**Fluorescent D-amino-acids reveal bi-cellular cell wall modifications important for *Bdellovibrio bacteriovorus* predation**

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Modification of essential bacterial peptidoglycan (PG)-containing cell walls can lead to antibiotic resistance; for example, β-lactam resistance by \(\text{L, D-transpeptidase}\) activities. Predatory *Bdellovibrio bacteriovorus* are naturally antibacterial and combat infections by traversing, modifying and finally destroying walls of Gram-negative prey bacteria, modifying their own PG as they grow inside prey. Historically, these multi-enzymatic processes on two similar PG walls have proved challenging to elucidate. Here, with a PG-labelling approach utilizing timed pulses of multiple fluorescent D-amino acids, we illuminate dynamic changes that predator and prey walls go through during the different phases of bacteria:bacteria invasion. We show formation of a reinforced circular port-hole in the prey wall, \(\text{L, D-transpeptidase}\) mediated D-amino acid modifications strengthening prey PG during *Bdellovibrio* invasion, and a zonal mode of predator elongation. This process is followed by unconventional, multi-point and synchronous septation of the intracellular *Bdellovibrio*, accommodating odd- and even-numbered progeny formation by non-binary division.

Peptidoglycan (PG) is a shape-determining macromolecule common to the bacterial domain. The mature PG wall of bacteria is made by glycan polymerization and peptide crosslinking of a D-amino acid-rich muramyl pentapeptide subunit (Fig. 1a). These crosslinks give the PG wall its essential load-bearing properties against the bacterial cell's turgor pressure and are made in two basic ways; either 3–4 crosslinks catalysed by normally essential and common penicillin-binding proteins or 3–3 crosslinks catalysed by normally disposable, variable, L, D-transpeptidases (Ldt) (Fig. 1b)\textsuperscript{1,2}.

Although penicillin-binding proteins and Ldt are evolutionarily and structurally distinct transpeptidases, research in diverse bacteria showed that both enzyme types can exchange a range of naturally occurring D-amino acids (DAAs) with the fifth- and fourth-position \(\text{D-alanines in the peptide stems of PG subunits, respectively}^{2–4} (\text{Fig. 1b)}\). Such exchanges are associated with changes in a variety of biophysical properties of the wall\textsuperscript{2}, in particular the strength (as determined by osmolarity challenge\textsuperscript{6–7}) in some bacteria. Substrate promiscuity of these transpeptidases toward a diverse set of DAAs\textsuperscript{4} has allowed the development of fluorescent D-amino acids (FDAAs) and their implementation as a means to visualize PG dynamics in situ\textsuperscript{6–12}.

*Bdellovibrio bacteriovorus* (approximately 1.0×0.3\(\mu\)m) prey on (larger) Gram-negative bacterial species by breaching the prey outer membrane, residing in the modified prey periplasm (forming the ‘bdelloplast’), rescaling and growing within\textsuperscript{14–15}, before finally bursting out to invade more prey (Fig. 1c). The prey are killed some 20 min into predation when electron transport ceases as predator molecules pass across the prey inner membrane\textsuperscript{15}; however, the prey bdelloplast is kept intact for 4h to allow ‘private dining’ and consumption of prey contents by the predator. Early electron microscopic work\textsuperscript{16–18} led to the assumptions that the invading *B. bacteriovorus* would squeeze through the outer layers of the prey bacterium, degrading some type of entry pore in the prey PG-containing cell wall, re-sealing this, and modifying the rest of the prey PG. However, as the biochemically similar walls were obscured at the points of contact between the two bacterial cells, this bi-cellular multi-enzymatic process has, until now, been difficult to analyse. Therefore, other than recent work showing the mechanisms of prey cell rounding\textsuperscript{18}, self-protection from auto-rounding\textsuperscript{19} and marking of the wall for later destruction\textsuperscript{20}, *B. bacteriovorus* wall-invasion dynamics and enzymology have remained a subject of conjecture.

Here, we combine three differently coloured FDAAs\textsuperscript{20} in a timed series (Fig. 1d,e) to illuminate dynamic PG modifications during bacterial predation, simultaneously, in two bacterial species. Three-dimensional structured illumination microscopy (3D-SIM), resolved the *B. bacteriovorus* processes of: breaching the prey PG; constructing a reinforced port-hole in the prey cell wall; re-sealing the port-hole after entry; modifying the prey PG with L, D-transpeptidases; and eventually achieving filamentous, intra-bacterial zonal cell growth and synchronous, multi-site septation.

**Results**

Multi-colour-FDAA microscopy reveals prey versus predator cell wall modifications during invasion. A synchronous predatory...
Fig. 1 | Background and introduction to experimental procedures. a, Biosynthesis of PG starts in the cytoplasm by sequential addition of \( \alpha \)-alanine, \( \alpha \)-glutamic acid, a diamino acid and a dipeptide of \( \alpha \)-alanine–\( \alpha \)-alanine to disaccharide units. This subunit is then incorporated into the murein sacculus by glycan polymerization via transglycosylases. The \( \alpha \)-alanine at position 5 can also be cleaved by the actions of \( \alpha \)-\( \beta \)-carboxypeptidases. b, \( \alpha \)-\( \beta \)-transpeptidases cleave the \( \alpha \)-alanine from position 4 and utilize the energy from cleaving this bond to form a 3-3 crosslink with another acyl-acceptor stem peptide or replace the \( \alpha \)-alanine with a free \( \alpha \)-amino acid such as a fluorescent \( \alpha \)-amino acid (FDAA). c, Timed stages of the predatory cycle of \( B. \) bacteriovorus (black) bacteria invading \( E. \) coli prey (grey). At 0–15 min post-mixing of \( B. \) bacteriovorus and prey, \( B. \) bacteriovorus attach and begin to enter the outer layers of the prey. At 30 min, most of the \( B. \) bacteriovorus have entered the prey periplasm, modifying the prey cell to form a rounded ‘bdelloplast’. At 1–3 h, \( B. \) bacteriovorus growth occurs at the expense of the prey cell contents in the form of elongation as a filament. At 4 h, this filament fragments into smaller attack-phase cells that break out from the bdelloplast. d, FDAA used in this study; colours are representative of emission maxima. e, Multi-coloured-FDAA labelling scheme with time points observed by wide-field epifluorescence microscopy. Predator and prey cells were pre-labelled separately with BADA and TADA, respectively, before being washed and then mixed. Samples of this mixed infection were then pulse-labelled with HADA for 10 min before each time point before being fixed, washed, and then microscopically observed. f, Phase contrast and epi-fluorescent microscopy images of the early stages of \( B. \) bacteriovorus predation. The \( B. \) bacteriovorus are false-coloured in green, the \( E. \) coli prey cells are false-coloured in red and the pulsed HADA signal is false-coloured in blue. Each channel is displayed independently in white and with all three fluorescence channels merged in an epifluorescence (EPI) overlay. HADA fluorescence signal on the prey wall has an intense focus at each point of \( B. \) bacteriovorus contact and spreads from this point across the rest of the wall. Scale bars, 1 μm. The two images are representative of between 321 and 10,546 cells for each time point, detailed in Supplementary Table 1.
invasion co-culture of *Escherichia coli* prey cells pre-labelled with a red FDAA, TADA, and *B. bacteriovorus* predator cells pre-labelled with a green FDAA, BADA, was established, and this invasive culture was further pulse-labelled with a blue FDAA, HADA, for 10 min at key points during the predation process. The cells were then fixed, washed and imaged (Fig. 1e).

Total cell wall fluorescence of now-dead prey cells (TADA) showed no appreciable change through the invasive process (Supplementary Fig. 1); however, both labelling patterns and signal intensities of pulsed HADA fluorescence showed dramatic differences depending on the stage of predation.

HADA pulses early in the infection, 15 or 30 min post-mixing of predators with prey, resulted in labelling of various subcellular features. In particular, intense, localized, focal HADA marks on the prey PG (and a gradient of blue HADA signal from that focal point) were seen associated with attached *B. bacteriovorus* cells, revealing the entry point of the *B. bacteriovorus* during the earliest predator–prey interaction (Fig. 1f).

To further characterize these subcellular features in early predation, we imaged these labelled cells with high-resolution 3D-SIM. 3D-SIM resolved most of these focal marks of HADA labelling as annular ring structures (~25% of all HADA-bright prey cells investigated at the earliest predation point, Fig. 2, Supplementary Table 2 and Supplementary Movie 1) having a width (~0.24 µm; Supplementary Table 2) slightly less than that of a *B. bacteriovorus* cell (~0.33 µm) at the point of predator invasive cell pole–prey contact. This is consistent with the *B. bacteriovorus* ‘squeezing through the entry pore’ idea suggested by electron micrographs in earlier work.\(^{16,21,22}\) Therefore, these HADA foci probably indicate the specific modification of the prey cell wall by the predator during entry (Fig. 2a).

The ring of HADA modification was on the prey PG rather than the predator PG, as it was always observed at the point of the prey PG, whether the predator was on the outside, inside, or partially entering the prey cell (Supplementary Fig. 2a–c). Furthermore, rare instances were observed where the predator had become detached from the prey but the HADA foci were still visible, confirming that these foci were indeed on the prey PG (Supplementary Fig. 2d).

To establish that the dark channel in the HADA focal mark was indeed an entry pore in the prey PG, we needed to detect the reduction of prey-PG material at the HADA channel centre. Using a more outer-membrane-permeable *E. coli imp4213* mutant strain as an alternative prey allowed us to label the prey PG uniformly and more completely with otherwise poorly outer-membrane-permeable TADA. In these cells, dark pores in the TADA signal (arrowheads, TADA channel, Fig. 3a) were present, coincident with, and central within, the HADA ring (Fig. 3a and Supplementary Table 3). These results represent a direct observation of *B. bacteriovorus* generating a ringed pore in the prey PG; a process that had previously been only inferred from indirect evidence.\(^{16,21,22}\)

Our approach also allowed us to distinguish clear deformations of the prey cell wall at the point where the *B. bacteriovorus* cell had entered (arrowheads, Fig. 2b; arrowheads, HADA channel, Supplementary Fig. 3; and Supplementary Table 2), clarifying visually previous suggestions that *B. bacteriovorus* enzymatic modifications of prey cell walls may act to soften them.\(^{16,21,22}\)

To investigate dynamic changes in pores after invasion, we analysed (Supplementary Table 2, Fig. 2c and Supplementary Fig. 2e), ~400 HADA-labelled *E. coli S17-1* bdelloplasts. In 27% of these containing internalized *B. bacteriovorus*, there was a HADA ring similar to the entry pore on bdelloplasts, located at the prey–predator contact point on the prey wall–proximal pole of the internalized *B. bacteriovorus* cells (red arrowheads, Supplementary Fig. 2e and Supplementary Table 2). In some cases (4%), the HADA patches were filled discs (white arrowheads, Fig. 2c and yellow arrowheads, Supplementary Fig. 2e). Such discs were also coincident with dark pores in the TADA label of *E. coli imp4213* mutant bdelloplasts (Fig. 3c and Supplementary Table 3) suggesting that they are sealing discs made by internalized *B. bacteriovorus* to close the prey, keeping the bdelloplast intact for predator consumption of contents.

*B. bacteriovorus* establishment inside prey is accompanied by an L,D-transpeptidase-mediated prey wall modification. As the *B. bacteriovorus* cells enter the prey periplasm, the prey cells become rounded (Fig. 2a), forming a bdelloplast.\(^{13}\) During this period, the extent of HADA incorporation to the whole rounding wall of the (now dead) prey substantially increased and peaked around 45 min post-mixing, with ~2 to 4 times more HADA signal intensity (blue line, Fig. 4a, see Methods for details) than the mean HADA labelling at later 2, 3 and 4 h predation time points.

Previous global transcriptomic work had shown that the predicted *B. bacteriovorus* L,D-transpeptidase (*ldt*) genes, *bd0886* and *bd1176*, are transcriptionally upregulated at 30 min from the start of predation about fivefold and sixfold, respectively.\(^{25}\) These predicted L,D-transpeptidases, therefore, are good candidates for prey wall modification enzymes during bdelloplast establishment. Reverse transcription-PCR analysis confirmed that the expression of both genes peaked at 15–30 min into predation (Fig. 4b); time points at which HADA incorporation to the prey walls begins (blue line, Fig. 4a). Deletion of both of these *ldt* genes (leaving 17 *ldt* homologues intact) resulted in a Δ*bd0886bd1176* predator (named Δ2ldt) that caused ~2–4 times less prey HADA incorporation activity than the wild type (blue line versus orange line, Fig. 4a and representative images in Fig. 4c versus Fig. 4d). This significant difference suggests
Bdelloplast wall modification is largely by the action of *B. bacteriovorus* enzymes that act on uncrosslinked tetrapeptides of the prey PG. To test the nature of the bdelloplast wall modification, we quantified HADA incorporation in bdelloplasts formed by *B. bacteriovorus* predation on different *E. coli* prey lacking different PG modification functionalities. The prey strain *E. coli* BW25113 Δ6LDT lacks all of the 6 *E. coli* L,D-transpeptidases (and therefore any L,D-transpeptidation activity). It lacks tripeptides, 3-3 crosslinks and PG-attached Lpp, and is rich in tetrapeptides. The prey strain *E. coli* BW25113 ΔdacA lacks the major *E. coli* D,D-carboxy-peptidase DacA and so contains more pentapeptides in its PG. The prey strain *E. coli* BW25113 Δ6LDTΔdacA lacks all L,D-transpeptidases and the D,D-carboxy-peptidase DacA and so contains mainly tetrapeptides, some pentapeptides, and lacks the modifications introduced by L,D-transpeptidases. Compared with the wild-type prey strain *E. coli* BW25113 WT, predation of these strains by *B. bacteriovorus* and pulse labelling with HADA at 35–45 min post-mixing of predator and prey resulted in significantly more HADA incorporation for both prey strains lacking the L,D-transpeptidase activity (Δ6LDT and Δ6LDT ΔdacA, Fig. 5a), but with no significant difference for prey lacking DacA alone (Fig. 5a). In the absence of *B. bacteriovorus* predation, prey cells in Ca/HEPES buffer pulsed with HADA showed a fraction of the HADA incorporation when compared with the prey strains subjected to *B. bacteriovorus* predation (~1.5–14.6% of HADA incorporation, controls versus + Bds, Fig. 5a). The majority of the *E. coli* self-labelling (in controls in the absence of *B. bacteriovorus*, Fig. 5a) was absent in the *E. coli* BW25113 Δ6LDT, showing the LdtEC to be responsible for this small amount of labelling. That predation of this strain actually resulted in more HADA incorporation further supports the notion that this incorporation is by *Bdellovibrio*-encoded enzymes rather than those of the prey. Altogether, these results suggest that a significant proportion of the strong HADA incorporation observed on the prey PG during predation involves predator L,D-transpeptidase activity on tetrapeptides of the prey bdelloplast PG (and not D,D-transpeptidase activity on pentapeptides). These data, along with Bd1176–mCherry and Δ2ldt data above, show that this activity comes from L,D-transpeptidases secreted by the *B. bacteriovorus* and not due to lingering activities of prey Ldt enzymes.
1,6-D-transpeptidase mediated prey wall modification confers bdelloplast physical robustness. To determine the role of the 1,6-D-transpeptidase activity, we assayed the stability of bdelloplasts produced by wild-type *B. bacteriovorus* or by Δ2ldt mutant predator under osmotic challenge using the β-galactosidase substrate chlorophenyl red-β-d-galactopyranoside (CPRG) method to screen for damage to bacterial cell walls.

Bdelloplasts, at the point where peak Ldt FDAAs transfer was observed (1 h post-synchronous infection of *E. coli* S17-1 lacZ prey) were subjected to osmotic upshock or downshock. We observed increased β-galactosidase activity (Fig. 5b) in the supernatant from shocked bdelloplasts formed by Δ2ldt mutant predators relative to wild type in all conditions tested, including a small (but significant) increase in levels from bdelloplasts formed by Δ2ldt predators, subjected only to the stress of centrifugation and resuspension in buffer (Fig. 5b). These data suggest that Bd0886 and Bd1176 l,d-transpeptidase activities strengthen the bdelloplast wall to resist bursting during periods of *B. bacteriovorus* predatory intrabacterial growth, after prey entry.

To investigate whether this Ldt modification had any effect on the bdelloplast morphology, we measured the sizes and shapes of the prey and bdelloplasts. Early bdelloplasts (45–60 min) formed by the Ldt mutant *B. bacteriovorus* were slightly, but significantly (*p < 0.0001*), less round than those formed by the wild type.
they do not replicate outside prey. On the other hand, after 2–3 h than could have been accrued from just one invading Bdellovibrio. While potentially fascinating, quantifying this inter-wall transfer proved impossible to resolve with current reagents. The high level of E. coli + Bd WT alone) or prey cells (Bd + ΔΔ2ldt) with controls of uninvaded E. coli prey cells (E. coli alone) or B. bacteriovorus cells alone (Bd WT alone). Red colour from positive CPRG reaction was measured by spectrophotometry at 574 nm and readings were normalized to each experiment. Bddelplasts formed by wild-type (E. coli + Bd WT) or bdelloplasts

(Fig. 5 | Plots showing HADA incorporation in the PG of prey E. coli mutants upon B. bacteriovorus predation and showing the damage by osmotic shock to bdelloplasts formed by B. bacteriovorus Ldt mutants. a. Chart of mean HADA fluorescent signal of prey strains preyed on by B. bacteriovorus (+Bd), and pulsed with HADA at 35–45 min post-mixing (the time point of maximal HADA incorporation for E. coli S17-1). Controls were in Ca/HEPES buffer without B. bacteriovorus predation, but pulsed with HADA at the same time point. Measurements are total mean background-corrected fluorescent signal of prey cells, reported in relative fluorescent units measured by MicrobeJ. Prey cells lacking all six LΔ-transpeptidases (∆6LDT) accumulated more HADA fluorescence following predation by B. bacteriovorus. Control samples without B. bacteriovorus predation accumulated considerably less HADA fluorescence. Controls of ∆6LDT prey cells without Bdellovibrio predation accumulated negligible HADA fluorescence. Data are from two (for the controls) or three independent repeats. Error bars are s.e.m. WT; E. coli BW25113 wild-type strain YB7421; Δ6LDT: E. coli BW25113 Δ6LDT strain deficient in all six LΔ-transpeptidases; ∆dacA: E. coli BW25113 strain YB7423 deficient in dacA; ∆6LDTΔdacA: E. coli BW25113 Δ6LDTΔdacA strain YB7439 deficient in all six LΔ-transpeptidases and dacA. NS, not significant; all other comparisons were significant *P < 0.0001, with the one exception shown, by the Mann–Whitney test. b. CPRG β-galactosidase assay measuring cytoplasmic leakage of shocked E. coli bdelloplasts formed by wild-type (E. coli + Bd WT) or bdelloplasts formed by ΔΔ2ldt mutant B. bacteriovorus (E. coli + Bd ΔΔ2ldt) with controls of uninvaded E. coli prey cells (E. coli alone) or B. bacteriovorus cells alone (Bd WT alone). Red colour from positive CPRG reaction was measured by spectrophotometry at 574 nm and readings were normalized to each experiment. Bdelloplasts were harvested by centrifugation and shocked by resuspension in Ca/HEPES buffer for no shock, except centrifugation alone (Buffer), Ca/HEPES buffer supplemented with 750 mM NaCl (Upshock) or upshock followed by further centrifugation and resuspension in water (Downshock). Error bars are s.e.m. Statistical significance was determined by Student’s t-test (two-tailed) *P < 0.05, **P < 0.01, ***P < 0.001. Data are the mean of seven independent repeats.

(Supplementary Fig. 5). We hypothesize that the less robust bdelloplasts formed by the Ldt mutant result in more flexible walls that warped more by the invading B. bacteriovorus cell, visible at the earlier stage of invasion after the B. bacteriovorus cell squeezed into the full prey cell. At later stages of invasion (2–4 h), degradation of prey cell content may be why the differences between bdelloplasts formed by the mutant or the wild type are no longer significant.

Multi-coloured-FDAA labelling provides direct evidence for the zonal mode of elongation and synchronous division of B. bacteriovorus growing inside prey. B. bacteriovorus grow without binary fission, as a single multi-nucleoid filament inside prey39. At later time points, after 2 h post-mixing, we observed filamentous cell elongation of the B. bacteriovorus within bdelloplasts (Fig. 6a)39. Attack-phase B. bacteriovorus were added in excess to ensure efficient predation in our experiments and attack-phase predator cells that did not enter prey can be seen to retain substantial initial BADA labelling (Fig. 6a and yellow arrowheads, Fig. 6b), because they do not replicate outside prey. On the other hand, after 2–3 h post-mixing, we observe some green BADA transfer into the prey bdelloplast structure (BADA signal on bdelloplasts, Fig. 6a), which may represent a predator-to-prey DAA turnover and transfer event as the growing B. bacteriovorus make new PG during elongation. While potentially fascinating, quantifying this inter-wall transfer proved impossible to resolve with current reagents. The high level of BADA accumulation in these bdelloplast walls appears to be more than could have been accrued from just one invading Bdellovibrio. This may be a slow accumulation into the prey PG of free BADA present in the medium. This BADA may have been released from excess non-invading Bdellovibrio due to their self-PG turnover, and/or releasing of BADA transiently accumulated in their cell envelopes. This pool of free BADA would be present throughout the 4 h predatory cycle and so could incorporate into prey over a longer time compared with the 10 min pulses of HADA availability.

3D-SIM imaging showed that B. bacteriovorus cells elongate along the filament with numerous, focused zones of growth (labelled with HADA, red arrowhead, Fig. 6b) covering the entire cell surface except the apparently inert poles (preserving the original BADA signal, green arrowheads, Fig. 6b). Later, around 3 h post-mixing, new HADA incorporation appears as defined narrow foci along the filament (Fig. 6a and red arrowheads, Fig. 6c), at points in B. bacteriovorus where new division septa would be expected to form synchronously39. After 4 h post-mixing, these foci become the points of septum formation (Fig. 6a and yellow arrowheads, Fig. 6d). Finally, newly released, attack-phase B. bacteriovorus daughter cells (white arrowheads, Fig. 6d) incorporate pulsed HADA all over the cell and can therefore be distinguished from excess BADA-labelled predators that did not enter prey cells by the presence of a strong HADA fluorescent signal, but low BADA fluorescent signal.

Discussion

Here, using multi-coloured-FDAA labelling and super-resolution imaging, we directly visualize subcellular modifications by B. bacteriovorus on E. coli PG cell walls and their effects during predation. Our data define an entry port structure by which a B. bacteriovorus cell accesses the cytoplasmic membrane face of the prey cell wall and seals itself in. We also show the sites of PG growth in the non-binary fission mode of predator growth. In addition, we show that
l,d-transpeptidases from the *B. bacteriovorus* modify the PG of prey during residency of the predator to establish a stable intracellular niche.

Pioneering enzymology of prey bdelloplast extracts in the 1970s had detected bulk enzyme activities suggestive of extensive predator modification of prey PG. These included solubilization of 25% of the meso-diaminopimelic acid (*m*-DAP) residues on the PG and the addition of free *m*-DAP back to the bdelloplast, *m*-DAP is a residue native to PG that has both l- and d-amino acid properties. Therefore, we see FDAA signals in our studies acting as visible substrates for these enzymatic, fresco-like changes to the walls of invaded prey.

The initial ring of intense FDAA incorporation matches with the gap on the prey cell wall at the contact point with the *B. bacteriovorus* pole (Supplementary Tables 2 and 3, and Figs. 2a and 3a). Such a remodelling of the prey PG probably strengthens the predator entry point. We also show here (Figs. 2c and 3b) that such entry ports have accumulated centralized FDAA signal after *B. bacteriovorus* entry, which might represent a gradual ring-to-disc re-sealing activity of this pore; a process that had previously been only inferred by indirect evidence of ‘scars’ left behind on the prey cell wall at the point of entry.

The most extensive prey cell wall modification occurs 30–45 min after mixing *B. bacteriovorus* with the prey; involving the l,d-transpeptidases with major contributions from 2 of the 19 Ldt genes encoded by the genes *bd0886* and *bd1176* (Fig. 4a). These observations may be due to pulsed FDAA signals mimicking the incorporation of previously solubilized *m*-DAP reported in early *B. bacteriovorus* studies, but this is beyond the scope of our present work. While we were able to isolate fluorescent FDAA-labelled sacculi, the amounts were not sufficient for mass spectrometry-based identification of sites of d-amino acid incorporation in *Bdellovibrio* or *E. coli* (Supplementary Fig. 7). Incorporation of non-canonical d-amino acids into the cell wall is a stress response in *Vibrio cholerae*, which is shown to stabilize the PG integrity of the cells in stationary phase. The incorporation of native *m*-DAP and/or d-amino acids into the prey cell wall by *B. bacteriovorus* Ldts early in the predation (15 min–1 h) could represent an analogous means of forming a stabilized and stress-resistant bdelloplast. The susceptibility of bdelloplasts formed by the Δ2ldt mutant predator to bursting during osmotic stress (Fig. 5b) supports this hypothesis.

FDAA labelling also elucidated the growth of the intraperiplasmic *B. bacteriovorus* predator directly (Fig. 6). Growth starts in patches along the length of the *B. bacteriovorus* cell, but not at the poles (Figs. 6a,b). After *B. bacteriovorus* septation, final predator self-PG-modification produces attack-phase *B. bacteriovorus* (Fig. 6d) that each emerge with one flagellated and one piliated pole. These experiments provide evidence that both predator poles can carry out bilateral growth, along the length of the cell, rather than one ‘old’ pole remaining attached to the membrane and growth emanating solely from specific regions. Synchronous septum construction (that results in odd or even progeny numbers) is seen along the length of the filamentous *B. bacteriovorus* growing within the bdelloplast (Fig. 6a,c,d), confirming earlier movies of this synchronous division.

In conclusion, the ability to distinctly label the PG-containing cell walls of two different genera of interacting bacteria with different coloured FDAs has illuminated a series of dynamic molecular modifications that predatory *B. bacteriovorus* make to prey cell
walls and self cell walls during their intraperiplasmic lifestyle. These modifications (pore formation and resealing without bacterial bursting and PG remodelling with free small molecules, that is, DAAs, in dual-cell systems) are previously uncharacterized in bacteria, and are key mechanisms of *B. bacteriovorus* predation. Given the inherent promiscuity of virtually all PG-containing bacteria to incorporate DAAs in situ[8,9], we expect this general approach to be helpful for visualizing interactions of other complex bacterial communities, such as microbiota. Accordingly, we would not be surprised if this and similar approaches illuminate other examples of inter-generic PG modifications with novel functions.

**Methods**

**RNA isolation from predatory cycle and RT-PCR analysis.** Synchronous predatory infections of *B. bacteriovorus* HD100 on E. coli S71-1 in Ca/HEPES buffer (2 mM CaCl₂, 25 mM HEPES, pH 7.6), or strain S71-1 suspended in Ca/HEPES alone, were set up as previously described[10] with samples throughout the time course being taken and total RNA isolated from them. This semi-quantitative PCR allows the evaluation of specific predator transcripts in the presence of fluctuating levels of prey RNA as the predator degrades it. RNA was isolated from the samples using a Promega SV Total RNA isolation kit with the RNA quality being verified by an Agilent Bioanalyzer using the RNA Nano kit. RT-PCR was performed with the Qiagen One-step RT-PCR kit with the following reaction conditions: one cycle 50 °C for 30 min, 95 °C for 15 min, then 25 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, a 10 min extension at 72 °C after the 30 cycles, and finally a 4 °C hold. Two independent repeats were carried out. Primers to anneal to the region of *bd1176* were 5'-AGCCTCTACATGGGTGCAAG-3' and 5'-AACCTTGCTGCTATACCAACC-3'. Primers to anneal to *bd1176* were 5'-GCCAACCGCCGTTAATG-3' and 5'-GGCCGCGTTAGTTGTCG-3'.

**Generation of gene deletion mutants in *B. bacteriovorus*.** Markless deletion of both the *bd0886* and *bd1176* genes from *B. bacteriovorus* HD100 was achieved sequentially as described previously[11,12]. Primers designed to amplify to the upstream region of *bd0886* were: B0886F 5'-ACGGGGTACCGGATGTGATTCATG-3' and Bd1176-R 5'-ACGGGGTACCGGATGTGATTCAT-3'. Primers designed for a PCR amplification of the downstream region of *bd0886* were: Delbd0886F 5'-GATTGCCAGCTCCCCTATGTCTAGAAATCCTCCGAAG-3' and Delbd1176R 5'-GCCAACCGCCGTTAATG-3'. Primers designed to amplify to the downstream region of *bd1176* were: Delbd1176F 5'-AGCCTCTACATGGGTGCAAG-3' and Delbd1176R 5'-GCCAACCGCCGTTAATG-3'.

**Construction of an E. coli strain lacking all six l.d.-transpeptidases.** E. coli BW25113Δl.dLT was lacking all five previously published l.d.-transpeptidase genes (erfK, ybnT, ybnF, ycbB, ycbA) as well as a sixth gene encoding a putative l.d.-transpeptidase, *yfK*. Gene deletions were generated and combined by transferring kan-marked alleles from the Keio *E. coli* single-gene knockout library[20] into relevant background strains using PI phage transduction[40]. The Keio *E. coli* pKD13-derived marked alleles from the Keio background strains using P1 phage transduction[40]. The Keio pKD13-derived markerless Δl.dLT 5.1 prey to incorporate FDAAs in situ[9,35, we expect this general approach to be helpful for visualizing interactions of other complex bacterial communities, such as microbiota. Accordingly, we would not be surprised if this and similar approaches illuminate other examples of inter-generic PG modifications with novel functions.

**HADA pulse-labeling of *B. bacteriovorus*.** HADA was used to label the PG of *B. bacteriovorus* in situ. Strains were grown on LB plates (1000 µg ml⁻¹ kanamycin sulfate) for 12 h at 30 °C, serially diluted, and plated on LB plates containing 500 µg ml⁻¹ kanamycin sulfate. The plates were incubated for 24 h at 30 °C, after which the colonies were transferred into a solution of 1000 µg ml⁻¹ kanamycin sulfate in fresh LB and incubated for an additional 24 h. The cells were then washed twice in Ca/HEPES buffer before being resuspended in an equal volume of Ca/HEPES buffer. E. coli S71-1 or E. coli imp4213 cells were grown for 16 h in LB at 37 °C with shaking at 100 r.p.m. and were back diluted to OD₆₀₀₃.₀ in fresh LB, (yielding ~10 x 10⁶ cfu ml⁻¹) and labelled with a final concentration of 500 µM HADA (by addition of 5µl of a 50mM stock in dimethyl sulfoxide (DMSO)) for 30 min at 30 °C. The cells were then washed twice in Ca/HEPES buffer before being resuspended in an equal volume of Ca/HEPES buffer. E. coli BW25113 strains were grown as for strain S71-1, except strains Y7423, Y7424 and Y7479 were supplemented with 50µg ml⁻¹ kanamycin sulfate for incubation and washed of this by centrifugation at 5,000 for 5 min, resuspension in fresh LB and further centrifugation at 12,000 × g for 5 min, before back-dilution to OD₆₀₀₃.₀ in Ca/HEPES buffer. This resulted in similar numbers of cells for each strain: E. coli BW25113 Δl.dLT 5.1 x 10⁶ ± 3.6 x 10⁵, Y7423 5.2 x 10⁶ ± 1.8 x 10⁶, Y7424 4.9 x 10⁶ ± 2 x 10⁶, Y7479 4.3 x 10⁵ ± 1.6 x 10⁵ as determined by colony-forming units.

**RT-PCR analysis.** For quantification of fluorescent signal, the exposures were chosen to give values that did not exceed the maximum, so that saturation was not reached for any of the fluorescent channels. Images were processed in the Equipment and settings section in the Supplementary Information.

**Quantification of fluorescent signal.** For quantification of fluorescent signal, images were acquired as above, but with varying exposure and gain settings. The exposures were chosen to give values that did not exceed the maximum, so that saturation was not reached for any of the fluorescent channels. Images were analysed using the Microbel plugin for the ImageJ (FIJI) distribution software (http://www.indiana.edu/~microbel/index.html), which automates detection of boundaries within an image. The E. coli prey cells and *B. bacteriovorus* cells were detected using the resulting binary mask from both the phase contrast and either the TADA bacteria within an image. The shape measurements including the angularity, area, aspect ratio, circularity, curvature, length, roundness, sinuosity, solidity and width were measured for each type of cell. Background-corrected mean fluorescent intensity was measured for each cell and then the mean

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of these measurements was determined for each cell type, for each independent experiment. Typically, 500–5,000 cells were measured at each time point for each independent experiment (details of n for each sample in each experiment are presented in Supplementary Table 1).

Code availability. The images and the data were analysed by Microbel (5.11v), a freely available and open-source software. The code source is available upon request from A.Du.

CPRG assay of leakage of osmotically shocked bdelloplasts derived from predation by Ldt mutant versus wild-type B. bacteriovorus. To evaluate whether DAA transfer to prey bdelloplast cells altered the physical stability of those walls to osmotic changes, an assay for leakage of cytoplasmic contents, including β-galactosidase, was used, with the CPRG as a detection reagent. E. coli S17-1 (ac') prey cells were grown for 16 h in YT broth at 37 °C with 200 µg/mL shaking, before being supplemented with 200 µM IPTG for 2 h to induce expression of lacZ. These prey cells were then centrifuged at 5,100g for 5 min and resuspended in Ca/HEPES buffer (2 mM CaCl2, 25 mM HEPES, pH 7.6) and then diluted to OD600 1.0 in Ca/HEPES buffer. Bdellovibrio bacteriovorus HD100 or ΔΔldt strains were grown predatorily for 16 h at 29 °C on stationary-phase E. coli S17-1 prey cells until they were fully lysed, and then B. bacteriovorus were filtered through a 0.45 µm filter, concentrated 50× by centrifugation at 5,100g for 20 min and resuspended in Ca/HEPES buffer total protein concentration of these concentrated suspensions was determined by Lowry assay, and matched amounts of 50 µg/mL of each strain were used for semi-synchronous infections (see above), and 284 µL of concentrated suspension made up to a total of 800 µL in Ca/HEPES buffer) with 400 µL of diluted E. coli S17-1 prey cells. This resulted in a multiplicity of infection (MOI of B. bacteriovorus cell:E. coli cell) of 1.4 to 10.5 for the wild-type strain HD100 as determined by plaque assay. The excess of predators resulted in an MOI of 200 prey cells rounds of invasion HD100 invasion and ΔΔldt mutant of prey cells rounded by invasion of ΔΔldt mutant after incubation at 29 °C for 1 h with shaking at 200 r.p.m.

A control of prey alone (400 µL diluted prey cells with 800 µL Ca/HEPES buffer) resulted in no rounded prey cells and a control of wild-type B. bacteriovorus HD100 cells alone (50 µg/mL in a total of 1,200 µL Ca/HEPES buffer) was included. After incubation, bdelloplasts (or cells in the controls) were harvested by centrifugation at 17,000g for 2 min and supernatant was removed. The pellets were resuspended in: Ca/HEPES buffer supplemented with 20 µg/mL CPRG (Sigma) for centrifugation shock alone; Ca/HEPES buffer supplemented with 750 mM NaCl and 200 µg/mL CPRG for upshock; Ca/HEPES buffer supplemented with 750 mM NaCl, incubated for 30 min at 29 °C by centrifugation at 17,000g for 2 min and supernatant removed, then the pellet resuspended in water supplemented with 20 µg/mL CPRG for downshock. These were then incubated for 30 min at 29 °C before purifying the supernatant, containing any bdelloplast leakage products, for β-galactosidase assay by removing cells by centrifugation at 17,000g for 2 min followed by filtration through a 0.2 µm filter. The β-galactosidase assay was carried out by incubation at 29 °C for 26 h and colour change was monitored by spectrophotometry at 574 nm. Data were normalized for each experiment.

Extra experimental considerations. The ΔΔldt mutant strain exhibited a plaquing phenotype, forming mostly very small plaques with ~1% forming plaques. In both cases, the prey cells were almost eradicated after 24 h with only 8–13 cells detected by Microbel in 20 fields of view for each experiment (reduced to 1.0±0.4 % of starting values for HD100 and 3.3±0.8 % for the ΔΔldt mutant).

Data availability. The raw data that support the findings of this study are available from the corresponding author upon request.

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**Author contributions**

E.K. and R.E.S. conceived the study and carried out the experiments along with C.L. and A. De. J.G. and J.B. performed muropeptide analysis in the laboratory of W.V. using reagents constructed by M.V.N. and J.R., and bacterial strains constructed by R.T. E.K. and R.E.S. wrote the manuscript with input and comments from the other authors.

**Competing interests**

The authors declare no competing financial interests.

**Additional information**

Supplementary information is available for this paper at doi:10.1038/s41564-017-0029-y. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to R.E.S.

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» Experimental design

1. Sample size
   Describe how sample size was determined.
   No sample-size calculation was performed but large numbers (85-4966) of bacterial cells were measured at each time-point, which was sufficient to support the statistical difference (if any) between samples in our conditions.

2. Data exclusions
   Describe any data exclusions.
   No data were excluded from the analyses.

3. Replication
   Describe whether the experimental findings were reliably reproduced.
   All attempts at replication in the stated conditions were successful.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   No experimental group was defined in our study.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   Since no group allocation was performed in our study, blinding was not relevant.

   Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters
   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   n/a Confirmed
   - [ ] The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
   - [ ] A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
   - [ ] A statement indicating how many times each experiment was replicated
   - [ ] The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
   - [ ] A description of any assumptions or corrections, such as an adjustment for multiple comparisons
   - [ ] The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
   - [ ] A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
   - [ ] Clearly defined error bars

   See the web collection on statistics for biologists for further resources and guidance.
Software

7. Software

Describe the software used to analyze the data in this study.

The images and the data were analyzed by MicrobeJ (5.11v), a freely available and open-source software [Ref44] in our manuscript Ducret, A., Guardokus, E. M., & Brun, Y. V. (2016). MicrobeJ, a tool for high throughput bacterial cell detection and quantitative analysis. Nature Microbiology, 1(7), 16077. http://doi.org/10.1038/nmicrobiol.2016.77. The code source is freely available upon request from Dr Adrien Ducret at the email in the paper.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

Available upon request and proper material transfer agreement to Michael Van Nieuwenhze, Indiana University. FDAA synthesis protocol is detailed in: Kuru, E., Tekkam, S., Hall, E., Brun, Y. V. & Van Nieuwenhze, M. S. Synthesis of fluorescent D-amino acids and their use for probing peptidoglycan synthesis and bacterial growth in situ. Nature protocols 10, 33-52, doi:10.1038/nprot.2014.197 (2015).

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

N/A

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

N/A

b. Describe the method of cell line authentication used.

N/A
c. Report whether the cell lines were tested for mycoplasma contamination.

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N/A

Animals and human research participants

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

N/A

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

N/A