iGEM 2015 – Microbiology – BMB – SDU

Project type: Construction **Project title: Constructing a CyaA knockout**	**Creation date: 9/6-2015** **Written by:** ADK, CEM, EMT **Performed by:** EMT, ADK, AC

1. SOPs in use

iGEM 2013 SOP010 Phusion PCR
iGEM 2015 SOP0013 Electroporation
iGEM 2013 SOP0021 Colony PCR with My Taq
iGEM 2015 SOP0004 Phage Transduction
iGEM 2014 SOP0009 Transformation

2. Purpose

To create a knockout of the CyaA gene in MG1655. This is done to get a bacterial strain which we can use to use and test the Two-Hybrid System.

3. Overview

Date (DD.MM.YY)	**Person(s) (initials)**	**Experiments**	**SOPs**
09.06.15	MMM,EMT,KAT	Phusion PCR on DNA template	iGEM2013_SOP010_v01_ phusion PCR
10.06.15	MMM,EMT	Phusion PCR on DNA template	iGEM2013_SOP010_v01_ phusion PCR

11.-29.06.15	MMM, EMT, AC	- Phusion gradient PCR - Phusion PCR (GC buffer) - MyTag PCR - MyTag PCR gradient - MyTag PCR (new program 1 DEL) - MyTag PCR (new program 1 DEL, lower conc. of primer) - Physion PCR (new program 2 DEL, + GC/HF buffer + MgCl2)	iGEM2013_SOP010_v01_ phusion PCR
02.07.15	ADK	Electroporation	iGEM 2015 SOP0013
03.07.15	ET, AC	Culture transfer	
07.07.2015	ADK, EMT	Electroporation	iGEM 2015 SOP0013
09-07-2015	AC, ADK	Electroporation	iGEM 2015 SOP0013
10-07-2015	ADK, EMT	PCR (Need more product)	iGEM2013_SOP010_v01_ phusion PCR
11-07-2015	ADK	PCR - the concentration from yesterday wasn't high enough Elektroporation	iGEM 2015 SOP0013
12-07-2015	CEM	PCR of PDK3 and PDK4	iGEM2013_SOP010_v01_ phusion PCR
13-07-2015	ADK	PCR of PDK3 and PDK4	iGEM2013_SOP010_v01_ phusion PCR
15-07-2015	ADK	PCR of PDK3	iGEM2013_SOP010_v01_ phusion PCR
16-07-2015	ADK, TBA	Electroporation	iGEM 2015 SOP0013
17-07-2015	EMT	PCR of PDK3	iGEM2013_SOP010_v01_ phusion PCR
18-07-2015	ADK, TBA	Electroporation	iGEM 2015 SOP0013
22-07-2015	TBA	colony PCR with MyTaq and primer 011 and 012 (cyaA F and cyaA R)	iGEM2013_SOP0021_v01_ My Taq

06-08-2015	ADK, TBA	PCR with new primers of PDK3, PDK4, MG1655 (downstream and upstream)	iGEM2013_SOP010_v01_ phusion PCR iGEM2013_SOP0021_v01_ My Taq
07-08-2015	ADK, TBA, JSP	Phusion PCR of pKD4+upstream and pKD4+downstream	iGEM2013_SOP010_v01_ phusion PCR
08-08-2015	ADK	phusion PCR of pKD3+upstream and pKD3+downstream	iGEM2013_SOP010_v01_ phusion PCR
08-08-2015	TBA	phusion PCR of pKD4-upstream+downstream, pKD4-downstream+upstream, G25 primer 21+22, G25 primer 23+24	iGEM2013_SOP010_v01_ phusion PCR
09.08.2015	ADK, TBA	MyTaq PCR of downstream MG1655, upstream MG1655 to verify if we use the right primers.	iGEM2013_SOP0021_v01_ My Taq
09.08.2015	ADK, TBA	MyTaq PCR of pKD3+upstream, pKD3+downstream, pKD4+upstream, pKD4+downstream	iGEM2013_SOP0021_v01_ My Taq
09.08.2015	ADK, TBA	MyTaq PCR of -pKD3-upstream+downstream -pKD4-upstream+downstream -pKD3-downstream+upstream -pKD4-downstream+upstream	iGEM2013_SOP0021_v01_ My Taq
10.08.2015	ADK, TBA	Electroporation of pDK4+up (G33/G40), pKD4+down (G34/G41), pKD3+up (G39)	iGEM 2015 SOP0013
10.08.2015	ADK, TBA	MyTaq PCR of the colonies pDK4+up (G33/G40), pKD4+down (G34/G41), pKD3+up (G39) from the electroporation	iGEM2013_SOP0021_v01_ My Taq
12.08.2015	EMT	Phagh transduktion on BW25113:delta-CyaA :: KAN BW25113:delta-CyaA :: CLM	iGEM2015_SOP0004_v01
14.08.2015	EMT	Phagh transduktion on BW25113:delta-CyaA :: KAN BW25113:delta-CyaA :: CLM	iGEM2015_SOP0004_v01

15.08.2015	EMT	Colony PCR with MyTaq on MG1655:delta-CyaA :: KAN and MG1655:delta-CyaA :: CLM	iGEM2013_SOP0021_v01
16.08.2015	EMT	Transformation of MG1655:delta-CyaA :: KAN and MG1655:delta-CyaA :: CLM with pcp20	iGEM2014_SOP0009_v01
17.08.2015	EMT	Colony PCR with MyTaq on MG1655:delta-CyaA :: KAN MG1655:delta-CyaA :: CLM MG1655 MG1655:delta-CyA x 16	iGEM2013_SOP0021_v01

4. Materials required.

Materials in use

Name	**Components (Concentrations)**	**Manufacturer / Cat. #**	**Room**	**Safety considerations**
	Primer: 013 and 014 DNA template: pKD3 and pKD4			
	Primer: 013 and 014 DNA template: pKD3 and pKD4			
	E. coli strain BW25113, DNA G12 LB media Arabinose			
	Primer: 013 and 014 DNA template: G12			
	E. coli Strain MG1655 Primer: 013 and 014, 021 and 022,			

	023 and 024 DNA template: PDK3 and PDK4			

5. Experiment history

Date (DD.MM.YY)	**Person(s) (initials)**	**Alterations to SOPs and remarks to experiments**	**SOPs**
09.06.15	MMM,EMT,KAT	Phusion PCR on DNA template	iGEM2014_SOP0010_v01_Phusion PCR
10.06.15	MMM,EMT	Phusion PCR on DNA template	iGEM2014_SOP0010_v01_Phusion PCR
11.-29.06.15	MMM, EMT, AC	- Phusion gradient PCR temp range 55-65 C - Phusion PCR (GC buffer) - MyTag PCR - MyTag PCR gradient temp range 55-65 - MyTag PCR (new program 1 DEL) - MyTag PCR (new program 1 DEL, denaturation temp 95 C, annealing temp 55-65 , lower conc. of primer) - Physion PCR (new program 2 DEL, 5 x cycles denaturing 98 C annealing temp 55 C, 30x cycles denaturing 95 C annealing temp 65 C, + GC/HF buffer + MgCl2)	iGEM2014_SOP0010_v01_Phusion PCR
02-07-2015	ADK, EMT, CEM	Done according to the SOP, zap with 200Ω, 1,8kv, 25µFD	
03-07-2015	EMT, AC	Wrong selection plates used (only one antibiotic), cultures transferred to liquid media containing chloramphenicol and ampicillin.	

04-07-2015	AC	1 mL culture transferred to solid selection media.	
07-07-15	ADK, EMT	Done according to the SOP, zap with 200Ω, 1,8kv, 25µFD - Plate out on 30ᵞamp/ 25ᵞcml plates	SDU iGEM 2015 SOP0013
09-07-15	AC, ADK	Done according to the SOP, but in the previous we have used the wrong curvets and we incubated at 30 degrades, here we used the rights curvets and incubated at 37degrades - Plate out on 25ᵞcml plates - zap with 200Ω, 1,8kv, 25µFD	SDU iGEM 2015 SOP0013
10-07-2015	EMT, ADK	- Physion PCR (new program 2 DEL, 5 x cycles denaturing 98 C annealing temp 55 C, 30x cycles denaturing 95 C annealing temp 65 C, + GC/HF buffer + MgCl2)	iGEM2014_SOP0010_v01_Phusion PCR
11-07-2015	ADK	- Physion PCR (new program 2 DEL, 5 x cycles denaturing 98 C annealing temp 55 C, 30x cycles denaturing 95 C annealing temp 65 C, + GC/HF buffer + MgCl2) and made a MyTaq PCR - MyTaq: 4µlDNTP, 2µl forward primer, 2µl reverse primer, 1µl template, 10,5µl mytaq Elektroporation: Used 98ng, 198,4ng, and 294ng DNA cyaA (G14), put aribinose in at OD_{450} =0,613 Made 2 curvets without DNA, to control, one to a cml plate and one to a clean plate	iGEM2014_SOP0010_v01_Phusion PCR
12-07-2015	CEM	Plated out 100µl of the sample from yesterday, on plates with cml. (And on plates with amp, by mistake) - for longer phenotype ekspression PCR of pKD3 and pKD4	

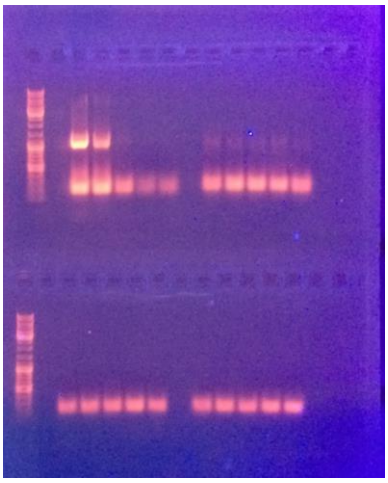
13-07-2015	ADK	PCR of pKD3 and pKD4 - we have a suspicion that we might have messed up the sample - Made with MyTaq and with phusion (like 11-07-15)	iGEM 2013 SOP0021
15-07-2015	ADK	PCR of pKD3 made to mastermix with: 50µl CG buffer, 20µl dnt mix, 10µl forward primer, 20µl reverse primer, 5µl pdk3 template, 10µl MgCl, 140µl H_2O, 2.5µl DNA polymerase - program: Del	iGEM 2013 SOP0021
16-07-2015	ADK, TBA	Electroporation at two different ODs; OD = 0,3 and OD = 0,6. Four different samples; negative control (-), positive control ('+', containing 10 ng of R35), 240 ng of G17 (pKD3) and 480 ng of G17 (pKD3). Phenotypical expression for >2 hours in incubator. For OD = 0,3: the cells where spund down, the supernantant discarded and resuspended in remaining supernantant. 100 µL where plated out on Cml-plates with 0,2% glucose. For the negative control, the cells where plated out on regular LA-plates (without glucose). For OD = 0,6: 100 µL where plated out from the 1 mL cell solution (the negative control on regular LA). The remaining of the four samples was left at room temperature for the following day. All 8 plates where left in the incubator @ 37 degrees	iGEM 2015 SOP0013
18-07-15	ADK, TBA	Added arabinose at OD=0,3, let the culture incubated with the aribonse for hour instead of a half. Made four different samples, one with 935ng of G19, one with 467,5ng of G19, a negative (-) control, and a positive control ('+', containing 10 ng of R35) - Plated the sample out on cml and glucose - plated a little of the 935ng G19 samlpe out on a kan+ glucose to check if somehow messed up PDK3 with PDK4 - Left some of the samples to plate	iGEM 2015 SOP0013

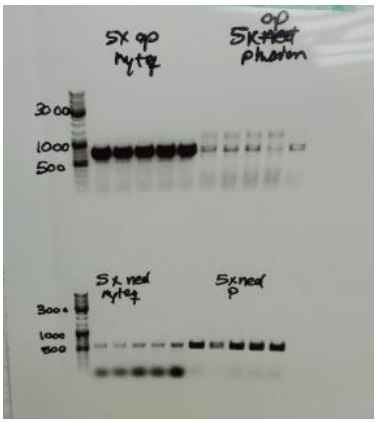
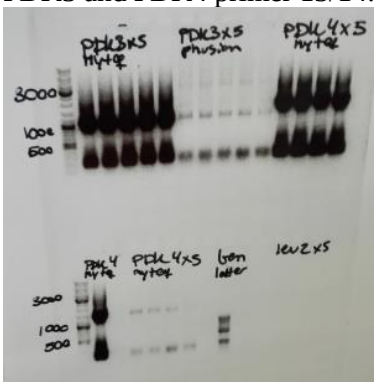
		tomorrow, for ekstra phenotype ekspression	
06-08-2015	ADK	-All PCR reaction were made both with phusion PCR and MyTaq. - Phusion PCR:10µl HF buffer, 1µl dNTPs, 0,5µl DNA template, 2,5µl FW-primer, 2,5µl R-primer, 4µl $MgCl_2$, 0,5µl DNA polymerase, 29µl H_2O - MyTaq: 25µl MyTaq, 5µl FW-Primer, 5µl R-primer, 15µl H_2O, 0,5 DNA template Program (DEL) for PDK3 and PDK4: 95^0C 3min, (95^0C 20S - 55^0C 20S - 72^0C 1min) x6, (96^0C 30S - 65^0C 20S - 72 1min) x31, 72^0C 5min Program for MG1655 up- and downstrem: 98^0C 2min, (98^0C 10S, 55^0C 20S, 72^0C 20S) x30, 72^0C 7min, 4^0C	iGEM2013_SOP0021_v01_ My Taq iGEM2013_SOP010_v01_ phusion PCR
07-08-2015	TBA, JSP	Phusion PCR on results from yesterday. 4 PCR mixes were prepared, 4 µL of MgCl2 added; 1: templates = downstream cyaA (1µL from G25 added) + pKD3 (1µL from G31 added) (kan-cassette) using primers = 13 + 24 2: templates = upstream cyaA (1 µL from G27 added) + pKD3 (1 µL from G31 added) (kan-cassette) using primers = 14 + 21 3: templates = downstream cyaA (1 µL from G25 added) + pKD4 (0,5 µL from G30 added) (cml-cassette) using primers = 13 + 24 4: templates = upstream cyaA (1 µL from G27 added) + pKD4 (0,5 µL from G31 added) (cml-cassette) using primers = 14 + 21 Elongation time changed to 1:15 min	iGEM2013_SOP010_v01_ phusion PCR
08.08.15	ADK	phusion PCR of: - pKD3+upstream, made from G32 and G28, primer 014 and 021 - pKD3+downstream, made from	iGEM2013_SOP010_v01_ phusion PCR

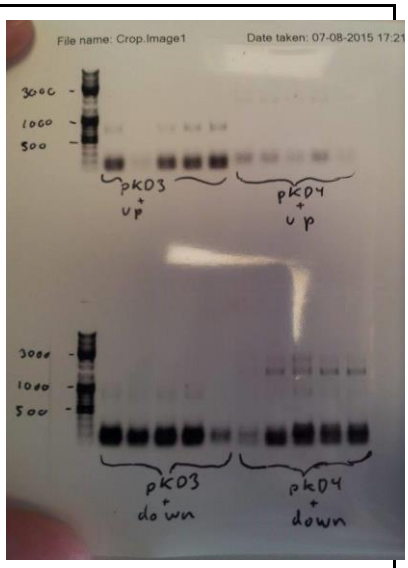
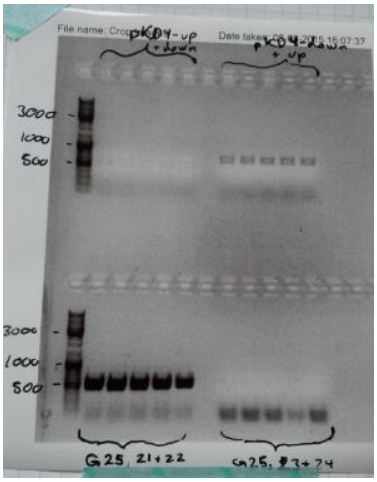
		G32 and G25, primer 013+024 - Phusion PCR Mix:10µl HF buffer, 1µl dNTPs, 0,5µl DNA template, 2,5µl FW-primer, 2,5µl R-primer, 4µl $MgCl_2$, 0,5µl DNA polymerase, 29µl H_2O - Program: 98^0C 2min, (98^0C 10S, 55^0C 20S, 72^0C 20S) x30, 72^0C 7min, 4^0C	
08-08-15	TBA	phusion PCR of pKD4-upstream+downstream made from G33+G25 (primer 21 +24), pKD4-downstream+upstream made from G34+G28 (primer 21 + 24). G25 primer 21+22, G25 primer 23+24 to check if we use the right primers	iGEM2013_SOP010_v01_ phusion PCR
09.08.2015	ADK, TBA	MyTaq PCR of downstream MG1655, upstream MG1655 to verify if we use the right primers. Used Primer 21/22 to upstream, primer 23/24. Mix: 25µl MyTaq, 5µl primer, 5µl primer, 15µl H_2O, 0.5 template	iGEM2013_SOP0021_v01_ My Taq
09.08.2015	ADK, TBA	MyTaq PCR of pKD3+upstream (primer 14/21), pKD3+downstream (primer 13/24), pKD4+upstream (13/21), pKD4+downstream (13/24). Mix: 25µl MyTaq, 5µl primer, 5µl primer, 15µl H_2O, 0.5 template	iGEM2013_SOP0021_v01_ My Taq
09.08.2015	ADK, TBA	MyTaq PCR of -pKD3-upstream+downstream (21/24) -pKD4-upstream+downstream (21/24) -pKD4-downstream+upstream (21/24)	iGEM2013_SOP0021_v01_ My Taq
10.08.2015	ADK, TBA	Electroporation of pDK4+up (G33/G40), pKD4+down (G34/G41), pKD3+up (G39) in *E. coli* BW25113. Done according to the SOP. Made a positive control with plasmid R71, and a negative control without insert. Will be plated out on LA plates with KAN/CML according to the backbones resistance.	iGEM 2015 SOP0013

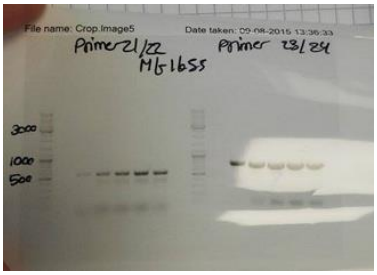
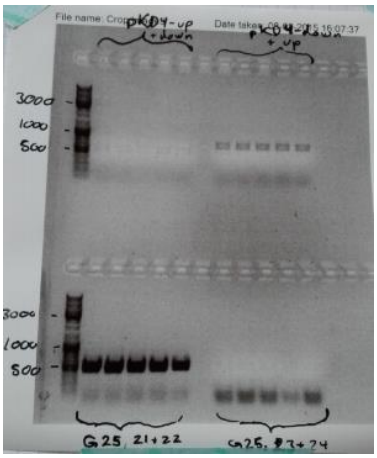
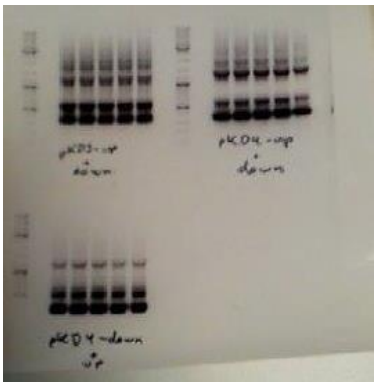
11.08.2015	ADK, TBA	MyTaq PCR of the colonies pDK4+up (G33/G40), pKD4+down (G34/G41), pKD3+up (G39) from the electroporation. Made according to the SOP.	iGEM2013_SOP0021_v01_ My Taq
12.08.2015	EMT	Phag transduction: Lysate made in BW25113:delta-CyaA::KAN and BW25113:delta-CyaA::CML with P1. BW25113:delta-CyaA::CML (was lost) Done according to SOP. Transduction of BW25113:delta-CyaA::KAN lysate in MG1655	iGEM2015_SOP0004_v01
14.08.2015	EMT	Lysate made in BW25113:delta-CyaA::KAN and BW25113:delta-CyaA::CML Done according to SOP Phag transduction: Lysate of BW25113:delta-CyaA::KAN and BW25113:delta-CyaA::CML were use to make 4 sapmpels each (done according to SOP)	iGEM2015_SOP0004_v01
15.08.2015	EMT	Colony PCR with MyTaq 3 colonies on each plate were used, giving a total of 12 samples. given the nr. 1-12 (done according to SOP)	iGEM2013_SOP0021_v01
16.08.2015	EMT	Transformation done according to SOP, on MG1655:delta-CyA:KAN and MG1655:delta-CyA:CML	iGEM2014_SOP0009_v01
17.08.2015	EMT	Colony PCR with MyTaq a total of 19 samples were made: 1- WT 2- MG1655:delta-CyA::KAN 3- MG1655:delta-CyA::CML 4 to 16 MG1655: delta-CyA	iGEM2013_SOP0021_v01
18.08.2015	EMT	Transformation of MG1655:delta-CyA with R49	
19.08.2015	EMT	Transformation of MG1655:delta-CyA with R81, R82 and R81+R82	

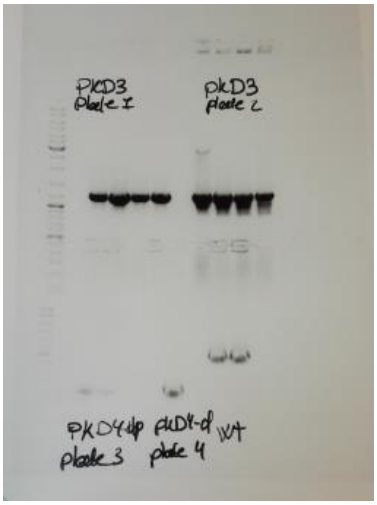
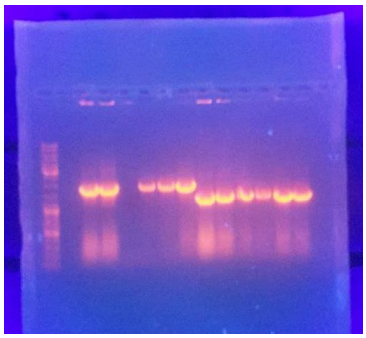
6. Results

Date (DD.MM.YY)	Picture	Comments
9/6-15	Gel: Failed no bands at all	
10.06.15	Gel: Failed no bands at all	
11.-29.06.15	Gel: all attemps failed, showing broad band of primer dimer Gel: Physion PCR (new program 2 DEL) gave result for PDK3 + GC	
03-07-2015	Electroporation and Culture transfer	Failed
09-07-2015	No cultures on plates	
10-07-2015	No cultures on plates PCR: 8,5ng/µl	
11-07-2015	PCR: 24,5 ng/µl Elektroporation: No culture on the plate	
13-07-2015	Elektroporation: No culture on the plate PCR from yesterday: Primer dimers PCR from today: PDK3 with phusion worked, the other failed. Concentration:15ng/µl	
15-07-2015	phusion PCR of PDK3, concentration: 38,5ng/µl	
16-07-2015	colonies on positive and negative control - the rest failed	
17-07-2015	PCR of G17 got two concentration G18=13,9ng/µl, G9=37,4ng/µl	

20-07-2015	Electroporation - got colonies on positive control plates and on kanamycin + glucose.	
22-07-2015	colony PCR: got 3 bands from the PCR - will try with an aneling temp. on 60^0 instead of 55^0.	
23-07-2015	We concluded that the electorporation haven't worked at any time. We will make new primers, so we can make longer overhangs. We designed primers to 500bp downstream from CyaA and 500bp upstream from CyaA	
06.08.2015	PCR with new primers of PDK3, PDK4, MG1655 (downstream and upstream) - all PCR reactions were successful - MyTaq worked best on PDK4, and PKD3	Upstream (primer 23/24)/ downstream (primer 21/22) MG1655:  PDK3 and PDK4 primer 13/14: 

07-08-2015	Inconclusive results on the PCR-reactions on pKD3 with upstream or downstream cyaA. Bands extracted from pKD4 + up and stored as G33 Bands (the ones in 'the middle') extracted from pKD4+down and stored as G34	
08.08-15	phusion PCR of pKD3+upstream, pKD3+downstream - purified the samples from the gel, got bad concentrations: pKD3+Upstream (G35)=5,4ng/µl, pKD3+downstream (G36)=4,9ng/µl - we will continue with pKD4 up/down	
08.08.15	Not certain about the results, need to discuss the results in the group - have saved the enterie gel in the refrigerator.	
09.08.2015	Primer 21/ 22: upstream Primer 23/24: downstream	

		
09.08.2015	MyTaq PCR of pKD3+upstream (primer 14/21), pKD3+downstream (primer 13/24), pKD4+upstream (13/21), pKD4+downstream (13/24). Got following concentration: - pKD4+UP (G33)= 7,8ng/µl - pKD4+down (G34)=8,8ng/µl - pKD3+UP(G35)=5,4ng/µl - pKD3+down (G36)=4,9ng/µl	 
09.08.2015	MyTaq PCR of -pKD3-upstream+downstream -pKD4-upstream+downstream -pKD4-downstream+upstream Failed to get appropriate bands.	

11.08.2015	Electroporation. Got colonies of BW25113 with G39 (pKD3-up)2µl and 10µl, G40(pKD4-up)2µl, G41(pKD4-down) 2µl. MyTaq colony pcr verified the colonies. WT=BW25113	
12.08.2015	Phagh transduction; failed MG1655:delta-CyaA::KAN - No colonies	
14.08.2015	Phagh transduction succeeded	
15.08.2015	Colony PCR of Phagh transduction MG1655:delta-CyaA::KAN MG1655:delta-CyaA::CLM As seen on the gel, well 5 did not work, but the rest of the PCR's did. The first wel is Mix-lader, the 3 to 8 wel is MG1655:delta-CyaA::KAN and well 9-14 is MG1655:delta-CyaA::CLM	
17.08.2015	Colony PCR with MyTaq:	
18.08.2015	Transformation with R49 failed, the culture was contaminated.	
19.08.2015		

7. Appendices