

MINI-REVIEW

D. L. Gutnick · H. Bach

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

Received: 2 February 2000 / Received revision: 2 June 2000 / Accepted: 3 June 2000

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.

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, non-toxic 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

D. L. Gutnick (✉) · H. Bach
Department of Molecular Microbiology and Biotechnology,
George S. Wise Faculty of Life Sciences, Tel-Aviv University,
Tel-Aviv 69978, Israel
e-mail: davidg@post.tau.ac.il
Tel.: +972-3-6409834
Fax: +972-3-6425786/6409407

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 only 160 $\mu\text{mol/g}$ 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		Reference
			(mg/g polymer)	($\mu\text{mol/g}$ polymer)	
Group II alkaline earth metals					
Mg^{2+}	<i>Escherichia coli</i> K-12	Peptidoglycan ^a	0.9	35	Hoyle and Beveridge 1984
		Peptidoglycan ^a , LPS ^b , proteins	6	256	Beveridge and Koval 1981
	<i>Enterobacter</i> sp. 11870	EPS ^c	8	329	Geddie and Sutherland 1993
	<i>Klebsiella aerogenes</i> 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
	<i>Rhizobium meliloti</i> YE-2	EPS ^e	5.4	222	Geddie and Sutherland 1993
	<i>Zooglea ramigera</i>	Zooglan ^f	5.5	226	Geddie and Sutherland 1993
	<i>Bacillus liqueniformis</i>	γ -Glutamyl capsular polymer ^g	1.8	73	McLean et al. 1990
Ca^{2+}	<i>E. coli</i> K-12	Peptidoglycan ^a	1.5	38	Hoyle and Beveridge 1984
		Peptidoglycan ^a , LPS ^b , proteins	1.4	35	Beveridge and Koval 1981
	<i>Enterobacter</i> sp. 11870	EPS ^c	11.8	295	Geddie and Sutherland 1993
	<i>K. aerogenes</i> type 54 strain A3 (sl)	EPS ^d	9.1	228	Geddie and Sutherland 1993
	<i>R. meliloti</i> YE-2	EPS ^e	7.2	180	Geddie and Sutherland 1993
	<i>Zooglea ramigera</i>	Zooglan ^f	4.5	113	Geddie and Sutherland 1993
	<i>B. liqueniformis</i>	γ -Glutamyl capsular polymer ^g	41.8	1044	McLean et al. 1990
	<i>B. subtilis</i> 168	Peptidoglycan ^a	30	750	Doyle et al. 1980
Sr^{2+}	<i>E. coli</i> 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+}	<i>E. coli</i> K-12	Peptidoglycan ^a	9.7	71	Hoyle and Beveridge 1984
Transition elements I and II					
Sc^{3+}	<i>E. coli</i> 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+}	<i>E. coli</i> K-12	Peptidoglycan ^a	299.5	2156	Hoyle and Beveridge 1984
		Peptidoglycan ^a , LPS ^b , proteins	10.8	78	Beveridge and Koval 1981
	<i>Pseudomonas aeruginosa</i> type A ⁺ B ⁻	LPS ^b	31.8	229	Langley and Beveridge 1999
Ce^{3+}	<i>E. coli</i> K-12	Peptidoglycan ^a	308	2198	Hoyle and Beveridge 1984
		Peptidoglycan ^a , LPS ^b , proteins	14	100	Beveridge and Koval 1981
Pr^{3+}	<i>E. coli</i> K-12	Peptidoglycan ^a , LPS ^b , proteins	8.2	58	Beveridge and Koval 1981
Sm^{3+}	<i>E. coli</i> K-12	Peptidoglycan ^a , LPS ^b , proteins	1.7	11	Beveridge and Koval 1981
UO_2^{2+}	<i>Acinetobacter lwoffii</i> RAG-1	Apoemulsan ^h	243	1021	Zosim et al. 1983
		Apoemulsan ^h in emulsanosol	958	4025	Zosim et al. 1983
	<i>E. coli</i> K-12	Peptidoglycan ^a	2.4	10	Hoyle and Beveridge 1984
		Peptidoglycan ^a , LPS ^b , proteins	15.7	66	Beveridge and Koval 1981
	<i>Pseudomonas</i> sp. EPS-5028	EPS ⁱ	96	403	Marques et al. 1990
		EPS ^j	46	193	Marques et al. 1990
	<i>Thiobacillus ferrooxidans</i> TF1-35	LPS ^b	0.2	1	Dispirito et al. 1983
	<i>Z. ramigera</i>	Zooglan ^f	370	1554	Norberg and Persson 1984
ZrO^{2+}	<i>E. coli</i> K-12	Peptidoglycan ^a	134	1469	Hoyle and Beveridge 1984
		Peptidoglycan ^a , LPS ^b , proteins	19.3	212	Beveridge and Koval 1981
HfO^{2+}	<i>E. coli</i> K-12	Peptidoglycan ^a	1416	7932	Hoyle and Beveridge 1984
		Peptidoglycan ^a , LPS ^b , proteins	167.8	940	Beveridge and Koval 1981
Cr^{3+}	<i>Bacillus liqueniformis</i>	γ -Glutamyl capsular polymer ^g	48.9	940	McLean et al. 1990
MoO_2^{2+}	<i>E. coli</i> K-12	Peptidoglycan ^a	532	5545	Hoyle and Beveridge 1984
		Peptidoglycan ^a , LPS ^b , proteins	21.6	225	Beveridge and Koval 1981
Mn^{2+}	<i>E. coli</i> K-12	Peptidoglycan ^a	2.9	52	Hoyle and Beveridge 1984
		Peptidoglycan ^a , LPS ^b , proteins	7.7	140	Beveridge and Koval 1981
	<i>B. liqueniformis</i>	γ -Glutamyl capsular polymer ^g	3.9	71	McLean et al. 1990
	<i>B. subtilis</i> 168	Peptidoglycan ^c	40.7	741	Doyle et al. 1980
Transition elements III					
Fe^{3+}	<i>Bradyrhizobium japonicum</i> USDA 110	EPS ⁱ	59.2	1060	Corzo et al. 1994
	<i>Bradyrhizobium (chamaecytisus)</i> strain BGA-1	EPS ⁱ	24	430	Corzo et al. 1994
	<i>E. coli</i> K-12	Peptidoglycan ^a	5.6	100	Hoyle and Beveridge 1984
		Peptidoglycan ^a , LPS ^b , proteins	11.2	200	Beveridge and Koval 1981
	<i>P. aeruginosa</i> type A ⁻ B ⁺	LPS ^b	90	1611	Langley and Beveridge 1999
	<i>B. liqueniformis</i>	γ -Glutamyl capsular polymer ^g	74.8	1340	McLean et al. 1990

Table 1 (Contd.)

Metal	Strain	Biopolymer	Bound metal		Reference
			(mg/g polymer)	($\mu\text{mol/g}$ polymer)	
Fe ²⁺	<i>E. coli</i> K-12	Peptidoglycan ^a , LPS ^b , proteins	3.2	57	Beveridge and Koval 1981
Co ²⁺	<i>E. coli</i> K-12	Peptidoglycan ^a	2.5	42	Hoyle and Beveridge 1984
		Peptidoglycan ^a , LPS ^b , proteins	10.5	178	Beveridge and Koval 1981
		γ -Glutamyl capsular polymer ^g	5.9	100	McLean et al. 1990
Ni ²⁺	<i>E. coli</i> K-12	Peptidoglycan ^a	1.1	19	Hoyle and Beveridge 1984
		Peptidoglycan ^a , LPS ^b , proteins	0.1	2	Beveridge and Koval 1981
		γ -Glutamyl capsular polymer ^g	4.7	80	McLean et al. 1990
		Peptidoglycan ^a	37.5	639	Doyle et al. 1980
Ru ³⁺	<i>E. coli</i> K-12	Peptidoglycan ^a , LPS ^b , proteins	9.1	90	Beveridge and Koval 1981
OsO ₄	<i>E. coli</i> 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⁺	<i>E. coli</i> K-12	Peptidoglycan ^a , LPS ^b , proteins	0.4	2	Beveridge and Koval 1981
Transition elements IV					
Cu ²⁺	<i>E. coli</i> K-12	Peptidoglycan ^a , LPS ^b , proteins	5.7	90	Beveridge and Koval 1981
		EPS ⁱ	13.2	207	Bitton and Freihofer 1978
		LPS ^b	14	220	Langley and Beveridge 1999
		Xanthan ^k	7.81	123	Mittelman and Geesey 1985
		Zooglan ^f	323	5083	Norberg and Person 1984
		γ -Glutamyl capsular polymer ^g	56.5	890	McLean et al. 1990
		Fresh water sediments	EPS ⁱ	16	253
Au ³⁺	<i>E. coli</i> K-12	Peptidoglycan ^a , LPS ^b , proteins	11	56	Beveridge and Koval 1981
		LPS ^b	108	548	Langley and Beveridge 1999
Zn ²⁺	<i>E. coli</i> K-12	Peptidoglycan ^a , LPS ^b , proteins	25.5	390	Beveridge and Koval 1981
		γ -Glutamyl capsular polymer ^l	9.7	149	McLean et al. 1990
Cd ²⁺	<i>K. aerogenes</i>	EPS ⁱ	11	98	Bitton and Freihofer 1978
		Zooglan ^f	1.9	19	Park et al. 1999
		EPS ⁱ	0.9	8	Scott and Palmer 1988
		Apoemulsan ^h	141	1250	Solomon 1997
		Apoemulsan in apoemulsanosol ^h	282	2250	Solomon 1997
Hg ²⁺	<i>E. coli</i> K-12	Peptidoglycan ^a , LPS ^b , proteins	12.8	64	Beveridge and Koval 1981
In ³⁺	<i>E. coli</i> K-12	Peptidoglycan ^a , LPS ^b , proteins	0.114	1	Beveridge and Koval 1981
Pb ²⁺	<i>E. coli</i> 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 apo-

emulsan 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

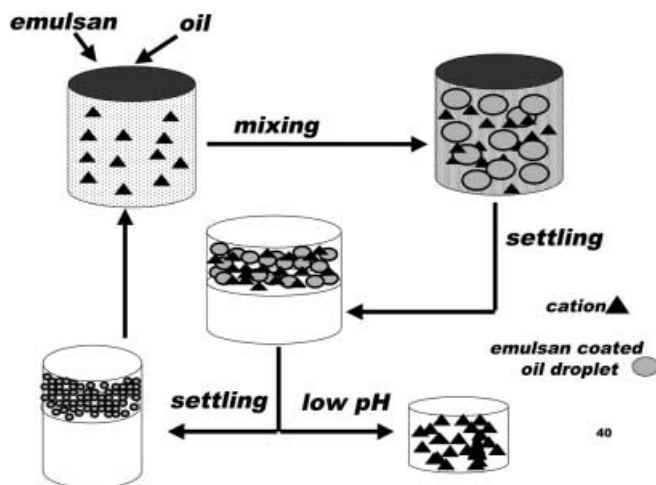


Fig. 1 Scheme for metal recovery and recycling of emulsanosols. An oil/water emulsion is formed by adding a preparation of emulsan and oil to a tank or other source of water containing heavy metals. After emulsification a cream layer, the emulsanosol, is formed during the settling process. The metal-containing 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, pyruvyl 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 β -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 emulsions, 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 emulsionsol technology as illustrated in Fig. 1. Emulsionsols are highly stable and can be recycled once the initial emulsion is formed. Previous results have demonstrated that once formed, emulsionsols 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 emulsionsols 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 emulsionsols 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, emulsionsols 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).

References

- Alexander M (1999) Biodegradation and bioremediation. Academic Press, San Diego
- Alon RN, Gutnick DL (1993) Esterase from the oil-degrading *Acinetobacter lwoffii* RAG-1: sequence analysis and over-expression in *Escherichia coli*. FEMS Microbiol Lett 112: 275–280
- Alon RN, Mirny L, Sussman JL, Gutnick DL (1995) Detection of alpha/beta-hydrolase fold in the cell surface esterases of *Acinetobacter* species using an analysis of 3D profiles. FEBS Lett 371: 231–235
- Asthana RK, Chatterjee S, Singh SP (1995) Investigations on nickel biosorption and its remobilization. Process Biochem 30: 729–734
- Avery SV, Tobin JM (1993) Mechanism of adsorption of hard and soft metal ions to *Saccharomyces cerevisiae* and influence of hard and soft anions. Appl Environ Microbiol 59: 2851–2856
- Bach H, Berdichevsky Y, Gutnick D (1998) Use of a recombinant enzyme for the production of amphipathic polysaccharide bioemulsifiers. In: 8th Int Symp Genet Ind Microorg, Jerusalem, Israel
- Belsky I, Gutnick DL, Rosenberg E (1979) Emulsifier of *Acinetobacter* RAG-1: determination of emulsifier-bound fatty acids. FEBS Lett 101: 175–178
- Bernhard F, Schullerus D, Bellemann P, Nimitz M, Coplin DL, Geider K (1996) Genetic transfer of amylovoran and stewartan synthesis between *Erwinia amylovora* and *Erwinia stewartii*. Microbiology 142: 1087–1096
- Beveridge TJ (1989) Role of cellular design in bacterial metal accumulation and mineralization. Annu Rev Microbiol 43: 147–171
- Beveridge TJ, Koval SF (1981) Binding of metals to cell envelopes of *Escherichia coli* K-12. Appl Environ Microbiol 42: 325–335
- Beveridge TJ, Murray RG (1980) Sites of metal deposition in the cell wall of *Bacillus subtilis*. J Bacteriol 141: 876–887
- Beveridge TJ, Forsberg CW, Doyle RJ (1982) Major sites of metal binding in *Bacillus licheniformis* walls. J Bacteriol 150: 1438–1448
- Bitton G, Freihofer V (1978) Influence of extracellular polysaccharides on the toxicity of copper and cadmium toward *Klebsiella aerogenes*. Microb Ecol 4: 119–125
- Brown MJ, Lester JN (1979) Metal removal in activated sludge: the role of bacterial extracellular polymers. Water Res 13: 817–837
- Bugert P, Geider K (1995) Molecular analysis of the *ams* operon required for exopolysaccharide synthesis of *Erwinia amylovora*. Mol Microbiol 15: 917–933
- Chen JH, Czajka DR, Lion LW, Shuler ML, Ghiorse WC (1995a) Trace metal mobilization in soil by bacterial polymers. Environ Health Perspect 103 [Suppl 1]: 53–58
- Chen JH, Lion LW, Ghiorse WC, Shuler ML (1995b) Mobilization of adsorbed cadmium and lead in aquifer material by bacterial extracellular polymers. Water Res 29: 421–430
- Cheng MH, Patterson JW, Minear RA (1975) Heavy metals uptake by activated sludge. J Water Pollut Control Fed 47: 362–376
- Colwell RR (1994) Scientific foundation of bioremediation and gaps remaining to be filled. Res Microbiol 145: 40–41

- Corzo J, Leon-Barrion M, Hernando-Rico V, Gutierrez-Navarro AM (1994) Precipitation of metallic cations by the acidic exopolysaccharides from *Bradyrhizobium japonicum* and *Bradyrhizobium (Chamaecytisus)* strain BGA-1. *Appl Environ Microbiol* 60: 4531–4536
- Cozzi D, Desideri PG, Lepri L (1969) The mechanism of ion exchange with alginic acid. *J Chromatogr* 40: 130–137
- Diels L, Vanroy S, Somers K, Willems I, Mergeay M, Springael D, Leysen R (1995) The use of bacteria immobilized in tubular membrane reactors for heavy-metal recovery and degradation of chlorinated aromatics. *J Membr Sci* 100: 249–258
- Diels L, De Smet M, Hooyberghs L, Corbisier P (1999) Heavy metals bioremediation of soil. *Mol Biotechnol* 12: 149–158
- DiSpirito AA, Talgani Jr JW, Tuovinen OH (1983) Accumulation and cellular distribution of uranium in *Thiobacillus ferrooxidans*. *Arch Microbiol* 135: 250–253
- Doyle RJ, Matthews TH, Streips UN (1980) Chemical basis for selectivity of metal ions by the *Bacillus subtilis* cell wall. *J Bacteriol* 143: 471–480
- Edwards KJ, Jay AJ, Colquhoun IJ, Morris VJ, Gasson MJ, Griffin AM (1999) Generation of a novel polysaccharide by inactivation of the *aceP* gene from the acetan biosynthetic pathway in *Acetobacter xylinum*. *Microbiology* 145: 1499–1506
- Ferris FG, Beveridge TJ (1986) Site specificity of metallic ion binding in *Escherichia coli* K-12 lipopolysaccharide. *Can J Microbiol* 32: 52–55
- Foureste E, Roux C (1992) Heavy-metal biosorption by fungal mycelial by-products – mechanisms and influence of pH. *Appl Microbiol Biotechnol* 37: 399–403
- Fristoe BR, Nelson PO (1983) Equilibrium chemical modelling of heavy metals in activated sludge. *Water Res* 17: 771–778
- Geddie JL, Sutherland IW (1993) Uptake of metals by bacterial polysaccharides. *J Appl Bacteriol* 74: 467–472
- Glombitza F, Eckhart L, Hummel A (1997) Fundamentals of the application of biosorption to the separation of uranium from mining drainage waters. *Res Microbiol* 148: 517–518
- Gorkovenko A, Zhang J, Gross RA, Allen AL, Kaplan DL (1997) Bioengineering of emulsifier structure: emulsan analogs. *Can J Microbiol* 43: 384–390
- Gutnick DL (1987) The emulsan polymer – perspectives on a microbial capsule as an industrial product. *Biopolymers* S26: 223–240
- Gutnick DL (1994) Microbiological treatment of contaminated storage containers. *Res Microbiol* 145: 56–60
- Gutnick DL (1997) Engineering polysaccharides for biosorption of heavy metals at oil/water interfaces. *Res Microbiol* 148: 519–521
- Gutnick DL, Allon R, Levy C, et al (1991) Applications of *Acinetobacter* as an industrial microorganism. In: Towner KJ, Bergogne-Berezin E, Fewson CA (eds) *The biology of Acinetobacter – taxonomy, clinical importance, molecular biology, physiology, industrial relevance*. Plenum Press, New York, pp 411–441
- Gutnick DL, Bach H, Nakar D (1999) Production and application of amphipathic biopolymers for application in oil industry. In: 5th Int Symp Biol Process Fossil Fuels
- Hoyle BD, Beveridge TJ (1984) Metal binding by the peptidoglycan sacculus of *Escherichia coli* K-12. *Can J Microbiol* 30: 204–211
- Juni E, Heym GA (1964) Pathways for biosynthesis of a bacterial capsular polysaccharide. *J Bacteriol* 87: 461–467
- Kaplan N, Rosenberg E (1982) Exopolysaccharide distribution and bioemulsifier production by *Acinetobacter calcoaceticus* BD4 and BD413. *Appl Environ Microbiol* 44: 1341
- Kaplan N, Zosim Z, Rosenberg E (1987a) Reconstitution of emulsifying activity of *Acinetobacter calcoaceticus* BD4 emulsan by using pure polysaccharide and protein. *Appl Environ Microbiol* 53: 440–446
- Kaplan D, Christiaen D, Arad S (1987b) Chelating properties of extracellular polysaccharide from *Chlorella* spp. *Appl Environ Microbiol* 53: 2953–2956
- Kasan HC, Baecker AAW (1989) An assessment of toxic metal biosorption by activated sludge from the treatment of coal-gasification effluent of a petrochemical plant. *Water Res* 23: 795–800
- Kotrba P, Doleckova L, De Lorenzo V, Ruml T (1999) Enhanced bioaccumulation of heavy metal ions by bacterial cells due to surface display of short metal binding peptides. *Appl Environ Microbiol* 65: 1092–1098
- Kratochvil D, Volesky B (1998) Advances in the biosorption of heavy metals. *Trends Biotechnol* 16: 291–300
- Langley S, Beveridge TJ (1999) Effect of O-side-chain-lipopolysaccharide chemistry on metal binding. *Appl Environ Microbiol* 65: 489–498
- Ledin M, Pedersen K (1996) The environmental impact of mine wastes – roles of microorganisms and their significance in treatment of mine wastes. *Earth-Sci Rev* 41: 67–108
- Lovley DR, Coates JD (1997) Bioremediation of metal contamination. *Curr Opin Biotechnol* 8: 285–289
- Macaskie LE, Dean ACR, Cheetham AK, Jakeman RJB, Skarnulis AJ (1987) Cadmium accumulation by a *Citrobacter* sp.: the chemical nature of the accumulated metal precipitate and its location on the bacterial cells. *J Gen Microbiol* 133: 539–544
- Macaskie LE, Empson RM, Cheetham AK, Grey CP, Skarnulis AJ (1992) Uranium bioaccumulation by a *Citrobacter* sp. as a result of enzymically mediated growth of polycrystalline HUO_2PO_4 . *Science* 257: 782–784
- Marques AM, Bonet R, Simon-Pujol MD, Fuste MC, Congregado F (1990) Removal of uranium by an exopolysaccharide from *Pseudomonas* sp. *Appl Microbiol Biotechnol* 34: 429–431
- Matis KA, Zouboulis AI (1998) Biosorptive flotation for separation of zinc and cadmium cations from dilute solutions. *Resour Environ Biotechnol* 2: 117–136
- Matis KA, Zouboulis AI, Grigoriadou AA, Lazaridis NK, Ekaeteriniadou LV (1996) Metal biosorption-flotation. Application to cadmium removal. *Appl Microbiol Biotechnol* 45: 569–573
- McLean RJ, Beauchemin D, Clapham L, Beveridge TJ (1990) Metal-binding characteristics of the gamma-glutamyl capsular polymer of *Bacillus licheniformis* ATCC 9945. *Appl Environ Microbiol* 56: 3671–3677
- McLean RC, Fortin D, Brown DA (1996) Microbial metal-binding mechanisms and their relation to nuclear waste disposal. *Can J Microbiol* 42: 392–400
- Mittelman MW, Geesey GG (1985) Copper-binding characteristics of exopolymers from a freshwater-sediment bacterium. *Appl Environ Microbiol* 49: 846–851
- Mullen MD, Wolf DC, Ferris FG, Beveridge TJ, Flemming CA, Bailey GW (1989) Bacterial sorption of heavy metals. *Appl Environ Microbiol* 55: 3143–3149
- Muzzarelli RA, Tubertini O (1969) Chitin and chitosan as chromatographic supports and adsorbents for collection of metal ions from organic and aqueous solutions and sea-water. *Talanta* 16: 1571–1577
- Nies DH (1999) Microbial heavy-metal resistance. *Appl Microbiol Biotechnol* 51: 730–750
- Norberg AB, Persson H (1984) Accumulation of heavy-metal ions by *Zoogloea ramigera*. *Biotechnol Bioeng* 26: 239–246
- Park JK, Jin YB, Chen JH (1999) Reusable biosorbents in capsules from *Zoogloea ramigera* cells for cadmium removal. *Biotechnol Bioeng* 63: 116–121
- Pines O, Gutnick D (1984) Specific binding of a bacteriophage at a hydrocarbon-water interface. *J Bacteriol* 157: 179–183
- Pines O, Gutnick D (1986) A role for emulsan in growth of *Acinetobacter calcoaceticus* on crude oil. *Appl Environ Microbiol* 51: 661–663
- Rayman MK, MacLeod RA (1975) Interaction of Mg^{2+} with peptidoglycan and its relation to the prevention of lysis of a marine pseudomonad. *J Bacteriol* 122: 650–659
- Reddy PG, Allon R, Mevarech M, Mendelovitz S, Sato Y, Gutnick DL (1989) Cloning and expression in *Escherichia coli* of an esterase-coding gene from the oil-degrading bacterium *Acinetobacter calcoaceticus* RAG-1. *Gene* 76: 145–152
- Reuber TL, Walker GC (1993) Biosynthesis of succinoglycan, a symbiotically important exopolysaccharide of *Rhizobium meliloti*. *Cell* 74: 269–280

- Roane TM, Pepper IL, Miller RM (1996) Microbial remediation of metals. In: Crawford RL, Crawford DL (eds) *Bioremediation principles and application*. Cambridge University Press, New York, pp 312–340
- Rosenberg E, Perry A, Gibson DT, Gutnick DL (1979a) Emulsifier of *Arthrobacter* RAG-1: specificity of hydrocarbon substrate. *Appl Environ Microbiol* 37: 409–413
- Rosenberg E, Zuckerberg A, Rubinovitz C, Gutnick DL (1979b) Emulsifier of *Arthrobacter* RAG-1: isolation and emulsifying properties. *Appl Environ Microbiol* 37: 402–408
- Rosenberg M, Gutnick D, Rosenberg E (1980) Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol Lett* 9: 29–33
- Scott JA, Palmer SJ (1988) Cadmium bio-sorption by bacterial exopolysaccharide. *Biotechnol Lett* 10: 21–24
- Shabtay Y, Pines O, Gutnick D (1985) Emulsan: a case study of microbial capsules as industrial products. In: Laskin A (ed) *Developments in industrial microbiology*. Soc Ind Microbiol, Richmond, pp 291–307
- Silver S (1994) Exploiting heavy metal resistance systems in bioremediation. *Res Microbiol* 145: 61–67
- Solomon O (1997) Cadmium binding to apoemulsan and apoemulsanosol produced by the bacteria *Acinetobacter lwoffii* RAG-1. PhD thesis, Tel-Aviv University
- Sousa C, Cebolla A, De Lorenzo V (1996) Enhanced metalload-sorption of bacterial cells displaying poly-His peptides. *Nat Biotechnol* 14: 1017–1020
- Sousa C, Kotrba P, Ruml T, Cebolla A, De Lorenzo V (1998) Metalload-sorption by *Escherichia coli* cells displaying yeast and mammalian metallothioneins anchored to the outer membrane protein LamB. *J Bacteriol* 180: 2280–2284
- Sterritt RM, Lester JN (1981) The influence of sludge age on heavy metal removal in the activated sludge process. *Water Res* 15: 59–65
- Stevenson G, Andrianopoulos K, Hobbs M, Reeves PR (1996) Organization of the *Escherichia coli* K-12 gene cluster responsible for production of the extracellular polysaccharide colanic acid. *J Bacteriol* 178: 4885–4893
- Strain SM, Fesik SW, Armitage IM (1983) Structure and metal-binding properties of lipopolysaccharides from heptoseless mutants of *Escherichia coli* studied by ¹³C and ³¹P nuclear magnetic resonance. *J Biol Chem* 258: 13466–13477
- Taghavi S, Mergeay M, Nies D, van der Lelie D (1997) *Alcaligenes eutrophus* as a model system for bacterial interactions with heavy metals in the environment. *Res Microbiol* 148: 536–551
- Tolley MR, Strachan LF, Macaskie LE (1995) Lanthanum accumulation from acidic solutions using a *Citrobacter* sp. immobilized in a flow-through bioreactor. *J Ind Microbiol* 14: 271–280
- Valls M, Gonzalez-Duarte R, Atrian S, De Lorenzo V (1998) Bioaccumulation of heavy metals with protein fusions of metallothionein to bacterial OMPs. *Biochimie* 80: 855–861
- Vanderslice RW, Doherty DH, Capage MA, et al (1989) Genetic engineering of polysaccharide structure in *Xanthomonas campestris*. In: Crescenzi V, Dea ICM, Paoletti S, et al (eds) *Biomedical and biotechnological advances in industrial polysaccharides*. Gordon and Breach, New York, pp 145–156
- Volesky B (1990) *Biosorption of heavy metals*. CRC Press, Boca Raton
- Volesky B, Holan ZR (1995) Biosorption of heavy metals. *Biotechnol Prog* 11: 235–250
- White C, Sayer JA, Gadd GM (1997) Microbial solubilization and immobilization of toxic metals: key biogeochemical processes for treatment of contamination. *FEMS Microbiol Rev* 20: 503–516
- Whitfield C, Roberts IS (1999) Structure, assembly and regulation of expression of capsules in *Escherichia coli*. *Mol Microbiol* 31: 1307–1319
- Zosim Z, Gutnick DL, Rosenberg E (1982) Properties of hydrocarbon-in-water emulsions stabilized by *Acinetobacter* RAG-1 emulsan. *Biotechnol Bioeng* 24: 281–292
- Zosim Z, Gutnick DL, Rosenberg E (1983) Uranium binding by emulsan and emulsanosols. *Biotechnol Bioeng* 25: 1725–1735
- Zouboulis AI, Solari P, Matis KA, Stalidis GA (1995) Toxic metals removal from dilute solutions by biosorptive flotation. *Water Sci Technol* 32: 211–220
- Zuckerberg A, Diver A, Peeri Z, Gutnick DL, Rosenberg E (1979) Emulsifier of *Arthrobacter* RAG-1: chemical and physical properties. *Appl Environ Microbiol* 37: 414–420