WP_Contruction of interspecific expression system (IES)

Date: 2020-02-05
Tags: *Cloning IES*
Created by: Maarten Verhoeven

Summary: In order to enable genetic modification of bacteria in wastewater treatment a system for expressing heterologous genes in both gram positive and gram negative bacteria is required. The present workplan describes work planned to design and construct the vector containing a interspecific expression system that allows for expression of selection markers in multiple species.

Background: Wastewater treatment currently relies on complex microbial communities that are formed from microorganism originated from the environment. Through application of selection pressures (e.g. sludge retention time, aeration rate, temperature etc..). While this method is excellent for removal of most of the organic compounds that are present at high(er) quantities in wastewater, micro-pollutant (small molecules present in low quantities) are often not removed sufficiently. As a result these pollutants are often released into the environment, which in some cases can have severe detrimental effects. A cost-effective option to remove micro-pollutants could be through bio-augmentation, the introduction of microbial species or strains that have capacity to degrade specific pollutants into the community. The greatest challenge for successful bio-augmentation is in obtaining microorganisms that can degrade the pollutants while concurrently also sustaining growth within the activated sludge community. Introduction of exogenous microorganisms have so far had limited success due to their low survival after prolonged cultivation. An alternative approach is to generate recombinant strains from species that already have a dominant presence in the activated sludge environment. Recombinant engineering has initially been successful for a relatively small set of organisms, with *Escherichia coli* and *Saccharomyces cerevisiae* most commonly used. Due to advancements in DNA sequencing and synthetic biology, the number of genetically accessible microorganisms has increased gradually. However, apart from one study in 2002, the utilization of genetic modification in wastewater treatment has been largely unexplored. As part of the Rubicon project I will be exploring whether we can apply recombinant technology to construct strains that can be used to augment wastewater treatment plants. Successful recombinant bio-augmentation requires that the host microorganism is genetically accessible.Therefore, a novel screening method will be developed that can be used to select the species capable of expressing transfected DNA.

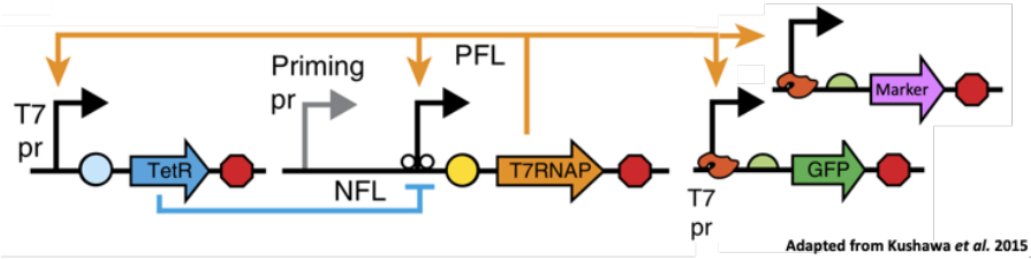
Aim of the workplan: The screening method that will be developed will require an gene expression system that allows for expression in a wide variety of host organisms. The aim of the experiments that are associated to this workplan is to design and construct an interspecific expression system (**IES**) that allows for expression of a green fluorescent protein (GFP) in wide variety of bacterial species. Expression of GFP can subsequently be used to identify possible bio-augmentation candidates that are genetically accessible and thus could be used to introduce pathways for

WP_Contruction of interspecific expression system (IES)

Date: 2020-02-05
Tags: *Cloning IES*
Created by: Maarten Verhoeven

degradation of micro-pollutants.

Approach and activities: The IES will require several components to allow for expression of GFP regardless of the host's own expression machinery. Kushawa *et al.* 2015 describes such a system in their study on the design and testing of a portable expression system. In the present study we will adapt their design to use it for screening of genetic accessibility. For the IES to work properly, several components will need to be selected, designed and constructed:



Schematic overview of the IES. The selection marker and GFP are expressed using the T7 promoter (T7 pr). A broad-host-range priming promoter activates T7 RNA polymerase (T7RNAP) expression. Stable non-toxic expression of T7RNAP is ensured through positive and negative feedback loops (PFL and NFL) that utilize the tetracycling repressor (TetR) and T7 promoter itself.

- GFP coding sequence (744bp): The GFP coding sequence is used widespread. While such a sequence should in theory work fine, it could be worth it to check the codon usage this the chosen CDS using codon optimization tools such as JCat.
- Selection marker sequence: In order to be able to select successful transformants after transfection the IES will include a antibiotic selection marker. The choice of the selection marker will be highly dependent on the results of meta-genome assembled genome (MAG) data mining. This should be able to provide an overview on which antibiotic resistance (e.g. Ampiciline, Kanamycine, Chloramphenicol) are commonly present in WWTP samples. based on this overview one or more selection markers can be selected, codon optimized and synthesized.
- Transcription priming promoter sequence : While expression of the IES should operate independently from the host cell transcription machinery once active, the system does require initial activation (i.e. the transcription of the T7 RNA polymerase). Kushawa *et al*. employed a priming promoter sequence for this (see figure below). This sequence was originated from *S. cerevisiae* and contains several bacterial putative transcription starting sites. In their research

WP_Contruction of interspecific expression system (IES)

Date: 2020-02-05
Tags: *Cloning IES*
Created by: Maarten Verhoeven

this sequence was able to start transcription sufficiently for the T7 RNA polymerase (see attached supplementary data file from Kushawa *et al.*, figure 1 for actual sequence). For our study the same or a similar sequence could be used. Important is that the sequence contains one or more putative transcription starting sites.

- T7 promoter sequence: With only 23 bp in length the T7 polymerase in convenient for cloning purposes. Additionally, it should only be recruiting the T7 RNA polymerase, making the expression mostly independent from the host transcription machinery. An additional T7 promoter will be needed that contains the tetracycline binding domain.
- T7-RNA polymerase coding sequence (2697bp): The T7 polymerase should be regulated used both a T7 promoter as well as a repressor to avoid too high expression of the polymerase. Both these positive and negative feedback loops should stabilize the expression of the polymerase. Kushawa et. al. did not use codon optimization for this sequence. In our case we could opt to do so.
- Tetracycline repressor (669bp): The tetracycline repressor ensures that expression of the T7 polymerase (which is self-driven) does not get out of hand.
- Terminator sequence(s): Kushawa *et al.* used short transcription termination signals BBa_B0015 and BBa_B1002 that are only 129bp and 45bp respectively (See supplementary material table 4 for more details).
- Vector replication origin: In order to ensure that the vector is sustained in both cells after division a replication origin sequence is required. In the table below an overview of the most used broad-host-range plasmids are shown. In order to maximize the probability that the expression vector is replicated in a preferred host, a cocktail of multiple vector could be used to transform the activated sludge samples. Alternatively a system based on the Phi29 bacteriophage replisome could be used. While this system has not yet been used in-vivo, in vitro engineering on lipid vesicles showed that it has the potential to yield fully orthogonal replication of DNA iGEM TU-Delft. If time permits an initial design using the Phi29 system will be taken along in the assembly process.

WP_Contruction of interspecific expression system (IES)

Date: 2020-02-05
Tags: *Cloning IES*
Created by: Maarten Verhoeven

List of species in which extensively used broad-host-range plasmids are known to replicate

Plasmid group	Species	References
IncP	*Achromobacter parvulus*, *Acinetobacter* spp., *Aeromonas* spp., *Agrobacterium* spp., *Alcaligenes* spp., *Aliivibrio salmonicida*, *Anabaena* spp., *Azospirillum braziliense*, *Azotobacter* spp., *Bordetella* spp., *Caulobacter* spp., Enterobacteriaceae, *Gluconacetobacter xylinus*, *Haemophilus influenzae*, *Hypomycrobium* X, *Legionella pneumophila*, *Methylophilus methyltrophus*, *Methylococcus methanolicus*, *Methylosinus trichosporium*, *Myxococcus xanthus*, *Neisseria* spp., *Paracoccous denitrificans*, *Pseudomonas* spp., *Rhizobium* spp., *Rhodopseudomonas* spp., *Rhodospirillum* spp., *Shewanella* spp., *Thiobacillus* spp., *Xanthomonas campestris*	((14), unpublished data)
IncQ	*Acinetobacter calcoaceticus*, *Actinobacillus pleuropneumoniae*, *Actinomyces naeslundii*, *A. viscosus*, *Aerobacter aerogenes*, *Aeromonas hydrophila*, *Agrobacterium tumefaciens*, *Alcaligenes eutrophus*, *Azotobacter vinelandii*, *Brevibacterium methylicum*, *Caulobacter crescentus*, *Desulfovibrio vulgaris*, *Erwinia carotovora*, *E. chrysanthemi*, *Escherichia coli*, *Gluconacetobacter xylinus*, *Gluconobacter* spp., *Hyphomicrobium* spp., *Klebsiella aerogenes*, *K. pneumoniae*, *Methylophilus methylotrophus*, *Moraxella* spp., *Mycobacterium aurum*, *M. smegmatis*, *Paracoccus denitrificans*, *Pasteurella multocida*, *Porphyromonas gingivalis*, *Proteus mirabilis*, *Providencia* spp., *Pseudomonas* spp., *Rhizobium leguminosarum*, *R. meliloti*, *Rhodobacter sphaeroides*, *R. capsulatus*, *Rhodopseudomonas spheroides*, *Salmonella* spp., *Serratia marcescens*, *Streptomyces lividans*, *Synechococcus* spp., *Thiobacillus ferrooxidans*, *Vibrio salmonicida*, *Yersinia enterocolitica*, *Xanthomonas campestris*, *X. maltophilia*	(39–42)
IncW	*Acinetobacter calcoaceticus*, *Aeromonas liquefaciens*, *A. salmonicida*, *Agrobacterium tumefaciens*, *A. rhizogenes*, *Alcaligenes eutrophus*, *Enterobacter* sp., *Erwinia amylovora*, *E. carotovora* subsp. *Carotovora*, *E. herbicola*, *E. rubrifaciens*, *E. stewartii*, *Escherichia coli*, *Klebsiella* spp., *Legionella pneumophila*, *Methylophilus methylotrophus*, *Myxococcus virescens*, *M. xanthus*, *Proteus rettgeri*, *P. mirabilis*, *Providencia stuartii*, *Pseudomonas* spp., *Rhizobium leguminosarum*, *R. trifolii*, *Salmonella enteritidis*, *S. typhimurium*, *S. ordonez*, *Serratia marcescens*, *Shigella* spp., *Vibrio cholerae*, *Xanthomonas campestris* pv. *campestris*, *X. campestris* pv. *malvacearum*, *Zymomonas mobilis*	(10, 43)
pBBR1 based	*Alcaligenes eutrophus*, *Bartonella bacilliformis*, *Bordetella* spp., *Brucella* spp., *Caulobacter crescentus*, *Escherichia coli*, *Gluconacetobacter xylinus*, *Paracoccous denitrificans*, *Pseudomonas fluorescens*, *P. putida*, *Rhizobium meliloti*, *R. leguminosarum* by. *viciae*, *Rhodobacter sphaeroides*, *Salmonella typhimurium*, *Vibrio cholerae*, *Xanthomonas campestris*	(44, 45)

The list is not exhaustive

All DNA vector designs will be made and tested using the Geneious Prime software suite. Most of the components described in above can be ordered as synthetic constructs. Depending on the length and complexity different methods of DNA synthesis could be used including gBlock oligo synthesis and full vector assembly. DNA synthesis service providers that might be suitable are: IDT, GeneArt, TWIST or SigmaAldrich. After retrieving the synthesized fragments, depending on the supplier additional cloning into a topovector and subsequent quality control using sequencing might be required.

Final assembly of all the components will be preformed using Golden Gate Cloning. All cloning step

WP_Contruction of interspecific expression system (IES)

Date: 2020-02-05
Tags: *Cloning IES*
Created by: Maarten Verhoeven

will be done in E.coli. The final plasmids will need to be verified by DNA sequencing. In order to verify if the IES does perform independent from other expression signals, several control plasmid will have to be constructed in parallel (i.e. without the T7 polymerase or priming promoter sequencing).

the final verification of the expression method will be done in both E. coli and a pseudomonas species as described earlier by Kushawa et al.

Planning:

Activity:	week 1	week 2	week 3	week 4	week 5	week 6	week 7	week 8	week 9	week 10
Obtain Geneious software for *in-silico* design										
Design the IES in Geneious										
Order the DNA fragments from supplier and clone into TOPO-vectors										
Use GGC to assemble fragments into expression vectors										
Verify the system in *E.coli* and *Pseudomonas* species										

Data management:

DNA construct designs will be stored both in the elabjournal database and within Geneious Prime.

Risk mitigation:

Description:	**Preventive action:**	**Alternative action:**

WP_Contruction of interspecific expression system (IES)

Date: 2020-02-05
Tags: *Cloning IES*
Created by: Maarten Verhoeven

The selected selection markers do not result in a adequate selection pressure to prevent growth of non-transfected cells.	In silico search in the MAGs that are available will be done to assess which antibiotic resistance genes are predicted to be already present in typical WWTP communities. Based on this information a pre-selection of suitable antibiotic selection markers can be made.	MIC (Minimum Inhibitory Concentration) tests could be done to determine whether, and if so, at which concentration of added antibiotics microorganisms will be present.
The replication origin that was chosen does not function properly in the desired bio-augmentation candidate(s)	Cassettes will be for the four different replication origin sequences. With golden gate cloning it will be fairly straightforward to build the transformation with the desired replication sequence	It might be possible to construct a species independent replication system using the components from the Phi29 bacteriophage. Combined with the Cre/LOX homologous end joining system this could result in an interesting method of plasmid replication.
The chosen plasmid designs do not yield function expression systems in E.coli or B. subtillus.	All plasmids are based on previously confirmed parts and will be sequenced prior to utilisation	Controls will be taken along for each of the components in the IES and replication sequences. This will be able to shed light on which parts might need redesigns.

Required materials (and non-standard equipment):

- Geneious Prime software: will be used to design the DNA construct *in-silico.*
- Synthesized DNA fragments, can be ordered as fully assembled vectors or as gblocks
- Depending on the specifications of the ordered DNA a blunt-end TOPO cloning vector is required to store the DNA in *E.coli*.
- Golden gate cloning enzyme: BsaI and T4 ligase should be sufficient for this particular cloning method.
- Chemically competent *E.coli* cells.

WP_Contruction of interspecific expression system (IES)

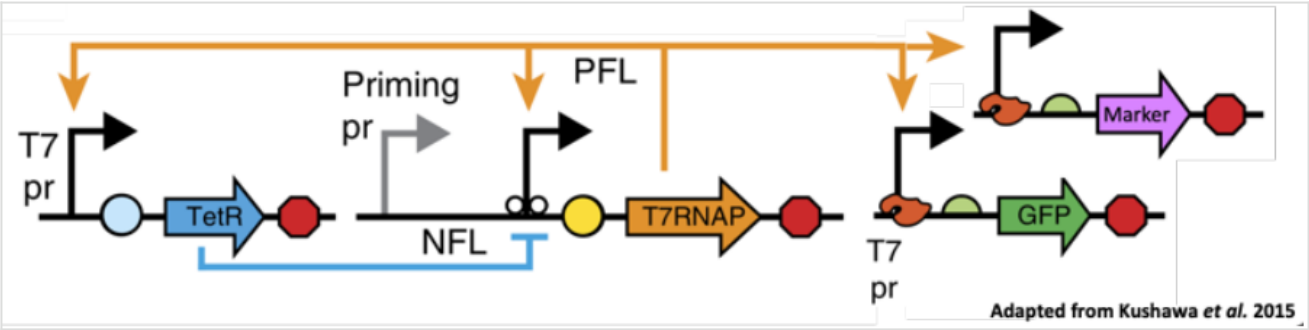
Date: 2020-02-05
Tags: *Cloning IES*
Created by: Maarten Verhoeven

Steps

- Obtain Geneious software for in-silico design (2020-03-16 09:18:06)
- Design the IES in geneious (2020-04-22 10:57:54)
- Order the DNA fragments from supplier (2020-04-22 10:57:58)
- Use GGC to assemble fragments into expression vectors (2020-10-21 11:26:50)
- Verify the system in E.coli and Pseudomonas species (2020-10-21 11:26:58)

Attached files

Untitled.png
sha256: f6708ab3ddde26c1c646d4ac8c8b29350648193545e567998ccbc5caf864edb6



ncomms8832.pdf (Research article, Kushawa et al. 2015)
sha256: 831ede7bd96319eda04e3ea07a36af498c5ed6d06a13b93f83c349109849b829

41467.2015.BFncomms8832.MOESM551.ESM.pdf (Supplementary Data, Kushawa et al.)
sha256: 86f9092ea708f683830d5dfa0c928d46f07a4802fef5180f4316a3982f4e624a

Table.2.vectors.png
sha256: 6cd69e6459d62c92f600fafaccecf1f563eb7b1fee510fd6107144d4df68bbb2

WP_Contruction of interspecific expression system (IES)

Date: 2020-02-05
Tags: *Cloning IES*
Created by: Maarten Verhoeven

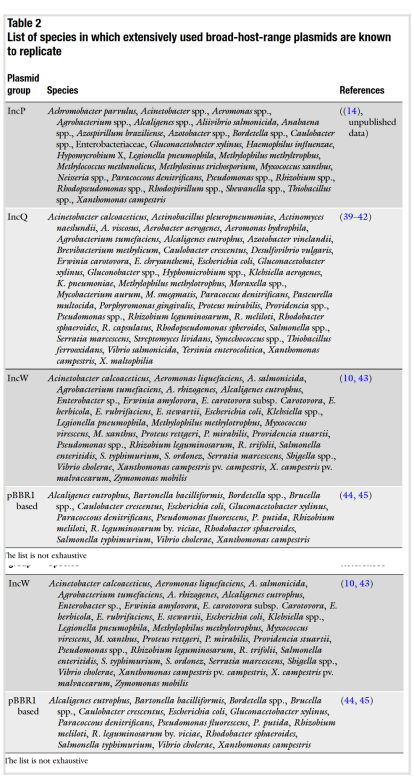


Table 2
List of species in which extensively used broad-host-range plasmids are known to replicate

Plasmid group	Species	References
IncP	*Achromobacter parvulus, Acinetobacter* spp., *Aeromonas* spp., *Agrobacterium* spp., *Alcaligenes* spp., *Allivibrio salmonicida, Anabaena* spp., *Azospirillum brasilense, Azotobacter* spp., *Bordetella* spp., *Caulobacter* spp., Enterobacteriaceae, *Gluconacetobacter xylinus, Haemophilus influenzae, Hyponycrobium* X, *Legionella pneumophila, Methylophilus methylotrophus, Methylococcus methanolicus, Methylosinus trichosporium, Myxococcus xanthus, Neisseria* spp., *Paracoccus denitrificans, Pseudomonas* spp., *Rhizobium* spp., *Rhodopseudomonas* spp., *Rhodospirillum* spp., *Shewanella* spp., *Thiobacillus* spp., *Xanthomonas campestris*	((14), unpublished data)
IncQ	*Acinetobacter calcoaceticus, Actinobacillus pleuropneumoniae, Actinomyces naeslundii, A. viscous, Aerobacter aerogenes, Aeromonas hydrophila, Agrobacterium tumefaciens, Alcaligenes eutrophus, Azotobacter vinelandii, Brevibacterium methylicum, Caulobacter crescentus, Desulfovibrio vulgaris, Erwinia carotovora, E. chrysanthemi, Escherichia coli, Gluconacetobacter xylinus, Gluconobacter* spp., *Hyphomicrobium* spp., *Klebsiella aerogenes, K. pneumoniae, Methylophilus methylotrophus, Moraxella* spp., *Mycobacterium aurum, M. smegmatis, Paracoccus denitrificans, Pasteurella multocida, Porphyromonas gingivalis, Proteus mirabilis, Providencia* spp., *Pseudomonas* spp., *Rhizobium leguminosarum, R. meliloti, Rhodobacter sphaeroides, R. capsulatus, Rhodopseudomonas spheroides, Salmonella* spp., *Serratia marcescens, Streptomyces lividans, Synechococcus* spp., *Thiobacillus ferrooxidans, Vibrio salmonicida, Yersinia enterocolitica, Xanthomonas campestris, X. maltophilia*	(39–42)
IncW	*Acinetobacter calcoaceticus, Aeromonas liquefaciens, A. salmonicida, Agrobacterium tumefaciens, A. rhizogenes, Alcaligenes eutrophus, Enterobacter* sp., *Erwinia amylovora, E. carotovora* subsp. *Carotovora, E. herbicola, E. rubrifaciens, E. stewartii, Escherichia coli, Klebsiella* spp., *Legionella pneumophila, Methylophilus methylotrophus, Myxococcus virescens, M. xanthus, Proteus rettgeri, P. mirabilis, Providencia stuartii, Pseudomonas* spp., *Rhizobium leguminosarum, R. trifolii, Salmonella enteritidis, S. typhimurium, S. ordonez, Serratia marcescens, Shigella* spp., *Vibrio cholerae, Xanthomonas campestris* pv. *campestris, X. campestris* pv. *malvacearum, Zymomonas mobilis*	(10, 43)
pBBR1 based	*Alcaligenes eutrophus, Bartonella bacilliformis, Bordetella* spp., *Brucella* spp., *Caulobacter crescentus, Escherichia coli, Gluconacetobacter xylinus, Paracoccous denitrificans, Pseudomonas fluorescens, P. putida, Rhizobium meliloti, R. leguminosarum* by. *viciae, Rhodobacter sphaeroides, Salmonella typhimurium, Vibrio cholerae, Xanthomonas campestris*	(44, 45)
The list is not exhaustive		
IncW	*Acinetobacter calcoaceticus, Aeromonas liquefaciens, A. salmonicida, Agrobacterium tumefaciens, A. rhizogenes, Alcaligenes eutrophus, Enterobacter* sp., *Erwinia amylovora, E. carotovora* subsp. *Carotovora, E. herbicola, E. rubrifaciens, E. stewartii, Escherichia coli, Klebsiella* spp., *Legionella pneumophila, Methylophilus methylotrophus, Myxococcus virescens, M. xanthus, Proteus rettgeri, P. mirabilis, Providencia stuartii, Pseudomonas* spp., *Rhizobium leguminosarum, R. trifolii, Salmonella enteritidis, S. typhimurium, S. ordonez, Serratia marcescens, Shigella* spp., *Vibrio cholerae, Xanthomonas campestris* pv. *campestris, X. campestris* pv. *malvacearum, Zymomonas mobilis*	(10, 43)
pBBR1 based	*Alcaligenes eutrophus, Bartonella bacilliformis, Bordetella* spp., *Brucella* spp., *Caulobacter crescentus, Escherichia coli, Gluconacetobacter xylinus, Paracoccous denitrificans, Pseudomonas fluorescens, P. putida, Rhizobium meliloti, R. leguminosarum* by. *viciae, Rhodobacter sphaeroides, Salmonella typhimurium, Vibrio cholerae, Xanthomonas campestris*	(44, 45)
The list is not exhaustive		

2011.Book.StrainEngineering.pdf

Unique eLabID: 20200205-9a5f6c867b0cf79886b360c3692b26df6a459649
Link: https://130.225.39.29:443/database.php?mode=view&id=2