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Characterization of Dps proteins from *Nostoc punctiforme* in *Escherichia coli*

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Introduction

Cyanobacteria

Cyanobacteria are together with higher plants and microalgae the only organisms capable of carrying out oxygenic photosynthesis. According to the endosymbiotic theory the chloroplasts in photosynthetic organisms evolved from cyanobacteria. Cyanobacteria are a diverse group of gram-negative bacteria. They are a widely spread group with most species living in marine or fresh water habitats, but they are also found in terrestrial environments and in symbiosis with many different plants and fungi. Cyanobacteria can be either unicellular or filamentous and show a broad spectrum of sizes and morphologies. Among the filamentous species there is some degree of cellular differentiation. When resources are scarce and the vegetative cells cannot proliferate normally, some of the cells in the filament can answer to the stress by differentiating into other cell-types better adapted to the specific stress. The possible cell-types are the spore-like akinetes, the motile hormogonia and the nitrogen-fixing heterocysts. This is highlighted more below [2, 4, 5, 6, 8].

Nostoc punctiforme

The *Nostoc punctiforme (N. punctiforme)* is a filamentous cyanobacterium that lives as vegetative cells when resources are not scarce. However, when the vegetative cells cannot proliferate normally, they can answer to the stress by differentiating into other cell-types. The cell-types existing in Nostoc are the spore-like akinetes, the motile filaments called hormogonia and the nitrogen-fixing heterocysts. Phosphate limitation induces differentiation into akinetes. Akinetes are spore-like and more resistant to cold and desiccation. It is a temporary state and can re-differentiate back into vegetative cells. The differentiation of vegetative cells into hormogonia is not caused by one single factor. Instead there are many growth limiting environmental factors, such as depleted levels of nutrients and low light conditions that are leading to this transient cell-type. The stress signal leading to heterocyst formation is lack of ionic nitrogen compounds. About one of every 15 vegetative cells along linear filaments is differentiated into this irreversible cell-type (see figure 1). But the heterocyst content can be as high as 50 percent when growing in symbiosis with roots of plants. Heterocysts possess nitrogenases which are able to fix atmospheric nitrogen, which is subsequently reduced into soluble NH_4^+. Nitrogenases are very oxygen-sensitive, so the heterocysts have mechanisms of avoiding oxygen. The envelope has three extra cell wall layers, which reduce the diffusion of oxygen and other gases. The respiratory system is very active, and by being membrane bound, it can consume entering oxygen before it reaches the nitrogenase and other oxygen-sensitive enzymes in the cytoplasm. Further, the water-splitting oxygen-producing photosystem II is down-regulated. As a consequence the heterocysts, with only a functioning photosystem I, are not able to fix CO_2.

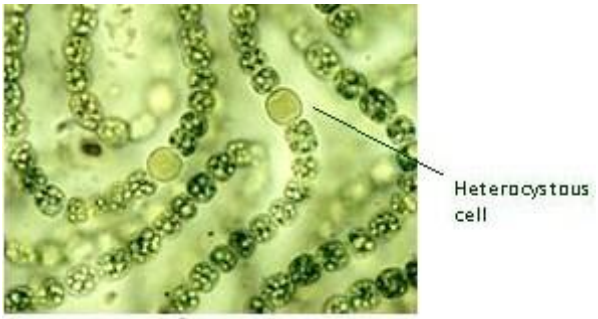

Figure 1. Filaments of vegetative cells with some cells differentiated into heterocysts. Modified from [17].

The vegetative and heterocystous cells are mutually interdependent. The vegetative cells are in need of a source of nitrogen and the heterocysts are in need of fixed carbon and reductants. This exchange is accomplished by intermembrane channels between the vegetative and heterogenous cells. Nitrogen is transported as glutamine to the vegetative cells and carbon compounds and reductants are transferred from the vegetative cells to the heterocysts (see figure 2).

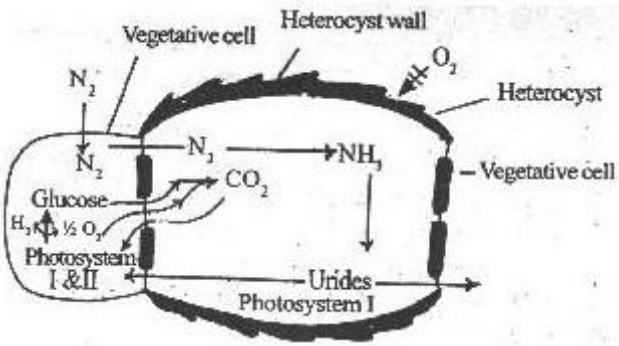

Figure 2. A vegetative and heterocystous cell. Their traded metabolites and main functional differences are highlighted. Modified from [16].

There is an increasing interest in cyanobacteria as potential biofuel producers. As photosynthetic organisms they only require solar energy, CO_2 and H_2O for growth. Unlike the current methods for biofuel production, using cyanobacteria would not compete with food production. Also, the overall energetic efficiency would be enormous compared to the existing technologies where heterotrophs are used to ferment small fractions of high-energy biomass.

The enzymes in fermenting pathways are extremely oxygen-sensitive. For them to function in the oxygen-producing photosynthetic organisms, the processes must be separated. *N. punctiforme* and other heterocystous cyanobacteria are interesting in that way, providing the anaerobic environment in the heterocysts while photosynthesis still can continue in the connected vegetative cells [2, 3, 4, 5, 6, 7, 8, 15].

Dps proteins

DNA-binding proteins from starved cells (Dps) are widely distributed among bacteria and are of major importance in protection of the DNA against oxidative damage and other stresses. The protein got its name in 1992 when Kolter et al. isolated it from three-day-old cells of *Escherichia coli (E. coli*). The protein was more abundant in these starved cells and they were found to bind non-specifically to DNA, forming stable complexes.

Dps proteins belong to the ferritin superfamily and have been found to have two main functions. The first is as a source of iron storage. Iron is an essential element, playing an important role in vital processes such as respiration, oxygen transport and DNA biosynthesis. However, the available free cytosolic Fe(II) must be carefully regulated since excess Fe(II) can be toxic to the cells. The free Fe(II) is sequestered inside the negatively charged cavity of the Dps protein and a highly conserved ferroxidase center is responsible for a controlled oxidation of Fe(II), avoiding free Fe(II) to be available in the highly toxic hydroxyl radicals forming Fenton-reaction:

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + OH^\cdot$$

Instead, H_2O_2 is reduced to H_2O and the oxidized Fe(III) is stored in a mineralized form inside the Dps protein. The second Dps function is as a physical protection of the DNA by non-specifically binding to it, thus forming a condensed complex which protects the DNA against stresses such as starvation, high salt or radiation. It is also speculated that the Dps protein itself could be involved in gene regulation, controlling the expression of genes beneficial or deleterious to the stress resistance of the cells.

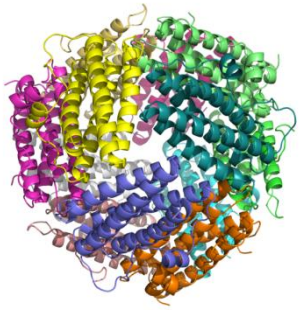

Figure 3. Structure of the Dps protein. Modified from [12].

Most bacteria contain only one Dps protein. In bacteria with multiple Dps proteins they have been shown to each respond to a specific stress stimulus. *N. punctiforme* contains five Dps proteins. By using the iTRAQ method based on isobaric tagging of peptides, global differences in protein abundance between vegetative cells and heterocysts were analyzed, giving the conclusion that four out of the five Dps proteins in *N. punctiforme* were more abundant in heterocysts than in vegetative cells [9, 10, 11].

E. coli as the model system

E. coli was chosen as the model organism for over-expression and functional studies of the five *N. punctiforme* Dps proteins. *E. coli* has advantages as a model system because of its user-friendliness and its well characterized properties. For the purpose of this study, *E. coli* is a suitable model organism because it has only one Dps protein, making the functional studies of introduced Dps proteins less complicated to interpret. For future experiments, the *E. coli* Dps protein could easily be knocked-down for a Dps-free background and clearer effects of the introduced Dps proteins. After characterization in *E. coli* the knowledge can be used for studies in *N. punctiforme* [13, 14, 15].

The purpose of this project

The purpose of this project was to investigate the five Dps proteins of *N. punctiforme* and characterize their roles in protection against different reactive oxygen species (ROS) and oxygen. A characterization of these Dps proteins is of special interest since they could have important relevance in keeping low ROS/oxygen levels in the heterocysts. This could be exploited for protecting oxygen-sensitive enzymes, with possibly improved biofuel production as a result.

Results

The purpose of this project was to investigate the effect of five Dps proteins on growth at various conditions. Five constructs with the different *dps* genes were made by using the Gibson assembly method, giving the possibilities to make complex constructs in one single reaction. The constructs should produce the Dps proteins at high levels upon induction. Therefore, the *lac* promoter (Plac) was chosen to drive the expression since it is inducible by IPTG in *E. coli* and is relatively strong. To terminate the expression of the Dps proteins, the double terminator BBa_B0015 was chosen. The Strep-tag simplifies future gene product purifications and western blots. For the ribosomal binding site we chose the relatively strong BBa_B0034 from registry of standard biological parts [1]. For the vector part the choice fell on pSB1A3. It is a plasmid used for Bio-Brick cloning, has a high copy-number and is ampicillin resistant. A total of seven constructs were made, five encoding the different Dps proteins and two controls, one with Plac in empty vector and one encoding green fluorescence protein (GFP). The Plac in empty vector (Plac_Empty) construct will show the effect the empty plasmids have upon growing in *E. coli* cells, thus making it possible to estimate the effect the Dps proteins have on the *E. coli* cells. The plasmid with the well-characterized GFP gives general information about how a high production of a protein affects the *E. coli* cells.

Primer design

Primers for Gibson assembly should be designed as to contain the sequence for PCR amplification and the sequence for the overlap with the target sequence. The standard protocol for the relatively new Gibson assembly method says the overlapping regions should be 40 base pairs. However, experiments in our lab have shown that around 25 base pairs are sufficient. The Gibson assembly method can be used to assemble fragments of a size of minimum 200 base pairs. Therefore, the Ribosomal Binding Site (RBS) and the Strep-tag sequences, which are too small, were constructed by being parts of the overlapping regions of the primers (see figure 4). Otherwise, the primers were made by means of trying to avoid hairpin-structures and avoiding the GC content to be too high or low and similar between the forward and reverse primers.

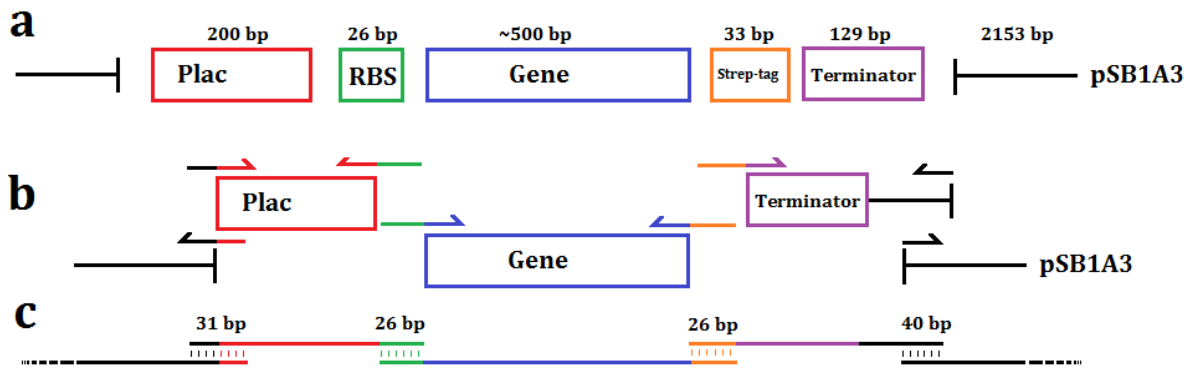

Figure 4. A shows the overall setup of the constructs to be made, B the primer design setup and C the sizes of the overlapping primers.

Table 1. The fragment composition of each construct and their corresponding negative controls (marked with *) with dH_2O instead of Gibson assembly mix.

Sample	Construct	P_{lac}	P_{lac}_Empty	Gene	Terminator	pSB1A3
1	1	+		Npun_F3730	+	+
2	2	+		Npun_F6212	+	+
3	3	+		Npun_R3258	+	+
4	4	+		Npun_R5701	+	+
5	5	+		Npun_R5799	+	+
6	6	+		GFP	+	+
7	7		+		+	+
8	1*	+		Npun_F3730	+	+
9	6*	+		GFP	+	+
10	7*	+	+		+	+

Gibson fragment amplification

The Gibson fragments to be assembled were made by PCR amplification using the designed primers on the different templates needed. A problem was the amplification of the vector fragment. However, by initially running a few cycles at lower annealing temperature (cold-start PCR), the vector could be made. All PCR-products were treated with Dpn1. Dpn1 cleaves methylated DNA; therefore the template-DNA will be degraded, whereas the PCR-product will be unaffected. Table 2 shows the correct sizes the different fragments for the Gibson assembly should have and figure 5 shows the gel-pictures thereof.

Table 2. The wanted sizes of the different fragments for the Gibson assembly.

Fragment	Plac	Npun_ F3730	Npun_ F6212	Npun_ R3258	Npun_ R5701	Npun_ R5799	Plac_ Empty	GFP	Terminator	pSB1 A3
Size (bp)	239	548	491	596	629	611	272	773	331	2044

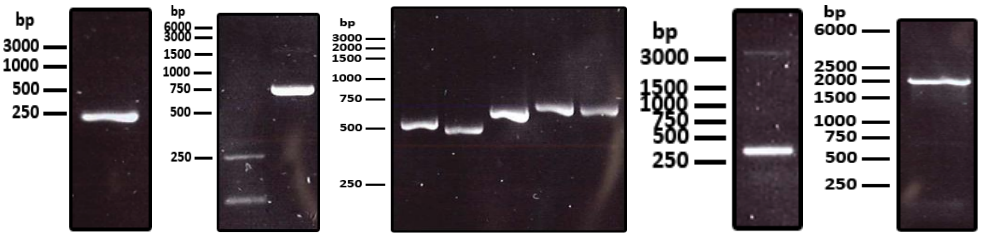

Figure 5. Gel pictures of a) Plac b) Plac in empty vector and GFP c) *dps* genes 1-5 d) Terminator and e) pSB1A3

Gibson assembly

Figure 6 shows the gel-picture of one round of Gibson assembly for all seven samples and their corresponding negative controls.

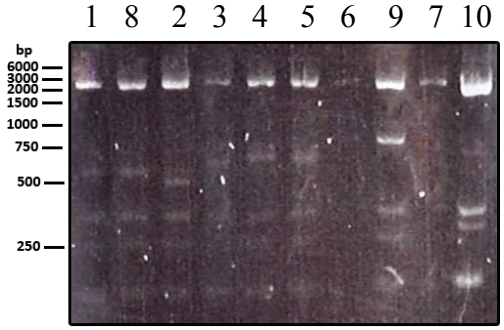

Figure 6. Gel-picture of the constructs after Gibson assembly, lane numbers corresponding to the sample numbering in Table 1.

No obvious difference between the constructs containing the Dps proteins and their negative control was seen. Construct 6 with the GFP gene gave only a weak band at the size corresponding to the vector part, while none of the smaller fragments were seen. Probably this was due to an error during loading the gel, rather than they had been chewed up in the Gibson assembly reaction. For construct 7 a clear band at the top of the gel was depicted, while no bands corresponding to the smaller fragments were shown. This seemed promising for being an assembled product. All samples were transformed into XL1-Blue competent cells and spread on LB + ampicillin plates and incubated at 37° C over night. The number of colonies on the different plates is depicted in Table 3.

PCR - screening

PCR-screening on colonies from the plates with many colonies compared to their corresponding negative control plates (Table 3) was made using the VF2 and VR as primers. The correct size of the fragment for each construct using these primers is depicted in Table 4.

Table 3. Number of colonies on the different plates after transformation. Numbering of samples according to Table 1.

Sample	1	2	3	4	5	6	7	8	9	10
Colonies	224	2	2	6	7	3	18	3	6	2

Table 4. The wanted correct sizes (bp) for the whole assembled plasmid, for the fragments after PCR-screening and after restriction enzyme (RE) cutting.

Construct	1	2	3	4	5	6	7
Whole size (bp) of the plasmid	2984	2990	3095	3128	3110	3272	2558
Correct size (bp) after PCR-screening	1198	1141	1246	1279	1261	1423	706
Correct size (bp) after RE-cutting	2141 + 843	2141 + 849	2141 + 954	2141 + 987	2141 + 969	2141 + 1131	2141 + 417

Six colonies from the plate with possibly assembled construct 7 products (plate 7) were picked for the PCR-screening and the results are shown in Figure 7.

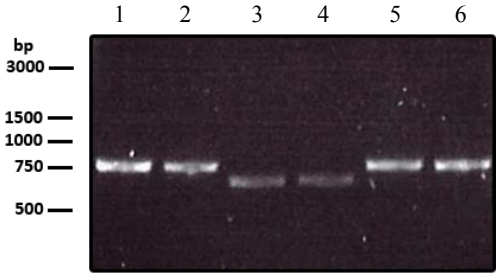

Figure 7. Gel-picture of the PCR-screening of six colonies from plate 7.

A correct construct 7 should have a fragment size of 706 bp (see Table 4), therefore colony 1, 2, 5 and 6 seemed promising.

Restriction enzyme digestion

For an additional screening method, the colonies from the PCR-screening were digested with the restriction enzymes (RE) XbaI and PstI. Non-cleaved samples or incorrect fragment size between the two restriction sites would imply false positive results from the PCR-screening. See Table 3 for the correct sizes the fragments should have after RE-digestion.

Below is shown the gel-picture after the RE-cutting of PCR-screened plate 7 colonies (Figure 6). Colony 3 and 4 were used as negative controls.

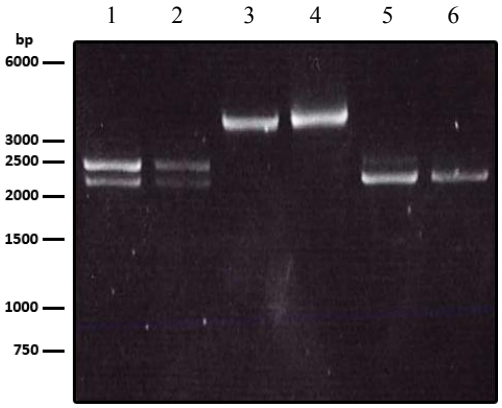

Figure 6. Gel-picture of the fragments after RE-digestion
with XbaI and PstI of the PCR-screened colonies from plate 7.

Characterization of Npun_F3730

To investigate the effect of over-expressing Npun_F3730 in *E.coli*, a growth experiment was carried out. Duplicates of wild-type and Npun_F3730 mutant cell-cultures were grown with and without 1 mM H_2O_2 during exponential phase. Isopropyl-b-D-thiogalactopyranoside (IPTG) was added to the mutant cell-cultures two hours prior the start of the experiment to a final concentration of 0,2 mM to induce the lac promoter. OD600 was measured at time 0, indicating the time of the addition of H_2O_2 and the start of the experiment, and subsequently approximately every 60 minutes (see table 5 for exact values). The growth rate (μ) of the wild-type cell-culture with H_2O_2 was 79,2 % of the growth rate (μ) for the wild-type cell-culture without H_2O_2. Similarly, the growth rate (μ) for the Npun_F3730 mutant cell-culture with H_2O_2 was 94,1% of the growth rate (μ) for the Npun_F3730 mutant cell-culture without H_2O_2 This indicates a strong stress protection by Npun_F3730. After 50 minutes there was a clear decrease in growth rate for the wild-type cell-culture with H_2O_2 compared to the wild-type cell-culture without H_2O_2 and this indicates that it takes some time before the cells are affected by the H_2O_2. The wild-type cell-culture without H_2O_2 and the mutant cell-culture without H_2O_2 had similar growth rate curves. This indicates that the over-expression of the Npun_F3730 does not stress the cells.

Table 5. The average OD600 of duplicates for each cell culture at different time points.

Time (mins)	0	66	129	195	255
Wt H2O2	0,20935	0,455	0,64445	0,7881	0,97905
Wt Ctrl	0,2869	0,6359	1,0668	1,5451	1,84615
Npun_F3730 H2O2	0,1469	0,3743	0,64235	0,9069	1,20005
Npun_F3730 Ctrl	0,1838	0,48765	0,89075	1,3556	1,6417

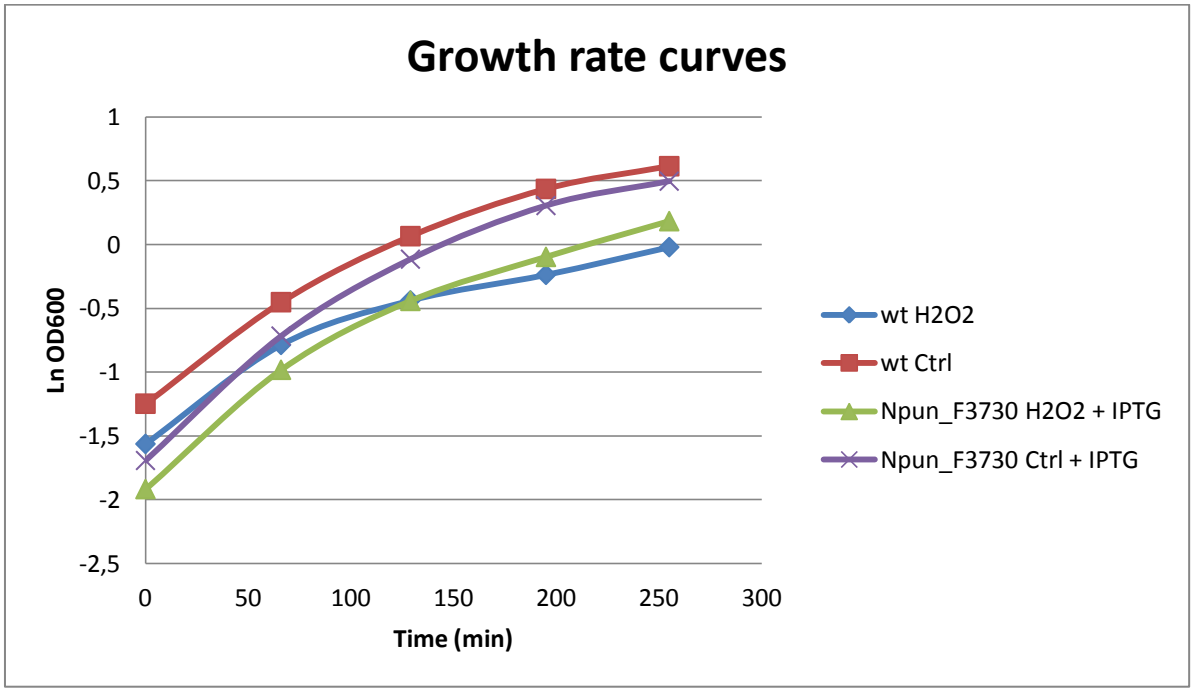

Figure 5. Growth rate curves on a logarithmic scale for wild-type and Npun_F3730 mutant cell-cultures with or without 1 mM H_2O_2 were depicted by measuring their OD600 at different time points.

Discussion

The results give a strong indication that at least one out of the five *N. punctiforme* Dps proteins are directly involved in protection against the ROS H_2O_2. However, we cannot exclude that the over-expressed Dps protein is not protecting against other ROS such as superoxide. This is something we need to investigate further. Furthermore, the expression level of the Dps protein needs to be verified by Western blots using antibodies against the strep-tag which was fused into the construct.

E. coli has one functioning Dps protein and its effect will constitute an unknown background value in the investigation of the *N. punctiforme* Dps proteins to different ROS. For future experiments it would be better to knock-out the *E. coli* Dps protein to get a Dps-free background and clear effects of the different *N. punctiforme* Dps proteins.

The results from the growth experiment on Npun_F3730 in *E. coli* should be seen as preliminary. The start cultures should be of larger volumes to avoid making the surface exposed to the air noticeably larger after the removal of cell-culture for OD600 measurement. More air would have a positive effect on growth. Further, all cultures need to have the same OD600 at the start of the measurements to ensure a possibility to interpret the differences in growth rate. The cultures were grown in duplicates. More trustworthy results would be obtained by using triplicates.

The lac promoter is very strong. It is possible that the over-expression of the Dps adds an extra stress factor to the cells. This could lead to an underestimation of the Dps effect on the H_2O_2- stressed cells. Further, people at our lab working with the same *E. coli* strain observed some leakage of the lac promoter in its non-induced state. Therefore it would be a good idea to grow mutant cell-cultures without inducing them with IPTG to see if the lac promoter really is fully repressed in its non-induced state. It is possible that only low levels of Dps are needed for a protection against H_2O_2. A change to a less strong promoter, fully repressed in its non-induced state, could be a better choice. Or to use an *E. coli* strain in which the lac promoter repression is stronger.

Materials and Methods

PCR

For PCR amplification of the different Gibson fragments the primers designed are shown in Table 4. The Phusion high-fidelity polymerase (Finnzymes) was used for the PCR amplifications according to the manufacturer's guidelines

Table 4. Primers designed for amplification of Gibson fragments.

Primers	Sequences 5'to 3'	Size(bp)	Annealing size	Tm(˚C)
Plac_For	GCCGCTTCTAGAGCAATACGCAAACCGCCTCTCCC	35	22	72.4
Plac_Rev	CTAGTATTTCTCCTCTTTCTCGAGTATGTGTGAAATTGTTATCCGCTCAC	50	24	67.05
Plac_Emp_Rev	TTATTTTTCGAATTGGGGATGACTCCATGCGGCCTAGTATTTCTCCTCTTTCTCG	55	22	56.2
Npun_F3730_For	TACTCGAGAAAGAGGAGAAATACTAGATGTCATCAAAAGTAACAGTCAAAAATG	54	28	64.14
Npun_F3730_Rev	TTATTTTTCGAATTGGGGATGACTCCATGCGGCTTCTTCCAGTAAACTTCTCAACATCC	59	26	64.65
Npun_F6121_For	TACTCGAGAAAGAGGAGAAATACTAGATGCAAGAACTTGACTATAAAAAGACTATC	56	30	62.29
Npun_F6121_Rev	TTATTTTTCGAATTGGGGATGACTCCATGCGGCGCTAAAATCGCGTAGCATCTTT	55	22	63.56
Npun_R3258_For	TACTCGAGAAAGAGGAGAAATACTAGATGCGTGCGATAAACATTGGGTTG	50	24	72.1
Npun_R3258_Rev	TTATTTTTCGAATTGGGGATGACTCCATGCGGCCACACCCACAGGAGTCTTAGCC	55	22	67.28
Npun_R5701_For	TACTCGAGAAAGAGGAGAAATACTAGATGAGCGACAACAGCATTGC	46	20	66.72
Npun_R5701_Rev	TTATTTTTCGAATTGGGGATGACTCCATGCGGCTTTTACAGCAGCAGGAATCTTTTG	57	24	65.41
Npun_R5799_For	TACTCGAGAAAGAGGAGAAATACTAGATGTCTGAAACGCAAACTTTGTTACG	52	26	67.84
Npun_R5799_Rev	TTATTTTTCGAATTGGGGATGACTCCATGCGGCGCTTTGAGCCGCTTGGAC	51	18	66.48
GFP_For	TACTCGAGAAAGAGGAGAAATACTAGATGAGTAAAGGAGAAGAACTTTTCA	51	25	60.62
GFP_Rev	TTATTTTTCGAATTGGGGATGACTCCATGCGGCTTTGTATAGTTCATCCATGCCATG	57	24	64.7
Term_For	GGAGTCATCCCCAATTCGAAAAATAACCAGGCATCAAATAAAACGAAAGGC	51	25	70.59
VR	GCTCACTCAAAGGCGGTAAT	20	20	63.48
pSB1A3_For	CGTTCGGCTGCGGCGAGCGG	20	20	82.47
pSB1A3_Rev	GAGGCGGTTTGCGTATTGCTCTAGAAGCGGCCGCGAAT	38	20	69.86

The DNA from the PCR-reactions was treated with Dpn1 restriction endonuclease to get rid of template DNA. Each sample was digested with the Dpn1 FastDigest (Fermentas) and incubated at 37°C for 1 h.

Digested samples were purified using PCR clean-up kit (Fermentas).

Gibson assembly

300 fM DNA instead of 30 fM as the standard protocol was used for the Gibson assembly (Collins B, Copley H, Emmrich P, Handley W, Hohmann A, Knott E, Masset P, Reeve B and Sanderson T (2010). http://www.synbio.org.uk/gibson/downloads/files/RFC57.pdf) since better results had been obtained at our lab using this higher concentration.
The molar ratio for each fragment to be assembled in each construct was set as 1:1. The Gibson master mix was made by mixing 32 µl 5X isothermal buffer, 3,2 µl T5 exonuclease (0,1 units/µl), 16 µl Taq ligase (40 units/µl), 2 µl Phusion hot start DNA polymerase (2 units/µl) and 66,8 µl dH_2O to a final volume of 120 µl. 20 µl Gibson master mix was used for each construct to be assembled. The samples were incubated at 50°C for 1 h.

Transformation

After Gibson assembly 2 µl of the mix was used to transform 100 µl of XL1-Blue competent cells. The samples were incubated on ice for 30 minutes and then heat-shocked at 42°C for 1 minute. The samples were then put on ice for 5 minutes before 900 µl of LB medium was added to each sample and put on shaking at 37°C for 1 h. The samples were subsequently centrifuged to get pellets. The pellets were resuspended and approximately 100 µl of each sample was plated on LB+ampicillin and incubated at 37°C overnight.

Screening

Dream-taq polymerase was used for the PCR-screening of promising colonies after transformation according to the manufacturer's protocol (Fermentas). Each colony to be PCR-screened was re-streaked on new LB+ampicillin plates. The DNA was visualized on 1% agarose TAE-gel. The colonies that gave promising results were grown overnight in LB+ampicillin and the plasmids were extracted using the Plasmid Mini Prep Kit (Fermentas). The concentration of DNA was measured with spectrophotometer.

As an extra screening method the extracted plasmids were treated with the restriction enzymes (RE) XbaI and PstI. A reaction mixture with 10X FastDigest buffer, 1 µl of each RE and DNA (200 ng/20 µl) to a total volume of 20 µl was prepared. The mixture was incubated at 37°C for 10 minutes. The DNA was visualized on 1 % agarose TAE-gel.

Growth experiment

A growth experiment with cell-cultures of Npun_F3730 mutant and XL1-Blue *E.coli* wild-type was done. The medium used throughout this experiment was LB for the wild-type cell-cultures and LB with 100 µg/ml ampicillin for the mutant cell-cultures. For growing seed cultures, cells were inoculated to 5 ml medium and put on shaking at 37°C overnight.

The next day 1 ml from the mutant cell-cultures was transferred into four different flasks with 19 ml of new medium. Similarly, 1 ml from the wild-type cell-cultures was transferred into four different flasks with 19 ml of new medium. They were subsequently put on shaking at 37°C and their OD600 were measured until they reached the target OD600 of approximately 0,2. For the lac promoter to be fully induced at the start of the experiment IPTG (0,2 mM) was added the mutant cell-cultures approximately 2 hours before the OD600 reached 0,2. At the start of the experiment two of the four mutant cell-cultures were treated with 1 mM of H_2O_2. The OD600 was measured approximately every 60 minutes for a total of four times, almost throughout the whole logarithmic phase. See Table 5 in appendix for exact OD600 and time points.

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