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Bacterial Inactivation of the Anticancer Drug Doxorubicin

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SUMMARY

Microbes are exposed to compounds produced by members of their ecological niche, including molecules with antibiotic or antineoplastic activities. As a result, even bacteria that do not produce such compounds can harbor the genetic machinery to inactivate or degrade these molecules. Here, we investigated environmental actinomycetes for their ability to inactivate doxorubicin, an aminoglycosylated anthracycline anticancer drug. One strain, *Streptomyces* WAC04685, inactivates doxorubicin via a deglycosylation mechanism. Activity-based purification of the enzymes responsible for drug inactivation identified the NADH dehydrogenase component of respiratory electron transport complex I, which was confirmed by gene inactivation studies. A mechanism where reduction of the quinone ring of the anthracycline by NADH dehydrogenase leads to deglycosylation is proposed. This work adds anticancer drug inactivation to the enzymatic inactivation portfolio of actinomycetes and offers possibilities for novel applications in drug detoxification.

INTRODUCTION

Soil microbes, and particularly the actinobacteria, are prolific producers of natural products. Many of these compounds demonstrate antibacterial or anticancer activity and so have been applied to human medicine through clinical use as antibiotics or antineoplastic drugs. In the environment, many soil organisms harbor enzymes that modify or degrade these products to avoid their toxic effects. Therefore, environmental bacteria may represent a previously uninvestigated source of unique strategies to detoxify antineoplastics.

On this basis, we sought to probe a library of environmental isolates for the ability to catalyze the detoxification of antineoplastic drugs. Understanding how environmental bacteria might detoxify anticancer therapeutics could have many benefits. The degradation of these toxic products could be applicable to safely discarding waste from the pharmaceutical industry, including remediating spills; might be life-saving if used to remove excess drug administered as an accidental overdose; or, most tantalizing, careful targeting of drug inactivation to nontumor tissues could potentially remove the side effects for patients undergoing chemotherapy without compromising the efficacy of the treatment. The downstream potential of a novel environmental detoxification mechanism would depend on its precise nature; the first step must be the identification of new catalysts capable of drug detoxification.

For this proof-of-principle study, the anthracycline antineoplastic doxorubicin was selected because it is a natural product of an actinobacteria, *Streptomyces peucetius* var. *casieus.* Doxorubicin also has antibiotic activity, providing a simple resistance assay for screening of environmental bacteria. Doxorubicin is the most commonly used anthracycline drug and is chosen for treatment of a range of cancers, including carcinomas, soft tissue sarcomas, and hematological malignancies. Anthracyclines are thought to inhibit the growth of cancer cells through multiple mechanisms, including intercalation in DNA, which leads to inhibition of both DNA replication and RNA transcription; generation of free radicals, resulting in DNA damage and lipid peroxidation; DNA binding and alkylation; DNA cross-linking; interference with DNA unwinding, DNA strand separation, and helicase activity; direct membrane damage due to lipid oxidation; and inhibition of topoisomerase II (reviewed in Gewirtz, 1999). The use of any anthracycline drug, however, is limited, due to cumulative toxicity. For doxorubicin, the maximum cumulative dose is approximately 550–700 mg/m^2 for otherwise healthy adults (Novopharm, 1996). Although several potential side effects are known, including mucosal toxicity, alopecia, and secondary malignancy, cardiotoxicity is the most troubling. This toxicity is thought to be due to free radical damage and disruption of iron homeostasis initiated by the drug and enhanced by the cardiac tissues' diminished production of reactive oxygen species (ROS)-degrading enzymes, such as superoxide dismutase, glutathione peroxidase, and catalase (Licata, et al., 2000; Minotti, et al., 2000; Singal, et al., 2000).

In bacteria, anthracyclines like doxorubicin inhibit growth through similar mechanisms to those that cause cytotoxicity in humans, particularly the induction of DNA/RNA damage. Some resistance has been documented in *E. coli* K-12, but strains deficient in antibiotic efflux due to mutation or deletion of the *acr* locus were found to be drug sensitive (Kaur, 1997). For our purposes, we did not want to investigate efflux strategies, but rather wished to focus on identifying new catalysts capable of drug detoxification. Therefore, we screened a collection of

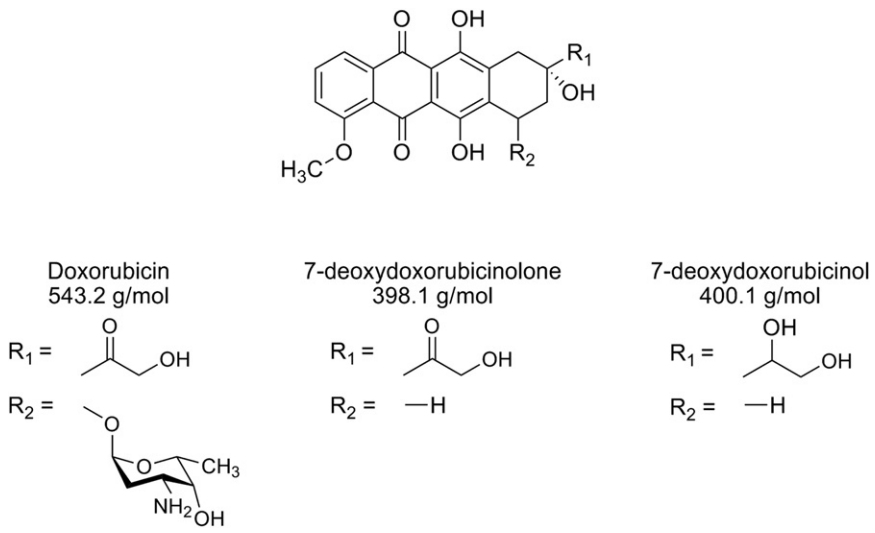

Figure 1. Structures of Doxorubicin and Aglycone Products Detected in This Study
Structure of 7-deoxydoxorubicinol is supported by MS/MS data (Figure S1), and the structure of 7-deoxydoxorubicinolone is supported by ^{13}C NMR data (Figure S2). See also Figures S1 and S2.

environmental actinobacteria for resistance to doxorubicin and then screened a subset for degradation activity. After purification and identification of the doxorubicin degradation proteins, we report that the inactivation of anticancer anthracyclines can occur through reductive deglycosylation mediated by the NADH dehydrogenase component of the respiratory electron transport complex I. This understanding of anticancer drug inactivation in bacteria gives proof-of-principle for the screening of environmental isolates to identify novel degradation strategies for antineoplastics.

RESULTS

Screen of Environmental Actinomycetes for Anthracycline Degradation

Drug tolerance to doxorubicin among bacterial strains was estimated by calculating the minimum inhibitory concentrations (MIC) for several sample strains from our set of actinomycetes collected from various locales (D'Costa, et al., 2006). For planktonic cells growing in liquid broth, MICs ranged from less than 2 μg/ml to over 200 μg/ml; the median of 12 strains was 3.2 μg/ml. Strains were more resistant to doxorubicin when grown on solid media, so a concentration of 150 μg/ml was used in solid agar to screen a library of 626 soil actinomycetes for resistance to doxorubicin. The screen showed that 67% of strains were resistant to 150 μg/ml doxorubicin; selected strains were subsequently assessed for their MIC using liquid media. One of these strains, WAC04685, had a broth MIC > 200 μg/ml and was determined to be related to *Streptomyces zaomycecius* and *Streptomyces phaeochromogenes* by 16S rDNA analysis (99% sequence identity). This strain was selected for further study because of its robust growth and ability to degrade anthracyclines (detailed below).

The methanolic extracts of cultures of *Streptomyces* WAC04685 grown in the presence or absence of doxorubicin were analyzed by reverse phase high performance liquid chromatography (HPLC) with mass spectrometry detection (LC/MS). A new peak that retained the anthracycline UV/visible spectroscopic properties, namely an absorbance maximum at 480 nm, was observed in the extract from cultures grown with the drug (data not shown). The 401.7 *m/z* ion, detected in positive ion mode, is consistent with the mass of 7-deoxydoxorubicinol (400.1 g/ml), which is a deglycosylated alcohol derivative of doxorubicin (Figure 1). The predicted structure was confirmed by tandem MS/MS analysis on both doxorubicin and the reaction product purified from HPLC, showing that both molecules share the same decomposition fragments (305, 321, and 346 *m/z*) that are consistent with anthracyclines (Figure S1 available online). This metabolism of doxorubicin was observed regardless of when the drug was introduced, implying that the degradation machinery is constitutively expressed.

Purification and Characterization of an Anthracycline Degradation Enzyme and Its Products

Purification of the putative anthracycline degradation enzyme from natural abundance in *Streptomyces* WAC04685 was undertaken using ammonium sulfate precipitation in addition to a series of hydrophobic, dye affinity, gel filtration, cation exchange, and hydroxyapatite chromatographic steps. To quickly detect the presence of doxorubicin degradation activity in the resulting fractions after each separation technique, crude samples were reacted with doxorubicin then analyzed by thin layer chromatography (TLC). Empirical testing showed that degradation of doxorubicin was improved by adding nicotinamide adenine dinucleotide phosphate (NADPH) to the crude extracts along with doxorubicin. Under the conditions used, two different product spots were well separated and the intrinsic red color from the anthracycline core was sufficient for detection. The sensitivity of the TLC was comparable to that of LC/MS for the detection of doxorubicin degradation products (data not shown). Analysis of active fractions by LC/MS in positive ion mode also demonstrated two product peaks at 399 and 402 *m/z*, which had a difference of two mass units. The masses of the two products are consistent with 7-deoxydoxorubicinolone (398.1 g/mol) and 7-deoxydoxorubicinol (400.1 g/mol) (Figure 1). Despite further efforts at purification, while using NADPH as a cofactor, it was not possible to separate activity producing only 7-deoxydoxorubicinolone or 7-deoxydoxorubicinol; all reactions produced some of both products.

Purified active protein fractions were visualized by SDS-PAGE and resulted in the identification of three protein bands with the apparent sizes of 24, 38, and 50 kDa (Figure 2A). Native PAGE of the same purified fractions also indicated the presence of three bands with different apparent abundance (Figure 2B).

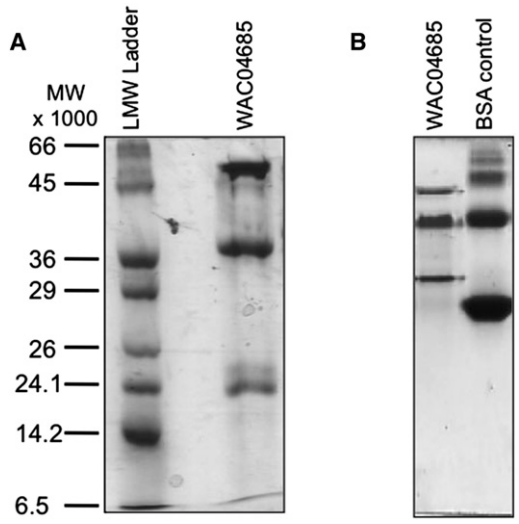

Figure 2. Polyacryamide Gel Electrophoresis of Purified Doxorubicin Degradation Enzymes

(A) Denaturing SDS-PAGE.

(B) Native PAGE. These bands were excised and identified as members of the NADH dehydrogenase complex (NuoE, NuoF, and NuoG) by mass spectrometry of proteolytic fragments. See also Table S1.

These three proteins could not be further separated while maintaining activity, suggestive of an enzyme complex.

Tryptic digestion followed by peptide sequencing by mass spectrometry from both the native and SDS-PAGE gels identified similar peptides (Table S1). The best score for one of the proteins on both gel types was for NuoF; this protein was identified as the top hit for all three of the protein bands present on the native PAGE gel. NuoF is a peripheral protein that is part of the NADH: ubiquinone oxidoreductase complex and is thought to be involved in NADH binding (Friedrich and Scheide, 2000). Other related proteins, including NuoG and NuoE, were also detected, although with lower scores, from the native PAGE and SDS-PAGE bands.

Purified NADH dehydrogenase, consisting of NuoEFG from *Streptomyces* WAC04685, was reacted with NADPH and doxorubicin; using one-dimensional (1D) and two-dimensional (2D) nuclear magnetic resonance (NMR) spectroscopy, the enzyme-modified product was found to be consistent with 7-deoxydoxorubicinolone (Figure S2). The other product that had been detected in crude protein preparations, 7-deoxydoxorubicinol, was not detected in NMR experiments. Purified NADH dehydrogenase was also used for kinetic analyses in reactions that initially contained NADPH, but during optimization of the kinetic experiments, NADH was found to be a much better cofactor. When NuoEFG complex was reacted with excess NADH, it was found to have a K_m of 95 ± 16 μM and V_{max} of 2.06 μmol/s for doxorubicin, demonstrating a k_{cat} of 720/s.

The product purified from *Streptomyces* WAC04685 NADH dehydrogenase reactions, 7-deoxydoxorubicinolone, lacks antimicrobial activity as assessed by liquid MIC tests against several *Streptomyces* strains, even those that are doxorubicin-sensitive (Table 1).

Genome Sequencing of *Streptomyces* WAC04685

Initially, peptide-mass identification of NuoE, NuoF, and NuoG from *Streptomyces* WAC04685 was based on comparison to proteins from *Streptomyces coelicolor.* It was unclear, based on this data, if all three proteins were involved or required for the degradation of doxorubicin in *Streptomyces* WAC04685. Thus, having established that WAC04685 catalyzes the degradation of doxorubicin into 7-deoxydoxorubicinolone, a thorough analysis of the strain itself was desired. Seeking information about taxonomy, possible production of anthracyclines, as well as the doxorubicin resistance mechanisms, a draft genome sequence of WAC04685 was determined on the Illumina platform. After assembly, the genome was revealed to be about 7.8 Mbp and was in 1,916 contigs, encoding 7,716 open reading frames. No plasmids were detected. The N50 was 8,566, and the percentage guanine + cytosine base pairs in nucleotide sequence (GC%) was 71.37%.

From the genome sequence, 16S rDNA analysis indicated that WAC04685 is closely related (99% sequence identity) to both *Streptomyces zaomycecius* and *Streptomyces phaeochromogenes*. Little has been reported about these two strains, but neither has been documented to produce or be resistant to anthracyclines.

WAC04685 copiously produces an orange-red pigment when cultured, which is reminiscent of the color of anthracyclines. However, WAC04685 is unlikely to be an anthracycline producer, because a search for homologs of *Streptomyces peucetius* DpsF (polyketide cyclase) and DnrD (catalyzes closure of the final anthracycline ring) (Grimm, et al., 1994; Madduri and Hutchinson, 1995a, 1995b) identified no genes above the cutoff of e^{-10}.

The toxicity of anthracyclines is in part due to the generation of reactive oxygen species (ROS), so the WAC04685 genome was searched for homologs of superoxide dismutase, catalase, and glutathione peroxidase. Two distinct genes were found with similarity to superoxide dismutases, one with similarity to glutathione peroxidase, and at least two genes with similarity to catalase. Comparatively, analysis suggests that *S. coelicolor* M145 encodes three distinct genes with similarity to superoxide dismutases, one with similarity to glutathione peroxidase, and four genes with similarity to catalase. Surveying other sequenced *Streptomyces* indicates that, in all genomes so far, there is at least one representative of each of the ROS-detoxifying genes queried. This analysis gives no estimate of total expression levels for these putative protein products, but at least documents that *Streptomyces* are generally well-equipped to deal with reactive oxygen stress.

Finally, the assembled *Streptomyces* WAC04685 draft genome was searched for homologs of the *nuo* genes; three contigs were identified that contained segments of the expected 14 gene locus. Based on similarity, these contigs contained *nuoA-nuoF*, fragments of *nuoG*, and *nuoJ-N*. Intracontig gaps were bridged by PCR and sequenced to assemble the *nuoABCDEFGHIJKLMN* locus that constitutes the electron transport complex I. No additional homologs of *nuo* genes were identified with >40% similarity, leading to the prediction that there is a single copy of each of the *nuo* genes located in a single continuous locus. Comparison of 14

Table 1. MIC for Selected *Streptomyces* Strains Tested against Anthracycline Drugs Doxorubicin, Daunorubicin, and the Doxorubicin Aglycone Product, 7-Deoxydoxorubicinolone

	Compound		
Strain	Doxorubicin	7-Deoxydoxorubicinolone	Daunorubicin
Streptomyces sp. Mg1[a]	1.56	>200	ND
S. sp. Wasp[a]	25	>200	0.78–1.56
S. sp. SPB74[a]	0.78	>200	n.d.
S. sp. SPB78[a]	1.56	>200	n.d.
S. sviceus ATCC 29083[a]	0.78	>200	n.d.
Saccharopolyspora erythraea 2338[a]	6.25	>200	1.56
WAC04685[b]	>200	>200	12.5
WAC04741[b]	12.5	>200	n.d.
Streptomyces coelicolor M145	3.125	ND	0.78
Streptomyces peucetius var. *casius* ATCC 29050	3.125	ND	0.78

ND, not determined.
[a]Broad strain collection.
[b]Wright actinomycete collection.

sequenced *Streptomyces* strains shows that almost all have either one or two BLAST-detectable copies of each of the *nuo* genes. Most (>75%) strains have only one copy of *nuoE* and *nuoG,* while about half of the strains investigated have two copies of *nuoF.* This, together with the high predicted sequence coverage and small average gap size of the genome assembly, supports our hypothesis that there is a single Nuo locus.

Investigations of the *Streptomyces* WAC04685 *nuo* Locus

Among the NuoE, NuoF, and NuoG proteins that make up the NADH dehydrogenase, only NuoF is predicted to have NADH binding function (Friedrich and Scheide, 2000). Our prior analysis had showed that there was a single copy of *nuoF* in WAC04685, so it was targeted for insertional inactivation in order to determine if doxorubicin could still be inactivated in the absence of intact NuoF. Disruption of *nuoF* resulted in a strain that was no longer capable of doxorubicin inactivation (Figure 3), confirming the role of NADH dehydrogenase in doxorubicin degradation. In these reactions, NADH was used as the cofactor with crude lysate and confirmed the production of a sole product, 7-deoxydoxorubicinolone, by the wild-type WAC04685 under these conditions (Figure 3). The *nuoF*-inactivated strain nevertheless retained its drug resistance phenotype (MIC > 200 μg/ml doxorubicin), suggesting additional modes of drug resistance present in *Streptomyces* WAC04685.

Doxorubicin Degradation Ability among Other Bacteria

Based on the findings that NADH dehydrogenase is responsible for doxorubicin degradation in *Streptomyces* WAC04685 and knowledge that the *nuoEFG* genes are widespread, additional *Streptomyces* strains were tested for doxorubicin degradation ability. WAC04741, *Streptomyces coelicolor* M145, and *Streptomyces peucetius* var. *casius* ATCC 29050 all demonstrated the ability to degrade doxorubicin in TLC and LC/MS analysis (data not shown).

DISCUSSION

Doxorubicin Resistance in Environmental Bacteria

The initial screen of our environmental actinomycete library showed that the majority of strains (67%) tested were resistant to doxorubicin at 150 μg/ml; this is the first report, to our knowledge, of anticancer drug resistance among environmental bacteria. The prevalence of resistance was surprising and the mechanisms of this resistance have not been elucidated. For our purposes, we hoped to discover mechanisms of drug degradation among the isolates, so we elected to focus on the novel doxorubicin inactivation identified in *Streptomyces* WAC04685. Here, the enzymatic conversion of doxorubicin to 7-deoxydoxorubicinolone has been documented and 7-deoxydoxorubicionolone has been shown to be many times less toxic than doxorubicin itself; thus, a bacterial doxorubicin degradation and detoxification system was successfully identified. Additional strains and other species of bacteria were shown to catalyze the same process, indicating that the degradation activity is not unique to *Streptomyces* WAC04685. This provides proof-of-principle for the concept of bacterial resistance to anticancer drugs, and future studies could target additional antineoplastic drugs outside of the anthracycline group.

Isolation of the NADH Dehydrogenase Complex Consisting of NuoE, NuoF, and NuoG

Activity-based purification of the doxorubicin degradation protein led to the identification of NuoE, NuoF, and NuoG. Together, NuoE, NuoF, and NuoG make up the NADH dehydrogenase module of the 14-subunit respiratory electron transport complex I. The NADH dehydrogenase module protrudes from the rest of the membrane-anchored complex into the cytosol and is unique to bacterial NADH:ubiquinone oxidoreductases (reviewed by Friedrich and Scheide, 2000). During purification, the NADH dehydrogenase module was removed from the other complex I proteins.

The role of NuoF in NADH binding (Friedrich and Scheide, 2000) is likely why the complex showed affinity for Blue

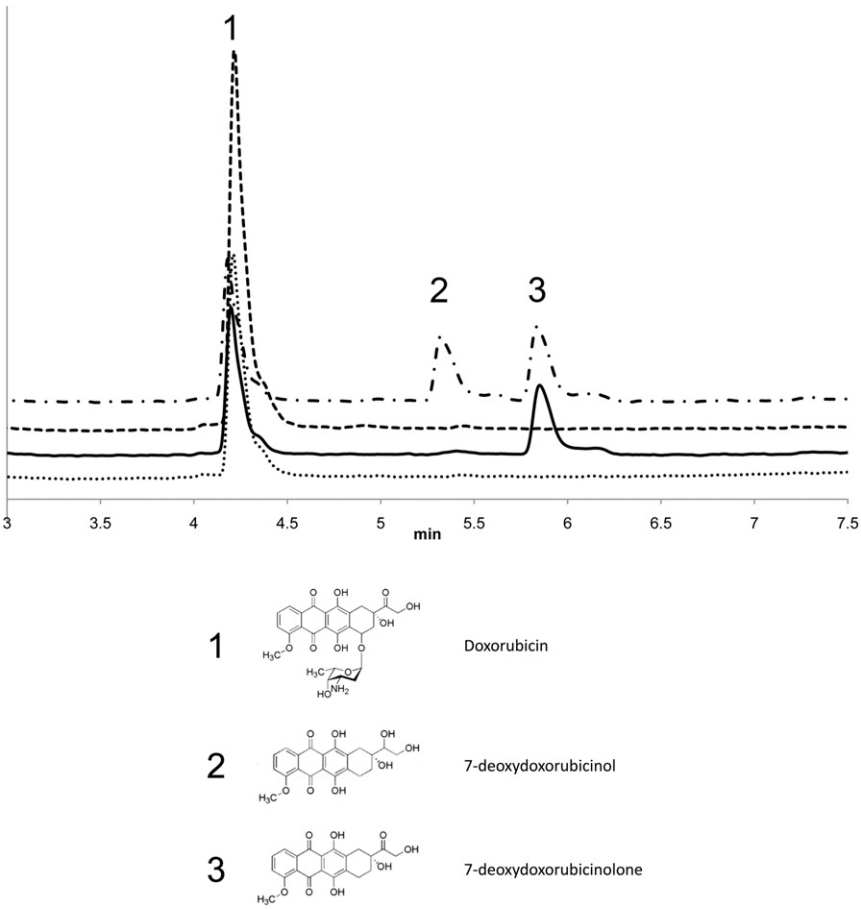

Figure 3. HPLC Spectra of Doxorubicin Degradation by *Streptomyces* WAC04685 Wild-type and *nuoF*::pSET152 Strains

Intact doxorubicin (dashed line) was applied as a control. Degradation reactions were performed using crude extracts of *Streptomyces* WAC04685 wild-type with either NADH (solid line) or NADPH as a cofactor (dashed and dotted line) or *nuoF*::pSET152 with NADH (dotted line), demonstrating the loss of degradation ability in the knockout. See also Tables S2 and S3.

Sepharose and why NuoF was the best hit for all three protein bands on the two PAGE gel types (Figure 2). Blue Sepharose was used in one step of the purification, and the Cibacron blue dye is an analog of nicotinamide adenine dinucleotide (NAD)/nicotinamide adenine dinucleotide phosphate (NADP) that frequently is used to purify NAD/NADP-requiring enzymes. Likely, NuoF was immobilized on the Blue Sepharose, and the other NADH dehydrogenase components, NuoE and NuoG, were purified by virtue of their interaction with NuoF. On the SDS-PAGE gel, NuoE, NuoF, and NuoG migrate faster than their expected sizes of 25.6, 48.8, and 78.8 kDa, respectively. On the native PAGE gel, the three bands likely reflect different proportions of NuoF interacting with NuoE and/or NuoG.

Activity of Streptomyces WAC04685 Extracts against Doxorubicin with NADH and NADPH

During the experiments, both NADPH and NADH were used as cofactors for the enzymatic inactivation of doxorubicin by the NuoEFG complex. Initially, NADPH was used because its empirical addition to crude extracts of WAC04685 improved the production of two aglycone degradation products: 7-deoxydoxorubicinol and 7-deoxydoxorubicinolone. Use of NADPH was continued until the identification of the degradation enzyme as NADH dehydrogenase (NuoEFG complex). Since the well-studied NADH dehydrogenase of the respiratory chain from *E. coli* is strictly restricted to using NADH, not NADPH (Dancey, et al., 1976), the activity of NADH dehydrogenase from WAC04685 was re-evaluated with NADH as a cofactor. Not only did purified NADH dehydrogenase show improved conversion of doxorubicin to 7-deoxydoxorubicinolone when NADH was used, but crude lysates reacted with NADH showed only the production of 7-deoxydoxorubicinolone, whereas crude lysates in the presence of NADPH produced 7-deoxydoxorubicinolone and 7-deoxydoxorubicinol.

The two aglycone products, 7-deoxydoxorubicinol and 7-deoxydoxorubicinolone, differ only in the state of the side chain carbonyl group at C-13 (Figure 1). Analogous to 7-deoxydoxorubicinol, C-13 alcohol forms of glycosylated anthracycline drugs are known. The drug daunorubicin is an anthracycline closely related to doxorubicin, differing in that the hydroxyl at C-14 is absent in daunorubicin. The reduced C-13 alcohol forms of the drugs are called daunorubicinol or doxorubicinol, respectively. In humans, the liver cytosol contains carbonyl reductase, aldose reductase, and dihydrodiol dehydrogenase 2, which require NADPH and convert daunorubicin to daunorubicinol (Ohara, et al., 1995). Daunorubicinol is less toxic than the ketone form of the drug, and carbonyl reductase in particular was strongly induced in the presence of daunorubicin, leading to a drug-resistant phenotype (Ax, et al., 2000). BLAST analysis of our *Streptomyces* WAC04685 genome sequence indicates the presence of genes encoding potential homologs of carbonyl reductase, aldose reductase, and dihydrodiol dehydrogenase 2 (identity > 30%, e values > e^{-11}). In reactions with crude lysate, these proteins could convert 7-deoxydoxorubicinolone to 7-deoxydoxorubicinol in the presence of NADPH.

Based on these findings, NADH dehydrogenase uses NADH as the cofactor to produce 7-deoxydoxorubicinolone from doxorubicin. When NADPH was used with purified NADH dehydrogenase, 7-deoxydoxorubicinolone was the sole product, which may suggest that some NADH was copurified with the enzyme complex. When NADPH was used with crude lysates, 7-deoxydoxorubicinol was produced in addition to 7-deoxydoxorubicinolone, likely because the NADPH was stimulating the action of carbonyl reductase or similar proteins

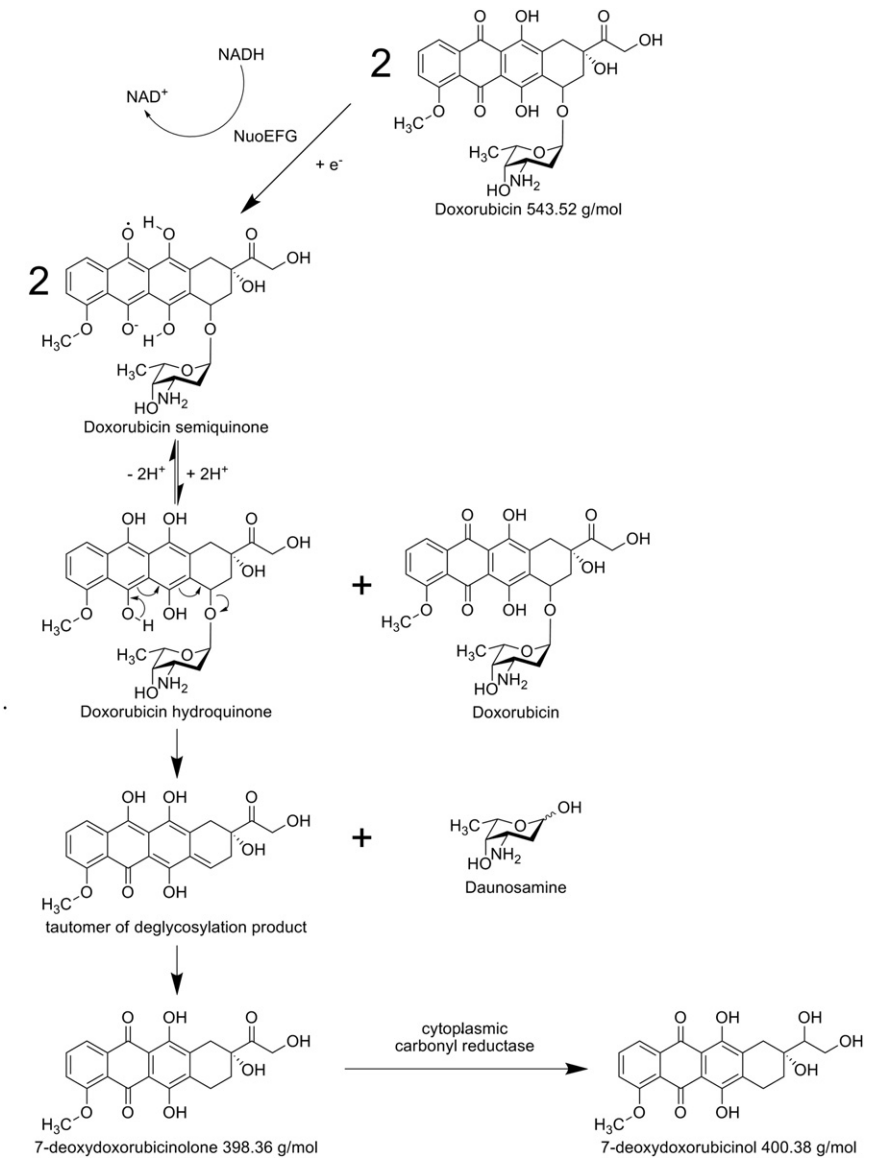

Figure 4. Scheme for the Mechanism of Inactivation of Doxorubicin by NADH Dehydrogenase

Two doxorubicin semiquinones disproportionate to form the doxorubicin hydroquinone, which is unstable and decomposes to 7-deoxydoxorubicinolone and the sugar daunosamine (Lea et al., 1990; Gille and Nohl, 1997). 7-deoxydoxorubicinolone can be converted to 7-deoxydoxorubicinol by the action of cytoplasmic NADPH-dependent carbonyl reductases; therefore, 7-deoxydoxorubicinol was only observed in reactions with NADPH and crude cell extracts.

present in the lysate. These reductases are less able to function with NADH as a cofactor, and so reactions with NADH and crude lysate do not show the presence of 7-deoxydoxorubicinol. This establishes that NADH dehydrogenase is involved in the deglycosylation of doxorubicin, generating the aglycone 7-deoxydoxorubicinolone. The observation of 7-deoxydoxorubicinol in some experiments was likely due to the use of NADPH cofactor in assay of crude lysates containing carbonyl reductase or similar proteins; 7-deoxydoxorubicinol is not a direct product of NADH dehydrogenase.

Role of NADH Dehydrogenase in Doxorubicin Degradation

Our findings indicate that NADH dehydrogenase is an enzyme complex essential for the degradation of doxorubicin in *Streptomyces* WAC04685. This enzyme initiates the mechanism that converts doxorubicin into nontoxic 7-deoxydoxorubicinolone. Purification of NADH dehydrogenase components NuoE, NuoF, and NuoG from *Streptomyces* WAC04685 demonstrate that the enzymes are sufficient to achieve the degradation of doxorubicin in vitro. Inactivation of *nuoF* in *Streptomyces* WAC04685 showed that, in the absence of the normal *nuo* locus, the ability to degrade doxorubicin is impaired. Therefore, we conclude that NADH dehydrogenase is responsible for degrading doxorubicin.

A scheme for the enzymatic production of doxorubicin aglycone catalyzed by the exogenous NADH dehydrogenase of mammalian cardiac tissue had previously been proposed based on one-electron reduction of the anthracycline quinone ring, leading to the loss of the daunosamine sugar (Gille and Nohl, 1997). The intermediates of this reaction had been the subject of prior pulse-radiolysis studies documenting the chemistry of the one- and two-electron reduction mechanisms (Lea, et al., 1990). The production of 7-deoxydoxorubicinolone from bacterial NADH dehydrogenase likely follows the same scheme (Figure 4). This process of reductive deglycosylation is significant for two main reasons: anthracycline aglycones are much more hydrophobic than the glycosylated counterparts, and therefore can enter membranes, and anthracycline semiquinones can give rise to $O_2^{\bullet -}$ radicals by autoxidation (Doroshow and Davies, 1986; Gille and Nohl, 1997). Anthracycline aglycone semiquinones can also be formed by NADH dehydrogenase-catalyzed reduction of the quinone ring (Gille and Nohl, 1997). The anthracycline aglycone semiquinones then divert an electron to H_2O_2, forming $HO^{\bullet}$ and OH^-; these reactive oxygen species (ROS) then cause toxicity to the host cell (Gille and Nohl, 1997). To our knowledge, this is the first report of NADH dehydrogenase action on anthracyclines operating in bacteria and suggests that bacteria could be used as model systems for additional studies of dehydrogenase-based anthracycline toxicity.

Comparison of Biological Activity of 7-Deoxydoxorubicinolone in Prokaryotes and Eukaryotes

Our characterization of the effect of anthracycline aglycones on intact cells in MIC experiments showed that these are less toxic than intact anthracyclines in bacteria, in contrast to aglycone-linked human cardiac toxicity. In humans, cardiac tissue is uniquely susceptible to anthracycline semiquinone toxicity, because of the production of an exogenous NADH dehydrogenase (Rasmussen and Rasmussen, 1985) and because of catalase activity that is 2% to 4% of that in liver or kidney (Thayer, 1977). Essentially, the exogenous NADH dehydrogenase converts doxorubicin to 7-deoxydoxorubicinolone, but then 7-deoxydoxorubicinolone can be used as a substrate for further one-electron reduction by NADH dehydrogenase with concomitant production of ROS. This reductive process does not degrade the aglycones, and so they can continue to redox cycle, produce ROS, and cause damage.

The higher levels of catalase, superoxide dismutase, and glutathione peroxidase in bacterial cells relative to human cardiac tissue may account for the different response to the same compound between bacterial and eukaryotic cardiac cells. Bacterial cells are much more competent at dealing with the outcomes of anthracycline semiquinone oxidation-reduction cycling and the resulting reactive oxygen species (ROS) evolved. The aglycone molecules may accumulate in membranes due to their hydrophobicity; the rapid growth and division of bacterial cells relative to eukaryotic cardiac tissue may also explain the differential effect of anthracycline aglycones in prokaryotes and eukaryotes.

Physiological Effects of Doxorubicin Degradation in Bacteria or Potential Therapeutics

Streptomyces WAC04685 has been demonstrated to degrade doxorubicin. However, this degradation may play a subordinate role in resistance compared to other factors, such as efflux. *Streptomyces* WAC04685 is highly resistant, with an MIC > 200 μg/ml; but inactivation of *nuoF* had no impact on resistance, despite being unable to convert doxorubicin to 7-deoxydoxorubicinol in vitro. This shows that the degradation of doxorubicin is not essential for the survival of these bacteria, and so the degradation does not strongly affect the resistance of the organism. Nonetheless, based on these findings, novel strategies to improve the quality-of-life of cancer patients could be envisaged.

The essential issue with chemotherapy is that the drugs do not selectively target tumor tissue, and therefore numerous side effects occur that significantly impact quality-of-life for chemotherapy patients. Our findings reveal the mechanism for the bacterial inactivation of doxorubicin. This could be exploited to detoxify the drug in nontumor tissues. Recently, new vectors to allow the overexpression of target proteins in lactobacilli have been developed in order to allow in situ production of biotherapeutics by intestinal lactobacilli (Duong et al., 2011). Thus, future efforts could attempt to engineer probiotic bacteria that overproduce NADH dehydrogenase in the gut of treated humans, with the goal of reducing active drug concentration in the gut. This in situ degradation of drug in nontarget tissues could be protective against the gastrointestinal side effects of chemotherapy with anthracyclines. In the future, we will continue to survey environmental bacteria for additional strategies for the degradation of these antitumor drugs. Decolorizing reactions, which would involve breaking down the anthracycline core, could be of additional interest. Environmental bacteria, like *Streptomyces* WAC04685, have also been revealed here to be of potential utility for fermenting anthracyclines into nontoxic products, perhaps if bulk disposal was desired.

For the first time, a systematic screen of environmental bacteria has been shown to identify strains able to resist and metabolize antineoplastic compounds. This expands our understanding of the antibiotic resistome (D'Costa, et al., 2006, 2007) to include other drugs, such as anticancer compounds, that also have antibiotic activity. Further screening of environmental bacteria for the resistance or ability to metabolize additional antitumor drugs could give even more new strategies to improve the quality-of-life of cancer patients undergoing chemotherapy.

SIGNIFICANCE

Environmental bacteria are exposed to a wide variety of potentially lethal compounds, as some organisms are producers of antibiotic and/or antineoplastic compounds. This study establishes that nonproducer soil isolates can harbor the biological machinery required to degrade and inactivate anticancer therapeutics. A library of environmental isolates was screened for resistance to doxorubicin, a widely used human chemotherapy drug. In particular, the isolate WAC04685 was selected and has been shown to degrade doxorubicin into 7-deoxydoxorubicinolone in a reaction catalyzed by NADH dehydrogenase, a three-protein complex. The identity of purified 7-deoxydoxorubicinolone was verified by NMR and shown to be nontoxic to bacteria at concentrations over 200 μg/ml.

Production of deglycosylated anthracyclines, like 7-deoxydoxorubicinolone, have also been documented in treated mammalian tissues. Prior mechanistic work suggested that exogenous NADH dehydrogenase of cardiac tissue also catalyzes the reductive deglycosylation of anthracyclines. To our knowledge, our study is the first report of an analogous system operating in bacteria. This establishes that bacteria could be used as a model system for further studies probing the toxicity of anthracycline drugs.

This work establishes the proof-of-principle that environmental bacteria can modify or degrade anticancer drugs, and adds antineoplastic drug inactivation to the enzymatic inactivation portfolio of actinomycetes. The tremendous diversity of chemical modification ability represented in our soil isolate library, which can be employed to modify or degrade therapeutic drugs, can be screened for novel activity on any number of therapeutic drugs. Selective detoxification of drugs in noncancerous tissues could have positive quality-of-life outcomes for chemotherapy patients.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions

Strains used for cloning or conjugation were *E. coli* TOP10 (Invitrogen) and *E. coli* ET12567/PU8002, respectively. *E. coli* were grown and maintained at

37°C in Luria-Bertani (LB) media or LB agar (LB with 1.5% agar) supplemented with 100 μl/ml ampicillin, 50 μg/ml kanamycin, and/or 50 μg/ml apramycin, when required.

Streptomyces strains were obtained from the spore stocks of the actinomycete library constructed by D'Costa et al. (2006). Additional *Streptomyces* strains were obtained from the genome-sequenced collection at the Broad Institute.

Streptomyces strains were grown and maintained at 30°C in liquid Streptomyces isolation medium (D'Costa, et al., 2006) or Bennett's medium (modified from Jones, 1949; 1% potato starch, 0.2% cassamino acids, 0.18% yeast extract, and 2 ml of Czapek mineral mix [per 100 ml: 10 g KCl, 10 g $MgSO_4 \cdot 7H_2O$, 12 g $NaNO_3$, 0.2 g $FeSO_4 \cdot 7H_2O$, and 200 μl concentrated HCl]). Solid media used in culture plates were Bennett agar (Bennett with 1.5% agar) or Manitol-Soy agar (2% mannitol, 2% soya bean meal, 10 mM $MgCl_2$, 1.5% agar) supplemented with apramycin (50 mg/ml) and nalidixic acid (30 mg/ml), when required.

Anthracyclines doxorubicin (Novopharm) and daunorubicin (Erfa Canada) were purchased from McMaster Hospital pharmacy; additional doxorubicin was obtained from AK Scientific. Antibiotics were obtained from Sigma or BioShop.

Screen for Anthracycline Resistance among Soil Isolates

The spore stocks generated by D'Costa et al. (2006) were used as the inoculum for *Streptomyces* strains. These stocks were diluted 10X to create a daughter library plate, from which 10 μl were applied to three 96-well plates containing 200 μl solid Bennett's agar supplemented or not with doxorubicin (150 μg/ml). The screen was performed in duplicate, screening a total of 626 individual actinomycete strains. Plates were incubated for 3 days at 30°C before scoring by eye. Growth was ranked on a scale from 0 to 3 (no growth to dense growth), and resistant strains were identified as those that ranked two to three on both replicates.

To follow up on the mechanism of resistance in strains, 5 ml cultures in Bennett's medium were grown in the presence or absence of 150 μg/ml doxorubicin. After growth at 30°C with shaking at 250 rpm in the absence of light, the cultures were lyophilized. Drug and metabolites were extracted by the addition of 200–500 μl of methanol followed by centrifugation at 16,100 × g for 10 min. Extracts were stored in the dark at 4°C until samples were analyzed.

Methanol extracts were analyzed by high-pressure liquid chromatography (HPLC) and subsequent liquid chromatography with mass spectrometry (LC/MS).

HPLC and LC/MS Analysis

Fractions were analyzed by HPLC and LC/MS using a guard plus Dionex Acclaim 120 C18 column (3 μm 120 Å 4.6 × 150 mm). For HPLC, the separation was performed using a flow rate of 1 ml/min under a gradient of acetonitrile complemented with 0.05% trifluoroacetic acid (solvent B) in water complemented with 0.05% trifluoroacetic acid (solvent A), and analyses were made under UV light at various wavelengths (200 nm, 220 nm, 259 nm, 300 nm, and 340 nm). For LC/MS, the separation was performed using a flow rate of 1 ml/min under a gradient of acetonitrile complemented with 0.05% formic acid (solvent B) in water complemented with 0.05% formic acid (solvent A), and analyses were made under UV light at wavelengths from 220 to 500 nm.

Anthracycline Degradation Tests

Doxorubicin degradation test reactions contained 150 μg/ml doxorubicin and a cofactor recycling system consisting of 1 mM $NADP^+$, 40 mM glucose 6-phosphate, and 0.3 units of glucose 6-phosphate dehydrogenase, in addition to various quantities of protein fractions (usually 50–300 μg total protein, as judged by Bradford assay). The reactions were incubated from 3 to 16 hr at 30°C. After incubation, methanol was added to the reaction mix (50:50 vol/vol), and the results were analyzed by TLC and LC/MS. TLC was performed on silica plates (Algram SIL G/UV_{254} 0.22 mm Silica gel 60) with a mixture chloroform:methanol (9:1), as the mobile phase. The plates were then visualized by eye and scanned under normal light conditions. The results were confirmed by LC/MS (Qtrap, Applied Biosystems) in the same conditions described before.

Enzymatic Preparation of Doxorubicin Derivatives for NMR Analysis

To prepare for NMR, 6 mg of doxorubicin were mixed with 40 mM glucose-6-phosphate, 1 mM NADP+, 120,000 units G6P dehydrogenase, and 5 mg of purified NuoEFG complex. This mixture was incubated overnight at 30°C and lyophilized. The lyophilized pellet was resuspended in 100% DMSO and passed through a reverse phase column (C18). The fractions were then analyzed by NMR. 1D and 2D NMR experiments (correlated spectroscopy, heteronuclear single quantum coherence spectroscopy, and heteronuclear multiple-bond correlation spectroscopy) were carried out in DMSO-d_6 (Cambridge Isotope Laboratories Inc.) using a Bruker AVIII 700 MHz instrument. Chemical shifts are reported in ppm relative to tetramethylsilane using the residual solvent signals at 2.50 and 39.50 ppm as internal references for the ^1H and ^{13}C spectra, respectively.

Activity-Based Purification of the Putative Anthracycline Degradation Enzyme

WAC04685 was grown in liquid Bennett media for 3 days at 30°C with shaking. After lysis by cell disruptor, ammonium sulfate precipitation was used to concentrate the target proteins. The precipitate from 10% ammonium sulfate was discarded, and the precipitate from the fraction between 10% and 60% ammonium sulfate was collected. Precipitated material was resuspended in the minimal volume of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.5, 1 M ammonium sulfate possible. The resuspended material was loaded onto a hand-poured XK-50 FPLC column containing 60 ml of phenyl Sepharose 6 FF (GE Healthcare) resin, and the proteins were eluted using a gradient of 50 mM HEPES pH 7.5. The active fractions were pooled and loaded onto a hand-poured XK-16 column containing 60 ml of Cibacron Blue Sepharose and the proteins were eluted using a gradient of 50 mM HEPES pH 7.5, 1 M NaCl. The active protein pool was then desalted and separated on an analytical Superdex 200 10/300 GL gel filtration column (GE Healthcare). Pooled active protein was loaded onto a 1 ml Q Sepharose HP column (GE Healthcare) and eluted with 50 mM HEPES pH 7.5, 1 M NaCl. Finally, the active fractions were pooled and separated by gravity chromatography through 1 ml hydroxyapatite gel (BioGel HTP gel, Biorad), and elution was with 50 or 100 mM sodium phosphate buffer pH 7. All active fractions were analyzed by SDS-PAGE and native PAGE gel, and the gels were stained by Coomassie blue or silver.

Peptide Mass Fingerprinting of the Putative Anthracycline Degradation Enzyme

The protein gel spots/bands were destained with 50 mM ammonium bicarbonate and water until clear, then rinsed with water. The spots were then reduced with 10 mM DTT at 55°C for 30 min and alkylated with 100 mM iodoacetamide at room temperature for 15 min in the dark. Proteins were digested in situ with 30 μl (13 ng/μl) trypsin (Promega Corporation, Madison WI) in 50 mM ammonium bicarbonate at 37°C overnight, followed by peptide extraction with 30 μl of 5% formic acid, and then 30 μl of acetonitrile.

Nanoscale Microcapillary Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry

The pooled extracts were concentrated to less than 5 μl on a SpeedVac spinning concentrator and then brought up in 0.1% formic acid/5% acetonitrile for protein identification by microflow liquid chromatography electrospray tandem mass spectrometry (microLC-ESI-MS/MS) using a ThermoFisher LTQ-XL-Orbitrap Hybrid Mass Spectrometer (ThermoFisher, Bremen, Germany) coupled to an Eksigent nanoLC-2D HPLC system (Eksigent, Dublin, CA). Chromatography was performed using 0.1% formic acid in both the A solvent (98% water, 2% acetonitrile) and B solvent (80% acetonitrile, 10% isopropanol, 10% water) and a 5% B to 95% B gradient over 30 min at 5 μl/min through an Eksigent capillary (CSP-3 C18-100, 0.3 m × 100 mm) column. The LTQ-XL-Orbitrap mass spectrometer experiment was set to perform a Fourier transform (FT) full scan from 200-2000 m/z with resolution set at 60,000 (at 500 m/z), followed by linear ion trap MS/MS scans on the top five ions. Dynamic exclusion was set to 2 and selected ions were placed on an exclusion list for 30 s. The lock-mass option was enabled for the FT full scans using the ambient air polydimethylcyclosiloxane ion of m/z = 445.120024 or a common phthalate ion m/z = 391.284286 for real time internal calibration.

Data Processing and Database Searching

Tandem mass spectra were extracted and converted in extensible markup language and Mascot-generating files. All MS/MS samples were analyzed using X! Tandem and Mascot database. X! Tandem and Mascot were set up to search the National Center for Biotechnology Information and Swissprot database (699,052 entries), assuming the digestion enzyme Trypsin. X! Tandem and Mascot were searched with a fragment ion mass tolerance of 0.40 Da and a parent ion tolerance of 10.0 ppm. The oxidation of methionine and the iodoacetamide derivative of cysteine were specified in X! Tandem and Mascot as variable modifications. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least three identified peptides.

Kinetics of Doxorubicin Degradation

To determine the kinetic constants of the degradation complex, we followed the oxidation of NADH at 340 nm. To do so, 12.5 mmol of NADH were mixed with 0.47 mg of protein complex in the presence of various amounts of doxorubicin (from 0 to 700 μM), and the reaction was monitored using a microplate reader (Spectramax 384 plus, Molecular Devices) for a period of 360 s. The rates were then plotted using Graphit 1.0 software.

Genome Sequencing of Strain WAC04685 and Manipulation of Its NuoF Homolog

Genomic DNA was prepared using a DNEasy Blood and Tissue Kit (QIAGEN), following the Gram-positive bacteria protocol. Sequencing on the Illumina platform was performed at Ambry Genetics (Aliso Viejo, CA). From this, 3,560,391 paired-end reads (7,120,872 reads total) as 72-mers were used for assembly for a total of 512,702,784 bp. The average length of fragments was 325 bp, for an average insert length of 181 bp. Assembly of Illumina reads was accomplished using Velvet (version 0.7.63; http://www.ebi.ac.uk/~zerbino/velvet/). Values for the length parameter (k) and the coverage cutoff (kmer) were 31 and 8, respectively. After assembly, the genome was revealed to be about 7.8 Mbp and was in 1,916 contigs (about 1,700 bp/contig). The N50 was 8,566, and the GC% was 71.37%.

nuoF Disruption in WAC04685

A suicide plasmid was generated to disrupt *nuoF* by single recombination in WAC04685. Therefore, a 978 bp internal portion of *nuoF* was amplified by PCR with the WAC04685 genomic DNA as template, using the primers 5′ATA CTG ATC AAG CTT GGA CTC CGG TCT GCG CGG 3′ and 5′ATA ATA GAA TTC TTG TCG AGG TCG GAC ATC ACG 3′, containing, respectively, the *Hind* III (Fermentas) and EcoRI (Fermentas) restriction sites. The amplicon was digested and ligated to the pSET152 vector digested by the same enzymes, generating the pEM1 plasmid. The plasmid was then used to transform *E. coli* ET/PU by electroporation (Dunican and Shivnan, 1989). Mobilization of plasmids from *E. coli* to *Streptomyces* WAC04685 strains was done essentially as described by Schäfer et al. (1990). Transconjugants were selected on MS agar and overlaid with apramycin (50 mg/ml) and nalidixic acid (30 mg/ml). The presence of integration was analyzed further by growing the transconjugant colonies on Bennett agar in the presence of apramycin (50 mg/ml), and the disruption of *nuoF* was confirmed by PCR performed on the genomic DNA of the mutants.

Activity Tests for the Disruption Mutants

The WAC04685 mutants containing pEM1 were grown in 200 ml of Bennett liquid media supplemented with apramycin (50 mg/ml) during 3 days at 30°C. The cells were then collected, resuspended in HEPES 50 mM, pH 7.5, and disrupted with a cell disruptor. The lysate was centrifuged, and an anthracycline degradation test was made on the supernatant, as described above.

ACCESSION NUMBERS

The Genbank accession number for the WAC4685 *nuo* cluster reported in this paper is JX297339.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2012.08.011.

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