Controllable Adhesion of Microbial Reactors

iGEM team Delft University of Technology 2011

Summary

For this year's iGEM competition, the team of Delft University of Technology has developed a procedure for engineering a microbial cell of interest to both the industry and academia. The idea is to use a protein with adhesive properties to be able to arrange and cluster cells in a controllable way. With a look at possible industrial applications, we envision solutions for environmental issues (e.g. waste water treatment), for instance by making biofilm formation controllable in order to bind cells to a surface, whenever desired. This can for instance simplify biomass removal from an environment. Furthermore, a controllable cellular array can be made in order to detect pollutants on a micro scale, which makes the process faster and cheaper.

Controllable configuration of cells can also be interesting for fundamental sciences. Controlling spatial arrangements of cell populations can lead to a more detailed knowledge of cell-to-cell communication. This can be very interesting for applications in tissue development: how do cells influence each other in making a specific configuration?

From a technical standpoint, in order to use the selected adhesive protein to make cellular binding controllable, it needs to be anchored and active in the outer side of the cell, preferably in one specific location: here lies exactly the major challenge of this research project aimed at engineering a bacterial cell.

Background and Rationale

The basis of our project proposal is a synthetic toolbox for bioadhesion. Bioadhesion is a natural phenomenon that is especially relevant for biofilm formation (Hall-Stoodley et al. 2004), and which can be exploited in various engineering application: microbial process improvement by biomass retention in reactors, bioarray design, and bioconstruction. Microbial production processes mainly rely on submerged cultures made up of single cells. They are used in production processes because of their stability, predictability and controllability. Single cells ensure that the whole cellular surface can be accessed and that high transport rates are achieved. Nevertheless, retention of the biomass is beneficial for many production processes. Instead of fixing carbon in biomass, the carbon can be channeled exclusively to the desired product. Usually, retention using a biofilm is not desired, because the extracellular matrix and the thick multi cell-layer rapidly face transport limitations. In nature, however, mixed cultures are the norm and they are predominantly present in the form of biofilms (Hall-Stoodley et al. 2004). Biofilms are generally located on an abiotic surface and remain on that throughout time. This seemingly basic ability is actually coveted in industry and academia, preferably without the need of a mixed culture (Groboillot et al. 1997). Control of the in situ adhesion properties of cells can be used to retain or remove cells from a solution in which they were previously freely dissolved. When able to control the attachment and detachment of cells, in industry one could use this to simplify biomass retention and removal.
Synthetic adhesion can be used to fixate active biomass in a specific spatial configuration, which can be used for bioarray design as well as for a sequence of bioconversion processes (production line design). More fundamental applications are also possible, such as the localization of specific colonies or even spatial arrangement and movement of cell populations.
In general, this control holds promising possibilities for industry and academia alike. As a proof of principle, we want to develop a pollution-sensing array. The array will consist of several different pollutant-sensing E. coli, each attached to a specific spot on a simple plastic surface. An easy to read output signal (fluorescence or pigment) is used to show the presence of a certain pollutant (see Figure 1).
This sets the stage for a cheap and simple detection array. We foresee the use of these arrays in detection of possible harmful side products of waste water streams.

Building on the work of last year's TU Delft iGEM team (project Alkanivore), the process combined with our adhesion system can be used in waste water treatment, removing the need of complicated biomass removal steps. With the addition of the adhesion mechanism, the bacteria will attach themselves to a given surface once their task is done, or whenever a specific signal is sensed. The treated water can then be moved to the next purification step, while the bacteria remain in their place. Furthermore, these can then be physically removed if bound to certain carriers, while using the detachment will allow for the reuse or the uncontaminated washout of any biomass left. Retaining biomass would be very advantageous in processes using slow-growing bacteria, a property which is not uncommon to pollutant-consuming organisms. Once an adequate biomass concentration is reached, it can then be easily retained for future purifications without the need of additional chemicals, particles or filters.

Controlling attachment requires a synthetic control of the cell's ability to attach and detach. The natural mechanisms used for adhesion would require engineering a large intricate network of genes (Karatan and Watnick 2009). Therefore, we propose to not engineer the natural biofilm network, but to design an alternative adhesion system.

A solution is available outside the natural bacterial biofilm mechanisms. Using a heterologous adhesive protein, we will be able to control the adhesion of cells to other cells, as well as that of cells to surfaces. This protein is called Mussels foot protein 5 (Mfp5). This protein owns its adhesion mechanism to L-DOPA groups in the protein, created by conversion of Tyrosine to L-DOPA, greatly increasing its adhesive abilities (Weinhold et al. 2006). These L-DOPA groups can subsequently be converted to o-quinone groups by oxidation, significantly decreasing the adhesive abilities. These distinct forms are what allow the control of adhesion within one protein.

Despite the fact that it originated in a multicellular eukaryote, Mfp5 has been expressed in the well-known bacterial workhorse Escherichia coli and proven to be functional (Hwang et al. 2004). This allows us to combine the unique properties of Mfp5 with any other biocatalyst functions available in E. coli. Specifically the possibility to bind in aqueous environments, ensuring its use in microbial solutions and the controllability of its adhesion, allowing attachment and detachment at a desired time or place, are unique to Mfp5.
In perspective, we will be able to fully control localization of cell colonies.

Objectives of Proposed Research

The ability to combine the unique properties of the Mfp5 protein with the workhorse *E. Coli* has led us to three parallel subprojects; cell-cell controllable attachment, cell-surface controllable attachment and the optimization and characterization of Mfp5.
The total project holds promising possibilities for industry and academia.

Aim

Our aim of competing in the 2011 iGEM competition is to engineer a bacterial strain to give it new properties in order to achieve controllable adhesion of microbial reactors. The main theme to achieve this aim is to control cell-cell attachment and cell-surface attachment in such a way that it is applicable for industry and fundamental research.

Long-term goals with corresponding, specific required objectives

Some long-term goals require overlapping specifying objectives, which allows us to work parallel on the projects (see also research design and methods).

Cell - Surface attachment:

- Simplification and full control of biomass removal, applicable in several industrial processes
- Detection arrays; we would like to design detection arrays for possible harmful by-products in oil refinery waste streams.
- The production line; attachment of *E. coli* cells with different properties in a desired ranking, for example organism A converts a substrate to a product, which is subsequently used by organism B as a substrate and so on.

Cell - Cell attachment

- Localization of cells to create a desired configuration; this is a more fundamental approach which is very interesting in understanding communication and other interactions between cells.
- Control of spatial movements of cell populations

Optimization and characterization of Mfp5

We choose to focus on the variation of amount of DOPA[1] and the influence of pH on the adhesiveness. Both parts will contribute to better understanding of chemical controllability of the protein.

Specific objectives

- Overexpression and secretion of tyrosine, the activator of Mfp5
- Controllable attachment – detachment of the cells by regulating the amount of adhesive protein and by regulating the adhesiveness.
- Target the Mfp5 integration in the membrane to a specific site of the cell in such a way that desired configurations are possible to create.
- Control the amount of DOPA to control adhesiveness.
- Determine the influence of pH on the adhesiveness
- Combine controllable Mfp5 and other desired characteristics in *E. coli*. (Including last year's alkanivore properties, sensing devices etc)

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[1] Molecule that gives the protein its adhesive properties when activated

Research Outline and Methods

The project can be defined in several work packages: (1) Mfp5 expression and activation, characterization of adhesion, (2) Protein engineering to improve adhesion, (3) Target the protein to specific positions in the membrane.

Mfp5 Production

Using the available Mfp5 BioBrick made by the Berkeley 2009 iGEM team, we will transform *E. coli* competent cells and use the standardized marker for screening to check for transformants on agar plates. Purchased tyrosinase will be added to the culture, activating external Mfp5. This should lead to aggregation of cells and adhesion to vessel walls. This is the quick and easy route – if no aggregation or adhesion is observed, we will switch to a more detailed approach. This would include the modification of the Berkeley BioBrick to include the cleavage signal for the auto-transporter system, which will cause the Mfp5 not to be incorporated into the membrane, but to be excreted. Modification will be done with simple restriction and ligation techniques, since all parts are known to be available and well characterized. If secretion is successful but functional membrane integration is not, other spacers than those used by Berkeley may solve this problem.
A very similar approach will be used for tyrosinase production. For tyrosinase no BioBrick is available so we will synthesize the sequence shown to function in *E. coli* by (Cabrera-Valladares et al., 2005). When available, cloning will allow for further extensive use of the gene. A problem here could be uncontrolled tyrosine oxidation. To prevent this a transport system forcing tyrosinase to remain unfolded should be used. Improper extracellular folding may be solved by parameter or environment tuning.

When possible, we will deal with the targeting of tyrosinase to the inner membrane. This will allow for activation of Mfp5 inside the cell without making intracellular aggregates. The approach would be similar as that described for Mfp5, except that one needs to discern internal from outer membrane integration. This can be checked by adding substrate for tyrosinase to the medium and by measuring conversion rates.

Adhesion

We want to engineer the adhesive abilities of Mfp5 as soon as secretion and/or membrane integration has been achieved. The major cause of adhesion is the L-DOPA group resulting from the hydroxylation of tyrosine present in the protein. Therefore an intuitive approach would be to increase the tyrosine content. Although an increase in DOPA content does not guarantee better adhesion, it was shown to improve performance (Lin et al. 2007). Other protein engineering targets are the flexible portions and spacer sequences influencing the folding of the protein. The actual engineering of the sequence could be done by basic restriction and ligation techniques but synthesis is preferred for its precision and predictability.

Cell-to-Surface attachment

An approach to test the attachment may be that proposed by (Hwang et al. 2004), in which specific slides were used to check the attachment. Exertion of a small flow can readily show which cells are attached and an increasing flow rate will increase the shear force, giving a practical measure of the strength. For the control of the attachment, an inducible promoter should be used, whereas for a proof-of-principle we will probably use IPTG as a signal

molecule. To detect a change in the binding strength one should first check it in a static environment, by analysing also a negative control without inducer. More interesting would be a real-time binary system, in which one could change between a freely dissolved culture to, upon induction, attachment of cells to the vessel wall reducing cell density measured by spectrophotometry. The presence of cells on the wall can be checked by adding lysing agents. Subsequently, the absence of the signal should result in a slow release of the cells due to Mfp5 degradation and replacement by its inactive form. A faster method would be increasing the pH, resulting in the oxidation of L-DOPA to *o*-quinone, decreasing its binding strength.

Cell-to-Cell attachment

One of our interests is the formation of controllable cell aggregates using membrane-bound Mfp5. We will use micro fluidic devices to study the cell-to-cell interaction at the micron scale. By directing Mfp5 towards one direction of the cell, it will become possible to form structures like a line of single cells (Figure 2). The difficulty here lies in finding any direction in an *E. coli* cell. We intend to utilize the flagella localization machinery, since the cell has a unique flagella motor and since previous iGEM teams have developed experience in engineering the flagellar machinery. The existence of any directional binding should be observable using high resolution fluorescence microscopes.

Final Goals

If all of the systems described above are working correctly, we can aim towards a possible application. The major determining factor for getting this far is time availability. We have the beneficial position of all members having lab experience. Another way to save time is depicted by the ancient saying “time is money”: for example, when more funds are available we can order constructs or competent cells we would otherwise have to make, which can be tricky and unstable. An increase in funds would also increase the total amount of experiments we can run, since the chemicals determine our major expenses. We have three systems in mind, ranging from fundamental to health, environment and industry. In the fundamental system we would use the cells with Mfp5 in one specific direction for the production of a spatial configuration. In the health/environmental based application, we are aiming towards a pollutant sensing mechanism. Therefore, we need to combine our Mfp5 attachment system with a sensing and signalling mechanism available from the iGEM database. In the industrial system we intend to use the attachment/detachment system to let cells attach to the reactor wall, flush the vessel, and release.

Significance and Future Directions

Our project is based on the use of the unique adhesive properties of mussel foot proteins, aiming towards their improvement and control, and combining them with the growing need to use pure culture biofilms for many relevant applications. The excretion of the recombinant adhesive protein is definitely feasible. The challenge in this experiment is to achieve the differentiation of the adhesive strength of the protein to engineer cells with different adhesive properties. There are many successful previous studies about the expression of these proteins and of their properties and also about their potential applications. With this grant proposal, we present a new perspective on the usage of these recombinant proteins combined with engineered bacteria in order to control adhesion of microbes in different environments and applications. Our study will be not only the basis but also the first and the most important step for the implementation of these goals in a number of areas of the industry (e.g. water treatment and oil treatment, fermentation processes, medical implants), as well as in academia.

As mentioned above, the achievement of completion of this project will pave the way for large scale applications of this study, mainly for environmental use, such as a solution to collect plastics from the plastic garbage patch and the oil removal from water. This could be possible by combining organisms that degrade or remove from the environment this kind of substances.

Ethics

Obtaining specific organisms and unique properties by engineered modification is one of the main objectives of Synthetic Biology. These modifications may involve risks that either have influence on the working environment of scientists or on society in general. These risks need to be taken into account by the scientific community. The effect of these risks differs and depends on the type of organism used as a microbial workhorse, the environment in which it is applied, the techniques used in the lab, and on the properties that scientists target to change. Risks should be in balance with the benefits that applications of research might lead to. Therefore, all possible involved risks should be evaluated in respect to the scientist's safety and to society.

In our specific project we are employing *E. coli* as host for a protein derived from Mussels. As a prime example of Synthetic Biology, it also carries all related risks. With regards to the organism used there are no major risks since *E. coli* has a Generally Regarded As Safe status and Mussels are not known for pathogenic activity. Still, use of any genetically modified organism in an uncontrolled environment includes risks and therefore a careful approach is necessary in both the lab and in future applications.

Figures

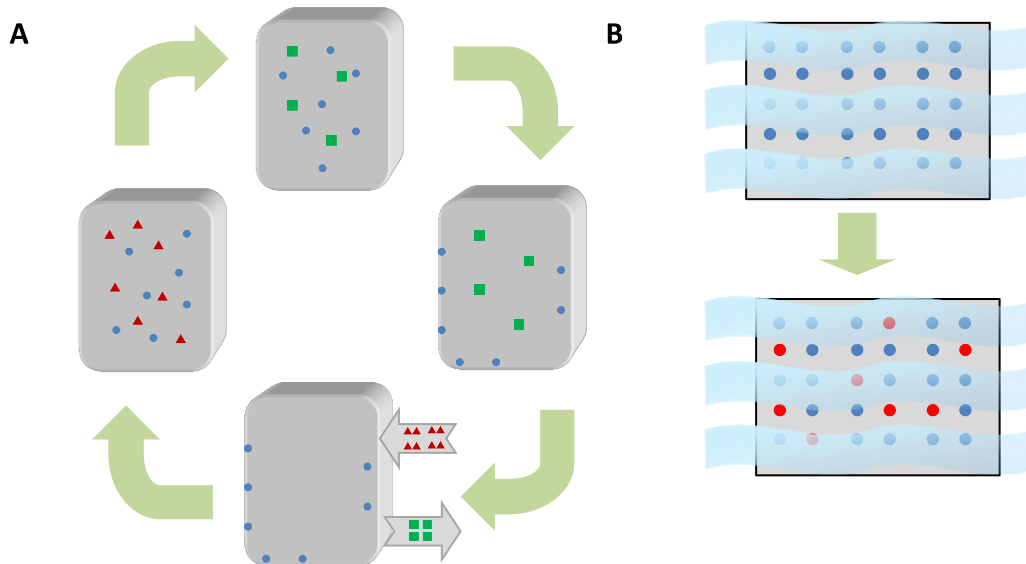

Figure 1: a) Synthetic adhesion for biomass retention: Clockwise arrows indicate, starting from top-left: i) Active bioconversion, ii) Substrate depletion triggers cell adhesion, iii) Product removal and substrate addition, iv) Presence of substrate triggers cell release. Triangle: substrate, Square: product, Circle: biomass.
b) Bioarray design: Different cell colonies (blue) are fixed to specific spots in the array. When exposed to a stream containing pollutants, they will give a corresponding visual signal (red colonies).

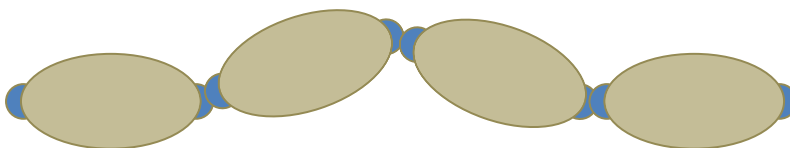

Figure 2: Bioconstruction: Using membrane targeting of the adhesive protein, cell lines can be constructed. When the proteins are located on one side, an internal gradient can be used to determine the opposite side. With proteins on two opposite sides in all cells, a line will be formed by the preferential binding from protein to protein.