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Optimized *E. coli* expression strain LOBSTR eliminates common contaminants from His-tag purification

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Abstract

His-tag affinity purification is one of the most commonly used methods to purify recombinant proteins expressed in *E. coli*. One drawback of using the His-tag is the co-purification of contaminating histidine-rich *E. coli* proteins. We engineered a new *E. coli* expression strain, LOBSTR (low background strain), which eliminates the most abundant contaminants. LOBSTR is derived from the *E. coli* BL21(DE3) strain and carries genomically modified copies of *arnA* and *slyD*, whose protein products exhibit reduced affinities to Ni and Co resins, resulting in a much higher purity of the target protein. The use of LOBSTR enables the pursuit of challenging low-expressing protein targets by reducing background contamination with no additional purification steps, materials, or costs, and thus pushes the limits of standard His-tag purifications.

Keywords

BL21(DE3); *E. coli* protein expression strain; His-tag affinity purification; LOBSTR

INTRODUCTION

Many methods of recombinant protein purification have been developed. One of the most widely used techniques is the His-tag affinity purification¹. A small His-tag (usually 6 or 10 histidines) is fused to either the N or C terminus of the target protein, enabling capture by nickel or cobalt ions coordinated on a variety of commercially available resins. The small size of the His-tag, low cost, and ease of use have made it the most popular affinity-tag available. Expression of recombinant His-tagged proteins is largely carried out in *E. coli* because it is easy to culture and it allows for the production of target proteins with high yield. However, one major drawback of His-tag affinity purification of proteins expressed in *E. coli* is the presence of naturally histidine-rich host proteins, resulting in co-purification of these contaminants^{2,3}. The two most common *E. coli* contaminants are ArnA, a bifunctional enzyme involved in the modification of lipid A phosphates with aminoarabinose⁴, and SlyD, a peptidyl-prolyl cis/trans-isomerase^{1,5}. ArnA has several non-consecutive histidine

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COMPETING INTERESTS STATEMENT

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residues, which are surface exposed and form clusters within the hexameric structure^{2,3,6}. In contrast, SlyD is characterized by a 48 amino acid unstructured C-terminal tail containing 15 histidines^{4,7}. Because the Ni-binding mechanism of ArnA and SlyD mimics that of the His-tag, both proteins are co-purified along with the target protein in His-tag affinity purifications. For well expressing recombinant proteins, these endogenous proteins are a small problem because they are out-competed by the sheer amount of the protein of interest. However, many proteins, including human proteins, large, multi-domain proteins and co-expressed protein complexes are ignored as viable targets for *in vitro* studies because they express poorly and consequently cannot be isolated in sufficient amounts or with high purity. When protein expression is low, host proteins, especially ArnA and SlyD, have a similar abundance and compete for binding on Ni or Co resins. As a result, ArnA and SlyD are purified in nearly equal amounts when compared to the target protein. The most effective means to increase the purity of the target protein is to use additional affinity tags or multiple purification steps, however this lowers the yield and increases the purification time and cost. Because both *arnA* and *slyD* knockout strains suffer growth defects, these strains are not viable options for recombinant protein expression^{8,9}. To address these problems, we designed a new *E. coli* expression strain named LOBSTR (low-background-strain), which features genomic modifications in *arnA* and *slyD* based on surface engineering. LOBSTR maintains normal cell growth but significantly reduces the Ni- and Co-binding affinities of both host proteins. LOBSTR drastically reduces ArnA and SlyD contamination, thus enabling the purification even of poorly expressing target proteins.

MATERIALS AND METHODS

Wild type *arnA* was PCR amplified from *E. coli* genomic DNA with *NdeI* and *XhoI* restriction site overhangs on the 5' and 3' ends, respectively, using primers 1F and 1R (See all primer details in Table S1), and cloned into the bacterial expression vector pColaDuet (EMD Millipore). Two serine point mutations were introduced at site 1 (H359S and H361S) using primers 2F and 2R. Two additional serine point mutations were introduced at site 2 (H592S and H593S) using primers 3F and 3R to generate the final *arnA* mutant containing a total of four histidine to serine mutations.

The *arnA* knockout strain was generated with the *E. coli* recombineering technique¹⁰, using the pKD4 plasmid as a template for the selectable marker and BL21(DE3) as the parental strain. The forward and reverse primers, 4F and 4R, were designed to maintain the reading frame of *arnB*, which shares its start codon with the stop codon of *arnA* within the *arn* operon¹¹ (also called pmrHFIJKLM operon¹²). A slightly modified scheme was used to introduce the *arnA* mutant back into the *arnA* knockout strain at the original locus (Fig. S1). First, mutant *arnA* was amplified and combined with the amplified selectable marker in a second PCR step. The resulting PCR product containing mutated *arnA* and the selectable marker was transformed into the *arnA* knockout strain for recombination using the λ Red recombinase plasmid (pKD46). The selectable marker was eliminated using the FLP plasmid (pCP20). For the modification in *slyD*, the *arnA* mutant strain was transformed with a PCR product (generated using primers 5F and 5R) containing a selectable marker flanked by homologous overhangs that, after recombination, result in the elimination of the 46-residue C-terminal, histidine-rich segment of SlyD. Again, the selectable marker was later

removed using pCP20. Proper genomic integration was confirmed by PCR and sequencing. The RIL plasmid (Agilent Technologies) encoding rare tRNAs was transformed into the final expression strain to improve the expression of our eukaryotic target proteins.

The binding affinity of wild type and mutant ArnA were assessed by immobilizing purified protein onto a 1 ml His-Trap FF column (GE Healthcare) equilibrated in 50 mM potassium phosphate pH 8.0, 300 mM NaCl, and 5 mM beta-mercaptoethanol. Protein was eluted with a linear gradient of 0–150 mM imidazole. The imidazole concentration at the elution peak of each protein was recorded and compared.

Growth analysis was performed at 18, 25 and 37°C for both LOBSTR and the BL21(DE3) strains carrying the same test expression plasmid (See table S2 for a list of all test constructs). Cultures of 1L were grown in LB medium supplemented with 0.4% (w/v) glucose and antibiotic selection at 37°C to OD600 ~0.7. Protein expression was induced with 0.2 mM IPTG 20 minutes after the cultures were shifted to the desired expression temperature. OD600 was measured from the initial synchronization time and until the cells were harvested ~20–22 hours after induction.

To test protein purification, BL21(DE3) and LOBSTR cultures were started at 37°C in LB medium supplemented with 0.4% (w/v) glucose and appropriate antibiotic selection. At OD600 ~0.7, cultures were shifted to 18°C and induced with 0.2 mM IPTG ~20 min later. Cultures were harvested after 18–20 hours. For each strain and construct tested, a total of ~3.5g of cells were resuspended in 50 mL of resuspension buffer (40 mM potassium phosphate pH 8.0, 150 mM NaCl, 40 mM imidazole, and 3mM beta-mercaptoethanol) and lysed with a cell disrupter (Constant Systems). Lysates were cleared for 25 min at 9500×g and the soluble fraction was incubated with 400 µl bed volume of Ni Sepharose 6 Fast Flow (GE Healthcare) resin for 1 hour while stirring at 4°C. The resin was collected and washed with 6 mL of resuspension buffer and eluted with 2 mL of elution buffer (40 mM potassium phosphate pH 8.0, 150 mM NaCl, 250 mM imidazole, and 3 mM beta-mercaptoethanol). Elution fractions were analyzed on a 4–15 % SDS-PAGE gradient gel (Bio-RAD) and stained with Coomassie Blue R250. Purifications using Ni-NTA (Qiagen) and Talon (Clontech) resins were performed using resuspension buffer containing 20 mM or 5 mM imidazole, respectively, following manufacturer's recommendations.

RESULTS

We designed surface engineered forms of *E. coli* ArnA and SlyD based on their crystal and NMR structures, respectively^{6,7} (Fig. S2). Both proteins have exposed histidine-rich surfaces that result in binding to immobilized metal-affinity resins. ArnA is a hexamer, formed by a dimer of trimers. The structure revealed two prominent surface-exposed patches of histidine residues. One of the patches is at a trimer interface and results in a cluster of 9 histidines per trimer (Fig. S2, site 1). We mutated histidine residues 359 and 361 to serines to abolish this histidine-rich surface. The second cluster of surface-exposed histidines was removed by mutating histidines 592 and 593 to serines (Fig. S2, Site 2). To determine whether the histidine to serine mutations resulted in weaker Ni-binding affinity, both recombinant wild type and mutant ArnA were first purified in batch. Subsequently, pure

protein was loaded onto a His-trap Ni-column and eluted with a linear imidazole gradient. Wild type ArnA eluted at a concentration of ~60 mM imidazole, while mutant ArnA showed significantly weaker binding affinity, eluting at ~30 mM imidazole (Fig. S3). Thus, mutating four histidine residues to serines in ArnA (24 per hexamer) lowers the Ni-affinity to a level comparable to non-specific binding. Similarly, analysis of the SlyD NMR structure showed that all of the clustered histidine residues reside in an unstructured tail at the very C terminus of the protein (Fig. S2). A previous study suggested that deleting this tail has little effect on cell growth^{13,14}. Thus, we truncated SlyD at residue 150, thereby maintaining the structural integrity of the catalytic N-terminal domain while removing the entire unstructured tail. Using a modified recombineering¹⁰ approach, we then replaced the genomic copies of *arnA* and *slyD* in the host strain BL21(DE3) with our mutant versions to create LOBSTR (overview Fig. S1). To confirm that the combined genetic modifications in LOBSTR also maintain normal growth, we monitored and compared its growth rate to the parental BL21(DE3) strain at 18, 25 and 37°C. A test construct (See table S2 for a list of all test constructs) was expressed over the duration of the growth analysis. No significant difference in growth rate at any of the induction temperatures was observed between LOBSTR and BL21(DE3), and the final OD600 of the cultures after overnight induction were very similar (Fig. 1).

To verify that LOBSTR reduces ArnA and SlyD contamination, we performed small-scale purifications of seven different protein constructs in the parental BL21(DE3) strain and in LOBSTR. The seven constructs (Table S2) were chosen to represent a wide range of potential targets, including low- and higher-expressing constructs, monomeric proteins, dimeric complexes, 6×- and 10×His-tagged proteins. Most of our test constructs contain a SUMO-tag fused to the N terminus to increase protein solubility. In the BL21(DE3) strain background, high levels of contamination by both ArnA and SlyD can be seen in the elutions (Fig. 2). Illustrating the low expressions levels of target proteins, ArnA and SlyD are purified in amounts nearly equivalent to that of the target protein, as seen in constructs 2, 4, and 5. However, in LOBSTR, the vast majority of contaminants are eliminated, with the target protein now being the most prominent protein. Purification of construct 1, a heterodimeric complex with one binding partner carrying a 6×His-tag, is also greatly enhanced in LOBSTR. Furthermore, the amounts of all target proteins purified are similar between the BL21(DE3) strain and LOBSTR. Since the initial purity is much greater, fewer subsequent purification steps are required to obtain pure protein, resulting in equivalent, if not greater, final yields from LOBSTR. Curiously, a secondary contaminant, indicated by a double asterisk (***) in Fig. 2, is also reduced in LOBSTR. This protein, identified by mass spectrometry as Hsp15, is reported to bind nucleic acids¹⁵. While no modifications have been made to this protein in LOBSTR, we speculate that it may have non-specific binding affinity to SlyD, which is highly negatively charged. To ensure that the results seen here are reproducible on a variety of commercially available resins, we purified constructs 1 and 5 on two additional commonly used resins, Ni-NTA (Qiagen) and Talon (Clontech) (Fig. 2B). Both resin manufacturers recommend lower imidazole concentrations in the binding and washing buffers compared to the Ni Sepharose 6 FF resin (GE Healthcare), which was used for the purifications above. Still, nearly complete elimination of ArnA and SlyD contamination is observed on these resins as well (Figure 2B).

DISCUSSION

LOBSTR enables the pursuit of poorly expressed protein targets in *E. coli* by lowering the background contamination of ArnA and SlyD. Previously, constructs yielding only 0.1–1 mg of target protein per liter of culture could be considered inadequate for in vitro studies. At such low levels of expression, ArnA and SlyD compete for the binding capacity of the metal affinity resin and are co-purified in equivalent or even greater amounts. LOBSTR enables a significantly higher yield and purity of poorly expressed target protein eluted from Ni or Co resins. Protein purity is of key importance for most downstream purposes, whether the protein is used in medical applications, binding studies, functional assays, or structural studies (EM, SAXS, NMR, and crystallography). An alternate approach to eliminate *E. coli* host contaminants has been developed previously⁹. Here, ArnA, SlyD, and Can were genomically tagged with a chitin-binding domain and eliminated over chitin beads, pulling out the contaminants and leaving the target protein in the flow-through. In addition, GlmS is mutated to reduce binding to Ni and Co. While this method is successful in removing the contaminants, it requires an additional purification step as well as an additional resin, increasing both the time and cost of each purification. However, LOBSTR only requires a one-step purification to eliminate the major *E. coli* contaminants ArnA and SlyD with no additional costs and is specifically designed for low-expressing proteins. An alternate purification strategy is to simply perform a second IMAC step after cleaving off the His-tag from the protein of interest so that contaminants are rebound while the cleaved protein remains in the flow-through. While this method is successful when the contaminants make up only a small fraction of the total immobilized protein in the first IMAC step, it is highly inefficient if the contaminants are abundant and thus substantially reduce the initial yield. LOBSTR instead, incorporates genomic modifications to *arnA* and *slyD* in order to reduce the affinity of their gene products for metal affinity resins, eliminating them from co-purification with recombinant proteins of interest. Thus, proteins that were previously ignored as targets for recombinant expression and purification are now accessible.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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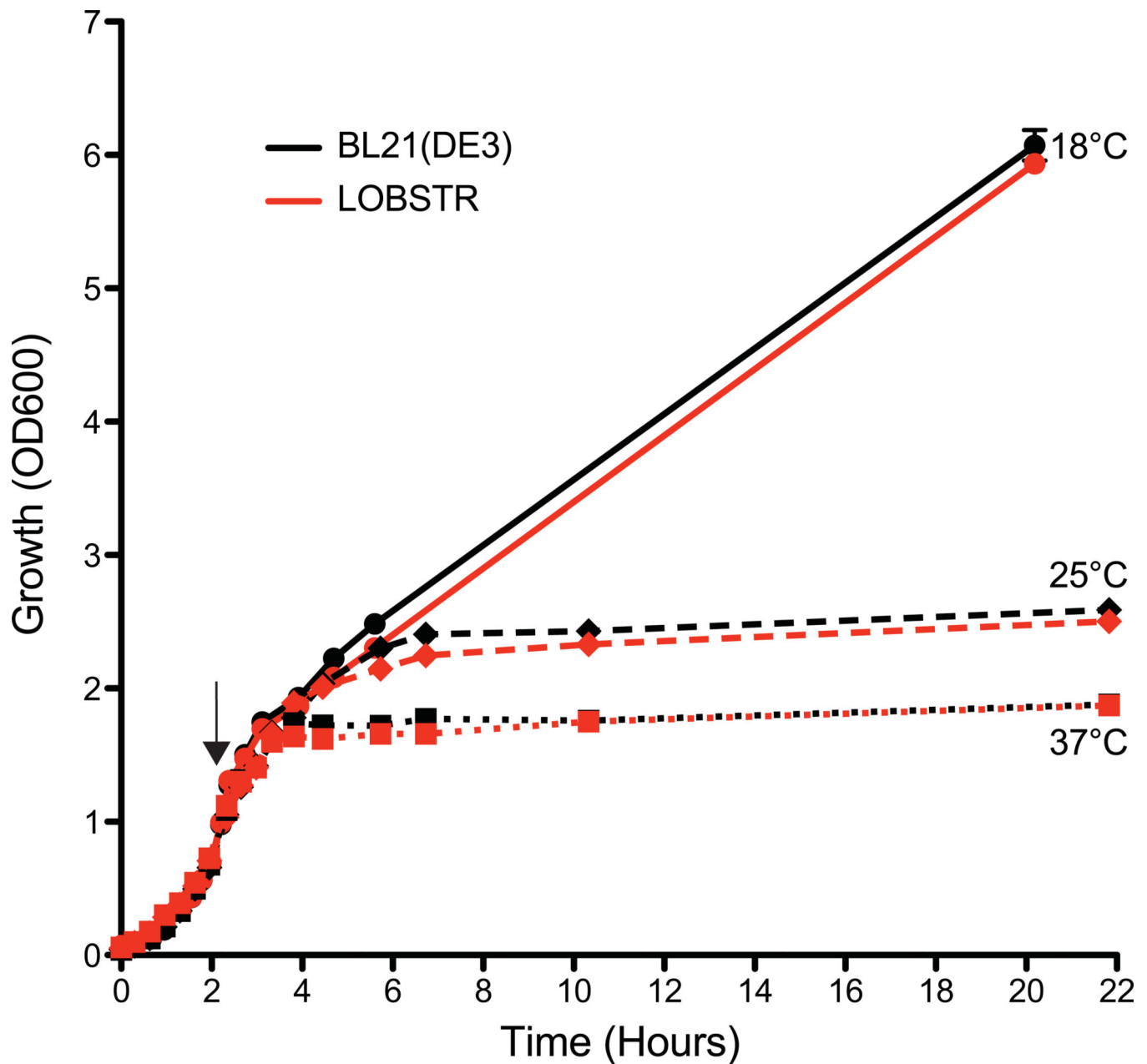


Figure 1. LOBSTR and the parental BL21(DE3) strain show comparable growth

The growth (OD600) of both LOBSTR and the parental BL21(DE3) strain was measured from initial synchronization at 0 hours until the final harvest. Both strains carried the same expression plasmid and were grown at 37°C until an OD600 ~0.7, at which point protein expression was induced at 18, 25 and 37°C (black arrow). The growth curves for LOBSTR and BL21(DE3) are shown in red and black, respectively. Cell growth during log phase and final cell density is similar for both strains.

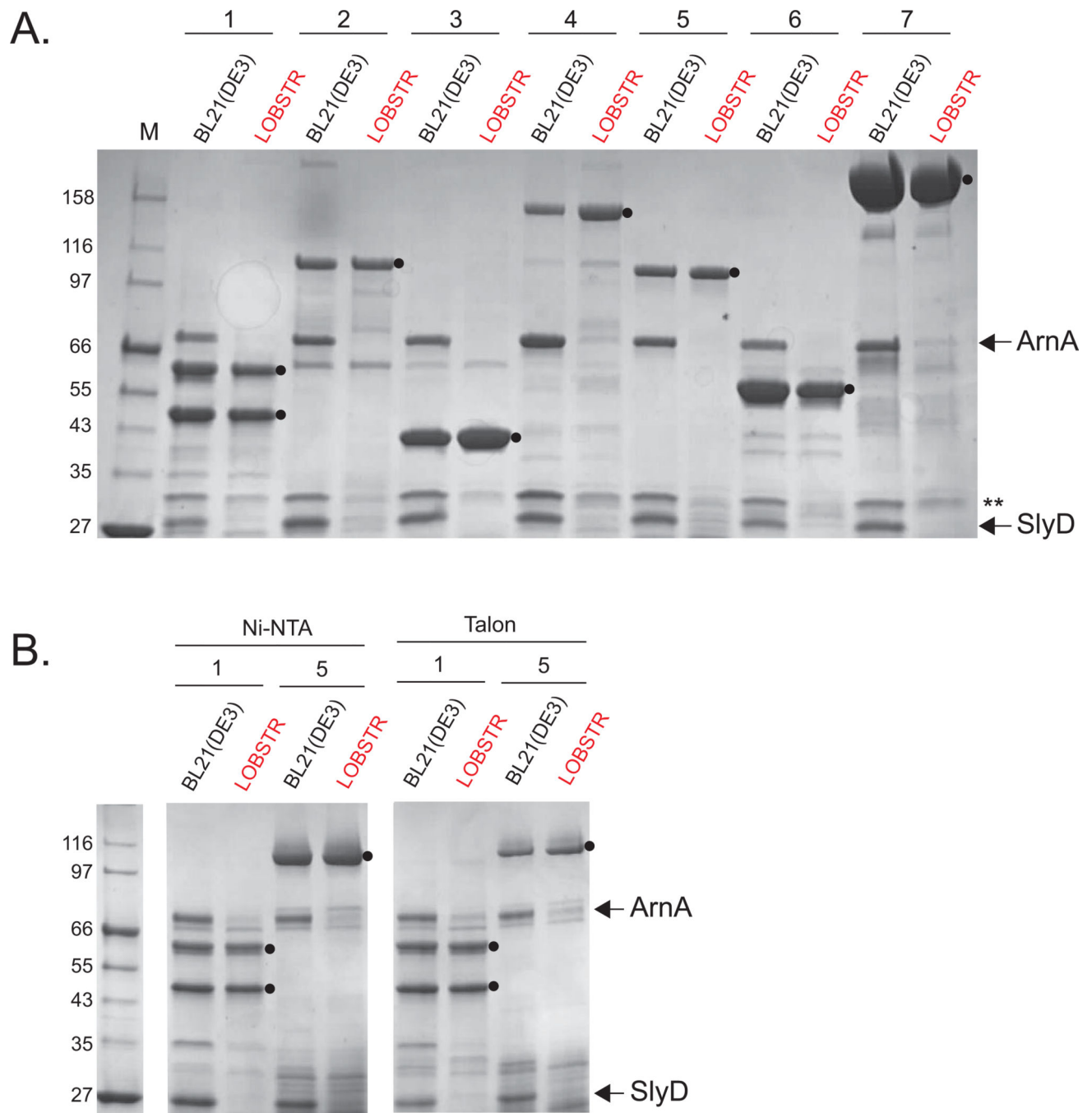


Figure 2. ArnA and SlyD are eliminated from His-tag purifications from LOBSTR

Elution samples of test purifications from BL21(DE3) and LOBSTR using common metal affinity resins are shown. **A.** Seven protein constructs were purified from both the parental BL21(DE3) strain and LOBSTR using Ni Sepharose 6FF resin (GE Healthcare). The constructs are numbered 1–7, and contain either a 6×His-tag (1 and 4) or a 10×His-tag (2,3,5–7). See table S2 for a list of all test constructs. The elution samples were run on an SDS-PAGE gel and stained with Coomassie Blue R250. ArnA and SlyD are indicated by arrows and target proteins indicated with a black circle (●). The double asterisk (**)

indicates Hsp15, another protein showing reduced Ni-binding affinity in LOBSTR. **B.** Purifications of constructs 1 and 5 from BL21(DE3) and LOBSTR were also carried out on two additional commonly used resins, Ni-NTA (Qiagen) and Talon (Clontech). In each case, ArnA and SlyD are successfully eliminated in LOBSTR.