INACTIVATION OF THE LIPOPEPTIDE ANTIBIOTIC DAPTOMYCIN

BY HYDROLYTIC MECHANISMS

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Running title: Daptomycin Resistance by Enzymatic Hydrolysis

ABSTRACT

The lipopeptide daptomycin is a member of the newest FDA-approved antimicrobial class, exhibiting potency against a broad range of Gram-positive pathogens, with only rare incidences of clinical resistance. Environmental bacteria harbor an abundance of resistance determinants orthologous to those in pathogens, and thus may serve as an early warning system for future clinical emergence. A collection of morphologically diverse environmental actinomycetes, demonstrating unprecedented frequencies of daptomycin resistance and high levels of resistance by antibiotic inactivation, was characterized to elucidate modes of drug inactivation. In vivo
studies revealed that hydrolysis plays a key role, resulting in one or both of the following structural modifications: ring hydrolysis resulting in linearization (in 44% of inactivating isolates), or deacylation of the lipid tail (29%). Characterization of the mechanism in actinomycete WAC4713 (Streptomyces sp. with MIC 512 µg/ml) demonstrated a constitutive resistance phenotype and established daptomycin’s circularizing ester linkage as the site of hydrolysis. Characterization of the hydrolase responsible revealed it to be a likely serine protease. These studies suggested that daptomycin is susceptible to general proteolytic hydrolysis, which was further supported by studies using proteases of diverse origin. These findings represent the first comprehensive characterization of daptomycin inactivation in any bacterial class, and may not only presage a future mechanism of clinical resistance, but suggest strategies for the development of next generation lipopeptides.

INTRODUCTION

The escalating incidences of infections caused by multi-drug resistant bacteria (29, 40) have resulted in an urgent need for the development of novel antimicrobial therapies. In response to this call for new drugs, the lipopeptide daptomycin was approved by the FDA in 2003. This drug represents the first and (so far) only antibiotic of its class to be clinically implemented. Daptomycin exhibits potent and rapid bactericidal activity against an exceptionally broad range of Gram-positive bacteria including multi-drug resistant pathogens such as methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant enterococci (VRE) (4, 12).

Although daptomycin’s mechanism of action has not fully been elucidated, it is known to act by insertion into the bacterial cell membrane in a Ca²⁺-dependent manner resulting in rapid membrane depolarization (38). Daptomycin is synthesized by the actinomycete Streptomyces
roseosporus. Its structure is comprised of thirteen amino acids cyclized through an ester linkage between the secondary hydroxyl group of an internal threonine and the carboxyl of the C-terminal kynurenine residues (Fig. 1B). The N-terminal Trp is capped with a C10 fatty acyl tail. These structural components have been demonstrated to be essential for antimicrobial activity (6, 31). Incidences of decreased susceptibility to daptomycin in clinical settings have been rare thus far, and mechanisms of resistance in Gram-positives outside of the pathogenic cocci family have been relatively unexplored.

Environmental bacteria harbor an abundance of resistance genes orthologous to those found in clinical pathogens (15, 21, 24, 30), for example the three-gene operon that confers resistance to vancomycin in enterococci (21, 30) and the CTX-M class of β-lactamases (36). These bacteria represent a deep and important reservoir of resistance determinants that have the potential for mobilization into the greater microbial community, including clinically important strains.

We have shown that soil actinomycetes, renowned for their capacity to synthesize antibiotics, are exceptionally antibiotic resistant (13). Mechanistic studies on such a reservoir have the potential to not only serve as an early warning system for the emergence of new clinical resistance mechanisms, but also guide the development of next-generation therapies. A screen of a morphologically diverse library of actinomycetes demonstrated unprecedented frequencies of resistance to daptomycin (almost universal ineffectiveness) and exceptionally high levels of resistance by means of antibiotic inactivation (approximately 80%) (13).

In this work, we characterize the molecular mechanisms of daptomycin inactivation within our actinomycete library and the associated prevalence of each strategy. These studies identified several distinct hydrolytic mechanisms of inactivation including ring opening esterase and lipase-like removal of the fatty acyl tail. Additionally, studies on a collection of diverse
proteases demonstrated the potential for daptomycin inactivation to occur outside the actinomycete subset of bacteria.

**MATERIALS AND METHODS**

**Actinomycete Minimum Inhibitory Concentration Determination**

Sixty daptomycin-resistant spore-forming actinomycetes from a previously characterized soil isolate library (13) were selected for study. To assess susceptibility to daptomycin (Cubist Pharmaceuticals), minimal inhibitory concentrations (MICs) were performed according to previously established protocols (13) using two-fold increments from 4 to 512 µg/mL. All media were supplemented with 1.5 mM CaCl₂.

**Survey of in Vivo Daptomycin Inactivation**

Liquid cultures of Ca²⁺-supplemented *Streptomyces* Isolation media (SIM) (13) (1.5 mL volume) containing glass beads to promote aeration and disrupt mycelia were inoculated in the presence and absence of 20 µg/mL daptomycin, and incubated at 30 °C and 250 rpm. After incubation for 2 and 4 days, aliquots of 250 µL were taken aseptically for subsequent analysis. To assess antimicrobial activity, disk diffusion assays were performed on tryptone soya agar supplemented with 1.5 mM CaCl₂. Inocula were prepared to the 0.5 McFarland standard using the direct colony suspension method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (11), with *Micrococcus luteus* as a susceptible test organism. Culture supernatants (spent medium) were spotted on sterile filter disks, and plates were incubated at 30 °C for 2 days. The strains capable of degrading daptomycin were identified by the absence of a zone of inhibition in the disk diffusion assays.
For analysis of the inactivation products, clarified culture supernatants by centrifugation (13,000 rpm for 5 minutes) from Day 4 were analyzed by liquid chromatography coupled mass spectrometry (LC/MS) (Agilent 1100 Series LC System and QTRAP® LC/MS/MS system, Applied Biosystems) using a C18 column (Dionex, Acclaim™ 120, 3 µm, 120 Å, 4.6 x 150 mm) and a linear gradient of dH2O and acetonitrile, both supplemented with 0.05% formic acid. Separation was monitored in the 200-400 nm range. Inactivation products were identified by a shift in retention time, a change in mass-to-charge (m/z) ratio and a detectable absorbance at 350 nm, characteristic of derivatives containing the kynurenine chromophore (20, 41). Where no primary or secondary inactivation product was detectable, the sample from Day 2 was analyzed.

Characterization of Actinomycete Isolate WAC4713

The actinomycete isolate WAC4713, which exhibited robust inactivation of daptomycin by ring hydrolysis, was selected for further study. To assess phylogeny, a region of the 16S rDNA was amplified from genomic DNA for sequencing as described in D’Costa et al. (13). The primers utilized for amplification were: 5’–AGAGTTTGATC(A/C)TGGCTCAG and 5’–CGG(C/T)TACCTTGTTACGACTT. The resulting construct was sequenced at the Mobix Central Facility (McMaster University, Hamilton, Canada).

Time Dependency of WAC4713-mediated Daptomycin Inactivation

To investigate time-dependent inactivation of daptomycin, 50 mL cultures of SIM supplemented with Ca²⁺ and 20 µg/mL daptomycin were inoculated with 5 µL of purified spore suspensions (7 x 10⁹ CFU/mL) and incubated at 30 °C and 250 rpm. At various time points from 0 to 48 hours, 500 µL aliquots were taken aseptically and stored at -80 °C for subsequent analysis. Antimicrobial activity was monitored by disk diffusion assay and inactivation products were
assessed by reverse-phase high-performance liquid chromatography (HPLC). Products of interest were purified for LC/MS analysis.

**Purification of the Daptomycin Inactivating Hydrolase from WAC4713**

Seed cultures of 5 mL liquid SIM were inoculated from single colonies and incubated for 3 days at 30 °C and 250 rpm. Each was subcultured into 200 mL of liquid SIM using a 1% (v/v) inoculum, and subsequently into 1 L cultures of liquid SIM (16 L total). All cultures were supplemented with 60 µg/mL trimethoprim to prevent contamination. After 3 days, the cultures were filtered through Whatman Grade 4 filter paper (15 cm diameter, qualitative), and the filtrates were pooled. Culture filtrates from 16 L of cell growth medium were supplemented with 0.25 mM 1,10-phenanthroline and concentrated using tangential flow filtration (Ultrasette™ Omega™ 10 kDa NMWL, Pall Life Sciences) to approximately 1 L and subjected to fast-performance liquid chromatography using cation exchange (SP Sepharose Fast Flow 500 mL, GE Healthcare). Purification was performed using a linear gradient of buffers A (50 mM N-(2-hydroxyethyl)-piperazine-N’-(2-ethanesulfonic acid) (HEPES), pH 7.5) and B (50 mM HEPES, pH 7.5, 1 M NaCl). Fractions containing the protein of interest were concentrated using a centrifugal filter device (Centricon Plus-70 10 kDa NMWL, Millipore), and applied to a size-exclusion column (Superdex™-200 24 mL, GE Healthcare) equilibrated with 50 mM HEPES, pH 7.5.

To assay enzyme activity, reactions were prepared in 50 mM HEPES pH 7, supplemented with 100 µg/mL daptomycin and 1.5 mM CaCl₂. Reactions were initiated by addition of the eluate, and incubated at 30 °C. Daptomycin inactivation was assessed by LC/MS (AutoPurification HPLC System and Micromass ZQ 2000 MS system, Waters) using a C18 column (Waters, Atlantis HILIC Silica, 5 µm, 100 Å, 4.6 x 50 mm) and the following solvent systems: dH₂O and...
acetonitrile (Solvents A and B respectively), each supplemented with 0.01% trifluoroacetic acid. To resolve daptomycin and the inactive ring-hydrolyzed product, an isocratic separation was performed using 60% Solvent A for 5 minutes. An injection volume of 20 µL was used. For each purification step, fractions that exhibited complete conversion of daptomycin after 2 hours were selected.

To identify the daptomycin hydrolase, an in-gel enzyme assay was developed. Concentrated fractions were resolved in two replicates by standard SDS-PAGE. Half the gel, representing one set of replicates, was incubated in renaturation buffer (Bio-Rad) for 30 minutes at room temperature. Lanes were sliced according to various molecular weight ranges, and each band was immersed in a 250 µL reaction mixture containing the following components: 50 mM HEPES, pH 7.5, 100 µg/mL daptomycin, and 3 mM CaCl₂. Reactions were incubated overnight at 30 °C, and analyzed by LC/MS. The gel section containing the second set of replicates was stained with Colloidal blue (Invitrogen), and bands corresponding to those inactivating daptomycin were excised for MS/MS peptide analysis. Analysis was performed by reverse-phase HPLC nano-electrospray tandem mass spectrometry (μLC/MS/MS, Thermo LTQ-Orbitrap Mass Spectrometer) at the Harvard Microchemistry and Proteomics Analysis Facility (10). Associated genes were identified using BLAST (1) analysis of the draft genome sequence of isolate WAC4713.

For quantification purposes, total protein concentration was determined by the Bradford method and enzyme activity as described above. Reactions were incubated for 2 hours at 30 °C, stopped with 30% (v/v) methanol, and analyzed by LC/MS. Activity was defined by the increase in hydrolyzed daptomycin, as observed by peak area.

**Genome Sequencing of Isolate WAC4713**
The WAC4713 genome draft was obtained using a whole-genome shotgun strategy (18) and Illumina Genome Analyzer technology. The genomic DNA was prepared from 2 mL of liquid culture using the DNeasy® kit (Qiagen, USA). The sample library preparation and sequencing using Illumina Genome Analyzer IIx were performed by Ambry Genetics (Aliso Viejo, CA, USA). From sequencing, 7 475 486 paired end reads (totaling ~531 MB) were obtained. The paired reads were assembled using Velvet version 1.1.02 (44) (hash length 41, coverage cutoff 10), which generated 1682 contigs with an N50 of 16446 bp and total consensus of 9 MB; based upon the assembly results we estimate ~59-fold coverage.

Characterization of WAC4713-mediated Daptomycin Inactivation

To assess the site of ring hydrolysis, a large-scale purification of the linearized inactivation product was necessary for nuclear magnetic resonance (NMR) analysis and tandem mass spectrometry. A 20 mL reaction containing 50 mM HEPES pH 7.0, 500 µg/mL daptomycin, 1.5 mM CaCl₂ and 0.5 µg/mL of partially purified hydrolase from isolate WAC4713 was incubated at 30 °C for 24 hours. The completion of the reaction was analyzed by LC/MS as described above. The reaction volume was reduced by lyophilization, and the sample was resuspended in dH₂O. The sample was then desalted on Sep-Pak® Plus C18 cartridge (Waters) according to manufacturer’s protocol. Daptomycin and chemically hydrolyzed daptomycin were used as reference compounds. Metal ions associated with daptomycin were removed using Chelex 100 resin (Biorad) according to manufacturer’s protocol. Chemically hydrolyzed daptomycin was generated by incubating 1 mM of daptomycin in a solution of 20 mM LiOH at room temperature for 1 hour. The reaction was quenched by adding hydrochloric acid to attain a neutral pH. The hydrolysate was desalted on Sep-Pak® Plus C18 cartridge (Waters). 1D and 2D NMR experiments (COSY, HSQC, HMBC) were carried out in D₂O using a Brucker AVIII 700 MHz.
instrument. Structural analyses by tandem mass spectrometry were performed using the parameters described in the Supplementary experimental procedures.

**Determination of Enzyme Kinetic Parameters**

The hydrolysis of daptomycin was monitored by LC/MS, as previously described. A reaction was typically performed in a final volume of 250 \( \mu \)L containing 50 mM HEPES pH 7.0, 1.5 mM CaCl\(_2\), 0.1 \( \mu \)g/mL of partially purified enzyme and daptomycin (between 1 mM and 10 \( \mu \)M). The reactions were initiated with daptomycin, incubated at 30 °C for 120 minutes and stopped with 30% (v/v) methanol; each reaction was performed in triplicate. Nonlinear regression analyses of dependences of the enzyme initial velocity on substrate concentration \( (v = V_{\text{max}} \cdot [S]/K_m + [S]) \) were performed using GraphPad Prism 5 software.

**Enzyme Inhibition: WAC4713 Daptomycin Hydrolase**

*In vitro* inhibition studies were performed using active enzyme purified by SP Sepharose. Each reaction contained 50 mM HEPES, pH 7, 100 \( \mu \)g/mL daptomycin and 0.1 \( \mu \)g/ml of partially purified enzyme. Inhibition reactions were supplemented with the following concentrations of protease inhibitors: EDTA (5 mM), 1,10-phenanthroline (5 mM), pepstatin (1 \( \mu \)M), chymostatin (100 \( \mu \)M), leupeptin (10 \( \mu \)M), phenylmethylsulfonyl fluoride (PMSF, 1 mM), 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF, 2 \( \mu \)M), and benzamidine (1 mM). Reactions were preincubated at 30 °C for 3 h in the absence of daptomycin and for an additional 2 h subsequent to initiation. Assays were stopped with 30% (v/v) methanol and analyzed by LC/MS.

**In Vitro Daptomycin Inactivation: Commercial Protease Survey**

The following commercially available proteases were tested: subtilisin A (from *B. licheniformis*), thermolysin (from *B. thermoproteolyticus*), type I protease (bovine), *S. griseus* proteases (mixture), \( \alpha \)-chymotrypsin (bovine), trypsin (porcine, recombinant), elastase (porcine), and *S.
aureus V8 protease. All enzymes were purchased from Sigma-Aldrich Co., with the exception of trypsin (Promega, Corp.). Reactions were prepared in 2.5 mM Tris, pH 7.5, supplemented with 20 µg/mL daptomycin and 3 mM CaCl$_2$. Each reaction was initiated by addition of 10 U enzyme and incubated at 30 °C overnight. Daptomycin inactivation was assessed by antimicrobial disk diffusion assays and reverse-phase HPLC to monitor daptomycin disappearance. For enzymes capable of partially or completely inactivating daptomycin, time course analyses were performed. Reactions were prepared in 2.5 mM HEPES, pH 7.5, supplemented with 40 µg/mL daptomycin and 3 mM CaCl$_2$ (2 mL volume), initiated by addition of 100 U enzyme, and incubated at 30 °C. Aliquots of 200 µL were taken from the reaction mixture for subsequent analysis at various time points from 0-48 hours.

RESULTS

Hydrolytic Mechanisms of Daptomycin Inactivation by Soil-dwelling Bacteria

Susceptibility analyses of sixty actinomycete strains from the previous study (13) indicated high levels of resistance to daptomycin (MICs ≥ 256 µg/mL). With respect to mechanism, isolates exhibiting drug inactivation were screened to assess the nature of the detoxified products. Daptomycin derivatives were identified from LC/MS data based on the following criteria: retention time, mass spectrometric data, and absorbance at 350 nm characteristic of derivatives containing the kynurenine chromophore (20, 41). Representative analyses of several selected isolates are shown in Fig. 1A, and frequency data are summarized in Fig. 1C. Two dominant primary inactivation products were observed. The most prevalent product, more hydrophilic in nature than daptomycin, was detected in 44% of all daptomycin inactivating isolates (Fig. 1B and 1C). Its mass increase of 18 Da in comparison to the daptomycin reference is a signature
indicator of ring-opening hydrolysis ($m/z$ 1639). The second inactivation product ($m/z$ 1280), detected in 29% of daptomycin modifying isolates, corresponded to the peptide core of daptomycin resulting from hydrolytic deacylation. Subsequent degradation products corresponding to further cleavage along the peptide chain were also detected. For 9% of isolates capable of daptomycin inactivation, no product was detected, which may reflect rapid hydrolysis and subsequent degradation. The order of daptomycin degradation is unknown for most isolates, but at least in one case, only deacylation of the intact cyclic peptide was observed under the conditions described in Materials and Methods.

**Characterization of Daptomycin Ring Hydrolysis by Actinomycete Isolate WAC4713**

Actinomycete isolate WAC4713 was selected for further characterization of the novel ring hydrolysis mechanism. It exhibited robust growth in the presence of daptomycin and efficient drug inactivation. BLASTn analysis of partial 16S rDNA indicated sequence similarity to *Streptomyces cirratus* (99% sequence identity) known for its synthesis of the 16-membered macrolide antibiotic cirramycin (42).

Growth analyses performed on *Streptomyces* WAC4713 (daptomycin MIC 512 µg/mL) indicated uninterrupted growth upon supplementation with subinhibitory concentrations of daptomycin (Fig. S1), regardless of the time of addition, consistent with a constitutive mechanism of resistance (25).

Time course analysis in liquid growth medium confirmed the absence of intact daptomycin by 12 hours of incubation (Fig. 2), an observation that was associated with the loss of antimicrobial activity and appearance of a single primary inactivation product ($m/z$ 1639) consistent with the addition of a water molecule to the antibiotic. The subsequent decrease of this product was coupled with the accumulation of hydrophilic products that absorb at 350 nm. Mass
spectrometry analysis of the derivatives indicated secondary inactivation products corresponding
to the deacylation ($m/z$ 1485, peak E Fig 2) and cleavage of the N-terminal Trp ($m/z$ 1299, peak F Fig 2) of hydrolyzed daptomycin.

To elucidate the structure of the primary inactivation product and deduce the site of hydrolysis, NMR and tandem mass spectrometry experiments were performed on the purified inactive product B ($m/z$ 1639), in addition to reference compounds daptomycin and chemically hydrolyzed daptomycin. 1D and 2D NMR experiments were performed, and the assignment of $^1$H and $^{13}$C chemical shifts of all samples was achieved by analysis of the COSY, HSQC and HMBC spectra and comparison with published data (2). A number of differences were observed upon comparison of the spectra of daptomycin and its hydrolytic derivatives. In the $^1$H NMR spectra of the primary inactivation product and chemically hydrolyzed daptomycin, the unique low field resonance at 5.45 ppm assigned to the side chain Thr-$H$β due to the ester bond linkage was shifted to 4.26 ppm (Fig 3). The same phenomenon was observed in the $^{13}$C spectrum: the chemical shift corresponding to Thr-$C$β was shifted from 70.68 ppm to 66.52 ppm, indicating loss of the ester linkage. Tandem mass spectrometry analysis confirmed the loss of the ester linkage between Thr and Kyn (Fig. S2 and Table S1).

**Characterization of the Daptomycin Ring-hydrolyzing Esterase from Isolate WAC4713**

The ring hydrolyzing esterase from *Streptomyces* strain WAC4713 was partially purified from 16 L of culture supernatant. Purification strategies, which included cationic exchange and gel exclusion, resulted in a $1.6 \times 10^3$-fold increase in specific activity (Table S2), where activity was defined by the ability to convert daptomycin to its inactive, ring-hydrolyzed product. An in-gel antibiotic inactivation assay, developed to assist in the identification of the enzyme of interest
Active site class was assessed using a panel of protease inhibitors against the partially purified enzyme from isolate WAC4713. The daptomycin ring hydrolysis activity was inhibited by the addition of Ser protease inhibitors PMSF and AEBSF (Fig. 4). The steady-state kinetic parameters were determined in triplicate by varying the daptomycin concentration between 0 and 1 mM (Fig. S3). The $K_{m}$ for daptomycin was determined to be $154 \pm 9 \mu M$.

In order to evaluate if the partially purified hydrolase was responsible for the degradation of daptomycin subsequent to ring hydrolysis previously observed (Fig. 2), a time course experiment (between 0 – 48 h) with daptomycin and hydrolyzed daptomycin was performed. The partially purified hydrolase from isolate WAC4713 was able to cleave the daptomycin ester bond between Thr and Kyn, however it was not capable of utilizing hydrolyzed daptomycin as a substrate for further degradation.

The effect of calcium on daptomycin ring hydrolysis activity was tested at 0.15 mM and 2 mM daptomycin by varying the calcium:daptomycin ratio between 0:1 and 4:1. Calcium had no effect on daptomycin ring hydrolysis activity (Fig. S4).

**Daptomycin is Susceptible to Hydrolysis by Diverse Proteases**

To assess the intrinsic susceptibility of daptomycin to proteolytic hydrolysis, a small library of commercially available proteases was screened against daptomycin as a substrate. This included enzymes of both bacterial and mammalian origin, and spanned all three major classes of proteases. *In vitro* inactivation of daptomycin, whether partial or complete, was demonstrated by two of the eight proteases studied (Fig. S5), including the bovine-derived chymotrypsin and type I proteases.
To investigate the mode of daptomycin inactivation, time course studies were performed using each of the two proteases, coupled with analysis by antimicrobial disk diffusion assay and LC/MS. For each enzyme, inactivation of daptomycin was correlated with the appearance of the linearized product (m/z 1639). Secondary degradation products were not observed with the two assayed proteases.

**DISCUSSION**

The lipopeptide daptomycin has proven to be exceptional as a therapy for multi-drug resistant infections caused by many Gram-positive pathogens (4, 12, 19, 39, 43). Surveillance programs have identified only rare cases of clinical resistance to date (12, 33, 37). Previous work has shown that a subset of environmental Gram-positive bacteria, the actinomycetes, demonstrate near universal resistance and exceptionally high levels of enzymatic drug inactivation (13). It is imperative to understand the modes of inactivation, the enzymes responsible, and the potential host organism diversity, as resistance by antibiotic detoxification can present challenges upon clinical emergence (14).

Examination of our collection of actinomycetes revealed significant resistance levels (MICs ≥256 µg/ml) and showed two primary modes of daptomycin inactivation: ring hydrolysis leading to linearization of the cyclic compound (44% of inactivating isolates), and deacylation of the lipid tail (29% of drug inactivating isolates). These findings are significant because they indicate environmentally widespread novel mechanisms of potential daptomycin resistance.

Daptomycin modification by deacylation has previously been identified in a subset of *Actinoplanes* species as a means of generating a peptide nucleus for subsequent semi-synthetic chemical modifications of the core peptide (7, 16). Deacylation by *Actinoplanes utahensis* is
attributed to a heterodimeric protein with an exceptionally broad substrate specificity that includes the cyclic hexapeptide antifungal echinocandin B, the cyclic antifungal pseudomycin, and the glycopeptide antibiotic teicoplanin (27). Sequence analysis revealed a number of candidate deacylases within the genomes of actinomycetes, potentially accounting for the deacylation observed in the library. Collectively, these data indicate that hydrolytic deacylation of daptomycin is not restricted to Actinoplanes species, and is more broadly distributed within the bacterial population than previously suggested.

Daptomycin inactivation by ring hydrolysis represents a novel mechanism of inactivation (Fig. 1). Our results suggest that daptomycin resistance is not inducible in Streptomyces isolate WAC4713. This observation however does not preclude the possibility of multiple mechanisms of resistance, a strategy commonly employed by actinomycetes (22, 32, 35). With respect to the site of inactivation, enzyme-catalyzed linearization of daptomycin can be accomplished through hydrolysis of one of nine amide linkages or its lone, thermodynamically more sensitive ester. Structural analysis confirmed the site of hydrolase-mediated inactivation to be the ester bond between Thr and Kyn.

Characterization of the ring-opening hydrolase from isolate WAC4713 proved to be extremely challenging. The low abundance of this secreted enzyme necessitated large-scale purification from culture supernatants, and concentration of the enzyme required for purification resulted in increased rates of auto-degradation that destabilized the drug inactivating activity. Additionally, while MS/MS peptide sequencing (10) and data correlation with genome sequences of model Streptomyces species and draft genome of WAC4713 was successful at identifying candidate esterases (Tables S3 and S4), subsequent in vitro studies of the purified recombinant enzymes could not identify an active protease, as determined by both casein and gelatin zymography and
in vitro daptomycin inactivation assay (refer to Supplementary Material). This suggested that although heterologous expression in *E. coli* yielded soluble enzyme for purification, the recombinant enzymes were inactive, potentially due to protein misfolding. The selective inhibition of the native enzyme by PMSF and AEBSF indicates that a secreted serine hydrolase is responsible for inactivation of daptomycin. Therefore, among the candidate esterases identified, the putative subtilisin-like protease and truncated peptidase S8 and S53 subtilisin/kexin/sedolisin (Table S3) are the most likely candidates. Daptomycin requires Ca$^{2+}$ for its antimicrobial activity (3, 23, 34), and has been shown to be present in two different forms depending on the presence or absence of Ca$^{2+}$: the micelle form (14-16 daptomycin units) and the apo-daptomycin (monomeric form) respectively (34). The resistance esterase activity is however not affected by daptomycin conformation (Fig S4).

The majority of actinomycete library strains surveyed exhibited further degradation of primary daptomycin inactivation products (Fig. 1). While the mechanisms for these degradation processes are yet to be elucidated, it is likely that the cleavage reactions are enzyme-catalyzed. With regard to isolate WAC4713, the partially purified hydrolase showed only ring hydrolysis activity against daptomycin, indicating that another enzyme is likely responsible for degradation following linearization (Fig. 2). Enzymatic degradation of several cyclic peptide compounds has been demonstrated by Gram-negative *Sphingosinicella* species (8, 26). Given the data collected from analysis of the actinomycete library, a proposed actinomycete degradation scheme for daptomycin is presented in Fig. 5.

We have demonstrated that multiple modes of daptomycin inactivation are evident in environmental actinomycetes, with hydrolysis playing a fundamental role in each process. It is important to note that while the frequencies observed for each mode of inactivation are
unprecedented, they likely represent an underestimate of the genetic potential available to bacteria. With respect to the environmental resistome as a whole, the diversity of daptomycin inactivating enzymes uncovered in this work is only partially reflective of the true extent of this reservoir, as this study was limited to spore-forming actinomycetes. Additionally, it is important to note the potential clinical significance of this mechanism. Recent characterizations have identified rapidly growing *Mycobacterium* (5) and several *Nocardia* clinical isolates exhibiting robust resistance to daptomycin (28), characteristic of the environmental actinomycetes from this study. Further work is required to confirm the mechanism of resistance as well as the determinants responsible.

The finding of daptomycin inactivation by multiple actinomycete-derived hydrolases suggests that its molecular structure is susceptible to general hydrolytic cleavage. To provide evidence to validate this hypothesis, an *in vitro* screen performed on a small library of commercially available proteases demonstrated daptomycin inactivation by two out of eight of the enzymes screened, independent of the host origin from the previously characterized actinomycete hydrolase. This illustrates that daptomycin inactivation can be accomplished without the evolution of a highly specific resistance enzyme, and predicts that enzymatic inactivation by other Gram-positives outside the actinobacterial subset is possible in the future.

This study represents the first comprehensive analysis of daptomycin inactivation as a mechanism of resistance in any class of bacteria. The frequency of high-level resistance identified in actinobacteria (13, 15) is an illustration of the potential of Gram-positive bacteria to develop resistance to lipopeptides. The finding of high frequencies of proteolytic inactivation among actinomycetes is consistent with their known capacity to secrete an abundance of hydrolytic enzymes including proteases (9). However, with that in consideration, the spectrum
of bacteria acknowledged for extracellular protease synthesis includes the pathogen *S. aureus* (17, 45), for which daptomycin is an approved treatment. While *S. aureus* isolates have yet to exhibit resistance to daptomycin by enzymatic hydrolysis, it is conceivable that secreted proteases could evolve the capacity to accommodate daptomycin as a substrate or that daptomycin hydrolase genes could be transferred to sensitive organisms paralleling the history of acquired resistance elements to the majority of clinically used antibiotics.

We note that the finding of instability of daptomycin to environmental organisms is also beneficial. The fact that there are multiple mechanisms of inactivation predicts that unlike other antibiotics, daptomycin will not accumulate in the environment creating a selective pressure for resistance dissemination.

Finally, these findings have the potential to significantly benefit the drug development process by informing the rational design of second-generation lipopeptides. We have shown that enzyme-catalyzed hydrolysis of daptomycin can be achieved by hydrolases of diverse origin, and that this modification occurs at the ester linkage. While comparative studies between representatives of the depsipeptide and peptide subclasses of lipopeptides are necessary, it is likely that replacement of daptomycin’s ester linkage by a more hydrolytically stable linkage will result in a lipopeptide less susceptible to ring hydrolysis. Together, these findings not only provide novel insights into the future clinical efficacy of daptomycin, but also may serve as a foundation for research aimed at preserving lipopeptides as an effective class of antimicrobials.
ACKNOWLEDGMENTS

This work was supported by a Canadian Institutes of Health Research Grant (MT-13536) to G.D.W and scholarship to V.M.D, Cubist Pharmaceuticals, and the Canada Research Chairs program. We thank Inga Kireeva for assistance in peptide mapping.

REFERENCES


Figure 1. Daptomycin inactivation profile by environmental actinomycetes. A, representative HPLC chromatograms of clarified culture media supplemented with daptomycin was analyzed after 4 days of growth. Isolate WAC4666 (I), isolate WAC4674 (II), isolate WAC4670 (III), isolate WAC4713 (IV) and daptomycin (V). The letters on top of the each peak indicate a specific daptomycin product (refer to panel B for cleavage site): A, m/z 1621-
daptomycin; B, m/z 1639-ring cleavage, most probable at site 1; C, m/z 1450-cleavage at site 1 and 3; D, m/z 1467-cleavage at site 2; E, m/z 1485-cleavage at site 1 and 2; F, m/z 1299-cleavage at site 1 and 4. B, daptomycin structure indicating the most common sites of cleavage observed in the screen. The kynurene residue is highlighted in blue. C, Summary of daptomycin inactivation phenotypes of 60 actinomycetes. Each circle in the Venn diagram represents inactivation products deduced, where percentages of all resistant strains assayed are indicated.
Figure 2. Time course analysis of daptomycin inactivation by *Streptomyces* strain WAC4713. HPLC chromatograms and disk diffusion assay of WAC4713 clarified culture media supplemented with daptomycin at different incubation time (control, daptomycin alone). The letter near each peak indicates a specific daptomycin product (refer to Fig.1 panel B for cleavage site): A, *m/z* 1621-daptomycin; B, *m/z* 1639-cleavage at site 1; E, *m/z* 1485-cleavage at site 1 and 2; F, *m/z* 1299-cleavage at site 1 and 4.
Figure 3. $^1$H-NMR spectra of daptomycin and ring-opened inactivation product. 700 MHz

$^1$H-NMR spectra of daptomycin (1), base hydrolyzed daptomycin (2) and ring-opened daptomycin by isolate WAC4713. A, $^1$H-NMR between 5.1 and 7.9 ppm. B, $^1$H-NMR between 0.9 and 4.4 ppm. The spectra show the shift of Thr-\textit{H}β due to the ester bond linkage from 5.45
ppm in daptomycin to 4.26 ppm in the ring-opened product, indicated by the arrows. 

Chemical structure of ring-opened daptomycin, the Thr-Hβ is highlighted in bold. Abbreviations: Orn, ornithine; MeGlu, L-3-methylglutamic acid; kyn, kynurinine.
Figure 4. Inhibition of the daptomycin hydrolase from *Streptomyces* WAC4713. *A*, protease inhibitor profile for WAC4713 hydrolase. Control reactions were incubated in the absence of inhibitor and each reaction was performed in triplicate. Note the following abbreviations: Phen = 1,10-phenanthroline, PMSF = phenylmethylsulfonyl fluoride, AEBSF = 4-(2-aminoethyl)benzenesulfonyl fluoride. *B*, IC<sub>50</sub> curve for PMSF (720 ± 31 µM) and AEBSF (541 ± 22 µM).
Figure 5. Proposed daptomycin degradation schemes by actinomycetes. The actinomycetes described within this work demonstrated one or more of the following pathways.