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The Albicidin Resistance Factor AlbD Is a Serine Endopeptidase That Hydrolyzes Unusual Oligoaromatic-Type Peptides

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Supporting Information

ABSTRACT: The *para*-aminobenzoic acid-containing peptide albicidin is a pathogenicity factor synthesized by *Xanthomonas albilineans* in infections of sugar cane. Albicidin is a nanomolar inhibitor of the bacterial DNA gyrase with a strong activity against various Gram-negative bacteria. The bacterium *Pantoea dispersa* expresses the hydrolase AlbD, conferring natural resistance against albicidin. We show that AlbD is a novel type of endopeptidase that catalyzes the cleavage of albicidin at a peptide backbone amide bond, thus abolishing its antimicrobial activity. Additionally, we determined the minimal cleavage motif of AlbD with substrates derived by chemical synthesis. Our results clearly identify AlbD as a unique endopeptidase that is the first member of a new subfamily of peptidases. Our findings provide the molecular basis for a natural detoxification mechanism, potentially rendering a new tool in biological chemistry approaches.

Bacteria of the genus *Xanthomonas* belong to a large group of exclusively plant-associated pathogens causing enormous losses worldwide in various economically important crops.[1] *Xanthomonas albilineans* is a xylem invading plant pathogen, provoking leaf scald disease in sugar cane plants. In previous studies, it has been found that the major phytotoxin of *X. albilineans* is correlated with a small antibiotic called albicidin.[2] Albicidin inhibits chloroplast DNA replication, which results in blocked chloroplast differentiation and is characterized by white foliar stripes on sugar cane leaves.[3] Apart from the significance of albicidin for the phytopathogenicity of *X. albilineans*, albicidin shows remarkable antibiotic activity at nanomolar concentrations to a wide range of Gram-positive and Gram-negative bacteria, e.g., *Enterobacter aerogenes*, *Escherichia coli*, *Haemophilus influenza*, *Klebsiella pneumonia*, *Shigella sonnei*, and *Staphylococcus aureus*.[4] The molecular target of albicidin is the GyrA subunit of the DNA gyrase (topoisomerase II), which is present in chloroplasts of plant cells and in bacteria.[3] Recently, we succeeded in elucidating the structure of albicidin.[5] Albicidin (**1**) is a linear oligoaromatic-type peptide (Figure 1A), which is assembled by a hybrid polyketide synthase/non-ribosomal peptide synthetase (PKS/NRPS) complex.[2,6] Albicidin is composed of an N-terminal PKS-derived 2-methylcoumaric acid (MCA-1) attached to an NRPS-derived pentapeptide composed of two 4-aminobenzoic acids (*p*ABA-2 and *p*ABA-4), the unusual α-amino acid β-cyano-L-alanine (Cya-3), and two 4-amino-2-hydroxy-3-methoxybenzoic acids (*p*MBA-5 and *p*MBA-6). Owing to its unique properties, albicidin is considered as a lead structure, potentially rendering a clinically useful next-generation antibiotic. At this stage, understanding of bacterial resistance factors has a significant impact on drug development. For albicidin, various resistance strategies have already been described in literature, including resistance conferred by reduced uptake as well as non-catalytic detoxification processes.[7−12] In search of biocontrol agents for leaf scald disease of sugar cane, Zhang and Birch reported on a resistance mechanism from *Pantoea dispersa*, a Gram-negative Enterobacterium.[13,14] Accordingly, the *P. dispersa* enzyme AlbD was found to detoxify albicidin and to attenuate pathogenicity of *X. albilineans*. AlbD had been classified as an esterase and discussed as a possible antipathogenic approach for disease control by its expression in transgenic sugar cane plants.[15] However, the exact mechanism of albicidin detoxification remained unknown due to the lack of knowledge of the molecular structure of albicidin. The recent success in elucidating the chemical structure and developing the total synthesis of albicidin paved the way for reinvestigating the AlbD-mediated detoxification mechanism as well as its substrate specificity.

We expressed recombinant AlbD from a synthetic gene in *E. coli* and purified the protein to homogeneity (see Supporting Information (SI)). We then tested the bioactivity of purified AlbD *in vitro* by co-incubation with synthetic albicidin.[5] Agar diffusion assays revealed that purified AlbD is capable of efficiently abolishing the antibiotic activity of albicidin (SI Fig. 1). To monitor the reaction catalyzed by AlbD, we performed *in vitro* assays with albicidin and analyzed the hydrolysate by means of HPLC-HR-ESI-(+)-Orbitrap-MS. Incubation of **1** (molecular formula $C_{44}H_{39}O_{12}N_6$, ($[M - H]^-$ = 841 Da) with AlbD yields two cleavage products (Figure 1B,C): fragment **2** (molecular formula $C_{28}H_{24}O_6N_4$, $[M - H]^-$ = 511.18 Da) and fragment **3** (molecular formula $C_{16}H_{16}O_7N_2$, $[M - H]^-$ = 347.10 Da). Based on the structure of albicidin and the fragment masses observed, our findings show that AlbD is an endopeptidase rather than an esterase. The endopeptidase cleaves albicidin at the central amide bond between the building

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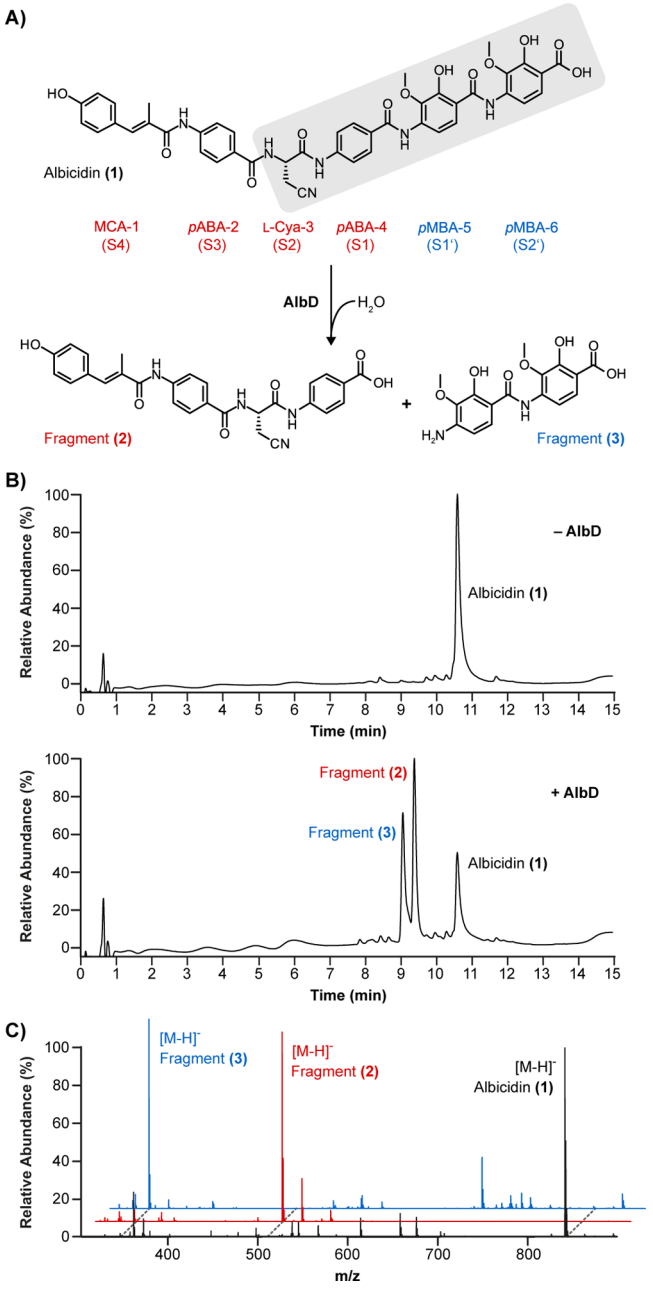

Figure 1. Hydrolysis of albicidin by AlbD. (A) Structure of albicidin (**1**) and the two fragments (**2** and **3**) observed upon incubation with AlbD: the N-terminal acyl-tripeptidyl fragment (**2**, MCA-*p*ABA-Cya-*p*ABA) and the C-terminal dipeptidyl fragment (**3**, *p*MBA-*p*MBA). Residues according to Schechter and Berger are given in parentheses.[16] The minimal cleavage motif is highlighted in gray. (B) HPLC-UV traces (308 nm) of albicidin samples incubated for 3 h at 28 °C in the absence (top) and presence of AlbD (bottom). (C) Staggered overlay of the mass spectra (ESI, negative mode) of the corresponding peaks using the same color code.

blocks *p*ABA-4 and *p*MBA-5 (Figure 1A), resulting in fragment MCA-*p*ABA-L-Cya-*p*ABA (**2**) and fragment *p*MBA-*p*MBA (**3**).

Subsequently, we addressed elucidating the minimal cleavage motif of AlbD, which is defined as the smallest structural motif of albicidin still being recognized and hydrolyzed by the enzyme. This information is of interest for understanding the mode of resistance triggered by AlbD. For this purpose, truncated and terminally modified albicidin derivatives were synthesized (see SI).[5] These substrates were incubated with purified AlbD *in vitro*, and cleavage reactions were thereafter analyzed by HPLC-ESI-MS (see SI). Based on the active-site classification of peptidases by Schechter and Berger, substrate residues were named S1−S4 in the N-terminal direction of the cleavage site, whereas residues in the C-terminal direction of the cleavage site are referred to as S1′ and S2′ (Figure 1A).[16]

Table 1 highlights cleavage efficiencies of AlbD for representative substrates (results for all tested substrates are summarized in SI Tab. 4). With albicidin as the reference substrate, the minimal cleavage motif is confined as a tripeptide being composed of either *p*ABA-4 (S1)-*p*MBA-5 (S1′)-*p*MBA-6 (S2′) or L-Cya-3 (S2)-*p*ABA-4 (S1)-*p*MBA-5 (S1′) (Figure 1A). It thus seems that AlbD requires either the S2 or S2′ position occupied to efficiently cleave the substrate, since dipeptides comprising only the *p*ABA-4 (S1)-*p*MBA-5 (S1′) motif are not processed by AlbD. Interestingly, *enantio*-albicidin (*ent*-**1**), containing D-Cya-3, was revealed to be one of the weakest substrates for AlbD. This reflects the importance of the stereochemical arrangement of substrates in the S2 position (L-Cya-3) for efficient substrate cleavage (Table 1). As *ent*-**1** partially retains antibacterial activity and inhibitory activity toward DNA gyrase,[5] these are important insights for the design of AlbD-resistant albicidin analogues. N-Terminally truncated peptides, e.g., tetrapeptide **4** being devoid of residues MCA-1 (S4) and *p*ABA-2 (S3), appear to be better substrates than albicidin itself (Table 1 and SI Tab. 4). This result may reflect the fact that albicidin is not the natural substrate of AlbD. Since homologues of *albD* are found in several *Pantoea* species that are not associated with *X. albilineans* and in other genera than *Pantoea* (SI Fig. 5), the natural substrate of AlbD would be rather another unknown compound commonly found in *Pantoea*'s niches. The evolution of the albicidin-detoxifying activity of AlbD could then be attributed only to the chance encounter between *P. dispersa* and *X. albilineans* in sugar cane.

The absence of an N-terminally located positive charge in tripeptide **8** (residues S2, S1 and S1′) seems crucial for AlbD activity, as validated by using synthetic derivatives with acetylated N-termini (tripeptides **7** vs **8**). By contrast, replacing the C-terminal carboxy moiety of *p*MBA-5 by an amide (tripeptides **8** vs **9**) does not influence the cleavage efficiency significantly. A negative charge near the C-terminus, as it is present in albicidin, thus appears to be less important for molecular recognition. Interestingly, all truncated versions of albicidin-derived peptides (**4**, **5**, **7**−**10**) lack antibacterial activity (see SI). Accordingly, the structural integrity of the pentapeptide backbone is a requirement for antibacterial activity.

Furthermore, we studied the AlbD-mediated hydrolysis of modified tripeptide **6**, in which the *p*MBA building blocks found in albicidin had been replaced with 4-amino-3-isopropoxybenzoic acids (*p*IBA). A similar substitution pattern is observed in the structurally related cystobactamids—recently identified gyrase inhibitors produced by *Cystobacter* sp. Cbv34.[17] Our results show that cystobactamid-derived peptide **6** (Table 1 and SI Tab. 4) is also efficiently cleaved by AlbD, thus indicating that the sterically more demanding isopropyl substituent does not efficiently prevent peptide hydrolysis. Therefore, we deduce that the promiscuous endopeptidase AlbD most likely confers resistance against cystobactamids as well.

Given the high homology of AlbD to the family of serine endopeptidases of the α/β-fold hydrolase superfamily, the

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Table 1. Selected Substrates for the Determination of the Minimal Cleavage Motif of AlbD[a]

Substrate	Substrate residues						RCR[(1)]	SD[(2)]
	S4	**S3**	**S2**	**S1**	**S1'**	**S2'**		
albicidin (**1**)	MCA	*p*ABA	L-Cya	*p*ABA	*p*MBA	*p*MBA	1.00	0.00
ent-albicidin (ent-**1**)	MCA	*p*ABA	D-Cya	*p*ABA	*p*MBA	*p*MBA	0.35	0.05
tetrapeptide (**4**)			L-Cya	*p*ABA	*p*MBA	*p*MBA	1.77	0.03
tripeptide (**5**)				*p*ABA	*p*MBA	*p*MBA	1.44	0.01
modified tripeptide (**6**)				*p*ABA	*p*IBA	*p*IBA	1.21	0.15
tripeptide (**7**)			L-Cya	*p*ABA	*p*MBA		-0.25	0.34
acetylated tripeptide (**8**)		Ac-	L-Cya	*p*ABA	*p*MBA		1.77	0.03
amidated tripeptide (**9**)			L-Cya	*p*ABA	*p*MBA	-NH_2	0.35	0.07
dipeptide (**10**)				*p*ABA	*p*MBA		-0.13	0.22

[a]Residues are named according to the Schechter and Berger nomenclature.[16] The cleavage site is indicated by the red line. Ac = acetylated N-terminus, NH_2 = amidated C-terminus, RCR[(1)] = relative cleavage ratios determined by HPLC-MS in triplicate, SD[(2)] = standard deviation. For calculation of RCR, see SI. Note that an RCR value of 1.77 corresponds to complete hydrolysis of truncated derivatives and that RCR values represent end-point data (after 3 h incubation). Differences in the cleavage efficiency of better AlbD substrates compared to albicidin are thus not resolved in these experiments.

active site of this enzyme was identified as a putative catalytic triad: a nucleophilic serine (Ser105), a hydrogen-accepting histidine (His200), and an aspartic acid (Asp169) stabilizing the reaction complex (SI Fig. 2). To verify the function of the amino acids assigned to the catalytic triad of AlbD, we generated the AlbD variants S105A, H200A, and D169A (see SI), respectively. Far-UV circular dichroism (CD) spectra of these AlbD variants are highly comparable to the CD spectrum of the wild-type protein, displaying pronounced α-helical conformation (SI Fig. 3). These data are in agreement with the computational prediction of secondary structural elements (SI Fig. 2) and support the structural integrity of the protein variants. The AlbD variants were assayed for their ability to degrade albicidin in agar diffusion assays, demonstrating that none of the variants is able to impair the antibiotic activity of albicidin (SI Fig. 1). This ability was further tested by co-incubation of albicidin with AlbD and its Ala-substitution variants (AlbD-S105A, AlbD-D169A, and AlbD-H200A) under optimized conditions and subsequent LC/MS analysis to relatively quantify the conversion rate. The hydrolytic activity of AlbD-S105A and AlbD-H200A is completely abolished, whereas AlbD-D169A retains a minimal residual activity compared to wild-type AlbD (SI Tab. 3). These results confirm a direct involvement of the corresponding residues in the catalytic triad of AlbD. The residual activity of AlbD-D169A can be rationalized by partial stabilization of the reaction complex through other acidic residues in close proximity of His200.[18] Intriguingly, homology modeling using two different template structures yields a structural model of AlbD in which the identified catalytic triad Ser105-His200-Asp169 appears to be correctly positioned to exert hydrolytic activity (SI Fig. 4).

Based on the oligopeptidic character of albicidin, we reclassify AlbD as a serine endopeptidase. Consulting the MEROPS homology database,[19] which provides the most comprehensive overview of peptidases, led to a preliminary classification of AlbD as a member of the SC clan and the peptidase family S9. MEROPS summarizes the biological function of S9 peptidases as mainly degrading biologically active compounds. Members of the SC clan share the above-mentioned α/β-hydrolase fold as a common and superior structural element. The S9 family comprises four subfamilies, whereas the catalytic core motif of AlbD shows the closest homology to the S9D subfamily (S9D-stereotypical catalytic core motif: GGH**S**YGAFMT) but also exhibits specific sequence features (catalytic core motif of AlbD: VGH**S**L-GSVLL). Phylogenetic analysis based on currently available genome sequences indicates that AlbD shares at most 35% sequence similarity with members of the S9 family but reveals clades (50−95% sequence similarity) that potentially bear endopeptidase activity similar to that of AlbD of *P. dispersa* (SI Fig. 5).

To the best of our knowledge, AlbD is the only known example of a peptidase that is able to cleave an amide bond between two δ-amino acids, in which the α-carbon atom of residue n as well as the δ-carbon atom of residue n+1 are integral parts of the aromatic benzene ring. Given these specifications and the fact that the catalytic core motif which is characteristic for each peptidase subfamily differs especially in the C-terminal region, we suggest a novel subfamily of the peptidase family S9, with AlbD as a type example. Knowledge of the promiscuity of AlbD and its homologues from other bacterial strains, as well as more detailed investigations on natural and synthetic substrates, might support application of these endopeptidases as a tool for biological chemists. Engineered AlbD variants capable of processing amide-linked aromatic compounds other than albicidin could find useful applications in various areas. Based on the uniqueness of AlbD as an endopeptidase, future work is envisaged to obtain atomistic structure information on AlbD. Such data will help researchers to understand the mechanism of albicidin detoxification and can be exploited for improving albicidin as an antibacterial lead structure. This could be achieved by the directed synthesis of bioactive derivatives of albicidin, no longer vulnerable to the cleavage by peptidases like AlbD. Moreover, the design of more potent biocontrol agents and the creation of transgenic sugar cane cultivars will support the stable generation of clean and renewable energy sources in the future.

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■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, compound characterization, and supplementary figures. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b04099.

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Notes

The authors declare no competing financial interest.

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