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Bacillus subtilis Class Ib Ribonucleotide Reductase Is a Dimanganese(III)-Tyrosyl Radical Enzyme

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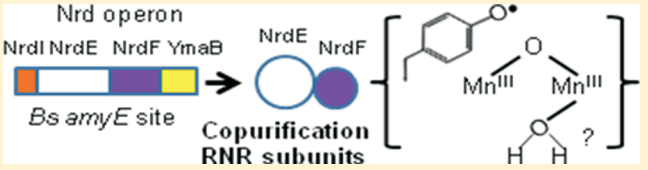
Supporting Information

ABSTRACT: *Bacillus subtilis* class Ib ribonucleotide reductase (RNR) catalyzes the conversion of nucleotides to deoxynucleotides, providing the building blocks for DNA replication and repair. It is composed of two proteins: α (NrdE) and β (NrdF). β contains the metallo-cofactor, essential for the initiation of the reduction process. The RNR genes are organized within the *nrdI-nrdE-nrdF-ymaB* operon. Each protein has been cloned, expressed, and purified from *Escherichia coli*. As isolated, recombinant NrdF (rNrdF) contained a diferric-tyrosyl radical [$Fe(III)_2$-$Y^\bullet$] cofactor. Alternatively, this cluster could be self-assembled from apo-rNrdF, Fe(II), and O_2. Apo-rNrdF loaded using 4 $Mn(II)/\beta_2$, O_2, and reduced NrdI (a flavodoxin) can form a dimanganese-(III)-$Y^\bullet$ [$Mn(III)_2$-$Y^\bullet$] cofactor. In the presence of rNrdE, ATP, and CDP, $Mn(III)_2$-$Y^\bullet$ and $Fe(III)_2$-$Y^\bullet$ rNrdF generate dCDP at rates of 132 and 10 nmol min^{-1} mg^{-1}, respectively (both normalized for 1 $Y^\bullet/\beta_2$). To determine the endogenous cofactor of NrdF in *B. subtilis*, the entire operon was placed behind a Pspank(hy) promoter and integrated into the *B. subtilis* genome at the *amyE* site. All four genes were induced in cells grown in Luria-Bertani medium, with levels of NrdE and NrdF elevated 35-fold relative to that of the wild-type strain. NrdE and NrdF were copurified in a 1:1 ratio from this engineered *B. subtilis*. The visible, EPR, and atomic absorption spectra of the purified NrdENrdF complex (eNrdF) exhibited characteristics of a $Mn(III)_2$-$Y^\bullet$ center with 2 Mn/β_2 and 0.5 $Y^\bullet/\beta_2$ and an activity of 318−363 nmol min^{-1} mg^{-1} (normalized for 1 $Y^\bullet/\beta_2$). These data strongly suggest that the *B. subtilis* class Ib RNR is a $Mn(III)_2$-$Y^\bullet$ enzyme.



Ribonucleotide reductases (RNRs) catalyze the conversion of nucleotides (UDP, ADP, GDP, and CDP) to deoxynucleotides, providing the monomeric building blocks required for DNA replication and repair.[1] RNRs have been classified on the basis of their metallo-cofactor, essential for the initiation of nucleotide reduction. The class I RNRs use dinuclear metal clusters and have been further subdivided (Ia, Ib, and Ic) on the basis of their metal composition (Figure 1).[2] This composition has remained an issue because of the expression of the RNRs in heterologous bacterial systems and addition of metal to crude cell extracts, prejudicing loading of the apoprotein that is often expressed.[2] Our recent studies have suggested that biosynthetic pathways are important for class Ia and Ib cofactor assembly and that often proteins in the pathway are absent or not expressed in recombinant hosts.[2,3] In this paper, we report the isolation and characterization of the *Bacillus subtilis* (*Bs*) class Ib RNR from recombinant (r) and endogenous (e) sources and the characterization of their metallo-cofactors.

All class I RNRs contain two proteins, α and β, which in the class Ia prokaryotic systems are homodimers and form active $\alpha_2\beta_2$ complexes. α is the site of nucleotide reduction and allosteric effector binding (dATP, TTP, dGTP, and ATP) that controls the specificity of reduction. It is named NrdA for the class Ia and Ic RNRs and NrdE for the class Ib RNRs. β houses the metal cofactor essential for the initiation of reduction and is named NrdB for the class Ia and Ic RNRs and NrdF for the Ib RNRs. All class I β proteins are structurally homologous, and the class Ia and Ib RNRs contain identical metal ligands (compare panels A and B of Figure 1).[4,5]

There are a number of important distinctions between the class Ia and Ib RNRs. One is the presence of the N-terminal ATP cone domain (activity site) that binds ATP and dATP and regulates the rate of nucleotide production in NrdA, which is absent in NrdE. A second is the fact that the class Ib RNR genes, *nrdE* and *nrdF*, are almost always found in an operon with *nrdI* encoding an unusual flavodoxin that plays an essential role in the formation of the metal cofactor assembled in NrdF in vivo.[6−8]

Finally, the most intriguing distinction between the class Ia and Ib β proteins is the metal composition of the cofactors active in nucleotide reduction. The class Ia RNRs contain only a diferric-tyrosyl radical [$Fe(III)_2$-$Y^\bullet$] cofactor (Figure 1A). It can be formed by self-assembly in vitro from apo-NrdB, Fe(II), and O_2[9] and is identical to the cofactor isolated from endogenous sources.[9,10] The class Ib NrdFs, however, can form either an $Fe(III)_2$-$Y^\bullet$ or a dimanganese(III)-$Y^\bullet$ [$Mn(III)_2$-$Y^\bullet$] cofactor in vitro, and both are active in nucleotide reduction.[7,11,12] The NrdF $Fe(III)_2$-$Y^\bullet$ cofactor can also be formed by self-assembly in vitro, with varying degrees of success, in a fashion similar to that of class

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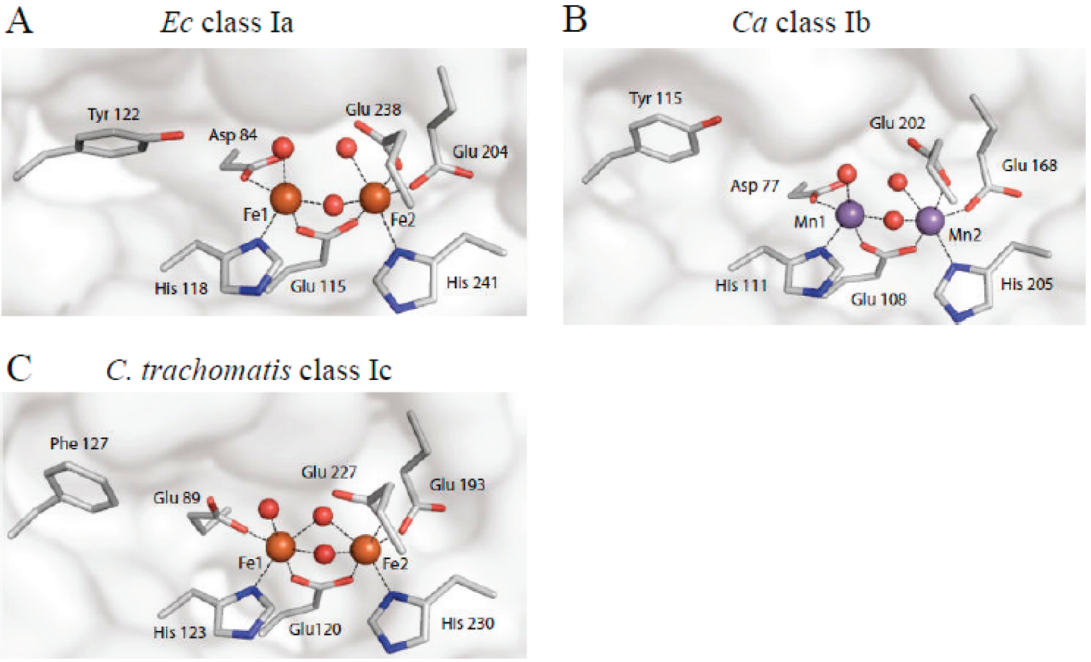

Figure 1. Structures of the metallo-cofactors of the three class I RNRs (adapted from ref 2). Iron and manganese ions are depicted as brown and purple spheres, respectively. (A) *Ec* class Ia $Fe(III)_2$ cluster.[5] (B) *Ca* Ib $Mn(III)_2$ cluster.[4] (C) *Chlamydia trachomatis* class Ic $Fe(III)_2$ cluster.[45] Note that *C. trachomatis* class Ic RNR is active with a $Mn^{IV}Fe^{III}$ cofactor,[46] but a structure is not yet available.

Ia NrdB.[2] However, assembly of the $Mn(III)_2$-$Y^\bullet$ cofactor in vitro requires in addition to apo-NrdF and O_2, Mn(II) and NrdI with the hydroquinone form of its FMN cofactor ($NrdI_{hq}$).[7] $NrdI_{hq}$ is unable to assist Mn(II)-loaded NrdB in assembly of a Mn-$(III)_2$-$Y^\bullet$ cofactor.[7] Finally, recent studies of NrdF isolated from endogenous sources, *Escherichia coli* (*Ec*) and *Corynebacterium ammoniagenes* (*Ca*), have shown that they both possess only a $Mn(III)_2$-$Y^\bullet$ cofactor (Figure 1B).[4,8,12] The activity of β proteins with $Fe(III)_2$-$Y^\bullet$ and $Mn(III)_2$-$Y^\bullet$ cofactors raises several issues. One is whether the active form of the cofactor can be modulated by growth conditions and, if so, how metalation (mismetalation) is controlled.[2,3] A second is whether all class Ib RNRs utilize $Mn(III)_2$-$Y^\bullet$ cofactors or whether there are yet to be identified conditions under which active iron clusters are generated.

We decided to identify the metallo-cofactor of the class Ib RNR from *Bs* for several reasons. First, like *Ec*, *Bs* is a well-studied model organism with accessible genetics and biochemistry. Second, in contrast to *Ec* that contains both class Ia and Ib RNRs, where the Ia enzyme is essential for normal aerobic growth, *Bs* contains a single, class Ib, RNR that is essential for normal aerobic growth. Finally, many human pathogens [*Bacillus anthracis* (*Ba*), *Mycobacterium tuberculosis* (*Mt*), *Staphylococcus aureus* (*Sa*), and *Streptococcus pyogenes* (*Sp*)] also contain a single, class Ib RNR,[13] and thus, knowledge about the *Bs* enzyme might be informative with respect to the metallo-cofactor in these other organisms.

To determine the active form of the class Ib RNR in *Bs*, NrdF was isolated from a strain in which the operon containing the *nrdI*, *nrdE*, *nrdF*, and *ymaB* genes was cloned into pDR111 under a Pspank(hy) promoter and then integrated into the *Bs* genome in the *amyE* site.[14] All four proteins were expressed, and their concentrations were upregulated. Levels of NrdE and NrdF were increased 35-fold relative to that of the wt strain, facilitating RNR purification. Surprisingly, NrdF was copurified with NrdE in a 1:1 ratio (eNrdF is the complex of NrdEF isolated from *Bs*). UV−vis and electron paramagnetic resonance (EPR) spectra of the purified eNrdF resemble those recently reported for *Ec* $Mn(III)_2$-$Y^\bullet$ and *Ca* $Mn(III)_2$-$Y^\bullet$ NrdF.[4,7,8,12] eNrdF in this complex contained 2 Mn/β_2 and 0.5 $Y^\bullet/\beta_2$. The four genes from the operon were also individually cloned, expressed, and purified from *Ec* (recombinant proteins isolated from *Ec* are hence designated by an r before the protein name). $Mn(III)_2$-$Y^\bullet$ and $Fe(III)_2$-$Y^\bullet$ cofactors in rNrdF were reconstituted in vitro from apo-rNrdF, Mn(II), $NrdI_{hq}$, and O_2 in the former case and Fe(II) and O_2 in the latter case. The $Mn(III)_2$-$Y^\bullet$ rNrdF has activity (132 nmol min^{-1} mg^{-1}) that is ~36% of that of the endogenous enzyme purified from *Bs* (363 nmol min^{-1} mg^{-1}) and is 13 times greater than that of the $Fe(III)_2$-$Y^\bullet$ rNrdF (10 nmol min^{-1} mg^{-1}) . The results together strongly suggest that the $Mn(III)_2$-$Y^\bullet$ cofactor is the active cofactor in the *Bs* class Ib RNR in vivo and that NrdI plays an essential role in its formation.

MATERIALS AND METHODS

Cloning, Expression, and Purification of *Bs* N-Terminally Tagged His_6-rNrdE, -rNrdF, -rNrdI, and -rYmaB from *Ec*. Details of the cloning, expression, and purification of rNrdE, rNrdF, rNrdI, and rYmaB are provided as Supporting Information. Briefly, the *Bs* NrdE, NrdF, NrdI, and YmaB genes were amplified by polymerase chain reaction using the wt *Bs* JH624 genomic DNA [a generous gift from A. Grossman (Massachusetts Institute of Technology)] as a template. The amplified DNA fragments were then cloned into pET14b (Novagen). In this construct, each protein has a His_6-SSGLVPRGSH tag at its N-terminus. The resulting plasmids were sequenced and then transformed into BL21 codon Plus cells (Stratagene). A nickel

nitrilotriacetic acid (Ni-NTA) affinity column (Qiagen) was employed to purify each His-tagged protein from cells induced at an A_{600} of 0.8 with 0.2 mM isopropyl β-D-thiogalactoside (IPTG). To purify rNrdI to homogeneity, an additional DEAE-Sepharose chromatography step was required and is described in the Supporting Information. All four proteins were purified to >95% homogeneity. The specific activity (SA) of His_6-rNrdE was 2 nmol min^{-1} mg^{-1} with a 10-fold excess of $Mn(III)_2$-$Y^\bullet$ His_6-rNrdF (73 nmol min^{-1} mg^{-1}). The SA of $Fe(III)_2$-$Y^\bullet$ His_6-rNrdF as isolated (0.2 $Y^\bullet/\beta_2$) was 4.5 nmol min^{-1} mg^{-1}.

Antibodies. Polyclonal rabbit antibodies (Abs) against His_6-rNrdE, -rNrdF, -rNrdI, and -rYmaB were produced by Covance Research Products (Denver, PA).

Metal Content. Manganese and iron concentrations were determined using a Perkin-Elmer Analyst 600 atomic absorption spectrometer with manganese and iron standards from Fluka. The iron content in heterologously expressed and reconstituted NrdFs was quantitated by the ferrozine method.[15]

Protein Concentration. Concentrations of purified His_6-rNrdE, -rNrdF, and -rYmaB were determined by A_{280} using ε_{280} [79.5, 54.8, and 18.5 mM^{-1} cm^{-1}, respectively, and 134.2 mM^{-1} cm^{-1} for eNrdF calculated using Protparam in ExPASy[16] (http://expasy.org/tools/protparam.html)]. To determine the His_6-rNrdI protein concentration, trichloroacetic acid (TCA) was added to a final concentration of 3%.[17] The FMN was removed by centrifugation at 20000*g*, and the pellet was washed with 3% TCA in buffer. The colorless pellet was isolated by centrifugation and redissolved in 500 μL of 50 mM Tris-HCl (pH 7.6) with 0.1% SDS. The apo-NrdI concentration was determined by A_{280} using an ε_{280} estimated to be 10 mM^{-1} cm^{-1}.[16]

Characterization of His_6-NrdI. The method for determining ε_{449ox} and ε_{610sq} of FMN bound to NrdI was conducted as previously described[18] and is detailed in the Supporting Information.

Construction of the *Bs* 1B-UP Strain. Two primers, 5′-TATACTGCTAGCAGTGTTAGTGATGAGAGATTCA-3′ and 5′-AACCGGGCTAGCTTATTCAAGAATATCAACAA-CAAAC-3′, each containing an NheI site (underlined), were used to amplify the entire class Ib operon (4.1 kb) using wt *Bs* JH624 genomic DNA as a template. The amplified fragment was cloned into pDR111 [a gift from David Rudner (Harvard Medical School, Boston, MA)],[14] a plasmid containing *lacI* and *specR* [a gene encoding spectinomycin (Spect) resistance], under a Pspank(hy) promoter that is repressed by LacI. The resulting plasmids were isolated and digested with *Hin*dIII (NEB) to select for the correct orientation of the insert and designated pDR111-1B-UP. This plasmid was used to transform *Bs* wt strain JH642 (pheA1 trpC2),[19] and the transformants were selected on Luria-Bertani (LB) agar plates containing 100 μg/mL Spect. Transformants were then streaked on starch plates and grown at 37 °C for 12 h before the contents of the plates were stained with iodine to select for starch+ colonies. A starch+ phenotype indicates that homologous recombination has occurred at the *amyE* site and that the *amyE* gene has been disrupted. Disruption of the *amyE* gene removes the ability of *Bs* to break down starch, which can be assayed with iodine as described by Cutting and Vander-horn.[20]

Cell Growth and Protein Purification of eNrdF. The *Bs* 1B-UP strain was grown in 5 mL of LB medium containing 100 μg/mL Spect overnight at 37 °C. The starter culture was diluted in 2 L of the same medium containing 1 mM IPTG in a 6 L baffled flask and grown at 37 °C with shaking at 200 rpm. At an A_{600} of 0.8, the cells were harvested by centrifugation at 3000*g* for 10 min. The cell paste (2.9 g) was resuspended in 10 mL of buffer A [50 mM Tris-HCl (pH 7.6) and 5% glycerol] supplemented with 1 tablet of protease inhibitor cocktail (Complete Mini, EDTA free, Roche). Cells were then lysed with a French press at 14000 psi, and the cell debris was removed by centrifugation at 48000*g* for 30 min. $(NH_4)_2SO_4$ (40% saturation, 243 g/L) was added gradually over 30 min to the cell lysate that was being stirred. The supernatant was collected after centrifugation at 48000*g* for 30 min. Additional $(NH_4)_2SO_4$ was added to 60% saturation (142 g/L) while the mixture was being stirred over 30 min, followed by centrifugation at 48000*g* for 30 min. The supernatant was discarded. The pellet was redissolved in 10 mL of buffer A containing 40% $(NH_4)_2SO_4$ (buffer B). This protein solution was loaded on a Butyl Sepharose column (10 mL, 2.5 cm × 2 cm), and the column was washed with 100 mL of buffer B at a rate of 2.25 mL/min. The protein was eluted with an 80 mL × 80 mL linear gradient [buffer B to buffer A, 40 to 0% $(NH_4)_2SO_4$] and further eluted with 50 mL of buffer A at a flow rate of 3.5 mL/min. Fractions were collected every 3 min, and those containing RNR activity were pooled and concentrated using an Amicon Ultra YM30 centrifugal concentrator (Millipore). The solution was diluted with 10× buffer A, reconcentrated to 1 mL, frozen in liquid N_2, and stored at −80 °C.

The protein solution (7.5 mg/mL, 1 mL) was then thawed on ice and chromatographed using a Poros HQ 20 FPLC anion exchange column (Applied Biosystems, 1.6 cm × 10 cm, 20 mL, flow rate of 2 mL/min). The column was equilibrated with 40 mL of buffer A containing 300 mM NaCl. After being loaded, the column was washed with 40 mL of buffer A containing 300 mM NaCl, and the protein was eluted with a 120 mL linear gradient, from 300 to 550 mM NaCl in buffer A, followed by 60 mL of 1.5 M NaCl in buffer A. Fractions (2 mL) were collected. NrdEF-containing fractions were identified by SDS−PAGE and RNR activity assays. Fractions 39−42 were pooled and concentrated to 420 μL as described above. This protocol resulted in NrdEF purified to ∼80% homogeneity.

Enzyme Activity Assays. All activity assays for rNrdF or eNrdF contained, in a final volume of 220 μL, NrdF [eNrdF (0.2 μM) or rNrdF (1 μM)], 2−10 μM rNrdE (10-fold excess relative to eNrdF or rNrdF), 0.3 mM dATP (or 1 mM ATP), 20 mM DTT, 10 mM NaF, and 0.5 mM [^{3}H]CDP (ViTrax, 5700−21000 cpm/nmol), in 50 mM Hepes (pH 7.6) at 37 °C. Aliquots (52 μL) were removed every 5 min over 15 min. Each sample was then incubated with 10 units of alkaline phosphatase (Roche, from calf intestine), and the amount of dCDP produced was analyzed by the method of Steeper and Steuart.[21]

A typical assay of the fractions from the Butyl Sepharose and Poros HQ 20 columns contained, in a final volume of 50 μL, 20 μL of protein solution from each fraction, 6 μM rNrdE (SA of 2 nmol min^{-1} mg^{-1}), 0.3 mM dATP, 20 mM DTT, 10 mM NaF, and 0.5 mM [^{3}H]CDP (ViTrax, 5700 cpm/nmol), in 50 mM Hepes (pH 7.6). The reaction mixture was incubated at 37 °C for 30 min, and the reaction was then stopped when the mixture was heated at 100 °C for 2 min. The workup for dCDP formation is as described above.

EPR Spectroscopy. EPR spectra of NrdF were recorded on a Bruker EMX X-band spectrometer at 77 or 4 K using a liquid N_2 finger dewar or an Oxford Instruments liquid helium cryostat, respectively. Acquisition parameters were as described previously.[8] Spin quantitation was performed by double integration of the signal and comparison with a copper standard[22] or an *Ec* NrdB sample

with a known $Y^{\bullet}$ concentration. Analysis was conducted using WinEPR (Bruker).

Western Blot Analyses of NrdE, NrdF, NrdI, and YmaB in *Bs* wt and 1B-UP Strains. *Bs* wt and 1B-UP cells grown in LB medium and LB medium with 1 mM IPTG, respectively, were harvested at an A_{600} of ~0.8, and the cell pellets were resuspended in buffer A (2 mL/g of cell paste) supplemented with a protease inhibitor cocktail (Roche). The cells were lysed with the French press and centrifuged at 48000*g* at 4 °C for 20 min to remove cell debris. The protein concentration of the supernatant was determined using a Bradford assay and BSA as a standard. The supernatant was diluted with 2× Laemmli buffer[23] and boiled for 5 min at 100 °C. Standard curves were generated using known amounts of purified His_6-rNrdE, -rNrdF, -rNrdI, and -rYmaB (Figure S4 of the Supporting Information). All samples were loaded onto either a 10% (for NrdE and NrdF) or a 15% (for NrdI and YmaB) SDS−PAGE gel (Bio-Rad).[23] The gel was run at 200 V for 45 min, and the proteins were transferred to nitrocellulose membranes (Whatman) at 100 V and 4 °C for 80 min in 50 mM Tris, 40 mM glycine, 20% (v/v) methanol, and 0.03% (w/v) SDS. Primary Abs were added at a 1:500 dilution. Stabilized peroxidase-conjugated goat anti-rabbit (H + L) secondary Ab (Thermo Scientific) was used at a 1:5000 dilution. The nitrocellulose membranes were developed using Super-Signal West Femto Maximum Sensitivity Substrate (Thermo scientific). A CCD camera (ChemiDoc XRS Bio-Rad) was used to record the blotting signals. The proteins were quantified by analyses of the signal intensities with Quantity One (Bio-Rad).

RESULTS

***Bs* rNrdF Heterologously Expressed in *Ec* Contains an $Fe(III)_2$-$Y^{\bullet}$ Cofactor and Is Enzymatically Active.** *Bs* rNrdE and rNrdF, with a His_6 tag and 10-amino acid spacer appended to their N-termini, were cloned from the *Bs* genomic DNA and expressed in *Ec*. Each protein was purified to >95% homogeneity using a Ni-NTA agarose column (Figure S1A,B of the Supporting Information). The UV−vis spectrum of the purified rNrdF (Figure S1C of the Supporting Information) exhibited absorbance features at 325, 365, and 410 nm, associated with an $Fe(III)_2$-$Y^{\bullet}$ cluster. The EPR spectrum at 77 K (Figure S1D of the Supporting Information) was identical to that of the iron-loaded *Ba* rNrdF[24] with additional hyperfine structure that is not observed in spectra reported for the iron-loaded, class Ib, rNrdFs from *Ca*, *Mt*, *Ec*, and *Sp*.[6,11,18,25] The protein as isolated had 0.2 $Y^{\bullet}/\beta_2$. When Fe(II) was added to the crude cell lysate prior to its purification, the $Y^{\bullet}$ content increased to 0.8 $Y^{\bullet}/\beta_2$ (Table 1).[18] Incubation of iron-loaded rNrdF with 10 equiv of NrdE, [^{3}H]CDP as the substrate, and 1 mM ATP or 0.3 mM dATP as an effector gave an activity of 27 or 19 nmol min^{-1} mg^{-1}, respectively (normalized for 1 $Y^{\bullet}/\beta_2$) (Table 1). Thus, an $Fe(III)_2$-$Y^{\bullet}$ active cluster can self-assemble in *Bs* rNrdF, although its activity and those of many other class Ib iron-loaded rNrdFs are low in comparison with that of class Ia $Fe(III)_2$-$Y^{\bullet}$ NrdBs (*Ec*, 6000 nmol min^{-1} mg^{-1}).[2]

***Bs* rNrdI Contains FMN and Exhibits Redox Properties Similar to Those of *Ec* NrdI.** The His_6-tagged *Bs nrdI* gene was cloned, and the protein was expressed and purified to 95% homogeneity by Ni-NTA and anion exchange chromatography as described in the Supporting Information (Figure S2A). The ε_{449ox} for NrdI with FMN ($NrdI_{ox}$), which has a λ_{max} at 449 nm, was established to be 9.6 mM^{-1} cm^{-1} by determining the amount of dithionite required to reduce $NrdI_{ox}$ to $NrdI_{hq}$ (Supporting Information and Figure S2B) and using denatured NrdI to determine the amount of protein. NrdI as isolated contained 1 FMN/monomer. The ε_{610sq} for $NrdI_{sq}$ (Figure S2B of the Supporting Information) was determined to be 4.9 ± 0.7 mM^{-1} cm^{-1} as previously described for *Ec* NrdI.[18] The maximal percentage of FMN stabilized in the sq form (24%) (Figure S2B of the Supporting Information) is similar to that observed for *Ec* NrdI (30%)[18] and lower than that reported for *Ba* (60%) and *Bc* (100%) NrdI.[26,27]

Table 1. Specific Activities of $Fe(III)_2$-$Y^{\bullet}$ rNrdF and $Mn(III)_2$-$Y^{\bullet}$ eNrdF

	$Fe(III)_2$-$Y^{\bullet}$ rNrdF (0.8 $Y^{\bullet}$)[a]		$Mn(III)_2$-$Y^{\bullet}$ eNrdF (0.4 $Y^{\bullet}$)[b]	
	ATP	dATP	ATP	dATP
SA[c]	22	15	160	52
SA[c] per $Y^{\bullet}$	27	19	363	117

[a] Purified from *Ec* and reconstituted by addition of Fe(II) to crude extracts. [b] Purified from *Bs*. [c] SA was measured with ATP or dATP as the allosteric effector and is given in nanomoles per minute per milligram.

rNrdI Is Required for the Formation of an Active $Mn(III)_2$-$Y^{\bullet}$ Cluster in Vitro from rNrdF. Apo-rNrdF was isolated using 1,10-phenanthroline in the growth medium[18,28] and purified to homogeneity. The assembly of the manganese cofactor was conducted via incubation of Mn(II)-loaded β_2 (25 μM) and $NrdI_{hq}$ (50 μM) followed by addition of 1 mM O_2[7] and incubation for 3 min at room temperature. Kinetic studies showed that the reaction is complete within this time (data not shown). The mixture was then transferred to an EPR tube and analyzed at 77 K (Figure S3A of the Supporting Information, blue).[7] Chelex-100 (Na^+ form) was then added to the sample to remove unreacted Mn(II), and the EPR spectrum was again recorded and the spin quantitated (Figure S3B of the Supporting Information). A control in the absence of NrdI showed the spectrum of $Mn(H_2O)_6^{2+}$ (Figure S3A of the Supporting Information, red). The amount of Mn in the sample was quantitated by atomic absorption. The $Y^{\bullet}/\beta_2$ and Mn/β_2 ratios [0.55 and 1.9, respectively (Table S1 of the Supporting Information)] were both substoichiometric, but the $Y^{\bullet}/\beta_2$ ratio is higher than those recently reported for the $Mn(III)_2$-$Y^{\bullet}$ cofactor in endogenous NrdFs isolated form *Ec* and *Ca*.[4,7,8,12]

Apo-rNrdF was also reconstituted with Fe(II) and O_2 to give the $Fe(III)_2$-$Y^{\bullet}$ cofactor with 0.9 $Y^{\bullet}/\beta_2$ and 2.6 Fe/β_2 (Table S1 of the Supporting Information). The EPR spectrum for the $Fe(III)_2$-$Y^{\bullet}$ cluster is distinct (Figure S3C of the Supporting Information) from that of the $Mn(III)_2$-$Y^{\bullet}$ cluster, as previously demonstrated with *Ec* NrdF.[7]

Enzyme activity assays were performed with the reconstituted Mn- and Fe-loaded rNrdFs. Assays were typically conducted with 1 μM rNrdF, 10 μM His_6-rNrdE (SA of 2 nmol min^{-1} mg^{-1}), 0.5 mM CDP, 1 mM ATP as the effector, and 20 mM DTT as the reductant.[21] The activity of Mn-rNrdF was 132 nmol min^{-1} mg^{-1}, while that of Fe-rNrdF was 10 nmol min^{-1} mg^{-1} (Table S1 of the Supporting Information, normalized for 1 $Y^{\bullet}/\beta 2$). Thus, both Fe- and Mn-loaded rNrdFs are active, with the activity of the latter being approximately 13 times greater than that of the former after normalization for $Y^{\bullet}$. These results are similar to those recently reported for *Ec* NrdF.[7]

Construction of a *Bs* Strain (1B-UP) with the Four Genes in the Class Ib Operon Overexpressed. To facilitate purification

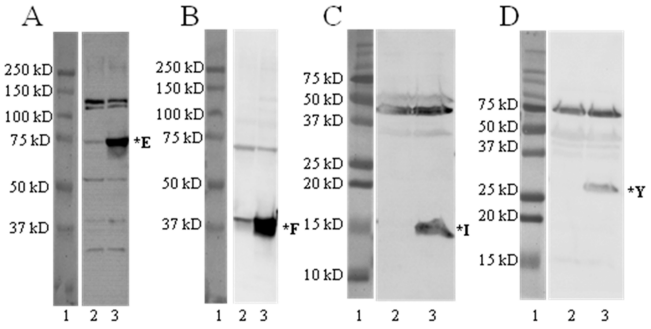

Figure 2. Western blots of NrdE (A), NrdF (B), NrdI (C), and YmaB (D) in wt and 1B-UP *Bs* strains: lane 1, molecular mass standards; lane 2, wt strain; lane 3, 1B-UP strain.

of NrdF from *Bs* in sufficient quantities to characterize its metallo-cofactor, we constructed a strain in which the entire class Ib operon (4.1 kb) was cloned under the Pspank(hy) promoter in pDR111 and integrated into the *amyE* site of the *Bs* JH624 strain (see Materials and Methods) to give *Bs* 1B-UP.[14,19] The entire operon was cloned in an effort to maintain the relative expression levels of each gene in the operon that could be crucial for metallo-cofactor assembly. Activity assays and Abs raised to rNrdE, rNrdF, rNrdI, and rYmaB were used to evaluate the success of our strategy.

The activity of NrdF in crude cell lysates of the wt and 1B-UP strain was determined by addition of excess His_6-rNrdE. The value of 0.05 nmol min^{-1} mg^{-1} for wt *Bs* is 1600-fold higher than that previously reported,[29] and the value of 0.9 nmol min^{-1} mg^{-1} for 1B-UP is 18 times greater than that of the wt strain. Western blots of crude cell extracts in both strains revealed that the levels of all four proteins were clearly elevated in the 1B-UP strain relative to the wt strain (in Figure 2, compare lanes 2 and 3). Quantitative Western blots were also performed (Figure S4 of the Supporting Information) using the recombinant protein standards, and the NrdE:NrdF:NrdI:YmaB molar ratio in the 1B-UP strain was 64:64:8:1. The ratio of NrdE and NrdF relative to the wt strain was increased 35-fold. The level of YmaB was elevated ~10-fold, while the level of NrdI increased 80−190-fold relative to that of the wt strain. The atypical ribosome binding site of NrdI is likely responsible for its inefficient translation in the wt strain.[30] The Pspank(hy) promoter in the 1B-UP strain has resulted in an increased level of transcription as well as more efficient translation.

Purification of eNrdF from the 1B-UP Strain. The purification of eNrdF from the 1B-UP strain is summarized in Table 2 and Figure 3. Western blotting revealed that both NrdF and NrdE precipitated between 40 and 60% $(NH_4)_2SO_4$ and were separated from YmaB and NrdI, which predominantly precipitated with 40% $(NH_4)_2SO_4$ (data not shown). The resulting pellet was redissolved in a 40% $(NH_4)_2SO_4$ solution and purified using Butyl Sepharose chromatography (Figure S5A of the Supporting Information). Fractions containing RNR activity were pooled, desalted, and then purified further using Poros HQ20 FPLC anion exchange chromatography. The elution profile is shown in Figure S5B of the Supporting Information. The appropriate fractions were pooled and stored at ~1 mg/mL and −80 °C.

SDS−PAGE analysis of each step in the purification is shown in Figure 3A and revealed a band at 75 kDa that copurified with NrdF. As our His_6-rNrdE migrated at 75 kDa (actual molecular mass of 80 kDa), we wondered if the proteins migrating in this region could contain NrdE. Abs to NrdE and Western analysis revealed its presence. The ratio of NrdE to NrdF was determined in crude cell extracts by quantitative Western analysis and on purified protein (Figure 3A, lane 5) by Coomassie staining in comparison with known concentrations of His_6-rNrdE and His_6-rNrdF. In both cases, the ratio was 1:1 and the purified eNrdF was judged to be ~80% pure (Figure 3A, lane 5). The copurification of NrdE and NrdF was unexpected and exciting as in general, the subunit interactions of class I RNRs are weak and the subunits separate during chromatography.[31,32] A unique opportunity for structural characterization crystallographically and by cryoEM thus presents itself.

Table 2. Purification of eNrdF from the *Bs* 1B-UP Strain[a]

step[b]	volume (mL)	concn (mg/mL)	protein (mg)	SA[c] (units/mg)	TA[d] (units)	yield (%)
lysate	10	15.2	152	0.85	129	100
AS	10.7	7	74.5	0.65	48.1	37.3
butyl	1	7.5	7.5	1.94	14.5	11.2
FPLC	0.42	1.1	0.5	17.2[e]	8.1	6.3

[a] Purified from 2.9 g of cell paste. [b] Lysate, AS, butyl, and FPLC denote crude cell lysate, ammonium sulfate precipitate, protein after Butyl Sepharose chromatography, and protein after FPLC Poros HQ20 chromatography, respectively. [c] SAs were measured using dATP as an allosteric effector and are given in nanomoles per minute per milligram of total protein. [d] Total activity. [e] Purified eNrdF. The SAs for NrdF and eNrdF are therefore 52 and 117 nmol min^{-1} mg^{-1}, respectively (normalized for 1 $Y^{\bullet}$) (Table 1).

eNrdF from the 1B-UP Strain Contains a $Mn(III)_2$-$Y^{\bullet}$ Cofactor. A UV−vis spectrum of purified eNrdF is shown in Figure 3B and its inset. The spectrum is similar to that of the manganese-reconstituted rNrdF with a sharp peak and broad shoulder at 410 and 390 nm, respectively, indicative of $Y^{\bullet}$. The light scattering obscures the broad features observed with the *Ec* $Mn(III)_2$-$Y^{\bullet}$ cofactor from 500 to 600 nm, indicative of oxidized dimanganese clusters.[2] The 325 and 365 nm features associated with the diferric cluster (Figure S1C of the Supporting Information) are absent.[7] eNrdF was also analyzed by EPR spectroscopy at 77 and 4 K (Figure 4A,B), and spin quantitation revealed 0.4−0.5 $Y^{\bullet}/\beta_2$ (four independent experiments). These spectra are very similar to that of our reconstituted $Mn(III)_2$-$Y^{\bullet}$ rNrdF and to those reported for *Ec* and *Ca* NrdFs.[4,7,8,12] Atomic absorption studies revealed that NrdF contained 1.8−2.4 Mn/β_2 and 0.14 Fe/β_2 (Table 3). Thus, in all cases of $Mn(III)_2$-$Y^{\bullet}$ cofactor detected, metal loading and $Y^{\bullet}$ are substoichiometric.

Activity of $Mn(III)_2$-$Y^{\bullet}$ rNrdF Compared with That of $Fe(III)_2$-$Y^{\bullet}$ rNrdF. The assay for the class Ib NrdF was based on extensive studies of class Ia RNRs. For the Ia RNRs, the α and β subunits separate during purification because of their weak interactions,[32] and as a consequence, each subunit is assayed in the presence of an excess of the second subunit to ensure 100% "active" complex formation. In addition, the oxidation of two cysteines in the active site of α to a disulfide accompanies substrate reduction,[1] and thus, a mechanism for disulfide reduction is essential for measuring multiple turnovers of NDP to dNDP. Because the reductant in vivo for most class Ib RNRs has not been extensively studied, the nonphysiological reductant dithiothreitol (DTT) is typically used.[33] The activity with DTT relative to the endogenous reductant is unknown. Finally, the allosteric regulation of the class Ib enzymes is not as well studied

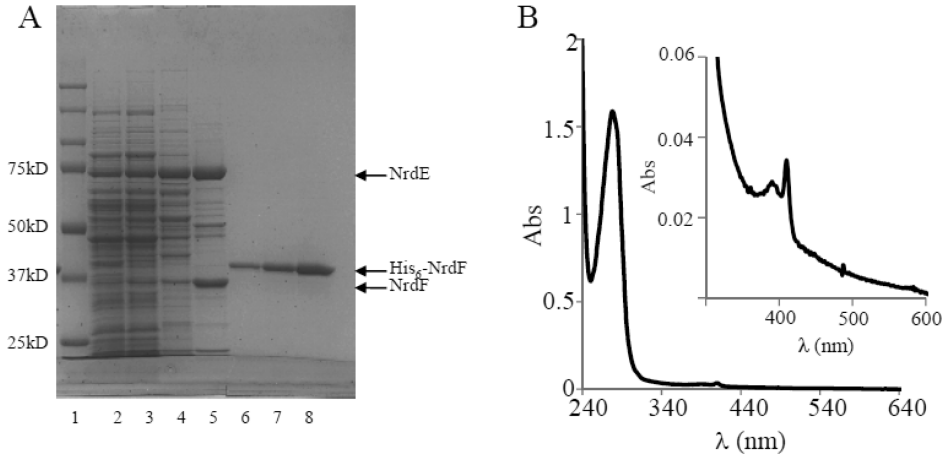

Figure 3. Purification of eNrdF from the *Bs* 1B-UP strain. (A) Samples from each step of the purification were loaded on a 10% SDS−PAGE gel: lane 1, protein molecular mass markers; lane 2, 30 μg of crude cell extracts; lane 3, 30 μg after ammonium sulfate precipitation; lane 4, 15 μg after Butyl Sepharose chromatography; lane 5, 10 μg after the FPLC Poros HQ20 column; lanes 6−8, 1, 2, and 4 μg of His_6-rNrdF, respectively. (B) Visible spectrum of eNrdF. The inset is an enlargement to magnify the features of the $Mn(III)_2$-$Y^\bullet$ cluster.

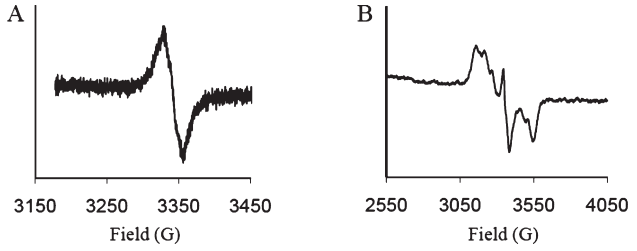

Figure 4. X-Band EPR spectra of eNrdF at 77 (A) and 4 K (B). (A) The spectrum was recorded at 77 K and 9.4 GHz, with a 1 mW power, a 2.52×10^4 gain, a 1.5 G modulation amplitude, and a 100 kHz modulation frequency. (B) The spectrum was recorded at 4 K and 9.4 GHz, with a 1 mW power, a 1.26×10^4 gain, a 4.0 G modulation amplitude, and a 100 kHz modulation frequency.

Table 3. Metal Content of rNrdF and eNrdF

	$Fe(III)_2$-$Y^\bullet$ rNrdF[a]	$Mn(III)_2$-$Y^\bullet$ NrdF[b]
$Y^\bullet/\beta_2$	0.18	0.44
Mn/β_2	0.038	1.8−2.4
Fe/β_2	0.9	0.14

[a] Purified from *Ec*. [b] Purified from *Bs*.

as that of the Ia enzymes and remains to be examined in a case by case study with the physiologically relevant metallo-cofactor.

In the assays for the Fe- and Mn-loaded rNrdF, a 10-fold excess of rNrdE was used, CDP was the substrate, ATP or dATP was the effector, and DTT was the reductant. The ratio of Mn-loaded rNrdF to Fe-loaded rNrdF activity, when normalized for 1 $Y^\bullet/\beta 2$, is 132:10. We note that both rNrdE and rNrdF are N-terminally tagged. Removal of the tag from rNrdE and rNrdF, leaving three amino acids (GSH), however, had little effect on activity.

In the assays of eNrdF activity, there is the added complexity that NrdE and NrdF are copurified, and thus, initially no NrdE was added to the assay mixtures. The activity was, however, only 32 nmol min^{-1} mg^{-1} (normalized for 1 $Y^\bullet/\beta_2$). Thus, eNrdF was also titrated with variable amounts of rNrdE (Table S2 of the Supporting Information). With a 10-fold excess, the SA ranged from 318 (Table S2 of the Supporting Information) to 363 nmol min^{-1} mg^{-1} (Table 1, both normalized for 1 $Y^\bullet/\beta_2$). This number is 3 times higher than that of the rNrdF [132 nmol min^{-1} mg^{-1} (Table S1 of the Supporting Information)] and is 10- and 1000-fold lower than the activities reported recently for $Mn(III)_2$-$Y^\bullet$ NrdF isolated from endogenous *Ec* and *Ca*, respectively.[4,8,12]

The basis for the requirement for excess rNrdE to optimize the activity eNrdF is not understood. The most reasonable explanation is that DTT was excluded from buffers during the purification of eNrdF to prevent reduction of the $Y^\bullet$ in NrdF.[34] NrdE is susceptible to oxidation (five essential cysteines),[35] and thus, the low activity may be associated with irreversible oxidation of NrdE in eNrdF. As noted above, the physiological reductant for the *Bs* RNR would likely increase activity. Finally, rYmaB, the protein in the RNR operon of unknown function,[30] has no effect on catalytic activity.

Although the *Bs* class Ib activity assay requires further optimization, several conclusions are apparent. First, that CDP is more rapidly reduced with ATP than with dATP as the allosteric effector (Table S2 of the Supporting Information and Table 1). These results are distinct from those reported previously for the $Fe(III)_2$-$Y^\bullet$ class Ib RNRs.[36,37] The second and major result is that the $Fe(III)_2$-$Y^\bullet$ rNrdF generated in vitro has only ∼10% of the activity (27 nmol min^{-1} mg^{-1}) of $Mn(III)_2$-$Y^\bullet$ NrdF isolated in eNrdF (363 nmol min^{-1} mg^{-1}) (Table 1). This result is similar to the observations we recently reported with *Ec* NrdF.[7] While both the iron and manganese cofactors of rNrdF are active in nucleotide reduction, the manganese cofactor is much more active and is the one assembled in vivo.

DISCUSSION

The construction of the *Bs* 1B-UP strain resulted in increased amounts of the four proteins encoded by the *Bs nrdI-nrdE-nrdF-ymaB* operon, facilitating purification of active eNrdF.[30] Abs raised to each of the independently expressed recombinant proteins revealed that the levels of NrdE and NrdF in 1B-UP were elevated 35-fold relative to that of the wt strain. The 18-fold increase in RNR activity in crude cell extracts of 1B-UP relative to

the wt strain demonstrated that NrdF has an active cofactor assembled. The visible and EPR spectra of the purified RNR (Figures 3 and 4) demonstrate the presence of the $Mn(III)_2$-$Y^\bullet$ cofactor, similar to that recently characterized in *Ec* and *Ca*.[4,7,8,12] In each of these cases, however, manganese was added to the growth medium to obtain loaded NrdF in sufficient quantities for biophysical characterization of the metal center,[4,7,8,12] while in the *Bs* case, no addition of Mn(II) was required. The $Y^\bullet$ content ($0.44\ Y^\bullet/\beta_2$) was also higher than that purified from *Ca* ($0.36\ Y^\bullet/\beta_2$) and *Ec* ($0.2\ Y^\bullet/\beta_2$).[4,7,8,12] Thus, the $Mn(III)_2$-$Y^\bullet$ center is also the active cofactor of RNR in *Bs*.

Several surprising observations resulted from the purification of eNrdF from the 1B-UP strain. One was the fact that isolation of NrdF was accompanied by NrdE at each step during the purification. This result is consistent with an early report that fractionation of *Bs* crude cell extracts by size exclusion chromatography gave RNR activity eluting as a large complex.[29] However, contradictory studies of the quaternary structure of the *Ca* RNR[12,31] and our own recent studies using recombinant *Bs* proteins (data not shown) indicate that the quaternary structure(s) of active class Ib RNRs requires further investigation. We proposed that the quaternary structure and the inability to correctly assemble active NrdEF from rNrdF and rNrdE are, in part, responsible for the differences in activities observed between rNrdF and eNrdF.

Our recent studies showed that *Ec* NrdF interacts strongly with *Ec* NrdI, an observation that facilitated its purification from endogenous levels in an engineered *Ec* strain.[8] Thus, the second interesting aspect of the purification of *e*NrdF was that, in contrast to the *Ec* results, *Bs* His_6-NrdI was unable to enrich NrdF from the 1B-UP strain cell lysate by Ni-NTA affinity chromatography. While it is possible that the copurification of eNrdEF could disrupt its interaction with NrdI, our recent structural studies of the *Ec* NrdINrdF complex suggest that NrdE can be docked onto this complex with no steric interference, using the class Ia RNR docking model ($\alpha_2\beta_2$).[38] Differences in interactions between NrdI and NrdF might have been anticipated from sequence alignments of NrdIs. All NrdIs have a loop that covers the FMN cofactor (designated the 50S loop). In *Bacillus*, this loop contains only three amino acids, distinct from the two additional subclasses of NrdIs, in which the loop is glycine rich and much longer.[18,26,27,38] A recent publication describing the structure of *Ba* NrdI supports this distinction, as a K_d for its interaction with *Ba* NrdF was reported to be weak (50 μM).[26] These studies suggest that the affinity of *Bs* NrdI for NrdF is distinct from the affinity of these interactions in *Ec*.

Additional distinctions between NrdIs from different sources are based on titration studies with dithionite to maximize FMN_{sq}. Results with *Bs* NrdI (24%) are similar to those reported for *Ec* NrdI (30%) but different from those for *Ba* (60%) and *Bc* (100%) NrdI.[18] Our biochemical and structural studies with the *Ec* NrdI·Mn-NrdF complex[7,38] suggested that HOO(H) is the oxidant of manganese. Whether the increased amounts of FMN_{sq} observed in the *Bc* and *Ba* NrdI titrations are indicative of an alternative Mn(II) oxidant, $O_2^{\bullet-}$, remains to be established.[2]

Further insight into the role of *Bs* NrdI in $Mn(III)_2$-$Y^\bullet$ cluster assembly is provided by Western analyses of crude cell extracts of wt and 1B-UP strains. NrdI is present at a much lower level than NrdEF and thus likely acts catalytically. We have recently reported a similar observation for the *Ec* class Ib RNR.[8] In neither case has the reductase that re-reduces $NrdI_{ox}$ (or $NrdI_{sq}$) been identified. Thus, the observed substoichiometric $Mn(III)_2$-$Y^\bullet$ cluster assembly might be associated with insufficient reductase to recycle NrdI.

Finally, the *Bs* class Ib RNR activity assay requires further investigation. The activity of *Bs* NrdEF and the observation that addition of additional rNrdE increases the activity are likely associated with the absence of DTT during the purification of eNrdF and the use of the nonphysiological reductant in the assay. While glutaredoxin or the glutaredoxin-like protein NrdH has been proposed to be the physiological reductant for NrdE in *Ca*, *Ll*, *St*, *Sa*, and *Ec*,[33,36,37,39,40] the absence of NrdH in the *Bs* class Ib operon/genome requires an alternative candidate. In *Bs*, the *yosR* gene encodes a glutaredoxin-like protein and is located near the RNR operon with a prophage origin (SPβ *bsnrdEF*).[30] While we cannot rule out the possibility that YosR is the endogenous NrdE reductant, we disfavor this proposal as this operon is present only in the genomes of some *Bs* subspecies and *yosR* is nonessential.[41,42] By process of elimination, we consider thioredoxin and thioredoxin reductase as the best candidates for the NrdE reductant, as both genes (*trxA* and *trxB*, respectively) are essential.[43] The recent discovery of a small thiol molecule, bacillithiol,[44] in some Gram-positive bacteria, including *Bs*, suggests that this molecule may also be a candidate for NrdE reduction. Ongoing experiments will answer this question.

In summary, regardless of the source of the $Mn(III)_2$-$Y^\bullet$ cofactor, reconstituted or endogenous, this cofactor is 13 times more active than the $Fe(III)_2$-$Y^\bullet$ cofactor when assayed under the same conditions. Moreover, the isolation of $Mn(III)_2$-$Y^\bullet$ eNrdF from *Bs* cultured in LB medium without manipulation of the metal content suggests that *Bs* NrdF is an obligate manganese enzyme. The fact that the $Fe(III)_2$-$Y^\bullet$ rNrdF is also enzymatically active establishes that cells have developed mechanisms for either enhancing insertion of the correct metal or preventing mismetalation. Such mechanisms could have significant medical implications as these studies further suggest that many pathogenic organisms require a $Mn(III)_2$-$Y^\bullet$ cluster to produce deoxynucleotides and probably to escape host defense strategies of limiting iron for the pathogens.[13]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Experimental details and data about cloning, expression, and purification of His_6-rNrdE, -rNrdF, -rNrdI, and -rYmaB, characterization of rNrdI, in vitro assembly of active $Fe(III)_2$-$Y^\bullet$ and $Mn(III)_2$-$Y^\bullet$ rNrdF, and chromatography elution profiles of *Bs* eNrdF purification. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

RNR, ribonucleotide reductase; α_2, ribonucleotide reductase large subunit; β_2, ribonucleotide reductase small subunit; r, recombinant; wt, wild type; e, endogenous; eNrdF, NrdF isolated in a complex with NrdE from *Bs*; $Y^{\bullet}$, tyrosyl radical; CDP, cytidine 5′-diphosphate; dATP, deoxyadenosine 5′-triphosphate; *Bs*, *B. subtilis*; *Ec*, *E. coli*; $Fe(III)_2$-$Y^{\bullet}$, diferric-tyrosyl radical; $Mn(III)_2$-$Y^{\bullet}$, dimanganese-tyrosyl radical; *Ca*, *C. ammoniagenes*; *Ba*, *B. anthracis*; *Mt*, *M. tuberculosis*; *Sa*, *S. aureus*; *Sp*, *St. pyogenes*; SA, specific activity; $NrdI_{hq}$, NrdI, hydroquinone form; $NrdI_{ox}$, NrdI, oxidized form; EPR, electron paramagnetic resonance; FPLC, fast protein liquid chromatography; BSA, bovine serum albumin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; Ni-NTA, nickel nitrilotriacetic acid; IPTG, isopropyl β-D-thiogalactoside; Abs, antibodies; Spect, spectinomycin; DTT, dithiothreitol; *Ll*, *Lactococcus lactis*; Amp, ampicillin; β-ME, β-mercaptoethanol; CV, column volume; PMSF, phenylmethanesulfonyl fluoride.

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