Supplementary Information

Cytochrome P450-catalysed L-tryptophan nitration in thaxtomin phytotoxin biosynthesis

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Supplementary Results

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Supplementary Figure 1: Biosynthesis of thaxtomin A and deletion of txtE in *S. turgidiscabies*. A: Model for txtE replacement in *S. turgidiscabies* using marker-exchange mutagenesis. B: Southern hybridization of 32P-labeled internal 993 bp txtE probe (left) and p2XP450 targeting vector (right) with EcoRI-digested genomic DNA isolated from *S. turgidiscabies* wild type (lane 1) and ΔtxtE (lane 2).
| Strain                     | Thaxomin[^a] | μg culture[^1] |
|---------------------------|--------------|----------------|
| WT                        | 111.73±6.16  |
| WTpIJ8600+T               | 71.66±10.81  |
| WTpIJ8600tTxtD+T          | 61.63±2.79   |
| WTpIJ8600TxtE+T           | 161.69±14.14 |
| ΔtxtE                     | ND           |
| ΔtxtEplJ8600+T            | ND           |
| ΔtxtEplJ8600tTxtD+T       | ND           |
| ΔtxtEplJ8600TxtE+T        | 6.05±0.14    |
| ΔtxtD                     | 6.25±0.17    |
| ΔtxtDplJ8600+T            | 7.06±1.5     |
| ΔtxtDplJ8600tTxtD+T       | 29.74±0.42   |
| ΔtxtDplJ8600TxtE+T        | 5.85±0.06    |

[^a]: mean ± standard deviation, n=3. ND = not detected

**Supplementary Table 1:** Complementation analysis of *txtE* and *txtD* in *S. turgidiscabies* Car8 (WT). Strains were grown in OBB for 7 days. The thiostrepton inducible promoter on plasmid pIJ8600 was induced using 10 μg/ml thiostrepton (+T) 3 days following inoculation.
### CLUSTAL 2.1 multiple sequence alignment

| Description | Sequence 1 | Sequence 2 | Sequence 3 | Sequence 4 | Sequence 5 | Sequence 6 |
|-------------|------------|------------|------------|------------|------------|------------|
| C Helix     | CAAGLKDPRLTADRG--TEVLAAKFQGFIPNFRFWTNVVMTDPFRFAKLSTSRVR | CAAGLKDPQFTADRG--TEVLAAKFQGFIPNFRFWTNVVMTDPFRFAKLSTSRVR | AGFTAAHGYQDQLKQYVAHDLVASQPAG---ATEIDAVPAALAEPLGAVAHFGEYEE | DTVIRLAAKQAQDGGLTEETPFTQTVHQLATVFAIAGTEPQSLDSLGSFLAARNGRQERFL | DTVIRLAAKQAQDGGLTEETPFTQTVHQLATVFAIAGTEPQSLDSLGSFLAARNGRQERFL | AGFTAAHGYQDQLKQYVAHDLVASQPAG---ATEIDAVPAALAEPLGAVAHFGEYEE |
| St Helix    | CAAGLKDPQFTADRG--TEVLAAKFQGFIPNFRFWTNVVMTDPFRFAKLSTSRVR | CAAGLKDPQFTADRG--TEVLAAKFQGFIPNFRFWTNVVMTDPFRFAKLSTSRVR | AGFTAAHGYQDQLKQYVAHDLVASQPAG---ATEIDAVPAALAEPLGAVAHFGEYEE | DTVIRLAAKQAQDGGLTEETPFTQTVHQLATVFAIAGTEPQSLDSLGSFLAARNGRQERFL | DTVIRLAAKQAQDGGLTEETPFTQTVHQLATVFAIAGTEPQSLDSLGSFLAARNGRQERFL | AGFTAAHGYQDQLKQYVAHDLVASQPAG---ATEIDAVPAALAEPLGAVAHFGEYEE |
| P450eryf    | -----MTTVPDLESDSFHVD-------WYRTYELRETAPVTFPVRLG-QDLATWVGDYSE | -----MTTVPDLESDSFHVD-------WYRTYELRETAPVTFPVRLG-QDLATWVGDYSE | -----MTTVPDLESDSFHVD-------WYRTYELRETAPVTFPVRLG-QDLATWVGDYSE | ----MTTVPDLESDSFHVD-------WYRTYELRETAPVTFPVRLG-QDLATWVGDYSE | ----MTTVPDLESDSFHVD-------WYRTYELRETAPVTFPVRLG-QDLATWVGDYSE | -----MTTVPDLESDSFHVD-------WYRTYELRETAPVTFPVRLG-QDLATWVGDYSE |
| P450nor     | --MAQGAPSFFPSRSSQGPEE------PAPEAKLRTANFVSQKVLDFGSALWTVKhK | --MAQGAPSFFPSRSSQGPEE------PAPEAKLRTANFVSQKVLDFGSALWTVKhK | --MAQGAPSFFPSRSSQGPEE------PAPEAKLRTANFVSQKVLDFGSALWTVKhK | --MAQGAPSFFPSRSSQGPEE------PAPEAKLRTANFVSQKVLDFGSALWTVKhK | --MAQGAPSFFPSRSSQGPEE------PAPEAKLRTANFVSQKVLDFGSALWTVKhK | --MAQGAPSFFPSRSSQGPEE------PAPEAKLRTANFVSQKVLDFGSALWTVKhK |
| P450terp    | VMQTRQKGPQFLSNAGSE-SEILYDQNNFPAAPNRG169GCPFVIGDGLTSMQPHTFAVL | VMQTRQKGPQFLSNAGSE-SEILYDQNNFPAAPNRG169GCPFVIGDGLTSMQPHTFAVL | VMQTRQKGPQFLSNAGSE-SEILYDQNNFPAAPNRG169GCPFVIGDGLTSMQPHTFAVL | VMQTRQKGPQFLSNAGSE-SEILYDQNNFPAAPNRG169GCPFVIGDGLTSMQPHTFAVL | VMQTRQKGPQFLSNAGSE-SEILYDQNNFPAAPNRG169GCPFVIGDGLTSMQPHTFAVL | VMQTRQKGPQFLSNAGSE-SEILYDQNNFPAAPNRG169GCPFVIGDGLTSMQPHTFAVL |
| P450cam     | GTETIQSNAANLAPPHVHEVFLVPFDNYNP5RNLSA0G3QVQAWAVLQESVPDVPDWTNG | GTETIQSNAANLAPPHVHEVFLVPFDNYNP5RNLSA0G3QVQAWAVLQESVPDVPDWTNG | GTETIQSNAANLAPPHVHEVFLVPFDNYNP5RNLSA0G3QVQAWAVLQESVPDVPDWTNG | GTETIQSNAANLAPPHVHEVFLVPFDNYNP5RNLSA0G3QVQAWAVLQESVPDVPDWTNG | GTETIQSNAANLAPPHVHEVFLVPFDNYNP5RNLSA0G3QVQAWAVLQESVPDVPDWTNG | GTETIQSNAANLAPPHVHEVFLVPFDNYNP5RNLSA0G3QVQAWAVLQESVPDVPDWTNG |
| Cys Pocket  | RPNDFDLDRPNSAR-HLSFGQGVHACLAAQLISLQLKWFYVALLNRFPGIRT---AGEPI388 | RPNDFDLDRPNSAR-HLSFGQGVHACLAAQLISLQLKWFYVALLNRFPGIRT---AGEPI388 | RPNDFDLDRPNSAR-HLSFGQGVHACLAAQLISLQLKWFYVALLNRFPGIRT---AGEPI388 | RPNDFDLDRPNSAR-HLSFGQGVHACLAAQLISLQLKWFYVALLNRFPGIRT---AGEPI388 | RPNDFDLDRPNSAR-HLSFGQGVHACLAAQLISLQLKWFYVALLNRFPGIRT---AGEPI388 | RPNDFDLDRPNSAR-HLSFGQGVHACLAAQLISLQLKWFYVALLNRFPGIRT---AGEPI388 |
| I Helix     | ADACQVDTOANEGLVRYNASQFTW-VAAKDVEHMVRERIAEQGTA1FLGSA-NADME332 | ADACQVDTOANEGLVRYNASQFTW-VAAKDVEHMVRERIAEQGTA1FLGSA-NADME332 | ADACQVDTOANEGLVRYNASQFTW-VAAKDVEHMVRERIAEQGTA1FLGSA-NADME332 | ADACQVDTOANEGLVRYNASQFTW-VAAKDVEHMVRERIAEQGTA1FLGSA-NADME332 | ADACQVDTOANEGLVRYNASQFTW-VAAKDVEHMVRERIAEQGTA1FLGSA-NADME332 | ADACQVDTOANEGLVRYNASQFTW-VAAKDVEHMVRERIAEQGTA1FLGSA-NADME332 |
| K Helix     | RPNDFLDLPNFSAR-HLSFCVH-ALQALISLKLW4FYVALLNRFPGIRT---AGEPI388 | RPNDFLDLPNFSAR-HLSFCVH-ALQALISLKLW4FYVALLNRFPGIRT---AGEPI388 | RPNDFLDLPNFSAR-HLSFCVH-ALQALISLKLW4FYVALLNRFPGIRT---AGEPI388 | RPNDFLDLPNFSAR-HLSFCVH-ALQALISLKLW4FYVALLNRFPGIRT---AGEPI388 | RPNDFLDLPNFSAR-HLSFCVH-ALQALISLKLW4FYVALLNRFPGIRT---AGEPI388 | RPNDFLDLPNFSAR-HLSFCVH-ALQALISLKLW4FYVALLNRFPGIRT---AGEPI388 |
| Oys Pocket  | TPWNLIRSQLSRLSLSLSL | TPWNLIRSQLSRLSLSLSL | TPWNLIRSQLSRLSLSLSL | TPWNLIRSQLSRLSLSLSL | TPWNLIRSQLSRLSLSLSL | TPWNLIRSQLSRLSLSLSL |
| L Helix     | WRENLEFRSLRSLRSLSLR--------406 | WRENLEFRSLRSLRSLSLR--------406 | WRENLEFRSLRSLRSLSLR--------406 | WRENLEFRSLRSLRSLSLR--------406 | WRENLEFRSLRSLRSLSLR--------406 | WRENLEFRSLRSLRSLSLR--------406 |
| P450eryf    | WRSLSLLRLGIDQVPLVRLG-------404 | WRSLSLLRLGIDQVPLVRLG-------404 | WRSLSLLRLGIDQVPLVRLG-------404 | WRSLSLLRLGIDQVPLVRLG-------404 | WRSLSLLRLGIDQVPLVRLG-------404 | WRSLSLLRLGIDQVPLVRLG-------404 |
| P450nor     | YTPLDRGVDGLVPVFLF--------403 | YTPLDRGVDGLVPVFLF--------403 | YTPLDRGVDGLVPVFLF--------403 | YTPLDRGVDGLVPVFLF--------403 | YTPLDRGVDGLVPVFLF--------403 | YTPLDRGVDGLVPVFLF--------403 |
| P450terp    | LVATNFGGPKPNVPFAK------428 | LVATNFGGPKPNVPFAK------428 | LVATNFGGPKPNVPFAK------428 | LVATNFGGPKPNVPFAK------428 | LVATNFGGPKPNVPFAK------428 | LVATNFGGPKPNVPFAK------428 |
| P450cam     | QRKGIVSOS4QVQLPVLDVFATTKAV414 | QRKGIVSOS4QVQLPVLDVFATTKAV414 | QRKGIVSOS4QVQLPVLDVFATTKAV414 | QRKGIVSOS4QVQLPVLDVFATTKAV414 | QRKGIVSOS4QVQLPVLDVFATTKAV414 | QRKGIVSOS4QVQLPVLDVFATTKAV414 |
**Supplementary Figure 2:** Alignment of the sequences of TxtE from *S. scabies* 87.22 (Ss_TxtE) and *S. turgidiscabies* car8 (St_TxtE) with the sequences of CYPs of known structure [P450eryF (CYP107A1) from *Saccharopolyspora erythraea*, P450cam (CYP101) from *Pseudomonas putida*, P450nor (CYP55A1) from *Fusarium oxysporum* and P450terp (CYP108) from *Pseudomonas* sp.]. Catalytically important conserved residues are highlighted in yellow. Residues highlighted in red are deviations from the consensus in TxtE and residues in bold are deviations from the consensus in the reference sequences. Some of the helices in structurally determined CYPs are denoted by grey shading. The C helix (HxxR) involved in heme propionate binding is conserved. The highly conserved and structurally important salt bridge forming K helix sequence (ExxR) is conserved. The Cys pocket sequence (FxxGx(H/R)xCxG) is almost conserved except for the mutation of G to A which is shared with P450nor (CYP55A1). The L helix conserved glutamate (E) residue involved in the proton relay is mutated to glutamine (Q in TxtE). The catalytically important I helix consensus sequence ((A/G)x(E/D)T) is significantly different in TxtE (LFxPT) although the threonine (T) is conserved. The two proline residues (P and P) present in the I “helix” of TxtE are highlighted. The proline (P) residue that functions to mark the end of the I “helix” is highlighted and conserved.

**Supplementary Methods**

**General**

All reagents and chemicals were purchased from Sigma-Aldrich unless otherwise stated. Partially purified ferredoxin (1 mg/mL solution in Trizma pH 8) and ferredoxin reductase (8.5 units made to 0.5 mL in Tris 25 mM, pH 8, 0.1 M NaCl, 20% glycerol) from *Spinacia oleracea* were purchased from Sigma and were used without further purification. 2-(N,N-diethylamino)-diazenolate 2-oxide hydrate (DEANO) was used to make a stock solution (100 mM) in 10 mM sodium hydroxide which was stored at -20 °C.

NMR spectra were recorded with Bruker DPX400, DRX500, AVIII700 instruments. MALDI-TOF mass spectrometry was carried out on a Bruker Ultraflex II TOF/TOF spectrometer. IR spectra were recorded on a Perkin Elmer Spectrum One FT-IR spectrophotometer. High resolution mass spectra were measured on Bruker MicroTOF or MaXis mass spectrometers. UV-Vis spectroscopy was carried out on a Perkin Elmer Lambda 35 UV-Vis spectrometer.

**Cloning and overexpression of txtE in *E. coli***

The primers used to amplify *txtE* from *S. scabies* 87.22 genomic DNA were as follows: *forward primer* (5'\- CACCGTGACCGTCCCCCTCG-3'); *reverse primer* (5'-TCAGCGGAGGCTGAGCGGCA-3'). A CACC sequence was introduced before the GTG start codon in the forward primer, to allow directional TOPO cloning of blunt-end PCR products into pET151/D-Topo. The PCR mixture (50 μL) contained *S. scabies*
genomic DNA as template (50 ng), 2 μM of each primer, 0.1 mM of each dNTP (Fermentas), 5% dimethyl sulfoxide and 3.5U Expand high fidelity DNA polymerase (Roche) in 1X Expand reaction buffer with MgCl₂. Reaction conditions consisted of an initial denaturation step at 95°C for 2 min followed by 30 cycles of 94 °C for 45 s, 59 °C for 45 s, and 72 °C for 90 s. The 1225 bp PCR product containing the 1221 bp **txtE** gene was separated on a 1% agarose gel (SeaKem LEagarose, Rockland, USA), visualised by staining with 0.2 μg/mL ethidium bromide and extracted using a QIAquick Gel Extraction Kit (Qiagen).

The purified PCR product (~30 ng) was ligated with the pET151/D Topo vector (15-20 ng), using the Champion pET Directional TOPO Expression Kit (Invitrogen). The reaction mixture was used to transform One Shot TOPO10 chemically competent *E. coli* cells. The transformation mix was plated on LB plates supplemented with ampicillin (50 μg/mL). Clones were isolated from ampicillin resistant colonies. The sequence of the cloned gene in the pET151/D-TOPO expression vector, was confirmed by DNA sequencing (GATC-biotech). One correct clone (pSB13) was used to transform *E. coli* BL21Star(DE3) (Invitrogen) for expression of the full-length **txtE** gene as an N-terminal His₆ in-frame fusion protein.

For overexpression of the **txtE** gene, LB medium (300 mL) supplemented with ampicillin (100 μg/mL) was inoculated with 1.5 mL overnight culture of *E. coli* BL21Star(DE3)/pSB13 and the resulting culture was incubated at 37 °C with shaking at 180 rpm. Incubation was continued until the optical density at 600 nm of the culture reached 0.6-0.7, at which time isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM to induce expression. The culture was incubated overnight at 15 °C (180 rpm).

**Purification of His₆-TxtE**

Protein purification (Ni-NTA and gel filtration) was carried out on a GE Healthcare Akta Purifier. Purification Buffers were as follows:
Buffer A: 20 mM Tris buffer-HCl, pH 8.0, 100 mM NaCl, 20 mM imidazole, 1 mM DTT and 10% glycerol.
Buffer B: 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 200 mM imidazole, 1 mM DTT, 10% glycerol.
Buffer C: 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM DTT and 10% glycerol.
Buffer D: 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM DTT and 30% glycerol.

Cells were harvested by centrifugation and the pellet was resuspended in Buffer A (10 mL). The cells were lysed in the presence of 1mM phenylmethylsulphonyl fluoride (PMSF) using a French Press (17,000 psi internal cell pressure) followed by sonication for 2 min (Ultrasonic processor, Jencons). After removal of the cellular debris by centrifugation (18,000 x g for 20 min, at 4 °C), the supernatant was applied to a 1 mL HiTrap™ HP affinity column (Nickel Sepharose High Performance, GE Healthcare) equilibrated with Buffer A (flow rate 0.5 mL/min). Unbound proteins were removed by washing with Buffer A (20 mL) and the His\textsubscript{6}-TxtE fusion protein was eluted with ~4 mL of Buffer B.

Fractions containing His\textsubscript{6}-TxtE were yellow to orange in colour. They were pooled, diluted to 10 mL with Buffer C and concentrated to ~500 µL using Amicon® Ultra centrifugal filtration device with a 30,000 NMWL membrane (Millipore). The protein was further purified by gel filtration using Superdex 12 prep grade resin (110 mL) equilibrated with Buffer C. The sample was applied to the column and eluted with 64 mL of Buffer C (Flow rate 0.75 mL/min, column dead volume 40 mL). The fractions were analysed by SDS-PAGE (10%) and those containing His\textsubscript{6}-TxtE were pooled, and concentrated to 250 µL as described above. The solution was diluted with Buffer D to give the pure protein as a red solution in a final volume of 500 µL which was analysed by electrophoresis (10% SDS-PAGE, Supplementary Fig. 3). Pure His\textsubscript{6}-TxtE was aliquoted and frozen at -80 °C. The protein was used in subsequent experiments without further purification.
Supplementary Figure 3: SDS-PAGE analysis of TxtE purification. Lane 1: total protein; Lane 2: total soluble protein; Lane 3: After Ni-NTA chromatography; Lane 4: after gel filtration; Lane 5: 7 μL PAGE Ruler Plus (Fermentas). The predicted molecular weight of His₆-TxtE is 48,534 Da

Confirmation of TxtE identity and analysis of native oligomerization state

Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard. Heme concentration was calculated using the pyridine hemochromogen assay. The oligomerisation state of His₆-TxtE was determined by gel filtration on a 110 mL Superose 12 prep grade gel filtration resin poured in an XK 16/50 column (Amersham Biosciences), equilibrated with Buffer C, at a flow rate of 0.75 mL/min. The column was calibrated with the Kit for MolecularWeights 12,000-200,000 (Sigma), consisting of cytochrome c (12,400 Da), carbonic anhydrase (29,000 Da), bovine serum albumin (66,000 Da), alcohol dehydrogenase (150,000 Da), β-amylase (200,000 Da), and blue dextran (2,000,000 Da). His₆-TxtE eluted after 64 mL indicating it is a monomer. The identity of purified His₆-TxtE was confirmed by MALDI-TOF mass spectrometry on tryptic digests of the protein carried out using Trypsin Profile IGD kit (Sigma). 10 predicted tryptic fragments of TxtE (37.8% coverage) were identified in this analysis.

UV-Vis spectroscopic analysis of TxtE

TxtE was confirmed as a cytochrome P450 by UV-Vis spectroscopic analysis of its ferrous C=O complex. To cuvette A was added a solution of TxtE (8 μM) in Tris (25 mM, pH 8) and to it was added sodium dithionite (10 μL, 1 M) and a UV-Vis spectrum was measured. Carbon monoxide gas was bubbled through Tris buffer (1 mL, 25 mM, pH
8) for 2-3 min. This solution (120 µL) was used to dilute TxtE (8 µM final concentration). The resulting solution was added to cuvette B followed by addition of sodium dithionate (10 µL, 1 M). A UV-Vis spectrum was measured. Spectrum B was subtracted from spectrum A to give the C=O difference spectrum. Addition of 10 mM DTT to cuvette A and B and repeating the measurements gave increased absorbance at 450 nm and reduced it at 420 nm (Supplementary Fig. 4).

The TxtE Fe(III)-NO complex was observed by adding DEANO (5 µL, 100 mM) to a solution of TxtE (8 µM) in Tris buffer (25 mM, pH 8) and measuring the UV-Vis spectrum. This species exhibited a $\lambda_{\text{max}}$ of 431 nm in the UV-Vis spectrum (Supplementary Fig. 4). The TxtE Fe(II) complex was generated by adding sodium dithionite (5 µL, 1 M) to a solution of TxtE (8 µM) in Tris buffer (25 mM, pH 8). This species exhibited a $\lambda_{\text{max}}$ of 404 nm in the UV-Vis spectrum (Supplementary Fig. 4).

**Supplementary Figure 4:** UV-Vis spectroscopic analysis of TxtE. A: TxtE + CO, sodium dithionite (18 mM); B: TxtE + CO, sodium dithionite (18 mM), DTT (10 mM). C: UV-Vis spectroscopic analysis ofTxtE. TxtE (25 mM Tris pH 8.0) black; TxtE reduced with sodium dithionite (blue); TxtE-NO complex (red).


**Qualitative Binding Assays - Type I difference spectra**

Assays were carried out to examine which compounds bind to TtxE. For each compound two cuvettes A and B were charged with equal volumes of a solution of TtxE (~4 µM) in Tris buffer (25 mM, pH 8). To cuvette A was added the potential substrate (1.7 mM final concentration) and to cuvette B was added an equal volume of Tris buffer (25 mM, pH 8). Spectra were recorded on a dual beam spectrophotometer to give difference spectra. (Supplementary Fig. 5A)

**Determination of dissociation constant of L-Tryptophan from TtxE.**

A solution of TtxE (400 µL, 6 µM) in Tris buffer (25 mM, pH 8) was divided equally between two cuvettes. The cuvettes were placed in a dual beam UV-Vis spectrophotometer and an absorbance spectrum was measured. L-tryptophan was added to the sample cuvette (0.5 µL, 20 mM solution) and an equal volume of water was added to the reference. A difference spectrum was measured. This was repeated until the protein was saturated with L-Trp. The added substrate solution did not exceed 5% of the total volume (Supplementary Fig. 5A). This procedure was repeated three times.

The difference in absorbance of each spectrum at the $\lambda_{\text{max}}$ (390 nm) and the $\lambda_{\text{min}}$ (420 nm) was calculated for the three data sets and the average was plotted against the concentration of the substrate (Supplementary Fig. 5B). The data were fitted to a one site binding model using Origin 8, data were recorded in triplicate and from these an average and standard deviation was calculated. The dissociation constant for L-Trp was estimated as $60 \pm 6$ µM from this binding curve.
Supplementary Figure 5: Analysis of L-tryptophan binding to TxtE by UV-Vis spectroscopy. A: Type I spectra from titration of TxtE (6 μM) with L-tryptophan (0 to 769 μM), λ<sub>max</sub> 390 nm, λ<sub>min</sub> 420 nm. B: The plot shows the difference in absorbance at 390 nm and 420 nm versus L-tryptophan concentration. The data were fitted to a one-site binding model using Origin 8. Points are the mean of three measurements and error bars show ± one standard deviation.

Analysis of TxtE-catalyzed L-Trp nitration. TxtE (1.5 μM) was incubated with DEANO (0.5 mM), NADPH (1 mM), ferredoxin (2 μL), ferredoxin reductase (2 μL) and L-tryptophan (0.5 mM) in a total volume of 200 μL of Tris buffer (25 mM, pH 8). The reaction was incubated at room temperature for 90 min during which time the solution became yellow. HCl (3 μl, 5 M) was added to precipitate the protein and the solution was filtered (0.2 μm microspin filter tubes) and analysed by LC-MS (Supplementary Fig. 6), (LC-MS elution profile given in Supplementary Table 2).
**Supplementary Figure 6:** LC-MS (conditions in Supplementary Table 2) extracted ion chromatograms ($m/z = 205$ and 250) for enzymatic nitration reaction and controls. a, nitration reaction (see methods for details) shows presence of compound with $m/z = 250$ consistent with nitrated tryptophan. b, control – NADPH. c, control –DEANO. d, control –ferredoxin (Fd). e, control –reductase (Fr). f, control with boiled TtxE.

**Purification of L-4-nitrotryptophan 2 from the enzymatic reaction**

Five scaled up reactions were set up as described above to a final volume of 1 mL Tris buffer (25 mM, pH 8). The reactions were incubated at room temperature for 2 h then pooled and filtered. L-4-Nitrotryptophan 2 was purified from the reaction mixture by semi-preparative reverse phase HPLC using the elution conditions described in Supplementary Table 3. The UV-Vis spectrum of DL-4-nitrotryptophan indicated that it absorbs at both 210 nm and 400 nm so these wavelengths could be used to monitor elution during HPLC. The molecular formula of the product was confirmed by HRMS (calculated for $[C_{11}H_{12}N_{3}O_{4}]^{+}$: 250.0822; found: 250.0820). The MS/MS fragmentation pattern for a synthetic standard of DL-4-nitrotryptophan and the enzymatic product are identical (Supplementary Fig. 7).
Direct comparison of the $^1$H NMR spectra of synthetic DL-4-nitrotryptophan and the L-4-nitrotryptophan 2 isolated from the enzymatic reaction, recorded at approximately the same concentration, under the same conditions and on the same instrument (50 mM DCl in D$_2$O, 700 MHz), indicated that they have identical planar structures (Supplementary Fig. 8A). We were, however, cautious about drawing the conclusion that the two compounds were identical on the basis of this data alone, because it has previously been reported that pH and concentration differences can alter the chemical shifts of aromatic protons in nitro-tryptophans. To unambiguously confirm that the two compounds have identical planar structures, 200 μL of each sample were mixed and a $^1$H NMR spectrum was recorded of the mixture. The spectrum of the 1:1 mixture showed only one set of signals confirming that the compounds have the same constitution (Supplementary Fig. 8B).
**Supplementary Figure 7:** MS/MS fragmentation patterns: (A) synthetic standard of DL-4-nitrotryptophan, (B) L-4-nitrotryptophan 2 isolated from the enzymatic reaction.
Supplementary Figure 8: $^1$H NMR spectra of synthetic and enzymatic 4-nitrotryptophan. A: Comparison of the aromatic regions of the $^1$H NMR spectra of the isolated product of the enzymatic nitration reaction (top spectrum) and synthetic dl-4-nitrotryptophan (bottom spectrum). B: Aromatic region of the $^1$H NMR spectrum of a mixture of synthetic dl-4-nitrotryptophan and L-4-nitrotryptophan 2 isolated from the enzymatic reaction.

**LC-MS analyses and purification of enzymatic products using semi-preparative HPLC**

LC-MS analyses were carried out on an Agilent 1100 HPLC instrument equipped with a diode array detector and connected to a Bruker HCT+ mass spectrometer. An Agilent ZORBAX Eclipse XDB-C18 (4.6 x 150 mm, 5 µm) column was used. The flow rate was 1 mL/min and the elution profile is summarised in Supplementary Table 2. Semi-preparative HPLC was carried out on an Agilent 1100 instrument equipped with a Zorbax SB-C18 (21.2 x 100 mm, 5µm) column monitoring absorbance at 210 nm. The flow rate was 5 mL/min and the elution conditions are given in Supplementary Table 3.

| Time (min) | Solvent          |
|-----------|------------------|
|           | Water/ MeOH      |
|           | TFA (0.1%) TFA (0.1%) |
| 0         | 100              | 0               |
| 30        | 0                | 100             |
| 40        | 0                | 100             |
| 60        | 100              | 0               |

**Supplementary Table 2:** HPLC elution conditions for analysis of enzymatic reactions.
**Supplementary Table 3:** HPLC elution conditions for purification of L-4-nitrotryptophan 2 from enzymatic reactions.

| Time (min) | Solvent                  |
|------------|--------------------------|
|            | Water/ TFA (0.1%) | MeOH/ TFA (0.1%) |
| 0          | 100                      | 0                |
| 5          | 80                       | 20               |
| 30         | 80                       | 20               |
| 45         | 0                        | 100              |
| 60         | 100                      | 0                |

**TtxE-catalyzed incorporation of $^{18}$O$_2$ into L-4-nitrotryptophan 2**

Reaction buffer [Tris (25 mM, pH 8), glycerol 5%, L-tryptophan 0.5 mM] was stirred overnight with continuous bubbling of nitrogen gas. All reaction vials were degassed by evacuating on a Shlenck line followed by flushing with argon (x 3). A solution of NADPH (25 mM) was made in a vial that had been flushed previously with argon, using degassed reaction buffer. Gas tight syringes were used to transfer liquids.

Degassed reaction buffer (400 µL) was transferred via syringe to a sealed and degassed 2mL vial and $^{18}$O$_2$ (Cortecnet) was bubbled into the buffer for 5-10min. Solutions of TtxE (1.5 µM final concentration), ferredoxin (5 µL) and ferredoxin reductase (5 µL) were then added followed by a solution of DEANO (2.5 µL, 0.5 mM final concentration) that had been purged with argon for 5min. The vial was placed in a water bath (27 ºC) and a solution of NADPH (20 µL, 1 mM final concentration) was added to start the reaction. The reaction was incubated at 27 ºC for 120 min and aliquots of 100 µL were removed from the reaction after 15, 30, 90 and 120 min. 10% TFA (10 µL) was added to each aliquot, which was then flash frozen using liquid nitrogen and analyzed by high resolution LC-MS on a Sigma Ascentis Express column (C18, 150 x 2.1 mm, 2.7 µm) attached to a Dionex 3000RS UHPLC coupled with a Bruker MaXis Q-TOF mass spectrometer. Mobile phase was water containing 0.1% formic acid and mobile phase B was methanol containing 0.1% formic acid. The flow rate was 0.2 ml/min and absorbance at 220 nm was monitored. The following elution profile was used: 0-5 min – 100% A; 5-17.4 min – 100% A to 100% B; 17.4- 22.4 min – 100% B; then equilibrate back to 100%
A. The mass spectrometer was operated in electrospray positive mode with a scan range of 50-2,000 m/z. Source conditions were: end plate offset at -500 V; capillary at -4500 V; nebulizer gas (N\textsubscript{2}) at 1.6 bar; dry gas (N\textsubscript{2}) at 8 L/min; dry temperature at 180 °C. Ion transfer conditions were: ion funnel RF at 200 Vpp; multiple RF at 200 Vpp; quadruple low mass at 55 m/z; collision energy at 5.0 eV; collision RF at 600 Vpp; ion cooler RF at 50-350 Vpp; transfer time was set to 121 µs; pre-Pulse storage time was set to 1 µs. Calibration was carried out with sodium formate (10 mM) through a loop injection of 20µL of standard solution at the beginning of each run.

**Incubation of TxtE with H\textsubscript{2}\textsuperscript{18}O**

Tris buffer (2 µL, 2.5 M, pH 8) was added to H\textsubscript{2}\textsuperscript{18}O (185 µL) to make the reaction buffer (Tris 25 mM, pH 8). Solutions of TxtE (4 µL, 1.5 µM), ferredoxin (2 µL), ferredoxin reductase (2 µL), L-tryptophan (0.5 mM), DEANO (0.5 mM) and NADPH (1 mM) were added to give a final volume of 200 µL. The same reaction mixture with unlabelled H\textsubscript{2}O in place of H\textsubscript{2}\textsuperscript{18}O and 4-nitro-DL-tryptophan in H\textsubscript{2}\textsuperscript{18}O buffer (Tris 25 mM, pH 8) were prepared as controls. The reactions were incubated at 27 °C for 90 min. 100 µL of each reaction was diluted in 2 mL of H\textsubscript{2}O. The resulting solution was freeze dried, and the residue was dissolved in 100 µl of H\textsubscript{2}O and analysed by LC-MS as described for analysis of TxtE-catalysed L-Trp nitration (Supplementary Fig. 9).
Supplementary Figure 9: Investigation of H$_2^{18}$O incorporation into L-4-nitrotryptophan 2. Extracted ion chromatograms (EICs) at m/z = 250 (corresponding to unlabeled 2) and 252 (corresponding to 2 containing a single $^{18}$O label) from LC-MS analyses of synthetic DL-4-nitro-tryptophan in reaction buffer made with H$_2^{18}$O, but lacking TxtE (traces a and b, respectively); TxtE-catalyzed nitration of L-Trp in reaction buffer made with H$_2^{16}$O (traces c and d, respectively); and TxtE-catalyzed nitration of L-Trp in reaction buffer made with H$_2^{18}$O (traces e and f, respectively).

Incubation of TxtE with L-tryptophan in the absence of oxygen.
Degassed reaction buffer and NADPH solution were prepared as described for TxtE-catalyzed incorporation of $^{18}$O$_2$ into L-4-nitrotryptophan 2.
Degassed reaction buffer (60 µL) was added to a sealed vial followed by solutions of TxtE (1.5 µM final concentration), ferredoxin (10 µL) and ferredoxin reductase (10 µL). The solution was gently purged with argon for ~5 min and degassed reaction buffer (400 µL) was added. The vial was placed in a water bath (27 ºC) and a solution of DEANO (2.5 µL, 0.5 mM final concentration) was added followed by a solution of NADPH (20 µL, 1 mM final concentration). The reaction was incubated at 27 ºC for 120 min. The reaction was stopped by addition of 10% TFA (10 µL), and the resulting solution was flash frozen using liquid nitrogen and analyzed by high resolution LC-MS as described for TxtE-catalyzed incorporation of $^{18}$O$_2$ into L-4-nitrotryptophan 2. No L-4-nitrotryptophan 2 was detected (Supplementary Fig. 10).
Supplementary Figure 10: Dependence of TtxE-catalyzed nitration on oxygen. Extracted ion chromatograms at $m/z = 250.08 \pm 0.005$ (corresponding to L-4-nitrotryptophan) from LC-MS analyses of TtxE-catalyzed nitration of L-Trp in the presence of oxygen (top trace) and in the absence of oxygen (bottom trace).

Synthesis of DL-4-nitrotryptophan

DL-4-nitrotryptophan was synthesized in five steps from commercially available 4-nitroindole\(^7,\, 8\). 4-nitroindole was converted to the corresponding gramine \(3\) then elaborated to the DL-4-nitrotryptophan in four further steps (Supplementary Figure 11). The product was purified by semi-preparative reverse phase HPLC using the elution conditions described in Supplementary Table 4.
Supplementary Figure 11: Synthesis of DL-4-nitrotryptophan. (i) NMe₂, CH₂O, AcOH, 20 °C, 17h, 61%; (ii) MeI, EtOH, 20 °C, 98%; (iii) NaOH, toluene, Δ, 17h, 54%; (iv) EtOH/H₂O, 2M NaOH, Δ, 6h; (v) 5M HCl(aq), Δ, 17h, 85%.

Dimethyl-(4-nitro-1H-indol-3-ylmethyl)-amine 3

AcOH (2.5 mL) was cooled to 0°C and to it was added formaldehyde (39%, 0.19 mL, 1.7 mmol) followed by dimethylamine (2 M in THF, 0.85 mL, 1.7 mmol). The solution was stirred at 0°C for 10 min then added to solid 4-nitroindole (250 mg, 1.54 mmol). The suspension was allowed to warm to 20 °C and was stirred for 17 h during which time the solid dissolved to give a yellow solution. The solution was diluted with H₂O (10 mL) and washed with EtOAc (2 x 5 mL). The aqueous phase was made basic with NaOH and extracted with EtOAc (3 x 5 mL). The combined organic extracts were washed with brine, dried over MgSO₄ and concentrated to dryness in vacuo. The crude solid was tritutrated with diethyl ether to give the product as a yellow solid (230 mg, 68%).

v_max (neat, cm⁻¹) 1513, 1455, 1370, 1353, 1319, 1297, 1279, 854, 824, 784, 726.
δ_H (300MHz, MeOD) 2.17 (6H, s, N(CH$_3$)$_2$), 3.76 (2H, s, CH$_2$N), 7.22 (1H, dd, J=8Hz, J=8Hz, C$^6$H) 7.48 (1H, s, C$^2$H), 7.72 (1H, dd, J=1Hz, 8Hz, C$^5$H or C$^3$H), 7.82 (1H, dd, J=1Hz, 8Hz, C$^5$H or C$^4$H)  
δ_C (100MHz, MeOD) 44.73 (N(CH$_3$)$_2$), 56.80 (CH$_2$N), 112.42, 118.33 (CH), 118.67 (CH), 120.08, 121.15 (CH), 131.42 (CH), 141.12, 144.30 (CNO$_2$).

HRMS (ES$^+$) calculated for [C$_{11}$H$_{13}$N$_3$NaO$_2$]$^+$: 242.0900, found: 242.0890.

**Trimethyl-(4-nitro-1H-indol-3-ylmethyl)-ammonium iodide 4**

![4](image)

MeI (108 μL, 1.1 mmol) was added to a solution of the amine 3 (220 mg, 1.0 mmol) in ethanol (1.5 mL). The solution was stirred at 20 °C for 17 h. The resulting suspension was centrifuged and the supernatant was decanted. The solid was washed with EtOAc (2 x 0.5 mL) and centrifuged to give the product as a yellow solid (95%, 340 mg) which was used directly in the next step without further purification or characterization.

HRMS (ES$^+$) calculated for [C$_{12}$H$_{16}$N$_3$O$_2$]$^+$: 234.1237, found: 234.1241

**Diethyl 2-acetamido-2-((4-nitro-1H-indol-3-yl)methyl)malonate 5**

![5](image)

Dry toluene (3 mL) was added to pulverised NaOH (60 mg, 1.5 mmol) and the suspension was refluxed at 110 °C for 1 h. Diethylacetamidomalonate (220 mg, 1.0 mmol) was added as a solid and the reaction was stirred for 5 min. The ammonium salt 4 (340 mg, 0.9 mmol) was added and the reaction was stirred at reflux for a further 3h. The reaction was then allowed to cool to 20 °C and stirred for 17 h. The reaction solution was
diluted with EtOAc (10 mL) and washed with H₂O (3 mL) and brine (3 mL), dried over MgSO₄ and reduced in vacuo to give the crude product as a brown oil. The crude product was purified by flash column chromatography (1:1 hexane:EtOAc) to afford the product as a yellow solid (46 %, 160 mg).

υₘₐₓ (neat, cm⁻¹) 1743, 1727, 1653, 1624, 1517, 1298, 1280, 1210, 1194, 784, 797.

δ_H (400 MHz, CDCl₃) 1.23 (6H, t, J = 7.1 Hz, CH₂CH₃), 1.81 (2H, s, COCH₃), 3.96 (2H, s, CH₂) 4.21 (4H, m, CH₂CH₃), 6.60 (1H, s, NH), 7.15 (1H, dd, J = 8.0 Hz, J = 8 Hz, C₆H), 7.40 (1H, d, J = 2.0 Hz C₅H), 7.56 (1H, d, J = 8.0 Hz, C₇H), 7.83 (1H, d, J = 8.0 Hz, C₅H), 9.45 (1H, s, NH).

δ_C (400 MHz, MeOD) 14.18 (2(CH₂CH₃)), 22.36 (COCH₃), 31.34 (CH₂CN), 63.35 (2(CH₂CH₃)), 69.15 (C(CO₂Et)₂), 109.13 (C₃), 118.67 (C₅ or C₇), 118.82 (C₅ or C₇), 120.43 (C₅), 121.00 (C₆), 131.62 (C₇), 140.75 (C₈), 144.18 (C₄), 169.21 (COOEt), 172.53 (COMe).

HRMS (ES⁺) calculated for [C₁₈H₂₁N₃NaO₇]+= 414.1272, found: 414.1265

**DL-4-Nitrotryptophan**

The ester 5 (100 mg, 0.26 mmol) was dissolved in EtOH (1 mL) to give a yellow solution and NaOH (5 M, 0.5 mL) was added resulting in a deep red solution. The solution was refluxed for 17 h at 110 °C. The red solution was acidified, at which point it became yellow and was reduced in vacuo. The residue was triturated with EtOAc/MeOH (10:1) and the organic phase was concentrated in vacuo. The residue was dissolved in a minimum of THF (0.2 mL) and HCl (5 M, 1 mL) was added. The solution was refluxed at 100°C for 17 h and then concentrated in vacuo. The resulting residue was dissolved in acidified H₂O (50 mM) and purified by HPLC using conditions described in Supplementary Table 4, to give the product as a yellow crystalline solid (45 mg, 69 %).
$\nu_{\text{max}}$ (neat, cm$^{-1}$): 3350, 2920, 1726, 1504, 1443, 1365, 1318, 1295, 1262, 1203, 803, 789.

$\delta_{\text{H}}$ (500 MHz, 100mM DCl in D$_2$O) 3.37 (1H, dd, $J= 8.6$ Hz, $J=15$ Hz, C$_{1^{'}}$-HH), 3.73 (1H, dd, $J=6$ Hz, $J=15$ Hz, C$_{1^{'}}$-HH), 4.24 (1H, dd, $J= 8.6$ Hz, $J=6$ Hz C$_{2^{'}}$H), 7.31 (1H, dd, $J=8$Hz, $J=8$Hz, C$_{6}$H), 7.55 (1H, s, C$_{2}$H), 7.87 (1H, d, $J=8$Hz, C$_{7}$H), 8.02 (1H, d, $J=8$Hz, C$_{5}$H).

$\delta_{\text{C}}$ (125 MHz, 100mM DCl in D$_2$O): 29.27 (C$_{1^{'}}$), 55.00 (C$_{2^{'}}$), 106.84 (C$_{3}$), 118.82 (C$_{8}$), 119.60 (C$_{5}$), 120.31 (C$_{7}$), 121.50 (C$_{6}$), 132.14 (C$_{2}$), 140.07 (C$_{9}$), 141.88 (C$_{4}$), 172.26 (C$_{3}$).

m/z (ES$^+$) [MH]$^+$ 250, [M-17]$^+$ 233, [M-34]$^+$ 215, [M-45]$^+$ 204.

HRMS (ES$^+$) calculated for [C$_{11}$H$_{12}$N$_{3}$O$_{4}$]$^+$: 250.0822, found: 250.0817.

**Purification of synthetic DL-4-nitro-tryptophan using semi-preparative HPLC**

Semi-preparative HPLC was carried out on an Agilent 1100 instrument equipped with a Zorbax SB-C18 (21.2 x 100 mm, 5 µm) column monitoring absorbance at 400 nm. The flow rate was 5 mL/min and the elution conditions are given in Supplementary Table 4.

| Time (min) | Solvent         |
|------------|-----------------|
|            | Water/ TFA 0.1% | MeOH/ TFA 0.1 |
| 0          | 100             | 0             |
| 20         | 60              | 40            |
| 35         | 60              | 40            |
| 45         | 0               | 100           |
| 60         | 100             | 0             |

**Supplementary Table 4:** HPLC elution conditions for purification of synthetic DL-4-nitrotryptophan.

**Thaxtomin A production.** *S. turgidiscabies* cultures were grown in 5 ml of oat bran broth (OBB)$^9$ in 6-well plates. Each well was inoculated with $10^5$ spores and plates were incubated at 25°C and shaken at 120 rpm for 7 days. Cultures were then filtered and thaxtomin A was extracted by applying the filtrate to C18 SPE columns (Alltech, Deerfield, IL) washed with 25% ethanol, eluted with 50% methanol, and quantified using
HPLC (column: 5 μm C18; 250 x 4.6 mm; mobile phase: MeCN:H$_2$O (40:60), 1 ml/min, detection at 380 nm).

**Molecular genetic manipulations.** DNA and RNA manipulations were performed according to standard techniques.$^{10,11,12}$ *S. turgidiscabies* Car8 is a plant pathogenic strain that has been described previously.$^{13}$ DNA was extracted from *Streptomyces* cultures grown in CRM medium.$^{14}$ Total genomic DNA was extracted using a modification of the procedure of Rao et al.$^{15}$ as specified in Keiser et al.$^{11}$. A *txtE* targeting vector was constructed using *txtE* flanking regions ligated to a fragment containing *tsr* (encoding thioestrepton resistance) in vector pOJ260.$^{11}$ The 5’ (3022 bp) and 3’ (1597 bp) regions flanking *txtE* were PCR amplified using primers 5’P450BamHI (5’-CTTGGATCCAGGCTGGCGCTCTACCC-3’), 5’P450HindIII (5’-ACACAAGCTTGATCTCCCCTGGCTCTACG-3’) and 3’P450BamHI (5’-TTCAGGATCTGTGGCGAGCGGCGACGATTTAT-3’), 3’P450EcoRI (5’-TCTAGAATTCCCGCCTTCGCCACCTACTACGAC-3’), respectively (Fig. 1C). A 1.8 kb fragment containing *tsr* was generated by BamHI digestion of pKK2007.$^{10}$ The three fragments were directionally cloned into pOJ260 linearized by HindIII and EcoRI, resulting in p2XP450. p2XP450 was propagated in the methylation-deficient *E. coli* ET12567 and subsequently used to transform *S. turgidiscabies* Car8 protoplasts. Transformant colonies were screened for an apramycin sensitive, thioestrepton resistant phenotype.

**NO and L-4-nitrotryptophan feeding experiments.** Cultures were grown in 6-well plates in 5 ml of OBB medium inoculated with 10$^5$ spores at 25°C and shaken at 120 rpm. At the onset of thaxtomin production, as detected by development of a yellow coloration in the medium of *S. turgidiscabies* Car-8 cultures (approximately 3 days after inoculation), 50 μl of 100 mM diethylamino nitric oxide (DEANO) (Invitrogen, Carlsbad, CA) in water (1 mM, final concentration), 15 μg of L-4-nitrotryptophan in water, or 50 μl of water were added to cultures. L-4-nitrotryptophan was produced, purified and characterized as previously described.$^{16}$ At 7 days the cultures were harvested and thaxtomin were extracted and quantified by HPLC as described previously.$^{16,17}$
**S1 Nuclease mapping**

S1 nuclease protection assays were performed according to standard techniques\(^\text{11}\). The transcriptional start site of *txtD* was evaluated using a 416 bp probe possessing a 131 bp heterologous 5’ tail in order to distinguish probe-probe annealing artifacts from read-through transcription (Supplementary Fig. 12). This probe was generated using pGEMT (Promega) as a PCR template using a \(\gamma^{32}\text{P}-\text{dATP}-\text{labeled downstream primer JK61 (5’GCACGGGGCGGGCAAGGAAGG)}\) that annealed to a cloned 512 bp insert and an unlabelled upstream primer JK13F (5’CCAGCTGGCGAAAGGGGATGTG) that annealed to the pGEMT vector. Primer JK61 was \(\gamma^{32}\text{P}-\text{dATP}-\text{labeled using T4 polynucleotide kinase according to the manufacturer’s instructions (Invitrogen).}\)

The transcriptional start site of *txtE* was determined using a 405 bp probe generated by PCR using JK66F (TTGGGGTAGTCGAAAAGGAAT) and \(\gamma^{32}\text{P}-\text{dATP}-\text{labeled downstream primer JK66R (CGCAGTACCGTAGCAGAAC)}\). A DNA sequencing ladder was generated using a DNA di-deoxyterminal sequencing kit (USB) using primer JK66R (Supplementary Fig. 12).
Supplementary Figure 12: Transcriptional organization of the txtE-txtD operon. A, Genetic organization of the txtE-txtD locus showing probes used in the S1 nuclease mapping experiments. B, Nucleotide sequence of the txtE promoter region, showing the txtE transcription start point, putative -10 and -35 core promoter motifs (double underlined), a 9 bp inverted repeat (underlined), and the translational start point of TxtE (bold). C, The txtE promoter regions of S. turgidiscabies Car8 and S. scabies 87.22. The putative core promoter motifs are double underlined. D, High resolution S1 nuclease mapping of the 5' end of the txtE transcript using probe A, demonstrating the transcriptional start point of txtDE (asterisks). RNA was isolated from a 3 day S. turgidiscabies culture grown in OBB. Lanes labelled G, A, T, and C represent a
di-deoxy sequencing ladder generated using the same oligonucleotide used to generate probe A. E. S1 nuclease protection of the \textit{txtE-txtD} polycistronic transcript. RNA was isolated from a 3 day \textit{S. turgidiscabies} culture grown in OBB with or without 10% glucose (thaxtomin biosynthesis is catabolite repressed). Probe B provided full length protection of a 285 bp fragment spanning the 3’ terminus of \textit{txtE} and 5’ translational start of \textit{txtD}, indicating that transcription of \textit{txtD} likely occurs upstream of \textit{txtE}. Probe B contains a 135 bp 5’ heterologous tail to distinguish probe-probe annealing artifacts.

**Southern Blot Hybridisation**

Deletion of \textit{txtD} was confirmed using Southern blot hybridization. \textit{Eco}RI digested genomic DNA was resolved on a 0.7% agarose gel using horizontal gel electrophoresis and then transferred to Hybond-N nylon membranes (Amersham) using the manufacturer’s alkaline transfer protocol. An internal 993 bp \textit{txtD} probe was generated by PCR amplification using primers JK50F1 (GCACCCCCTGGCCGAACGACA) and JK50R1 (TCCGGCGCGACCCCATCCACT). The $\alpha$-32P-dATP-labeled DNA probes were generated using a random primer labeling kit (Invitrogen). Hybridization was performed at 65°C in Hyb-9® hybridization solution (Gentra Systems). The most stringent wash was 0.2X SSC and 0.5% SDS for 20 min at 65°C. Autoradiography was performed with X-OMAT AR X-ray film (Eastman Kodak) at -80°C.

**Roles of TtxE and TtxD in vivo**

The contributions of TtxE and TtxD to thaxtomin production were evaluated by cloning \textit{txtE} under the control of a thiostrepton inducible promoter (p\textit{tipA}) in pIJ8600. pIJ600TXTD was described previously. \textit{txtE} was PCR amplified using primers JK33F\_NdeI (5’- TGGACATATGCTCGCCTCCTGCGCTTTCTGGAATCCC) and JK33R\_BamHI (5’- ATTCGGATCCTCAGCGGAGGCTGAGCGGCAGTGA), and the PCR product was digested using \textit{NdeI} and \textit{BamHI}. Plasmid vector pIJ8600 was propagated in \textit{E. coli} ET12567 and digested with \textit{NdeI} and \textit{BglII} restriction endonucleases. The resulting JK33FR insert and pIJ8600 vector were ligated using T4 DNA ligase, and the ligation reaction was used directly to transform \textit{S. turgidiscabies} wild type, \textit{$\Delta$txtE}, and \textit{$\Delta$txtD} strains using polyethylene glycol-mediated protoplast transformation. The sequence of \textit{tipA::txtD} in \textit{S. turgidiscabies} was confirmed by colony PCR amplification using primers JK58F (5’- CGTGAGGAGGCAGCGTGGAC)
and JK58R (5’- TTATGCGTGGGCGATGGTTGTTGT) and DNA sequencing of the PCR product.

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