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Consequences of Ca^{2+} Deficiency on Macromolecular Synthesis and Adenylate Energy Charge in *Yersinia pestis*

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At 37 but not 26°C virulent *Yersinia pestis* is known to require at least 2.5 mM Ca^{2+} for growth; this requirement is potentiated by Mg^{2+}. After shift of log-phase cells (doubling time of 2 h) from 26 to 37°C in Ca^{2+}-deficient medium, shutoff of net ribonucleic acid synthesis preceded that of protein and cell mass. With 2.5 mM Mg^{2+}, about two doublings in cell mass and number occurred before restriction with synthesis of sufficient deoxyribonucleic acid to account for initiation and termination of two postshift rounds of chromosome replication. Temperature shift with 20 mM Mg^{2+} resulted in a single doubling of cell mass and number with one round of chromosome replication. Subsequent to shutoff of ribonucleic acid accumulation, ribonucleoside but not deoxyribonucleoside triphosphate pools became reduced to about 50% of normal values and the adenylate energy charge fell from about 0.8, typical of growing cells, to about 0.6. Excretion of significant concentrations of adenine nucleotides under both permissive and restrictive conditions was observed. Only trace levels (<0.01 μmol/g [dry weight]) of guanosine 5′-diphosphate 3′-diphosphate accumulated under restrictive or permissive conditions; guanosine 5′-triphosphate 3′-diphosphate was not detected. Return of fully restricted cells from 37 to 26°C without Ca^{2+} resulted in prompt growth, whereas addition of Ca^{2+} at 37°C was ineffective. This finding indicates that the observed temperature-sensitive lesion in ribonucleic acid synthesis that results in restriction can be prevented but not reversed by cultivation with Ca^{2+}.

Ca^{2+} promotes essential organelle-mediated reactions in eucaryotic cells which thus possess a general nutritional requirement for the cation (4, 21). In contrast, vegetative growth of procaryotes usually occurs in Ca^{2+}-deficient medium although a requirement may exist for performance of ancillary functions including morphogenic change (11, 18, 27, 41), gene exchange (10, 35, 39), stabilization of surface structures (7, 13, 24), and activity of degradative exoenzymes (30). Ca^{2+} is actively transported during sporulation in *Bacillus* (12) but is removed from the cytoplasm of this and other growing bacteria via a specific energy-dependent exit reaction (38). The cation is excluded by those transport systems that accumulate essential divalent metallic cations (29, 36, 37), and the existence of Ca^{2+}-dependent mutants analogous to those blocked in uptake of such essential cations (29) has not been reported. Since only trace levels of Ca^{2+} normally exist within bacteria (38), much of that found in dried preparations of whole cells (0.23% in *Escherichia coli* [19]) is probably associated with the envelope (3, 7, 13, 34). This concentration may be influenced by the environment; for example, the content of Ca^{2+} in mycoplasma membranes paralleled that added to the medium (17).

Wild-type *Yersinia pestis*, the causative agent of bubonic plague, and other yersiniae exhibit a nutritional requirement for Ca^{2+} which is unique among procaryotes. Sustained growth in vitro of these enteric facultative intracellular parasites at 37°C (host temperature) is dependent upon addition of that amount of Ca^{2+} (2.5 mM) normally present in mammalian plasma; the cation is not required for division at room temperature (6). Ca^{2+} could be replaced by equimolar Sr^{2+} or Zn^{2+} but not Mg^{2+} which, when present at the concentration of intraleukocytic fluid (20 mM), potentiated the requirement (15). During cultivation at 37°C in various media simulating intraleukocytic fluid with respect to Ca^{2+} (not added) and Mg^{2+} (20 to 40 mM), *Y. pestis* ceased synthesis of DNA (44), became swollen (14),

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contained nucleoids in the form of axial filaments (14), and released V antigen (a 90,000-dalton protein [20]) and W antigen (a 145,000-dalton lipoprotein [20]). The anatomical origin and physiological role of V and W antigens are unknown; their release from some component of the envelope seems likely (6). These restrictive conditions are selective for Ca^{2+}-independent mutants which fail to produce V and W antigens and are avirulent (6, 16).

The purpose of this report is to describe the physiological consequences of Ca^{2+} deficiency in *Y. pestis*. We demonstrate that the adenylate energy charge of Ca^{2+}-starved cells is significantly reduced. However, this evident lesion in energy metabolism may not reflect the primary cause of restriction since shutoff of net RNA synthesis occurred before significant reduction of both energy charge and ribonucleoside triphosphate pools.

MATERIALS AND METHODS

Bacteria. *Y. pestis* EV76 or isogenic derivatives were used in all experiments. Cells of this isolate are avirulent due to a presumed lesion in iron metabolism unrelated to Ca^{2+} dependence (6). A Ca^{2+}-independent mutant was isolated on magnesium oxalate agar (16), and a *pyr his* auxotroph was obtained by stepwise induction with UV light and selection with penicillin (5).

Medium and cultivation. A modification (44) of a defined liquid medium containing fermentable carbohydrate and 15 amino acids (15) was prepared containing appropriate concentrations of $MgCl_2$ and $CaCl_2$. The medium was dispensed into Erlenmeyer flasks (10%, vol/vol) which, after inoculation, were aerated at 200 rpm on a model G76 gyratory water bath shaker (New Brunswick Scientific Co., Inc., New Brunswick, N.J.). Cells were routinely adapted for use in experiments by prior growth for 10 generations at 26°C in Ca^{2+}-deficient medium (2.5 mM Mg^{2+}).

Measurement of growth. Optical density of cultures, appropriately diluted in 0.033 M potassium phosphate buffer, pH 7.0 (phosphate buffer), was determined at 620 nm with a model 2000 spectrophotometer (Gilford Instruments Laboratories, Inc., Oberlin, Ohio). At 26°C, an optical density unit of 1 corresponded to 1.5×10^8 total cells per ml. Colony-forming units were measured by spreading samples of culture, appropriately diluted in phosphate buffer, on the surface of solid blood agar base (BBL Microbiology Systems, Cockeysville, Md.); colonies were counted after incubation at 26°C for 48 h. Dry weight was assayed by centrifuging 40 ml of culture (17,000 × *g* for 10 min at 4°C) and washing the cells twice with 0.15 M ammonium acetate buffer, pH 7.0. The cells were then quantitatively transferred into tared vessels and brought to dryness at 90°C and then to constant weight at 110°C. Total cells were counted by direct observation in a Petroff-Hausser counting chamber.

Degradation of macromolecules. Log-phase cells grown at 26°C (2.5 mM Mg^{2+}, no added Ca^{2+}) were pulsed for 30 min with carrier-free [6-^{3}H]thymine (10 μCi/ml), [5-^{3}H]uracil (1 μCi/ml), or L-[3-^{3}H]histidine (1 μCi/ml) and then chased for 2 h by addition of the respective unlabeled compound (0.1 μmol/ml). The organisms were then diluted into fresh medium containing the same concentration of unlabeled precursor and incubated under permissive and restrictive conditions. Trichloroacetic acid-insoluble radioactivity was measured in samples prepared for counting as described below.

Net synthesis of macromolecules. Cells of a *pyr his* auxotroph were grown at 26°C (2.5 mM Mg^{2+}, no added Ca^{2+}) in medium supplemented with 1.0 mM L-histidine and 1.0 mM [2-^{14}C]uracil (0.05 μCi/μmol). These labeled organisms were diluted into identical medium (containing radioactive precursor) before appropriate addition of Ca^{2+} or increased Mg^{2+} upon shift to 37°C. Net synthesis of nucleic acids in these subcultures was measured by use of a modification (32) of the Schmidt-Thannhauser procedure (33). Total incorporation of isotope was determined by addition of 1.0 ml of culture to 1.0 ml of cold 10% trichloroacetic acid. After storage for 30 min in an ice bath, the precipitate was collected on a membrane filter (0.22-μm pore size; Millipore Corp., Bedford, Mass.), washed with 10 ml of cold 5% trichloroacetic acid, dried, and prepared for counting. Accumulation of DNA was measured by addition of 1.0 ml of a parallel sample to 0.1 ml of 5.5 N NaOH. After incubation at 37°C for 18 h, this digest received 0.1 ml of 6 N HCl and 1.2 ml of cold 10% trichloroacetic acid. After storage for 30 min in an ice bath, the precipitate was collected and counted as above. This value was subtracted from that obtained for total incorporation to yield counts specific for RNA.

Net synthesis of protein was similarly determined by growing the *pyr his* isolate for 10 generations in medium containing added 1.0 mM uracil and 1.0 mM L-[*U*-^{14}C]histidine (0.01 μCi/μmol). Upon dilution of cells into the same medium (containing isotope) and cultivation under permissive and restrictive conditions, samples of 1.0 ml were removed and prepared for counting as described for total nucleic acids.

Nucleotide pools. Cells were grown for about seven generations at 26°C in medium (20 mM Mg^{2+}, no added Ca^{2+}) containing 0.21 mM $^{32}P_i$ (237 μCi/μmol) where buffering capacity was maintained by addition of 0.025 M *N*-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid. After transfer to identical medium (containing isotope) and further cultivation under permissive or restrictive conditions, samples of 0.5 ml were removed and extracted by the procedure of Bagnara and Finch (2). Ribo- and deoxyribonucleoside triphosphates were chromatographed as described by Randerath and Randerath (31), and guanosine 5'-diphosphate 3'-diphosphate (ppGpp) and guanosine 5'-triphosphate 3'-diphosphate (pppGpp) were determined by the method of Cashel (8).

Determination of radioactivity. Oven-dried membrane filters were counted in toluene base containing 0.4% 2,5-diphenyloxazole and 0.005% 1,4-di-2(5-phenyloxazolyl)benzene with a model 3320 Tri-Carb scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). All samples were counted for sufficient time to yield at least 10^3 counts above

background. Areas on polyethyleneimine-cellulose sheets containing ^{32}P-labeled nucleotides were removed by cutting and counted directly.

Adenylate energy charge. Adenine nucleotides were extracted by addition of 4 volumes of culture to 1 volume of cold 35% $HClO_4$ (43) in 67 mM EDTA (22). After centrifugation, 2.0 ml of supernatant was neutralized by addition of 0.7 ml of 0.58 M $KHCO_3$ in 2.6 M KOH (43). ADP and AMP were determined after their enzymatic conversion to ATP (9). The latter was assayed by the luciferase procedure (42) in a scintillation vial containing 1.8 ml of buffer (3 mM $MgSO_4$ in 40 mM glycylglycine, pH 7.4), 2.0 ml of culture extract, and 75 μl of partially purified luciferase and luciferin (25); after 15 s of incubation, the light emitted over a period of 6 s was measured in a Packard Tri-Carb scintillation spectrometer. Intracellular nucleotide concentrations were calculated by subtracting values obtained with comparable filtered (0.22-μm pore size, Millipore Corp.) cultures. Quintuplicate assays were performed on each sample, and the mean value was used to calculate adenylate energy charge. Analysis of external standards treated identically to culture samples resulted in recoveries of 101, 107, and 91% for ATP, ADP, and AMP, respectively. Activity of partially purified luciferase with GTP and ADP was less than 2% of that obtained with ATP.

Miscellaneous. Radiochemicals were purchased from New England Nuclear Corp., Boston, Mass., and firefly lantern extract (FLE-50) was obtained from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Kinetics of restriction. Use of cells fully adapted to the defined medium and strict control of temperature, rate of aeration, and concentrations of Ca^{2+} and Mg^{2+} were necessary to obtain reproducible patterns of growth and restriction. A lag period of 4 to 6 h observed at 26°C upon inoculation from blood agar base was reduced but not eliminated after subculture at this temperature (Fig. 1A). Ca^{2+} was not required for growth at 26°C where concentrations of Mg^{2+} ranging from 0.25 to 25 mM supported full-scale increases in cell mass (about 10 optical density units). However, shift of Ca^{2+}-deficient cultures to elevated temperatures (34 to 40°C) resulted in a progressive reduction in cell yield. In this case, the onset of restriction was hastened by increased concentrations of Mg^{2+} (Table 1). The presence of 2.5 mM Ca^{2+} or Sr^{2+} (but not Mn^{2+}, Fe^{2+}, or Fe^{3+}) permitted growth to continue at elevated temperatures regardless of the concentration of Mg^{2+}. Some of these responses are shown in Fig. 1B. Mutant Ca^{2+}-independent cells exhibited normal growth under all of these conditions.

An immediate increase in ratio of optical density to cell dry weight occurred at high rates of aeration (>250 rpm) after shift of Ca^{2+}-deficient cultures to 37°C. This change was followed by a marked decrease in the ratio commencing at the time cell division had ceased. These deviations were largely eliminated by reducing the aeration rate to 200 rpm. Under this condition, cells restricted at 37°C with 2.5 and 20 mM Mg^{2+} yielded a respective 3- to 4-fold and 1.5- to 2-fold postshift increase in both cell mass and number. Since maximum increases in both parameters were obtained after shift during early logarithmic growth, the cells were routinely incubated for 2 to 3 h at 26°C before transfer to 37°C.

Parameters of a typical response of *Y. pestis* to restriction in Ca^{2+}-deficient medium are

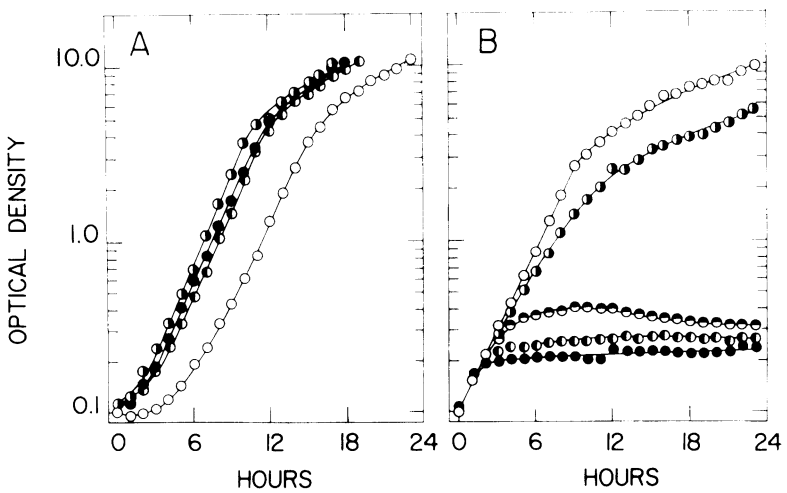

FIG. 1. *Growth of Y. pestis EV76 in Ca^{2+}-deficient medium with 2.5 mM $MgCl_2$ at 26°C (A) upon inoculation from blood agar base (○) and after one (●), two (◑), and three (◐) transfers, and at 37°C (B) after two transfers at 26°C with added 2.5 mM $CaCl_2$ (○), 2.5 mM $SrCl_2$ (◑), 2.5 mM $MnCl_2$ (◐), increase of $MgCl_2$ to 20 mM (●), and without addition (◓).*

shown in Fig. 2. The cells were fully adapted by prior growth at 26°C for 10 generations and either maintained in the same environment (2.5 mM Mg^{2+}, no Ca^{2+}) or shifted to 37°C after 2 h (2.5 mM Mg^{2+} and no Ca^{2+}, or 2.5 mM Mg^{2+} and 2.5 mM Ca^{2+}) or 3 h (20 mM Mg^{2+}, no Ca^{2+}) of growth at 26°C. The ratio of optical density to dry weight remained uniform during subsequent growth although microscopic examination showed that some early aggregation of cells occurred, especially in media containing only 2.5 mM Mg^{2+}. Pronounced agglutination, reflected by a corresponding reduction in colony-forming units compared to total cells, occurred at 37°C upon restriction or after entry into the stationary phase following growth with Ca^{2+}. As shown by direct observation, a typical fourfold (5×10^7 to 2×10^8) and twofold (1×10^8 to 2×10^8) postshift increase in cell numbers occurred during restriction with 2.5 mM and 20 mM Mg^{2+}, respectively.

Degradation of macromolecules. Cells were pulsed with radioactive precursors and then incubated with excess unlabeled precursor. No detectable net degradation of DNA or RNA occurred under permissive or restrictive conditions. Radioactive histidine was lost from both normal and restricted cells at a rate of 1.5% per h.

Net synthesis of macromolecules. Cells of a *pyr his* auxotroph were grown for sufficient time (10 generations) with radioactive uracil or L-histidine to undergo uniform labeling. Subsequent incorporation of these precursors, present at the same specific activity, into macromolecules was then compared in subcultures incubated under permissive and restrictive conditions.

The first detectable event associated with restriction was shutoff of net RNA synthesis, which occurred about 1 and 2 h after shift with 20 mM and 2.5 mM Mg^{2+}, respectively (Fig. 3C). Similar reduction in accumulation of DNA (Fig. 3B), protein (Fig. 4B), and cell mass (Fig. 3A and 4A) was not observed until after an additional hour of incubation. During restriction with 20 mM Mg^{2+}, sufficient DNA accrued from the time of shift (34 cpm) until growth ceased (96 cpm) to account for termination of the ongoing round of chromosome replication plus completion of another round. Similarly, sufficient postshift DNA accumulated in the Ca^{2+}-deficient culture containing 2.5 mM Mg^{2+} (33 cpm to 190 cpm) to permit termination of an additional round of replication.

Comparison of rates of net synthesis (Fig. 3 and 4) shows that ratios of all macromolecular species to cell mass remained parallel under permissive conditions; during restriction, those of DNA and protein increased and that of RNA

TABLE 1. *Number of doublings in optical density by Y. pestis EV76 after shift from 26°C to elevated temperatures in Ca^{2+}-deficient medium containing various concentrations of Mg^{2+}*

Temp of incubation (°C)	Concn of Mg^{2+} (mM)		
	0.25	2.5	25
34	3.5	3.2	2.0
35.5	3.0	2.3	1.5
37	2.3	1.8	1.1
38.5	1.9	1.4	1.0
40	1.8	1.1	1.0

[a] Cells in log-phase growth were shifted at an optical density of 0.2.

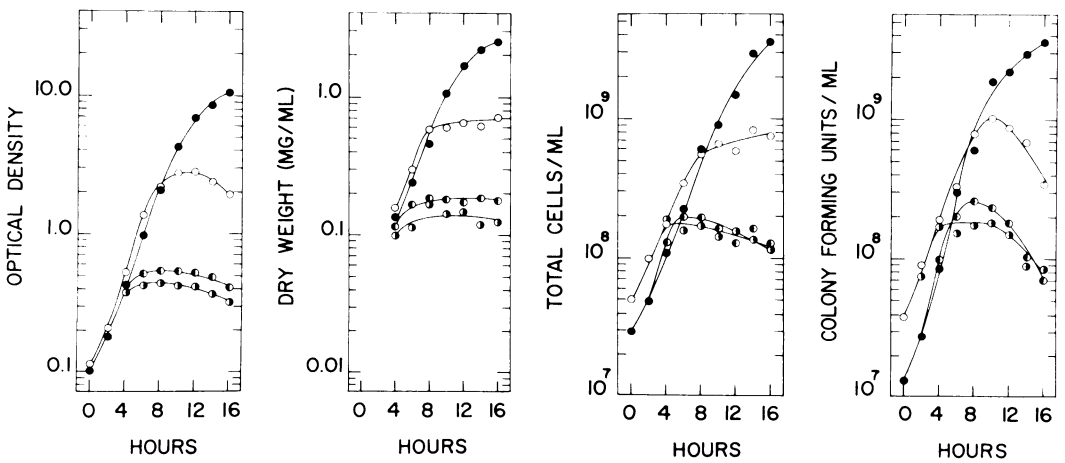

FIG. 2. *Optical density, dry weight, total cells, and colony-forming units of Y. pestis EV76 upon continuous cultivation at 26°C with no added Ca^{2+} and 2.5 mM Mg^{2+} (●), or upon shift from 26 to 37°C after 2 h with 2.5 mM Ca^{2+} and 2.5 mM Mg^{2+} (○) or with no added Ca^{2+} and 2.5 mM Mg^{2+} (◐), or after 3 h with no added Ca^{2+} and 20 mM Mg^{2+} (◑).*

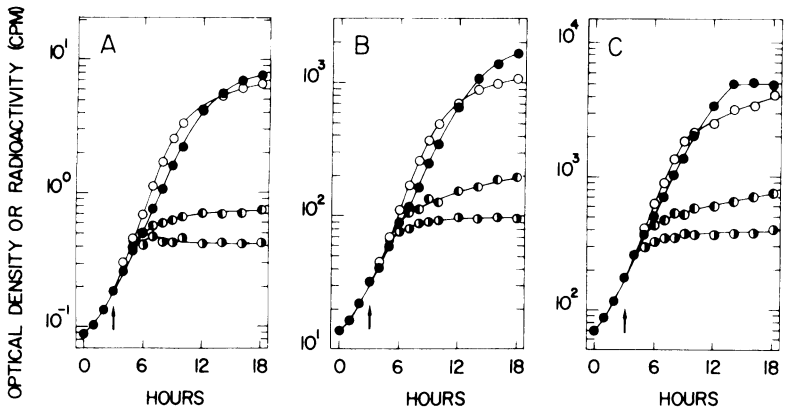

FIG. 3. *Optical density (A) and net synthesis of DNA (B) and of RNA (C) by cells of Y. pestis EV76 grown at 26°C and maintained at 26°C with 2.5 mM $MgCl_2$ and no added $CaCl_2$ (●) or shifted to 37°C (arrow) with 2.5 mM $MgCl_2$ and 2.5 mM $CaCl_2$ (○), with 2.5 mM Mg^{2+} without added $CaCl_2$ (◐), or with 20 mM $MgCl_2$ without added $CaCl_2$ (◑).*

decreased. These changes closely resembled those observed in control cells during entry into the stationary phase.

Nucleotide pools. Preliminary studies showed that patterns of growth and restriction in *N*-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid-buffered medium containing reduced phosphate, necessary for labeling with ^{32}P, were similar to those already described. The deoxyribonucleoside triphosphate pools were not significantly altered 6 h after shift to restrictive conditions (20 mM Mg^{2+}, no added Ca^{2+}), whereas the ribonucleoside triphosphate pools were reduced by about 50% (Table 2). No significant drop in ATP or GTP occurred until after the rate of RNA accumulation had become significantly reduced. For example, 4 h after shift, the ATP and GTP pools were 3.55 and 1.74 μmol/g (dry weight), respectively. The level of these nucleotides subsequently decreased and then stabilized after 6 h at the values shown. Extracts of growing and restricted cells contained only trace levels of ppGpp (<0.01 μmol/g of cells [dry weight]); pppGpp was not detected.

Adenylate energy charge. Intracellular concentrations of ATP, ADP, and AMP and the adenylate energy charge of normal and restricted cells are shown in Table 3. The energy charge of Ca^{2+}-starved organisms was significantly lower than that of growing cells. This decrease primarily reflected a reduced ATP pool, although increases in AMP and ADP also occurred during restriction. A normal energy charge of about 0.8 was maintained for 4 h after shift to restrictive conditions.

Even under permissive conditions, yersiniae released significant extracellular AMP and ADP (Table 4). About twice as much AMP accumulated during restriction as was released during normal growth. Incubation at 37°C also resulted in excretion of ATP except in the highly restrictive Ca^{2+}-deficient medium containing 20 mM Mg^{2+}.

Release from restriction. Cells in a series of parallel cultures (2.5 mM Mg^{2+}, no added Ca^{2+}) were brought into log-phase growth at 26°C, shifted to 37°C, and then provided periodically with 2.5 mM Ca^{2+} (Fig. 5B) or returned to 26°C (Fig. 5A). The latter promptly resumed division even after 10 h of prior cultivation at 37°C. However, addition of Ca^{2+} was ineffective in promoting growth at 37°C after the cells had become restricted.

DISCUSSION

During Ca^{2+} starvation at 37°C, the percentage of DNA and protein per unit of cell mass increased at the same ratio while that of RNA decreased. Restriction with 20 mM and 2.5 mM Mg^{2+} resulted in approximately one and two respective postshift doublings in cell mass and number. Similarly, sufficient DNA accumulated to account for initiation and termination of one and two rounds of replication, respectively, in addition to that in progress at the time of shift (assuming that each chromosome contained a maximum of one division fork as occurs in *E. coli* grown at a similar doubling time of about 120 min [40] and that the average log-phase cell contained $\sqrt{2}x$ or $1.4x$ chromosomes, where x is the chromosome number immediately after division). These observations indicate that restriction is not a consequence of unbalanced growth but rather reflects an ordered downshift of normal anabolic functions (23).

The first observed event associated with this

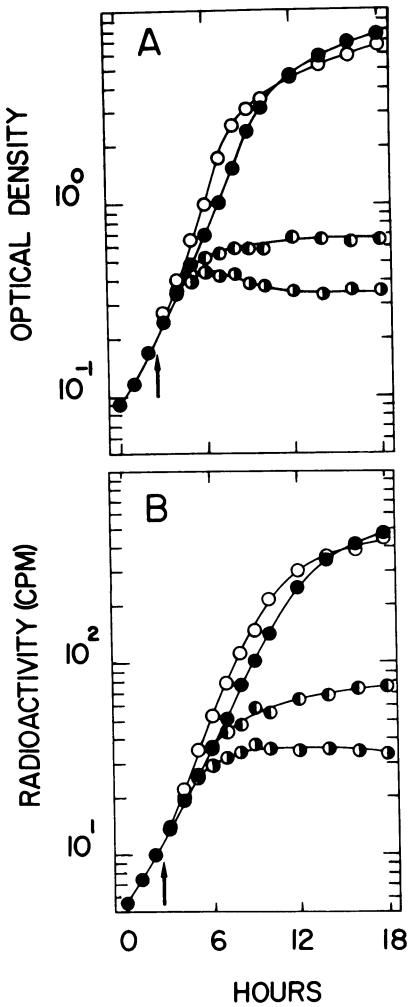

FIG. 4. *Optical density (A) and net synthesis of protein (B) by cells of Y. pestis as described for Fig. 3.*

process was shutoff of net RNA synthesis. Prior results obtained by DNA:RNA hybridization showed that rRNA and tRNA were not transcribed during restriction although synthesis of some messenger RNA was detected (W. T. Charnetzky and R. R. Brubaker, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, D48, p. 59). The block in RNA accumulation reported here therefore refers to stable transcripts. Similarly, restricted cells exhibited a reduction in ribonucleoside triphosphate pools and adenylate energy charge. Of interest was the finding that yersiniae, unlike *E. coli* (43), released significant levels of extracellular adenine nucleotides. Shutoff of stable RNA synthesis with depletion of nucleotide pools occurs in *E. coli* after starvation for a number of nutrients including essential amino acids. However, this type of stepdown as well as depletion of fermentable carbohydrate caused

TABLE 2. *Nucleoside triphosphate pools of Y. pestis EV76 grown under permissive and restrictive conditions*

Nucleoside triphosphate	Nucleotide level (μmol/g of bacteria [dry wt]) under various conditions of cultivation[a]		
	26°C; no Ca^{2+} [b]	37°C; no Ca^{2+} [b]	37°C; plus 2.5 mM Ca^{2+} [b]
GTP	2.07	0.62	1.52
ATP	4.15	1.63	2.78
CTP	1.17	0.57	1.37
UTP	1.58	0.73	1.35
dGTP	0.79	0.62	0.70
dATP	0.70	0.69	0.73
dCTP	0.66	0.63	0.53
TTP	1.32	1.09	1.13

[a] All cultures contained 20 mM $MgCl_2$.

[b] Optical density at time of extraction (6 h of incubation): 26°C and no Ca^{2+}, 0.610; 37°C and no Ca^{2+}, 0.599; 37°C plus 2.5 mM Ca^{2+}, 0.572.

TABLE 3. *Effect of cultural conditions on the intracellular adenylate pool of Y. pestis EV76*[a]

Incubation temp (°C)	Additions (mM)[b]		Nucleotides (μmol/g [dry wt])[c]				Energy[d] charge
	Ca^{2+}	Mg^{2+}	ATP	ADP	AMP	Total	
26	0	2.5	3.2 ± 0.4	1.7 ± 0.3	<0.1	4.9 ± 0.2	0.82 ± 0.04
26	0	20	3.1 ± 0.3	1.4 ± 0.4	<0.1	4.5 ± 0.2	0.84 ± 0.04
37	2.5	2.5	2.4 ± 0.3	2.4 ± 0.3	<0.1	4.7 ± 0.2	0.75 ± 0.06
37	2.5	20	2.3 ± 0.2	1.5 ± 0.2	<0.1	3.8 ± 0.2	0.80 ± 0.01
37[e]	0	2.5	1.6 ± 0.2	2.0 ± 0.2	0.3 ± 0.2	3.9 ± 0.2	0.67 ± 0.04
37[e]	0	20	1.3 ± 0.2	2.8 ± 0.2	0.5 ± 0.2	4.6 ± 0.2	0.57 ± 0.02

[a] All cultures were extracted at an optical density of 0.6.

[b] Present in the growth medium.

[c] Determined in quintuplicate.

[d] Defined as [(ATP) + ½ (ADP)]/[(ATP) + (ADP) + (AMP)].

[e] Values obtained after 6 h of incubation.

TABLE 4. *Effect of cultural conditions on release of extracellular adenine nucleotides by Y. pestis EV76*[a]

Incubation temp (°C)	Additions (mM)		Nucleotide (μmol/g [dry wt])		
	Ca^{2+}	Mg^{2+}	ATP	ADP	AMP
26	0	2.5	0.05	0.37	1.09
26	0	20	0.05	0.28	0.44
37	2.5	2.5	0.83	0.37	1.16
37	2.5	20	0.63	0.44	1.19
37	0	2.5	0.96	0.60	2.23
37	0	20	<0.05	0.76	2.44

[a] Results obtained from same determination shown in Table 3.

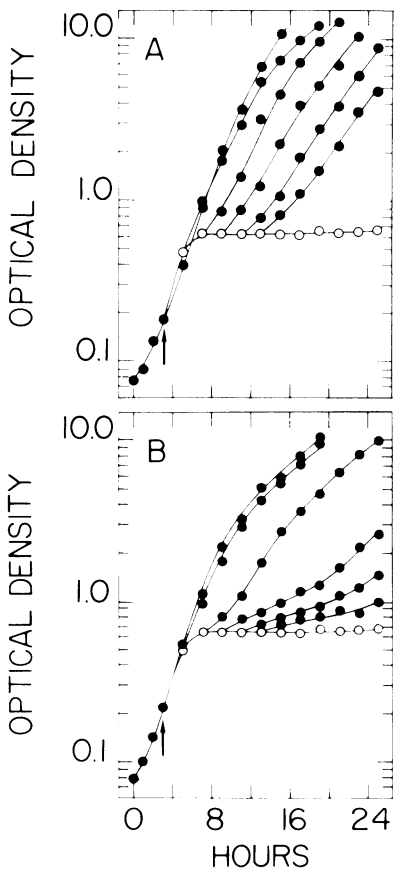

FIG. 5. *Restriction and reinitiation of growth of cells of Y. pestis EV76. Cells were initially grown with 2.5 mM $MgCl_2$ without added Ca^{2+} at 26°C, shifted to 37°C (arrow), and either returned to 26°C (A) or supplied with 2.5 mM $CaCl_2$ at 37°C (B) at intervals of 2 h (●); a control culture was maintained under restrictive conditions (○).*

significant production of ppGpp (26) which did not occur in restricted yersiniae. The latter, also unlike starved *E. coli* (26), maintained normal deoxyribonucleoside triphosphate pools. Active metabolism permitting maintenance of these pools may account for the prolonged process of postshift DNA synthesis observed in this study. Experiments designed to measure formation of (p)ppGpp in yersiniae starved for nitrogen and carbohydrate are in progress and may help explain the absence of these nucleotides during restriction. Since stable RNA synthesis ceased before significant reduction of energy charge and ribonucleoside triphosphate pools, restriction may reflect loss of some factor that stimulates transcription of stable RNA (see reference 26) rather than a primary lesion in ability to maintain an energy charge compatible with growth (43).

Growth of fully restricted cells could not be initiated at 37°C by addition of Ca^{2+}. This finding indicates that the cation must be continuously present to prevent metabolic shiftdown at host temperatures. Since Ca^{2+} interacts primarily with the bacterial envelope and can cause profound temperature-dependent changes in membrane structure (28), further study of its possible influence on biological transport and oxidative phosphorylation was initiated. Results of preliminary experiments suggest that restricted yersiniae may resemble mutants of *E. coli* lacking membrane-bound ATPase activity (1) and further show that normal growth can occur at 37°C in Ca^{2+}-deficient medium supplemented with exogenous ATP (R. J. Zahorchak and R. R. Brubaker, unpublished observations). Accordingly, during residence within the Ca^{2+}-deficient environment of host cells, yersiniae may be able to utilize and possibly even depend upon exogenous energy-rich compounds.

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