The biofilm matrix

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Abstract | The microorganisms in biofilms live in a self-produced matrix of hydrated extracellular polymeric substances (EPS) that form their immediate environment. EPS are mainly polysaccharides, proteins, nucleic acids and lipids; they provide the mechanical stability of biofilms, mediate their adhesion to surfaces and form a cohesive, three-dimensional polymer network that interconnects and transiently immobilizes biofilm cells. In addition, the biofilm matrix acts as an external digestive system by keeping extracellular enzymes close to the cells, enabling them to metabolize dissolved, colloidal and solid biopolymers. Here we describe the functions, properties and constituents of the EPS matrix that make biofilms the most successful forms of life on earth.

Biofilm
A loose definition for microbial aggregates that usually accumulate at a solid–liquid interface and are encased in a matrix of highly hydrated EPS. Included in this definition are cell aggregates such as flocs (floating biofilms) and sludge, which are not attached to an interface but which share the characteristics of biofilms. Multispecies biofilms can form stable microconsortia, develop physiochemical gradients, and undergo horizontal gene transfer and intense cell–cell communication, and these consortia therefore represent highly competitive environments.

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doi:10.1038/nrmicro2415
Published online 2 August 2010

Microorganisms do not live as pure cultures of dispersed single cells but instead accumulate at interfaces to form polymicrobial aggregates such as films, mats, flocs, sludge or 'biofilms' (REF. 1). In most biofilms, the microorganisms account for less than 10% of the dry mass, whereas the matrix can account for over 90%. The matrix is the extracellular material, mostly produced by the organisms themselves, in which the biofilm cells are embedded. It consists of a conglomeration of different types of biopolymers — known as extracellular polymeric substances (EPS) — that forms the scaffold for the three-dimensional architecture of the biofilm and is responsible for adhesion to surfaces and for cohesion in the biofilm. The formation of a biofilm allows a lifestyle that is entirely different from the planktonic state. Although "the precise and molecular interactions of the various secreted biofilm matrix polymers … have not been defined, and the contributions of these components to matrix integrity are poorly understood at a molecular level" (REF. 2), several functions of EPS have been determined (TABLE 1), demonstrating a wide range of advantages for the biofilm mode of life.

EPS immobilize biofilm cells and keep them in close proximity, thus allowing for intense interactions, including cell–cell communication, and the formation of synergistic microconsortia. Owing to the retention of extracellular enzymes, a versatile external digestive system is generated, sequestering dissolved and particulate nutrients from the water phase and allowing them to be utilized as nutrient and energy sources. The matrix also acts as a recycling centre by keeping all of the components of lysed cells available. This includes DNA, which may represent a reservoir of genes for horizontal gene transfer. EPS can also serve as a nutrient source, although some components of EPS are only slowly biodegradable and, owing to the complexity of EPS, complete degradation of all components requires a wide range of enzymes. The matrix protects organisms against desiccation, oxidizing or charged biocides, some antibiotics and metallic cations, ultraviolet radiation, many (but not all) protozoan grazers and host immune defences. Ecologically, competition and cooperation in the confined space of the EPS matrix lead to a constant adaptation of population fitness.

It is unclear whether the matrix confers an ecological advantage on all cells in the biofilm, in particular those that are furthest from the surface. Simulations of competition in a biofilm revealed a strong evolutionary benefit for polymer producers at the expense of non-producers, possibly because polymers push the daughter cells of polymer producers closer to oxygen-rich environments[3].

EPS have been called 'the dark matter of biofilms' because of the large range of matrix biopolymers and the difficulty in analysing them[4]. EPS can vary greatly between biofilms, depending on the microorganisms present, the shear forces experienced, the temperature and the availability of nutrients. EPS were initially denoted 'extracellular polysaccharides' but were renamed, as it became clear that the matrix also contains proteins, nucleic acids, lipids and other biopolymers such as humic substances[1,5]. Extracellular bacterial structures such as flagella, pili and fimbriae can also stabilize the matrix[6]. Membrane vesicles derived from outer membranes of Gram-negative bacteria can contain a range of enzymes and DNA and can alter matrix properties[7], sometimes acting as 'killer vesicles' targeted at competing biofilm organisms.

Table 1 | **Functions of extracellular polymeric substances in bacterial biofilms**

Function	Relevance for biofilms	EPS components involved
Adhesion	Allows the initial steps in the colonization of abiotic and biotic surfaces by planktonic cells, and the long-term attachment of whole biofilms to surfaces	Polysaccharides, proteins, DNA and amphiphilic molecules
Aggregation of bacterial cells	Enables bridging between cells, the temporary immobilization of bacterial populations, the development of high cell densities and cell–cell recognition	Polysaccharides, proteins and DNA
Cohesion of biofilms	Forms a hydrated polymer network (the biofilm matrix), mediating the mechanical stability of biofilms (often in conjunction with multivalent cations) and, through the EPS structure (capsule, slime or sheath), determining biofilm architecture, as well as allowing cell–cell communication	Neutral and charged polysaccharides, proteins (such as amyloids and lectins), and DNA
Retention of water	Maintains a highly hydrated microenvironment around biofilm organisms, leading to their tolerance of dessication in water-deficient environments	Hydrophilic polysaccharides and, possibly, proteins
Protective barrier	Confers resistance to nonspecific and specific host defences during infection, and confers tolerance to various antimicrobial agents (for example, disinfectants and antibiotics), as well as protecting cyanobacterial nitrogenase from the harmful effects of oxygen and protecting against some grazing protoza	Polysaccharides and proteins
Sorption of organic compounds	Allows the accumulation of nutrients from the environment and the sorption of xenobiotics (thus contributing to environmental detoxification)	Charged or hydrophobic polysaccharides and proteins
Sorption of inorganic ions	Promotes polysaccharide gel formation, ion exchange, mineral formation and the accumulation of toxic metal ions (thus contributing to environmental detoxification)	Charged polysaccharides and proteins, including inorganic substituents such as phosphate and sulphate
Enzymatic activity	Enables the digestion of exogenous macromolecules for nutrient acquisition and the degradation of structural EPS, allowing the release of cells from biofilms	Proteins
Nutrient source	Provides a source of carbon-, nitrogen- and phosphorus-containing compounds for utilization by the biofilm community	Potentially all EPS components
Exchange of genetic information	Faciliates horizontal gene transfer between biofilm cells	DNA
Electron donor or acceptor	Permits redox activity in the biofilm matrix	Proteins (for example, those forming pili and nanowires) and, possibly, humic substances
Export of cell components	Releases cellular material as a result of metabolic turnover	Membrane vesicles containing nucleic acids, enzymes, lipopolysaccharides and phospholipids
Sink for excess energy	Stores excess carbon under unbalanced carbon to nitrogen ratios	Polysaccharides
Binding of enzymes	Results in the accumulation, retention and stabilization of enzymes through their interaction with polysaccharides	Polysaccharides and enzymes

EPS, extracellular polymeric substances.

Globally, EPS represent a dominant fraction of the reduced-carbon reservoir in soils and in sediments, and suspended aggregates in oceans and freshwater. There, they serve as nutrients and thus play an important part in microbial ecology[8–12].

In this Review, we focus on the role of these matrix components in the architecture of bacterial biofilms, discuss the challenges of isolating EPS and describe the different components of biofilms. BOX 1 provides information about EPS of other organisms.

EPS and biofilm architecture

Cells in a biofilm are surrounded by EPS, which constitute the immediate environment of these cells. Some EPS, in particular those forming capsules, are associated more closely with cell surfaces than others. The formation and maintenance of structured multicellular microbial communities crucially depend on the production and quantity of EPS[13]. The concentration, cohesion, charge, sorption capacity, specificity and nature of the individual components of EPS, as well as the three-dimensional architecture of the matrix (the dense areas, pores and channels), determine the mode of life in a given biofilm. The resulting biofilm morphology can be smooth and flat, rough, fluffy or filamentous, and the biofilm can also vary in its degree of porosity, having mushroom-like macrocolonies surrounded by water-filled voids. All of these morphologies have the same effect: to transiently immobilize biofilm cells and allow the existence of long-term mixed-species microconsortia, with their interactions and gradients; this provides very diverse habitats on a small scale, favouring biodiversity.

Extracellular polymeric substances
Hydrated biopolymers (including polysaccharides, proteins, nucleic acids and lipids) that are secreted by biofilm cells to encase and immobilize microbial aggregates. These biopolymers are responsible for the macroscopic appearance of biofilms, which are frequently referred to as 'slime'.

Box 1 | Extracellular polymeric substances from fungi, algae and archaea

Extracellular polymeric substances (EPS) are not unique to bacteria. Some of the most abundant EPS producers are microalgae (in particular, diatoms)[103]. Microalgal EPS play important parts in the stabilization of sediments[104] and the entrainment of sand[105], but they are also involved in marine fouling. The green alga *Penium margaritaceum* has been shown to produce large amounts of EPS (predominantly polysaccharides[106,107]) that, in turn, support the growth of heterotrophic bacteria which use EPS as a substrate. Fungi (yeasts and moulds) also produce EPS. Examples are certain *Candida* spp.[108] that produce EPS which are involved in the processes of flocculation, adhesion and biofilm formation[109]. The archaeon *Sulfolobus solfataricus* produces polysaccharides in response to adhesion[110]; other than this, there is surprisingly little information about the EPS matrices of archaea.

The use of microelectrodes (to monitor oxygen levels, for example) revealed spatial heterogeneity in biofilms on a micrometre scale[14] (FIG. 1). On the basis of staining with lectins and imaging with confocal laser scanning microscopy to differentiate various EPS components and biofilm organisms, it was concluded that the EPS matrix provides a physical structure that segregates microdomains[15]. These regions harbour different biochemical environments that are enzymatically modified in response to changing conditions. For further investigation of the matrix architecture, a reliable allocation of the binding sites of lectins is crucial. Chemical analyses can possibly be put into a spatial context by combining confocal laser scanning microscopy and Raman microscopy[16] (BOX 2).

The architecture of biofilms is influenced by many factors, including hydrodynamic conditions, concentration of nutrients, bacterial motility and intercellular communication as well as exopolysaccharides and proteins, as demonstrated by the altered morphology of biofilms produced by mutants lacking components of EPS. For example, exopolysaccharides of *Vibrio cholerae*[17] and colanic acid of *Escherichia coli*[18] are involved in the formation of a three-dimensional biofilm architecture. The *Bacillus subtilis* biofilm matrix consists of an exopolysaccharide and the secreted protein TasA, both of which are required for the structural integrity of the matrix and the development of biofilm architecture in the form of fruiting body-like structures[19]. During aggregation of the soil bacterium *Myxococcus xanthus*, the polysaccharide component of the extracellular matrix forms a scaffold within the fruiting-body structure[20]. One of the best studied exopolysaccharides involved in biofilm formation is alginate in the biofilms of mucoid strains of the opportunistic pathogen *Pseudomonas aeruginosa*[21,22]. Alginate is not essential for *P. aeruginosa* biofilm formation[23], but it has a notable effect on biofilm architecture when it is present. Under conditions in which alginate producers form structurally heterogeneous biofilms, non-mucoid strains develop flat and more homogeneous biofilms (FIG. 2a–c).

Acetyl groups are common substituents of exopolysaccharides, and they increase the adhesive and cohesive properties of EPS and alter biofilm architecture. The modification of alginate with acetyl groups strongly influences the aggregation of bacteria into microcolonies and determines the structurally heterogeneous architecture of mature biofilms[21,22] (FIG. 2e,f). Biofilm architecture can also be strongly influenced by the interaction of anionic EPS, containing carboxylic groups, with multivalent cations. For example, Ca^{2+} can form a bridge between polyanionic alginate molecules, stimulating the development of thick and compact biofilms with increased mechanical stability[24] (FIG. 2d).

Humic substance
A component of the natural organic matter in soil and water enviroments. Humic substances are mixtures of compounds that are formed by limited degradation and transformation of dead organic matter and that are resistant to complete biodegradation. They can be divided into three main fractions: humic acids, fulvic acids and humin. They usually include phenolic and polyaromatic compounds (containing peptide and carbohydrate moieties with carboxylic substituents), providing the acidic character.

Flagellum
A long, thin, helically shaped bacterial appendage that provides motility. A flagellum consists of several components and moves by rotation, much like a propeller. The motor is anchored in the cytoplasmic membrane and the cell wall.

Pilus
A bacterial surface structure that is similar to a fimbria but is typically a longer structure, and that is present on the cell surface in one or two copies. Pili can be receptors for bacteriophages and also facilitate genetic exchange between bacterial cells during conjugation. Type IV pili mediate twitching motility, which is a flagella-independent form of bacterial translocation over surfaces, and can be involved in biofilm development.

Isolation of EPS

The identification of EPS components depends on the isolation method used. However, efficient EPS isolation is challenging, particularly for EPS from environmental biofilms, which can contain an immense range of components that each require different extraction methods. In a mixed-species biofilm, many members of the microbial community contribute their own (and often specific) EPS that then merge into a complex mixture[11] and remain in the matrix even after their producers have died or left the biofilm. Furthermore, it is next to impossible to quantitatively isolate EPS from a given biofilm, because some of the EPS fraction remains bound to the bacteria, and because the isolation procedure damages cells, causing intracellular material to leak into the matrix.

There is no universal EPS isolation method — the extraction procedure has to be adapted to the specific type of biofilm under investigation. Centrifugation, filtration, heating, blending, sonication, and treatment with complexing agents and with ion exchanger resins have been described[25,26], and the use of sodium hydroxide has even been reported[27], although this method almost certainly leads to contamination with cytoplasmic components. One popular method uses a cation exchanger resin[28], which removes the cations that bridge the negatively charged groups of the polysaccharide and protein moieties of EPS. Alginate from *P. aeruginosa* is comprised solely of uronic acids, which are not found inside the cells and can therefore be used as EPS markers during isolation[29]. The presence of intracellular enzymes, such as glucose-6-phosphate 1-dehydrogenase (G6PD, also known as Zwf), indicates contamination with cellular components. Following extraction, a common concentration step is to precipitate solubilized EPS by adding ethanol or acetone[11]; however, this method primarily precipitates polysaccharides, leading to an underestimation of the other components of EPS.

Common EPS isolation techniques inherently select for water-soluble EPS and lose insoluble EPS, including cellulose, which is an important constituent of the matrices of many bacteria. Cellulose plays an important part in biofilm-related infections caused by *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter* spp., *Citrobacter* spp. and *Salmonella enterica* subsp. *enterica* serovar Typhimurium[6,30–32]. Isolation of cellulose requires harsh conditions, such as treatment with acetic acid and nitric acid at 95 °C[3].

Exopolysaccharides

Polysaccharides are a major fraction of the EPS matrix[28,29]. Most are long molecules, linear or branched, with a molecular mass of 0.5×10^6 daltons to 2×10^6 daltons.

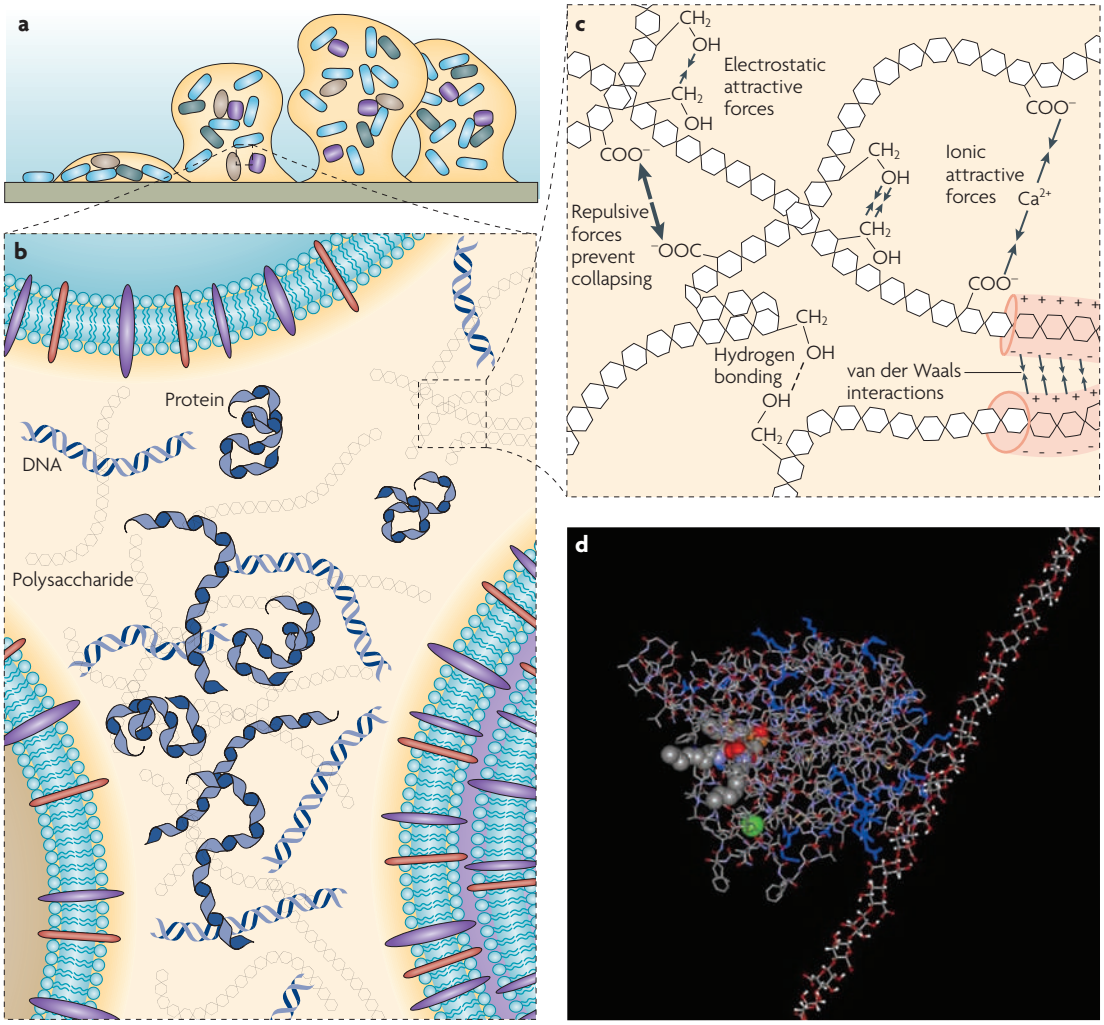


Figure 1 | **The extracellular polymeric substances matrix at different dimensions. a** | A model of a bacterial biofilm attached to a solid surface. Biofilm formation starts with the attachment of a cell to a surface. A microcolony forms through division of the bacterium, and production of the biofilm matrix is initiated. Other bacteria can then be recruited as the biofilm expands owing to cell division and the further production of matrix components. **b** | The major matrix components — polysaccharides, proteins and DNA — are distributed between the cells in a non-homogeneous pattern, setting up differences between regions of the matrix. **c** | The classes of weak physicochemical interactions and the entanglement of biopolymers that dominate the stability of the EPS matrix[47]. **d** | A molecular modelling simulation of the interaction between the exopolysaccharide alginate (right) and the extracellular enzyme lipase (left) of *Pseudomonas aeruginosa* in aqueous solution. The starting structure for the simulation of the lipase protein was obtained from the Protein Data Bank[117]. The coloured spheres represent 1,2-dioctylcarbamoyl-glycero-3-*O*-octylphosphonate in the lipase active site (which was present as part of the crystal structure), except for the green sphere, which represents a Ca^{2+} ion. The aggregate is stabilized by the interaction of the positively charged amino acids arginine and histidine (indicated in blue) with the polyanionic alginate. Water molecules are not shown. Image courtesy of H. Kuhn, CAM-D Technologies, Essen, Germany.

Several polysaccharides have been visualized by electron microscopy as fine strands that are attached to the cell surface and form complex networks. Microscopic techniques in combination with specific carbohydrate staining using fluorescently labelled lectins or antibodies (BOX 2), as well as biochemical analyses for independent verification, have demonstrated the ubiquity of matrix polysaccharides not only in biofilms from natural marine, freshwater and soil environments and from man-made water systems, but also in biofilms associated with chronic infections in humans and in pure-culture experimental biofilms. In recent years, exopolysaccharides from an extensive range of bacterial species from diverse environments have been isolated and characterized[33].

Several exopolysaccharides are homopolysaccharides, including the sucrose-derived glucans and fructans produced by the streptococci in oral biofilms, and cellulose formed by *Gluconacetobacter xylinus*, *Agrobacterium tumefaciens*, *Rhizobium* spp. and various species from the Enterobacteriaceae[6] and Pseudomonadaceae families[29]. However, most exopolysaccharides are

Fimbria
A filamentous structure composed of one or a few proteins that extends from the surface of a cell and can have diverse functions. Fimbriae are involved in attachment to both animate and inanimate surfaces and in the formation of pellicles and biofilms. They assist in the disease process of some pathogens, such as *S. enterica*, *Neisseria gonorrhoea* and *Bordetella pertussis*.

Membrane vesicle
A vesicle that is formed from the outer membrane of Gram-negative bacteria, is secreted from the cell surface and contains extracellular enzymes and nucleic acids. These vesicles may represent mobile elements in the EPS matrix.

Capsule
A discrete polysaccharide (sometimes also protein) layer that is firmly attached to the surface of a bacterial cell, closely surrounding it, in contrast to less compact, amorphous slime that is shed into the more distant extracellular environment.

Lectin
A protein or glycoprotein of plant, animal or microbial origin that binds to carbohydrates with a characteristic specificity. Fluorescently labelled lectins can be used as probes to investigate EPS composition, enabling the microscopic *in situ* detection of EPS and their distribution in biofilms.

Raman microscopy
A spectroscopic technique based on inelastic light scattering (Raman scattering) of monochromatic laser light in the near-ultraviolet range, revealing vibrational, rotational and other low-frequency modes in a system. The technique is used for the analysis of chemical bonds and is suitable for very small volumes, allowing spectra and chemical information to be obtained for the molecules present in that volume.

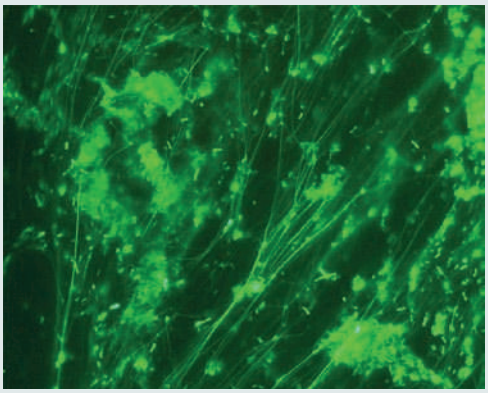
Box 2 | ***In situ* detection of extracellular polymeric substances**

The most important tool for non-destructive *in situ* detection of components of the extracellular polymeric substances (EPS) in biofilms is confocal laser scanning microscopy (CLSM) in combination with fluorescent dyes[15]. Using fluorescently labelled lectins, exopolysaccharides are visualized according to their interaction with specific target sugars. Such approaches have revealed the complex composition and arrangement of EPS in natural biofilms[15]. Fluorescently labelled antibodies against exopolysaccharides have been used in a similar way[111]; this technique is well established for use with pure cultures. In an excellent overview of the topic, Neu and Lawrence[112] reported clear-cut specific multilabelling but also nonspecific binding patterns with both lectins and antibodies.

A promising approach is the use of CLSM-based lectin-binding analysis in combination with Raman microscopy[16]. This combination gives a more in-depth insight into EPS composition. However, the allocation of spectra to individual lectin-stained clusters remains a challenge and requires substantial further development.

An approach to localizing enzymatic activity in biofilms is direct microscopic visualization by staining with fluorogenic substrates[43]. Phosphatase activity in laboratory biofilms and in activated sludge flocs was detected using the water-soluble substrate ELF-97 phosphate, which yields an insoluble fluorescent precipitate upon cleavage by the enzyme. This method allowed the spatial distribution of phosphatase activity to be studied in whole flocs and in vertical sections of biofilms. Extracellular redox activity was visualized by reduction of the tetrazolium salt 5-cyano-2,3-di-4-tolyl tetrazolium chloride (CTC) to CTC formazan crystals at the point of reaction.

Extracellular DNA can be detected with dye specific for nucleic acid. For example, a 4-day-old culture of the gammaproteobacterium strain F8 (which was isolated from the Saskatchewan river, Canada) was grown on freshwater basal-medium agar and subsequently stained with the dye SYTO9 to visualize the DNA (see the figure). Aside from the DNA, the bacteria are visible as small rods between the DNA strands. Image courtesy of U. Boeckelmann and U. Szewzyk, Technische Universität Berlin, Germany.



heteropolysaccharides that consist of a mixture of neutral and charged sugar residues. They can contain organic or inorganic substituents that greatly affect their physical and biological properties. Owing to the presence of uronic acids (and, in some cases, ketal-linked pyruvate or, rarely, sulphate), many known exopolysaccharides, including alginate, xanthan and colanic acid, are polyanionic. Polycationic exopolysaccharides also exist, such as intercellular adhesin, which is composed of β-1,6-linked *N*-acetylglucosamine with partly deacetylated residues. This adhesin was discovered in important nosocomial pathogens such as *Staphylococcus aureus* and *Staphylococcus epidermidis*, which can colonize medical implants and lead to biofilm-related infections[34], and it has since been detected in a range of other bacteria[35].

Exopolysaccharides can be diverse even between strains of a single species; for example, various *Streptococcus thermophilus* strains produce heteropolysaccharides of different monomer compositions and ratios and different molecular masses[36]. *P. aeruginosa*, one of the best studied models for biofilm formation, produces at least three distinct exopolysaccharides that contribute to biofilm development and architecture: alginate, Pel and Psl[37]. Alginate is one of the most extensively studied exopolysaccharides, but it consists of only uronic acids and so is not representative of all exopolysaccharides. Alginate is a high-molecular-mass, unbranched heteropolymer consisting of 1,4-linked uronic residues of β-D-mannuronate and α-L-guluronate. These components are arranged in homopolymeric blocks of polymannuronate and heteropolymeric sequences with a random distribution of guluronate and partially *O*-acetylated mannuronate residues. Overproduction of alginate is characteristic of mucoid strains and is usually as a result of mutations in the gene encoding the σ-factor AlgU negative regulator (MucA). Alginate is involved in the establishment of microcolonies at the beginning of biofilm formation, but it is also responsible for the mechanical stability of mature biofilms. However, in non-mucoid wild-type strains, which do not express alginate biosynthesis genes, the polysaccharides Pel and Psl are involved in the establishment of biofilms. Pel is a glucose-rich polysaccharide, whereas Psl consists of a repeating pentasaccharide containing D-mannose, D-glucose and L-rhamnose[38]. Pel is essential for the formation of biofilms (called pellicles) at air–liquid interfaces and biofilms that are attached to a surface, and Psl is involved in the adherence to abiotic and biotic surfaces and in the maintenance of biofilm architecture. During attachment, Psl is anchored to the cell surface in a helical pattern, possibly promoting cell–cell interactions[39]. It then accumulates in the periphery of microcolonies during biofilm maturation, preparing a Psl-free cavity in the microcolony centre for the subsequent dispersal stage (BOX 3), during which this matrix cavity contains swimming cells together with dead cells and extracellular DNA (eDNA)[39].

In many bacteria, exopolysaccharides are indispensable for biofilm formation, and mutants that cannot synthesize exopolysaccharides are severely compromised or unable to form mature biofilms[17,18,39] (although bacteria may still attach to surfaces and form microcolonies to a limited extent). However, in mixed-species biofilms the presence of a species that produces exopolysaccharides may lead to the integration of other species that do not synthesize matrix polymers[13]. Therefore, the proportions of different exopolysaccharides in mixed biofilms do not necessarily reflect the proportions of the cells present, nor do the different exopolysaccharides add equally to the structure and properties of the resulting biofilms[40].

Extracellular proteins

The biofilm matrix can contain considerable amounts of proteins that, together, can far exceed the polysaccharide content, on a mass basis[28,41]. This has been reported for environmental biofilms as well as for activated sludge and biofilms in sewers[42].

Enzymes. Various extracellular enzymes have been detected in biofilms, many of which are involved in the degradation of biopolymers. The substrates of these extracellular enzymes include water-soluble polymers (such as many polysaccharides, proteins and nucleic acids) and water-insoluble compounds (such as cellulose,

chitin and lipids), as well as organic particles that are trapped in biofilms[43] (TABLE 2). The presence of enzymes that degrade EPS components makes the matrix an external digestive system that breaks down biopolymers to low-molecular-mass products that can then be taken up and utilized as carbon and energy sources. In addition, some enzymes can be involved in the degradation of structural EPS to promote the detachment of bacteria from biofilms. Other enzymes act as virulence factors in medical biofilms during infectious processes.

Some extracellular enzymes from bacteria (and some from fungi) are of commercial interest and are produced on a large scale industrially. In addition, extracellular enzymes carry out self-purification processes in soils, sediments and water, and these processes have been adopted for the biological treatment of drinking water and waste water, using biofilms and flocs to degrade organic substances.

Extracellular enzymes are also used for the degradation of synthetic polymers by degrading additives such as plasticizers (for example, terephthalates) or antioxidants, or by attacking the polymer backbone[44]. Furthermore, extracellular redox enzymes play a part in microbially influenced corrosion[45].

Extracellular enzymes can be efficiently retained in the biofilm matrix by their interaction with polysaccharides[43,46]. For example, the association of extracellular lactonizing lipase (LipA; also known as Lip) with alginate produced by *P. aeruginosa* is based on weak binding forces[47]; this hypothesis is supported by molecular modelling (FIG. 1d). Such interactions result in a matrix of exopolysaccharides that are biochemically activated by the attached enzymes. This arrangement retains the enzymatic activity close to the cell and keeps the diffusion distances of enzymatic products short, thereby optimizing their uptake by bacteria. Moreover, the interactions between enzymes and structural exopolysaccharides enhance the thermostability of the enzymes and their resistance to proteolysis[40].

EPS-modifying enzymes. Various enzymes can potentially degrade EPS components during starvation, targeting EPS made by the bacterium that produces the enzyme or EPS made by other species[29,48]. Examples are the dextran, inulin and levan that are formed by oral streptococci[49] and the levan that is present in the matrix voids of *Pseudomonas syringae* biofilms[50]. Exopolysaccharides are degraded mainly by hydrolases and lyases[31,50], but degradation is generally slow. In marine stromatolites, EPS polysaccharides and proteins are secreted by the bacteria and are then rapidly fragmented and rearranged by degradation, specifically by sulphate-reducing bacteria, to a more refractory polymer[11]. Furthermore, a very important stage in biofilm development is the dispersion of sessile cells from the biofilms, which allows new biofilms to be formed[39,51]. This dispersion occurs in response to environmental changes; it can be induced by nutrient starvation[52] or sudden nutrient availability[51] and requires modification of the matrix by enzymes secreted from the bacteria[51]. An example of an enzyme that degrades exopolysaccharides to allow detachment and dispersal of biofilm cells is *N*-acetyl-β-hexosaminidase (encoded by *dspB*), which is produced by the periodontal pathogen *Actinobacillus actinomycetemcomitans*[53]. A *dspB* mutant formed biofilms that could not release cells.

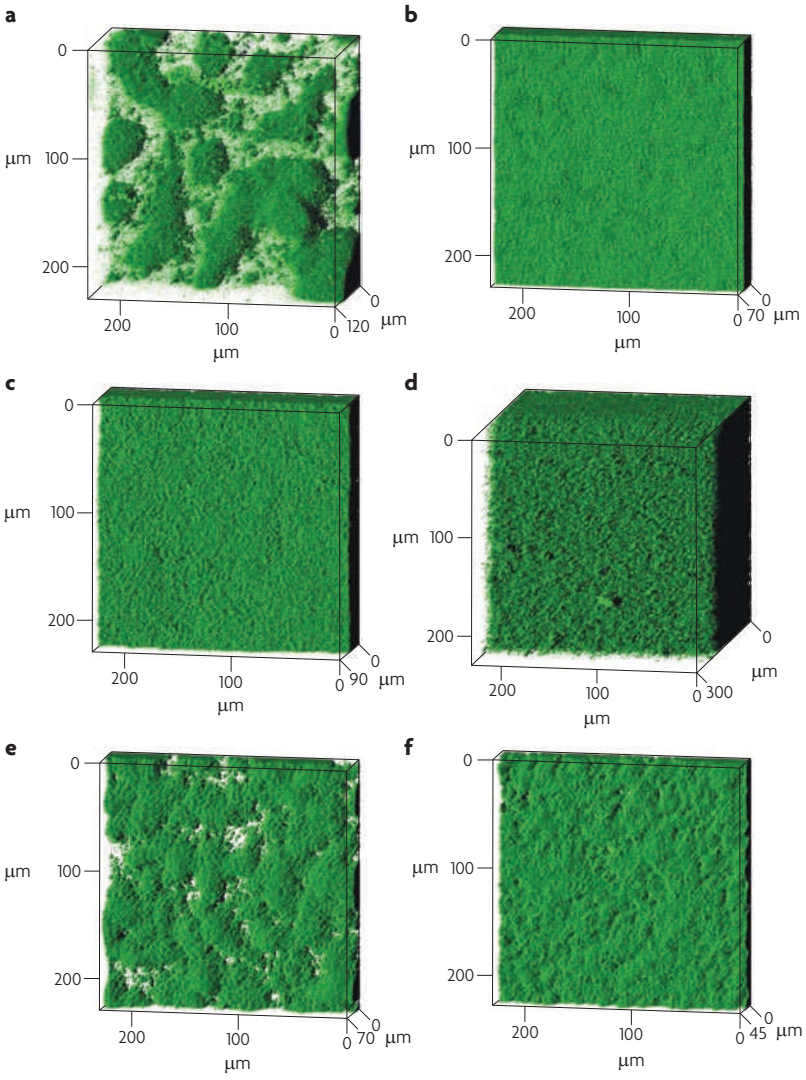


Figure 2 | **Dynamics of *Pseudomonas aeruginosa* biofilm architecture.** Confocal laser scanning microscopy images (×325 magnification) of *Pseudomonas aeruginosa* biofilms grown for 70 h on glass in a flow cell at 30 °C (with a flow rate of 20 ml per h). Biofilms were stained with DNA-binding dye SYTO9, and cells are green. **a** | A mucoid (alginate-overproducing) environmental strain, *P. aeruginosa* str. SG81 (REF. 118), which produces uneven, lumpy biofilms. **b** | A spontaneous non-mucoid revertant strain, *P. aeruginosa* str. SG81R1 (derived from *P. aeruginosa* str. SG81), which has lost the ability to produce alginate. The lack of alginate leads to the formation of smoother and flatter biofilms. **c** | The typically non-mucoid wild-type strain *P. aeruginosa* str. PAO1, which is widely used in biofilm research. This strain also produces smooth, flat biofilms. **d** | The effect of Ca^{2+} (1 mM Ca^{2+} in the growth medium) on biofilm architecture is shown for a mucoid biofilm of *P. aeruginosa* str. SG81. Ca^{2+} stabilizes the crossbridges between alginate, allowing a thicker and more stable biofilm to be formed. **e** | The dependency of mucoid biofilm architecture on the presence of *O*-acetyl groups in alginate: the clinical strain *P. aeruginosa* str. FRD1, which produces *O*-acetylated alginate, forms biofilms similar to the mucoid strain shown in part **a**. **f** | The mutant strain *P. aeruginosa* str. FRD1153 produces alginate in similar amounts to the parent strain, *P. aeruginosa* str. FRD1, but is defective in alginate acetylation and loses it mucoid phenotype. Biofilms of this strain resemble biofilms of the non-mucoid strains shown in parts **b** and **c**. Part **a**–**d** images courtesy of M. Strathmann, IWW Water Centre, Germany. Part **e** and **f** images are reproduced, with permission, from REF. 21 © (2004) Elsevier.

Box 3 | **Dispersal of biofilms**

Mixtures of enzymes for the dispersal of biofilms are described and covered by various patents, but they have poor long-term efficacy in the process of anti-fouling. Dispersion by the induction of a prophage followed by cell death and subsequent cell cluster disaggregation have been observed[100]. A substituted fatty acid, *cis*-11-methyl-2-dodecenoic acid (called 'diffusible signal factor'), was recovered from *Xanthomonas campestris* and found to be responsible for virulence as well as for inducing the release of endo-β-1,4-mannanase[113]. It has been suggested that certain species may encode stress regulons involved in biofilm dispersion. It was reported that *cis*-2-decenoic acid produced by *Pseudomonas aeruginosa* may act as a fatty acid messenger that can induce the dispersion of biofilms formed by *Escherichai coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Bacillus subtilis* and the yeast *Candida albicans*[114]. Such a 'universal biofilm disperser' is of great interest in medical and technical systems and may be of environmental concern. Very recently, biofilm disassembly was reportedly triggered in *B. subtilis*, *P. aeruginosa* and *S. aureus* by a mixture of D-amino acids, releasing amyloid fibres that linked the cells together[115]. This process could be a strategy used by biofilm bacteria to create pores and channels locally, leading to better mass transport within the biofilm.

Bacteriophages supply a wide range of polysaccharide-degrading enzymes. However, these enzymes are very specific and rarely act on more than a few closely related polysaccharide structures. Phages and bacteria can coexist stably in biofilms, suggesting that phages would make poor tools for the control of biofilm formation. However, combinations of phage enzymes and disinfectants have been recommended as possible control strategies under certain conditions[116], and adding the phage and then the disinfectant is more effective than adding either alone.

The existence of an organism or enzyme that could disperse all biofilms would lead to a global environmental disaster, because it would find its substrates on a global scale and could compromise the self-purification function of soils and sediments as well as the functions of all biological water treatments. It is the great variability of EPS that protects biofilms and, in turn, limits the success of enzymatic anti-fouling strategies. For fast and complete biofilm removal — as is required in anti-fouling strategies, for example — existing enzymes are too slow and their activities are too limited. However, for destabilization of the matrix, they may have their virtues.

However, there is no single enzyme or simple enzyme mixture that can degrade all of the polysaccharides in a biofilm matrix.

Structural proteins. The non-enzymatic proteins in the matrix, such as the cell surface-associated and extracellular carbohydrate-binding proteins (called lectins), are involved in the formation and stabilization of the polysaccharide matrix network and constitute a link between the bacterial surface and extracellular EPS. Examples include glucan-binding proteins in biofilms of the dental pathogen *Streptococcus mutans*[54], lectin-like proteins in the matrices of activated sludge flocs[55], outer-membrane lectins of *Azospirillum brasiliense*[56] and the galactose-specific lectin LecA and fucose-specific lectin LecB[57,58] of *P. aeruginosa*, both of which have been implicated in biofilm formation. Synthetic high-affinity multivalent ligands that target LecB inhibit *P. aeruginosa* biofilm formation and induce complete dispersion of established biofilms[59], underscoring the stabilizing effect of LecB in intact biofilms of *P. aeruginosa*. The secreted protein CdrA was shown to bind directly to Psl in *P. aeruginosa* biofilms[19], leading to the suggestion that extracellular CdrA cross-links Psl molecules and thereby strengthens the matrix, whereas cell-associated CdrA anchors the cells to Psl in the matrix. The extracellular protein TasA is required for the structural integrity of *B. subtilis* biofilms, along with an exopolysaccharide[20]. Interestingly, complementation studies with *tasA* mutants and exopolysaccharide synthesis mutants revealed that TasA and the exopolysaccharide were assembled correctly outside the cells to yield a functional matrix even when each of the two EPS components was produced by different cells in the same biofilm.

Another group of extracellular proteins are biofilm-associated surface protein (Bap) from *S. aureus* and the Bap-like proteins. These are high-molecular-mass proteins on the bacterial cell surface that promote biofilm formation in several bacterial species[60]. They contain a core domain of tandem repeats that is required for the formation of a biofilm and plays a part in bacterial infectious processes. Other ubiquitous proteinaceous components of the matrix are amyloids. These compounds have been defined as orderly repeats of protein molecules arranged as fibres of indefinite length in a cross-β structure, in which the β-strands are perpendicular to the fibre axis[61]. Functional amyloids of bacterial origin have been detected in various habitats, including freshwater lakes, brackish water, drinking-water reservoirs and waste-water treatment plants[61]. Amyloids are involved in adhesion to inanimate surfaces and host cells, with subsequent invasion of the host cells, and they also function as cytotoxins for both plant cells and bacteria[61].

Lastly, proteinaceous appendages such as pili, fimbriae and flagella can also act as structural elements by interacting with other EPS components of the biofilm matrix. For example, type IV pili of *P. aeruginosa* bind DNA[62] and so possibly act as cross-linking structures. In *S.* Typhimurium and *E. coli*, the co-production of thin aggregative fimbriae and cellulose results in the formation of a rigid, hydrophobic extracellular matrix, whereas the production of either fimbriae or cellulose results in a fragile network[6], underlining the functional role of fimbriae for matrix stabilization.

Extracellular DNA

Biofilms of various origins have been found to contain eDNA, but it is reported to occur in particularly large amounts in waste-water biofilms[28], although the amount produced can vary even between closely related species. eDNA is a major structural component in the biofilm matrix of *S. aureus*, whereas it is only a minor component of biofilms formed by *S. epidermidis*[63].

Role of eDNA. Although eDNA was initially seen as residual material from lysed cells, it has become increasingly clear that it is in fact an integral part of the matrix[1] and of the biofilm mode of life[64]. The importance of nucleic acids in microbial aggregation was observed in a species from the genus *Rhodovulum*, members of which are self-flocculating bacteria; this species produces EPS consisting of carbohydrates, proteins and nucleic acids[65]. Treatment of flocculated cells with nucleolytic enzymes resulted in deflocculation, whereas polysaccharide-degrading and protein-degrading enzymes had no effect. eDNA is also a major matrix component in *P. aeruginosa* biofilms, in which it functions as an intercellular connector[66]. In addition, DNase inhibits the formation of biofilms in *P. aeruginosa*[67], and *Bacillus cereus* uses DNA as an adhesin[68].

Matrix void
A pore or channel in the biofilm matrix that contains liquid water and is not filled with hydrated EPS molecules.

Stromatolite
A laminated microbial mat that is typically built from layers of filamentous cyanobacteria and other microorganisms that become fossilized. Stromatolites are the oldest records of life on Earth, dating back 3.5 billion years.

Table 2 | **Biofilm enzymes in natural and man-made aquatic environments***

Enzyme	Type of biofilm
Protein-degrading enzymes	
Protease	River biofilms and activated sludge
Peptidase	Drinking-water biofilms, river biofilms, waste-water biofilms, sewer biofilms, marine aggregates and activated sludge
Polysaccharide or oligosaccharide-degrading enzymes	
Endocellulase	River biofilms
Chitinase	River biofilms and estuarine-sediment biofilms
α-glucosidase	River biofilms, sewer biofilms, stream sediment biofilms, lake sediment biofilms, waste-water biofilms, marine aggregates and activated sludge
β-glucosidase	River biofilms, biofilms from trickling biofilters, sewer biofilms, stream sediment biofilms, lake sediment biofilms, marine aggregates and activated sludge
β-xylosidase	River biofilms and lake sediment biofilms
N-acetyl-β-D-glucosaminidase	River biofilms, marine aggregates and activated sludge
Chitobiosidase	Marine aggregates
β-glucuronidase	Activated sludge
Lipid-degrading enzymes	
Lipase	Marine aggregates and activated sludge
Esterase	River biofilms, lake sediment biofilms, drinking-water biofilms, sewer biofilms, stream sediment biofilms and activated sludge
Phosphomonoesterases	
Phosphatase	River biofilms, sewer biofilms, stream biofilms, marine aggregates and activated sludge
Oxidoreductases	
Phenol oxidase	River biofilms
Peroxidase	River biofilms
Extracellular redox activity	Activated sludge

*Data from REF. 46.

eDNA also has antimicrobial activity, causing cell lysis by chelating cations that stabilize lipopolysaccharide and the bacterial outer membrane[69].

Localization of eDNA. The localization of eDNA can vary widely between biofilms. In *P. aeruginosa* biofilms eDNA forms a grid-like structure[70], whereas in an aquatic bacterial isolate (a gammaproteobacterium called strain F8) eDNA forms a filamentous network[71] (BOX 2). In nontypeable *Haemophilus influenzae* biofilms, eDNA is present as a dense network of fine strands as well as in individual, thicker 'ropes' that span water channels[72]. The eDNA seems to localize in a time-dependent manner in the stalks of mushroom-shaped microcolonies in biofilms. Particularly high concentrations of eDNA were found in the outer parts of the stalk, thus forming a border between stalk-forming and cap-forming *P. aeruginosa* subpopulations[70]. It was speculated that DNA in the mushroom stalks might cause the accumulation of migrating bacteria, resulting in the formation of mushroom caps.

Origin of eDNA. The origin of eDNA seems to differ between species. In gammaproteobacteria strain F8, biofilm eDNA has similarities to but also distinct differences from genomic DNA[71], indicating that this eDNA is not simply released by lysed cells. However, in *P. aeruginosa* and *Pseudomonas putida* biofilms, eDNA and genomic DNA seemed to be identical[73]. In *S. epidermidis* biofilms, eDNA is generated through the lysis of a subpopulation of the bacteria, mediated by bifunctional autolysin (AtlE). This eDNA promoted biofilm formation of the remaining population, supporting the concept of a structural function for eDNA, as suggested by Molin and Tolker-Nielsen[64]. However, lysed cells are not the only source of eDNA, and active excretion of DNA cannot be excluded.

Surfactants and lipids

Extracellular polysaccharides, proteins and DNA are highly hydrated hydrophilic molecules, but other EPS have hydrophobic properties. For example, a *Rhodococcus* sp. strain[74] that possesses a capsule but no fimbriae can adhere to Teflon and colonizes waxy leaf surfaces using EPS with hydrophobic properties. The hydrophobic character of the EPS was attributed to substituents such as polysaccharide-linked methyl and acetyl groups[75].

Lipids are also found in the matrix[41]. Lipopolysaccharides are crucial for the adherence of *Thiobacillus ferrooxidans* to pyrite surfaces[76], and *Serratia marcescens* produces extracellular lipids with surface-active properties (known as 'serrawettins')[77]. Other surface-active EPS include surfactin, viscosin and emulsan, which can disperse hydrophobic substances and make them bioavailable. They may be useful for microbially enhanced oil recovery and for bioremediation of oil spills.

Biosurfactants can have antibacterial and antifungal properties and are important for bacterial attachment and detachment from oil droplets[78]. The quest for 'green' chemicals may enhance further work on this class of molecule[79,80]. Biosurfactants generated by microorganisms at the air–water interface of surface waters obviously have an important role, influencing surface tension and, thus, the gas exchange between oceans and the atmosphere[81]. Interestingly, rhamnolipids, which can act as surfactants, have been found in the EPS matrix of *P. aeruginosa*[82]. They display surface activity and have been proposed to act in initial microcolony formation, facilitating surface-associated bacterial migration and the formation of mushroom-shaped structures, preventing colonization of channels, and playing a part in biofilm dispersion[83,84].

Water

Water is by far the largest component of the matrix, leading K. C. Marshall to call biofilms 'stiff water'. The EPS matrix provides a highly hydrated environment that dries more slowly than its surroundings and therefore buffers the biofilm cells against fluctuations in water potential. Many EPS are hygroscopic and seem to retain water entropically rather than through specific water-binding mechanisms. It has been proposed that EPS result in hydraulic decoupling during rapid wetting or drying events, protecting the biofilm-embedded bacteria

Surface-active property
The ability of a molecule to alter the interface of two different phases. Substances with surface-active properties (surfactants) are amphipatic molecules with both hydrophilic and hydrophobic (generally hydrocarbon) moieties. They partition preferentially at the interface between fluid phases with different degrees of polarity and hydrogen bonding, such as oil–water interfaces.

in unsaturated soils, for example[85]. When embedded in EPS, the cyanobacterium *Nostoc commune* maintains its photosynthetic activity during drying and rehydration, whereas EPS-depleted *N. commune* was notably impaired under these conditions[86].

Bacteria actively respond to desiccation by producing EPS[87]. Desiccation seems to be one of the environmental conditions under which EPS provides global benefits to both EPS producers and other members of the biofilm community[88]. Desiccation concentrates EPS, increasing the number of nonspecific binding sites that can react with each other (compared with the number that can react when EPS components are separated at higher water content) and reducing biofilm volume. This can be easily observed on phototrophic biofilms attached to walls, which curl up when they dry out.

The EPS matrix can act as a molecular sieve, sequestering cations, anions, apolar compounds and particles from the water phase[89]. EPS contain apolar regions, groups with hydrogen-bonding potential, anionic groups (in uronic acids and proteins) and cationic groups (for example, in amino sugars)[90]. Owing to this stickiness of the matrix, particles and nanoparticles can be trapped and accumulated. Interestingly, heavy metals such as Zn^{2+}, Cd^{2+}, and Ni^{2+} bind to cell walls of bacteria in activated sludge, whereas hydrophobic compounds such as benzene, toluene and xylene are present in the matrix[91]. The response of biofilms to absorbed substances can be complex; for example, toluene induces enhanced production of carboxylic groups in *P. putida* biofilms[92].

EPS and mechanical properties of biofilms

Although biofilms are commonly referred to as 'slime', which implies that they are not rigid structures, their mechanical stability is important. Interestingly, it seems to be mainly the exopolysaccharides in the matrix that provide this feature. The process of anti-fouling, which entails the removal of unwanted biofilms, is carried out by overcoming the cohesive and adhesive forces of the matrix. In the case of biofilms in catheters, matrix stability determines biofilm detachment and the size of the resulting embolus[93]. During the treatment of waste water, the cohesion of flocs and biofilms determines the stability of several important processes, including flocculation, settling and dewatering[94]. In natural environments, EPS play a crucial part in the stabilization of sediments[95]. Furthermore, biofilms in stagnant waters can be disrupted by the low shear forces, and extremely stable bacterial biofilms with a rubber-like appearance serve as holdfasts for members of the family Podostemaceae (the riverweeds) at waterfall impact points[96]. The mechanical properties of biofilms can be influenced by shear forces, suggesting that biofilms can undergo phenotypic adaptation[93]. Bacterial microcolonies have been observed rolling along surfaces when under steady shear forces[97].

In general, biofilms display viscoelastic properties. They undergo both reversible elastic responses and irreversible deformation, depending strongly on the forces acting on the EPS matrix. Compression experiments with *P. aeruginosa* biofilms revealed that in response to pressure the biofilms go through a phase of elastic behaviour until a break point is reached, after which the biofilm behaves like a viscous fluid[24]. This raised the concept of fluctuating binding points between EPS components that are kept together by weak physicochemical interactions such as hydrogen bonds, van der Waals forces and electrostatic interactions. Entanglement of biopolymers further contributes to matrix stability[24]. *S. aureus* biofilms show elastic-solid-like response to short-timescale stimuli and viscous-fluid-like response to long-timescale stimuli[97]. Elastic materials absorb stress energy through deformation, and transient stress events might be resisted by reversible deformation. The result is a rearrangement of the biofilm to mitigate exposure to external shear stress. It is possible that, on an intermediate timescale, a biofilm can increase the strength of its structural matrix in response to mechanical stresses by increasing EPS production[98]. The interaction of multivalent inorganic ions with EPS can greatly influence the mechanical properties of biofilms. For example, the presence of Ca^{2+} increased the mechanical stability of mucoid *P. aeruginosa* biofilms; this effect was explained by the Ca^{2+}-mediated cross-linking of polyanionic alginate molecules[24]. When the rheological properties of a biofilm were examined on a microscale level using a novel microfluidic device, finite element analysis and confocal laser scanning microscopy, increased cohesion under shear stress (known as strain hardening) was observed for biofilms of *Klebsiella pneumoniae* and *S. epidermidis*[99].

Data about the cohesive strength of biofilms vary greatly, depending on the method used to measure it. Although the magnitude of the elasticity modulus and the viscosity vary among mixed-species biofilms[94], the qualitative viscoelastic responses to shear stress are consistent[93]. Stress relaxation time (usually around 18 minutes) is similar in a wide range of environmental biofilms[98], and it was speculated that this is the shortest period over which a biofilm can mount a phenotypic response to transient mechanical stress. However, stress relaxation time can be much shorter than this in certain examples; in the case of *S. epidermidis* biofilms, it was determined to be only 13.8 seconds[99].

Conclusions

Put simply, there is no biofilm without an EPS matrix — EPS are essential for biofilm formation and make possible a lifestyle that is entirely different from the planktonic state.

However, despite much research on biofilms, basic questions remain. One of these questions concerns the extracellular enzymatic activity of the biofilm matrix and its quantitative contribution to the carbon cycle. This contribution obviously has global relevance, because these enzymes render enormous quantities of dissolved polymers and particulate substrates bioavailable for further decomposition, but this has not yet been quantified on a global scale.

How similar types of EPS influence biofilm development in different bacterial species is largely unknown. A first approach was recently reported for the staphylococcal biofilm matrix polymers poly-*N*-acetylglucosamine (PNAG; a polysaccharide) and eDNA[63], which were

Biosurfactant
A substance that is synthesized by living cells (mostly bacteria and yeasts) and that is surface active. Biosurfactants reduce surface tension, stabilize emulsions, promote foaming and are generally non-toxic and biodegradable. When grown on hydrocarbon substrates as a carbon source, microorganisms can synthesize a wide range of biosurfactants, such as glycolipids and phospholipids. These chemicals are apparently synthesized to emulsify the hydrocarbon substrate and facilitate its transport into the cells. In some bacterial species, such as *P. aeruginosa*, biosurfactants are also involved in a group movement behaviour called swarming motility.

Hydraulic decoupling
The formation of areas that have virtually no exchange of water content with their environment. An example is a desiccated EPS layer that covers an area with a high water content but has very low water transport through the layer, retaining the water underneath.

Elasticity modulus
The tendency of an object or material to reversibly develop an elastic force in response to deformation. Mathematically, the elasticity modulus is the proportionality factor between the force and the deformation, or, in other words, the slope on a plot of stress versus strain in the elastic deformation region. Stiff materials have a higher elasticity modulus, whereas soft materials have a lower one.

Stress relaxation
A deviation from the ideal elastic behaviour of a material due to an internal relief of stress under constant strain. Some materials, when put under mechanical tension, undergo internal flow processes (termed 'creep') that are at least partially irreversible and lead to a constant deformation of the test specimen.

found to have completely different structural roles in biofilms of *S. aureus* and *S. epidermidis*. PNAG made a considerable contribution to biofilm integrity in *S. epidermidis*, whereas this function was served by eDNA in *S. aureus* biofilms. Furthermore, the amount and the temporal sequence of EPS formation in response to various physical and biological conditions are largely unknown for environmental biofilms. One intriguing theory is that cell lysis and subsequent local decomposition of the EPS matrix might be advantageous for the biofilm population, creating new pores and channels that improve nutrient access[100]. The weakest point in current EPS research is the potential to predict EPS production, a prerequisite for which is the elucidation of the underlying regulatory processes. It has already become clear in several Gram-negative bacteria that cell-to-cell communication mechanisms (such as quorum sensing) and the intracellular level of the second messenger cyclic di-GMP are involved in regulating biofilm formation and the production of matrix components such as certain polysaccharides and proteins, DNA, and rhamnolipids[101,102]. A better understanding of the regulation of EPS production in mixed-species biofilms, as well as a spatial and temporal dissection of the phases in EPS production, will reveal important aspects of the oldest, most successful and widespread form of life on Earth.

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Acknowledgements
We are grateful for the inspiring cooperation with partners in the research group on 'Physico-chemistry of Biofilms': W. Borchard, K.-E. Jaeger, H. Kuhn, C. Mayer and W. Veeman. We also acknowledge financial support by the German Research Foundation to various EPS research projects. Furthermore, constructive, critical and stimulating comments and discussions with I. Sutherland are highly appreciated.

Competing interests statement
The authors declare no competing financial interests.

DATABASES
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FURTHER INFORMATION
Hans-Curt Flemming's and Jost Wingender's homepage: http://www.uni-due.de/biofilm-centre
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