Exoprotease activity in Osnat’s marine bacteria – 25/3/25 – finished summarizing 19/06/25

**Goal:** One of the referees for Osnat’s paper suggested we try to better connect the specific strains we used to the phenotypes of her models. One way of doing this is trying to see whether there is a correlation between exoenzymatic activity of the strains and the predicted activity in the model. To do this, we will focus on ,easuring exproteolytic activity, since this is a mechanism which could transform inaccessible “DON” to accessible “DIN” (actually amino acids).

I chose to use the following substrates:

1. **For Leu-aminopeptidase**: Leu-AMc (L-Leucine-7-amido-4-methylcoumarin hydrochloride, Merck L2145 – note written sometimes as “amino-4” and not amido). It has been used to look at Synechococcus exopeptidase activity (Martinez and Azam, AEM 1993), particle-associated activity (Smith et al, Nature 1992) and, more recently, in natural samples across latitudes (Balmonte et al, L&O 2021).
2. **For endo-acting chemotrypsins**: ALA-ALA-PHE 7-AMIDO-4-METHYLCOUMARIN. This has been used in the methods paper and the Balmonte et al paper.
3. **For endo-acting trypsin**: N-T-BOC-LEU-SER-THR-ARG 7-AMIDO-4. This is from the methods paper (the Balmonte et al paper uses different substrates).
4. **For the calibration curve**: 7 amino 4 methyl coumarine (AMC)

The information on these substrates, including how to dilute them into DMSO stock solutions, is found in the Excel file “Exoenzymatic activity template from Tinkara revised 250325”.

Osnat, Dikla and Waseem could not find Ruegeria pomeroyii, so this strain was not tested in this experiment.

\*\* Get growth data from Dikla \*\*

**Preparing stock solutions:** 

Stock solutions for each substrate should be added at a final concentration of 100uM, so 4uL/well

**Strains, growth and cell counts**: Growth and counting of the bacteria was performed by Dikla. The 8 bacterial strains were revived from frozen stocks into 5ml MB, allowed to revive for ~48 hours at 25 degrees C (with shaking 90 rpm). They were then transferred to 60 ml ProMM and grown for 48 hours at 25 degrees C (with shaking 90 rpm). Growth curves for these starters are found in the excel file – it seems that some (e.g. CIP, Nitzch, 1A3) are likely in stationary stage, whereas others may still be exponentially growing. Before the start of the experiment, the cultures were centrifuged at 7500 rpm/g for 5 minutes, the growth media was decanted, and the cells were resuspended in ~5 ml unamended sterile natural seawater. The cultures were counted using a Cytoflex (Beckman Coulter) flow cytometer after staining with Sybr Green. The results are found in the excel file “Cytoflex 250325”. Used these numbers to calculate the number of cells to take (excel file “Exoenzyme activity 250325:

**Experimental setup:**

Bacteria were first diluted 1:50 in seawater, and these were used to prepare the triplicate 5,000,000 cells/ml wells (columns 1-3), which were then serially diluted 10-fold (20uL into 200uL, row 1 into row 4 and row 7, etc). Experiment was performed in black 96-well plates (agilent 204626-100 cell plt 96 blk ps tc lid irr). These are tissue-culture treated (need to check what this means), and not low-binding.

Heat-killed cells were prepared by diluting 1:50, then preparing tubes with 5,000,000 cells/ml (~600uL), and these were heated to 96 degrees for ~20 min. There was not enough volume for the Phaeobacter and MED4 so I did only duplicates (hence cells F3, 6, 9 and H3, 6, 9 should be like the blanks).

Due to lack of time I decided to start on the first day only with one substrate – Leu-AMC, for leucine-aminopeptidase.

*Prepare cells - Dilute to 5X10^6 cells/ml, then serial dilutions*.

\*\* noticed day later that final volume was 200uL and not 220, meaning that the cell numbers are a bit lower and the substrate concentration a bit higher – incorporate into calculation \*\* added 190625 – the Excel file had corrected numbers (e.g. 220uL). Not sure if I set up the experiment with 200 or 220. In any case since we will probably use the second day results I will not deal with this at this stage\*\*



*Prepare heat-killed samples*:

\*\* Added more volume for experiment on day 2



Waseem added 4uL of the Leu-APA, incubated at room temperature for 1 and 2 hours. First hour was by mistake in the light. Measured also t=0, using Tal’s plate reader.

Results analysis:

1. Calibration curve is linear from about 20nM to about 20uM. Very similar slope after 1 and 2 hours.

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| Figure 1: Calibration curve after 2hours, showing the full curve, and the region to 20uM and 100nM | | |

1. The blank (with added substrate) is about 0.5uM, regardless of whether after one or two hours. Since the regression line is very similar this seems to mean that while there is a background, there is very little actual protease activity in the water.
2. Subtracted the blank from all values (starting Q28 in 1h and 2h tabs), then calculated the uM AMC substrate (starting Q39), then divided by hours (Q51, copied the 1h data to Q62 of the 2h tab). There was a clear signal above the noise only in the 5,000,000 cells/ml at both time points, for Alteromonas, Pseudoalteromonas, Roseovarius, Phaeobacter and MArinobacter. Sulfitobacter was closer to the LOD in the 1h incubation (one well had negative numbers) but the signal seemed stronger after 2h. Marinovum might be getting close to the LOD after 2h. MED4 had no signal at either time. The heat-killed negative controls had no activity
3. When both times were above LOD, the rates of proteolysis were similar after 1h and 2h, with the exception of Marinobacter, where the rates were higher after 2h (t-test results in Q 85, tab 2h).
4. There was a signal above LOD for Alteromonas also in 2/3 wells in the the 500,000 cells sample, but it was probably very close to the LOD, and was not scaled 1:10 compared to the 5,000,000 cells sample (order of magnitude lower).
5. Alteromonas had by far the highest aminopeptidase activity per cell – almost 3-fold higher than the next highest (Pseudoalteromonas and Phaeobacter). Subacter, Marinovum and MED4 were close to or below the LOD.
6. \*\*20/6/25 – re-checked the results as I was working on the 26/3/25 data and noticed that I made a 3-order-of-magnitude mistake. Dividing umole/L\*h (degradation rate) by cells which are in cells/ml means I need to change the umole to nmole. The results are then in fmole/cell\*h rather than pmole. This also makes sense in comparison with the literature, e.g. Martinez et al AME 1996 (in my papers as “enzymatic activity marine bacteria AME 1996”), where the values are between ~4-3000 amole substrate cell-1 h-1 (0.004-3 fmole substrate cell-1 h-1 – our cells range from 0-0.7 fmole cell-1 h-1).

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| Figure 2: aminopeptidase activity in cells several hours after transfer into seawater, units corrected on 20/6/25 |

As a reminder, these results are in cells incubated in SW within 2-3 hours of growing exponentially. Worth repeating tomorrow in starved cells?

**26/3/25: repeating exoprotease experiments with cells after 24h in seawater (nutrient starvation).**

1. Take cells from incubator in culture room. Measure OD600 if possible. Take samples for FCM (5uL Glut in 1mL cells).
2. Prepare 1:50 dilution in 15ml tubes (so there is sufficient volume for all experiments and heat-killed controls) – 100ul cells + 5ml seawater
3. Prepare two sets of heat-killed cells (5 reactions each – we need a total of 9, for the three substrates), this time with larger volumes:  
   
4. Prepare plates as yesterday, with updates map:



(written 220625, based on written notes):

1. MED4 was diluted only 1:25 in prep for FCM
2. Plate 1 was Leu-AMC, plate 2 was AAP-AMC, plate 3 was MT-BOC, plate 4 was standards (as was originally planned for experiment 1, the day before).
3. There was not enough substrtate for column 9 in the AAP plate. Indeed the FL from this column is at baseline levels.
4. BOC was at 30mM (not enough substrate – calculated anc changed on the fly?).
5. Heat-killed cells were cooled before loading on the plate to avoid condensate.
6. A different column is used for the blank of each substrate: column 3 for Leu-AMC, column 4 for AAP-AMC, column 5 for MT-BOC. This means that the blank needs to be adjusted for each plate

**Results summary** – written (unfortunately) several months later, on 19/6/25 and 22/6/25.

Overall experiment was performed as planned, with the three substrates. Measurements were taken at ~11:12 (t=0), 12:04 (t=1h), 13:30 (t=2.5h) and 18:06 (t=5h, by Waseem). Results are found in the Excel file “proteolysis 26032025”.

**FCM cell counts** – found in the file “10CC Osnat and DS” sent by Dikla by email on 6/4/25, copied to the tab “plate counts and setup” and compared with the measurements from the day before. All strains had higher cell counts on the second day, with 5 strains were <more or less> on the 1:1 line. There were three exceptions: MED4, HP15 and Roseo, which were much higher when measured on the second day. This could, in principle, be because the cells had continued to grow, but I am worried that this is (again) a problem with the cell counts, potentially the cultures not having been diluted sufficiently on the first day. Nevertheless, I will continue with the analyses with the new cell counts.

Since the experiment was performed with the old cell numbers (aiming for 5x10^6 cells/ml) I calculated from the corrected cell numbers what the actual numbers were in the first dilution (cell N3-N10 in the “Cell counts and setup” tab of the Excel file). The numbers ranged from ~5x10^6 to 6x10^7. Copied these to the “plate setup” map.

Checked the calibration curves (copied the same analysis from the 25/3/25 file) – all time-points were highly linear (R2>0.99 from 28nM to 20uM) with a very similar slope.

(220625) – copied the calibration curves and data from the first analyzed plate to all time-points. Stuff to notice: In plate 3, there was originally clearly activity in the Pseudoalteromonas that was above background (including after removal of the blank), but the calibration curve calculates a negative result. The problem was a mistake in assigning the correct blank.

There was clearly enzymatic activity observed already at t=0 in plates 1 and 3 – disregarding these data?

Results summary:

**Leucine aminopeptidase**: There was strong leucine aminopeptidase activity already at t=0, and relatively little difference over time – potentially a small decline in Pseudoalteromonas and increases in the strains with low activity – Sulfitobacter, Marinovum and Marinobacter. In Marinovum and Marinobacter the measurements were clearly above background only after almost 7 hours incubation. This suggests that ecto-acting aminopeptidase is the strongest activity, which is also supported by the actual values (up to 0.4 fmole substrate cell-1 h-1 – 10-fold higher than any activity except chemotrypsin on Alteromonas, which was ~3-fold lower in the ~7h incubation.

**Endo-acting (chemotrypsin) activity** was only observed after 2.3 hours, and then only in Alteromonas. It took almost 7 hours to observe activity in Pseudoalteromonoas and Phaboacter. There was positive fluorescence already at 2.3 hours in Alteromonas, Pseudoalteromonas and Phaeobacter, but this was translated to a negative concentration in all strains but Alteromonas. If the calibration curve was modified to use only up to 6uM, the R2 was higher and the intercept lower the graphs were closer to the observed – e.g. now Pseudoalteromonas and Phaobacter had measurable activity at t=2.3, so I used the modified curve for all plots. Notably, the activity increase in all strains also between 2.3 to 6.8h, although not other strain increased above background noise. It seems that this activity is low but potentially still there in other organisms.

**Endo-actiing (Trypsin) activity** was only observed after at least 2.3 hours, but increased only slightly there.

**Overall summary**: I chose to use the 2.3h data, since ~7 could be considered too long, with potential mechanisms such as cell lysis coming into play. The only major difference if using the 7h data would be a higher chemotrypsin activity in Alteromonas.

**The “strong” phenotype organisms, Alteromonas and Pseudoalteromonas, had the highest per-cell activity in the Leucine aminopeptidase assay, followed by Phaeobacter (inhibitory).** All other strains had much lower (5-10 fold) activity. This was consistent with the experiment performed on the previous day, although here the activity of some other strains was also high (Roseovarius, Marinobacter). This could be due to problems in the cell counting or to a change between cells right after transfer to seawater and those starved for 24h (activity, viability). **A similar pattern was observed in the endo-acting (chemotrypsin) activity, where these three strains were the only ones above LOD**. **Alteromonas and Pseudoalteromonas also had measurable Endo-acting (Trypsin) activity, although here Marinovum (weak) had a higher activity**. Sulfitobacter, Roseovarius, Marinobacter and Prochlorococcus MED4 had low or unmeasurable activity in all proteolytic assays under these conditions. **Therefore, overall, there seemed to be an agreement between proteolytic activity and co-culture phenotype**.

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| Figure 3 – proteolytic activity after 24h. Different color bars show different incubation times. The upper-right panel is Figure 2 (experiment performed previous day), for comparison. |