AmiA is a penicillin target enzyme with dual activity in the intracellular pathogen *Chlamydia pneumoniae*

Anna Klöckner1,*, Christian Otten1,*, Adeline Derouaux2,†, Waldemar Vollmer2, Henrike Bühl1, Stefania De Benedetti1, Daniela Münch1, Michaele Josten1, Katja Mölleken3, Hans-Georg Sahl1 & Beate Henrichfreise1

Intracellular *Chlamydiaceae* do not need to resist osmotic challenges and a functional cell wall was not detected in these pathogens. Nevertheless, a recent study revealed evidence for circular peptidoglycan-like structures in *Chlamydiaceae* and penicillin inhibits cytokinesis, a phenomenon known as the chlamydial anomaly. Here, by characterizing a cell wall precursor-processing enzyme, we provide insights into the mechanisms underlying this mystery. We show that AmiA from *Chlamydia pneumoniae* separates daughter cells in an *Escherichia coli* amidase mutant. Contrary to homologues from free-living bacteria, chlamydial AmiA uses lipid II as a substrate and has dual activity, acting as an amidase and a carboxypeptidase. The latter function is penicillin sensitive and assigned to a penicillin-binding protein motif. Consistent with the lack of a regulatory domain in AmiA, chlamydial CPn0902, annotated as NlpD, is a carboxypeptidase, rather than an amidase activator, which is the case for *E. coli* NlpD. Functional conservation of AmiA implicates a role in cytokinesis and host response modulation.
Chlamydiaceae are characterized by an obligate intracellular lifestyle and a unique biphasic developmental cycle. Despite the economic and public health importance of ocular, respiratory and sexually transmitted diseases caused by Chlamydiaceae, our knowledge about their biology at a molecular level is still limited. One crucial aspect of chlamydial biology that remains to be elucidated is the long known, yet still enigmatic chlamydial anomaly: for endobacteria such as Chlamydiaceae, there is no need to maintain osmotic stabilization by means of a cell wall, and a functional cell wall has not been detected in these pathogens. Nevertheless, antibiotics that target cell wall biosynthesis are active against Chlamydiaceae. Penicillin has no bactericidal effect on Chlamydia trachomatis, Chlamydia pneumoniae, C. trachomatis, C. pneumoniae and, the two membrane-linked steps (MraY and Mrg) of cell wall precursor synthesis has been demonstrated. Chlamydiaceae genomes retained two periplasmic enzymes that are involved in side-wall (PB2P) and septal (Ftsl (PBPF)) incorporation of lipid II building blocks in free-living rod-shaped bacteria such as E. coli. Moreover, the human pathogens harbour a rudimentary set of divisome proteins, lacking the almost ubiquitous central organizer FtsZ, but including FtsW, FtsI (PBPF) and FtsK, and possess, despite their spherical shape, the cytoskeletal protein MreB. AmiA is the only amidase encoded by Chlamydiaceae genomes and cell wall remodelling enzymes such as endopeptidases and lytic transglycosylases have not been identified.

The bacterial mesh-like cell wall consists of peptidoglycan (PG), a polymer of sugar chains that are cross-linked via flexible peptide bridges. PG is evolutionary conserved in eubacteria with the exception of some obligate intracellular species. Biosynthesis of PG takes place in three compartments of bacterial cells. In the cytoplasm, formation of the soluble precursors UDP-N-acetylmuramic and UDP-N-acetylmuramyl-pentapeptide is catalysed. At the inner leaflet of the cytoplasmic membrane, the two precursors are assembled with the lipid carrier bactoprenol-phosphate (undecaprenyl-P) to form lipid II, the completed cell wall-building block. Lipid II is then translocated to the outside of the cell and incorporated into the PG network releasing undecaprenyl-PP. PG is evolutionary conserved in eubacteria with the exception of some obligate intracellular species. Biosynthesis of PG takes place in three compartments of bacterial cells. In the cytoplasm, formation of the soluble precursors UDP-N-acetylmuramic and UDP-N-acetylmuramyl-pentapeptide is catalysed. At the inner leaflet of the cytoplasmic membrane, the two precursors are assembled with the lipid carrier bactoprenol-phosphate (undecaprenyl-P) to form lipid II, the completed cell wall-building block. Lipid II is then translocated to the outside of the cell and incorporated into the PG network releasing undecaprenyl-PP.

Prokaryotic cell division is driven by >10 essential proteins, which assemble at the septum to form the divisome. Briefly, the following stages of cell division are recognized in E. coli: the tubulin orthologue FtsZ forms a ring-like structure (Z ring) at midcell and initiates divisome assembly by attracting a set of ‘early’ proteins to form a cytoplasmic apparatus linked to the membrane; then enzymes for lipid II biosynthesis and presumably for PG biosynthesis localize to the midcell to carry out ‘preseptal’ elongation, a process in which the actin orthologue MreB might participate; additional elements co-ordinate chromosome segregation and build an extracellular connector for outer membrane constriction; finally, proteins involved in the incorporation (for example, transpeptidase FtsI (peptillin-binding protein (PBPF) 3) and flippase FtsW) and remodelling (amidases) of septal PG assemble. The process ends with cell segregation.

The three periplasmic N-acetylmuramyl-L-alanine amidases AmiA, AmiB and AmiC have been shown to be important for cell separation in E. coli. Double- and triple-amidase mutants form cell chains to varying extents. The chain-forming amidase mutants constrict the inner membrane, fail to split septal PG, are connected by shared PG layers and partially constrict their outer membranes. In contrast to AmiB and AmiC, AmiA is not localized to the septum. Nevertheless, E. coli double mutants with only AmiA are still able to partially separate. AmiA, AmiB and AmiC are members of the zinc-dependent N-acetylmuramyl-L-Ala amidase 3 family and catalyse the release of peptide moieties from PG by cleaving the amide bond between the lactyl group of muramic acid and the amino group of L-alanine. In E. coli the amidases AmiA, AmiB and AmiC need to be activated by LytM domain proteins to efficiently hydrolyse septal PG. EnvC has been shown to serve as a specific activator for AmiA and AmiB, and NlpD specifically activates AmiC. In previous studies, E. coli AmiA has been shown to hydrolyse polymeric PG, as well as glycan strands of varying chain length consisting of at least two transglycosylated lipid II cell wall-building blocks in a zinc-dependent fashion in vitro. Monomeric lipid II was not degraded by E. coli AmiA, suggesting that the enzyme requires at least a tetrasaccharide as a substrate.

Despite massive reduction in the genome size, a nearly complete pathway for lipid II biosynthesis has been found in Chlamydiaceae genomes, and activity of enzymes catalysing cytoplasmic steps (MurA, MurC/Ddl, CT390, DapF, MurE, GlyA and MurF) and the two membrane-linked steps (MraY and Mrg) of cell wall precursor synthesis has been demonstrated. Chlamydiaceae genomes retained two periplasmic enzymes that are involved in side-wall (PB2P) and septal (Ftsl (PBPF)) incorporation of lipid II building blocks in free-living rod-shaped bacteria such as E. coli. Moreover, the human pathogens harbour a rudimentary set of divisome proteins, lacking the almost ubiquitous central organizer FtsZ, but including FtsW, FtsI (PBPF) and FtsK, and possess, despite their spherical shape, the cytoskeletal protein MreB. AmiA is the only amidase encoded by Chlamydiaceae genomes and cell wall remodelling enzymes such as endopeptidases and lytic transglycosylases have not been identified.

We discussed that maintaining biosynthesis of the cell wall-building block lipid II in cell wall-lacking ‘minimal bacteria’ such as Chlamydiaceae may reflect a vital role of the lipid II pathway in prokaryotic cell division. Moreover, we proposed that MreB is involved in orchestration of lipid II biosynthesis at the septum and as such may be necessary for maintaining a functional divisome machinery in Chlamydiaceae. Recently, a cell wall sacculus was detected in the evolutionary earlier, genomically less reduced environmental chlamydiae Protochlamydia. However, consistent with our model described above, MreB and its interacting partner RodZ from the environmental chlamydial genus Waddlia have been shown to localize to the septum, and in Chlamydiaceae no cell wall but a distinct circularly shaped structure was found. This structure was suggested to consist of rudimentary PG-like material that is localized to the septum of dividing cells.

The aim of this study was to gain first mechanistic insights in processing of chlamydial lipid II at the division site. Here, we demonstrate that amidase AmiA is functionally conserved in Chlamydiaceae and capable of using the monomeric cell wall-building block lipid II as a substrate for amidase and novel penicillin-sensitive DD-carboxypeptidase (DD-CPase) activity in vitro. AmiA-driven processing of lipid II might be crucial for a complete lipid II biosynthesis and recycling cycle, and thus for maintaining coordinated cell division in Chlamydiaceae. Moreover, lipid II processing might modulate host response to muropeptides.

Results
AmiA from C. pneumoniae is functional in E. coli. AmiA from C. pneumoniae (AmiACp) and E. coli show 32% amino-acid sequence identity. If AmiACp is capable of degrading PG, as shown before for the homologue of E. coli, the enzyme might lyse bacterial cells. Overexpression of AmiACp in the periplasm of E. coli was achieved by fusion to the OmpA leader peptide and resulted in lysis of the producer strain after induction (Fig. 1a). To investigate functionality of AmiACp in cell separation of free-living bacteria, we performed complementation assays using an E. coli ΔamiABC triple-knockout mutant harbouring an arabinose-inducible amiC gene on a plasmid (Fig. 1b). When grown in the presence of glucose, the cells fail to separate during cell division and form long chains (Fig. 1c). The expression of AmiACp resulted in separated rod-shaped cells (Fig. 1c). Quantitative analysis of the experiments revealed that virtually all cells of the amidase triple mutant were present in chains (Fig. 1d). Upon induction of AmiACp expression, the separation
of daughter cells was rescued and 55% of the cells were single or paired (Fig. 1d). These data are in agreement with 30–40% chains observed in an E. coli ΔamiABC mutant containing AmiA6. In total, these findings indicate that AmiA can perform daughter cell separation in E. coli, presumably by the degradation of septal PG during cell division.

**AmiACP shows amidase activity on the PG and lipid II precursor.** Purified AmiACP was tested for degradation of PG using a remazol dye-release assay. As shown before for E. coli amidases8, AmiACP released remazol-stained reaction products into the supernatant from the PG sacculi (Fig. 2a,b). A control protein (DD-CPase VanY) that removes D-Ala from the peptide side chains, did not release the dye. Chlamydiaceae do not contain detectable quantities of PG but retained the biosynthesis pathway of the cell wall-building block lipid II13. We tested whether chlamydial AmiA can remove the pentapeptide side chain from the membrane-bound cell wall precursor. Thin-layer chromatography (TLC) analysis of lipid II reaction products combined with mass spectrometry (MS) revealed that AmiACP was capable of using lipid II as a substrate for amidase activity and cleaved the peptide side chain from the sugar moieties of lipid II (Fig. 2c–f).

In summary, AmiACP is an N-acetylmuramyl-L-alanine amidase using polymeric PG and monomeric lipid II as a substrate in vitro.

**AmiACP has an additional function as DD-CPase.** Surprisingly, our TLC/MS analysis showed that chlamydial AmiA exhibited dual enzymatic activity on lipid II in vitro. In addition to the amidase product, we identified lipid II with a tetrapeptide, indicating that cleavage of the terminal D-Ala residues had occurred (Fig. 2c–f). Therefore, AmiA has also DD-CPase activity. It was possible that the heterologous AmiA produced in E. coli was contaminated by host DD-CPases (class C PBPs). To test this possibility, we performed several control experiments. A mock protein purification from the E. coli strain harbouring the empty vector had no detectable DD-CPase activity (Fig. 2c) and we did not detect any PBPs in the AmiACP preparation when labelling with fluorescent penicillin bocillin FL (Supplementary Fig. 1), making it unlikely that the DD-CPase detected was due to contaminating host PBPs. The enzyme had no beta-lactamase activity
in a nitrocefin hydrolysis-based test system (Supplementary Fig. 2), indicating that the detection of possibly contaminating PBPs was not impaired by hydrolysis of bocillin FL. Of note, DD-CPase activity of AmiA<sub>Cp</sub> was sensitive to penicillin and blocked in a molar ratio of 1:1 (protein:inhibitor), whereas amidase activity was not impaired in the presence of the beta-lactam (Fig. 2d; Supplementary Fig. 3). Our results identify AmiA<sub>Cp</sub> as a novel DD-CPase and, besides PBP2, PBP3 and PBP6, as an additional so far unknown target of beta-lactams in Chlamydiaceae.

Identification of the DD-CPase active site. Penicillin-sensitive PBP DD-CPases, including VanY<sub>D</sub>, DD-CPase from vancomycin-resistant Enterococcus faecalis (VanY<sub>DF</sub>) (Fig. 4a).

Table 1. Masses of lipid II fragments. The masses are calculated and measured as [M-H]<sup>-</sup> using Q-TOF MS. "Symbol" indicates the presence (x) or absence (o) of amidase activity. VanY<sub>DF</sub>, VanY<sub>Sa</sub> (Vancomycin-resistant Staphylococcus aureus), AmiA<sub>Cp</sub>, and AmiA<sub>Ac</sub> (Adriamycin-resistant A. caviae) were used as control enzymes.

| Sample                                                                 | m/z calculated [M-H]<sup>-</sup> | m/z measured [M-H]<sup>-</sup> | Symbol |
|------------------------------------------------------------------------|----------------------------------|--------------------------------|--------|
| Undecaprenyl-pyrophosphoryl-MurNAc-(GlcNAc)-pentapeptide (Lipid II)   | 1,875.24                        | 1,875.59                       | x      |
| Undecaprenyl-pyrophosphoryl-MurNAc-(GlcNAc)-tetrapeptide              | 1,804.14                        | 1,804.60                       | o      |
| Undecaprenyl-pyrophosphoryl-MurNAc-(GlcNAc)                           | 1,383.71†                       | 1,383.51†                      | x      |
| Pentapeptide                                                          | 510.53                          | 510.37                         | o      |

†Mass of the decarboxylated sodium adduct.

Figure 2 | In vitro activity of AmiA<sub>Cp</sub>. The enzyme used PG as well as monomeric lipid II as a substrate. Dye-release assay on the degradation of PG: released reaction products after incubation of remazol-stained PG with control enzyme VanY<sub>Sa</sub> and AmiA<sub>Cp</sub> (a) and photometric analysis of supernatants (b). Error bars indicate ± s.d. (n = 3). Unpaired t-test revealed statistical significance in comparison with VanY control, two-tailed ***P-value ≤ 0.0001. TLC analysis (c,d) and MS (e,f) of lipid II reaction products. AmiA<sub>Cp</sub> showed novel amidase and DD-CPase activity on lipid II. The latter function was sensitive to penicillin. *The O-1-carboxyethyl-residue at position 3 in the MurNAc moiety of the amidase reaction product undecaprenyl-pyrophosphoryl-MurNAc-GlcNAc is decarboxylated, as usually observed with the matrix 6-Aza-2-thiothymine<sup>46</sup>. Additional peaks in the spectrum for the decarboxylated undecaprenyl-pyrophosphoryl-MurNAc-GlcNAc sodium adduct are attributed to the addition of water to conjugated double bonds in the undecaprenyl moiety (mass shift of 18 Da). (GlcNAc: N-acetylglucosamine; MurNAc: N-acetylmuramic acid).
AmiA from E. coli and selected Chlamydiales species. The amidase active site of E. coli AmiA contains three zinc-coordinating residues (H65, E80 and H133), and E242 that is predicted to serve as a general base catalyst. These active site residues (in bold and boxed) are highly conserved in the Chlamydiales species. Of note, the Chlamydiales AmiA proteins lack a domain with predicted autoregulatory functions (underlined and in italics) that is exclusively found in cell division amidasises AmiA, AmiB and AmiC and contains an α-helix (S157–V173 in E. coli AmiA, corresponding to D280–L296 in AmiB from B. henselae) occluding the active site. During cell division, the regulatory domain is proposed to interact with LytM domain factors to relieve autoinhibition by a conformational switch. PBP DD-CPases have three typical motifs, SxxK, S(Y)xN and K(H,R)T(S)G, corresponding to D280–L296 in AmiB from B. henselae amidase, AmiB from Bartonella henselae and AmiC and contains an SRRxFLK (with x being a polar amino acid) consensus motif (boxed and in italics) that directs AmiA to the T at motif.

Figure 4 | Characterization of active sites. Studies of the amidase active site (a). The zinc-coordinating His residues are essential for the lytic activity of AmiACp. Error bars indicate ± s.d. (n = 3). Discovery of the amino acids involved in DD-CPase activity (b). The exchange of residue S96 in the SxxK tetrad leads to a loss of function.
This zinc metalloprotease family is dominated by mammalian enzymes but comprises a few proteins of bacterial origin such as the γ-D-glutamyl-(L)-mDAP-hydrolysing peptidase I from Bacillus sphaericus\textsuperscript{27}. To identify amino acids that are involved in the DD-CPase function of AmiACP, we replaced the serine residues S96 and S140 in the two PBP homologous motifs SxxK and SxN as well as the residue S185, which is conserved in AmiA from E. coli and Chlamydiaceae and found in peptidase I from B. sphaericus. The replacement of S96 abolished DD-CPase activity, whereas the other mutations had no effect (Fig. 4b). These results provide further proof that the observed activity was not owing to a contaminating host PBP in the purified protein. The penicillin-sensitive DD-CPase function seems to be conferred solely by an SxxK motif independently from the apparently not essential SxN triad and the absent KTG motif. This is different from conventional bacterial PBPs whose activity depend, in addition to the SxxK tetrad, on a functional SxN motif and which typically contain a KTG motif as well\textsuperscript{28}. The DD-CPase active site mutant AmiAS96ACp was not impaired in amidase activity, providing further evidence that both enzymatic activities of AmiACP are independent from each other (Supplementary Fig. 4b).

**Secretion of AmiACP.** AmiA from E. coli contains a twin-arginine translocation (Tat) motif and is transported to the periplasm by the Tat system\textsuperscript{29}. Consistent with the absence of a Tat system in Chlamydiaceae, AmiACP does not have an SRRxFKL Tat consensus motif (Fig. 3)\textsuperscript{30,31}, but contains a putative signal peptide. Without being fused to the OmpA leader peptide, the overexpressed AmiACP did not accumulate efficiently in the periplasm to lyse the E. coli producer strain (Fig. 1a, AmiACP). Nevertheless, in the complementation assay, native AmiACP was able to rescue cell separation in the E. coli amidase triple mutant (Fig. 1c,d), whereas AmiAASPCp, which lacks the putative signal peptide, failed to complement the mutant (Supplementary Fig. 5). MS of the heterologously expressed native AmiACP revealed cleavage of the signal peptide in E. coli between residues 37 and 38 and not further downstream as frequently found for Tat signal peptides and predicted by SignalP (Fig. 3)\textsuperscript{32}. Thus, our data indicate that AmiACP contains a functional Sec signal peptide.

**Chlamydial AmiA lacks an autoregulatory domain.** The active sites of the cell division amidases AmiA, AmiB and AmiC from Gram-negative bacteria are likely to be occluded by a conserved α-helix, as previously shown for AmiB from B. henselae\textsuperscript{25}. These amidases have low basal in vitro activity and need to be activated by EnvC or NlpD. The LytM-containing septal ring factors are thought to specifically interact with the autoregulatory domain of the corresponding enzymes (EnvC with AmiA and AmiB and NlpD with AmiC) to promote the release of the regulatory helix from the active site\textsuperscript{25}. Sequence analysis showed that AmiACP...
Figure 6 | Proposed model for the role of the conserved lipid II pathway in maintaining a functional cell division machinery and contributing to modulation of host response in Chlamydiaceae. A complete cycle of lipid II biosynthesis, processing and recycling needs to be maintained for coordinated function of the cell division machinery. Structural protein MreB (in interaction with RodZ) functionally organizes MurF, MraY and MurG, three key components in lipid II biosynthesis, at the septum. The synthesized precursor is translocated to the outside and processed by a rudimentary cell wall biosynthesis/cell division machinery. DD-CPase activity of AmiA, together with PBP6 and Cpn0902 (NlpD), might orchestrate DD-CPase functions in chlamydial cell division. Amidase activity of AmiA would play a central role in processing lipid II by releasing the pentapeptide side chain to allow for (i) transpeptidation of pentapeptides catalysed by monofunctional transpeptidases Ftsl and PBP2 and for (ii) regulation of host immune response due to blocking of Nod2 sensing. FtsZ as well as transglycosylases, endopeptidases and pyrophosphorylases are not found in chlamydial cell division. Amidase activity of AmiA would play a central role in processing lipid II by releasing the pentapeptide side chain to allow for (i) transpeptidation of pentapeptides catalysed by monofunctional transpeptidases Ftsl and PBP2 and for (ii) regulation of host immune response due to blocking of Nod2 sensing. FtsZ as well as transglycosylases, endopeptidases and pyrophosphorylases are not found in chlamydial cell division. 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are unknown. Moreover, recycling of bactoprenol-P remains understood physiological role for these peptidases. In addition, bactoprenol-P is known to be a common pathogenic strategy to subvert muropeptide recognition and host innate immunity has been described for various human pathogens as well. Macromolecular pattern recognition receptors Nod1 and Nod2, which sense bacterial cell wall fragments to signal through a MyD88-independent pathway and mediate Nod1 and Nod2, which sense bacterial cell wall fragments to signal through a MyD88-independent pathway and mediate activation of the transcription factor NFκB. The amidase activity provided by AmiA could be involved in host response regulation, and it has been shown to be important for the induction of cytokines during infection. Induction of cytokines during infection might contribute to sustaining long-term residence in infected cells and thus to pathogenicity. Induction of cytokines during infection might contribute to sustaining long-term residence in infected cells and thus to pathogenicity. The concept of PG degradation provided by AmiA could be involved in host response regulation. The concept of PG degradation provided by AmiA could be involved in host response regulation.

**Methods**

**Bacterial strains and growth conditions.** E. coli JM83 carrying AmiA expression plasmids and E. coli ADE24 were maintained on Luria Bertani (LB) agar plates containing 100 μg ml⁻¹ ampicillin and 30 μg ml⁻¹ chloramphenicol, respectively.

**Growth kinetics.** E. coli JM83 harbouring expression vectors was grown in LB and induced with 200 ng ml⁻¹ 1-anhydrotetracycline (AHT) at an OD₆₀₀ of 0.6.

**Construction of E. coli ADE24.** E. coli MHD41 carries a markerless deletion in amiB. We first deleted amiA by P1 transduction with JW4228 from the Keio E. coli mutant strain collection, followed by the excision of the kanamycin resistance gene. Next, the amiC gene was deleted by P1 transduction with JW5449 from the Keio collection, followed by removal of the kanamycin resistance gene. The amiC gene was amplified by PCR from pBADAmiC and used for transformation into E. coli JM83. The resulting pBAD33-amiC was transformed into the triple-amidase mutant to obtain strain ADE24.

**Cloning of amiA.** The amiA gene from C. pneumoniae Gii was amplified by PCR using the primers listed in Supplementary Table 1 and cloned into pASK-IBA2 and pASK-IBA3 using the SacI and HindIII restriction sites upstream and downstream, respectively, of amiC. The amiA gene was then cloned in plasmid pBAD33 and the resulting pBAD33-amiC was transformed into the triple-amidase mutant to obtain strain ADE24.

**Site-directed mutagenesis.** H67, S96, H136, S140, S185 and E207 in AmiAcP were changed to Ala using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Germany). The respective sense and antisense primers listed in Supplementary Table 1 were used according to the manufacturer’s instructions. Correct base changes were confirmed by sequencing.

**In vivo complementation assay.** E. coli ADE24 harbours an E. coli amiC encoding pBAD33 vector and shows an inducible chain-forming phenotype in the presence of glucose (Fig. 2; Supplementary Table 1). E. coli ADE24 was transformed with vector pBAD33-amiAcP allowing for expression of AmiAcP including its native N-terminal signal peptide upon induction by AHT. Cells were grown in the same medium used for overproduction of AmiAcP containing 30 μg ml⁻¹ chloramphenicol, 100 μg ml⁻¹ ampicillin, 0.8% glucose and 200 ng ml⁻¹ AHT at 32 °C. Samples were taken at an OD₆₀₀ of 0.5 and complementation was monitored by microscopy.

**AmiA overproduction and purification.** E. coli JM83 cells transformed with IB2-amiAcP were grown in no-salt LB in presence of 300 mM sucrose at 30 °C until they reached an OD₆₀₀ of 0.6 and induced by the addition of 200 ng ml⁻¹ AHT at 37 °C. Stained sacculi (20 μl) including its native N-terminal signal peptide upon induction by AHT. Cells were harvested and purification was performed using the manufacturer’s (IBA, Germany) protocol for cleared lysates. OmpA cleavage was monitored by Western blotting.

**AmiA activity assay.** Remazol staining of E. coli W3110 PG sacculi and dye-release assays were performed as described previously with slight modifications. Stained sacculi (20 μl) were incubated at 37 °C for 16 h with 4 μM AmiA in 200 μl 50 mM Hepes, pH 7.5, 20% dimethyl sulfoxide. In vitro activity assays using lipid II as a substrate were carried out in a final volume of 4 μl containing 4 μl purified protein, 2 nmol lipid II, 200 mM MgCl₂, pH 7.5 and 5% dimethyl sulfoxide and incubated for 4 h at 37 °C. Reaction products were extracted with 40 μl of n-butanol/pyridine acetate (2:1, v/v, pH 4.2) and analysed by TLC and MS as described previously and below, respectively. For TLC, silica was used as the stationary phase, whereas the mobile phase consisted of chloroform-methanol-water-ammonia carbonate (88:48:10:1). Spots were visualized by phosphomolybdic acid (PMA) staining. For penicillin inhibition assays, the AmpR resistance marker in the AmiAcP expression vector was exchanged with CamR resistance marker.
For reaction products 1 ml of matrix solution (2,5-DHAP/DAC), this ternary mixture was pipetted up and down until crystallization started. The suspension (1 μl) was spotted onto an AnchorChip 600 target and allowed to dry at RT. The spectra were recorded in the linear positive mode on a biflex III mass spectrometer.

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**Author contributions**

B.H., A.K., C.O. and H.-G.S. designed research; experiments were performed by A.K., C.O., A.D., H.B., S.D.B., D.M., M.J. and K.M.; A.D. and W.V. developed analytical tools; C.O., A.K., B.H., H.-G.S., W.V., M.J., A.D., H.B., S.D.B., D.M. and K.M. analysed data; B.H., A.K., C.O., W.V. and H.B. wrote the paper.

**Additional information**

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