Genotypic Identification of Biofilm forming Bacteria on Stainless Steel

Tracking the Stainless Steel Stainers

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Introduction

Each year corrosion induced damages amount to billions of Dollars. Not only does the weather and other environmental determinants favor the corrosion of metal surfaces but microorganisms accelerate and often even induce this costly abrasion through their metabolic activity. Nonetheless, the microbial communities that live on metals were scarcely investigated, so far, and there is still a lack of foolproof and quick methods for examining them.
Our course project deals with the molecular analysis of corrosion inducing bacterial biofilms growing on metal surfaces. It is part of an ongoing research project on Corrosive Biofilm formation, see: http://www.microeco.unizh.ch/projects/indexpro.htm

Methods (2)

We processed 16S rDNA from biofilms grown on stainless steel. The biofilm communities were investigated without prior cultivation of the individual populations (1). We started our work with bacterial plasmids from E.coli clones which included 16S rDNA inserts originating from biofilms grown on metal coupons in flow cell reactors and focused on the analysis of organisms present in biofilms grown on metal surfaces. Fig. 1 summarizes the major steps in the molecular analysis process. The grey highlighted boxes show our actual work, based on the work of Marcello Marchiani. We worked with bacterial plasmids from reactors 2 and 7 (R_2 and R_7 respectively) both originally inoculated with a biofilm from an actual corrosion case (Gebäude 211) grown on the surface of stainless steel metal discs or originating from the floating phase of reactor 2 (clones from R_2).
In this work we reproduced a process that enables one to identify by molecular analyses the types of organisms involved in such a biofilm. Our goal is to provide preliminary data for the creation of a DNA database that allows the creation of sure and easy to use probes for quick identification.

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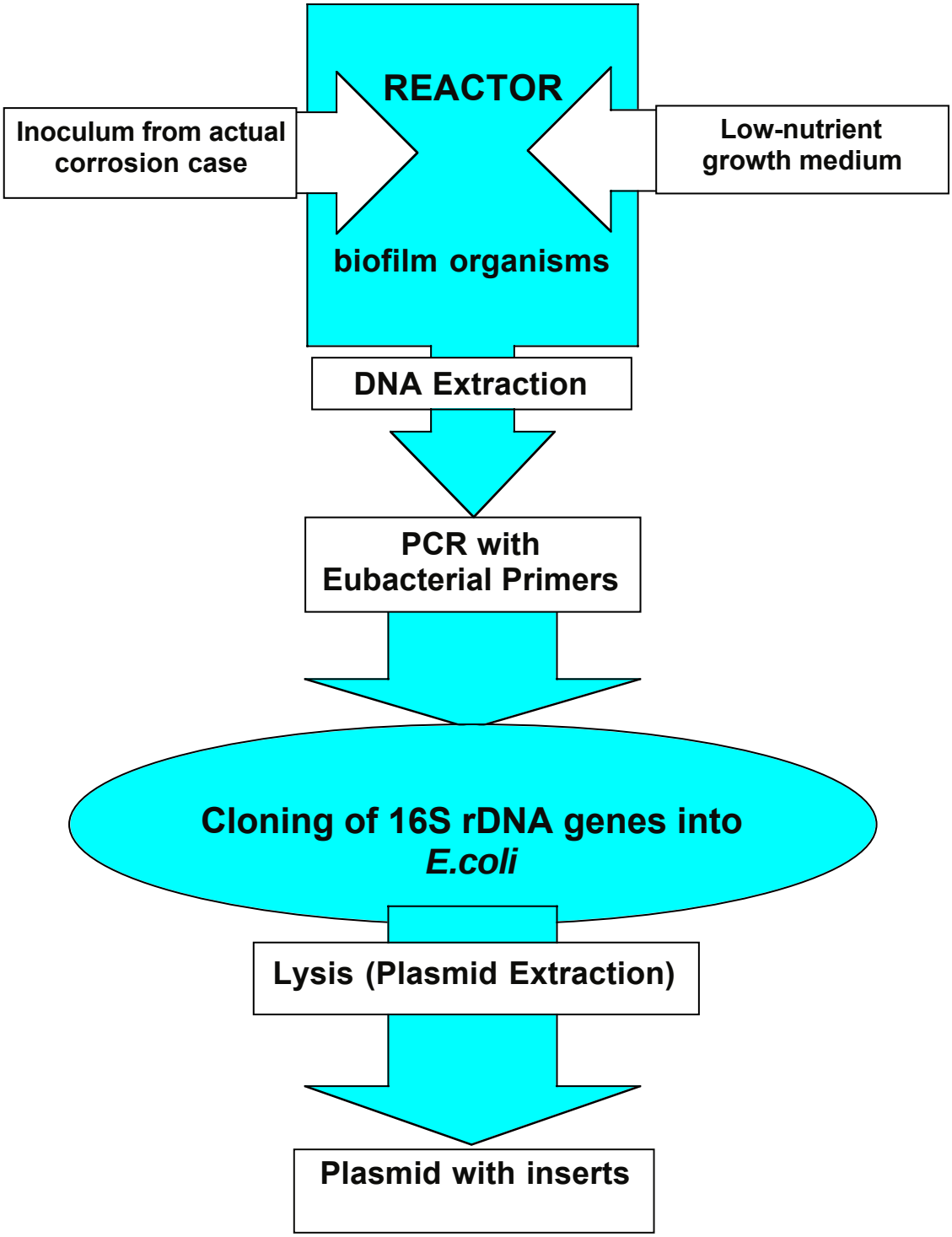
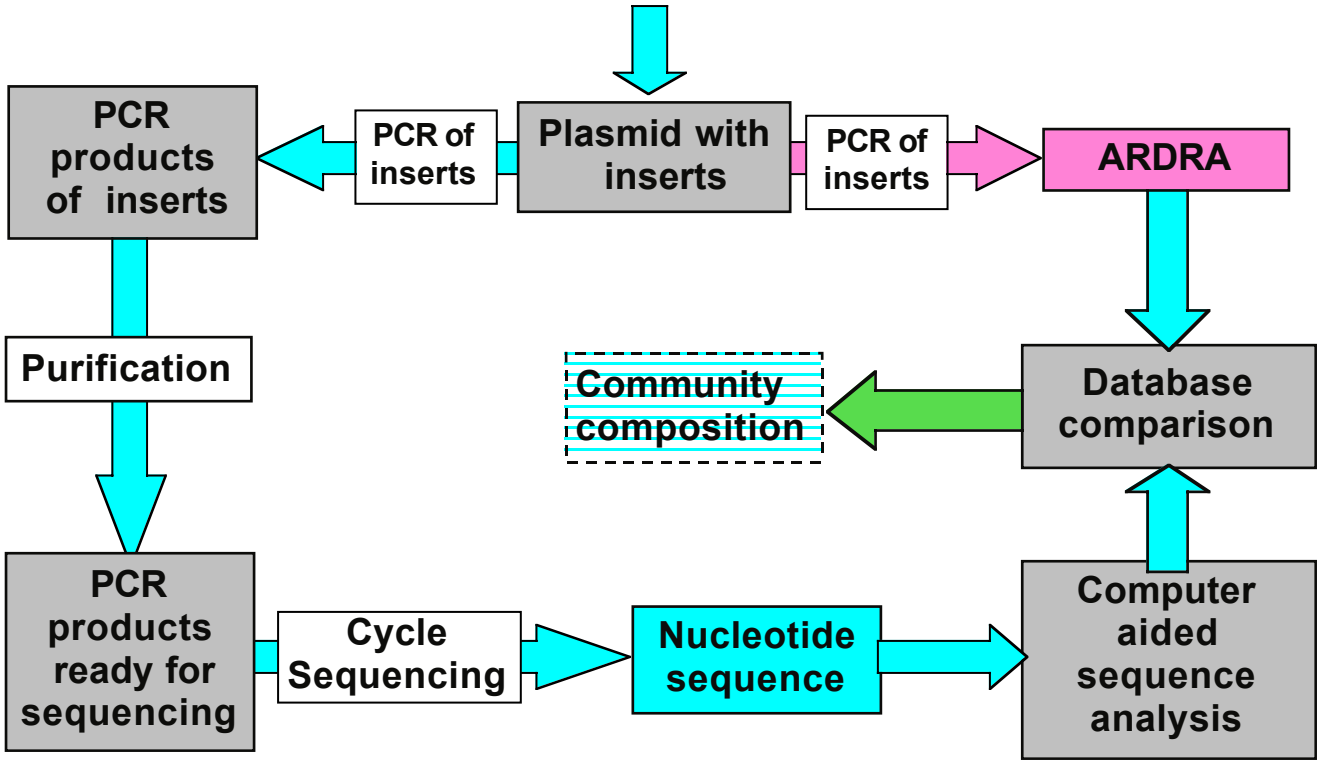
Work flow sheet (Fig. 1)





PCR amplification of 16S rDNA subclone inserts

Lysates of the cloned 16S rDNA inserts (in *E.coli)* were amplified by **PCR using M13** forward and reverse **primers**. M13 primers are small DNA fragments with sequences which are complementary to each strand of the edge of the original M13 vector, just up to the region where the restriction enzymes made their cut.

Analysis/Verification of the amplification products

The products were detected by **gel electrophoresis** in an 1% agarose gel using the following electrophoresis conditions: Tris-Acetate-EDTA (TAE) buffer 0.5 x, gel run for 30 min at 60 volts. 1 µl of sample DNA solution and 4 µl of standard loading buffer were loaded into each slot of the gel per sample. Detection of DNA was performed by staining the gels in ethidium bromide solution (final concentration: 1 µg/ml).

Testing the polymorphism of the inserts through ARDRA (Amplified Ribosomal DNA Restriction Analysis), (3). Selection of clones

PCR products of the amplification with M13 primers were **digested by the RsaI restriction enzym**e using standard procedure. The digested products were separated in a 2 % agarose gel by electrophoresis with the following conditions: Tris-Acetate-EDTA (TAE) buffer 0.5x, gel run for 60 min at 60 volts. 6µl of digested DNA together with 4 µl standard loading buffer was loaded into each slot of the gel.

Purification of the PCR products

The PCR products were purified, i.e. primer, unincorporated nucleotides and salts were removed. **Purification** was performed with **Amicon colums (Millipore).**

Verification of PCR-products

The purified products were analysed as described above (electrophoresis in an 1% agarose gel using the following electrophoresis conditions: Tris-Acetate-EDTA (TAE) buffer 0.5 x, gel run for 30 min at 60 volts. 1 µl of sample DNA solution and 4 µl of standard loading buffer were loaded into each slot of the gel per

sample. Detection of DNA was performed by staining the gels in ethidium bromide solution (final concentration: 1 µg/ml).

Sequencing of 16S rRNA genes

In the **sequencing reaction (see protocol)** only one primer is used, i.e. only one strand is copied. Therefore the number of copies of one strand of the gene increases linearly.
Sequencing was performed with ABI Prism® BigDye Terminator Cycle Sequencing Ready Reaction kit. Dideoxy nucleotides are added to the sequencing reaction components and forward and backward primers in separate PCR tubes We used M13 forward and reverse primers, in contrast to other groups who used internal primers. Electrophoresis and detection of the products was performed on a ABI Prism 377 Sequencer, (Perkin Elmer, Applied Biosystems).

Purification of Sequencing PCR products

Unincorporated dye was removed by passing the samples through **Sephadex** columns.

Separation of the labelled DNA molecules and detection on the automated Sequencer

The mixture of strands fluorescently labelled with ddNTP are separated on their size in a polyacrylamide gel by electrophoresis.
By passing a laser beam at the bottom of the gel, the fluorescently labelled fragments can be detected. Since each base has its own color, i.e. its own excitation and emission wavelenghts, the sequencer can detect the order of the bases in the sequenced gene.

Searching the Databases for analysis and identification of sequences

The obtained sequences were compared with known sequences deposited in the NCBI (National Center for Biotechnology Information) databank. Search query was perfomed with the Nucleotide Blast® (Basic Local Alignment Search Tool) program (http://www.ncbi.nlm.nih.gov/blast/).

Results and Conclusions

PCR-Amplification and ARDRA (Amplified Ribosomal DNA Restriction Analysis)

Results of amplification of inserts with M13 primers are shown in Fig. 2.

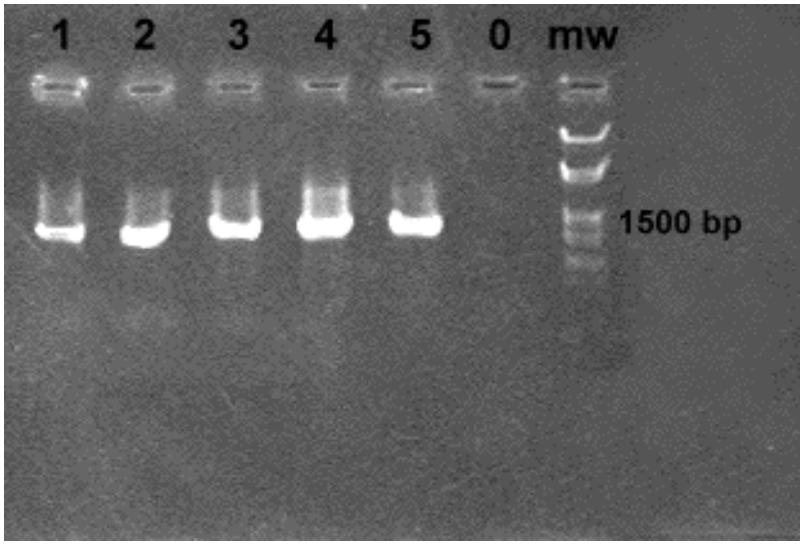


Fig. 2 Gel electrophoresis of (16s rDNA) **PCR products** after amplification of the inserts with M13 primers. 1: clone 4_{R2}, 2: clone 5_{R2}, 3: clone 9_{R2}, 4: clone 17_{R7}, 5: positive control, 0: negative control (sterile ddH_2O instead of template DNA)

ARDRA analysis of clones 4, 5, 9 from reactor 2 revealed different restriction fragment patterns (picture not shown). Clones 1, 2 ,4 and 5 from reactor 7 had the same restriction fragment pattern (Fig. 3), suggesting identity. For verification another restriction enzyme will be used. Clones 4,5,9 from R2 and clone 5 from R7 were further processed.

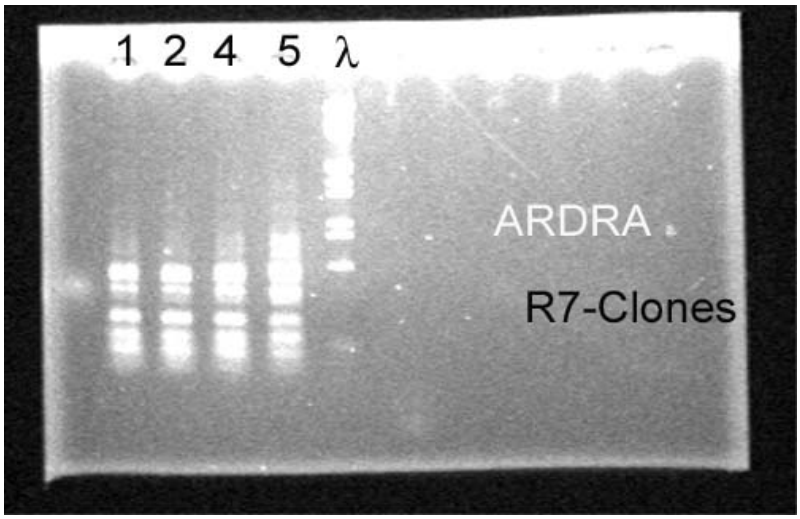


Figure 3: ARDRA of the previously amplified PCR products of clones 1,2,4,5 from reactor R_7. l is the molecular weight marker.

Sequences

Search with the Nucleotide Blast program gave us hints on the group of microorganisms to which our clones belong to.
Clone 5 from reactor 7 (5_{R7}) is a member of a beta-subgroup proteobacterium and has 98 % similarity with an unidentified clone Wuba72.
Clone 4 from reactor 2 (4_{R2}) revealed 94% similarity with *Ralstonia taiwanensis;* clone 5 from reactor 2 (5_{R2}) 96% similarity with a *Sphingomonas* species (alpha-proteobacterium); clone 9 showed >90% similarity with *Ralstonia solanacearum*

Applying this analysis pathway to all the clones of the respective reactors (ca. 50-100 for each reactor) will give us data on all the organisms that are found in the community. The collection of data will be assembled to form a genetic library of metal corroding bacteria. Such libraries will enable us to create specific probes for *in situ* identification of the target organisms on specimen of actual corrosion cases (1).

Literature

1. Amman R.I., Ludwig W., and Schleifer K.H. 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* 59, 143-169.

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