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Acidic Acetonitrile for Cellular Metabolome Extraction from *Escherichia coli*

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Cellular metabolome analysis by chromatography−mass spectrometry (MS) requires prior metabolite extraction. We examined a diversity of solvent systems for extraction of water-soluble metabolites from *Escherichia coli*. Quantitative yields of ∼100 different metabolites were measured by liquid chromatography−tandem MS and displayed in clustered heat map format. Many metabolites, including most amino acids and components of central carbon metabolism, were adequately extracted by a broad spectrum of solvent mixtures. For nucleotide triphosphates, however, mixtures of acidic (0.1 M formic acid-containing) acetonitrile/water (80:20) or acetonitrile/methanol/water (40:40:20) gave superior triphosphate yields. Experiments involving isotopic tracers revealed that the improved triphosphate yields in the acidic acetonitrile were in part due to reduced triphosphate decomposition, which is a major problem when extracting with other solvent systems such as methanol/water. We recommend acidic solvent mixtures containing acetonitrile for extraction of the *E. coli* metabolome.

Most methods of cellular metabolome analysis, such as gas chromatography−MS or liquid chromatography−tandem MS (LC−MS/MS), require extraction of metabolites from cells. Accordingly, quenching metabolism and extracting metabolites are critical components of metabolomic procedures.[1−5] Due to its importance as both a model organism and human pathogen, *Escherichia coli* is an important target organism for metabolome analysis.[6] To date, however, optimized procedures for extracting metabolites from *E. coli* have been lacking.

Maharjan and Ferenci evaluated different methods of metabolome extraction from *E. coli* using thin layer chromatography as the analytical readout.[2] They concluded that cold methanol/water (50:50) enabled detection of more metabolites than hot alcohol, hot potassium hydroxide, cold perchloric acid, or cold chloroform/methanol. These results, combined with the presumptive lower risk of metabolite degradation with cold methanol extraction compared to procedures involving high temperatures or extremes of pH, have resulted in cold methanol/water being a leading approach for *E. coli* metabolome extraction.

Recently, however, we found that extraction of *E. coli* with cold 50:50 methanol/water resulted in marked decomposition of nucleotide triphosphates.[7] The decomposition was catalyzed by *E. coli* components, as it occurred much faster than for isolated triphosphates. The triphosphate losses were associated with concomitant production of less phosphorylated nucleotide derivatives (e.g., monophosphates, nucleosides, bases). This pattern of nonphysiological metabolite losses and gains is extremely detrimental in metabolomic studies, leading to underestimation of triphosphate levels, overestimation of the levels of other nucleotides and bases, and substantial misestimation of the cellular energy charge. Thus, new extraction approaches are required to enable more effective global metabolic analysis in *E. coli* and related organisms.

Here we applied metabolome quantitation-mass spectrometry (quantitative LC−MS/MS involving multiple reaction monitoring [MRM] for numerous metabolites) on a triple-quadrupole mass spectrometer to investigate potential alternative means of metabolome extraction from *E. coli*. Metabolite yields were determined under a variety of different extraction conditions and visualized in clustered heat map format. The most striking metabolite cluster was highly enriched in triphosphates, whose levels were much higher when extracting using acetonitrile-containing mixtures relative to methanol/water or other tested solvent systems. Acidic acetonitrile-containing solvent mixtures were particularly preferred for effectively extracting triphosphates while minimizing their degradation during the extraction process.

EXPERIMENTAL SECTION

Culture Conditions. *E. coli* K-12 strain NCM 3722 was cultured in minimal complete media[8] with 10 mM ammonium chloride and 0.4% glucose at 37 °C. For liquid cultures, exponentially growing cells at an optical density at 650 nm (OD_{650}) of ∼0.35 were harvested by centrifugation at room temperature for 4 min at 5000*g*, and the resulting cell pellet was extracted immediately as described below.

Filter culture—growth of cells on top of a filter sitting on an agarose-media support—has certain advantages for metabolomic studies, e.g., enabling fast quenching of metabolism and nondis-

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Table 1. Extraction Solvents

solvent mixture	culture type tested[a]	temperature of first extraction step (°C)[b]
methanol/water (80:20)[c]	both	−80
chloroform/methanol (67:33)	liquid	−80
ethyl acetate (100)	liquid	4
acidic methanol/water (80:20 + 0.1 M formic acid)[d]	liquid	−80
basic methanol/water (80:20 + 0.1 M NH_4OH)	liquid	−80
acidic acetonitrile/methanol/water (40:40:20 + 0.1 M formic acid)[d]	both	−20
basic acetonitrile/methanol/water (40:40:20 + 0.1 M NH_4OH)	both	−20
acetonitrile/methanol/water (40:40:20)	both	−20
acidic acetonitrile/water (80:20 + 0.1 M formic acid)[d]	both	4
basic acetonitrile/water (80:20 + 0.1 M NH_4OH)	both	4
acetonitrile/water (80:20)	both	4

[a] Both = both liquid cultures and filter cultures; liquid = liquid cultures. [b] The temperature used for the first extraction step was the coldest convenient temperature given the freezing and phase separation behavior of the solvent mixtures. For example, at <0 °C, acetonitrile/water (80:20) tends to separate into two phases and the aqueous-rich phase freezes; accordingly, this solvent mixture was used at 4 °C. [c] For extraction of filters with methanol/water, the first extraction step employed pure methanol (ref 10), and the subsequent steps used methanol/water (80:20). [d] Because certain metabolites have limited stability at acidic pH, the acidic extracts were neutralized using ammonium hydroxide immediately following completion of the extraction process. Head-to-head comparison of neutralized versus non-neutralized samples revealed generally better metabolite yields in the neutralized samples. Similar neutralization (using formic acid) was also tested for the basic pH samples but was not routinely conducted because it did not improve the results.

ruptive alteration of the cellular nutrient environment.[9,10] To establish the filter cultures, exponentially growing *E. coli* (5 mL/filter, $OD_{650} \sim 0.1$) were filtered onto 82 mm, 0.45 μm pore size nylon membrane (MAGNA, GE Osmonics, Minnetonka, MN). The filters were then placed face up on agarose plates loaded with complete minimal media. To prepare the plates, ultrapure agarose was washed three times in cartridge-purified water to remove trace contaminants and then added to the desired liquid media at 1.5% by weight. Filter cultures were harvested at $OD_{650} \sim 0.35$, with the OD determined by washing the filter-bound cells vigorously into 5 mL of media; the cells reach OD_{650} 0.35 at ~2.5 h after loading them onto the filter. To quench metabolism and initiate the extraction process, the cell-loaded filters were dropped into extraction solvent as described below.

Metabolite Extraction. For cells grown in liquid culture, 300 μL of extraction solvent was added to the collected cell pellet to quench metabolism and initiate the extraction process. After 15 min at the solvent temperature indicated in Table 1, the mixture was spun in a microfuge for 5 min at maximum speed and 4 °C to separate insoluble materials from the extracted metabolites. The resulting pellet was then re-extracted twice with 200 μL of solvent at 4 °C. All three of the supernatants were combined to yield 700 μL of final extract.

For filter extractions, the cell-loaded filter membrane was quenched by placing it cell side down in a 100 mm polystyrene tissue culture dish filled with 2.5 mL of extraction solvent at the temperature indicated in Table 1. After 15 min, the cell−solvent mixture was removed from the dish and set aside. An additional 1 mL of solvent was used to wash the filter to ensure that all the cellular material had been removed, and the resulting solution was combined with the initial ~2 mL extract. The resulting ~3 mL volume was split into two Eppendorf tubes and spun in a microfuge for 5 min at maximum speed and 4 °C, and the soluble extract was set aside. The residual pellets were each re-extracted twice with 50 μL of solvent at 4 °C for a total extraction volume (after absorptive solvent losses) of ~3 mL.

For the water immiscible solvents, the extraction procedure was the same as above, except that the two phases were divided after conclusion of the extraction steps. The phases were then analyzed separately by LC−MS/MS. For the chloroform/methanol mixture, the upper (aqueous) phase was ~200 μL of the ~700 μL total volume. For ethyl acetate, the lower (aqueous) phase was ~100 μL of the ~700 μL total volume. For almost all of the studied (generally hydrophilic) compounds, the vast majority of the signal was associated with the aqueous phase. Reported yields for the water immiscible solvents (Figure 1) accordingly refer to the aqueous portion of the extract and are corrected for small fractional volume of that phase (e.g., for ethyl acetate, reported signal = observed signal × 100 μL/700 μL).

Metabolome Quantitation. All cell extracts were analyzed by LC−MS/MS on a Quantum Ultra triple-quadrupole MS (Thermo Electron, Waltham, MA). LC separation used an aminopropyl column (Luna, Phenomenex, Torrance, CA) at pH 9.4 in hydrophilic interaction chromatography (HILIC) mode as described previously.[11] Mass spectrometry parameters were as previously described,[11] with the exception of some updated MRMs (Supporting Information Table 1). Samples were analyzed via separate runs in positive and negative ion mode. Except when otherwise noted, for positive mode the column dimensions were 100 mm × 1 mm and the gradient was $t = 0$ min, 85% B; $t = 12$ min, 0% B; $t = 24$ min, 0% B; $t = 26$ min, 85% B; $t = 35$ min, 85% B. The flow rate was $t = 0$ min, 50 μL/min; $t = 19$ min, 50 μL/min; $t = 20$ min, 70 μL/min; $t = 33$ min, 70 μL/min; $t = 34$ min, 50 μL/min; $t = 35$ min, 50 μL/min. For negative mode, the column dimensions were 50 mm × 2 mm and the gradient was $t = 0$ min, 85% B; $t = 3$ min, 40% B; $t = 10$ min, 0% B; $t = 20$ min, 0% B; $t = 21$ min, 85% B; $t = 27$ min, 85% B. The flow rate was $t = 0$ min, 100 μL/min; $t = 16$ min, 100 μL/min; $t = 18$ min, 200 μL/min; $t = 25$ min, 200 μL/min; $t = 26$ min, 100 μL/min; $t = 27$ min, 100 μL/min. Solvent A is 20 mM ammonium acetate + 20 mM ammonium hydroxide in 95:5 water/acetonitrile. Solvent B is acetonitrile. All samples were analyzed within 16 h of their preparation.

Data Analysis. Quadruplicate samples (duplicates on each of two different days) were collected and analyzed for each extraction condition. Raw peak heights (in units of ion counts) were averaged over the four samples, with peaks falling below the lower limit of detection in a particular sample or extraction

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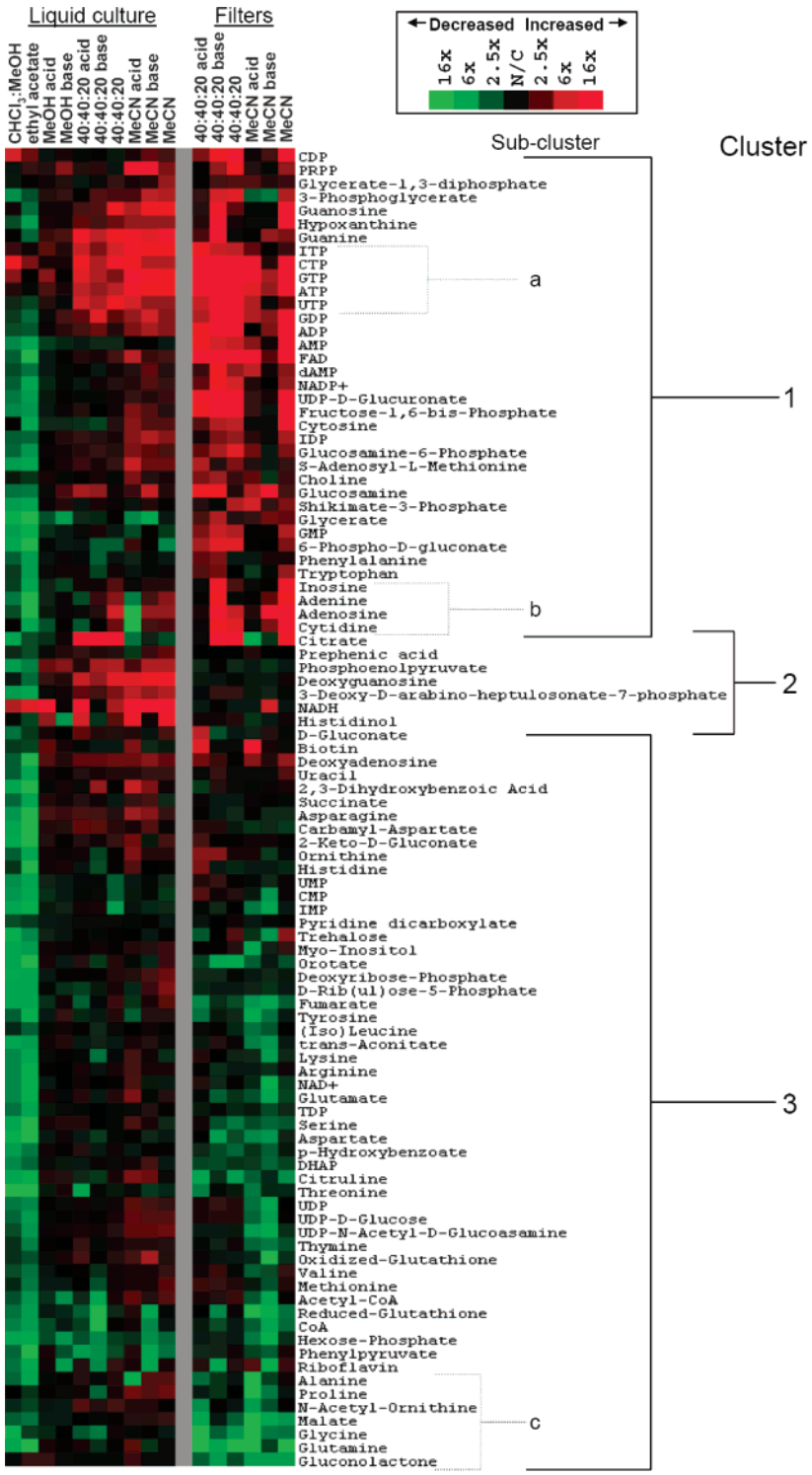

Figure 1. Clustered heat map of metabolite yields with different extraction solvents. Columns represent different extraction solvent mixtures applied either to cells pelleted from liquid culture or to filter cultures. Rows represent individual metabolites. Fold-change is relative to extraction with methanol/water (80:20) with red = larger measured metabolite yield from indicated solvent and green = larger yield from methanol/water. For details on the solvent mixtures, see Table 1. Abbreviations: $CHCl_3$/MeOH, chloroform/methanol (67:33); MeOH, methanol/water (80:20); 40:40:20, acetonitrile/methanol/water (40:40:20); MeCN, acetonitrile/water (80:20); acid, 0.1 M formic acid; base, 0.1 M ammonium hydroxide.

condition assigned a value of 300 ion counts, the upper bound of the typical instrument noise. All peak heights for the pellet samples were normalized against the corresponding peak height for the methanol/water (80:20) pellet extract; filter culture peak heights were normalized against the corresponding peak height for the methanol/water (80:20) filter extract. The normalized peak heights were $\log_2$-transformed prior to hierarchical clustering by metabolite and heat map display using the Princeton Microarray database (http://puma.princeton.edu)[12] as follows:

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$$\text{Value used for clustering and display} = \log_2 [\text{average}_{N=4} (\text{peak height for metabolite in test extract}) / \text{average}_{N=4} (\text{peak height for metabolite in methanol/water extract})]$$

Supporting Information Table 2 provides the $\log_2$-transformed data in Excel format organized as required for clustering using the Princeton Microarray Database software.

To assess reproducibility across samples, relative standard deviations (RSDs) of the quadruplicate samples were calculated as follows:

$$\text{RSD} = [\text{standard deviation}_{N=4} (\text{peak height for metabolite}) / \text{average}_{N=4} (\text{peak height for metabolite})]$$

The statistical significance of the differences across extraction conditions were evaluated by two-tailed, unpaired *t* test.

Isotope Labeling and Carbon Starvation. For ^{13}C-labeling studies, cells were grown a minimum of 25 generations on uniformly ^{13}C-glucose (>99.9%; Cambridge Isotope Laboratories, Andover, MA). For experiments involving spiking of the extraction solvent with unlabeled standard compounds, the concentrations of spiked compound were as follows: ATP (10 μg/mL), ADP (12 μg/mL), AMP (10 μg/mL), CTP (1 μg/mL), GTP (1 μg/mL), NADH (3.5 μg/mL), NADPH (3.5 μg/mL). The absolute cellular amounts of adenosine nucleotides was determined as previously described[13] based on the extraction volume, the ratio of the ^{13}C-labeled (cellular) to unlabeled (spiked standard) peak heights, the concentration of the unlabeled standard, and the mass of *E. coli* extracted. *E. coli* culture at $OD_{650} = 0.35$ had a cell dry weight (CDW) of 0.16 mg/mL.

For the transient N-labeling and carbon starvation studies, cells in filter culture were transferred to agarose plates containing media in which either unlabeled ammonia had been replaced by an equal concentration of ^{15}N-ammonia (99%; Cambridge Isotope Laboratories) (N-labeling) or in which glucose had been omitted (C-starvation). For C-starvation, the cells were transferred serially to two different, identical agarose plates lacking glucose (the time sitting on the first C-starvation plate was 2 min); the serial transfer minimized carryover of glucose from the complete minimal media plate to the second C-starvation plate. After growth on the modified medium for 10 min (C-starvation) or 15 min (N-switching), the cultures were quenched and extracted and the signals for unlabeled metabolites were measured. Unlabeled metabolite levels in the test cultures were normalized to those in control cultures to determine either the fraction of metabolite remaining unlabeled (N-switch) or the fractional change in metabolite concentration (C-starvation). The statistical significance of the resulting metabolomic changes were evaluated by two-tailed, unpaired *t* test.

RESULTS AND DISCUSSION

Solvent Mixtures Containing Acetonitrile Improve Metabolite Yields. We examined the ability to extract known water-soluble cellular metabolites from *E. coli* with a spectrum of different solvent mixtures, all under cold conditions.[2,3] Experiments were performed for both liquid cultures harvested by centrifugation and for filter cultures, which have the critical advantage over cell pellets of enabling rapid quenching of *E. coli* metabolism without metabolome perturbation.[6] The precise solvent mixtures and extraction temperatures are listed in Table 1. Compounds were grouped by hierarchical clustering,[14] and data was plotted in heat map format, normalized against extraction with 80:20 methanol/water, a common current approach (Figure 1). The data in Figure 1 are means of $N = 4$ independent biological replicates. The median inter-replicate RSD was 33% for the liquid culture samples and 41% for the filter culture samples, with similar RSDs found across different extraction conditions (with the exception of the extraction of filter cultures with acidic acetonitrile/water, for which the RSD was uniquely large with a median of 62%). These RSDs, while nontrivial, are small relative to the differences between the extraction conditions (the scale of the heat map is from 16-fold decreased to 16-fold increased).

The left-most columns in Figure 1 show results for extraction of *E. coli* pellets with chloroform/methanol (67:33), ethyl acetate, or acidic or basic methanol/water (80:20). None of these mixtures had major advantages in terms of metabolite yields over 80:20 methanol/water, as indicated by the paucity of red boxes in these columns. Accordingly, these solvents were not studied further.

The columns in the center and right of Figure 1 show analogous data for extraction of *E. coli* pellets and filter cultures with solvent mixtures containing acetonitrile/water. Each acetonitrile-containing mixture gave markedly enhanced yields of a substantial number of compounds compared to methanol/water (cluster 1; notice the high proportion of red boxes for this cluster in the acetonitrile-containing columns). The extraction power of the acetonitrile-containing solvent mixtures was absolutely reliant on the inclusion of water—pure acetonitrile gave very low metabolite yields (Supporting Information Figure 1). It was also reliant on immediate extraction with a solvent mixture containing acetonitrile. Initial extraction with methanol/water followed by an acetonitrile-containing mixture gave results more similar to methanol/water alone (Supporting Information Figure 2).

Acetonitrile/Methanol/Water Mixtures Are Preferred for Extracting Filter Cultures. Compounds that extract preferentially with acetonitrile-containing solvent mixtures are enriched in cluster 1 of Figure 1. For most of these compounds, similar solvent preferences were found for cell pellets and for filter cultures. Also, extraction with acetonitrile/water (80:20) and with acetonitrile/methanol/water (40:40:20) generally gave similar results. For the seven compounds in cluster 2, acetonitrile extraction was preferred for cell pellets but not substantially for filter cultures.

Compounds without a substantial preference for acetonitrile cluster in the lower half of Figure 1 (cluster 3). For many of these compounds, extraction of filter-bound cells with acetonitrile/water alone was somewhat deficient (as indicated by the high fraction of green boxes in the three right-most columns of cluster 3). This deficiency was largely rectified with acetonitrile/methanol/water,

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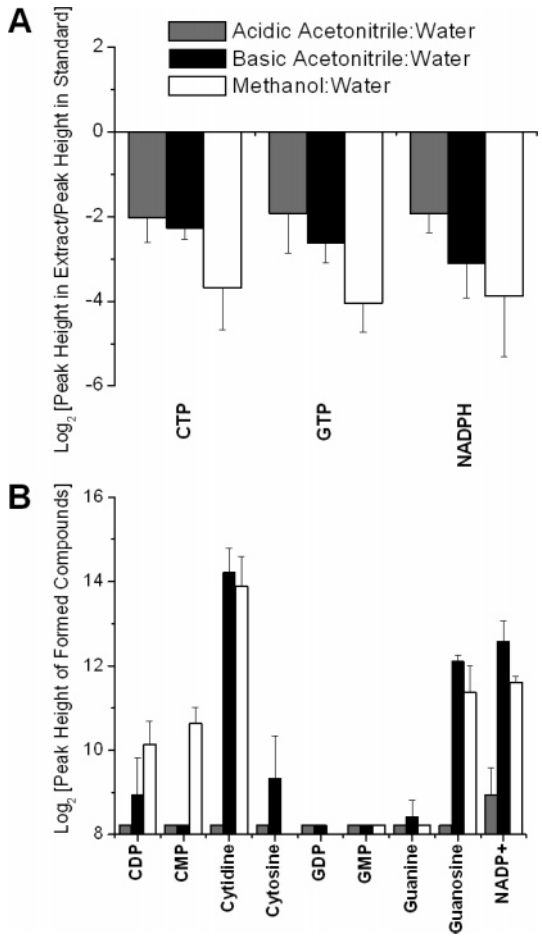

Figure 2. Extraction with acidic (0.1 M formic acid-containing) acetonitrile/water (80:20) minimizes degradation of high-energy into low-energy compounds during the extraction process. Cells were grown in uniformly ^{13}C-labeled glucose to differentiate endogenous metabolites from spiked, unlabeled CTP, GTP, and NADPH and their unlabeled degradation products. (A) Spiked compound losses as a function of extraction solvent. (B) Formation of degradation products of the spiked CTP, GTP, and NADPH as a function of extraction solvent. Error bars are SD of $N = 4$ independent replicates.

which is accordingly a preferred solvent mixture for extraction of filter cultures. Acetonitrile/methanol/water also has a lower freezing point than acetonitrile/water, which is advantageous for rapidly cooling cells to quench metabolism.

The reasons for the differences in the preferred extraction solvents for cell pellets versus filter-bound cells are not currently clear. They are not due solely to metabolite binding to the filter surface, as standards spiked onto the filter were recovered efficiently using any of the tested methanol/water and acetonitrile/(methanol)/water mixtures. Thus, it appears that these differences emerged due to a three-way interaction between the cells, filter, and solvent mixture. A possible underlying cause involves different solvent mixtures differentially impacting cell adherence to the filters. The potential for differential extraction of filter-bound cells versus cell pellets, irrespective of the underlying cause, is a complication that needs to be taken into account in the development of metabolomic sampling procedures.

Nucleotide Triphosphates Strongly Prefer Acetonitrile-Containing Solvent Mixtures. Nucleotide triphosphates (subcluster "a" in Figure 1) generally showed ≥5-fold higher yields with acetonitrile-containing extraction solvent mixtures than with methanol/water. The extent of the increase was statistically significant (for each triphosphate, $p < 0.02$ for liquid cultures and $p < 0.0002$ for filter cultures). Remarkably, hierarchical clustering based on the pattern of metabolites yields (without explicit consideration of metabolites structures or biological roles) automatically grouped together all five of the measured nucleotide triphosphate species based on their strong preference for extraction with solvent mixtures containing acetonitrile. The probability of such coclustering of triphosphates based on chance alone is $<10^{-7}$.

Acidic Acetonitrile Minimizes Triphosphate Degradation. In general, the effects of methanol versus acetonitrile were substantially larger than those of solvent pH. One notable difference, however, between the acidic acetonitrile-containing mixtures and the unbuffered or basic mixtures was lower yields of nucleosides and bases in the acidic conditions (subcluster "b" in Figure 1). We have previously shown that extraction of *E. coli* with methanol/water results in nonphysiological formation of nucleosides and bases by triphosphate degradation.[7] Accordingly, we were curious whether the apparently poor yields of nucleosides and bases in the acidic acetonitrile conditions reflected worse extraction (undesirable) or less triphosphate degradation (highly desirable).

To distinguish between these possibilities, we grew *E. coli* in uniformly ^{13}C-glucose (to differentiate endogenous compounds from spiked, unlabeled standards) and extracted with solvent containing several high-energy unlabeled compounds: CTP, GTP, and NADPH. Extraction with acidic acetonitrile minimized losses of the spiked compounds (Figure 2A; note that the lower loss of NADPH in the acidic than the basic condition contrasts with literature reports of better NADPH stability in base than acid[11,15,16] and may relate to the presence of acetonitrile). The presence of acid also decreased the degradation of the spiked CTP and GTP into nucleotide monophosphates, nucleosides, or bases (Figure 2B). Similar trends were obtained also with acetonitrile/methanol/water (40:40:20) (Supporting Information Figure 3). Thus, the acidic acetonitrile-containing extraction mixtures desirably prevent triphosphate decomposition.

More Complete Metabolite Extraction Increases the Risks of Ion Suppression. Many compounds gave bigger signals with the acidic acetonitrile-containing extraction mixtures; relatively few gave substantially smaller ones. Nevertheless, we were curious as the origin of the apparently poor extraction of a subset of compounds, most notably glutamine and glycine (subcluster "c" in Figure 1), with acidic acetonitrile.

Visual inspection of the insoluble debris left after metabolome extraction revealed that the acetonitrile-containing mixtures much more completely solubilized *E. coli* than did methanol/water. This raised the possibility that, by extracting a broader spectrum of molecules, the acetonitrile-containing mixtures might be generating more complex extracts which were more prone to induce ion suppression artifacts in LC−MS/MS analysis.

To examine this possibility, we compared the signals for a variety of compounds from acidic acetonitrile/methanol/water versus methanol/water extracts, after separation on a 10 cm versus

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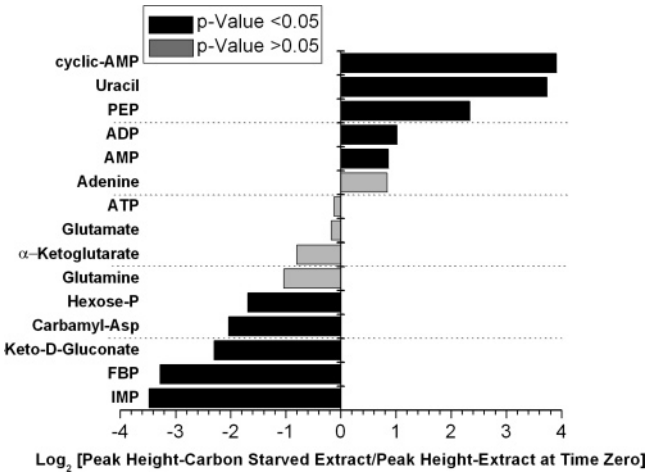

Figure 3. Metabolome changes in response to carbon starvation of *E. coli* for 15 min. Data are from filter cultures extracted with acidic acetonitrile/methanol/water (40:40:20). The compounds shown were selected for either their general metabolic importance or for changing markedly upon starvation. For data on all measured compounds, see Supporting Information Table 3.

25 cm chromatography column (Supporting Information Figure 4). The longer column, by providing better separation (at the expense of reduced signal-to-noise, especially for strongly retained compounds), rescued the apparently poor yields of amino acids in the acidic acetonitrile-containing condition. Thus, compared to methanol/water, acidic acetonitrile is not deficient in extraction of any of the studied compounds. The richness of the acidic acetonitrile extract is, however, a double-edged sword—for certain metabolites, higher quality separation is required due to the increased extract complexity.

Acidic Acetonitrile Extraction and Macromolecule Decomposition. A potential artifactual source of apparently effective metabolite extraction is production of free metabolites by macromolecule decomposition during the extraction process. Because the acid can catalyze breakage of peptide or phosphate bonds, we wished to rule out the possibility that macromolecule decomposition was artificially inflating metabolite yields for the acidic acetonitrile-containing extraction mixtures. To this end, we switched exponentially growing *E. coli* filter cultures from unlabeled ammonia to ^{15}N-ammonia and measured the levels of unlabeled intracellular metabolites 15 min thereafter. The doubling time of *E. coli* on minimal media is 75 min. Thus, macromolecules should be <25% labeled over 15 min. Nevertheless, the measured N-containing small-molecule metabolites were generally >50% labeled (as evidenced by a >2-fold decay in the unlabeled signal) within this time period, indicating that the measured compounds are indeed largely intracellular metabolites, not macromolecule degradation products. Both the most abundant amino acid (glutamate) and nucleotide (ATP) showed >90% labeling.

Initial Biological Tests of Acidic Acetonitrile Extraction. To further examine the reliability of acidic acetonitrile/methanol/water extraction of *E. coli* filter cultures, we determined the absolute yields of adenosine nucleotides, by extracting uniformly ^{13}C-labeled cells with solvent spiked with known quantities of ATP, ADP, and AMP. We found the absolute concentrations of ATP, ADP, and AMP to be 12 μmol/gCDW, 0.64 μmol/gCDW, and 0.32 μmol/gCDW, respectively. The resulting adenylate energy charge, ([ATP] + [ADP]/2)/([ATP] + [ADP] + [AMP]), was 0.95. Notably, careful prior studies found somewhat lower ATP and higher ADP and AMP levels in *E. coli* and, accordingly, a substantially lower adenylate energy charge.[17,18] This likely reflects slow metabolism quenching and/or some degradation of ATP to ADP and AMP during previous extraction procedures.

We also examined the metabolome of *E. coli* after 15 min of carbon starvation via acidic acetonitrile/methanol/water extraction of filter cultures. As shown in Figure 3 and Supporting Information Table 2, the most prominent metabolome changes include rising cAMP, uracil, and phosphoenolpyruvate, and falling FBP and IMP. Consistent with glucose withdrawal leading to a drop in the adenylate energy charge, there was a substantial (~2×) rise in AMP and ADP; however, ATP levels fell only a trace amount on this time scale, consistent with previous literature.[19] In general, the metabolome responses to glucose removal were consistent with those reported previously.[10]

CONCLUSIONS

A key challenge in extraction of *E. coli* is the propensity for bacterial components to catalyze triphosphate decomposition even after exposure of the cells to organic solvent. This complication, which we have found also for yeast, albeit it to a lesser extent than for *E. coli* (E.K. and J.D.R., unpublished results), necessitated the development of the acidic acetonitrile/(methanol)/water extraction method presented here. The acidic acetonitrile-containing solvent mixtures minimize losses of high-energy metabolites and their conversion into low-energy derivatives during the *E. coli* extraction process. Its efficacy is probably due to the combination of acetonitrile and acid eliminating (via denaturation or a related process) residual catalytic activity of *E. coli* components.

The acidic acetonitrile/methanol/water mixture is compatible with our previously described filter culture methodology,[9,10] which enables fast quenching of *E. coli* metabolism and is robust to the tendency of bacteria to leak metabolites very quickly upon exposure to cold solvent.[20,21] The combination of the filter culture technique and the acidic acetonitrile-containing extraction mixture enables markedly improved measurement of key physiological parameters, such as cellular energy charge, in *E. coli*.

Currently, the extent to which the present acidic acetonitrile/methanol/water approach is adequate for extraction of the full scope of the *E. coli* metabolome remains unknown. It is likely that other extraction procedures will prove superior for certain classes of metabolites. In addition, the extent to which the optimum metabolome extraction procedures will prove to be organism-specific[22−24] remains unclear. Beyond providing an

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improved approach to extraction of *E. coli*, the present work provides a battery of assays that can be used to address these larger analytical issues.

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SUPPORTING INFORMATION AVAILABLE

Supporting Information Table 1, MRM parameters; Supporting Information Table 2, Microsoft Excel file containing data shown graphically in Figure 1; Supporting Information Table 3, complete data for 15 min C-starvation of *E. coli*; Supporting Information Figure 1, extraction with acetonitrile alone vs acetonitrile/water; Supporting Information Figure 2, immediate extraction with acetonitrile/water vs serial extraction with methanol/water followed by acetonitrile/water; Supporting Information Figure 3, extraction with acidic acetonitrile/methanol/water minimizes degradation of high-energy into low-energy compounds during the extraction process; Supporting Information Figure 4, apparently poor yield of glutamine with acidic acetonitrile extraction is due to ion suppression. This material is available free of charge via the Internet at http://pubs.acs.org.

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