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The UvrA-like protein Ecm16 requires ATPase activity to render resistance against echinomycin

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Abstract

Bacteria use various strategies to become antibiotic resistant. The molecular details of these strategies are not fully understood. We can increase our understanding by investigating the same strategies found in antibiotic-producing bacteria. In this work, we characterize the self-resistance protein Ecm16 encoded by echinomycin-producing bacteria. Ecm16 is a structural homolog of the nucleotide excision repair protein UvrA. Expression of *ecm16* in the heterologous system *Escherichia coli* was sufficient to render resistance against echinomycin. Ecm16 binds DNA (double-stranded and single-stranded) using a nucleotide-independent binding mode. Ecm16's binding affinity for DNA increased by 1.7-fold when the DNA is intercalated with echinomycin. Ecm16 can render resistance against echinomycin toxicity independently of the nucleotide excision repair system. Similar to UvrA, Ecm16 has ATPase activity, and this activity is essential for Ecm16's ability to render echinomycin resistance. Notably, UvrA and Ecm16 were unable to complement each other's function. Together, our findings identify new mechanistic details of how a refurbished DNA repair protein Ecm16 can specifically render resistance to the DNA intercalator echinomycin.

KEYWORDS

antibiotic resistance, DNA intercalator, echinomycin, Ecm16, nucleotide excision repair, SOS, UvrA

1 | INTRODUCTION

The current crisis with antibiotic resistance has become one of the biggest public health challenges of our time (Centers for Disease Control and Prevention, CDC). Bacteria can acquire antibiotic resistance through various mechanisms that include the upregulation of efflux pumps, modification of antibiotics, and modification and protection of the antibiotic target. Notably, many of these antibiotic resistance mechanisms have also been found in bacteria that are themselves producers of antibiotics, which are utilized for self-protection (Ogawara, 2019; Wencewicz, 2019). Some genes involved in these antibiotic resistance mechanisms have been proposed to originate from antibiotic-producing bacteria shared with pathogenic bacteria through transformation, transduction, or conjugation (Benveniste & Davies, 1973; Martinez, 2018; Ogawara, 1981; Walker & Walker, 1970). This potential sharing of genetic information highlights the importance of identifying the mechanistic details of self-resistance found in antibiotic-producing bacteria.

Amanda Erlandson and Priyanka Gade contributed equally to this study.

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About 80% of our known antibiotics are produced by *Streptomyces spp*. (de Lima Procópio et al., 2012). One such antibiotic produced by *Streptomyces* is echinomycin, the first identified DNA bisintercalator (Waring, 1992). DNA intercalators bind reversibly to the DNA double helix in a sequence independent manner by inserting one or more planar, fused aromatic ring groups in between adjacent base pairs (Fletcher & Fox, 1996). Echinomycin, a member of the quinomycin family of antibiotics, contains two quinoxaline chromophores attached to a cyclic octadepsipeptide (Chen & Patel, 1995; Dawson et al., 2007; Dell et al., 1975) (Figure 1a). The quinoxaline chromophores intercalate between DNA bases pairs, with preference for CpG sites, while the depsipeptide core binds to the DNA minor groove (Dawson et al., 2007; Van Dyke & Dervan, 1984). Structural studies show that binding of echinomycin leads to DNA unwinding by ~20°, (Wu et al., 2018). Echinomycin bound to DNA has been shown to inhibit transcription in bacteria and chromatin condensation and DNA replication in eukaryotes, ultimately leading to cell cycle arrest (Fok & Waring, 1972; Krasinska & Fisher, 2009; May et al., 2004; Sato et al., 1967; Ward et al., 1965; Waring & Makoff, 1974). Quinomycins have been shown to possess antimicrobial, antiviral, and antitumor activities (Carter et al., 1954; Chen et al., 2021; Dolma et al., 2003; Sato et al., 1969; Yoshida et al., 1961).

Echinomycin was originally discovered in *Streptomyces echinatus* but has since been found to be produced by several species of *Streptomyces* (Fernández et al., 2014; Foster et al., 1985). In one of these species, *Streptomyces lasolacidi* (formerly known as *S. lasaliensis*), the echinomycin biosynthetic gene cluster lies within a 36 kilobase region of a 520 kilobase giant linear plasmid (Kinashi, 2011; Kinashi et al., 1987; Watanabe et al., 2006). This gene cluster consists of eight genes involved in quinoxaline-2-carboxylic acid synthesis, five genes for octadepsipeptide backbone synthesis (Watanabe et al., 2006), and five genes that encode proteins proposed to have regulatory or unknown functions (Fernández et al., 2014). One of the genes of unverified function, *ecm16*, encodes a homolog of the prokaryotic nucleotide excision repair (NER) protein UvrA. Although the function of Ecm16 has not been analyzed in any of its native *Streptomyces species*, the significance of Ecm16 has been postulated for the ability of other bacteria to synthesize echinomycin. In an *E. coli* system capable of total biosynthesis of echinomycin, the absence of *ecm16* resulted in hampered growth suggesting that Ecm16 could serve as a self-resistance protein against echinomycin (Watanabe et al., 2006). Interestingly, biosynthetic clusters involved in the synthesis of other DNA intercalator drugs also include genes that encode UvrA-like proteins (Ogawara, 2019). However, mechanistic understanding of how these proteins render resistance against DNA intercalators remains limited.

UvrA is part of the NER system responsible for repairing diverse types of DNA damage in bacteria, including pyrimidine dimers (Sancar & Rupp, 1983; Setlow & Carrier, 1964), unpaired T and G residues (Thomas et al., 1986), backbone modifications such as

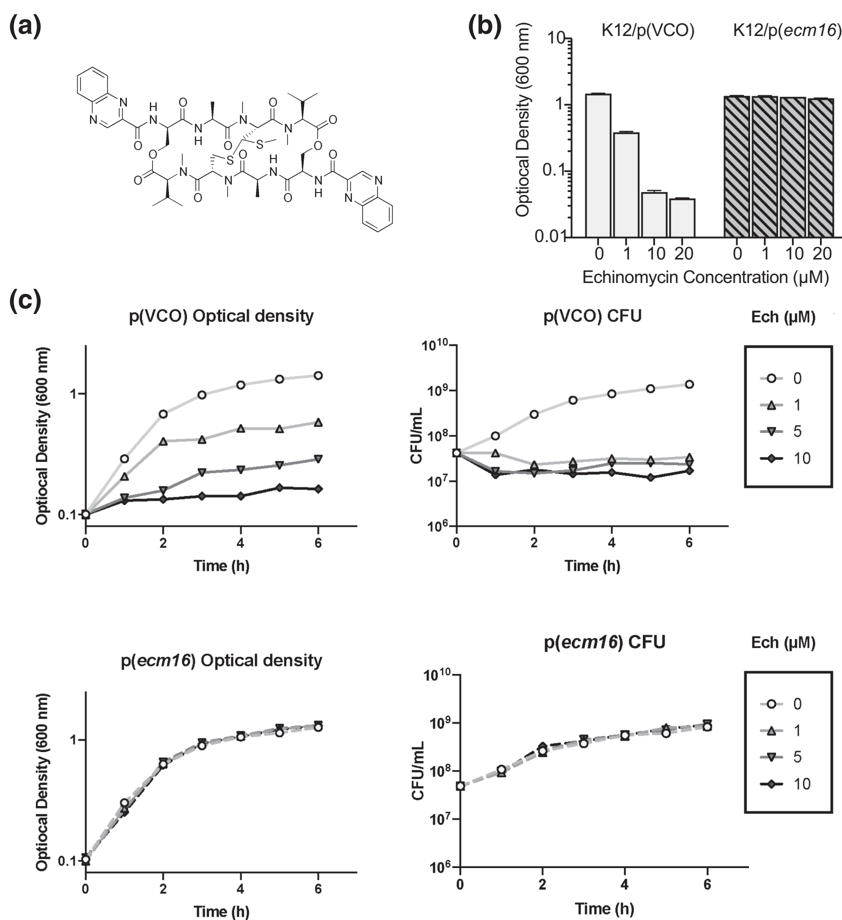


FIGURE 1 Expression of *ecm16* gives resistance against echinomycin. (a) Structure of echinomycin. (b) Optical density after 6 h. Exposure to echinomycin results in reduction of *E. coli* growth in liquid media. Maximum optical densities were determined after 6 h exposure to echinomycin concentrations ranging from 1 μM to 20 μM. Echinomycin was added at time zero to cultures at 0.2 OD_{600nm}. *E. coli* K12 strains with pBAD vector-control-only (VCO) or pBAD-*ecm16* (p(*ecm16*) were used for comparison. Error bars represent standard error for duplicate replicates of one trial, analysis is representative of three independent trials. (c) Plots of optical density and corresponding colony forming units (CFUs) of K12/p(VCO) and K12/p(*ecm16*) strains. Overnight cultures were diluted to 0.1 OD_{600} and grown over a 6-hour period in LB containing 0, 1, 5, or 10 μM echinomycin. Samples taken every hour were serially diluted and plated on LB agar plates. The count (log CFU/ml) was determined from plates grown overnight at 37°C using MicrobeJ.

single nucleotide gaps and nicks (Delagoutte et al., 2002; DellaVecchia et al., 2004; Moolenaar et al., 2000; Nazimiec et al., 1992), and damage conferred by anthramycin (Gunz et al., 1996; Krugh et al., 1989; Nazimiec et al., 1992), cholesterol adducts (Gomez-Pinto et al., 2004), and fluorescein (DellaVecchia et al., 2004). The NER protein involved in recognizing DNA damage is UvrA, a dimeric protein with a total of four ATP-binding sites, two UvrB-binding domains, and a single DNA binding groove (Husain et al., 1986; Jaciuk et al., 2011; Pakotiprapha et al., 2008, 2009; Timmins et al., 2009). ATPase activity conferred by the ATP-binding domains is necessary for UvrA's interaction with DNA, dissociation of dimer into monomers, and interaction with UvrB (Husain et al., 1986; Oh et al., 1989). Two models have been proposed regarding the detection of damaged DNA: (Ogawara, 2019) UvrA alone scans the DNA until it detects a lesion, at which point it stalls and complexes with UvrB (Stracy et al., 2016) and (Wencewicz, 2019) a $UvrA_2$-$UvrB_2$ complex searches and locates damaged DNA (Kad et al., 2010; Kad & Van Houten, 2012). In either model, UvrA is the first protein in the NER system to bind DNA and the one responsible for promoting conformational changes of DNA and UvrB for both to bind to each other (Kraithong et al., 2021). Defining the molecular mechanism used by UvrA to recognize the wide range of DNA damage types has remained elusive in the field.

In addition to the canonical UvrA, several bacterial phyla (including *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*) also express a class II UvrA, which are further subdivided into class IIa and IIb (Marszałkowska et al., 2013). The UvrA homologs found in *Streptomyces* that produce DNA intercalator drugs are class IIa UvrA (Ogawara, 2019; Zhang et al., 2013). Compared to the class I UvrA protein, class IIa UvrA lacks the first zinc finger motif and the UvrB-binding domain (Marszałkowska et al., 2013). Class IIa UvrA proteins share ~40% sequence identity with the class I UvrA. The specific functions of class IIa UvrA proteins in various bacterial species remain unclear. For instance, in *Deinococcus radiodurans*, the expression of both class I and class IIa *uvrA* genes is upregulated following ionizing radiation (Liu et al., 2003). However, the deletion of class IIa *uvrA* gene had no effect on the cell's UV sensitivity (Tanaka et al., 2005). In *Xanthomonas axonopodis* and *Pseudomonas putida*, the double knockout of class I and class IIa *uvrA* displayed slightly higher sensitivity to high UV radiation compared to the single knockout Δ*uvrA* (class I) suggesting that class IIa UvrA can contribute, although at a minor level, to the repair of UV-induced DNA damage (Shen et al., 2007). In *P. putida*, class IIa UvrA was also proposed to be involved in mutagenesis mechanisms during the stationary growth phase (Tark et al., 2008). Overall, our knowledge about the specific roles that class IIa UvrA proteins play in bacteria and whether they can substitute the activity of the canonical UvrA remain limited.

In this study, we present the in vivo and in vitro characterization of Ecm16, a class IIa UvrA protein from *Streptomyces lasalocidi* (Ogawara, 2019; Zhang et al., 2013). We demonstrate in vivo that the expression of *ecm16* is sufficient to render the echinomycin-sensitive *E. coli* resistant to relatively high levels of echinomycin (20 μM). In vitro, we showed that Ecm16 preferentially binds echinomycin-containing DNA. Similar to class I UvrA, Ecm16 can hydrolyze ATP, suggesting that class IIa UvrA proteins require ATPase activity for detecting/repairing DNA perturbations caused by echinomycin. Furthermore, Ecm16's drug resistance activity does not require any of the other components of the NER pathway. Ecm16 was unable to complement an *E. coli* class I *uvrA* knockout strain recovering from UV-induced DNA damage. Collectively, our work provides new insights into how class IIa UvrA proteins catalyze drug resistance independently of the evolutionarily related class I UvrA.

2 | RESULTS

2.1 | Expression of *ecm16* provides resistance against echinomycin

Although previously proposed, there is no direct experimental data in the literature confirming that Ecm16 renders resistance against echinomycin (Watanabe et al., 2006). To provide this evidence and characterize Ecm16, we used *E. coli* K-12 cells as a heterologous host system to express *ecm16*. The natively echinomycin-sensitive *E. coli* cells were transformed with a low-copy vector encoding *ecm16* under the arabinose inducible promoter [p(*ecm16*)]. In the absence of echinomycin, *E. coli* cells with vector-control-only (VCO) reached saturation (OD_{600nm} ~ 1.0) within a 6 h growth period (Figure 1b). The same VCO cells grown in the presence of 1 μM echinomycin only reached ~0.35 OD_{600nm} within the same 6 h period. Almost no detectable growth was observed for VCO cells at echinomycin concentration of 10 μM or higher. In contrast, *E. coli* cells expressing *ecm16* reached equivalent maximal densities when grown in the presence or in the absence of supplemented echinomycin. Using growth curve analyses, we determined the effect of *ecm16* expression on bacterial doubling time (Figure S1). In the absence of echinomycin, *E. coli* with or without the expression of *ecm16* were able to double every ~0.54 h under our growth conditions. However, in the presence of echinomycin, the doubling time of VCO cells increased nearly 4-fold at the highest concentration used in this study. Remarkably, cells expressing *ecm16* were able to maintain their normal doubling time up to levels of 20 μM echinomycin. Furthermore, we confirmed that Ecm16 renders resistance against echinomycin using survival assays under titrated levels of echinomycin (Figure 1c). These data revealed that echinomycin is bacteriostatic in *E. coli*.

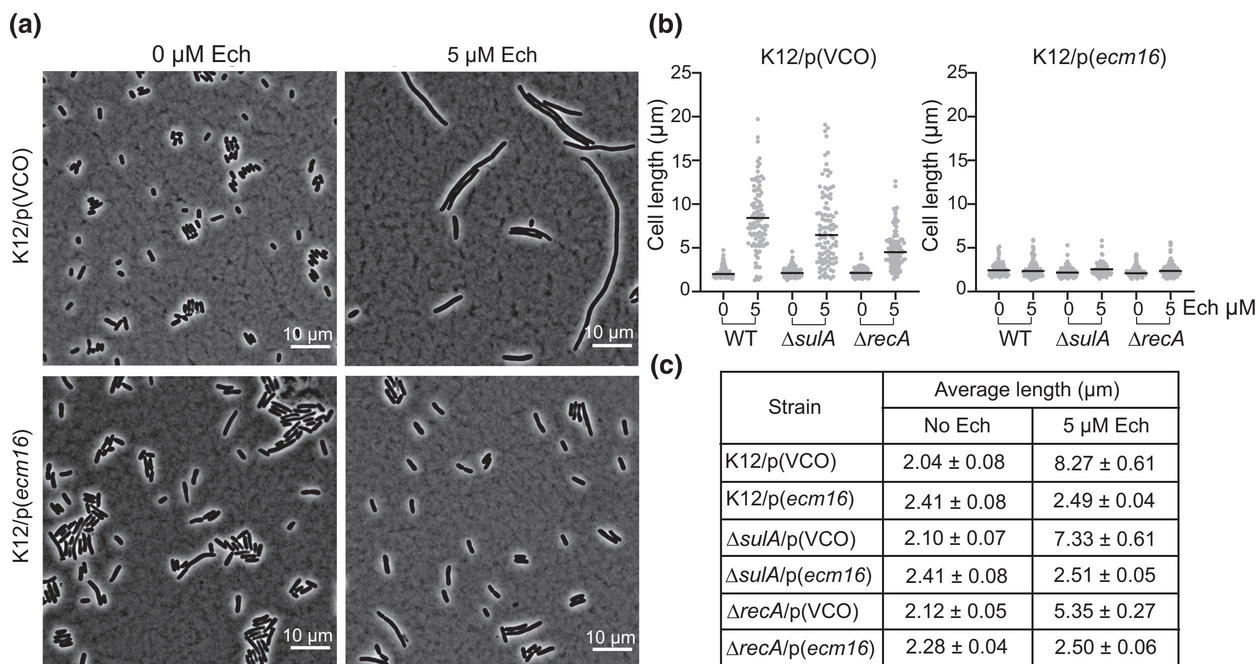
2.2 | Ecm16 prevents cellular filamentation caused by exposure to echinomycin

E. coli adopt filamentous shapes in response to a variety of stressful environments, including DNA damage and exposure to antibiotics (Adler & Hardigree, 1965; Kantor & Deering, 1966; Miller et al., 2004; Suzuki et al., 1967). To determine whether Ecm16 can prevent cellular filamentation, we analyzed the cell length of *E. coli* after exposure to echinomycin. The mean cell length of *E. coli* was determined to be ~2 μm when grown in the absence of echinomycin.

Exposure to echinomycin resulted in cells with a broad cell length distribution (Figure 2a). We observed that the expression of *ecm16* alone in the absence of echinomycin resulted in a ~15% increase in mean cell length compared to the VCO. However, when echinomycin was supplemented to cells expressing *ecm16*, cell length was not altered compared to the no echinomycin control treatment. To further characterize the potential mechanism involved in the filamentation observed in cells exposed to echinomycin, we analyzed the cell length of SOS mutant strains. Upon DNA damage, the SOS response is triggered to simultaneously accomplish at least two goals: repair the damage and halt cytokinesis while DNA repair is underway. RecA activates the SOS response by deactivating the repressors of SOS (Murli & Walker, 1993). SulA is made during SOS induction to prevent FtsZ assembly that ultimately inhibits cytokinesis (Bi & Lutkenhaus, 1993; Cordell et al., 2003; Gottesman et al., 1981; Huisman & D'Ari, 1981; Mizusawa et al., 1983). For our analyses, we used two *E. coli* strains with knockouts of genes encoding these two SOS regulators: *recA* and *sulA* (Keio Collection) (Baba et al., 2006). Our cell length analyses revealed that both mutant strains retained their filamentation phenotype upon exposure to echinomycin, suggesting that echinomycin triggers primarily an SOS-independent filamentation in *E. coli* (Figure 2b,c). Based on average cell length, we observed a decrease in filamentation in response to echinomycin in the *sulA* and *recA* strains compared to the wildtype background, suggesting echinomycin can also trigger an SOS-dependent filamentation, albeit to a lesser extent. Notably, the expression of *ecm16* in both mutant strains *recA* and *sulA* prevented any filamentation. In summary, our in vivo analyses revealed that the expression of *ecm16* in *E. coli* cells results in protection against the toxicity caused by echinomycin exposure.

2.3 | Ecm16 binds DNA in a nucleotide sequence-independent manner

We investigated how the local DNA structure influences Ecm16's ability to recognize its substrate. The NER UvrA from *E. coli* and *Mycobacterium tuberculosis* has been shown to exhibit stronger affinity for single-stranded DNA (ssDNA) versus double-stranded DNA (dsDNA) (Rossi et al., 2011; Seeberg & Steinum, 1982). Using electrophoretic mobility shift assay, we characterized the DNA binding activity of purified recombinant Ecm16 to various DNA substrates (Table S1) (Figure 3a). The DNA substrates (32-mer ssDNA and dsDNA oligonucleotides) used in these experiments were designed with sequences previously shown to be UvrA substrates (Jaciuk et al., 2011) modified at the center to contain a central echinomycin binding sequence (5-ACGT-3′). These substrates were chemically synthesized (Integrated DNA Technologies, Coralville, Iowa) and incubated over a range of Ecm16 concentrations. Slower mobility species corresponding to DNA-protein complex were generated as a result of DNA binding to Ecm16 (Figure 3a). Ecm16 displayed similar binding affinity to dsDNA ($K_d = 34.8$ nM) and ssDNA ($K_d = 39.6$ nM)



Strain	Average length (μm)	
	No Ech	5 μM Ech
K12/p(VCO)	2.04 ± 0.08	8.27 ± 0.61
K12/p(*ecm16*)	2.41 ± 0.08	2.49 ± 0.04
Δ*sulA*/p(VCO)	2.10 ± 0.07	7.33 ± 0.61
Δ*sulA*/p(*ecm16*)	2.41 ± 0.08	2.51 ± 0.05
Δ*recA*/p(VCO)	2.12 ± 0.05	5.35 ± 0.27
Δ*recA*/p(*ecm16*)	2.28 ± 0.04	2.50 ± 0.06

FIGURE 2 *E. coli* cells expressing *ecm16* do not filament after echinomycin treatment. (a) Phase contrast microscopy of strains K12/p(VCO) (top panels) and K12/p(*ecm16*) (bottom panels) grown in the presence of inducer (arabinose 0.2%) supplemented with or without 5 μM echinomycin (ech). Cells were grown in the presence of echinomycin for 5 h and subsequently spotted on 1% agarose minimal media pads for imaging. Scale bar = 10 μm. (b) Scatter dot plots of cell size distribution of strains K12/p(VCO) and K12/p(*ecm16*) under wild-type (WT), D*sulA*, and D*recA* background supplemented with or without 5 μM echinomycin (ech). (c) Average cell length and standard error for 100 cells per condition are shown. 3% of cells displayed cell lengths over 25 μm (not included in plot). Cell lengths were measured using the software MicrobeJ. Representatives of three independent replicates are plotted.

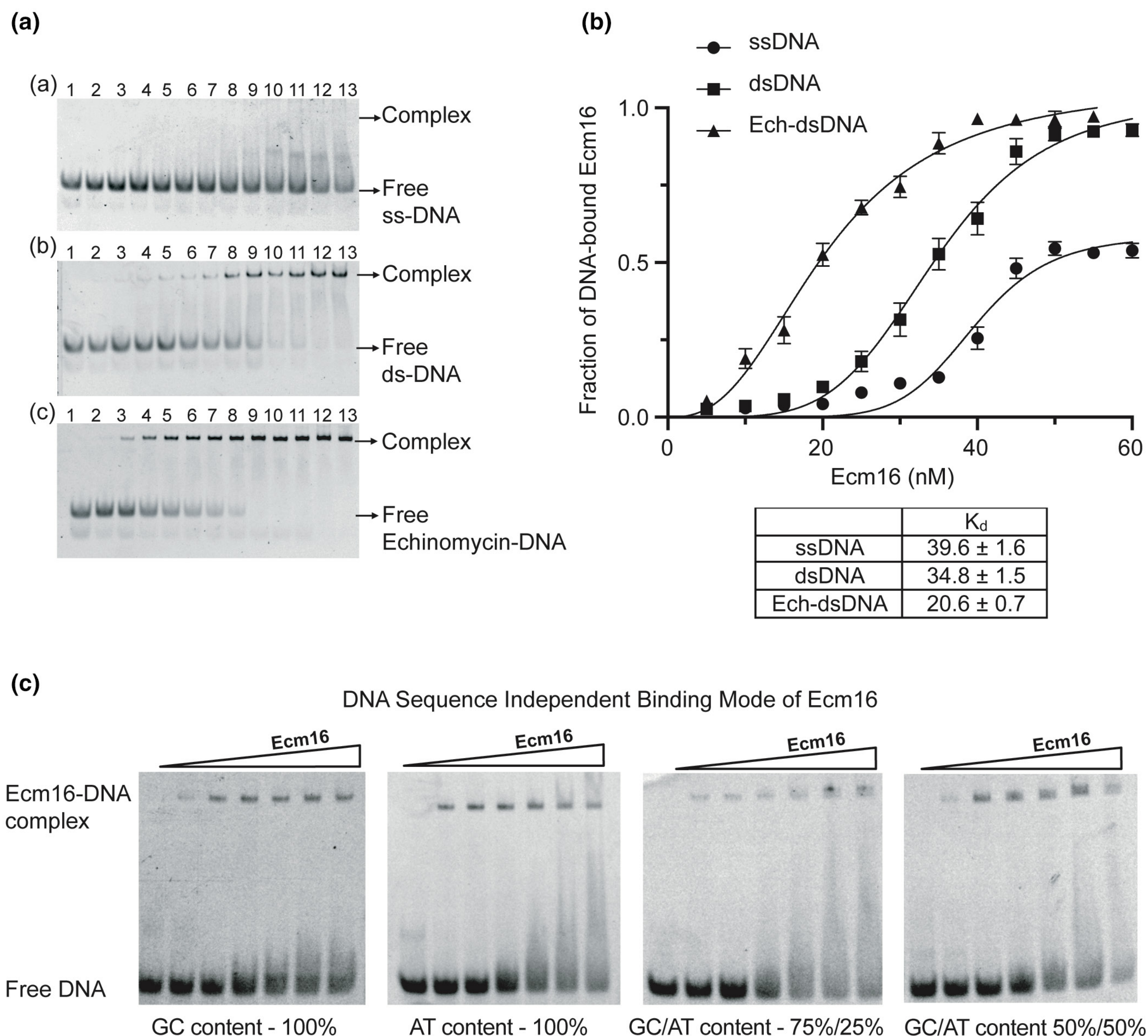


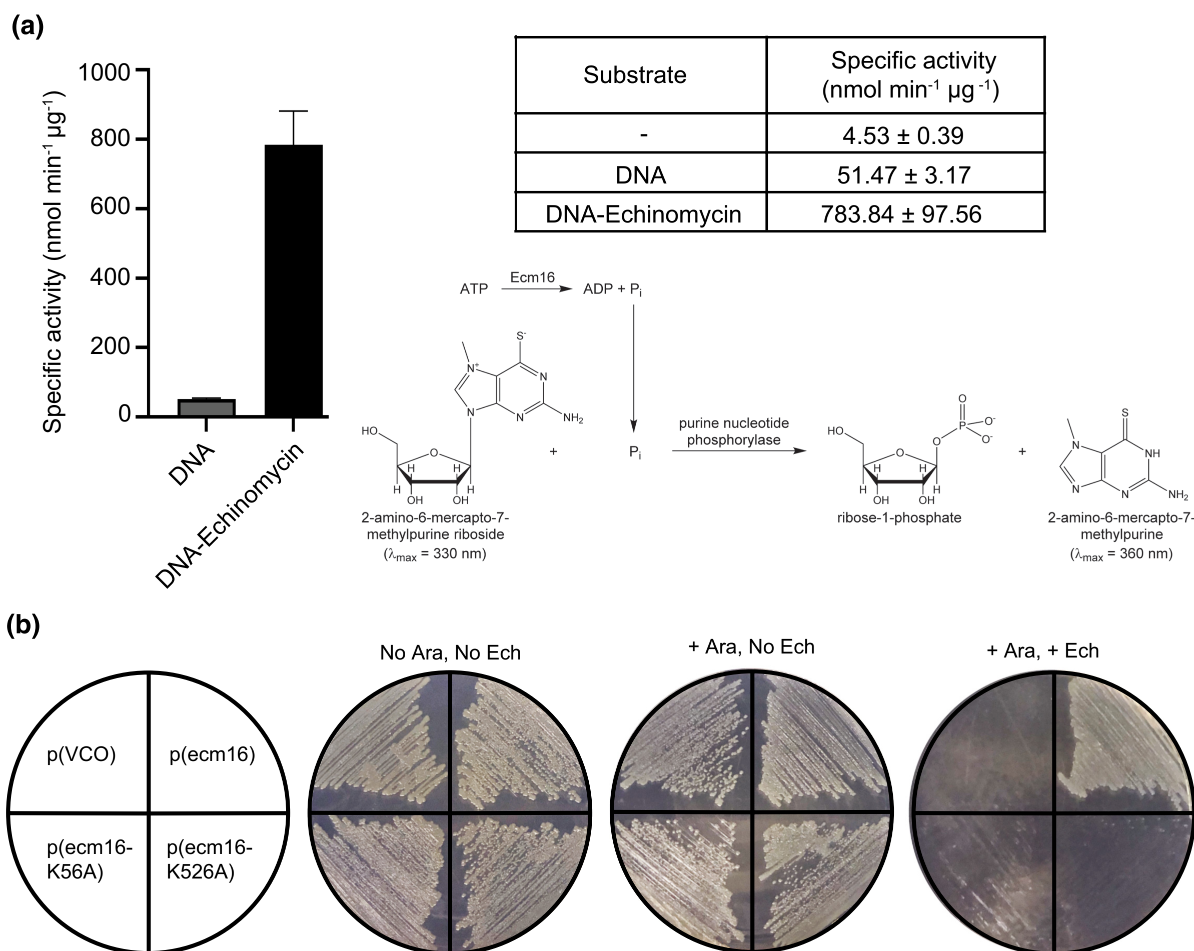
FIGURE 3 DNA binding activity of Ecm16. (a) Reaction mixtures contained 0.25 nM DNA substrate in the absence (lane 1) or presence of 5 to 60 nM Ecm16 with increment of 5 nM (lanes 2–13), (a) ssDNA; (b) dsDNA; (c) echinomycin-DNA. (b) Fraction of ssDNA (panel (a)), dsDNA (panel (b)), echinomycin-DNA (panel (c)) bound to Ecm16 is plotted against the indicated amounts of Ecm16. ssDNA (filled circles); dsDNA (filled squares); echinomycin-DNA (filled triangles). Each point on the curves represents the mean of three separate experiments. (c) DNA binding activity of Ecm16 in presence of 100% GC and AT, 75%/25% and 50%/50% GC/AT composition 0.25 nM DNA substrates in the absence (lane 1) or presence of 5 to 30 nM Ecm16 with increment of 5 nM (lanes 2–7).

(Figure 3b). The substrate affinity of Ecm16 increased when dsDNA was combined with echinomycin (K_d = 20.6 nM) (Figure 3a,b). Class I UvrA proteins are also known to display higher binding affinity for damaged DNA compared to non-damaged DNA (Jaciuk et al., 2011; Rossi et al., 2011). Given that echinomycin intercalates DNA with a preference for CpG sites (Dawson et al., 2007; Van Dyke & Dervan, 1984), we tested whether Ecm16 displays stronger affinity for specific DNA sequences. We analyzed the binding of Ecm16 to DNA sequences with differing AT/GC content using electrophoretic mobility shift assays (Figure 3c). Our results revealed no significant differences in Ecm16's substrate binding with varying AT/GC content, suggesting that Ecm16 primarily interacts with the DNA backbone, and not the bases. These data indicate that Ecm16 has substrate specificity and/or higher binding affinity for DNA-echinomycin and binds DNA in a nucleotide sequence-independent mode.

2.4 | Ecm16 displays ATPase activity that is essential for echinomycin resistance

The ATPase activity of UvrA is required for UvrA's ability to recognize DNA damage and initiate the repair mechanism (Oh et al., 1989; Thomas et al., 1985). Ecm16, similar to other UvrA homolog proteins, has high conservation of amino acids involved in ATP binding

and hydrolysis, including those found in Walker A, Walker B, and the alpha-helical ABC signature sequence (Figure S2). To determine whether Ecm16 displays ATPase activity and whether this activity is required in protection against echinomycin, we first characterized Ecm16's ability to hydrolyze ATP in vitro. We used an enzyme-coupled assay that utilizes the purine nucleoside phosphorylase (PNP) (Figure 4a). During the reaction, PNP combines inorganic phosphate (Pi) produced by Ecm16's ATPase with 2-amino-6-mercapto-7-methylpurine (MESG) to form 2-amino-6-mercapto-7-methylpurine. This enzymatic conversion of MESG results in a spectrophotometric shift that is monitored using a spectrophotometer. Ecm16 shows basal level ATP hydrolysis activity in the absence of any substrate, a characteristic which is also observed in class I UvrA (Rossi et al., 2011). The addition of dsDNA increased Ecm16's ATPase specific activity ~10-fold, indicating that the protein shows DNA-dependent ATP hydrolysis activity. The addition of echinomycin-dsDNA complex increased Ecm16's ATPase activity ~200-fold (Figure 4a). We next tested whether Ecm16's ATPase activity is involved in its ability to render echinomycin resistance in vivo. To do that, we targeted two conserved lysine residues in UvrA-type proteins that have been shown to be essential for scanning DNA and discriminating between native and damaged DNA (Kraithong et al., 2017; Pakotiprapha et al., 2012; Thiagalingam & Grossman, 1991, 1993). We engineered two Ecm16 variants: one at the proximal ATP-binding site with the conserved residue Lys56 changed to an alanine, and the other at the distal site with Lys526 changed to an alanine. Our data revealed that *E. coli* cells exclusively expressing either Ecm16^{K56A} or Ecm16^{K526A} failed to rescue cells from echinomycin toxicity (Figure 4b). These data suggest that Ecm16 requires the energetics of ATP hydrolysis to render echinomycin resistance.



Substrate	Specific activity (nmol min^{-1} μg^{-1})
-	4.53 ± 0.39
DNA	51.47 ± 3.17
DNA-Echinomycin	783.84 ± 97.56

FIGURE 4 ATPase activity of Ecm16. (a) Specific activity of 0.2 μM purified Ecm16 in the presence or absence of 1 μM DNA and DNA-echinomycin substrates. Error bars represent standard error of three independent experiments. The reaction mixture contained 2-amino-6-mercapto-7-methylpurine (MESG) and purine nucleoside phosphorylase, 1 mM ATP and 10 mM $MgCl_2$. A phosphate standard was measured to calibrate the UV absorbance signal to the amount of inorganic phosphate release. (b) Streak plates containing *E. coli* cultures containing a plasmid with WT *ecm16* (p[*emc16*]), *ecm16* ATP-binding variant (p[*emc16*-K526A]), or vector control (p(VCO)). Cultures were grown overnight and normalized to 0.5 OD_{600} before streaking on LB ampicillin plates containing 0.2% arabinose or 1 μM echinomycin (with 0.2% arabinose) and were incubated at 37°C overnight. Plates are representative of 3 independent trials.

2.5 | Ecm16's echinomycin resistance activity does not require components of the NER system

Based on similarities between Ecm16 and UvrA, we examined whether Ecm16's mechanism of protective action against echinomycin resembles the activity of the DNA repair protein UvrA. In *E. coli*, UvrA or $UvrA_2$-$UvrB_2$ detects DNA damage and recruits the rest of the NER system (UvrB/C/D) (Caron et al., 1985; Kisker et al., 2013; Orren & Sancar, 1989; Yeung et al., 1986). To address the possibility that Ecm16's ability to render cells echinomycin resistant requires the function of NER components, we analyzed *E. coli* strains with knockouts of each individual component (*uvrA*, *uvrB*, *uvrC*, and *uvrD*; Keio Collection) (Baba et al., 2006) (Figure 5a). To each of these strains, we transformed the replicating plasmid encoding *ecm16* or VCO. We tested the ability of these strains to grow in the presence of echinomycin concentrations that were tolerated by cells expressing *ecm16*. Our growth analyses revealed that cells encoding the NER system and cells without components of the NER system displayed

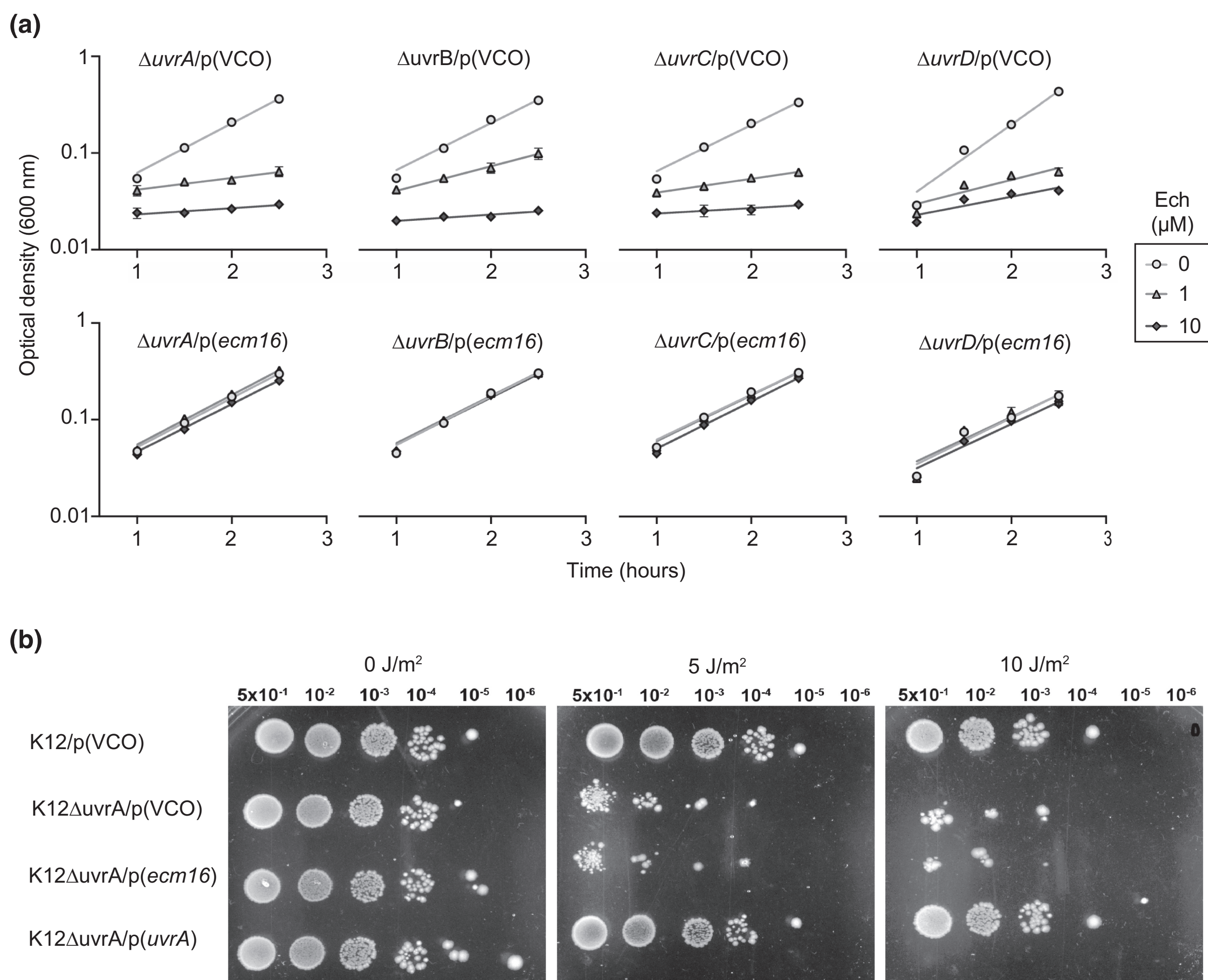


FIGURE 5 Ecm16 is not associated with classic NER function. (a) Exponential state growth curves ($n = 3$) of *E. coli* K12 strains with different components of the native nucleotide excision repair system (NER) knocked out (Δ*uvrA*, Δ*uvrB*, Δ*uvrC*, or Δ*uvrD*) and carrying vector-control-only or vector encoding *ecm16*. Cultures (2 ml) were set to OD_{600nm} ~ 0.2 in rich media (LB) supplemented with the inducer (0.2% arabinose) and varying concentrations of echinomycin (0–10 μM). Growth was monitored by measuring the absorbance at 600 nm every 30 min. *E. coli* K12 NER knockouts show similar patterns of resistance to echinomycin in the presence of inducer for the expression of ecm16. Results are shown for exponential growth phase with exponential trend line, error bars represent SEM of duplicate replicates. All results shown are representative of three independent replicates. (b) Colony forming unit (CFU) assays after UV radiation. 5 μl of cultures grown to OD_{600nm} ~ 0.2 were serial diluted (dilution factor = 5×10^{-1} to 10^{-6}) and spotted on LB plates supplemented with 0.2% arabinose. Cultures included the following strains: Control sample (top row) are wild-type cells with vector-control-only, test samples (middle 2nd and 3rd rows) are strains with the native *E. coli*'s uvrA gene knocked out and with vector-control-only or vector encoding *ecm16*, complementation control strain (bottom row) encodes *E. coli*'s native *uvrA* gene in the same pBAD vector. Freshly spotted plates were exposed to UV radiation and then incubated at 37°C for 18 h prior to imaging. The data shown are a representative of three independent replicates.

similar doubling rates when grown in the presence of echinomycin. Ecm16 was able to provide the same level of echinomycin resistance in the absence of NER.

2.6 | Ecm16 and UvrA cannot complement each other's function

In *E. coli*, the activity of UvrA is essential for repairing various types of DNA damage including thymine dimers resulting from exposure to UV radiation (Boyce & Howard-Flanders, 2003). To determine whether Ecm16 can complement UvrA's activity, we examined the ability of various strains to recover from UV radiation by performing colony-forming unit (CFU) assays (Figure 5b). *E. coli* cells with their native *uvrA* gene and VCO can effectively recover from 10 J/m^2 of UV radiation exposure as evidenced by the similar number of CFUs compared to the no-radiation control. However, the cell's ability to recover from UV radiation is significantly reduced when *E. coli*'s native *uvrA* gene is knocked out. Once *uvrA* was expressed *in trans* from a replicating plasmid under the control of the arabinose inducible promoter, the ability to repair DNA damage was fully recovered. These data are in accordance with previous analyses of *uvrA* knockout strains (Springall et al., 2018). However, when the gene encoding for Ecm16 was expressed *in trans* from the same replicating plasmid controlled by the arabinose promoter, *E. coli* cells were unable to recover from UV radiation. The number of CFUs of the strain expressing *ecm16* was as diminished as the CFUs observed on the *uvrA* knock out strain. The same trend of UV resistance was also observed among the *E. coli* strains when lower levels of radiation (5 J/m^2) were used. The inability of Ecm16 to complement an *uvrA* knockout suggests that Ecm16 is unable to recognize thymine dimers and/or recruit the rest of the NER system for proper DNA repair.

Given that *E. coli* K-12 cells display sensitivity to echinomycin suggested that UvrA is unable to protect from echinomycin toxicity. To determine whether increasing the cellular levels of UvrA would result in protection against echinomycin, we constructed an *E. coli* strain with two copies of *uvrA*: its native copy and a second copy expressed *in trans* from a replicating plasmid (same vector used for *ecm16* expression). Cells expressing *uvrA* from the inducible promoter grown in the presence of echinomycin display the same growth defect as the empty vector control (Figure S3). These results confirm that UvrA and Ecm16 cannot complement each other's function due to either differences in damage detection and/or repair.

3 | DISCUSSION

In this study, we provide in vivo and in vitro characterization of Ecm16, a class IIa UvrA protein. Our data demonstrate that the expression of *ecm16* is sufficient to confer echinomycin resistance to an otherwise echinomycin sensitive bacterium, *E. coli* K-12. We show that ATP hydrolysis of Ecm16 is required for its ability to render resistance. Furthermore, Ecm16 was able to confer echinomycin resistance to host cells that are deficient in UvrA, UvrB, UvrC, or UvrD, indicating that Ecm16 does not depend on the NER machinery for its function. Finally, our data reveal that despite the sequence similarity between UvrA and Ecm16, they are unable to complement each other's function. Collectively, our data expand our current understanding of the various mechanism that class IIa UvrA proteins utilize for their function.Our work provides further insights into the potential mechanism used by Ecm16 to render resistance from echinomycin toxicity. In the case of NER, UvrA recognizes DNA lesions and promotes the recruitment of the rest of the NER system to excise the damaged DNA. However, Ecm16 retained its ability to render echinomycin resistance in the absence of any of the NER proteins. This observation posits two potential models where Ecm16 can either work with other housekeeping proteins found in *E. coli* or Ecm16 can work alone. The former model is plausible given that UvrA has been shown to work collaboratively with non-NER proteins (Mazon et al., 2009; Tubbs et al., 2009). In the scenario where the latter model is correct, we envision Ecm16 to use ATP hydrolysis to restore the native DNA helix properties. UvrA has been proposed to subject DNA to a 'stress test' where UvrA unwinds and stretches (or compresses) DNA during the detection of damage (Yang, 2011). Ecm16 could be using a version of this 'stress test' mechanism but in doing that it would potentially release the echinomycin bound from DNA. We are currently testing between these two potential models.

Based on primary sequence conservation, various drug-producing bacteria have been proposed to encode UvrA class IIa proteins to counteract DNA intercalators (Ogawara, 2019). For example, daunorubicin and triostin A include a UvrA-homolog encoding gene in their biosynthetic clusters: *drrC* and *trsM* (Furuya & Hutchinson, 1998; Keller et al., 2010; Ogawara, 2019; Praseuth et al., 2008; Watanabe et al., 2006; Zhang et al., 2013). DrrC has been shown to render self-protection in *Streptomyces peucetius* against daunorubicin (Moolenaar et al., 2000; Prija & Prasad, 2017). The production of doxorubicin increased ~5-fold in a *S. peucetius* strain overexpressing *drrC* compared to that of the parental strain (Malla et al., 2010). Notably, the high degree of sequence similarity among class IIa UvrA proteins in drug-producing bacteria suggest that Ecm16's mechanism for self-protection may be broadly conserved. However, the diversity of substrates in these class IIa UvrA proteins suggest that these proteins must include a mechanism for detecting specific substrates. Self-protection UvrA class IIa proteins may also share substrates. For example, *Streptomyces triostinicus* encoding the biosynthetic cluster for triostin A was shown to produce not only triostin A but also echinomycin (Praseuth et al., 2008), suggesting that TrsM protected *S. triostinicus* from both drugs. More research is necessary to understand how these various UvrA class IIa proteins recognize their distinct substrates and to determine whether their mechanisms of action are conserved.

Although multiple models have been proposed to explain how the NER system recognizes DNA damage, conclusively addressing the mechanism of recognition remains a major gap in our knowledge in both bacterial and eukaryotic NER systems. We propose that the understanding of the mechanism of substrate recognition by Ecm16

can provide insights into how the NER system recognizes its substrates. This is especially the case given that UvrA and Ecm16 were unable to promote each other's function. UvrA has been proposed to distinguish between native and damaged DNA by sensing changes in various properties of DNA (structural, electrostatic, dynamics, stability, flexibility) (Kraithong et al., 2021). Our data revealed that class I UvrA is unable to render echinomycin resistance possibly because the distortions of DNA caused by echinomycin are not recognized by UvrA. This scenario would be surprising given the wide range of DNA distortions that class I UvrA proteins are known to recognize. Another scenario is that the NER system is unable to repair any potential DNA damage caused by echinomycin toxicity. Regardless of which mechanism is at play, figuring out why class I UvrA proteins are unable to render resistance against echinomycin has the potential to advance our understanding of the mechanisms of the universally conserved NER system.

4 | EXPERIMENTAL PROCEDURES

4.1 | Bacterial growth conditions

E. coli strains were grown in Luria Bertani broth (LB) (Fisher BioReagents, NJ, USA). All cultures grown in liquid media were grown at 37°C with orbital shaking at 200 rpm. Strains cultured on solid media were plated on LB agar (Fisher BioReagents, NJ, USA) and incubated at 37°C. Ampicillin (amp) resistant strains were grown with 50 μg amp/ml liquid media (Ampicillin sodium salt prepared in H_2O, Amresco, Ohio, USA) and 100 μg amp/ml solid media. Kanamycin (kan) resistant strains were treated with 30 μg kan/ml for liquid media (kanamycin sulfate prepared in H_2O, IBI Scientific, NJ. USA), and 50 μg kan/ml for solid media. For induction, 0.2% L-(+)-arabinose (Alfa Aesar, G.B.) was added to either liquid or solid media. Strains grown with echinomycin were treated with 1 μM-20 μM of echinomycin diluted in H_2O from a 908 μM echinomycin (Sigma, USA) solution prepared in methanol (Fisher Chemical, NJ, USA). All strains and primers used for this study are listed in Tables S2 and S3.

4.2 | Growth analyses

Cultures were grown in liquid media with ampicillin overnight from frozen stocks, incubated at 37°C and 200 rpm. Saturated cultures were diluted with fresh medium and induced with 0.2% arabinose for 60 min and used to inoculate 2 ml duplicate replicate samples at a starting optical density of 0.02 OD_{600} in 13 mm glass tubes. Cultures were grown in rich liquid media (LB), with ampicillin and 0.2% arabinose, incubated at 37°C and 200 rpm. Optical density readings were taken every 30 min for 6 h using Thermo UV–Vis Spectrophotometer. Maximum growth in echinomycin was determined by evaluating serial dilutions of echinomycin in H_2O when added to LB media inoculated with K-12 *E. coli* culture, using 1 μM to 20 μM ech. K-12/pBAD (vector control) and K-12/pBAD-*ecm16* were grown overnight from frozen stocks at 37°C and 200 rpm, and prepared as in growth curves. Endpoint readings were taken after 6 h of incubation.

4.3 | Imaging

K-12 strains were grown from frozen stocks and used to inoculate 3 ml of LB/Amp/0.2% arabinose to 0.2 OD_{600}. Cells were induced with 0.2% arabinose 30 min prior to dilution. Following inoculation, cultures were treated with 5 μM echinomycin and grown for 5 h. Cultures were spotted on 0.2% agarose (Ultrapure, Invitrogen, Spain) discs, and visualized with phase contrast microscopy using Zeiss Axio Observer 2.1 inverted microscope with a Plan-Apochromat 100×/1.40 Oil Ph3 M27 (WD = 0.17 mm) objective, AxioCam 506 mono camera and ZEN software. Cell size was calculated using ImageJ/FIJI with MicrobeJ (Ducret et al., 2016; Schindelin et al., 2012; Schneider et al., 2012).

4.4 | UV-radiation sensitivity test

Cultures were grown overnight incubated at 37°C and 200 rpm in liquid media with ampicillin and used to inoculate samples to 0.3 OD_{600} the following day. 0.2% arabinose was added to each sample and cells were incubated for 2 h. Cells were then serially diluted by factors of 10 and 5 μl of cultures were spotted on LB/ampicillin plates with 0.2% arabinose. Plates were exposed to ultraviolet radiation (nm) at 5 J/m^2 or 10 J/m^2 or no UV as a negative control.

4.5 | Bacterial survival curves

Cultures inoculated from frozen stocks were grown overnight in LB media supplemented with ampicillin at 37°C. Overnight cultures were diluted to 0.1 OD_{600} and grown in LB containing various concentrations of echinomycin. Samples taken every hour were analyzed for optical density and plated for CFU quantification. For CFU quantification, samples were serially diluted and plated on LB agar plates containing Amp. Plates were incubated at 37°C overnight. Number of colonies on each plate were counted using ImageJ software to determine Log CFU/ml.

4.6 | Purification of Ecm16

Recombinant proteins were expressed by transforming *E. coli* BL21 (de3) (Novagen, Merck Millipore) host strains with the recombinant pET28a constructs encoding Ecm16 wild-type. Cells were cultured at 37°C in 5 ml of LB medium supplemented with kanamycin to an optical density of ~0.6–0.8 at 600 nm. Protein expression was performed by inducing the cells with 0.1 mM isopropyl-β-D-thiogalactoside, and growth was continued for a further 16 h at 18°C. The cell pellet was resuspended in lysis buffer containing

50 mM HEPES pH 7.5, 200 mM NaCl, 10% glycerol, 10 mM imidazole, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg/ml DNase, and 10 mM $MgCl_2$. Cells were disrupted using sonication (Branson Ultrasonics) and cell debris was discarded by centrifugation at 15,000*g* for 45 min at 4°C. Cleared cell lysate was applied on 5 ml His-Trap Crude column (GE Healthcare) equilibrated with loading buffer (50 mM HEPES, pH, 7.5 and 200 mM NaCl). The protein was eluted with a step gradient of imidazole (250 mM) using elution buffer (50 mM HEPES pH 7.5, 200 mM NaCl, 500 mM Imidazole). Ecm16 eluted from the nickel column was diluted up to 10-fold using dilution buffer (50 mM HEPES pH 7.5, 50 mM NaCl). The diluted protein was applied to 5 ml HiTrap Q HP anion exchange column (GE Healthcare). Protein was eluted with 5 column volumes (CV) of linear NaCl gradient from 50 mM to 500 mM concentration. The protein was concentrated to 4–6 mg/ml and gel filtration was performed on a Superdex 200 10/300 GL (GE Healthcare) column equilibrated with storage buffer (50 mM HEPES pH 7.5, 50 mM NaCl). The eluted protein sample was concentrated to 8–10 mg/ml, flash-frozen in liquid nitrogen and stored at −80°C.

4.7 | Electrophoretic mobility shift assay

Stock solutions of echinomycin (908 μM) (Cayman Chemical) were prepared by dissolving the echinomycin powder in 100% methanol. DNA substrates were purchased PAGE-purified from Integrated DNA Technologies. A 32-mer palindromic DNA oligonucleotide containing 5′-ACGT-3′ echinomycin binding site at the center was used for the assay (Table S1). DNA-echinomycin complex was allowed to form by incubating 1.0: 1.1 molar ratio of DNA: echinomycin for 15 min at room temperature. 10 μl reactions containing Ecm16 protein at concentrations ranging from 5 to 60 nM was incubated with 25 nM ssDNA or dsDNA or Ech-dsDNA substrates for 20 min at room temperature in EMSA reaction buffer (50 mM HEPES pH 7.5, 50 mM NaCl). The protein-DNA complexes were electrophoretically separated at 4°C on 6% native polyacrylamide gels (Invitrogen) in 1× TBE buffer (90 mM Tris base, 90 mM Boric acid, 2 mM Na_2EDTA) at 75 V/cm. The gels were stained for 10 min in 1× TBE buffer containing 5 μl of SYBR gold nucleic acid gel stain (Invitrogen). The band intensity corresponding to the free DNA and protein-DNA were visualized by ultraviolet transilluminator (Azure c200) and the bands were quantified using ImageJ software (Schneider et al., 2012). The fraction of DNA bound was calculated as the ratio of bound DNA to free DNA, except for the ssDNA substrate. The binding of ssDNA to Ecm16 resulted in a smear, therefore, the fraction of DNA bound was calculated using a ratio of free DNA remaining in the given lane to the protein-free lane. The data sets were subjected to a nonlinear regression analysis model with a single-site binding curve fit with hill slope. The fraction bound is reported as the mean ± standard deviation of three independent experiments. EMSA using oligonucleotides containing 100% GC and AT, 75%/25% and 50%/50% GC/AT composition was performed using similar experimental conditions.

4.8 | ATPase activity assay

Adenosine triphosphate (ATP) was dissolved in storage buffer (50 mM HEPES pH 7.5, 50 mM NaCl) to prepare a 100 mM stock solution. Hydrolysis rate of ATP by Ecm16 was measured in a 96-well plate using an enzyme-coupled assay where the substrate 2-amino-6-mercapto-7-methylpurine riboside (MESG) is enzymatically converted to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine by purine nucleoside phosphorylase (PNP) in the presence of inorganic phosphate (Pi). This enzymatic conversion of MESG results in a spectrophotometric shift in maximum absorbance of 360 nm for the product formed. The reaction mixture (100 μl) contained 50 mM HEPES pH 7.5, 50 mM NaCl, 0.5 mg/ml BSA, 10% glycerol, 10 mM $MgCl_2$, 1 mM ATP, 0.2 mM MESG, 1× reaction buffer (50 mM tris HCl pH 7.5, 10 mM MgCl2, 0.1 mM sodium azide), 1 U/ml PNP and/or 0.5 μM Ecm16 in presence of DNA substrates. A 32-mer palindromic DNA oligonucleotide containing single echinomycin binding site was used as DNA substrate (Table S1). DNA-echinomycin complex was allowed to form by incubating 1.0: 1.1 molar ratio of DNA: echinomycin for 15 min. The rate of hydrolysis was calculated from the linear change in absorbance at 360 nm and 22°C for 60 min. The absorbance at 360 nm were corrected for background absorbance and the nanomoles of inorganic phosphate released after ATP hydrolysis of Ecm16 were calculated using KH_2PO_4 as the source for the inorganic phosphate. The effect of DNA on ATP hydrolysis of Ecm16 was studied by adding 2.5 μM 32-mer dsDNA or Ech-dsDNA substrate in the assay mixture. The data are reported as the mean rate (M/min) ± the standard deviation of the mean, $n = 3$.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

ETHICS STATEMENT

The work presented here did not include human or animal subjects nor human or animal material or data. Thus, no formal consent or approval was necessary.

AUTHOR CONTRIBUTIONS

P.E.M. and C.-Y.K. conceived and supervised the project. A.E. and I.P.M. performed bacterial growth and microscopy studies. P.G. conducted ATP hydrolysis and EMSA measurements. A.E., P.G., C.-Y.K., and P.E.M. wrote the manuscript.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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