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RESEARCH ARTICLE

Genetic basis of destruxin production in the entomopathogen *Metarhizium robertsii*

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Abstract Destruxins are among the most exhaustively researched secondary metabolites of entomopathogenic fungi, yet definitive evidence for their roles in pathogenicity and virulence has yet to be shown. To establish the genetic bases for the biosynthesis of this family of depsipeptides, we identified a 23,792-bp gene in *Metarhizium robertsii* ARSEF 2575 containing six complete nonribosomal peptide synthetase modules, with an *N*-methyltransferase domain in each of the last two modules. This domain arrangement is consistent with the positioning of the adjacent amino acids *N*-methyl-L-valine and *N*-methyl-L-alanine within the depsipeptide structure of destruxin. *DXS* expression levels in vitro and in vivo exhibited comparable patterns, beginning at low levels during the early growth phases and increasing with time. Targeted gene knockout using *Agrobacterium*-mediated transformation produced mutants that failed to synthesize destruxins, in comparison with wild type and ectopic control strains, indicating the involvement of this gene in destruxin biosynthesis. The destruxin synthetase (*DXS*) disruption mutant was as virulent as the control strain when conidial inoculum was topically applied to larvae of *Spodoptera exigua*, *Galleria mellonella*, and *Tenebrio molitor* indicating that destruxins are dispensable for virulence in these insect hosts. The *DXS* mutants exhibited no other detectable changes in morphology and development.

Keywords *Metarhizium robertsii* · Destruxins · NRPS · Secondary metabolism

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Introduction

Destruxins (DTX) are a family of cyclic depsipeptides produced by multiple plant and insect fungal pathogens (Pedras et al. 2002) (Fig. 1). The vast majority of the more than 35 members of the destruxin family have been isolated from strains belonging to the genus *Metarhizium* (Kodaira 1962; Pedras et al. 2002). These metabolites have been linked to a plethora of biological activities. Exogenously introduced DTX are highly toxic against a wide range of insect species (Amiri et al. 1999; Sree et al. 2008) and have been reported to accumulate in fungus-infected individuals (Suzuki et al. 1971; Vey and Goetz 1986). Injection, ingestion or topical application of purified DTX causes tetanic and flaccid paralysis, an effect linked to their abilities to depolarize Ca^{2+} gradients across the muscle plasma membrane (Samuels et al. 1988b; Dumas et al. 1996b; Hinaje et al. 2002), and the one that closely resembles symptoms observed in insects infected with destruxin-

producing *Metarhizium* strains (Kodaira 1962; Samuels et al. 1988a). Intriguingly, DTX injected into insects also negatively impact both their cellular (Dumas et al. 1996b; Vilcinskas et al. 1997; Vey et al. 2002) and humoral (Pal et al. 2007) immune responses, which reinforces the notion of their potential contribution to *Metarhizium* virulence. These metabolites also induce oxidative stress (Sree and Padmaja 2008), damage midgut epithelium, disrupt salivary gland integrity (Sowjanya Sree and Padmaja 2008), and have a powerful inhibitory effect on fluid secretion by Malpighian tubules in various arthropods (James et al. 1993; Dumas et al. 1996b; Ruiz-Sanchez et al. 2010). Reports of different levels of DTX production in vitro by several *Metarhizium* strains and differential insect sensitivity to the metabolites have provided correlative data to explain the host range of those isolates, as well as their rapidity of killing (Samuels et al. 1988a; Kershaw et al. 1999; Moon et al. 2008; Wang et al. 2009). All of these results have contributed to the status of DTX as *de facto* virulence factors for those fungi that produce them. However, definitive genetic proof of their contributions to virulence has been lacking. In this paper, we describe the identification and inactivation by targeted gene knockout of a nonribosomal peptide synthetase (NRPS) gene from *M. robertsii* ARSEF 2575, demonstrate its role in DTX biosynthesis, and show that abolition of DTX production does not alter its virulence against larvae of the susceptible insect hosts *Spodoptera exigua*, *Galleria mellonella*, and *Tenebrio molitor*.

Materials and methods

Strains, culture conditions, and *Agrobacterium tumefaciens*-mediated transformation (ATMT)

Metarhizium robertsii ARSEF 2575 (formerly known as *M. anisopliae*) (Bischoff et al. 2009) and its derivatives were grown on ¼-strength Sabouraud dextrose agar with yeast extract (SDAY/4) as previously described (Moon et al. 2008). For the analyses of destruxin production and in vitro gene expression, cultures were started with 1×10^6 conidia/100 ml HB broth (Giuliano Garisto Donzelli et al. 2010) and incubated in 250 ml flasks at 28°C in the dark on a rotary shaker at 150 rpm. ATMT and subsequent transformant handling were carried out as previously described, except that *A. tumefaciens* strain EHA105 was used instead of GV3101 (Moon et al. 2008; Giuliano Garisto Donzelli et al. 2010).

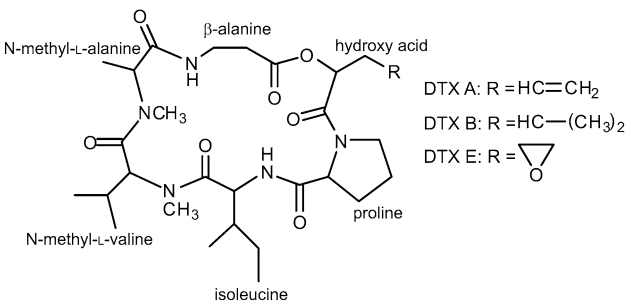


Fig. 1 Chemical structure of destruxin A, B and E

Cloning of the destruxin synthetase gene (*DXS*)

Based on the NCBI accession AJ273779 (Freimoser et al. 2003), we designed primers AJ273779F and AJ273779R (Table 1) and PCR screened a bacterial artificial chromosome (BAC) library constructed using *M. robertsii* ARSEF 2575 genomic DNA. BAC DNA from the positive clone 11F5 was partially sequenced at the Cornell University DNA Sequencing and Genotyping facility. The genomic region harboring the partial BAC sequence was identified using a draft of the *M. robertsii* ARSEF 2575 genome (Donzelli et al., unpublished).

Construction of pBDU-NRPS4KO

The binary vector pBDU-NRPS4KO was designed to target and disrupt the *DXS* gene. Two fragments from the *DXS* coding region (NRPS4-A and NRPS4-B, Fig. 5a), located 157 bp apart, were PCR amplified with the primer pairs NRPS4KO-A-F/NRPS4KO-A-R2 and NRPS4KO-B–F/NRPS4KO-B-R, respectively (Table 1), using ARSEF 2575 total DNA as the template. The *bar* expression cassette, which confers resistance to the drug bialaphos, was PCR amplified from pBARKS1 (Pall and Brunelli 1993) using the primers BarExprS-F and BarExprS-R-KS1 (Table 1). The binary vector pBDU-NRPS4KO was produced by assembling NRPS4-A, NRPS4-B, and *bar* into the *Xba*I- and *Nt.Bbv*CI-linearized vector pBDU in the presence of the Uracil-Specific Excision Reagent or USER enzyme as suggested by the manufacturer (NEB, Ipswich, MA). pBDU is a pPK2 (Covert et al. 2001) derivative modified as suggested by NEB (http://www.neb.com/nebecomm/products/productN5504.asp.) to allow USER cloning. PCR amplifications for USER-based cloning were conducted as described (Nour-Eldin et al. 2006; Geu-Flores et al. 2007; Frandsen et al. 2008).

Screening for homologous recombination events at the *DXS* locus

Screening for homologous recombination events at the *DXS* locus was conducted by PCR and subsequently confirmed by Southern analysis. Screening primers for primary transformants were C6-ScreenF and Ptrpc80R (Table 1;

Table 1 Primers used in this study

Name	Sequence 5′ → 3′	Remarks
AJ273779F	AGT TAC TGG GCG ACT TTG GA	Identification of BAC clone
AJ273779R	CCA TGA GCG ATT GCA AAA TA	Identification of BAC clone
NRPS4KO-A-F	GGG AAA GdUG GCA TGG TAG TCT GTA TC	pBDU-NRPS4KO construction
NRPS4KO-B-F	ACT TGT GGdU CAA GCG GCT ACC CAC AA	pBDU-NRPS4KO construction
NRPS4KO-B-R	GGA GAC AdUC AAC CTT GCC ATT CTC GT	pBDU-NRPS4KO construction
NRPS-KO-A-R2	ATC ATC CdUT CCG TGG TTC GTA AGC	pBDU-NRPS4KO construction
BarExprS-F	AGG ATG AdUA GAA GAT GAT ATT GAA GGA	pBDU-NRPS4KO construction
BarExprS-R-KS1	ACC ACA AGdU CCA ATT CGC CCT ATA GT	pBDU-NRPS4KO construction
C6-ScreenF	GCG AGC TAC TTG CCG TCT ACT	Initial transformant screening
Ptrpc80R	CCG CCT GGA CGA CTA AAC C	Initial transformant screening
NRPS4KO-screen-F	GGA GCT TTT CGA TGC GGA CAC TTA	KO and monoconidial purification confirmation; amplification of *DXS* probe; RT-PCR
NRPS4KO-screen-R	TGC TCT GCT GGC TTG GAA CTG TGT	KO and monoconidial purification confirmation; amplification of *DXS* probe; RT-PCR
BarF	GTC TGC ACC ATC GTC AAC	*Bar*-specific probe (Moon et al. 2008)
BarR	CGT CAT GCC AGT TCC CGT	*Bar*-specific probe (Moon et al. 2008)
Baw-Act-275F	CCC CAT CGA GCA CGG TAT CAT CA	*S. exigua* β-tubulin (Giuliano Garisto Donzelli et al. 2010)
Baw-Act-439R	ACA TGG CGG GGG AGT TGA AGG TCT	*S. exigua* β-tubulin (Giuliano Garisto Donzelli et al. 2010)
Ma-Btub-F1	CGT CGA CGA TAA TCC GCC AAC AT	ARSEF 2575 β-tubulin (Giuliano Garisto Donzelli et al. 2010)
Ma-Btub-R18	TTC AGG TCA CCG TAC GAA GGG T	ARSEF 2575 β-tubulin (Giuliano Garisto Donzelli et al. 2010)

dU deoxyuridine

Fig. 5a). PCR confirmation of both the *DXS* knockout (KO) event and purity of single conidial isolates made use of the primers NRPS4KO-screen-F and NRPS4KO-screen-R (Table 1; Fig. 5a). Southern analyses were carried out as previously described (Moon et al. 2008; Giuliano Garisto Donzelli et al. 2010) on *Sac*I- or *Sal*I-digested *M. robertsii* ARSEF 2575 total DNA. A PCR DIG Probe Synthesis Kit (Roche Applied Science, Indianapolis, IN), together with the primer couples NRPS4KO-screen-F/NRPS4KO-screen-R or BarF/BarR (Table 1), was used to synthesize DNA probes specific for either the NRPS4 (Probe 1, P1, Fig. 5a) or the *bar* (Probe 2, P2, Fig. 5a) coding regions, respectively.

RT-PCR for *DXS* expression

In vitro *DXS* expression was evaluated during growth in HB broth 19, 24, 48, 72, 96, 120, 168 and 240 h after medium inoculation with 1 ml of 10^6 conidia/ml harvested from 1-week-old cultures grown on SDAY/4. RNA extraction and reverse transcription conditions were as described (Giuliano Garisto Donzelli et al. 2010). Expression of *DXS* within fungus-infected *S. exigua* larvae at post-inoculation times of 28, 52, 72, 98, and 122 h, and 122-h non-motile larvae (early stage mummies, M1), 172-h non-motile larvae (late stage mummies, M2), and from controls 72 h after a mock inoculation followed a method previously described (Giuliano Garisto Donzelli et al. 2010). Primer couples used for the analyses were Baw-Act-275F/Baw-Act-439R, Ma-Btub F1/Ma-Btub-R18, and NRPS4KO-screen-F/NRPS4KO-screen-R, which anneal to the *S. exigua* β-tubulin, *M. robertsii* β-tubulin, and *DXS* genes, respectively (Table 1).

Detection of DTX

Production of DTX by the WT strain ARSEF 2575, the *DXS* KO strains 2.1 and 4.1, and the ectopic integrants 1.1 and 5.1 was determined by quantitative HPLC analysis of the major DTX components (A and B) in solid phase-extracted culture filtrate. All samples analyzed were from HB broth cultures grown for 5 days with rotary shaking (150 rpm) at 28°C. Solid phase extraction (SPE) was accomplished by loading 5 ml aliquots of culture filtrates onto a C18-SPE cartridge (200 mg; Alltech #40515) conditioned with 10 column volumes each of MeOH, and then deionized H_2O. The charged cartridges were rinsed with 10 ml water and then eluted with 2 ml MeOH. The MeOH eluates were then dried under a nitrogen stream with gentle heating and reconstituted for analysis in 10 ml of MeOH for the positive controls (extracts from ARSEF 2575 and ectopic integrants), and 1 ml for the DTX-deficient extracts (*DXS* KO strains and uninoculated broth extracts). Thus, extracts of the KO strains and culture medium alone were

analyzed at a tenfold higher concentration (relative to the culture filtrate volume) than those of the WT and ectopic strains, to insure detection of trace amounts of DTX, if present, in the former samples. The samples were analyzed by two different HPLC methods: (1) a relatively insensitive method using UV detection to estimate DTX production by the WT and ectopic strains, and (2) a more sensitive method using detection by MS–MS multiple reaction monitoring (MRM) to establish a lower limit of detection (LOD) for extracts of the KO strains.

In method 1, 10-µl aliquots of extract were injected onto an RP C18-A column (Varian Polaris, 250 mm × 4.6 mm, 5-µm particle, 180-Å pore), and eluted with acetonitrile:water (1:1) at a flow rate of 1 ml/min (Waters 600 pump), with detection by UV absorbance at 220 nm (extracted from a 190–350 nm scan on a Waters 996 diode array detector). DTX A and B were estimated using a standard curve for each compound. The LOD (established as $S/N = 3$) was 7- and 9-ng on-column, or 135- and 186-µg/L broth, for DTX A and B. DTX standards were purified from WT culture filtrates, and their identities were verified by ^{1}H NMR on a Varian INOVA 600 spectrometer. DTX E, the third major component of the *M. robertsii* destruxin profile, has a labile epoxide group in the hydroxy acid side chain. It appears to co-vary quantitatively with DTX A and DTX B but, due to its instability, it was not included in the quantitative analysis.

In method 2, 5-µl aliquots were injected onto an RP-C18 column (Phenomenex Prodigy ODS3, 150 × 2 mm, 5-µm particle, 100-Å pore) eluted with acetonitrile:water:formic acid (650:350:1) at 0.25 ml/min, with detection by low resolution electrospray mass spectrometry on an ABI-Sciex Q-Trap 2000 spectrometer operated in positive ion mode. The pseudomolecular ions [M + H]+ of DTX A and B were observed at *m/z* 578 and 594, respectively. Accordingly, six MS–MS parent ion → product ion transitions were monitored, 578 → 465, 437, and 342 for detecting DTX A, and 594 → 481, 453, and 368 for detecting DTX B (as well as DTX E, which is isobaric to DTX B at unit mass resolution). Declustering and collision voltages used for all MS–MS experiments were 71 and 39 V, respectively. The LOD ($S/N = 3$) for this method was 12- and 16-pg on-column, or 0.5- and 0.6-ng/L broth for DTX A and B, respectively.

Insect virulence assays

Eggs of *S. exigua* were obtained from Benzon Research (Carlisle, PA). Newly hatched larvae were reared on BAW diet that contained chlortetracycline, methyl paraben and potassium sorbate (product no. F9220B, Bio-Serv, Frenchtown, NJ) at 25°C and 15:9 h light:dark regime until reaching the 2nd instar. Larvae were then transferred to Southland Beet Armyworm (BAW) diet containing chlortetracycline (Southland Products Inc., Lake Village, AR) as the only antibiotic until assay setup (Moon et al. 2008) and then moved to Southland BAW diet without chlortetracycline for the duration of the virulence assays.

Spodoptera exigua 2nd instar larvae were inoculated immediately after molting. *G. mellonella* and *T. molitor* larvae were purchased from Berkshire Biological (Westhampton, MA) and 4th instar individuals were inoculated within a few hours of arrival.

Conidia were harvested from 10-day-old cultures by addition of 10 ml of 0.1% Silwet L-77 (Loveland Industries Inc., Greeley, CO) and gently scraping the surface with a sterile inoculation loop to dislodge conidia. Conidial suspensions were pipetted from the plate and filtered through two layers of cheesecloth into sterile 50-ml tubes (Becton–Dickinson Falcon, Sparks, MD) and vortexed for 5 min. All larvae were dipped for 10 s in conidia resuspended in 0.1% Silwet L-77 at 10^4–10^7 conidia/ml, depending on the assay (Table 3). Larvae dipped in 0.1% Silwet-L-77 alone were used as controls. In all instances, 24 larvae/treatment were assayed. Mortality and time-to-death inflicted by the WT and the *DXS* KO strain 4.1 were evaluated up to 7 days after inoculation. Two or three independent assays were carried out for *S. exigua* (two), *G. mellonella* (three) and *T. molitor* (three). After dipping, larvae were placed individually in 24-well plates with diet and incubated at 25°C with a 15:9 h light:dark photoperiod. Larvae were subsequently transferred to clean plates and provided fresh diet every day to avoid uncontrolled re-inoculation from fungus growing on diet, frass and exuviae.

Data analysis

Identification of functional domains in *DXS* and surrounding genes was carried out using InterProScan (Quevillon et al. 2005) and PFAM (Finn et al. 2010). *N*-methyltransferase domains were identified using motifs previously described (Weber et al. 1994; Ansari et al. 2008) and confirmed by comparing *N*-methyltransferase domains extracted from NRPSs with known *N*-methylated products to *C*- and *O*-methyltransferase domains from other proteins. To this end, multiple sequence alignment was carried out with Muscle (Edgar 2004), followed by cluster analysis based on the Maximum Likelihood method and WAG model, which assumed a Gamma distribution calculated on five discrete categories and a shape parameter of 4.3882, calculated using 180 positions in the available dataset and 500 bootstrap replicates. Both steps were conducted with the appropriate modules provided in MEGA5 (Tamura et al. 2011).

Data from insect virulence assays were analyzed using the survival analysis module provided with JMP 9.0.2

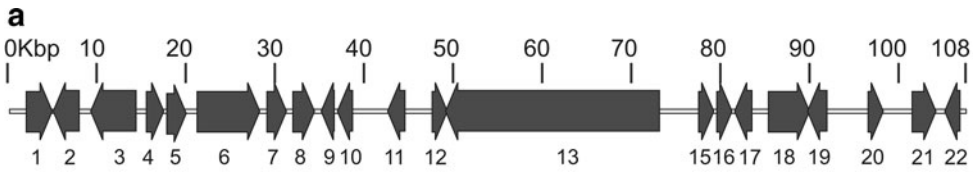
(SAS Institute Inc. Cary, NC). Survival curves calculated for insects inoculated with either the *DXS* KO strain 4.1 or the WT were compared with the Log-rank test.

Results

DXS gene isolation and structure

Initial cloning of the DXS gene was carried out using the cDNA sequence of NCBI accession number AJ273779. This clone was previously identified (Freimoser et al. 2003) from *M. anisopliae* ARSEF 2575 grown for 24 h in minimal medium containing 1% cockroach cuticles. Partial sequencing of a BAC clone containing AJ273779 yielded a ~14,000-bp sequence, which included the 3′ end of an uncharacterized NRPS containing two *N*-methyltransferase domains. Complete gene structure, determined using data from an early draft of the *M. robertsii* ARSEF 2575 genome (Donzelli et al., unpublished data), revealed a 23,792-nt ORF, likely interrupted by one intron, which encoded a predicted 7,913 aa polypeptide (GenBank accession **JN805540**). The gene (*DXS*) was located in a 107,640-nt contig of the ARSEF 2575 genome in which a second, 2-module GliP-like NRPS and a polyketide synthase (PKS) predicted to synthesize a reduced polyketide were also present (Fig. 2a). BlastN similarity searches indicated that the region is nearly perfectly conserved in *M. anisopliae* ARSEF 23 (scaffold 50), while no significant matches were detected in the *M. acridum* CQMa 102 genome (Gao et al. 2011).

DXS contains 6 complete NRPS modules, with an *N*-methyltransferase domain in each of the last two modules (Fig. 2b). Both domains were aligned with corresponding amino acid sequences retrieved from several fungal NRPSs producing *N*-methylated products and with *C*-methyltransferase domains from several hybrid PKS–NRPSs. Similar to what has been described by others (Ansari et al. 2008), both putative *N*-methyltransferase domains from *DXS* clustered with their homologs in other organisms and displayed motifs identified previously (Weber et al. 1994; Ansari et al. 2008) (Fig. 3a). The position of the *N*-methyltransferase domains and the directionality of peptide synthesis were used to infer



ID	Product description	Size (aa)	Best non-*Metarhizium* BlastX match in the nr database		
			Accession	E-value	Organism
1	benzoylformate decarboxylase	501	YP_002754411	7.7E-154	*Acidobacterium capsulatum*
2	iron transport multicopper oxidase	563	EFQ29622	1.3E-180	*Glomerella graminicola*
3	polyketide synthase	1,594	XP_002487169	0.0	*Talaromyces stipitatus*
4	cytochrome p450	547	XP_002487170	1.9E-108	*Talaromyces stipitatus*
5	cytochrome p450	484	XP_002487173	1.3E-101	*Talaromyces stipitatus*
6	nonribosomal peptide synthetase-GliP like	2,139	XP_002487175	0.0	*Talaromyces stipitatus*
7	cytochrome p450	511	EFQ26043	0.0	*Glomerella graminicola*
8	GPI ethanolamine phosphate transferase	713	XP_962669	0.0	*Neurospora crassa*
9	branched-chain-amino-acid aminotransferase	365	XP_001390686	2.7E-89	*Aspergillus niger*
10	Zn6Cys2 zinc finger domain protein	473	XP_002487176	2.2E-22	*Talaromyces stipitatus*
11	peptidase S33 family protein	624	XP_001395907	6.8E-113	*Aspergillus clavatus*
12	phytanoyl-dioxygenase family protein	319	XP_002478037	2.1E-97	*Talaromyces stipitatus*
13	nonribosomal peptide synthetase	7,913	AAX63399	0.0	*Hypocrea virens*
14	cytochrome p450	538	XP_001821718	1.6E-109	*Aspergillus oryzae*
15	aldo-keto reductase	384	XP_003044437	1.1E-163	*Nectria heamatococca*
16	glutamate decarboxylase	495	EFQ36242	0.0	*Glomerella graminicola*
17	vacuolar ATP synthase subunit b	450	EFQ31673	0.0	*Glomerella graminicola*
18	ABC transporter	1,332	XP_003052249	0.0	*Nectria haematococca*
19	conserved hypothetical protein	567	XP_001211968	7.2E-19	*Aspergillus terreus*
20	winged helix and SAM-dependent methyltransferase	352	XP_001228816	3.2E-42	*Chaetomium globosum*
21	MFS transporter	505	XP_868838	1.1E-159	*Aspergillus nidulans*
22	hypothetical protein	268	-	-	no match

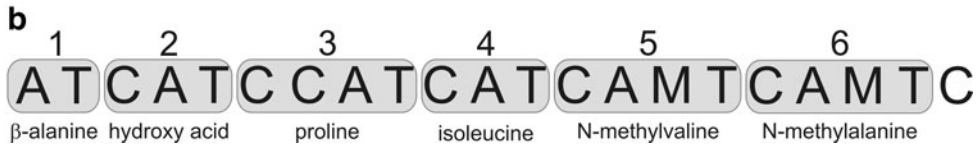
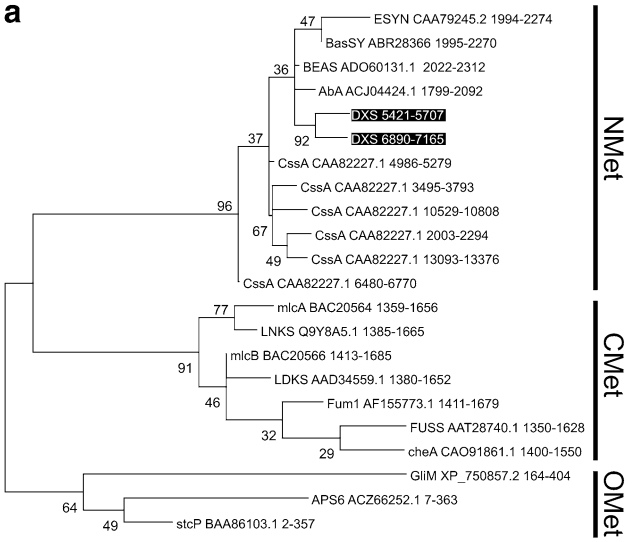


Fig. 2 **a** Structure and predicted specificity of each module of DXS. *A* adenylation domain, *T* thiolation domain, *C* condensation domain, *M* *N*-methyltransferase domain. **b** Gene models identified in the genomic region surrounding DXS. For each predicted gene, the putative protein product, its predicted amino acid length, and the best non-Metarhizium BlastX match in the nr database are reported

a



b

Protein	A#[a]	Preferred ligand	Selectivity-conferring residues[b]										Predicted specificity[c]
			235	236	239	278	299	301	322	330	331	517	
DXS	**1**	**β-ala**	**D**	**I**	**F**	**Y**	**A**	**I**	**A**	**T**	**A**	**K**	**phe=trp=phg=tyr=bht-like**
DXS	**6**	**ala**	**D**	**V**	**W**	**I**	**Y**	**A**	**A**	**V**	**I**	**K**	**gly=ala=val=leu=ile=abu=iva-like**
CssA	11	ala	D	I	F	I	Y	A	A	I	L	K	
DXS	**2**	**hac[d]**	**G**	**A**	**N**	**L**	**I**	**G**	**A**	**T**	**V**	**K**	**No predictions**
ABA	1	d-hmp[e]	G	A	L	L	V	G	I	T	V	K	
feESYN	1	d-hiv[f]	G	A	L	H	V	V	G	S	I	K	
DXS	**3**	**pro**	**D**	**L**	**H**	**E**	**I**	**G**	**I**	**I**	**S**	**K**	**pro=pip-like**
ABA	5	pro	D	V	W	V	F	S	A	I	Q	K	
DXS	**4**	**ile**	**D**	**G**	**L**	**F**	**I**	**G**	**I**	**P**	**V**	**K**	**gly=ala=val=leu=ile=abu=iva-like**
fsESYN	2	leu/ile	D	A	W	F	A	G	V	M	I	K	
Ba1	1	ile	D	G	F	F	F	G	V	V	Y	K	
DXS	**5**	**val**	**D**	**A**	**W**	**F**	**Y**	**G**	**G**	**T**	**F**	**K**	**gly=ala=val=leu=ile=abu=iva-like**
ABA	7	val	D	A	W	M	F	A	A	I	L	K	
ABA	9	val	D	A	W	M	F	A	A	I	L	K	
CssA	9	val	D	A	W	M	F	A	A	I	L	K	
CssA	4	val	D	A	W	M	F	A	A	I	I	K	
ABA	2	val	D	A	W	M	F	A	A	I	L	K	

[a] Adenylation domain number from protein N-terminus
[b] Amino acid position within the adenylation domain according to Stachelhaus et al. (1999)
[c] Prediction from NRPSpredictor (Rausch et al. 2005)
[d] hac: variable hydroxy acid
[e] d-hmp: 2(R)-hydroxy-3(R)-methylpentanoic acid
[f] d-hiv: D-2-hydroxyvaleric acid

◀ **Fig. 3** **a** Cluster analysis of *N*-methyltransferase (NMet) domains extracted from NRPSs having known *N*-methylated products, which included destruxin, *DXS*, cyclosporine, CssA (Weber et al. 1994), aureobasidin A, AbA (Slightom et al. 2009), enniatin, ESYN (Haese et al. 1993), beauvericin, BEAS (Xu et al. 2008), and bassianolide, BasSY (Jirakkakul et al. 2008); *C*-methyltransferase domains extracted from several PKSs encoding *C*-methylated products (Skellam et al. 2010), including compactin, mlcA and mlcB (Eisfeld 2009), lovastatin, LNKS (Hendrickson et al. 1999) and LDKS (Kennedy et al. 1999), fusarin C, FUSS (Song et al. 2004), cytochalasan, CheA (Schumann and Hertweck 2007); and the *O*-methyltransferases involved in the biosynthesis of gliotoxin, GliM (Gardiner and Howlett 2005), aflatoxin, stcP (Kelkar et al. 1996), and apicidin, APS6 (Jin et al. 2010). Note that the four NMet domains in AbA are 100% identical to each other at the amino acid level, so only one was used for this analysis. The tree was inferred using the Maximum Likelihood method based on the WAG model and 500 bootstrap replicates. The bootstrap value (percentage of replicate trees showing that same clade) is reported at each node of the tree. The GenBank accession number and the protein segment used for the analysis are reported at the end of each branch. **b** Comparison of the 10-aa code (Stachelhaus et al. 1999) of the six *DXS* adenylation domains with those from other fungal synthetases having known metabolites. CssA: cyclosporine synthetase from *Tolypocladium inflatum* (CAA82227), AbA: aurobasidin A from *Aureobasidium pullulans* (ACJ04424), feESYN: enniatin synthetase from *Fusarium equiseti* (CAA79245.2), fsESYN: enniatin synthetase from *Fusarium sambucineum* (Pieper et al. 1992; Xu et al. 2009), and Ba1: bacitracin synthetase 1 from *Bacillus licheniformis* (O68006). The 10-aa code was identified using NRPSpredictor (Rausch et al. 2005)

the specificity of each adenylation domain (Figs. 2b, 3b). The assignment is further supported by the sequence divergence of the putative hydroxy acid (hac) adenylation domain (A2) from other typical amino acid-activating A domains (Fig. 3b).

In vitro and in vivo expression of *DXS*

Expression of *DXS* was characterized by RT-PCR both in vitro and during the interaction with the *S. exigua* host. In both cases, the transcription pattern was relatively simple: *DXS* expression was low during early growth phases (i.e., after the first 48 h in HB broth and 72 h into *S. exigua* infection), increased with time under both conditions, and reached a steady high level at later stages (Fig. 4a, b). RT-PCR also indicated the presence of *DXS* transcripts in conidia (Fig. 4a, time 0).

Identification of *DXS* knockout strains

Initial PCR-screening of bialaphos-resistant colonies and subsequent confirmation of *DXS* disruption after selection of single conidial progeny (Fig. 5a, b) lead to the identification of strains 2.1 and 4.1 as homologous integrants at the *DXS* locus. The bialaphos-resistant strains 1.1 and 5.1, in which *DXS* appeared to be intact, were retained as phenotypic controls. Southern analysis confirmed the PCR analysis demonstrating that 2.1 and 4.1 carried a single

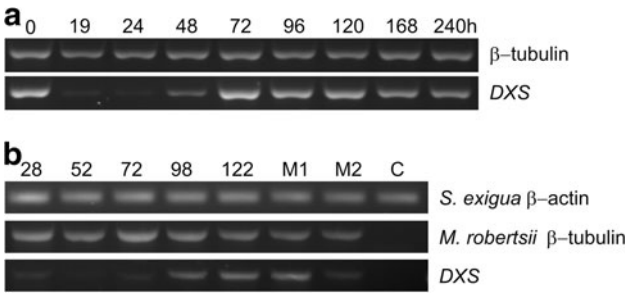


Fig. 4 Detection of *DXS* expression by RT-PCR in *Metarhizium robertsii* **a** conidia (time 0) or mycelia 19, 24, 48, 72, 96, 120, 168 and 240 h after inoculation into HB medium; and **b** in fungus-infected motile *Spodoptera exigua* larvae 28, 52, 72, 98, and 122 h after inoculation, and from fungus-infected non-motile larvae 122 h (M1) and 172 h (M2) after inoculation. RNA extracted from mock-inoculated larvae at 72 h was used as a control (*C*)

T-DNA insertion at the *DXS* locus (Fig. 5c). Southern analysis also confirmed the integration of the T-DNA outside the targeted region in strains 1.1 and 5.1 (Fig. 5c).

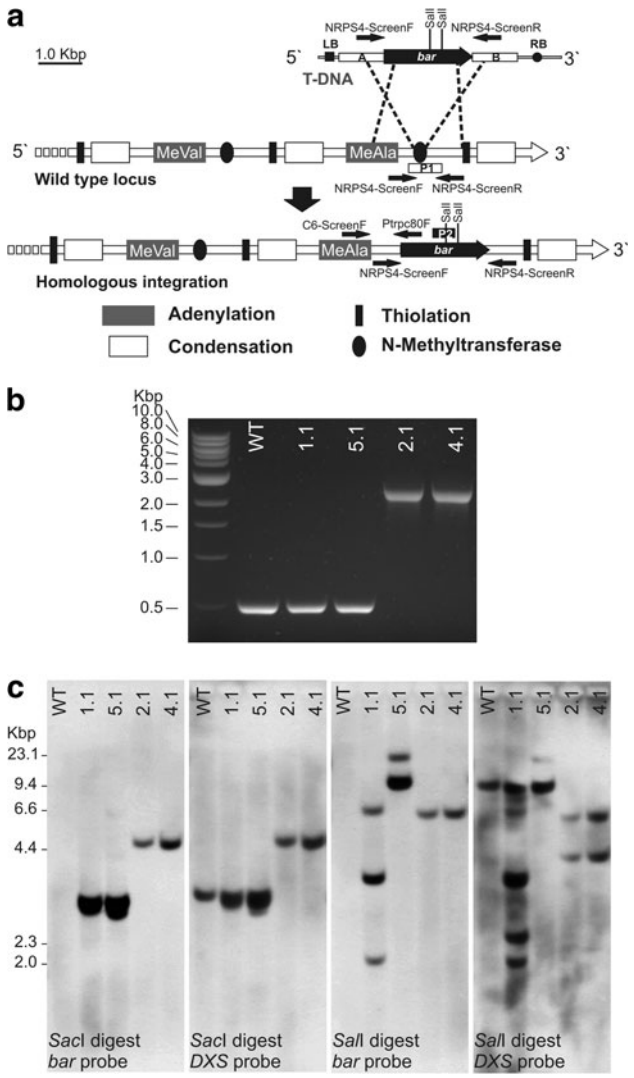


Fig. 5 **a** Recombination of the *bar* gene within the *DXS* gene to create *DXS* knockout (KO) strains. Position of the primers used for PCR identification and confirmation of the *DXS* KO events is marked by small black arrows. Position of the probes used for Southern analyses is also indicated and marked as *P1* (*DXS* probe) or *P2* (*bar* probe). The predicted amino acid specificity of the depicted adenylation domains is reported. **b** *DXS* disruption detected by PCR in strains 2.1 and 4.1 using the primers NRPS4KO-screen-F and NRPS4KO-screen-R (Table 1). Wild type (WT) strain ARSEF 2575 and ectopic integrants 1.1 and 5.1 were used as the negative controls. T-DNA insertion within the *DXS* coding region is revealed by the increased size to 2,242 bp due to the insertion of the 1,867-bp *bar* expression cassette into the 2.1 and 4.1 amplicons compared to the 532-bp amplification product obtained from the wild type (WT) and ectopic integrants. **c** Southern analyses comparing WT, the ectopic integrant strains 1.1 and 5.1, and the homologous integrant strains 2.1 and 4.1. Genomic DNA of each strain (2 μg) was digested with either *Sal*I or *Sac*I and hybridized with either the *DXS* or *bar* probe after gel separation and transfer onto nylon membrane

Phenotypic effects of the *DXS* knockout

DXS KO strains 2.1 and 4.1 were analyzed for production of DTX and both failed to yield detectable amounts of the metabolites (Fig. 6; Table 2). The ectopic integrants 5.1 and 1.1, on the other hand, retained the ability to synthesize these metabolites at WT levels (Fig. 6; Table 2). No other obvious phenotypic changes were observed in the mutants, including pigmentation, growth rate, or morphology (data not shown). Abolition of DTX biosynthesis had no significant effect on virulence levels of *M. robertsii* ARSEF 2575 against larvae of *S. exigua* (2nd instar), *G. mellonella* (4th instar) and *T. molitor* (4th instar) at any of the wide range of inoculum concentrations tested and in independently replicated assays with one exception. In assay #1 with *T. molitor*, the KO strain was significantly less virulent than the WT when the lowest inoculum dose was applied (Table 3), but this result was not repeated in the following two assays (Table 3).

Discussion

Destruxins A, B and E are the primary constituents reported from fermentation broths of *M. robertsii*, and they are by far the most exhaustively researched toxins of entomopathogenic fungi. Using targeted gene knockout, we identified *DXS* as the primary gene responsible for DTX biosynthesis. *DXS* encodes for an NRPS, a family of large multifunctional, multimodular enzymes. Each NRPS module contains domains needed for the activation (adenylation domain), propagation (thiolation domain), and incorporation (condensation domain) of one monomer contained in the final metabolite. In some cases, the process can be iterative and one module introduces an amino acid multiple times in the final molecule but, in the most frequent cases to date, fungal NPRSs assemble the peptide in a linear fashion, adding one amino acid for each module in the protein (Eisfeld 2009). Consistent with DTX structure, *DXS* encodes for a large protein carrying six NRPS modules, where each module accounts for the incorporation of one of the six monomers (five amino acids and one hydroxy acid) constituting the DTX backbone structure (Fig. 1). The presence of *N*-methyltransferase domains in the last two *DXS* modules fits well with the presence of *N*-methyl-L-valine and *N*-methyl-L-alanine in the DTX molecule (Fig. 1), while their locations allow the putative attribution of the amino acid specificity of each *DXS* module, as shown in Fig. 2b. Heterogeneity in the DTX family of cyclic depsipeptides is conferred by variation in the hydroxy acid and the amino acid substitutions in positions 1–4. We anticipate that fungi capable of producing structural variants of DTX not found in *M. robertsii*

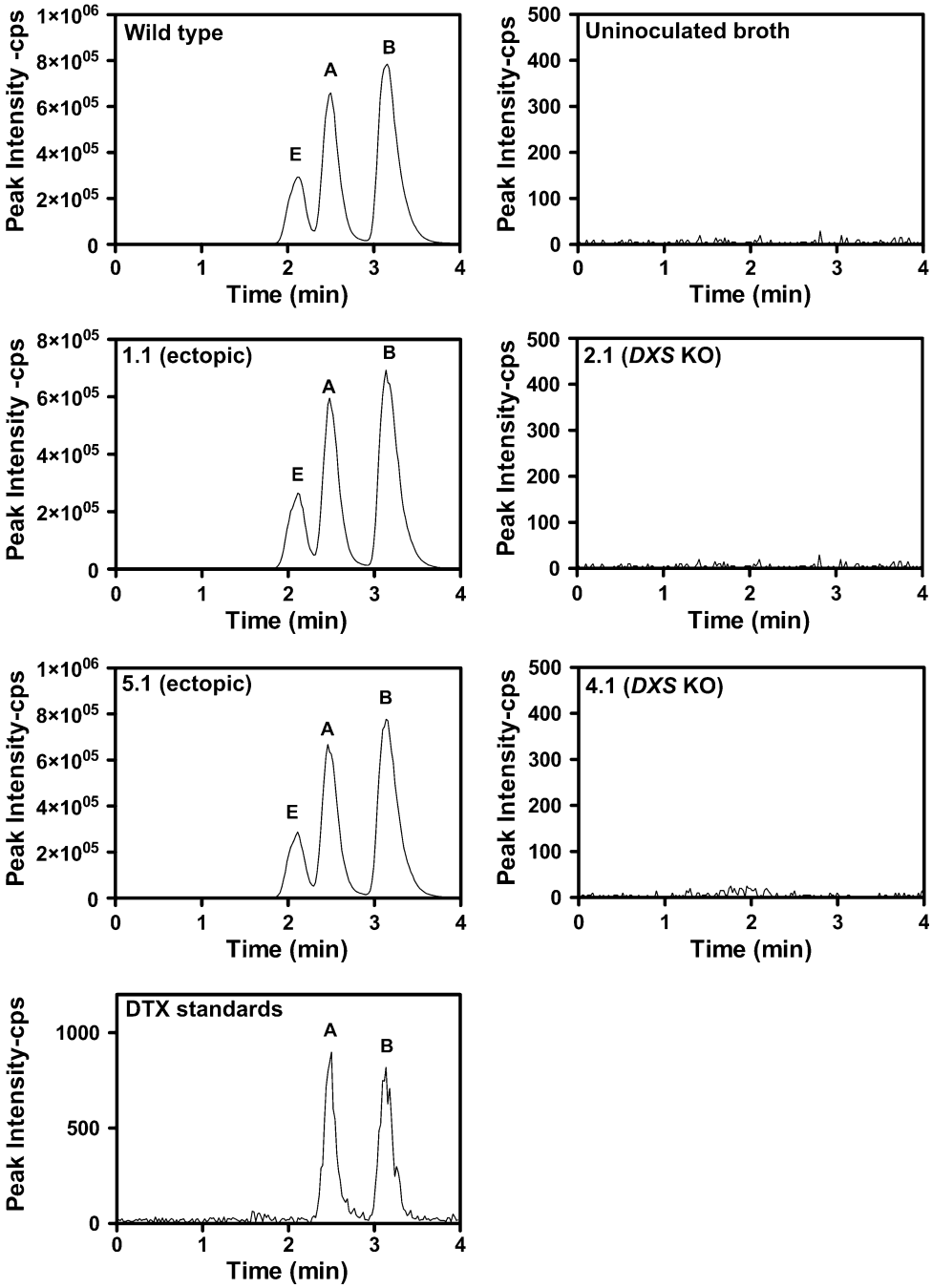


Fig. 6 HPLC-MS–MS analyses of culture filtrate extracts from the wild type (WT) ARSEF 2575 (2.5 µl broth equivalent), the ectopic integrants 1.1 and 5.1 (2.5 µl broth equivalent), and the *DXS* null mutants 2.1 and 4.1 (25 µl broth equivalent), grown in HB broth for 5 days; an extract of the uninoculated broth was included as a negative control (25 µl broth equivalent). Note that the KO strain samples and the broth control were injected at a tenfold higher concentration relative to the extracted broth volume than that of the WT and ectopic samples. Also included for comparison is a standard composed of pure destruxins A and B (500 pg each loaded onto the column). Chromatograms are summed ion traces from 6 MS–MS transitions (594 → 481, 453, 368 for detecting DTX E and B; 578 → 465, 437, 342 for detecting DTX A). Peak intensity is measured in ion counts per second (cps). The DTX A, B and E peaks are marked with the corresponding letters

ARSEF 2575 will likely have slightly modified modules that account for the reported amino acid substitutions (Pedras et al. 2002).

The role of *DXS* in DTX biosynthesis is supported by the absence of the metabolites in independently disrupted mutants as compared to the WT and ectopic strains. In addition, the genome of *M. acridum*, known to be a non-producer of DTX (Kershaw et al. 1999; Moon et al. 2008), does not contain any homologs of this gene and its surrounding region (Gao et al. 2011).

The similarity of the in vivo and in vitro expression patterns of *DXS*, as observed in Fig. 4, suggests a developmental regulation of this gene, similar to what has been reported with other secondary metabolites (Calvo et al. 2002; Kato et al. 2003; Yu and Keller 2005; Fox and Howlett 2008) including those from *M. robertsii* (Giuliano Garisto Donzelli et al. 2010). *DXS* transcripts were also detected in conidia produced on SDAY/4, hinting at either the presence of the metabolites in these propagules or a possible accumulation of transcripts during conidium formation.

Table 2 Dextruxin production in the wild type (WT) *Metarhizium robertsii* ARSEF 2575, ectopic integrant controls (Ect), and *DXS* knockout (KO) mutants

Sample	DTX A	DTX B
WT	64 ± 5	83 ± 4
1.1 (Ect)	56 ± 1	59 + 2
5.1 (Ect)	55 ± 5	65 ± 4
2.1 (KO)	n.d.	n.d.
4.1 (KO)	n.d.	n.d.
HB medium control	n.d.	n.d.

Cultures were grown for 5 days in HB broth. Filtrates were processed using C18-SPE, then analyzed on HPLC with UV detection at 220 nm (Method 1 described in the "Materials and method" section). Values are mean ± SEM in mg/L

n.d. not detected

Loss of DTX production had no phenotypic effect in our experimental settings. This included no stable changes in virulence against any of the three insect species assayed, which is surprising considering the existing body of work supporting the insecticidal effects of DTXs. Others have drawn a tentative link between the ability of some *Metarhizium* strains to rapidly kill their hosts (Samuels et al. 1988a; Amiri-Besheli et al. 2000) and their production of secondary metabolites, including DTX. However, the relationship between fungal secondary metabolism and pathogenicity is far from understood and, for the vast majority of cases, the roles of small molecules in these complex processes remain elusive. For instance, hybrid PKS–NRPS pathways leading to the biosynthesis of tenellin in *Beauveria bassiana* and the mutagenic fusarin-like NG-39X compounds in *M. robertsii* were found not to contribute significantly to virulence against *G. mellonella* and *S. exigua* larvae, respectively (Eley et al. 2007; Giuliano Garisto Donzelli et al. 2010). Similarly, targeted gene disruption experiments with *M. robertsii* ARSEF 2575 to test the role of serinocyclin, a spore-derived peptide, revealed that KO strains were as virulent as WT when assayed on larvae of *S. exigua* or *Leptinotarsa decemlineata*, and no differences in morphology or physiology could be demonstrated (Moon et al. 2008). In contrast, mutants of *B. bassiana* with targeted disruptions of the peptide synthetase genes responsible for the biosynthesis of beauvericin and bassianolide were morphologically indistinguishable from WT, but the KO strains showed decreased virulence when tested against *S. frugiperda*, *Helicoverpa zeae*, and *G. mellonella* larvae (Xu et al. 2008, 2009). Similarly, targeted gene knockout of a geranylgeranyl diphosphate synthase abolished the production of helvolic acid and reduced virulence of *M. anisopliae* NAFF635007 against two genera of insect larvae (Singkaravanit et al. 2010).

The finding that the genetic abolishment of DTX production in *M. robertsii* had no measurable effect on the virulence of the fungus when conidia were applied topically to *S. exigua*, *G. mellonella* or *T. molitor* larvae is in stark contrast to the outcome predicted by a large body of in vitro toxicological studies suggesting DTX are key virulence factors for *Metarhizium* invertebrate pathogens (Pal et al. 2007; Sree and Padmaja 2008). Resolving this incongruity will require further investigation. For instance, some insects can recover from sub-lethal DTX applications, and sensitivity to DTX has been shown to vary with the host species (Samuels et al. 1988a; Kershaw et al. 1999). However, little is known about possible detoxification mechanisms of most arthropods and whether they are constitutive or induced as part of the immune response mounted by the insect after fungal infection (Rohlfs and Churchill 2011). It is also possible that DTXs are contributory virulence factors that affect only hosts carrying specific-molecular targets, as seen for some secondary metabolites produced by plant pathogenic fungi (Sindhu et al. 2008; Sweat et al. 2008), or their presence may be redundant for virulence because of the action of additional unknown compounds produced in amounts sufficient to ensure efficient killing, the effects of which mask a specific role for DTX. This is supported by the occasional inconsistent correlation between strain virulence and in vitro DTX production (Samuels et al. 1988a; Kershaw et al. 1999; Amiri-Besheli et al. 2000; Moon et al. 2008).

It has been reported that loss of a dispensable chromosome in *M. anisopliae* strain V275 resulted in several biochemical changes including the inability to produce DTX; the V275 strain showed reduced virulence on *T. molitor*, but there were no differences against *G. mellonella*. However, the number of phenotypic changes associated with dispensable chromosome loss does not allow one to attribute the observed difference in virulence solely to the loss of DTX production (Wang et al. 2003). Our data suggest that the virulence changes observed in strain V275 were likely unrelated to the abolishment of DTX production, since we report here no loss in virulence of a DTX-deficient KO strain against *T. molitor* larvae. In addition, there is an abundance of genes encoding potentially toxic proteins and putative secondary metabolites predicted from the recently sequenced *M. anisopliae* ARSEF 23 and *M. robertsii* ARSEF 2575 genomes, many of which might be new candidates for playing causal roles in virulence (Gao et al. 2011; Donzelli et al., unpublished). Alternatively, the relationship of DTX to virulence may be coincidental, and the compounds may play roles in other unknown functions, or cause subtle effects in the host, not measurable with traditional pathogenicity assays. Our study neither confirms nor refutes these hypotheses definitively but clearly demonstrates the dispensability of DTX

Table 3 Results of insect virulence assays comparing wild type (WT) and the *DXS* knockout (KO) strain 4.1 against *Tenebrio molitor*, *Galleria mellonella* and *Spodoptera exigua*

Host	Assay #	Dose[a]	Survival (days)						Log-rank test		
			Median[b]		Mean		Standard error				
			WT	KO	WT	KO	WT	KO	χ^2	*df*	*P* value
T. molitor	**1**	**1×10^5**	**7**	**–**	**6.4**	**6.9**	**0.2**	**0.1**	**9.341**	**1**	**0.002**
	1	1×10^6	7	7	6.3	6.8	0.2	0.1	0.665	1	0.415
	1	1×10^7	6	6	5.8	5.6	0.2	0.2	0.147	1	0.702
	2	1×10^5	–	–	6.4	6.7	0.1	0.2	0.187	1	0.666
	2	1×10^6	7	–	6.5	6.8	0.2	0.1	3.319	1	0.069
	2	1×10^7	6	6	5.8	5.7	0.2	0.3	0.030	1	0.862
	3	1×10^5	–	–	7.0	5.9	0.1	0.1	2.301	1	0.129
	3	1×10^6	6.5	6.5	6.3	6.2	0.2	0.2	0.163	1	0.686
	3	1×10^7	5.5	6	5.7	6.0	0.2	0.2	0.741	1	0.389
G. mellonella	1	1×10^5	5.5	5	5.4	5.3	0.2	0.2	0.576	1	0.448
	1	1×10^6	4	4	4.0	4.1	0.1	0.2	0.414	1	0.520
	1	1×10^7	3	3	3.0	3.0	0.0	0.0	1.958	1	0.162
	2	1×10^5	–	–	6.9	6.5	0.1	0.2	0.233	1	0.629
	2	1×10^6	5	5	4.5	5.1	0.2	0.3	0.928	1	0.335
	2	1×10^7	3	3	3.3	3.5	0.1	0.2	0.298	1	0.585
	3	1×10^5	7	–	5.7	6.5	0.3	0.3	0.964	1	0.326
	3	1×10^6	4	4.5	4.8	4.5	0.4	0.2	0.517	1	0.472
	3	1×10^7	3	3	3.3	3.3	0.2	0.2	0.058	1	0.811
S. exigua	1	5×10^5	3	3	3.9	3.4	0.3	0.1	1.802	1	0.179
	1	6.2×10^5	3	3	3.1	3.2	0.1	0.1	0.397	1	0.529
	1	7.5×10^5	3	3	3.0	3.1	0.0	0.1	0.019	1	0.889
	1	1×10^6	3	3	2.9	2.9	0.1	0.1	0.000	1	1.000
	1	5×10^6	3	3	2.5	2.6	0.1	0.1	0.083	1	0.773
	2	1×10^4	–	–	6.9	7.0	0.2	0.0	0.197	1	0.657
	2	5×10^4	–	–	6.1	5.5	0.3	0.2	0.087	1	0.769
	2	1×10^5	6	5	5.6	5.2	0.3	0.3	0.717	1	0.397
	2	5×10^5	3	3.5	3.8	3.7	0.2	0.2	0.003	1	0.955
	2	1×10^6	3	3	3.1	3.0	0.1	0.1	0.177	1	0.674

Assays displaying statistically significant difference between WT and KO are in boldface. Mortality of the mock-inoculated controls never exceeded 8% at the end of the assay

[a] Dose is number of conidia/ml

[b] Median survival time cannot be estimated when the smallest survival time calculated by the survivor function is >0.5

as virulence factors of *M. robertsii* ARSEF 2575 as a pathogen of *S. exigua*, *T. molitor*, and *G. mellonella*.

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