A Novel Mycolactone from a Clinical Isolate of *Mycobacterium ulcerans* Provides Evidence for Additional Toxin Heterogeneity as a Result of Specific Changes in the Modular Polyketide Synthase

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Mycolactones are macrocyclic polyketide toxins produced by the pathogen *Mycobacterium ulcerans*, the etiologic agent of the emerging human disease known as Buruli ulcer.[1] The disease is characterised by large necrotic skin lesions, and currently surgical intervention is the only realistic therapy.[2] Mycolactone appears to play a key role in infection, since, in an animal model, subcutaneously injected purified mycolactone reproduces the pathology of the disease, while *M. ulcerans* strains deficient in mycolactone production do not provoke lesions.[1] Mycolactones thus appear to provide the first example of a polyketide virulence factor in a human pathogen.[3] Mycolactone also has immunosuppressive properties and appears to induce apoptosis.[4,5]

The structures of mycolactones A and B have been determined[1,6] to be, respectively, the *Z*- and *E*- isomers of a 12-membered macrocyclic polyketide to which a second highly unsaturated polyketide chain is appended via an ester linkage (Scheme 1). The complete structures and their absolute configuration have been confirmed by chemical synthesis.[7,8] Further work has revealed the existence, in culture extracts of a typical strain of *M. ulcerans*, of small amounts of other mycolactones that differ from mycolactones A and B only in the side chain[9–11] and whose structures very largely reflect the aberrant operation of a specific cytochrome P450 hydroxylase required for mycolactone biosynthesis.[3,10,12] Scrutiny of 34 different clinical isolates also indicated very little heterogeneity, again restricted to the side chain, although the structures were not examined in detail.[11]

The genetic basis for mycolactone biosynthesis has recently been revealed.[12] *M. ulcerans* contains a 174 kb megaplasmid that harbours, in addition to a number of auxiliary genes, several very large genes encoding type I modular polyketide synthases that closely resemble the actinomycete PKSs that

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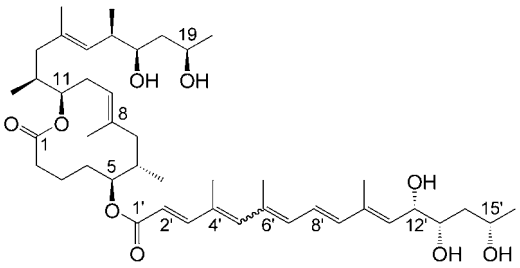

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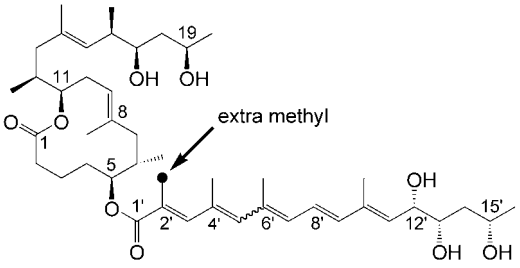

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Scheme 1. *The structures of mycolactone A (Z-$\Delta^{4',5'}$) and B (E-$\Delta^{4',5'}$) from the African strain MUAgy99 (**1**) and the Chinese strain MU98912 (**2**).*

govern the biosynthesis of erythromycin, rapamycin and other macrocyclic polyketides, in which each module of fatty acid synthase-related enzymes catalyses a specific cycle of polyketide chain extension.[13,14] Genes *mlsA1* (51 kbp) and *mlsA2* (7 kbp) encode the PKS for production of the 12-membered core lactone, while *mlsB* (42 kbp) encodes the side-chain PKS.

The availability of this sequence has prompted us to investigate the structural differences between mycolactones A/B, from an African isolate (MUAgy99) and the mycolactones produced by another pathogenic strain of *M. ulcerans*, to see whether any variant mycolactones in the latter strain might be accounted for by changes within the PKS rather than changes in processing steps. To characterise the mycolactone metabolites, we used our recently described method of LC-sequential mass spectrometry (LC-MS^n), performed on an ion-trap mass spectrometer.[10] Ion-trap mass spectrometry (by using either FTICR or a quadrupole ion trap) allows multistage collision fragmentation of target molecules to yield detailed structural information. We report that mycolactones from a pathogenic strain of *M. ulcerans* from China (MU98912) all possess an extra methyl group at C2′ compared to mycolactone A (see Scheme 1), as the apparent result of the recruitment of a single catalytic domain of altered specificity in the mycolactone PKS.

For details of the growth of *M. ulcerans* strains and extraction of metabolites, see the Experimental Section. Preliminary LC-MS analysis of the cell extract showed that normal mycolactones, with characteristic values of $m/z = 765$, 763, 749, and 747, were not produced by the Chinese strain, MU98912. However, at least three new components at $m/z = 779$, 777 and 761, were detected. When on-line LC-MS/MS analyses were performed on these ions, they showed fragmentation patterns surprisingly similar to that of normal mycolactone A/B (see Figure 1). All the MS/MS spectra of the mycolactones from MU98912 contained fragment ions corresponding to fragment ions A and B in MUAgy99, which are characteristic of the core lactone and the polyketide side chain, respectively.[10] Fragment ion A was conserved in all the spectra, while fragment ion B varied exactly in accordance with the variation in the mass of the precursor ion. It therefore appears that the core lactone is identical in the mycolactones from MUAgy99 and MU98912, and structural variations are restricted to the polyketide side chain.

To obtain further information about such structural variations, off-line accurate-mass analyses and deuterium-exchange experiments were performed on these newly identified mycolactones. The results, when compared to those from the classic mycolactones from MUAgy99 (Table 1) clearly showed that mycolactones from MU98912 have the same number of exchangeable protons but an extra methylene group compared to their counterparts from MUAgy99. We reasoned that these results might be accounted for if there were an extra *C*- or *O*-linked methyl substituent in the side chain of all the mycolactones from the MU98912.

Table 1. *Comparison of molecular formulae and of numbers of exchangeable protons in mycolactones from the African strain MUAgy99 and the Chinese strain MU98912.*

MUAgy99[a]			MU98912				
Metabolite $[M+Na]^+$	Formula	n[b]	Metabolite $[M+Na]^+$	Formula	Observed Mass	Error (ppm)	n[b]
765	$C_{44}H_{70}O_9Na$	5	779	$C_{45}H_{72}O_9Na$	779.5022	−6.0	5
763	$C_{44}H_{68}O_9Na$	4	777	$C_{45}H_{70}O_9Na$	777.4922	1.3	4
747	$C_{44}H_{68}O_8Na$	3	761	$C_{45}H_{70}O_8Na$	761.4943	3.0	3

[a] The data for mycolactones from MUAgy99 are taken from ref. [10]. [b] Number of deuterons after exchange.

To test this idea, and to locate the exact position of such an extra methyl group within the side chain, detailed comparisons were carried out between the MS/MS spectra of mycolactones from the two strains. In the MS/MS spectra of mycolactones from MUAgy99 (a representative MS/MS spectrum (of $m/z = 765$) is shown at the top of Figure 1), the fragment ion at $m/z = 565$ is always seen. We have previously proposed that this conserved fragment, designated fragment ion C,[10] arises as a result of cleavage of the C6′–C7′ bond. In addition to fragment ion C, conserved fragment ions at $m/z = 579$ (ion D) and 631 (ion E) arise from the mycolactones from MUAgy99, and are identified by deuterated MS/MS analysis (data not shown) as resulting from cleavage of C7′–C8′ and C10′–C11′, respectively (see Scheme 2). In comparison, in the MS/MS spectra of mycolactones from MU98912, deuterated MS/MS analysis showed the counterpart of ion E ($m/z = 631$) increased by 14 mass units to $m/z = 645$; this suggests that there is an extra methyl group and that it lies within the span C2′ to C10′. However, no fragment 14 mass units higher than fragment ion D ($m/z = 579$) was seen. Instead of both ion C ($m/z = 565$) and ion D ($m/z =$

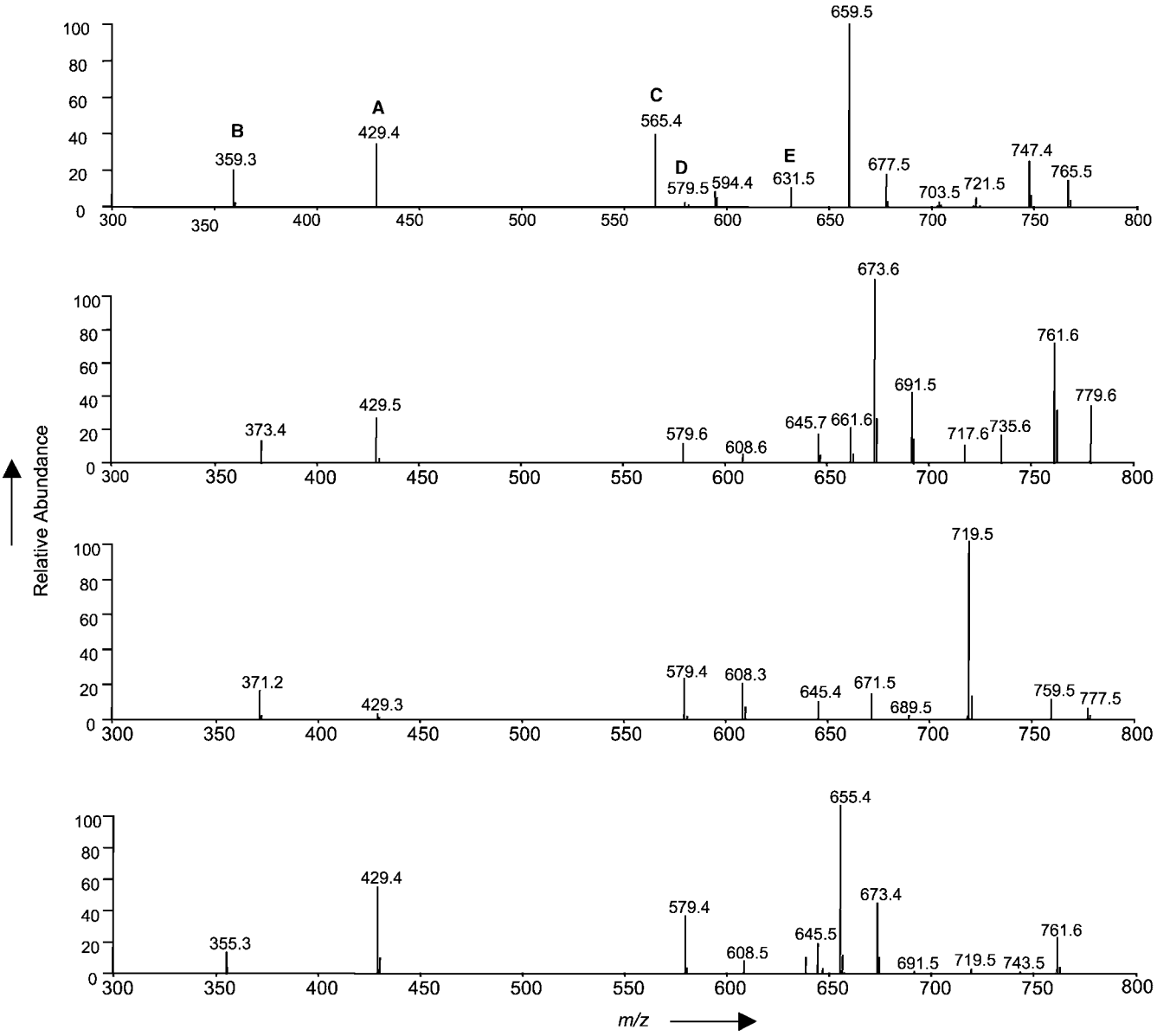
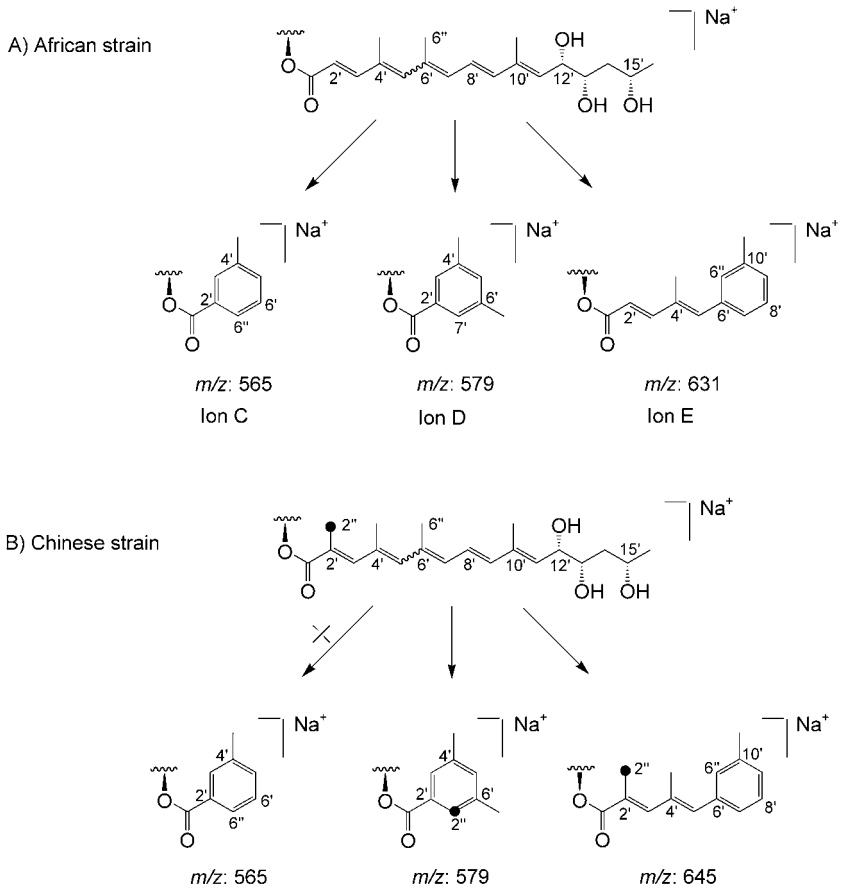


Figure 1. *The MS/MS spectra of mycolactone precursor ions (from top to bottom) at* m/z *= 765 (MUAgy99) and at* m/z *= 779, 777 and 761 (MU98912).*

579), only a fragment ion at $m/z = 579$ (14 mass units higher than fragment C) was seen. This important information provides strong evidence that there is an extra *C*-linked methyl group at the C2′ position.

In the light of this specific structural difference between the mycolactones from MUAgy99 and MU98912, nucleotide-sequence analysis of the appropriate part of the mycolactone biosynthetic genes was carried out. Preliminary restriction-mapping analysis of the *M. ulcerans* megaplasmid bearing the mycolactone biosynthetic genes showed (as expected) no evident differences between MUAgy99 and MU98912. We then specifically amplified by PCR and sequenced the DNA-encoding extension module 7 of the PKS MlsB, which governs the insertion of the last polyketide extension unit to provide carbons C1′ and C2′ of the side chain. For the bulk of this module, there were no significant amino acid-sequence differences between the two strains (overall DNA sequence identity $> 99.3\,\%$). However, the acyltransferase domain AT7 showed highly significant differences, as shown in Figure 2. The sequence of AT7 from MU98912 is identical to a typical methylmalonyl-CoA-specific AT domain from elsewhere in the mycolactone PKS, such as the extension module 6 of MlsB,[12] and differs markedly over much of its length from the sequence of the (malonyl-CoA-specific) AT7 of MUAgy99. In particular, the sequence motifs highlighted are all highly diagnostic of differences between substrate specificity for methylmalonyl- or malonyl-CoA.[15–18]

It has been recently demonstrated that the substrate specificity of an acyltransferase domain in a modular PKS can be widened to accommodate both methylmalonyl-CoA and ma-



Scheme 2. *The proposed structures of fragment ions C, D and E from the African strain MUAgy99 and of the corresponding fragment ions from the Chinese strain MU98912.*

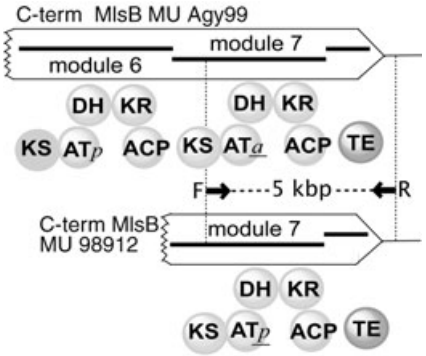


Figure 2. *Schematic representation of the domain structure of extension modules 6 and 7 in MlsB from MUAgy99 and module 7 from MU98912 showing the position of the oligonucleotides used for PCR and the altered AT7 domain substrate specificity identified by DNA sequencing of the PCR product from strain MU98912 compared with strain MUAgy99.*

lonyl-CoA by the specific alteration of very few key active-site residues.[16–18] Figure 3 illustrates the fact that AT domains in the mycolactone PKS that are specific for malonyl- or methylmalonyl-CoA show much more deep-seated differences and are only mutually identical in sequence at their N and (particularly) C termini. There is thus an apparent replacement of a large portion of the side chain PKS module 7 AT domain in one *M. ulcerans* strain compared to the other. The evolutionary pathway by which these changes occurred remains obscure, but the discovery of this natural difference is prefigured by the strategy of AT "domain swapping", which has been widely used to switch the chemical specificity of modular PKSs.[19,20] Whatever natural mechanisms are at work, the additional heterogeneity of mycolactones revealed in this study suggests that mycolactone-related metabolites from various *M. ulcerans* strains might form a much larger structural family than first appreciated. It remains to be explored whether this structural variation influences the biological properties of the mycolactone products.

Experimental Section

Microbiological methods: The two clinical isolates of *M. ulcerans* used in this study, MUAgy99 and MU98912, were obtained from patients in Ghana and China, respectively.[21] MU98912 was kindly provided by F. Portaels. All necessary permissions have been obtained for the distribution of these strains. The growth of strains and the preparation of cell extracts were performed as previously described.[10] For DNA sequence analysis, the DNA-encoding module 7 of the PKS MlsB was PCR-amplified from each strain by using genomic DNA as template with the forward primer ALLKS-CTERM-F 5′-CCTCATCCTCCAACAACC-3′ (corresponding to the C-terminal end of the KS7 domain of MlsB) and the reverse primer MLSB-intTE-R 5′-GCTCAACCTCGTTTTCCCCATAC-3′ (corresponding to a position just downstream of the mlsB stop codon as shown in Figure 2). A 5 kbp product was obtained in both cases and was fully sequenced on both strands by primer walking. The DNA sequence obtained from MU98912 has been deposited in Genbank under the accession No. AY743331.

LC-MS analysis: LC-MS and LC-MS/MS analyses were carried out on a Finnigan LCQ instrument, essentially as previously described.[10] Accurate-mass analyses were performed on an API QSTAR pulsar (Applied Biosystems). Deuterium-exchange experiments were carried out as previously described.[10]

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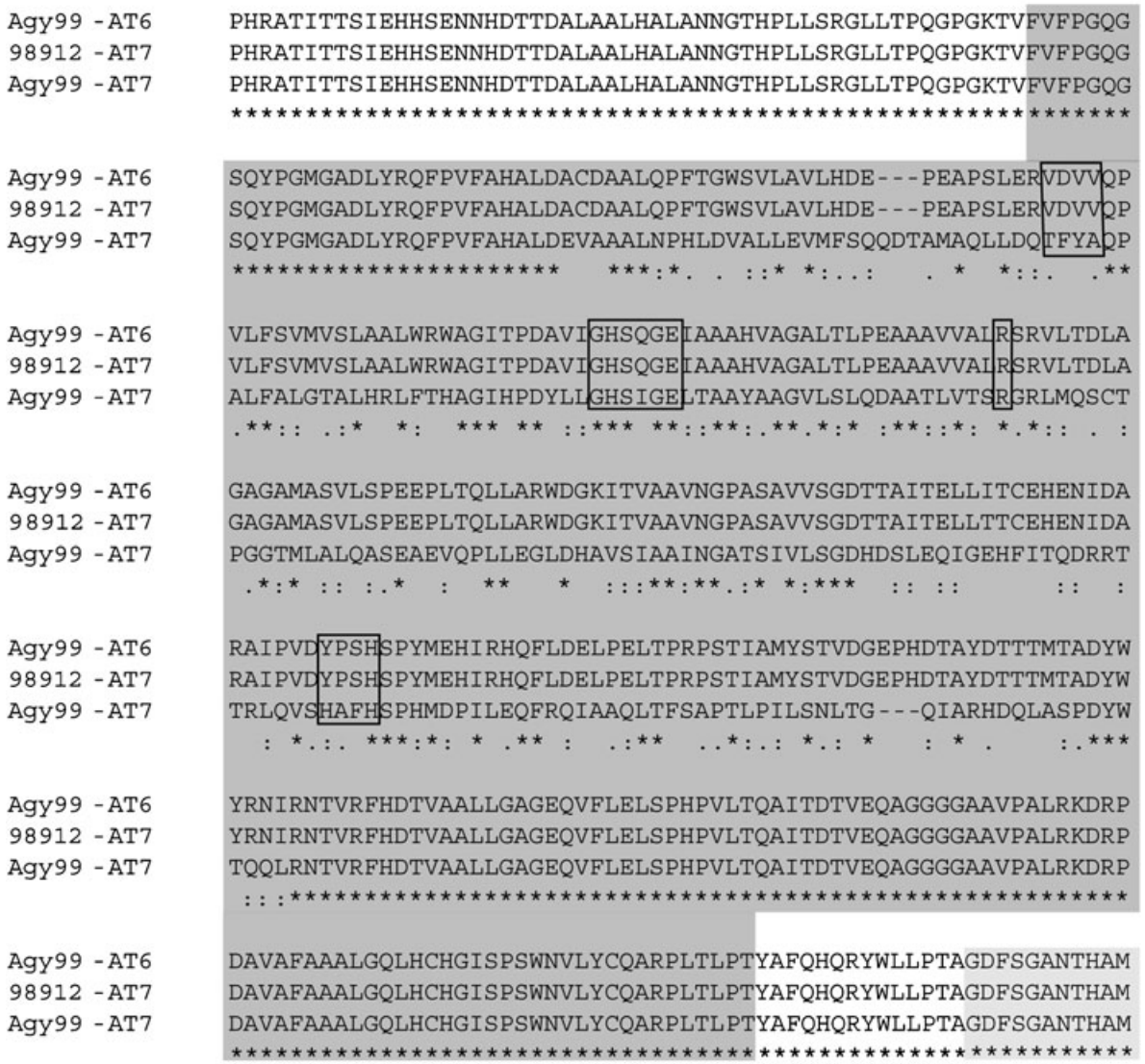

Figure 3. *Amino acid sequence comparisons between the AT6 and AT7 domains of MUAgy99 with the AT7 domain of MU98912. Dark grey shading indicates the AT domain. Boxed sequences are residues known to be critical for AT substrate specificity. Light grey shading indicates the start of the DH domain.*

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