

Dynamic thiolation–thioesterase structure of a non-ribosomal peptide synthetase

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Non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) produce numerous secondary metabolites with various therapeutic/antibiotic properties¹. Like fatty acid synthases (FAS), these enzymes are organized in modular assembly lines in which each module, made of conserved domains, incorporates a given monomer unit into the growing chain. Knowledge about domain or module interactions may enable reengineering of this assembly line enzymatic organization and open avenues for the design of new bioactive compounds with improved therapeutic properties. So far, little structural information has been available on how the domains interact and communicate. This may be because of inherent interdomain mobility hindering crystallization, or because crystallized molecules may not represent the active domain orientations². In solution, the large size and internal dynamics of multidomain fragments (>35 kilodaltons) make structure determination by nuclear magnetic resonance a challenge and require advanced technologies. Here we present the solution structure of the apo-thiolation–thioesterase (T–TE) di-domain fragment of the *Escherichia coli* enterobactin synthetase EntF NRPS subunit. In the holoenzyme, the T domain carries the growing chain tethered to a 4′-phosphopantetheine whereas the TE domain catalyses hydrolysis and cyclization of the iron chelator enterobactin. The T–TE di-domain forms a compact but dynamic structure with a well-defined domain interface; the two active sites are at a suitable distance for substrate transfer from T to TE. We observe extensive interdomain and intradomain motions for well-defined regions and show that these are modulated by interactions with proteins that participate in the biosynthesis. The T–TE interaction described here provides a model for NRPS, PKS and FAS function in general as T–TE-like di-domains typically catalyse the last step in numerous assembly-line chain-termination machineries.

The *Escherichia coli* enterobactin synthetase (Ent) synthesizes the siderophore enterobactin, a virulence factor used by *E. coli* to infect iron-limited microenvironments of vertebrate hosts. Enterobactin is obtained by three iterative condensations of dihydroxybenzoate (DHB) with serine, followed by macrocyclizing release of the macro-lactone that can form high-affinity hexadentate complexes with ferric iron^{3,4} (Fig. 1). Ent is a two-module (EntB/EntE and EntF), three-protein assembly line that combines the features of both a type I NRPS, in which all domains interact *in cis* in a single protein chain (EntF), and a type II NRPS, in which the domains interact *in trans* as free-standing units (EntB/EntE). The dedicated phosphopantetheinyl transferase (PPTase), EntD, loads the apo form of the T domain with 4′-phosphopantetheine (4′-PP), which tethers the growing acyl chain while allowing the bound substrates to navigate between the catalytic sites (Fig. 1a). The EntE and EntF adenylation (A) domains select the appropriate substrates (DHB or serine) and activate them

as acyl-adenosine-monophosphates. These are then loaded on the 4′-PP arms attached to Ser 245 of the holo-EntB aryl carrier protein (ArCP) and to Ser 48 of holo-EntF T domains, respectively (Fig. 1b). The EntF condensation (C) domain catalyses the DHB–Ser amide-bond formation (Fig. 1c). The DHB–Ser chains are next transferred to the active site Ser 180 of the thioesterase (TE) domain, thereby making the 4′-PP arm of the T domain available for the next cycle of condensation (Fig. 1d). In this step of the reaction, the 4′-PP arm (wavy line in Fig. 1d) has to reach from Ser 48 of the T domain to Ser 180 of the TE domain, in which the triad Ser 180, Asp 207 and His 313 catalyses the oxoester bond formation between the substrate and Ser 180. The steps shown in Fig. 1b–d are repeated twice to produce the tethered linear DHB–Ser trimer before its intramolecular cyclization by the TE domain of EntF, which constitutes the release of the enterobactin trilactone (Fig. 1e). The structural basis of communication between the T and TE domains in chain-release steps at the end of NRPS assembly lines has been so far unknown.

Here we describe the structure of the 37 kDa apo-EntF T–TE di-domain in which the active site Ser 48 (of the T domain) was replaced with Ala to enable production of a homogeneous sample. This mutation is isomorphous as the NMRs of the T–TE di-domain are unchanged except for the immediate environment of residue 48, and the interdomain contacts are identical (see Methods and Supplementary Figs 1 and 2). It represents the physiological structure before CoA addition as indicated at the top of Fig. 1a. The mutated apoprotein allows us to distinguish domain interactions from domain/substrate contributions (including the 4′-PP arm), a crucial knowledge for successful assembly line reprogramming by domain swap. The T and TE domains have a well-defined relative orientation with a primarily hydrophobic interface (Fig. 2a, c). This results in a 1,300 Å² buried surface area not including the linker, which interacts with both domains and contributes to a further 200 Å². The globular T domain forms a three-helical bundle that is wedged between the globular core of the thioesterase and two α -helices (α_{4TE} – α_{5TE}) protruding from this core. These helices resemble two webbed fingers that form a lid covering the T and TE active sites. Ser48Ala and Ser 180 are found 17 Å apart at both ends of a canyon formed by loop L12_{TE} and helix α_{4TE} . This allows the 4′-PP arm, which is tethered to Ser 48 of the T domain in the holoprotein and can span up to ~20 Å, to reach Ser 180 of the TE domain. Dynamics data indicate that the two helices (α_{4TE} – α_{5TE}) form a mobile flap that is able to open to accommodate the 4′-PP arm (see below). Thus, the structure should not be interpreted as rigid but rapidly fluctuating to allow loading of the 4′-PP arm and the growing enterobactin chain. This resembles many other proteins that have to undergo major opening fluctuations to allow binding of target proteins or substrates, such as HIV protease⁵.

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The T domain contacts TE via the beginning of helix α_{2T} that contains Ser48Ala, the end of loop L3_T, and the single turn helix α_{1T} (Fig. 2c). The region near Ser48Ala interacts with both the core of the TE domain, via L2_{TE}, and the tips of the helical fingers, which thus cover the T active site (Fig. 2b). In EntF, helix α_{1T} stabilizes the hydrophobic core of the T domain with a phenylalanine side chain (Phe41) anchored in the heart of the helical bundle. An adjacent phenylalanine (Phe42) interacts with hydrophobic residues of β_{1TE} . Point mutations of either phenylalanines lead to a disruption of the T/TE domain interaction and an unfolded T domain (data not shown). α_{1T} is absent in the only other structure of an (excised) T domain^{6,7}, but has been observed in related FAS and PKS acyl carrier proteins (ACP) and may contribute to domain recognition^{8–10} (see Supplementary Information). Loop L3_T, which interacts with β_{1TE} , features helical characteristics and is mobile as indicated by the low intensities of the corresponding NMR signals (Supplementary Fig. 8).

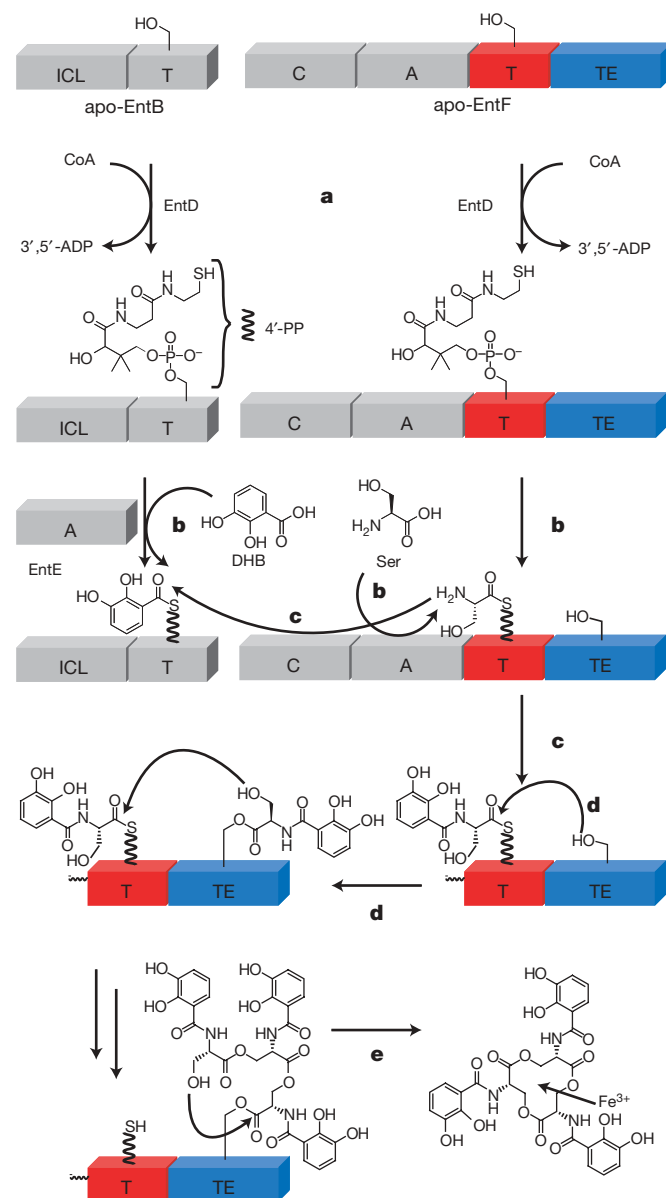


Figure 1 | Summary of enterobactin synthesis. The grey, red and blue boxes represent individual domains; the C, A, T and TE domains are described in the text. The EntB ICL domain is an isochorismate ligase that participates in the conversion of chorismate to DHB, a preliminary step to the synthesis of enterobactin. Initially, EntD loads the phosphopantetheinyl arm (4'-PP, represented as a wavy line) onto the active-site serines of apo-EntB and apo-EntF. The synthesis steps (a–e) are described in the text.

In general, the T domain shows internal mobility as further supported by fast NH exchange (Supplementary Fig. 9). Such dynamics have been observed for single-domain ACP-type T domains in FAS systems¹¹, and may be a general property of acyl and peptidyl carrier proteins (see Supplementary Information).

The TE domain adopts the overall fold of α/β -hydrolases. However, the 'finger' region differs in the two systems for which TE structures have been determined^{12,13}. Helix α_{4TE} faces the catalytic triad, located in a conserved region, and delimits part of the 'cyclization bucket' previously described for substrate macrocyclization in surfactin type I thioesterase¹² (TEI; Fig. 2b, grey and white). For surfactin TEI, two conformers have been observed for this helix¹², whereas the electron density was too low in fengimycin TEI to allow any structural interpretation¹³ (see Supplementary Information). Moreover, NMR studies of the free standing surfactin type II thioesterase (TEII) indicated two conformations in the corresponding lid¹⁴. Accordingly, many residues in this region of the EntF TE domain show reduced intensities in their NMR signals, which is indicative of slow modulations of their environments (Supplementary Fig. 8). Furthermore, unlike the core of the TE domain, all amide protons of the finger region exchange rapidly with

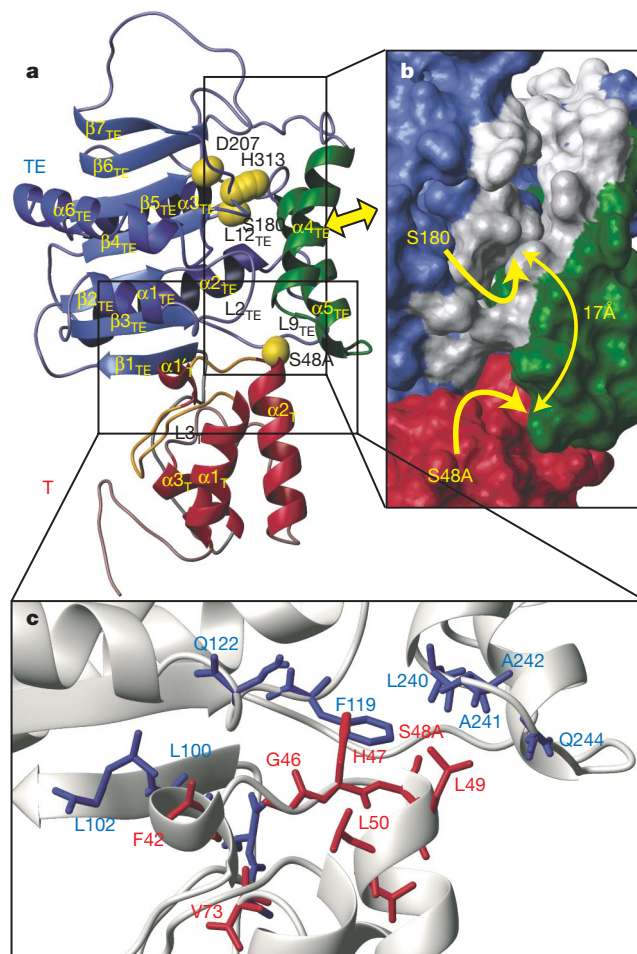


Figure 2 | Structure of the EntF T-TE fragment. **a**, A ribbon diagram is shown. The T domain (red) is wedged between the core (blue) and the lid (green) of the TE domain. Active sites are shown as yellow spheres. The double-headed arrow emphasizes that the flap formed by the α_{4TE} and α_{5TE} helices is relatively mobile, opening frequently, which seems to be necessary to accommodate the 4'-PP arm in the processes depicted in Fig. 1. **b**, Surface representation of the region containing the canyon (grey) which must open to accommodate the 4'-PP arm in the holoprotein. The cyclization bucket¹² is shown in white and grey. In this conformation, Ser48Ala and Ser180 are buried. **c**, The domain interface is shown. The side chains of all residues giving rise to interdomain nuclear Overhauser effects (NOEs) are shown.

solvent (Supplementary Fig. 9), indicative of breathing/opening motions. Such mobility may be necessary to allow for access to both T and TE active sites, which would be occluded in a more rigid canyon structure (see later). The mobility also provides conformational plasticity to the TE active site to accommodate the various intermediates stored on Ser 180 and allow the 4'-PP arm to navigate in and out of the canyon in the holoprotein, which would not be possible in a static interpretation of our solution structure.

In addition to the intradomain dynamics reported above, we observed an open form of the T-TE molecule, T-TE⁰, in which TE and T interact little or not at all (see Supplementary Information and Fig. 3). Indeed, although ACP-type T domains have recently been observed in FAS^{15,16}, they have previously been reported to be invisible in the crystal structures of FAS or PKS due to low electron density¹⁷⁻¹⁹, in agreement with dynamic domain interactions. To investigate the significance of these dynamic events, the T-TE fragment was titrated with binding partners that participate in different steps of enterobactin synthesis. To become functional as carrier proteins, T domains first need to be primed with 4'-PP arms by a PPTase (Fig. 1a). When the broad-specificity PPTase Sfp²⁰ was added to a solution of T-TE, a binding site was identified around the active-site residue 48, as expected⁶ (Fig. 3a, green). In addition, however, the subset of signals of the T-TE⁰ conformer increased, suggesting that Sfp drives the equilibrium towards the dislodged form of the di-domain (Fig. 3). This is reasonable because Ser48Ala is partially covered by the tips of the fingers in the di-domain (Fig. 2b). The

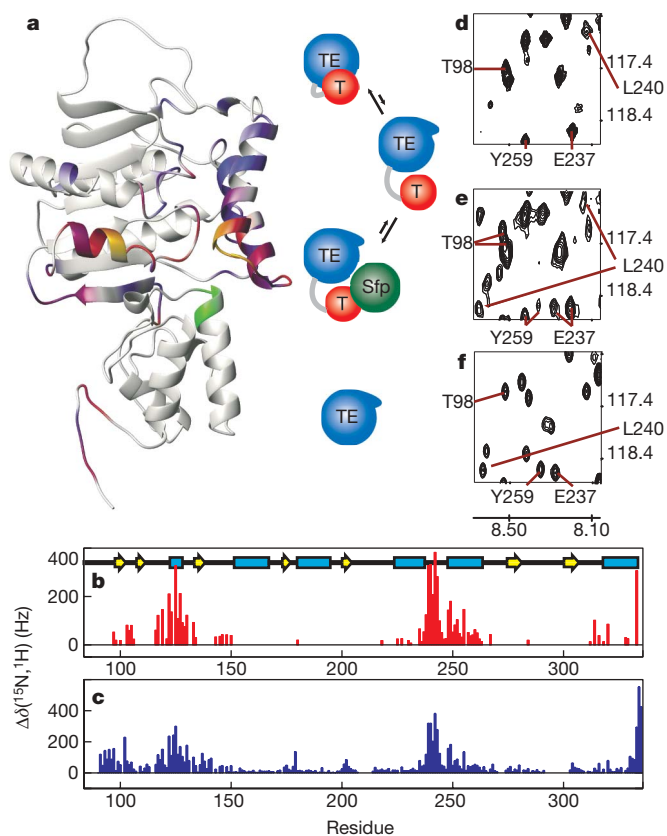


Figure 3 | Interaction with Sfp. **a**, Residues subject to two different environments. The colour code represents small (blue), medium (red) and large (yellow) chemical shift differences between the two forms as reported in **b, c**. Chemical shift differences between the major conformer of free T-TE and the excised TE domain. **d-f**, Spectral signatures of the Sfp interaction. **d**, The reference spectrum of Ser48Ala. The open form is only visible with longer acquisitions (Supplementary Fig. 3). On addition of Sfp, the signals of the residues in green (see **a**) disappear from the spectrum, and those of the second form appear (**e, f**). Corresponding region of the spectrum of the excised TE.

simultaneous observation of new signals and shifts of resonances (Supplementary Fig. 11) indicates the presence of complex dynamic processes involved in the function of this enzyme. The EntF-specific EntD enzyme²⁰ caused similar selection of the open state; in contrast, the ACP-specific AcpS PPTase²¹ did not (Supplementary Fig. 12), highlighting the specificity of the mechanism.

The EntF C domain²² condenses the substrates that are covalently attached as thioesters to 4'-PP arms on the EntB and EntF T domains (Fig. 1c). Specific chemical shift changes on addition of the EntF C domain to T-TE showed the C-domain-binding face on T (Fig. 4), and only minor effects were observed on TE. Because the T/C surface does not overlap with the TE interaction surface, no disruption of the T/TE interface is necessary and only a modulation of their interaction is observed. Notably, no interaction was observed when the TycC5 (the fifth module of the tyrocidine synthetase C subunit) C domain was added to a T-TE solution, suggesting that this T-C recognition is EntF specific (see Supplementary Fig. 13). For both C-domain and Sfp interactions, perturbations on TE are not limited to the interface, but affect the finger region and the TE active site, showing that the binding events probably influence the dynamics and/or the conformations in these areas. These observations apply to the apo form of the molecule and thus only report on domain-domain interactions. It is probable that during biosynthesis the 4'-PP arm, loaded with various intermediates, also affects these dynamic effects.

The structure of the T-TE di-domain provides a starting point for elucidating the communication between domains in NRPS systems. Understanding how the central carrier domains interact with their partner catalytic domains is a prerequisite for understanding the NRPS assembly logic in chain initiation, elongation and termination steps. Our data document a well-defined, yet dynamic, interface between the T and TE domains, consistent with the idea that the T domain must interact with the upstream C and A domains as well as

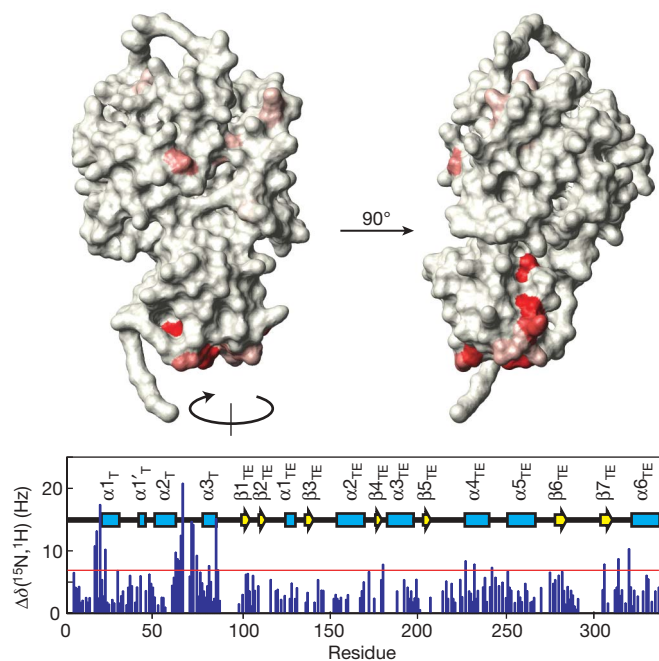


Figure 4 | Interaction with the C domain. Many resonances experience shifts on addition of the C domain (bottom), indicating a modulation of the environment of the corresponding residues. Those belonging to the T domain form an interaction surface that does not overlap with the T-TE interface (top). Thus, no disruption of the T-TE interaction is observed. The effects on the TE domain may be due to weak, indirect interactions with the C domain, or to a secondary effect due to fluctuations of the dynamics or modifications of the conformations in these regions. Residues with shifts larger than one s.d. from the mean (red line, bottom) are colour coded with a gradation from white to red.

this downstream TE domain. The PPTase and C domain, both of which must visit the T domain, modulate the interaction between the T and TE domains as well as the conformational and/or dynamic properties of each domain. Notably, the *in trans* interaction between surfactin TEII and the TycC3 T domain uses a different domain orientation but places the two active sites at a comparable distance¹⁴. Moreover, the *in trans* domain interaction selects subsets of conformational states similarly to the *in cis* situation described here. NRPS domains need to communicate with each other at specific points during the peptide synthesis, hence natural product assembly lines must be dynamic entities. The observed malleability may serve to regulate this interplay by stabilizing a given interaction during the course of the corresponding reaction.

METHODS SUMMARY

Recombinant protein was produced in an *E. coli* expression system. The Ser48Ala mutation was used because it was difficult to produce exclusively holo- or apo-T-TE, rather than a mixture, in bacterial expression systems tested. Moreover, wild-type apo-T-TE exists in two inter-converting conformational states for the T domain (data not shown); a similar phenomenon is observed for the apo-T domain of tyrocidin synthetase, TycC3, in which the Ser48Ala mutation locks the protein in a single conformation⁶. Out of the 337 backbone resonances and the associated side-chain resonances, 313 were assigned with transverse relaxation optimized spectroscopy (TROSY) versions of conventional experiments²³ sometimes with non-uniform sampling²⁴. A double TROSY hNcaNH was recorded to facilitate the assignment²⁵. Most distance restraints were obtained from time-shared nuclear Overhauser enhancement spectroscopy (NOESY) experiments²⁶. High-resolution structures were obtained with the program CYANA²⁷ (backbone root mean square deviations (r.m.s.d.) of 1.2 Å for residues in secondary structure elements, with corresponding r.m.s.d. for the single domains at 0.6 Å (T domain) and 1.0 Å (TE domain; Fig. 2 and Supplementary Fig. 3)). The presence of complex dynamic process prevented the use of residual dipolar couplings^{28,29}. Titrations were conducted with a constant T-TE concentration and with decreasing concentration of the titrant, to ensure that no degradation was induced by the titration. The determination of solvent accessible residues was obtained by changing the solvent from H₂O to D₂O with a PD10 column (GE healthcare).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions D.P.F., G.W. and C.T.W. designed research and wrote the manuscript. D.P.F. conducted the research including protein expression, resonance assignment and structure calculation. H.A. prepared various samples and helped during the acquisition of NMR experiments. D.A.V. prepared various samples. A.K. helped during the structure calculation and prepared the C domains EntD and Sfp samples. A.E.B. participated in early stages of the project.

Author Information Atomic coordinates have been deposited in the Protein Data Bank under accession code 2ROQ. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to G.W. (gerhard_wagner@hms.harvard.edu), C.T.W. (christopher_walsh@hms.harvard.edu), or D.P.F. (dominique_frueh@hms.harvard.edu).

METHODS

Protein expression and purification. A pET30a+ plasmid containing the EntF T–TE gene, with a C-terminal His₆ tag, was transformed into *E. coli* BL21(DE3) cells. Isotope enrichment of protonated samples was produced by overexpression in M9 minimal media in H₂O containing ¹⁵NH₄Cl or ¹³C glucose or both. Deuterated samples (including methyl labelled samples) were prepared in D₂O containing ²H-¹³C glucose. Partially deuterated samples were prepared in recycled D₂O with ¹³C glucose. ‘ILV methyl labelled’ samples, which are selectively protonated on methyls of Leu, Val and Ile (δ1), were prepared as described in^{26,30}, with α-ketobutyrate and α-ketoisovalerate precursors either uniformly enriched in ¹³C, for assignments of side-chain signals, or only ¹³C enriched at the methyls of the precursors, for the NOE experiments. Samples selectively enriched in ¹⁵N for a given amino acid were prepared by supplying the medium with a single ¹⁵N-enriched amino acid and 19 unlabelled amino acids³¹. The growth times were 4 h in H₂O, 6 h in recycled D₂O and 12 h in D₂O. Induction times were 2 h in H₂O, 4 h in recycled D₂O and 6 h in D₂O. For all samples, the cells were lysed by sonication and the protein was purified using Ni–NTA resin (Qiagen) followed by FPLC using a Sephadex gel-filtration column (S75 or S200). The final sample concentrations were approximately 300 μM in 20 mM phosphate buffer (pH = 6.7) with 150 mM NaCl, and 1 mM EDTA and dithiothreitol.

Assignment of backbone resonances. TROSY versions of backbone experiments^{23,32,33} were used in a 70% deuterated ²H-¹⁵N-¹³C sample. This led to the assignment of 50% of the signals, 30% of which were assigned with the automated assignment program IBIS³⁴. HN-TROSY-HSQC³⁵ spectra were recorded on samples selectively enriched in ¹⁵N for Ile, Leu, Val, Lys, Phe, Tyr and Gly (that partially scrambles into Ser). These spectra were combined with the ¹H, ¹⁵N projections of a CCONH spectrum of a ILV methyl-labelled sample and with the ¹H, ¹⁵N projections of an HNCOCACB spectrum. These combinations enabled the identification of Ile, Leu, Val, Lys, Phe, Tyr, Gly and Ser residues that were neighbours of Ile, Leu, Val and Gly residues. Similarly, a non-uniformly sampled²⁴ HNCOCACB spectrum was recorded on a sample uniformly labelled in ¹⁵N and selectively labelled on carbonyl carbons of prolines. In addition, a non-uniformly sampled double-TROSY-hNcaNH experiment²⁵ was designed to correlate triplets of amide nitrogens belonging to contiguous residues. All of the information together led to 70% of the backbone assignment. A different strategy was used to assign residues with non-exchangeable amide protons, which constitute 30% of the TE domain. Non-uniform sampling was used to record high-resolution HNCOCACB, HNCACB and HNCOCACB spectra of a ¹⁵N-¹³C-labelled sample at 600 MHz with a good signal-to-noise ratio in spite of the fast relaxation due to the sample protonation. A ¹³C-detected MQ-HACACO³⁶ was then used to assign Hα signals. These experiments were used together with a ¹⁵N-dispersed NOESY-TROSY-HSQC leading to the assignment of 93% of the backbone resonances (313 out of 337 residues).

Assignment of side-chain resonances. HCCONH and CCONH²⁷ experiments were recorded on a 70% deuterated, uniformly ¹⁵N- and ¹³C-enriched sample. These experiments were also recorded on a ILV-methyl-labelled sample together with HCCH-total correlation spectroscopy (TOCSY)³⁸. An HCCH-COSY³⁹ was recorded on a ¹⁵N-¹³C-enriched sample to assign the signals of residues with non-exchangeable amide protons. Spin systems obtained with this experiment were related to backbone assignments by the MQ-HACACO experiment. Further assignments were obtained with NOESY spectra described below, after initial structure calculations.

Measurement of NMR constraints. Torsion angles constraints were obtained with the program TALOS⁴⁰. Most of the distance constraints were measured with time-shared three-dimensional (3D) TS-NOESY-HN-TROSY/NOESY-HC-PEP-HSQC and 4D HN-TROSY/HC-HSQC-NOESY-HN-TROSY/HC-PEP-HSQC experiments²⁶ with a mixing time (τ_m) of 200 ms, recorded on a ILV-methyl-labelled sample that also contained protonated phenylalanine⁴¹. A time-shared HSQC-NOESY (D.P.F., unpublished data), with high resolution for the dimension featuring the cross-peaks, was recorded to provide accurate integration of the NOE signals. A NOESY-HN-TROSY spectrum (τ_m = 90 ms) was recorded on a protonated sample to measure restraints involving non-exchangeable amide protons.

NMR data acquisition. All experiments were conducted on Bruker spectrometers equipped with cryoprobes. For the assignment of backbone resonances, experiments were recorded on a 900 MHz spectrometer, except for those involving ¹³C-¹⁵N samples and selectively labelled samples, which were measured at 600 MHz, and the MQ-HACACO experiment, which was recorded on a 500 MHz spectrometer equipped with a TXI cryoprobe. Side-chain experiments were measured at 600 MHz, except for the HCCH-COSY, which was recorded at 900 MHz. NOESY spectra were recorded at 750 MHz (3D and 4D time-shared experiments) and 900 MHz (3D time-shared HSQC-NOESY and 3D NOESY-TROSY). All spectra were processed with nmrPipe⁴² and analysed with CARRA⁴³.

Structure calculation. The structures of each domain were first solved individually, with constraints measured on the T–TE fragment. For the T domain, a homology model with the TycC3 T domain (PDB code 1JMK²⁰) could be produced⁴⁴. For the TE domain, sequential alignment with the surfactin TE1 (1DNY¹²) provided erroneous results. However, we could use secondary structure alignment, on the basis of TALOS predictions and NOE patterns, to produce a model with the program DeepView^{44,45}. The program CYANA²⁷ was used to predict NOEs on the basis of these models. The quality of the model could immediately be inspected in the various NOESY spectra and numerous corrections were made, in particular with help from 4D spectra. This resulted in a first low-resolution structure (r.m.s.d. of about 4 Å in each domain and 10 Å for the T–TE fragment). This structure was then used to identify new distance constraints and to make further corrections. This was repeated for each new structure. One-hundred-and-eighty-one hydrogen bond constraints were added for residues in secondary structured regions, once the local r.m.s.d. were shown to be under 0.5 Å, and 497 torsion angle constraints were included when they supported the existing structure. In the end, 4,383 distance constraints (1,163 intraresidues, 2,546 medium range and 935 long range) with 34 interdomain distance constraints were used to produce a structure with a global r.m.s.d. of 2.2 Å. The structural refinement in explicit water was performed using the RECOORD⁴⁶ scripts, after converting the CYANA derived structures into the CNS/XPLOR⁴⁷ format.

Titration. See Supplementary Information. The reported chemical shift differences were calculated by $\Delta\delta(^1\text{H}, ^{15}\text{N}) = ((\Delta\omega_{\text{H}})^2 + ((1/10)\Delta\omega_{\text{N}})^2)^{1/2}$, where $\Delta\omega_i$ is the chemical shift difference between the two species for nucleus *i*. A red dashed line on the resulting figures indicates a threshold of 1 s.d. that was used to single out outliers.

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