracellular concentrations of Na^+, Cl^-, and L-glutamic acid are avoided and that an adequate adenylate energy charge is assured by addition of extra fermentable carbohydrate.

MATERIALS AND METHODS

Bacteria. An Lcr^+ but nonpigmented (22) isolate of *Y. pestis* KIM (9) and its isogenic Lcr^- mutant, known to be cured of the Lcr plasmid (47), were used in all experiments. Cells of this strain lack the ca. 100-kb chromosomal segment mediating absorption of hemin and high-affinity uptake of iron (22, 35) and are thus virulent only via intravenous injection (51). They lack detectable aspartase (20) but possess the species-specific (5, 21) ca. 10-kb pesticin (47) and ca. 100-kb capsule or exotoxin plasmids (47).

Media. The chemically defined medium of Higuchi et al. (28) as subsequently modified (56) was used in initial experiments to identify Na^+ and L-glutamic acid as significant variables. This medium was then modified to permit independent analysis of these and other nutrients by maintaining neutral pH by addition of the artificial buffers piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) and Tris base. To prepare 1 liter of the basic medium, 500 ml of amino acid stock solution (9.0 mM DL-alanine, 7.6 mM L-isoleucine, 4.0 mM L-leucine, 3.2 mM L-methionine, 4.8 mM L-phenylalanine, 2.8 mM L-threonine, 13.6 mM L-valine, 2.0 mM L-arginine–HCl, 14.0 mM L-proline, 2.2 mM L-lysine–HCl, 5.2 mM glycine, 2.2 mM L-tyrosine), 10 ml of 0.25 M K_2HPO_4, and 260 ml of distilled water were added to a 2-liter Erlenmeyer flask fitted with a cotton stopper. After sterilization by autoclaving, the flask received by aseptic transfer 100 ml of filter-sterilized stock salt solution (100 mM citric acid, 200 mM MgO, 250 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], 1.0 mM $FeSO_4$, 0.1 mM $MnSO_4$), 100 ml of filter-sterilized vitamin stock solution (0.03 mM thiamine, 0.04 mM pantothenic acid, 0.02 mM biotin), 10 ml of filter-sterilized 1.0 M potassium D-gluconate, 10 ml of filter-sterilized 0.1 M L-cysteine-HCl, and 10 ml of filter-sterilized 10 mM L-tryptophan. Prepared in this manner, the medium exhibited a pH of 6.75 and contained per liter a total of 15 meq of added K^+, 40 meq of added Mg^{2+}, about 3 meq of added Cl^-, 30 meq of citric acid, 25 meq of HEPES, and no added Na^+ or Ca^{2+}. The values obtained for both of these cations by flame absorption spectroscopy were <0.1 mM. Ca^{2+} was added at a concentration of 4.0 mM to ensure full repression of V antigen.

The basic medium defined above provided full-scale growth of Lcr^- cells at 37°C with and without 150 mM Tris-PIPES, thus ensuring that both buffers were biologically inert at concentrations used to maintain neutrality of pH after addition of Na^+, Cl^-, and L-glutamic acid. Information obtained from these determinations enabled construction of a variation of the new Ca^{2+}-deficient medium that provided a worst-case scenario for vegetative growth of Lcr^+ cells at 37°C (WCS). To prepare 1 liter of WCS, the basic medium received 125 mmol of NaCl, 5 mmol of KCl, 30 mmol K-PIPES, and 25 mmol of sodium L-glutamate, thus resulting in a total of 150 mM Na^+, 50 mM K^+, ca. 133 mM Cl^-, 20 mM Mg^{2+}, and <0.1 mM Ca^{2+}. A parallel Ca^{2+}-deficient medium that provided the best-case scenario for vegetative growth of Lcr^+ cells at 37°C (BCS) was also devised. To prepare 1 liter of BCS, the basic medium received 130 mmol of K-PIPES and 10 mmol of Tris–L-aspartate, thus resulting in a total of <0.1 mM Na^+, 145 mM K^+, ca. 3 mM Cl^-, 20 mM Mg^{2+}, and <0.1 mM Ca^{2+}. The ability of increasing concentrations of potassium D-gluconate to influence growth in WCS and BCS was evaluated by adding this carbohydrate at the expense of an equimolar amount of K-PIPES, thereby keeping the concentration of K^+ constant.

Cultivation. Bacteria, stored as described previously in buffered glycerol at −20°C (4), were inoculated directly onto the surface of slopes of tryptose blood agar base (Difco Laboratories, Detroit, Mich.) and incubated for 2 days at 26°C. The organisms were then removed in sterile 0.033 M potassium phosphate buffer (pH 7.0) and used to inoculate cultures contained in stoppered Erlenmeyer flasks at an optical density of 0.1 to 0.2 (620 nm). After sufficient aeration at 200 rpm at 26°C on a model G76 water bath shaker (New Brunswick Scientific Co., Inc., New Brunswick, N.J.) to achieve late-logarithmic-phase growth, yersiniae were diluted into a fresh culture of the same medium at an optical density of 0.1 to 0.2. This second transfer was brought to the late logarithmic phase of growth at 26°C and then used to inoculate, at an optical density of 0.1, a third culture of sufficient volume so that it could later be divided into subcultures for use in experiments. These subcultures were prepared after the third transfer had achieved an optical density of 0.2 to 0.25, at which time they were shifted to 37°C. In most cases, third transfers consisted of 10 ml of medium per 125-ml Erlenmeyer flask, although cultures of 25 ml of medium per 250-ml flask were used for estimation of antigen production.

Miscellaneous. Monospecific rabbit polyclonal anti-V antigen and anti-antigen 5 (17) have been described elsewhere (12, 36–38) and were used for semiquantitative assays of cell extracts by diffusion in agar (12, 13, 33). Yersiniae were harvested by centrifugation at 10,000 × *g* for 30 min, suspended in 0.05 M Tris-HCl buffer (pH 8.0), and then disrupted by treatment with a sonic probe (MSE Instruments, Ltd., London, England); extracts were clarified by centrifugation as described above prior to use in assays. Units of antigen, defined as the reciprocal of the highest dilution that yielded a detectable band of precipitate, are expressed in terms of specific activity (units per milligram of protein) where protein was determined by the method of Lowry et al. (34). Accumulation and destruction of amino acids in cultures were determined by paper chromatography as described previously (20).

RESULTS

The intent of the study described below was to develop a Ca^{2+}-deficient medium capable of supporting full-scale vegetative growth with maximum production of V antigen. This end was accomplished by adding sufficient Mg^{2+} (20 mM) to ensure full induction of this virulence factor while providing nutrients capable of ensuring an adenylate energy charge consistent with cell division.

Nutritional modulators of the low calcium response. Typical plots of optical density versus time obtained for Lcr^+ and Lcr^- yersiniae cultivated at 37°C in the chemically defined medium of Zahorchak et al. (56) are shown in Fig. 1A and B, respectively. Lcr^+ cells achieved a maximum optical density of

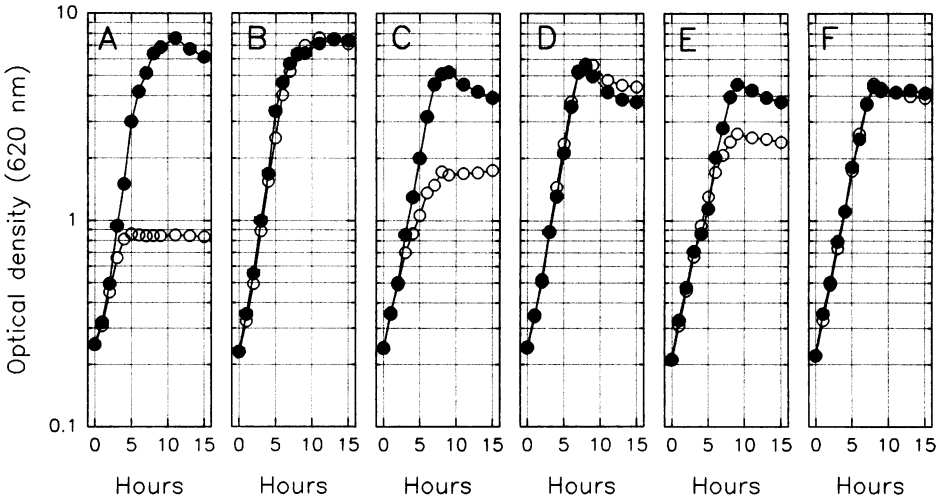

FIG. 1. Growth of Lcr^+ (A, C, and E) and Lcr^- (B, D, and F) cells of *Y. pestis* KIM at 37°C in the chemically defined medium of Higuchi et al. (29), as modified by Zahorchak (56), containing 20 mM Mg^{2+} and 81.1 mM L-glutamic acid with cationic equivalents supplied as Na^+ (A and B), K^+ (C and D), or K^+ (without L-glutamic acid) (E and F) either with (●) or without (○) added 4.0 mM Ca^{2+}. Bacteria were previously subcultured twice at 26°C in the same media.

about 8, hereafter termed full-scale growth, with added Ca^{2+} but could not complete two doublings in its absence. Replacement of all Na^+ added to this medium with K^+ enabled Lcr^+ cells grown without added Ca^{2+} to undergo an extra generation (Fig. 1C); the response of Lcr^- organisms was not influenced by Ca^{2+} (Fig. 1D). Further elimination of added L-glutamic acid as well as Na^+ and Ca^{2+} enabled Lcr^+ cells to attain an additional half generation of growth (Fig. 1E); omission of this amino acid slightly decreased the maximum optical densities achieved by Lcr^+ cells augmented with Ca^{2+} (Fig. 1E) and those observed for both Ca^{2+}-supplemented and Ca^{2+}-deficient cultures of Lcr^- mutants (Fig. 1F).

Refined medium for independent analysis of nutritional modifiers. The basic medium described in Materials and Methods was developed to permit independent analysis of the influence of Na^+, K^+, and L-glutamic acid on expression of the low calcium response. Maximum optical densities of Lcr^- cells grown with increasing concentrations of NaCl and KCl were first determined in this medium at 26°C. No nutritional requirement for NaCl was detected, although addition of 10 to 50 nmol/ml slightly enhanced growth; maximum optical density occurred at the physiological level (150 mM), and a concentration of 300 mM was toxic (Fig. 2A). The organisms exhibited a typical obligatory nutritional requirement for about 0.5 mM KCl, and as with NaCl, addition of 150 mM was optimal and addition of 300 mM was toxic (Fig. 2B). Identical results were obtained when these determinations were repeated by replacing the anionic equivalents supplied by Cl^- with PIPES (data not shown). This information provided appropriate physiological ranges for evaluating the ability of Na^+, K^+, and Cl^- to modulate the low calcium response.

Dose dependence of Na^+ toxicity. Lcr^+ cells grew at 37°C in Na^+- and Ca^{2+}-deficient medium to maximum optical densities of about 2 and 3 with and without added L-glutamate, respectively (Fig. 3A). Addition of 50 mM NaCl resulted in corresponding decreases to about 0.8 and 2 (Fig. 3B). Continued increase of NaCl to concentrations of 100 (Fig. 3C), 150 (Fig. 3D), 200 (Fig. 3E), and 250 (Fig. 3F) mM did not cause significant additional reduction in maximum optical densities during growth with L-glutamate, although a final increase to 300 mM was inhibitory (Fig. 3G). A corresponding NaCl-dependent inhibition was observed in Ca^{2+}-deficient cultures lacking L-glutamate. Extreme concentrations of NaCl (200 to 300 mM) were also toxic to Lcr^- cells as judged by observation of patterns identical to those shown in Fig. 3 for Lcr^+ organisms grown with Ca^{2+} (data not shown).

Specificity of Na^+ toxicity. An experiment was performed to determine if K^+ and Cl^- shared the capability of Na^+ to selectively inhibit growth of Lcr^+ cells at 37°C in Ca^{2+}-deficient medium containing L-glutamic acid. The basic medium, prepared with 50 mM Tris–L-glutamate, was either left unsupplemented or brought to 150 mM with respect to total Na^+, K^+, or Tris by using Cl^- to maintain neutrality of pH. As shown in Fig. 4A, both unsupplemented medium and those containing KCl or Tris-Cl permitted maximum optical densities to approach 2 (although the rate of growth with KCl was

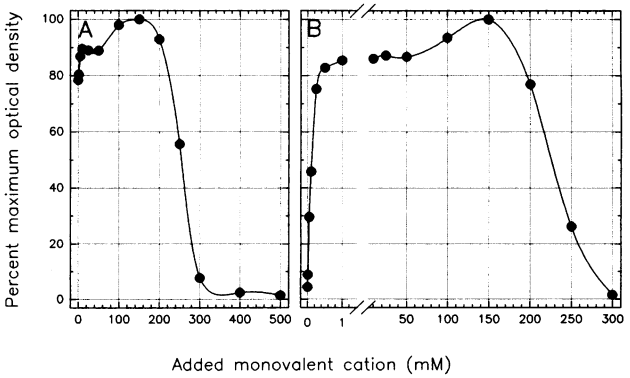

FIG. 2. Growth of Lcr^- cells of *Y. pestis* KIM at 26°C in basic defined medium modified so as to lack added L-glutamic acid while containing 2.5 mM $KHPO_4^-$, 10 mM potassium D-gluconate, and controlled levels of NaCl (A) or 2.5 mM $NaHPO_4^-$, 5 mM magnesium D-gluconate, and controlled levels of KCl (B); maximum optical densities set at 100% were 7.44 for Na^+ (150 mM) and 6.45 for K^+ (150 mM). Bacteria were previously subcultured twice to the mid-logarithmic growth phase in the same media containing no added Na^+ (A) or 0.5 mM K^+ (B).

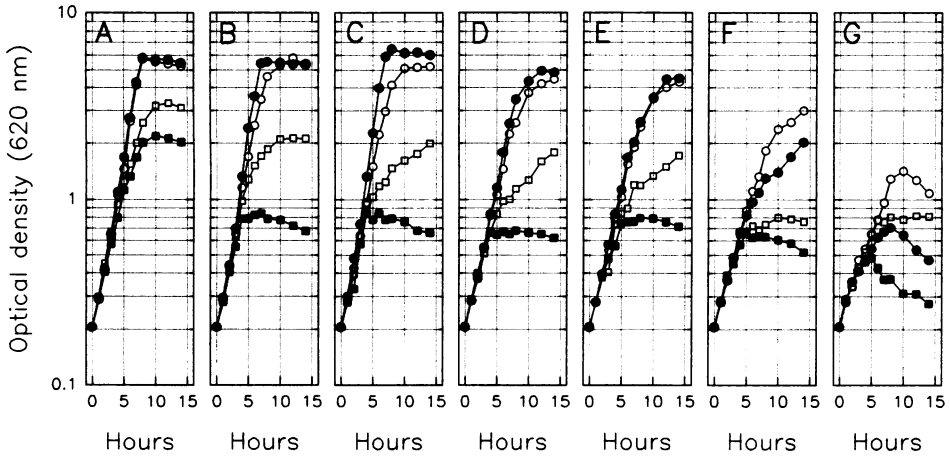

FIG. 3. Growth of Lcr$^+$ cells of *Y. pestis* KIM at 37°C in the basic medium containing Na^+ added at 0 (A), 50 (B), 100 (C), 150 (D), 200 (E), 250 (F), or 300 (G) mM with either 50 mM Tris-Cl and 4.0 mM Ca^{2+} (○), 50 mM Tris–L-glutamate and 4.0 mM Ca^{2+} (●), 50 mM Tris-Cl and no added Ca^{2+} (□), or 50 mM Tris–L-glutamate and no added Ca^{2+} (■). Bacteria were previously subcultured twice at 26°C in the same media (containing either added Tris-Cl or Tris–L-glutamate) without added Na^+ or Ca^{2+}.

reduced), whereas organisms cultivated with NaCl failed to complete two doublings. NaCl was not inhibitory after addition of Ca^{2+} (Fig. 4B). In contrast, addition of anionic equivalents in the form of PIPES rather than Cl^- both increased the growth rate of cells in cultures supplemented with K^+ and enabled those in the culture containing Na^+ to achieve an extra doubling (Fig. 4C). Full-scale growth was obtained after addition of Ca^{2+} to cultures neutralized with PIPES (Fig. 4D). Yields of Lcr$^-$ cells in these media resembled those shown in Fig. 4 for cultures of Lcr$^+$ cells containing added Ca^{2+} (data not shown). These findings verify that the combination of Na^+ and L-glutamic acid is toxic to Lcr$^+$ cells in the absence of Ca^{2+} and further demonstrate that Cl^- contributes to this inhibition.

Dose dependence of L-glutamate toxicity. Lcr$^+$ cells were grown at 26°C with 0.05 M NaCl and either 10 mM Tris–L-glutamate or Tris-PIPES. Upon entering the late log phase (when L-glutamate in the supplemented culture became exhausted), the organisms were appropriately diluted into flasks with fresh medium containing increasing concentrations of L-glutamate (0 to 80 mM), which were then incubated at 37°C. The maximum optical density of these noninduced bacteria typically exceeded 2 (regardless of the concentration of L-glutamate added to the final transfer), and those of the cultures receiving low levels of this amino acid (0 and 1 mM) approached 4 (Fig. 5A). Full-scale growth of noninduced yersiniae always occurred with added Ca^{2+}, and generation

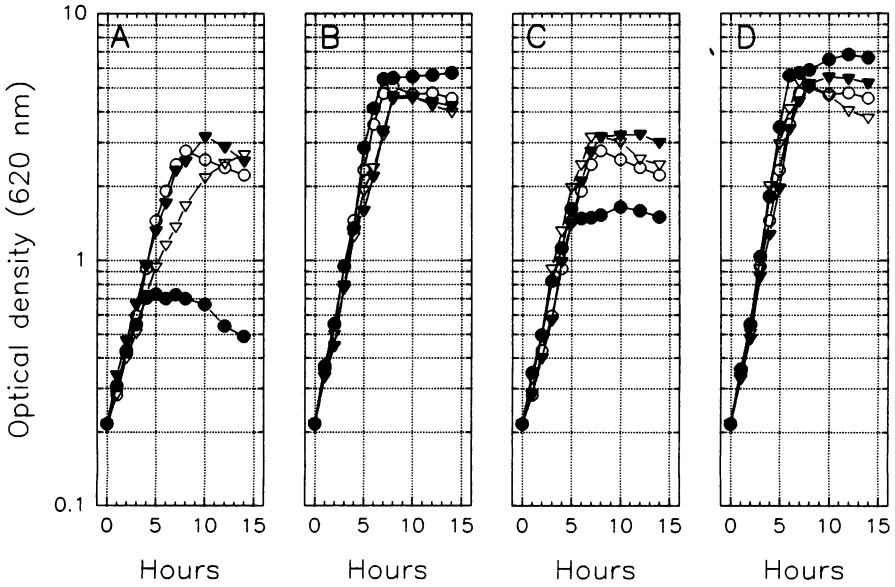

FIG. 4. Growth of Lcr$^+$ cells of *Y. pestis* KIM at 37°C in the basic medium plus 50 mM Tris–L-glutamate either without (A) or with (B) added 4.0 mM Ca^{2+} containing either no additional monovalent cation (○), 150 mM NaCl (●), 150 mM KCl (▽), or 150 mM Tris-Cl (▼) and in the basic medium plus 50 mM Tris–L-glutamate either without (C) or with (D) added 4.0 mM Ca^{2+} containing either no monovalent cation (○), 150 mM Na-PIPES (●), 150 mM K-PIPES (▽), or 150 mM Tris-PIPES (▼). Bacteria were previously subcultured twice at 26°C in the same medium (lacking added Ca^{2+}, Na^+, Cl^-, or PIPES).

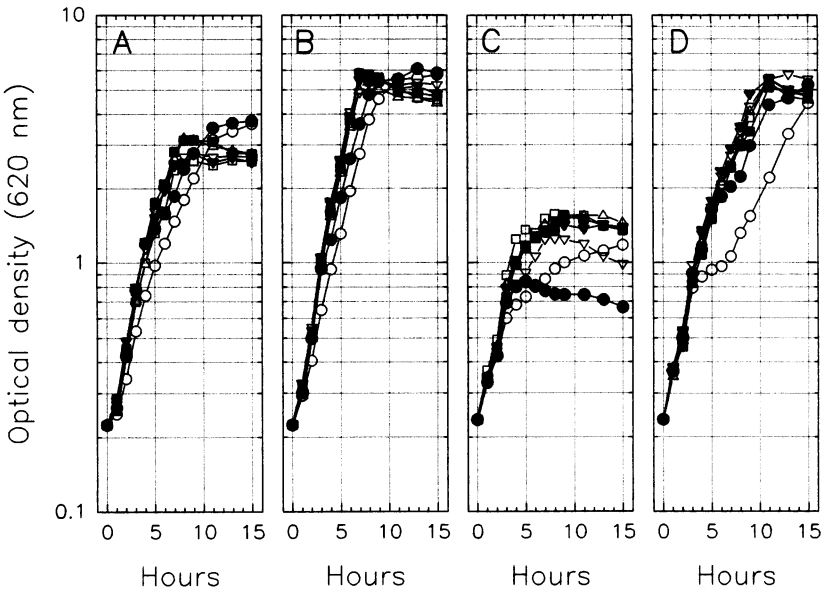

FIG. 5. Growth of Lcr$^+$ cells of *Y. pestis* KIM at 37°C in the basic medium plus 50 mM NaCl and 100 mM K-PIPES either without (A) or with (B) added 4.0 mM Ca^{2+} after two prior transfers without added L-glutamic acid and in the same medium either without (C) or with (D) added 4.0 mM Ca^{2+} after two prior transfers with 10 mM Tris–L-glutamate; subcultures contained either 0 (○), 1 (●), 5 (▽), 10 (▼), 20 (□), 50 (■), or 80 (△) mM Tris–L-glutamate.

times were significantly decreased in the presence of 5 to 80 mM L-glutamate (Fig. 5B). In contrast, induced organisms failed to achieve maximum optical densities of 2 regardless of the concentration of added L-glutamate, and the onset of restriction occurred more rapidly in the presence of low concentrations of the amino acid (Fig. 5C). Full-scale growth of induced organisms was observed with Ca^{2+}, although a pause was noted after about two and three doublings in the cultures initially containing 0 and 1 mM L-glutamate, respectively (Fig. 5D). Results obtained with Lcr$^-$ mutants were similar to those shown in Fig. 5 for Lcr$^+$ cells grown with Ca^{2+} (data not shown). These results indicate that bacteriostasis is contingent upon the continued presence of a minimal level of L-glutamate and that about two generations are required for conversion to the induced physiological state.

Specificity of L-glutamate toxicity. An experiment was undertaken to determine if some selected analogs could substitute for L-glutamate in promoting bacteriostasis. Lcr$^+$ cells were cultivated at 26°C in Ca^{2+}-deficient medium with 10 mM α-ketoglutarate or equimolar amounts of physiologically related L-amino acids. After appropriate dilution into the same medium, the bacteria were incubated at 37°C in the presence and absence of added Ca^{2+}. The onset of restriction in the control culture containing L-glutamate (Fig. 6B) was abrupt,

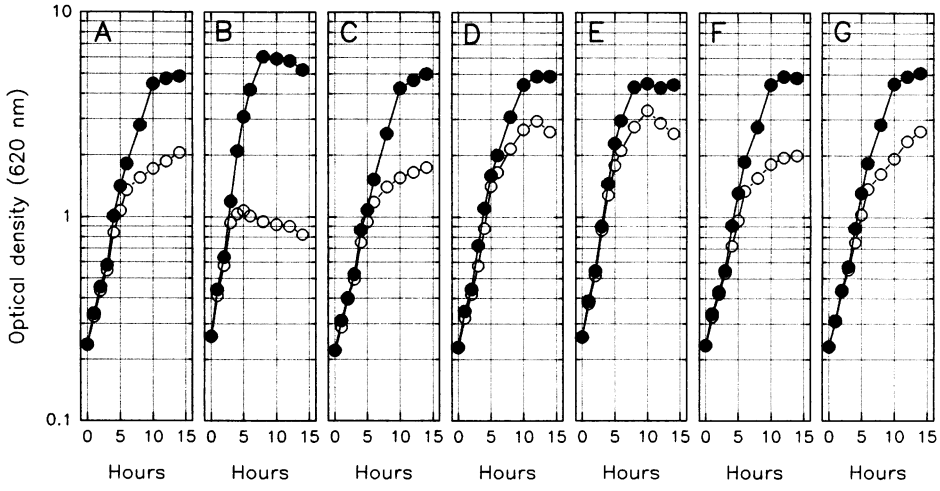

FIG. 6. Growth of Lcr$^+$ cells of *Y. pestis* KIM at 37°C in the basic medium plus 50 mM NaCl and 100 mM K-PIPES either without (○) or with (●) added 4.0 mM Ca^{2+}; subcultures were not supplemented (A) or received 10 mM additions of Tris–L-glutamate (B), L-glutamine (C), Tris–α-ketoglutarate (D), Tris–L-aspartate (E), L-asparagine (F), or L-alanine (G). Bacteria were previously subcultured twice at 26°C in the same medium (lacking added Ca^{2+} or supplement).

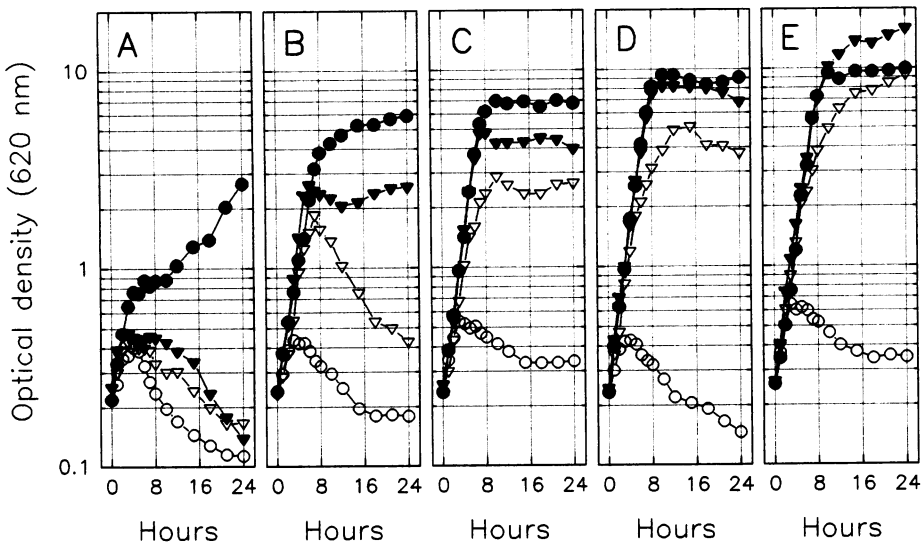

FIG. 7. Growth of Lcr$^+$ cells of *Y. pestis* KIM at 37°C in the WCS variation of the basic medium without (○) or with (●) 4.0 mM added Ca^{2+} and in the BCS variation of the basic medium without (▽) or with (▼) 4.0 mM added Ca^{2+}; subcultures received 1 (A), 5 (B), 10 (C), 20 (D), or 40 (E) mM potassium D-gluconate. Bacteria were previously transferred twice at 26°C in the same media (defined in Materials and Methods) containing 10 mM potassium D-gluconate before dilution to inoculate subcultures.

whereas vegetative growth continued at a reduced but constant rate in the absence of L-glutamate (Fig. 6A) or with L-glutamine (Fig. 6C) or L-asparagine (Fig. 6F). Significantly increased maximum optical densities were achieved with α-ketoglutarate (Fig. 6D) or L-aspartic acid (Fig. 6E); L-alanine also stimulated growth to a lesser extent (Fig. 6G). These substitutions were not toxic, as shown by the occurrence of maximum optical densities exceeding 4 upon addition of Ca^{2+}. In contrast, 10 mM D-glutamic acid exhibited marked toxicity in the presence or absence of added Ca^{2+} (data not shown). These results indicate that the ability to promote Na^+-dependent bacteriostasis at 37°C in Ca^{2+}-deficient medium is specific to L-glutamic acid.

Role of fermentable carbohydrate. The level of fermentable carbohydrate added to Ca^{2+}-deficient media, designed to either inhibit (i.e., WCS) or favor (i.e., BCS) growth of Lcr$^+$ cells at 37°C, was increased in an attempt to prolong an adenylate energy charge consistent with growth. Elimination of D-gluconate altogether from these media resulted in prompt and extensive lysis of both Lcr$^+$ and Lcr$^-$ organisms immediately after shift from 26 to 37°C (data not shown). Lcr$^+$ cells always exhibited abrupt shutoff of vegetative growth with attendant lysis in Ca^{2+}-deficient WCS regardless of the concentration of added D-gluconate. However, the molarity of added D-gluconate prompted dramatic differences in maximum optical densities obtained in Ca^{2+}-deficient BCS. For example, the immediate lysis that occurred in this medium containing 1 mM D-gluconate (Fig. 7A) was postponed upon increase to 5 mM until the organisms had accomplished two doublings (Fig. 7B). A further increase to 10 (Fig. 7C) or 20 (Fig. 7D) mM resulted in proportional increases in cell yield without subsequent lysis, and an increase to 40 mM D-gluconate enabled the organisms to achieve full-scale growth, as evidenced by a maximum optical density of about 10 (Fig. 7E). Results obtained for Lcr$^-$ mutants in WCS and BCS supplemented with increased levels of D-gluconate always resembled those shown for Lcr$^+$ cells incubated with Ca^{2+} (data not shown).

Expression of V antigen. Comparable titers of V antigen and chromosomally encoded antigen 5 were determined after cultivation at 37°C for 6 h (mid-logarithmic growth phase for all permissive environments) and 18 h (stationary phase) in WCS and BCS. V antigen was not detected in disrupted Lcr$^-$ cells or in extracts of Lcr$^+$ organisms grown with added Ca^{2+}. Typical specific activities of 2 and 1.5 were obtained for V antigen after 6 h of cultivation in Ca^{2+}-deficient WCS and BCS, respectively. After 18 h, the corresponding value for WCS fell to 1 (evidently due to the occurrence of partial bacterial lysis), whereas that determined for BCS was 6, a specific activity typical of full induction in unfractionated extracts of *Y. pestis* (12). In all cases, specific activities obtained for antigen 5, included as an internal control, ranged between 1 and 1.5. The results of this determination demonstrate that V antigen was amply induced during the course of full-scale growth in Ca^{2+}-deficient BCS at 37°C.

DISCUSSION

As reflected by its smaller genome (35), cells of *Y. pestis* lack many regulatory functions that permit *Escherichia coli* to rapidly adjust to environmental changes or extremes. For example, the latter can grow in about 0.7 M NaCl (26), whereas, as shown in this study, yersiniae were unable to tolerate 0.3 M NaCl even at 26°C, where the low calcium response is not expressed. Furthermore, the genome of *Y. pestis*, as opposed to those of the enteropathogenic yersiniae, also harbors multiple copies of insertion sequences (22, 39). Perhaps as a consequence of this invasion, plague bacilli lack certain enzymes of intermediary metabolism that are necessary for efficient catabolic flow of carbon (11), including aspartase (20), and constitutively express enzymes of the glyoxylate bypass (30), suggesting lack of a complete tricarboxylic acid cycle. These lesions are obviously not deleterious in vivo but may account for the marked shutoff of vegetative growth known to occur at 37°C in Ca^{2+}-deficient medium; this is in contrast to the enteropathogenic yersiniae, where additional doublings occur (*Y. pseudotuberculosis*) or growth continues at a reduced rate (*Y. enterocolitica*) (14).

The finding that the population shift resulting in emergence

of Lcr^- mutants of *Y. pestis* could be delayed by cultivation with CO_2 (18) prompted further study showing that yersiniae catalyze fixation of CO_2 into oxalacetate via phosphoenolpyruvate carboxykinase and an irreversible phosphoenolpyruvate carboxylase (3). The importance of these reactions was appreciated upon later discovery that cells of *Y. pestis* lack the almost ubiquitous procaryote enzyme aspartase and are thus unable to convert L-aspartic acid into the tricarboxylic acid cycle intermediate fumaric acid (20). A major consequence of this lesion is that the L-aspartate generated in *Y. pestis* by transamination of other amino acids with oxalacetate is unable to recycle into the tricarboxylic acid cycle, as occurs in the case of the enteropathogenic yersiniae. This block became especially severe in the presence of L-glutamate, which, as a result of such transamination, promoted massive accumulation of extracellular L-aspartate at the expense of the oxalacetate pool (20). The latter must thus be regenerated by alternative mechanisms, especially those requiring fixation of CO_2 into oxalacetate, to ensure formation of citric acid for continued operation of the tricarboxylic acid cycle and attendant generation of ATP. Accordingly, the process of supplying CO_2 to Ca^{2+}-starved yersiniae at 37°C (18) or, as found in the present study, of eliminating L-glutamate from the medium favors maintenance of the oxalacetate pool, thereby ensuring an adenylate energy charge consistent with vegetative growth.

The nature of available sources of energy was shown in early studies to influence the ability of wild-type yersiniae to grow in Ca^{2+}-deficient environments. For example, massive lysis of Lcr^+ but not Lcr^- cells resulted if a sugar capable of being rapidly catabolized (e.g., D-glucose) became exhausted during the logarithmic growth phase (54). Identical results were obtained in this study, where lysis of Lcr^+ yersiniae occurred in Ca^{2+}-deficient BCS containing 5 mM D-gluconate, a rapidly catabolized carbohydrate, whereas medium containing 40 mM D-gluconate yielded full-scale growth. This observation, plus the finding that a combination of Na^+, L-glutamic acid, and Cl^- is selectively toxic to Lcr^+ cells at 37°C during Ca^{2+} privation, is consistent with the prior hypothesis that restriction of growth reflects an inadequate adenylate energy charge (57).

Concentrations of Na^+ determined for mammalian intracellular fluid and blood are 10 and 142 mM, respectively; corresponding values for K^+ are 150 and 4.2 mM (31). Na^+ (but not K^+) is a metabolic inhibitor that is eliminated from bacterial cytoplasm via an energy-dependent exit reaction (46). A full explanation of why the extracellular concentration of Na^+ is inhibitory in vitro to Lcr^+ yersiniae grown at 37°C in the absence of Ca^{2+} is beyond the scope of this paper, although we suspect that results of further study may reveal that the sodium pump is normally dismantled in vivo during residence of Lcr^+ cells in necrotic lesions. It is appropriate, however, to note here that the toxic combination of high Na^+ and no Ca^{2+} is artificial in the sense that it is not known to exist in vivo (although it is typical of the oxalated culture media commonly used to select Lcr^- mutants [29]). In summary, these findings illustrate that media simulating mammalian vascular fluid with respect to Na^+ and Cl^- are toxic to Lcr^+ cells of *Y. pestis* (unless the extracellular concentration of Ca^{2+} is provided) and that Ca^{2+} is unnecessary for growth in media simulating cytoplasm (where K^+ replaces Na^+).

Despite claims that V antigen serves as a monofunctional (6, 27) or bifunctional (40) regulator of the low calcium response, no evidence indicating that this protein binds to DNA or otherwise directly influences transcription or translation of Yops or vegetative protein has yet been reported. The observation reported here that V antigen was fully induced during Ca^{2+}-independent growth in BCS also argues against a role as direct regulator of cell division. Nevertheless, the correlation remains that full induction of V antigen and bacteriostasis occur together as long as the medium contains appreciable levels of Na^+, L-glutamate, and Cl^-. A number of models have been proposed to define the molecular basis of the low calcium response (6, 40, 43, 49). Although many features of these schemes are undoubtedly correct, they fail to predict the phenotypes defined in the present study and will thus require modification to account for the roles of heretofore uncontrolled variables, especially Na^+. If full-scale growth of Lcr^+ yersiniae can now be achieved at 37°C in Ca^{2+}-deficient medium, similar results may occur with Ca^{2+}-blind mutants (2, 41, 45, 55). This prospect as well as the assumption that Yops, like V antigen, undergo full induction at 37°C in BCS lacking added Ca^{2+} is presently under investigation.

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