Biofilm formation is intrinsic to the origin of life

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Abstract

Biofilm formation the build up of multicellular, often surface-associated, communities of autonomous cells, is the natural mode of growth of up to 80% of microorganisms living on this planet. Their tolerance against multiple environmental stresses makes biofilms refractory towards antimicrobial treatment strategies and the actions of the immune system. But how did biofilm formation arise? Here, I argue that the origin of the biofilm lifestyle has its foundation in fundamental surface-triggered chemical reactions and energy preserving mechanisms that enabled the development of life on earth. Subsequently, prototypical biofilm formation has evolved and diversified concomitantly in composition and regulation with the expansion of prokaryotic organisms and their radiation by occupation of diverse ecological niches. This ancient origin of biofilm formation thus indicates that harnessing conditions have been the rule rather than the exception in microbial life, while upon the emergence of the association of microbes with higher organisms including recent human pathogens, although being in a nutritional and stress-protecting heaven, some of these basic mechanisms of biofilm formation have been surprisingly conserved to promote sustained survival in new environments.

Keywords: serpentinization, fermentation, respiration, electron transfer, energy conservation, biofilm formation, extracellular matrix

Introduction

Global devastating acute and even chronic bacterial infections such as medieval plague caused by *Yersinia pestis*, pandemic cholera caused by *Vibrio cholerae*, whooping cough caused by *Bordetella pertussis* and tuberculosis caused by *Mycobacterium tuberculosis* had previously contributed to imprint our anthropocentric view of microbes acting in pure culture infecting as single-cell planktonic organisms. Upon closer inspection of the disease process, however, these and other infectious agents and microbes in nature are, rarely, if at all, observed as single planktonic microbial cells. Multicellular biofilm forming microbes which display as surface, interface or self-attached cell aggregates, consisting of autonomous cells of diverse phylogenetic origin constitute the majority of microbial life. The success of this multicellular mode of growth during earth time is reflected by the fact that even up to 80% of human bacterial infections are biofilm-associated according to the National Institutes of Health [1]. Thereby, biofilm formation might be the major virulence factor of otherwise more benign micororganisms or play a role only in a part of the infection process. May it be in marine sediments, in the continental subsurface or in association with higher organisms such as plants, invertebrates and humans, biofilm forming organisms are predominant and drive to 100% geochemical processes [1]. Biofilm defined by Bill Costerton as 'microbes adhering to each other and/or to surfaces or interfaces with the aid of a self-produced (or environmentally based) extracellular matrix' [2] include per definition initial adherence, microcolonies and monolayers of cells. These initial structures and mature biofilms as multicellular assemblies of self-autonomous cells with tissue-like properties undergo sophisticated genetically and environmentally programmed developments and can enter into a life cycle between multicellularity and the planktonic state. This life style transition is directed by the integration of a multitude of environmental and intrinsic signals on various regulatory levels [3-7]. Rather the rule than the exception, more than one distinct pathway leading to biofilm formation can be encoded by a microbial genome with biofilm pathways and biosynthetic entities to build up genetically-programmed modular biofilms which are highly flexible with respect to the contributing components [8-13]. Considering this flexibility, the genetic plasticity of biofilm genes and their lateral transfer, it might be challenging to develop strategies to tackle microbial biofilm infections, although commonly acting activators of biofilm formation such as the ubiquitous second messenger cyclic di-GMP have been identified [14-16]. Refractoriness might thus be founded in the origin of biofilm formation which is proposed to be intimately coupled to the origin of life itself. To understand the fundamentals of ancient metabolisms which are preserved until today even in evolved organisms might also aid tackling of chronic infections [17, 18].

Evolution of life in close contact to surfaces

After the birth of our solar system and the proto-earth 4.6 and 4.5 billion years ago, respectively, life has been dated to originate approximately 3.8 billion years ago. First indications for biofilm forming organisms have been estimated to be as old as 3.5 to 3.8 illion years [19, 20]. Named as stromatholites in the case of cyanobacteria or more general microbialites, these are observed as surface-spread monolayers of microbial consortia blended with inorganic sediments which were formally described as early as 1883 [21, 22]. Here, I argue that biofilm formation defined in an original sense as the close association of microbial cells with surfaces is in fact intrinsic to the prerequisites and molecular mechanisms that enabled emergence of life (Figure 1).

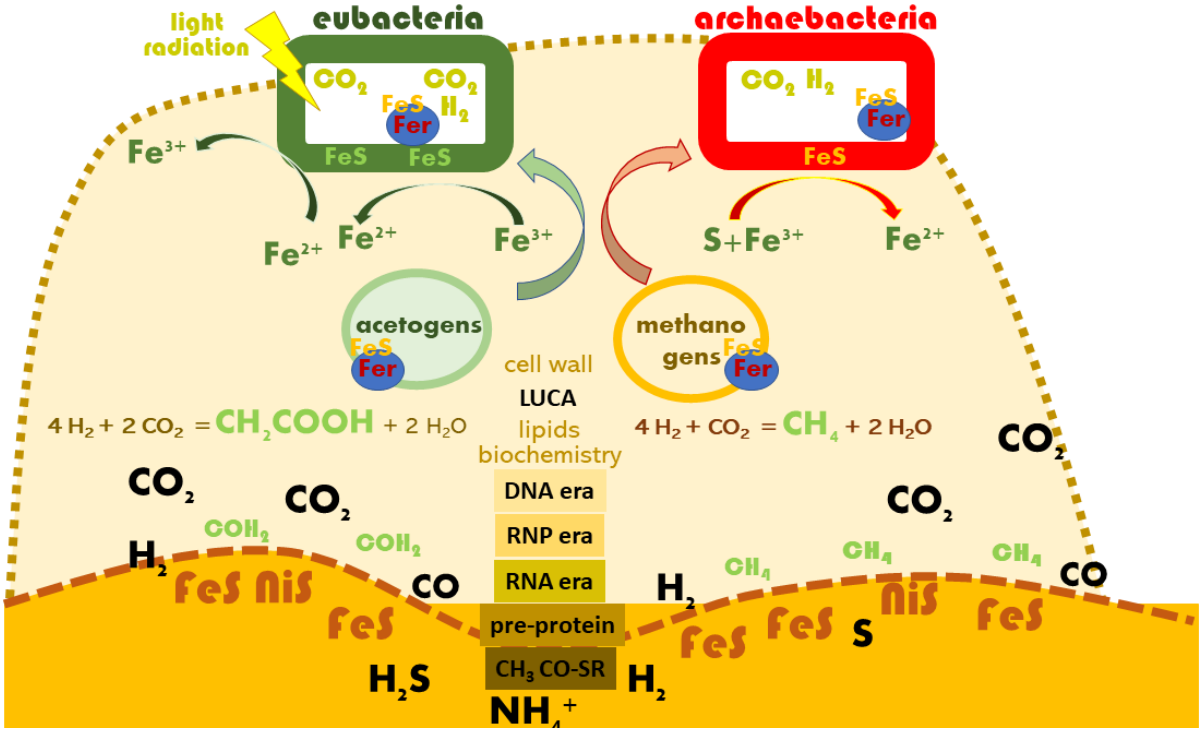

Figure 1. The biofilm mode of life has its origin in the development of life on catalytic surfaces (minerals) such as FeS, FeNiS or FeS/SiO_2 clusters that are intimately associated with its emergence. On these and/or similar redox-active clusters in the presence of molecules such as hydrogen, H2; carbon dioxide, CO_2; carbon monoxide, CO; ammonium, NH_4^+: and hydrogen sulfide, H_2S; created by geochemical reactions such as serpentinization, the first organic molecules including formamide, acetate and methane and high-energy acetyl-thioesters arised. Subsequently, upon the consecutive development of more complex organic molecules, the RNA, RNP (ribonucleoprotein) and DNA era, perhaps preceeded by pre-proteins with metal cofactors similar to the ancient ferredoxin fold emerged. The first biological membranes (organized around semipermeable energized FeS or FeS/SiO_2 walls with built up ion gradient) and cell walls in protected compartments and a last universal common ancestor (LUCA) were similarly facilitated in contact with FeS and/or similar redox-active mineral clusters resulting in first primodal biotic energy metabolisms [23]. Evolution would have eventually led to the incorporation of FeS clusters into respiration-associated proteins with the ferredoxin fold (Fer) central to the origin of ancient redox metabolism, as still universally found in all domains of life [24]. Of note, though, the third domain Eukarya synthesizes Fe-S protein only in mitochondria and plastids [25]. Suggested primeval (prokaryotic) forms of life, with an acetogenic and methanogenic life form on mineral surfaces would have been sessile, essentially constituting ancestral biofilms of the eubacterial and archaeabacterial line. Respiration with subsequent transformation of minerals using ferrous and ferric iron as one of the most ancient redox pairs, followed soon by sulfur and sulfur molecules in different redox stages. Over time, diversification of cellular life has led to populations of cells specialized on biofilm or planktonic lifestyles dependent on the availability of redox pairs and possibilities for electrohomeostasis. In the presence of respiratory electron acceptors, some bacterial species or strains take over the niche from competitors through (planktonic) population increase, thereby taking prevalent control of the metabolic resources available at the site. Inspired by [19].

Emergence of life at all of its stages including the fundamental principles of metabolism and energy conservation to the emergence of cellular organization to arise required solid catalytically effectual surfaces. Thus already far before branching into two structurally fundamental different, conceptually highly similar branches of early life, the eubacterial and archaebacterial protocells from the last universal common ancestor (LUCA), cells emerged from inorganic template predecessors not only in intimate surface-association or even being embedded within [26-29], but with the active contribution of reactive inorganic surfaces to the development of metabolism and as a cell template. Which of various environmental conditions have been most beneficial for the emergence of life and thus can be regarded as its most likely origin is still debated [30-33]. Among the scenarios are conditions similar as found today in alkaline hydrothermal vents considered to provide appropriate prerequisites for the cradle of life such as chemical disequilibria, natural gradients and transition metals at different redox stages for initial catalysis [34-36]. With its foundation in fundamental geochemical processes such as serpentinization, the transformation of minerals and water, the production and accumulation of inorganic molecules provided the basis for the transition to biochemical processes and the emergence of organic molecules [37]. Catalytic, electron-donating and/or accepting iron/nickel sulfur clusters subsequently enabled the acceleration (relative to Earth time) of chemical reactions with inorganic gases CO, CO_2, H_2 and NH_3 as substrates to provide the first organic molecules such as formate, methane, acetic acid, lactate, pyruvate and subsequently even amino acids. Indeed these ancient inorganic and organic molecules or variants of it are even today central to carbon fixation and energy metabolism [18, 38-40]. Furthermore, included in sediments, this type of environment led to the concentration of small molecular compounds and limited the diffusion of molecules [26].

With energy conservation as one of the prerequisites of life, initial chemical conservation of energy has already been possible abiotic on energized semipermeable FeS walls or semi-permeable membranes which enabled synthesis of simple organic molecules such as high energy acetyl-thioesters [41]. This type of energy conservation has subsequently been replaced by substrate-level phosphorylation. and chemiosmotic energy conserving mechanisms at membranes evolved. This type of chemical energy conservation is coupled to the synthesis of ATP as a universal energy currency, driven by the built-up of ion, Na^+ and/or H^+ gradients, over an impermeable membrane which allowed also transport against a concentration gradient, uphill redox reactions and fundamental energy-requiring processes such as cell division and motility [42]. In the primeval Earth before the onset of life, these energy-conserving reactions have been initially facilitated through catalysis of the redox reactions on iron-sulfur mineral walls or mineral walls of similar transition metal composition and perhaps mixed with silica, with conservation of energy in chemical form as a proton gradient over those semi-permeable inorganic walls [26, 34]. Nevertheless, these principles of bioenergetics are basically valid until today and organisms with rudimentary mechanisms of energy conservation are even found in today's organisms [43]. As such obtain many organisms the energy for life in the form of the ubiquitous currency ATP by fermentation, substrate-level phosphorylation of energetically high molecules such as acetyl-phosphate and conserve energy over membranes without the sophisticated respiratory chains widespread today among bacteria. Nevertheless, the first cells have almost certainly developed slowly as even today's microbes in energy-poor subseafloor sediments have an estimated doubling time up to 1000 years [44, 45], and can have subsequently optimized acquisition of energy [46].

With the emergence of respiration, accompanied further by ultimate oxidation of carbon sources to CO_2, more energy has been conserved and, with the energy of the

electron flow used to build up sodium and proton gradients by spatially separated energetically favored redox reactions.[46] This spatially separated redox reactions require membrane diffusible lipid soluble redox compounds such as quinones and methanophenazine and membrane-associated proteins containing iron-sulfur clusters, flavins and heme containing iron, with ferredoxin and cytochrome c, respectively, as the most ancient protostructures [24, 47-50]. Respiration, which leads to a higher energy gain than fermentation has been originally anaerobic and upon the availability of oxygen as an electron acceptor, enabled aerobic metabolism. The use of oxygen as an electron acceptor significantly extended the range of available redox pairs, energy gain and ecological opportunities [51-53] equally as the lateral transfer of respiratory modules [54, 55]. With the conservation of energy in a chemiosmotic H^+ or Na^+ membrane gradient, chemical energy in the form of ATP is ubiquitously gained by conceptually similar, structurally distinct ATP synthases [56, 57]. However, there are indications that even today fermentation can be coupled to extracellular electron transfer which promotes more rapid proliferation and metabolism indicating that intrinsic redox reactions feedback on metabolism [58]. Similarly to the origin of simple central biomolecules and its energy conservation, emergence of other cellular components such as lipids are supposed to have required catalysis on autocatalytic surfaces [59, 60].

Extending this scenario, primitive forms of cellular life can have intimately evolved as inherently sessile surface-associated life forms in direct contact with minerals or within sediments. Indeed, also other fundamental metabolic processes such as anoxygenic photosynthesis thought to represent one of the earliest forms of metabolism and an alternative mode of energy conservation using light as the external energy sources is based on the oxidation of Fe^{2+} to Fe^{3+} [61, 62]. Furthermore, Fe^{3+} has been recognized as the most ancient and a central electron acceptor [63] and Fe^{2+} is in a reverse reaction also used as an electron donor [64]. Furthermore, biofilms form even today on metal sulfides and sulfur, the second central redox-active compound enabling ancient metabolism. Thus, surface association enabled various modes of energy conservation with subsequent development of a protocell and its growth connecting biofilm formation intimately to the origin of life. The early type of surface associated biofilm formation has been evolutionary maintained until today in microbes with microbes still respiring extensively using different iron and sulfur redox stages in minerals [65-67]. Considering the harsh conditions upon life emergence also puts forward the hypothesis that resource-restricted damaging-exposed environmental conditions, commonly called stress, has been the rule rather than the exception as experienced by some microbes today [68]. Thus, elevated repair and persistence mechanisms, which are a hallmark of today's biofilms were necessary to evolve concomitantly with the emergence of microbial (biofilm) life [69]. Biofilm formation and the physiology of microbes has, however, evolved and diversified with the radiation of the two fundamentally different prokaryotic forms of life, the eubacteria and the archaebacteria. On the phylogenetic level, collectively, those organisms have access to all thermodynamically possible redox couples in minerals and in soluble form. The introduction of oxygen as an electron acceptors during the Great Oxygenation Event widened further the spectrum of potential redox pairs.

The most ancient metabolisms, as found in acetogens, carbon fixation via CO_2 which is subsequently reduced by H_2 to acetate, and methanogens, fixation of CO_2 subsequently reduced by H_2 to methane (Figure 1), do neither possess heme, quinones and only rudimentary respiratory chains. Nevertheless built up of chemically stored energy requires ion pumps, membranes, protein-framed iron-sulfur clusters such as in ferredoxin to facilitate redox reactions [41, 70, 71]. Thereby, energy provided by light or radiation equally as electron bifurcation, the coupling of energetically favourable with

unfavourable redox reactions, can enable carbon fixation and energy storage [72, 73]. Eventually, energy storage evolved towards quinone- or methanophenazine based proton gradient generation using heme containing cytochromes in respiratory chains including their regulatory components [74-76]. In fact, the fundamental origin of the components of the respiratory chain is reflected by their combinatorial assembly using a redox protein construction kit [77].

Planktonic cells evolved secondary to surface-associated cells

What does the 'emergence of life as an intrinsic biofilm' hypothesis has as consequence? Accordingly and most importantly, planktonic, not surface attached, cells evolved secondary to surface attached cells. Prerequisites that enabled the development of planktonic cells include the availability of electron donors and acceptors in solution (and subsequently on biotic surfaces) combined with high affinity terminal reductases.

Biofilm microbes with their intimate surface association have developed various modes of direct and indirect electron transfer to communicate with abiotic surfaces and, through abiotic electron transfer, with each other ([78]; Figure 2). Such deliver nanotubes, membrane extrusions with nanotubes and nanowires, proteinaceous type IV pili or archaella appendages, respectively, electrons to abiotic surfaces and, today, in biotechnological applications for current generation to electrons [79-82]. Furthermore, diffusible small redox active molecules emerge either synthesized endogenously and/or are provided exogenously from the environment to contribute efficiently to extracellular electron transfer. Example are the antibiotic pyocyanin and the humic model compound anthraquinone-2,6-disulfonate, respectively [83]. The biological significance of extracellular electron transfer might be equal as to the secretion of small molecular metabolic waste products that can be catabolically used by other microorganisms as carbon source and thus contribute to the optimal use of energized electrons and energetically higher molecules for energy conservation resulting in a macroorganism, for example, in cell dense sediments..

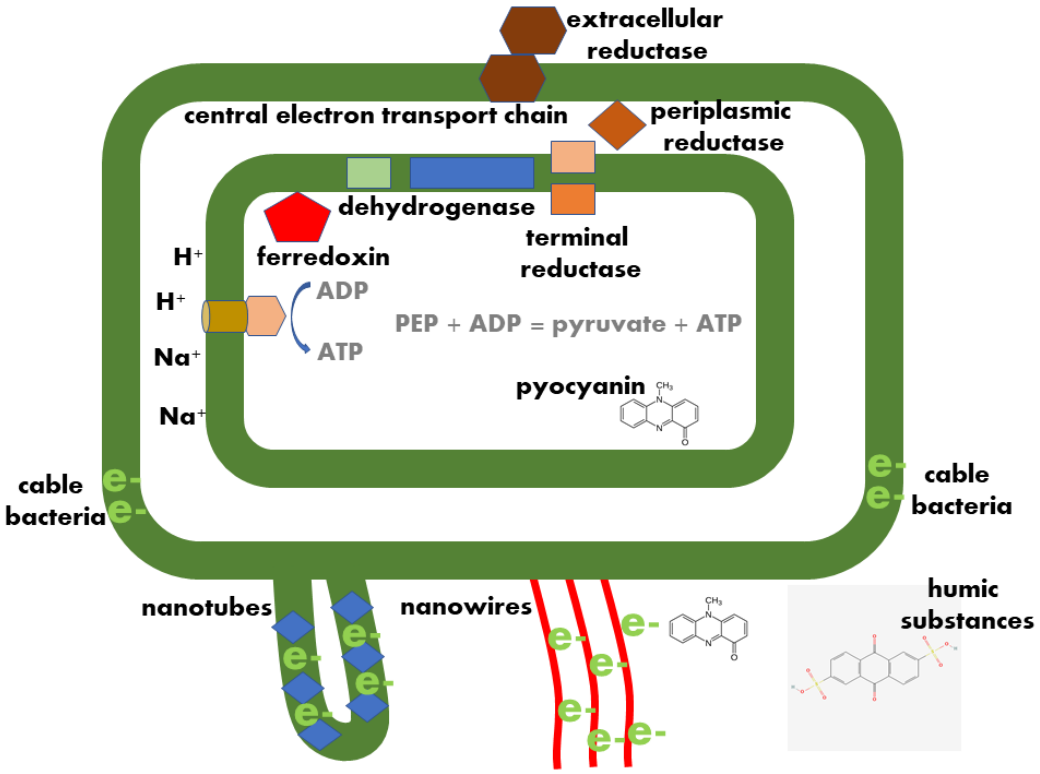

Figure 2. Different modes of electron transfer for energy gain. In respiration, an electron transport chain in the cytoplasmic membrane consisting of FeS cluster, flavin and heme containing integral membrane proteins and membrane diffusible quinone-based redox compounds transfer electrons along the redox gradient with the subsequent built up of an ion gradient, H^+ or Na^+, over the membrane for the production of the energy equivalent ATP. Substrate level phosphorylation is an alternative way to general ATP. The input into the central electron transport chain comes from different metabolic processes such as the catabolism of sugars and nucleotide biosynthesis performed by oxidoreductases, the most prominent the NAD(P)H/NAD^+ dehydrogenase complex, the succinate and formate dehydrogenase complex, hydrogenases, but also the dihydroorotate dehydrogenase in the nucleogenesis pathway and alternative oxidoreductases [84]. The presence and the concentration of the terminal electron acceptor determines the expression of the terminal reductase [53]. For example, in *Escherichia coli*, there exist at least ten terminal reductases. These integral membrane or periplasmic proteins, dedicated for the respiration of substrates such as oxygen, dimethylsulfoxide, trimethylamine-N-oxide, fumarate, nitrate and nitrite are expressed upon substrate availability. Reductases can, however, also be located in the outer membrane with the catalytic center facing the exterior. The periplasmic located CymA reductase of *Shewanella oneidensis* has been shown to respire ferric iron not only in its ingenious host, but also in *E. coli* [85]. Other means to transport electrons along the redox gradient are intercellular nanotubes, nanowires (type IV pili in eubacteria or archaella in archaebacteria), intrinsic and extrinsic diffusible redox active substances such as pyocyanin and the humic model compound anthraquinone-2,6-disulfonate, respectively, as well as an unknown mechanism in cable pili. PEP, phosphoenolpyruvate.

Thus the surface associated protocells might have been a naked cells emerging from a prebiotic gel [28] without an extracellular matrix surrounding them. This aspect is distinct from the hallmark characteristic for todays' biofilms, which are defined as 'matrix enclosed microbial populations adherent to each other' [2]. It cannot be excluded though that a primordial musilace existed [22, 36] or extracellularly secreted DNA, an extracellular component of biofilms even today [86], might have early served as a biofilm extracellular matrix component. This is not unlikely considering the higher permeability of early membranes [41]. So why and how did extracellular matrix production of surface attached biofilm cells evolve. While the 'how' cannot be answered directly, the production of an extracellular matrix certainly has provided an additional layer of protection. More diversified metabolic pathways emerged in surface-attached cells or their cellular predecessors might have required protection from radiation, fast or uncontrollable redox reactions or interfering metal ions. Indications for an early arisal of a polysaccharide-based extracellular matrix are a poly-1,6-beta-N-acetylglucosamine/cellulose synthase like glycosyltransferase family 2 protein encoding genes among the genes most likely present in the last universal common ancestor of bacteria and archaea [87]. Those gene products encoded in today's genomes by members of deepest branching bacterial phyla such as Dictyoglomota, Coprothermobacterota, Caldiserica and Bipolaricaulota equally as in Archaea, although shorter in length, structurally resemble cellulose synthases in its core functional domain (Figure 3A and data not shown). Obviously, the advantages of extracellular matrix production were higher than the disadvantage; which are, for example, to be at a larger physical distance to the respective solid electron donors and acceptors. To overcome this hurdle, one can hypothesize that biofilm extracellular matrix components can associate with respective electron donors/acceptors for efficient energy gain [88], as inorganic matter is integrated for stabilization of biofilm structures [88]. As another aspect, upon division of protocells attached to a surface, not all cells in

multicellular structures have equal access to the redox pairs which has perhaps severely limited the proliferation of merely surface-attached protocells. Production of matrix and other extracellular structures would thereby have enabled physical closeness with efficient transfer of electrons and small molecules and enabled an extension of the biofilms [89, 90].

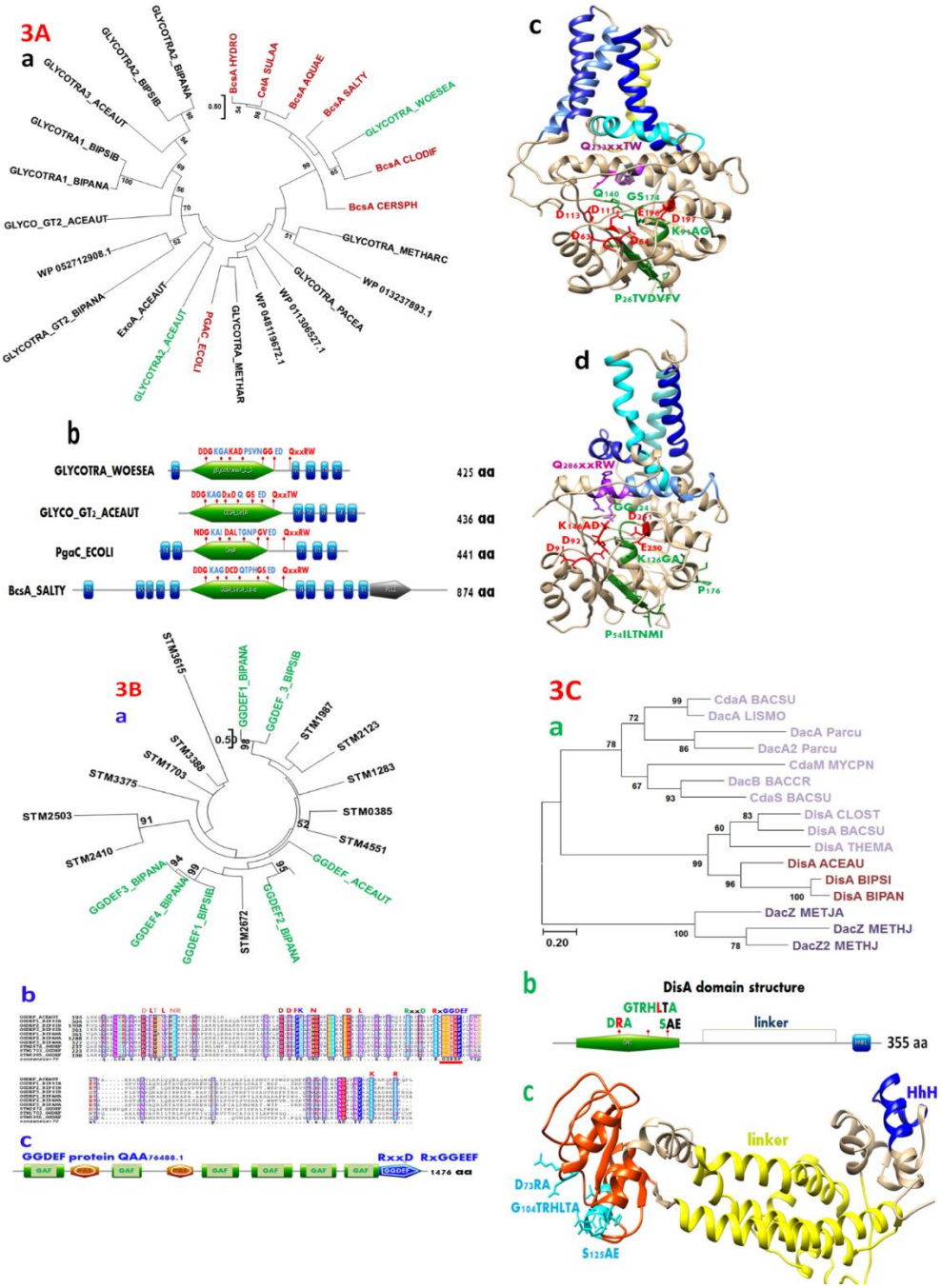

Figure 3. Presence of biofilm gene products in deeply branching bacteria and/or archaea indicate an ancient origin of molecular mechanisms of biofilm formation. **A** poly-1,6-beta-N-acetylglucosamine/cellulose/alginate synthase genes have been predicted to be present in LUCA of bacteria and archaea [87]. a) Phylogenetic position of the core catalytic domain of representative glycosyltransferases identified by Blast searching [91], archaeal and bacterial deeply branching phyla with the poly-beta-1,6-N-acetyl-glucoseamine synthase PgaC of *Escherichia coli* and the cellulose synthase BcsA of *Aquifex aeolicus*. The three (nearly) complete genomes of Candidatus *Acetothermum autotrophicum* (ACEAUT),

Candidatus *Bipolaricaulis sibiricus* Ch78 (BIPSIB) and Candidatus *Bipolaricaulis anaerobius* Ran1 (BIPANA) and one identified homologous glycosyltransferase each from DPANN archaea and *Methanosarcina* were also included in the phylogenetic analysis. b) Domain structure and glycosyltransferase 2 (GT2) characteristic motifs and c) structural models of an archaeal (MBP4151113.1, Woesearchaeota archaeon) and a Bipolaricaulota representative (BAL58984.1, Candidatus *A. autotrophicum*). The structural models were constructed with Phyre2 [92]. In blue and yellow, transmembrane helices as predicted by TMHMM 2.0 [93]. The GT2 domains are named according to the domain with lowest Expect values.

BcsA_HYDRO (A0A432RG04, *Hydrogenothermus* sp.), CelA_SULAA (*Sulfurihydrogenibium azorense* Az-Ful), BcsA_AQUAE (O67406, *Aquifex aeolicus*), BcsA_SALTY (Q93IN2, *S. typhimurium*), BcsA_CLODIF (AJP12292.1, *Clostridioides difficile* 630), BcsA_CERSPH (*Cereibacter sphaeroides* ATCC 17023), GLYCOTRA_WOESEA (MBI4151113.1), GLYCOTRA_METHARC (CAG0966249.1), WP 013237893.1 (*Clostridium ljundahlii*), GLYCOTRA_PACEA (MBI5803293.1), WP 011306527.1 (*Methanosarcina barkeri* DSM804), WP 048119672.1 (*M. barkeri* 227), GLYCOTRA_METHAR (MBI4151113.1), PGAC_ECOLI (P75905, *E. coli* K-12), GLYCOTRA2_ACEAUT (BAL58984.1), ExoA_ACEAUT (BAL60196.1), GLYCOTRA_GT2_BIPANA (WP 122030831.1), WP 052712908.1 (*M. barkeri* 227), GLYCO_GT2_ACEAUT (BAL60260.1), GLYCOTRA1_BIPANA (WP 157959473.1), GLYCOTRA1_BIPSIB (QAA76168.1), GLYCOTRA3_ACEAUT (BAL59581.1), GLYCOTRA2_BIPSIB (QAA76636.1), GLYCOTRA2_BIPANA (WP 122030981.1).

B. Phylogenetic position of GGDEF domains of GGDEF domain proteins of representative Bipolaricaulota species. a) Blast searching [91] the three (nearly) complete genomes of C. *A. autotrophicum*, C. *B. sibiricus* and C. *B. anaerobius* with the diguanylate cyclase AdrA of *Escherichia coli* recognized one, three and four GGDEF domain proteins. GGDEF_ACEAUT (BAL59883.1); GGDEF1_BIPSIB (QAA76087.1); GGDEF2_BIPSIB (QAA76488.1); GGDEF3_ BIPSIB (QAA76751.1; GGDEF1_BPIANA (WP_122030618.1); GGDEF2_BIPANA (WP_122031498.1); GGDEF3_BIPANA (WP_162297725.1); GGDEF4_BIPANA (SQD92144.1). GGDEF domain proteins of *Salmonella typhimurium* are described [94]. b) Alignment of GGDEF domains of GGDEF domain proteins of C. *A. autotrophicum*, C. *B. sibiricus* and C. *B. anaerobius* with selected GGDEF domains of *S. typhimurium* proteins. Underlined in red is the conserved catalytic GGDEF motif. c) Domain structure and conserved residues of the GGDEF domain protein QAA76488.1 of C. *B. sibiricus* from the Bipolaricaulota phylum.

C. Diadenylate cyclase proteins of representative Bipolaricaulota species, C. *A. autotrophicum*, C. *B. sibiricus* and C. *B. anaerobius* as identified by Blast searching with the diadenylate cyclase DisA of *T. maritima*. a) Phylogenetic position of the diadenylate cyclase (DAC) domain of DisA proteins from C. *A. autotrophicum* (BAL58991.1), C. *B. sibiricus* (QAA77389.1) and C. *B. anaerobius* (WP_122030516.1) in relation to the DAC domain of representative proteins of the distinct and characterized diadenylate cyclase groups DisA, CdaA (DacA), CdaM, CdaS (DacB), CdaZ (DacZ) [95, 96]. b) All currently identified diadenylate cyclase proteins of Bipolaricaulota species belong to the DisA group with an identical domain structure of DAC-linker-(helix-hairpin-helix) non-specific DNA binding motif class 1 as exemplified by the DisA protein of C. *A. autotrophicum*. Catalytic residues are partially conserved in DisA of of C. *A. authtrophicum* with the DGA motif changed to DRA, the RHR motif in the context of GTRHRxA being GTRHLTA and the catalytic serine conserved in the context of SAE. DisA proteins of C. *B. sibiricus* and C. *B. anaerobius* have the RHR motif conserved. c) Structural model of the DisA protein of C. *A. autotrophicum* constructed with Phyre2 [92] and displayed in Chimera [97]. CdaA_BACSU (Q45589, *Bacillus subtilis* 168); DacA_LISMO (Q8Y5E4, *Listeria monocytogenes* serovar

1/2a ATCC BAA-679); DacA_Parcu (A0A419G9B5, Candidatus *Parcubacteria* bacterium); DacA2_Parcu (NCO15553.1, Candidatus *Parcubacteria* bacterium); CdaM_MYCPN(A0A8D9FKA8, *Mycoplasma pneumoniae* ATCC 29342); DacB BACCR (Q812L9, *Bacillus cereus* ATCC 14579); CdaS_BACSU (O31854, *B. subtilis* 168); DisA_CLOST (WP_013240715.1, *Clostridium ljungdahlii* DSM 13528); DisA_BACSU (P37573, *B. subtilis* 168); DisA THEMA (Q9WY43, *T. maritima* ATCC 43589); DisA_ACEAU (BAL58991.1, Candidatus *A. autotrophicum*); DisA_BIPSI (QAA77389.1, Candidatus *B. sibiricus*); DisA_BIPAN (WP_122030516.1, Candidatus *B. anaerobius*); DACZ_METJA (Q58408, *Methanocaldococcus jannaschii* ATCC 43067); DacZ_METHJ (Q2FNI7, *Methanospirillum hungatei* JF-1 ATCC 27890); DacZ2_METHJ (Q2FM57, *Methanospirillum hungatei* JF-1); DacZ_METJA (Q58408, *Methanocaldococcus jannaschii* ATCC 43067).

Cellulose, a 1,4 beta-glucan composed of solely the most chemically inert sugar alpha-D-glucose might have been one of the early polysaccharide extracellular matrix components. Indeed, cellulose is considered to serve as a biosignature in rock records as an indication of microbial life [98] and is produced by microbes from deep braches of the phylogenetic tree of the phylum Aquificales such as representatives *Aquifex aeolicus* and *Hydrogenothermus*. What is again surprising though is the maintenance and modified reuse of such ancient components, as cellulose production has been conserved in evolved human pathogens as the gamma-proteobacterium *S. typhimurium* where the microbes form cellulose-based biofilms within immune cells to restrict virulence [99-101]. In metabolic agreement with an ancient origin of cellulose is the fact that UPD-glucose for cellulose biosynthesis is provided by the gluconeogenesis pathway in *S. typhimurium* [102], an anabolic pathway, more ancient than catabolic glycolysis, for the supply of sugar molecules from acetate synthesized by the Wood-Ljungdahl pathway of carbon fixation from CO_2 reduced by H_2 [103].

Preliminary bioinformatics analyses indicated also the presence of the ubiquitous biofilm activator, the second messenger molecule cyclic di-GMP, to be of ancient origin as present already in representatives of deepest branching bacterial phyla such as Dictyoglomota, Coprothermobacterota, Caldiserica and Bipolaricaulota equally as in clostridial acetogens (Figure 3B and data not shown). Cyclic di-GMP intercedes the shift between the fundamental single-cell life styles sessility and motility and concurrently regulates other viable physiological pathways such as DNA repair and photosynthesis [14]. The absence of this heat-stable molecule in the archaeal lineage is not an argument for the lack of cyclic di-GMP signaling in a last ubiquitous common ancestor as this second messenger can rapidly disappear on evolutionary time scales even in free-living bacterial genera without close association with a host [104-106]. Although today less abundant with respect to overall copy number of synthesizing and degrading gene products, cyclic di-AMP can be the (or among) common second messenger signaling molecules as it regulates most fundamental essential physiological processes such as monitoring DNA integrity combined with subsequent surveillance of repair mechanisms equally as regulating potassium homeostasis [14, 107, 108]. Cyclic di-AMP signaling affectgs biofilm formation by regulation of cell wall biosynthesis and eDNA secretion which might be related to cell membrane/envelope permeability [109, 110]. Importantly, genes for cyclic di-AMP synthesizing enzymes are common to Archaea and deepest branching bacterial phylua such as Dictyoglomota, Caldiserica and Bipolaricaulota and present in model acetogen *Clostridium ljungdahlii* among other acetogens (Figure 3C and data not shown). However, such genes might not be recognized as most ancient vertically evolved genes due to early interdomain recombination events which placed the enzymatic domains under the posttranslational control of a diversity of intra- and extra-cellular signal domains and due to lateral gene transfer which targets cyclic di-GMP signaling more

frequent than expected on average [87, 95, 111, 112]. Indeed, one can hypothesize that biofilm and respective regulatory genes must have been subject of extended early lateral gene transfer to adapt the capability to form a diversity of distinctly regulated biofilms adapted to respective environmental conditions. Furthermore, one might ask the question why in particular cyclic di-AMP and cyclic di-GMP had been selected to ubiquitously direct biofilm related physiology and metabolism. As an argument from a chemical perspective, in comparison to the monocyclic cyclic AMP and cyclic GMP, the covalent bonds in the di-cyclic molecules experience less tension and thus lead to more stable molecules [113] On the other hand, a wide spectrum of diverse cyclic di-nucleotides, cyclic oligo-nucleotides and even pyrimidine-based cyclic nucleotides have been recently unravelled to occur [114-116]. To what extent these and other nucleotide-based signaling molecules play a role in physiology and metabolism of the majority of under-investigated environmental microbes beyond phage defense systems remains to be determined.

Diversification of the biofilm response

The biofilm response in the presence of solid electron acceptors and other energy-gain-related signals has extended and diversified. Proposed as the fundamental reaction and still observed as a prerequisite for direct energy gain from solid mineral electron acceptors [117, 118] is the promotion of sessility (biofilm establishment) as previously hypothesized in this opinion paper to have originated concomitantly with life and being the most fundamental behavior towards the ancient solid electron donors and acceptors. Motility and chemotaxis can be required for modern free living microbes to approach a diversity of solid electron acceptors and organic matter [119], and the motility lifestyle opposite biofilm formation to trigger biofilm dispersal. However, the respiratory responses have diversified and being subject to lateral gene transfer, while the response towards certain electron acceptors have even reverted [55, 120, 121]. For example does nitrate respiration of the gastrointestinal pathogen *S. typhimurium* lead to reduced biofilm formation, but triggers motility in order to promote invasion of the gut epithelial cell line and virulence, while in other bacterial species nitrate respiration promotes biofilm formation [122-125]. Such a differentiated microbial behavior can extend to the strain level to ensure maintenance of a species in most possible ecological breadth through strain-dependent differential occupation of ecological niches and most beneficial responses to diverse environments [125, 126].

Discussion

While the early origin of life spanning from the emergence of first organic molecules to fundamental energy and carbon fixation mechanisms in bacteria and archaea has been thoroughly studied, development of alternative aspects of microbial physiology and metabolism such as the time line of origin, evolution and diversification of surface dependent energy conservation processes and biofilm formation with its cell differentiation into, for example, persister cells, has not been performed. Identification of the ancient inventory and the phylogeny of biofilm genes including diversification of mechanisms and regulation of biofilm formation in the deepest branching bacterial and archaeal phyla equally as the evolution of energy preserving modules with the integration of additional ancient energy-gaining pathways such as anaerobic sulfur respiration [127], can give a first glimps on these processes. On the other hand, while the presence of biofilm components such as the genetic modules for the production of the poly-beta-1,6-N-acetyl-glucoseamine and cellulose exopolysaccharide have been realized to occur in bacteria throughout the phylogenetic tree, its origin has not been defined [101, 128, 129]. Equally, although a Gram-negative acyl-homoserine quorum sensing system has also identified in

archaea [130], deep phylogenetic origin of biofilm-regulating quorum sensing systems remains elusive [131]. However, an early bacterial ancestor has been suggested to have been flagellated microbe with two membranes [132]. Extended such analyses might, however, provide novel insights into the treatment of biofilm infections [133, 134]

In many bacterial species, an agar-grown biofilm model has been identified. These colony morphotype biofilm models with dense association of individual cells embedded into a honey-bee comb like extracellular matrix [122, 135-137] can be seen in a light rather different than a laboratory curiosity resembling tissue development in higher organisms [5, 6]. In fact, the dense association of microbial cells, a readily accessible and genetically screenable biofilm model, might not only be a physiologically and ecologically relevant model for biofilm formation in microbial-dense environments such as the colon, but also serve as a model for ancient environmental biofilms on surfaces [138-140]. The subsequent free living planktonic life style is considered a temporary condition in the life cycle of most microbes [141], which does, however, not contradict the multiple independent gain of multicellularity in eukaryotes [142].

The fundamentally ancient process of energy conservation involving solid reversible redox active surfaces has many applications.. One of the most prominent applications is the gain of energy in microbial fuel cells [143, 144]. Another example is the gain of energy by electrosynthesis [145, 146]. In a reverse process, biofilms that protect steel from corrosion may be developed [147]. Furthermore, the understanding of these ancient molecular mechanisms of biofilm formation might also aid to modulate biofilm formation [148, 149] and tackle chronic infections [150, 151]. Integrating further the knowledge on these ancient processes of energy acquisition and its preservation in modern microbes might lead to innovative biofilm treatment strategies and new application processes.

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