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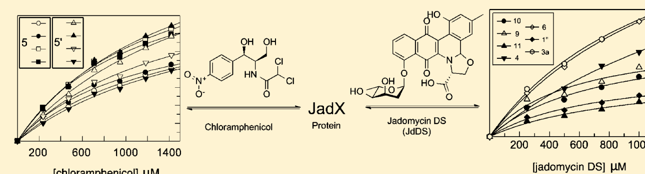
JadX is a Disparate Natural Product Binding Protein

Andrew W. Robertson,[†] Stephanie M. Forget,[†] Camilo F. Martinez-Farina,[†] Nicole E. McCormick,[‡] Raymond T. Syvitski,[†,§] and David L. Jakeman*[,†,‡]

[†]Department of Chemistry and [‡]College of Pharmacy, Dalhousie University, Halifax, Nova Scotia B3H 4R2, Canada
[§]Institute for Marine Biosciences, National Research Council of Canada, Halifax, Nova Scotia B3H 3Z1, Canada

Supporting Information

ABSTRACT: We report that JadX, a protein of previously undetermined function coded for in the jadomycin biosynthetic gene cluster of *Streptomyces venezuelae* ISP5230, affects both chloramphenicol and jadomycin production levels in blocked mutants. Characterization of recombinant JadX through protein−ligand interactions by chemical shift perturbation and WaterLOGSY NMR spectroscopy resulted in the observation of binding between JadX and a series of jadomycins and between JadX and chloramphenicol, another natural product produced by *S. venezuelae* ISP5230. These results suggest JadX to be an unusual class of natural product binding protein involved in binding structurally disparate natural products. The ability for JadX to bind two different natural products in vitro and the ability to affect production of these secondary metabolites in vivo suggest a potential role in regulation or signaling. This is the first example of functional characterization of these JadX-like proteins, and provides insight into a previously unobserved regulatory process.



■ INTRODUCTION

Biosynthetic pathways producing natural products require enzymes and regulatory proteins to work cooperatively to furnish complex stereospecific structures. Genes essential for individual secondary metabolite production are often found in gene clusters with genes localized in close proximity to one another.[1,2] The biosynthesis of these natural products is often subject to complex regulation, controlled by one or more regulatory proteins coded for within their respective biosynthetic gene clusters.[3−5] Many strains of *Streptomyces* are capable of producing multiple secondary natural products.[6−8] There is a growing body of evidence that the production of many of these compounds is regulated via complex cross-regulation, in which cluster-situated regulatory proteins from one pathway affect gene expression in other secondary metabolite pathway.[1,3,4,9] Examples of this multinatural product cross-regulation can be found in the model organism *Streptomyces coelicolor*, regulating production of undecylprodigiosin and actinorhodin,[10] and more recently, *Streptomyces venezuelae* ISP5230 (ATCC 10712), regulating production of the angucycline family of natural products known as the jadomycins (Jd) and chloramphenicol (Cm).[11,12] It was reported by Tan and co-workers that JadR2 is responsible for repressing Jd production by inhibiting the transcription of *jadR1*, whose gene product is responsible for activation of Jd production.[11,12] There are a number of regulatory genes within the Jd biosynthetic gene cluster, but none in the Cm gene cluster.[12,13] Tan and co-workers identified that regulation of Cm biosynthesis is accomplished by Jd-cluster-situated regulators JadR1 and JadR2 via cross-regulation between the pathways, and JadR2 has been shown to bind both Cm and JdB end products further substantiating this cross talk between pathways.[12] It is rare that a secondary metabolite biosynthetic pathway is completely elucidated, and despite extensive research conducted toward the mapping of Jd biosynthesis, many of the gene products involved only have putatively assigned functions or have yet to have any function ascribed to them.[14,15] Despite the wealth of genomic data available, assignment of function via homology, especially in complex biosynthetic pathways, can lead to misrepresentation of function.[16] This problem is compounded when little or no known homology is identified or when a protein exists in a family of structurally similar proteins with diverse functions.[17] Characterization of these proteins remains one of the most challenging problems associated with mapping biochemical processes. Identification of ligands for these proteins is a very important first step for identifying function, but requires fast, reliable, efficient screens for potential ligands. For secondary metabolite biosynthetic systems, a sensitive, nondestructive method is beneficial to avoid loss of valuable, often difficult to purify, natural products. The ability to observe binding in the presence of impurities or other compounds is highly advantageous, especially if purification is challenging. NMR binding methodologies offer these desired experimental parameters and have been used extensively in drug design[18−20] and more recently the functional assignment of putatively characterized proteins.[16] This methodology could offer a powerful tool for streamlining ligand screening as an initial step to aid in the characterization of proteins of unknown function.

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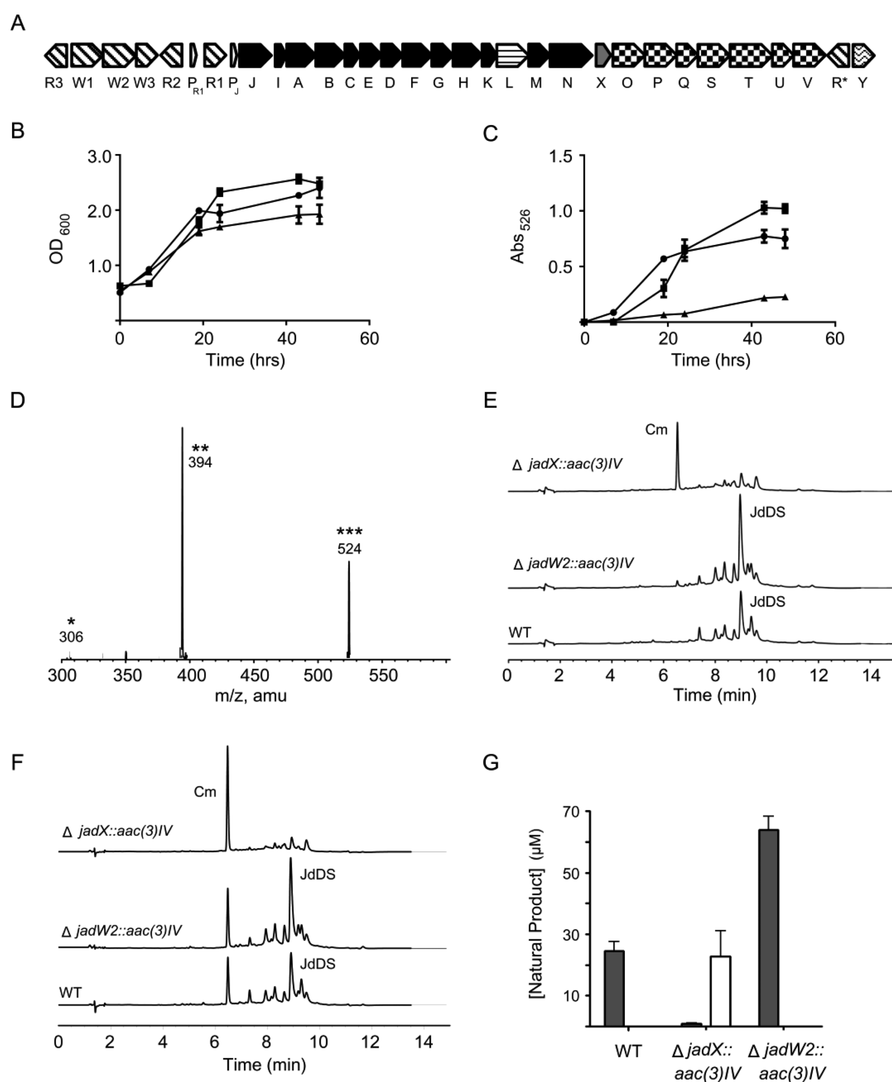

Figure 1. (A) Genetic organization of *S. venezuelae* ISP5230 Jd gene cluster showing ORFs coding for regulatory proteins (diagonal stripes), predicted promotor regions (white), Jd aglycone structural enzymes (black), dideoxysugar structural enzymes (checkered), an efflux protein (horizontal stripes), a cofactor supplying reductase (wavy lines), and JadX (gray). A complete list of all gene products and their putative/assigned functions can be found in Table S1. (B) Cellular growth curves (OD_{600}) of *S. venezuelae* ISP5230 WT (●), *S. venezuelae* ISP5230 VS1099 (■), and *S. venezuelae* ISP5230 VS1085 (▲). (C) Absorbance of clarified growth media at λ = 526 nm (Abs_{526}), estimating colored compound production of *S. venezuelae* ISP5230 WT (•), *S. venezuelae* ISP5230 VS1099 (■), and *S. venezuelae* ISP5230 VS1085 (▲). (D) LC-MS/MS fragmentation pattern of JdDS, illustrating $[M + H]^+$ (***), cleavage of the sugar $[M + H - \text{digitoxose}]^+$ (**), and the amino acid group $[M + H - \text{digitoxose} - R]^+$ (*). (E) HPLC traces of reversed-phase phenyl column growth media extracts after 48 h. (F) HPLC traces of reversed-phase phenyl column growth media extracts after 48 h spiked with 200 μM Cm. (G) Final JdDS (gray bars) and Cm (white bars) concentrations from strains after 48 h growth.

Herein, we identified and quantified ligand binding to JadX, a gene product coded for within the *S. venezuelae* Jd biosynthetic cluster, using NMR binding methodologies. The ligands were identified as Jd and Cm. The data presented illuminate the function of JadX as a new class of nuclear transport factor 2 (NTF2)-like protein involved in binding multiple secondary metabolites and likely involved in the complex cross-regulation of Jd and Cm biosynthesis, acting by responding to endogenous natural product signals. Reoccurrence of these JadX-like proteins coded for in many secondary metabolite gene clusters suggests a role in the biosynthesis of a wide array of natural products.

■ RESULTS

JadX Homology Studies. The *jadX* gene was identified by Vining and co-workers immediately upstream of the dideoxysugar tailoring region of the Jd biosynthetic gene cluster (Figure 1A).[14] A BLASTP search identified that JadX is included in the NTF2-like superfamily.[17] This superfamily represents a large group of proteins containing a similar fold but diverse functions, making putative functional analysis by homology ambiguous. The BLASTP search identified a series of similar proteins coded for in other secondary metabolite biosynthetic pathways including azicemicin,[21] geldanamycin,[22] herbimycin,[23] kinamycin,[24] and methylenomycin (Figure S1).[25,26] A full sequence alignment of these JadX homologues is presented in the Figure S2. All of these natural products are polyketide-derived natural products, except methylenomycin. Azicemicin and kinamycin are, together with Jd, angucyclines. The putative function of these proteins is described as either unknown or JadX-like. Interestingly, as opposed to the jadomycin biosynthetic pathway in which *jadX* is located

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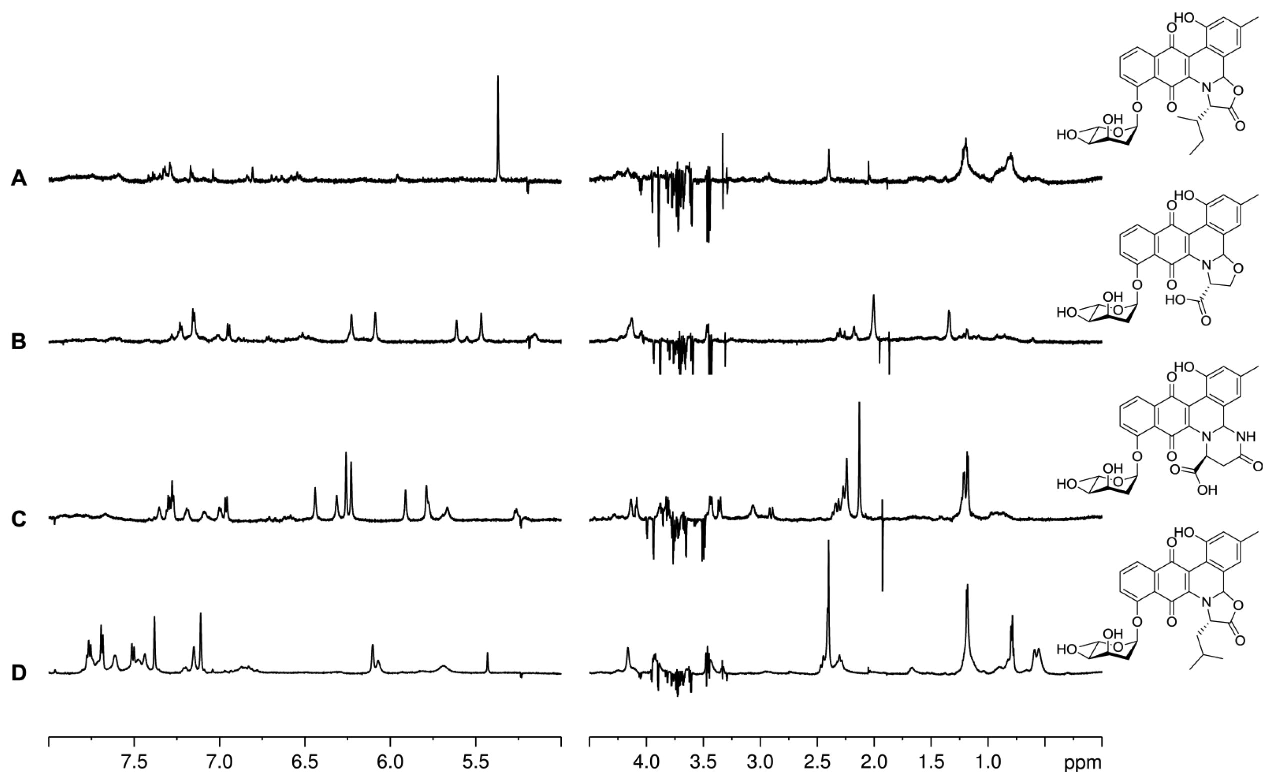

Figure 2. Overlaid WaterLOGSY spectra (700 MHz, 1:9 dPBS/PBS, pH 7.6) of JadX (0.05 mM) in the presence of the nonbinding standard D-galactose (5 mM) and (A) JdB (500 μM), (B) JdDS (750 μM), (C) JdN (750 μM), and (D) JdL (1 mM) illustrating specific JadX binding to a series of jadomycin analogues. Binding ligands (jadomycins) are phased positively, and negative control nonbinding compounds (D-galactose) are phased negatively.

between the PKS and the dideoxysugar gene cluster isolated away from the regulatory genes, many of the *jadX*-like genes are located slightly upstream or downstream of their entire biosynthetic clusters, often in close proximity to the respective regulatory genes.[21,23] It can be postulated that the reoccurrence of these JadX-like proteins coded for in many secondary metabolite gene clusters suggests an important role within their respective pathways.

JadX Disruption Shifts *S. venezuelae* Natural Product Profile. To test the importance of *jadX* for production of Jd, comparative Jd production growths were conducted for *S. venezuelae* ISP5230 VS1085 (WT) and *S. venezuelae* VS1085 mutant (Δ*jadX::aac(3)IV*), a mutant strain in which *jadX* is disrupted via insertion of an apramycin-resistance cassette.[14] Our analysis of other apramycin-blocked mutants in the dideoxysugar pathway resulted in the characterization of metabolites not initially identified in previous studies, including a differentially glycosylated jadomycin.[27] We therefore reconfirmed by PCR the location of the apramycin-resistance cassette in the jadomycin biosynthetic cluster for the Δ*jadX::aac(3)IV* mutant. The mutant strain *S. venezuelae* ISP5230 VS1099 (Δ*jadW2::aac(3)IV*) was selected as a positive Jd control. The *jadW2* gene is involved in regulation of Jd production.[28] This mutant shows an increase in Jd production by a factor of 2−5 compared to that of WT and has been successfully utilized as a production strain to increase yields of several Jd analogues.[29−32] To aid in both the analysis of the growth media and NMR binding studies, growth was carried out in the presence of D-serine (60 mM) as the sole nitrogen source. It is well-established in the literature that growth of *S. venezuelae* in the presence of serine (D-, L-, or DL-) as the sole nitrogen source slows cellular growth while facilitating higher natural product production, especially Cm.[33−35] When subjected to shock conditions (ethanol), the bacteria switches natural product production to JdDS (structure found in Figure 2B).[36,37] We have found that this compound is produced at higher levels than is JdB, allowing for straightforward detection by LC-MS/MS analysis. All three strains were successful in utilizing the D-serine minimal media as a nutrient source, growing to comparable OD_{600} values (Figure 1B). This suggested that any changes in natural product profiles were not a result of differences in cell proliferation. The jadomycin family of natural products are colored compounds typically absorbing light at ∼526 nm. Measuring A_{526} qualitatively estimates production of jadomycin and jadomycin-like compound production within the growth media.[32] A_{526} values illustrated a significant reduction in the production of colored compounds from Δ*jadX::aac(3)IV* compared to that of the Δ*jadW2::aac(3)IV* and WT strains. LC-MS/MS analysis of the growth media after 48 h identified that media concentrations of JdDS were highest for Δ*jadW2::aac(3)IV* (∼64 μM) followed by that for the WT strain (∼25 μM) (Figure 1G). The Δ*jadX::aac(3)IV* mutant produced concentrations of JdDS below the limit of accurate detection by LC-MS/MS analysis (∼2 μM). These results were recapitulated using L-isoleucine as the nitrogen source, and a comparable loss in JdB production was observed (Figure S3D).

To determine whether the natural product HPLC profile of Δ*jadX::aac(3)IV* was altered, a crude purification was performed by passing clarified growth media down a reversed-phase phenyl column to trap natural products. These natural products were eluted with methanol and analyzed by

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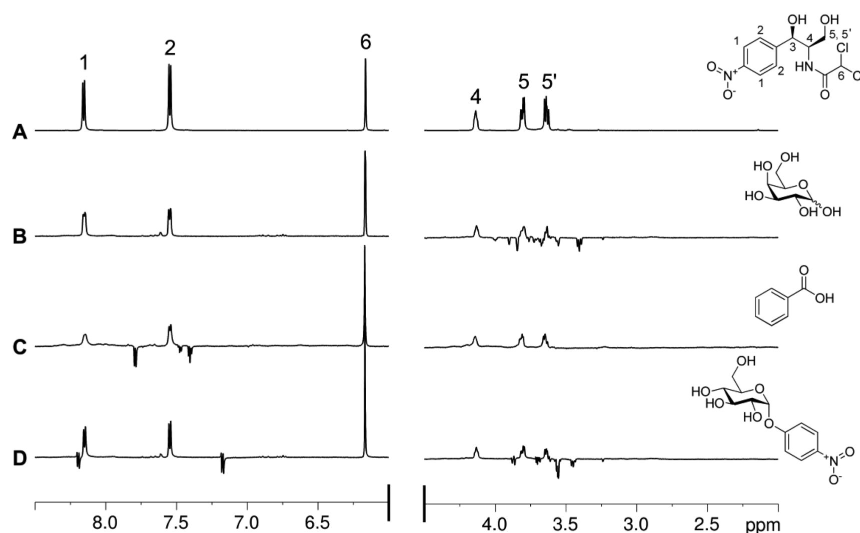

Figure 3. (A) Standard ^{1}H NMR spectrum of Cm (2 mM); (B−D) Overlaid WaterLOGSY NMR spectra (700 MHz, 1:9 dPBS/PBS, pH 7.6) with JadX (0.1 mM) illustrating JadX-Cm (2 mM) binding (positive phasing) in the presence of nonbinding compounds (2 mM, negative phasing) (B) D-galactose, (C) benzoic acid, and (D) 4-nitrophenyl α-D-glucopyranoside.

HPLC (Figure 1E). Both the Δ*jadW2::aac(3)IV* and WT showed a JdDS peak at 9 min. As expected on the basis of the LC-MS/MS analysis, the Δ*jadX::aac(3)IV* HPLC profile showed an absence of JdDS, but a new signal appeared at 6.5 min (Figure 1E). This peak was identified as Cm via HPLC-spiking experiments (Figure 1F) and LC-MS/MS analysis. LC-MS/MS analysis quantitating Cm production was performed with crude media from each strain. It was found that neither the WT nor Δ*jadW2::aac(3)IV* produced quantifiable amounts of Cm. The Δ*jadX::aac(3)IV* mutant produced Cm to a final concentration of 23 μM after 48 h (Figure 1G). This value is comparable to reported chloramphenicol production by *S. venezuelae* ISP5230 in similar media.[38] The complete loss of JdDS and JdB production illustrates the importance of JadX for Jd biosynthesis. Cm production by the Δ*jadX::aac(3)IV* mutant may be explained by the loss of production of Jd. The two antibiotics are heavily cross-regulated; it is believed that this regulation is antagonistic in fashion, with Jd production eliminating Cm biosynthesis and vice versa.[12] This may account for the shift in the natural product profile of the Δ*jadX::aac(3)IV* mutant, further illustrating the complex cross-regulation of these compounds.

JadX Binds Jadomycins and Chloramphenicol. In an effort to provide further insight into the function of JadX, protein−ligand binding by NMR methodologies was conducted. JadX-His$_6$ was cloned and recombinantly expressed in *Escherichia coli* BL21(DE3) and purified. The two ligand-observed NMR methods tested for their screening capabilities were saturation transfer difference (STD) NMR[39,40] and water ligand-observed via gradient spectroscopy (WaterLOGSY)[41] methods previously utilized in our lab.[42,43] WaterLOGSY proved to be a more sensitive method compared to STD-NMR and as such was used exclusively for this study. WaterLOGSY also benefits from being able to observe directly both nonbinding (phased negatively) and binding (phased positively) compounds simultaneously in a 1D spectrum, allowing for the inclusion of nonbinding controls to aid in data interpretation. Several small molecules were screened as potential JadX ligands. The only compounds found to bind with JadX were Jds and Cm. It was found that JadX was capable of binding a diverse series of Jds. Both the hydrophobic (L and B) and hydrophilic (DS and N) Jds showed interaction with JadX (Figure 2A−D). JdDS possessed excellent water solubility, was easily purified, and was used for all subsequent binding studies. We were confident that the JadX−JdDS interaction was specific because of the ability of JadX to bind the selection of Jds. Additionally, JadX did not bind benzoic acid, illustrating that the interaction was not dominated by the carboxylic acid present on JdDS (Figure 3C) or a nonspecific binding event mediated by aromatic ring systems. More interesting was the unexpected result that JadX bound the antibiotic Cm (Figure 3B). To test the specificity of this interaction, WaterLOGSY experiments were conducted with JadX in the presence of Cm and a series of other small molecules. In the presence of known nonbinders, D-galactose and benzoic acid, JadX showed selective binding to Cm (Figure 3B−D). In the presence of a structural mimic, 4-nitrophenyl α-D-glucopyranoside, JadX bound Cm preferentially (Figure 3D). This illustrated that the JadX−Cm interaction is specific and is not dictated by the presence of a para-nitrophenyl group. It also suggests that the chlorinated amide "tail" may play an important role in binding. Tan and co-workers have previously shown the Jd regulator JadR2 to bind Cm, JdA, and JdB, resulting in release from the Jd gene cluster promoter, upregulating the transcription of *jadR1*.[12] The *jadR1* gene product then goes on to inhibit Cm production directly by binding a promoter within the Cm gene cluster. The cross-regulation of secondary metabolite biosyntheses in *Streptomyces* is becoming a well-established phenomenon. Because JadX has no documented catalytic activity and is capable of binding the two end product secondary metabolites coupled with loss of Jd production and an increase of Cm production in the Δ*jadX::aac(3)IV* mutant, we suggest that it possesses an important regulatory or signal receptor function involved in the biosynthetic coordination of these two pathways. This work is the first to identify JadX, and likely other JadX-like homologues, as a new class of antibiotic receptor proteins involved in the modulation of natural product biosynthesis.

Quantification of JadX-Ligand Binding. With the discovery of Cm and JdDS as JadX ligands, our focus shifted

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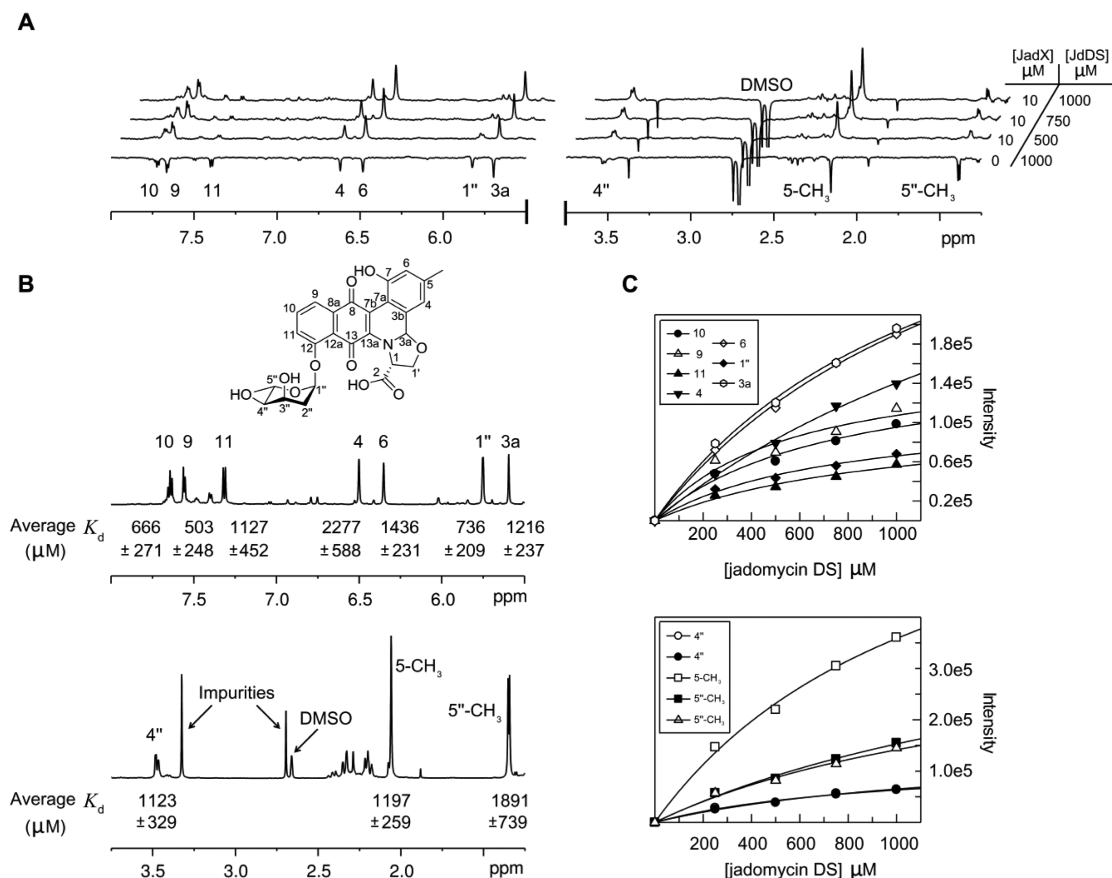

Figure 4. (A) Labeled overlaid WaterLOGSY NMR spectra of varying JdDS concentrations in the absence of (negative control) and presence of (binding) JadX. Concentrations are listed to the right of each spectrum. Nonbinding compounds are phased negatively, and binding compounds are phased positively. (B) Labeled structure of JdDS and labeled ^{1}H NMR spectrum of JdDS with the average K_d values associated with each signal listed below. K_d values of doublets and triplets are an average of the K_d values associated with each individual peak. Errors associated with each K_d are listed below. (C) Select dose−response curves including best fit curves (solid lines) used to calculate K_d values.

to measuring ligand affinity. WaterLOGSY is not only a qualitative screening tool for protein−ligand interactions but also can be implemented to determine dissociation constants (K_d) of protein−ligand systems.[44,45] This is accomplished through a series of experiments keeping protein concentration constant while varying ligand concentration. Signal intensity can be plotted versus ligand concentration giving a hyperbolic-like curve. Control experiments for ligand in the absence of protein must also be generated to correct the loss of signal intensity associated with free ligand. Taking the difference of these experiments produces a typical dose−response curve (Figure 4) that can be fitted using nonlinear regression, estimating K_d (Equation S1).[44] Individual peak intensities can be plotted as a function of ligand concentration giving a series of K_d values associated with individual protons on the ligand.

Signal intensities of JdDS and Cm were measured over a range of concentrations in the presence and absence of JadX. Because of solubility issues with both ligands, NMR samples were supplemented with 5% (v/v) DMSO-d_6 to improve natural product solubility. DMSO-d_6 also served as a non-binding control, phasing in the opposite orientation compared to that of binding molecules in the resultant spectra (Figure 4A and 5A). This allowed for easier interpretation of the results because the DMSO-d_6 peak always phased negatively in the presence or absence of JadX. ^{1}H-signals for both natural products that produced acceptable dose response curves were analyzed (Figure 4C and 5C). For multiplets (doublets and triplets), each peak was analyzed separately, and the listed K_d values represent the average of all peaks within a given multiplet (Figure 4B and 5B). Calculated values for JdDS ranged from 503 to 2277 μM, with an average value of 1217 μM (Figure 4B,C). The majority of these values were associated with protons on the polyaromatic backbone. Unfortunately, not all signals could be analyzed. Peaks close to the HOD had inconsistent signal intensities, whereas other observed changes in intensities were not large enough to generate accurate dose−response curves, which explains the spread of calculated K_d values. Of the difficult to quantitate signals, protons associated with L-digitoxose were particularly challenging, with many suffering poor difference intensities. This likely illustrates JadX selectively binding to the aromatic backbone, although the L-digitoxose sugar may not be directly bound. Impurities associated with the JdDS sample also consistently phased negatively in the presence and absence of JadX, further illustrating selective protein−ligand interaction (Figure 4A). For Cm, all signals, with the exception of H3 and H4 (too close to HOD signal), were suitable for K_d quantification. Values ranged from 897 to 1670 μM with an average of 1181 μM (Figure 5A−C). These values are comparable to JdDS K_d values, suggesting that JadX has comparable affinity for both natural products.

Competitive Ligand Binding Experiments. To test whether Jd and Cm are bound in a competitive fashion, JadX WaterLOGSY experiments in the presence of both JdDS and

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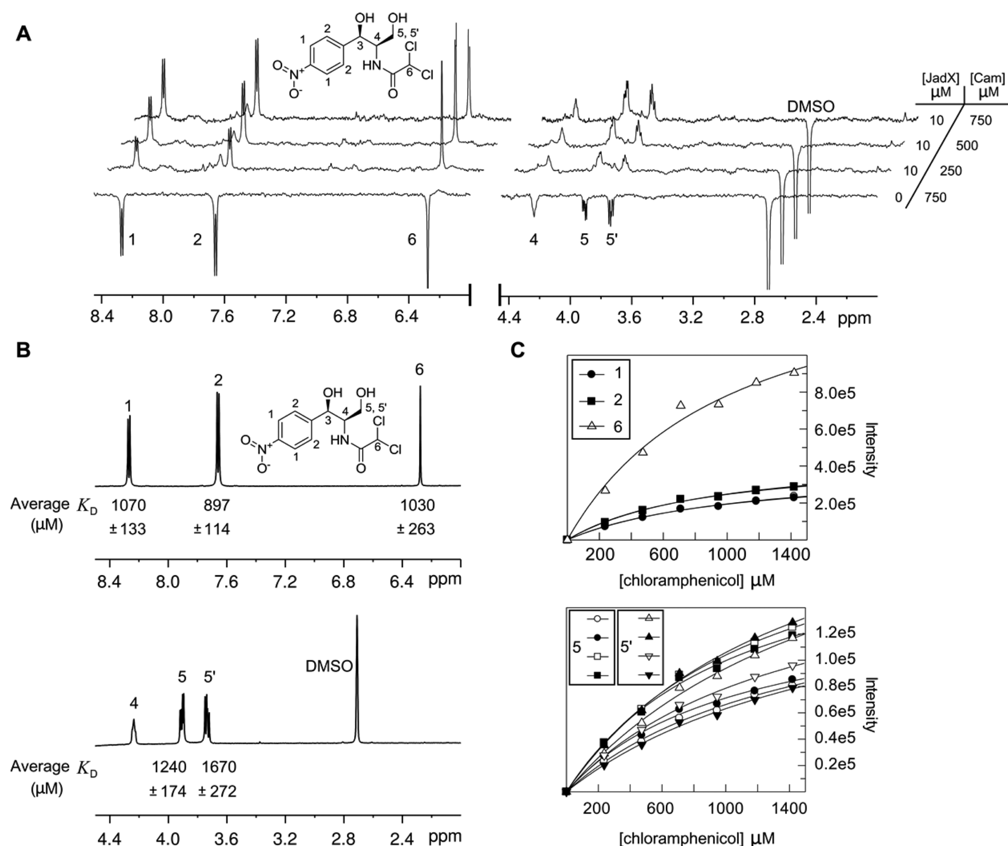

Figure 5. (A) Labeled overlaid WaterLOGSY NMR spectra of varying Cm concentrations in the absence of (negative control) and presence of (binding) JadX. Concentrations are listed to the right of each spectrum. Nonbinding compounds are phased negatively, and binding compounds are phased positively. (B) Labeled structure of Cm and labeled ^{1}H NMR spectrum of Cm with the average K_d values associated with each peak listed below. K_d values of doublets and triplets are an average of the K_d values associated with each individual signal. Errors associated with each K_d are listed below. (C) Select dose−response curves including best fit curves (solid lines) used to determine K_d values.

Cm were conducted. By keeping one ligand at a high constant concentration while varying the other in the presence of JadX, K_d values can be estimated and compared to values in the absence of the other ligand. If a known binder phases negatively in the presence of high concentrations of another ligand, then it illustrates a competitive binding interaction where both ligands are competing for the same site. For both sets of experiments, both natural products appeared to simultaneously bind, with signals from each compound phasing positive in the WaterLOGSY spectra (Figure S12−S13 and S15−S16). Monitoring the signal intensities of the natural product at lower concentrations resulted in typical dose−response curves. For both sets of experiments, K_d values were marginally higher. For Cm binding JadX in the presence of JdDS the K_d value was 1426 μM (versus 1219 μM in the absence of JdDS), and for JdDS binding JadX in the presence of Cm, the K_d value was 3262 μM (versus 1532 μM in the absence of Cm, Table S3).

The fact that both ligands appear to bind JadX simultaneously may be explained through a noncompetitive binding model in which each ligand has a distinct binding site and binding is independent of one another. The dual binding may also be an artifact of the NMR experiment, similar to the INPHARMA experiments conducted by Carlomagno and coworkers.[46] The first natural product could bind, receive the magnetization transfer from the protein, and then leave the binding pocket opening the site for the second natural product, resulting in both phasing positively. Unfortunately, this ligand-observed binding approach does not conclusively determine the presence of several binding sites or identify whether ligands interact with different residues on the protein.

Protein Observed Ligand Binding. To complement the WaterLOGSY data and to identify if Cm and JdDS interact differentially with JadX, K_d determinations by ^{1}H−^{15}N HSQC chemical shift perturbations were conducted. Singly labeled ^{15}N-JadX-His$_6$ was prepared and purified.[47] At pH 7.6, there were few ^{1}H−^{15}N HSQC cross peaks. To address this, the pH of the PBS was dropped to pH 6.6 to slow H−D exchange, resulting in an increase in the observable number of cross peaks (Figure S18). To ensure no appreciable loss in binding associated with the pH change, ^{15}N-JadX-Cm WaterLOGSY binding experiments were conducted at both pH values. It was found that ^{15}N-JadX bound Cm at both pH values, with minimal loss in signal intensities (Figure S19). With WaterLOGSY results comparable at pH 6.6 and 7.6, a series of ^{1}H−^{15}N HSQC experiments were recorded with ^{15}N-JadX in the presence of increasing concentrations of Cm or JdDS. In both series of HSQC titrations, JdDS and Cm resulted in perturbations of different ^{1}H−^{15}N-JadX cross-peaks (Figure 6A,B). Unfortunately, none of the Cm-induced chemical shift perturbations were large enough to quantify reliably (Figure 6A). Varying the JdDS concentration resulted in a more pronounced shift in the cross peaks (Figure 6B), with one signal (Figure 6C) undergoing a significant chemical shift change of 30−40 Hz between the no-ligand control and the maximum ligand/protein (3:1) ratio. Plotting $\Delta\delta_{obs}$ as a function of ligand concentration gave a protein−ligand binding

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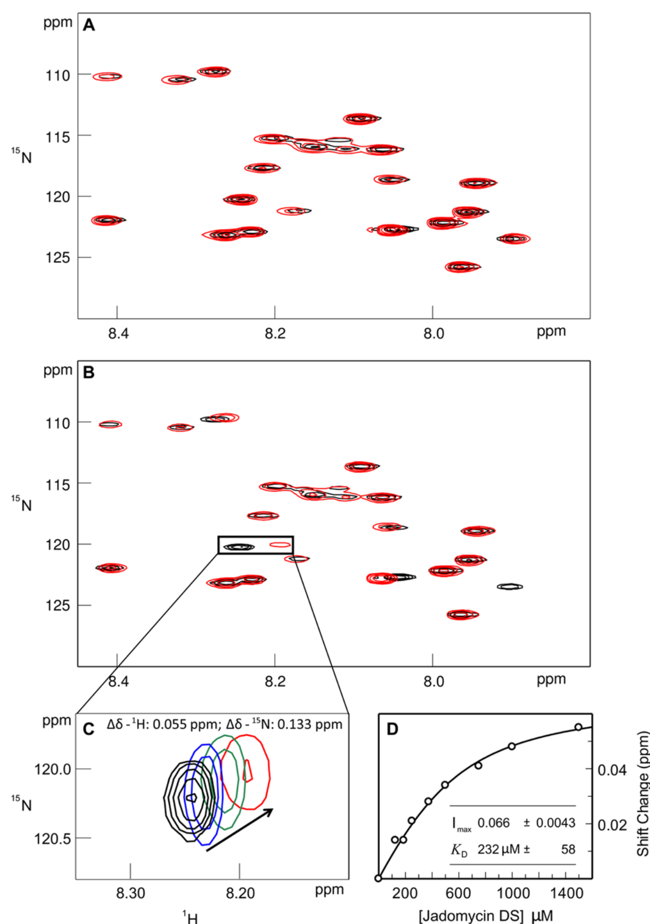

Figure 6. (A) Overlaid ^{15}N−^{1}H HSQC spectra of ^{15}N-JadX (500 μM, pH 6.6) in the absence (black) and presence (red) of Cm (2000 μM). (B) Overlaid ^{15}N−^{1}H HSQC of ^{15}N-JadX (500 μM, pH 6.6) in the absence (black) and presence (red) of JdDS (1500 μM). (C) Overlaid ^{15}N−^{1}H HSQC spectra of ^{15}N-JadX (500 μM) in the presence of varying JdDS concentrations: 0 μM (black), 187.5 μM (blue), 500 μM (green), and 1500 μM (red). The maximum ^{1}H and ^{15}N $\Delta\delta$ values are shown. (D) Representative dose−response curve with best-fit curve (solid line). The estimated K_d and maximum $\Delta\delta$ (I_{max}) are shown.

curve (Figure 6D) that was fit using nonlinear regression (Equation S2).[48] The measured K_d value of 232 μM was lower than that observed with the WaterLOGSY methodology and significantly tighter than the nonspecific binding[48] we have recently observed for JdDS interactions with a series of unrelated enzymes.[49] K_d values determined by WaterLOGSY have been reported to underestimate affinity because of experimental variables.[48,50] Additionally, pH differences between the two experiments may have affected the apparent K_d values. It is likely that the K_d calculated by ^{1}H−^{15}N HSQC chemical shift perturbations (232 μM) is more representative of the true dissociation constant associated with JadX−JdDS binding. This concentration is within the realm of measured jadomycin concentrations associated with fermentations of the WT and Δ*jadW2::aac(3)IV* strains. Additionally, these concentrations are final concentrations of the natural products in free solution and are potentially lower relative to concentrations within the bacterial cells, likely making the calculated K_d more physiologically relevant.

The binding of both JdDS and Cm to JadX was also monitored by ^{1}H−^{15}N HSQC titration through a series of experiments where a sample of constant JdDS and JadX was titrated with increasing Cm (Figure S20). Under these conditions, one cross peak shifted by 38 Hz (proton dimension) that was not observed to shift upon titration of Cm into JadX. The same cross peak was observed to broaden and disappear, but not to shift (Figure S20B) upon titration of JdDS into JadX. The broadening may be indicative of a slow or conformational change.[48]

A comparison of the ^{1}H−^{15}N HSQC spectra recorded with both ligands versus a spectrum free of ligand or in the presence of a single ligand showed several changes in chemical shift (Figure S21). These observations are supportive of simultaneous binding of Cm and JdDS to JadX. That Cm induces a significant perturbation only in the presence of JdDS may hint at a change in protein conformation that ties into a cross-regulatory function for JadX. Additional mechanistic insight from a structural perspective is underway and will be reported in due course.

■ DISCUSSION

Functional characterization of proteins involved in natural product biosynthesis with little or no sequence homology to current protein sequence databases remains a challenging functional scientific problem. Modeling of these pathways is very important from an industrial perspective as strong understanding of the enzymatic and regulatory systems in a biosynthetic pathway facilitates bioengineering approaches to improve yields, to shift production from one natural product to another, or to biosynthesize new derivatives.

Our finding that *jadX* disruption led to a complete loss of Jd production suggests a more important role for JadX in Jd biosynthesis than that previously established.[14] A similar finding associated with the JadX homologue MmyY, coded for in the methylenomycin gene cluster, has been observed.[26] A disruption of *mmyY* with an apramycin-resistance cassette (Δ*mmyY::aac(3)IV*) resulted in complete loss of production of methyleneomycin.[26] This further illustrates that these JadX-like homologues play vital roles in their respective biosynthetic pathways. Disruption of *jadX* also led to a switch in natural product biosynthesis from JdDS to Cm, suggesting a role in regulation of both natural products. There are six known regulatory genes in Jd biosynthesis (*jadW1*, *W2*, *W3*, *R1*, *R2*, *R3*, and *R**) located at the beginning and end of the Jd gene cluster.[14,28,51−53] The cluster-situated regulatory gene products of *jadR1* and *jadR2* are involved in cross-regulation of Jd and Cm biosynthesis and are located upstream of the polyketide synthase structural genes.[12,54] We have identified that *jadX* located upstream of the sugar biosynthetic genes likely codes for an unusual natural product binding protein. It also plays a significant role in the levels of Jd produced in the *S. venezuelae* ISP5230. Protein−ligand NMR binding methodologies qualitatively identified Jds and Cm as JadX ligands, with binding shown to be specific. WaterLOGSY methodologies also quantified K_d values of these protein−ligand interactions and showed JadX to have similar affinity for these structurally diverse natural products. Protein observed binding was also illustrated using ^{15}N-JadX to support the WaterLOGSY results. These results imply that JadX is likely a nonenzymatic protein capable of selectively binding the end product Jd family of natural products and Cm with similar affinity, coupled with the lack of production of Jd in the blocked mutant strain. This suggests that JadX may have a regulatory or cell signaling role and responds to these natural product signals to elicit a physiological response. Both the competitive binding and

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$^{1}H-^{15}N$ HSQC experiments identified a likely noncompetitive model for the two ligands with two separate JadX binding sites. The data also highlights the complex cross-regulation of these two disparate natural products. It is likely that other JadX-like gene products coded for in their respective biosynthetic gene clusters may function as natural product binding proteins. Our research illustrates that protein−ligand binding by NMR spectroscopy can be applied to proteins of unknown function in complex biosynthetic systems to aid in functional characterization.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b11286.

> Homology alignment, experimental procedures for JadX cloning and recombinant production, media, characterization data, and binding data are described. (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*david.jakeman@dal.ca

Notes

The authors declare no competing financial interest.

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