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A Role for Exopolysaccharides in the Protection of Microorganisms from Desiccation

TSLIL OPHIR AND DAVID L. GUTNICK*

Department of Molecular Microbiology and Biotechnology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv 69978, Israel

Received 4 August 1993/Accepted 12 November 1993

Mucoid strains of *Escherichia coli*, *Acinetobacter calcoaceticus*, and *Erwinia stewartii* were significantly more resistant to desiccation than corresponding isogenic nonmucoid mutants (survival rates of up to 35% in mucoid strains and between 0.7 and 5% in nonmucoid variants), even in colonies containing both cell types. Desiccation was found to bring about an induction of β-galactosidase in Lon$^-$ strains of *E. coli* K-12 carrying transcriptional *lac* fusions in the capsule biosynthetic (*cps*) regulon. This induction was dependent on the transcriptional activators RcsA and RcsB. Induction was lower in cells carrying mutations in the membrane sensor protein RcsC.

Colanic acid is the major class I highly viscous capsular polysaccharide produced by a number of organisms such as *Salmonella* spp., *Klebsiella* spp., and certain mucoid variants of *Escherichia coli*. We have found that mucoid strains of *E. coli* K-12 can produce as much as 30% of the cell dry weight as colanic acid capsule and slime (2a). The biopolymer consists of a hexasaccharide repeating unit containing D-glucose, D-galactose, L-fucose, and D-glucuronic acid in a ratio of 1:2:2:1 (11). In *E. coli* K-12, mucoid strains arise primarily as the result of mutations in the ATP-dependent Lon protease leading to the induction of transcription of the *cps* (capsule biosynthetic) gene cluster. Mucoidy can also arise as a result of a specific mutation in the membrane protein RcsC (*rcsC137*) (1). A model for the regulation of this system (5) involves the participation of two positive regulators, RcsA (the Lon-sensitive transcription activator) and RcsB. Recent studies suggest that there is an interaction between these two proteins which enhances the stability of RcsA in Lon$^+$ cells (18). In addition, sequence homologies lead to the conclusion that RcsB and RcsC constitute a two-component effector-sensor regulatory system (17). Recently, it has been suggested that a third protein, RcsF, may be involved in the positive regulation of *cps* expression by phosphorylating RcsB, although this has not been directly demonstrated (3).

In plant pathogens, type I exopolysaccharides (EPS) have been shown to serve as virulence agents in various strains of *Pseudomonas*, *Erwinia*, and *Xanthomonas* (9), while in rhizobia they have been shown to be involved in various symbiotic events such as infection of root hairs, nodule invasion, nitrogen fixation, and determination of host range (10). Although the regulation of colanic acid synthesis has been intensively studied in *E. coli*, little is known about its biological function. It has been suggested that the biopolymer may function to protect cells under conditions of stress such as exposure to toxic elements or desiccation (4, 16). Recently it has been shown that EPS can absorb large amounts of water when immobilized on sand particles, thereby slowing the drying rate (15). Those authors suggest that a similar situation may occur on the microbial cell surface, leading to the protection of the cell from desiccation. In this regard, we have shown that a partially purified preparation of colanic acid isolated from a mucoid *lon* mutant of *E. coli* K-12, strain JT4000 (Table 1), is a highly viscous, water-soluble biopolymer which exhibits reduced viscosities as high as 3,500 ml/g (12a). This indicates that colanic acid binds many times its weight in water and may play a role in maintaining an appropriately humid environment surrounding the cell surface. It was of interest, therefore, to investigate the possibility that bacterial EPS can protect cells from desiccation.

Desiccation survival assay. To study the possible role of colanic acid in protecting cells from desiccation, a number of mucoid and nonmucoid strains (Table 1) were tested for their survival under conditions of dehydration. Cells were grown on either minimal medium or Luria broth (LB) to mid-log phase as described previously (6). Ten to twelve aliquots, each containing 100 to 200 cells from each strain, were spotted onto Millipore filters which were placed onto LB plates and incubated at 30°C for various periods prior to desiccation. Cells from the individual colonies were harvested and dried (see below) at various intervals following inoculation. Between 0 and 4 h virtually no survival (<1 CFU) was observed for cells from either mucoid or nonmucoid strains. By 8 h after inoculation, when the number of viable cells had reached about 5×10^6 per colony, 2 to 4% of both mucoid and nonmucoid strains survived desiccation. Between 8 and 16 h, survival of the mucoid strains increased to about 10% (10^8 cells per colony), while survival of the cells from the nonmucoid strain showed no increase. Maximum differences between survival rates of mucoid and nonmucoid cells were observed in colonies which had been allowed to develop for 20 h (8×10^8 to 2×10^9 cells per colony). Between 20 and 72 h the number of cells per colony remained relatively constant. Data reported in Tables 2 and 3 were obtained with colonies harvested after 20 h of incubation.

The filter on which the colonies were grown was cut so that a small piece containing a single colony could be added to an Eppendorf microcentrifuge tube. The colonies in five such tubes were resuspended separately and the viable count (VC) was determined. Five colonies from a second set were each distributed to Eppendorf tubes and dried under vacuum at room temperature in a Speed-Vac concentrator (Savant) for 5 h. No further significant decrease in VC was observed for longer periods (up to 10 h) of vacuum desiccation. Cells were

* Corresponding author. Phone: 972-3-6409834. Fax: 972-3-6425786. Electronic mail address: Davidg@ccsg.tau.ac.il.

TABLE 1. Bacterial strains

Strain	Genotype (background)	Source (reference) or derivation
E. coli K-12		
JT4000	*lon510* Δ*lac* (SG20250)	S. Gottesman (18)
SG20247	*lon*$^+$ *rcsC137*	S. Gottesman
SG20250	*lon*$^+$ *cps*$^+$ Δ*lac* (MC4100)	S. Gottesman (6)
SG20780	*lon510 cpsB*::*lac* (SG20250)	S. Gottesman (19)
SG20811	*lon*$^+$ *cpsB*::*lac rcsC*::Tn*10*	S. Gottesman
SG21064	*lon*$^+$ *rcsB*::Tn*5*	S. Gottesman
SG21085	*lon510 rcsA*::Tn*10*	S. Gottesman
VS12005	*cpsE*::Tn*10* (C600)	V. Stout
VS12006	*lon510 sulA cpsD*::Tn*10* (C600)	V. Stout
VS12007	*lon510 sulA cpsC*::Tn*10* (C600)	V. Stout
ZO13	*cps*::*lac* (SG20250)	P1 transduction from SG20780
ZO4005	*cpsE*::Tn*10* (SG20250)	P1 transduction from VS12005
ZO4006	*cpsD*::Tn*10* (SG20250)	P1 transduction from VS12006
ZO4007	*cpsC*::Tn*10* (SG20250)	P1 transduction from VS12007
ZO4064	*rcsB*::Tn*5* (SG20250)	P1 transduction from SG21064
ZO78011	*lon cpsB*::*lac rcsC*::Tn*10* (SG20250)	P1 transduction from SG20811
ZO78016	*lon*$^+$ *cpsB*::*lac* (SG20250)	P1 transduction from ATC12016
ZO78047	*lon cpsB*::*lac rcsC137* (SG20250)	P1 transduction from SG20247
ZO78064	*lon cpsB*::*lac rcsB*::Tn*5* (SG20250)	P1 transduction from SG21064
ZO78085	*lon cpsB*::*lac rcsA*::Tn*10* (SG20250)	P1 transduction from SG21085
E. stewartii		
DC283	Nal^r	D. Coplin (2)
DM14101	*rcsC*::pGD704 (*rcsC* Amp^r) (DC283)	D. Coplin
PJD1910	*cpsC*::Tn*5* (DC283)	D. Coplin
A. calcoaceticus		
BD4	Wild type	E. Juni (7)
BD413	*trp*, minicapsule mutant of BD4	E. Juni (7)
BD4-R7	Capsule-negative mutant of BD4	N. Kaplan (8)

suspended by vigorous mixing in a Vortex Genie mixer for 1.5 min at maximum speed. No cell aggregates or clumps were observed upon microscopic examination of the resuspended cells. Routine incubation of the washed filters on LB plates showed that there were only between one and five cells remaining on the filters. The desiccated colonies were then resuspended, and the VC was determined. The survival rate was calculated simply as $VC_{\text{before drying}}/VC_{\text{after drying}} \times 100$. Similar survival rates were observed for the *E. coli* strains when colonies were dried slowly by removing the filters from the agar plates and incubating them in empty plates for up to 24 h at 30°C. This killing was not due to simple starvation, since cells which had been incubated for the same period on agar medium lacking nutrients necessary for growth showed survival rates of about 90% (data not shown).

Survival of mucoid and nonmucoid strains. Typical survival results are presented in Table 2 for (i) the parental strain SG20250, as well as mucoid and nonmucoid mutants of *E. coli* K-12; (ii) the soil organism *Acinetobacter calcoaceticus* BD4, its miniencapsulated mutant *A. calcoaceticus* BD413, and the nonmucoid strain *A. calcoaceticus* BD4-R7; and (iii) the mucoid plant pathogen *Erwinia stewartii* DC283 (the parental strain), the mucoid strain *E. stewartii* DM14101, which carries plasmid pGP704, and *E. stewartii* PJD1910, a nonmucoid mutant of strain DC283. In the case of the *E. coli* strains, a survival rate of more than 20% was observed for the heavily encapsulated strain JT4000. Similar results were obtained for the mucoid strain SG20247, which contains the *rcsC137* allele. In sharp contrast, the *E. coli* parental strain SG20250, which produces very little colanic acid in a *lon*$^+$ background, and the nonmucoid mutants showed survival rates as low as 0.6%. In *E. coli* this was the case regardless of whether the lack of mucoidy was due to a mutation in the *cps* cluster or in the *rcsA* or *rcsB* alleles. Furthermore, the *cps* mutants VS12005, VS12006, and VS12007, which carry mutations in the *cpsE*, *cpsD*, and *cpsC*, genes, respectively, in a genetic background of *E. coli* C600, exhibited survival rates similar to those of strains ZO4006 and ZO4007, in which the same mutations were introduced into an *E. coli* MC4100 background.

The enhanced survival of capsule-bearing cells was observed in other strains as well. For example, cells from the heavily

TABLE 2. Survival of mucoid and nonmucoid strains

Strain	Mucoidy	Survival rate (%)[a]
E. coli		
JT4000	+	23.0 ± 5.1
SG20247	+	21.0 ± 3.5
SG20250	−	3.0 ± 0.8
ZO4006	−	2.2 ± 0.4
ZO4007	−	3.4 ± 0.8
ZO4064	−	2.8 ± 0.4
E. stewartii		
DC283	+	5.1 ± 1.0
DM14101	+	2.7 ± 0.5
PJD1910	−	0.6 ± 0.1
A. calcoaceticus		
BD4	+	33.3 ± 1.4
BD413	±	9.4 ± 0.5
BD4-R7	−	5.7 ± 0.3

[a] Data are mean values ± standard deviation.

TABLE 3. Survival of mucoid and nonmucoid cells in mixed colonies

Strain	% Survival	VC ratio (mucoid/nonmucoid)	
		Before drying	After drying
E. coli			
JT4000	34	0.1	1.0
ZO4006	5		
JT4000	37	0.1	1.7
ZO4007	4		
JT4000	32	0.1	1.3
ZO4064	2		
E. stewartii			
DM14101	2.5	0.1	1.0
PJD1910	0.5		
A. calcoaceticus			
BD4	45	0.2	10.0
BD413	1		

encapsulated soil organism *A. calcoaceticus* BD4 exhibited a survival of over 30%, while cells from a nonmucoid mutant, *A. calcoaceticus* BD4-R7, survived at an ~6-fold-lower rate. The minicapsular derivative *A. calcoaceticus* BD413 exhibited survival rates about twofold higher than those of the nonproducing mutant. As expected, the mucoid intracellular plant pathogen *E. stewartii* DC283 was much more sensitive to desiccation than either the *E. coli* or the *Acinetobacter* strains. Similar rates of survival were obtained with mucoid colonies of *E. stewartii* DM14101, a *rcsC* mutant of DC283. In contrast, cells from the nonmucoid mutant *E. stewartii* PJD1910 were significantly more sensitive to the desiccation treatment.

Survival in mixed colonies. The results presented in Table 2 indicated that the presence of a capsule (and/or slime) can protect cells in a colony from desiccation. It was of interest to determine whether this protection could be afforded to neighboring cells which are defective in capsule production. Cells of mucoid and nonmucoid strains were mixed, spotted on filters in a ratio of between 1:4 and 1:9 (mucoid to nonmucoid cells), and incubated for 20 h as described above (Table 3). The colonies which developed appeared mucoid before desiccation. The antibiotic-resistant nonmucoid strains were enumerated either on plates containing the appropriate antibiotic (*E. coli* and *E. stewartii* strains) or on the basis of their tryptophan auxotrophy (*A. calcoaceticus* strains). The mucoid cells were readily distinguished on LB plates. Following desiccation, the mixed colonies were resuspended by vigorous mixing as described above. The results in Table 3 demonstrate that despite the mucoid morphology of the mixed colonies prior to desiccation, there was no enhancement of the survival rate of the nonmucoid strains. Thus, the treatment resulted in a dramatic increase of 5- to 10-fold in the ratio of mucoid to nonmucoid strains in the case of the *E. coli* and *Erwinia* colonies and an increase of more than 40-fold in the ratio of the mucoid to nonmucoid cells in the colonies of *A. calcoaceticus*.

Distribution of cells in mixed colonies. One explanation of the results in Table 3 is that the EPS must be attached correctly to the cell surface so that only the biopolymer-producing organism is protected. This was found to be the case for the oil-degrading organism *Acinetobacter lwoffii* RAG-1, in which the cell-bound amphipathic capsular biopolymer emulsan was shown to be required for growth on crude oil; this requirement was not removed by supplementation with the cell-free polymer in *trans* (14). Similarly, the exogenous addition of colanic acid to developing colonies or to the colonies immediately prior to desiccation had no effect on the enhancement of survival of the nonmucoid mutants. An alternative explanation is that in the developing mixed colony, there was an actual physical separation of the mucoid and the nonmucoid cells.

Colonies consisting of cells either from single strains or from mixtures of mucoid and nonmucoid strains were examined with an Olympus model BH2 microscope fitted with an Olympus C-35 AD-4 camera (Fig 1). In contrast to the colonial morphology from the single strains, which appeared quite uniform (Fig. 1A and B), the mixed colonies (Fig. 1C through E) displayed distinct pockets of either mucoid or nonmucoid cell aggregates, depending on the initial ratio of mucoid to nonmucoid cells. Pockets of mucoid cells appeared as droplets of slime (Fig. 1D), while cell aggregates of the nonmucoid strain which carried a *cpsB-lac* fusion appeared as dark areas on LB plates supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Fig. 1C). A similar pattern was observed when colonies were allowed to develop on the agar itself rather than on filters; cells of JT4000 appeared as dark mucoid areas, while the nonmucoid SG20780 cells were present in the transparent, lighter nonmucoid areas (Fig. 1E). In addition, a very distinctive slime layer, which did not appear to be in contact with any of the nonmucoid cells, was formed at the outside of the colony (Fig. 1C and D). Thus, the apparent independent development and physical separation of cells from the two strains within the same aggregate could account for the differences in survival of the cell types within the same colony.

Induction of *cps*::*lac* fusions by desiccation. The regulation of transcription of synthesis of *cps* genes in *E. coli* involves the participation of a two-component membrane sensor-effector pair (17). While a number of environmental factors appear to affect the development of mucoidy, no specific environmental signal leading to the induction of transcription has been described. It was therefore of interest to test the effect of desiccation on the expression of the *cps* genes. Colonies formed from *lon*$^+$ and *lon* mutant strains, each containing the *cpsB*::*lac* fusion, were allowed to develop on filters as described above. Triplicate samples of colonies from each strain were dried in a Speed-Vac concentrator, and at various intervals the samples were removed and the cells were resuspended and assayed for β-galactosidase activity as previously described. As shown in Fig. 2, β-galactosidase activity in colonies of strain SG20780 (*lon cpsB*::*lac*) increased with drying time over a period of 50 min to about 50 Miller units (12), which represents an ~2-fold induction over the initial level. During this induction period, no cell death was observed. In sharp contrast to strain SG20780, strain ZO78016, which is *lon*$^+$, showed no such induction. It was of particular interest that no induction (as indicated by the arrow in Fig. 2) was observed in strains ZO78064 and ZO78085 (*lon rcsB* and *lon rcsA*, respectively), strongly suggesting that the regulatory pathway of *cps* gene expression under conditions of desiccation is the same as that described previously (5). Finally, it was of interest to test the induction in strains mutated in the *rcsC* gene, particularly in light of the sequence homology of the RcsC protein to other transmembrane protein kinase-phosphatase sensor proteins (17). As illustrated in Fig. 2, very little induction was observed with the mutant strain ZO78011, which carries a null mutation in *rcsC*. Cells from strain ZO78047 (*rcsC137*) also showed reduced induction of β-galactosidase.

The results presented in this report indicate a role for EPS in protecting microbial colonies from desiccation. It has been suggested that soil organisms may exploit their ability to produce EPS in order to generate their own microenvironment

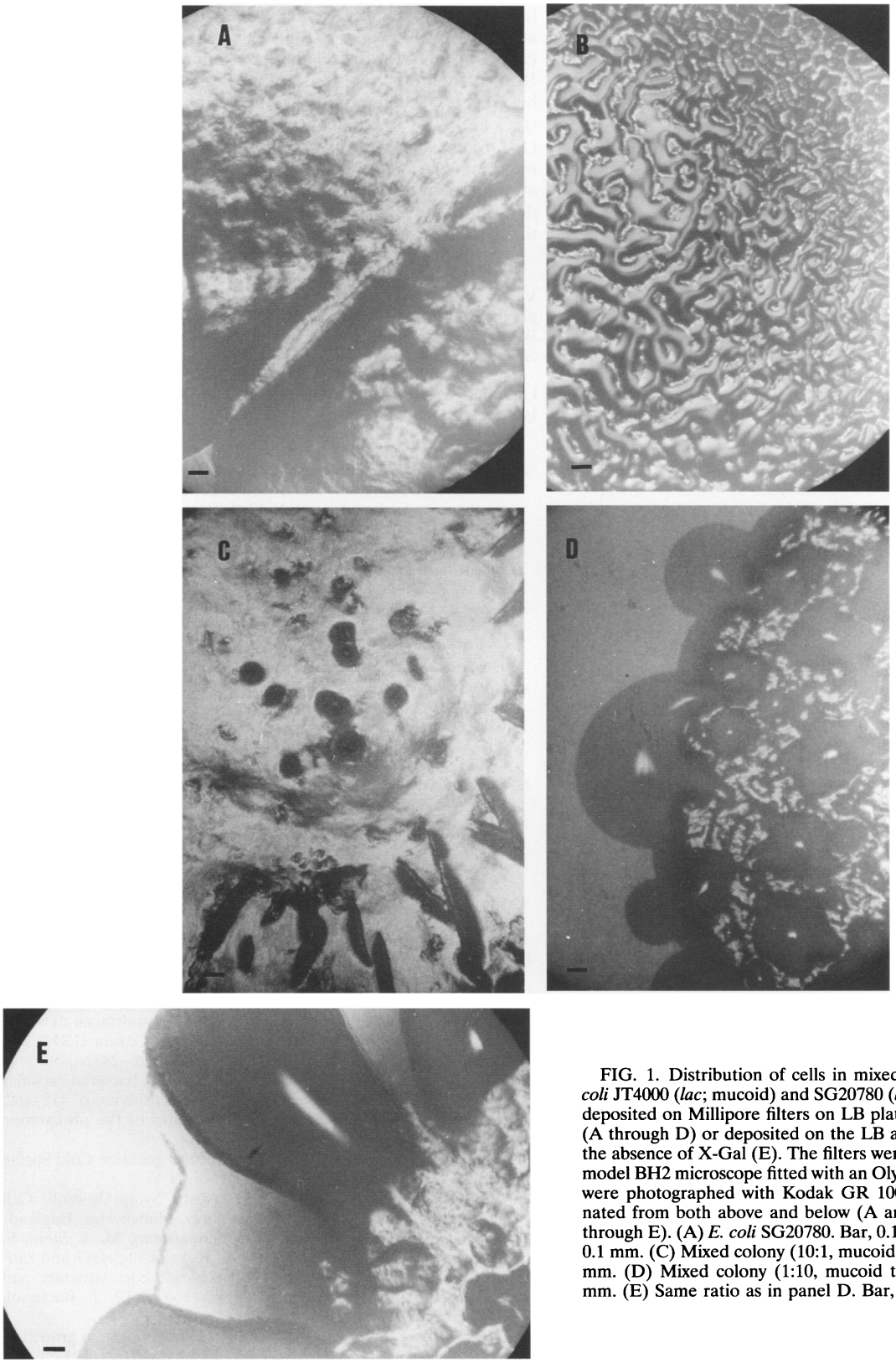

FIG. 1. Distribution of cells in mixed colonies. Cells of strains *E. coli* JT4000 (*lac*; mucoid) and SG20780 (*lac*$^+$; nonmucoid) were either deposited on Millipore filters on LB plates supplemented with X-Gal (A through D) or deposited on the LB agar directly and incubated in the absence of X-Gal (E). The filters were examined with an Olympus model BH2 microscope fitted with an Olympus C-35 AD-4 camera and were photographed with Kodak GR 100 film. Colonies were illuminated from both above and below (A and B) or from above only (C through E). (A) *E. coli* SG20780. Bar, 0.1 mm. (B) *E. coli* JT4000. Bar, 0.1 mm. (C) Mixed colony (10:1, mucoid to nonmucoid cells). Bar, 0.4 mm. (D) Mixed colony (1:10, mucoid to nonmucoid cells). Bar, 0.4 mm. (E) Same ratio as in panel D. Bar, 0.1 mm.

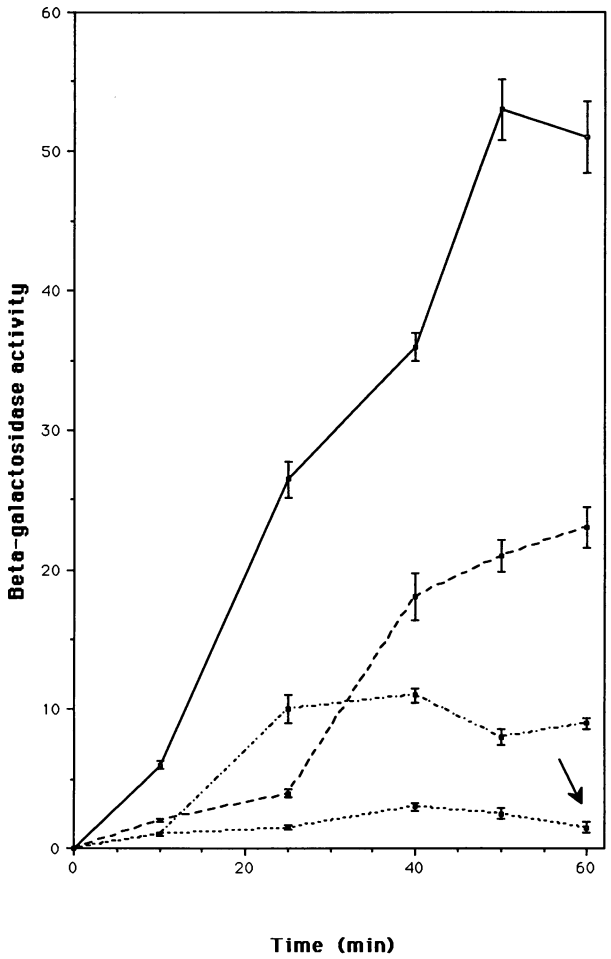

FIG. 2. Induction of *cps*::*lac* fusions by desiccation. Triplicate samples of colonies were dried in a Speed-Vac concentrator. At various intervals the samples were removed, and the cells were resuspended and assayed for β-galactosidase activity. Data are the averages of measurements from six different colonies. —, SG20780 (*lon cpsB*::*lac*); ▪▪▪▪▪, ZO78016 (*lon*$^+$ *cpsB*::*lac*); ▪-▪-, ZO78011 (*lon cpsB*::*lac rcsC*); ---, ZO78047, *lon cpsB*::*lac rcsC137*). The arrow indicates the values of β-galactosidase activity (Miller units) in colonies of strains ZO78064 (*lon rcsB*) and ZO78085 (*lon rcsA*) after desiccation for 1 h.

in the presence of a complex substratum (15). The fact that almost no protection is afforded nonmucoid cells even in the presence of large amounts of biopolymer in mixed colonies (Table 3) may stem from dramatic differences in the cell surface properties of the two strains (Fig. 1). This property is likely to be of importance for the survival of microorganisms in a variety of natural environments and may play a significant role in determining the composition of biofilms, particularly in arid regions.

Exposure to desiccation led to an induction of *cps* transcription in *lon* mutant cells. This induction is apparently regulated via the pathway previously described for *cps* transcription (5), since (i) both RcsA and RcsB were required, and (ii) induction was observed only in *lon* mutant cells. It remains to be determined whether the same promoters are involved. However, desiccation-induced transcription also appears to require a functional RcsC protein. Recently, it has been suggested that RcsC simply serves as a phosphatase to inactivate (dephosphorylate) the effector RcsB and that the RcsC137 protein lacks the phosphatase activity, which in turn increases the amount of activated RcsB in the cell (3). This explanation cannot account for the results reported here, in which an intact RcsC protein was required for *cps* induction. In this regard, it has recently been reported that lipopolysaccharide mutants *rfaG* and *rfaH* are mucoid provided that the cells are *rcsC*$^+$ (13). Since such mutations may produce changes in the outer membrane of the cells, RcsC may be sensing similar physical alterations in response to desiccation. It will be of interest to determine the specificity associated with RcsC signal recognition and the potential role of cross-talk in this process.

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