The Gene *cal*C Encodes for a Non-Heme Iron Metalloprotein Responsible for Calicheamicin Self-Resistance in *Micromonospora*

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Calicheamicin γ_1^I (Figure 1a, **1**)[1] from *Micromonospora echinospora* spp. *calichensis* is over 1000 times more potent than adriamycin,[2] clinically one of the most useful antitumor agents available. Of the two distinct structural regions within **1**,[3] the aryltetrasaccharide is comprised of a unique set of carbohydrate and aromatic units which serves to site-specifically deliver the metabolite into the minor groove of DNA,[4] while the aglycon, or "warhead", consists of a highly functionalized bicyclo[7.3.1]-tridecadiynene core structure with an allylic trisulfide serving as the triggering mechanism.[5] Once the aryltetrasaccharide is firmly docked, aromatization of the bicyclo[7.3.1]tridecadiynene core structure, via a 1,4-dehydrobenzene diradical (**5**), results in the site-specific oxidative double-strand scission of the targeted DNA.[3a,4a] This extraordinary reactivity has sparked considerable interest in the pharmaceutical industry, leading to the recent success of **1**−antibody conjugates (CMA-676) to treat acute myelogenous leukemia (AML).[6] Yet, nothing is known about how *Micromonospora* constructs **1** or controls the toxic effects of this extremely reactive metabolite. In an ongoing effort to unravel these mysteries, we report the discovery and characterization of a gene (*cal*C) from *Micromonospora echinospora* spp. *calichensis* which specifically confers resistance to **1** in vivo and demonstrate that its encoded protein (CalC) is a non-heme iron metalloprotein which inhibits **1**-induced DNA cleavage in vitro. This work represents the first cloning and characterization of a resistance gene for any non-chromoprotein enediyne.

To isolate the gene(s) responsible for **1** resistance in *Micromonospora*, clones conferring **1** resistance were selected by growth of a *Micromonospora* genomic bifunctional cosmid library[7] on Luria Bertani plates containing ampicillin (50 μg mL^{-1}) and **1** (0.25 μg mL^{-1}). In this selection, six clones (3a, 4a, 4b, 10a, 13a and 16a) displayed resistance to **1**, and restriction mapping of these clones localized the desired phenotype to a ∼2-kb *Pst*I−*Sac*I fragment of DNA (Figure 1b, pJT1214).[8] Nucleotide sequence analysis of the *Pst*I−*Sac*I fragment suggested it contained four possible open reading frames (Figure 1b), only one of which (*cal*C) encoded the desired phenotype.[9] The subsequent IPTG-inducible overexpression of *cal*C using the pMAL-C2 vector (pRE7, generating a maltose-binding protein (mbp)−CalC fusion protein) in *Escherichia coli* increased **1**-resistance 10^2-fold in vivo (50 μg mL^{-1} **1** when induced with 0.5 mM IPTG).[10] The overexpressed mbp−CalC was purified from pRE7/*E. coli* to homogeneity as judged by SDS−PAGE, the typical yield of which was 5.1 mg of mbp−CalC per 1.0 g of wet cells.[11]

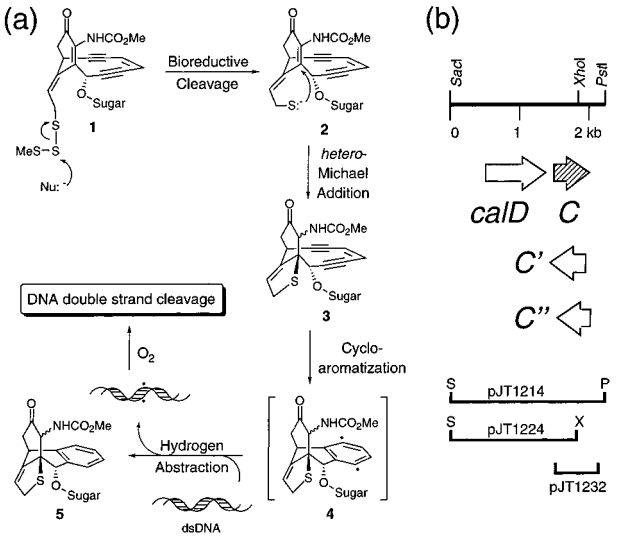

Figure 1. (a) Mechanism by which calicheamicin cleaves double-stranded DNA. **(b)** Map of the *cal*C locus and schematic representation of subclones.

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(8) For the plasmids described in the text, the maximum tolerated concentrations of **1** (in micrograms per milliliter) on LB agar plates containing 50 μg mL^{-1} ampicillin in *E. coli* are as follow: cosmids 3a, 4a, 4b, 10a, 13a, and 16a, 0.5; pJT1214 and pJT1232, 5.0; pRE7, 20.0; induced pRE7, 50.0; and pJT1224, pAP6, pRE1, and control plasmids pUC18, pBluescript, and pMAL-C2, <0.01. Given that the cosmid clones which carry *cal*C are known to carry both polyketide synthase and deoxy sugar genes, this work also verifies that we have cloned the **1** biosynthetic gene cluster (Whitwam, R. E.; Ahlert, J.; Shepard, E.; Thorson, J. S., unpublished results).

(9) The translation of *cal*D (CalD) revealed the presence of three amino acid motifs typically conserved in *S*-adenosylmethionine-utilizing *O*-methyltransferases. BLAST analysis of the translation of *cal*C (CalC) and *calC″* (CalC″) revealed no homology with known proteins, while the translation CalC′ displayed a weak alignment with apoproteins of the chromoprotein enediynes. A convenient *Xho*I site provided a subclone (pJT1224),[8] containing truncated *cal*C, *calC′*, and *calC″* but intact *cal*D, which could not confer resistance to **1**. PCR amplification of the putative *cal*C region gave plasmid pJT1232 which could, once again, confer resistance to **1**.[8]

(10) CalC′ (pAP6) or CalC″ (pRE1) overexpression in *E. coli* could not confer resistance to **1**.[9]

(11) An overnight LB culture (containing 50 μg mL^{-1} ampicillin and 50 ng mL^{-1} **1**) from a fresh pRE7/*E. coli* colony was grown at 37 °C, 250 rpm, to A_{600} = 0.5, induced with 0.5 mM IPTG, and growth continued overnight. Cells were harvested (4000*g*, 4 °C, 20 min), resuspended in buffer A (50 mM Tris-Cl, pH 7.5, 200 mM NaCl, 1 mM EDTA), and disrupted by sonication. The cell debris was removed by centrifugation (5000*g*, 4 °C, 20 min) and the supernatant chromatographed on an amylose affinity column (1.5 × 7.0 cm, 1 mL min^{-1}). The desired mbp−CalC was eluted with buffer A containing 10 mM maltose, concentrated, and resolved on S-300 (50 mM Tris·Cl, pH 7.5, 200 mM NaCl).

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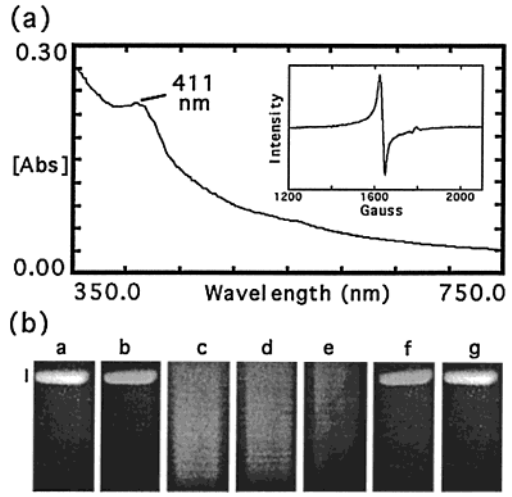

Figure 2. (a) UV−visible absorption spectra of purified mbp−CalC (52 μM mpb−CalC; 10 mM Tris·HCl, pH 7.5) and low-temperature (4.3 K) X-band EPR of CalC (inset; 250 μM mpb−CalC containing 0.5 mol of Fe per mol of CalC, 10 mM Tris·HCl, pH 7.5). (Spectrometer settings: field set, 2050 G; scan range, 4000 G; time constant, 82 s; modulation amplitude, 16 G; microwave power, 31 μW; frequency, 9.71 GHz; gain, 1000; determined spin quantitation, 90 ± 10 μM Fe.) **(b)** Results of the mbp−CalC in vitro assay (I, supercoiled DNA): (a) pBS DNA (1 μg) and **1** (30.0 nM); (b) pBS (1 μg) and DTT (833 μM); (c) pBS (1 μg), DTT (833 μM), and **1** (30.0 nM); (d) pBS (1 μg), DTT (833 μM), **1** (30.0 nM), and mbp (15 nM); (e) pBS (1 μg), DTT (833 μM), **1** (30.0 nM), and *apo*-mbp−CalC (15 nM); (f) pBS (1 μg), DTT (833 μM), **1** (30.0 nM), and mbp−CalC (15 nM); and (g) pBS (1 μg), DTT (833 μM), **1** (30.0 nM), *apo*-mbp−CalC (15 nM), and 1 mM Fe^{3+} (reconstitution with Fe^{2+} not shown).

Purified mbp−CalC displayed a yellow color in concentrated form, and subsequent metal analysis, using inductively coupled plasma atomic mass spectrometry (ICP-MS), revealed the presence of Fe. Determination of the Fe stoichiometry, accomplished in conjunction with quantitative amino acid hydrolysis,[12] indicated 2.23 ± 0.3 mol of Fe per mol of mbp−CalC.[13] The electronic absorption spectrum of mbp−CalC is shown in Figure 2a. In addition to the A_{280} protein absorbance ($\epsilon_{280} = 99\,300\ M^{-1}\ cm^{-1}$), a clear absorbance maximum at 411 nm ($\epsilon_{411} = 6000\ M^{-1}\ cm^{-1}$) can be observed. Furthermore, X-band EPR measurements on the oxidized protein exposed a standard rhombic EPR signal at $g = 4.3$ ($E/D = 0.33$) (Figure 2a, inset). The metal content was 90 ± 10 μM Fe (approximately 72 ± 10% of total iron as seen by ICP-MS, Figure 2b). Consistent with the lack of cysteines in the primary sequence of CalC, the spectroscopic evidence indicates the presence of a mononuclear Fe^{3+} center in CalC.[14]

Given that **1** leads to dsDNA cleavage and CalC provides **1**-resistance in vivo, it was expected that the addition of CalC to an in vitro **1**-induced DNA cleavage assay would inhibit DNA cleavage. To test this postulation, preliminary assays utilized supercoiled pBluescript plasmid DNA (pBS) as the template, DTT as the reductive initiator, and the DNA cleavage products were resolved by gel electrophoresis.[15] As illustrated in Figure 2b, no DNA cleavage was observed in the absence of DTT or **1** (lanes a and b), while efficient cleavage was demonstrated in the presence of DTT and **1** (lane c). As expected, the addition of mbp−CalC completely inhibited **1**-induced DNA cleavage (lane f), while the addition of mbp alone (lane d) as a control failed to inhibit **1**-induced DNA cleavage. Furthermore, preincubation of mbp−CalC with DTT,[15] or *apo*-mbp−CalC (lacking the Fe cofactor), also failed to inhibit **1**-induced DNA cleavage (lane e). However, the addition of Fe^{2+} or Fe^{3+} to the *apo*-mbp−CalC assay could reconstitute CalC activity (lane g).[16]

A number of mechanisms are known for the detoxification of reactive secondary metabolites.[17] While the precise CalC **1**-deactivation mechanism is unclear, one compelling hypothesis consistent with both the presented spectroscopic evidence and established oxidative **1**−DNA cleavage mechanism is that CalC catalyzes radical disproportionation in a manner reminiscent of bacterial iron superoxide dismutases (Fe-SODs).[18] A second mechanism consistent with the reported evidence is that CalC catalyzes oxidative modification of **1** in a manner similar to mononuclear iron-containing oxygenases,[19] thereby interfering with (i) DNA-binding, (ii) the reductive triggering event (Figure 1, **1**−**3**), and/or (iii) cycloaromatization (**4**). Finally, CalC may simply be involved in the noncovalent sequestration of **1**, believed to be the method of self-resistance for chromoprotein enediyne (e.g., neocarzinostatin) producers.[20] In contrast to CalC, however, the apoproteins in these systems do not depend on a metal cofactor and are required in roughly a 20-fold excess to achieve inhibition of DNA cleavage in vitro.[21] The present work clearly demonstrates that CalC both is a non-heme iron metalloprotein and can inhibit DNA cleavage at substoichiometric concentrations in vitro.[15] Thus, paralleling the postulated biosynthetic distinctions between the chromoprotein and nonchromoprotein enediynes,[1a,b] nature may have also evolved distinct mechanisms of self-resistance.

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(12) The precise mbp−CalC concentration was determined by quantitative amino acid hydrolysis by the Rockefeller University Protein/DNA Technology Center. Trace metal content of an aliquot of the hydrolysate was subsequently determined by ICP-MS on four distinct mbp−CalC preparations with buffer alone and/or maltose-binding protein alone, analyzed in parallel as controls. Results were independently confirmed by methodology for spectrophotometric Fe determination (Fish, W. W. *Methods Enzymol*. **1988**, *158*, 357−364).

(13) Based upon the monomeric molecular weight of 63 576 calculated from the known nucleotide sequence of the mbp−*cal*C gene fusion, which is consistent with the subunit molecular weight determined by SDS−PAGE.

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(15) In a typical assay, purified mbp−CalC (15.0 nM) and 30.0 nM **1** were preincubated for 15 min in a total volume of 25 μL of 40 mM Tris·Cl, pH 7.5, at 37 °C. To the assay solution were added 2.5 μL of supercoiled pBluescript II SK− double-stranded DNA stock (400 ng μL^{-1}) and 2.5 μL of 10 mM dithiothreitol stock solution, and the assay was incubated for an additional 1 h at 37 °C. DNA fragmentation was assessed by electrophoresis on a 1% agarose gel stained with ethidium bromide. Using this assay, mbp−CalC could completely inhibit **1**-induced DNA cleavage at concentrations nearing a 10^3-fold excess of **1**. Preincubation of mbp−CalC and DTT, protein removal via forced dialysis (Microcon-10), and the subsequent use of the DTT solution as reductant did not noticeably affect the amount of DNA cleavage.

(16) Extended dialysis of CalC against buffer containing 1 mM EDTA could not remove Fe; however, storage of CalC for extended periods at 4 °C led to the eventual loss of Fe as determined by UV−vis and metal analysis.[13] Reconstitution of *apo*-mbp−CalC was accomplished by preincubation with 1 mM $FeSO_4$ (Fe^{2+}) or $FeCl_3$ (Fe^{3+}) prior to the activity assay; see ref 15.

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