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

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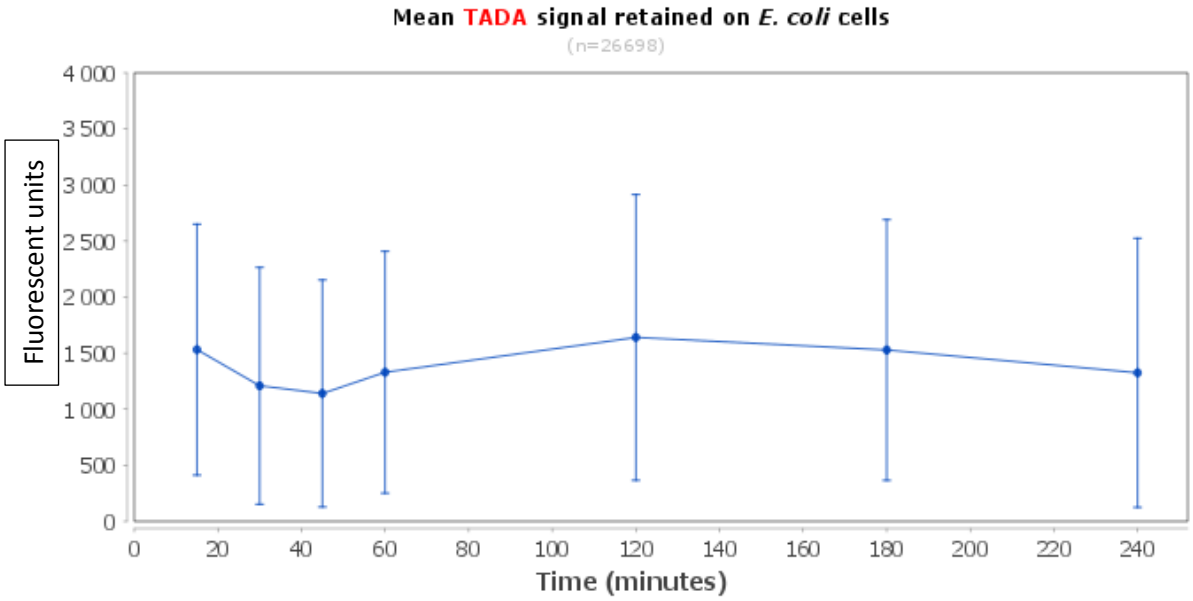
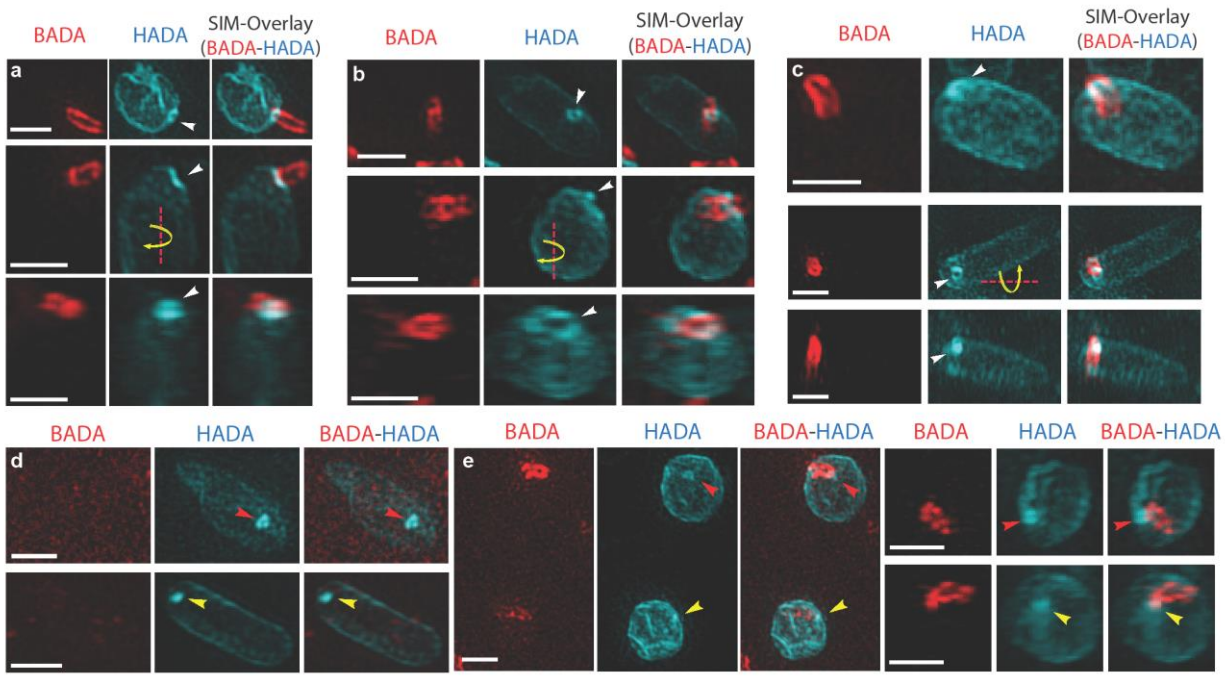
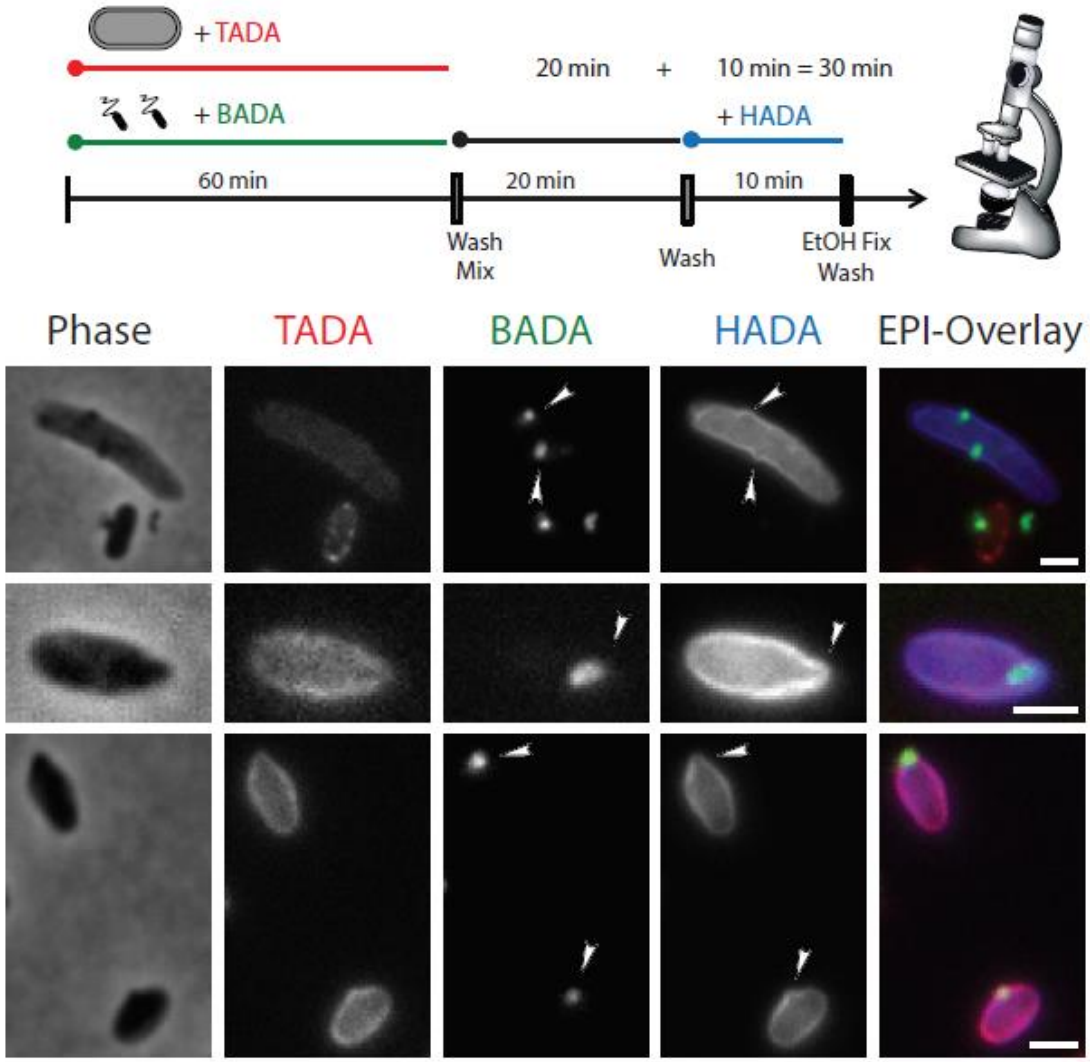


Supplementary Figure 1 Plot of mean TADA fluorescent signal of cells against time throughout the predation cycle. Measurements are total mean TADA fluorescent signal from free uninvaded *E. coli* prey cells or invaded prey bdelloplast. Time is in minutes post-mixing of predator and prey and fluorescence is in relative fluorescent units measured by MicrobeJ (see methods). n= 26,698. Details of numbers of cells analysed at each time point are elaborated below. Error bars are one standard deviation.



Supplementary Figure 2 3D-SIM images of early predation by *B. bacteriovorus* (pre-labelled with BADA, false-coloured red) on prey *E. coli* cells after a pulse labelling for 10 minutes with HADA (false-coloured cyan) to show early modification of cell walls. **a-** Examples of *B. bacteriovorus* cells in the very earliest stages of prey entry through a pore of HADA modification. Here, only the very front of the predator has penetrated the prey PG. **b-** Examples with the *B. bacteriovorus* cell half way through the HADA labelled pore. **c-** Examples of *B. bacteriovorus* cells mostly through the HADA labelled pore; only the very rear of the predator cell has not passed through the prey PG. The lowest panels in **a-c** show the same cells as the middle panels from a different angle. Yellow arrows indicate the direction of rotation around the axis represented by the red dashed line to move from the angle shown in the middle panel to the angle shown in the lower panel. **d-** Examples of HADA modification on the prey cell wall in the absence of a *B. bacteriovorus* cell. Either a ring (red arrowheads) or disc (yellow arrowheads) of HADA modification can be seen. The lookup tables for the BADA channel have been adjusted until the background signal is very high to show that there is not any *B. bacteriovorus* cells with low level labelling attached. Instead, these seem to be examples where the *B. bacteriovorus* cell has become detached soon after modifying the prey

PG, likely as a result of the centrifugation and washing steps of the labelling procedure. This shows that the HADA rings of modification are indeed on the prey PG rather than waves of modification moving along the entering *B. bacteriovorus* cell. **e-** Examples of the two forms of seal highlighted by HADA labelling. The internalised *B. bacteriovorus* cell has a contact point with the prey PG where a small ring of HADA is visible (red arrowheads, top cells) or a filled disc of HADA labelling (yellow arrowheads, lower cells). Scale bars are 1µm. Data are representative of two independent repeats.



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959 **Supplementary Figure 3-** Phase contrast and epi-fluorescent microscopy images of the early
960 stages of *Bdellovibrio* predation. The *Bdellovibrio* were pre-labelled with BADA and are false-
961 coloured in green, the *E. coli* prey cells were pre-labelled with TADA and are false-coloured
962 in red. The cells were pulse-labelled for 10 minutes before each acquisition time point with
963 HADA, which is false-coloured in blue. Each channel is displayed independently in white and
964 with all 3 fluorescence channels merged in EPI-overlay. The prey cell wall bulges around the
965 invading *Bdellovibrio* cell to allow the predator entry to the confined space of the periplasm
966 (arrowheads). Data are representative of five independent repeats. Scale bars are 1 µm.

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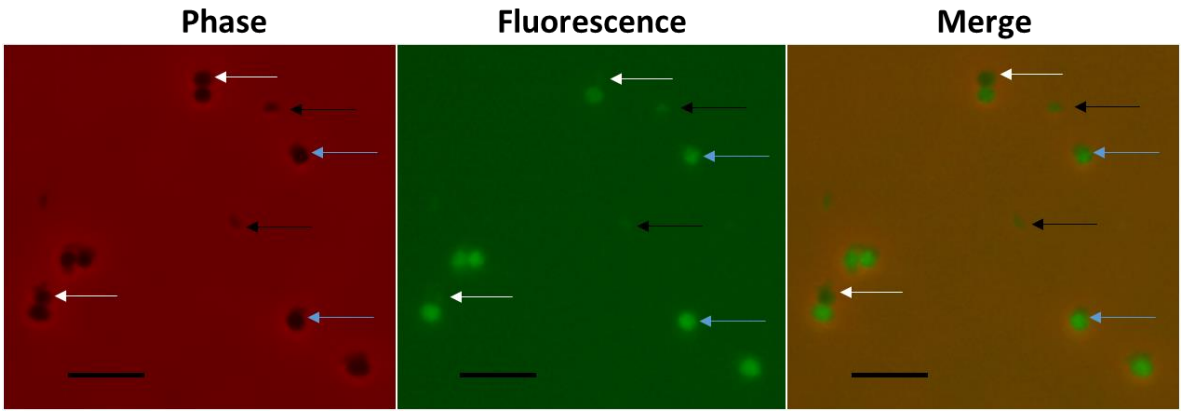
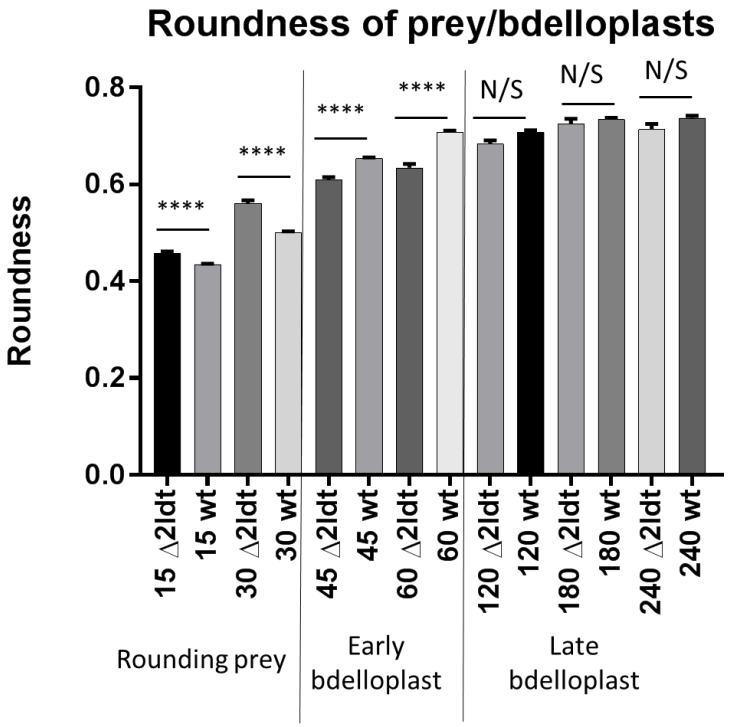


Supplementary Figure 4 Epifluorescent images of *E. coli* invaded by *B. bacteriovorus* HD100 with C-terminally mCherry tagged Bd1176 protein at 45 minutes post-infection. Fluorescence was acquired with a two second exposure and maximum sensitivity gain. mCherry fluorescence has been false-coloured in green. The fluorescent tag was localised to the bdelloplasts (blue arrows) indicating that the protein was exported into the bdelloplast periplasm and/or cytoplasm, consistent with it acting on the prey cell wall. Some bdelloplasts showed low levels of fluorescence (white arrows) as they were likely very recently formed bdelloplasts in this semi-synchronous infection and this is in agreement with the variable levels of HADA incorporation effected by this protein and Bd0886. The *Bdellovibrio* cells themselves (black arrows) also showed a low level of fluorescence suggesting that the protein is either pre-produced in an inactive form, or that the *Bdellovibrio* have a mechanism to control its activity within the *Bdellovibrio* periplasm, such as an associated immunity protein. An immunity protein has recently been described which protects *B. bacteriovorus* from its own predatory DacB enzymes which round up the bdelloplast[1] Scale bars are 5 µm. Data are representative of three independent repeats.



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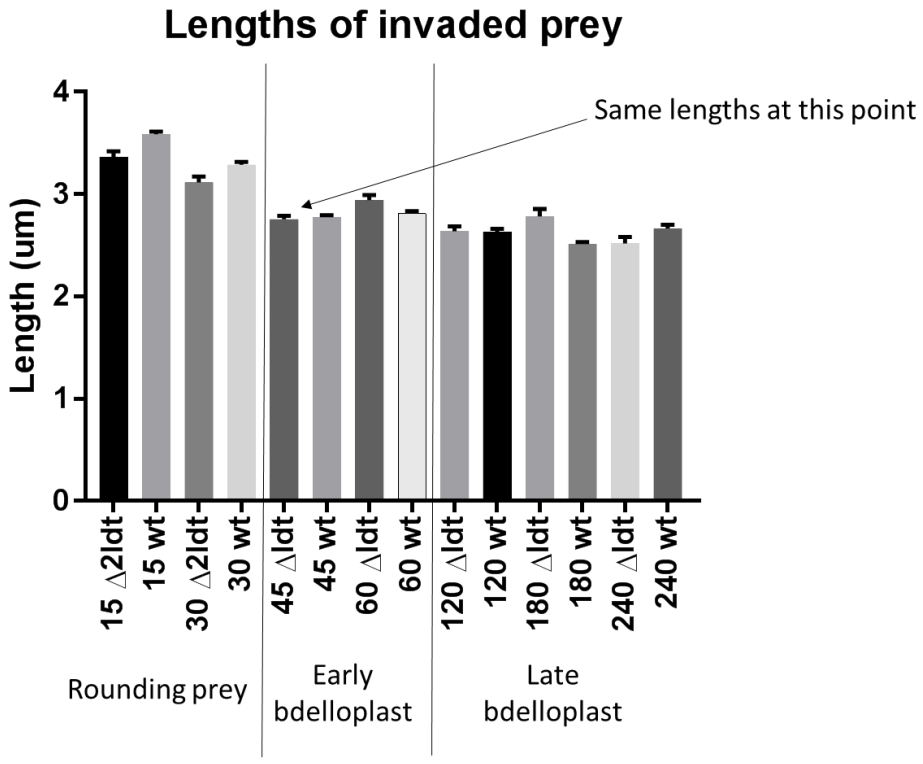

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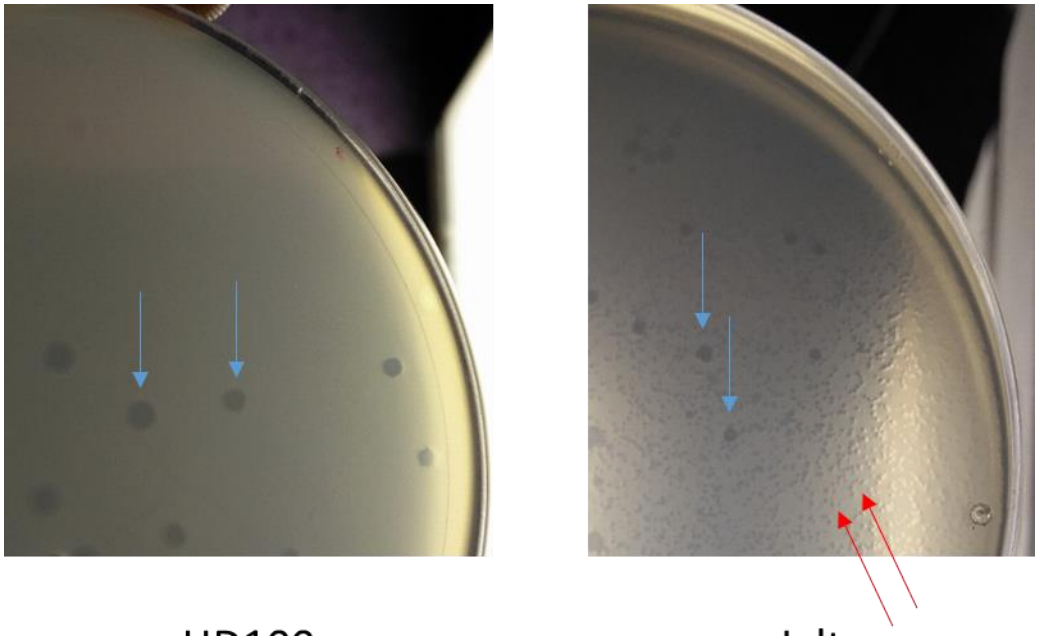
989 **Supplementary Figure 5**- Morphology of prey and bdelloplasts formed by predation by

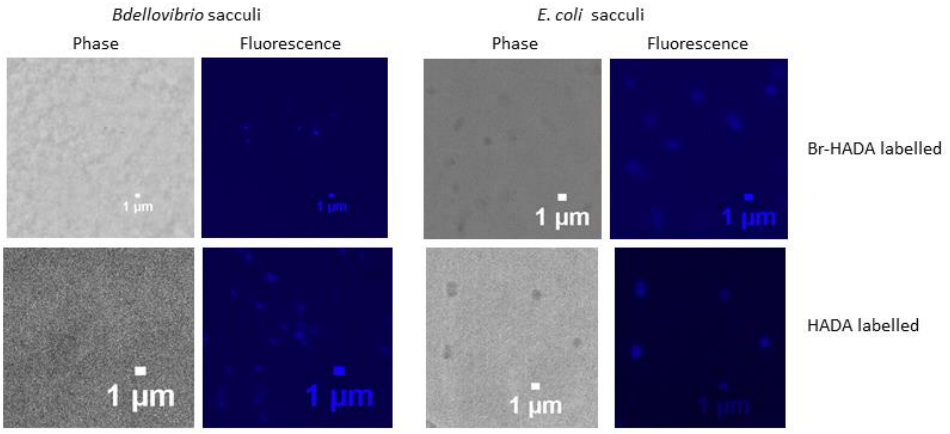
990 *Δ2ldt* mutants compared to wild type. **a-** roundness **b-** lengths as measured by the MicrobeJ

plugin for ImageJ. (Prey gradually transform and shorten, from rod shaped to rounded, by the action of predators and eventually reach maximum morphological modification by predators which are now internal). At the 15 and 30 minute timepoints, prey cells mixed with wild-type predator are less round and longer than the prey cells mixed with *Δ2ldt* predators. This is likely the result of slight asynchrony of the predatory process in these experiments with the *Δ2ldt* mutants preying slightly faster than the wild-type. At the 45 minute timepoint, the lengths of the prey of the wild type and *Δ2ldt* predator are equal suggesting that at this point, virtually all of the prey have formed bdelloplasts. At this timepoint and at 60 minutes, the bdelloplasts formed by the *Δ2ldt* mutant are significantly less round than those formed by the wild-type. We hypothesise that this may be the result of the wild-type predator Ldt action strengthening the bdelloplast wall and the weaker bdelloplast wall formed by the *Δ2ldt* mutant predator being more warped by the invading predator cells. This difference was not observed to be significant at later timepoints; this could be due to predator degradation of the prey contents relieving outward osmotic pressure within the bdelloplast. **** $p<0.0001$ by the Mann-Whitney test, between prey mixed with *Δ2ldt* and wild-type predators. Roundness is measured in arbitrary units defined by the MicrobeJ plugin as $4 \times [Area] \,/\, (\pi \times [Major\ axis]^2)$. Data are from two independent repeats for the *Δ2ldt* mutant and five independent repeats for the wild-type. Error bars are SEM.

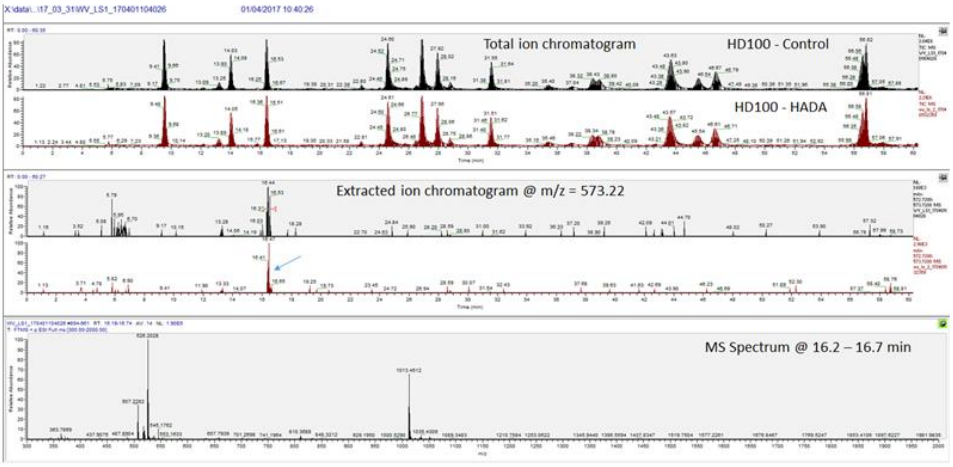


Supplementary Figure 6- Double-layer overlay agar petri dishes demonstrating plaque formation by wild-type (HD100) and mutant *Δ2ldt Bdellovibrio.* YPSC media was used with 1% agar in the bottom layer and 0.6% agar in the top layer, which is supplemented with 100 µl of stationary phase prey *E. coli* to form a lawn and 100 µl of 10^{-4} to 10^{-7} dilutions of concentrated *Bdellovibrio* samples (see methods) to form plaques. Plaques form as regions of clearing in the prey lawn, becoming 4-8 mm in diameter in the wild-type and a minority of plaques formed by the *Δ2ldt* mutant after 5-8 days incubation at 29°C (blue arrows). The majority of plaques formed by the *Δ2ldt* mutant only grow to 1-3 mm in this incubation time (red arrows). Images are representative of plates from 7 independent repeats.

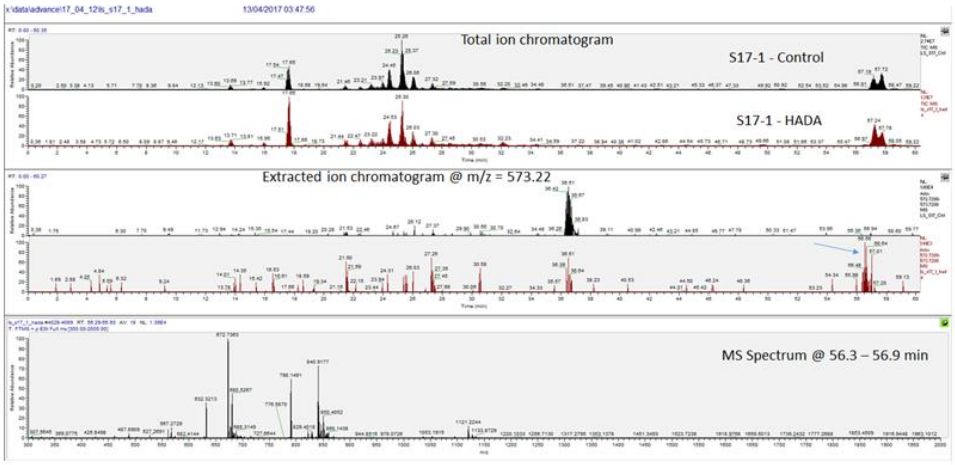
A


Bdellovibrio sacculi
E. coli sacculi
Phase
Fluorescence
Phase
Fluorescence
Br-HADA labelled
HADA labelled
1 µm

B


Total ion chromatogram
HD100 - Control
HD100 - HADA
Extracted ion chromatogram @ m/z = 573.22
MS Spectrum @ 16.2 – 16.7 min

C


Total ion chromatogram
S17-1 - Control
S17-1 - HADA
Extracted ion chromatogram @ m/z = 573.22
MS Spectrum @ 56.3 – 56.9 min

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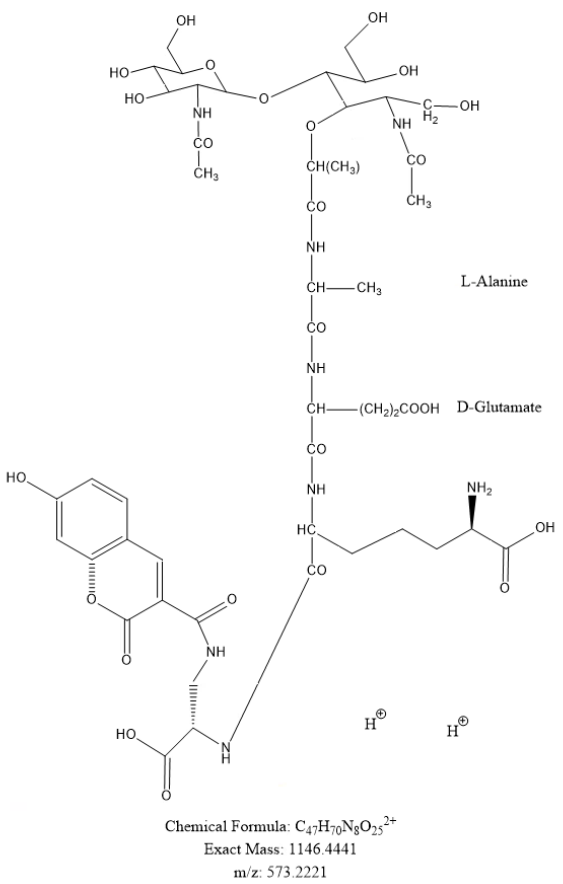
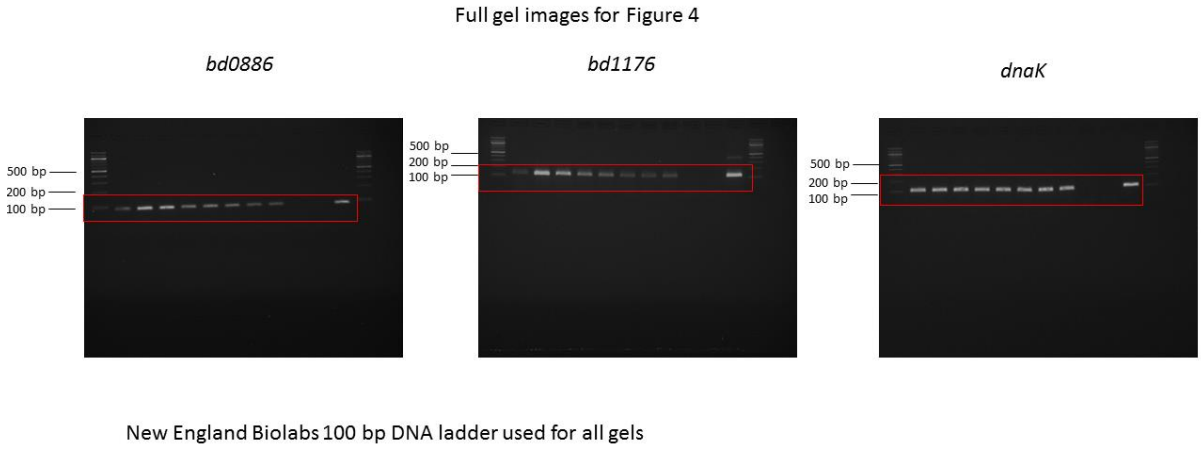
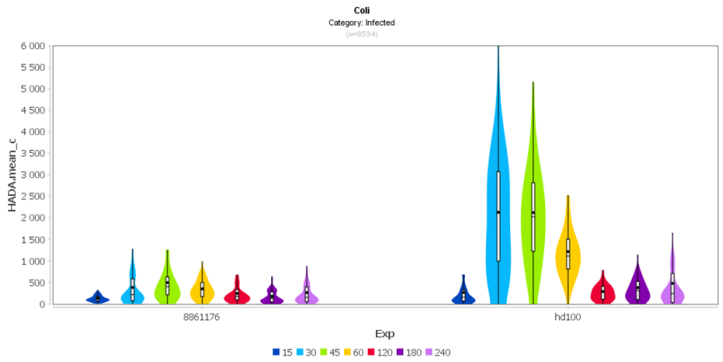


Supplementary Figure 7- Analysis of PG sacculi from *Bdellovibrio* and *E. coli* cells labelled *in vivo* with FDAAs. (A) Live cells of *Bdellovibrio* and *E. coli* were labelled with Br-HADA or HADA. PG sacculi were isolated and visualized by phase contrast and epifluorescence microscopy. Images are representative of one attempt of labelling with each FDAA. Sacculi from *E. coli* (B) or *Bdellovibrio* (C) were digested with cellosyl and the resulting muropeptides were reduced and analysed by LC-MS/MS. The top panels show total ion chromatogram traces for both the unlabelled control (black) and HADA-labelled (red) samples respectively. The middle panels show plots of extracted ion chromatograms (selected m/z = 573.22) for the same samples. The mass filter was set at m/z = 573.22 to correspond with the $2H^+$ charged ion of the expected major HADA-labelled muropeptide [shown in (D)]. The bottom panels in (B) and (C) show the mass spectra observed at the retention times (blue arrow, middle panel) where the 573.22 ion species was most intense in the corresponding HADA-labelled samples (between 16.2 and 16.7 min and 53.6 and 53.9 min respectively). In neither

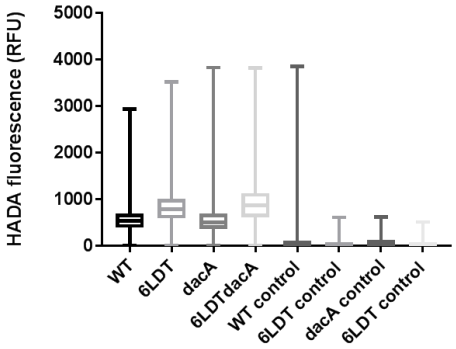
instance was sufficient ion signal present to permit positive confirmation of the desired HADA-labelled muropeptide.



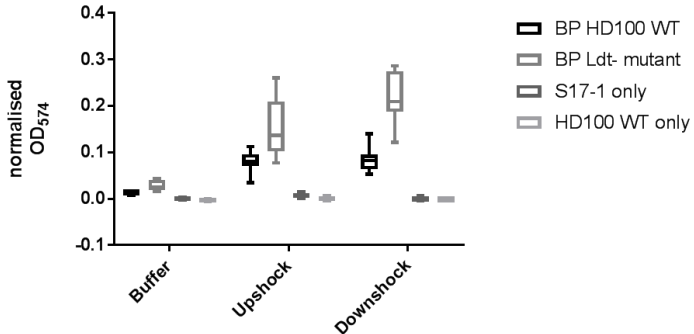
Supplementary Figure 8- Full, uncropped images of the gels used for Figure 4 with the cropped regions highlighted. Primers designed to anneal specifically to the gene labelled above each gel were used for RT-PCR.



Plot to show distribution of data for figure 4a



Plot to show data distribution in Figure 5a



Plot to show data distribution in Figure 5b

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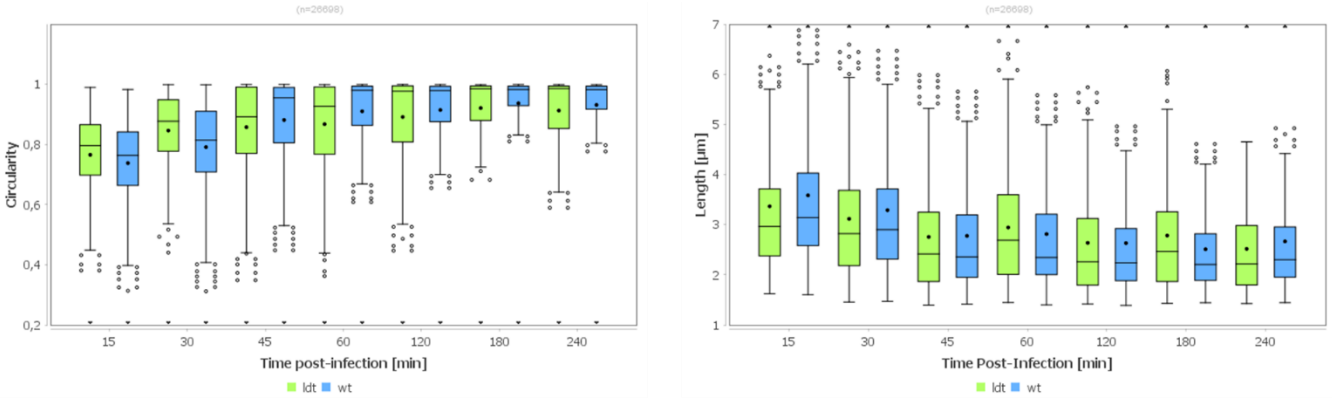

Plots to show data distribution in Supplementary figure 5

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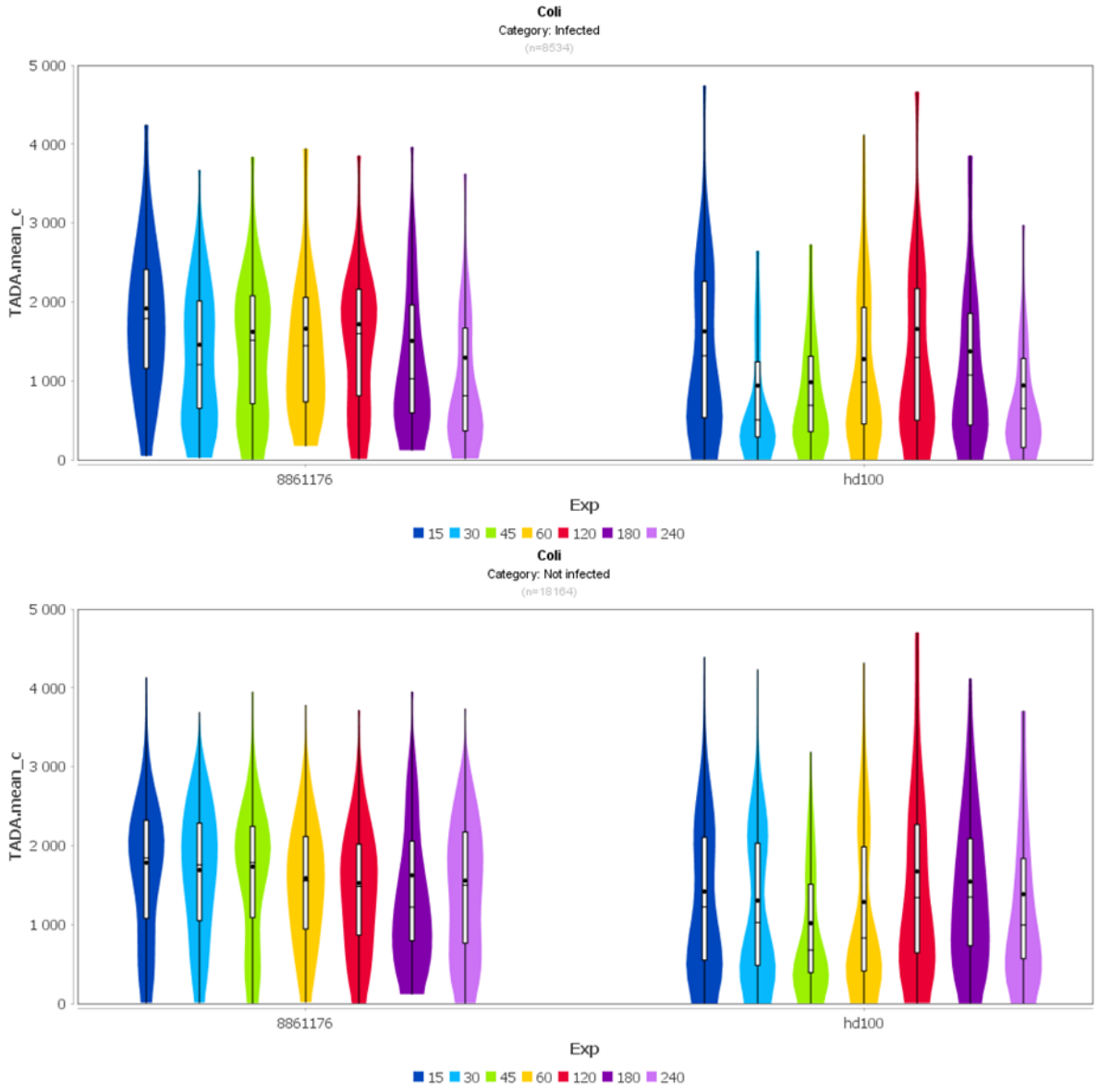

Plot to show data distribution in Supplementary Figure 1

Supplementary Figure 9- Plots to show data distribution.

Mutant prey experiments	**WT keio**	**6LDT**	**dacA**	**6LDTdacA**	**WT control**	**6LDT control**	**dacA control**	**6LDTdacA control**
n1	208	220	239	338	111	105	40	92
n2	106	76	38	30	204	77	46	57
n3	344	260	45	247	-	-	-	-
totals	658	556	322	615	315	182	86	149
Timepoint (minutes) S17-1 experiments		**15**	**30**	**45**	**60**	**120**	**180**	**240**
Prey cells/bdelloplasts	HD100	395	1210	1879	1528	141	203	190
	Δ2ldt	159	226	510	237	187	89	85
Bdellovibrio cells	HD100	4381	2388	2034	3721	1348	3379	4966
	Δ2ldt	1511	2286	2555	933	2411	1062	1912
Timepoint (minutes) morphology experiments		**15**	**30**	**45**	**60**	**120**	**180**	**240**
Prey cells/bdelloplasts	HD100	3692	3632	5511	3639	1994	1993	1110
	Δ2ldt	1063	642	1387	627	818	321	269

Supplementary Table 1- Numbers of cells analysed for each experiment. Numbers of cells analysed using the MicrobeJ plugin for ImageJ (FIJI release) for the different experiments. For the FDAA labelling of mutant prey experiment: WT- *E. coli* BW25113 wild-type strain YB7421, 6LDT- *E. coli* BW25113 Δ6LDT deficient in all 6 L,D transpeptidases, dacA- *E. coli* BW25113 strain YB7423 deficient in DacA, 6LDTdacA- *E. coli* BW25113 strain YB7439 deficient in all 6 L,D transpeptidases and DacA. Three independent repeats were carried out with *Bdellovibrio* and 2 independent repeats were carried out in control experiments without *Bdellovibrio* added. For the FDAA labelling of predation on *E. coli* S17-1 experiments and for the prey cell/bdelloplast morphology experiments, five independent repeats were carried out for the HD100 wild-type experiments and two independent repeats were carried out for the *Δ2ldt* mutant experiments. RT-PCR experiments were carried out on RNA isolated from two independent repeats, with the expression patterns for test and control genes in agreement in both experiments.

	instances	% of instances	Average long axis diameter in nm	Std-dev	instances	% of instances	Average long axis diameter in nm	Std-dev
Timepoint	**15 min**				**30 min**			
Number of HADA-bright prey cells investigated	202	-	-	-	229	-	-	-
# of invaded prey (half-entered counted as 0.5)	147	72.8	-	-	180	78.6	-	-
# of predator outside AND touching (half-entered counted as 0.5)	47.5	23.5	-	-	44.5	19.4	-	-
# of prey with **multiple** predators inside	13	6.4	-	-	19	8.3	-	-
Predation related cellular features in HADA-bright prey cells investigated								
# of bdelloplasts	158	78.2	-	-	201	87.8	-	-
# of bdellopasts with deformations toward the predator inside	129	63.9	-	-	176	76.9	-	-
Predation related sub-cellular features in HADA-bright prey cells investigated								
# of HADA Rings (from outside or half-entered)	51	25.2	235.84	28.40	17	7.4	243.43	38.54
# of HADA Discs (from outside)	28	13.9	213.42	31.81	20	8.7	188.50	47.52
# of HADA Disc Seals (from inside)	39	19.3	176.87	33.64	57	24.9	158.51	35.56
# of HADA Ring Seals (from inside)	7	3.5	228.57	55.98	7	3.1	234.00	33.72
Predation related miscellaneous features in HADA-bright prey cells investigated								
# of predator inside, but no clear discs/seal/ring	78	38.6	-	-	107	46.7	-	-
# discs/rings on prey OR bdelloplasts without any clearly labelled predator	17	8.4	-	-	21	9.2	-	-

Supplementary Table 2- Systematic analysis of 3D reconstructed cells from 3D-SIM (see **Figure 2, Supplementary Movie** and **Supplementary Figure 2** for examples of these). HADA Rings are defined as clearly resolved rings of HADA modification on the prey PG proximal to the point of contact with the predator on the outside or inside of the prey. HADA Discs are defined as regions of HADA at the point of predator prey contact that were not resolved into rings, likely due to early contact by the predator. Seals are defined as patches of HADA at the point of contact with an internalised predator, likely indicative of the re-sealing of the hole in the prey PG. These Seals were in the form of either 'Disc Seals'; filled discs of HADA (see **Supplementary Figure 2e- yellow arrowheads**) or 'Ring Seals'; rings of HADA of slightly smaller diameter than the initial entry pore (see **Supplementary Figure 2e- red arrowheads**).

	Instances	% of instances	Average long axis diameter (nm)	Std-dev	Instances	% of instances	Average long axis diameter (nm)	Std-dev
	15 minutes				30 minutes			
Number of interacting cells investigated	45				63			
# of HADA RINGS	37	82.2	205.24	35.29	56	88.8	210.98	54.86
# of co-incident HADA RING-TADA Pore	17	37.8	145.23	42.19	43	68.3	179.35	40.34
# of HADA Discs					7	11.1	254.27	55.07
# of co-incident HADA Disc-TADA Pore					4	6.3	243.0	40.75
# of HADA patches	8	17.8	231.63	52.20	2	3.2	259	43.84

Supplementary Table 3- Systematic analysis of 3D reconstructed cells from 3D-SIM (See **Figure 3**) with *E. coli imp4213*$^{-}$ permeable mutant strain. HADA Rings were clearly resolved rings of HADA modification on the prey PG proximal to the point of contact with the predator on the outside or the inside of the prey. In these experiments using this more permeable strain of prey, pores in the TADA were visible co-incident with the HADA Rings. Patches were regions of HADA at the point of predator prey contact that were not resolved into rings (Likely early contact by the predator at 15 minutes or nearly complete Disc at 30 minutes). Discs were patches of HADA at the point of contact with an internalised predator, likely the re-sealing of the hole in the prey PG and with this prey strain were seen co-incident with the patch of HADA.

Supplementary Reference

1 Lambert, C. *et al.* Ankyrin-mediated self-protection during cell invasion by the bacterial predator *Bdellovibrio bacteriovorus*. *Nature communications* **6**, 8884, doi:10.1038/ncomms9884 (2015).