Amyloid Based Ceramic Biofilm and its Applications in Building Materials

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Abstract

Biofilm-Integrated Nanofiber Display (BIND) (Nguyen, Botyanszki, Tay, & Joshi, 2014a) is a platform for the engineering of the bacterial extracellular matrix material in amyloid. In this platform an engineered version of the CsgA protein can be functionalized in a variety of ways by fusing different peptide domains to it. We thought to exploit this mechanism by engineering the CsgA protein in order to create a ceramic coating which resample the properties of nacre and can therefore potentially exhibiting corrosion and mold protection, as well as fire resistance. Due to its biological means of production, the resulting coating is emission-free, non-toxic and would require far less caloric input than typical ceramic processes, which are famously energy intensive.

Microbiologically induced calcium carbonate precipitation (MICP) is a natural process which can be mimicked and used for a number of applications, such as metal remediation, concrete restoration and carbon sequestration. The carbonate precipitation induced by these microbes can be achieved via Urease or Carbonic Anhydrase (CA). The latter is one of the fastest known catalysts and is the enzyme responsible for the reversible hydration of CO$_2$ and can therefore constitute an interesting tool for CO$_2$ sequestration.

For this reason it was decided to fuse CA to CsgA in order to assist the biomineralization process, thereby creating an amyloid based ceramic biofilm. Different sequences of CA from different species: *Gallus gallus* α-CAII, *Bacillus subtilis* XF-1 β-CA, *e. Coli* β-CA, ζ-CA from *Thalassiosira weissflogi* were successfully cloned using Gibson Assembly into a previously described plasmid (Dorval Courchesne, Duraj-Thatte, Tay, Nguyen, & Joshi, 2016).

All four different CAs cloned showed enzymatic activity at pH above 8.5 making these the first set of active enzymes directly fused to CsgA.
Furthermore, all the constructs have been showing the ability of retaining the precipitation of calcium carbonate attached to their biofilm even after extensive washing. It is concluded that this is due to the presence of the enzyme, given that in the positive control the precipitation was rinsed away after washing.

Altogether these results demonstrate that the enzymes fused to CsgA are capable not only of assisting in the precipitation, but also in retaining the precipitants on the biofilm, confirming thus the importance of the enzyme in the biomineralization process and therefore making it an indispensable element in the creation of a ceramic biofilm.
Author’s Biographical Sketch

I am a researcher at the Joshi lab at the Wyss Institute for Biologically Inspired Engineering at Harvard University, where this thesis has been developed as part of the Degree of Master of Liberal Arts in Extension Studies in the Field of Biology.

A native of Italy and a licensed architect in the EU, prior to entering into the field of biology I practiced architecture in Europe and in the US with among others Zaha Hadid Architects, Foster+Partners and UNStudio. During these years in architectural practice, I became acutely aware of how much is to be gained in the construction industry through more efficiently performing materials and with less wasteful production processes. Crucial to this is a community in the architectural world who not only possesses the skillset necessary for realizing complex building projects, but who can take the larger questions posed during the process and translate them into hard science. With this at hand, I seek to merge diverse fields of investigation and to leverage expertise from multiple disciplines in design and science.

Combining lifelong passions both for science and for the built environment, my long-term aim is that of creating advanced active and passive materials for architectural applications via biological means.
Dedication

To my father, who unfortunately will never be able to read this but who would have been extremely proud to see this evolution from architecture into science and engineering, all of which were passions of his.
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Chapter I.
Introduction

Biofilms are formed by colonies of microorganisms together with the extracellular matrix (ECM) which they secrete and in which they grow. They are capable of adhering to an array of substrates creating a cohesive surface coating. Biofilms are ubiquitous and are capable of growing in extreme conditions – from hot springs to glaciers. Although biofilms are usually associated with tenacious infections such as pneumonia, the creation of the Biofilm-Integrated Nanofiber Display (BIND) platform (Nguyen, Botyanszki, Tay, & Joshi, 2014b) by Prof. Joshi's group at the Wyss Institute for Biologically Inspired Engineering at Harvard University shows how the bacteria's ability to create a tenacious biofilm can be exploited and engineered for beneficial purposes by reprogramming the bacterial ECM material.

This research aims to exploit the ability of the bacteria to create a programmable surface by combining the ability of BIND to strongly adhere to the substrates, and therefore to aggregates, together with microbiologically induced calcite precipitation (MICP) which will lead to calcium carbonate precipitation. We intend to accomplish the above by directly fusing carbonic anhydrase - responsible for the carbonate precipitation - to curli functional amyloid fibers which is the major protein component in the creation of the biofilm, and the fundamental element in the biofilm’s adhesion to its substrate. The ultimate aim hereof is the engineering of highly durable coatings for building construction materials.

Stemming directly from a growing public awareness over climate change and other human and environmental health hazards, policy and code changes are dictating an ever increasing environmental compliance in building construction techniques, materials
and design. In this context, over the past several decades ever more stringent requirements have arrived on the legislative level concerning material chemistry and means of material production, as well as on the energy efficiency and emissions of buildings in use. Yet, a majority of widely used building materials are still non-renewable, limited in their recyclability, produced by energy-intensive and environmentally damaging means, and/or are toxic during production, construction and end use. In the latter half of the 19th century, steel and reinforced concrete were developed. This represented a fundamental and permanent shift in building technology, and, still to this day, those materials and their derivatives dominate global building construction. Bioengineering, being a rapidly evolving yet relatively young field, holds similar potential to fundamentally change building construction as did material science during those decades following the industrial revolution. New building materials that arrive on the market need to comply with existing environmental and worker safety legislation while still maintaining, at minimum, a core offering the same standards of well tested and proven materials. In parallel there is increased public scrutiny and criticism of numerous extraction processes in anything from harvesting of tropical hardwood, through mining for stone or precious metals and all the way to fossil fuel based materials like plastics. With any given material, the trade-off between its production process and life cycle vis-a-vis its desired properties whether they be mechanical, chemical, tactile, visual or other, comes largely down to material chemistry and therefore molecular makeup.

With this at hand, it seems plausible that bioengineering can provide us with an unprecedented opportunity to tailor the ideal material to a particular set of needs,
mitigating many compromises that previously would have heavily influenced the process, and thereby redefining the very way we design and construct buildings.

Figure 1. Micro to Macro

The conceptual potential scalar relationship between a customized bioengineered microorganism, biofilms to architectural components and our built environment.
Project Description

Statement of Research Hypothesis

This research hypothesizes that biofilms produced by certain strains of Escherichia coli (E. coli) can be engineered to produce different type of coatings and technologies for Architecture Engineering Construction (AEC) applications. The overall aim is to mitigate specific problems such as corrosion mold formation and fire protection in buildings and more broadly to reduce harmful emissions caused by AEC related activity.

In order to achieve the above this research intends to use BIND technology to engineer bacteria to induce carbonate precipitation by controlling their production of carbonic anhydrase, with the final aim of creating a ceramic-like coating capable of protecting its substrate.

Specific Aims

Through achievement of evaluation of the results of the five Specific Aims, below, we seek, with this thesis to achieve a better understanding of the application and suitability of the BIND biofilm as building construction material.

- Aim 1. Identify and display different classes of CA on curli fibers and show its activity.
• Aim 2. Identify the ability of the enzyme displayed on curli fibers to facilitate the precipitation of calcium carbonate. Would the resulting biofilm be potentially useful as tool for CO₂ sequestration?

• Aim 3. Investigate upon the possibility to use the biofilm in order to create 3D architectures.

• Aim 4. Investigate upon the possibility to create a ceramic biofilm with BIND.

• Aim 5. Would the above mentioned ceramic coating constitute a suitable fire retardant in material such as timber, textile and, in general, in cellulose based materials? Furthermore could this material constitute a suitable protection from corrosion in metal such as rebar (the tensile steel component used in reinforced concrete)?

As the duration of this project is highly limited, aim 5 has to be seen as speculative work based upon the results obtained in the first 4 aims to determine future investigations and work trajectories.

Work Significance

We believe the possible outcome of this study presents a viable, novel option for surface treatment of building components as a durable, non-toxic, corrosion and fire resistant material.

In theory, by resembling nacre properties, the proposed system would have a density 1/3 that of steel and a degree of surface toughness approaching that of advanced engineering ceramics (Heinemann, Launspach, Gries, & Fritz, 2011), making the product
not only applicable to the building industry but also in the naval or aerospace fields along with several other industries where these properties are required. The resulting product would also be biocompatible and could therefore be used in areas such as tissue engineering. Because of the similar biomineralization process and resemblance to enamel, it could be used for instance as alternative product for fillings in dentistry and as coating for bone replacement.

Generally, ceramics are highly resistant to redox reactions (rapid (burning) and slow (corrosion)) because the elements composing the compounds are already very tightly bound and changing the compounds through the separation of compound and reduction and oxidation of the elements realizes very little, if any, negative change potential energy of the final state. Due to the inability of wood to resist high temperatures, a ceramic coating for this material has to date been an impossible proposition. Wood is also a material which is extensively used in building construction, although since the decades following the industrial revolution it has been relegated to mainly smaller structures in low-density or rural settings. However contemporary architecture and engineering are gradually expanding the use of wood for larger buildings in urban settings, and building codes are incrementally evolving accordingly. We believe that a positive result would create a basis for a highly innovative material, and one which holds potential to fill a presently untapped market segment.

Furthermore, during the aggregation process, CO$_2$ - being one of the components of the chemical reaction that occurs - is sequestered from the surrounding air. The only by-product of this reaction is H$_2$O, radically setting the process apart from conventional
forms of ceramic coating, which are typically either applied at great energy expenditure or, in the case of ambient cured systems, cause harmful emissions.

Background of the Problem

Building construction is one of society’s most polluting activities, accounting for a significant portion of total global greenhouse gas emissions – by some estimates upward of 40% (Un-Habitat, 2012). A lot is to be gained in this industry through more efficiently performing materials and with less wasteful production processes. A possible solution to this problem would be that of using nature’s ability to self-assemble materials.

Limitations of existing cladding materials

Building envelope materials have to adhere to a series of building code requirements and fulfill various engineering constraints, including:

- Strength
- Weight
- Fire safety
- Maintenance regime
- Durability
- Heat transfer
- Moisture management
- Impact on human health
- Environmental compliance in sourcing, manufacturing and operation
All of this puts limitations constraints on the production, procurement and use of any given building material. These can relate to environmental concerns, such as emissions harmful to workers (e.g. vinyl); harmful to humans as well as the environment itself (e.g. brick and concrete); and extraction and/or regrowth and its impact on the natural environment (e.g. wood and stone). It can be a performative limitation such as corrosion (e.g. metals and polymers); flammability (e.g. wood products and certain polymers); or onerous maintenance. For all of these materials there are then additional inherent constraints as to how they can be molded into shape and made into fit-for-purpose building components.

Limitations of existing coating technology

Ceramic coatings are generally used on metal components for wear, corrosion and friction resistance as well as thermal protection. Currently, there are several application technologies competing in this space. The majority of these and other application technologies require highly controlled environments and in most cases also some sort of gaseous or liquid fuel for combustion.

Proposed solutions

Since previous studies (Nguyen et al., 2014a) have shown that BIND is capable of selectively binding to specific materials, including steel, we believe that this technology can be used as anticorrosive coating in rebar, among other substrates. We are interested in testing this type of coating on different materials, including, as previously touched upon, those for which a ceramic coating represents an impossible proposition due to their inability to resist high temperatures, such as wood and fabrics. We are particularly
interested in BIND in this instance due to its customizability. In principle, much of the
aforementioned can be achieved using for instance cellulose or alginate, both of which
however would need much more extensive testing and on a case-by-case basis before
compatibility can be ensured. We believe for this reason that BIND offers an important
advantage in comparison, inasmuch as whatever may be the desired properties of
adherence and other performance, these can be identified and BIND can from the start be
tailored accordingly.

Biofilms, Curli fibers and Biofilm-Integrated Nanofiber Display (BIND)

Biofilms are usually associated with the recalcitrance of many infections to
antimicrobial therapy (Gilbert, Maira-Litran, McBain, Rickard, & Whyte, 2002). This is
due to the ability of the bacteria to secrete a protective polymeric matrix made of
polysaccharides, protein, DNA and lipids (Flemming & Wingender, 2010) which protects
the cell from external agents.

Many different strains of bacteria are capable of secreting protein nanofibers,
which form amyloid plaques in the ECM. These amyloids constitute the major
compponent of the biofilm’s biomass, and are responsible for the adhesion to its substrate.
After the initial reversible attachment, the adhesion becomes irreversible, allowing for
different stages of maturation and, finally, dispersion of new colonies. This allows the
biofilm to colonize neighboring surfaces, making it increasingly resistant to antibiotics.

Curli fibers are amyloid nanofibers produced by Enterobacteriaceae's nucleation-
precipitation secretion pathway (Barnhart & Chapman, 2006). The Curli assembly is
controlled by different Curli-specific genes called *csg*, which are encoded by 2
divergently transcribe operons - *csgDEFG* and *csgBAC* - where *CsgD* is the master
regulator necessary for the transcription of the \textit{csgBAC} operon. In this system the cell secretes a \textit{CsgA} protein through the out-of-membrane pore \textit{CsgG}. \textit{CsgA} is then nucleated by another Curli fiber subunit protein, \textit{CsgB}, which anchors \textit{CsgA} to the cell surface (Blanco, Evans, Smith, Badtke, & Chapman, 2012) (Fig. 2).

BIND works by exploiting this system. A laboratory strain of \textit{E. coli} containing a deletion of the \textit{CsgA} gene (\textit{E. coli ΔcgsA}) is transformed with a plasmid encoding an engineered version of the \textit{CsgA} protein, which can be functionalized in a variety of ways by fusing different peptide domains. In other words, the \textit{CsgA} protein can be engineered in order to display different types of coating treatments.

Some of the advantages discussed in Nguyen et al. (2014) of using this system are:

1. It represents an easier and more flexible method compared to engineering the exopolysaccharide part of the biofilm, something that would require a multiple step pathway and which would have less chemical tolerance.

2. \textit{CsgA} is capable of forming fibers between 4 and 7 nm in diameter, and of creating a dense matrix. Also, a recent study on Amyloid Fibrils show that the strength of these fibers (0.6 +/- 0.4 GPa) is comparable to that of steel and their mechanical stiffness (3.3 +/- 0.4 GPa) is comparable to that of silk (J. F. Smith, Knowles, Dobson, MacPhee, & Welland, 2006). Furthermore it has been shown that Curli fibers are highly resistant and require treatment such as formic acid (FA) or hexafluoro-2-propanol (HFIP) in order to liberate the monomers from their fibers (Nordstedt et al., 1994). These properties make this self-assembling
biofilm an interesting platform for the development of coatings for application to materials which are in constant or prolonged exposure to harsh environments.

3. Curli fibers can constitute up to 40% of the biomass of many biofilms and can therefore be engineered to create a biofilm, expressing the added functionalization in a large portion of the resulting engineered biofilm.

4. *E. coli* derived curli fibers are one of the most studied type of amyloids which is therefore an advantage in comparison with other, less known types of amyloids produced by many other bacteria.

5. Another mechanism implemented by BIND technology is the split-adhesin system (Zakeri et al., 2012). This system allows for larger proteins to be bonded; it uses a 13 amino-acid peptide SpyTag that binds with high specificity to the 15 kDa protein Spy-Catcher fused to the protein of interest.
Figure 2. Model of curli biogenesis

Excluding CsgD, the master curli regulator, all Csg proteins have Sec-dependent signal sequences allowing their secretion into the periplasm. The lipoprotein CsgG forms a pore-like structure in the outer membrane. The major subunit protein CsgA and the nucleator CsgB are secreted to the cell surface in a CsgG- and CsgE-dependent manner. CsgF associates with the outer membrane and is required for cell association of the minor curli fiber subunit CsgB. Situated at the cell surface, CsgB nucleates soluble, unstructured CsgA into a highly ordered amyloid fiber. Curli production can be visualized by CR binding, which is absent in a CsgA mutant, and by transmission electron microscopy (left inserts). Also shown are two CsgA subunits interacting in a cross-β conformation, with the R1–R5 interaction depicted (right inset). (Blanco et al., 2012)
Nacre

Nacre - the organic-inorganic composite material also known as mother-of-pearl – has a toughness greater than steel and close to that of advanced engineering ceramics, engineered polymers and some alloys. It also exhibits composite action far superior to any man-made material (Cartwright & Checa, 2007) (Z. Huang & Li, 2013) (Yao, Epstein, Liu, Sauer, & Yang, 2009) (Espinosa, Rim, Barthelat, & Buehler, 2009) (Sun & Bhushan, 2012). For this reason nacre has long been a biomimetic model material, inspiring material scientists to design and develop many advanced materials in the last two decades (Luz & Mano, 2009) (Schmieden, Meyer, & Aubin-Tam, 2016) (Barthelat, 2010) (Finnemore et al., 2012).

What is particularly interesting about this material, is the fact that its alternation of mineral (95%) and protein (5%) build-up gives nacre a toughness of about three orders of magnitude greater than the mineral it is made of, aragonite (Barthelat, 2010). In fact, due to the mineral-protein buildup of nacre, it achieves a high degree of strength and toughness by capitalizing on the hardness of the mineral component but where the organic interlayers prevent the material from being brittle by relieving stresses and therefore the propagation of cracking. In a sense the material works as a brick and mortar assembly, in which the “soft” mortar separates the “hard” components from one another and allows them to move independently, while absorbing stresses.

Furthermore a study published in Nature in early 2017 (Das, Thomas, Moeller, & Walther, 2017) demonstrates that a nacre-inspired self-assembly brick-wall nanostructure made from sodium carboxymethyl cellulose (CMC) / montmorillonite (MTM) can be used as fire retardant of textiles. This shows how highly loaded polymer/clay nano-
composites with layered structures, which mimics nacre’s structure and composition, can be used as robust fire retardant surface coating.

Nacre offers a series of advantages relative to aforementioned limitations, exhibiting corrosion and mold protection, fire resistance and increased material toughness while providing no shape limitations as well as a time-efficient, emission-free and non-toxic manufacturing environment due to its biological means of production. Process wise, we therefore see advantages of synthetic nacre over ceramic fabrication techniques – it would for instance quite likely require less caloric input than ceramic processes, which are famously energy intensive.

Microbiologically induced calcite precipitation (MICP)

The extraordinary ability of some strains of bacteria, such as Sporosarcina pasteurii, to induce ureolysis leading to calcium carbonate precipitation, has already been exploited in the building industry in order to make bio-bricks like bioMASON and in self-healing concrete (Wiktor & Jonkers, 2011). Some previous studies have also shown how MICP can be use as alternative to conventional methods of cementation, as a method in liquefaction prevention to improve the stability of the soil. (Cheng, Cord-Ruwisch, & Shahin, 2013) (Whiffin, van Paassen, & Harkes, 2007) (Cheng & Cord-Ruwisch, 2012)

This research will add onto these technologies the ability of BIND to selectively adhere to specific materials by engineering the bacteria in order, for instance, to absorb contaminants from acidic rain to stop them from entering the soil, or perhaps by absorbing the salt present in air and water to prevent them from corroding metals such as rebar in reinforced concrete.
In addition, even though some interesting processing such as freeze casting has shown to be a promising technique for mimicking nacre in a bulk material, so far nacreous processing techniques have been limited to creating a \(200\mu m\) layer material (Wegst et al., 2014). We believe that by using a combination of BIND and MICP we would be able to scale up the same process used for coating in order to produce bulk materials with ceramic resemblance.

**BIND - based Additive Manufacturing via biological production means.**

Additive Manufacturing (AM) is a technology by which three-dimensional objects are created through depositing material layer-by-layer. Some main aspects that sets AM apart from other traditional modes of making are that it has much fewer limitations in shape of final product, while producing little to no manufacturing waste. The proposed technology implements AM via biological production means. It works via self-assembly, meaning that the material once activated literally grows layer by layer on top of any geometric shape until stopped. In this way it can act as surface reinforcement, or protect from fire and corrosion materials, such as wood, alloys or fiberglass.

The aim is to engineer the bacteria in order to regulate the CA production ultimately producing nacre synthetically. To achieve this we engineered the *E. coli* \(\Delta CsgA\) in order to secrete fusion proteins with an amyloid domain, necessary for the self-assembly of the protein into fibers, which will be bonded together with a CA enzyme, where the latter will be responsible for producing the aragonite build up. This way we hope to obtain the typical alternation of aragonite and protein layers of nacre responsible for the toughness of this material.
Carbonic Anhydrase

Carbonic Anhydrase (CA) is an enzyme responsible for the reversible hydration of CO\(_2\) (CO\(_2\) + H\(_2\)O → HCO\(_3^−\) + H\(^+\)). The enzyme was discovered in 1933 at Cambridge University and described in an article concluding that the enzyme was necessary for the transit of HCO\(_3^−\) from the erythrocytes to the pulmonary capillary (Meldrum & Roughton, 1933).

The conversion of carbon dioxide and water to bicarbonate and protons occurs spontaneously but slowly (Badger & Price, 1994), however CA catalyzes the reaction of a million molecules per second, \((k_{cat}^{CO_2} \approx 10^6 \text{ s}^{-1})\) \(t \frac{k_{cat}}{K_m^{CO_2}} \approx 10^8 \text{ M}^{-1} \text{ s}^{-1}\), (Chegwidden, Carter, & Edwards, 2000) making it one of the fastest known catalysts. Since this reaction is fundamental to many physiological processes, the enzyme can be found in all kingdoms of life. In the animal kingdom it is important in the respiration and transport of CO\(_2\)/bicarbonate, in electrolyte secretion, calcification and bone resorption, in gluconeogenesis, lipogenesis, ureagenesis and tumorigenicity. In algae, plants and certain bacteria CA plays an important role in photosynthesis, in marine diatoms it is important for carbon fixation. In protozoa, although still not clear, it is involved in certain de novo biosynthetic pathways (Krungkrai, Krungkrai, & Supuran, 2007). Perhaps its ubiquity as catalyst is the reason why the scientific community keeps making discoveries on the evolution and mechanisms of this enzyme. For instance, five different families of CA were first identified: α-CA, β-CA, γ-CA, δ-CA and ζ-CA to which one more was added in 2014: η-CA (Del Prete et al., 2014). All the CAs are metalloenzymes which evolved independently, α, β, δ and η-CAs use Zn ions at the active site while it has been suggested that γ-CAs uses a different transition metal than zinc (Alber et al., 1999). ζ-
CAAs are cambialistic enzymes which can be active with either Cd or Zn ions (Supuran & Capasso, 2016) (Fig. 3 and 5).

Figure 3. The reversible hydration of carbon dioxide to bicarbonate

The reversible hydration of carbon dioxide to bicarbonate catalyzed by CAs by means of a metal (M)-hydroxide mechanism. Modified from Berg [17]. (1) The release of a proton from the zinc-bound water generates the zinc-bound OH$^{-}$; (2) A CO$_2$ molecule binds to the active site and is positioned for optimal interaction with the zinc-bound OH$^{-}$; (3) The hydroxide ion attacks the carbonyl of CO$_2$, producing HCO$_3^{-}$; (4) The release of HCO$_3^{-}$ regenerates the enzyme. (Lionetto, Caricato, Giordano, & Schettino, 2016)
The $\alpha$-Carbonic Anhydrases

$\alpha$-CAs are monomers involving three histidine ligands and can be found in the animal kingdom but also in bacteria and green algae. The catalysis happens via the nucleophilic attack of the $\text{Zn-OH}^-$ on $\text{CO}_2$. Due to its importance in human physiology, this class is by far the most studied. There are at least 16 isoforms found in mammals, which can be subdivided into cytosolic CAs (CA-I, CA-II, CA-III, CA-VII and CA-XIII), mitochondrial CAs (CA-VA and CA-VB), secreted CAs (CA-VI), membrane-associated (CA-IV, CA-IX, CA-XII, CA-XIV XV) and CA-related proteins (CA-RP VIII, X and XI) which exhibit no CA activity (Pan, Rodriguez, & Parkkila, 2007).

The $\beta$-Carbonic Anhydrases

$\beta$-CA have two conserved cysteines and one conserved histidine (Kimber & Pai, 2000) and are polymeric, with a dimer as the basic building block: they can in fact be dimers, tetramers, hexamers, and octamers, and have a wide range of molecular masses reaching up to 200 kDa.

They were initially thought to be found only in plants but can be found in bacteria, plant chloroplasts, red and green algae, and in the Archaea. $\beta$-CA is the only family known to exhibit allostery (Rowlett, 2010a). The first reported crystal structure of $\beta$-CA is dated in 2000, when it was determined in the red alga $P.purpureum$ (Mitsuhashi et al., 2000). So far 12 different crystal structures can be found. $\beta$-CAs have a unique $\alpha/\beta$ fold which creates dimers, or in some cases pseudo dimers composed by a monomer and a short polypeptide linker. With the exception of Holothio bacillus
neopolitanus, which is missing one active site, all β-CAs are zinc metalloenzymes with one Zn ion per monomer.

The β-CAs can be separated in two classes or types, determined by the ligation of the active site and the orientation and organization of nearby residues (Rowlett, 2010b). Type I has a Zn(Cys)$_2$(His)(X) where X is an exchangeable ligand, which can be water, or an inorganic or organic ion, whereas type II are allosteric (Frost & McKenna, 2014). Furthermore type II has a steep pH-rate profile: it has been shown that Escherichia coli, Salmonella enterica, Haemophilus influenzae and Mycobacterium tuberculosis Rv3588 have a lower activity at pH below 8.0.

Haemophilus influenzae (HICA) is the archetype dimer of type II with an intact Arg-Trp-Tyr with residues involved in the allosteric switching of two different conformations: a T-state, the inactive state (typical of type II) and an active R-state (presumably structurally similar to that of the type I β-CAs) (Fig. 4). β-CA is active only when cellular bicarbonate concentrations are low (Fig. 4); in β-CA the bicarbonate ion is both an inhibitor and a product of the CO$_2$ hydration reaction, β-CA essentially acts as a bicarbonate regulator (Rowlett, 2010b). This allosteric regulation has only been demonstrated in two different β-CA: HICA and in E. coli CA (ECCA) which is required for aerobic growth under atmospheric CO$_2$ concentrations (Merlin, Masters, McAteer, & Coulson, 2003), however the physiological significance of this allosteric mechanism has yet to be demonstrated.
Figure 4. Allosteric regulation


The γ-Carbonic Anhydrases

γ-CAs are composed of only one omotrimeric enzyme and are so far only found in Bacteria, Archaea and plants. They are similar to the alpha class, since they also have three histidine ligands, however these ligands come from a different monomer. Their evolution is dated between 3 and 4.5 billion years ago. Although they are probably the oldest class, only two crystal structures are reported, both in the Archaea domain: one called Cam, found in Methanosarcina thermophila (Kisker, Schindelin, Alber, Ferry, & Rees, 1996), the other called CamH, a homolog from P. horikoshii (Jeyakanthan et al., 2008). Furthermore γ-CA has so far only been characterized biochemically in M. thermophila (Alber & Ferry, 1994).
It was considered to use Zn ion, however this conclusion was made based on the overproduction of the enzyme in E. coli. A recent study (MacAuley et al., 2009) had shown how the methanoarchaea such as *M. thermophile* - which, being anaerobic, live in ferro-rich and oxygen-free environments - when synthesized in vivo contains iron in its active site. In fact, when Cam uses iron its activity is much higher than the Cam aerobically purified from E. coli using Zn ion. Therefore Fe^{2+} is the physiological metal cofactor of γ-CAs (Tripp, Bell, Cruz, Krebs, & Ferry, 2004), making γ-CAs the only class using iron in its active site.

**The δ- and ζ- Carbonic Anhydrases**

δ-CA and ζ-CA are to date both only found in marine diatoms and they both play a very important role in carbon fixation in marine environments. In the late 90s a CA from diatom, *Thalassiosira weissflogii* (TWCA1), was purified (Roberts, Lane, & Morel, 1997) with the active site being similar to the alpha class. However the sequence was different from any of the enzymes found in this class, so the δ-class was proposed as a new class for TWCA1 (Soto et al., 2006). Another study found that whenever the concentration of Zn ions were low, and in the presence of either cadmium ions or low CO₂ pressure, this type of diatom can produce another form of CA; the CDCA1 (Lane et al., 2005). As CDCA1 resembles the active site of β class, but has no similarity to it in terms of neither structure nor gene sequence, a new enzymatic class, ζ-CA, was added. δ-CA resembles α-CAs involving three histidine ligands and Zn ion as metal cofactor where ζ-CA is a cambialistic enzyme which can be active with either Cd or Zn, and which uses two cysteine and one histidine.
The η-Carbonic Anhydrases

η-CA uses two histidine and one glutamine ligand and Zn ion bound within the active site. It was discovered in 2014 (Del Prete et al., 2014) in the malaria pathogen *Plasmodium falciparum*, one of the most severe forms of malaria. This pathogen needs purines and pyrimidines for DNA/RNA synthesis (De Simone, Di Fiore, Capasso, & Supuran, 2015) which the pathogen needs to synthesize de novo from HCO$_3^-$ adenosine-5-triphosphate (ATP) and glutamine (Gln), since they are both very low in concentration in human erythrocytes (Del Prete et al., 2016). HCO$_3^-$ is generated from CO$_2$ via η-CA and novel ways to inhibit this enzyme are under study in order to target this severe form of malaria.
Figure 5. The metal ion coordination pattern in the CAs

(A) α-, γ- and δ-CAs (in the α- and δ-classes the coordinating residues are from the same monomer, whereas in γ-CAs the third His is from an adjacent monomer, see also later in the text). (B) β-CAs (type I, opened active site). (C) β-CAs (type II, closed active site, an aspartate residues as the fourth zinc ligand). (D) ζ-CAs with Cd(II) bound within the active site. (E) η-CAs. "(Supuran, 2016)
Chapter II.
Material and Methods

Identification of coding sequences of carbonic anhydrase in different species

Considering the diversity of CAs in nature we decided to identify different sequences coming from a variety of organisms. In order to make sure that the cloned CA sequence of interest would be able to be translated together with the CsgA portion, we decided to concentrate on smaller sequences. Previous successful cloning of larger sequences were around 350 amino acid (AA), so we aimed for sequences under or around the same size. We cloned different CA coming from almost all different classes: four from $\alpha$-CA, three from $\beta$-CA, one from $\gamma$-CA, and the CDCA1 from *Thalassiosira weissflogii*, the cadmium-specific CA, $\zeta$-CA.

Sequences cloned from $\alpha$-CA

*Neisseria gonorrhoeae* $\alpha$-CA

The CA gene from *N. gonorrhoeae* encodes a 252-residue polypeptide. It is a high-activity CA with a CO$_2$ hydration turnover number of $1 \times 10^6$ s$^{-1}$ at pH 9 and 25°C and its Km is 2.5 fold the human CA type II. The crystal structure reported for this protein is a monomer (S. Huang et al., 1998) (Fig. 6) similar to that of human isozyme II. This CA was heterologously produced as a periplasmic protein lacking N-terminal 26 residues in in *E. coli* (Chirica, Elleby, Jonsson, & Lindskog, 1997). As it was previously cloned in *E. coli* we decided to choose this as possible candidate.
Figure 6. Crystal structure of the *N. gonorrhoeae* α class carbonic anhydrase.

"**A**: View of the monomeric *N. gonorrhoeae* structure. The active site Zn is shown in blue. The α-helices are shown in yellow and the β-strands are shown in blue. Residues Cys-28 (shown in red) and Cys-181 (shown in green) are completely conserved in prokaryotic α class carbonic anhydrase sequences and form a disulfide bond. The crystal structure model was produced by Insight II (Molecular Simulations, San Diego, CA, USA) using the atomic coordinates of the 1.78-Å structure [78]. **B**: View of the active site. The active site Zn is shown in magenta and the coordinating residues (His-92, His-94 and His-111) are shown in red. His-66, thought to act as a proton shuttle analogously to His-64 of human CA II, is shown in blue. Glu-98 and Thr-177 of the hydrogen bond network are shown in yellow and green, respectively. The active site model was produced by Rasmol (Biomolecular Structure, Glaxo Research and Development; Middlesex, UK) using the atomic coordinates of the 1.78-Å structure [78]." (K. S. Smith & Ferry, 2000)

**α-Carbonic anhydrase II from *Gallus gallus* (Chicken) (CHCA)**

This CA is an α-CA which has an important role in the eggshell’s formation: its activity has been measured to be of 19,000 units/mg protein using the Wilbur and Anderson method (Nishita et al., 2011) (Karl M. Wilbur and Norman G. Anderson, 1948). This is one of the first CA identified in Aves (Bernstein, Nevalainen, Schraer, & Schraer, 1968) and has been the subject of extensive study ever since. It is one of the
sequences chosen due to its ability to biomineralize calcium carbonate and because it is a monomer with a small sequence of only 260 AA.

**Nacrein**

Before describing the other two sequences of α-CA cloned, it is important to introduce some of the studies done on nacrein since this protein works in a different way compared to the rest of the CAs. Nacrein is a soluble organic matrix protein which can be found in the nacreous layer of oyster pearls. The protein contains two functional domains: a CA and a repeat domain, Gly-Xaa-Asn (glycine, an unspecified amino acid, which can be either Asparagine or Aspartic acid and Asparagine). Interestingly it has been shown that where the CA is responsible for the catalysis of HCO$_3^-$, the repeated domains are responsible for binding calcium and therefore contribute to the formation of the calcium carbonate crystal (Miyamoto et al., 1996).

The above has been confirmed by a coeval study on abalone shells. In the abalone shells layers of calcite crystals and aragonite are alternated and complemented by the synthesis of polyanionic proteins, proteins which alone can control the switching between aragonite and calcite. Nacrein can therefore control hierarchical biomineral growth (Belcher et al., 1996).

**Pinctada fucata α-CA**

As mentioned in the previous chapter nacrein plays a very important role in the formation of the shell of many bivalves and gastropods, one of these being the *Pinctada fucata*, also known as the Akoya pearl oyster. It has been shown that nacrein protein has the ability to bind Ca$^{2+}$ and that it can function both as a calcium concentrator and as an
enzyme required for production of carbonate ions, which are assembled at mineralization sites in CaCO₃. For the above reasons we decided to include the sequence in the pool of potential successful cloning.

Nacrein-like protein F1 from *Crassostrea gigas* (Pacific oyster) α-CA

The Pacific oyster *Crassostrea gigas* is a marine bivalve belonging to the phylum Mollusca, two different nacrein-like proteins – F1 and F2 - were found in this organism. In both these proteins the Gly-X-Asn repeat domain is replaced by a series of acidic amino acids (D/E). As it shows a different but similar mechanism to that of nacrein, and as their expression levels are consistent with the increase of the calcite portion of the shell, we decided to include the sequence of one of the two nacrein-like proteins - F1 - in the pool. F1 consists of 1,631 bp encoding 428 amino acids from a 1284-nucleotide with a molecular weight of 49.53 kDa. It exhibits typical catalytic sites of α-CAs with three histidine residues and 25 active sites (Song, Wang, Li, & Zhang, 2014).

Sequences cloned from β-CA

Only three sequences were cloned from this class, due mainly to the fact that these types of CAs are polymeric. Our concerns were that the protein cloned would have been too large to be expressed together with CsgA, also the presence of the CsgA fibers could have interfered with their assembly into larger and more complex geometries, thereby compromising the activity of the enzyme. However some strains of E. coli already use this enzyme and therefore it was considered as potential candidate.
Carbonic anhydrase 1 *Escherichia coli* (strain K12) (ECCA) β-CA

*E. coli* CA (ECCA) is needed for biosynthesis of arginine, pyrimidines, and purines, for fatty acid biosynthesis and in central metabolism. Its expression is higher in slow-growing cultures and at higher culture densities, and it is also elevated in starvation and under heat stress (Merlin et al., 2003).

ECCA activity is highly pH-dependent: it is active at pH 8.4 to pH 9.1 but not active at neutral pH, possibly due to the lack of coordination of water at the zinc cation (Merlin et al., 2003). Moreover it can adopt two distinct conformations displaying very different catalytic rates. Crystallography studies show that the enzyme is a homo-tetramer with dihedral symmetry and where the monomer structure has a central four-stranded parallel beta-sheet and five major alpha-helical segments. Furthermore the EECA monomer has 3 components: an N-terminal arm, a conserved zinc-binding core and an alpha-helix C-terminal subdomain (Fig. 7) (Cronk, Endrizzi, Cronk, O’neill, & Zhang, 2001).
"(B) Ribbon diagram of ECCA monomer, with secondary structure elements labeled. Helices are shown in red, β-strands in yellow, and connecting segments are blue. The magenta sphere denotes the location of the zinc ion. (C) Tetrameric association observed in ECCA crystal lattices. Two dimers, the bottom one oriented as shown in B, associate along two perpendicular crystallographic twofold axes, forming a tetramer with 222 point symmetry. Each monomer is represented as a different colored ribbon, and the zinc atoms are shown as magenta spheres." (Cronk et al., 2001).

*Neisseria sicca* β-CA

This enzyme was the first CA purified from a prokaryote (Veitch & Blankenship, 1963) and is a monomer with a mass of 28.6 kDa and one mole of Zn. Maximal activity was found at 7.1 pH and the enzyme was expressed under low pCO₂ (CO₂ pressure) conditions (Adler, Brundell, Falkbring, & Nyman, 1972). Although it has a weak esterase activity (10% of human CAII) it is one of the few monomeric enzymes in bacteria and was therefore considered as one of the possible successful candidates.
Bacillus subtilis XF-1 (BACCA) β-CA

The Bacillus subtilis is a gram-positive plant-associated bacterium capable of forming complex mineralized biofilms (fig 8 and 9). Its matrix consists of exopolysaccharides and amyloid-like fibers, produced by the tapA-sipW-tasA operon (henceforth tapA). A recent publication has shown that the mineral scaffolds 3D architecture is necessary to hold the extracellular matrix together with the bacterial cells (Oppenheimer-Shaanan et al., 2016) (Fig. 8 and 9). Furthermore the study shows how bacteria can grow and form these calcified structures in an agar plate, with calcium acetate as source of calcium and in both CO₂ enriched and not enriched conditions, however the precipitation was lacking in anaerobic condition.

B. subtilis has a small monomeric β-CA of 197 AA and 21.7 kD. This is clearly an interesting example and a useful reference for the purpose of this research and some of the experiments were repeated on all the strains successfully cloned including the CA from B. subtilis strain here above.
Figure 8. Calcite precipitation in *B. subtilis*.

"Complex colony morphology correlates with calcite precipitation in *Bacillus subtilis*. (a–c) Top view of a colony of an undomesticated strain of wild-type *B. subtilis* (NCIB 3610). The colonies were grown on solid biomineralization-promoting medium without (a) or with (a and b) a calcium source, for 3 and 7 (a), 21 (b) days, at 30 °C, in a CO$_2$-enriched environment. (b) Top view of a colony (left) and a magnification of calcite crystals at the periphery (right upper) or centre (right lower) of the colony. Images were taken by Stereo microscope with an objective of $\times0.5$ (a and b, left) or $\times1$ (b, right) Scale bar corresponds to 2 mm (a and b, left) or 100 µm (b, right). (c) The FTIR spectra of calcium carbonate minerals precipitated at the edges of the colony. $v_2$ and $v_3$ indicate characteristic vibrations. The results are of a representative experiment out of five independent repeats." (Oppenheimer-Shaanan et al., 2016)
Figure 9. Calcite precipitation in *B. subtilis*

"Calcite and amorphous calcium carbonate have a distinct spatio-temporal organization within the biofilm. (a, b) Upper panel: Top view of a biofilm of wild-type *B. subtilis*. The biofilms were grown at 30°C, in a CO₂-enriched environment. (a) On solid biomineralization-promoting medium with a calcium source for 1, 2, 5 days or without a calcium source for 5 days. (b) The biofilms were grown on solid biomineralization-promoting medium with a calcium source for 10, 21 days or on MSgg and mLB medium for 5 days. Images were taken with a stereo microscope with an objective ×1. Scale bar corresponds to 500 µm. Lower panel: MicroCT images of *B. subtilis* biofilms. Scale bar corresponds to 2 mm. (c) Images representing the thickness of the calcium carbonate buildup underneath the wrinkles of wild-type and lcfA mutant. The images were obtained from the microCT by 2D slice cutting through the biofilm. Scale bar corresponds to 3.5 mm. The results are of a representative experiment out of three independent repeats." (Oppenheimer-Shaanan et al., 2016)
Sequences cloned from $\gamma$-CA and $\zeta$-CA

$\gamma$-CA *Bacillus marisflavi*

*Bacillus marisflavi* is a gram-positive Bacillus with its CAs belonging to the $\gamma$ CA family. As mentioned above, this is the only class using iron in its active site. Previous studies have shown that this strain of Bacillus is capable of precipitating calcium carbonate (Silva-Castro et al., 2015) and was therefore considered a candidate.

*Thalassiosira weissflogii* cadmium-specific carbonic anhydrase (cdca1) $\zeta$-CA (DIACA)

Diatoms are unicellular photoautotrophic algae belonging to the Chromista, a large eukaryotic group. They are responsible for a large fraction of CO$_2$ sequestration in deep seawater and their importance in the environment makes them one of the primary organisms to study in order to understand the physiology and ecophysiology of carbon fixation.

As previously mentioned, $\zeta$-CA is a cambialistic enzyme which can be active with either Cd or Zn. Cadmium CA (CDCA) is a result of diatoms’ adaptation to marine life. This sequence was chosen as it seemed an interesting and exploitable mechanism, as well as due to its flexibility in using different metal ions and its small monomeric structure.
Molecular Cloning with Gibson Assembly

Cell Strains and Plasmids

The different sequences described in the above chapter were cloned using Gibson Assembly into a previously described plasmid (Dorval Courchesne et al., 2016), with a single operon, csgBACEFG, under the control of the T7 promoter, where the nucleator protein CsgB, responsible for connecting curli fibers to the external surface of the bacteria, was deleted. This plasmid was created using the two divergent operons regions, the csgBAC and csgEFG, which were PCR isolated from the W3110 strain of *E. coli* K12 and cloned by overlap extension into the pET21d plasmid.

Of the nine different sequences of CA from different species: *Gallus gallus* α-CAII, *Neisseria gonorrhoeae* α-CA, Nacrein-like protein F1 α-CA, the *Pinctada fucata* nacrein α-CA, *Bacillus subtilis* XF-1 β-CA, *e. Coli* β-CA, *Neisseria sicca* β-CA, ζ-CA from *Thalassiosira weissflogii* (cadmium-specific CA (cdca1)) and γ-CA Bacillus marisflavi, only four (*Gallus gallus* α-CAII, *Bacillus subtilis* XF-1 β-CA, *e. Coli* β-CA, ζ-CA from *Thalassiosira weissflogii*), were successfully synthesized, we attribute this to the fact that the unsuccessful sequences have extensive repeating regions.

Protein expression was performed on all the successful plasmids in a curli operon deletion mutant, PQN4. The PQN4 is an *E. coli* strain derived from LSR10 (MC4100, ΔCsgA, λ(DE3), CamR) which was constructed to knockout the curli operon.
Curli Nanofiber Expression

Curli nanofiber expression was performed by following the protocol described in a recent paper from the Joshi Lab (Dorval Courchesne et al., 2016). The 4 transformed PQN4 cells were plated onto lysogeny broth (LB) agar plates with addition of 0.5% (m/v) glucose (for catabolite repression of T7RNAP) and 100 μg/mL carbenicillin and growth over night at 37 °C. The day after the colonies were picked and inoculated in 5 mL LB containing and 100 μg/mL carbenicillin and 2% (m/v) glucose for overnight growth at 37 °C. The following day the cultures were diluted 100-fold in fresh LB medium with 100 μg/mL carbenicillin and 2% (m/v) glucose and cultured at 37 °C. The media Optical Density (OD\textsubscript{600 nm}) was measured and when it reached between 0.6 to 0.8 the cells were pelleted at 4000 g and gently re-suspended in LB containing 0.4 mM IPTG and 100 μg/mL carbenicillin and 100μM of zinc acetate but no glucose. The culture was incubated overnight at 37 °C for protein expression.

Confirmation of fiber production via Congo Red Amyloid Staining

Standard amyloid-staining colorimetric dye, Congo Red (CR)(Marcus, Sadimin, Richardson, Goodell, & Fyfe, 2012) was used for quantification of curli production in the four mutants. This was performed periodically as previously described (Nguyen et al., 2014b). One ml of the engineered bacteria culture was centrifuged at 4000 g for 10 minutes and suspended in phosphate buffer with the addition of 0.00015% of Congo Red. This was incubated at room temperature for 10 minutes and then again centrifuged for 10 minutes at maximum speed. The absorption of the supernatants was measured at 490 nm in order to quantify the amount of Congo Red which did not bind to the cells.
Confirmation of fiber production via SEM imaging

Curli nanofiber expression was performed as per above protocol and the following day the culture was vacuum-filtered onto 47 mm polycarbonate filter membranes with 10 μm pores (EMD Millipore) until complete clogging, in order to retain enough fiber on the filter. The filter was then prepared for SEM imaging in order to confirm the presence of the fibers.

Confirmation of Cloning via SDS PAGE

Standard SDS-PAGE protocol was performed on all constructs to identify whether the different carbonic anhydrases were successfully expressed in the PQN4 cells. 150 μl of bacteria culture at OD$_{600\text{ nm}}$ = 100 was centrifuged at maximum speed for 3 minutes. Supernatant were removed and re-suspended in 950μl of Laemmli buffer and 50 μl of beta mercaptoethanol. Sample were then placed in a Denville Incu-Block block heater at 95 C for 10 min and then centrifuged again at max speed for 30 seconds. All the samples were then loaded in a precast gel (Mini-Protean TGX from Bio-Rad) and run in a Mini-PROTEAN Tetra Cell vertical mini gel electrophoresis machine.

Hydrogel production of the engineered mutants

A protocol for vacuum filtration and purification used for free standing film production was previously described (Dorval Courchesne et al., 2016). A variation of the protocol using SDS (sodium dodecyl sulfate) but not guanidinium chloride (GdmCl) nor benzonase was performed on the engineered constructs, in order to test whether we could have been able to obtain a similar film. 50ml of each bacteria culture was vacuum-filtered
on a 47 mm polycarbonate filter membrane with 10 μm pores (EMD Millipore). This was then rinsed with 5 ml of DI water and at this point 5 ml of 5% (m/v) SDS was incubated for 5 min on the filter. This was followed by a second rinse, again using 5 ml of DI water. The remaining gel was collected using a spatula.

Direct Screening of carbonic anhydrase via Plate Assay using p-NPA

A method for screening the presence of CA in bacteria directly on plates was recently described and used on different strains of bacteria including *C. freundii* SW3 (Ramanan et al., 2009). The colonies which had an active enzyme were described in this study as yellow in color compared to the normal color of the control. We thought the experiment was a quick and effective way to screen for the presence and activity of CA in our different constructs and so the same protocol was performed.

All different constructs were plated in Luria agar containing 10 mM p-NPA to screen for the presence of CA enzyme. The experiment was repeated several times but unfortunately none of the plates had colonies. We attribute this to the fact that p-NPA needs to be dissolved in 100% ethanol (100 mg/ml), and possibly the strain used by us was too sensitive to the presence of ethanol in the agar plates.

Direct screening of carbonic anhydrase and calcium carbonate crystal production via plate assay using calcium acetate as source of calcium.

A recent publication in Nature (Oppenheimer-Shaanan et al., 2016) has shown the ability of *Bacillus subtilis* and *Mycobacterium smegmatis* to biomineralize and form
calcite in biofilm development. We decided to use the same method in order to assess whether a similar biofilm could have been produced by our engineered strains.

Beside bicarbonate (HCO$_3^-$) calcium carbonate precipitation demands free Ca$^{2+}$ and as per the above study, calcium acetate was used as source of calcium. Agar plates containing different concentrations of calcium acetate (0.25% and 2.5%) were used and 20μl of bacteria culture at OD$_{600\text{ nm}} = 0.3$ was streaked on the plate and allowed to grow at 37°C in both a CO$_2$ enriched environment and a non-enriched environment. The growth was monitored every 3 days for the first week (Fig. 10) and after that weekly. The above mentioned study showed crystal formation after 15 days in both CO$_2$ enriched conditions and -not, with clear crystal structures after 30 days from plating. Furthermore, in order to assay the pH changes during biofilm formation, phenol at 0.0025% of final concentration was added to a duplicate of each plate (Marvasi, Visscher, Perito, Mastromei, & Casillas-Martínez, 2010). Moreover, as some CA performed better at higher pH, the same experiment was performed with plates at pH 8.5.
Figure 10. Plated assay of the direct screening method

Above with phenol red, below without phenol red. Plates with 0.25% calcium acetate (right) and 2.5% (left)
Enzyme Activity Assays

I. Wilburn and Anderson Electrometric Method

A variation of the Wilbur–Anderson electrometric method, a well-established protocol to test CO₂ hydration activity (Karl M. Wilbur and Norman G. Anderson, 1948) used by different studies on CA, biomineralization and carbon sequestration (Barbero et al., 2013) (Kim et al., 2012) (Jo, Kim, Seo, Kang, & Cha, 2013), was performed in order to test the activity of the enzyme. A reaction buffer was made using Tris–HCl, pH 8.8 (0.02 M) and 30 mL of this buffer was added to a glass beaker to which 1 ml of bacteria culture was added. A pH electrode was calibrated and allowed to reach equilibrium after which the reaction was initiated by addition of 20 ml of CO₂ solution. Data was recorded at 1-second intervals for a minute using Lab 850 Benchtop pH Meter from SI Analytics; the experiment was performed both at room temperature and in a container with ice to keep the temperature at around 3°C.

WT with addition of 1 mg/ml lyophilized powder from Sigma of carbonic anhydrase from bovine erythrocytes (specific activity ≥3,500 W-A units/mg protein) was used as positive control and WT without the addition of the enzyme was used as negative control.
II. Colorimetric Method using Phenol Red

Another well-established colorimetric method based on pH/dye indicator was used in order to assess the activity of the enzyme (Khalifah, 1971) as well as a variation of the protocol performed by study on CA in *E. coli* (Cronk et al., 2001).

A buffer solution containing 25 mM HEPES, 100μM phenol red (pH 7.5, $\lambda_{\text{max}} = 558$ nm) and 100 mM sodium sulfate - to maintain ionic strength of the reaction medium - was used in order to test CA activity. Bacteria culture containing 100μM of zinc acetate was centrifuged at 4000 g for 30 sec and re-suspended in HEPES buffer at OD$_{600 \text{ nm}} = 3$, this was done in order to avoid compromising the test since the color of the media would have altered that of the phenol red. 200 μl of the re-suspended bacteria was added to a 1.5 ml semi-macro cuvettes from Brand, as well as 500 μl of the phenol red buffer. The cuvette was then placed in a UV-Vis spectrometer CARY (CARYUVVIS) and the reaction, and therefore the reading, was started by manual addition of 500 μl of CO2 (aq).

200 μl of WT re-suspended in HEPES buffer with addition of 1mg/ml lyophilized powder (from Sigma) of carbonic anhydrase from bovine erythrocytes (specific activity ≥3,500 W-A units/mg protein) was used as positive control and WT also re-suspended in HEPES buffer without the addition of the enzyme was used as negative control. Data were acquired at 0.1 sec intervals for 1 minute at 558 nm wavelength. A cuvette containing only HEPES buffer was used for correction, spectra of the dye in the buffer was recoded before the introduction of CO$_2$ (red base form) and after the introduction of CO$_2$ (yellow acid form) (Fig. 11)
III. Colorimetric Method using m-cresol purple

The same protocol described in the above paragraph was also performed using a different dye, m-cresol purple (pH 8.4, $\lambda_{\text{max}} = 578$ nm). Different types of CA work at different pH including ECCA which works best at pH above 7.5, we therefore thought using an indicator which works at higher pH to be appropriate. The buffer in this case was made using 25 mM TAPS, 100$\mu$M m-cresol purple (pH 8.4, $\lambda_{\text{max}} = 578$ nm) and 100 mM sodium sulfate. The bacteria culture containing 100$\mu$M of zinc acetate were re-suspended in TAPS OD$_{600\text{nm}} = 3$. As per above experiment 200 $\mu$l of the re-suspended bacteria was added to a 1.5 ml semi-macro cuvettes as well as 500 $\mu$l of the m-cresol purple buffer.

200 $\mu$l of WT re-suspended in TAPS buffer with addition of 1mg/ml lyophilized powder (from Sigma) of carbonic anhydrase from bovine erythrocytes (specific activity $\geq 3,500$ W-A units/mg protein) was used as positive control and WT also re-suspended in TAPS buffer without the addition of the enzyme was used as negative control. The reaction was again initiated by the addition of 0.5 ml of CO$_2$ (aq), and data were acquired from T=0 coinciding with the manual addition of carbon dioxide in aqueous solution at 0.1 sec intervals for 1 minute at 578 nm wavelength. The correction was done using cuvette containing TAPS instead of HEPES. As per above experiment spectra of the acid and base form of the dye was recorded (Fig. 11).
Figure 11. Phenol red and m-cresol purple spectra in both acid and base form
Precipitation Assay

Calcium carbonate exhibits different polymorphism (Fig. 12). In addition to the well known polymorph forms: calcite, aragonite and vaterite, there are several less understood Amorphous Calcium Carbonate (ACC) in both hydrous and anhydrous form. In biologically induced crystallization the anhydrous form can arise from stabilized hydrous precursors (monohydrocalcite, ikaite and amorphous calcium carbonate ACC) (Cartwright, Checa, Gale, Gebauer, & Sainz-Díaz, 2012). Of the different polymorphs calcite is the most stable and least soluble where vaterite is the least stable and most soluble (Boulos et al., 2015). Generally speaking biogenetic ACC is distinguishable from its synthetic counterpart because its structural features have a distinct short-range which is similar to that of calcite (calcitic ACC) aragonite (aragonitic ACC) and monohydrocalcite (MHC-like ACC) (Cartwright et al., 2012).

We used two different previously described methods to test calcium carbonate precipitation in bacteria in order to quantify the possibility of using the engineered strain to form a ceramic biofilm. The first one uses 50 ml of Tris HCl 8.3 pH (20mM), 1.5 ml of CaCl$_2$ (30 mM); and the second one uses 30 ml mixture containing 200mM Tris Buffer, 100mM CaCl$_2$ solution (Kim et al., 2012) (Wei et al., 2015). A 47 mm polycarbonate filter membrane with 10 μm pores (EMD Millipore) was use in order to collect the fibers: the bacteria culture was vacuum-filtered until clogging in order to have the maximum amount of fibers on the filter. The filter was then placed in glass beaker and the two different solutions above were added, followed by the addition of CO$_2$ gas at uniform flow rate for 5 hours (Fig.12). The filters placed in the beakers were let dry at room temperature for few days. The precipitants were collected and analyzed using SEM,
Energy-dispersive X-ray spectroscopy (EDS) and Fourier-transform infrared spectroscopy (FTIR). Wild type and CA from bovine erythrocytes were used as negative and positive control, respectively. The experiment was performed at both pH 7.5 and 8.5.

Figure 12. Precipitation of calcium carbonate
Figure 13. Precipitation assay
Chapter III.

Results

SDS-PAGE

SDS-PAGE was performed on the constructs in order to understand whether the cloning was successful. Three clear bands were found for Bacillus subtilis XF-1 β-CA (21.714 kD), Gallus gallus α-CAII (29.008 kD) and E. coli β-CA (23.764 kD). However, the band for the Thalassiosira weissflogii cadmium-specific CA (cdca1), the ζ-CA (18.784 kD), was not visible. For this reason transformation protocol was performed again followed by SDS PAGE to confirm that the cloning was successful (Fig. 14).

Figure 14. SDS PAGE gel
Congo Red

As mentioned under methods, amyloid fibers can be stained using Congo Red - this method used to understand the amount of fiber produced by the mutants was performed routinely as previously outlined under methods. All the engineered mutants showed curli fiber production (Fig.15). The assay was performed in both leaky and non-leaky expression as well as in zinc enriched bacteria media. 0.00015% of Congo Red was used as control.

Figure 15. Congo Red results
Hydrogel production of the engineered mutants

The protocol for hydrogel production of the engineered mutants described under methods was performed in all constructs and all constructs were able to produce hydrogel (Fig. 15). Interestingly, one of the constructs, BACCA gave a different and stiffer gel. The mechanical properties of the different gels will be investigated at a later stage.

Figure 15. Hydrogel of the engineered mutants
Wilburn and Anderson Electrometric Method and Colorimeter Assays

The protocol described under methods from Wilburn and Anderson, where an electrode is measuring the change in pH over time, was performed in order to confirm the activity of the enzymes in the different mutants.

This type of assay is universally used to determine CA activity, however this method is far less precise than the colorimeter methods used here below. This is due to the precision of the instrument itself. The electrometric assay can only measure within a 1-second interval, whereas the colorimeter assay uses a photometer which can be set to a 0.1 second interval. Unfortunately this experiment didn't give a positive result, as can be inferred from the graph (Fig. 16) all the contracts show a very similar performance to that of the negative control. We think this is due to the fact that the experiment was executed at pH 8 which, as mention under methods, could possibly be too low to the enzyme in our engineered construct to be active (refer to below results from colorimeter assay for further explanation).
Figure 16. Wilburn and Anderson Electrometric Method

Wilburn and Anderson Electrometric Method and Colorimeter Assays

- 30 ml Tris HCL (2mM) + 20 ml CO2 (aq) at 4°C
- NEGATIVE: 1ml WT
- 1 ml ECCA
- 1ml CHCA
- 1 ml DIACA
- 1ml BACCA
- POSITIVE 1ml LB with 1mg Bovine CA

- 30 ml Tris HCL (2mM) + 20 ml CO2 (aq) at room temperature
- NEGATIVE: 1ml WT
- 1 ml ECCA
- 1ml CHCA
- 1 ml DIACA
- 1ml BACCA
- POSITIVE 1ml LB with 1mg Bovine CA
It is important to give a small introduction on the two colorimeter assays performed: the studies mentioned under methods have been performed using purified enzymes, however this was not possible in our case since we wanted to show the activity of the enzyme directly fused to CsgA. For this reason, the assay was performed under less than perfect conditions. In fact, although the bacteria culture was spun and re-suspended in buffer in order to have a clearer solution, the fibers present in the buffer conferred a cloudy aspect, which possibly diminished the reading through Mie scattering.

As previously shown, CA in E. coli is highly pH dependent and works best at a pH above 8 (Cronk et al., 2001). This could possibly be the reason as to why the two colorimeter assays gave such markedly different results.

As shown in the graph of the assay performed at 7.5 pH using phenol red (Fig. 17), all the mutants have a possible low activity. The reaction time of the positive was around 15 seconds; the negative control reaction time was between 40 and 45 seconds, and all the mutants showed a time reaction of around 35 seconds. We found this to be inconclusive and therefore performed a similar assay using m-cresol purple at pH 8.4 as described under methods (Fig. 18). In the latter case the time reaction of the positive control was assessed at around 15 seconds and the negative one at around 45 seconds. All the mutants with exception of the CHCA - which shows a similar slope but a much slower time reaction of 20 seconds - show a clear activity. The three mutants: BACCA, ECCA and DIACA have a reaction time of 12 to 15 seconds; DIACA shows a fairly similar slope to the positive control and a reaction time which possibly surpasses that of the positive control.
Figure 17. Colorimeter activity assay

*Phenol red (above) and m-cresol purple (below)*

Colorimetric Method
Phenol Red
pH 7.5  
$\lambda_{\text{max}} = 558 \text{ nm}$

Sec     Absorption

0    1.3
20    1.1
40    1.0
60    0.9
80    0.8
100   0.7
120   0.6

Colorimetric Method
M-cresol Purple
pH 8.5  
$\lambda_{\text{max}} = 578 \text{ nm}$

Sec     Absorption

0    1.3
20    1.1
40    1.0
60    0.9
80    0.8
100   0.7
120   0.6
Light Microscopy Results

Light microscopy images of the filter used in the precipitation assay were taken in order to see whether any precipitation was visible. The images were taken in both assays performed, and at pH 7.5 as well as at pH 8.4. The images from filter used in the experiment with lower concentration of calcium chloride (50 ml of Tris HCL 8.3 pH (20mM), 1.5 ml of CaCl$_2$ (30 mM)) show slightly darker areas around the fibers, but it was not possible to determine if this was due to the possible presence of precipitants (Fig. 18). However all the filters used for the precipitation assay at higher concentration of calcium chloride (30 ml mixture containing 200mM Tris Buffer, 100mM CaCl$_2$ solution) at both pH of 7.5 (Fig. 19) and 8.5 (Fig. 20) show a clear presence of crystal structures.
Figure 18. Fiber and crystals, light microscopy.

*Left: control no precipitation, right: diatom construct, protocol performed with lower concentration of calcium chloride and at pH 7.5*
Figure 19. Light microscopy images of the engineered mutants

*Filters used for precipitation assay at higher concentration of calcium chloride and at pH 7.5.*
Figure 20. Light microscopy images of the engineered mutants and controls

*Filters used for precipitation assay at higher concentration of calcium chloride and at pH 8.5.*
Figure 21. Filters after precipitation assay (top).

*Higher concentration of calcium chloride and at pH 7.5.*

Figure 22. Filters after precipitation assay (bottom).

*Higher concentration of calcium chloride and at pH 8.5.*
Figure 23. Powder collected from one of the samples
Confirmation of Fiber Production and Precipitation via SEM

The presence of fibers was confirmed in all the samples by SEM imaging, the light microscopy image here above (Fig. 18) also confirmed the result.

In order to assess if the fibers were able to assist in the precipitation of calcium carbonate, the filters used here above where SEM imaged. The assay was performed as described under methods at both pH 7.5 and 8.5 and both high and low concentration of calcium chloride was used. A duplicate of each condition was made in order to have, of each, a washed and not washed sample. The washed sample was created by immersing the filter in a beaker containing 100 ml of DI water and placed on the shaker for 24h. The filter was then picked from the beaker and the powder that was washed away from the filter was decanted and SEM imaged (Fig. 23). The assay done at the lower concentration of calcium chloride and at pH 7.5 showed a possible presence of calcium carbonate, but this was found to be inconclusive via SEM EDS. However the filters used in the experiments at higher concentration of calcium chloride at both higher and lower pH showed a clear mineral presence. The unwashed filter had a markedly different crystal structure compared to that of the washed one. What is to be noticed (Fig. 25) in the unwashed samples is that, although the samples where created in different pH conditions and although the colony wasn't the same (in fact each colony comes from two different plates of the same construct), each construct gives a crystal structure which is very similar within the same construct, and which differs completely from the others. BACCA gave in both cases (at pH 7.5 and 8.5) large irregular shapes; CHCA gave triangulated shapes; DIACA showed a fractal branch like structure, which is interestingly similar to that of both the positive controls. Finally ECCA showed a mixture of different irregular shapes.
However FTIR didn't confirm these as calcium carbonate. We therefore believe this to be a layer of calcium chloride, which when dissolved in water revealed the presence of calcium carbonate underneath in the washed samples. FTIR assay of the same calcium chloride used in the experiment, placed over the same filter used in the precipitation assay, will need to be performed in order to confirm whether this is indeed a layer of calcium chloride. Although not part of the main research theme, it would nevertheless be interesting to understand whether the crystal structures seen in the unwashed samples are due to the different size and shape that the fibers of each construct is exhibiting, and whether each differing fiber typology can have an effect on the formations of calcium chloride crystals.

As mentioned here above, the washed samples were completely different from the unwashed ones (Fig. 26). What is interesting in this case is the fact that only the mutants had the ability to retain the precipitants after washing, whereas in both the positive and negative WT and TFF2 the precipitation was washed away. This was confirmed by the fact that the powder collected had presence of calcium carbonate.

This is clearly a very important result as it appears that the enzyme is capable not only of assisting in the precipitation, but also in fixing the precipitants on the biofilm, confirming thus the importance of the enzyme in the biomineralization process and therefore making it an indispensable element in the creation of a ceramic biofilm.
Figure 24. SEM images of washed filters

*Lower calcium chloride concentration and at pH 7.5.*
Figure 25. SEM images of unwashed filters.

*Higher calcium chloride concentration and at both pH 8.5 and 7.5.*
Figure 26. SEM images of washed filters.

*Higher calcium chloride concentration and at pH 8.5.*
Confirmation of Precipitation via SEM EDS

SEM EDS was used in order to assay the possible presence of the precipitants by quantifying the amount of calcium and carbon compared to the amount of chloride and other elements. We decided to assay only the washed filters at both concentrations of calcium chloride and at both pH 7.5 and 8.5. All the filters were imaged and only the filters used at higher pH gave a positive result (Fig. 27 through 30).

Confirmation of Precipitation via FTIR

FTIR confirmed the presence of calcium carbonate in all the washed samples, with exception of both positive and negative control of wild type and TFF2 (Fig. 31 and 32). Wild type and TFF2 had similar peaks to that of the filter: only BACCA, CHCA, DIACA and ECCA have clear peaks at 1474, 878 and 709, congruous with the typical spectra of calcium carbonate (Saraya & Rokbaa, 2016). This is again confirming that the calcium carbonate was able to be retained only by the engineered mutants.
Figure 27. Energy-dispersive X-ray spectroscopy of samples

*BACA at lower concentration of calcium chloride and at pH 7.5*
Figure 28. Energy-dispersive X-ray spectroscopy of samples

*BACCA washed filter at higher concentration of calcium chloride and at pH 8.5*
Figure 29. Energy-dispersive X-ray spectroscopy of samples

*TFF2 washed filter and powder at higher concentration of calcium chloride and at pH 8.5*
Figure 30. Energy-dispersive X-ray spectroscopy of samples

WT negative - washed filter at higher concentration of calcium chloride and at pH 8.5, possible presence of calcified bacteria cells.
Figure 31. FTIR of washed filters

BAC back washed 8.5

CH washed 8.5

DIA 8.5 washed

EC washed 8.5

This study

FTIR spectra of calcium carbonate from Saraya & Rokbaa, 2016
Figure 32. FTIR of washed filters

- Filter only
- WT 8.5
- TFF2 positive washed 7.5 back

This study
FTIR spectra of calcium carbonate from Saraya & Rokbaa, 2016
Result of direct screening of carbonic anhydrase and calcium carbonate crystal production via plate assay using calcium acetate as source of calcium

This assay was performed in order to evaluate whether the colony would be able to create a ceramic film directly. It was performed at both pH 7.5 and 8.5. The plates at lower pH didn't show any precipitation, however one of the constructs -ECCA- had white spotted areas which appear to be quite different from the rest of the colonies plated. This will require further investigation in order to assess whether the colony in fact has traces of calcium carbonate (Fig.33). In the event of a positive result further tuning of the amount of calcium acetate, of the pH level and of the amount of CO$_2$ will have to be accomplished.

![Plate assay of crystal production.](image)

Possible precipitation in sample called ECCA
3D structures

Assembly of Films via Wet and Heat Welding

Both wet and heat welding was used in order to test the possibility of creating 3D structures using the gel made with previously engineered constructs.

Gel protocol as described under methods was performed and the resulting gel was then cast in silicone molds and let dry overnight into a dry film. Two films were welded together by adding 10 μl of water drops between the two films and let dry (Fig. 34). SEM images of the films were taken in order to show the integration (Fig. 35).

A similar process was used with heat welding (Fig.34). Again, per above, the gel was put in a mold and the two dry films were then weld together using 100-240V/15W Craft Hot Knife 10CM Styrofoam Cutting Pen, and SEM images were subsequently taken to show the integration of the two films (Fig. 35).

In order to demonstrate the flexibility of the system to create 3D structures we decided to create a small object. Given that a main subject of the present thesis is the application of BIND in building construction, the archetypal house was used as the 3D object. The different parts of the object were created by casting the gel into silicone molds and let dry overnight. These were then assembled through addition of water as described under methods. Photographs of the final object show the result (Fig. 36).
The use of the engineered hydrogel as ink in 3D printing

We also wanted to demonstrate the ability of the gel to be used as ink in 3D printing and therefore placed some of the gel into a syringe and by singular addition of a thin layer of gel (Fig. 37) attempted to create different structures which were then let dry.

Figure 34. Wet and heat welding.
Figure 35. SEM images of junction between two pieces of thin film

The two pieces are connected via wet (left) and heat (right) welding.
Figure 36. 3D construction using thin films connected via wet welding.

Figure 37. 3D printing using engineered hydrogel.
Another method to assess the possibility of creating 3D structures was also explored: a polystyrene ball was coated with the engineered hydrogel and let dry overnight. The following day the ball was immersed in chloroform, which, being a non-polar solvent, allowed the polystyrene ball to dissolve while leaving the coated film intact (Fig. 38).

Figure 38. 3D structure via coating

*A polystyrene sphere is coated with the engendered hydrogel, let dry overnight and then dissolved in chloroform leaving only the dry hydrogel behind*
Something else we wanted to highlight is the recyclability of the material as well as its ability to imprint shapes and patterns. We therefore let some of the gel dry on a recycling symbol on polystyrene, and then melted the polystyrene in chloroform as described above. The resulting film was then dissolved in water and recycled (Fig. 39).

Figure 39. Recycling the engineered hydrogel.
Substrate studies

Adherence of the gel coating was tested on different substrates wood, metal and fabric in order to understand possible future direction of this research. The gel created with the engineered culture was applied on the surfaces allowing the coating to adhere to the substrate SEM images were taken on each sample and they all show a clear integration of the gel into the material (Figs 40 through 42).

Figure 40. SEM images of uncoated (left) and coated (right) metal mesh.
Figure 41. SEM images of uncoated (left) and coated (right) cardboard.

Figure 42. SEM images of uncoated (left) and coated (right) pine wood.
Fire retardant Testing

Bench-scale vertical flame test (VFT) was performed of control and treated fabric after 5 seconds of ignition, per ASTM D6413-94. This test entails subjecting the fabric sample to fire, recording the burn time and the glow time, determining the char length from burn edge to unaffected areas (Fig. 43). The test was performed on linen fabric coated with the engineered hydrogel, a positive control was created by coating the same fabric with a commercially available fire retardant spray for fabric, and finally a non coated linen specimen was used as negative control.

The test was performed using a previously engineered mutant from The Joshi Lab, TFF2 (Nash, 2015) in order to see if the fibers by themselves would be able to act as protective coating, unfortunately this gave a negative result, the gel wasn't capable of protecting the fabric from the fire nor delaying the burning process. However we believe that the mineral presence in the variation of a gel created with the new mutants, and via precipitation assay of these, would yield a potential positive result.
Figure 43. Fire break through test per ASTM E 136 / EN ISO 11925-2.

*Left to right: Positive control and same fabric coated with TFF2*
Chapter IV.

Discussion

This work demonstrates the first set of active enzymes directly fused to CsgA. Furthermore the enzyme is not only showing to be active, and assisting in the precipitation of calcium carbonate, but it is also capable of retaining the precipitants in the biofilm, making it an indispensable element in the process of creating a ceramic film via biomineralization.

We believe that since this research demonstrates, for the first time, the possibility of directly fusing an enzyme to amyloid fibers, it could be of potential interest to future applications on amyloids.

Future investigations

The length of the study didn't allow enough time for the full array of desired tests to be carried out on the biofilm when applied to different substrates. Future investigations will be performed in order to test the possible capability of the system of forming a layer of ceramic on wood, steel or textile on which standard test methods, described below, will be performed. This can be achieved in different ways: by growing the biofilm directly on the substrate, or by coating the substrate using the engineered hydrogel. We would like to test both options in a later stage in order to assess the suitability of each for larger applications.
Substrate studies

When considering potential materials to act as a substrate, wood seems relevant and interesting. One major obstacle to wood’s use as a structural or cladding material is its properties with regards to combustibility and resistance to biological agents like bacterial mold or fungi. For instance, many local building codes require any building envelope or structural wood components in walls, floors and roofs to be covered in non-combustible material. The solutions typically used are cementboard, gypsumboard or liquid fire retardant treatment. All of these come with their separate implications in terms of adding weight and bulk, being non-renewable or containing toxic ingredients. Future investigations of this research could in this instance address these shortcomings.

Corrosion Testing on Substrates

Another set of tests we think to be important in order to determine the possible use of the engineered coating in building construction are the standard tests for atmospheric corrosion of steel (per ASTM G50–10) and the corrosion potential of uncoated reinforcing steel in concrete (per ASTM C876-15). The latter method uses a half cell meter to evaluate the potential for both passive and active (chloride-induced galvanic reaction) corrosion of rebar by undesired exposure to water and oxygen infiltrating the concrete surface. The concrete creates a unique environment for the steel and this is therefore a different test to the atmospheric corrosion test.

Mechanical testing

Mechanical testing of the thin film created via dehydration of the hydrogel will also be performed on the constructs. We intend to derive a number of key properties of
each construct, most notably pull-off strength of coatings (per ASTM D4541 – 17),
determination of the elongation, tensile strength, and stiffness/modulus of elasticity (per
ASTM D2370 – 16) and scratch hardness (per ASTM G171 - 03(2017)).
Appendix 1.
Definition of Terms

- Amyloid is a β-sheet-rich fold extracellular proteinaceous deposit with a filamentous morphology. Amyloids grow in length by adding one monomer at the time, forming fibrils.
- Fibrils are aggregates of amyloids.
- Enteric bacteria or Enterobacteriaceae are a family of bacteria that can be both harmless or pathogenic.
- Escherichia coli or *E. coli* is a type of enteric bacteria.
- Biofilm "is an assemblage of microbial cells that is irreversibly associated (not removed by gentle rinsing) with a surface and enclosed in a matrix of primarily polysaccharide material. Noncellular materials such as mineral crystals, corrosion particles, clay or silt particles, or blood components, depending on the environment in which the biofilm has developed, may also be found in the biofilm matrix. Biofilm-associated organisms also differ from their planktonic (freely suspended) counterparts with respect to the genes that are transcribed. Biofilms may form on a wide variety of surfaces, including living tissues, indwelling medical devices, industrial or potable water system piping, or natural aquatic systems." (Donlan, 2002)
- Extracellular Matrix (EMC), "Complex network of polysaccharides (such as glycosaminoglycans or cellulose) and proteins (such as collagen) secreted by cells. Serves as a structural element in tissues and also influences their development and physiology." (Alberts et al., 2002)
- Curli fibers are "the major proteinaceous component of a complex extracellular matrix produced by many Enterobacteriaceae. Curli were first discovered in the late
1980s on Escherichia coli strains that caused bovine mastitis, and have since been implicated in many physiological and pathogenic processes of *E. coli* and *Salmonella* spp. Curli fibers are involved in adhesion to surfaces, cell aggregation, and biofilm formation. Curli also mediate host cell adhesion and invasion, and they are potent inducers of the host inflammatory response. The structure and biogenesis of curli are unique among bacterial fibers that have been described to date. Structurally and biochemically, curli belong to a growing class of fibers known as amyloids."(Barnhart & Chapman, 2006)

- Extracellular nucleation-precipitation secretion pathway the process under which *CsgA* is secreted into the extracellular milieu and nucleated into a fiber by *CsgB*.
- Ureolysis a chain of chemical reactions leading to the formation of calcium carbonate precipitates.
- *Sporosarcina pasteurii* "is a gram-positive bacterium able to survive in highly alkaline environments (pH~10) and is one of the bacterial species that can become a causative agent of a phenomenon called Microbiologically Induced Calcite Precipitation (MICP)" (Bhaduri, Debnath, Mitra, Liu, & Kumar, 2016)
- Carbonic anhydrase an enzyme responsible to accelerate the catalysis of carbon dioxide and water into bicarbonate and protons and for the biomineralization process of some organism such as the aragonite build up in the nacreous layer in oyster pearls.
- Biofilm-Integrates Nanofiber Display (BIND) a platform for the "molecular programming of the bacterial extracellular matrix material by genetically appending peptide domains to the amyloid protein *CsgA*, the dominant proteinaceous component in *Escherichia coli* biofilms." (Nguyen et al., 2014b)
- *CsgA* major structural subunit of Curli assembly
• CsgB the nucleator protein of Curli assembly

• CsgD the master curli regulator, CsgD contains an N-terminal receiver domain and a C-terminal helix-turn-helix DNA binding domain. (Barnhart & Chapman, 2006)

• CsgG is an outer membrane (OM) lipoprotein that is required for the stability and secretion of CsgA and CsgB (Chapman et al., 2002) (Loferer, Hammar, & Normark, 1997)

• Operon is "a functioning unit of genomic DNA containing a cluster of genes under the control of a single promoter." (Sadava, 2011)

• csgBAC operon an operon which encodes the major structural subunit, CsgA, and the nucleator protein CsgB

• csgDEFG operon encodes four accessory proteins required for curli assembly, CsgD, CsgE, CsgF, CsgG.

• Microbiologically Induced Calcite Precipitation MICP "is a process wherein precipitation of calcium carbonate is induced by certain microbes under suitable environmental conditions." (Bhaduri et al., 2016)

• Nacre, popularly known as mother of pearl, is the organic-inorganic material which is naturally produced by some mollusks such as bivalves, cephalopods and gastropods and constitute the inner portion of the shell

• Additive Manufacturing (AM) is a process to make three dimensional shapes by adding layer over layer of material.

• Computer Aided Design (CAD) "is the use of technology to aid the design and, particularly, the drafting of a part or product, including entire buildings. It is both a visual - or drawing - and symbol-based method of communication, following standard
conventions to a specific technical field, such as architecture or engineering." (Aouad, 2012)

- Computer Aided Manufacturing (CAM) the use of software and different types of machine to automate the manufacturing process.

- Computer Numerical Control (CNC) "is a machine whose movements are controlled by a computer through a process known as Computer Numerical Control. Some examples of common CNC machines are routers, milling machines, 3D printers, vinyl cutters, laser cutters, pick-and-place machines, and many others." (Ford, 2016)
Baclulus subtilis XF-1 P-CA

Appendix 2.

Sequences
Thalassiosira weissflogii cadmium-specific carbonic anhydrase (cdca1) ζ-CA
References


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