YbiB from *Escherichia coli*, the Defining Member of the Novel TrpD2 Family of Prokaryotic DNA-binding Proteins*

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**Background:** YbiB belongs to the uncharacterized family of TrpD2 proteins.

**Results:** YbiB binds to DNA with high affinity. The *ybiB* gene is under LexA control and induced by DNA-damaging agents.

**Conclusion:** The TrpD2 proteins are a novel family of prokaryotic DNA-binding proteins.

**Significance:** TrpD2 proteins may be part of the LexA-controlled SOS response in bacteria.

We present the crystal structure and biochemical characterization of *Escherichia coli* YbiB, a member of the hitherto uncharacterized TrpD2 protein family. Our results demonstrate that the functional diversity of proteins with a common fold can be far greater than predictable by computational annotation. The TrpD2 proteins show high structural homology to anthranilate phosphoribosyltransferase (TrpD) and nucleoside phosphoribosyl class II enzymes but bind with high affinity (Kᵰ = 10–100 nM) to nucleic acids without detectable sequence specificity. The difference in affinity between single- and double-stranded DNA is minor. Results suggest that multiple YbiB molecules bind to one longer DNA molecule in a cooperative manner. The YbiB protein is a homodimer that, therefore, has two electropositive DNA binding grooves. But due to negative cooperativity within the dimer, only one groove binds DNA in *in vitro* experiments. A monomerized variant remains able to bind DNA with similar affinity, but the negative cooperative effect is eliminated. The *ybiB* gene forms an operon with the DNA helicase gene dinG and is under LexA control, being induced by DNA-damaging agents. Thus, speculatively, the TrpD2 proteins may be part of the LexA-controlled SOS response in bacteria.

The emergence of novel enzymatic functions is frequently coupled to gene duplication. Selection pressure can only be imposed upon one of the twin genes, whereas the other is free to mutate and drift to a novel function (1). Such pairs of homologous genes are frequently found in genomes, and the investigation of the unconventional homolog is a method of choice to discover novel enzymatic activities. In this study, we have started to elucidate the function of the new family of TrpD2 proteins, which have two well characterized homologues: AnPRT (anthranilate phosphoribosyltransferase; also termed TrpD) and NP-II (nucleoside phosphorylase class II) families (Fig. 1). TrpD2 was initially found as a duplicate of the TrpD enzyme, also termed TrpD (on average 17% sequence identity). This enzyme, from the metabolic pathway of tryptophan biosynthesis, catalyzes the condensation of the nitrogenated base anthranilate and the phosphoribosyl donor 5′-phosphoribosyl-α1-pyrophosphate (PRPP). Crystal structures of TrpD have been solved (2–5), and its physicochemical and catalytic properties have been studied in detail (6–9). The other homologue to TrpD2 (on average 10% sequence identity) is the NP-II (10–13). NP-II proteins act in metabolic pyrimidine salvage pathways, catalyzing the phosphorolytic cleavage of nucleosides to free pyrimidine bases and deoxyribose-1-phosphate. The reactions of the TrpD and NP-II enzymes resemble each other chemically; TrpD synthesizes a glycosidic bond, whereas NP-II enzymes phosphorylate it. Despite sharing a low sequence similarity (on average 12% sequence identity) and displaying some significant differences, TrpD and NP-II proteins are both members of the phosphoribosyl transferase class III superfamily and have a similar fold, their active sites share common features, and both enzymes have a similar quaternary structure, where homodimers are formed by head-to-head interactions of equivalent secondary structure elements (2, 3).

Whereas TrpD and NP-II proteins are broadly distributed enzymes (TrpD is not present in animals), TrpD2 proteins are limited to prokaryotes and primarily present in proteobacteria, firmicutes, actinobacteria, cyanobacteria, aquificales, spirochaetales, and some archaea like halobacteria. The TrpD2 protein family shares an average sequence identity of about 40%, although the most distant members exhibit low conservation with only 19% identity. Here, we have studied the structural and biochemical properties of a defining representative of the TrpD2 family, the *Escherichia coli* protein YbiB. Our findings show that, despite having a common fold, TrpD2 enzymes do not share catalytic functions with TrpD and NP-II enzymes but are a new class of DNA-binding proteins. The dimeric protein has two DNA binding sites, but only one of them is occupied by DNA due to negative cooperativity. The *ybiB* gene is located in

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* The authors declare that they have no conflicts of interest with the contents of this article.

This article contains supplemental Movie S1.

The atomic coordinates and structure factors (code 4MUO) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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4 The abbreviation used is: PRPP, 5′-phosphoribosyl-α1-pyrophosphate.
a LexA-controlled operon in many species and is induced upon DNA damage. A participation of the TrpD2 proteins in the LexA-controlled mechanism of SOS response in bacteria might be hypothesized, but this remains to be confirmed.

Experimental Procedures

Phylogenetic Analysis and Multiple Sequence Alignment—Sequences that represent the full range of sequence variation within each family were manually selected from individual alignments of the TrpD, TrpD2, and NP-II families. A T-COFFEE (14) alignment was created, and MEGA (15) was used to generate and evaluate a phylogenetic tree based on this multiple sequence alignment. The tree was calculated using the neighbor-joining algorithm, based on Poisson-corrected distances, and analyzed by bootstrapping.

To generate a sequence logo-based multiple sequence alignment of the TrpD and TrpD2 families, E. coli YbiB was used as a query for a BLAST search with a limit of 80% identity (82 TrpD and 100 TrpD2 sequences left). Inconsequential N- and C-terminal extensions were deleted as well as sequences with non-conserved insertions, and sequence logos (17) were generated from the remaining sequences (81 TrpD and 93 TrpD2 sequences). The two sequence logos were then realigned with the remaining sequences (81 TrpD and 93 TrpD2 sequences).

Assays for Phosphoribosyltransferase and Phosphorylase Activity—Steady state kinetic measurements for TrpD activity were carried out as described by Schlee et al. (6) with a MgCl2 concentration of 2 mM. S. saccharomyces TrpD served as positive control.

Tests for nucleoside phosphorylase activity were carried out using different deoxyribo- and ribonucleosides (daa, A, dC, G, and T), as well as dNMPs and NMPs (dAMP, dCMP, dGMP, GMP, TMP, AMP, CMP, GMP, and UMP). 5 μM YbiB was mixed with 0.2 mg/ml substrate in 50 mM potassium phosphate buffer, pH 7.0. The mixtures were incubated for 12 h at room temperature. Afterward, protein was removed by acetone precipitation, and the supernatants were freeze-dried. The remainder was resuspended in buffer and separated by reversed phase HPLC (20) to discriminate between nucleosides and the free bases that would have been liberated by a phosphorylase activity. The NP-II from E. coli, DeoA, served as a positive control.

Nucleoside phosphoribosyltransferase activity of YbiB was measured in lysogeny broth (LB) medium supplemented with 75 μg/ml kanamycin and 100 μg/ml ampicillin at 37 °C up to an A600 of 0.6–0.8. Protein expression was induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside, and culture growth continued for an additional 6 h at 30 °C. Cells were harvested by centrifugation. Pellets were resuspended in a lysis buffer containing 100 mM phosphate buffer, pH 8.0, 300 mM KCl, 1 mM DTT and lysed by sonication in the presence of 0.01 mg/ml DNase I. Lysates were clarified by centrifugation, and supernatants containing the target protein were applied to a Ni2+-chelating Hi-Trap HP column (GE Healthcare) equilibrated in lysis buffer. Elution used 500 mM imidazole. Resulting samples were concentrated by ultrafiltration, and the buffer was simultaneously exchanged to 50 mM Tris/HCl, pH 7.5. The protein was then concentrated to ~6 mg/ml (Bradford assay) (18) and stored at 4 °C until further use.

The production of selenomethionyl-labeled samples was as above but using metabolic inhibition in M9 minimal medium supplemented with l-selenium-methionine (Sigma). Here, expression after induction used further growth at 20 °C for 18 h overnight. Purification of labeled samples was as that of the native protein.

To produce a monomeric YbiB variant, the amino acid exchanges L40E and I51E were introduced by QuikChange™ mutagenesis (19). The mutant variant YbiB_L40E,I51E was expressed and purified as described for wild-type YbiB, but cells were grown at 25 °C overnight. Lysis buffer was 50 mM potassium phosphate, pH 7.5, 300 mM KCl, 10 mM imidazole, and the protein was stored in 50 mM Tris/HCl, 2 mM potassium phosphate, pH 7.5.

The trpD gene from Saccharomyces cerevisiae and the trpD2 gene from Aquifex aeolicus (aattroP2) were cloned into pET28a, expressed in E. coli T7 Express cells (30 or 37 °C, overnight), and purified by nickel chelate chromatography using the same conditions as for YbiB_L40E,I51E.

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night with PRPP (1 mM) in 50 mM Tris/HCl, pH 7.5, 1 mM MgSO₄ in the presence of YbiB (1 μM) at room temperature. Transfer of the phosphoribosyl moiety to the bases was analyzed by reversed phase HPLC as described above. The NMPs of all tested bases served as a reference.

**Crystallization**—Crystals of YbiB were grown using the hanging drop method in Hampton VDX™ 24-well plates at room temperature. The best crystals grew from solutions containing 30% PEG 600, 100 mM sodium citrate, pH 5.5, in drops consisting of 2 μl of protein solution and 2 μl of reservoir suspended over 1 ml of mother liquor. Crystals with plate morphology grew in 2 days to approximate dimensions of 250 × 250 × 100 μm³. Crystals from selenomethionyl-derivated YbiB grew from 20% PEG 600, 100 mM sodium citrate, pH 5.0, at a protein concentration of ~9.2 mg/ml. Here, crystal morphology was that of rods with frayed termini. Data collection from derivatized (but not native) crystals required cryoprotection by soaking in solutions containing 26% PEG 600, 100 mM citrate, pH 5.0, for 20 min. Long soaking times were required to permit the reannealing of the lattice that became initially disrupted.

**Crystal Structure Elucidation**—Native x-ray diffraction data were collected at 100 K on beamline X06SA at SLS, Villigen (Switzerland). Data corresponded to a total rotation range of 180° with an oscillation angle of 0.5°/frame and exposure time of 0.5 s/image. Data processing used XDS/XSCALE (21). Data processing statistics are given in Table 1. Extensive attempts at phasing by molecular replacement were unsuccessful. Finally, phases were obtained from a 3λ multiple anomalous dispersion experiment using two Se-Met crystals to collect (i) remote and (ii) peak and inflection wavelength. Multiple anomalous dispersion data statistics are listed in Table 2.

Anomalous scatterers were located using SOLVE (22) (21 selenium sites were located of 24 present in the asymmetric unit). The positions were validated using VECTOR (CCP4). Density modification and automated model building were carried out with RESOLVE (22). This yielded an initial model containing 395 residues (of 640 total residues in the asymmetric unit) that were placed in several independent fragments. The order of the fragments was manually determined by comparison with the structure of ssAnPRC (3). Subsequently, NCS averaging and phase extension was carried out in DM (23) using NCS masks calculated in MAMA (Uppsala Software Factory). Further automatic model building used ARP/wARP warpNtrace (24), where sequence docking was performed manually. The solvent structure was built with ARP/wARP and COOT (25). Final model refinement was in PHENIX (26), applying TLS refinement, where four TLS groups were defined that corresponded to the domain definitions of YbiB. Structure visualization and superpositions were done with PyMOL (version 1.6.0.0, Schrödinger, LLC, New York).

**Electrophoretic Mobility Shift Assays**—To prepare 32P-labeled double-stranded DNA (dsDNA) probes (≤58 bp), two complementary single-stranded oligonucleotides (Table 3) were hybridized, whereas the 260-bp dsDNA probe was created by standard PCR amplification. All dsDNAs included 5’ TA overhangs to increase the efficiency of the subsequent 32P-labeling by T4 polynucleotide kinase. In the case of the 260-bp fragment, the overhangs were generated by digestion with the restriction enzyme NdeI. For labeling, the single- and double-stranded DNA probes (10 pmol) were incubated with 30 μCi of [γ-32P]ATP (3 Ci/μmol) and T4 polynucleotide kinase (New England Biolabs) in a total volume of 20 μl at 37 °C for 1 h. The 260-base single stranded probe was labeled by an asymmetric amplification of a 260-bp dsDNA template (0.1 pmol) in the presence of 0.3 μM [α-32P]dCTP (3 Ci/μmol) under otherwise standard PCR conditions.

YbiB was mixed with the labeled probes in 50 mM Tris/HCl, 2 mM potassium phosphate, pH 7.5. The samples were incubated for 30 min at room temperature and analyzed on native 6 or 10% polyacrylamide gels in 1× TAE, pH 7.0. The gel was autoradiographed by phosphorimaging (Cyclone phosphor imager, PerkinElmer Life Sciences). Because the concentration of DNA (<0.3 nm) was much lower than the concentration of the protein, the dissociation constant Ka corresponds to the protein concentration where half of the DNA is bound to the protein, giving a shifted band. To determine apparent Ka values and Hill coefficients, the signal of the free probe in each lane was quantified by densitometry with OptiQuant version 3.0 (PerkinElmer Life Sciences) and normalized to the total lane density. The normalized signals were plotted against the YbiB concentration, and the Hill function (y = axn/Ka + xⁿ), where a represents maximum signal and n is Hill slope) was fitted to the data to obtain apparent Ka values (SigmaPlot version 12). Log-
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### TABLE 3
Oligonucleotides used for electrophoretic mobility shift assays

| Name                      | Sequence (5’→3’) | Notes                                      |
|---------------------------|------------------|--------------------------------------------|
| 28-base ssDNA             | GCCGCTATGATTTACGACG | Hybridized with 28-base dsDNA 1a          |
| 28-base dsDNA 1a          | TACGGCTATGATTTACGACG | Hybridized with 28-base dsDNA 1a          |
| 28-base ssDNA 1a          | TAAATGATGATTTACGACG | Hybridized with 28-base dsDNA 1a          |
| 58-base dsDNA 1           | GCCGCTATGATTTACGACG | Hybridized with 58-base dsDNA 1           |
| 58-base dsDNA 1a          | TACGGCTATGATTTACGACG | Hybridized with 58-base dsDNA 1a          |
| 260-base Base p1          | GACTCAATGCTGTTATAATCACGACG | Amplification of a 260-base ssDNA or 260-bp precursor dsDNA probe |
| 260-base ssDNA p2         | GAATCAATGCTGTTATAATCACGACG | Amplification of a 260-base ssDNA or 260-bp precursor dsDNA probe |

**Notes**

*Boldface letters indicate either 5’ TA overhangs, which arise after hybridization of the respective oligonucleotides, or from NdeI recognition sites.*

### TABLE 4
Oligonucleotides used to produce DNA probes for fluorescence titration

| Name                      | Sequence (5’→3’) | Notes                                      |
|---------------------------|------------------|--------------------------------------------|
| 14-base dsDNA I           | GAACCTACGAAACGACG | Hybridized with 14-base dsDNA II           |
| 14-base dsDNA II          | GCTGCTATGCTGACGTC | Hybridized with 14-base dsDNA II           |
| 28-base dsDNA Ib          | TCCCGCGATGATACCCGATCCACG | Hybridized with 28-base dsDNA Ib          |
| 28-base dsDNA Iib         | GATGGTGATGACGTACGATCCCGCGCA | Hybridized with 28-base dsDNA Iib         |
| 20-base random ssDNA      | GCTTGTGATGACGTACGATCCCGCGCA | Hybridized with 28-base dsDNA Iib         |

**Notes**

*Fluorescence Titrations—The binding of DNA or RNA to YbiB and aaTrpD2 was monitored by the quench of protein fluorescence resulting from a tryptophan located at the edge of the binding groove (Fig. 4B). 2 μM protein (subunit concentration) was mixed with increasing amounts of oligonucleotide (oligo(dT) or oligo(U) of specified length, random sequences; see Table 4) in 50 mM Tris/HCl, pH 7.5. dsDNA substrates were generated by annealing two complementary oligonucleotides. 2 mM potassium phosphate was added because otherwise YbiB degraded during the course of the measurement. Control experiments were carried out to assess the influence of salt concentration on nucleotide binding (Fig. 8). The samples were incubated for several min at 25 °C, and then YbiB was excited at 280 nm, and fluorescence was recorded at 340 nm, using a Jasco FP-6500 spectrofluorimeter. For aaTrpD2, excitation was carried out at 295 nm, and binding was monitored at 320 nm to avoid excess signal by tyrosine fluorescence. The measured fluorescence was corrected by the inner filter effect of DNA or RNA and normalized. The slope of linear fluorescence decrease caused by this inner filter effect was determined at concentrations higher than titration saturation. The absolute changes in fluorescence after subtracting the inner filter effect were between 7 and 21%, depending on the length of the DNA/RNA ligand, or 4% in case of aaTrpD2. To calculate the thermodynamic binding constant \( K_{DNA/RNA} \), the fluorescence intensity was plotted against the total ligand (DNA/RNA) concentration and evaluated by fitting (Sigma Plot 12) Equation 1,

\[
F = F_0 + (F_{max} - F_0) \times 0.5 \times (1 + (L_0 + K_{DNA/RNA})/E_0) - (((1 + (L_0 + K_{DNA/RNA})/E_0)^2 - 4L_0/E_0)^{0.5}) \quad (\text{Eq. 1})
\]

where, in the general case, \( F \) is the corrected fluorescence after each titration step, \( F_{max} \) is the saturation value, \( F_0 \) is the starting fluorescence, \( L_0 \) is the total ligand concentration at each titration step, and \( E_0 \) is the total protein concentration (concentration of binding sites) (27). In the specific case of YbiB binding to DNA, multiple YbiB molecules (\( c_{YbiB, monomer} = 2 \mu \text{M} \)) bind to one DNA molecule at different stoichiometries \( n \), depending on DNA length. Here, \( E_0 \) is equivalent to the concentration of the YbiB-DNA complex at saturation, and \( E_0 \times n = c_{YbiB, monomer} \). Because \( E_0 \) is provided by the fitting algorithm, the stoichiometry (number of YbiB monomers that bind to one DNA molecule) can be calculated as \( n = c_{YbiB, monomer}/E_0 \).

RT-PCR Analysis to Test for Co-transcription of ybiB and Its Neighbors—Residual DNA was removed from an E. coli RNA preparation by digestion with DNase. Reverse transcription was performed with SuperScript III reverse transcriptase (Invitrogen). To blind out endogenous priming, individual “anchor primers” were used for reverse transcription that consisted of a 5’ constant 21-base anchor sequence and a 3’ 20-base genespecific sequence (downstream primers 1–4). Subsequent
PCRs were then performed with gene-specific upstream primers 1–4 and the constant anchor primer. Primer pairs 1 and 3 are positive controls and test for intact mRNAs containing *dinG* or *ybiB*. Primer pair 2 tests for co-transcription of *dinG* and *ybiB*, and primer pair 4 tests for co-transcription of *ybiB* and *ybiC*.

Analysis of ybiB Expression by Western Blotting—An *E. coli* K12 MG1655 derivative with a His<sub>6</sub> tag genomically fused 3’ to *ybiB* was generated using a homologous recombination technique. A linear PCR fragment with the 3’ sequence of *ybiB*, the joined His tag sequence, and a cat resistance cassette was amplified using the pKD3 plasmid as a template (28). Genome modification was carried out by λ Red-mediated homologous recombination using the *E. coli* strain DY329 (29). Successful recombination was verified by PCR and sequencing. Modified genomic *ybiB* was transferred to *E. coli* MG1655 via P1 transduction (30). These *E. coli* cells containing a genomically His<sub>6</sub>-tagged *ybiB* gene were inoculated to an *A<sub>600</sub>* of 0.1 and grown for 1.5 h at 37 °C without antibiotics, following the addition of varying amounts of mitomycin C and incubation at 37 °C for an additional 2 h. Cells were harvested and disrupted by ultrasonication in 50 mM potassium phosphate, pH 7.5, 10 mM imidazole. Lysates were cleared by centrifugation, and overall protein concentrations were determined by a Bradford assay (18). A small sample of the lysate was removed, and equal amounts of total protein were subjected to Western analysis to determine the expression of the housekeeping protein GAPDH (31), which served as a loading control. GAPDH was detected with a primary monoclonal mouse anti-GAPDH antibody (Abcam ab125247) and a peroxidase-conjugated secondary polyclonal goat anti-mouse antibody (Sigma-Aldrich). To enrich and quantify the His<sub>6</sub>-tagged YbiB protein, Ni<sup>2+</sup>-nitrilotriacetic
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acid beads (Thermo Scientific) were added to the rest of the lysate, and the mixtures were incubated for 1 h at 4 °C. The beads were washed three times with 50 mM potassium phosphate, pH 7.5, 300 mM KCl, 10 mM imidazole. The supernatants were discarded, and the beads were supplemented with SDS-PAGE loading buffer. Subsequently, sample volumes corresponding to equal amounts of total protein were separated by SDS-PAGE, and upon blotting, YbiB was detected with a peroxidase-conjugated anti-His6 antibody (Roche Applied Science).

Results

The Crystal Structure of YbiB Reveals a Conserved Fold with Divergent Substrate Binding Features—To elucidate the function of the TrpD2 family, we recombinantly produced and characterized its representative in E. coli, YbiB. Size exclusion chromatography suggested that it forms a homodimer (Fig. 2), in agreement with the known oligomerization states of TrpD and NP-II homologues. To investigate whether TrpD2 shares a catalytic activity with the latter enzymes, recombinant YbiB was then tested for TrpD activity, nucleotide salvage activity, and nucleoside phosphorylase activity. No catalytic turnover could be detected for any of the reactions.

To gain insight into the function of YbiB, we elucidated its three-dimensional structure at 1.94 Å resolution using x-ray crystallography (Figs. 3 and 4 and Tables 1 and 2). The crystal structure reveals that YbiB folds into a bilobal architecture characteristic of the phosphoribosyltransferase class III superfamily (3). This fold is organized into non-sequential N- and C-terminal domains, where the smaller N-terminal α-helical domain is formed by six helices and provides the contact interface for homodimerization, whereas the larger C-terminal α/β-domain comprises a central β-sheet that is surrounded by seven additional α-helices. In relation to TrpD, the closest structural homolog, YbiB, lacks the two C-terminal helices. To evaluate the structural similarity between TrpD and TrpD2 proteins, we compared YbiB and TrpD from S. sulfataricus (Protein Data Bank code 1O17). These superimpose with a root mean square deviation of 1.6 Å for 60 Cα atoms in the N-terminal domain and 2.6 Å for 148 Cα atoms in the C-terminal domain (Fig. 4A). The crystal form used in this study contained two YbiB protomers in its asymmetric unit, corresponding to a biological dimer (Fig. 3A). The two molecular copies are essentially identical (protomer superimposition yields a root mean square deviation of 0.25 Å). The oligomerization state of YbiB in the crystal agrees with that derived from analytical gel filtration (Fig. 2). The dimeric arrangement of YbiB closely resembles that of TrpD and NP-II enzymes. As in those cases, YbiB dimerizes via the N-terminal domain in a head-to-head fashion, with the interface being largely hydrophobic and formed by the helices α1, α3, and α9 of each subunit.

Interestingly, two symmetrically arranged large grooves are formed at the contact interface between the protomers. The grooves contain clusters of arginine and lysine residues and are highly positively charged (Fig. 3B and supplemental Movie S1). The exact position of positively charged residues in these is not always conserved across the TrpD2 family, but the high density of basic residues in the grooves is a common feature (Fig. 5). The C-terminal rim of the groove is formed by a flexible loop that is disordered in the crystal structure and that predictably becomes ordered upon ligand binding to the groove. Almost all conserved amino acids can be found along the groove and the adjacent deep surface cavity (Fig. 4C). However, the active site of TrpD and NP-II, located at the rim of the cavity, is not completely abolished (Fig. 3C and supplemental Movie S1). Resi-
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FIGURE 5. Multiple sequence alignment of the TrpD and TrpD2 families, shown as sequence logos. The E. coli YbiB sequence is shown as a reference. All Arg and Lys residues in E. coli YbiB that contribute to the charged groove are colored in blue. The conserved GTGGD motif in TrpD, representing the PRPP binding loop, and the residues involved in the binding of anthranilic acid (A) are marked in orange at the top. The sequence logo algorithm fades out columns that are represented in a few sequences only, resulting in seemingly empty gap columns. In consequence, gap columns indicate variable protein regions in this representation.

TrpD2 Proteins Bind DNA without Sequence Specificity—The shape of the grooves and the distribution of positive charges instantly implied the binding of polynucleic acids to YbiB, mediated by electrostatic interactions. One groove would comply with the spatial requirements of a single-stranded or (partially unwound) double-stranded DNA fragment of 6–10-base pair length. To test this hypothesis, we performed electrophoretic mobility shift assays (EMSAs) with 28-, 56-, and 260-bp-long dsDNA oligonucleotides of random sequence and ssDNA probes of corresponding sequence and determined the binding constants (Fig. 6). The results showed a strong interaction with only a slight difference between dsDNA and ssDNA. Almost all apparent $K_D$ values were in the 0.1–1 μM range. Affinity slightly increases with the length of the DNA, and ssDNA shows an only slightly higher (2–5-fold) affinity than dsDNA. Whereas the binding of YbiB to long probes shows a significant cooperativity (with respect to the binding of multiple protein molecules to one DNA molecule), this effect is reduced with short probes, particularly with dsDNA. We showed in subsequent experiments (below) that three YbiB dimers bind to the 28-base probe, and consequently, about 20 YbiB dimers bind to the 260-base probes. The homologous...
The TrpD protein from *S. cerevisiae* showed no binding to DNA in EMSAs.

The nucleic acid binding properties of YbiB were analyzed more accurately in solution by fluorescence titration (Fig. 7 and Table 5) that exploited the native signal of a tryptophan residue at the edge of the predicted DNA binding groove (Fig. 4B). The results confirmed that there is no significant difference between the binding to dsDNA or ssDNA (Fig. 7, A and B). The reason for this behavior might be that binding is mediated by the DNA backbone only or that dsDNA is even partially melted by YbiB to form a bubble. The insignificant difference between dsDNA and ssDNA binding allowed us to study the binding to nucleic acids of different length in detail with ssDNA only, in order to exclude stoichiometric artifacts by melting short dsDNA probes. The cooperative effect of multiple YbiB binding to one DNA molecule is not detectable in these experiments because of the quite high protein concentrations we had to use to obtain a good signal/noise ratio.

Based on the crystal structure, we estimate that the binding groove of YbiB can accommodate DNA chains of 6–10 bases in length. However, we did not evaluate the titration data of the 6-base-long ssDNA (Fig. 7C) because the signal was low and noisy. We assume that such short DNAs bind but do not significantly influence the fluorescence signal of the tryptophan at the outer edge of the binding groove. The dissociation constants for all longer probes were in the nanomolar range ($K_D$ of 0.02–0.09 M) and were nearly constant throughout the whole range of probe length tested (Table 5). This is an ~10-fold higher affinity than found in the EMSA experiments. We ascribe this effect to a disturbance of the equilibrium during electrophoresis, and such differences have also been reported for other DNA-binding enzymes like the glycosylase Mug (32).

A 20-base-long dT oligonucleotide and one of random sequence showed comparable affinities (Fig. 7D), suggesting that nucleic acid binding by YbiB is not sequence-specific.

We next studied whether YbiB also binds ssRNA. Both a 12-base-
and 20-base-long ssRNA probe were tested, yielding dissociation constants and stoichiometries comparable with those of corresponding ssDNA probes (Fig. 7E and Table 5). Thus, YbiB does not discriminate between RNA and DNA in vitro. If both kinds of single-stranded nucleic acids do not similarly serve as substrates, then other currently unidentified factors must provide substrate specificity. These observations also suggest that the binding is predominantly mediated by Coulomb interactions between the negatively charged backbone of the nucleic acid and the positively charged residues located along the groove of YbiB. Along these lines, the addition of increasing amounts of free orthophosphate leads to an inhibition of the binding between YbiB and DNA, with increased dissociation constants being observed (Fig. 8A). To test binding of YbiB to DNA under physiological conditions with elevated salt concentrations, we added 50 or 100 mM NaCl (Fig. 8B). This leads to a decrease of affinity by a factor of 3 or 5 only, indicating that YbiB is likely to bind to DNA also within the cell. Last, we showed that a second member of the TrpD2 family, the protein from *A. aeolicus* (aaTrpD2), also binds to single-stranded nucleic acids with a similar affinity (Fig. 7F and Table 5).

**Stoichiometry and Mode of Binding of the YbiB-DNA Complex—Oligonucleotides**

Oligonucleotides with a length of 12 bases or less bind one dimer of YbiB (Figs. 7 and 9 and Table 6). Remarkably, the extension of an ssDNA probe from 12 to 14 bases allows two YbiB dimers to bind one oligonucleotide simultaneously. This stoichiometry is persistent up to an oligonucleotide length of 24 bases. DNA probes of 26 bases and longer bind three YbiB dimers. Our experiments with dsDNA give congruent results; a 14-bp dsDNA binds two YbiB dimers, and one of 28 bp binds...
### TABLE 5
Dissociation constants for the binding of YbiB and aaTrpD2 to various DNA and RNA oligonucleotides

| Oligonucleotide | YbiB $K_D$ ($\mu M$) | aaTrpD2 $K_D$ ($\mu M$) | YbiB, L40E, I51E $K_D$ ($\mu M$) |
|-----------------|-----------------------|-------------------------|-------------------------------|
| 8-Base poly(dT) | 0.07 ± 0.04           | 0.06 ± 0.02             | 0.09 ± 0.02                   |
| 8-Base poly(U)  | 0.04 ± 0.02           | 0.02 ± 0.01             | 0.05 ± 0.02                   |
| 10-Base poly(dT) | 0.08 ± 0.04           | 0.01 ± 0.00             | 0.03 ± 0.01                   |
| 10-Base poly(U) | 0.04 ± 0.02           | 0.01 ± 0.00             | 0.03 ± 0.01                   |
| 12-Base poly(dT) | 0.04 ± 0.02           | 0.01 ± 0.00             | 0.03 ± 0.01                   |
| 12-Base poly(U) | 0.04 ± 0.02           | 0.01 ± 0.00             | 0.03 ± 0.01                   |
| 12-Base random ssDNA | 0.04 ± 0.02 | 0.01 ± 0.00 | 0.03 ± 0.01 |
| 14-Base poly(dT) | 0.03 ± 0.02           | 0.01 ± 0.00             | 0.02 ± 0.01                   |
| 14-Base poly(U) | 0.03 ± 0.02           | 0.01 ± 0.00             | 0.02 ± 0.01                   |
| 14-Base random ssDNA | 0.03 ± 0.02 | 0.01 ± 0.00 | 0.02 ± 0.01 |
| 14-bp ssRNA     | 0.04 ± 0.02           | 0.01 ± 0.00             | 0.02 ± 0.01                   |
| 14-bp dsDNA     | 0.04 ± 0.02           | 0.01 ± 0.00             | 0.02 ± 0.01                   |
| 20-Base poly(dT) | 0.01 ± 0.00           | 0.03 ± 0.00             | 0.01 ± 0.00                   |
| 20-Base poly(U) | 0.01 ± 0.00           | 0.03 ± 0.00             | 0.01 ± 0.00                   |
| 20-Base random ssDNA | 0.01 ± 0.00 | 0.03 ± 0.00 | 0.01 ± 0.00 |
| 20-Base dsDNA   | 0.01 ± 0.00           | 0.03 ± 0.00             | 0.01 ± 0.00                   |
| 24-Base poly(dT) | 0.00 ± 0.00           | 0.04 ± 0.00             | 0.00 ± 0.00                   |
| 24-Base poly(U) | 0.00 ± 0.00           | 0.04 ± 0.00             | 0.00 ± 0.00                   |
| 24-Base random ssDNA | 0.00 ± 0.00 | 0.04 ± 0.00 | 0.00 ± 0.00 |
| 24-Base dsDNA   | 0.00 ± 0.00           | 0.04 ± 0.00             | 0.00 ± 0.00                   |
| 26-Base poly(dT) | 0.00 ± 0.00           | 0.04 ± 0.00             | 0.00 ± 0.00                   |
| 26-Base poly(U) | 0.00 ± 0.00           | 0.04 ± 0.00             | 0.00 ± 0.00                   |
| 26-Base random ssDNA | 0.00 ± 0.00 | 0.04 ± 0.00 | 0.00 ± 0.00 |
| 26-Base dsDNA   | 0.00 ± 0.00           | 0.04 ± 0.00             | 0.00 ± 0.00                   |
| 28-Base poly(dT) | 0.00 ± 0.00           | 0.04 ± 0.00             | 0.00 ± 0.00                   |
| 28-Base poly(U) | 0.00 ± 0.00           | 0.04 ± 0.00             | 0.00 ± 0.00                   |
| 28-Base random ssDNA | 0.00 ± 0.00 | 0.04 ± 0.00 | 0.00 ± 0.00 |
| 28-Base dsDNA   | 0.00 ± 0.00           | 0.04 ± 0.00             | 0.00 ± 0.00                   |

* $K_D$ values are refer to the concentration of binding sites on the oligonucleotide. Oligonucleotides up to 12 bases bind 1 YbiB molecule; 14–24-base oligonucleotides bind 2 YbiB molecules; and >26-base oligonucleotides bind 3 YbiB molecules (see Figs. 7 and 9 and Table 6). Values without S.D. result from single experiments; all other values represent the mean of three experiments.
same lengths of the probe as for the dimeric protein. We regard this as a further support that the DNA binding region of dimeric YbiB is (at least for nonspecific DNA binding) not much extended beyond one of the charged grooves and that binding mode (iii) might be the native way of YbiB-DNA interaction.

**YbiB Expression Is under LexA Control in the Bacterial Cell—On the E. coli chromosome, ybiB is located outside the trp operon, like the trpD2 genes in all other species. Downstream, the yet uncharacterized ORF ybiC is found. In γ-proteobacteria, the gene upstream, dinG, encodes a DNA helicase, whose expression is controlled by the DNA damage-induced SOS response system (34), mediated by the LexA repressor (Fig. 10A). DinG has initially been described as a helicase that is active on D-loops, R-loops, and forked structures and might be involved in recombinational DNA repair or the resumption of blocked replication (35, 36). A recent study shows that DinG is also involved in resolving stalled transcription complexes (37). Furthermore, E. coli DinG activity is stimulated by the single-stranded DNA-binding protein (SSB) (38), and in some organisms, a nuclease domain is fused to DinG so that the fusion protein may act as a nuclease rather than as a helicase to produce ssDNA (39).

The absence of known promotor elements specific to ybiB and the only 28-base pair gap between the stop codon of dinG and the start codon of ybiB suggested that these two genes form a transcriptional unit, as already deduced from a genome-wide transcription analysis (40). An RT-PCR analysis on *E. coli* RNA confirmed this assumption and demonstrated that ybiC is not part of this transcriptional unit (Fig. 10B). DNA-damaging conditions (e.g. the addition of mitomycin C to the growth medium) stimulate ybiB expression, as shown by Western blotting (Fig. 11). We conclude from these experiments that YbiB is regulated by the LexA-induced SOS response system and co-expressed with a DNA helicase.

**Discussion**

The rapid sequencing of whole microbial genomes over recent years has revealed a large number of proteins of unknown function. Biochemical characterization of such ORFs offers the opportunity to identify new and unpredicted protein functions. Our results contribute to demonstrating that the functional diversity of proteins with a common fold can be far greater than predictable by computational annotation. Although all known proteins with the nucleoside phosphorylase/phosphoribosyltransferase fold are metabolic enzymes, YbiB is the defining member of the nucleic acid-binding TrpD2 family within this fold. We have shown that, in contrast to TrpD and NP-II enzymes, the TrpD2 proteins bind DNA with high affinity. The affinity constants are similar to what has been observed for other DNA-binding enzymes like glycosylases (32, 41), and like for these enzymes, binding is dependent on salt concentration. This indicates that the binding is mediated by electrostatic interactions of the DNA backbone with basic residues that are located in two symmetric grooves. These grooves are rudimentarily present in TrpD but enlarged in TrpD2 and functionalized for DNA binding. Some catalytically relevant residues within the active site of TrpD and NP-II are conserved in TrpD2, but we postulate that the putative active site of TrpD2 enzymes is much larger and extended by an adjacent large cavity. Both the grooves and the cavity are formed by the two protomers in concert. We therefore assume that dimerization has functional purposes in TrpD2 and does not only serve for protein stability as in TrpD (7).

The DNA binding properties of the TrpD2 proteins and their genomic organization together with the DNA helicase DinG in a LexA-controlled operon imply a role in the bacterial SOS response. Alternatively, however, TrpD2 might only profit from the expression level within the DinG operon but serve a
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TABLE 6
Stoichiometries for the binding of YbiB to DNA of different length

| 8-Base ssDNA | 10-Base ssDNA | 12-Base ssDNA | 14-Base dsDNA | 14-bp ssDNA | 24-Base ssDNA | 26-Base ssDNA | 28-Base ssDNA | 28-bp dsDNA |
|--------------|--------------|--------------|--------------|-------------|--------------|--------------|--------------|-------------|
| YbiB molar ratio* | 2.3 ± 0.1 | 1.9 ± 0.1 | 1.9 ± 0.2 | 4.2 ± 0.2 | 3.8 ± 0.1 | 4.3 ± 0.1 | 5.6 ± 0.1 | 5.9 ± 0.1 | 6.6 ± 0.1 |
| YbiB_L40E,I51E molar ratio* | 1.0 ± 0.1 | 2.0 ± 0.2 | | | | | | | |

* Indicating the YbiB subunits or monomers that are bound by one molecule of ss/dsDNA simultaneously. Each value represents the mean of three experiments (shown in Fig. 9) and the S.D.

Figure 10. Operon organization of ybiB. A, a genomic organization of ybiB and surrounding genes. The dinG promoter (gray box) is controlled by the LexA repressor. Primer pairs 1–4 used for RT-PCR are depicted as arrows. B, RT-PCR analysis to test for co-transcription of ybiB and its neighbors. Primer pairs 1 and 3 are positive controls and test for intact mRNAs containing dinG or ybiB. Primer pair 2 tests for co-transcription of dinG and ybiB, and primer pair 4 tests for co-transcription of ybiB and ybiC.

Figure 11. Influence of mutagenic agents on ybiB expression. Increasing amounts of mitomycin C (MMC, given in µg/ml above each lane) were added during cultivation. Equal amounts of total cellular protein were separated by SDS-PAGE, blotted and YbiB (36 kDa) detected by an anti-His$_6$ antibody (Roche Applied Science). The GAPDH protein (36 kDa) was used as a loading control.

During cultivation. Equal amounts of total cellular protein were separated by SDS-PAGE, blotted and YbiB (36 kDa) detected by an anti-His$_6$ antibody (Roche Applied Science). The GAPDH protein (36 kDa) was used as a loading control.

different function in the cell, a phenomenon called “genomic hitchhiking” (42). Using different experimental approaches, we are currently investigating whether YbiB has catalytic activity and, if it were an enzyme, what its function on DNA might be. Unfortunately, a ybiB knock-out strain shows no specific phenotype even under DNA damage conditions. We assume that either putatively interacting domains of the SOS response machinery could complement the ybiB knock-out or that the TrpD2 proteins are involved in completely different cellular processes. Thus, further work is required to elucidate the cellular function of TrpD2 enzymes.

Author Contributions—D. S. established and carried out YbiB purification, expression analysis, and DNA binding assays and drafted the manuscript. W. K. refined and extended all of these data. Therefore, D. S. and W. K. should be regarded as joint first authors. C. S. crystallized YbiB and solved the crystal structure. A. H. monomerized YbiB. O. M. solved the crystal structure of YbiB and revised the manuscript. P. B. conceived of the study, coordinated experiments, carried out sequence/phylogenetic analysis and some of the expression analyses, and drafted the manuscript. All authors read and approved the final manuscript.

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