Searching for PCA Reducers and Oxidizers

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Introduction

Living organisms use the transfer of electrons between donor and acceptors to fuel their metabolism. For most organisms, redox reactions occur intracellularly. Some microbes, however, are able to transfer electrons from and to external electron donor or acceptors, in a process known as External Electron Transfer (EET) (Kato 2016). EET can be facilitated by various mechanisms, such as direct cell contact (Hernandez and Newman 2001), conductive nanowires (Qian et al. 2011; Malvankar et al. 2011; Leang et al. 2010), or by redox-active electron shuttles (Von Canstein et al. 2008; Marsili et al. 2008).
Bacteria can take advantage of diverse array of redox-active molecules for electron shuttling. Such molecules can be actively secreted by the cells like flavins (Von Canstein et al. 2008) or phenazines (Glasser, Kern, and Newman 2014), or be abundant molecules in the environment, like humic substances (Lovley et al. 1999; Benz, Schink, and Brune 1998; Coates et al. 2002). Phenazines are one group of redox-active molecule which is secreted by *Pseudomonas aeruginosa*, and can help it either transfer electrons to iron as an electron acceptor (Wang et al. 2011), and help cells survive in anaerobic microenvironments within biofilms (Glasser, Saunders, and Newman 2017). Additionally, Phenazines help maintain redox balance (Price-Whelan, Dietrich, and Newman 2007) and conserve energy during fermentation the cell during anaerobic fermentation (Glasser, Kern, and Newman 2014).
Most of the research on phenazine has focused on *Pseudomonas*, but many more organisms are predicted to interact with phenazines (Glasser, Saunders, and Newman 2017). We therefore set out to enrich for, and ultimately isolate, organisms able to reduce or oxidize phenazines. We focus here of phenazine-1-carboxylic acid (PCA), as it is known to be less toxic than other phenazine species, and serves as the precursor for many other phenazines (Mavrodi et al. 1998).

Materials and Methods

Source of soil samples

Fresh soil samples were collected from the top 15 cm in three different locations in the Cape Cod area. The first sample was collected from agricultural soil in a farm located at (41°36'58.9"N 70°34'31.2"W). The second sample was taken from soil at the side of the bike path connecting Woods Hole and Falmouth (41°32'42.8"N 70°37'52.4"W). The third sample was collected from forest soil (41°31'34.3"N 70°39'05.3"W). All samples were collected to a 50 mL Falcon tube and brought to the lab. Soil samples were ground with a mortar and pestle and then sieved through a 2mm sieve to remove large rocks and organic matter. The samples were quickly moved to an anaerobic chamber (Coy) to keep condition as anaerobic as possible.

Medium and culturing conditions

Standard anaerobic culturing techniques were used throughout. Enrichment cultures were incubated in room temperature either in 200 µL in 96 well plates or in balch tubes sealed with thick butyl rubber stoppers. Liquid media were autoclaved to remove dissolved O_2 and then moved to an anaerobic chamber. The basal medium used is based on the Freshwater ("FW") Base (see Appendix), supplemented with 50 µM sodium sulfide as the sulfur source, 10 mM of ammonium chloride as the Nitrogen source, 1 mM of potassium phosphate as the phosphorous source, 1x of 13-Vitamin solution (see Appendix), 1x of HCl-dissolved trace elements (see Appendix) and 45 mM of Sodium bicarbonate as a buffer. Our liquid enrichment medium for phenazine reducers was supplemented with 100 µM sodium acetate as a carbon sources, 10 mM sodium fumarate, and 1 mM of oxidized phenazine-1-carboxylic acid (PCA). Our liquid enrichment medium for phenazine oxidizers was supplemented with 100 µM sodium acetate as a carbon sources, 10 mM sodium nitrate or 10 mM sodium fumarate as the electron acceptor, and 1 mM of reduced PCA. Vitamin mix, sodium sulfide, sodium bicarbonate, sodium nitrate, and sodium fumarate were added from anaerobic stock solutions after autoclaving. Reduced PCA was prepared by gassing balch tubes containing 2 mM of oxidized PCA with H_2-CO_2 (80:20, vol/vol) in the presence of palladium-covered aluminum chips. Plastic tubing was inserted into the batch tubes and connected to the gas source through syringe needles to ensure gas flow over the palladium chips. Gassing was stopped when the color of the liquid turned yellow-green (indicating the reduction of PCA). Solid medium used for preparation of shake-tubes had the same composition of the original enrichment media, and was supplemented with 1.5% Bacto agar. Solid medium for isolation of single colonies from enrichment cultures was the basal medium supplemented with 5mM sodium acetate 1.5% Bacto agar, and either 10 mM of sodium fumarate or 10 mM of sodium nitrate. Sodium sulfide was not added to the medium but the plates were incubated under **COMPOSITION**.

Analytical methods

For analysis of the concentrations of organic acids in the enrichment cultures, 1 mL aliquots were taken from each enrichment and filtered through a 0.2 µm filter. Aliquots were acidified by adding 10% (vol/vol) of 5N H_2SO_4. Concentrations of organic acids were measured high-performance liquid chromatography (HPLC, Shimadzu LC-2010C) with an organic acid analysis column (Aminex HPX-87H) with an eluent of 5 mM H2SO4. Both UV absorbance detector and refractive index detector were used.

For analysis of the concentrations of ions in the enrichment cultures, 50 µL aliquots were taken from each enrichment and diluted into 5 mL of DI water, and then filtered through a 0.2 µm filter. The concentration of nitrate and nitrite were determined with an ion chromatograph (Dionex ICS-2000), with the standard settings used by the course (see Appendix).

The oxidation state of PCA in our cultures was determined qualitatively by a color change. PCA in the oxidized state is clear, whereas reduced PCA is yellow-green. The reduction state of PCA is also correlated to absorbance in 440 nm wavelength. To quantify the reduction state of PCA, we used a spectrophotometer set to 445 nm, and measured the absorbance of our cultures.

Cell concentrations were estimated by filtering aliquots from cultures on 0.22 μm polycarbonate filters, staining them with DAPI, and imaging with epifluorescent microscope under 100x magnification. Cell concentration represents the average of three measurements of cell number on a 0.01 mm^2 grid.

Results

Initial enrichment for PCA oxidizers

Our initial goal was to isolate PCA oxidizing microbes. Enrichments were established by transferring 1-g subsamples from each of the freshly collected soil samples into 9 ml of anoxic medium in a balch tube in an anaerobic chamber, or by transferring 20 mg of fresh soil samples into 190 μL anoxic medium in a 96 well plate. Both nitrate and fumarate were chosen as electron acceptors for PCA oxidation. Positive enrichments were identified by color change of the medium from yellow-green to clear. The following day after inoculating the enrichments, two out of the three initial samples showed a color change in the medium where nitrate served as the electron acceptor, but not when fumarate was the electron acceptor. In addition, the color change was visible only in the 96-well plate format, but not in the balch tubes.

Identification of putative PCA reducing cultures

We hypothesized that the soil might be oxidizing PCA abiotically, and that in our enrichments where fumarate served as an electron acceptor PCA was actually subsequently reduced. To test this hypothesis, we transferred liquid from the fumarate containing media into fresh media with oxidized PCA and 10 mM fumarate. After 24-hour incubation, the culture changed color from clear to yellow-green, suggesting reduction of PCA. We hence started transferring 10% dilutions of this PCA reducing culture in an attempt to isolate PCA reducing bacteria. The phenotype (shift in PCA color) was reproducible throughout all transfers, seven in total. A quantitative measurement of the change in PCA reduction state by the enrichment is presented in Figure 1. The maximal cell concentration achieved by the enrichment was $\approx 3\times10^8$ cells mL^{-1}. Enrichments with the same media composition (100 μM acetate and 10 mM fumarate), but without PCA grew to comparable cells concentrations of $\approx 3\times10^8$ cells mL^{-1}.

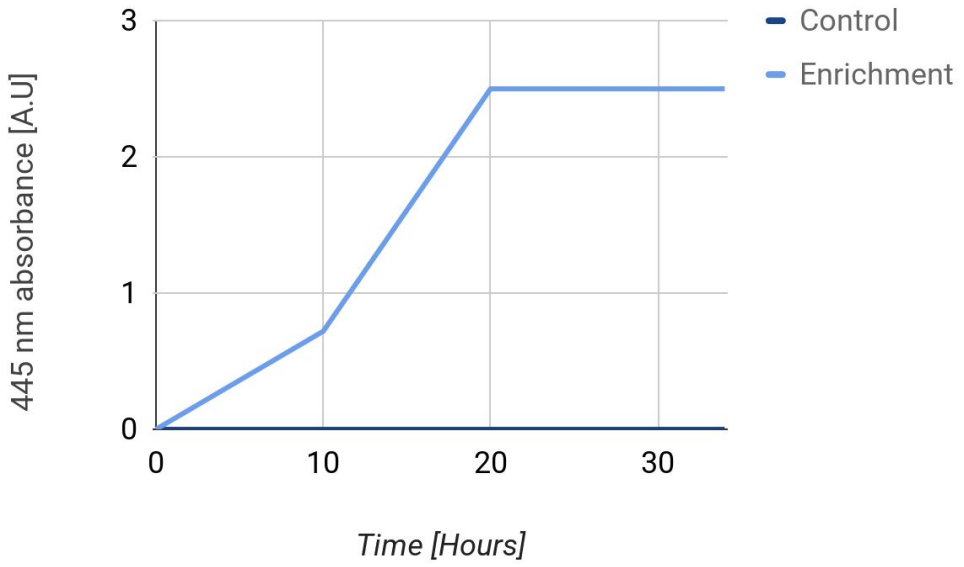

Figure 1 - Tracking the oxidation state of PCA in the putative PCA reducing enrichment. The oxidation state of PCA in control medium, as well as the enrichment were quantified by measuring absorbance at 445 nm. Reduction of PCA with time resembles a typical bacterial growth curve, suggesting reduction of the PCA in the medium by cells in the enrichment.

The similar growth patterns of fumarate containing enrichments with and without PCA suggested that PCA is not required for the growth of cells in the culture. To determine the specific metabolic process carried out by cells in the fumarate containing enrichments, we analyzed the organic acid composition of the medium just after inoculation, and 48-hours past inoculation, when cell concentration has reached it's maximum. The results, presented in Table 1, show almost complete consumption of the fumarate supplied in the medium (initial concentration was ≈15.5 mM), along with formation of equimolar amounts of succinate and acetate, and a small amount of lactate (0.3 mM). Notably, the presence of PCA in the medium did not affect the consumption of formation of metabolites. This raised the hypothesis that the reduction of PCA in the enrichment medium is nonspecific and occurs by promiscuous activity of an unknown cellular component. To test for the specificity of PCA reduction, we inoculated cells into an enrichment medium containing 100 µM acetate and 10 mM fumarate, along with 1 mM of oxidized pyocyanin (PYO), which is a different species of phenazine. The cultures did not seem to grow under these conditions, as no consumption of fumarate was observed, and no reduction of PYO was noticeable.

Table 1 - changes in the concentration of organic acids in the medium of the putative PCA reducing enrichments

Ion concentration [mM]	Control	PCA	No PCA	PYO	PCA without H_2
Fumarate consumption	0	15	15	0	15
Succinate	0	10	10	0	10

formation					
Acetate formation	0	10	10	0	10
Lactate formation	0	0.3	0.3	0	0.3

The apparent reaction performed by the culture is the conversion of fumarate into succinate and acetate. This can be achieved either by fumarate respiration, with H_2 from the headspace as the electron donor, or by fumarate disproportionation. To test which of the following occurs in our enrichment cultures, we repeated the experiment, but this time flushed the headspace of our balch tubes with N_2-CO_2 gas to remove H_2. Removal of H_2 had no effect on the consumption or production of metabolites in the medium, suggesting that H_2 is not an electron donor for the cells in our enrichments. Nevertheless, the stoichiometry of the metabolites consumed or produced does not form a redox balanced reaction. We further address this point in the discussion.
To further investigate the physiology of the putative PCA reducing cells, we attempted isolating single colonies from both agar plates or shake tubes. Single colonies were visible after 4-5 days. Single colonies from agar plates were inoculated into liquid media in 96-well format, and were amplified for 16S rRNA sequencing. We were only able to receive good quality 16S sequences for a single colony, which had 99% identity to *Raoultella planticola*, a *Gammaproteobacteria* known to occur in soils (Sharma et al. 2000). Our enrichment cultures were also plated on LB and 5YE plates aerobically, and colonies were visible after overnight incubation at 30 C. We did not verify the phenotype of LB or 5YE grown colonies.

Identification of candidate PCA reducing cultures

Five days after we saw a color change in the nitrate containing media in the 96-well plates, a similar color change was noticed in the balch tubes, indicating a possible biotic oxidation of PCA. The phenotype was maintained after 3 consecutive dilutions of 10% (vol/vol). The maximal concentration of cells we recorded for the enrichments was ~10^6 cells mL^{-1}. Aliquots from the first dilution (one 10% dilution from the initial inoculum) were taken for analysis of ion composition and for analysis of organic acids in the medium. The oxidation of PCA was accompanied with a depletion of the nitrate and ammonium pools (Table 2), and production of nitrate, suggesting reduction of nitrate, possibly by PCA.

Table 2 - Ion concentration in the putative PCA oxidizers enrichment, and control medium

Organic acid concentrations [mM]	Control	PCA oxidizers
Nitrate	16	2.8
Nitrite	0	4.6

Analysis of the concentration of organic acids in the putative PCA oxidizing enrichment revealed the formation of ≈5 mM acetate. No carbon source was introduced into the medium besides 100 µM acetate and PCA. These results imply breakdown of PCA instead of PCA oxidation, as degradation of reduced

PCA will result in the same phenotype as PCA oxidation (loss of the yellow-green color). To test whether PCA was degraded in the putative PCA oxidizing enrichments, we tried reo-reducing the medium the putative PCA oxidizing enrichments. The results, presented in Figure 2, show a partial re-reduction of the PCA in the medium. Interestingly, a day after re-reducing the medium, which shifted its color back to yellow-green, the medium was oxidized back to a clear medium. The partial re-reduction might suggest partial degradation of the PCA in the medium, while the re-oxidation of the medium after re-reducing it might suggest bona-fide PCA oxidation. We further discuss this conundrum in the discussion.

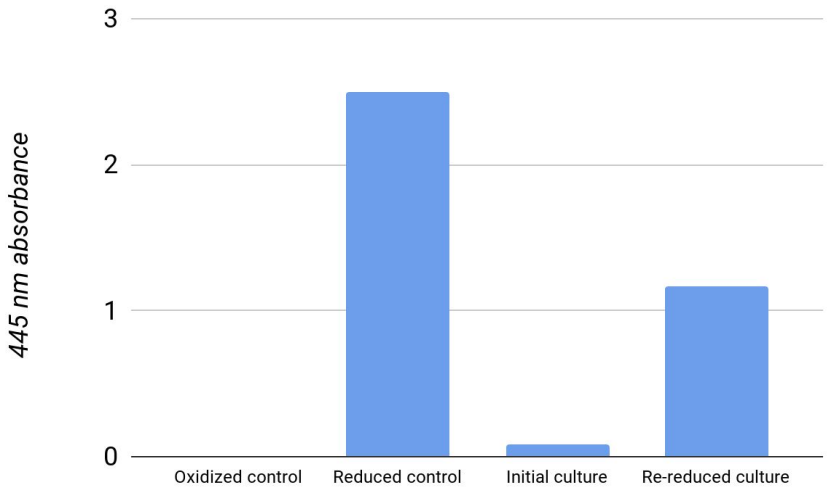

Figure 2 - Re-reduction of the putative PCA oxidizing enrichment. The reduction state of PCA in control media, as well as the enrichment before and after re-reduction was quantified by measuring absorbance of the medium at 445 nm. Partial re-reduction of the PCA in the enrichment is evident.

To further investigate the physiology of the putative PCA oxidizing or PCA degrading cells, we attempted isolating single colonies from both agar plates or shake tubes. Single colonies were visible after 4-5 days. Single colonies from agar plates were inoculated into liquid media in 96-well format, and were amplified for 16S rRNA sequencing. We were only able to receive good quality 16S sequences for a single colony, which had 98% identity to *Pseudocitrobacter anthropi* , a *Gammaproteobacteria* isolated from fecal samples (Kämpfer et al. 2014)**.**

Discussion

We set out to isolate from environmental samples microbial species who interact with phenazines. We chose to focus on isolating organisms able to reduce or oxidize PCA from soil samples collected in the Cape Cod area. After initial inoculation in defined media designed to enrich for the desired reactions, we saw indications of both PCA oxidation and reduction, evident from the change of color of the medium, which reflect the oxidation state of PCA. Transferring subsamples of our initial inoculum into fresh media allowed us to distill the biological component responsible for the reduction or oxidation of PCA, and

better characterize them. We performed initial physiological characterization of the metabolic reactions taking place in our enrichment cultures, as well as isolated single colonies, some of which are validated for the desired phenotypes. Further study might help answer some of the questions still unresolved both for PCA oxidizers and reducers. From our HPLC analysis, it is clear that the enrichment cultures for PCA reduction consume fumarate and produce mainly succinate and acetate. The stoichiometry of the reaction, however, is inconsistent with known pathways of fumarate fermentation (Dorn, Andreesen, and Gottschalk 1978). We ruled out fumarate respiration using H_2 as an electron donor. Possible explanations for the discrepancy are poor calibration of the standard curves for either fumarate, succinate or acetate. More careful measurements might reveal that the stoichiometric relations we measured are actually inaccurate. Alternatively, there might be additional possible metabolites not captured by our analysis which will be able to bridge the gap between our measurements and theoretical considerations.
An additional question that remains unresolved is the physiological relevance of PCA reduction to the metabolism of the cells in the enrichment cultures. No major phenotype was observed in the absence of PCA, suggesting either that the effect not significant enough to be resolved by our methods, or that the reduction is merely the result of nonspecific side reaction of some of the cellular machinery with PCA. We attempted to answer this question by replacing PCA with a different kind of phenazine, PYO, at equal concentration, but it seems as if this concentration of PYO is toxic to the cells and did not allow them to grow. Further studies using lower concentration of PYO and additional redox active molecules might help resolve the matter.
Regarding the putative PCA oxidation or degradation in our enrichment cultures, the results are inconclusive. The partial re-reduction of the PCA in the culture might suggest to partial degradation of PCA in the culture, whereas the swift re-oxidation of the culture after the re-reducing the PCA might suggest oxidation activity. While these two options are not mutually exclusive, the production of acetate in the absence of other carbon sources except PCA strongly suggests that some degradation of PCA is occurring. Measurement of the concentration of PCA in the enrichment might help substantiate the claim.
Overall, the work done in the project is a first step in characterizing two novel metabolic activities. Isolation of colonies with verified phenotypes and genotypes will greatly help expand our knowledge on those activities, and the role of phenazines, and possibly other redox active molecules in microbial metabolism.

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Appendix

Ion chromatography settings

Microbial Diversity 2017 – Ion Chromatography
Autosampler: Dionex ICS AS-DV
Anions:
System: Dionex ICS-2000
Columns: Dionex IonPac AS22 Fast 4 µm 4 mm x 150 mm (analytical)
Dionex IonPac AG22 Fast 4 µm 4 mm x 30 mm (guard)
Suppressor: Dionex AERS 500 4 mm
Eluent: 1.2 ml/min; isocratic; 15 min
1.4 mM sodium bicarbonate (Dionex, Thermo Fisher Scientific)
4.5 mM sodium carbonate (Dionex, Thermo Fisher Scientific)
Suppression: 29 mA
Standard: Dionex Seven Anion Standard II (Thermo Fisher Scientific)
Column temperature: 30°C
Conductivity cell: 35°C
Cations:
System: Dionex ICS-2000
Columns: Dionex IonPac CS19 4 µm 2 mm x 250 mm (analytical)
Dionex IonPac CG19 4 µm 2 mm x 50 mm (guard)
Suppressor: Dionex CERS 500 2 mm
Eluent: 0.3 ml/min; isocratic; 15 min
7 mM Methanesulfonic Acid (Sigma Aldrich)
Suppression: 8 mA
Standard: Dionex Six Cation-II Standard (Thermo Fisher Scientific)
Column temperature: 30°C
Conductivity cell: 35°C
Software: Chromeleon 7.2 SR4

Media composition

100X Freshwater ("FW") Base (per liter)

Component	Amount	FW	100X Conc.	Final Conc.
NaCl	100 g	58.44	1711 mM	17.1 mM
$MgCl_2 \cdot 6H_2O$	40 g	203.30	197 mM	1.97 mM
$CaCl_2 \cdot 2H_2O$	10 g	147.02	68 mM	0.68 mM
KCl	50 g	74.56	671 mM	6.71 mM

Keep on shelf in clean bottle, not sterile

1000x "2017 HCl-Dissolved Trace Element" Stock Solution (per liter)

Component	Amount	FW	1000X Conc.	Final Conc.
20 mM HCl	1.7 ml conc. HCl	na	20 mM	20 µM
$FeCl_3 \cdot 6H_2O$	2027 mg	270.30	7.5 mM	7.5 µM
H_3BO_3	30 mg	61.83	0.48 mM	0.48 µM
$MnCl_2 \cdot 4H_2O$	100 mg	197.91	0.5 mM	0.5 µM
$CoCl_2 \cdot 6H_2O$	190 mg	237.93	6.8 mM	6.8 µM
$NiCl_2 \cdot 6H_2O$	24 mg	237.69	1.0 mM	1.0 µM
$CuCl_2 \cdot 2H_2O$	2 mg	170.48	12 µM	12 nM
$ZnCl_2$	68 mg	136.30	0.5 mM	0.5 µM
Na_2SeO_3	4 mg	172.94	23 µM	23 nM
*Na_2MoO_4	30.9 mg	205.92	150 µM	150 nM

* Ideally, for the enrichment of bacterial with alternative nitrogenases, all added Mo would be left out entirely, a decision that would not be without caveats and pitfalls.

1000x 13-Vitamin Solution

Component	Amount	1000X Conc.	Final Conc.
10 mM MOPS, pH 7.2	1000 ml	10 mM	10 µM
Riboflavin	100 mg	0.1 mg/ml	0.1 µg/ml
Biotin	30 mg	0.03 mg/ml	0.03 µg/ml
Thiamine HCl	100 mg	0.1 mg/ml	0.1 µg/ml
L-Ascorbic acid	100 mg	0.1 mg/ml	0.1 µg/ml
d-Ca-pantothenate	100 mg	0.1 mg/ml	0.1 µg/ml
Folic acid	100 mg	0.1 mg/ml	0.1 µg/ml
Nicotinic acid	100 mg	0.1 mg/ml	0.1 µg/ml
4-aminobenzoic acid	100 mg	0.1 mg/ml	0.1 µg/ml
pyridoxine HCl	100 mg	0.1 mg/ml	0.1 µg/ml
Lipoic acid	100 mg	0.1 mg/ml	0.1 µg/ml
NAD	100 mg	0.1 mg/ml	0.1 µg/ml
Thiamine pyrophosphate	100 mg	0.1 mg/ml	0.1 µg/ml
Cyanocobalamin	10 mg	0.01 mg/ml	0.01 µg/ml

-Titrate with 5 M NaOH (5-10 drops) until dissolved.
-Filter sterilize (0.2 µm filter) and refrigerate in the dark in 10 ml aliquots.

16S sequencing results

Putative PCA oxidizer colony

GGGATCAAGTTCTCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCGAACCGCTGGCA
ACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGAC
GACAGCCATGCAGCACCTGTCTCACGGTTCCCGAAGGCACCAATTCATCTCTGAAAAGTTCC
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TAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGT
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CATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTG
TGGCTGGTCATCCTCTCAGACCAGCTAGGGATCGTCGCCTAGTGAGCCGTTACCCCACCTAC
TAGCTAATCCCATCTGGGCACATCCGATGGCAAGAGGCCCGAAAGGTCCCCTCTTTGGTCTT
GC

Putative PCA reducer colony

GGGTTAACTTCCTTCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCGGACCGCTGGCA
ACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGAC
GACAGCCATGCAGCACCTGTCTCAGAGTTCCCGAAGGCACCAAAGCATCTCTGCTAAGTTCT
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AGTCTTTGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCATTTCACCG
CTACACCTGGAATTCTACCCCCCTCTACAAGACTCAAGCTTGCCAGTTTCAAATGCAGTTCCC
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GTAATTCCGATTAACGCTTGCACCCTCCCGAATTACCCGCGGCTGCTGGCACG