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Abstract

In liquid culture, eight typical Gram-negative bacteria were ca. 10,000-fold more sensitive to cationic detergents than to the anionic detergent sodium dodecyl sulfate. Cetyltrimethylammonium bromide (CTAB) was inhibitory at concentrations ranging from 0.0006% to 0.01%. Four pseudomonads able to form biofilms were ca. 1000-fold more resistant to CTAB on Luria–Bertani agar plates than they were in liquid culture. A *lasI* mutant of *Pseudomonas aeruginosa* was only able to tolerate 0.1% CTAB on Luria–Bertani agar plates but could tolerate 5% CTAB when supplemented with homoserine lactone containing culture supernatants.

Keywords: sodium dodecyl sulfate, cetyltrimethylammonium bromide, bacterial detergent resistance, homoserine lactones, *Pseudomonas* biofilms.

Résumé

En culture liquide, huit bactéries Gram négatif typiques étaient ca. 10,000 fois plus sensible aux détergents cationiques qu'au détergent anionique dodécyl sulfate de sodium. Le bromure de cétyltriméthylammonium (CTAB) était inhibiteur à des

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concentrations allant de 0,0006% à 0,01%. Quatre pseudomonades capables de former des biofilms étaient ca. 1000 fois plus résistant au CTAB sur des plaques de gélose Luria – Bertani qu'en culture liquide. Un mutant lasI de Pseudomonas aeruginosa ne pouvait tolérer que 0,1% de CTAB sur des plaques de gélose Luria-Bertani, mais pouvait tolérer 5% de CTAB lorsqu'il était complété par des surnageants de culture contenant de l'homosérine lactone.

Mots clés: sulfate de sodium dodécyle, bromure de cétyltriméthylammonium, résistance aux détergents bactériens, homosérine lactones, biofilms de Pseudomonas.

Introduction

Bacteria have developed specific responses to a great many chemical and environmental stresses (Storz and Hengge-Aronis 2000). Of these, we are interested in the chemical insults provided by detergents and have used sodium dodecyl sulfate (SDS) resistance as our model system (Nickerson and Aspedon 1992). Following our initial discovery that *Enterobacter cloacae* could grow in the presence of 25% SDS (Kramer et al. 1980), we learned the following. (*i*) Bacteria tolerate SDS rather than metabolize or modify it (Kramer et al. 1980). (*ii*) SDS resistance is a common feature among the *Enterobacteriaceae*, in that 200 of 208 strains grew well in the presence of 5% SDS (Kramer et al. 1984). (*iii*) SDS stress is accompanied by the synthesis of at least 19 unique or elevated SDS-induced proteins (Adamowicz et al. 1991). (*iv*) SDS resistance is energy dependent. The SDS-grown cells underwent rapid lysis when they ran out of energy or following the addition of sodium azide or dinitrophenol (Aspedon and Nickerson 1993). (*v*) Part of this energy dependence appears to reside in the ClpAP and ClpXP energydependent intracellular proteases; *clpP* and *clpB* mutants of *Escherichia coli* were hypersensitive to SDS (Rajagopal et al. 2002). For wild-type *E. coli*, cellular ClpP levels, as determined by Western immunoblot analysis, increased ca. sixfold as the levels of added SDS increased from 0% to 2%. As a summation of these studies, we concluded that the cellular response to SDS occurs in at least five different locations (capsule, outer membrane, periplasm, cytoplasmic membrane, and cytoplasm) and that SDS resistance is a cooperative effort by these five different compartments of the Gram-negative cell (Rajagopal et al. 2002).

All the above studies examined the bacterial response to SDS, an anionic detergent. We next wanted to compare the cellular responses to cationic and anionic detergents. This subject had been broached in our survey of 208 strains of the *Enterobacteriaceae* (Kramer et al. 1984). None of the 200 SDS-resistant isolates could grow in the presence of ≥0.4% of three different cationic detergents. However, at that time we neglected to determine the actual threshold for sensitivity to cationic detergents, and accordingly, we have now reexamined that question. The present paper provides evidence that (*i*) in liquid culture Gram-negative bacteria are ca. 10 000 times more sensitive to cationic detergents than to anionic detergents, (*ii*) on solid media a few *Pseudomonas* spp. can tolerate high levels (3–5%) of cationic detergents, and (*iii*) these *Pseudomonas* spp. appear to do so, in part, via rudimentary biofilm formation. Mutants of *Pseudomonas aeruginosa* that are defective in biofilm formation exhibit the same sensitivity to cationic detergents on solid media as in liquid media.

Liquid culture

Our previous work on cationic detergents (Kramer et al. 1984) showed that none of the 208 strains of the *Enterobacteriaceae* examined were able to grow in the presence of ≥0.4% tetradecyltrimethylammonium bromide. However, at that time we did not establish the lower limits for growth inhibition. This omission has now been corrected (**Table 1**). Typical representatives were chosen from the genera *Serratia*, *Enterobacter*, *Escherichia*, and *Pseudomonas*. All showed the high levels of tolerance for SDS expected for Gram-negative bacteria (Kramer et al. 1984); all were exquisitely sensitive to cationic detergents, such as cetyltrimethylammonium bromide (CTAB), benzalkonium chloride (BZAC), and cetylpyridinum chloride (CPC) (Table 1). Depending on the strain, the maximum levels of CTAB tolerated ranged from 0.0008% to 0.006% (Table 1). Thus, there was a difference of ca. 10 000-fold between the levels of cationic and anionic detergents tolerated by typical Gramnegative bacteria. Note that for *E. coli* the same threshold value for cationic CTAB was obtained with three commonly used strains of *E. coli* (Table 1) and for strain MC4100 grown at 25, 30, and 37 °C (Table 1). This temperature independence makes it less likely that capsular colanic acid is

Table 1. Maximum concentration of detergent that permits bacterial growth in Luria–Bertani broth.

		Detergent concn.[a] (%)			
Bacterium strain	*Source*	*CTAB*	*BZAC*	*CPC*	*SDS*
Serratia marcescens 5384	D.S. Katz	0.005	0.002	0.0008	>10
Enterobacter cloacae VCK1	This lab	0.001	0.001	0.0004	25
Escherichia coli MC4100	S. Gottesman	0.0008[b]	0.001	0.0004	>10
Pseudomonas aeruginosa	T.A. Kokjohn	0.006	0.002	0.0008	>10
Pseudomonas aeruginosa lasI (PAO-JP1)	B.H. Iglewski	0.008	0.002	0.0008	>5
Pseudomonas putida	J. Alfano	0.006	0.002	0.0008	>10
Pseudomonas syringae	J. Alfano	0.002	0.002	0.0006	>5
Pseudomonas fluorescens	Sippiwissett[c]	0.004	0.002	0.0006	>10
Pseudomonas chlororaphis	Sippiwissett	0.006	0.002	0.0008	>10

a. Indicates the highest concentration of detergent at which growth occurred. The detergents used were cetyltrimethylammonium bromide (CTAB), benzalkonium chloride (BZAC), cetylpyridinium chloride (CPC), and sodium dodecyl sulfate (SDS). Multiple flasks contained Luria–Bertani broth supplemented with the indicated cationic detergent at concentrations ranging from 0.0001% up to 0.01%. All flasks were shaken at 200 r/min at 37 °C and growth was assessed after 24 h. All experiments were repeated at least twice. CTAB is also known as hexadecyltrimethylammonium bromide.

b. The identical threshold value was obtained for *E. coli* MC4100 grown at 25, 30, and 37 °C and for *E. coli* C600 and DH5α grown at 37 °C. The threshold for *E. coli* MC4100 grown at 42 °C was only 0.0001%.

c. Isolated by S. Rajagopal on TSA plates with 3% CTAB from mud samples obtained at Sippiwissett Bay, Mass., as part of the Microbial Diversity course, Marine Biology Lab, Woods Hole, Mass.

involved in any protection from the cationic detergents because the synthesis of colanic acid is turned on at temperatures ≤30 °C (Whitfield and Roberts 1999).

CTAB selection plates

Ten plates were prepared with trypticase soy agar supplemented with 3% CTAB, streaked with three mud samples from Sippiwissett Bay, and incubated at 30 °C for 3 days. Five mucoid colonies were obtained. All were oxidasepositive, Gram-negative rods that produced a pyocyanin-like pigment. Fatty acid analysis (Microbial ID, Inc., Newark, Del.) indicated that one colony was *Pseudomonas chlororaphis*, one was *Pseudomonas fluorescens* biotype A, and three were *P. fluorescens* biotype F. In liquid culture, all five *Pseudomonas* isolates were able to grow in the presence of 10% SDS, but even though they had been isolated by selection on 3% CTAB, they could not grow in liquid in the presence of even 0.01% CTAB. Two of the five *Pseudomonas* isolates

were then chosen for more precise determination of the maximum levels of CTAB they could tolerate (Table 1). Interestingly, their threshold values for CTAB tolerated were equal to or less than those for our lab strains of *P. aeruginosa* and *P. putida* (Table 1).

CTAB agar plates

Bacteria such as *P. fluorescens* can produce confluent biofilms on solid nonmetabolizable agar surfaces (Belova et al. 1995). Based on the solid versus liquid differences exhibited by our five CTAB selected isolates, we decided to examine the rest of our cultures in this manner. Overnight cultures were inoculated on Luria–Bertani (LB) agar plates supplemented with various concentrations of CTAB (**Table 2**). None of the enteric bacteria grew at >0.1% CTAB, whereas all *Pseudomonas* strains, except for *P. syringae* grew well on 5% CTAB-containing plates. Significantly, *Pseudomonas syringae* cannot form biofilms (Hirano and Upper 2000).

Uronic acid estimation

The extracellularpolysaccharide (EPS) matrix produced by *Pseudomonas* spp. is a complex biosynthetic polymer composed of poly-

Table 2. Maximum concentration of cetyltrimethylammonium bromide (CTAB) that permits bacterial growth on Luria–Bertani agar streak plates.[a]

	Concn. of CTAB in agar plates (%)							
Bacterium	*5*	*4*	*3*	*1*	*0.5*	*0.1*	*0.05*	*0.01*
Serratia marcescens	–	–	–	–	–	+	+	++
Enterobacter cloacae	–	–	–	–	–	–	+	++
Escherichia coli	–	–	–	–	–	–	–	++
Pseudomonas aeruginosa	+	+	++	++	++	+++	+++	+++
Pseudomonas putida	+	+	+	++	++	++	+++	+++
Pseudomonas syringae	–	–	–	–	–	+	+	++
Pseudomonas fluorescens[b]	±	±	+	+	+	++	+++	+++
Pseudomonas chlororaphis	+	+	+	+	+	++	++	+++

Note: +++, confluent growth; ++ good growth; +, growth; ±, minimal growth; –, no growth.

a. Plates were spot inoculated with 15 µL of a saturated overnight culture of each organism in Luria–Bertani broth. The detergent-containing agar plates were incubated for 3 days at 30 °C.

b. Pseudomonas fluorescens biotype F was used for all the studies on *P. fluorescens*.

saccharides, proteins, nucleic acids, and phospholipids (O'Toole and Kolter 1998; Stoodley et al. 2002). The amount of EPS produced by cells growing with increasing concentrations of CTAB was determined by the total carbohydrate and uronic acid contents. Uronic acid, which is a constituent of alginate EPS, was determined by the Asboe-Hansen method (Blumenkrantz and Asboe-Hansen 1973), total protein was estimated by the Bradford method (Bradford 1976), and total carbohydrate was determined by the phenol-sulfuric acid method (Hirs 1967). *Pseudomonas aeruginosa* PAO1 was inoculated in duplicate with 15 µL of an overnight culture on the detergent-containing plates and incubated at 30 °C for 3 days. Cell growth was then scraped off the agar surface using 2 mL of sterile water (for protein estimation) or 2 mL of 100% methanol (for uronic acid and carbohydrate determination) and the uronic acid – protein and the total carbohydrate–protein ratios were calculated. Compared with control cells grown on unsupplemented LB plates, the cultures grown with 5% CTAB showed a twofold increase in uronic acid content and a 2.5-fold increase in total carbohydrates per milligram of total cell protein.

Quorum sensing and biofilms

The data presented in Table 2 suggest a correlation: those bacteria able to form biofilms on solid surfaces are able to grow on agar plates containing ≥1% CTAB, while those that cannot form biofilms cannot grow on >0.1% CTAB. The importance of biofilms for cationic detergent resistance was supported by comparing wild-type *P. aeruginosa* PAO1 with the mutant *lasI* (PAO-JP1), which is defective in biofilm formation (Davies et al. 1998) because it is defective in the synthesis of the extracellular quorum-sensing molecule *N*-(3-oxododecanoyl)-L-homoserine lactone ($3OC_{12}$-HSL). *Pseudomonas aeruginosa* PAO1 requires the *lasI* gene product $3OC_{12}$-HSL to develop a normal differentiated biofilm, and when the autoinducer $3OC_{12}$-HSL is added to the medium of growing *lasI* mutant bacteria, these cells develop biofilms that are indistinguishable from the wild-type organism (Davies et al. 1998). Thus, the *lasI* mutant is unable to produce $3OC_{12}$-HSL but can respond to it if it is provided exogenously in the medium. Significantly, wild-type *P. aeruginosa* PAO1, the parent of *lasI*, grew on LB agar with up to 5% CTAB, but the *lasI* mutant was only able to tolerate 0.1% CTAB on unsupplemented LB agar plates (**Table 3**). However, it too

Table 3. Bacterial growth of wild type (PAO1) and *lasI* mutants (PAO-JP1) *Pseudomonas aeruginosa* PAO1 on Luria–Bertani (LB) agar plates with and without culture supernatant from *P. aeruginosa* PAO1.

	No supernatant[a]		*With supernatant*[b]	
% CTAB	*Wild type*	*lasI mutant*	*Wild type*	*lasI mutant*
0.00	+++	++	+++	+++
0.01	+++	++	ND	ND
0.05	+++	+	+++	+++
0.10	++	+	++	++
0.50	++	–	ND	ND
1.00	+	–	++	++
3.00	+	–	++	ND
5.00	±	–	++	++

Note: ND, not done; CTAB, cetyltrimethylammonium bromide. +++, confluent growth; ++, good growth; +, growth; ±, minimal growth; –, no growth.

a. Overnight culture (15 µL) was spot inoculated on LB agar plates with added cationic detergent of the appropriate concentration. All experiments were done in triplicate.

b. ell supernatants from a stationary phase culture of wild-type *P. aeruginosa* PAO1 were autoclaved and mixed in equal proportions with 2× strength LB. Agar (15 g/L) was added to the medium, followed by the cationic detergent of the appropriate concentration. Note that the longer chain homoserine lactones, such as those produced by *P. aeruginosa* and *Vibrio fischeri*, are well-known to be very heat stable (Eberhard 1972).

could tolerate 5% CTAB when supplemented with the culture supernatant containing heat stable (Eberhard 1972) $3OC_{12}$-HSL (Table 3). However, this extreme sensitivity of the *lasI* mutant was specific for the cationic detergents in that the mutant was still able to grow in the presence of >5% SDS (Table 1). In this regard, it is important to note the distinction in methodology between our work and that of Davies et al. (1998). We were looking at the ability of bacteria to grow in the presence of SDS or CTAB, whereas their SDS sensitivity was based on the SDS-induced detachment of *lasI* mutant biofilm cells from a glass surface (Davies et al. 1998).

Importance of biofilms

We have shown that in liquid culture eight typical Gramnegative bacteria were ca. 10 000-fold more sensitive to cationic detergents than to anionic detergents (Table 1). Of these, the four pseudomonads able to form biofilms were ca. 1000-fold more resistant to CTAB on agar plates where they could form biofilms (Table 2) than they were in

liquid culture. This value compares well with the factor of 1900 calculated by Grobe et al. (2002) for the greater resistance of *P. aeruginosa* in alginate beads to an alkyl dimethyl benzyl ammonium compound. In this regard, the inherent presence of some stationary-phase cells in an overnight inoculum is not sufficient for cationic detergent resistance. The importance of biofilms was amply illustrated by the inability of wild-type *P. aeruginosa* to tolerate CTAB in liquid culture (Table 1) or the *lasI* mutants of *P. aeruginosa* to tolerate CTAB on agar plates (Table 3). However, simply forming a capsule in planktonic cells was not sufficient for cationic detergent resistance. *Enterobacter cloacae* VCK1 was sensitive to low levels of CTAB (Table 1) even though it is a copious capsule producer; VCK1 colonies commonly form stalactites when plates are incubated upside down (Kramer et al. 1980). Similarly, synthesis of colanic acid by *E. coli* was turned on as the temperature is lowered to 30 °C (Whitfield and Roberts 1999) and yet the threshold value for CTAB sensitivity for *E. coli* was the same at 25, 30, and 37 °C (Table 1).

Thus, CTAB resistance, unlike SDS resistance, is a characteristic of biofilm-forming cells rather than of capsule-forming planktonic cells. Admittedly, the physiological status of cells growing as a lawn on the surface of nutrient agar plates does not generate uniform or ideal biofilms. However, the rudimentary biofilms produced (Belova et al. 1995) did allow the four *Pseudomonas* spp. to grow at 1000-times higher concentrations of CTAB than they could in liquid culture. Thus, the involvement of biofilms in some fashion is indicated. This view is strengthened by the elegant work of Campanac et al. (2002) who used a circulating tygon loop method to show that biofilm formation was involved in the resistance of *P. aeruginosa* to quaternary ammonium compounds. Of course, the involvement of biofilms does not mean that cationic detergent resistance is an exclusive property of biofilms. Other mechanisms, such as the induction of efflux pumps, may be involved as well.

The hallmark of bacterial biofilms that segregates them from bacteria that are simply attached to a solid surface is that biofilms contain extracellular polymers that completely surround the bacterial cells (O'Toole and Kolter 1998; Stoodley et al. 2002). These extracellular polymers can be highly diverse in their chemical composition and may include substituted and unsubstituted polysaccharides, substituted and unsubstituted proteins, nucleic acids, and phospholipids

(Stoodley et al. 2002). We do not yet know which aspect of biofilms is responsible for resistance to the cationic detergents. However, a recent study showed that mature biofilms of *P. aeruginosa* had radically different protein profiles from planktonic bacteria grown in chemostats; more than 800 proteins were shown to have a sixfold or greater change in expression level (Sauer et al. 2002). Thus, there are many possibilities to choose from regarding the actual biofilm-dependent mechanism for resistance to cationic detergents.

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