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MINI-REVIEW

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# Engineering bacterial biopolymers for the biosorption of heavy metals; new products and novel formulations

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**Abstract** Bioremediation of heavy metal pollution remains a major challenge in environmental biotechnology. One of the approaches considered for application involves biosorption either to biomass or to isolated biopolymers. Many bacterial polysaccharides have been shown to bind heavy metals with varying degrees of specificity and affinity. While various approaches have been adopted to generate polysaccharide variants altered in both structure and activity, metal biosorption has not been examined. Polymer engineering has included structural modification through the introduction of heterologous genes of the biosynthetic pathway into specific mutants, leading either to alterations in polysaccharide backbone or side chains, or to sugar modification. In addition, novel formulations can be designed which enlarge the family of available bacterial biopolymers for metal-binding and subsequent recovery. An example discussed here is the use of amphipathic bioemulsifiers such as emulsan, produced by the oil-degrading *Acinetobacter lwoffii* RAG-1, that forms stable, concentrated (70%), oil-in-water emulsions (emulsanosols). In this system metal ions bind primarily at the oil/water interface, enabling their recovery and concentration from relatively dilute solutions. In addition to the genetic modifications described above, a new approach to the generation of amphipathic bioemulsifying formulations is based on the interaction of native or recombinant esterase and its derivatives with emulsan and other water-soluble biopolymers. Cation-binding emulsions are generated from a variety of hydrophobic substrates. The features of these and other systems will be discussed, together with a brief consideration of possible applications.

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## Introduction

One of the major impacts of microbial metabolic processes on environmental biotechnology has been the exploitation of pathways for biodegradation and the consequent bioremediation of organic pollutants in the environment (Alexander 1999; Colwell 1994; Gutnick 1994, 1997). Both aerobic and anaerobic processes have been shown to lead to efficient combustion and/or biomineralization of the pollutant to a recalcitrant, nontoxic form. In contrast, heavy metal or radionuclide contamination, which is one of the major sources of pollution both in terrestrial and aquatic environments, presents quite a different challenge. In the case of metals and radionuclides, the metal ion can only be converted to the base metal (Lovley and Coates 1997), methylated (Lovley and Coates 1997; Silver 1994), precipitated (Beveridge 1989; Diels et al. 1999; Macaskie et al. 1987, 1992; McLean et al. 1996; Taghavi et al. 1997; Tolley et al. 1995; White et al. 1997), volatilized (Lovley and Coates 1997; Silver 1994; White et al. 1997), or complexed with an organic ligand. The development of technologies involving many of the processes listed above has been the subject of a host of basic and more applied projects (Brown and Lester 1979; Cheng et al. 1975; Diels et al. 1995, 1999; Fristoe and Nelson 1983; Kasan and Baecker 1989; Matis and Zouboulis 1998; Matis et al. 1996; Roane et al. 1996; Sterritt and Lester 1981; Zouboulis et al. 1995). Bioremediation technologies in general should be relatively inexpensive and simple because of the low added value associated with their commercial application (Nies 1999). One such approach for heavy metal remediation involves the formation of stable complexes between heavy metals and nuclides with microbial biomass (Ledin and Pedersen 1996; Kratochvil and Volesky 1998; Volesky 1990; Volesky and Holan 1995). These complexes are generally the result of electrostatic interactions between the metal ligands and negatively charged cellular biopolymers. For this application microbial biomass may simply constitute dried

cellular material which can be incorporated in a variety of configurations (i.e. columns, biofilters, packed resins, slurries etc.). Advantages of this technology include the ready availability of the inexpensive biomass and the development of technology for its immobilization. In contrast, the efficient exploitation of electrostatic interactions for binding heavy metals depends to a large extent on the specificity of interaction; a rather unpredictable and far from reproducible feature of ill-defined biomass. Moreover, the cation-binding capacity of a biomass preparation may depend on the nature and relative abundance of specific biopolymers. The characteristics of such materials are likely to vary over a wide range, depending on the physiology and history of the biomass preparation. Nevertheless, the ease of application and the demonstrated efficacy in certain field trials (Glombitza et al. 1997) have led to a large number of reports favoring this approach. Limited space does not permit a thorough treatment of this system within the context of this review. The reader is referred to a number of excellent recent publications dealing with the subject of cation-binding to microbial biomass and its application in bioremediation (Asthana et al. 1995; Avery and Tobin 1993; Foureste and Roux 1992; Volesky and Holan 1995). Another area which is not covered in this review is the binding of cations to specific protein molecules, such as metallothionein. This has been cloned into several microorganisms with a view towards engineering organisms that exhibit an enhanced binding of specific cations (Sousa et al. 1998; Valls et al. 1998). This approach has been used to generate overexpressed fusion proteins in which the metal-binding protein is fused to an extracellular domain of an outer membrane protein to enhance the binding capacity of the microbial biomass (Kotrba et al. 1999; Sousa et al. 1996).

In this review we will focus on the following features of metal biosorption to bacterial biopolymers:

1. Cation binding to specific bacterial biopolymers; presenting data related to range of cations bound, specificity and extent of binding.
2. Binding of cations by amphipathic biopolymers either free in solution or oriented at an oil-water interface in a stable emulsion. This feature allows for concentration and recovery of cations such as $Cd^{2+}$ $UO_2^{2+}$ from a relatively large volume of aqueous phase into an emulsified cream, by at least two orders of magnitude (Zosim et al. 1983).
3. Molecular approaches to biopolymer modification and formulation enhancing the range of preparations, which can be used to bind metal ligands at the oil/water interface.
4. Potential applications for metal remediation and/or recovery.

## Cation binding to bacterial biopolymers

A wide variety of microorganisms have been shown to produce various polysaccharides and other biopolymers which exhibit metal-binding properties (Chen et al. 1995a, b; Cozzi et al. 1969; Kaplan et al. 1987b). A representative sample of such biomolecules is shown in Table 1, from which it can be seen that biopolymers are produced in both gram-negative and gram-positive microorganisms. Prominent among the various polysaccharides and other organic biopolymers are peptidoglycan, water-soluble and amphipathic exopolysaccharides (EPS), capsular polysaccharides, capsular polyglutamic acid, teichoic and teichuronic acids, and lipopolysaccharides (LPS). For electrostatic interactions, the binding of cations to bacterial biopolymers generally occurs through interaction with negatively charged functional groups such as: (1) uronic acids (EPS from *Bradyrhizobium japonicum*, alginate, teichuronic acid, emulsan, or LPS from various sources), (2) phosphoryl groups associated with membrane components, or (3) carboxylic groups of amino acids. In addition to electrostatic interactions, there may also be cation-binding by positively charged polymers (Muzzarelli and Tubertini 1969), or coordination with hydroxyl groups (Beveridge and Murray 1980; Zosim et al. 1983). Such binding has been observed for eukaryotic polymers such as chitin or chitosan, presumably by chelation and coordination with hydroxyl groups (Muzzarelli and Tubertini 1969). These forms of non-electrostatic interaction may account for the greater-than-stoichiometric binding of cations at an oil/water interface discussed below (Zosim et al. 1983).

Despite the relatively few functional groups potentially involved in cation binding, microbial polymers differ widely both in specificity and in their metal-binding capacity. Table 1 presents some quantitative data on binding of different cations (grouped according to their position in the periodic table) to several bacterial biopolymer preparations. It should be noted that the various biopolymeric preparations presented in Table 1 may differ in their degree of purity. For example, in one case, cation binding to a purified preparation of peptidoglycan from *Escherichia coli* was investigated (Hoyle and Beveridge 1984), while in a second case the peptidoglycan was present in a less purified preparation containing a mixture of peptidoglycan, protein and LPS from the same organism (Beveridge and Koval 1981). Somewhat surprisingly, the two preparations differed both in their cation-binding specificity and in their cation-binding capacity (discussed below). In addition, in some cases binding to specific groups within the polymer was determined directly on isolated material, while in other instances the binding to specific functional groups was inferred from the results of measurements of binding before and after specific blocking or extraction of such groups from cell walls (Beveridge and Murray 1980). For example, a wall fraction from *Bacillus subtilis* was found to bind about 8 mmol Mg/g cell wall. After treatment with ethylene diamine to block carboxyl groups, Mg binding was reduced to only160 µmol/g cell wall. In sharp contrast, prior extraction with NaOH to remove teichoic acids resulted in a doubling of Mg

**Table 1** Binding of metals to bacterial biopolymers

| Metal | Strain | Biopolymer | Bound metal (mg/g polymer) | Bound metal (μmol/g polymer) | Reference |
|---|---|---|---|---|---|
| Group II alkaline earth metals | | | | | |
| $Mg^{2+}$ | *Escherichia coli* K-12 | Peptidoglycan[a] | 0.9 | 35 | Hoyle and Beveridge 1984 |
| | | Peptidoglycan[a], LPS[b], proteins | 6 | 256 | Beveridge and Koval 1981 |
| | *Enterobacter* sp. 11870 | EPS[c] | 8 | 329 | Geddie and Sutherland 1993 |
| | *Klebsiella aerogenes* type 54 strain A3 (sl) | EPS[d] | 7.4 | 304 | Geddie and Sutherland 1993 |
| | Marine pseudomonad B-16 | Peptidoglycan[a] | 5.6 | 230 | Rayman and MacLeod 1975 |
| | *Rhizobium meliloti* YE-2 | EPS[e] | 5.4 | 222 | Geddie and Sutherland 1993 |
| | *Zooglea ramigera* | Zooglan[f] | 5.5 | 226 | Geddie and Sutherland 1993 |
| | *Bacillus liqueniformis* | $\gamma$-Glutamyl capsular polymer[g] | 1.8 | 73 | McLean et al. 1990 |
| $Ca^{2+}$ | *E. coli* K-12 | Peptidoglycan[a] | 1.5 | 38 | Hoyle and Beveridge 1984 |
| | | Peptidoglycan[a], LPS[b], proteins | 1.4 | 35 | Beveridge and Koval 1981 |
| | *Enterobacter* sp. 11870 | EPS[c] | 11.8 | 295 | Geddie and Sutherland 1993 |
| | *K. aerogenes* type 54 strain A3 (sl) | EPS[d] | 9.1 | 228 | Geddie and Sutherland 1993 |
| | *R. meliloti* YE-2 | EPS[e] | 7.2 | 180 | Geddie and Sutherland 1993 |
| | *Zooglea ramigera* | Zooglan[f] | 4.5 | 113 | Geddie and Sutherland 1993 |
| | *B. liqueniformis* | $\gamma$-Glutamyl capsular polymer[g] | 41.8 | 1044 | McLean et al. 1990 |
| | *B. subtilis* 168 | Peptidoglycan[a] | 30 | 750 | Doyle et al. 1980 |
| $Sr^{2+}$ | *E. coli* K-12 | Peptidoglycan[a] | 2.2 | 25 | Hoyle and Beveridge 1984 |
| | | Peptidoglycan[a], LPS[b], proteins | 0.1 | 1 | Beveridge and Koval 1981 |
| $Ba^{2+}$ | *E. coli* K-12 | Peptidoglycan[a] | 9.7 | 71 | Hoyle and Beveridge 1984 |
| Transition elements I and II | | | | | |
| $Sc^{3+}$ | *E. coli* K-12 | Peptidoglycan[a] | 110.8 | 2464 | Hoyle and Beveridge 1984 |
| | | Peptidoglycan[a], LPS[b], proteins | 4.3 | 96 | Beveridge and Koval 1981 |
| $La^{3+}$ | *E. coli* K-12 | Peptidoglycan[a] | 299.5 | 2156 | Hoyle and Beveridge 1984 |
| | | Peptidoglycan[a], LPS[b], proteins | 10.8 | 78 | Beveridge and Koval 1981 |
| | *Pseudomonas aeruginosa* type $A^{+}B^{-}$ | LPS[b] | 31.8 | 229 | Langley and Beveridge 1999 |
| $Ce^{3+}$ | *E. coli* K-12 | Peptidoglycan[a] | 308 | 2198 | Hoyle and Beveridge 1984 |
| | | Peptidoglycan[a], LPS[b], proteins | 14 | 100 | Beveridge and Koval 1981 |
| $Pr^{3+}$ | *E. coli* K-12 | Peptidoglycan[a], LPS[b], proteins | 8.2 | 58 | Beveridge and Koval 1981 |
| $Sm^{3+}$ | *E. coli* K-12 | Peptidoglycan[a], LPS[b], proteins | 1.7 | 11 | Beveridge and Koval 1981 |
| $UO_2^{2+}$ | *Acinetobacter lwoffii* RAG-1 | Apoemulsan[h] | 243 | 1021 | Zosim et al. 1983 |
| | | Apoemulsan[h] in emulsanosol | 958 | 4025 | Zosim et al. 1983 |
| | *E. coli* K-12 | Peptidoglycan[a] | 2.4 | 10 | Hoyle and Beveridge 1984 |
| | | Peptidoglycan[a], LPS[b], proteins | 15.7 | 66 | Beveridge and Koval 1981 |
| | *Pseudomonas* sp. EPS-5028 | EPS[i] | 96 | 403 | Marques et al. 1990 |
| | | EPS[j] | 46 | 193 | Marques et al. 1990 |
| | *Thiobacillus ferrooxidans* TF1–35 | LPS[b] | 0.2 | 1 | Dispirito et al. 1983 |
| | *Z. ramigera* | Zooglan[f] | 370 | 1554 | Norberg and Persson 1984 |
| $ZrO^{2+}$ | *E. coli* K-12 | Peptidoglycan[a] | 134 | 1469 | Hoyle and Beveridge 1984 |
| | | Peptidoglycan[a], LPS[b], proteins | 19.3 | 212 | Beveridge and Koval 1981 |
| $HfO^{2+}$ | *E. coli* K-12 | Peptidoglycan[a] | 1416 | 7932 | Hoyle and Beveridge 1984 |
| | | Peptidoglycan[a], LPS[b], proteins | 167.8 | 940 | Beveridge and Koval 1981 |
| $Cr^{3+}$ | *Bacillus liqueniformis* | $\gamma$-Glutamyl capsular polymer[g] | 48.9 | 940 | McLean et al. 1990 |
| $MoO_2^{2+}$ | *E. coli* K-12 | Peptidoglycan[a] | 532 | 5545 | Hoyle and Beveridge 1984 |
| | | Peptidoglycan[a], LPS[b], proteins | 21.6 | 225 | Beveridge and Koval 1981 |
| $Mn^{2+}$ | *E. coli* K-12 | Peptidoglycan[a] | 2.9 | 52 | Hoyle and Beveridge 1984 |
| | | Peptidoglycan[a], LPS[b], proteins | 7.7 | 140 | Beveridge and Koval 1981 |
| | *B. liqueniformis* | $\gamma$-Glutamyl capsular polymer[g] | 3.9 | 71 | McLean et al. 1990 |
| | *B. subtilis* 168 | Peptidoglycan[c] | 40.7 | 741 | Doyle et al. 1980 |
| Transition elements III | | | | | |
| | *Bradyrhizobium japonicum* USDA 110 | EPS[i] | 59.2 | 1060 | Corzo et al. 1994 |
| $Fe^{3+}$ | *Bradyrhizobium (chamaecytisus)* strain BGA-1 | EPS[i] | 24 | 430 | Corzo et al. 1994 |
| | *E. coli* K-12 | Peptidoglycan[a] | 5.6 | 100 | Hoyle and Beveridge 1984 |
| | | Peptidoglycan[a], LPS[b], proteins | 11.2 | 200 | Beveridge and Koval 1981 |
| | *P. aeruginosa* type $A^{-}B^{+}$ | LPS[b] | 90 | 1611 | Langley and Beveridge 1999 |
| | *B. liqueniformis* | $\gamma$-Glutamyl capsular polymer[g] | 74.8 | 1340 | McLean et al. 1990 |

**Table 1** (Contd.)

| Metal | Strain | Biopolymer | Bound metal (mg/g polymer) | Bound metal (μmol/g polymer) | Reference |
|---|---|---|---|---|---|
| $Fe^{2+}$ | *E. coli* K-12 | Peptidoglycan[a], LPS[b], proteins | 3.2 | 57 | Beveridge and Koval 1981 |
| $Co^{2+}$ | *E.coli* K-12 | Peptidoglycan[a] | 2.5 | 42 | Hoyle and Beveridge 1984 |
| | | Peptidoglycan[a], LPS[b], proteins | 10.5 | 178 | Beveridge and Koval 1981 |
| | *B. liqueniformis* | γ-Glutamyl capsular polymer[g] | 5.9 | 100 | McLean et al. 1990 |
| $Ni^{2+}$ | *E. coli* K-12 | Peptidoglycan[a] | 1.1 | 19 | Hoyle and Beveridge 1984 |
| | | Peptidoglycan[a], LPS[b], proteins | 0.1 | 2 | Beveridge and Koval 1981 |
| | *B. liqueniformis* | γ-Glutamyl capsular polymer[g] | 4.7 | 80 | McLean et al. 1990 |
| | *B. subtilis* 168 | Peptidoglycan[a] | 37.5 | 639 | Doyle et al. 1980 |
| $Ru^{3+}$ | *E. coli* K-12 | Peptidoglycan[a], LPS[b], proteins | 9.1 | 90 | Beveridge and Koval 1981 |
| $OsO_4$ | *E. coli* K-12 | Peptidoglycan[a] | 3.8 | 20 | Hoyle and Beveridge 1984 |
| | | Peptidoglycan[a], LPS[b], proteins | 197.8 | 1040 | Beveridge and Koval 1981 |
| $Pt^{4+}$ | *E. coli* K-12 | Peptidoglycan[a], LPS[b], proteins | 0.4 | 2 | Beveridge and Koval 1981 |
| Transition elements IV | | | | | |
| $Cu^{2+}$ | *E. coli K-12* | Peptidoglycan[a], LPS[b], proteins | 5.7 | 90 | Beveridge and Koval 1981 |
| | *K. aerogenes* | EPS[i] | 13.2 | 207 | Bitton and Freihofer 1978 |
| | *P. aeruginosa* type $A^{-}B^{+}$ | LPS[b] | 14 | 220 | Langley and Beveridge 1999 |
| | *Xanthomonas campestris* | Xanthan[k] | 7.81 | 123 | Mittelman and Geesey 1985 |
| | *Z. ramigera* | Zooglan[f] | 323 | 5083 | Norberg and Person 1984 |
| | *B. liqueniformis* | γ-Glutamyl capsular polymer[g] | 56.5 | 890 | McLean et al. 1990 |
| | Fresh water sediments | EPS[i] | 16 | 253 | Mittelman and Geesey 1985 |
| $Au^{3+}$ | *E.coli* K-12 | Peptidoglycan[a], LPS[b], proteins | 11 | 56 | Beveridge and Koval 1981 |
| | *P. aeruginosa* type $A^{-}B^{+}$ | LPS[b] | 108 | 548 | Langley and Beveridge 1999 |
| $Zn^{2+}$ | *E. coli* K-12 | Peptidoglycan[a], LPS[b], proteins | 25.5 | 390 | Beveridge and Koval 1981 |
| | *B. liqueniformis* | γ-Glutamyl capsular polymer[i] | 9.7 | 149 | McLean et al. 1990 |
| $Cd^{2+}$ | *K. aerogenes* | EPS[i] | 11 | 98 | Bitton and Freihofer 1978 |
| | *Z. ramigera* 115 | Zooglan[f] | 1.9 | 19 | Park et al. 1999 |
| | *Arthrobacter viscosus* | EPS[l] | 0.9 | 8 | Scott and Palmer 1988 |
| | *A. lwoffii* RAG-1 | Apoemulsan[h] | 141 | 1250 | Solomon 1997 |
| | | Apoemulsan in apoemulsanosol[h] | 282 | 2250 | Solomon 1997 |
| $Hg^{2+}$ | *E. coli* K-12 | Peptidoglycan[a], LPS[b], proteins | 12.8 | 64 | Beveridge and Koval 1981 |
| $In^{3+}$ | *E. coli* K-12 | Peptidoglycan[a], LPS[b], proteins | 0.114 | 1 | Beveridge and Koval 1981 |
| $Pb^{2+}$ | *E.coli* K-12 | Peptidoglycan[a] | 10.3 | 49.7 | Hoyle and Beveridge 1984 |
| | | Peptidoglycan[a], LPS[b], proteins | 31.5 | 152 | Beveridge and Koval 1981 |

[a] β (1,4)-Linked *N*-acetyl glucosamine-*N*-acetylmuramic acid crosslinked (L)-Ala-D-glucose (L)-meso-diamino pimelic acid-D-alanine residues
[b] Lipopolysaccharide
[c] Glucose, fucose and glucuronic acid; ratio:1.5:1:0.8
[d] Glucose, fucose, glucuronic acid and acetate; ratio:2.1:1:0.83:0.43
[e] Glucose, galactose, acetate, pyruvate and succinate; ratio:7:1:0.63:0.76:1
[f] Glucose, galactose, acetate, pyruvate and succinate; ratio:2:1:0.64:0.44
[g] γ-Glutamic acid
[h] [D-Galactosamine, D-galactosamine uronic acid, bacillosamine (2,4) diamino, 6-deoxy glucose] apoemulsanosol concentrated emulsion (70% w/v hexadecane in water); see text
[i] Exopolysaccharide
[j] Deacylated exopolysaccharide
[k] Glucose, mannose, glucuronate, acetate and pyruvate
[l] D-Glucose, D-galactose and D-mannuronic acid

binding to the walls, suggesting that not all the available binding sites were exposed on native walls, but were normally shielded by teichoic acid residues. Unlike the case with Mg binding, the blocking of carboxylic acid groups had little effect on Ca binding in the same system. However, Ca binding to *B. subtilis* walls lacking teichoic acid was reduced by a factor of ten once the teichoic acid was extracted. Similar approaches were used to examine cation binding to different walls in other organisms (Beveridge et al. 1982; Doyle et al. 1980; Ferris and Beveridge 1986; Geddie and Sutherland 1993; Langley and Beveridge 1999; Mullen et al. 1989; Strain et al. 1983), although binding differed both in terms of specificity and binding capacity. For example both Ca and Mg were found to bind to native walls of *Enterobacter* sp., *Klebsiella aerogenes*, *Rhizobium meliloti* and

*Zoogloea ramigera* (Geddie and Sutherland 1993). However, deacylation of such walls only slightly impaired the binding of either Ca or Mg when compared to results with *B. subtilis*. Although such comparative studies of cation binding to functional groups present in wall-associated polymers yielded some information regarding specificity, there have been only a few reports dealing with binding to walls prepared from specific mutants with altered surface properties. In one such report (Strain et al. 1983) $^{13}C$ and $^{31}P$ nuclear magnetic resonance (NMR) spectroscopy was used to characterize metal binding to LPS from a heptoseless mutant of *E. coli* K12. Low concentrations of $Ca^{2+}$, $Cd^{2+}$, $Gd^{3+}$, $La^{3+}$ and $Yb^{3+}$ all affected the $^{31}P$ NMR spectrum at low concentrations. The authors concluded that the LPS from this mutant contain a high affinity metal-binding site which involves the participation of a glycosidic diphosphate moiety. Langley and Beveridge (1999) exploited the fact that *Pseudomonas aeruginosa* PAO1 normally produces two chemically distinct types of LPS, the A-band and the B-band LPS, respectively. A series of isogenic strains were used including $A^{+}B^{-}$, $A^{-}B^{+}$ and $A^{-}B^{-}$ mutants. All strains bound small amounts of Cu onto the cell surface, suggesting that the binding may be to a common surface-binding site such as the phosphoryl groups on the core lipid A region. Mutants lacking the A-band LPS caused precipitation of Fe onto the cell surface, while mutants lacking B-band LPS gave rise to La crystals. The authors proposed that the binding of metal ions to LPS did not involve the direct involvement of *O*-antigen side chains, but that the B-band LPS may affect cell surface properties which enhance the precipitation of metals in specific regions on the cell surface.

## Cation binding to emulsan and other amphipathic biopolymers

As illustrated in Table 1, negatively charged biopolymers bind cations with different specificities and with different overall metal-binding capacities. Although the basis for such specificity is not clear, in one class of biopolymers there is a clear case of metal-binding enhancement under a condition which modifies the conformation of the biopolymer. One such biopolymer is the amphipathic, galactosamine-containing capsular bioemulsifier produced by the hydrocarbon-degrading organism *Acinetobacter lwoffii* RAG-1 (Gutnick 1987; Rosenberg et al. 1979b; Shabtay et al. 1985; Zuckerberg et al. 1979). The amphipathicity of emulsan is mediated by the presence of fatty acids (about 25% by weight) present in both ester and amide linkages (Belsky et al. 1979) and, in crude form, by the non-covalent association with several exocellular proteins. Protein-free emulsan, termed apoemulsan, retains partial emulsifying activity towards more polar hydrocarbons such as mixtures of aliphatics and aromatics, or crude or machine oil (Rosenberg et al. 1979a). Nevertheless, the apoemulsan does not emulsify very hydrophobic substances such as hexadecane or long chain waxes. Emulsification and emulsion stabilization are due to the tight affinity of emulsan for the oil/water interface; a property which allows the negatively charged water-soluble polymer to partition into a cream layer either after standing or following centrifugation. Such concentrated cream layers are oil-in-water emulsions in which the oil content can be as high as 70% by weight, yet water remains the bulk solvent. Such concentrated emulsan-stabilized creams are termed *emulsanosols* (Zosim et al. 1982). Emulsanosols are formed from the binding of emulsan to the oil/water interface, resulting in a polymer orientation such that the hydrophilic sugar residues (including the negative charges on the galactosamine uronic acid residues) face outward towards the aqueous solvent, while the hydrophobic groups are oriented towards the oil. The stabilization of the emulsion in the emulsanosol is thought to be due to the electrostatic charge repulsion between these uronic acid residues, thereby preventing droplet coalescence. The orientation of the polymer brings about a conformational change in the polysaccharide backbone, as evidenced by the fact that emulsan-specific phages adsorb to emulsan when it is either bound to an oil droplet or bound to the surface of the cell (Pines and Gutnick 1984, 1986). Interestingly, when purified apoemulsan was mixed with positively charged organic cations such as rhodamine, almost none of the cation remained bound to the biopolymer after dialysis. However, when the same experiment was performed, but this time substituting the water-soluble apoemulsan with an apoemulsanosol of hexadecane and water containing the same amounts of the bioemulsifier, about 3 μmol rhodamine/mg apoemulsan was bound at the oil/water interface (Zosim et al. 1982). It should be noted that if all the negative charges were saturated with cation, one would have expected only about 1.5 μmol rhodamine to have been bound. The results suggest that the orientation of the polymer not only stabilizes the cation-biopolymer interaction, but also results in the coordination of additional cations, perhaps through the interaction with hydroxyl groups on the amino-sugars in the polymer backbone. Enhanced binding of metal ions such as $Cd^{2+}$ or $UO_2^{2+}$ to apoemulsan was also observed at the oil/water interface. In a subsequent set of experiments, it was shown that cations bound to the emulsanosol could be completely removed to the aqueous phase when the pH was lowered to below the pK of the uronic acid residues ($<3.05$). Under these conditions, the emulsanosol remained stable as a concentrated oil-in-water emulsion and could be resuspended in an aqueous solution containing cations at neutral pH to generate an oil-in-water emulsion. This suggests the possibility of using emulsanosols as recyclable, water-soluble, cation-exchange complexes as illustrated in Fig. 1. The scheme represents a potential application for metal removal from large dilute aqueous volumes by concentration into a small, concentrated, water-in-oil emulsion layer. In this system, a mixture of emulsan or

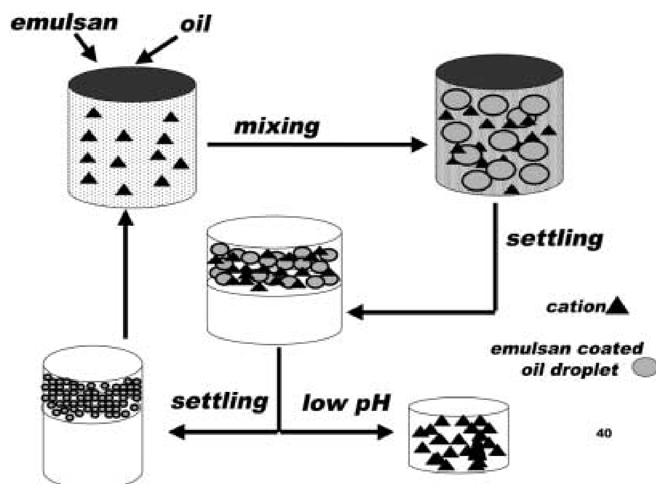


**Fig. 1** Scheme for metal recovery and recycling of emulsanosols. An oil/water emulsion is formed by adding a preparation of emulsan and oil to a tank or other source of water containing heavy metals. After emulsification a cream layer, the emulsanosol, is formed during the settling process. The metal-containing emulsanosol is subjected to low pH which causes the cations to dissociate into a separate container, while the emulsanosols are allowed to reform by settling. They are then returned directly to the polluted source to continue the cycle. Note that emulsan need only be added once to this system. Once formed, the emulsanosols are recycled through the metal-containing site for additional recovery. Metal ions are represented by *black triangles* and oil droplets by *grey ellipses*

apoemulsan and oil (composing about 0.1–2% of the total volume) can be used to generate an oil-in-water emulsion in a body of water polluted with heavy metal ions. At these concentrations of emulsan, more than 90% of the polymer adheres to the oil/water interface. Initial emulsion formation requires some input energy to form the emulsion. In our experience, continuous pumping of the emulsan-treated material can be used to generate the emulsion. Upon settling, the emulsion and adsorbed cations separate into a small concentrated emulsanosol. This cation-associated phase can be skimmed off the surface and transferred to a new container where the cations are removed by lowering the pH. The emulsanosol can then be recycled for another round of metal binding. The emulsanosols offer a number of potential advantages including stability, water compatibility, ability to be formed from waste oils and crude sludges with concomitant viscosity reduction of the oil and biodegradability.

## Novel esterase formulations and cation binding

Emulsan release from the cell surface is mediated by a cell-surface esterase enzyme which has been purified, cloned and sequenced (Alon and Gutnick 1993; Alon et al. 1995; Reddy et al. 1989). The overexpression of the esterase in *E. coli* led to its appearance in inclusion bodies, which could be isolated. The enzyme could be refolded using chaotropic agents, which were then removed by slow dialysis to yield an active enzyme (Bach et al. 1998). Modeling studies have shown that the enzyme most closely resembles proteins exhibiting an alpha-beta hydrolase fold in their tertiary conformation (Alon et al. 1995). This esterase is also one of several proteins released from the cell surface with the emulsan biopolymer and in fact can partially deesterify emulsan. Removal of the emulsan-associated proteins using any of a number of techniques, including hot phenol, proteases, or selective precipitation of the deproteinated polymer with cationic surfactants such as cetyl-trimethyl-ammonium bromide, yields a product termed apoemulsan. Apoemulsan exhibits a lower emulsifying activity, particularly towards very hydrophobic substrates such as very waxy oils, or straight chain aliphatics such as hexadecane. The addition of denatured protein to the apoemulsan restores the emulsifying activity towards hydrophobic substrates. Surprisingly, the addition of purified recombinant esterase to apoemulsan resulted in a formulation which showed emulsifying activity towards a host of hydrophobic substrates (Bach et al. 1998). Moreover, esterase-containing emulsanosols could be generated by centrifugation and exhibited the same stability as the concentrated emulsan-generated oil/water emulsions. This protein-mediated enhancement of emulsifying activity is accompanied by the enhanced binding of cations such as $Cd^{2+}$ or $UO_2^{2+}$ at the oil/water interface. It was also found that site-directed mutant esterase molecules, which are catalytically inactive, are still capable of enhancing the emulsifying activity and cation-binding properties of esterase-containing emulsanosols (Bach, in preparation, patent pending). In an attempt to map the portion of the esterase responsible for enhancing the emulsification, a series of peptides were generated by subjecting the emulsan to a series of proteolytic enzymes (Bach, in preparation). Both chymotrypsin and papain yielded peptides of about 10 kDa and 14 kDa, respectively. These peptides were also able to interact with apoemulsan, to reconstitute emulsification and to enhance cation binding at the oil/water interface. In addition, a series of fusion proteins were generated in which the maltose-binding protein (MBP) was fused at its C-terminus to various esterase fragments. These studies, which are currently in progress, demonstrated that after purification and cleavage of the MBP, at least two of the fragments were active in reconstituting apoemulsan-mediated emulsification towards hydrophobic substrates. Apoemulsanosols formed from these complexes were also able to bind the cations as efficiently as any other formulations. Current research in this direction includes the potential for using esterase derivatives fused to specific proteins to facilitate the interaction with emulsan/apoemulsan complexes in order to modify the amphipathicity of the emulsifying biopolymer. Finally, we have been able to use esterase derivatives and peptides to interact with a variety of water-soluble polysaccharides, thereby converting them to amphipathic bioemulsifiers which form emulsanosol-like emulsions (Bach and Gutnick, patent pending). Enhanced cation binding to these polyanions was also observed.

## Polysaccharide engineering and cation binding

In this section we suggest that recombinant DNA technology can be used to modify polysaccharide-encoding biosynthetic operons with a view towards producing biopolymers with enhanced cation-binding capacity. Genes encoding enzymes for the biosynthesis of polysaccharides usually occur in clusters (Bugert and Geider 1995; Reuber and Walker 1993; Stevenson et al. 1996; Vanderslice et al. 1989; Whitfield and Roberts 1999) Generally the biosynthetic regulons encode housekeeping enzymes for monomer synthesis, subunit construction (transglycosylases), polymerization, decoration with acetyl, succinyl, pyruvylyl and phosphoryl groups, and translocation to the outer surface of the cell (Vanderslice et al. 1989). It was noted that some of the xanthan-defective mutants of *Xanthomonas campestris* still produced small amounts of biopolymer with a modified structure. By introducing specific combinations of mutations into the chromosome, the authors were able to generate a family of viscous polymeric xanthan derivatives containing fewer numbers of sugars in the backbone with or without decoration, i.e. deacetylated, depyruvylated etc. Similar approaches were taken to produce mutant and recombinant derivatives of the viscous polyanionic biopolymer acetan produced by *Acetobacter xylinum* (Edwards et al. 1999). In another case (Bernhard et al. 1996), substitutions were made between biosynthetic genes for the production of EPS from *Erwinia amylovora* (amylovoran) and *Pantoea stewartii* (stewartan) respectively. Mutants of *P. stewartii* defective in stewartan synthesis could be complemented by cosmid libraries encoding the biosynthetic genes of amylovoran, in order to produce recombinant polysaccharides. Since many of the water-soluble polymers and EPS listed above are capable of interacting with recombinant esterase to generate amphipathic emulsifying formulations as described above, cation binding was examined at the oil/water interface and was found to be enhanced. It would be of interest in this regard to test the properties of recombinant polysaccharide derivatives which exhibit such enhanced cation-binding properties.

Two approaches have been used to prepare engineered derivatives of emulsan. In the first approach, mutants were prepared by modifying the fatty acid composition of the bioemulsifier, using a fatty acid auxotroph and feeding fatty acids directly into the medium (Gutnick 1997). Four derivatives were generated which contained 2-OH C14, palmitoleic, linoleic or linolenic acid, respectively, in place of the 2-OH C12 which is the main fatty acid of wild-type emulsan (Belsky et al. 1979; Gorkovenko et al. 1997). The derivatives were tested for emulsification, ability to remove microbes from hydrophobic surfaces (anti-adherence; Rosenberg et al. 1980) and binding of $UO_2^{2+}$ at the oil/water interface. The incorporation of linolenic acid into the polymer gave rise to a derivative which exhibited only about 10% of the emulsifying activity, compared to the wild-type polymer. The palmitoleic acid derivative was about half as active, while the linoleic acid mutant emulsan was about a third as active. As regards $UO_2^{2+}$ binding at the oil/water interface, all of the derivatives showed enhanced binding of the cations. These derivatives were recently tested using Cd binding and were found to be as effective as the parental bioemulsifier (unpublished observations). These experiments were conducted with the standard mixture of hexadecane-2-methyl naphthalene. However, when hexadecane or octadecane were used as hydrocarbon substrates for emulsification, the mutant biopolymers containing elevated levels of palmitoleic acid were about twice as active as those containing the hydroxy dodecanoic acids. It has recently been found that significant fatty acid changes in the acyl side chains of emulsan could be achieved by growing the cells of *Acinetobacter lwoffii* RAG-1 in the presence of fatty acids in the growth medium (Gorkovenko et al. 1997), although none of the activities of the biopolymer product were reported. These results may account for very old observations that the properties of the emulsan produced on oil substrates (so-called $\beta$-emulsans) differ significantly from those produced on ethanol.

The second approach is similar to those described above for other EPS and is currently in progress. Recently, the cluster encoding the biosynthetic pathway for emulsan synthesis was cloned and sequenced (Gutnick et al. 1999). On the basis of homology to other known sequences, emulsan most closely resembles the *O*-antigen of LPS, lacking keto-desoxyoctulosonic acid residues as well as the lipid A moiety of LPS. A series of emulsan-defective mutants were generated in the various biosynthetic genes and complemented with a genomic library from the organism *A. calcoaceticus* BD413. This strain produces a rhamnose-containing, extracellular, water-soluble capsular polysaccharide (Juni and Heym 1964) which normally does not exhibit any emulsifying activity (Kaplan et al. 1987a). This polymer, however, does exhibit emulsifying activity when it interacts with crude extracellular protein associated with it, or with recombinant esterase from *A. lwoffii* RAG-1 (Bach, in preparation). The biosynthetic pathway of the BD413 polymer includes a series of transglycosylation reactions, one of which transfers a UDP glucose to the membrane during subunit assembly (Kaplan and Rosenberg 1982). One of the complemented mutants was found to contain a glucose moiety in place of galactosamine. It is tempting to consider that by removing the galactosamine, potential sites for additional acylation could be lost, thereby altering the polarity of the molecule. In fact, this mutant derivative of emulsan exhibits about half as much emulsifying activity as the native bioemulsifier, but is still active in cation binding at the oil/water interface and in the removal of adherent organisms from hydrophobic surfaces (preliminary observations). It should be noted, however, that the overall yield of glucose-containing emulsan derivative is only about 10% of the wild-type yield, which is in agreement with previous

findings for xanthan derivatives (Vanderslice et al. 1989). It has yet to be determined whether this low productivity can be increased by optimizing fermentation conditions for production. The ability to modify a polysaccharide backbone by genetic techniques may pave the way to enhance the cation binding of the biopolymer, using derivatives which have a higher charge density. Moreover, the possibility of fusing active peptide fragments from the esterase with other proteins, such as metallothionein, may lead to the generation of novel formulations which both stabilize emulsanosols, and exhibit enhanced binding. This approach is currently being considered in our laboratory.

## Potential applications

Thus far, isolated biopolymers for heavy metal remediation have not been applied on a large scale, although synthetic polymers have been used for various precipitation treatments. It seems likely that incorporating polysaccharides into biofilter technology may provide useful applications for remediation, although much depends on the economics of such treatments. A biofilter for heavy metal removal from polluted water may involve the use of crude biomass, or it could involve a more selective cation-exchange process employing immobilized polysaccharides. Of course the economics will depend on the added value associated with enhanced specificity and cation-binding capacity. In addition, the use of defined materials in such a system enables the process to be modeled and simulated with better predictability.

An alternative approach could involve the use of emulsanosol technology as illustrated in Fig. 1. Emulsanosols are highly stable and can be recycled once the initial emulsion is formed. Previous results have demonstrated that once formed, emulsanosols can withstand the enormous shear forces of over 500 pump transits at a flow rate of 6,000 l/h at a pressure of 500 kPa through a 9 cm pipe. In terms of scaling-up, this stability could accommodate about 250,000 barrels of oil per day, in a 50–75 cm pipe without any inversion of the oil-in-water emulsion. Moreover, the use of emulsanosols for viscosity reduction in heavy oils and in the generation of homogenous emulsions for the efficient combustion of crude sludges and heavier materials has been scaled-up and demonstrated on a semi-industrial scale (Gutnick et al. 1991).

In line with this approach, novel formulation technology using highly active recombinant protein (or peptide)–polysaccharide complexes may provide an application for inexpensive excess polysaccharides, which need not be produced by fermentation and for which there is little industrial use. For example, the generic formulation might be applicable in designing specific emulsanosols in the remediation of particular polluted sites. The approach also offers the possibility of recovering heavy metals during the clean-up of sludges, tank bottoms and other oily wastes, using the same materials for both purposes. In addition, as emulsions with relatively low viscosity, emulsanosols may be more stable for applications in sites such as ships, barges and tank farms where the handling of pumpable liquids is generally easier than the handling of solids (Corzo et al. 1994; DiSpirito et al. 1983; Doyle et al. 1980; Hoyle and Beveridge 1984a; Marques et al. 1990; McLean et al. 1990; Mittelman and Geesey 1985; Norberg and Persson 1984; Park et al. 1999; Rayman and MacLeod 1975; Scott and Palmer 1988; Solomon 1997).

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