DTUiGEM-2015 Lab notebook: MAGE proof of concept in Bacillus subtilis W168

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Preparing electroporation competent

Preformed by Vilhelm, e-lab notebook: Viktor.

The protocol: electroporation competent *Bacillus subtilis 168* with following exceptions:

- Pellet was resuspended in 1/40 of original volume instead of 1/20.

Transformation of amyE_KO oligos in B. subtilis: 26.08.15

Preformed by Viktor and Vilhelm , Author of e-lab notes: Viktor

Purpose:

Transform different B. subtilis strains with an oligo. The hypothesis is that this oligo will introduce a stop codon in the *amyE* gene. As a result of this the bacteria will not be able to degrade starch. The bacteria will be grown on plates and by iodine staining, we will be able to see whether the *amyE* was knocked out.

The purpose is to test the efficiency of a single cycle of MAGE (transformation with ssDNA).

Protocol:

Electroporation of B. subtilis with ssDNA

Materials:

- Electroporation competen
- Recovery media
- LB
- LB + 5y neo + 1% starch
- Oligos: mage_amyE-1
- Iodine staing solution
  - 0.6 iodine
  - 50 ml 96% ethanol
-

Procedure:

Transformations		
Sample	Strain (B. subtilis 168)	Volumen of oligo (ul)
1	mutS::gp35_neoR	0.74
2	----||----	1.48
3	mutS::lambda_beta_neoR	0.74
4	----||----	1.48
5 (negative control)	mutS::gp35_neoR	No oligos
6 (negative control)	mutS::lambda-beta_neoR	----||----

7 (positive control)	amyE::gp35_neoR	----||----
8 (positive control)	amyE::lambda-beta_neoR	----||----

1. 100x, 1000x and 10000x dilutions was made of all samples

Plate	Medium	Sample	Volumen of sample (ul)	Dilutions
1	LB + 1% starch + 5y neo	1	100	100x, 1000x and 10000x
2		2		
3		3		
4		4		
5		5		100x and 1000x
6		6		
7		7		
8		8		

2. Plates was incubated O/N at 30 degC.

Iodine staining

3. 0.6 g iodine was mixed with 50 ml 96% ethanol in a fumehood. This was mixed till the iodine was completely dissolved.
4. 1 - 2 ml of iodine solution was added to one plate, let it sit for 1 - 2 min.
5. Count colonies that does not have a clearing zone.

Data:

Growth

All plates contained an uncountable number of colonies. On 1000x and 10000x single colonies was observed though they were close.

Iodine staining

Most colonies was observed to have clearing zones, but about 39 colonies didn't have clearing zones.

Results and Conclusion:

It seems like the method worked. Though it was hard to catch on pictures. There was a lot of colonies that we could not analyse because they were to close to together. If this was to be redone there should be used big dilutions and plated more volume (e.i. the bacteria will be more spread). 100,000x and 1,000,000x might be more suitable.

To overcome this problem in the first place replica plates was made of 100 colonies of plate, unfortunately no n of the picked colonies was transformants.

Two more experiments with the same setup was made. Unfortunately the data of which dilutions and number of CFUs got lost. We replicated the transformants onto LB, then iodine stained the starch plates. Some positive colonies was observed. We tried to locate those on the replicated to LB plates and restreake them onto LB + 5y neo + 1% starch, but they were now negative. This could be because we simply chose the wrong colonies from the replicated plate. The colonies could as well have been false positives, cause by that the colonies was in a state of growth where they have not expressed *amyE* yet.

MAGE - surfactin, streptomycin resistance and amyE: 28.08.15

Preformed by Viktor and Pernille e-lab notebook: Viktor.

Purpose

This is three different experiments done simultaneously:

1. Surfactin: introduce a change in the stachelhaus code to induce a change in the aminoacid sequence for NRP surfactin. To different oligos was tested.
2. Streptomycin (strp) resistance: introduce a point mutation in the ribosome which will make the transformants resistance to streptomycin.
3. *AmyE*: Introduce a stop codon in CDS for *amyE* and thereby knocking out the gene.

Materials

- Oligos
    - Surfactin: oligo_surf_asp->Asn_1 and oligo_surf asp->Asn_2_I
    - Streptomycin resistance: B_Sub_Mods0007.1mutationrpsL
    - amyE: mage_amyE-1
- Electroporation cuvettes (BioRad 0.2 cm gab)
- Recovery media
- Plates
    - LB
    - LB + 5y neo
    - LB + 5y neo + 1% starch
    - LB + 500y strp
- Electroporation competent cells
    - *ΔamyE::lambda-beta_neo*^{R}
    - *ΔamyE::gp35_neo*^{R}
    - *ΔmutS::lambda-beta_neo*^{R}
    - *ΔmutS::gp35_neo*^{R}
    - WT

Procedure

1. 0,5uL (5uM) oligo to was added to 100uL electro competent cells and was mixed gently by pipetting.
2. The sample was Incubated for 7 min.
3. Sample was added to electroporation cuvette and electroporated with 2.0 kV (for 0.2 cm cuvette).
4. Immediately after the electroporation 1 ml recovery medium was added to the electroporation cuvette this was mix by pippetting and the poured in to sterile plastic tubes.
5. For negative controls 100 ul of electroporation competent cells was added to 1 ml recovery media.
6. The plastic tubes were incubated for 4h at 30 degC at 220rpm.
7. 100 ul of 10^6 and 10^7 dilution was plated according to the following table.
8. 250 ul of each sample was mixed with 250 ul 40% glycerol and this was stored at -80 degC.

Strain	Oligo	Plate
ΔmutS::gp35	oligo_surf_asp->Asn_1	LB + 5y neo
ΔmutS::gp35	oligo_surf asp->Asn_2_I	
ΔmutS::gp35-neo^R	B_Sub_Mods0007.1mutationrpsL	
ΔmutS::lambda-beta_neo^R		
ΔamyE::gp35_neo^R		
ΔamyE::lambda-beta_neo^R		
WT		
ΔmutS::gp35-neo^R	mage_amyE-1	LB + 5y neo + 1% starch
ΔmutS::lambda-beta_neo^R		
ΔmutS::gp35-neo^R	No oligo	
ΔmutS::lambda-beta_neo^R		
ΔamyE::gp35_neo^R		
ΔamyE::lambda-beta_neo^R		

9. Plates were incubated O/N at 30 degC.

29.08.15 - Replating

1. There was no colonies on most plates.
2. Glycerol stock of transformants were diluted to 10^4 and 10^5 and this were plated on LB + 5y neo or LB + 5y neo + 1% starch.

30.08.15 - stamp replica plating

1. Plates with spectromycin transformants were stamp repleca plate onto LB + 500y strp.
2. Plates with amyE transformants were stamp replica plates onto LB
3. Plates was incubated at 30 degC

05.09.15 - replica colony picking

1. Single colonies was picked from streptomycin transformants plates (LB + 5y neo) using sterilized tooth picks and streaked on to both LB and LB + 500y strp.

14.09.15 - stamp replica plating of replica colony picking

1. Plates from replica colony picking was stamped onto LB and LB + 500y strep.
2. A plate with *B. subtilis* transformed with pDG268neo_gp35 was stamped onto LB and LB + 500y strep as a negative control

Data

Runtime from electroporation

Strain	Oligo	Runtime (ms)
ΔmutS::gp35	oligo_surf_asp->Asn_1	4.7
ΔmutS::gp35	oligo_surf asp->Asn_2_I	5.0
ΔmutS::gp35-neo^R	B_Sub_Mods0007.1mutationrpsL	4.6
ΔmutS::beta_neo^R	------------||-----------	4.8
ΔamyE::gp35_neo^R	------------||-----------	4.6
ΔamyE::beta_neo^R	------------||-----------	4.9
WT	------------||-----------	4.0
ΔmutS::gp35-neo^R	mage_amyE-1	4.5
ΔmutS::beta_neo^R	mage_amyE-1	5.3

Streak replica plating

CFU count		
	LB + 500y Strep	**LB + 5y neo**
ΔmutS::beta_neo^R	7	52
ΔmutS::gp35-neo^R	1	100

Conclusion

The runtime for the electroporation were not optimal, since it should be between 5.0 and 6.0. The reason for this in unknown.

Stamp replica plating - very hard to analyze, because the colonies were smeared.

Replica colony picking - colonies growing on 500y strep plates, this indicates that the oligos has been able to integrate the changes in the genome of B. subtilis, e.i. our method seems to work.

Stamp replica plating of replica colony picking plates - It was verified that the colonies could grow on 500y strep and no colonies was growing on negative control.

cPCR of Surfactin MAGE 1: 03.09.15

Preformed by Viktor and Vilhelm e-lab notebook: Viktor.

Purpose

The purpose is to electroporate oligos into *ΔmutS::gp35*. We used only this strain because we expected this to be the most efficient. The oligos will introduce a change in the stachelhaus code to induce a change in the composistion of the NRP surfactin. To different oligos was tested.

Material

- Oligos
    - Surfactin: oligo_surf_asp->Asn_1 and oligo_surf asp->Asn_2_I
- Electroporation cuvettes (BioRad 0.2 cm gab)
- Recovery media
- Plates
    - LB
    - LB + 5y neo
    - LB + 5y neo + 1% starch
    - LB + 500y strp
- Electroporation competent cells
    - *ΔmutS::gp35_neo*^{R}
    - WT
- Phusion polymerase kit NEB

Procedure

01.09.15:

1. Single colonies of possible transformants was restreaked on LB + 5y ne and the the coloni was inoculated in 10 ml ddH2O.
2. This was boiled for 10 min. at 95 degC and stored overnight at -20 degC

02.09.15: pilot cPCR

1. A gradient cPCR was set up using the positive control primers. A1-A8 templates was used and all of them was done with both GC og HF buffer. Gradient was set to +/- 10 from 65 degC

2. The optimal conditions was found to be annealing temp.: 65 degC and GC buffer (seems like the HF buffer tube wasn't made properly).
    a. *This is of course the optimal conditions for the positive control primer, but the properties of the positive control and the cPCR primer are really similar.*

03.09.15: pool cPCR

1. To make waste less cPCRs we started by doing pools of 8 sample.
    a. 1 ul of 8 different samples was mixed and 1 ul of this mix was used as template for cPCR.
    b. Positive controls of all reactions was made this makes a total of 32 reachtions
    c. *Negative controls was forgotten, but done in the next experiment.*
2. To mastermix for 16 reactions was made: one for samples and another for positive controls. Receipt for 16 reactions mastermix'
    a. 12.6 x 16 ul ddH2O
    b. 4 x 16 ul GC buffer
    c. 0.4 x 16 ul dNTPs
    d. 2 x 16 ul of each primer
    e. 0.2 x 16 ul polymerase
3. 19 ul mastermix was used for each reaction.
4. 1 ul of boiled cells (from 01.09.15) was used as template.

Data

Pool cPCR:

All positive controls was positive. All samples, but one was positive. This was more than expected, but it is possible to have one or two mutants every 8 colonies. It might be false positive due to that the primer is able to bind even though the insert has not been done.

cPCR of pool G

To check the result from the pool cPCR are false positives all 8 samples from pool G (which was positive in the pool cPCR) was run in their own PCR reaction. This time including a negative control: WT B. subtilis with test primers. One positive control was made

PCR tubes				
Tube	**Template**	**Fwd primer**	**Rev primer**	**Expected band size (bp)**
1	G1	cPCR_surfactin-F	cPCR_surfactin-R-onlyanneals	775
2	G2	----||-----	----||-----	----||----

3	G3	----||-----	----||-----	----||----
4	G4	----||-----	----||-----	----||----
5	G5	----||-----	----||-----	----||----
6	G6	----||-----	----||-----	----||----
7	G7	----||-----	----||-----	----||----
8	G8	----||-----	----||-----	----||----
9	G5	----||-----	cPCR_surfactin-R-positive_cont	450
10	WT	----||-----	----||-----	No band
11	WT	----||-----	cPCR_surfactin-R-onlyanneals	No band

Conclusion

Unfortunately the primers could also bind to the wild type, e.i. this screening method did not work. It should be possible to find an annealing temperature where the primer will only anneal if there is an insert, but we do not have time to do this optimization. Instead all the colonies will be send for sequencing using the positive control primer since this fragment will contain the targeted nucleotides.

4 cycles of MAGE using strep oligos: 06.09.15 and 07.09.15

Preformed by Vilhelm and Viktor, e-lab notes: Viktor.

Purpose

Because the transformation efficiency earlier has been very low we tried to do more cycle of MAGE and thereby increasing the efficiency. We plated sample every cycle to see if the efficiency increased with the number of cycles. The difference in efficiency for 3 of the 4 different MAGE ready strains was tested as well (*amyE::Lambda-beta* was left out only because we did not have any more competent cell of this strain). To different *ΔmutS::gp35-neo*^{R} was used. The only difference between those two is that they are from two different batches of electron competent cells.

Protocol:

MAGE in Bacillus subtilis 168

Materials:

- Electroporation competent cell
  - *ΔamyE::gp35-neo*^{R}
  - *ΔmutS::gp35-neo*^{R}
  - *ΔmutS::beta-neo*^{R} *new*
  - *ΔmutS::beta-neo*^{R} *old*
  - *WT*
- Oligos
  - B_Sub_Mods0007.1mutationrpsL
- Linearized pDG268neo_lambda-beta (positive control)
- Electroporation kuvettes
- Elekctroporation media
- Recovery media
- Plates
  - LB
  - LB + 5y neo
  - LB + 500y strp

Procedure:

MAGE cycle 1

10. 0,25uL (5uM) oligo to was added to 100uL electro competent cells and was mixed gently with pipet or flicking the tubes.
11. The sample was Incubated for 7 min.
12. Sample was added to electroporation cuvette and electroporated with 2.2kV (for 0.2 cm cuvette).
13. Immediately after the electroporation 1 ml recovery medium was added to the electroporation cuvette this was mix by pippetting and the poured in to sterile plastic tubes containing 2 ml recovery media.
14. The plastic tubes were incubated for 5h and 10 min. at 30 degC at 220rpm.
15. Glycerol stocks of all samples was made (If would be used if we didn't hit a good count able dilution).
    - 500 ul of cells was added to 500 ul 40% glycerol in a cryotube.
    - This was mixed by pipetting and stored at -80 degC.
16. 10^4 and 10^5 dilutions was made using ddH2O. These was plated on LB + 5y neo, incubated at 37 degC.

MAGE cycle 2

1. All samples was centrifuged at 5000g for 10min, and washed in 100uL electroporation medium (make a 1/20 decrease in volume). This procedure was repeated 4 times.
    a. *ΔmutS::beta-neo*^{R} *new* and *old* were mixed by mistake.
2. Electroporation was carried out as in MAGE cycle 1.
3. Positive control was linearized pDG268neo_lambda-beta was used instead of oligo.
4. After 4 hours and 30 min of incubation
5. Glycerol stocks of all samples was made (If would be used if we didn't hit a good count able dilution).
    a. 500 ul of cells was added to 500 ul 40% glycerol in a cryotube.
    b. This was mixed by pipetting and stored at -80 degC.
6. 10^4 and 10^5 dilutions was made using ddH2O. These was plated on LB + 5y neo.

MAGE cycle 3

1. Protocol was followed with following exceptions
    a. Cells from MAGE cycle 2 was incubated for 5 hours.
2. Glycerol stocks of all samples was made (If would be used if we didn't hit a good count able dilution).

a. 500 ul of cells was added to 500 ul 40% glycerol in a cryotube.

b. This was mixed by pipetting and stored at -80 degC.

3. 10^4 and 10^5 dilutions was made using ddH2O. These was plated on LB + 5y neo and incubated

4.

MAGE cycle 4

1. Protocol was followed with following exceptions

   a. Cells from MAGE cycle 3 was incubated for 4 hours and 20 min.

2. Glycerol stocks of all samples was made (If would be used if we didn't hit a good count able dilution).

   a. 500 ul of cells was added to 500 ul 40% glycerol in a cryotube.

   b. This was mixed by pipetting and stored at -80 degC.

3. 10^4 and 10^5 dilutions was made using ddH2O. These was plated on LB + 5y neo and incubated at 37 degC

Replica colony picking plating

7. Where it was possible single colonies was picket from the plates and replicated to LB and to LB + 500y strep.

8. Positive and negative control were not replicated.

Replating

a. Since we were not able to select single colonies *ΔmutS::gp35-neo*^{R} from MAGE cycle 1 and 2 or of *ΔamyE::gp35-neo*^{R} from MAGE cycle 3 and 4. Glycerol stock of those was diluted in LB to 10^2, 10^3, 10^4 and 10^5 and plated onto LB and LB + 500y strep.

Data

	Runtime for MAGE cycle 1	**Runtime for MAGE cycle 2**	**Runtime for MAGE cycle 2**	**Runtime for MAGE cycle 4**
Strain	**Runtime (ms)**			
WT (Negative control)	4.9	5.4	5.3	5.6
WT (positive control)	5.0	5.4	5.4	5.3
***ΔamyE::gp35-neo*^{R}**	5.2	5.4	5.6	5.2
***ΔmutS::gp35-neo*^{R}**	5.1	5.3	5.3	5.2
***ΔmutS::beta-neo*^{R}**	5.2	5.5	5.4	5.4

new				
ΔmutS::beta-neo^R *old*	5.1	5.5	5.4	5.4

	OD_{600} for MAGE cycle 1	**OD_{600} for MAGE cycle 2**	**OD_{600} for MAGE cycle 3**	**OD_{600} for MAGE cycle 4**
Strain	**OD_{600}**			
WT (negative control)	1.19	0.95	1.31	0.9
WT (positive control)	1.30	0.9	0.93	0.31
ΔamyE::gp35-neo^R	1.08	0.70	1.17	0.76
ΔmutS::gp35-neo^R	1.29	0.61	0.62	0.16
ΔmutS::beta-neo^R ***new***	1.18	0.92	1.25	1.02
ΔmutS::beta-neo^R ***old***	1.40	1.25	1.4	1.17

Counting CFUs

a. Positive control had 100+ colonies for all cycles

b. Negative control had no colonies for all cycles

	CFU count of replica colony picking plates from MAGE cycle 1	**CFU count of replica colony picking plates from MAGE cycle 2**	**CFU count of replica colony picking plates from MAGE cycle 3**	**CFU count of replica colony picking plates from MAGE cycle 4**
Strain	**Number of colonies (on 500y strep : on LB)**			
ΔamyE::gp35-neo^R	0 : 100	4 : 48	N/A	N/A
ΔmutS::gp35-neo^R	N/A	N/A	1 : 200	1 : 100
ΔmutS::beta-	0 : 300	17 : 166	22 : 91	1 : 7

neo*^{R} *new				
ΔmutS::beta-neo*^{R} *old		22 : 187	87 : 152	1 : 20

Replating - 8 CFUs was observed on "MAGE4 *ΔmutS::beta-neo*^{R} *old* 500y strep. 10^3". No CFUs was observed on any other plates.

Results and Conclusion

It seems that mutS::beta is better than the wild type, mutS::GP35 and amyE::GP35. We cannot conclude that more MAGE cycles gives a higher yield than a single cycle, because this result is not constant. The experiment will need to be run with a better method of testing if the insertion has been incorporated into the genome.

Preparing electroporation competent B. subtilis strains: 09.09.15

Preformed by Vilhelm and Viktor, e-lab notes: Viktor.

Purpose

The purpose is to make electroporation competence cells of the four different B. subtilis strains.

- *ΔamyE::gp35-neo*^{R}
- *ΔmutS::gp35-neo*^{R}
- *ΔmutS::beta-neo*^{R} *new*
- *ΔmutS::beta-neo*^{R} *old*
- *WT*

Protocol:

Electroporation competent Bacillus subtilis 168

Materials:

- Overnight cultures
  - *ΔamyE::gp35-neo*^{R}
  - *ΔamyE::beta-neo*^{R}
  - *ΔmutS::gp35-neo*^{R}
  - *ΔmutS::beta-neo*^{R}
- Growth media (GM)
- Elekctroporation media (EM)
- L-Threonin
- Glycine
- Tween80

Procedure:

1. 7 ml O/N culture of all the different strains was mixed with 112 ml (16-fold) GM in 500 ml Erlenmeyer flasks.
2. Incubated in 37 degC shaking at 180 - 200 rpm
3. OD was measured regularly
4. *ΔmutS::gp35-neo*^{R} was unfortunately grown to a to high OD. The culture was then started over.
   a. 7 ml of overgrown culture was inoculated in 112 ml GM in a fresh Erlenmeyer flask
   b. Incubated shaking at 37 degC

5. After 3 hours of incubation glycine, L-threonin and tween80 was added to a final concentration of 1.0%, 2.0% and 0.03%, respectively, to *ΔmutS::gp35-neo*^{R} and *ΔmutS::beta-neo*^{R}.
    a. Not to *ΔamyE::beta-neo*^{R} since it had not reached the right OD yet
    b. This was added to *ΔamyE::beta-neo*^{R} after 3 hours and 25 min. of incubation
    c. glycine, L-threonin and tween80 was added to the new batch of after 1 hour and 37 min of incubation.
6. After 1 hour of incubation the samples was put on ice for 5 min.
7. Cultures was spun for 10 min at 5000 rpm at 4 degC supernatant was disposed and additional culture was added to the tubes at spun at same conditions.
8. Pellet was resuspended in 1/20 of original volume of EM and spun for 10 min at 5000 rpm at 4 degC.
    a. This was repeated 4 times. After a fifth resuspention, this was divided into eppendorf tubes and stored at -80 degC.
    b. *While resuspending the cells the looked more white than they used to, e.i. something might have gone wrong, ex. the cells have been lyzed.*

Data

OD measurements						
Strain\Time (hours:min.)	01:05	02:15	02:35	02:58	03:10	03:25
***ΔamyE::beta-neo*^{R}**	0.16	0.29	0.44	0.68	0.72	0.84
***ΔamyE::gp35-neo*^{R}**	0.22	0.54	0.78	0.98	N/A	N/A
***ΔmutS::beta-neo*^{R}**	0.23	0.58	0.79	0.96	N/A	N/A
***ΔmutS::gp35-neo*^{R}**	0.31	0.85	1.16	N/A	N/A	N/A

Marked with green is when glycine, L-threonin and Tween80 was added. Marked with red OD > 0.9.

OD measurements						
Strain\Time (hours:min.)	00:18	01:06	01:20	01:29	01:33	01:37
***ΔmutS::gp35-neo*^{R}**	0.09	0.29	0.44	0.64	0.79	N/A

Marked with green is when glycine, L-threonin and Tween80 was added.

Results and conclution

Cells were not tested.

MAGE optimization: length and number of mismatches: 11.09.15

Preformed by Vilhelm and Viktor, e-lab notes: Viktor.

Purpose

The purpose is to test the which effect the length of the oligo and the number of mismatches in the oligo has on the efficiency of MAGE.

Protocol:

MAGE in Bacillus subtilis 168

Materials:

- Electroporation competent cell
    - *ΔmutS::beta-neo*^{R}
- Oligos
    - rpsL 1mm
    - rpsL 2mm
    - rpsL 3mm
    - rpsL 4mm
    - rpsL 5mm
    - rpsL 6mm
    - rpsL 50nt
    - rpsL 60nt
    - rpsL 70nt
    - rpsL 80nt
    - rpsL 100nt
- Linearized pDG268neo_lambda-beta (positive control)
- Electroporation cuvettes (from Bio Rad 0.2 cm gap)
- Elekctroporation media
- Recovery media
- Plates
    - LB
    - LB + 5y neo
    - LB + 500y strp

Procedure:

1. Following oligos were transformed into *ΔmutS::beta-neo*^{R}
    a. Variation in length: 50nt, 60nt, 70nt, 80nt and 100nt
    b. Variation in numbers of mismatches (mm): 1, 2, 3, 4, 5 and 6
2. Positive control was used linearized pDG268neo_gp35
3. Negative control: no oligo was used
4. Protocol was followed with following exceptions:
    a. Mistake: the double amount of 70 nt oligo and 100 nt oligo were added.
    b. 5 mm oligo transformation was shocked 4 times before the shock went through the cuvette
        i. The first 3 time the shock was making sparks.
5. After incubation OD600 was measured
6. 10^3, 10^4 and 10^5 dilutions was made of all samples.
7. 200 ul of dilution were plated on 500y strep and LB
8. Plates were incubated at 37 degC O/N.
9. Single colonies from LB plates were selected and restreaked onto LB and LB + 500y strep.

Data:

Oligo	Runtime (ms)	OD_{600} (measured after incubation)
1 mm	5.5	0.98
2 mm	5.6	0.97
3 mm	5.6	0.37
4 mm	5,6	0.52
5 mm	5.5	0.23
6 mm	5.6	0.46
Positive control	5.6	0.52
Negative control	5.6	0.49
50 nt	5.6	0.47
60 nt	5.6	0.51
70 nt	5.5	0.53

80 nt	5.6	0.57
100 nt	5.4	0.71

Oligo mm and length	**CFU**	Total number of colonies picked
1mm	0	100
2 mm		
3mm	0	100
4mm	3	100
5 mm	4	100
6 mm	3	100
50 nt	2	100
60 nt	2	100
70 nt		
80 nt	3	100
100 nt	0	100

Results and Conclusion:

The experiments indicate that that 5 mismatches of with a length of 80nt and using 5uM of oligo would optimize the MAGE method. The data is of pure quality and nothing certain can be concluded from this experiment.

MAGE optimization: Amount of oligo: 11.09.15

Preformed by Vilhelm and Viktor, e-lab notes: Viktor.

Purpose

The aim of this experiment is to give an indication what the optimal amount of oligo is for MAGE in B. subtilis. Previously we have found that B. subtilis *ΔmutS::beta-neo*^{R} is the most efficient strain. This is the only strain that will be tested. Apart from oligo amount, the correlation between OD600 and concentration of B. Subtilis will be calculated.

Protocol:

MAGE in Bacillus subtilis 168

Materials:

- Electroporation competent cell
  - *ΔmutS::beta-neo*^{R}
- Oligos
  - B_Sub_Mods0007.1mutationrpsL
- Linearized pDG268neo_lambda-beta (positive control)
- Electroporation cuvettes (from Bio Rad 0.2 cm gap)
- Elekctroporation media
- Recovery media
- Plates
  - LB
  - LB + 5y neo
  - LB + 500y strp

Procedure:

1. The following amounts of oligo was tested in duplicates
   - 0.2 ul, 5 ul, 10 ul, 20 ul and 25 ul
2. Only *ΔmutS::beta-neo*^{R} and WT (positive control) were used
3. Electroporation was done following the protocol with following exceptions:
   - Only one cycle was done.
   - Final incubation was extended to 5 hour because of logistics in the lab
   - After final incubation cell was on ice for 1 hour prior to plating, due to problems in the lab.

4. OD was measured for all the samples.
5. Dilution that was plated was chosen individually for each sample based on the OD measurement.
6. Three different dilutions was picked for each sample and 100 ul were plated on LB plates.
7. 200 ul of each non-diluted sample was plated on strep plates

Replica colony picking

10. Single colonies were picked using sterile tooth picks and streaked onto both LB and LB + 500y strep.
11. Plates were incubated for 48 hours at 37 degC

Data:

Measured runtime	
Oligo amount	**Runtime (ms)**
0.2	5.30
0.2	5.10
5	5.20
5	5.00
10	5.10
10	5.20
20	5.30
20	5.20
25	4.90
25	5.80
Positive control	5.8
Negative control	5.9

Colony count:

Plates was grown to uncountable amount of colonies. Single colonies could be located.

Colony count from colony picked plates

Amount of oligo (ul)	Number of colonies in total	Number of colonies of strep.
0.2	200	70
5	300	54
10	200	53
20	200	98
25	300	56

Results and Conclusion:

We were not able to tell anything about the efficiency because that we were not able to count the the colonies. Therefore the plate will be replicated by colony picking.

Replica colony picking

Colonies showed a large variation in size and morphology. The fact that there were about 90 out of 100 "transformants" on one of the plates made us strongly doubt the results. The lack of a negative control made it even harder to tell anything. Though we were able to count colonies that had a morphology of a growing Bacillus subtilis colony.

MAGE of surfactin with COS oligos: 13.09.15

Preformed by Vilhelm and Viktor, e-lab notes: Viktor.

Purpose

The purpose of this experiment is to use MAGE to introduce a mutation in the gene coding for NRPS that produces the NRP surfactin. Since we are not able to select for this rare event we will use a COS oligos for selection and the then the transformants will be screened using mass spectrometry looking for a specific change in the composition of the surfactin that the bacteria produces.

Protocol

MAGE in Bacillus subtilis 168

Materials:

- Electroporation competent cell
    - *WT*
    - *ΔmutS::beta-neo^R*
- Oligos
    - B_Sub_Mods0007.1mutationrpsL (used as COS oligo)
    - Oligo_surf_Asp->Asn_1_long
    - oligo_surf asp->Asn_2_I
- Linearized pDG268neo_gp35 (positive control)
- Electroporation cuvettes (from Bio Rad 0.2 cm gap)
- Elekctroporation media
- Recovery media
- Plates
    - LB
    - LB + 500y strp

Procedure:

1. In all sample *ΔmutS::beta-neo^R* was used. Duplicates with both Oligo_surf_Asp->Asn_1_long and oligo_surf asp->Asn_2_I was made.
2. For positive control we used WT strain treated with Linearized pDG268neo_gp35 and COS
3. Electroporation was done following the protocol with following exceptions:
    - Only one cycle was done.

- By mistake cells were shortly (approx. 1 min) put on ice 45 min before incubation was done
- After final incubation cell was on ice for 1 hour prior to plating, due to problems in the lab.

4. OD was measured for all the samples.
5. We made glycerol stocks of all the sample
    - 500 ul glycerol was mixed with 500 ul sample
6. Each sample was diluted to 10^3, 10^4 and 10^5 and 100 ul of this was plated on LB and LB + 500y strep
7. Plated were incubated O/N at 37 degC
8. Number of CFUs was counted 14.09.15

Data:

Sample (Oligo #duplicat)	Runtime (ms)
Oligo_surf_Asp->Asn_1_long #1	5.4
oligo_surf asp->Asn_2_I #1	5.6
Oligo_surf_Asp->Asn_1_long #2	5.4
oligo_surf asp->Asn_2_I #2	5,6
Positive control	5.2
Negative control	5.4

Sample (Oligo #duplicat)	OD600
Oligo_surf_Asp->Asn_1_long #1	0.31
oligo_surf asp->Asn_2_I #1	0.34
Oligo_surf_Asp->Asn_1_long #2	0.48
oligo_surf asp->Asn_2_I #2	0.57
Positive control	3.8
Negative control	0.53

CFUs on LB plates

Sample (Oligo #duplicat)	No dilution	10^3	10^4	10^5
Oligo_surf_Asp->Asn_1_long #1		Uncountable	202	25
oligo_surf asp->Asn_2_I #1		Uncountable	98	12
Oligo_surf_Asp->Asn_1_long #2		Uncountable	159	12
oligo_surf asp->Asn_2_I #2		Uncountable	154	13
Positive control	No growth			
Negative control	Over grown			

CFUs on 500y strep plates: No CFUs was observed (checked again 15.09.15, but no CFUs were observed).

Results and conclution

The experiment was not successful, e.i. the COS oligo did not get integrated. We will not screen any of the samples.

MAGE 2 of surfactin with COS oligos: 16.09.15

Preformed by Vilhelm and Viktor, e-lab notes: Viktor.

Purpose

This is a final try to see if we are able to change the NRP surfactin before the wiki freeze. Since the last attempt was not successful, we used a large amount of oligos. We tried to different amounts of surfactin oligos and COS oligos. Only *ΔmutS::beta-neo*^{R} was used as it to be the most efficient recombinase. The purpose is not to get any information about the efficiency, but only to see if we can get some transformants that we can screen using LC/MS.

Protocol:

MAGE in Bacillus subtilis 168

Materials:

- Electroporation competent cell
    - *WT*
    - *ΔmutS::beta-neo*^{R}
- Oligos
    - B_Sub_Mods0007.1mutationrpsL (used as COS oligo)
    - Oligo_surf_Asp->Asn_1_long
    - oligo_surf asp->Asn_2_I
- Linearized pDG268neo_gp35 (positive control)
- Electroporation cuvettes (from Bio Rad 0.2 cm gap)
- Recovery media
- Plates
    - LB
    - LB + 500y strp

Procedure:

1. In all sample *ΔmutS::beta-neo*^{R} was used. Duplicates with both oligo_surf_asp->Asn_1 and oligo_surf asp->Asn_2_I was made.
2. For positive control we used a WT strain treated with Linearized pDG268neo_gp35
3. Negative control we used a WT strain not treated with any oligo.
4. Following electroporations was done:

Strain	Sample (Oligo #duplicat)	Oligo amount (ul)	COS amount (ul)

ΔmutS::beta-neo^R	**Oligo_surf_Asp->Asn_1_long #1**	20	2
	oligo_surf asp->Asn_2_I #1		
	Oligo_surf_Asp->Asn_1_long #2	10	1
ΔmutS::beta-neo^{R*}	**oligo_surf asp->Asn_2_I #2**	5	0.5
WT	**Positive control**	20	0
	Negative control	0	0

*Only 50 ul of competent cells was left, this is why only half amount of oligo.

- Only one cycle was done.
- After final incubation cell was on ice for 30 min hour prior to plating, due to problems in the lab.

5. 200 ul of each sample (also controls) was plated onto LB and LB + 500y strep
6. Plated were incubated O/N at 37 degC
7. Number of CFUs was counted 17.09.15

Data:

Sample (Oligo #duplicat)	Runtime (ms)
Oligo_surf_Asp->Asn_1_long #1	4.6
oligo_surf asp->Asn_2_I #1	4.6
Oligo_surf_Asp->Asn_1_long #2	4.6
oligo_surf asp->Asn_2_I #2	5.2
Positive control	4.8
Negative control	4.3

CFU count		
Sample (Oligo #duplicat)	**LB**	**LB + 500Y STREP**
Oligo_surf_Asp->Asn_1_long #1	Over grown	
oligo_surf asp->Asn_2_I #1		1
Oligo_surf_Asp->Asn_1_long #2		1

oligo_surf asp->Asn_2_I #2		
Positive control		
Negative control		

Results and conclution

LB plates were over grown and two colonies on 500y strep plates had grown. These two colonies were resteaked onto 500y strep and was analyzed with LC/MS. The LB plates were send as well hoping that we will be able to differentiate the WT sufactin and the modified surfactin.