CHAPTER 4

Bioelectrocatalytic activity and electrochemical characterization of enriched electroactive microbial biofilms as a function of microbial inoculum and substrate

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Abstract

Different natural sources of microorganisms (inoculants) have been shown to yield electrochemically active biofilms. These biofilms often share distinctive electrochemical features pointing on the dominance of a limited number of microbial species. However, the qualitative and quantitative microbial composition of biofilms, especially in relation to the respective natural inoculates have been rarely reported. In this study, we demonstrated that the used inoculum and substrate considerably influences the bioelectrocatalytic activity and electrochemical characteristics of enriched anodic electroactive biofilms. Bioelectrochemical systems fed with different substrates (acetate and lactate) and inoculated with different microbial samples (primary waste water, activated sludge, primary sludge and secondary sludge) showed varied maximum current densities and electrochemical characteristics. Acetate-fed approaches showed the highest current density with primary waste water as an inoculum. Whereas, lactate resulted in comparatively low current output which might be due to its lower consumption or inability of consumption by electroactive bacteria enriched from identical waste water. In addition, the activated sludge also exhibited good performance in comparison to that of primary and secondary sludge. Furthermore, on the example of primary waste water derived biofilms fed with acetate we showed that flow cytometry provides an excellent tool for the analysis of the electrochemical driven microbial selection. The flow cytometry and t-RFLP analysis of the biofilms formed with primary waste water and acetate revealed the electrochemical selection and dominance of Geobacter sulfurreducens.
4.1 Introduction

Microbial bioelectrochemical systems (BES) exploit the energy available in a bioconvertable substrate through the catalytic activity of electrochemically active bacteria at the anode [1]. These bacteria, normally referred as electricigens [2] and exoelectrogens [3] form electroactive biofilms (EABs) at the anode. Anodic EABs are not only the key component of BES [4], they also play a crucial role in natural redox-cycles [5]. Commonly in BES, these biofilms facilitate the oxidation of the fuel (the substrate from the microorganism’s perspective) and transfer the released electrons to the anode that serves as microbial terminal electron acceptor. Up to now, a wealth of electron transfer mechanisms, including direct electron transfer and mediated electron transfer have been elucidated [6-8]. Apart from pure culture studies, devoted to the investigation of fundamental phenomena, most BES are run on mixed culture derived anodic biofilms. These biofilms are generally directly formed from the microorganisms present in waste water, as waste water treatment represents the most appealing application niche for BES [9], or from other bacterial sources like sewage sludge. During the last few years, it has been demonstrated that employing a already pre-selected bacterial community as bacterial source for biofilm formation, e.g. the effluent of a running BES [10], or a scrapped-off electrochemical active biofilm [11, 12] or placing the fresh electrode in the vicinity of an already active biofilm anode [13], results in high performing electrocatalytic microbial biofilms. Furthermore, the electrochemical characterization of these biofilms is gaining increasing interest as it allows the extraction of thermodynamic information on the anodic electron transfer processes [14-16]. Based on gene level analysis it has been shown that, the enriched bacterial communities and predominant species at anode vary with operational conditions, such as inoculum and substrate type etc. [3, 17-22]. In spite of these research advancements, it is constantly speculated that, in order to achieve a better performance with various BES, it is necessary to explore different microbial sources and to understand the bacterial community dynamics.

However, besides the growing research on other influential parameters, the microbial and molecular biological analysis of these biofilms is still in its infancy. Up to now, several techniques, providing different types and depth of information have been applied on single strain and mixed culture inoculum derived biofilms. In summary, the reported bacterial community composition among all studies differed significantly, however, Geobacteraceae and related species are said to play a key role (e.g. [18, 23-
It has to be stressed that the most frequently applied method of 16s rRNA fingerprinting like terminal restriction fragment length polymorphisms (T-RFLP) might give a good phylogenetic diversity resolution on natural sample composition but lack information on exact abundance of the community members due to varying effectivity in DNA extraction, amplification and the choice of restriction enzymes (e.g. [26, 27]). Nevertheless, all members of a complex community can be determined without any previous knowledge on their phylogenetic affiliation, even including uncultivable or unknown microbial species using universal fluorescent labeling and flow-cytometry [28]. Basically, every single cell can be characterized on its scatter characteristics providing information on its size and optical density as well as DNA content, which allows the identification of population subsets. Recently, it has been shown that T-RFLP and cytometric DNA/Scatter-Plot distributions provide equal as well as complementary information on natural community dynamics [29]. Thus, the combination of both techniques provides an excellent tool for the study of natural communities as it was demonstrated in this study for exemplary mixed culture derived EABs.

In addition to the microbial inoculum, improved mobilization of substrates is considered as an important aspect to expand application goals in addition to the improving BES performance [30]. It has been demonstrated that the substrate influences not only the integral composition of the bacterial community in the anode biofilm, but also the microbial fuel cell (MFC) performance including the power density and coulombic efficiency (CE) [30-32]. Glucose is considered as a substrate with relatively lower CE compared to lower molecular compounds, such as acetate, due to its fermentable nature [33]. Although the substrate type influences the current output, very few reports described the influence of different inoculants on the biofilm formation, their performance, electron transfer thermodynamics and community diversity in the enriched anodic EABs with similar substrate. Most of the previous studies were generally carried out with a single substrate or each study used different microbial inoculants at varying operational parameters, which makes it difficult to compare studies [30]. Therefore, in order to exclude influence of other operational variables and to investigate the sole effect of individual microbial inoculum source with acetate/lactate, the experiments were conducted with half cell set-ups under potentiostatic control. During this study, the influence of the used substrate and microbial inoculum on the general bioelectrocatalytic activity (current density) and the
voltammetric behavior was investigated. Furthermore, on the example of primary waste water derived biofilms fed with acetate we showed that flow-cytometry provides an excellent tool for the analysis of the electrochemical driven microbial selection.

4.2 Materials and Methods

4.2.1 General conditions

All microbiological and electrochemical experiments were conducted under strictly anoxic conditions. If not stated otherwise, all potentials provided in this article refer to the Ag/AgCl reference electrode (sat. KCl, 0.195V vs. standard hydrogen electrode, SHE).

4.2.2 Chemicals and electrodes

All chemicals were of analytical or biochemical grade and were purchased from Sigma–Aldrich and Merck. The working (anode) and counter (cathode) electrodes used throughout this study were graphite rods (CP-Graphite GmbH, Germany).

4.2.3 Growth medium and microbial inoculum

The bacterial growth medium used in all experiments was prepared as reported by Kim et al. [12]. It contained NH₄Cl (0.31 g L⁻¹), KCl (0.13 g L⁻¹), NaH₂PO₄·H₂O (2.69 g L⁻¹), Na₂HPO₄ (4.33 g L⁻¹) and trace metal (12.5 mL) and vitamin (12.5 mL) solutions [34]. Acetate or lactate (10 mM) served as a substrate. The final growth medium pH was adjusted to 6.8. Before start of the experiment the growth medium was purged with nitrogen for at least 20 min to ensure anoxic conditions. All microbial samples (primary waste water, activated sludge, primary sludge and secondary sludge) were obtained from the Waste water treatment plant (WWTP), Steinhof (Braunschweig) and were used as inoculum for the biofilm formation.

4.2.4 Electrochemical experimental set up

All half-cell electrochemical experiments were carried out under potentiostatic control, using a three-electrode arrangement consisting of the working electrode, a Ag/AgCl reference electrode (sat. KCl, Sensortechnik Meinsberg, Germany) and a counter electrode (Fig. 4.1). The experiments were conducted with VMP 3, multichannel potentiostat (Biologic Instruments, USA). Sealed glass vessels (250 mL) served as electrochemical cells which hosted the microbial growth medium, inoculum and the electrodes. All experiments were conducted at 35°C under potentiostatic control. Unless stated otherwise, the working electrode had a projected surface area of 8 cm².
4.2.5 *Biofilm growth with fed batch approach*

The biofilm formation procedure was followed as described by Liu et al [13]. For the biofilm formation 8 mL of individual microbial sample was inoculated into the sealed electrochemical cell that contained 200 mL of the stirred growth medium with substrate under study. A constant potential of 0.2 V was applied to the working electrode to facilitate the biofilm formation. The biofilm growth was monitored by measuring the bioelectrocatalytic oxidation current. For the initial (usually three) batch cycles, microbial inoculum was added to the medium. The experiments were performed at least in duplicates.

4.2.6 *Cyclic voltammetry*

Cyclic voltametry (CV) was performed with all biofilms formed with different inoculants and substrates during turnover and non-turnover conditions. The potentials were applied from -500 to 300 mV (vs. Ag/AgCl) at a scan rate of 1 mV/s with continuous monitoring of the current response.

4.2.7 *Microbiological analysis of biofilm formed with acetate and primary waste water*

4.2.7.1 *Flow-cytometry*

*Sample fixation and DNA staining*

Cells were harvested from the primary waste water sample and the enriched anodic biofilm. All samples were washed twice to remove any disturbing substances (cell
debris, organic material) with phosphate buffered saline (PBS: 0.4 M Na₂HPO₄/NaH₂PO₄, 150 mM NaCl, pH 7.2) by centrifugation at 3,200 x g for 10 min and conserved in fixation buffer (pH 7.0) containing: 5 mM BaCl₂ (BaCl₂.2H₂O; Laborchemie Apolda, Germany), 5 mM NiCl₂ (NiCl₂.6H₂O; Merck, Germany) and 10% sodium azide (Merck, Germany) dissolved in PBS (1 ml fixation buffer for app. 3 x 10⁸ cells ml⁻¹) for a maximum of 9 days. Aliquots of the fixed samples were washed twice in 2 ml PBS by centrifugation at 3,200 x g for 5 min and treated with 1 ml Tween 20 solution (0.5 g Tween 20 in 100 ml bidistilled water) for 20 min to facilitate dye penetration. Subsequently, the cells were washed, carefully resuspended in 2 ml DAPI solution (692 µl of 143 µM DAPI stock in 100 ml of 400 mM Na₂HPO₄, pH 7.0) and stained for at least 60 min in the dark at 20°C.

Multiparametric flow cytometry

Flow cytometric measurements were carried out using a MoFlo cell sorter (DakoCytomation, Fort Collins, CO, USA) equipped with two water-cooled argon-ion lasers (Innova 90C and Innova 70C from Coherent, Santa Clara, CA, USA). Excitation by 580 mW at 488 nm was used to analyze the forward scatter (FSC) and side scatter (SSC) as trigger signal at the first observation point, using a neutral density filter with an optical density of 2.3. DAPI dye was excited by 180 mW of ML-UV (333-365 nm) at the second observation point. The orthogonal signal was first reflected by a beam-splitter and then recorded after reflection by a 555 nm long-pass dichroic mirror, passage by a 505 nm short-pass dichroic mirror and a BP 488/10. DAPI fluorescence was passed through a 450/65 band pass filter. Photomultiplier tubes were obtained from Hamamatsu Photonics (models R928 and R 3896; Hamamatsu City, Japan). Amplification was carried out at linear or logarithmic scales, depending on the application. Fluorescent beads (Polybead Microspheres: diameter, 0.483 µm; flow check BB/Green compensation Kit, Blue Alignment Grade, ref. 23520, Polyscience, USA) were used to align the MoFlo (coefficient of variation – CV value - about 2%). Furthermore, an internal DAPI-stained bacterial cell standard was introduced for tuning the device up to a CV value not higher than 6%. Cell aggregation was not observed, thus clearly separated sub-populations were analyzed.

4.2.7.2 T-FRLP and Sequencing

For DNA extraction 200 µl of the fixed samples were processed with the Fast DNA Spin Kit for Soil (MP Biomedicals, Illkirch, France). PCR amplification of the 16S rRNA gene fragment with the universal primers 27F-FAM and 1492R [35] and the T-
RFLP analyses were performed as described elsewhere [36]. PCR product purification was done with QIAquick PCR Purification Kit (Qiagen, Venlo, The Netherlands). The restriction enzymes BstU I, Hae III, Msp I and Rsp I (New England Biolabs, Schwalbach, Germany) were used with the corresponding buffer. The genemapper V3.7 software (Applied Biosystems, Weiterstadt, Germany) was used to determine the length of the fluorescent terminal restriction fragments (T-RFs). Only peaks with a relative fluorescence intensity of 50 Units in the range of 50-500 bp were included in the analysis.

The PCR amplification product of biofilm samples were partial sequenced with the primers 27F and 1492R and the BigDye RR Terminator AmpliTaq Kit 1.1 (Applied Biosystems). An ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) was used for capillary electrophoresis and data collection. The data was analyzed with Abi Prism DNA sequencing analysis software. The sequence endings were trimmed and the two partial sequences of the 16S rRNA gene (27f with 479 bp and 1492r with 730 bp) were compared to published sequences with the BLAST tool (www.ncbi.nlm.nih.gov/BLAST) [37].

4.3 Results and Discussion

4.3.1 Bioelectrocatalytic activity of enriched microbial electroactive biofilms as a function of microbial inoculum and substrate

A significant difference in current generation was observed among BES with different inoculants and with acetate or lactate (Fig. 4.2). As shown in Fig. 4.2, the acetate-fed-reactor with primary waste water inoculum showed the highest current density (811.5 µA cm⁻²), followed by activated sludge based reactor (674.8 µA cm⁻²). Lactate resulted in the lower current outputs with all inoculants (except for primary sludge) in comparison to other systems fed with acetate which might be due to its lower consumption. The most possible reason for the low current densities with lactate could be the absence of high-current-producing exoelectrogenic microorganisms to develop biofilms either through competition with other microbes or an inability to use this specific substrate. Primary waste water also showed better bioelectrocatalytic performance (448.8 µA cm⁻²) with lactate, which was the highest amongst other microbial inoculants (with lactate). Furthermore, the high bioelectrocatalytic activity with activated sludge demonstrated its ability as efficient microbial inoculum source. The better performance with primary waste water and activated sludge indicated
selective enrichment of electrocatalytically active microbes on the anode and thus proved to be better candidates for the formation of mixed culture based EABs.

Figure 4.2: Bioelectrocatalytic performance of electroactive microbial biofilms derived from different inoculants with fed batch operation in potentiostatically controlled half-cell experiments (0.2 V vs. Ag/AgCl) at carbon rod electrodes. The substrate was 10 mM acetate or lactate. (Each data point represents mean of duplicate ± s.d.)

These experiments were performed with two independent replicates. Few amongst the used microbial inoculants showed differing bioelectrocatalytic performance with both runs which can be seen from fig. 4.2 (error bars). This is attributed to the complexity of these mixed culture inoculants. As shown here and in accordance with previous results [21, 30, 32, 38], acetate was the preferred substrate for electricity generation with different inoculants in MFCs. The low performance with primary and secondary sludge might be attributed to a low extent of bacterial adhesion to the anode which is necessary for better performance. It has been shown that electroactive biofilm formation at the anodes is an important factor to increase the current production [39]. Furthermore, these microbial inoculants contain a variety of non-electrogenic bacteria that compete with electrogenic bacteria for the growth, which probably slowed down the EAB formation process and thus overall bioelectrocatalytic performance.
Interestingly, primary sludge exhibited good performance with lactate than acetate which might be because of involvement of lactate utilizing microorganisms in this inoculum. This will be confirmed by further microbial community analysis.

**Figure 4.3**: Exemplary cyclic voltammograms of electroactive microbial biofilms derived from different inoculants recorded during turnover (A and B) and non-turnover conditions (C and D) conditions with acetate (10 mM). The scan scan rate used was 1 mV/s.

CV analysis during turnover and non-turnover conditions with all set-ups confirmed the biofilm associated current generation. Cyclic voltamograms (CVs) (see Fig. 4.3 and 4.4) on the example of exemplary primary waste water and secondary sludge based electroactive biofilms indicated the different electro-chemical behavior with both acetate and lactate. The formal potentials of the active site (bioelectrocatalysis) with primary waste water and secondary sludge were about -200 mV and -242 mV (vs. Ag/AgCl) respectively in case of acetate (derived from first derivative of CVs for turnover conditions of fig. 4.3 A and B). Whereas, in case of lactate (fig. 4.4 A and B) the formal potentials of the active site with primary waste water and secondary sludge were about –335 mV and -263 mV (vs. Ag/AgCl) respectively. This clearly indicates the enrichment of different microbes on the anode with different inoculants. This further demonstrates that the used substrate considerably
influences the enrichment of electrochemically active bacteria with an identical inoculum.

Figure 4.4: Exemplary cyclic voltammograms of electroactive microbial biofilms derived from different inoculants recorded during turnover (A and B) and non-turnover conditions (C and D) conditions with lactate (10 mM). The scan scan rate used was 1 mV/s.

Furthermore, the CV patterns during non-turnover conditions with both inoculants (Fig. 4.3, 4.4 C and D) showed very different and complex redox behaviour and thus electron transfer thermodynamics. This clearly indicated the probable enrichment of different microbes from the mixed culture communities. Recently, work on diversity of microbes in biofilms enriched with different substrates has been reported [38]. Besides various aspects, Chae et al. reported the lack of a single dominant bacterial species in the communities of anodic biofilms and the prominent presence of Geobacter-like species as important member of the bacterial community in MFCs fed with different substrates. Further analysis on enriched community structure and dynamics is under investigation with here tested microbial inoculants.
4.3.2 Electrochemical and microbiological analysis of exemplary biofilm formed with acetate and primary waste water

Figure 4.5 A & B depict the current production and cyclic voltammograms of acetate grown waste water derived exemplary electrochemically active biofilm. As can be seen, the biofilms produced a stable maximum current density of 600 $\mu$A cm$^{-2}$ (per projected surface area) for subsequent feeding cycles and possessed cyclic voltammetric characteristics (i.e. formal potential of the active site about - 200 mV vs. Ag/ AgCl, derived from first derivative; see Fig. 4.6) in accordance with numerous previous publications on waste water derived biofilms and pure cultures of *Geobacter sulfurreducens* (e.g. [13, 40]).

![Graph A: Maximum current densities of the biofilm (circles) obtained during consecutive fed-batch cycles.](image1)

![Graph B: Representative cyclic voltammetric curve of the respective biofilm for turnover conditions using a scan rate of 1 mV s$^{-1}$.](image2)

Figure 4.5: A) Maximum current densities of the biofilm (circles) obtained during consecutive fed-batch cycles, B) representative cyclic voltammetric curve of the respective biofilm for turnover conditions using a scan rate of 1 mV s$^{-1}$. 
Figure 4.6: First derivative of the cyclic voltamogram for turnover conditions (as shown in Figure 4.4 B) indicating the formal potential of the active site.

Subsequently, waste water inoculum and anode biofilms were analyzed by mult-parametric flow-cytometry using a DAPI-DNA staining. Figure 4.7 shows exemplary flow-cytometric patterns. A wealth of information on the complex structure of the waste water community (bacterial diversity and abundance) has been provided which allows community and population structure interpretation [28]. Therefore, Figure 4.7 A clearly reveals that the waste water sample is a complex mixture of numerous distinct bacterial sub-communities characterized by specific DNA contents and cell sizes which is in sharp contrast to the anodic biofilm. From Figure 4.7 B, showing the flow-cytometric pattern of the anodic biofilm, one can clearly deduce that the community consists of very similar microorganisms distributed into sub-populations of various chromosome equivalents per cell (see $C_{1n}$ to $C_{3n}$ sub-population). Such patterns are typical for pure strains and present live cycle events, as already described for biotechnologically and environmentally relevant species [41]. Concomitantly with the cytometric assessment the single species on the anode was identified as the deltaproteobacterium *Geobacter sulfurreducens* by T-RFLP and sequencing. Noteworthy, we also show (see Supplementary information) that *G. sulfurreducens* could not be detected in the waste water inoculum by T-RFLP and only mutedly by flow-cytometry, which indicates an
abundance in the lower single-digit percentage range. Therefore, the findings clearly demonstrate that the conditions at the anode lead to an electrochemical selection of one single microbial species, i.e. the formation of a microbial pure culture biofilm, from a mixed complex microbial source. The dominance of *G. sulfurreducens* is in line with the presented electrochemical data and in accordance with previous findings, (e.g. [24, 25]).

**Figure 4.7: Flow-cytometric patterns of A) waste water and B) the anode biofilm.**

Multiple sub-communities can clearly be differentiated with regard to cell size and DNA content within the complex structure of the waste water community (orange color). The small black oval gates, which were chosen according to the main sub-clusters in the right histogram of the anode biofilm do not engulf the similar sub-clusters in the waste water sample. This means that the chosen anode biofilm individuals are nearly not present in the waste water community (correlating to overall cell number the summed up gate cell numbers comprise 4.4%, but is definitively lower since other cell clusters overlap here, especially within the C$_{2n}$ gate). In contrast, the anode biofilm shows cell cycle related distributions of DNA/FSC sub-clusters of *G. sulfurreducens* with DAPI-DNA derived peak mean values of 22.99 (C$_{1n}$), 45.93 (C$_{2n}$), and 72.58 (C$_{3n}$). The DAPI-DNA peak distribution is also seen in the adjunct histograms (vertical) which clearly points to cell cycling in Fig. 4.7 B but not in Fig. 4.7 A. The abundance of *G. sulfurreducens* cells within the three sub-clusters inner cores is 27.75%, but comprise all in all 86.79% of all events. The lower left distributions in both histograms are due to the electronic noise of the flow cytometer.
Within this study we demonstrated the combination of flow-cytometry and TRFLP as a perfectly suited tool to reveal the electrochemical driven selection in microbial biofilms. By this selection, using acetate-based artificial waste water as the bacterial growth medium and real waste water as inoculum, pure culture biofilms of *G. sulfurreducens* were gained. This raises the question how distinctive environmental variables (e.g. the bacterial source and the substrate) influence the bacterial biofilm composition and dynamics. These and further follow-up questions are under investigation with flow-cytometry, allowing a high-throughput characterization of natural microbial communities without any previous knowledge on the bacterial composition. Thus, this first application of flow-cytometry to electrocatalytic biofilms paves the way to follow the community dynamics as well as bacterial activity states in response to micro-environmental changes in MFCs in high through-put.

### 4.4 References


Supplementary Information

4.1 T-RFLP-analyses & Sequences:

Figure 4.8: T-RFLP chromatograms of A1) waste water A2) biofilm samples after digestion with Rsa I B1) waste water and B2) biofilms samples after digestion with Msp I.
4.2 *Geobacter sulfurreducens* sequence identity:

**Partial Sequence 1 (27f)**

GGTGAAAGTGCGCAGGGGTAGTAAACGCCTGGATAATCTGCCCCAGGATT
TGAGTAAACATCTGGAAGGGGTAGTACATTACGAATAAGCCACGCGGTTCT
ACCCGATCTTTGCGGAGGAAAAAGGGGGGACCTTCGGGCCTCCTGTCCTTGGATG
AGTCTGCGTACCATTAGCTAGTTGGTGGGAATGCTACCAAGGCACAG
ATGTTAGCTGTTGAGAGATGATAGATCAGGACTTCGCTTGAAGAC
TCCAGACTCTCTACGGAGGAGCCAGCAGGCAGTTGGGAAATTTCGGCAATG
CCCTGAGCCAGCAACGCCCGTGGGTGAATAGGGGGGGACCTTCGGGCCTCCTGTCCTTGGATG
AGTCTGCGTACCATTAGCTAGTTGGTGGGAATGCTACCAAGGCACAG
ATGTTAGCTGTTGAGAGATGATAGATCAGGACTTCGCTTGAAGAC
TCCAGACTCTCTACGGAGGAGCCAGCAGGCAGTTGGGAAATTTCGGCAATG

**Partial Sequence 2 (1492r)**

GCTGCCTCCCACAAGGGGGTTAGCTCACGCACATTCCGGGACCAGTCAGCTCC
GTGGTGTGACGGGCGGTTGTGTAACAGGGGGGACGTATTCACCGCGGCA
TGCTGATCCGCGATATTTACCCGAGTTTTCTGCATTGCTCCACCTCTGCCTGTTTG
CCTGACTCTTTGTACCCGCTTTGTACGCAACGCTGCTGTCGACATTAC
CCATGAGGACTTTCAGCTCATTCCCACCTCTCCGGTGGTACCCGACGCGAGTT
TCATCGAGAGTCCCAACTCTAAATGATGCGAAGGGGTGGGTCGTC
GTGGCGGACTTAAACCAACATCTCACACGACACGAGCTGAGCCAGCAGCCATGC
AGCACCCTGTCTCAGCGCCTCCCCAGGGGCAACCCCTACTCCGCTCCGGGAGGGTT
CGTGGATGTACAGCCCAAGGTAGTCTCGCGCTTCTCGCAATTCACCA
CATGCTTCAAGGCAATAGTGCTGGGACCCGCCGCTAAATTTGCTTCCTTCTGCTTG
CGACCGTATTCCCGAGCCGAATCTACATTAAATGGTTAGCTACCCGACGACATGC
GGGTTCAATTCCGGCAGACCTAGTACTCCATCGGTATCCAGGCCGTAACCA
GGGATCTTAATCCTGTTGGAGTACCCACGCTTTCCGCTTCACAGCTATAC
GGTCCAGA