Viability and Metabolic Capability Are Maintained by *Escherichia coli*, *Pseudomonas aeruginosa*, and *Streptococcus lactis* at Very Low Adenylate Energy Charge

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Metabolic regulation by nucleotides has been examined in several bacteria within the context of the adenylate energy charge (EC) concept. The ECs of bacteria capable of only fermentative metabolism (*Streptococcus lactis* and the ATPase-less mutant *Escherichia coli* AN718) fell to less than 0.2 under carbon-limiting conditions, but the bacteria were able to step up the EC to greater than 0.8 upon exposure to nutrient sugars. Similarly, nongrowing *E. coli* 25922, whose EC had been artificially lowered to less than 0.1 by the addition of the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP), was able to immediately step up the EC to 0.8 to 0.9 upon the addition of glucose but was unable to respond to respiratory substrates. The EC of respiring bacteria (*E. coli* 25922 and *Pseudomonas aeruginosa* 27853) fell to 0.3 to 0.4 under certain limiting growth conditions, but the bacteria also responded immediately when challenged with succinate to give EC values greater than 0.8. These bacteria could not step up the EC with respiratory substrates in the presence of CCCP. For all bacteria, the loss of the ability to step up the EC was attributable to the loss of nutrient transport function. Mixtures of viable and HOCl-killed *E. coli* 25922 were able to step up the EC in proportion to the fraction of surviving cells. The data indicate that nucleotide phosphorylation levels are not regulatory in nongrowing bacteria but that the EC step-up achievable upon nutrient addition may be an accurate index of viability.

Nucleotides play an important regulatory role in cellular metabolism. Largely in recognition of the differential behavior of ATP against ADP or AMP as enzyme effectors, Atkinson proposed the adenylate energy charge (EC), defined as EC = ([ATP] + 1/2[ADP])/([ATP] + [ADP] + [AMP]), as a quantitative measure of nucleotide regulation (3). Accordingly, for a given set of environmental constraints, tight regulation of opposing anabolic and catabolic pathways is thought to maintain steady-state adenylate nucleotide levels within narrow concentration ranges. When external conditions, such as changes in nutrient supply, force changes in adenylate phosphorylation levels, metabolic fluxes then readjust in a manner dictated by the changing EC. A corollary is that the magnitude of EC accurately describes the metabolic state of the cell.

In vivo studies with a broad range of growing cells, including eucaryotes and procaryotes, have demonstrated predicted relationships among EC, protein synthesis, and cellular growth (6, 11). In general, cells with EC greater than 0.7 to 0.8 divide and synthesize protein at near-maximal rates, but these functions cease when the EC falls below this level. Furthermore, for several strains of bacteria deprived of energy for prolonged periods, the EC was reported to hold at about 0.5 until the cells began to lose viability, at which time further reduction of the EC paralleled cellular death (6, 11). This correlation between EC and viability has not been extensively tested, however, and several exceptions to the general behavior have been reported. Notably, the fermentative bacterium *Streptococcus cremoris* (16), the obligate intracellular bacterium *Rickettsia typhi* (19), and two microbial eucaryotes (4, 13) remained viable at EC less than 0.2 for extended periods during carbon starvation and rapidly returned to growing conditions (EC > 0.7) upon exposure to the limiting nutrient.

In this study, we demonstrate that several common bacterial strains possessing diverse metabolic capabilities are able to survive natural or artificially induced reduction in EC values to as low as 0.04 to 0.05 without loss of viability or apparent impairment of normal cellular function.

**MATERIALS AND METHODS**

Reagents. Radiolabeled [U-14C]p-glucose, [2,3-14C]succinate (sodium salt), and the scintillation fluor Aquasol II were purchased from New England Nuclear Corp. Ammonium phosphate (high-pressure liquid chromatography grade, monobasic) was obtained from Baker Chemical Co. Nutrient broth, tryptic soy broth, tryptic soy agar, Casamino Acids, and agar were from Difco Laboratories. Carbonyl cyanide m-chlorophenylhydrazone (CCCP) was from Aldrich Chemical Co. Hypochlorous acid (HOCl) was prepared by vacuum distillation of neutralized commercial bleach solutions (2). Reagent stocks were standardized by UV spectroscopy by using ε<sub>235</sub> = 100. All other chemicals were reagent grade and were used without further purification.

Bacterial strains. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were obtained from Difco as part of its MIC set; *Streptococcus lactis* ATCC 7962 was from the American Type Culture Collection, and the ATPase mutant (uncA) *E. coli* AN718 was generously given to us by Henry Rosen, Department of Infectious Diseases, University of Washington, Seattle. *E. coli* AN718 stocks were stored as frozen glycerol-broth suspensions at −75°C; the other strains were stored as received on disks at 4°C. *S. lactis* was grown in 30 g/liter of tryptic soy broth, and the
other bacteria were grown in 8 g/liter of nutrient broth. Cultures were grown as follows. Tryptic soy plates were streaked with cell suspensions obtained from stocks incubated overnight at 37°C in the appropriate medium. Slant tubes on tryptic soy agar were subsequently prepared with inocula from the plates. The slant tubes were replaced weekly and the plates were replaced monthly to maintain healthy cultures. For all experiments, 50 ml of broth was inoculated with cells from a slant tube and incubated aerobically overnight at 37°C in a shaker water bath. Five milliliters of this suspension was used as the inoculum for 1 liter of the same medium, which was supplemented with either 22 mM glucose or succinate in some of the experiments. Cells were grown aerobically to late-log phase, harvested, washed, and stored as previously described (1, 5), with the exception that for \textit{P. aeruginosa}, 10 mM magnesium chloride was added to all buffers and dilution blanks. The inclusion of Mg^{2+} was necessary to obtain reproducibly high cell viabilities. All cells were suspended in 100 mM sodium phosphate (pH 7.4, 4°C) containing 154 mM NaCl. The cell concentration of \textit{S. lactis} was adjusted to give an \(A_{600}/cm\) of 3, corresponding approximately to 0.5 mg (dry weight) per ml, and the other cells were adjusted to an \(A_{600}/cm\) of 6, i.e., 1 mg (dry weight) per ml. Viabilities (CFU per milliliter), measured by pour-plate analyses at these cell densities, were as follows: for \textit{E. coli} 25922, 1.7 \times 10^9 \pm 0.4 \times 10^9; for \textit{E. coli} AN718, 8.1 \times 10^8 \pm 0.6 \times 10^8; for \textit{P. aeruginosa} 27853, 2.4 \times 10^9 \pm 0.5 \times 10^9; and for \textit{S. lactis} 7962, 1.3 \times 10^8 \pm 0.4 \times 10^8. For experiments with partially inactivated cell suspensions, bacteria were flow-mixed with HOCI through a tangential 12-jet mixing chamber as previously described (2). Sodium thiosulphate was added 15 min after the bacteria were mixed to stop any residual chlorination reactions.

**EC measurements.** Portions (5 ml) of the cell suspensions were warmed with shaking for 5 min at 30°C in 20-ml glass vials. At timed intervals, 3 ml of the cell suspensions was quenched by rapid withdrawal by using a spring-loaded apparatus into a 5-ml gas-tight glass syringe (The Hamilton Co.) containing 1 ml of 2 M HClO\(_4\) (5). For step-up measurements, the cellular EC was allowed to reach a steady value, an energy source was added, and the EC was subsequently measured as described above at timed intervals. CCCP was prepared as a 20 mM solution in ethanol. Unless otherwise indicated, 12.5 \(\mu\)l of this solution was added to 5 ml of bacterial cell suspension to give a final CCCP concentration of 50 \(\mu\)M. The addition of 12.5 \(\mu\)l of ethanol alone did not affect the viabilities or cellular EC levels.

**Metabolite transport.** Intracellular accumulation of radio-labeled substrates was measured in parallel with EC determinations by using the same sample suspensions. Uptake was measured over 30 min by rapid filtration on cellulose triacetate membranes as previously described (1). Background counts were determined as the zero time intercept of counts per minute versus time plots and were subtracted from all measured values. Results are reported as the percentage of maximum accumulated counts per minute which was attained within 30 min. For cells exposed to CCCP, 100% maximum uptake was determined from untreated cells measured in parallel with the treated cells.

**Nucleotide determinations.** Bacterial nucleotide concentrations were measured by high-pressure liquid chromatography essentially as previously described (5) but with the following modifications. The instrument used was obtained from LDC/Milton Roy Co. and consisted of a Constametric 3G high-pressure pump, a Spectromonitor 3000 variable wavelength UV-vis detector, and a Chromatography Control Module programming and data analysis system. Separations were made by using a 25-cm C\(_{18}\) reversed-phase Spherosorb S50DS2 column (particle size, 5 \(\mu\)m). This system gave base-line resolution of all adenine nucleotides and superior resolution of other nucleotides. The EC was calculated directly from the respective chromatography peak areas.

**RESULTS**

**EC under starvation conditions.** When \textit{E. coli} 25922 was harvested, washed, suspended in buffer devoid of an energy source, and stored at 4°C, the EC remained constant at 0.5 to 0.6 for many hours. This value is consistent with the EC for nongrowing cells previously reported by others (7), but the EC exhibited culture-to-culture variations within that range and average values that were dependent upon the carbon source provided for growth. The highest values were obtained with cells grown on succinate-supplemented medium and the lowest with cells grown on glucose-supplemented medium. Growth on unsupplemented nutrient broth gave intermediate values. When the cell suspensions were warmed to room temperature, the EC slowly decreased to levels that were also carbon source dependent. After about 30 min, the EC stabilized at 0.4 to 0.5, 0.3 to 0.4, and 0.2 to 0.3 for cells grown on succinate, cells grown with no additional energy source, and cells grown with glucose, respectively. The EC varied from culture to culture within these ranges in a manner that was independent of growth rate, phase of growth from which the cells were harvested, concentration of added energy source, oxygenation rates, bacterial strain (\textit{E. coli} 25922 or \textit{E. coli} 11775), buffer strength or type (HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], Tris, and triethanolamine were also used), acidity (pH 6 to 8), buffer counterion (K\(^+\) or Na\(^+\)), harvest temperature, or shaker bath shaker speed.

The EC for \textit{P. aeruginosa} similarly declined upon warming from values of 0.5 to 0.6 to values of 0.3 to 0.4 for cells grown in unsupplemented medium. For \textit{P. aeruginosa}, the final EC values showed much less dependence on the composition of the growth medium than they did for \textit{E. coli}. The EC values for \textit{S. lactis} and the ATPase-deficient mutant \textit{E. coli} AN718 were already very low in the resting cells at 4°C, at 0.0 to 0.1 and 0.2 to 0.3, respectively, and did not show significant change upon warming. Typical results for these bacteria are summarized in Fig. 1A to D.

The adenine nucleotide pools in starved cells increased with time at rates of about 10%/h. Concentrations of guanine, uracil, hypoxanthine, inosine, guanosine, and GMP also increased; the bases were found exclusively in the extracellular medium, but all other compounds were intracellularly localized. These effects were not seen for cells maintained in an energy-rich environment, i.e., with an EC of 0.8 to 0.9. For all of the bacterial strains, cells remained 100% viable over the entire course of the starvation studies.

**EC step-up.** The addition of nutrients to energy-limited cells in their resting state caused a rapid increase in their EC to values greater than 0.8; typical responses to 22 mM glucose or succinate are given for several bacterial strains in Fig. 1. For \textit{E. coli}, this behavior is consistent with previously reported observations (7). In all cases in which the response was positive, the EC reached its maximum value within 5 min and was paralleled by uptake of the energy source. \textit{E. coli} 25922 grown in glucose-supplemented medium did not respond to succinate addition; this observation is consistent with reports that glucose represses succinate
transport (14). Similarly, E. coli AN718 did not respond to the addition of succinate, but the EC rose to expected values upon the addition of glucose (Fig. 1B). An identical response was observed (Fig. 1C) for S. lactis 7962, a bacterium that cannot respire. In contrast, the EC step-up in P. aeruginosa in response to succinate was rapid (Fig. 1D) irrespective of the carbon source used in the growth medium, but the response to glucose addition was negligible, even if the cells were grown in glucose-supplemented medium. As an obligate aerobe, P. aeruginosa is capable of rapid assimilation of respiratory substrates, but pathways for glucose catabolism are not induced in rich media, such as nutrient broth, even when glucose is included in the medium (17).

For the organisms examined, EC step-up responses have been repeatedly observed in conjunction with these and other studies conducted over a period of more than 1 year. The steady-state EC value attained upon the addition of glucose to glucose-grown E. coli was always greater than 0.8, and that for succinate-grown E. coli given succinate was in the range of 0.7 to 0.8. An EC step-up of similar magnitude was also invariably observed for the other bacteria when provided with a usable energy source.

**EC step-up in the presence of the respiratory uncoupler CCCP.** When respiring bacteria were preincubated with CCCP, the EC step-up induced upon subsequent exposure to respiratory substrates was lost. Thus, the EC for succinate-grown, CCCP-treated E. coli 25922 and P. aeruginosa 27853 did not respond to succinate. As expected, an EC step-up of normal magnitude was observed upon glucose addition to glucose-grown, CCCP-treated E. coli 25922, E. coli AN718, and S. lactis 7962. For E. coli 25922, results with glucose and succinate are given in Fig. 2.

CCCP also lowered the steady-state EC level maintained by succinate-grown E. coli 25922 cells in their resting state. This effect is illustrated in Fig. 2. Upon the addition of CCCP, the resting-cell EC value of 0.59 dropped to 0.16 after 35 min, which was identical to the EC level of glucose-grown cells. In contrast, the EC was 0.54 at this time in cells not treated with CCCP. The subsequent addition of succinate was not accompanied by an EC step-up of any significant magnitude, although in the absence of CCCP, the EC of
otherwise identically treated cells rose to 0.86. This effect was independent of both CCCP concentration over the measured range of 20 to 400 μM and medium acidity over the measured range of pH 6.0 to 7.4. Cells retained greater than 80% viability even after exposure to the highest CCCP concentrations used. If CCCP was added to glucose-grown *E. coli* 25922 in the presence of glucose, the normally high EC level of the cells fell slightly (from 0.92 to 0.81), whereas the addition of CCCP to succinate-grown cells in the presence of succinate caused the EC to decrease to less than 0.2 within 5 min (data not shown).

**EC step-up in chlorine-injured bacteria.** The effect of the oxidant HOCI on the ability of nongrowing *E. coli* 25922 to increase its EC in response to glucose is given in Fig. 3. The EC achievable in partially inactivated suspensions fell in parallel with viability. This relationship is further illustrated in the inset to Fig. 3, in which the EC is plotted against the viable cell fraction. We previously showed that HOCI-killed *E. coli* possess an EC of less than 0.1 (5). The overall EC can therefore be expressed to a good approximation by the simple formula EC = 0.9f, where f is the fraction of surviving cells.

**DISCUSSION**

The data demonstrate that various nongrowing bacteria with differing metabolic requirements are responsive to added nutrients over wide ranges of EC values. Even when the EC of *E. coli* 25922 was artificially lowered by the addition of CCCP, the cells were able to rapidly respond when challenged with glucose and were able to raise their EC to levels capable of supporting biosynthesis and growth.

The EC of nongrowing *E. coli* in our suspensions was lower than originally reported by Atkinson and co-workers (7) for aerobically grown glucose-limited *E. coli* B. The basis for this difference is not known, although in a subsequent review the authors remarked, concerning the relationship between EC of 0.5 to 0.6 and viability, that “further work has shown that modifications in the conditions of aging and in the composition of the plating medium can lead to rather high viability at somewhat lower energy charge values” (6).

Several controls indicate that the cells used in our studies were functionally normal. The bacteria were grown to minimize the accumulation of glycogen or other energy storage polymers (8, 18). Consequently, energy sources had to be derived from the transport of exogenous nutrients. In each instance, increases in EC were shown to be coincidentally linked to metabolite accumulation by normal mechanisms; conversely, the absence of response coincided with the absence of transport, as expected. We previously showed that the loss of viability in HOCl-treated *E. coli* coincides with both the loss of metabolite transport (1) and the inhibition of its proton-translocating ATP synthase (W. C. Barrette, Jr., unpublished observations). Other studies have shown that the phosphoenolpyruvate-dependent glucose phosphotransferase systems of normal *S. lactis* (15) and *E. coli* (10) remain poised to transport sugars under conditions of carbon starvation. For *E. coli* 25922, the gradual decline in steady-state EC levels following CCCP addition from values associated with the respiring organism to values typical of ATP generation primarily by substrate-level phosphorylation also suggests a normal metabolic response, as does the slow accumulation of hypoxanthine, inosine, and adenosine in the medium during carbon starvation (12). The latter phenomenon has been attributed to a mechanism for AMP excretion activated under these conditions (12). Therefore, the bacteria appear to be well regulated, although control by adenylate phosphorylation levels is negligible under these conditions.

Our interest in the relationship between EC and viability was prompted by the need for an accurate index of death that can be applied immediately to bacterial cell suspensions. Such need arises in studies of toxicities of chemical or biologically-derived oxidants, for example, in which a common criticism of the use of CFU is that an indeterminate amount of cellular repair might occur during the relatively long incubation period, giving an erroneous measurement of the extent of damage. For chlorine-injured *E. coli*, partially inactivated suspensions containing various proportions of viable and nonviable cells in the presence of energy sources established an average EC that corresponded to the value expected for the fraction of surviving cells if they possessed an EC of about 0.9. Chlorine-injured cells that retain the ability to replicate in a nutrient-rich environment therefore appear capable of maintaining an EC of 0.8 to 0.9, just as has been found for undamaged growing cells (6, 11), and the average EC of populations of cells examined under conditions that would otherwise support growth correctly assigned the relative number of viable and nonviable bacteria. On the other hand, the EC measured for all of the untreated bacteria under various medium conditions bore no relationship to their viabilities; i.e., the ability of the bacteria to respond metabolically to nutrients and to grow was independent of the EC over a wide range. This observation could have important consequences for current methodologies based upon EC values to gauge the metabolic well-being of cells, e.g., in fingerprinting mixed cultures in microbial ecology (9). A more useful general index, at least for viability, may be the EC achievable when the organisms are placed in a growth-supporting environment.
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