



Published in final edited form as:

Angew Chem Int Ed Engl. 2012 December 7; 51(50): 12519–12523. doi:10.1002/anie.201206749.

In situ* Probing of Newly Synthesized Peptidoglycan in Live Bacteria with Fluorescent D-Amino Acids.*

Mr. Erkin Kuru,

Indiana University, Bloomington, IN 47405 (USA)

Ms. H. Velocity Hughes,

Indiana University, Bloomington, IN 47405 (USA)

Dr. Pamela J. Brown,

Indiana University, Bloomington, IN 47405 (USA)

Mr. Edward Hall,

Indiana University, Bloomington, IN 47405 (USA)

Mr. Srinivas Tekkam,

Indiana University, Bloomington, IN 47405 (USA)

Prof. Felipe Cava,

Universidad Autonoma de Madrid, Campus de Cantoblanco Madrid, 28049 (Spain)

Prof. Miguel A. de Pedro,

Universidad Autonoma de Madrid, Campus de Cantoblanco Madrid, 28049 (Spain)

Prof. Yves V. Brun, and

Indiana University, Bloomington, IN 47405 (USA)

Prof. Michael S. VanNieuwenhze

Indiana University, Bloomington, IN 47405 (USA)

Yves V. Brun: ybrun@indiana.edu; Michael S. VanNieuwenhze: mvannieu@indiana.edu

Abstract

Tracking a bug's life: We describe the first direct and universal approach for labeling peptidoglycan (PG) of diverse bacteria by exploiting the surprising tolerance of cells for incorporating unnatural D-amino acids of various sizes and functionalities. These non-toxic D-amino acids preferably label the sites of active PG synthesis, enabling fine spatiotemporal tracking of cell wall dynamics in phylogenetically and morphologically diverse bacteria.

Keywords

bacteria; biosensors; D-amino acids; fluorescent probes; peptidoglycan

**We thank David Kysela for discussions and critical reading of the manuscript, Bill Turner and Alvin Kalinda for their help in simplifying the synthesis of FDAAs and Professor Patricia Foster and the members of her laboratory for their support during the initial stages of the project. MSV was funded by National Institutes of Health (NIH) grant AI 059327, YVB by NIH grant GM051986, MAP by Ministry of Education and Science (Spain) grant BFU2006-04574 and by the Fundación Ramón Areces, FC by MICINN (Spain) grant RYC-2010-06241 and P.J.B. by NIH National Research Service Award AI072992.

Correspondence to: Yves V. Brun, ybrun@indiana.edu; Michael S. VanNieuwenhze, mvannieu@indiana.edu.

Supporting information for this article is available on the WWW under <http://www.angewandte.org>.

Bacterial growth is controlled by the domain-specific peptidoglycan (PG) cell wall, a rigid and essential structure composed of glycan strands cross-linked by D-amino acid (DAA)-containing short peptides, whose biosynthesis machinery is a high-value target for antibiotics.^[1] Despite the incredible importance of this megapolymer, our knowledge of PG dynamics has been severely hampered by the lack of a global strategy for direct imaging of sites of PG synthesis in live cells. Significant limitations of current labeling methods, such as toxic effects and poor membrane permeability of the probes, have limited their applicability to only a small set of bacterial species.^[2] Commonly, these methods are labor-intensive and their sensitivity suffers from their indirect and multiple-step nature. To overcome these limitations, we sought a chemical biology approach that would enable the rapid and covalent incorporation and detection of a fluorescently derivatized PG component during cell wall synthesis in real time, in a wide range of bacterial species and in live cells.

Given the recently established role of PG biosynthetic machinery in incorporating various natural DAAs in the PG of diverse bacteria,^[3] we hypothesized that the mechanisms^[3b] for DAA incorporation in PG are common to the bacterial domain, but still highly selective for the D-center, and that molecules with various functionalities could be incorporated into the sites of new PG synthesis if attached to a DAA backbone. To test this hypothesis, we attached the relatively small fluorophores, 7-hydroxycoumarin 3-carboxylic acid (HCC-OH) and 4-chloro-7-nitrobenzofurazan (NBD-Cl), to a D-amino acid backbone, 3-amino-D-alanine, via a modular and simple synthetic scheme, generating HADA (emission maximum 450 nm, blue) and NADA (emission maximum 538 nm, green) (Scheme 1 and Figure S2).

Growth of the phylogenetically diverse model species *Escherichia coli*, *Agrobacterium tumefaciens* and *Bacillus subtilis* in the presence of these fluorescent D-amino acids (FDAAs) for as little as one generation (Figure S1a) resulted in strong peripheral and septal labeling of entire cell populations (Figures 1 and S3a, upper panels) without affecting growth rate (Figure S3b). Neither of the fluorescent L-amino acids (FLAAs, prepared from 3-amino-L-alanine, Figure S2c) resulted in significant labeling (Figure S3c), indicating that labeling is specific to the D-enantiomers. The labeling was exclusive to viable cells treated with the FDAAs and was not the result of non-specific interaction of FDAAs with the PG (Figure S4). Additionally, incorporation did not occur into teichoic acids for *B. subtilis* as indicated by identical labeling of wild-type and a $\Delta dltA$ mutant that does not D-alanyl-ate its teichoic acids (Figure S5a–b).

Retained fluorescence on the purified sacculi (Figures 1 and S3a, lower panels) demonstrated that the labeling of PG by the FDAAs was covalent. HPLC analyses of muropeptides isolated from labeled cells (Figures 2a–c, S6 and S7) revealed that 0.2% – 2.8% of total muropeptides were modified (Figure 2c), which is sufficient for detection in various experiments while avoiding possible toxicity issues that could result from abundant incorporation. Significantly, the FDAA-specific peaks, which were absent in samples treated with FLAAs, could be distinguished from unlabeled muropeptides at FDAA-specific absorption wavelengths (Figure 2b). MS/MS analyses of FDAA-modified muropeptides in *B. subtilis* indicated that FDAAs were exclusively incorporated in the fifth position of the stem peptide (Figures 2d, S7a, d and Table S1). Interestingly, in a $\Delta dacA$ mutant of *B. subtilis*, the fraction of labeled muropeptides and the fluorescent signal were substantially higher than in wild-type *B. subtilis*, which is likely due to the D,D-carboxypeptidase activity of DacA (Figure S5c–d). In contrast, the detectable incorporation was solely at the fourth position in *E. coli* and *A. tumefaciens* (Figures 2d and S7). These results are in agreement with the known sites of incorporation of various natural DAAs in these species and suggest that, similar to DAAs, FDAAs incorporate mainly through periplasmic exchange reactions with the muropeptides catalyzed either by D,D-transpeptidases, e.g. in *B. subtilis*, or by L,D-transpeptidases, e.g. in *E. coli* and *A. tumefaciens*.^[3b, 4] This DAA-like behavior together

with the ease of fluorescent detection make FDAAs a strong alternative to radioactive probes for studying *in vitro*^[3c] and *in vivo*^[5] activities of PG synthesis enzymes.

In contrast to current approaches,^[2b, 2f] pulse-chase experiments with HADA allowed the real-time tracking of new PG incorporation during growth via time-lapse microscopy. In *E. coli* and *B. subtilis* $\Delta dacA$ (Figure 3a and Supplementary Movies 1–2), the polar caps retained the HADA signal, but the signal from the lateral walls dispersed as the cells grew, in agreement with previous reports of cell wall growth along the length of the lateral walls.^[2f, 5]

Strikingly, short labeling times (2% – 8% of doubling time) using *E. coli* and *B. subtilis* $\Delta dacA$ with HADA resulted in preferential localization of the signal at the septal plane of predivisional cells and in punctate patterns on the lateral walls of elongating cells (Figure 4). Super-resolution microscopy of *E. coli* revealed reticulated hoop-like patterns of HADA labeling around the lateral wall (Figure 3b & Supplementary Movie 3), supportive of bursts of PG incorporation in the side-walls.^[6] This ability of FDAAs to resolve insertion of new PG provides the first direct detection of zones of PG synthesis in a structured rather than a random pattern in *E. coli*, consistent with recent results following the movement of the cell wall elongation machinery.^[7] Short labeling times with *A. tumefaciens*, whose growth occurs predominantly from a single pole and the site of cell division while the mother cell remains inert,^[4] resulted in polar and septal labeling. Super-resolution fluorescence microscopy of labeled cells further enhanced the spatial resolution of the site of active PG synthesis (Figure 3c & Supplementary Movie 4).

PG labeling in three evolutionarily distant species suggested that FDAAs could specifically label the active site of PG synthesis across the entire bacterial domain. When species representing diverse phyla and modes of growth were briefly incubated with FDAAs, we observed strong labeling at the sites of cell division in actively dividing cells (Figure 4 and Figure S1b). This septal probe incorporation was the sole mode in *Synechocystis* sp. PCC 6803, *Lactococcus lactis*, and *Staphylococcus aureus* as previously described.^[8] Super-resolution microscopy of *Staphylococcus aureus* further highlighted the different stages of these constricting septal rings (Figure 3d and Supplementary Movie 5). Labeling of *Streptococcus pneumoniae* occurred in single or split equatorial rings depending on the length of the cell, with peripheral labeling between the split rings as described.^[8] Labeling of *Streptomyces venezuelae* was predominantly apical as reported,^[9] with some weak labeling of vegetative septa and lateral walls suggestive of a low but continuous lateral PG synthesis. In *Caulobacter crescentus*, labeling occurred at the sites of septal elongation, lateral elongation, and stalk synthesis, as reported elsewhere.^[10]

Bacteria exhibit a myriad of growth patterns that provide selective advantages in the environment.^[11] The strong correlation between FDAA labeling and previously inferred regions of new PG synthesis in diverse model species has a number of important implications. First, FDAA labeling marks the sites of active PG synthesis and therefore provides a long sought broadly applicable tool to study the spatial dynamics of PG synthesis. Second, these results establish for the first time that the enzymes responsible for DAA incorporation in the PG are associated with active growth sites and that not all the surface of the sacculus is equivalent in terms of accessibility to these enzymes. Finally, FDAAs can be used to discover the growth modes of previously uncharacterized taxa. For example, our results show that that *Burkholderia phytofirmans* exhibits polar and midcell PG synthesis, that *Brachybacterium conglomeratum* exhibits prominent peripheral PG synthesis in addition to seemingly alternating perpendicular division planes, and that *Verrucomicrobium spinosum* exhibits strong peripheral PG synthesis and asymmetric septal labeling (Figure 4).

The efficient label incorporation in all the bacteria studied thus far also suggests that FDAA, therefore DAA, incorporation is common to the bacterial domain and FDAAs can thus be used to analyze natural bacterial populations, providing a convenient and quick standard to measure bacterial activity and to probe the diversity of growth modes in complex microbiomes.^[11] Indeed, labeling times with FDAAs as short as 2 hours revealed diverse modes of growth in saliva and freshwater samples in situ, but did not label dead cells as suggested by the strong correlation with Live-Dead staining (Figures S4b and S8).

Encouraged by the efficiency of FDAAs, we sought to increase our toolkit for PG detection and modification using differently functionalized unnatural D-amino acids. Following a similar approach, we derivatized the brighter and more versatile core fluorophore, fluorescein (emission maximum ~515 nm, green), and its analogue, carboxytetramethylrhodamine (TAMRA, emission maximum ~565 nm, red),^[12] with D-lysine in order to separate the bulky fluorophore from the DAA backbone, generating FDL and TDL (Scheme 1 and Figure S2a). Incubation of both *E. coli* and *B. subtilis* cells with FDL (536 Da) showed patterns similar to NADA, although labeling of *B. subtilis* was stronger than *E. coli*, presumably due to reduced permeability of the *E. coli* outer-membrane to molecules larger than ~500 Da^[13] (Figure S9a–c). Indeed, the larger TDL (560 Da) did not label *E. coli* cells, but labeling of *B. subtilis* was prominent and showed patterns similar to other FDAAs (Figure S9a–b). In order to expand the toolkit further, we used ‘clickable’ D-amino acids, namely ethynyl-D-alanine (EDA) or azido-D-alanine (ADA) (Scheme 1) that can be specifically captured by any molecule carrying the conjugate functional group via click-chemistry.^[14] Similar to FDAAs, these bioorthogonal DAAs, but not the L-enantiomer control ELA, labeled both *E. coli* and *B. subtilis* cells when captured by commercially available azido/alkyne fluorophores (Figures S2c–d and S9d–e).

Furthermore, custom D-amino acids containing different colored fluorophores can be used sequentially to enable “virtual time-lapse microscopy.” Since addition of each new probe indicates the location and extent of PG synthetic activity during the respective labeling periods, this approach provides a chronological account of shifts in PG synthesis of individual cells over time. This powerful method is exemplified here through click chemistry in gram-negative *A. tumefaciens* (Figures S1a and 3e), or by the use of TDL in gram-positive *S. venezuelae* (Figure 3f).

In contrast to the bacterial protein-making machinery’s inherent bias against unnatural amino acids,^[15] here we have shown that taxonomically diverse bacteria display a remarkable specific tolerance for incorporating D-amino acids with different sizes and functionalities into PG. Exploiting this tolerance provides a rapid, non-toxic, and universal method for real time tracking of PG at sites of active synthesis for the first time. Moreover, the combined utilization of two or more probes permits a temporal resolution never before achieved in cell wall growth studies. We expect that in combination with fluorescent protein fusions, mutational analysis, and chemical perturbations, this methodology will allow a comprehensive analysis of the regulation and coordination of bacterial growth. Furthermore, the tolerance for DAAs with substantial sizes or with bio-orthogonal handles will enable selective and specific modification of bacterial cell surfaces with various functionalities paving the way for development of DAA-based bacteria-specific diagnostic or therapeutic probes. Finally, when cells labeled with FDAAs are hybridized with fluorescent in situ hybridization (FISH) probes, this approach will allow the culture-independent and concurrent measurement of growth (with FDAAs) and taxonomy (with FISH) and the response of bacteria to varying conditions in medical or environmental microbiomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. Typas A, Banzhaf M, Gross CA, Vollmer W. *Nat Rev Microbiol.* 2012; 10:123–136. [PubMed: 22203377]
2. a) van Dam V, Orlachs N, Breukink E. *Chembiochem.* 2009; 10:617–624. [PubMed: 19173317] b) Daniel RA, Errington J. *Cell.* 2003; 113:767–776. [PubMed: 12809607] c) Tiyanont K, Doan T, Lazarus MB, Fang X, Rudner DZ, Walker S. *Proc Natl Acad Sci U S A.* 2006; 103:11033–11038. [PubMed: 16832063] d) Orlachs NK, Aarsman MEG, Verheul J, Arnusch CJ, Martin NI, Herve M, Vollmer W, de Kruijff B, Breukink E, den Blaauwen T. *Chembiochem.* 2011; 12:1124–1133. [PubMed: 21472954] e) Sadamoto R, Niikura K, Ueda T, Monde K, Fukuhara N, Nishimura SI. *J Am Chem Soc.* 2004; 126:3755–3761. [PubMed: 15038728] f) de Pedro MA, Quintela JC, Höltje JV, Schwarz H. *J Bacteriol.* 1997; 179:2823–2834. [PubMed: 9139895]
3. a) Lam H, Oh DC, Cava F, Takacs CN, Clardy J, de Pedro MA, Waldor MK. *Science.* 2009; 325:1552–1555. [PubMed: 19762646] b) Cava F, de Pedro MA, Lam H, Davis BM, Waldor MK. *EMBO J.* 2011; 30:3442–3453. [PubMed: 21792174] c) Lupoli TJ, Tsukamoto H, Doud EH, Wang TSA, Walker S, Kahne D. *J Am Chem Soc.* 2011; 133:10748–10751. [PubMed: 21682301]
4. Brown PJB, de Pedro MA, Kysela DT, Van der Henst C, Kim J, De Bolle X, Fuqua C, Brun YV. *Proc Natl Acad Sci U S A.* 2012; 109:1697–1701. [PubMed: 22307633]
5. Mobley HL, Koch AL, Doyle RJ, Streips UN. *J Bacteriol.* 1984; 158:169–179. [PubMed: 6232259]
6. Furchtgott L, Wingreen NS, Huang KC. *Mol Microbiol.* 2011; 81:340–353. [PubMed: 21501250]
7. a) Domínguez-Escobar J, Chastanet A, Crevenna AH, Fromion V, Wedlich-Söldner R, Carballido-López R. *Science.* 2011; 333:225–228. [PubMed: 21636744] b) Garner EC, Bernard R, Wang WQ, Zhuang XW, Rudner DZ, Mitchison T. *Science.* 2011; 333:222–225. [PubMed: 21636745]
8. a) Rippka R, Herdman M. *Ann Inst Pasteur/Microbiol.* 1985; 136:33–39. b) Zapun A, Vernet T, Pinho MG. *FEMS Microbiol Rev.* 2008; 32:345–360. [PubMed: 18266741]
9. Flärdh K. *Curr Opin Microbiol.* 2003; 6:564–571. [PubMed: 14662351]
10. Aaron M, Charbon G, Lam H, Schwarz H, Vollmer W, Jacobs-Wagner C. *Mol Microbiol.* 2007; 64:938–952. [PubMed: 17501919]
11. Young KD. *Microbiol Mol Biol Rev.* 2006; 70:660–703. [PubMed: 16959965]
12. Lavis LD, Raines RT. *ACS Chem Biol.* 2008; 3:142–155. [PubMed: 18355003]
13. Decad GM, Nikaido H. *J Bacteriol.* 1976; 128:325–336. [PubMed: 824274]
14. Prescher JA, Bertozzi CR. *Nat Chem Biol.* 2005; 1:13–21. [PubMed: 16407987]
15. a) Neumann H, Wang K, Davis L, Garcia-Alai M, Chin JW. *Nature.* 2010; 464:441–444. [PubMed: 20154731] b) Wang L, Brock A, Herberich B, Schultz PG. *Science.* 2001; 292:498–500. [PubMed: 11313494]

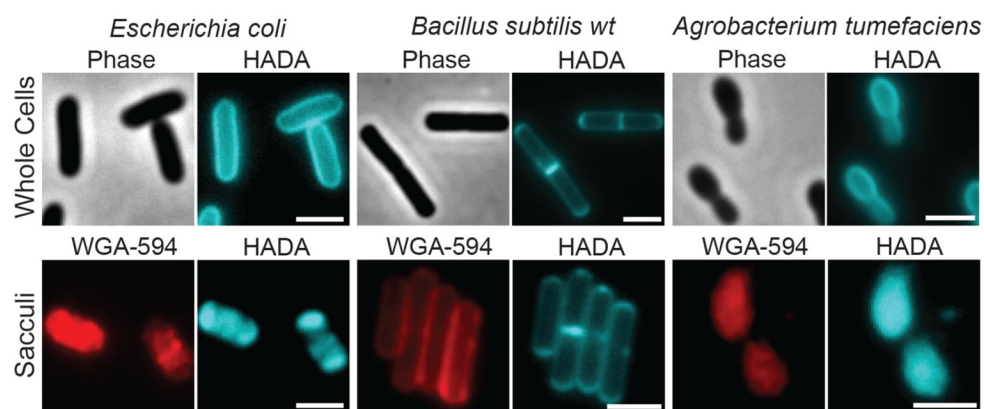
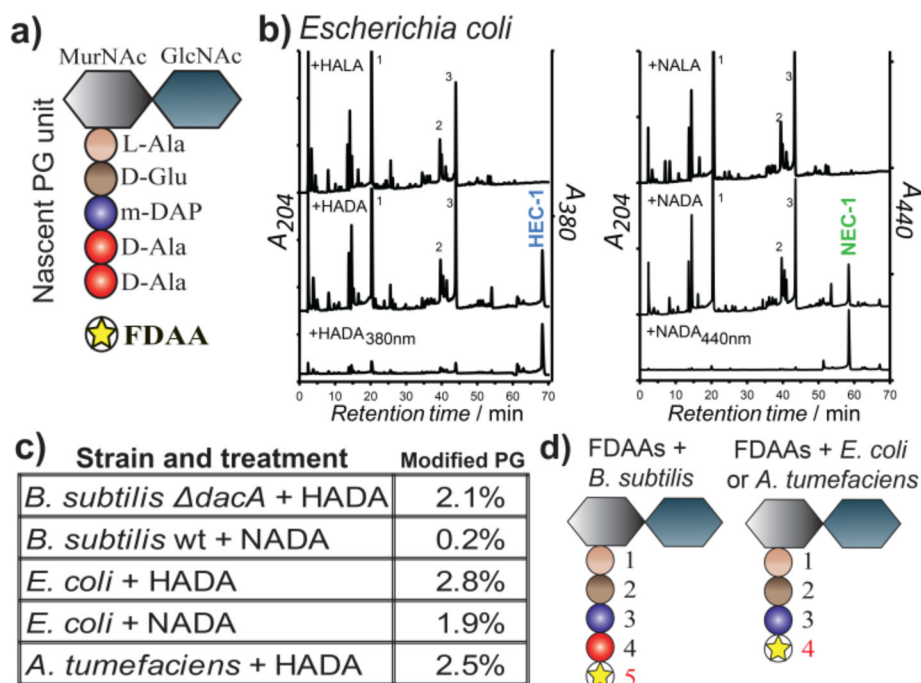
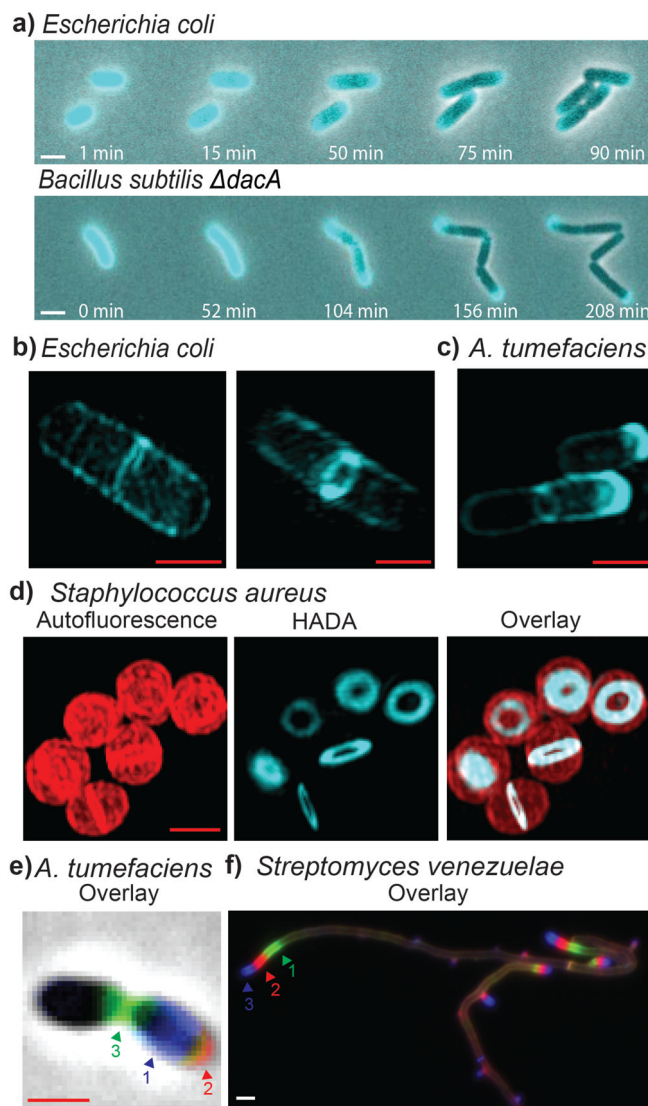


Figure 1. Long labeling pulses with HADA uniformly label PG in live cells of *E. coli*, *B. subtilis* and *A. tumefaciens*. The FDAA fluorescence is retained in isolated sacculi, which are also stained with the N-acetylglucosamine-specific WGA lectin conjugated to Alexa Fluor 594 (red). Scale bars, 2 μm .

**Figure 2.**

FDAA incorporation into the stem peptide of the PG subunit. a) Schematic representing the muramylpentapeptide precursor unit. b) HPLC detection of modified muuropeptides in *E. coli* incubated with HADA or HALA and NADA or NALA. Samples were monitored using a dual wavelength UV monitor set for general muuropeptide detection and for FDAA specific wavelengths. Peaks HEC-1 and NEC-1 correspond to the HADA or NADA modified muuropeptides in *E. coli* that were further characterized by electrospray ionization MS/MS (ESI-MS/MS). c) Percentage of FDAA incorporation into the total muuropeptides varies among different bacteria as revealed by HPLC analysis. d) MS/MS analyses show that FDAAs exclusively incorporate into the 4th position of muuropeptides in *E. coli* and *A. tumefaciens* and the 5th position in *B. subtilis*. See also Figures S6–7 and Table S1.

**Figure 3.**

FDAAs label diverse bacterial growth patterns. a) Time-lapse microscopy of HADA labeled *E. coli* and *B. subtilis* $\Delta dacA$ cells imaged during growth on LB agarose pads. Superresolution microscopy of b) *E. coli* and c) *A. tumefaciens* after short pulses with HADA. d) Superresolution microscopy of *S. aureus* cells after a short pulse with HADA. Autofluorescence is shown in red. e) Triple labeling of *A. tumefaciens* with HADA (blue), EDA (clicked with red sulfo-Cy3-azide), and NADA (green). f) Triple labeling of *S. venezuelae* with NADA (green), TDL (red), and HADA (blue). Arrows in the triple labeling panels indicate the sequence of labeling. White scale bars, 2 μm ; red scale bars, 1 μm .

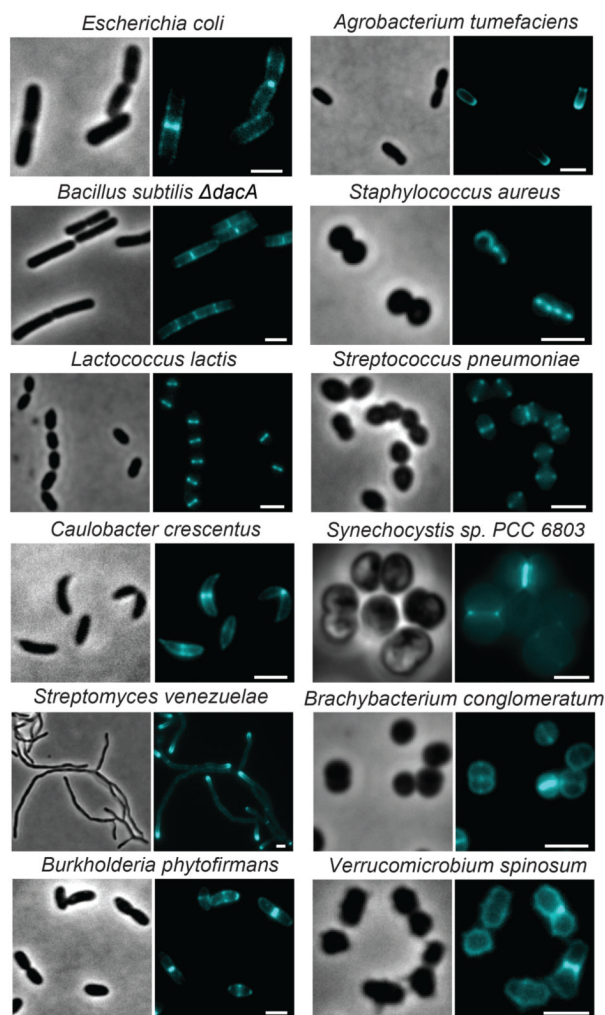
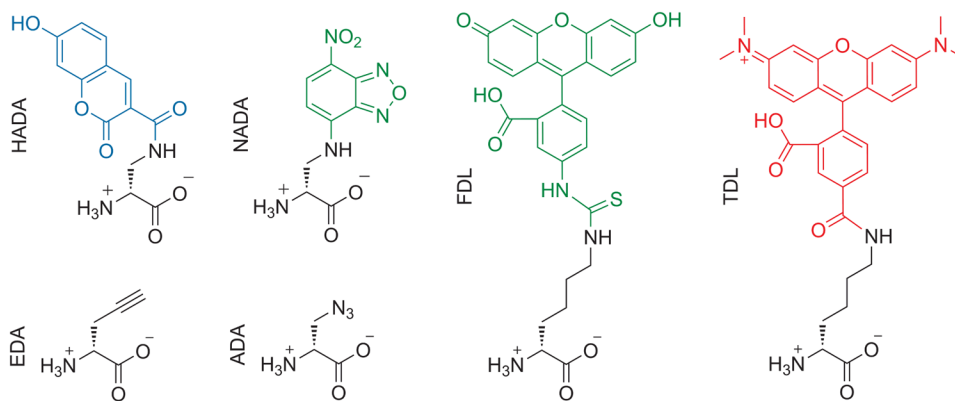


Figure 4. Short pulses of HADA label distinct modes of growth in diverse bacteria. Strains were labeled for ~2–8% of the doubling time: *E. coli* (30 s), *A. tumefaciens* (2 min), *B. subtilis* $\Delta dacA$ (30 s), *S. aureus* (2 min), *L. lactis* (2 min), *S. pneumoniae* (4 min), *C. crescentus* (5 min), *Synechocystis* sp. PCC 6803 (1 h), *S. venezuelae* (2 min), *B. conglomeratum* (8 min), *B. phytofirmans* (20 min), *V. spinosum* (10 min). Scale bars, 2 μm .

**Scheme 1.**

Modular structures of the fluorescent D-amino acids HADA, NADA, FDL and TDL and the 'clickable' D-amino acids EDA & ADA, color coded by the color of the fluorophores (blue, green and red).