Antibiotic Resistance

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Self-Resistance to an Antitumor Antibiotic: A DNA Glycosylase Triggers the Base-Excision Repair System in Yatakemycin Biosynthesis**

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Most of the antibiotics in clinical use to treat human disease are natural products produced by microorganisms. A general hypothesis is that antibiotic-resistance genes and mechanisms have co-evolved with antibiotic biosynthesis as a self-protection scheme.[1] Nature has evolved three major ways for avoidance of suicide in antibiotic-producing microbes:[2,3] antibiotic modification, target-protecting enzymes, and export pumps. However, other resistance mechanisms have yet to be discovered, especially for the highly toxic molecules that interrupt basic biological processes (for example DNA, protein, cell-wall biosynthesis).[4,5]

DNA-alkylating compounds represent one of the most abundant classes of mutagenic and genotoxic agents present in nature, including several families of DNA-targeting antibiotics for the treatment of cancer.[6] Yatakemycin (YTM, **1**, Scheme 1) is the most potent member among antitumor antibiotics belonging to the family of CC-1065 and duocarmycins, which are known to be DNA-alkylating agents (IC_{50} of 3 pM for the L1210 cell line).[7] Members of this family have been shown to selectively bind the minor groove of DNA in AT-rich sequences and alkylate adenine at the N3 position. YTM shows the most potent cytotoxicity against cancer cell lines, probably because of its unique "sandwiched" arrangement with a DNA-binding subunit located on each side of the central alkylation subunit; the adenine alkylation depends on both shape-selective recognition and shape-dependent catalysis.[8] It has also been reported that YTM selectively alkylates nucleosomal DNA and is almost completely occluded by histones.[9] So we have studied how the microorganism that produces YTM protects itself against this highly cytotoxic compound and avoids autotoxicity.

Currently, there is a great deal of attention for the chemical synthesis, DNA alkylation, and biological properties of the YTM family of natural products,[8] but little progress has been made on the biosynthetic pathway. Information on the biological resistance of this class of DNA-alkylating natural products has not been reported. Recently, the biosynthetic gene cluster of YTM in *Streptomyces* sp. TP-A0356 was sequenced and characterized to reveal 31 genes.[10] Among them, five genes, *ytkR2*, *ytkR3*, *ytkR4*, *ytkR5*, and *ytkR6*, have been proposed to be responsible for self-defense against YTM (Scheme 1 A). YtkR2 shows sequence homology to DNA glycosylase superfamilies, YtkR3 exhibits low sequence homology to xanthine phosphoribosyl-transferase, YtkR4 shows homology to a metal-dependent TatD family of DNase, and YtkR5 shows high sequence homology to AP endonuclease. These enzymes are all involved in the base-excision repair (BER) system, which cleaves damaged DNA bases within double-stranded DNA, producing an abasic site.[11] YtkR6 exhibits significant sequence homology to a family of drug-resistance transporters, such as ChaT1, a putative efflux protein in the chartreusin biosynthetic pathway.[12] Together, the functions of these enzymes might make up the network of self-protection in YTM-producing microorganisms, and they are also consistent with the discovery of multiple self-defense mechanisms in several actinomycetes, which produce antibiotics with high biological activity.[3,4] Herein, we describe the genetic and biochemical identification of YtkR2, an AlkD-like DNA glycosylase, involved in the BER system to confer self-resistance in YTM biosynthesis.

BLAST analysis showed that YtkR2 strongly resembles a structural DNA glycosylase containing Huntington/Elongation/A subunit/Target of rapamycin (HEAT)-like repeats, and one of the most significant homologues (26% identity and 48% similarity) is AlkD from *Bacillus cereus*.[13] DNA glycosylases have been identified in all organisms investigated and may be universally present in normal cellular metabolism, they catalyze the first step in the BER pathway by cleaving damaged DNA bases within double-stranded DNA to produce an abasic site.[11] The resulting abasic site is further processed by AP endonuclease, phosphodiesterase, DNA

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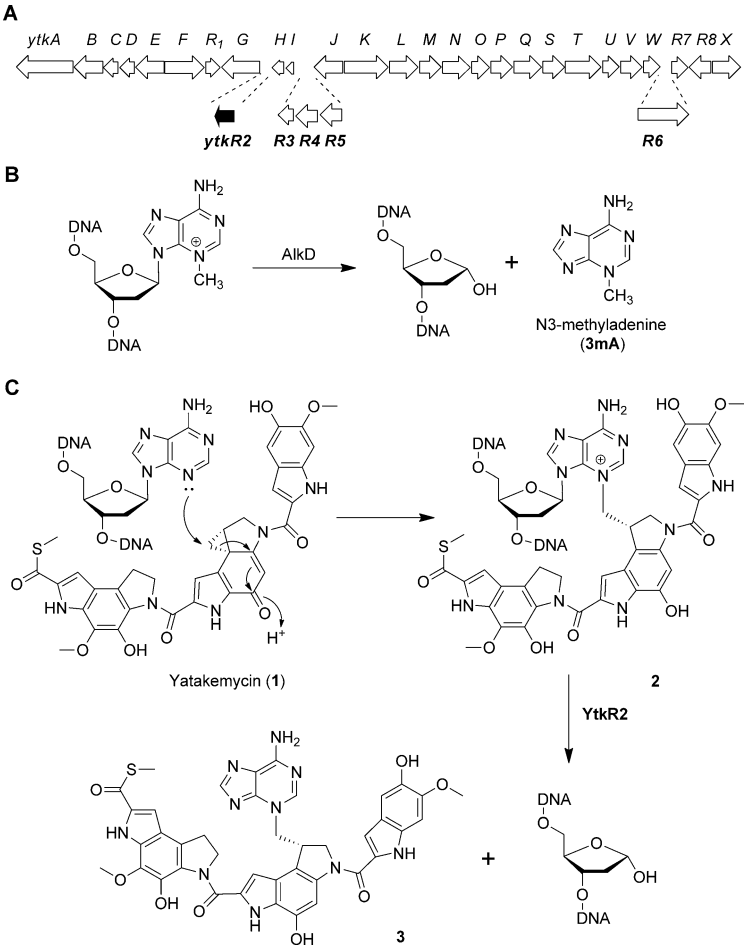

Scheme 1. Biosynthetic gene cluster for yatakemycin (YTM, **1**) production and base-excision repair of alkylated DNA by AlkD and YtkR2. A) The *ytkR2* gene is located in the YTM gene cluster. B) AlkD catalyzes the hydrolysis of the N-glycosidic bond to yield an abasic site and free 3-methyladenine. C) YtkR2 catalyzes the hydrolysis of the N-glycosidic bond to liberate an abasic site and free N3-YTM-alkylated adenine (**3**).

polymerases, and DNA ligase to restore the DNA to an undamaged state. The known structures for members of this family, AlkC and AlkD from *Bacillus cereus*, are distant homologues and are composed of six variant HEAT repeats.[14] They are specific for removal of 3-methyladenine (3mA) and 7-methylguanine (7mG) from the DNA by cleaving the N-glycosidic bond between the alkylated base and the deoxyribose moieties of the nucleotides, thus releasing the damaged base and creating an abasic (AP) site in the DNA (Scheme 1B). So, YtkR2 could confer resistance to the bacterial producer by specifically recognizing and cleaving the YTM-modified bases to initiate the BER pathway (Scheme 1C).

To validate our hypothesis, a gene-replacement plasmid pTG1311 was constructed, in which *ytkR2* was inactivated by deletion of a 705 bp fragment. Plasmid pTG1311 was introduced into *Streptomyces* sp. TP-A0356 by conjugation, to select for the double cross-over mutants *Streptomyces* sp. TG1308, and the genotype of this mutant was confirmed by PCR analysis (Supporting Information, Figure S1). YTM was still produced by the *ytkR2* gene-replacement mutant strain, TG1308, but the production was reduced by about 40%, as determined by HPLC analysis (Figure 1A). When a construct pTG1312 expressing an additional copy of *ytkR2* under the control of a constitutive *PermE** promoter was introduced into *Streptomyces* sp. TP-A0356 to afford strain TG1309. Analysis of the metabolites accumulated by this mutant revealed that the production of YTM was increased by 60% over the wild-type strain (Figure 1A).

Previous studies revealed that overexpression of resistance genes in *E. coli* could confer resistance to the corresponding antibiotic.[5,15] Therefore, we tested the function of YtkR2 in vivo by overexpressing YtkR2 in *E. coli* BL21 cells. The YtkR2-expression plasmid pTG1313 or empty vector pET37b were re-transformed into *E. coli* BL21 cells and plated on medium containing increasing amounts of YTM (Figure 1B) or a common alkylating agent methyl methanesulphonate (MMS; Figure 1C), respectively. The survival ratio suggested that in vivo YtkR2 is not only provides resistance to YTM but also to MMS. In contrast, AlkC and AlkD from *Bacillus cereus* only show resistance to MMS but not YTM. Together, the in vivo data unambiguously supported our hypothesis and prompted us to further characterize YtkR2 in vitro.

To explore the biochemical function of YtkR2, the gene was cloned into the pET37b vector and transformed into *E. coli* BL21 cells. Expression of YtkR2 was induced by IPTG to give a 28.4 kDa soluble protein with a His_8 tag at the C terminus, which was then purified on Ni-IDA agarose (Figure 2A). Initially an alkylated DNA fragment was used as a substrate. A 2.5 kb DNA fragment (plasmid pSP72 digested with *Eco*RI) was incubated with adequate YTM at room temperature for 36 h. The reaction mixture was subsequently treated with purified YtkR2 at 30°C for ten minutes. Compared with the unmodified DNA, the alkylated-DNA band shifted on the agarose gel with the addition of YtkR2 (Figure 2B), suggesting that YtkR2 might remove the YTM-modified bases leaving AP sites in DNA as we proposed in Scheme 1C. For further confirmation, hairpin DNA (hpDNA) with one ATTAA site (GCTAATTCTTTTTGAATTAGC)[16] was treated with YTM and then incubated with YtkR2 (see Supporting Information). The resulting products were analyzed by LC–MS at 385 nm (Figure 2C). In addition to the YTM (**1**) peak at 24 minutes, a new peak with *m*/*z* 815 in the mass spectra appeared at 13 minutes; this molecular weight corresponds to that of the YTM–adenine complex **3** (Scheme 1C). Next, HR-MALDI-Q-TOF-MS analysis of this enzymatic reaction yielded an $[M+H]^+$ ion at *m*/*z* 815.2367 (Figure 2D), which is consistent with the molecular formula for **3**, $C_{40}H_{34}N_{10}O_8S$ (calcd mass 815.2282). Further tandem-MS analysis showed that all the fragments detected were consistent with the proposed chemical formula for **3** (Figure 2E). It also confirmed that YtkR2 recognizes the

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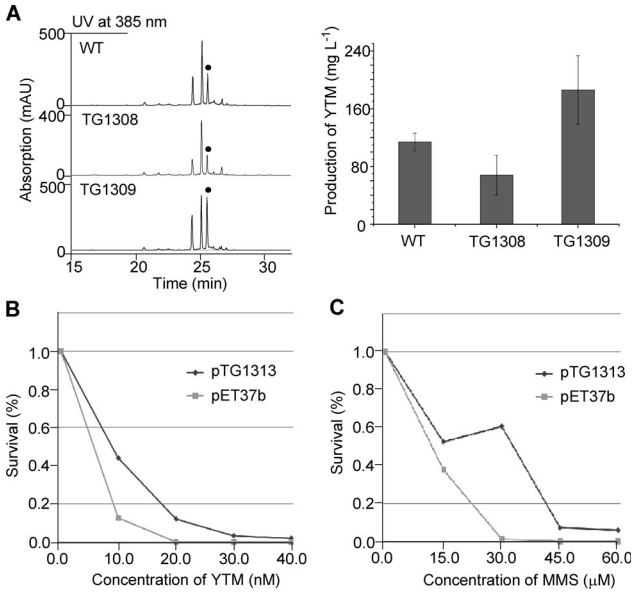

Figure 1. Functional validation of YtkR2 in vivo. A) Analysis of YTM (●) production from wild-type and recombinant strains. WT = wild-type *Streptomyces* sp. TP-A0356, with YTM yield of 113.7 ± 12.2 mg L^{-1}; TG1308 = mutant *Streptomyces* sp. TG1308 (Δ*ytkR2*), with YTM yield of 68.0 ± 27.6 mg L^{-1}; TG1309 = mutant *Streptomyces* sp. TG1309 (wild-type *Streptomyces* sp. TP-A0356 with the *ytkR2* expression plasmid pTG1312), with YTM yield of 186.0 ± 47.4 mg L^{-1}. B) YTM and C) MMS survival of *E. coli*. BL21 (DE3) cells transformed with expression plasmid pTG1313 (◆) or pET37b (■).

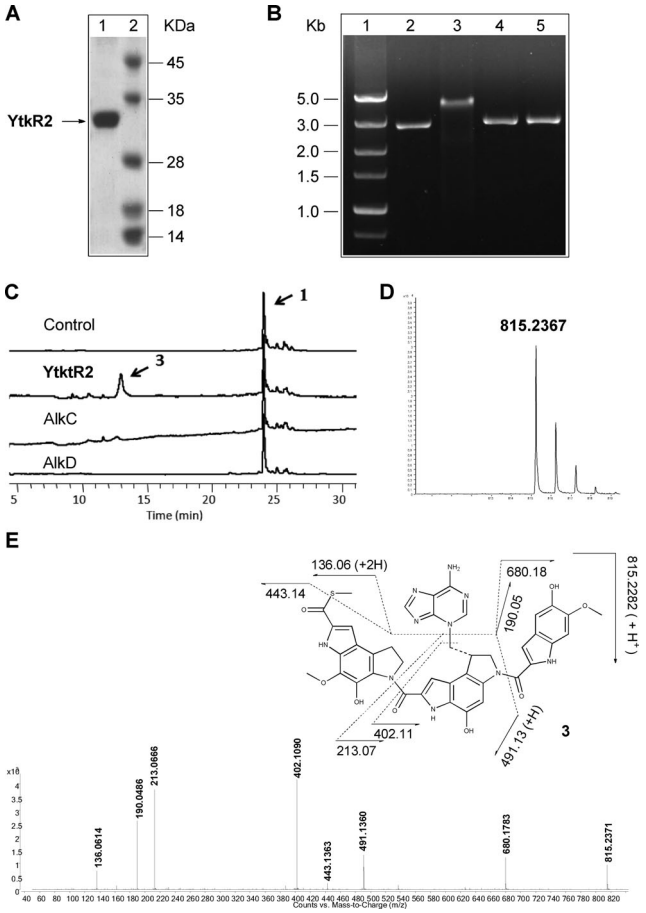

Figure 2. Biochemical characterization of YtkR2 in vitro. A) SDS/PAGE analysis of YtkR2 purified by Ni-IDA. Lane 1 = purified YtkR2; lane 2 = molecular markers. B) In vitro enzyme assay of YtkR2 with pSP72/*Eco*RI (2.469 kb) as substrate monitored by 1% agarose gel electrophoresis. Lane 1 = DNA markers; lane 2 = pSP72/*Eco*RI with YtkR2; lane 3 = YTM-alkylated pSP72/*Eco*RI with YtkR2; lane 4 = pSP72/*Eco*RI with denatured YtkR2; lane 5 = YTM-alkylated pSP72/*Eco*RI with denatured YtkR2. C) HPLC analysis of the enzymatic reaction products. D) High-resolution MALDI-MS analysis of the enzymatic product **3**. E) Tandem MS/MS spectrum analysis to confirm the enzymatic product **3**.

YTM-modified bases and releases the free YTM-alkylated adenine (Scheme 1C). However, the same substrate was also incubated with AlkC or AlkD and no production of **3** could be detected (Figure 2C).

Most DNA glycosylases examined to date utilize a similar strategy for binding DNA and flipping bases out of the helix despite their structural diversity.[17] Recently, AlkD has been crystallized in complex with DNAs containing alkylated, mismatched, and abasic nucleotides, which revealed how the HEAT-repeats domain distorts the DNA backbone to detect non-Watson–Crick base pairs without duplex intercalation.[18] We therefore proposed that YtkR2 might also bind DNA to form a relatively stable complex. To test the binding affinity, we measured YtkR2 binding and dissociation with hpDNA (GCTAATTCTTTTTGAATTAGC) by surface plasmon resonance (Figure 3A). Just as expected, YtkR2 was binding to this hpDNA with a rate of association $k_{as} = 6.49 \times 10^{-2}\,M^{-1}\,s^{-1}$, rate of dissociation $k_{dis} = 3.58 \times 10^{-4}\,s^{-1}$, and dissociation constant $K_d = 0.56$ μM, which indicated that the DNA-binding activity of YtkR2 is a bit higher than that of AlkD.[14]

While it appears that YtkR2 plays a role as a DNA glycosylase to resist YTM alkylation, we attempted to find further insight into the molecular basis of recognition and the enzymatic mechanism involved. Primary alignment of the sequences revealed that YtkR2 has the same conserved residues as AlkD, W113 (W109 for AlkD), D117 (D113 for AlkD), R151 (R148 for AlkD), and R198 (R190 for AlkD; Figure 3B), which have all been shown to be important for the biological activity of AlkD.[14,18] The activities of YtkR2 mutants were tested by monitoring the specific excision of **3** from the hpDNA substrate by HPLC. The results clearly showed that the activity of R198A was reduced significantly, and W113A, D117A, or R151A completely lost their biological activity (Figure 3F), which is consistent with the results from the mutation studies of AlkD.[14,18] Then the I-TASSER online homology-modeling server[19] was used to build a structural model of the YtkR2-YTM-DNA complex based on the structure of the AlkD-DNA complex.[18] Our modeling results (Figure 3C and Figure S2) show that the key residues and binding mode of YtkR2 with DNA are very similar to those of AlkD, which is also supported by the activity profiles of the YtkR2 mutants (Figure 3F). The predicted binding mode of YTM indicates that it resides between two side walls of the binding site, which are formed by residues L43/L118 and Y31/W113. Additionally, I82/L118/A114 form the bottom of the binding site, providing a hydrophobic environment to

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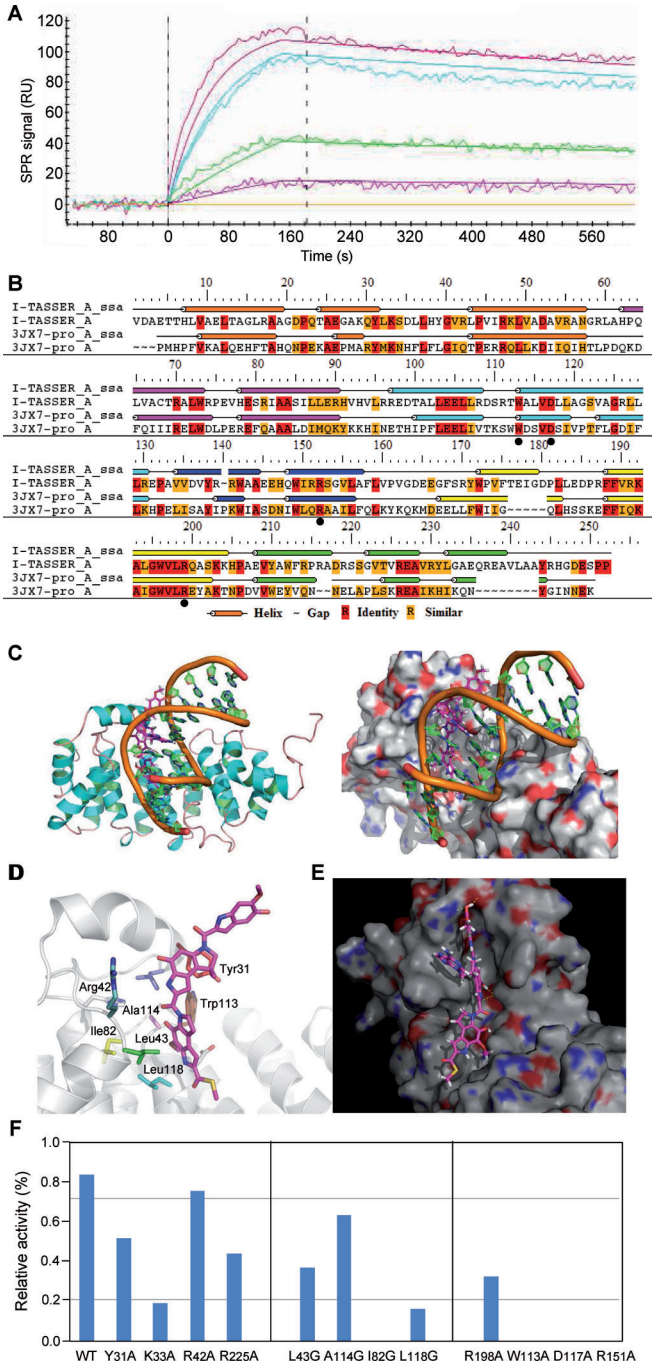

Figure 3. A) Analysis of YtkR2 association and dissociation with oligonucleotides measured by surface plasmon resonance. B) Sequence alignment of YtkR2 with AlkD (3JX7-proA) from *Bacillus cereus* by the I-TASSER_A server. The putative key residues mediating specific protein–DNA contacts are marked with black dots. C) Orthogonal views of the YtkR2 protein (left: in cartoon model; right: in molecular surface model) in complex with the DNA duplex and YTM (magenta) modeled by the I-TASSER server. D) Illustration of the key residues involved in YTM binding. E) A structural model of YtkR2 in complex with the enzymatic product **3** predicted by GOLD software. F) Relative biological activities of wild-type and mutant YtkR2 as monitored by HPLC.

host YTM (Figure 3D,E). So as expected, all of these residues were important for maintaining the biological activity of YtkR2. Indeed, each mutation, that is, L43G, L118G, Y31A, L118G, and A114G, resulted in a significant decrease in activity compared to the wild-type protein (Figure 3F). In particular, the I82G mutant completely lost activity. These results indicate that the hydrophobic residues on the bottom of the binding site are critical for excision of **3** by YtkR2, although they are likely not involved in any direct contacts with **3**.

In the biosynthetic study of YTM, the physiological role of YtkR2 was successfully identified as a novel DNA glycosylase that specifically recognizes and removes the N3-YTM-alkyladenine from the DNA by cleaving the N-glycosidic bond to initiate the BER pathway. Besides providing self-resistance mechanisms, the DNA repair system removes damage from the cells allowing them to live. This is the first example of a DNA glycosylase involved in the self-resistance to a DNA-targeting antibiotic.[3,4,20] Currently, the biological functions, structure, and mechanistic features of mammalian DNA glycosylases are being pursued, and several studies showed that DNA glycosylases confound cancer alkylation therapy by excising cytotoxic N3-methyladenine bases formed by DNA-targeting anticancer compounds.[21] Since YTM is a potent antitumor agent, the functional and structural elucidation of YtkR2 would help to understand the mechanism of this DNA-alkylating chemotherapy. On the other hand, the YtkR2 knock-out mutant still produces YTM, which indicates that there is another independent resistance mechanism (e.g. an efflux system). Also, the yield of YTM improved significantly when expression of YtkR2 was increased, which suggests that the self-resistance mechanism in the wild-type bacteria is not sufficient and restricts the production of YTM. Finally, given the fact that studying novel resistance mechanisms in microorganisms could benefit the understanding of the evolutionary dynamics of resistance and sensitivity in nature,[22] the work described herein provides a glimpse into the BER system for microbial secondary metabolism, which suggests that nature may have evolved several different mechanisms for self-defense.

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