Electronic Supplementary Material (ESI) for Molecular BioSystems

Characterization of AziR, a resistance protein of the DNA cross-linking agent azinomycin B

Jennifer Foulke-Abel, Gilbert T. Kelly, Huitu Zhang and Coran M. H. Watanabe

INVENTORY OF SUPPLEMENTAL INFORMATION

Figure S1. CLUSTALW multiple alignment of AziR and top six protein BLAST homologs.

Figure S2. Assessment of AziR phosphorylation/acetylation of azinomycin B using radiolabeled substrates and TLC separation.

Figure S3. Fluorescence titration curves and emission spectra, related to Figure 3.

Figure S4. Genetic maps of the plasmids used for AziR expression.

Figure S5. SDS-PAGE of purified AziR from heterologous hosts *S. lividans* and *E. coli*.

Supplemental Experimental Procedures. Details concerning radiolabeled TLC analysis, spectrophotometric phosphotransferase assay, and control fluorescence equilibrium titrations.

Supplemental Figures

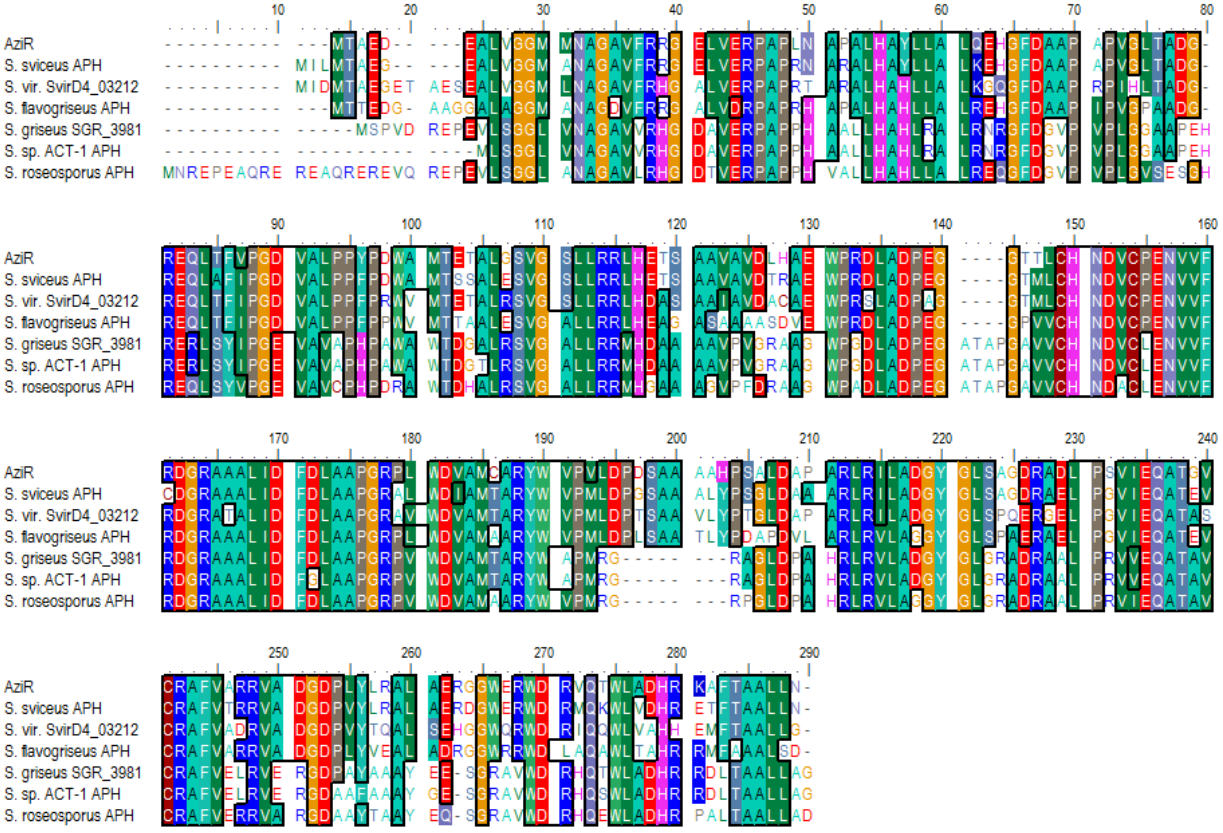

Figure S1. CLUSTALW multiple alignment of AziR and top six protein BLAST homologs. Graphic rendering generated using BioEdit.[1]

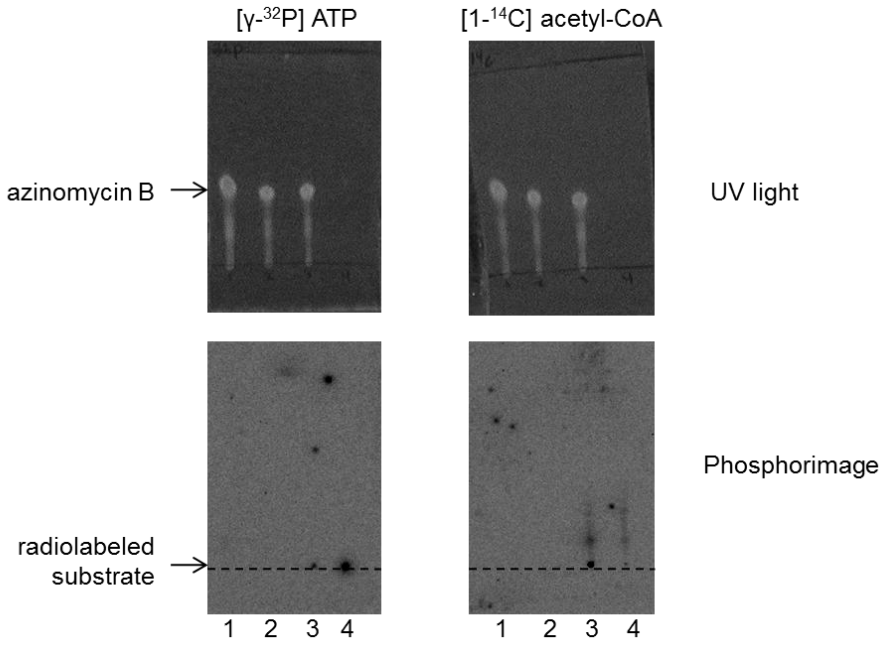

Figure S2. Assessing AziR phosphorylation/acetylation of azinomycin B using radiolabeled substrates. TLC separation and UV or phosphorimage detection of azinomycin B incubated with AziR and [γ-^{32}P] ATP or [1-^{14}C] acetyl-coenzyme A. Lane 1, azinomycin B; lane 2, azinomycin B + AziR; lane 3, azinomycin B + AziR + radiolabeled substrate; lane 4, radiolabeled substrate. Upper spots (not near baseline) on TLC phosphorimages are from stray radiation.

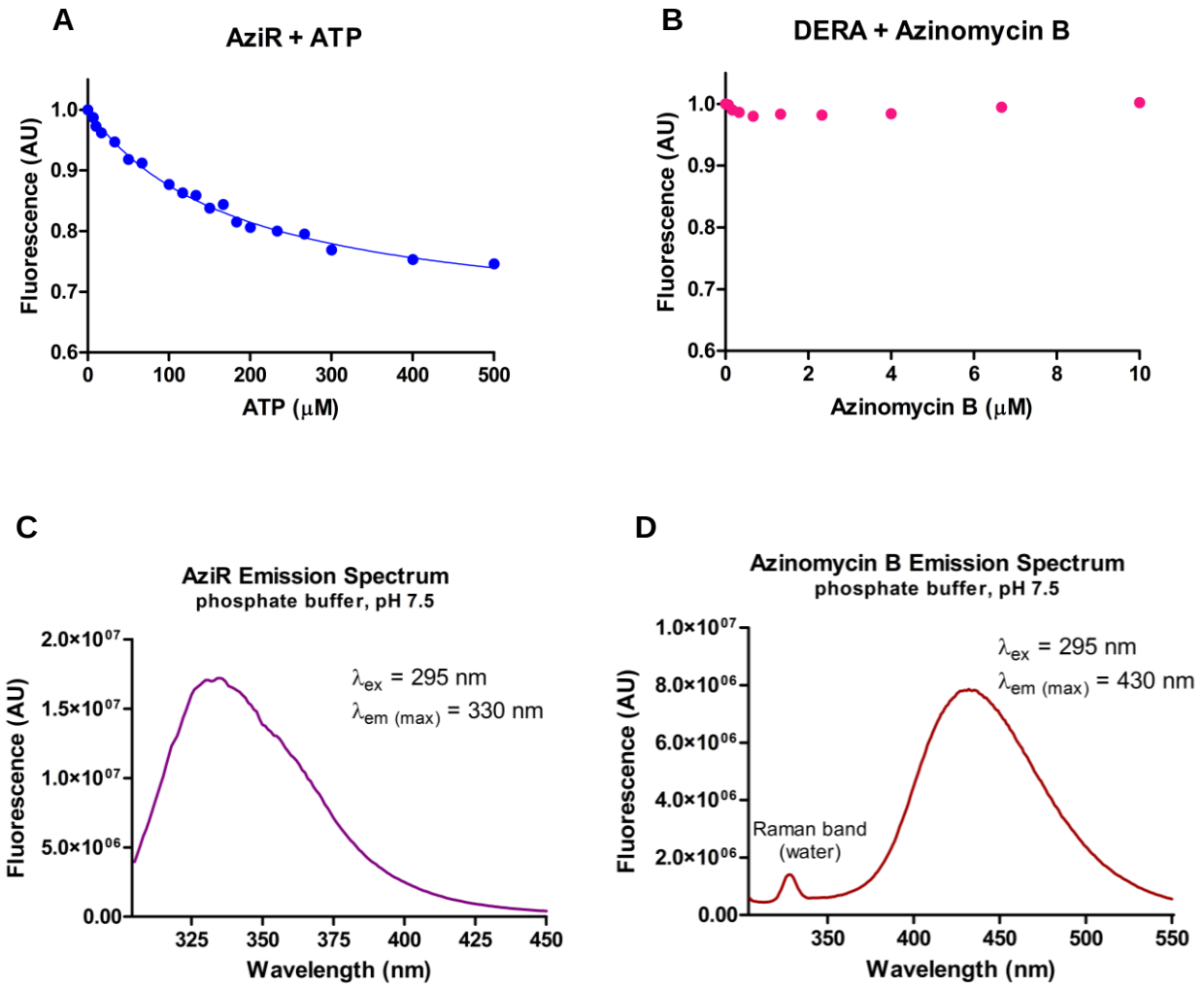

Figure S3. Fluorescence titration curves and emission spectra, related to Figure 3.
(A) AziR titrated with ATP, K_d = 185 ± 14 μM. (B) Non-specific *E. coli* protein 2-deoxyribose-5-phosphate aldolase (DERA) titrated with azinomycin B demonstrates lack of binding. (C) Fluorescence emission spectrum of AziR, λ_{ex} = 295 nm. (D) Fluorescence emission spectrum of azinomycin B, λ_{ex} = 295 nm.

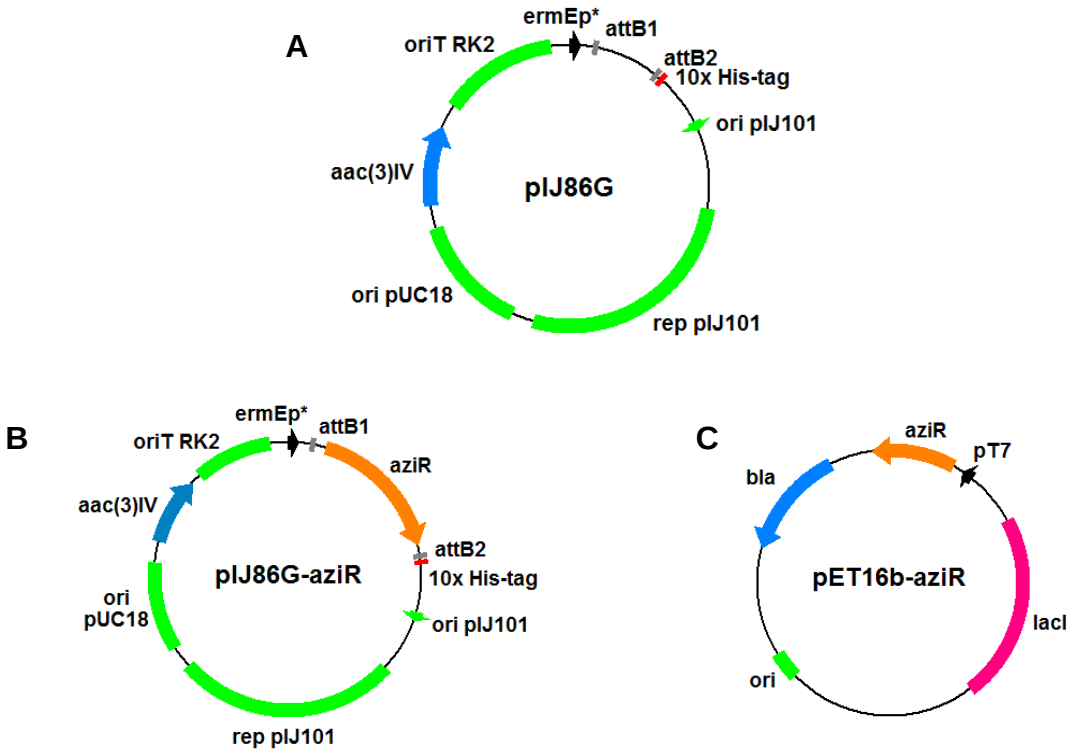

Figure S4. Maps of plasmids used in expression studies.
(A) pIJ86G, 7567 bp; (B) pIJ86G-aziR, 6607 bp; (C) pET16b-aziR, 6527 bp.

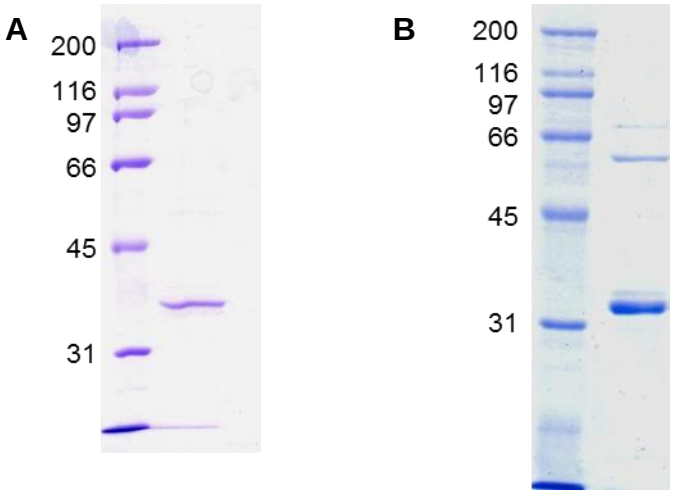

Figure S5. SDS-PAGE of purified AziR from heterologous hosts.
(A) *S. lividans* TK24 and (B) *E. coli* BL21(DE3). Ladder molecular weights are in kDa.

Supplemental Experimental Procedures

Phosphorylation/acetylation monitoring using radiolabeled cofactors

Reactions consisted of 500 µM azinomycin B and 500 µM radiolabeled substrate ([γ-^{32}P] ATP or [1-^{14}C] acetyl-coenzyme A; specific activity of ~0.1 µCi each), to which 100 µM AziR in 50 mM Tris pH 7.5, 10 mM NaCl and 10 mM $MgCl_2$ was added. After 1 h incubation at room temperature, reactions were extracted with dichloromethane and separated by TLC with 5% methanol in dichloromethane mobile phase. Plates were visualized with both UV light (254 nm) and phosphorimage.

Phosphotransferase activity assay

Purified AziR was subjected to assay with azinomycin B, kanamycin, streptomycin, or neomycin as described by McKay and coworkers.[2] Substrate phosphorylation was to be detected indirectly by coupling the release of ADP to the enzymatic reactions of pyruvate kinase and lactic acid dehydrogenase (PK/LDH), resulting in oxidation of NADH. Reaction progress was monitored at 340 nm on a Genesys 2 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Each reaction consisted of 885 µL buffer (50 mM Tris pH 7.5, 40 mM KCl, 10 mM $MgCl_2$, 0.5 mg/mL NADH, 2.5 mM phosphoenolpyruvate, 1 mM ATP) to which 10 µL of a 10 mM substrate stock was added, along with 5 µL of PK/LDH solution as obtained from the supplier (Sigma P0294, PK/LDH from rabbit muscle). After incubation for 20 min at 37 °C, AziR (100 µg) was quickly added to the reaction mixture just prior to spectroscopic monitoring. For all substrates tested, the rate of NADH oxidation was essentially zero, indicating a phosphorylation reaction did not take place.

Fluorescence equilibrium titration with DERA and azinomycin B

A protein not expected to bind azinomycin B, 2-deoxyribose-5-phosphate aldolase (DERA), was cloned, overexpressed in *E. coli*, and purified by methods described elsewhere.[3] Titration was carried out at an excitation wavelength of 295 nm using a 1 µM solution of DERA in phosphate buffer (20 mM NaH_2PO_4 pH 7.5, 50 mM NaCl, 5% glycerol, 1 mM dithiothreitol, 10 mM $MgCl_2$) and a 100 µM stock of azinomycin B. Data acquisition and analysis was identical to that described for the AziR and azinomycin B titration. The protein was not found to bind azinomycin B by the method described (**Figure S3B**).

Supplemental References

1 T. A. Hall, *Nucleic Acids Symp. Ser.*, 1999, **41**, 95-98.
2 G. A. McKay, P. R. Thompson and G. D. Wright, *Biochemistry*, 1994, **33**, 6936-6944.
3 G. DeSantis, J. Liu, D. P. Clark, A. Heine, I. A. Wilson and C.-H. Wong, *Biorg. Med. Chem.*, 2003, **11**, 43-52.