Engineering Escherichia coli BL21(DE3) Derivative Strains To Minimize E. coli Protein Contamination after Purification by Immobilized Metal Affinity Chromatography

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Received 19 January 2011/Accepted 4 May 2011

Recombinant His-tagged proteins expressed in Escherichia coli and purified by immobilized metal affinity chromatography (IMAC) are commonly coeluted with native E. coli proteins, especially if the recombinant protein is expressed at a low level. The E. coli contaminants display high affinity to divalent nickel or cobalt ions, mainly due to the presence of clustered histidine residues or biologically relevant metal binding sites. To improve the final purity of expressed His-tagged protein, we engineered E. coli BL21(DE3) expression strains in which the most recurring contaminants are either expressed with an alternative tag or mutated to decrease their affinity to divalent cations. The current study presents the design, engineering, and characterization of two E. coli BL21(DE3) derivatives, NiCo21(DE3) and NiCo22(DE3), which express the endogenous proteins SlyD, Can, ArnA, and (optionally) AceE fused at their C terminus to a chitin binding domain (CBD) and the protein GlmS, with six surface histidines replaced by alanines. We show that each E. coli CBD-tagged protein remains active and can be efficiently eliminated from an IMAC elution fraction using a chitin column flowthrough step, while the modification of GlmS results in loss of affinity for nickel-containing resin. The “NiCo” strains uniquely complement existing methods for improving the purity of recombinant His-tagged protein.

Over the past 25 years, several techniques and tools have been developed to express and purify recombinant proteins for protein structure-function studies, for the development of new drugs, or simply for the manufacture of enzymes. The most frequently used method for isolating recombinant protein from a cell lysate in a single purification step is immobilized metal ion affinity chromatography (IMAC). In the simplest application of this method, the target protein is tagged with a polyhistidine sequence (typically 6×His), which mediates chelation to immobilized divalent metal ions such as nickel or cobalt. Other studies have demonstrated that peptides with nonconsecutive histidines are also capable of chelation to immobilized divalent metal ions (5) (U.S. patent 7,176,298 [41] and U.S. patent application 2006/0030007 A1).

Escherichia coli is the most commonly used host for high-yield expression of recombinant protein, usually by exploiting the high promoter specificity and transcriptional activity of bacteriophage T7 RNA polymerase. However, several E. coli host proteins also contain nonconsecutive histidine residues exposed to the surface of their tertiary structure. In addition, metal binding motifs often mediate binding to nickel- and/or cobalt-containing purification resins. Such host proteins are routinely copurified during IMAC procedures and are therefore referred to as “contaminants.” Several metal binding proteins that behave as IMAC contaminants have been identified in recent years. For example, Bolanos-Garcia et al. reviewed this issue in detail by classifying the E. coli metal binding proteins according to their affinity for Ni-nitrilotriacetic acid (NTA) resin by determining the imidazole concentration required for elution (5). Among 17 E. coli IMAC contaminants described, 15 were reported to elute from Ni-NTA at an imidazole concentration of >55 mM, a concentration which is higher than advised for most IMAC column washing procedures. Thus, most of the cited contaminants are eluted only when the imidazole concentration is increased to a level that elutes the histidine-tagged protein of interest. Also, variable amounts of host protein contaminants are detected depending on the expression system used (genetic background of the strain and plasmid) and the culture conditions employed (medium, carbon source, oxygen, temperature, and cell density at the induction time and at the harvest time).

Various techniques for improving the purity of a His-tagged protein of interest have been described in the literature. First, an alternative to imidazole washing and elution is used to elute the protein of interest with an acidic buffer. At pH <6, the histidine side chain in most contexts becomes protonated and loses its affinity for divalent metals. However, the same is true for contaminant proteins enriched in histidine residues. Therefore, low-pH elution may not result in discrimination between elution of contaminants and elution of the protein of interest. Some impurities can also be reduced by adjusting growth conditions (culture conditions, medium composition, and the genetic background of the strain), but this method of addressing the problem is empirical. Secondary chromatographic steps, of course, may be carried out, e.g., size exclusion chromatography.

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† Supplemental material for this article may be found at http://aem.asm.org.
‡ Published ahead of print on 20 May 2011.
(31), protein specific chromatography (Heparin affinity chromatography described by Finzi et al. [10] or immunooaffinity chromatography described by Muller et al. [29]). But these approaches usually require time-consuming optimization procedures that are dependent on the properties of the target protein. Dual affinity tags, which imply the use of a second affinity column, also provide for improved target protein purity. For example polyhistidine has been combined with glutathione S-transferase (GST) or maltose binding protein (MBP) (36, 45). However, important concerns raised by employing double tags are risks of proteolysis or aggregation of the protein after cleavage of the first tag.

Deleting the genes of the most abundant contaminants also may not be a viable solution. For example, SlyD, a peptidyl prolyl cis-trans isomerase, is cited as the most frequent IMAC contaminant. However, E. coli SlyD suffer from a significant growth defect (38, 44). Furthermore, the majority of E. coli contaminants are critical for cell viability, especially under the stressed conditions caused by high-yield protein expression (5). Therefore, E. coli knockout strains for particular contaminants (such as chaperones or other stress response factors) have not been seriously considered as solutions for avoiding contamination of His-tagged target protein. As a new strategy, we engineered BL21(DE3), the most widely used E. coli strain for high-yield expression of recombinant protein, to express the major NiNTA binding proteins with an alternative tag. As a result, the tagged contaminants can be rapidly removed either before or after IMAC capture of the target protein.

**MATERIALS AND METHODS**

**Media and cultures.** All strains were routinely grown using Luria-Bertani (LB) liquid broth or agar (27) at 20°C, 30°C, or 37°C. All media were supplemented with appropriate antibiotics as follows: ampicillin at 100 µg/ml and chloramphenicol 4 µg/ml for maintaining the mini F<sup>+</sup> plasmid pFOS1-lacIq or 10 µg/ml for maintaining pMAK705 constructs. Tests with BL21(DE3) gms-CBD were performed on tryptone broth or agar (LT) containing 10 g tryptone, 5 g NaCl, and 15 g agar (22, 42), supplemented with appropriate antibiotics and 200 µg/ml N-acetylglucosamine, when necessary, and incubated at 37°C. The AceE purification was accomplished by purification on NiNTA agarose. The dissolved oxygen was kept above 20% of air saturation using proportional, integral, and differential control (PID) control of agitation (400 rpm to 600 rpm) and mixing pure oxygen with air (the gas flow rate was 0.5 vessel volume per minute [vvm]). The temperature was controlled at 37°C for both growth and induction. Once the culture reached an optical density at 600 nm (OD<sub>600</sub>) of 8 to 10, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to give a final concentration of 20 µM and the culture was incubated for an additional 3 h.

For shake flask expression of AlaRS in BL21(DE3) and NiCo21(DE3), cells carrying pQE30-alaRS and pFOS1-lacIq (which provides a source of Lac repressor to control expression from the T5lacO promoter) were grown in LB medium supplemented with 100 µg/ml of ampicillin. After outgrowth at 37°C to an OD<sub>600</sub> of ~0.8, 200 µM IPTG was added and the cultures were incubated for 4 h at 30°C (final OD<sub>600</sub> = 2.2 to 2.6; cell pellet, ~2 g).

For shake flask expression of GluRS in BL21(DE3), NiCo21(DE3), and NiCo22(DE3), cells carrying pET21a-glusRS were grown in 500 ml of LB medium supplemented with 100 µg/ml of ampicillin. After growth at 37°C to an OD<sub>600</sub> of ~0.5, 20 µM IPTG was added and the cultures were incubated for 4 h at 30°C (final OD<sub>600</sub> = 2.4 to 2.6). Cells containing pET21a were grown using the same procedure in order to prepare mock lysates for the lysate-mixing experiment (see Fig. 7).

**Enzymes and reagents.** Restriction enzymes and DNA-modifying enzymes were provided by New England Biolabs. Mutagenesis was carried out using a Phusion site-directed mutagenesis kit (New England Biolabs). DNA amplification procedures utilized either Phusion or Taq DNA polymerases.

**Strains and plasmids.** Bacterial strains and genotypes are listed in Table 1. Oligonucleotides and plasmids are described in Tables S1 and S2, respectively, in the supplemental material. The pMAK-CBD vector for 3<sup>rd</sup> end gene tagging was created by inserting the CBD open reading frame (ORF) from vector pTYB1 (New England Biolabs) into the polylinker region of pMAK705 (14). An amino acid linker region which encodes EQSSQ<sub>18</sub>, where the first LQ codons correspond to a PstI restriction site and the last Q5 codons contain a Sall restriction site, was inserted. A unique AsuI restriction site was inserted after the CBD ORF. All allele exchange procedures utilized derivatives of pMAK-CBD containing DNA fragments amplified from the BL21(DE3) chromosome. Genes of interest were cloned into HindIII, SphI, PstI, and/or SacI sites. Downstream DNA (3’ flanking sequence) was cloned into AsiSI, Acc65I, SacI, and/or EagI sites.

To create the strain BL21(DE3) slyD-CBD, pMAKslyD-CBD was transformed into BL21(DE3) and individual clones were grown in LB liquid medium with 4 µg/ml of chloramphenicol (CM). The allele exchange method of Hamilton et al. (14) was followed to replace the wild-type (wt) slyD gene with the slyD-CBD allele. The strains positive for allele exchange were cured of the pMAK vector carrying the wild-type slyD gene using a coumermycin treatment (8). PCR amplification with primers 4059For and 4060Rev confirmed that the slyD-CBD allele was present at the correct locus within the chromosome of BL21(DE3) slyD-CBD.

To create NiCo21(DE3), the allele exchange procedure was carried out in the same manner as that described for slyD-CBD except (i) <i>can</i> locus analysis was accomplished using primers 4841For and 2187Rev, (ii) analysis of the araA-arnD locus to confirm the araA-CBD-arnD allele was accomplished using primers...
directed-mutagenesis kit (New England BioLabs). The primer s1260 (Ptac forward), s1233 (pMAK705 polylinker forward), or s1224 (pMAK705 polylinker reverse).

Phage buffer A2. Elution was completed using a gradient from 20 mM to 400 mM sodium phosphate, pH 7.4, 500 mM NaCl, and 400 mM imidazole. A pool of eluted fractions was concentrated using a Vivaspin column (Vivascience) and then dialyzed against buffer A2 (20 mM sodium phosphate, pH 7.4, 300 mM NaCl, and 10 mM imidazole). The dialyzed His6-tagged target proteins were overexpressed from a vector that carries the chitin binding domain (CBD) sequence: AceE, GroES, and GroEL. Based on these previous studies, these previous reports were CRP, Fur, ArgE, DnaK, SlyD, AtfP, the Rho transcription terminator, CRP, and SlyD (data not shown). Several independent studies, performed using different conditions or different E. coli strains, also reported many of the same contaminants after Ni2+-affinity chromatography (5, 13, 18).

RESULTS

In order to confirm the major E. coli metal binding proteins cited in the literature, three different recombinant His-tagged target proteins were overexpressed from a vector in BL21(DE3). An automated AKTA FPLC system was used in combination with a 1-ml HisTrap column to perform a standard fractionation of the cell lysates. Mass spectrometry (MS) analysis of the target protein elution fractions revealed that the following E. coli host proteins also coeluted in significant amounts: DnaK, GlmS, AceE, EF-Tu, AraN, RnaseE, AtfP, the Rho transcription terminator, CRP, and SlyD (data not shown). Several independent studies, performed using different conditions or different E. coli strains, also reported many of the same contaminants after Ni2+-affinity chromatography (5, 13, 18). The most common E. coli proteins listed in these previous reports were CRP, Fur, ArgE, DnaK, SlyD, GlmS, GlgA, ODO1, ODO2, Can (YadF), AraN (YfbG), AceE, GroES, and GroEL. Based on these previous studies and our analysis, we chose the following consistent contaminants to tag with the chitin binding domain (CBD) sequence: SlyD, GlmS, Can, AraN, and AceE. The selection criteria also included the possibility of checking the activity of each tagged protein when expressed from the chromosome.

To create the desired expression strains, we first generated a plasmid encoding each candidate gene fused with the CBD ORF. The respective E. coli gene-CBD constructs were ex-
pressed in BL21(DE3) to examine the stability of the respective fusion protein. Anti-CBD immunoblots indicated that each fusion protein was not subject to in vivo proteolysis (data not shown), an outcome that would prevent the removal of the contaminant protein by tag-mediated chromatography. The plasmids encoding CBD fusion proteins were subsequently employed to modify the BL21(DE3) chromosome by homologous recombination at the native E. coli gene locus. The replacement of the native gene with the CBD fusion allele was performed using the allele exchange method described by Hamilton et al. (14). Efficient allele exchange occurs when the exchange vector contains at least 300 bp of homology to both 5′ and 3′ regions flanking the target site on the bacterial host chromosome. Thus, for each allele replacement step, homologous DNA sequences of at least 300 bp were cloned at the 5′ and the 3′ ends of the CBD sequence in the pMAK-CBD vector (see Materials and Methods). E. coli BL21(DE3) derivatives were generated by replacing each candidate allele one by one. The phenotype of each derivative strain was analyzed after each replacement step, and then each strain was tested as an expression host for one or more recombinant His-tagged proteins (E. coli alanyl-tRNA synthetase and/or E. coli glutamyl-tRNA synthetase).

(i) Major Ni-NTA contaminant SlyD (CBD-tagged) is removed by incubation with chitin beads. As a proof of principle, we chose first to address the most predominant Ni-NTA contaminant, SlyD. SlyD is a cytoplasmic protein originally identified in E. coli as a host factor required by bacteriophage phiX174 to induce cell lysis. The gene slyD (sensitivity to lysis) was first identified in a genetic selection for E. coli C strains resistant to lysis gene E of phage phiX174 (23). The protein was then isolated and characterized in a further study as a host factor required by bacteriophage phiX174 to induce cell lysis. The gene slyD contained 24 histidines, of which 15 are organized in four clusters, the N-terminal part (residues 1 to 146) containing the amidotransferase activity while the C-terminal domain contains the ketose/aldose isomerase activity. Respective active site residues are located at each extremity of the protein (position 2 for the amidotransferase and position 604 [of 609 residues] for the isomerase) (Fig. 3A). Since our attempt to place the CBD tag at the N-terminal end of GlmS was also unsuccessful, we assumed that the addition of the CBD tag at the N or C terminus of GlmS disturbs the respective active sites located at the termini of the protein. Rather than using a tag to eliminate GlmS from His-tagged protein fractions, we chose to alter GlmS affinity for divalent cations by generating a GlmS mutant in which the most exposed histidines would be replaced by alanines. After examining the three-dimensional (3D) structure of the GlmS dimer, the isomerase active form of GlmS (28), we selected six surface-exposed histidines to nickel. GlmS is a 67-kDa L-glutamine:D-fructose-6-phosphate aminotransferase involved in an essential step of bacterial cell wall biosynthesis. The enzyme utilizes D-fructose-6-phosphate and L-glutamate to form D-glucosamine-6-phosphate, which is a precursor for components of the peptidoglycan or lipopolysaccharide. Among 609 residues, GlmS contains 24 histidines, of which 15 are organized in four clusters of at least 3 histidines exposed on the surface, giving the protein a high potential for interacting with Ni2+ and Co2+ cations according to the crystal structure described by Mouilleron et al. (28).

When we replaced the glmS allele with the glmS-CBD allele on the chromosome of BL21(DE3), the resulting BL21(DE3) glmS-CBD strain was nearly nonviable (Fig. 3B). However, complementation of the GlmS-CBD defect was demonstrated using tryptone medium (LT) with addition of 200 μg/ml of glucosamine (GlcN) or N-acetylglucosamine (GlcNac) (Fig. 3B) (42, 43). GlmS is actually composed of two domains in which the N-terminal domain holds the glutamine amidotransferase activity while the C-terminal domain contains the ketose/aldose isomerase activity. Respective active site residues are located at each extremity of the protein (position 2 for the amidotransferase and position 604 [of 609 residues] for the isomerase) (Fig. 3A). Since our attempt to place the CBD tag at the N-terminal end of GlmS was also unsuccessful, we assumed that the addition of the CBD tag at the N or C terminus of GlmS disturbs the respective active sites located at the termini of the protein. Rather than using a tag to eliminate GlmS from His-tagged protein fractions, we chose to alter GlmS affinity for divalent cations by generating a GlmS mutant in which the most exposed histidines would be replaced by alanines. After examining the three-dimensional (3D) structure of the GlmS dimer, the isomerase active form of GlmS (28), we selected six surface-exposed histidines for mutagenesis (Fig. 3A): histidines 62 and 65 are located in the center of a cluster of 4 exposed histidines, histidines 432 and 436 occupy a central position in another cluster, and finally, histidines 466 and 467 are part of a large cluster of 6 histidines highly surface exposed and generated by the dimer interface (data not shown). The selected histidines (positions 62, 65, 432, 435, 466,
and 467) are all poorly conserved residues among GlmS homologs (data not shown).

We first examined the activity of the resulting GlmS 6His-Ala protein in a complementation assay using the strain BL21(DE3) glmS-CBD. As shown in Fig. 3B, in the absence of GlcN or GlcNAc, GlmS 6His-Ala is able to restore the growth of BL21(DE3) glmS-CBD with the same efficiency as wild-type GlmS. Both proteins, GlmS and GlmS 6His-Ala, were expressed from the low-copy-number plasmid pMAK705 (14). Next, to test the affinity of GlmS 6His-Ala for Ni-NTA resin, we performed a standard purification on a Ni-NTA column, using BL21(DE3) lysate as a control compared to BL21(DE3) overexpressing GlmS6His-Ala or GlmS from pMAK705. Cell lysates were loaded on a 1-ml HisTrap column using an AKTA FPLC system, followed by standard washing and elution steps (20 to 400 mM imidazole), and the elution fractions were analyzed by Coomassie blue SDS-PAGE and by MS. As an additional control, we included an intermediate mutant protein, GlmS2His-Ala, in which only the histidines at positions 62 and 65 were replaced by alanines. Figure 3C shows that the band migrating at the expected position of GlmS (67 kDa) is detected in the sample overexpressing GlmS (lane “wt”), while the same band is less intense for the GlmS2His-Ala sample (lane labeled “2 ala”). Most importantly, overexpression of the GlmS6His-Ala mutant protein does not result in binding and elution of a 67-kDa protein from the Ni-NTA resin (compare lane “0Ala” with lane “-” where cell extract was analyzed from BL21(DE3) carrying an empty plasmid). Additionally, MS
analysis of the Ni-NTA elution fractions supports the SDS-PAGE observations. When analyzing Ni-NTA binding proteins in the control samples, we found 38 times more spectral counts of GlmS when wild-type GlmS is overexpressed from a plasmid than for chromosomal expression in the empty-plasmid control strain. In contrast, overexpression of mutant GlmS6His-Ala from a plasmid results in only a modest increase of GlmS peptide in the imidazole elution fraction (4 spectral counts). These data indicate that the replacement of 6 surface-exposed histidines by alanines significantly decreases the affinity of GlmS for Ni-NTA resin, while the removal of only 2 histidines has a limited effect. The complementation studies show that this mutagenesis does not compromise the ability of GlmS to supply GlcNAc (Fig. 3B).

(iii) Construction of NiCo21(DE3) and NiCo22(DE3). Replacement of the glmS gene with the glmS6Ala allele was the last step in the construction of the NiCo21(DE3) and NiCo22(DE3) protein expression strains. NiCo21(DE3) additionally contains the slyD, can, and arnA genes tagged with the CBD ORF, while NiCo22(DE3) additionally contains the slyD, can, arnA, and aceE genes tagged with the CBD ORF. The can gene (yadF) encodes a β-class carbonic anhydrase (Can), a zinc metalloenzyme which interconverts carbon dioxide (CO₂) and bicarbonate. This protein of 25 kDa exhibits four zinc-binding sites that confer a significant affinity for metal chelating resins. Moreover, Can expression increases in high-density cultivation, during slow growth, or during stress and starvation, in other words, conditions typically encountered during recombinant-protein overexpression (26).

The arnA gene encodes a bifunctional enzyme of 74.3 kDa, a UDP-1-Ara4N formyltransferase/UDP-GlcA C-4”-decarboxylase, involved in the modification of the lipid A required for lipopolysaccharide biosynthesis. Also this modification of lipid A with 4-amino-4-deoxy-L-arabinose confers to Gram-negative bacteria a resistance to the cationic antimicrobial peptides and antibiotics such as polymyxin (6). ArnA (also called YfbG) is found as a recurring contaminant in IMAC, presumably due to the several clusters of surface-exposed histidines detected in the 3D structure of the active hexamer (12).

The aceE gene encodes subunit E1 of the pyruvate dehydrogenase multienzyme complex formed from 12 dimers of subunit E1, 24 subunits of AceF, and 6 LpdA dimers (2). The major role of pyruvate dehydrogenase in the tricarboxylic acid (TCA) cycle is the production of acetyl-coenzyme A (CoA) from pyruvate (37). Although AceE (99.7 kDa) is not essential for viability, its inactivation reportedly leads to disturbance of carbon metabolism (21). The protein displays three magnesium-binding sites that might explain its affinity for metal chelating resins.

For each intermediate strain, we examined the growth rate at diverse temperatures (20°C, 30°C, and 37°C) in order to detect any effect on cell viability. We confirmed the expression and tested the activity of each CBD tagged protein in vivo.

The anti-CBD Western blot demonstrates the stable expression of the CBD-tagged proteins in NiCo21(DE3) and NiCo22(DE3). However, we observed that after addition of the aceE-CBD allele, NiCo22(DE3) grew more slowly in liquid culture and on agar plates than BL21(DE3) and NiCo21(DE3) (Fig. 4B). This suggests that the addition of a CBD tag at the C terminus of AceE affects the activity of the individual AceE protein and/or the formation of the pyruvate dehydrogenase complex.

During NiCo strain construction and thereafter, genome stability was verified by PCR and sequence analysis of the loci containing the CBD-tagged genes. When identical DNA sequence tags are present at either 3 or 4 times in a genome, it
is certainly possible for recombination to occur, resulting in deletion of intervening sequence to occur. In the case of the NiCo strains, this type of event would result in cell death (and loss of such mutants from the culture) since intervening sequences are extensive and contain essential genes.

(iv) In vivo activity analysis of each CBD-tagged candidate protein. To assess the activity of the SlyD-CBD protein, we used an in vivo approach involving the lysis protein E originally expressed from the bacteriophage phiX174 genome. SlyD is required to induce lysis of *E. coli* after infection by the phage phiX174 or in other *E. coli* strains (background B, C, and K-12) when protein E is expressed from a multicopy plasmid (38, 46). Wild-type *E. coli* is sensitive to lysis in both situations, while a slyD knockout strain is shown to be resistant. The lysis is actually the consequence of the inhibition, by protein E, of MraY, a conserved phosphotransferase involved in the formation of intermediates for the peptidoglycan biosynthesis (4, 25, 47). The lysis phenotype in BL21(DE3) and in the strains carrying slyD-CBD was examined by monitoring OD600 following induction of expression of the protein E cloned on pMS119 plasmid. Figure 5A shows that all the strains display a sensitivity to lysis after induction of the protein E, indicating that SlyD-CBD is active. Lysis depends on lysis protein E, since it does not occur with empty-vector pMS119.
The Can protein is essential for growth under normal atmospheric conditions, even though cells depleted of Can are able to survive when supplied with CO2 or when cynT, a paralog gene of can, is activated upon induction with cyanate or azide (15, 26). The isolated strain BL21(DE3) slyD-CBD can-CBD did not show evidence of a growth defect on plates or under standard liquid culture conditions, suggesting that Can expressed with a C-terminal CBD tag is fully functional (data not shown).

Given that the enzymatic modifications carried out by ArnA confer polymyxin resistance to E. coli, we tested the activity of ArnA-CBD by restreaking the strains on LB medium supplemented with polymyxin B. The assays were performed at a concentration of 2 μg/ml of polymyxin B as described in the literature (7). Figure 5B shows that this is the MIC for NiCo21(DE3) at 30°C, but at 37°C, the strain is fully sensitive to the same concentration. This result suggests that ArnA-CBD is generally less active than wild-type ArnA. Interestingly, NiCo22(DE3), which also expresses ArnA-CBD but contains one more CBD-tagged protein (AceE-CBD), is resistant to polymyxin B at both 30°C and 37°C temperatures. The noticeable phenotype of NiCo22(DE3) is its lower growth rate than those of NiCo21(DE3) and BL21(DE3) (Fig. 4B). We consequently hypothesize that the activity of ArnA-CBD may be compromised and that slow growth allows for this protein to carry out its functions in cell envelope formation.

Depletion of AceE or elimination of its pyruvate dehydrogenase activity results in E. coli dependent on acetate for aerobic growth on glucose medium (21). We therefore performed a plate assay on minimal medium supplemented with glucose (0.2%) with and without potassium acetate (2 mM) to access AceE-CBD activity. As controls, we used two E. coli aceE mutants, the strains CGSC5477 and CGSC4823 (9, 16), which both show a growth defect in the absence of acetate. Despite the fact that NiCo22(DE3) exhibits a lower growth rate than BL21(DE3) and NiCo21(DE3), its growth is not acetate dependent (Fig. 5C). These results suggest that the CBD tag at the C terminus of AceE does not significantly affect AceE activity. If AceE-CBD activity was abolished, NiCo22(DE3) growth could be supported in the absence of acetate by a higher level of expression of the protein PoxB. PoxB is a nonessential pyruvate oxidase whose role of converting pyruvate into acetate and CO2 contributes significantly to growth under aerobic conditions. Abdel-Hamid et al. identified some aceE mutants able to grow without acetate due to an increased activity of endogenous PoxB throughout the growth cycle (with yet a 30% decrease in the growth rate compared to the level for the wild-type strain) (1). Although the nature of the constitutive expression has not been identified, they proposed that the poxB promoter lost its dependence on RpoS, a sigma factor (RpoS, σS, and σA) required to induce genes in stationary phase (1). To address this uncertainty, we sequenced the poxB promoter of BL21(DE3), NiCo21(DE3), and NiCo22(DE3) and found no mutations in the promoter region of these strains, suggesting that PoxB is still well regulated by RpoS in NiCo22(DE3).

(v) Recombinant protein expression by NiCo21(DE3) and NiCo22(DE3). NiCo21(DE3) and NiCo22(DE3) were used to express the E. coli His-tagged alanyl-tRNA synthetase (AlaRS) or the His-tagged glutamyl-tRNA synthetase (GluRS) using two conditions of expression (standard shake flask cultures and batch fermentation). The purification procedure was performed on immobilized nickel using either nickel beads (Superflow; Qiagen) or a HisTrap column managed by an AKTA FPLC system. In both cases, NiNTA elution fractions were incubated with chitin beads and the protein profiles of the resulting flowthrough were compared to that of the NiNTA elution pool (no chitin incubation). Proteins were characterized by Coomassie blue-stained SDS-PAGE gels by Western blot analysis and by MS.

Glutamyl-tRNA synthetase (GluRS-6His) was expressed in each expression strain using a high-density batch fermentation process carried out at 30°C in rich medium monitored for glucose concentration and oxygen (see Materials and Methods). Figure 6 shows the purification of the GluRS-6His on Ni-NTA resin (Superflow; Qiagen) followed by incubation with chitin beads. The profiles of proteins eluted after Ni-NTA (lanes E) are similar for the three strains (Fig. 6A). After incubation with chitin beads (lanes FT chitin), the collected flowthrough is void of the main contaminants for NiCo21(DE3) and NiCo22(DE3) samples. The Western blot shown in Fig. 6B confirms the removal of the CBD-tagged proteins after treatment on chitin beads (compare lane E and lane FT chitin). Finally, MS analyses of the samples taken after Ni-NTA elu-

**FIG. 4.** Growth curves and detection of CBD-tagged proteins. (A) Western blot analysis (anti-CBD) of total cell extracts from three independent clones (1, 2, 3) of NiCo21(DE3) and NiCo22(DE3). The CBD-tagged proteins are indicated by arrows. (B) Growth of BL21(DE3), NiCo21(DE3), and NiCo22(DE3) performed at 30°C in LB medium.
tion and after chitin flowthrough are consistent with our gel analyses, showing that, first, the CBD-tagged proteins identified in the elution after Ni-NTA are absent in the chitin flowthrough and, second, that GlmS6His-Ala is significantly reduced in the NiCo21(DE3) and NiCo22(DE3) samples compared with GlmS identified in BL21(DE3) (Fig. 6C).

To demonstrate the utility of the NiCo strains for poorly expressed proteins, GluRS was expressed in BL21(DE3), NiCo21(DE3), and NiCo22(DE3) and each cell lysate was then mixed with a “mock” lysate prepared from the same strain grown with an empty expression vector (pET21a). A mixture of 1 ml GluRS lysate to 15 ml empty-vector lysate was chosen so that GluRS would be present at a very low concentration relative to host proteins (see lysate load [L] lanes in Fig. 7).

The lysate mixture (1:15) corresponding to all three strains was subjected to standard Ni-NTA chromatography (wash with 8 CVs of 20 mM imidazole and elution with 1 CV of 250 mM imidazole). The Ni-NTA elution samples resulting from NiCo21(DE3) and NiCo22(DE3) were additionally incubated with chitin resin for 30 min. Lane 3 in Fig. 7 shows the protein profile of the Ni-NTA elution sample from the BL21(DE3) lysate mixture, while lanes 6, 7, 10, and 11 show the improvement in purity obtained when NiCo strain elution samples were further processed by chitin incubation. Visual inspection of the SDS-PAGE gel image shows that the NiCo samples (Fig. 7, lanes 7 and 11) have significantly less contaminants than the BL21(DE3) sample. To confirm the improvement in purity, we measured target protein purity using a Caliper LabChip GXII protein assay. Strikingly, the target protein is 90.74% and 87.48% pure after chitin incubation when purified from NiCo21(DE3) and NiCo22(DE3), respectively. When GluRS is expressed in BL21(DE3), the purity is only 56.01% when a standard Ni-NTA purification procedure is followed.

FIG. 5. Activity assays for SlyD-CBD, ArnA-CBD, and AceE-CBD. (A) SlyD-CBD activity is confirmed by lysis sensitivity of BL21(DE3), NiCo21(DE3), and NiCo22(DE3) upon induction of protein E. Cultures of these strains harboring the plasmid pMS119-pE or pMS119 were induced at an OD_{600} of 0.15 to 0.55 with 500 μM IPTG, indicated by an arrow, and monitored for optical density. (B) Bacterial growth assays with BL21(DE3), NiCo21(DE3), and NiCo22(DE3) were carried out by spotting 5 μl of overnight culture, diluted from 10^{-2} to 10^{-7}, on LB medium with or without 2 μg/ml polymyxin B. The plates were incubated for 24 h at 30°C or 37°C. (C) Bacterial growth assays with BL21(DE3), NiCo21(DE3), NiCo22(DE3), and two aceE mutants, CGSC5477 and CGSC4823, were carried out by spotting 5 μl of overnight culture, diluted 10^{-2} to 10^{-7}, on minimal medium with 0.2% glucose and supplemented with 2 mM potassium acetate when indicated.
In addition, we performed purification analyses using imidazole elution gradients and found that contaminants are equally problematic compared to single-step elution. Histagged alanyl-tRNA synthetase (AAlaRS) was purified by loading cell lysates on a 5-ml HisTrap column, followed by standard washing with 20 mM imidazole and elution with a 20 to 400 mM imidazole gradient using an AKTA FPLC system. Elution fractions with high concentrations of target protein consistently displayed multiple contaminating proteins. SlyD-CBD and ArnA-CBD were eluted at about 80 to 140 mM imidazole, while AceE-CBD was eluted all along the elution gradient (see Fig. S2 in the supplemental material). Only Can-CBD appears to be washed out before the elution step, as confirmed by Western blotting. Importantly, incubating the target protein pool with chitin beads resulted in the removal of CBD-tagged contaminant proteins (Fig. S2).

NiCo21(DE3) and NiCo22(DE3) were initially designed to remove the most common contaminants found after Ni-NTA and in the flowthrough after chitin bead treatment.

FIG. 6. Purification of the glutamyl-tRNA synthetase (GluRS) expressed in NiCo21(DE3) and NiCo22(DE3). Strains BL21(DE3), NiCo21(DE3), and NiCo22(DE3) expressing GluRS(6His) from pET21a were propagated in a high-density fermentor as indicated in Materials and Methods. One gram of each cell pellet was processed to purify GluRS(6His) using 1 ml Ni-NTA superflow resin followed by 1 h of batch incubation on chitin beads (1 ml). (A) Samples collected during the procedure were analyzed by SDS-PAGE. Lane M, molecular mass marker in kDa; lane L, cell lysate load; lane FT, flowthrough; lane W, first wash; lane B, boiled chitin beads; lane E, Ni-NTA elution. (B) Western blot analysis (anti-CBD) of the samples shown in panel A. GluRS, wild-type SlyD (SlyDwt), SlyD-CBD, ArnA-CBD, Can-CBD, and AceE-CBD are indicated by a symbol or an arrow. (C) The table indicates the number of spectra of each protein identified by mass spectrometry analysis in the elution fractions after Ni-NTA and in the flowthrough after chitin bead treatment.
**BL21(DE3) NiCo21(DE3) NiCo22(DE3)**

**FIG. 7. Utility of the NiCo strains for poorly expressed proteins.** Coomassie blue-stained SDS-PAGE gel of samples taken during the purification of GluRS on Ni-NTA superflow resin (2 ml) followed by 30 min of batch incubation on chitin beads (2 ml). The purifications were performed from a mixed cell lysate containing 1 ml GluRS lysate and 15 ml of empty-vector lysate from the same strain. Lanes 1, 4, and 8 represent lysate mixtures loaded (L) onto Ni-NTA columns. Each mixture contains a low concentration of GluRS which becomes the prominent band in the 250 mM imidazole elution sample (E). ft, Ni-NTA flowthrough; FT<sub>c</sub>, sample taken of the void volume after incubating the Ni-NTA elution sample with chitin beads.

puriﬁcation. In order to determine the efﬁciency of protein puriﬁcation on the alternative cobalt resin, we performed a comparison assay between Ni-NTA (Superflow; Qiagen) and cobalt (Talon superﬂow; Clontech) resins using the standard protocols recommended by the respective manufacturers. The amounts of puriﬁed His-tagged protein (glutamyl-tRNA synthetase) were equivalent from both resins, but the cobalt resin often gave elution fractions less contaminated by native *E. coli* proteins (data not shown). Although SlyD was reported as having a very weak afﬁnity for cobalt resin (24), we found after mass spectrometry analysis that the most abundant contaminant on cobalt resin is still SlyD (data not shown). Among the *E. coli* proteins copuriﬁed with His-tagged GluRS on cobalt, we also identiﬁed the elongation factor EF-Tu and the ferric uptake regulator protein (Fur) known to bind Ni-NTA resin.

**DISCUSSION**

In this work, we present the characterization of two protein expression strains designed to improve purity of target protein after immobilized metal afﬁnity chromatography (IMAC). BL21(DE3) is the most widely used *E. coli* strain for protein overexpression and often provides the highest yield of target protein relative to endogenous proteins. For this reason, BL21(DE3) was chosen as the parent for the NiCo strains. NiCo21(DE3) and NiCo22(DE3) were both engineered to express the most common contaminants (SlyD, Can, ArnA, GlmS, and AceE) either with a tag for removal by a rapid chromatography ﬂowthrough step or with mutations to decrease afﬁnity for divalent cations. We have demonstrated that all the CBD-tagged proteins (SlyD, Can, ArnA, and AceE) are efﬁciently removed from the Ni-NTA elution pool after batch incubation with chitin beads and that the modiﬁed GlmS<sub>His-Ala</sub> protein has lost its afﬁnity for nickel resin. NiCo21(DE3) and NiCo22(DE3) are thus preferred expression strains for obtaining recombinant His-tagged target proteins with reduced levels of host protein contamination.

We have demonstrated that the addition of a CBD tag at the C-terminal end of SlyD and Can protein does not affect their function in vivo. Importantly, addition of the CBD tag may have favorably altered the afﬁnity of Can for nickel resin since we routinely observed Can-CBD in the 20 mM imidazole ﬂowthrough or wash fractions (see Fig. S2 in the supplemental material), whereas Bolanos-Garcia et al. report that wild-type Can is eluted from Ni-NTA by 55 to 80 mM imidazole (5). In NiCo21(DE3), ArnA-CBD was observed to be partially active at 30°C but apparently inactive at 37°C according to the polymyxin B sensitivity phenotype. However, in the slow-growing strain NiCo22(DE3), the function of ArnA-CBD appears normal at 37°C. One explanation for this inconsistency is that at a higher growth rate, cell envelope formation (and resistance to polymyxin B) demands an ArnA protein with a higher speciﬁc activity. Therefore, our results suggest that the CBD tag only compromises and does not abolish the function of ArnA in vivo.

In the ﬁnal strain NiCo22(DE3), the CBD tag modiﬁcation of AceE seems to be responsible for the lower growth rate of this strain. However, the lack of acetate dependence for aerobic growth on glucose medium suggests that AceE-CBD is still active. On the other hand, we designed our acetate dependency assay based on data collected from experiments performed with *E. coli* K-12, since the two available control aceE mutants are K-12 strains (21). K-12 and B strains are known to contain major differences in their respective metabolic pathways, particularly in pyruvate and acetate production (33, 34). For example, in high-density cultures, *E. coli* K-12 excretes high levels of acetate in the presence of excess glucose concentrations, which affects its growth rate, while *E. coli* BL21 is much less sensitive to glucose concentration and produces lower levels of acetate (32). It seems that not only are the lower levels of production of pyruvate and acetate in *E. coli* BL21 the result of more-active carbon metabolism (TCA cycle, glyco-neogenesis, glyoxylate shunt, and anaerobic pathways), but glucose transport may be better controlled in BL21 than in K-12 (30, 33). In conclusion, the acetate assay indicates that AceE-CBD is most likely active.

One advantage of using chitin beads is their compatibility with diverse buffer conditions (HEPES, sodium phosphate, Tris-HCl, 50 mM to 2 M NaCl, 6 M urea, pH 6 to 9, 0.1 to 1% Triton X-100, and high imidazole concentrations) that allow direct loading of the elution fractions after IMAC without performing dialysis. In the experiment using cobalt resin, SlyD-CBD was directly cleared from the elution sample after 30 min batch incubation on chitin beads (data not shown). We routinely use 1-ml chitin beads to remove the CBD-tagged proteins from the Ni-NTA elution pool prepared from 1 g of cell pellet. For large cultures (2 liters or more) or especially when the recombinant His-tagged protein is expressed at a low level, batch incubation in a larger volume of chitin beads may help to efﬁciently remove all the CBD tagged proteins (1 ml chitin beads for every 0.1 liter of culture is another recommendation). However, increasing the incubation time with the beads...
We show that mutating surface-exposed histidines on GlmS is an effective strategy for eliminating binding to Ni-NTA. However, we have not followed this as a general approach, since we expect that many proteins would not tolerate removal of multiple histidines.

In summary, NiCo21(DE3) is indistinguishable from BL21(DE3) with respect to growth characteristics and the potential for recombinant protein expression. The NiCo strains consistently produced a high yield of recombinant protein in all of our studies, and the most common IMAC contaminants were removed by simply exposing the target protein pool to chitin beads, which are compatible with commonly used IMAC buffers. Therefore, we propose that the NiCo strains are superior alternatives to BL21(DE3) as protein production hosts, and we expect that these strains will perform well under all conditions routinely employed for the propagation of BL21(DE3).

ACKNOWLEDGMENTS

We thank Mern Sibley for introduction of the fhuA2 (T1 phage resistance) allele to BL21(DE3) and for providing the aceE mutant strains, Elisabeth Raleigh and Bill Jack for critical reading of the manuscript, Chris Taron for guidance throughout the project, and Don Comb and New England BioLabs for research support.

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