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Increasing the buffering capacity of minimal media leads to higher protein yield

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Abstract

We describe a general and simple modification to the standard M9 minimal medium recipe that leads to an approximate two-fold increase in the yield of heterologously expressed proteins in *E. coli* BL21(DE3) bacteria. We monitored the growth of bacteria transformed with plasmids for three different test proteins in five minimal media with different concentrations of buffering salts and/or initial media pH. After purification of the over-expressed proteins, we found a clear correlation between the protein yield and change in media pH over time, where the minimal media that were the most buffered and therefore most resistant to change in pH produced the most protein. And in all three test protein cases, the difference in yield was nearly two-fold between the best and worst buffering media. Thus, we propose that increasing the buffering capacity of M9 minimal media will generally lead to a similar increase for most of the proteins currently produced by this standard protein expression protocol. Moreover, we have qualitatively found that this effect also extends to deuterated M9 minimal media growths, which could lead to significant cost savings in these preparations.

Introduction

A prerequisite for structural and dynamics studies by biomolecular NMR techniques is the production of milligram quantities of stable isotopically labeled samples. While higher fields and cryogenically cooled probes have lowered the quantity of protein required for NMR studies, the fact remains that higher quality data will be acquired in a shorter amount of time on more concentrated samples. An initial barrier to NMR studies can be the sub-optimal quantities of protein produced from heterologous expression in *E. coli* bacteria. This problem is more acute when considering the loss in yield and the increase in cost associated with producing the highly deuterated samples that are required for TROSY-based NMR studies of larger (>30 kDa) macromolecules (Kay and Gardner 1997; Gardner and Kay 1998). A first step in undertaking NMR structural and dynamics studies on a new system is often to screen protein expression conditions, such as the concentration of IPTG for transcription induction of heterologous expression and/or the temperature and duration for protein expression, to maximize protein yield. Other optimizations may include the strain of cells used for protein expression (e.g., CodonPlus™ or Origami™), changing the plasmid backbone, or codon optimization of the gene of interest (Sahdev et al. 2008).

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When expressing difficult proteins, for instance eukaryotic or membrane proteins, or preparing highly deuterated, side chain methyl group $^{13}CH_3$-labeled samples, additional parameters may have to be improved for robust protein production. For example, the bacteria acclimatization process into minimal media and cell density at the time of expression can also be optimized. Early examples of acclimatizing bacteria include growing cells on agar plates or in liquid culture with increasing ratios of D_2O:H_2O (Venters et al. 1995; Gardner et al. 1997; Gardner and Kay 1998). Recently, several reports have appeared describing new methods for increasing protein yield using higher cell densities, fermentation techniques, or amino acid drop-out algae lysate supplements (Cai et al. 2016; O'Brien et al. 2018; Klopp et al. 2018). Whereas impressive gains in protein expression are noted in each, many of these methods require special equipment (e.g., fermenters) and none are as simple as the typical minimal media and methods for heterologous protein expression.

In this communication, we describe an extremely simple modification to the basic M9 minimal media recipe – increasing the buffering capacity – that provides an approximate two-fold increase in the amount of protein (tested herein) expressed from *E. coli* bacteria in protonated minimal media. For human ubiquitin and *Pyrococcus furiosus (P. furiosus)* Mre11 and $Rad50^{NBD}$, we find a good anti-correlation for the change in minimal media pH during bacterial growth and the amount of protein obtained after Ni^{2+}-affinity purification (i.e., the less the pH changes over time, the more over-expressed protein is obtained). We think that this is a general trend whereby similar expression enhancements will be obtained for the majority of proteins already being expressed in M9 minimal media for NMR studies. Since we are only adding extra buffer salts, the increased cost of this method (~$0.60/L of culture) is insignificant in the total cost of an isotopically labeled growth. Moreover, the two-fold increase means that the same quantities of protein can be obtained in correspondingly smaller culture volumes, representing a true cost savings in terms of isotopes. In our laboratory, we have found that increasing the buffering capacity of the minimal media also leads to nearly the same two-fold increase in protein yield when producing highly deuterated $^{13}CH_3$-side chain methyl group labeled samples. Here, the potential cost savings are more dramatic as the ^{2}H,^{12}C-D-glucose and D_2O isotopes used for these bacterial growths are much more expensive.

Materials and methods

Protein expression and purification

Table 1 lists the composition of each media used to screen for protein expression: (1) M9, which is based off the minimal medium originally formulated by Anderson, as described by Maniatis (Anderson 1946; Sambrook et al. 1989); (2) 2x M9, which has twice the buffer salt concentration of (1); and (3) N9, which also has twice the salt concentration as (1) with an adjusted ratio of Na_2HPO_4 and KH_2PO_4 to provide a slightly more basic medium (pH 7.1 vs pH 7.2). Two other minimal media were also used in the screen: M9 with an initial pH ~7.4 (adjusted with 1 M NaOH from the usual pH ~7.1 for normal M9 (1)) and M9+, a minimal medium recently described by Clore and co-workers (Cai et al. 2016). Because we were interested in the effects that the buffer composition and pH of the various media would have on cell growth and protein expression, the concentrations of NH_4Cl, D-glucose, trace metals,

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and vitamins (thiamine and biotin at 1 μg/mL final concentration each) were held constant across the various media being tested (see Table 1). For the preparation of each media, buffer salts (i.e., Na_2HPO_4, KH_2PO_4, K_2HPO_4, NaCl, $MgSO_4$, K_2SO_4, $MgCl_2$) were dissolved into 1 L H_2O and sterile filtered (0.2 μM bottle cap vacuum filtration device – MilliPore) into autoclaved bottles. NH4Cl, D-glucose, trace metals, and vitamins were then added from sterile stock solutions. Completed media were sterile transferred into autoclaved flasks for cell growth.

Ca^{2+}-competent *E. coli* C41(DE3) cells (Sigma) were transformed with plasmids encoding for 6x histidine-tagged human ubiquitin (pET29 – kan^R), *P. furiosus* Mre11 (pET22 – kan^R), or *P. furiosus* $Rad50^{NBD}$ (pRSETa – amp^R). C41(DE3) cells, which are used for overexpression of potentially toxic proteins (Miroux and Walker 1996; Dumon-Seignovert et al. 2004), were used here because initial expression screens for *P. furiosus* Mre11 and $Rad50^{NBD}$ revealed a requirement for this bacterial strain to get appreciable yield. Since we want to compare media and not different cell strains, we used this strain for ubiquitin as well. For each test protein, cells from the transformation plate were used to inoculate a 25 mL LB starter culture supplemented with the appropriate antibiotic (either 50 μg/mL kanamycin or 100 μg/mL ampicillin) in a 250 mL Erlenmeyer flask. LB starter cultures were grown at 37 °C with shaking (220 rpm). After ~6–8 hours, the LB starter cultures were used to inoculate 25 mL of each minimal media, supplemented with either 50 μg/mL kanamycin or 100 μg/mL ampicillin, to an optical density at 600 nm (OD_{600}) of ~0.05. The overnight minimal media cultures were grown at 37 °C with shaking (220 rpm) for ~18 hours (i.e., overnight) in 250 mL Erlenmeyer flasks. These overnight cultures were then used to inoculate 500 mL of the corresponding minimal media, supplemented with either 50 μg/mL kanamycin or 100 μg/mL ampicillin, to a starting OD_{600} of 0.15 (corresponding to volumes of ~10 – 25 mL depending on the OD_{600} of the overnight culture) in un-baffled 2.8 L Fernbach flasks. The cells continued to grow at 37 °C with shaking (220 rpm). At various timepoints, 3 mL samples of each culture were removed to measure the OD_{600} and media pH values. When the cell density of each culture reached OD_{600} ~1 for ubiquitin and Mre11 or ~0.7 for $Rad50^{NBD}$, 1 mM IPTG was added to each culture to induce protein expression, which lasted for three (Mre11) or four (ubiquitin and $Rad50^{NBD}$) hours at 37 °C. A lower OD_{600} for $Rad50^{NBD}$ induction was used because a primary screen of expression conditions revealed better yield after induction at a lower OD_{600}. The cells were removed from the media by centrifugation at 5500 rpm for 10 minutes in a Beckman JA-10 rotor. Cell pellets were resuspended in 40 mL of 50 mM $NaPO_4$, pH 8.0, 300 mM NaCl, 25 mM imidazole buffer; the cell suspension was frozen at −20 °C prior to protein purification.

Cell suspensions were thawed at room temperature, and PMSF (0.1 mg/mL) and lysozyme (0.25 mg/mL) were added to each. The cells were lysed by sonication for ubiquitin and Mre11 and homogenization (Avestin EmulsiFlex-C3) for $Rad50^{NBD}$. The cell lysates of Mre11 and $Rad50^{NBD}$ were subsequently heated at 65 °C for 30 minutes (*P. furiosus* is a hyperthermophilic organism). The insoluble material was removed by centrifugation at 17,000 rpm in a Beckman JA-20 rotor for 30 minutes. The soluble fraction was filtered through a 0.45 μm syringe filter (Sartorius) before loading onto a 5 mL HisTrap HP (GE Biosciences) column. After loading the protein onto the column, nonspecifically bound proteins/nucleic acids were removed by washing with 2 column volumes of 50 mM $NaPO_4$,

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pH 8.0, 1.5 M NaCl, 25 mM imidazole buffer. Protein was then eluted with 4 column volumes of 50 mM $NaPO_4$, pH 8.0, 300 mM NaCl, 300 mM imidazole.

Bacterial growth rates were determined with Eqn. 1, which describes discrete exponential growth. Linear least squares was used to find the slope of the linear portion of the natural log of the culture OD_{600} versus time, which was then used to find *r*, the growth rate for the population in the exponential phase.

$$\frac{d(\ln(OD_{600}))}{dt} = \ln(1 + r) \quad (1)$$

Changes in media pH (pH: the average pH decrease over time) for each condition were determined by subtracting the initial pH value at the time of inoculation from the pH at the time of cell harvest and dividing by the total time in which the data was collected (i.e., the total time the cells grew, which varied between eight to fourteen hours). The final protein concentrations and amounts were determined using the absorbance of the elution from the Ni^{2+}-affinity column at 280 nm (Abs_{280}), the final elution volume, and the Beer-Lambert Law: ubiquitin (ε = 1490 M^{-1} cm^{-1}, MW = 8565 Da), *P. furiosus* Mre11 (ε = 62800 M^{-1} cm^{-1}, MW = 49460 Da), *P. furiosus* $Rad50^{NBD}$ (ε = 35870 M^{-1} cm^{-1}, MW = 43050 Da).

Results and discussion

For each of the five minimal media tested (Table 1), we monitored the growth of the *E. coli* C41(DE3) cells via the optical density at 600 nm (OD_{600}; Fig. 1 panels A – C) and the change in the pH of the minimal media (Fig. 1 panels D – F) over time. Fig. 1 shows the results for human ubiquitin (panels A and D), *P. furiosus (Pf)* Mre11 (panels B and E), and the nucleotide binding domain (NBD) of *Pf* Rad50 (panels C and F). Qualitatively, the bacteria transformed with plasmids encoding ubiquitin or Mre11 grew similarly in M9, 2x M9, M9 with adjusted pH, and N9 media; whereas, the growth curves for bacteria in M9+ media are shifted to the right indicating that these cells grew at a slightly slower rate. In each of these cases, the bacteria grew with the typical lag, logarithmic, and stationary phases. Bacteria transformed with the plasmid encoding *Pf* $Rad50^{NBD}$ on the other hand grew with more complicated growth curves. While the cells grown in M9, 2x M9, and N9 media showed the typical bacterial growth patterns, the M9 with adjusted pH culture experienced a slowdown in growth partway through the logarithmic phase. Cells in M9+ on the other hand experienced a longer lag phase compared to cells in the other media before entering the logarithmic growth phase. Also note, that our growth curves for all of the media reach the stationary phase at the same cell density (OD_{600} ~4 for ubiquitin and Mre11 and OD_{600} ~2 for $Rad50^{NBD}$), depending on the transformed plasmid. This is quite a bit lower than the OD_{600} ~12 for the stationary phase reported by Clore and co-workers for M9+ (Cai et al. 2016). This difference is most likely due to the lower concentration of glucose used in this work (3 g/L *vs.* 18 g/L), and it implies that glucose concentration is limiting in our media. We used the data in Fig. 1, panels A – C and Eqn 1 to calculate growth rates during the logarithmic phase, which are listed in Table 2. Focusing on the bacterial cells transformed with ubiquitin and *Pf* Mre11, which displayed more typical growth curves, cells in M9

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(0.628 and 0.643 OD_{600}/hr for ubiquitin and Mre11, respectively) or 2x M9 (0.653 and 0.634 OD_{600}/hr, respectively) media grew more rapidly; whereas, the bacteria grew more slowly in M9 with adjusted pH (0.571 and 0.595 OD_{600}/hr, respectively) and the slowest in M9+ (0.519 and 0.543 OD_{600}/hr, respectively), indicating that the higher initial pH of these two media, pH ~7.5, may not be optimal for bacterial growth. However, we should point out that since the M9+ medium was designed to excel at higher OD_{600} (i.e., greater cell density), the growth rate of bacteria in the M9+ medium with additional glucose should accelerate over time as the OD_{600} increases and the pH of the medium decreases. The slower growth rate at lower OD_{600} (for lower concentrations of glucose) can be overcome by lowering the concentration K_2HPO_4 such that the initial pH is 7.2 rather than 7.5.

Fig. 1 also shows that for each of the media tested, the pH decreases over time. By and large, the decrease follows the lag, logarithmic, and stationary phases seen in the plots of OD_{600} over time and is expected for cells that are producing acidic byproducts (e.g., H^+ and acetate) from the metabolism of glucose (Roe et al. 1998). For each of three plasmid transformations, the largest decrease in pH is seen for cells grown in M9 and M9 with adjusted pH media. This behavior is expected since these media contain the lowest concentration of phosphate buffer (69.94 mM). Smaller decreases in pH over time were observed for the bacterial cells grown in the 2x M9, N9 and M9+ media, which contain 139.89, 143.26, and 209.21 mM phosphate buffer, respectively. Again, this result is expected given the higher concentrations of buffers. We then calculated an average pH decrease (pH) of the media, which is the difference between the final pH (measured at cell harvest) and the initial pH (measured before inoculation of the 500 mL culture) divided by the total time the cells were grown (ranging between 8 and 14 hours) and are given in Table 2. Unlike the growth rates, which were fairly uniform for each media across the different transformations, the pH values showed variation that is protein specific (Table 2). For example, cells transformed with *Pf* Rad50^{NBD} had the smallest change in pH (0.015 – 0.035 pH/hour), while cells transformed with *Pf* Mre11 had the largest change (0.031 – 0.080 pH/hour). We have also calculated pH rates over the time of protein induction (i.e., subtracting the pH at the time of induction from the pH at the time of cell harvest and dividing by the time for protein expression – three or four hours, data not shown). The trends during the protein overexpression time period follow the data presented in Fig. 1 and Table 2: M9 and M9 with adjusted pH media show the largest changes in pH (0.16 and 0.13, respectively, for *Pf* Mre11); whereas, the more buffered media showed lesser pH value – 0.10, 0.097, and 0.072 for *Pf* Mre11 grown in 2x M9, N9, and M9+ media, respectively. These results demonstrate a plasmid or heterologous protein specific effect on the metabolism of the *E. coli* cells (see below).

To see what effect the differences in growth rate and/or changes in pH have on protein yield, we induced over-expression of the three proteins by adding 1 mM IPTG (final concentration; the red lines in Fig. 1 panels A – C indicate the OD_{600} at time of induction). After a uniform amount of time, cells were separated from the media, lysed, and the expressed proteins were subsequently purified via Ni^{2+}-affinity chromatography. As shown in SDS-PAGE gels presented in the top of Fig. 2, good protein expression and purification are seen for each condition. However, qualitative differences can be seen in the expression of each protein. To understand these differences, we determined the total amount of protein that eluted from the

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Ni^{2+}-affinity column using the Abs_{280} and calculated molar extinction coefficients. For each of these three proteins, we see a clear anti-correlation between the amount of protein obtained after the Ni^{2+}-affinity column (in mg of purified protein) and the change in pH during bacterial growth and expression (Fig. 2, bottom). The clearest correlation between the amount of over-expressed protein and pH is obtained for ubiquitin (Pearson's correlation coefficient [R] = −0.84). Weaker correlations were seen for *Pf* Mre11 and $Rad50^{NBD}$ (R = −0.58 and −0.64, respectively); however, it is clear that more protein is obtained when the pH decreases less. No correlations were observed between growth rate and purified protein or between growth rate and the average decrease in pH (data not shown). We also compared the amount of over-expressed protein against the pH calculated from data over the three or four hours of protein induction. Again, a clear anti-correlation is seen between the amount of purified protein and the rate of change of the pH values during expression (data not shown). In all cases, more protein is obtained from media with extra buffering capacity (i.e., 2x M9, N9, and M9+), while the least amount of protein was purified from cells grown in M9 and M9 with adjusted pH media. In fact, we obtained 2.5 – 1.9 times more protein from the best performing media compared to the worse (M9 with adjusted pH; Table 2). Note, in our tests, there is no clear winner for which minimal media produces the most protein: for ubiquitin and Mre11, 2x M9 gave the higher yields, while M9+ produced more $Rad50^{NBD}$. Finally, unlike the bacteria growth rates, we find that the higher initial pH of the M9+ does not affect protein expression. Thus, the composition of the minimal medium, focusing on increasing the buffering capacity, should be tested during optimization of heterologous protein expression to increase protein yield.

Studier has observed that bacteria grown in high concentrations of phosphate (> 100 mM) can develop resistance to kanamycin (Studier 2005). However, we do not think that the bacteria grown in 2x M9, N9, or M9+, which all have phosphate concentrations greater than 100 mM, have developed a resistance to kanamycin (here, ubiquitin and Mre11). First, Studier observed this phenomenon in defined media that also contained amino acids and other nutrients, which are absent in minimal media (Studier 2005). Second, a resistance to the antibiotic would allow the bacteria to shed the plasmid resulting in less protein expression, and as Fig. 2 and Table 2 show, 2x M9 or M9+ provide the highest yield of over-expressed protein. Thus, we do not think that bacteria transformed with plasmids containing the kanamycin resistance marker will become resistant to the antibiotic in the high phosphate minimal media.

We used C41(DE3) cells for our media tests. As mentioned above (Materials and methods), we found a strict requirement to use these cells for heterologous expression of *Pf* Mre11 and $Rad50^{NBD}$. Since these cells were originally derived from *E. coli* BL21(DE3) for their ability to over-express a toxic membrane protein (Miroux and Walker 1996), we expect that similar increases in yield in higher buffered minimal media should be realized with the parental strain (i.e., BL21(DE3)) and its derivatives such as CodonPlus™ or Origami™, which are the predominant strains used in NMR studies. Also, our study utilized the genes of test proteins in pET and pRSET (pUC-derived) plasmids. pET- and pUC-based plasmids have several differences: pET vectors are generally larger and have a medium copy number (~15–60/cell) (Bolivar et al. 1977; Rosano and Ceccarelli 2014); on the other hand, pUC vectors are generally smaller and have a higher copy number (~500–700/cell) (Minton 1984;

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Rosano and Ceccarelli 2014). Protein expression from these vectors is driven by the *T7lac* (pET) and *T7* (pRSET) promotors. It is interesting to note that the bacteria grown in 2x M9 medium produced more protein from heterologous expression of genes in the pET vectors (ubiquitin and Mre11; Table 2), while cells grown in M9+ medium produced more $Rad50^{NBD}$, which is on the pRSET plasmid (Table 2). However, without more extensive testing it is impossible to know how general this trend is. The pET and pUC plasmid backbones are nevertheless the workhorse vectors used by NMR labs; thus, we expect that our observation will be generally applicable to the majority of protein systems studied by these groups.

Finally, while we have not extensively tested the effects of buffering capacity on bacterial growth and protein over-expression in deuterated minimal media, we have empirically found that we obtain on average ~1.66 times more *Pf* $Rad50^{NBD}$ when using deuterated 2x M9 compared to the yield from cells grown in M9. Furthermore, we get very little expression of *Pf* Mre11 in deuterated M9, which in fact led to this study. Of course, the extra buffered media (i.e., 2x M9 or M9+) should be tested to find optimal protein yield in highly deuterated methyl labeled samples in order to realize real cost savings. If found to follow the trends described herein (Fig. 2 and Table 2) and our empirical results, these increases in yields represent a real potential for cost savings as the same amount of protein could be obtained from two-thirds the amount of D_2O (~$400 per kg) and ^{2}H, ^{12}C-labeled D-glucose (~$100 per g) with the additional salts representing only $0.60/L extra cost.

Concluding remarks

We have shown that the simple act of increasing the buffering capacity of minimal media leads to higher yields of heterologous expressed proteins from standard *E. coli* bacteria. Instead of focusing on different medium acclimation techniques, driving up cell density, or using additives, we took the more straightforward approach of examining the buffering system of the medium. Therefore, our findings could be more general and subsequently extended to other bacterial cell growing protocols. For example, Wand and co-workers have described a method utilizing drop-out deuterated algal lysate (lacking isoleucine, leucine, and valine) for the production of highly deuterated, side chain methyl group labeled proteins in protonated medium (O'Brien et al. 2018). While that described procedure utilized 1.5x buffered minimal media and produced substantial gains, our results suggest that even higher yields could be achieved with 2x buffered media. Moreover, our modification is also readily applicable fermentation systems. Lastly, because our method is probably very general, every NMR spectroscopist can double the concentration of buffering salts in their minimal media, with no additional cost to the sample preparation, and easily obtain more protein for increased signal-to-noise and decreased experimental time.

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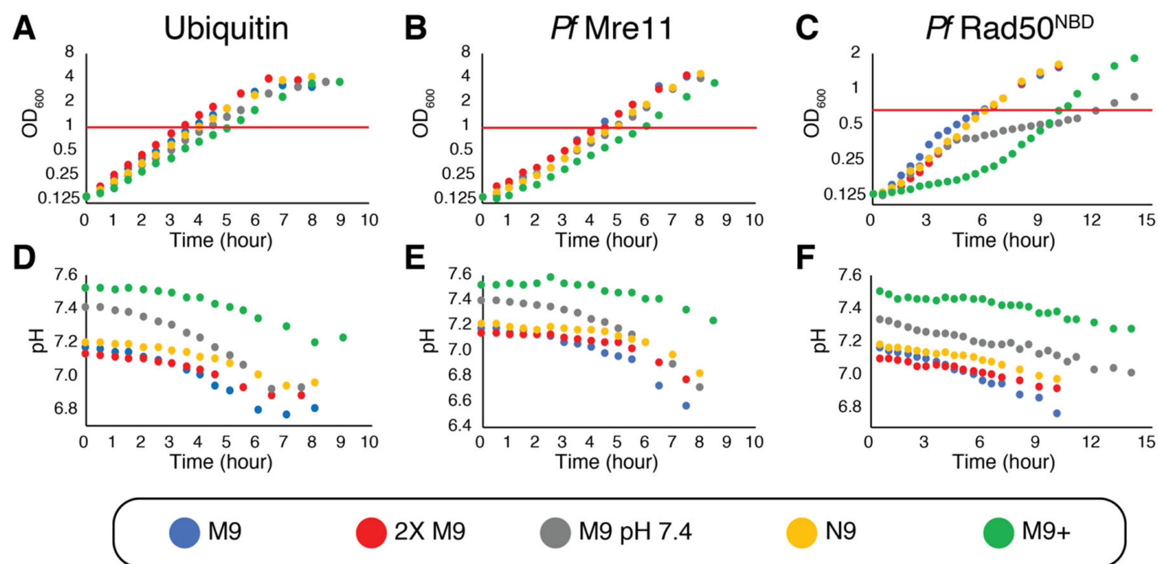

Fig. 1.

Growth and media pH curves for *E. coli* C41(DE3) cells transformed with human ubiquitin (A and D), *Pf* Mre11 (B and E), and *Pf* Rad50^{NBD} (C and F) heterologous expression plasmids. The upper panels (A – C) plot cell density, measured by OD_{600}, as a function of time; the lower panels (D – F) plot media pH as a function of time. In all panels, blue, red, grey, yellow, and green circles represent observations made from cells grown in M9, 2x M9, M9 with adjusted pH, N9, and M9+ minimal media, respectively. See Table 1 for minimal media compositions. The red lines in the growth curves (A – C) indicate the OD_{600} where the protein expression was induced.

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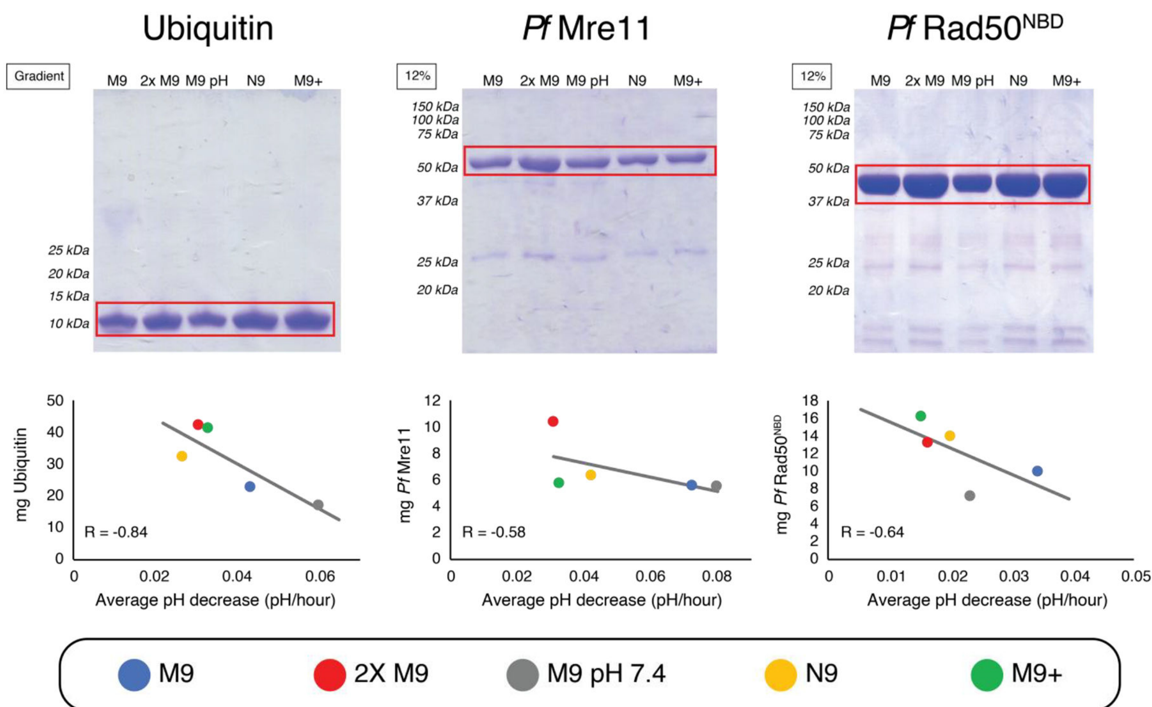

Fig. 2.

Expression of human ubiquitin, *Pf* Mre11, and *Pf* Rad50^{NBD} from *E. coli* grown in different minimal media. The top panels show SDS-PAGE of samples from the Ni^{2+}-affinity elutions for the three proteins: 3 μL of ubiquitin, 4 μL of Mre11, and 5 μL of Rad50^{NBD} elutions were loaded for each media test respectively on the gels. Bands corresponding to the given protein are outlined by red boxes and positions of the molecular weight standards are given on the left of each gel. The bottom panels show correlation plots of the quantity of protein obtained from the Ni^{2+}-affinity column versus the average pH decrease. The color of the points, as described in Fig. 1, represents the media in which the bacterial cells were grown. Pearson's correlation coefficient (R) for each graph is given in the lower left corner.

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Table 1

Composition of minimal media used in this study. The listed quantities are for 1 L of media.

Chemical	M9[d]	2x M9	N9	M9+[e]
Na_2HPO_4	6.8 g	13.6 g	14.6 g	9 g
KH_2PO_4	3 g	6 g	5.5 g	5 g
K_2HPO_4	-	-	-	19 g
NaCI	0.5 g	1 g	1 g	-
K_2SO_4	-	-	-	2.4 g
$MgSO_4 \cdot 7H_2O$	0.25 g	0.5 g	0.5 g	-
$MgCI_2$	-	-	-	0.95 g
$(NH_4)_2SO_4$[a]	1 g	1 g	1 g	1 g[e]
D-Glucose[a]	3 g	3 g	3 g	3 g[e]
Trace metals solution[b]	0.5 mL	0.5 mL	0.5 mL	0.5 mL
Vitamins[c]	1 mL	1 mL	1 mL	1 mL

[a]Unlabeled $(NH_4)_2SO_4$ and D-glucose were used for expression tests as the sole nitrogen and carbon sources, respectively.

[b]Trace metals solution contains 27 g $FeCl_3 \cdot 6H_2O$, 1.3 g $ZnCl_2$, 2.0 g $Na_2MoO_4 \cdot 2H_2O$, 2.5 g $CaCl_2 \cdot 2H_2O$, 2.0 g $CoCl_2 \cdot 6 H_2O$, 1.3 g $CuCl_2 \cdot 2H_2O$, 3.3 g $MnCl_2 \cdot 4H_2O$, 0.5 g H_3BO_3 and 100 mL HCl in 1 L of H_2O. The solution is sterile filtered, aliquoted, and stored at −20 °C until used.

[c]Stock solution of 1 mg/mL each thiamine and biotin.

[d]M9 medium with adjusted pH was made according to the M9 recipe. The pH was subsequently increased to pH ~7.4 with the addition of 1 M NaOH.

[e]Recipe for M9+ from Cai *et al* (Cai et al. 2016). The original medium composition contains 5 g/L NH_4Cl and 18 g/L D-glucose.

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Table 2

Comparison of bacterial growth rates, average pH change, and protein expression for the five minimal media that were tested.

	Ubiquitin			***Pf* Mre11**			***Pf* Rad50^{NBD}**		
	Growth rate[a]	**pH**[b]	**Yield**[c]	**Growth rate**[a]	**pH**[b]	**Yield**[c]	**Growth rate**[a]	**pH**[b]	**Yield**[c]
M9	0.63	0.043	22.7	0.64	0.073	5.5	0.22	0.035	9.9
M9 adjusted pH	0.57	0.060	16.9	0.60	0.080	5.5	0.25	0.023	7.1
N9	0.61	0.027	32.4	0.63	0.042	6.3	0.32	0.020	14.0
2x M9	0.65	0.031	42.2	0.63	0.031	10.4	0.35	0.016	13.1
M9+	0.52	0.033	41.4	0.54	0.033	5.7	0.37	0.015	16.2

[a] Growth rates were calculated from the logarithmic portion of the growth curves in Fig. 1 using Eqn. 1 and have units of OD_{600}/hour.

[b] The average pH change was calculated by subtracting the initial pH, when the cultures were inoculated, from the final pH, when the cells were harvested, and dividing by the total growth time. pH has units of pH/hour.

[c] Yield of the protein, in milligrams, after Ni^{2+}-affinity chromatography calculated from the extinction coefficient and molecular weight as described in Material and methods.