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Structure, function and immunochemistry of bacterial exopolysaccharides

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There has been much written on bacterial exopolysaccharides (EPS) and their role in virulence. Less has been published regarding EPS in free living species. This review focuses on that subject, emphasizing their functions in the environment and the use of antibody probes to study them.

Keywords: polysaccharides; bacterial capsule; biofilm

Composition and structure of bacterial polysaccharides

Bacterial surface polysaccharides come in two general forms, those bound to the cell surface by attachment to lipid A lipopolysaccharide (LPS), and those associated with the cell surface as a capsule, exopolysaccharide (EPS). EPS are very hydrated polymers with 99% of their wet weight comprised of water [111]. They have considerable heterogeneity, from the simple α, 1-4 linked, unbranched glucose polymers called dextrans, to the highly complex, branched, and substituted heteropolysaccharides made up of oligosaccharide repeating subunits such as xanthan and colanic acid [17,112]. EPS can also be substituted, normally ester or *N*-linked, with pyruvate, acetate, formate, sulfate, phosphate and other side groups [49].

Part of the structural diversity of EPS is due to the fact that two identical sugars can bond to form 11 different disaccharides. In contrast two identical amino acids can form only one dipeptide. Additionally, EPS contain a wide variety of sugars as for example glucose, mannose, glucuronic acid, and rhamnose in xanthan gum [32], galactose and glucose (*Rhizobium meliloti* [50]), xylose (*Cryptococcus neoformans* [9]), hexosamines, aminouronic acids, aldoses, diaminohexoses [56], 2,3-diamino-2,3-dideoxyuronic acid, and 5,7-diamino-3,5,7,9-tetradeoxynonulosonic acid [137]. Furthermore, the noncarbohydrate side groups that are found in bacterial EPS add to their heterogeneity that, as a consequence of all of these considerations, far exceeds that of proteins and is reflected in the hundreds of *O*-antigen serotypes of enterobacteria [49]. Functions for this heterogenicity have been ascribed to pathogens [24] but not environmental strains [106].

The molecular weights of LPS [15,39,43,78,92] and EPS [17,65,99,117] are also extraordinarily heterogeneous. With incomplete stringent control over the number of subunits added to a chain [6], long and short polymers are synthesized, although one molecular weight species predominates. EPS forms higher order structures [49]. Xanthan gum is thought to from double-strand antiparellel helices [84], while the EPS from various *Klebsiella* spp form left-handed helices [48]. The EPS component of many bacterial films forms thick fibers when examined with electron microscopy [3,8,94]. It has been suggested that these fibers are attached to discrete areas on the outer surface of the cell [8,94]. While EPS and LPS have partial synthetic pathway commonality in some species [58], the remainder of this review shall primarily be concerned with EPS.

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Transport attachment and localization of bacterial EPS

A major unanswered question is how the hydrophilic EPS is transported across the hydrophobic interior of the outer membrane to the outer surface of the outer membrane of Gram-negative bacteria. It has been suggested [8], but not documented [12,88], that adhesion sites (Bayer junctions) between the inner and outer membrane are sites of both LPS and EPS transport. Lipid A and *O*-antigen are synthesized separately on the inner face of the inner membrane and are joined on the periplasmic face of the inner membrane [82]. For *E. coli* EPS, a 60-kilodalton periplasmic protein is required for translocation to the outer surface of the outer membrane, whereas a protein is not required for LPS translocation [98,103]. Immunochemical analysis of *Pseudomonas* sp strain S9, suggests that EPS polymerization (or crosslinking) can occur on the outer surface of the outer membrane [135].

The fine structure of the fibrillar structures [3,8,94] radiating out from the cell surface suggests that EPS is bound at a limited number of discrete sites. Once these sites are filled, excess EPS may become the source of slime ([133]; EPS found free in the media). Alternatively, only EPS molecules of the correct length may bind to attachment sites, with larger and smaller molecules forming the slime [112]. Immunoelectron microscopy of a marine pseudomonad suggests that shorter EPS molecules are integrally bound to the outer membrane (integral capsule) while the longer polymers are loosely (peripherally) associated [34]. Without knowledge of export mechanisms it is difficult to theorize just how EPS may be site-specifically deposited. Along with caulobacters, hyphomicrobia are good models to study

mechanisms of EPS deposition. Since export is both polar and temporal, a rare occurrence in procaryotes [93,119,120,122,130], the machinery can be readily correlated with zones of production.

Regulation of bacterial EPS production

Many environmental factors can affect the rate of EPS synthesis in bacteria. They include increased oxygen [7], limitation of nitrogen [51,79] and cations (eg magnesium, sulfate, phosphate and calcium [27,51,112]), desiccation [87,112,129], low temperature [117], growth on minimal media [117] and growth phase [17,121,129,134]. In most of these cases, enhanced EPS synthesis is a response to environmental stress (eg nutrient limitation).

The question of regulation of EPS production has been approached using molecular techniques to analyze the genetic regulation of EPS synthesis in *E. coli* and *Alteromonas atlantica*. The regulatory circuit controlling colanic acid capsule synthesis in *E. coli* includes at least four proteins. RcsA and RcsB are positive *trans*-acting regulators of capsule synthesis and RcsC is a negative regulator [13,42,89]. RcsA is unstable due to its sensitivity to the Lon protease [116]. RcsC and RcsB are similar to many two-component (sensor-effector) regulatory systems [110]. Even though it is still not clear under what environmental stimulus RcsC (the sensor) activates RcsB (the effector), this is the first genetic evidence linking an environmental stimulus to increased EPS production.

The genetic mechanism for regulation of *A. atlantica* EPS synthesis differs markedly from that of colanic acid [5] and more closely resembles the antigenic phase variation described for *Salmonella, E. coli* and *Neisseria* [1,104,114]. These variable systems involve complex reversible genomic rearrangements which can turn EPS synthesis (or antigenic variation) off or on. Under conditions favorable for EPS production, those bacteria which are 'on' will predominate. If the conditions change so that EPS production is no longer favored, those bacteria which were already 'off' will out-compete the 'on' bacteria and predominate [5]. In caulobacters and hyphomonads, EPS regulation is temporal and less influenced by environmental factors [85,124]. In *Pseudomonas aeruginosa*, the intricate regulatory cascade of alginate expression is being worked out [30,35,97,131].

Functions of EPS

EPS is produced by the majority of Gram-negative bacteria, some of which invest more than 70% of their energy in its production [45]. Consequently many species grow faster on laboratory media after they mutate and stop producing EPS. This suggests that some environmental factor selects for continuous EPS production. The existence of a genetic switch in the marine bacterium *A. atlantica* [5] to insure the concurrent existence of both EPS-producing and non-producing forms of the organism emphasizes both the importance of EPS and its demand on cellular resources. Polar EPS synthesis, if EPS is an adhesin, may be another resource-saving mechanism.

As discussed in excellent earlier reviews by Decho [29] and Dudman [31a], many functions have been proposed for bacterial EPS (Table 1). They can be divided into four groups, functioning: a) as a physical protective barrier; b) as a response to environmental stress; c) in cell/cell recognition and interaction; or d) in biofilm formation/adhesion. The ability of EPS to act as a physical barrier has been demonstrated with pathogenic bacteria. Encapsulation of *E. coli, Klebsiella* sp, and pneumococci renders them resistant to phagocytosis, complement fixation, and antibody [37,102]. In fact, the pathogenicity of bacteria can be artificially increased by coating them with hog gastric mucin, a charged mucopolysaccharide [49]. Even though bacteria may be subjected to phagocytosis-like predation in the natural environment, they are not exposed to antibody or complement. There, EPS may protect against bacteriophage [118], hydrophobic toxins [127] and desiccation [81, 87].

In response to stress, when essential cations are required, anionic EPS would sequester them, increasing the gradient across the cell membranes [45]; or, excretion of the charged polymer may provide the driving force for importation of other charged ions [128]. Polymerization of EPS would also produce excess reducing power, used to drive high affinity or high energy uptake systems [45,115]. In symbiotic relationships, the EPS and LPS of some nitrogen-fixing bacteria, most notably *R. meliloti*, but also others [10,68,95], function in the host-specific, bacterial invasion of developing root nodules on leguminous plants [33,64]. The EPS is involved in the initial recognition and attachment of the bacteria, leading directly to morphogenic changes in the plant [10,33,64,95]. Kirchman *et al* [60] showed that *Pseudomonas marina* EPS is an inductive cue for the metamorphosis of the marine polychaete, *Janua brasiliensis*. The metamorphic trigger might involve the binding of a larval lectin to the EPS [59]. Other marine invertebrates however do not specifically bind with bacterial films prior to larval settlement and metamorphosis [125,126].

Nevertheless, it has long been recognized that there is an ordered sequence of periphytic succession for colonization of clean surfaces immersed in seawater. In the initial phase, after possible coating by organic matter [70], bacteria attach to a surface and begin to grow, forming microcolonies within several hours [19,25,31,36,77]. Subsequently, diatoms, fungi, protozoans, micro-algae and other microorganisms attach to the surface, adding to the primary slime layer [25,31,36,77,105]. This primary microbial colonization often appears to be a prerequisite for the final stage of succession in which large organisms, viz, invertebrates, attach and grow on the surface [23,26,139].

The biofilm/adhesion functions of EPS are extremely important medically and commercially. The importance of biofilm formation on bacterial growth in dilute nutrient environments has been recognized since Zobell and Anderson's [140] early work on the relationship between bacterial growth and solid surfaces. The involvement of EPS in initial adhesion of the cells [3,20,44], as the structural matrix of the biofilm and as an active metabolic component of the biofilm, has received much attention [eg 21,22,75,101,113]. Briefly, some EPS may function as an initial adhesion [3], more as a permanent adhesion [44] and many as the biofilm matrix [125].

Table 1 Exopolysaccharide and cell survival

Function	Survival advantage	References
Physical/protective barrier	Protection from desiccation, predation and the immune system. Resistance to toxins, antibiotics and poisons	[49,81,118,127]
Cell–cell recognition and interaction	Plant symbiosis, formation of nodules and microcolonies, invertebrate larvae settlement	[10,33,59,64,95]
Response to environmental stress	Sequestering and import of charged ions, production of excess reducing power	[45,115,128]
Adhesion and biofilm formation	Immobilization onto nutrient-rich surfaces, dissociation from nutrient-depleted surfaces	[3,44,125]

There are two major theories to explain the probability of bacterial attachment to a substratum in aqueous environments [76]. Each states that the adhesion of microorganisms to surfaces is influenced by long-range, short-range, and hydrodynamic forces. The DLVO (letters after first initials of surnames of its proposers) theory assumes that interaction between two objects is comprised of an attractive component, governed by Van der Waals forces, and a potential repulsive component due to overlap of electrical double layers associated with charged groups [70]. These yield two distances at which a particle may be attracted to the substratum. At a primary minimum (*ca* 1 nm), attractive forces are strong; at the secondary minimum (*ca* 15 nm), forces are weaker. These distances are divided by an intermediate repulsion barrier. Microorganisms may accumulate at the secondary minimum and much of the strategy in surface colonization is concerned with remaining at the secondary minimum and overcoming the repulsive barrier to reach the primary minimum [84]. Microorganisms synthesize a variety of tethers for this purpose. All have narrow diameter and sufficient length to minimize and 'break through' the repulsive layer [20]. Such structures have been reported [20] to include capsular exopolysaccharides, pili and flagella, eg long fibular EPS could form an adhesive bridge minimizing electrostatic repulsion [44].

A second, Stern, theory predicts that there will be a net charge distribution at any solid surface and that as a consequence, counter ions are held closely at the surface forming a Stern layer while the rest of the ions are less restricted forming a diffuse ionic atmosphere [70]. This model, probably less applicable in a marine habitat, also predicts a double layer of attractive domains sandwiching a repulsive barrier and would require similar structures to function as tethers as would the DLVO model.

Once bacteria are attached to the surface, multiple events can transpire to carry it to the primary minimum at which multiple bonds of a more permanent nature may be formed between the organism and substratum. This attachment is generally considered to involve hydrophobic bonds of outer membrane components of Gram-negative bacteria or more likely capsular EPS, cement-like biofilm. The roles of EPS in this process have been discussed with the conclusion that 'much more information is required' [16,74].

Christensen [16] points out though, that even with an explosion of new data, structure–function relationships remain largely untested. In a few species it was observed that EPS was not involved in, or inhibited, attachment [14,28,61,62,109,132,133]. An uncharged high molecular weight EPS synthesized by bacteria isolated from fish scales was not adhesive but instead served to decrease drag [100]. Whether an EPS functions as an adhesive or not may be dictated by its chemistry and that of the substratum. EPS is clearly important in the structural matrix of the biofilm. It can bind ions [67] and other nutrients and, thus, functions as more than a relatively inert 'cement'.

Immunochemistry of polysaccharides

Since capsules were recognized as an important virulence factor, the immunochemistry of polysaccharides has been intensively studied [11,47,55,57,73,86,108]. The unique immunological attributes of polysaccharide antigens are summarized in Table 2. Polysaccharides are unusual in that their immunogenicity varies with each animal, eg pneumococcal EPS is non-immunogenic in rabbits but an excellent immunogen in mice [72]. Polysaccharides are also unique in being T-independent antigens, ie they can directly stimulate B-cell antibody production and division without the help of T-cells [41] because their long repetitive structures can directly interact with and activate B-cells [41,52]. As a consequence, there may be little memory and subsequent challenge may not evoke an anamnestic response. It also means that anti-polysaccharide antibodies will be mostly IgM [41].

One of the major factors influencing the antigenicity of a polysaccharide is molecular weight [49]. Dextrans with an average molecular weight >90 000 are good immunogens while dextrans of an average molecular weight <50 000 are non-immunogenic [54]. The size of molecular aggregates [71,116] may be at least as important as polysaccharide chain length [55]. Kabat [53] working with anti-dextran antibodies was able to elucidate the size (six glucose residues) of the antigen-binding site on the antibody. In addition he showed that the nonreducing terminal glucose (designated the immunodominant sugar) contributed most to the antigenicity of the dextran, with each succeeding glucose contributing a smaller increment. Terminal nonreducing sugars on the side chains of the branched mannan antigens of yeast are also usually immunodominant [4,96]. Charge is another important factor in the antigenicity of polysaccharides, with the negatively charged moieties being immunodominant [49]. These include uronic acids, sulfate and phosphate groups [41]. Although phosphate and uronic acids, along with pyruvate and *O*-acetyl groups, can be part of the antigenic determinant, the specificity of the antibody is not always directed against the ionic portion of the polysaccharide [49]. Depyruvylation of xanthan abolishes the binding of anti-xanthan monoclonal anti-

Table 2 Special immunological characteristics of polysaccharide antigens

Characteristic	Postulated mechanism	References
T-independent immune response	Lack of MHC class II presentation of carbohydrate moities due to the inability of mammalian cells to degrade polysaccharides	[2]
Very poor memory	Due to lack of T-cell involvement; even very poor B-cell memory may be masked by antigen tolerance	[80]
IgM antibody isotype	T-independent immune responses generally don't induce the cytokines necessary for a class switch to IgA or IgG	[46]
Larger molecules are more immunogenic	Larger molecules may be more efficient at crosslinking receptors on the surface of B-cells which induces antibody production	[91]
Immunogenicity of polysaccharides varies between animals	Possibly due to the mimicking of host carbohydrate moities to which the host immune system is tolerant	[24]
Immunological paralysis (also caused by other types of antigens)	Caused by the persistence of polysaccharide in the animal (due to the failure of the host to degrade these molecules); also caused by the flooding of B-cell receptors during high zone tolerance preventing the membrane alterations necessary for B-cell stimulation	[49]

bodies [43], however it is not necessarily true that pyruvate directly interacts with the antibody. Pyruvate substitution of hexoses frequently imposes conformational rigidity and depyruvylation could abolish antibody recognition by changing the overall conformation of the polysaccharide. In fact, it may well be that conformational determinants, vs structural determinants, are important [53] and that antibodies recognize the overall three-dimensional shape of the antigenic determinant rather than a specific chemical property such as charge [41].

One of the simplest ways to examine the contribution of any specific portion of the EPS to its antigenicity is by selectively removing or degrading each substituent. The chemically modified EPS can then be tested for its ability to bind antibodies raised against the native molecule [49]. Methods which specifically alter polysaccharide substituents [66] include treatment with NaOH (breakage of ester bonds including *O*-acetyl, pyruvate, phosphate, sulphate), $NaBH_4$ (reduction of uronic acids), oxalic acid (depyruvylation) and periodic acid (oxidation of terminal non-reducing sugars and uronic acids) [66,107].

Although much work has been done on the immunochemistry of pathogenic bacterial EPS, to date many of these powerful approaches have not been applied to environmental bacterial EPS. This is especially the case with monoclonal antibody (hybridoma) technology [38]. Monoclonal antibodies have been produced against xanthan and algal alginate [123], but none has been reported against any marine bacterial EPS until D Sledjeski [106] in our laboratory made them against *Shewanella colwelliana*.

Recently, however, polyclonal antiserum was used as a probe for the microscopic investigation of EPS production by a marine pseudomonad during starvation [134], which synthesized both an integral and a peripheral EPS [34] of unknown functions. Integral EPS was constitutively produced while the peripheral EPS was synthesized as a response to starvation. It was speculated that the integral EPS was involved in adhesion while the peripheral EPS aided in detachment from the surface. Because polyclonal antisera were used, it was unclear whether these were the same EPS of different lengths or two structurally different EPS. Polyclonal antibodies have also been used to show that bacteria adhere differently to different surfaces (titanium or aluminum) based upon differences in the EPS structure [138]. In fact, although it is widely reported that different benthic species have different affinities for different surfaces, only rarely [63] has it been demonstrated that different EPS bind to different surfaces with varying affinities. In this scenario, EPS heterogeneity would be driven by the survival value of adhering a little better than others to specific surface habitats.

Lectins

Lectins are sugar-binding proteins of non-immune origin (from a wide variety of plants and animals) which agglutinate cells and/or precipitate glycoconjugates [40]. They are usually classified into categories according to their carbohydrate specificity: mannose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, L-fucose, *N*-acetylneuraminic acid, to name the most common [136]. These specificities are usually determined by assessing which monosaccharide(s) are the most effective inhibitors of agglutination of erythrocytes, or precipitation of carbohydrate-containing polymers by the lectin [69].

Lectins usually form dimers, with one sugar-binding site per subunit, although there are a few exceptions. The dissociation kinetics of lectin-carbohydrate complexes are very slow [69]. There is evidence for the importance of molecular shape in lectin-carbohydrate interactions; some lectins react poorly with monosaccharides, but combine readily with oligosaccharides. Some recognize only terminal nonreducing saccharides, while others also recognize internal sugar sequences [69]. At the present time, there are numerous applications for lectins, a number of which differ from those of antibodies. They are used without modifications as agglutinins; radiolabelled or conjugated with enzymes, biotin, fluorescent dyes, or colloidal gold as label and indicators for glycoproteins, or specific tissues or cell strains [69]. Lectins immobilized on chromatography resins or solid substrates are used to isolate or purify oligosaccharides, glycoproteins, and bacterial cells [90].

Directions

Important theoretical questions, intricately involving EPS, have been posed and briefly discussed in an earlier review [29]. Bacterial EPS have also been widely commercialized, having multiple uses [18] including burgeoning applications in metal bioremediation [12a]. Yet the extraordinary diversity of these polymers coupled with the fact that only a small percentage of environmental bacteria have yet been isolated, promises the discovery of new and unique EPS with different properties and applications. Antibodies and other molecular probes will be used to learn of their function and regulation, and for their purification.

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