Transport of Ca$^{2+}$ by *Yersinia pestis*†

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Low-calcium-response, or Lcr, plasmids of *Yersinia* are known to promote an in vitro nutritional requirement for 2.5 mM Ca$^{2+}$ at 37°C which, if not fulfilled, results in cessation of growth with induction of virulence functions (Lcr$^+$). The mechanism whereby Ca$^{2+}$ regulates this metabolic shift is unknown. Radioactive Ca$^{2+}$ was not actively accumulated by *Yersinia* but was excluded by an exit reaction analogous to those described for other bacteria. Nevertheless, cultivation at 37°C with 0.1 mM Ca$^{2+}$, a level insufficient to prevent restriction of cell division, promoted significantly more binding of the cation by Lcr$^+$ organisms than by plasmid-deficient Lcr$^-$ mutants. Accordingly, Lcr$^+$ *Yersinia* may possess unique ligands capable of recognizing Ca$^{2+}$.

Low-calcium-response, or Lcr, plasmids of wild-type *Yersinia pestis*, the causative agent of bubonic plague, and of the closely related enteropathogenic species *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* are known to promote an in vitro nutritional requirement at 37°C for 2.5 mM Ca$^{2+}$ (Lcr$^+$). An insufficiency of the cation results in cessation of cell division accompanied by maximum induction of virulence functions, including V and W antigens, certain outer membrane proteins of *Yersinia*, and a class of small acidic cytoplasmic peptides (4, 10, 16, 19). Restriction of growth in this environment is independent of phosphorylated guanosine regulatory nucleotides but nevertheless reflects ordered decreases in ribonucleoside triphosphate pools, reduction of adenylate energy charge, and shutdown of stable RNA synthesis (9, 21). The mechanism accounting for these changes has not yet been defined, but an alteration in the specificity of RNA polymerase is one of many possibilities. The studies presented here were performed to define the metabolism of Ca$^{2+}$ by *Yersinia* and thus to set limits to the role of the cation in regulating the growth and synthesis of virulence functions.

Slopes of tryptose blood agar base (Difco Laboratories, Detroit, Mich.) were incubated at 26°C for 1 day (*Y. pseudotuberculosis* PB1 [7] and *Y. enterocolitica* WA [8]) or 2 days (*Y. pestis* EV76 [6]) after direct inoculation from liquid stocks of buffered glycerol held at −20°C (3). Bacteria were suspended and appropriately diluted in 0.033 M potassium phosphate buffer (pH 7.0; phosphate buffer) for use as inocula or for determination of optical density and viability. The chemically defined medium of Higuchi et al. (13) as modified by Zahorck et al. (20) was used in all experiments. Ca$^{2+}$ was either omitted or added at a concentration of 4.0 mM to provide a permissive growth condition at 37°C; 20 mM Mg$^{2+}$ was present in all cases. At least one transfer at 26°C was performed to ensure full adaptation before the organisms were grown for use in experiments. Cultures were prepared in Erlenmeyer flasks in a liquid volume never exceeding 1/10 that of the flask; these cultures were aerated at 200 rpm in a model G76 Gyrotory water bath shaker (New Brunswick Scientific Co., Inc., Edison, N.J.). Bacteria were inoculated at an optical density of 0.1 at 620 nm.

*Yersinia* in phosphate buffer were used to prepare cultures in defined medium without added Ca$^{2+}$, and these cultures were incubated overnight at 26°C. Samples from this first transfer were used to inoculate second cultures in medium containing 4.0 mM or no added Ca$^{2+}$. After cultivation at 26 or 37°C, the organisms were harvested by centrifugation (10,000 × g at 4°C) when the optical density was 1; this value corresponded to the maximum obtained under restrictive conditions. The bacteria were then washed twice by centrifugation in a modified calcium transport buffer (17) composed of 0.4% D-glucose, 140 mM KCl, 10 mM Tris base, and 10 mM K$_2$HPO$_4$ adjusted to pH 7.8 with HCl; the final suspension was brought to an optical density of 1. Reaction mixtures were prepared in 125-ml Erlenmeyer flasks and consisted of 7.8 ml of cell suspension, 1.6 μmol of 45CaCl$_2$ (0.1 μCi/μmol; New England Nuclear Corp., Boston, Mass.), and sufficient distilled water to yield a final volume of 8.0 ml; in some cases the energy inhibitor cyanide m-chlorophenylhydrazine (CCCP) was added at a final concentration of 0.1 μmol/ml. After incubation at 37°C for 5 min, the reaction was started by the addition of 45Ca$^{2+}$. Samples (0.5 ml) were collected at intervals of 15 min, filtered (0.22-μm pore size; Millipore Corp., Bedford, Mass.), and washed twice with 5 ml of calcium transport buffer containing 2 mM unlabeled CaCl$_2$, and radioactivity was determined with a liquid scintillation spectrometer. Ca$^{2+}$ was not accumulated by either Lcr$^+$ or Lcr$^-$ cells of *Y. pestis* grown at 37°C either in the presence or absence of the cation (Fig. 1). In contrast, limited accumulation occurred if the organisms received CCCP. Essentially identical results were obtained with Lcr$^+$ and Lcr$^-$ *Y. pestis* grown at 26°C with or without added Ca$^{2+}$ and with *Escherichia coli* K-12 and Lcr$^+$ and Lcr$^-$ cells of *Y. pseudotuberculosis* cultivated at 26 or 37°C in the presence or absence of Ca$^{2+}$ (data not shown). These results demonstrated that neither growing nor restricted *Yersinia* accumulate significant Ca$^{2+}$.

To prove that the uptake of Ca$^{2+}$ by *Yersinia* poisoned with CCCP (Fig. 1) reflected inhibition of exit, organisms were grown through two transfers in defined medium at 26°C without added Ca$^{2+}$ and inoculated into fresh cultures in medium containing 4.0 mM or no added Ca$^{2+}$. After incubation for 6 h at 37°C, the bacteria were harvested, washed twice with calcium transport buffer lacking glucose, and then suspended in this buffer at an optical density of 2. The

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suspension was brought to 0.4 mM with $^{45}\text{Ca}^{2+}$ and incubated overnight at 4°C to permit accumulation of the cation. A 5-mL sample was added to 5 mL of buffer at 37°C in a 125-mL Erlenmeyer flask which then received either glucose or CCCP at a final concentration of 0.4% or 100 μM, respectively. The flasks were incubated at 37°C, and samples were removed and assayed for radioactivity as described above. Lcr− cells of Y. pestis rapidly eliminated internalized Ca$^{2+}$ after the temperature shift unless CCCP was present (Fig. 2), in which case additional cation accumulated in a manner resembling that shown for poisoned cells in Fig. 1. Analogous results were obtained for Lcr− cells of Y. pestis and for E. coli K-12 (data not shown). Results with Y. pseudotuberculosis PB1 and Y. enterocolitica WA were also similar except that less $^{45}\text{Ca}^{2+}$ accumulated during storage at 4°C, presumably because of an increased level of endogenous metabolism by these species at low temperature.

It was evident from the studies described above that organisms grown with unlabeled Ca$^{2+}$ accumulated less $^{45}\text{Ca}^{2+}$ in the cold than did those cultivated without the cation. This result would be expected if $^{45}\text{Ca}^{2+}$ added to washed cells was unable to compete for binding sites or to enter pools occupied by the unlabeled cation. To verify this point and to compare the binding capacity of Lcr+ and Lcr− cells of Y. pestis for the cation, organisms in phosphate buffer were inoculated into defined medium containing either 0.1 mM (0.6 μCi/ml) or 4.0 mM (2.0 μCi/ml) $^{45}\text{Ca}^{2+}$ and cultivated at 26°C for two transfers. The approximately 10 generations of growth that occurred during this process was sufficient to ensure that the bacteria had achieved a constant specific activity with respect to $^{45}\text{Ca}^{2+}$. The organisms were then inoculated into third transfers containing identical concentrations of $^{45}\text{Ca}^{2+}$, and optical density and radioactivity were monitored during growth of parallel cultures at 26 and 37°C. The lower concentration of $^{45}\text{Ca}^{2+}$ was not sufficient to permit growth of Lcr− yersiniae at 37°C but was in excess throughout the determinations of optical density and radioactivity. Samples (0.5 ml) were taken at intervals and filtered; the membranes were then washed twice with 25 mM Tris hydrochloride (pH 7.5) containing 2 mM unlabeled CaCl₂ before radioactivity was determined. Both Lcr+ and Lcr− organisms maintained a constant ratio of bound $^{45}\text{Ca}^{2+}$ to cell mass during growth under permissive conditions. For example, with added 0.1 mM $^{45}\text{Ca}^{2+}$, this value (nanomoles of bound Ca$^{2+}$ per unit of optical density) was about 0.25 for both Lcr+ and Lcr− organisms grown at 26°C (Fig. 3A) and for Lcr− cells grown at 37°C (Fig. 3C). Similarly, with added 4.0 mM $^{45}\text{Ca}^{2+}$, the ratio was approximately 3 for both Lcr+ and Lcr− organisms during growth at 26°C (Fig. 3B) and at 37°C (Fig. 3D). However, with added 0.1 mM $^{45}\text{Ca}^{2+}$, a significant increase in ratio from 0.25 to 0.35 was noted during restriction of Lcr− cells at 37°C (Fig. 3C).

Cursory attempts were made to define the ligands accounting for this ability of restricted Lcr+ yersiniae to bind about 70% more Ca$^{2+}$ than did Lcr− mutants. Although the results of initial determinations suggested that the organisms expressed calmodulinlike activity (5), these observations were not substantiated by further study. Similarly, attempts to show distinctions in the ability of lipopolysaccharides from Lcr+ and Lcr− yersiniae to bind Ca$^{2+}$ were not successful. Additional characterization of this interaction is in progress.

It is surprising that Lcr+ yersiniae require a millimolar level of Ca$^{2+}$ for multiplication at 37°C, since procaryotes can typically undergo vegetative growth in media containing only trace levels of this cation. Although Ca$^{2+}$ is actively transported by Bacillus species during sporulation, it is excluded from the cytoplasm of these species, E. coli, and presumably other bacteria during vegetative growth where it undergoes elimination via energy-dependent exit reactions (1, 12, 17). Before this report, the possibility existed that yersiniae might, in a reaction analogous to that in Bacillus species, accumulate Ca$^{2+}$ for some unique function such as carrying bicarbonate anions (18). The evidence presented here, however, demonstrated that the cation is metabolized in yersiniae by reactions typical of other procaryotes. Nevertheless, Ca$^{2+}$ does regulate complex changes in these organisms that probably serve to dictate distinct responses to Ca$^{2+}$-rich extracellular host environments and Ca$^{2+}$-deficient mammalian intracellular fluids (4, 15). Resolution of this role will require definition of the reaction that promotes
Lcr⁺-specific binding of Ca²⁺. Mutation to rifampin resistance is known to reflect alteration of RNA polymerase (14). It is probably significant that in yersinia this chromosomal mutation can promote expression of the Lcr⁺ phenotype (2) and that in E. coli it may result in a temperature-dependent requirement for Ca²⁺ (11).

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