TOO HIGH - TOO LOW PHOSPHATE

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Abstract: Phosphate is very limited in the natural environment and bacteria have developed several adaptation techniques in order to respond in slight fluctuations of nutrients. Usually secondary metabolites are produced in order to win the competition for limited nutrient acquisition. Phenazines is one of these, and give a very distinctive pigmented phenotype in Pseudomonas strain cultures. In this project, different phosphate concentrations were tested in *Pseudomonas chlororaphis* (*aureofaciens*) cultures. The main findings support that the cells are able to change their physiology, like growth, shape and stoichiometry according to the phosphate concentrations. Also the phenazine production is possibly regulated, with evidences from spectra absorbance that there is a negative correlation between phosphate and spectra absorbance.

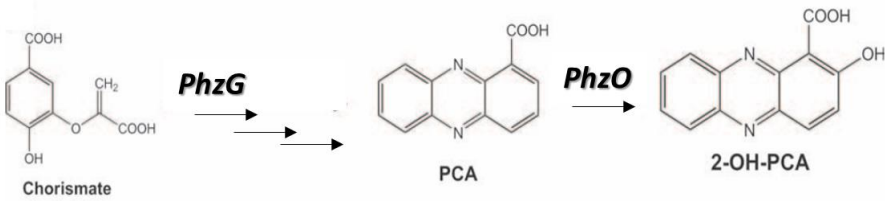
Introduction

In nature, phosphate is one of the most important growth limiting factor for organisms. But phosphate is essential for all living organisms, participating in critical biochemical processes, being a crucial component of the energy dynamics of cells and being a component of nucleic acids, phospholipids in membranes, and other biomolecules (Bains et al., 2012). Bacteria are able to monitor and respond to fluctuations in their microenvironment, adapting their metabolic and physiological potential to better suit their immediate requirements.

According to Chrzanowski et al. 1996, C:N:P ratio of bacteria was found to vary between 52:8:1 and 163:25:1 when N was abundant relative to P (N:P > 40:1). Inorganic phosphate (Pi) is the preferred source of phosphate for bacteria, and thus, adaptation to fluctuations in its extracellular concentration is an important biological trait. It has been found that the Pho regulon have roles in Pi transport and assimilation that facilitate adaptation to limiting Pi environments. Alkaline phosphatase (APase) is one of the most important enzymes, since liberates inorganic phosphate from a range of organic molecules and it is a well-conserved between different bacterial clades. The canonical Pho system links the detection of limiting Pi environments to regulatory mechanisms directing the adjustment of gene expression to specifically facilitate growth/survival in limited phsophate environment. The PhoX and PhoD are described as Pho-regulated phosphatases. PhoX is determined to be the major enzyme responsible for the phosphatase activity in both quantitative and qualitative assays for Pseudomonas species (Monds et al., 2006).

In order to deal with nutrient limitation conditions, bacteria are able to produce secondary metabolites for increasing the nutrient acquisition and winning competition battles. One well studied secondary metabolite is Phenazine (PCA), produced by a variety of bacteria, with the most known to be Pseuodmonas species. These functional group is largely responsible for differences in the physical and chemical properties of the individual phenazines and hence, their biological activity (Mavrodi et al. 2006). Phenazines merely serving as antibiotics, but also play important physiological roles, including one in nutrient acquisition and quorum sensing (Maddula et al., 2008). As Wand. Y and Newman. D (2008) show the different reduced forms of phenazines can have different reactivity for iron acquisition. Phenazines behave actually like electron shuttles, they are redox-active small molecules that are reduced within the bacterial cell and are oxidized outside the cell by terminal electron acceptors such as Fe(III) minerals (Wand. Y and Newman. D, 2008, Hernadez et al., 2004). Natural phenazines are produced almost exclusively by eubacteria; they often are excreted to very high levels (milligrams to grams per liter) during bacterial growth in vitro, and they typically are pigmented. This distinctive phenotype of Pseudomonas cultures, characterised phenazines as extracellular pigments. Phenazines are mainly produced during the transition from exponential to stationary growth phase, where the colour also starts to appear in the culture. Their absorption spectra that include two distinct maxima in the UV range and at least one in the visible range that varies according to the nature and position of substituents on the heterocyclic ring (Mavrodi et., 2006).

Chorismic acid was identified as the branchpoint to phenazine synthesis, with the phenazine nucleus formed by the symmetrical condensation of two chorismate molecules and the amide nitrogen of glutamine serving as the immediate source of nitrogen in the heterocyclic nucleus (Mavrodi et al., 2006). Seven genes have been found sufficient for synthesis of PCA from chorismate molecules, phzABCDEFG (Mavrodi et., 1998, 2006). Then, *phzO* can convert a monooxygenase, which is responsible for the primary yellow PZ derivative phenazine-1-carboxylic acid (PCA) into the orange 2-hydroxy- PCA (2-OH-PCA). This enzymatic reaction results in the partial conversion of PCA to 2-OH-PCA. Thus, *P*. *chlororaphis* produces primarily the yellow PCA (80 to 90%) and small amounts of the orange 2-OH-PCA (10 to 20%). Interestingly it is proposed by that alterations in the ratios of antibiotic secondary metabolites synthesized by an organism may have complex and wide-ranging effects on its biology (Maddula et al., 2008).



The question that was raised for the present mini-project was how a limiting environmental nutrient compound, like phosphate, could affect the physiology *Pseudomonas chlororaphis* (*aureofaciens*), a well-studied species with a very distinctive phenotype of phenazines.

Material and Methods

For this project the strain of *Pseudomonas chlororaphis* (*aureofaciens*) was used, culturing it in different phosphate concentrations. Initially the culture grew in LB media and then inoculated in the

media that is described in Table 1, according to Monds et al. 2006. The K2HPO4 was excluded from the autoclaved media, but after filter sterilize, it was added according to the targeting treatments.

Table 1: Media recipe according to Monds et al. 2006, used for Pseudomonas chlororaphis cultures in order to test different phosphate concentrations. The concentration in the parenthesis describe the final concentration in the media.

MOPS 50ml/L (0.5M)
20% Tryptone 10ml/L (10mM)
20% Glycerol 7.5ml/L
MgSO4 0.6ml/L (0.6mM)
Trace metals 1ml/L
+ K2HPO4 1ml/L (1mM) or +K2HPO4 100ml/L (0.1M)

The treatments that were tested in this project were four and all in triplicates

1. 0.1M
2. 1mM (optimal condition)
3. 0
4. 0 – old = Prolonged culture in Phosphate starvation

For the three first conditions the culture grew overnight in the described media with 1mM phosphate, in the fourth condition the culture was inoculated four times in media with 0 phosphate, hence it is described as prolonged phosphate starvation condition. All these cultures were pelleted, in order to reduce any possible addition of phosphate from the overnight growing culture. The experiment took place in flasks with 100ml media where 1ml culture was added (1:100 dillution) targeting to reach 10^6 cells per ml as initial point, in order to observe a full growth curve. The different techniques that were mainly used in this experiment are described in Table 2.

Table 2: Methods that were used during the growth curve characterisation in different time points during 24 hours. In parenthesis is described the amount of material that was used in each method.

	0hours	4hours	8hours	12hours	24hours
OD 550nm (2ml culture)	√	√	√	√	√
Microscopy	√	√	√		
Microscopy (live/dead staining)		√	√		
Spectrophotometer (1ml supernatant)	√	√	√	√	√
Ion chromatography (5ml supernatant)	√		√	√	√
qPCR (1ml pellet)		√	√	√	√

In the case of OD measurements cuvettes were used in spectrophotometer with absorbance in 550nm spectra. In the 24hour sampling the cultures were diluted 1:10.

For the microscopic analysis, 5ul was placed in a slide and observed under fluorescence microscope, where at least three pictures were taken per sample in phase-contrast. For the live-dead staining, the kit “L7012 LIVE/DEAD® BacLight Bacterial Viability Kit *for microscopy and quantitative assays” from

Invitrogen was used. 3ul of 1:1 mix (SYTO 9 dye : Propidium iodide) per 1ml sample was added and incubated in the dark for 15min. Then in the same away as for non-stained sample, the samples were observed under fluorescence microscope, but images were taken with GFP (live cells-green) and T-RED (dead cells-red) filters. The phase contrast images were analysed in image processing package FIJI in ImageJ. From the summary of measurements that were done, three characteristics were analysed further, average size, length, width and circularity of cells per image.

For the spectrophotometer measurements and the ion chromatography supernatant of the cultures were used after filteration through 0.2um filters, to ensure the exclusion of cells. In the spectrophotometer measurements, cuvettes were used with low light intensity lamp donor. The DARWin SP spftware was used for data acquisition of the absorbance of spectra.

For the Ion chromatography, the supernatant was diluted in different range according to the sample and then the dilution factor was multiplied to the raw data. Especially for the 0.1M phosphate treatment the supernatant was diluted 1:10 times. The quantification of the results were done by running standards in the beginning of the runs.

For the qPCR analysis five primer pairs were used for all the culture. The pellets were frozen in -80^{o}C very fast after sampling the cultures and pelleting. After unfreezing, RNA extraction from the pellet was done by Maxwell® RSC simplyRNA Cells Kit and Maxwell® RSC simplyRNA Tissue Kit. RNA was quantify by nanodrop, these measurements were used to estimate the amount of template that was ideal for each sample in the qPCR reaction, in order to not overload sample in a very sensitive equipment. The template for the qPCR reaction is a cDNA template that was obtained by combining the extracted RNA, random primers and GoScript Reaction Mix in a PCR reaction. Then this cDNA template is combined with the targeted primers for specific genes and GoTaq qPCR master mix under standard cycling conditions in qPCR machine. The protocol and the kits that were used were GoTaq® 2-Step RT-qPCR System. For this analysis, standard was one house keeping gene RpoD (RNA polymerase sigma factor) and the other four primer pairs were targeted genes of interest for this project, like PhoD, PhoX, PhzG, PhzO. Also a negative control was made by using as qPCR template, RNA just extracted without the cDNA. The low DNA amplification and the single CT values, suggested reliable data acquisition. The specific CT value of RpoD for the each sample was used to normalise the CT value of each sample that was obtained with the different primers. Then these numbers were calculated to log2 scale and for each primer the numbers were standardised to 1mM condition, which was considered as the optimal condition in this experiment. The target of this was to make a comparison among the samples, in order to understand up- and down- gene-regulations compare to the optimal condition.

Before the experimental set up, cultures were tested, hence to be familiarised with the techniques that were been used during the growth curve characterisation. Also the Environmental scanning electron microscope (E-SEM) was used before the experiment started. For this method, initially 0.2um filters, fixed liquid cultures (1.5% formaldehyde) or colonies where placed on stub with carbon tape. All of them were failed, possibly the cells were much more fragile than we imagined, and could not survive the drying vacuum. The next trial was made with sputter coating the cells with platinum. After fixing the cells for 2hours with 1.5% formaldehyde, they were filtered in Anodisc 13:0.2um filters and washed with MilliQ water twice. The filters were emerged in 30%, 50%, 70% and 100% ethanol for 20min, in order to dry any possible water. Extra drying process was done for these samples by Lilly microscope lab, before coat them with platinum. The target of using E-SEM was to observe endo-

cellular poly-phosphate formations, by scanning the cells for phosphorus, the coating method limits significantly this approach.

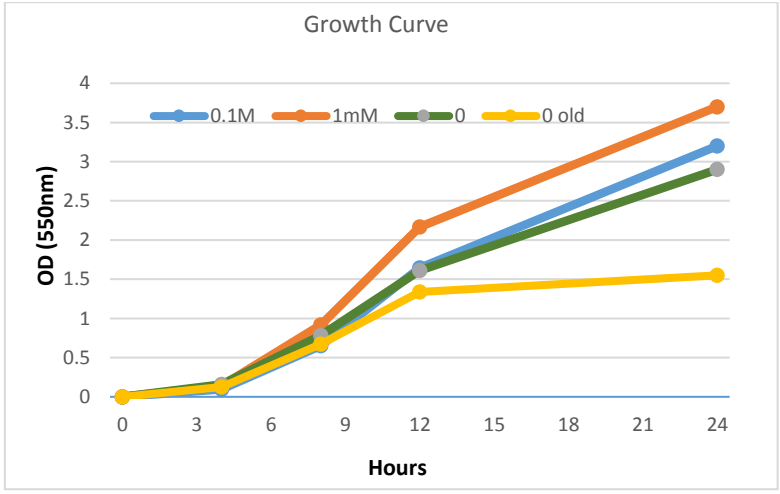
Also before the experiment started, the cultures were streaked on 5-Bromo-4-chloro-3-indolyl phosphate (BCIP), which is an artificial chromogenic substrate that is used to detect alkaline phosphatase activity. The cultures were tested in contrast to a Pseudomonas *fluorescens* and the mutant Delta*phoB*, which were obtained by Monds et al. 2006. The BCIP was diluted in dimethyl formamide (40mg/ml) and stored in glass flask without light, then was used in the media with final concentration 40ug/ml.

In the end, one culture of *Pseudomonas chlororaphis* (*aureofaciens*) was used as a potential phenazine mutant and it was obtained by transposon mutagenesis during the 2nd week of the course. The white colour of the culture was a strong evidence to support the idea of phenazine mutation, so it was tested in spectra absorbance.

All the above described methods and material were used in order to observe physiological differences of *Pseudomonas chlororaphis* (*aureofaciens*) under different phosphate treatments.

Results-Discussion

The main experiment took place during 24hours by following the growth curve of *Pseudomonas chlororaphis* (*aureofaciens*), which were treated in four different phosphate concentrations.



Graph 1: Growth curve of Pseudomonas chlororaphis under four different treatments (described in Material and Methods) for 24hours, OD absorbance 550nm. The absorbance is presented as average of the triplicates.

The growth curve proposes that the optimal condition of 1mM take over from 8hours until the end. The second highest is the too high phosphate treatment if 0.1M and the lowest is the prolonged starved culture 0-old. Suggesting that the very high phosphate does not necessarily increase the growth and that the long starvation can make a different condition compare to just not adding any phosphate in the media.

The cultures were observed under microscope in order to identify cell size or shape differences among the treatments or the growth phases.

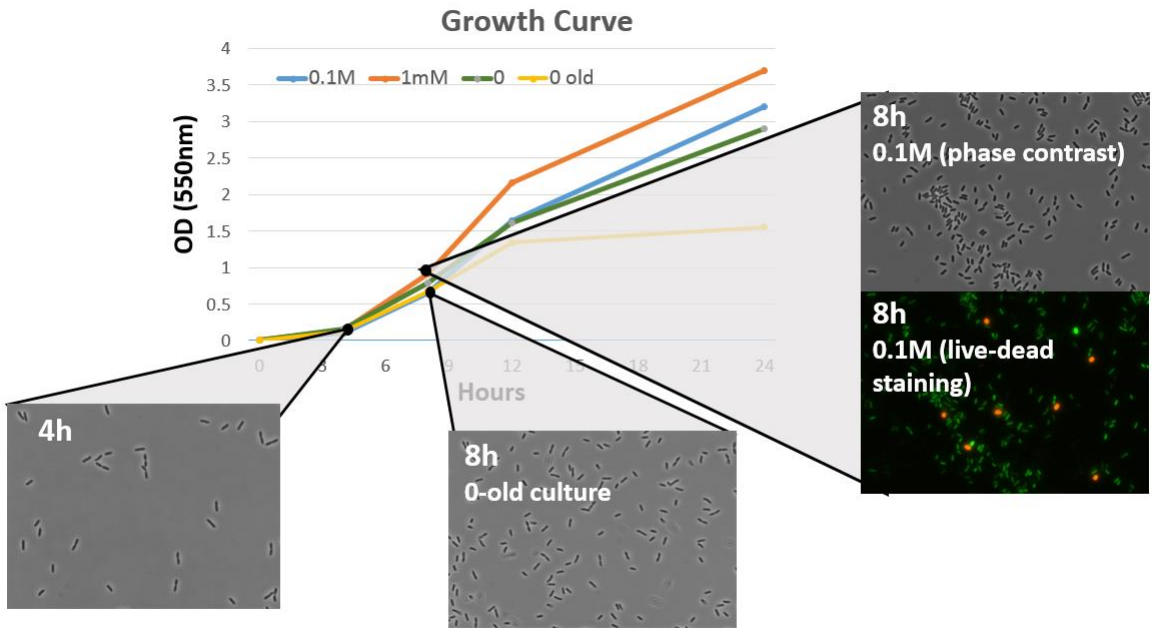
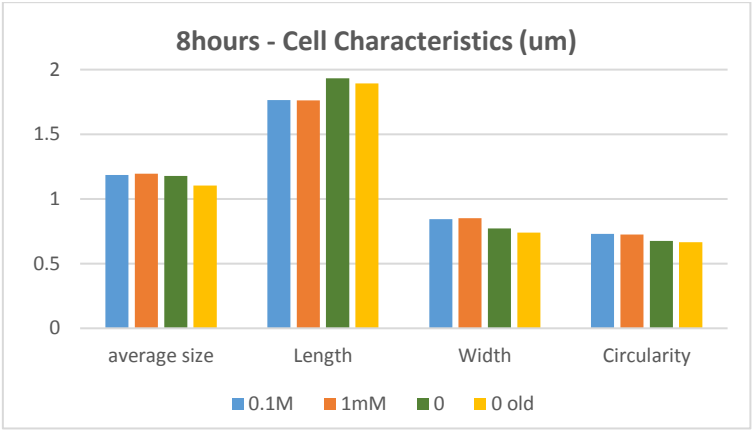


Figure 2: Growth curve of Pseudomonas chlororaphis under four different treatments (described in Material and Methods) for 24hours, OD absorbance 550nm, with microscope images from two sampling points (4h and 8h) in phase contrast and one with live-dead staining.

The low number of cells and the high percentage of doubling cells were very obvious in the beginning and the middle of the exponential phase. The live-dead staining helped to confirm the low numbers of dead cells in the exponential phase. No images were taken in the stationary phase, which could confirm a difference between exponential and stationary phase.

The microscopic images were mainly used in order to analyse cell size and shape differences among the different treatments in the exponential phase (8hours) culture, which were the latest images that were taken.



Graph 2: Microscopy image analysis with Fiji for average size, length, width and circularity of the cells per treatment (described in Material and Methods) in micrometres (um) for 8 hours cultures. The numbers are presented as average of the triplicates.

Graph 2 shows that the length is increase under starving phosphate conditions, like 0 and 0-old, and the width and circularity decrease. Proposing that the cells under limited phosphate can be more elongated than high or normal phosphate conditions. This could suggest a cellular strategy in order to increase diffusion ability (Nikaido et al. 1985) or could be lipid regulation strategy (Bains et al., 2012).

The above shape ideas were confirmed by images that were taken with E-SEM microscopy in x30000 magnification (Fig.2).

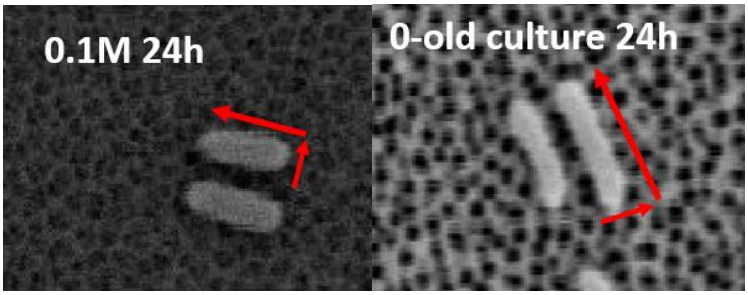


Figure 2: E-SEM images of Pseudomonas chlororaphis, taken under x30.000 magnification after fixation and sputter platinum coating cells. Red arrows added to emphasize the differences in length. First image presents 0.1M phosphate treatment and second 0-old phosphate starved treatment. Both cultures was inoculated in new media 24hours before they were fixed and coated.

In terms of the obvious phenotype of *Pseudomonas chlororaphis*, the phenazine production that gives a yellow-orange colour to the cultures. Differences in colour were very obvious (Fig.3), especially in 0.1M phosphate compare to the optimal 1mM phosphate treatment and a potential mutant for phenazines.

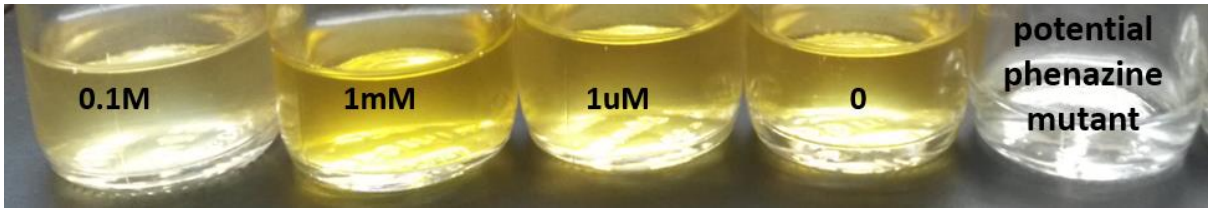


Figure 3: 20ml supernatant of 24hours cultures of Pseudomonas chlororaphis, growing in gradients of phosphate (0.1M, 1mM, 1um, and 0; without any phosphate addition) and one potential phenazine mutant.

These observations were in agreement with spectrophotometer absorbance measurements, as Figure 4 shows bellow.

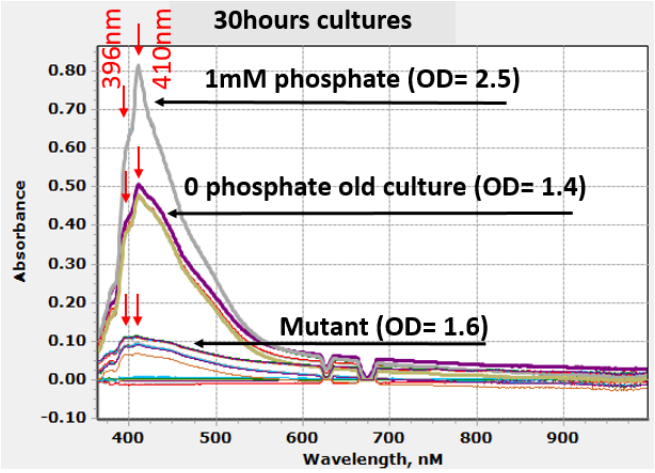


Figure 4: Spectra absorbance of supernatant from 30hours cultures of Pseudomonas chlororaphis, growing in phosphate treatments and one potential phenazine mutant. The grey line represents the optimal phosphate treatment of 1mM; with 2.5OD growth. The yellow and purple lines represents the prolonged phosphate starved culture; with 1.4OD growth and the lowest three lines represent the potential phenazine mutant; with 1.6OD growth. The red arrows added to emphasise the obvious high peak in 410nm and a shoulder in 396nm.

Figure 4 with the 410nm spectra absorbance, confirms the obvious differences in colours especially between 1mM and the potential mutant. The growth of the culture could definitely affect the colour (Mavrodi et., 2006). In these terms the difference between the growths of 2.5OD for the 1mM treatment and the 1.4OD for 0 treatment, excuse their difference in the absorbance peak. Suggesting that there is not necessarily difference in colour production. On the other hand, there is a colour and absorbance difference between the wild types and the potential phenazine mutant.

In the above presented peaks of Fig. 4 there is a shoulder in 398nm, something that was also observed during the growth curve spectra measurements (Fig. 5).

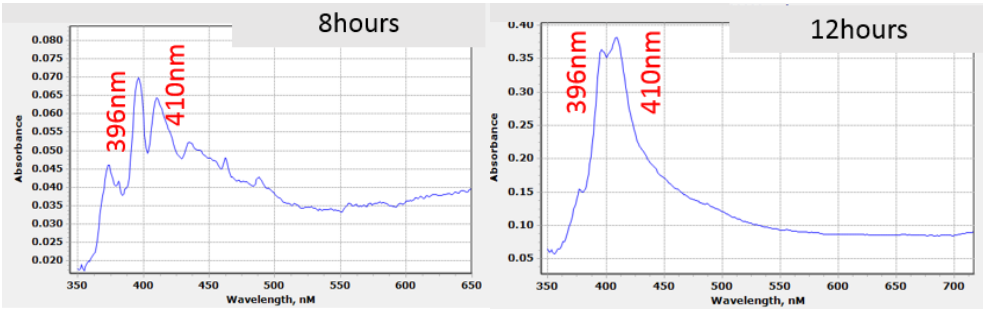
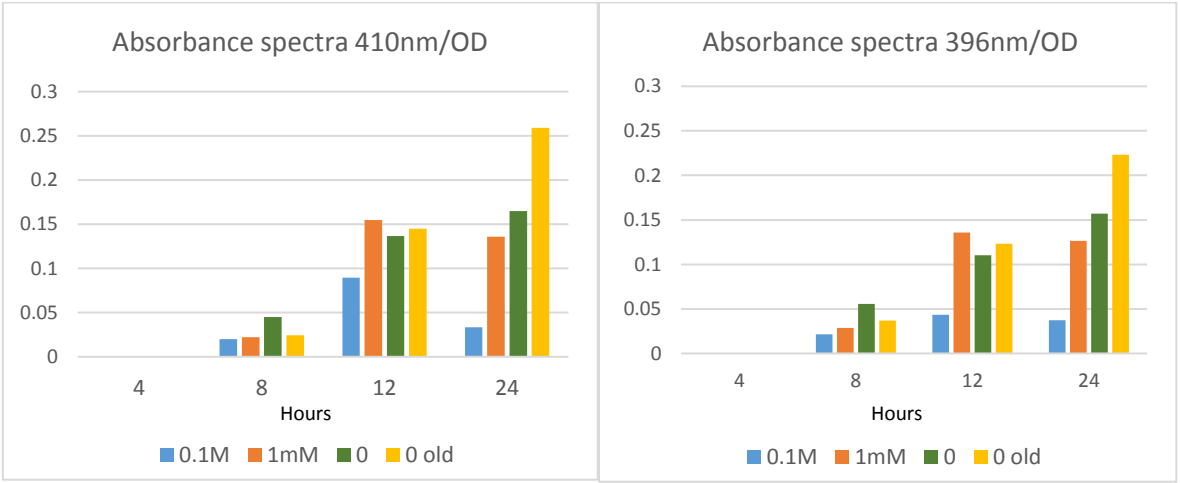


Figure 5: Spectra absorbance in two peaks; 396nm and 410nm of Pseudomonas chlororaphis culture supernatant, growing in 0 phosphate treatment. Measurement taken in 8 hours and 12 hours growing cultures. The scales of the graphs are not aligned, not comparison should be made.

Figure 5 was the first observation for spectra absorbance in two peaks, one in 396nm and one in 410nm. The 396nm spectra absorbance was higher than the 410nm in all the treatments the beginning of the exponential phase of the growing cultures, like 8 hours measurements. The 410nm took over in all the treatments during late exponential phase, like 12 hours. Proposing a possible shift of colour or metabolite production, like phenazines.

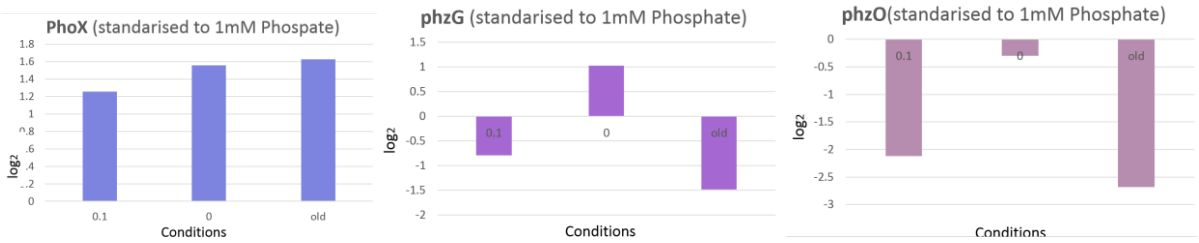


Graph 3: Spectra absorbance of Pseudomonas chlororaphis cultures supernatant under four different treatments (described in Material and Methods) divided to OD measurements during 24hours experiment. Left side: absorbance spectra 410nm. Right side: absorbance spectra 396nm. The measurements are presented as average of the triplicates.

Graph 12 shows that the colour of the culture start increasing after 12 hours, which is the late exponential phase and is high in late stationary phase. Something that agrees with previous observations for high phenazine production during stationary phase (Maddula et al., 2008), but also possibly production of more metabolites. As it has been mentioned before the colour should be

correlated with the growth of the culture. For this reason the absorbance of spectra divided to the OD measurements, correlate the growth and the colour production. 0-old phosphate starved culture reached quite high spectra absorbance compare to the 1mM treatment and the lowest absorbance was for the very high phosphate (0.1M). These are evidences for coloured metabolites that can be used for nutrient acquisition or as competition agents, like phenazines (Wand. Y and Newman. D, 2008), since they are not produced during high phosphate.

In order to understand better the phenazine production under the tested treatments, RNA qPCR was used targeting two phenazine genes (*PhzG- PhzO*) and two phosphate regulation genes (*PhoD-PhoX*). The *PhoD* primer had more than two CT values, so it was excluded for the analysis. The graphs bellow are normalised to a house keeping gene (*RpoD*) and then standardised to the optimal condition of 1mM.



Graph 4: Normalised CT values of RNA qPCR standardised to the optimal condition of 1mM treatment for each of the three types of primer pairs (PhoX, phzG, phzO) in a log2 scale for each treatment during the 12th hour of culture. The measurements are presented as average of the triplicates.

In the above graphs we can actually see upregulation of the PhoX genes and downregulation of the phenazine genes, except of 0 treatment for phzG, compare to the 1mM treatment. The graphs are not comparable to each other since the standardisation was according to the 1mM treatment for each primer pair. The highest upregulation was for the 0-old starved treatment, proposing that the culture respond positively to the starvation conditions. In terms of phenazine genes, the phzG genes are reaching differences up to log2(2) which is lower than the phzO. Proposing that the PCA form is more preferable than 2-OH-PCA, and especially in the 0 treatment where the phzG is even upregulated compare to the 1mM treatment. In high phosphate treatment (0.1M) the cells possibly do not need to produce a lot of phenazines, so the downregulation of phenazine genes are in agreement to the results from Fig. and Graph 3. The similar pattern of downregulation of phenazine genes for the high phosphate treatment (0.1M) and the 0-old phosphate starved culture, could propose a similar RNA pattern, even though the present results do not give any information about post-transcriptional regulation. A potential explanation could be that in too high and too low phosphate treatment, the cells behave similarly, since in the one they do not need to produce and in the other they cannot produce. That brings the assumption of recirculation of phenazines (Hernadez et al., 2004) for both cases.

In the end, some elemental analysis confirms that the above observations are correct, because the cultures they did have balanced stoichiometric C:N:P ratio. Firstly, the analysis in the E-SEMicroscope is presented in the figures bellow.

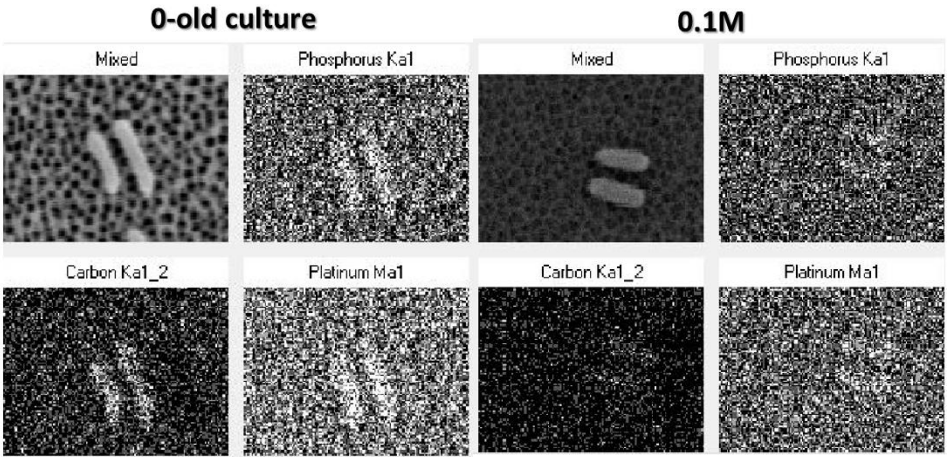
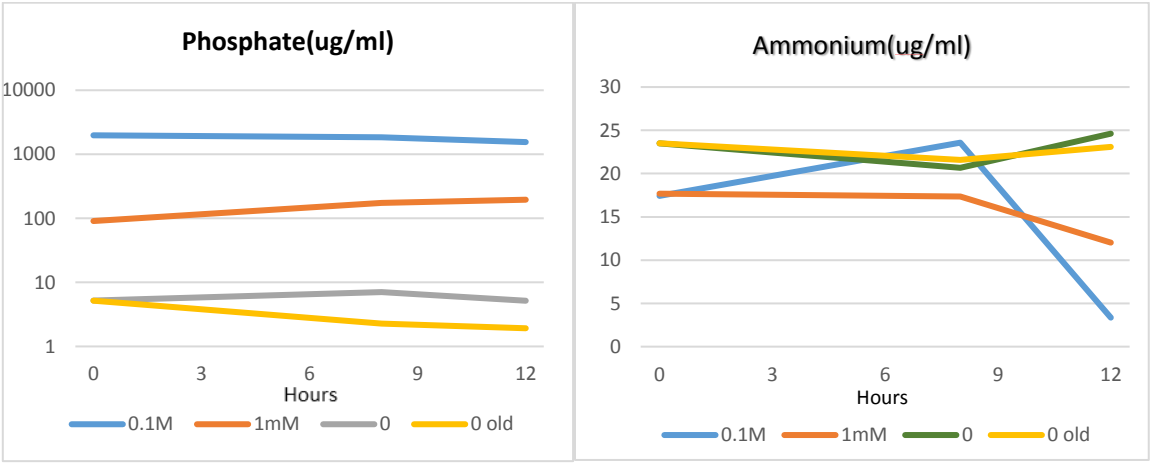


Figure 6: E-SEM images of Pseudomonas chlororaphis in 0-old phosphate starved culture (left side) and 0.1M treatment (right side) under 30.000 magnification with 600-300 frames scanning for Carbon, Phosphorous and Platinum atoms. The samples were sputter coated with platinum.

The E-SEM analysis for scanning for platinum and phosphorus was not very successful, since the ionisation energy of these two elements are very close. Even though it is expected that the platinum is evenly distributed in all the sample surface, so the accumulation in the cell biomass can be cellular phosphorus in addition to the sputtering coat levels. This is a clear suggestion for not using platinum sputter coating, but carbon if phosphorus analysis will need to be done. With the present images, the carbon scanning suggest a difference between the two different treatments that were analysed. The carbon content depicted accumulated in the 0-old phosphate starved cultures compare to the 0.1M treatment. This could be a sign that the carbon can be stored intracellular when there is nutrient limitation (Muhr et al., 2013; Huisman et al., 1991), like in this case phosphate. Oppositely, the cells does possibly uses all the carbon and not store during high phosphate conditions, like 0.1M.

Then in order to measure the N:P ratio, measurements of the treated cultures supernatant in ion chromatography were taken during the growth curve, as graphs bellow present.



Graph 5: Ion chromatography analysis of Pseudomonas chlororaphis cultures supernatant under four treatments (described in Material and Methods) during 24 hours experiment. Left side: Phosphate. Right side: Ammonium in ug/ml. The measurements are presented as average of the triplicates.

The Graph 5, shows that the phosphate started in different levels than the expected ones. In terms of the 0.1M and 1M treatments, the measurements propose lower amounts. On the other hand for the 0 treatments, phosphate were found in the media, even in media without inoculation, proposing that

it is very difficult to completely limit phosphate. During the experiment, the cultures slightly decreased the phosphate, except of the case of 1mM.

Then in the case of ammonium, all the cultures started from almost the same point, but 12 hours after there is an obvious pattern. For the 0 phosphate treatments, the ammonium was slightly increasing in the supernatant, suggesting that the cells probably liberate more extracellularly in order to low the levels intracellular and balance it with the limited phosphate. In contrast to that, in the 1mM and 0.1M treatments the ammonium was decreased in the supernatant, hence maybe it was used in a non toxic form intracellularly in order to reach the necessary levels for balancing the stoichiometry of C:N:P ratio.

All the above results support that there is a clear effect of phosphate levels on the cellular biology of *Pseudomonas chlororaphis,* but more research need to be done. In future terms, it will be appropriate to make statistical analysis on the present results, something that was not accomplished in the project due to time limitations. In this way, the evidences that are reported here, could have been presented as facts and new experiments could be based on them. If the experiments could be repeated, more accurate phenazine measurements would be used, qPCR could be done in at least two time points and microscope analysis in late stationary phase would be necessary to confirm the cellular shape differentiation between the treatments.

The main conclusions of this project are:

- ✓ Prolonged phosphate starvation gave different condition compare to a condition where no phosphate was added in a new inoculum.
- ✓ Too high phosphate did not increase the growth in higher levels than the optimal 1mM concentration, at least in the presently tested media.
- ✓ Too low phosphate possibly elongated the cell shape, which increasing their diffusion potential.
- ✓ Too high phosphate reduced the spectra absorbance, proposing that the secondary metabolites that are giving the colour to the culture were reduced. This can explain their decrease of the need to produce these metabolites that usually are used in nutrient acquisition and as competition agents, like phenazines.
- ✓ There was a balanced C:N:P ratio, with evidences for intracellular storage of Carbon and extracellular increase of Ammonium during limited Phosphate treatments.
- ✓ The PhzG gene was less downregulated than the PhzO compare to the optimal condition of 1mM treatment.
- ✓ Too high and too low phosphate could have similar pattern of phenazine gene expression, since the first does not need to produce and the other cannot produce. This could suggeste that phenazines can be recycled in both treatments, maybe in different efficiency.

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Appendix:

Q-pcr primers:

>RpoD size (bps): fragment 172 / gene 1848

Forward 1225 position

Reverse 1396 position

1. **RpoD-F**

GAAGGCAACATCGGTTTGAT

2. **RpoD-R**

TGAGCTTGTTGATCGTCTCG

>phzG size (bps): fragment 152/ gene 669

Forward 218 position

Reverse 369 position

3. **phzG-F**

GCATCGTGGTGATCAGTGAG

4. **phzG-R**

CTGGCCATTGAGGATGATCT

>phzO size(bps): fragment 150/ gene 1476

Forward 90 position

Reverse 239 position

5. **phzO-F**

AGGCCAAGTTGTTGAGGATG

6. **phzO-R**

TAGGCAGAAGAGGCCAGGTA

>phoX size(bps): fragment 224/ gene 1902

Forward 27 position

Reverse 250 position

7. **phoX-F**

TACCGACCTCGAAAAGATGG

8. **phoX-R**

TGATCAGCACCGAAGACTTG

>phoD size(bps): fragment 159/ gene 1542

Forward 777 position

Reverse 935 position

9. **phoD-F**

TGAGCGCTACACCAACAAAG

10. **phoD-R**