Amino Acid Labeling

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Metabolic Profiling of Bacteria by Unnatural C-terminated D-Amino Acids

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Abstract: *Bacterial peptidoglycan is a mesh-like network comprised of sugars and oligopeptides. Transpeptidases cross-link peptidoglycan oligopeptides to provide vital cell wall rigidity and structural support. It was recently discovered that the same transpeptidases catalyze the metabolic incorporation of exogenous D-amino acids onto bacterial cell surfaces with vast promiscuity for the side-chain identity. It is now shown that this enzymatic promiscuity is not exclusive to side chains, but that C-terminus variations can also be accommodated across a diverse range of bacteria. Atomic force microscopy analysis revealed that the incorporation of C-terminus amidated D-amino acids onto bacterial surfaces substantially reduced the cell wall stiffness. We exploited the promiscuity of bacterial transpeptidases to develop a novel assay for profiling different bacterial species.*

Tremendous strides have been made in the treatment and prevention of bacterial infections. However, two major hurdles in diagnostics continue to impede further progress: identification of the type of bacteria and the level of drug resistance. These two time-sensitive components often dictate the course of treatment. Methods that improve our ability to address these needs may have significant clinical utility. We envisioned a peptidoglycan metabolic labeling strategy that could form the basis of a precise and facile diagnostic test to determine these two components in a single step.

A large number of enzymatic transformations are necessary to properly assemble the peptidoglycan precursors. The lipid-anchored peptidoglycan precursors are then flipped to the outside of the cytoplasmic membrane where they are incorporated onto the growing peptidoglycan matrix. These covalent modifications are crucial for tuning the physical and mechanical properties of the peptidoglycan. The amount and nature of these modifications are inherently linked to the type of bacteria and may also be related to phenotypic differences within these species.

Chemical modifications of the peptidoglycan can mainly be attributed to the enzymatic processes by penicillin binding proteins (PBPs). The transpeptidase domains of PBPs are responsible for the cross-linking of neighboring stem peptides, a function that endows the peptidoglycan with increased rigidity and strength.[1] The cross-linking of the peptidoglycan is vital to bacteria. Interference with this process via β-lactam and glycopeptide treatment is lethal to many bacteria.[2] Recently, an alternate reaction of transpeptidase was discovered whereby terminal D-alanines were "swapped" with D-

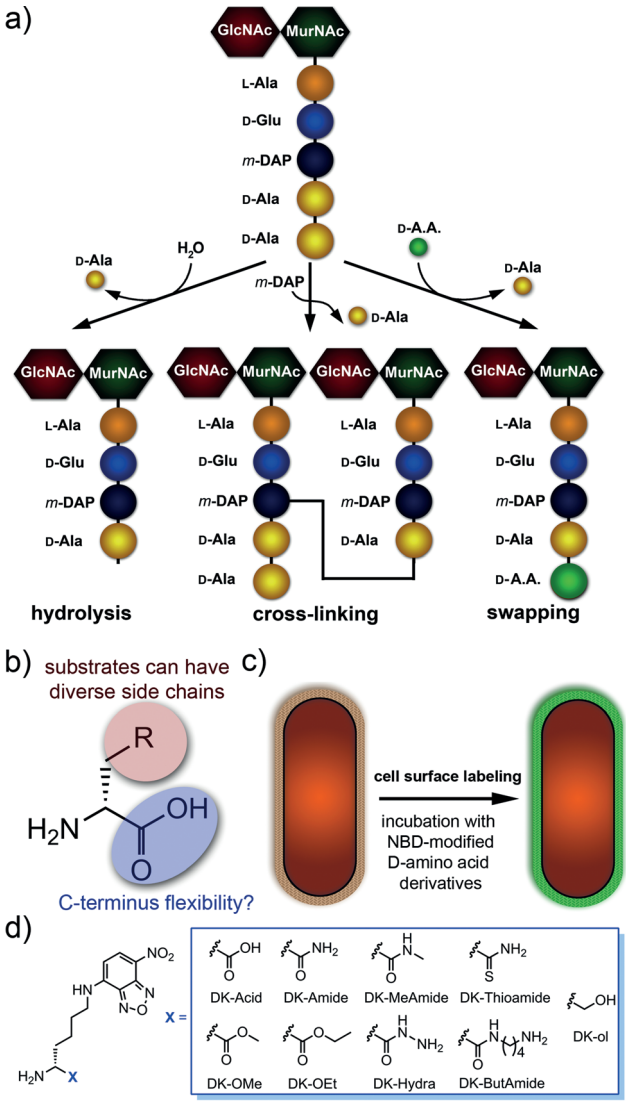

Figure 1. a) Representation of the peptidoglycan processing by PBP transpeptidase. GlcNAc = *N*-acetylglucosamine, MurNAc = *N*-acetylmuramic acid, *m*-DAP = *meso*-diaminopimelic acid. b) Basic unit of D-amino acid and derivatives that have been shown to be tolerated. c) Representation of the assay to assess D-amino acid incorporation. d) Chemical structures of D-amino acid derivatives that were synthesized and evaluated.

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amino acids from the surrounding medium (Figure 1a).[3] We and others have recently demonstrated that bacterial cell surfaces can be labeled with unnatural D-amino acids with expansive promiscuity in the identity of the side chain.[3,4] This led us to explore the possibility that non-native C-terminal variants are competent transpeptidase substrates (Figure 1b). Furthermore, we hypothesized that species- and strain-specific variations in the physical composition of the peptidoglycan (for example, thickness, charge, and cross-linking level) and peptidoglycan processing (for example, transpeptidase and carboxypeptidase domains of PBPs) could be probed and profiled with these C-terminal variants.

Herein, we show that non-native C-terminated D-amino acids are incorporated onto bacterial cell surfaces. Most importantly, these variants may provide a facile and sensitive platform to differentiate between bacterial species and phenotypes within individual species.

Initially, we synthesized a panel of fluorescently labeled D-amino acid derivatives to probe the promiscuity of the C-terminus by PBP transpeptidases (Figure 1d). The fluorescent nitrobenzoxadiazole (NBD) was chosen for its small size and we had previously shown that the native D-lysine (DK) carboxylic acid version (DK-Acid) is readily incorporated onto the surface of bacteria.[4h] This panel of variants was designed to allow the interrogation of the heteroatom specificity at the C-terminus, a requirement of a carbonyl group, and the ability to accommodate bulky functional groups.

We first evaluated the relative incorporation levels of the various D-amino acid derivatives using the Gram-positive bacterium *Bacillus subtilis* (*B. subtilis*) ΔdacA, which lacks the carboxypeptidase gene (*dacA*).[5] The dacA PBP carboxypeptidase enzyme catalyzes the hydrolysis of the fifth position D-alanine, thus effectively reducing the overall level of incorporation by exogenously supplemented D-amino acids. *B. subtilis* cells were incubated in the presence of the C-terminus variants from our panel of compounds and incorporation levels were quantified based on the NBD fluorescence using flow cytometry. As we had previously observed, the carboxylic acid variant (DK-Acid) is readily incorporated onto the bacterial surface (Figure 2).

Next, we probed the requirement of a negatively charged terminus by evaluating the neutral D-amino carboxamide derivate (DK-Amide). Remarkably, the incubation with DK-Amide led to a nearly two-fold increase in incorporation levels compared to DK-Acid.[4g,6] It is interesting to note that vegetative *B. subtilis* cells naturally possess a high level of amidated *meso*-diaminopimelic acid (*m*-DAP), which becomes the acyl acceptor upon cross-linking (Figure 1a).[7] Therefore, the final cross-linked product will also have a carboxamide group at the same location as the amide of a peptidoglycan swapped with D-amino carboxamide.

The similarly charged and sized carbothioamide variant (DK-Thioamide) highlights the importance of an oxygen atom at the carbonyl position for *B. subtilis* PBP transpeptidase. This requirement for the presence of a carbonyl group was further probed by the incubation of cells with a variant lacking carbonyl (DK-ol). The loss of fluorescence signal suggests that the carbonyl group is essential for

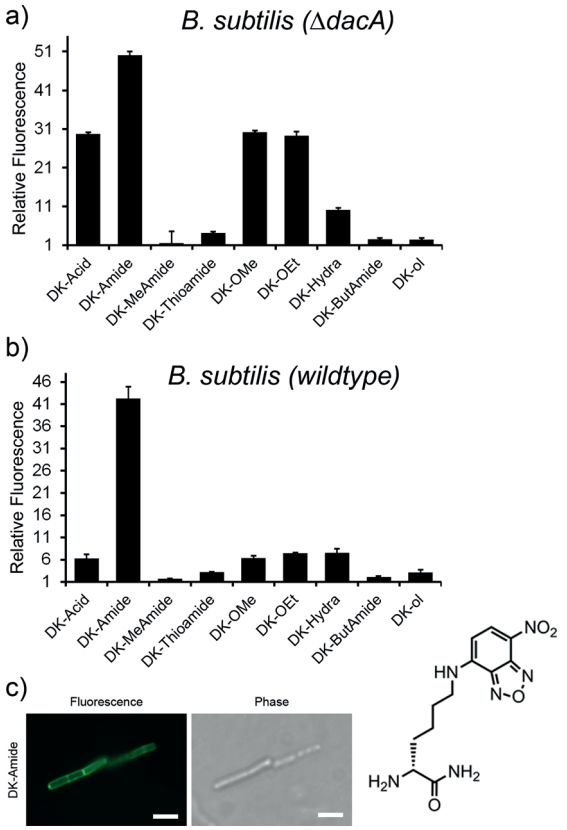

Figure 2. Incorporation of D-amino acid derivatives. Flow cytometry analysis of *B. subtilis* ΔdacA (a) and wildtype *B. subtilis* (b) incubated overnight in the presence of 100 μM of stated compounds. Data are represented as mean ± SD ($n = 3$). Relative fluorescence represents the fold increase over unlabeled cells. c) Fluorescence and phase-contrast microscopy imaging of *B. subtilis* wildtype cells labeled overnight with 200 μM DK-Amide. Scale bar represents 3 μm.

substrate recognition by PBP transpeptidase. A slight increase in steric bulk in the amidated terminus (DK-MeAmide) proved to be deleterious for surface labeling of *B. subtilis*, with a near complete loss of incorporation. The intolerance of the methyl group in DK-MeAmide suggests that the lack of charge and steric bulkiness combine to endow the compound with incompatible attributes as a substrate for transpeptidase-mediated swapping. The basic amide scaffold was investigated further by the extension of four methylene units with a terminal amino group (DK-ButAmide). This perturbation led to a complete loss of incorporation, which is presumably due to the size and charge reversal of the terminal modification.

The introduction of an additional amino group (DK-Hydra), generating a modification similar in size to methylamide, mostly restored the ability of the molecule to be incorporated onto bacterial surfaces (Figure 2). The site-specific surface display of hydrazide moieties provided by DK-Hydra should be compatible with bioorthogonal chemistries involving complementary aldehyde/ketone groups, thus opening up the possibility of the installation of a second and distinct molecule on the surface.[8] The esterification of the D-amino acid to the neutral C-terminus DK-OMe is also well-

tolerated as indicated by the level of surface labeling. The homologation of the methyl ester (DK-OEt) yielded a slightly bulkier variant that labeled bacteria to a comparable level to DK-OMe. As expected, the presence of dacA causes a reduction in incorporation levels for several of the D-amino acid variants (Figure 2). Additional experiments were performed to confirm the incorporation into the peptidoglycan by D-amino acid variants, possible side-chain tolerance, alternate pathways of C-terminal promiscuity, and growth phase effects (Supporting Information, Figures S1–S7).

The elevated incorporation levels observed with DK-Amide were explored next. Kinetics of incorporation and retention of labeling showed that DK-Amide is incorporated faster than DK-Acid and it was retained on the surface for hours after incorporation (Supporting Information, Figures S8, S9). These results suggest that amidation of the stem peptides by exogenous D-amino carboxamides may render them poor substrates for transpeptidase, thus effectively inhibiting subsequent swapping and cross-linking (Supporting Information, Figures S10, S11). Attenuation of cross-linking levels can, in turn, reduce the overall stiffness of the peptidoglycan. We set out to probe the peptidoglycan of bacterial cells treated with DK-Amide using atomic force microscopy (AFM).[9] We acquired AFM force-indentation curves (Figure 3) from wildtype *B. subtilis* cells subjected to

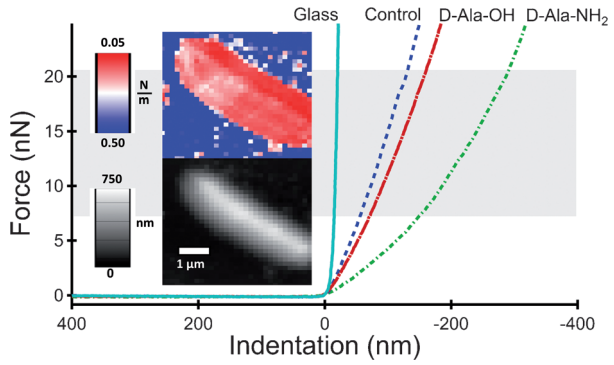

Figure 3. Typical force–indentation curves obtained on a poly-L-Lysine (PLL) coated glass surface, wildtype *B. subtilis* cells grown in standard medium, and wildtype *B. subtilis* incubated overnight in the presence of 10 mM of D-Ala-OH or D-Ala-NH_2. Grayed out area indicates the region used for all force curves in calculating the effective stiffness (for example, the slope of the force–indentation curve) of a given cell. PPL-coated glass appears incompressible. Inset: height image (bottom) and a color map of stiffness (top) calculated from the same force-volume data set. The color overlay represents the apparent stiffness of the cell probed at a given location. Note the greatly different apparent stiffness of the bacterial cell and the substrate.

different growth conditions using either standard medium (control) or D-Ala-OH/D-Ala-NH_2 containing media. While the presence of D-Ala-OH resulted only in minor changes in the mechanical properties of bacteria, the incorporation of D-Ala-NH_2 had a profound effect on the bacterial cells response to external load. Since constructing quantitative models and obtaining proper data to extract absolute values of Young's modulus is complicated, we opted to perform a relative characterization of the mechanical properties of the bacterial cell wall by measuring effective stiffness of *B. subtilis* cells under low to moderate compressions (50–300 nm). Due to cell-to-cell variations, analysis of the cell population (we analyzed 6–12 cells per sample; Supporting Information, Table S1) was used to detect changes in mechanical properties of the cell wall from disruption of the peptidoglycan cross-linking. We observed that effective stiffness of bacteria declined by about 25% when introducing D-Ala-NH_2 into the growth medium. The experimental stiffness values (with corresponding errors of the mean) for cells grown in as is (control), D-Ala-OH, and D-Ala-NH_2 spiked media were 148 ± 11 mN m^{-1} ($n = 9$), 131 ± 6 mN m^{-1} ($n = 11$), and 109 ± 13 mN m^{-1} ($n = 7$), respectively, for loads between 5 and 18 nN. These differences are even more pronounced at low loads (compare slopes at high and low forces in Figure 3), for example, another set of experiments with a different probe using loads under 5 nN showed a reduction in stiffness by about 45% from 128 ± 13 mN m^{-1} ($n = 5$) for normal peptidoglycan to 69 ± 5 mN m^{-1} ($n = 11$) for D-Ala-NH_2 modified peptidoglycan. The dramatic drop in the effective stiffness should be interpreted as a reduction of the Young's modulus of the peptidoglycan by approximately a third upon incorporation of D-Ala-NH_2.

We measured cell labeling by D-amino acid variants with six additional types of bacteria aside from *B. subtilis* to profile the labelling of cell surfaces across different species (Figure 4). Contrary to all other Gram-positive bacteria assayed, DK-Amide failed to extensively label the surface of Gram-negative *E. coli* and only DK-Acid displayed significant incorporation. These differences could potentially reflect poor permeation of the molecules to the site of the transpeptidase and/or inherent differences in the *E. coli* PBPs. Similarly, the Gram-negative human pathogen *Pseudomonas aeruginosa* (*P. aeruginosa*) was labeled poorly with all the compounds evaluated. Of note, the decreased β-lactam sensitivity (same protein target as D-amino acid variants) by Gram-negative organisms has been proposed to be primarily caused by the lack of outer membrane permeability.[9f] The incubation of Gram-positive *Listeria monocytogenes* (*L. monocytogenes*) with our panel of variants resulted in a labeling profile clearly distinct from *B. subtilis*. While DK-Amide retained the ability to efficiently label *L. monocytogenes* cells, DK-Thioamide labeling was slightly more efficient than the carboxylic acid variant DK-Acid, while DK-Hydra showed almost no incorporation. The Gram-positive *Staphylococcus epidermidis* (*S. epidermidis*) showed overall lower levels of labeling compared to *S. aureus*. Unique to the set of bacteria evaluated, DK-Hydra showed a twofold increase in labeling of S. epidermidis compared to DK-Acid.

Next, we evaluated our panel of compounds against *Lactobacillus casei* (*L. casei*), a symbiotic gut microorganism.[10] Incubation with DK-Amide led to high incorporation levels in *L. casei*, while DK-Acid and DK-Thioamide also labeled these cells efficiently. These findings are intriguing considering that the fifth position in the peptidoglycan of *L. casei* is D-lactic acid, not D-alanine.[11] Furthermore, to our knowledge this is the first evaluation of peptidoglycan labeling of symbiotic bacteria via unnatural D-amino acids and it may have implications on potential therapeutic

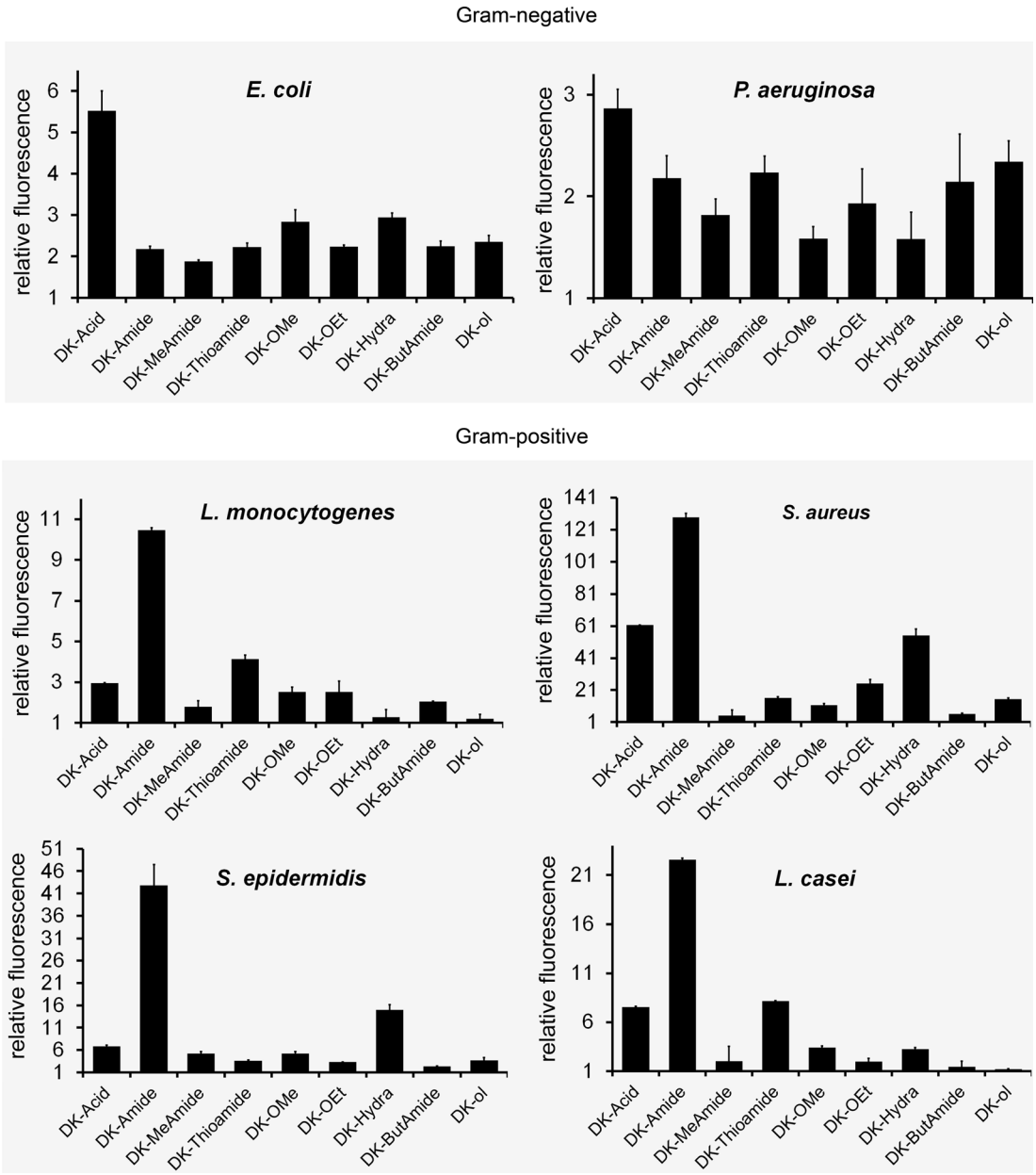

Figure 4. Differential profiling across several types of bacteria by flow cytometry analysis of specified strains incubated overnight in the presence of 100 μM of stated compounds. Relative fluorescence represents the fold increase over unlabeled cells. Data are represented as mean ± SD ($n = 3$).

application of this technology. The Gram-positive human pathogen *Staphylococcus aureus* (*S. aureus*) displayed the largest absolute fluorescence signals among the set of bacteria tested. It was observed that *S. aureus* was labeled with DK-Amide about twice as efficiently as with DK-Acid or DK-Hydra. It is interesting to note that unlike vegetative *B. subtilis* cells, *S. aureus* cells do not contain amidated *m*-DAP. Therefore, it appears that the higher labeling efficiency of DK-Amide can only be partially explained by its *m*-DAP mimicry.

In conclusion, we have shown that PBP transpeptidase exhibits remarkable flexibility in accepting unnatural D-amino acid derivatives as substrates for swapping surface-bound D-alanines. Most importantly, we demonstrate that subtle differences within and between bacterial species could be profiled within our panel of compounds. The incorporation profile has the potential to form the basis of a novel bacterial detection method.

Keywords: atomic force microscopy · bacteria · fluorescence assays · penicillin binding proteins · surface labeling

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[1] E. Sauvage, F. Kerff, M. Terrak, J. A. Ayala, P. Charlier, *FEMS Microbiol. Rev.* **2008**, *32*, 234–258.

[2] M. A. Kohanski, D. J. Dwyer, J. J. Collins, *Nat. Rev. Microbiol.* **2010**, *8*, 423 – 435.
[3] H. Lam, D. C. Oh, F. Cava, C. N. Takacs, J. Clardy, M. A. de Pedro, M. K. Waldor, *Science* **2009**, *325*, 1552 – 1555.
[4] a) F. Cava, M. A. de Pedro, H. Lam, B. M. Davis, M. K. Waldor, *EMBO J.* **2011**, *30*, 3442 – 3453; b) M. S. Siegrist, S. Whiteside, J. C. Jewett, A. Aditham, F. Cava, C. R. Bertozzi, *ACS Chem. Biol.* **2013**, *8*, 500 – 505; c) M. Pilhofer, K. Aistleitner, J. Biboy, J. Gray, E. Kuru, E. Hall, Y. V. Brun, M. S. VanNieuwenhze, W. Vollmer, M. Horn, G. J. Jensen, *Nat. Commun.* **2013**, *4*, 2856; d) P. Shieh, M. S. Siegrist, A. J. Cullen, C. R. Bertozzi, *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 5456 – 5461; e) E. Kuru, H. V. Hughes, P. J. Brown, E. Hall, S. Tekkam, F. Cava, M. A. de Pedro, Y. V. Brun, M. S. VanNieuwenhze, *Angew. Chem. Int. Ed.* **2012**, *51*, 12519 – 12523; *Angew. Chem.* **2012**, *124*, 12687 – 12691; f) T. J. Lupoli, H. Tsukamoto, E. H. Doud, T. S. A. Wang, S. Walker, D. Kahne, *J. Am. Chem. Soc.* **2011**, *133*, 10748 – 10751; g) M. D. Lebar, J. M. May, A. J. Meeske, S. A. Leiman, T. J. Lupoli, H. Tsukamoto, R. Losick, D. Z. Rudner, S. Walker, D. Kahne, *J. Am. Chem. Soc.* **2014**, *136*, 10874 – 10877; h) J. M. Fura, M. J. Sabulski, M. M. Pires, *ACS Chem. Biol.* **2014**, *9*, 1480 – 1489.
[5] K. F. Bott, *Science* **1994**, *263*, 546 – 548.
[6] During the preparation of this manuscript, Kahne et al. published a report showing that D-amino carboxamides are substrates of *B. subtilis* PBP transpeptidase and not *E. coli* PBP transpeptidase.[4g]
[7] a) A. Atrih, G. Bacher, G. Allmaier, M. P. Williamson, S. J. Foster, *J. Bacteriol.* **1999**, *181*, 3956 – 3966; b) V. V. Nemmara, S. A. Adediran, K. Dave, C. Duez, R. F. Pratt, *Biochemistry* **2013**, *52*, 2627 – 2637.
[8] a) I. Chen, M. Howarth, W. Y. Lin, A. Y. Ting, *Nat. Methods* **2005**, *2*, 99 – 104; b) D. Dutta, A. Pulsipher, W. Luo, M. N. Yousaf, *J. Am. Chem. Soc.* **2011**, *133*, 8704 – 8713; c) E. M. Sletten, C. R. Bertozzi, *Angew. Chem. Int. Ed.* **2009**, *48*, 6974 – 6998; *Angew. Chem.* **2009**, *121*, 7108 – 7133; d) L. K. Mahal, K. J. Yarema, C. R. Bertozzi, *Science* **1997**, *276*, 1125 – 1128.
[9] a) Y. F. Dufrêne, *J. Bacteriol.* **2002**, *184*, 5205 – 5213; b) Y. F. Dufrêne, *Nat. Rev. Microbiol.* **2004**, *2*, 451 – 460; c) M. E. Dokukin, N. V. Guz, I. Sokolov, *Biophys. J.* **2013**, *104*, 2123 – 2131; d) X. Yao, M. Jericho, D. Pink, T. Beveridge, *J. Bacteriol.* **1999**, *181*, 6865 – 6875; e) A. Touhami, B. Nysten, Y. F. Dufrene, *Langmuir* **2003**, *19*, 4539 – 4543; f) A. H. Delcour, *Biochim. Biophys. Acta Proteins Proteomics* **2009**, *1794*, 808 – 816.
[10] H. Licandro-Seraut, H. Scornec, T. Pedron, J. F. Cavin, P. J. Sansonetti, *Proc. Natl. Acad. Sci. USA* **2014**, *111*, E3101 – 3109.
[11] D. Billot-Klein, R. Legrand, B. Schoot, J. van Heijenoort, L. Gutmann, *J. Bacteriol.* **1997**, *179*, 6208 – 6212.

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