Immunoaffinity purification and characterization of RNA polymerase from Shewanella oneidensis

ARTICLE *in* PROTEIN EXPRESSION AND PURIFICATION · OCTOBER 2007

Impact Factor: 1.7 · DOI: 10.1016/j.pep.2007.03.020 · Source: PubMed

3 AUTHORS, INCLUDING:

Mitchell D Probasco
University of Wisconsin–Madison
8 PUBLICATIONS **793** CITATIONS
SEE PROFILE

Richard R Burgess
University of Wisconsin–Madison
237 PUBLICATIONS **14,510** CITATIONS
SEE PROFILE

Available online at www.sciencedirect.com
ScienceDirect
Protein Expression and Purification 55 (2007) 23–30
Protein Expression & Purification
www.elsevier.com/locate/yprep

Immunoaffinity purification and characterization of RNA polymerase from *Shewanella oneidensis*

Mitchell D. Probasco, Nancy E. Thompson, Richard R. Burgess *

McArdle Laboratory for Cancer Research, University of Wisconsin-Madison, Madison, WI 53706, USA

Received 20 December 2006, and in revised form 28 March 2007
Available online 10 April 2007

Abstract

Shewanella oneidensis is of particular interest for research because of its unique ability to use a variety of metals as final respiratory electron acceptors and reduce them into insoluble oxides. A collection of monoclonal antibodies (mAbs) that were prepared towards *Escherichia coli* RNA polymerase (RNAP) was tested for reactivity with proteins extracted from *S. oneidensis*. Two polyol-responsive monoclonal antibodies (PR-mAbs) were used to purify RNA polymerase from *S. oneidensis* using immunoaffinity purification techniques. A collection of mAbs towards *E. coli* sigma subunits was also examined for cross-reactivity with *S. oneidensis* proteins. Reactions were identified with mAbs to *E. coli* σ^{70} and σ^{54}. These mAbs will be useful tools for immunoaffinity purifying and studying the transcriptional machinery of *S. oneidensis*.

Keywords: RNA polymerase; Immunoaffinity chromatography; *Shewanella oneidensis*; Sigma factor; Monoclonal antibody

Shewanella oneidensis occupies many ecological niches, and is a dissimilatory metal reducing bacterium that can use a variety of metals (including iron, uranium, and chromium) as terminal electron acceptors. This unusual organism plays an important role in geobiology (see [1,2] for reviews). The genome of *S. oneidensis* has been sequenced [3]. Patterns of gene expression in this bacterium are beginning to be investigated by microarray analyses under various physiological conditions such as exposure to ionizing radiation [4], elevated salt concentrations [5], extremes of pH values [6], heat shock [7], cold shock [8], and exposure to different terminal electron acceptors [9,10]. However, while microarray analysis can help to determine the genes that are expressed under various experimental conditions, it does not characterize the transcriptional machinery.

Gene expression in bacteria is controlled, in part, by the population of different forms of the RNA polymerase (RNAP)[1] holoenzyme. Different holoenzymes all contain the core RNAP subunits (β′, β, α_2, and ω) but differ in the sigma subunit. Most bacteria have several different sigma subunits, with *Escherichia coli* having seven (σ^{70}, σ^{32}, σ^{54}, σ^{S}, σ^{F}, σ^{E}, σ^{FecI}). Different holoenzymes transcribe different classes of genes, depending upon the specificity of the promoter-binding sequence in different promoters. Availability of these different sigma subunits for the formation of the various holoenzymes can be controlled by various environmental stimuli, the most well-studied being the *E. coli* heat shock sigma factor, σ^{32} [11,12].

* Corresponding author. Fax: +1 608 262 2824.
E-mail address: burgess@oncology.wisc.edu (R.R. Burgess).

[1] *Abbreviations used:* mAbs, monoclonal antibodies; PR-mAb, polyol-responsive mAb; RNAP, RNA polymerase; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; ECL, enhanced chemiluminescence; TBS, trypticase soy broth; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PEI, polyethyleneimine; BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium; DEAE, diethylaminoethyl; CNBr, cyanogen bromide; BSA, bovine serum albumin; kDa, kilodalton.

1046-5928/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2007.03.020

Genome analysis of *S. oneidensis* indicates that the RNA polymerase of this bacterium contains genes that are the putative homologs of the core RNAP (β′, β, α_2, and ω) of *E. coli*. Likewise, there are at least six putative sigma subunits identified in the *S. oneidensis* genome database (σ^{70}, σ^{32}, σ^{54}, σ^{S}, σ^{F}, σ^{24}). A recent report described the isolation of transcription complexes from *S. oneidensis*, by affinity chromatography, using a genetically tagged α-subunit [13]; these researchers identified the other core subunits by the use of mass spectrometry. We have found that monoclonal antibodies (mAbs) that cross-react with components of the transcription machinery from different bacteria to be extremely useful in studying bacterial RNAPs. In this paper, we have used the collection of monoclonal antibodies (mAbs) that we developed against *E. coli* RNAP to examine the RNAP in *S. oneidensis*.

We used two of these mAbs to purify the RNA polymerase from *S. oneidensis*. These mAbs are described as "polyol-responsive" (PR-mAbs). PR-mAbs are very powerful tools in protein purification [14,15]. The term polyol-responsive mAbs refers to the characteristic of the mAb to release its antigen in the presence of a non-chaotropic salt and a polyol. This method is favorable because it is gentler than other techniques, and usually the protein can be eluted in a biologically active and structurally intact form. The mAbs to *E. coli* sigma factors [16] were also tested for cross-reactivity with *S. oneidensis* sigma factors.

Materials and methods

Media and buffers

TSB (*BBL Trypticase Soy Broth*): 30 g TSB (soybean casein digest broth, Becton–Dickinson, Sparks, MD) in 1 L H_2O; *LB Medium*: 10 g Bacto tryptone (Becton–Dickinson), 5 g Bacto yeast extract (Becton–Dickinson), 5 g NaCl in 1 L H_2O. *Antibody buffer*: 50 mM Tris–HCl, pH 6.9 and 25 mM NaCl; *Coupling buffer*: 0.1 M $NaHCO_3$, pH 8.3, 0.5 M NaCl; *Acetate buffer*: 0.1 M sodium acetate, pH 4, 0.5 M NaCl; *Blocking agent*: 1 M ethanolamine contained in coupling buffer, pH 8.3 with 6 N HCl; *Buffer A*: 50 mM Tris–HCl, pH 7.9, 0.1 mM EDTA, 50 mM NaCl, 5% glycerol and 0.1 mM DTT (also Buffer A may contain 0.3 M or 1 M NaCl); *Lysis buffer*: 260 μg lysozyme per ml (in Buffer A); *TE*: 50 mM Tris–HCl, pH 7.9, 0.1 mM EDTA (also TE may contain 0.15 M or 0.5 M NaCl); *Elution buffer*: TE buffer, 40% propylene glycol and 0.75 M ammonium sulfate (substitute 0.75 M NaCl for ammonium sulfate when using mAb 4RA2); *1X SDS sample buffer*: 62.5 mM Tris–HCl, pH 6.8, 3% SDS, 10% glycerol, 0.6 M 2-mercaptoethanol, 0.0125% (w/v) bromphenol blue; *TBST* (*Tris-buffered saline with Tween 20*): 10 mM Tris–HCl, 150 mM NaCl, 0.1% Tween 20; *Dulbecco's phosphate buffered saline* (Invitrogen, Carlsbad, CA): 1.5 mM KH_2PO_4, 8 mM $Na_2HPO_4{\cdot}7H_2O$, 2.7 mM KCl, 138 mM NaCl, pH 7.1.

Antibodies, bacterial strains, and antibody cross-reactivity

A culture of *S. oneidensis* (strain MR-1) was obtained from Dr. Brian Kay (Argonne National Laboratory). It was cultured in TSB (10 ml) at 30 °C overnight with shaking, and the cells were harvested by centrifugation (3000*g*) for 20 min. As a control for the mAb reactions, a culture of *E. coli* MG1655 was grown in 10 ml of TSB medium at 30 °C overnight with shaking and harvested by centrifugation. *E. coli* MG1655 is a well-studied strain of *E. coli* and has been sequenced [17]. This strain (a derivative of K12) was used as a source to purify *E. coli* core RNAP for use as an immunogen to produce the mAbs. In the preparation of the samples for Western blot analysis, 1 ml of 2× SDS sample buffer was added to the pellet, and the cells were sheared using a 22 gauge needle.

Western blot analysis was used to analyze the samples for cross-reactivity of the mAbs. The lysates were run on a 4–12% SDS–PAGE (Invitrogen) gel and transferred to nitrocellulose. Each Western blot was blocked with 1% BLOTTO (5 g non-fat dry milk in 500 ml of 1× phosphate-buffered saline) for a minimum of 1 h. The primary antibody, contained in ascites fluid, was then diluted 1:1000 in 1% BLOTTO. The secondary antibody used was goat anti-mouse IgG conjugated to horseradish peroxidase, also diluted 1:1000 in 1% BLOTTO. The primary and secondary antibodies were added separately and then allowed to incubate for about one hour each; after this hour the blot was rinsed with 3–5 washes of TBST. The washed blot was treated with the enhanced chemiluminescence (ECL) reagents (GE Healthcare, Piscataway, NJ) for about one minute and then removed. Film was exposed to the blot for varying time periods ranging from 5 to 15 s. For Western blots that contained purified RNAP, the secondary antibody was a goat anti-mouse IgG conjugated to alkaline phosphatase; BCIP/NBT (75 mM 5-bromo-4-chloro-3-indolyl phosphate and 20 mM nitro-blue tetrazolium) was then used as the substrate to develop the blot. Pre-stained molecular weight markers (Multimark, Invitrogen) were run on all gels.

Preparation of immunoaffinity columns

mAbs were purified from mouse ascites fluid [18]. Saturated ammonium sulfate (4.1 M) was added to the ascites fluid (45% of the total volume) and held at 4 °C overnight in order to precipitate the antibody. The solution was centrifuged (12,000*g*) for 10 min, and the antibody pellet was resuspended in antibody buffer (half of the original ascites volume) and dialyzed against antibody buffer overnight at 4 °C. After dialysis, the fluid was centrifuged (12,000*g*), the supernatant was run on a DE-52 DEAE-cellulose column. Under these conditions, most murine mAbs will flow through the DE-52, leaving most other proteins bound to the resin. The antibody-containing fractions (determined by SDS–PAGE analysis) were pooled, and the absorbance at 280 nm was measured to determine the concentration of

the protein. The purified antibody was dialyzed against coupling buffer overnight, and the antibody was conjugated to Sepharose that had been pre-activated with cyanogen bromide (CNBr), which was obtained from Sigma Chemical Co. (St. Louis, MO). Briefly, 1.0 g of the dried CNBr-activated Sepharose was allowed to swell in 1 mM HCl at room temperature for about 20 min. The resin was washed with about 200 ml of 1 mM HCl on a glass filter funnel. The Sepharose was quickly transferred to the coupling buffer containing the antibody. The ratio of antibody to Sepharose was about 2.5 mg antibody to 1 ml of packed, wet Sepharose; thus because 1 g of Sepharose yields about 3.5 ml of packed, wet Sepharose, 8.75 mg of antibody was used. The antibody and the CNBr-activated Sepharose (usually about 10 ml) were mixed end-over-end for 2 h at room temperature. The mAb-conjugated Sepharose was then collected on a glass filter funnel. The protein concentration in the filtrate was measured to ensure that the antibody had coupled to the Sepharose. The mAb-Sepharose was transferred to about 10 ml of blocking agent (1 M ethanolamine which had been adjusted to pH 8.3) for blocking the unreacted CNBr groups. The slurry was mixed end-over-end for 2 h at room temperature, and the mAb-Sepharose was collected on the glass filter funnel. The resin was then washed repeatedly with coupling buffer and acetate buffer (alternating) to remove any non-covalently bound proteins.

Purification of RNAP using an immunoaffinity column

The procedure used to purify the RNAP from *S. oneidensis* was adopted from the procedure to purify *E. coli* by immunoaffinity chromatography [18] with a few modifications. A general flow-chart for the purification is presented in Fig. 2a. Briefly, *S. oneidensis* was grown in 1 L of TSB at 30 °C and harvested in the late logarithmic phase of the growth curve (OD 0.7–0.8 at 600 nm). The cells (about 2–3 g wet weight) were then resuspended in 15 ml of lysis buffer, sonicated to break up the cells, and Triton X-100 (Pierce, Rockford, IL) was added to a final concentration of 0.1% and incubated on ice for 30 min. An additional 15 ml Buffer A was added, and the cell lysate was centrifuged (12,000*g*) for 20 min. polyethyleneimine (PEI) was added (900 μl of a 10% solution prepared in water and adjusted to pH 7.9) to the cell extract to precipitate the nucleic acids and acidic proteins, including RNA polymerase [19]. After adding the PEI, the solution was centrifuged (6000*g*) for 10 min. The pellet was washed by resuspending it in 20 ml of Buffer A +0.3 M NaCl. This solution was centrifuged again (6000*g*) and the pellet was resuspended in 20 ml Buffer A +1 M NaCl, which eluted the RNAP from the pellet. The mixture was centrifuged (6000*g*) again. The RNAP was precipitated from the supernatant with 7.6 g of enzyme grade ammonium sulfate per 20 ml supernatant and mixed at 4 °C for 10 min. This solution was centrifuged (4300*g*), and the ammonium sulfate pellet was resuspended in 10 ml of TE. Insoluble material was removed by centrifugation (4300*g*). The 8RB13- (or 4RA2-) conjugated Sepharose (2 ml packed volume) was added to the solution and allowed to incubate for about 1 h at 4 °C with mixing. The mAb-Sepharose was then placed in a 5-ml column and washed first with 20 ml TE + 0.15 M NaCl and then with 5 ml TE + 0.5 M NaCl. The RNAP was eluted off the column using elution buffer (TE containing either 0.75 M ammonium sulfate or NaCl and 40% propylene glycol). Fractions (1 ml) were collected and analyzed by 4–12% SDS–PAGE. The protein concentrations of the fractions and aliquots were determined using a Bradford Assay (Pierce) with bovine serum albumin as a protein standard. The peak fractions were pooled.

RNA polymerase activity assays

Activity of the immunopurified *S. oneidensis* core RNAP was determined essentially as described [20]. This assay uses denatured calf-thymus DNA as the template and measures the incorporation of nucleotides (containing a radiolabeled-nucleotide) into RNA. RNAP from *E. coli* MG1655 that was purified by 8RB13 chromatography [20] was used as the control.

Results

Reactivity of PR-mAbs

Like *E. coli*, *S. oneidensis* is classified as a γ-proteobacterium. It can be cultivated in the laboratory on complex media. Whole-cell lysates prepared from stationary-phase cells were run on SDS–PAGE, transferred to nitrocellulose, and probed with our different antibodies that react with *E. coli* core RNA polymerase [18,20,21]. Three mAbs (NT73, 8RB13, and 4RA2) were of particular interest because they are PR-mAbs and have been used to purify RNAP from different bacteria [18,20,21]. Using whole bacterial cell lysates, we examined the cross-reactivity of the various mAbs by Western blot analysis. PR-mAb, NT73, that reacts with the β′ subunit of *E. coli* [18], did not give a significant signal with *S. oneidensis* (data not shown). The epitope for this PR-mAb is known [22] and is not conserved in the *S. oneidensis* β′ subunit sequence [3]. However, PR-mAbs 8RB13 and 4RA2 reacted very well with what are likely to be the β- and α-subunits of *S. oneidensis*, respectively (Fig. 1) and were tested for the ability to purify polymerase from *S. oneidensis*.

Immunoaffinity purification of S. oneidensis RNAP

Shewanella oneidensis was first run on the 8RB13 column. A flow-chart of the purification scheme is shown in Fig. 2a. This mAb tends to purify the core form of the RNAP in preference to the holoenzyme [20]. The SDS–PAGE gel containing fractions from the purification is shown in Fig. 2a. This gel indicated a very high level of

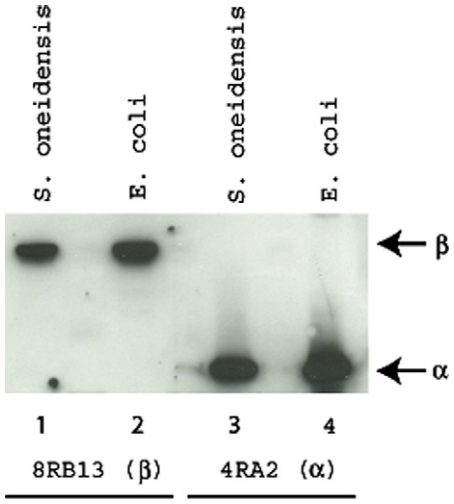

Fig. 1. Western blot using PR-mAbs and crude extracts. The gel was loaded with the approximate equivalent of 25 μl of *S. oneidensis* culture (Lanes 1 and 3) and 25 μl of *E. coli* (E) culture (Lanes 2 and 4). The Western blots were probed with PR-mAbs 8RB13 (β-subunit) and 4RA2 (α-subunit).

final purity in this single chromatographic step. The subunit composition of the RNAP isolated from *S. oneidensis* was very similar to that isolated from *E. coli* and other bacteria [20]. The core subunits (β′, β, α, and ω) are clearly identifiable (Fig. 2b). A few minor contaminants were also present, perhaps owing to a sub-optimal washing protocol for the *S. oneidensis* enzyme before elution from the immunoaffinity column. A Bradford assay was performed on the samples taken, and fractions were collected. The protein recovered was determined to be about 2.0 mg of total protein in the three peak fractions from the immunoaffinity column. Thus, from about 200 mg of total protein in the crude lysate, we recovered a total of 2.0 mg of *S. oneidensis* RNA polymerase at above 95% purity. This represents a greater than 70% recovery of RNAP from the lysate. This yield was very comparable to that obtained when an *E. coli* extract was run on 8RB13 columns [20].

To confirm the identity of the core subunits, equal amounts of RNAP from *E. coli* and *S. oneidensis* that had been purified on 8RB13-Sepharose were run a SDS–PAGE gel, and Western blots were performed, using a β-subunit mAb (8RB13) and an α-subunit mAb (4RA2). The stained gel in Fig. 3a shows that the core subunits of the two enzymes co-migrate. Importantly, enzyme purified from *S. oneidensis* with mAb 8RB13 also reacts with a mAb to the α-subunit (4RA2) in a Western blot (Fig. 3b). This confirms that the reactions observed in the Western blot using bacterial lysates (Fig. 1) are interpreted correctly.

The 4RA2 immunoaffinity column was also used to purify RNAP from *S. oneidensis* in the same manner as the 8RB13 purification; this mAb purifies the holoenzyme form of the RNAP. Fig. 4 shows the SDS–PAGE gel that was run after the purification by 4RA2. The yield of polymerase was considerably lower using the 4RA2 column

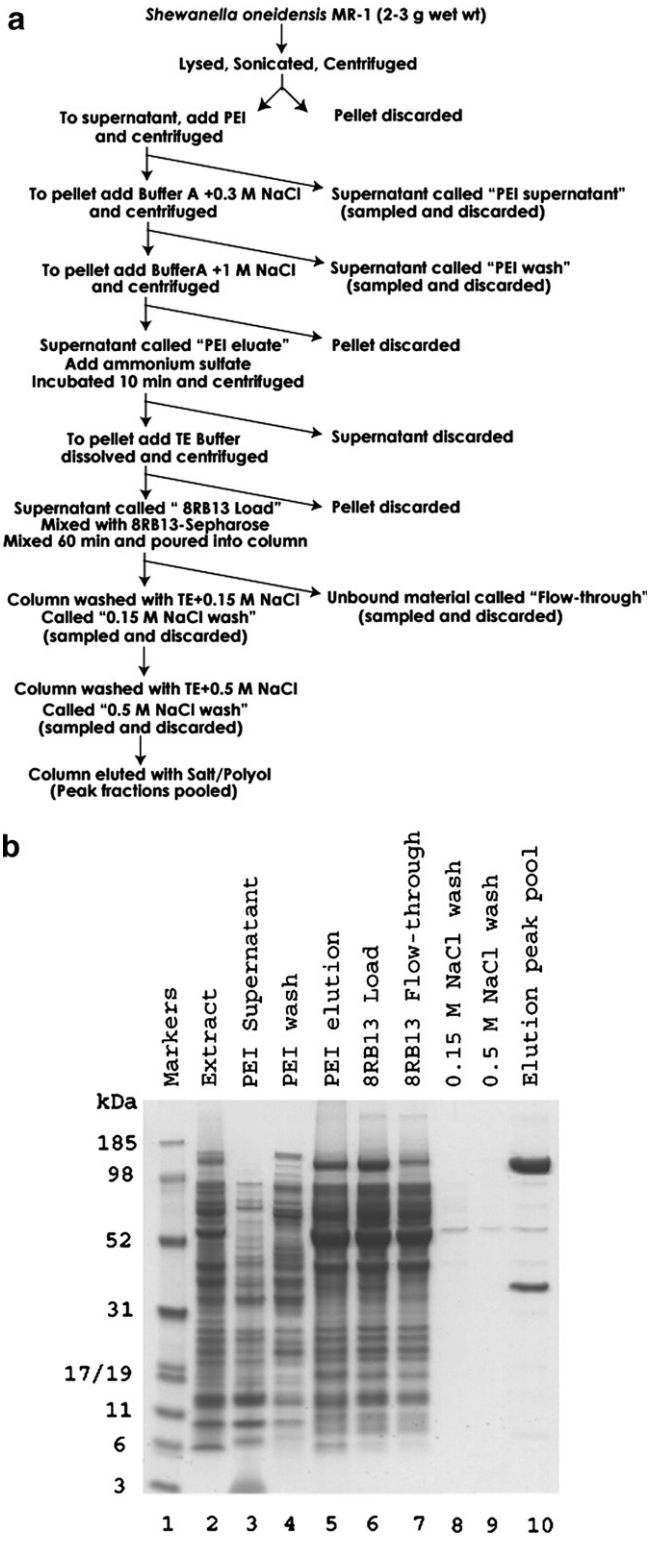

Fig. 2. Purification of *S. oneidensis* RNAP on 8RB13-Sepharose. (a) Flow-chart of the steps in the purification. (b) SDS–PAGE of samples from the purification. Lane 1: Molecular weight standards; Lane 2: Crude extract (diluted 1:5); Lane 3: PEI supernatant; Lane 4: PEI wash; Lane 5: PEI elution; Lane 6: Load for the 8RB13-Sepharose; Lane 7: Flow-through; Lane 8: TE + 0.15 M NaCl wash; Lane 9: TE + 0.50 M NaCl wash; Lane 10: Polyol/salt elution (peak fractions pooled).The gel was stained with GelCode (Pierce), a Coomassie blue-based stain.

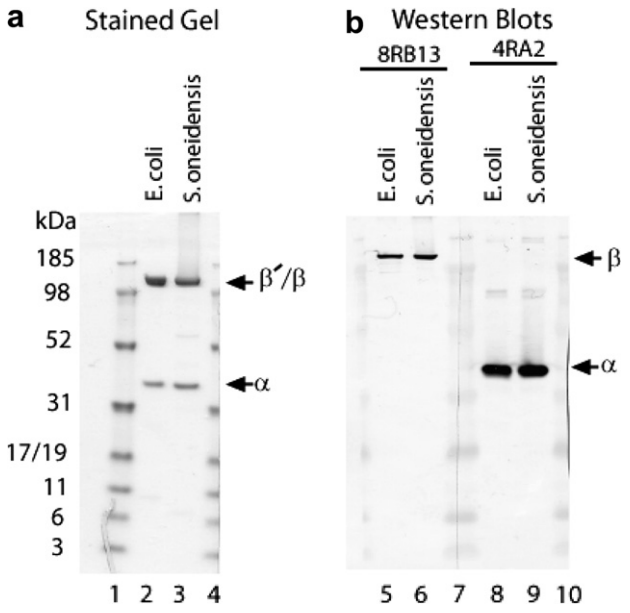

Fig. 3. SDS–PAGE and Western blots of RNAP purified from *E. coli* and *S. oneidensis* by chromatography on mAb 8RB13. (a) Stained gel containing 2 μg of RNAP from *E. coli* (lane 2) and *S. oneidensis* (lane 3). (b) Western blots containing 0.5 μg of RNAP from *E. coli* (lanes 5 and 8) and *S. oneidensis* (lanes 6 and 9) were reacted with the anti-β-subunit mAb (8RB13) and the anti-α-subunit mAb (4RA2) as indicated above the blot. Pre-stained molecular weight markers were run in lanes 1, 4, 7, and 10. These blots were processed using alkaline phosphatase-conjugated secondary antibody and BCIP/NBT substrate.

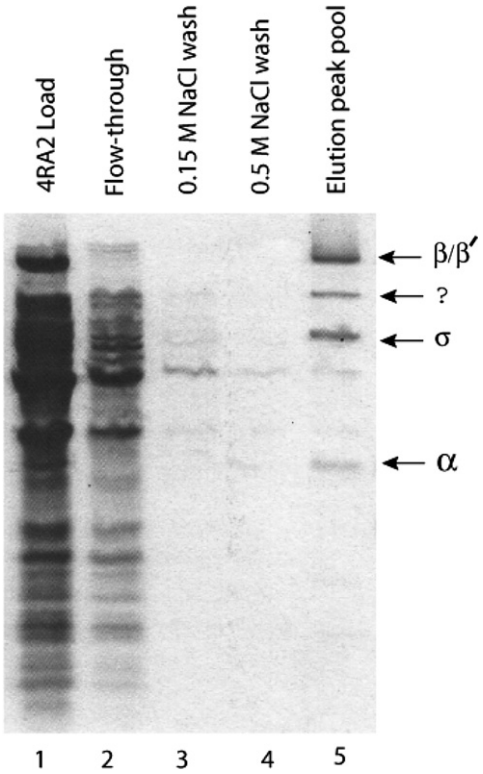

Fig. 4. SDS–PAGE of *S. oneidensis* RNAP from 4RA2-Sepharose purification. Lane 1: 4RA2-Sepharose load; Lane 2: Flow-through; Lane 3: TE + 0.15 M NaCl wash; Lane 4: TE + 0.5 M NaCl wash; Lane 5: Elution peak pooled. The gel was stained with GelCode.

than with the 8RB13 column. However, when the protein concentrations were compared with the literature values [21] for the 4RA2 purification, it was very similar to the purification of *Rhodobacterium spheroides* RNAP, using mAb 4RA2.

Activity of S. oneidensis core RNAP

The *S. oneidensis* core RNAP was assayed for the ability to transcribe RNA from a non-specific DNA template, under the conditions that we use for *E. coli* core RNAP. The RNAPs shown in Fig. 3a were used for this assay. This assay is appropriate because it is not dependent upon sigma-promoter interactions and the 8RB13 mAb purifies primarily the core form of the RNAP (Fig. 3 and [20]). As shown in Fig. 5, the *S. oneidensis* core RNAP was almost as active in DNA-template dependent incorporation of nucleotides into RNA as was the control *E. coli* core RNAP. The slightly lower activity of the *S. oneidensis* RNAP can likely be attributed to sub-optimal transcriptional conditions for the *S. oneidensis* enzyme, because the reaction conditions for the assay were those optimized for *E. coli* RNAP.

Reactivity of sigma mAbs

We also examined our collection of anti-σ antibodies that we felt were most likely to cross-react. Two anti-σ^{70} mAbs (2G10 and 3RD3) showed good cross-reactivity with a band of the size expected for the σ^{70} homolog of *S. oneidensis* (Fig. 6).

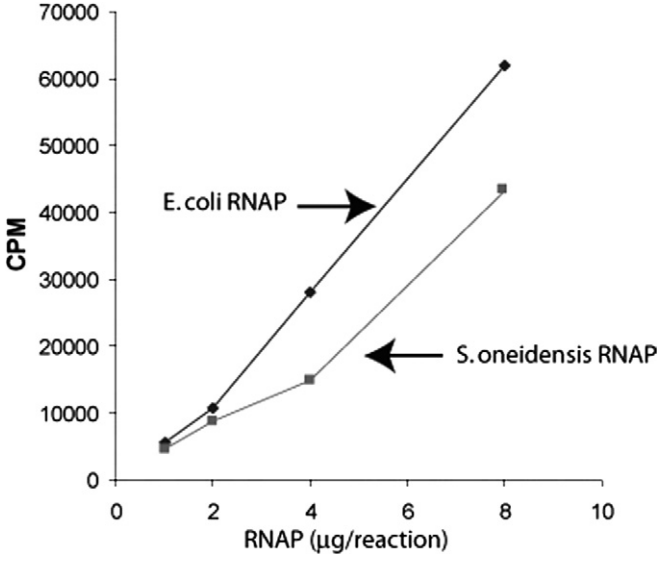

Fig. 5. Activity of *S. oneidensis* RNAP. Core RNAP purified on the 8RB13-Sepharose was assayed for the ability to incorporate NTPs into RNA in a DNA-template-dependent manner. *S. oneidensis* RNAP was compared with core RNAP purified from *E. coli* by 8RB13-chromatography. Data are expressed as counts per minute (cpm) of ^{32}P-UTP incorporated into RNA as a function of protein concentration. Each point is the average of duplicate samples.

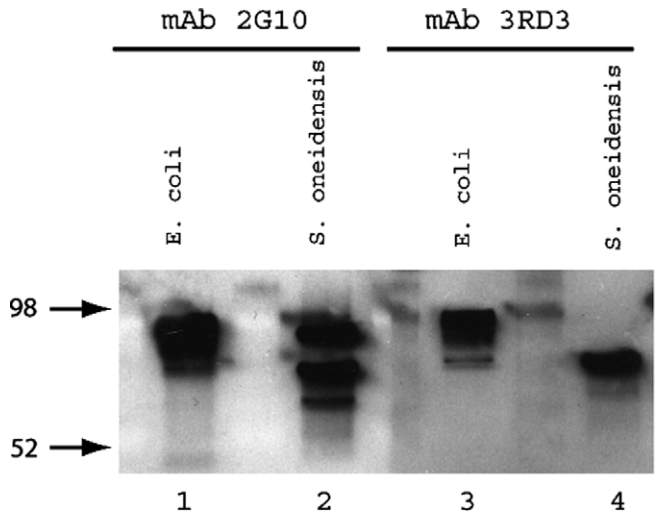

Fig. 6. Western blot using the anti-σ^{70} mAbs and crude extracts. The gel was loaded in the following matter: Lanes 1 and 3: equivalent of 12.5 μl of *E. coli* culture. Lanes 2 and 4: equivalent of about 12.5 μl of *S. oneidensis* culture. The Western blots were probed with the following mAbs: Lanes 1 and 2: mAb 2G10; Lanes 3 and 4: mAb 3RD3. Numbers on the left indicate the position of molecular weight markers (kDa).

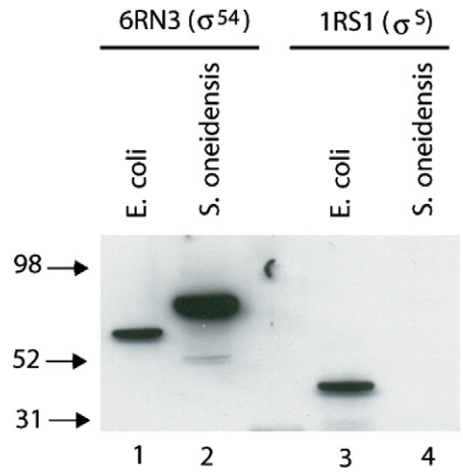

Fig. 7. Reactions of *S. oneidensis* proteins with σ^{54} and σ^{S} mAbs. The gel was loaded in the following manner: Lanes 1 and 3: equivalent of about 12.5 μl of *E. coli* culture. Lanes 2 and 4: equivalent of about 12.5 μl of *S. oneidensis* culture. Western blots were probed with the following mAbs: Lanes 1 and 2: mAb 6RN3 (*E. coli* σ^{54}); Lanes 3 and 4: mAb 1RS1 (*E. coli* σ^{S}). Numbers on the left indicate the position of molecular weight markers (kDa).

mAb 2G10 reacts with a conserved epitope in region 3.1 of *E. coli* σ^{70} and shows broad cross-reactivity with the major sigma factor from other bacteria [23]. Surprisingly, two bands were detected on the Western blot of *S. oneidensis* extract using the 2G10 antibody when only one was expected. The upper band has an apparent molecular weight on a SDS–PAGE gel of 95 kDa; the lower is about 70–75 kDa (*E. coli* σ^{70} runs at about 90 kDa on SDS–PAGE due to its high negative charge). These findings were compared with 3RD3, another σ^{70} antibody that reacts with an epitope in region 3 of *E. coli* σ^{70} [24]. 3RD3 gave a single band corresponding to the smaller of the two bands (70–75 kDa) that reacted with mAb 2G10 (Fig. 6). Thus, the 70–75 kDa band is likely the σ^{70} homolog.

Because the whole-cell lysates were prepared from stationary phase cells, a culture of *S. oneidensis* was grown and samples were taken throughout the growth curve and the whole-cell lysates were analyzed using both σ^{70} antibodies. The double band was present throughout the growth curve when detected with 2G10, but only one band was detected with 3RD3 (data not shown). *S. oneidensis* was also grown under anaerobic conditions at 30 °C, and Western blot analysis was then performed on the cell extract with mAbs 2G10 and 3RD3. mAb 2G10 again showed the 95 kDa band while mAb 3RD3 showed only the 70–75 kDa band (data not shown). These data indicate that the 95 kDa band is a protein that is present throughout the growth curve when *S. oneidensis* is cultured on complex media.

mAb 6RN3 reacts with σ^{54} from *E. coli* [16]. Although the epitope for this mAb is not known, it reacts strongly with a protein present in the *S. oneidensis* whole cell extract of the size expected for σ^{54} (Fig. 7). A mAb that reacts with *E. coli* σ^{S} (mAb 1RS1) did not react with any proteins in the *S. oneidensis* extract (Fig. 7), although considerable amounts of σ^{s} would be expected to be present in the overnight culture. Our mAbs to *E. coli* σ^{32}, σ^{F}, σ^{E}, and σ^{FecI} did not react with any protein in the *S. oneidensis* whole cell extract (data not shown), but these alternative sigma factors are often difficult to detect even in *E. coli* whole cell extracts (D. Bridges Jensen, Ph.D. Thesis, University of Wisconsin-Madison, 1998).

Discussion

From the experiments that we performed, we were able to determine that the core RNAP complex was predominantly conserved between *E. coli* and *S. oneidensis*. This makes many of the mAbs that were prepared against *E. coli* RNAP and its sigma factors very powerful tools for use with *S. oneidensis*. Included in these antibodies are 2G10 (σ^{70}), 3RD3 (σ^{70}), 6RN3 (σ^{54}), 8RB13 (β), and 4RA2 (α). These antibodies will prove to be valuable in transcriptional studies of *S. oneidensis*. *S. oneidensis* has a unique ability to use metals as final electron acceptors, but it can also be easily cultured aerobically in complex medium, making it a practical organism for transcriptional studies.

The immunoaffinity chromatography procedure, using mAb 8RB13, described in this paper, allows purification of milligram quantities of active core RNAP from *S. oneidensis* without any genetic modification of the organism. While the immunoaffinity chromatography using mAb 4RA2 was less promising, experimental conditions might be found that will improve the yield of the enzyme using this immunosorbent. Purified core RNAP is a very useful biological material for studying the transcriptional properties of RNAP. In addition, sigma factors and elongation

factors can be added to the core RNAP from a recombinant source and studied *in vitro*. The fact that the material purified by the broadly cross-reactive β-subunit PR-mAb (8RB13) also co-purifies a protein that reacts with the broadly cross-reactive α-subunit mAb (4RA2) supports the conclusion that we have purified RNAP from *S. oneidensis*, and that these mAbs prepared against *E. coli* RNAP are truly reacting with the *S. oneidensis* RNAP (Fig. 3). Lastly, the material obtained from the 8RB13-Sepharose shows RNAP activity comparable to that of *E. coli* (Fig. 5). These findings would be difficult to explain if the reactions observed in Fig. 1 were being misinterpreted.

The purification of *S. oneidensis* RNAP reported by Mayer et al. [13] required that one subunit of RNAP be modified with a tetracysteine tag. Those authors chose to tag the α-subunit. Our PR-mAb purification system does not require any genetic modification, although it does require that the PR-mAb is cross-reactive with the RNAP subunit from the organism of interest. PR-mAb 8RB13 shows wide cross-reactivity with the β-subunit of almost all of the bacteria that we have tested [20]. Consistent with the results obtained from purification of RNAP from *E. coli, Bacillus subtilis, Pseudomonas aeruginosa, and Streptomyces coelicolor* [20], purification of RNAP from *S. oneidensis* using 8RB13-Sepharose purifies primarily the core form of the RNAP (Fig. 2b). Recent epitope-mapping has shown that the epitope for mAb 8RB13 is contained within one of the major sites that core RNAP uses to bind sigma factors (unpublished data). Surprisingly, none of the RNAP-associated proteins identified by mass spectrometry in the Mayer study [13] were known sigma factors. While σ^{70} has been shown to cross-link to the α-subunit *in vitro* [25], in the Mayer study it is unlikely that the tag on the α-subunit would interfere with σ^{70} binding because the genetic fusion was not lethal to the cell.

The fact that two bands were detected by 2G10 (Fig. 6) was surprising, but presents two possibilities. The first possibility is that another sigma factor that runs at an apparent molecular weight of 95 kDa is present in *S. oneidensis*. This is a distinct possibility because *Mycobacterium smegmatis* has two major sigma factors, each of which reacts with mAb 2G10 [26]. In addition, mAb 2G10 cross-reacts with the alternative *E. coli sigma* factor, σ^{F} [23]. However, purification of RNAP using a 4RA2-Sepharose immunoaffinity column isolates holoenzymes; therefore, a Western blot was run on the peak fractions to test the presence of the double band using the two anti-σ^{70} antibodies. Only the 70–75 kDa band was detected by 2G10 after the purification on 4RA2-Sepharose, which coincided with the band that also reacted with mAb 3RD3 (data not shown). This finding indicates that the larger band was either not a second sigma factor that cross-reacts with mAb 2G10, or that it is a sigma factor that did not remain in complex with RNAP during the purification on 4RA2-Sepharose.

Another possibility is that there is a protein that is not a sigma factor in the whole cell extract of *S. oneidensis* that has an epitope recognized by mAb 2G10. mAb 2G10 recognizes an epitope within the sequence: MLQEMGREPTPEELAER [23]. We have determined that the underlined arginine residue is critical for reactivity of the epitope on a Western blot (unpublished data). A BLAST search of the *S. oneidensis* MR-1 genome [3], using the epitope sequence as a query, readily identified the *S. oneidensis* σ^{70} homolog (with a theoretical molecular weight = 61 kDa); the epitope in this protein deviated from the *E. coli* epitope by only one amino acid. All other proteins identified by this search had only very weak alignments. We identified four proteins with very weak sequence alignments to the epitope with theoretical molecular weights that are larger than *S. oneidensis* σ^{70}, and that also contain the conserved arginine residue: oligopeptidase A, 68 kDa; phosphoenolpyruvate carboxylase, 88 kDa; ankyrin domain protein, 82 kDa; and a conserved hypothetical protein, 161 kDa. It is possible that one of these proteins reacts with mAb 2G10 on Western blots. It would have been present in the crude extract that was used to test the antibodies (Fig. 1), but it is reasonable that it would not be present in the purified RNAP.

Acknowledgments

The authors thank Katherine Foley for her help with the production of the mAbs. This work was funded by Grant GM-28575 from the National Institutes of Health. In compliance with the University of Wisconsin-Madison's Conflict of Interest Committee oversight policies, the authors (R.R.B. and N.E.T.) acknowledge financial interest in the company, NeoClone LLC, Madison, Wisconsin, which markets mAbs 8RB13, 4RA2, 6RN3, and 2G10.

References

[1] J.M. Tiedje, *Shewanella*—the environmentally versatile genome, Nat. Biotechnol. 20 (2002) 1093–1094.
[2] K.H. Nealson, B. Andrea, B. McKee, Breathing metals as a way of life: Geobiology in action, Antonie van Leeuwenhoek 81 (2002) 215–222.
[3] J. Heidelberg, I. Paulsen, K. Nelson, E. Gaidos, W. Nelson, T. Read, J. Eisen, R. Seshadri, N. Ward, B. Methe, R. Clayton, T. Meyer, A. Tsapin, J. Scott, M. Beanan, L. Brinkac, S. Daugherty, R. DeBoy, R. Dodson, A. Durkin, D. Haft, J. Kolonay, R. Madupu, J. Peterson, L. Umayam, O. White, A. Wolf, J. Vanathevan, J. Weidman, M. Impraim, K. Lee, K. Berry, C. Lee, J. Mueller, H. Khouri, J. Gill, T. Utterback, L. McDonald, T. Feldblyum, H. Smith, J. Venter, K. Nealson, C. Fraiser, Genome sequence of the dissimilatory metal ion-reducing bacterium *Shewanella oneidensis*, Nat. Biotechnol. 20 (2002) 1118–1123.
[4] X. Qui, M.J. Daly, A. Vasilenko, M.V. Omelchenko, E.K. Gaidamakova, L. Wu, J. Zhou, G.W. Sundin, J.M. Tiedje, Transcriptome analysis applied to survival of *Shewanella oneidensis* MR-1 exposed to ionizing radiation, J. Bacteriol. 188 (2006) 1199–1204.
[5] Y. Liu, W. Gao, Y. Wang, L. Wu, X. Liu, T. Yan, E. Alm, A. Arkin, D.K. Thompson, M.W. Fields, J. Zhou, Transcriptome analysis of *Shewanella oneidensis* MR-1 response to elevated salt conditions, J. Bacteriol. 187 (2005) 2501–2507.
[6] A.B. Leaphart, D.K. Thompson, K. Huang, E. Alm, X.-F. Wan, A. Arkin, S.D. Brown, L. Wu, T. Yan, X. Liu, G.S. Wickman, J. Zhou,

Transcriptome profiling of *Shewanella oneidensis* gene expression following exposure to acidic and alkaline pH, J. Bacteriol. 188 (2006) 1633–1642.

[7] H. Gao, Y. Wang, X. Liu, T. Yan, L. Wu, E. Alm, A. Arkin, D.K. Thompson, J. Zhou, Global transcriptome analysis of the heat shock response of *Shewanella oneidensis*, J. Bacteriol. 186 (2004) 7796–7803.

[8] H. Gao, Z.K. Yang, L. Wu, D.K. Thompson, J. Zhou, Global transcriptome analysis of the cold shock response of *Shewanella oneidensis* MR-1 and mutational analysis of its classical cold shock proteins, J. Bacteriol. 188 (2006) 4560–4569.

[9] A.S. Beliaev, D.M. Klingeman, J.A. Klappenbach, L. Wu, M.F. Romine, J.M. Tiedje, K.H. Nealson, J.K. Fredrickson, J. Zhou, Global transcriptome analysis of *Shewanella oneidensis* MR-1 exposed to different terminal electron acceptors, J. Bacteriol. 187 (2005) 7138–7145.

[10] R. Bencheikh-Latmani, S.M. Williams, L. Haucke, C.S. Criddle, L. Wu, J. Zhou, B.M. Tebo, Global transcriptional profiling of *Shewanella oneidensis* MR-1 during Cr (VI) and U(VI) reduction, Appl. Environ. Microbiol. 71 (2005) 7453–7460.

[11] T. Yura, K. Nakahigashi, Regulation of heat-shock response, Curr. Opin. Microbiol. 2 (1999) 153–158.

[12] K. Zhao, M. Liu, R.R. Burgess, The global transcriptional response of *Escherichia coli* to induced σ^{32} protein involves σ^{32} regulon activation followed by inactivation and degradation of σ^{32} in vivo, J. Biol. Chem. 280 (2005) 17758–17768.

[13] M.U. Mayer, L. Shi, T.C. Squier, One-step, non-denaturing isolation of and RNA polymerase enzyme complexes using an improved multi-use affinity probe resin, Mol. Biosyst. 1 (2005) 53–56.

[14] N.E. Thompson, R.R. Burgess, Immunoaffinity purification of RNA polymerase II and transcription factors using polyol-responsive monoclonal antibodies, Methods Enzymol. 274 (1995) 513–526.

[15] R.R. Burgess, N.E. Thompson, Advances in gentle immunoaffinity chromatography, Curr. Opin. Biotechnol. 13 (2002) 304–308.

[16] L.C. Anthony, K.M. Foley, N.E. Thompson, R.R. Burgess, Expression, purification of, and monoclonal antibodies to σ factors from *Escherichia coli*, Methods Enzymol. 370 (2003) 181–192.

[17] F.R. Blattner, G. Plunkett 3rd, C.A. Bloch, N.T. Perna, V. Burland, M. Riley, J. Collado-Vides, J.D. Glasner, C.K. Rode, G.F. Mayhew, J. Gregor, N.W. Davis, H.A. Kirkpatrick, M.A. Goeden, D.J. Rose, B. Mau, Y. Shao, The complete genome sequence of *Escherichia coli* K-12, Science 277 (1997) 1453–1474.

[18] N.E. Thompson, D.A. Hager, R.R. Burgess, Isolation and characterization of a polyol-responsive monoclonal antibody useful for gentle purification of *Escherichia coli* RNA polymerase, Biochemistry 31 (1992) 7003–7008.

[19] R.R. Burgess, J.J. Jendrisak, A procedure for the rapid, large-scale purification of *E. coli* DNA-dependent RNA polymerase involving Polymin P precipitation and DNA-cellulose chromatography, Biochemistry 14 (1975) 4634–4638.

[20] V. Bergendahl, N.E. Thompson, K.M. Foley, B.M. Olson, R.R. Burgess, A cross-reactive polyol-responsive monoclonal antibody useful for isolation of core RNA polymerase from many bacterial species, Protein Express. Purif. 31 (2003) 155–160.

[21] J.R. Anthony, H. A Green, T.J. Donohue, Purification of *Rhodobacter sphaeroides* RNA polymerase and its sigma factors, Methods Enzymol. 370 (2003) 54–65.

[22] N.E. Thompson, T.M. Arthur, R.R. Burgess, Development of an epitope tag for the gentle purification of proteins by immunoaffinity chromatography: application to epitope-tagged green fluorescent protein, Anal. Biochem. 323 (2003) 171–179.

[23] M.J. Breyer, N.E. Thompson, R.R. Burgess, Identification of the epitope for a highly cross-reactive monoclonal antibody on the major sigma factor of bacterial RNA polymerase, J. Bacteriol. 179 (1997) 1404–1408.

[24] L. Rao, D.P. Jones, L.H. Nguyen, S.A. McMahan, R.R. Burgess, Epitope mapping using histidine-tagged protein fragments: application to *Escherichia coli* RNA polymerase σ^{70}, Anal. Biochem. 241 (1996) 173–179.

[25] S.A. McMahan, R.R. Burgess, Use of aryl azide cross-linkers to investigate protein–protein interactions: An optimization of important conditions as applied to *Escherichia coli* RNA polymerase and localization of a σ^{70}-α cross-link to the C-terminal region of α, Biochemistry 33 (1994) 12092–12099.

[26] M. Predich, L. Doukhan, G. Nair, I. Smith, Characterization of RNA polymerase and two sigma-factor genes from *Mycobacterium smegmatis*, Mol. Microbiol. 15 (1995) 355–366.