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Pseudocatabolite Repression of Type 1 Fimbriae of *Escherichia coli*

BARRY I. EISENSTEIN[1,2]†* AND DOUGLAS C. DODD[2]†

Departments of Medicine[1] and Microbiology,[2] University of Tennessee Center for the Health Sciences, Memphis, Tennessee 38163

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Previous work on the control of fimbriation in bacteria has demonstrated the importance of environmental factors such as static versus shaking broth and the absence versus the presence of glucose on the degree of fimbriation. When the Pil$^+$ K-12 strain of *Escherichia coli* CSH50 was grown in static broth, the bacteria grown with glucose were less fimbriate (as determined by electron microscopy) than those grown without glucose. In contrast, a derivative, the *pil-lac* operon fusion strain VL361, gave off similar proportions of Lac$^+$ and Lac$^-$ colonies when grown with or without glucose. Introduction of Δ*cya* into either CSH50 or VL361 did not affect synthesis of either fimbriae or β-galactosidase, respectively. When total synthesis of fimbriae by strain CSH50 was assayed, using an enzyme-linked immunosorbent inhibition test, glucose-grown bacteria made less antigen when they were grown in static broth but not when they were grown in shaking broth. When results are taken together, we interpret them as showing that glucose does not suppress fimbrial synthesis by classic catabolite repression but rather merely prevents the outgrowth of fimbriate bacteria in static broth.

Fimbriae (or pili) are proteinaceous organelles on the surfaces of many gram-negative bacteria that are important mediators of bacterial cell adherence to host mucosa (2, 9, 22, 23). In particular, type 1 fimbriae, which are found on the majority of clinical isolates of *Escherichia coli* (9), bind to mannose-containing receptors on epithelial cells (8, 19, 25) and on leukocytes (1). Since the presence of fimbriae may be advantageous or detrimental to the organism, depending on whether the organism is attaching to epithelial cells or to leukocytes (28, 29), the genetic control of fimbriation has become a subject of research interest in a number of laboratories.

Brinton and co-workers were the first to demonstrate the involvement of two separate mechanisms of control of fimbriation, phase variation and environmental regulation (3, 4, 32, 33). The first, phase variation, acts as an on-and-off switch in individual cells in the bacterial population. Using the in vivo operon fusion method (5, 6), we have shown that the switch is controlled at the level of transcription and that the oscillation between states of fimbriation and nonfimbriation occurs randomly at a frequency of approximately one switch per 1,000 cells per generation (10, 11). Although the exact mechanism of the switch is unknown at present, it appears to be functionally similar to the DNA-invertable element controlling flagellar phase variation in *Salmonella* spp. (30, 31, 35).

The second mechanism of fimbriation control, environmental regulation, determines the quantity of fimbriae per cell and is subject to the influences of nutrients and other growth factors in the environment. In addition, there may be environmental influences on the rates of phase variation in either or both directions. For example, Brinton has shown that lower growth temperatures increase the bidirectional rates of phase variation (3). Duguid, Old, and co-workers found that growth in static broth enhances the population of fimbriate bacteria (21), whereas the addition of glucose prevents this enhancement (22). Recently, Saier et al. showed that, in *Salmonella typhimurium*, glucose and other sugars repressed fimbrial synthesis (24). Since such catabolite repression is mediated, at least in part, by suppression of intracellular concentrations of cyclic AMP (cAMP) (14), it was assumed by Saier and co-workers that the glucose effect was cAMP dependent. In fact, these investigators found that a mutant strain deficient in adenyl cyclase, and thus unable to synthesize cAMP, was devoid of fimbriae unless cAMP was added to the cultures (24). Furthermore, cAMP appears to be important in the expression of other surface organelles such as K99 fimbriae in

† Present address: Department of Microbiology, University of Texas Health Science Center, San Antonio, TX 78284.

E. coli (15) and flagellae in *S. typhimurium* and *E. coli* (34).

With these studies in mind, we reexamined the effects of glucose and cAMP on the regulation of fimbriation in well-characterized *E. coli* strains. To better quantitate the effects of these substances on the transcription and final expression of fimbriae, we utilized both the in vivo operon fusion technique and a highly specific antifimbrial antiserum assay. Our results indicate that in these strains of *E. coli*, the glucose-induced suppression of fimbriation is cAMP independent and nongenetic in nature.

MATERIALS AND METHODS

Organisms and culture conditions. The genotypes and origins of the *E. coli* strains used are listed in Table 1. With the exception of clinical isolate VL1, all are derivatives of *E. coli* K-12. Table 1 describes the nomenclature for the Mu d and transposon insertions.

Bacteria were routinely grown in nutrient broth (Difco Laboratories, Detroit, Mich.), with or without added glucose (typically 1%). Cultures were grown with or without shaking (Gyrotory shaker [New Brunswick Scientific Co., New Brunswick, N.J.], 250 rpm). Solid media included LB agar (17) and lactose- or maltose-MacConkey agar (Difco). Growth temperatures were 30°C for temperature-sensitive strains containing Mu d; otherwise, they were 37°C. Cell growth was monitored at 550 nm on a Coleman Junior II Spectrophotometer (Coleman Systems, Irvine, Calif.).

Genetic methods. Generalized transduction was performed with P1 *vir* as described by Miller (17). The Δ*cya* derivative strains VL391 and VL393 were obtained by transducing strains CSH50 and VL361, respectively, with P1 *vir* grown on strain S10. Selection was for transduction of the closely linked *ilv*::Tn*5* marker, which allowed growth of transductants on LB agar containing 25 mg of kanamycin per ml. All Kan^r transductants were screened for *ilv* by replica plating onto minimal medium with or without isoleucine-valine. Putative *ilv*::Tn*5* derivatives were then tested for the Cya$^-$ phenotype by their growth as white colonies on maltose-MacConkey agar and their characteristic morphology as observed by electron microscopy.

Electron microscopy. Bacterial suspensions were negatively stained with phosphotungstic acid and examined with an EM 6B electron microscope (AEI Scientific Apparatus, Inc., Elmsford, N.Y.). The degree of fimbriation was analyzed by the method of Novotny et al. (18) and as previously described in detail (13).

Erythrocyte and yeast cell agglutination tests. Guinea pig erythrocytes and *Candida* yeast cells were prepared as previously described (13). Agglutination tests of equal portions of culture samples were performed as previously described (13).

β-Galactosidase assays. Enzyme assays of culture samples were performed by the sodium dodecyl sulfate-chloroform method of Miller (17), as previously described (12).

ELISA inhibition. An enzyme-linked immunosorbant assay (ELISA) for quantitative determination of fimbrial antigen was performed as described (D. Dodd and B. I. Eisenstein, Infect. Immun., in press). Basically, purified fimbriae were used to coat microtiter wells, and high-titer rabbit antifimbrial antiserum mixed with either a known amount of fimbriae or fimbria-containing bacteria was added to the wells, followed by a wash cycle. Subsequently, anti-rabbit goat antibody conjugated to horseradish peroxidase and substrate was added sequentially. The amounts of antigenically active fimbriae exposed on the surfaces of bacteria were determined by the amounts of ELISA inhibition that were observed compared with a standard inhibition curve, using purified fimbriae.

RESULTS

Suppression of fimbriation by glucose. Because of phase variation, there is in any given population of fimbriate bacteria a subpopulation of nonfimbriate cells. Putative environmental effects on the rates of phase variation should be detected as a change in the proportion of fimbriate to nonfimbriate bacteria. One way to determine this proportion is to examine by electron microscopy a bacterium sample that has been stained for fimbriae and then to calculate the percentage of fimbriate bacteria in the sample examined. Our first experiment was to see whether glucose altered phase variation. When *E. coli* strain VL1 was grown in static nutrient broth, with different amounts of glucose added, it was found that the population of cells with the highest proportion of fimbriate bacteria was grown without glucose (Table 2). These bacteria also synthesized the greatest amount of β-galactosidase per organism, as would be expected from the fact that the *lac* operon is catabolite sensitive. With growth in increasing amounts of glucose, both β-galactosidase synthesis and fimbrial expression decreased in parallel, suggesting that if glucose was acting at a genetic level, either the fimbrial operon is catabolite sensitive

TABLE 1. *E. coli* strains used

Strain	Relevant genotypes	Reference
CSH50	F$^-$ *ara* Δ(*lac-pro*) *rpsL thi*	Miller (17)
VL361	CSH50 *pil*::Mu d (Ap^r *lac*)[a]	This laboratory (10)
S10	F$^-$*lacZ relA lysA* Δ*cya-854 ilv*::Tn*5 argE*::Tn*10 su*$^-$ *spc*^{r}	P. Bassford
VL391	CSH50 Δ*cya-854 ilv*::Tn*5*	This study
VL393	VL361 Δ*cya-854 ilv*::Tn*5*	This study
VL1	Clinical isolate	This laboratory (13)

[a] Mu d and transposon insertions are denoted by the symbol::preceded by the gene into which the insertion has occurred. In the case of the Mu d insertion, a *pil-lac* operon fusion was constructed so that the transcription of *lacZ* would be under the control of the *pil* promoter. Such an operon fusion strain is phenotypically Pil$^-$.

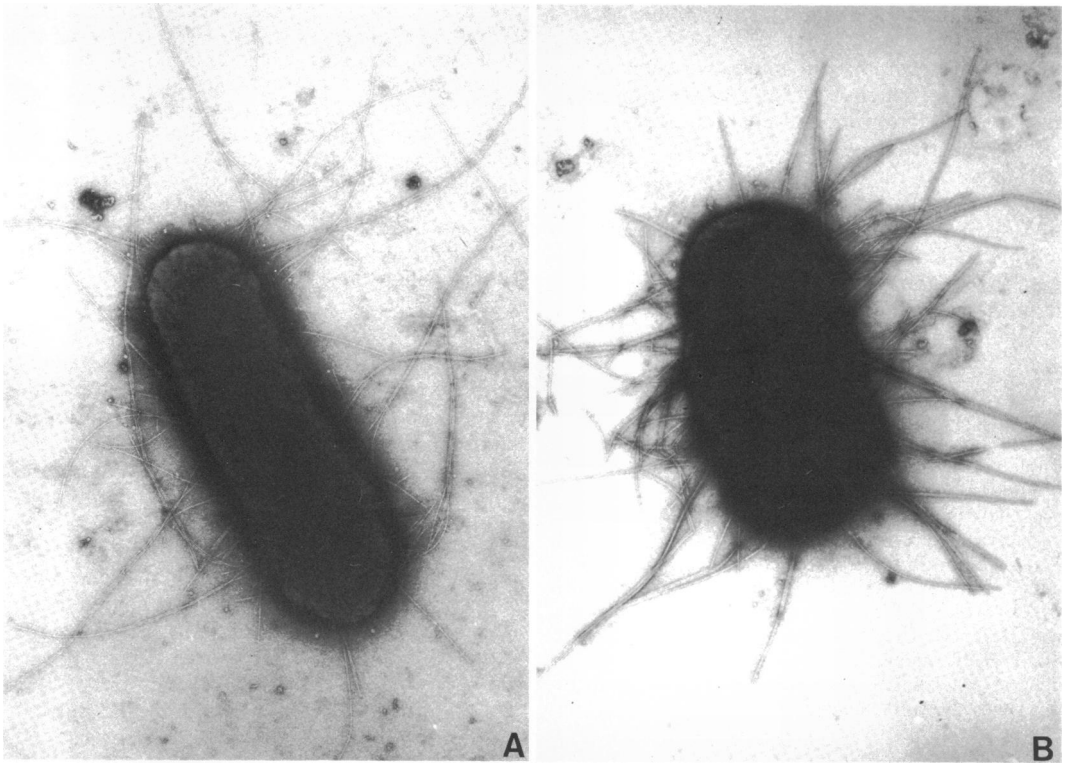

FIG. 1. Electron micrograph of *E. coli* strain VL1 grown statically in broth in the absence (A) or the presence (B) of 1% glucose and negatively stained with phosphotungstic acid. Although the proportion of glucose-grown bacteria that were fimbriate was reduced, as compared with bacteria grown without glucose, individual fimbriate bacteria possessed a normal number of fimbriae per cell. (Compare A with B.) ×15,000

or phase variation rates can be altered by glucose or its catabolites. In the former case, we would have expected all of the bacteria in the treated population to have exhibited a greatly reduced number of fimbriae per cell. Instead, we found a decreased proportion of fully fimbriate bacteria (Fig. 1).

Effects of acridine orange and cAMP on fimbriation. Catabolite-sensitive operons are suppressed in transcription by glucose in part via reduction of the intracellular concentrations of cAMP (14). Since these operons are dependent on cAMP for efficient transcription, it is to be expected that the synthesis of the gene products of such operons should be increased by the addition of exogenous cAMP and decreased by the addition of acridine orange, a DNA-intercalating dye that differentially inhibits cAMP-dependent promotors (26). To test whether the glucose-induced suppression of fimbriation is cAMP dependent, strain VL1 was grown in static broth that contained combinations of exogenous cAMP and acridine orange. When both the state of fimbriation (as measured by the proportion of fimbriate bacteria and the number of fimbriae per cell) and β-galactosidase production were assayed (Table 3), it was found that enzyme production followed a cAMP-dependent pattern (increased with exogenous cAMP, decreased with acridine orange), but fimbriation did not.

Fimbriation in a *cya* strain. To better demonstrate the cAMP-independent synthesis of fim-

TABLE 2. Glucose-mediated suppression of both fimbrial expression and β-galactosidase synthesis in *E. coli* strain VL1[a] grown in static nutrient broth for 48 h

Glucose in medium (g %)	Degree of fimbriation (% ± SD)	β-Galactosidase (U)
0	88 ± 4	5,900
0.1	79 ± 9	1,380
0.2	24 ± 7	15
0.5	28 ± 5	17
1	8 ± 1	13
2	10 ± 5	10

[a] Strain VL1 is a constitutive producer of β-galactosidase and was grown without inducer.

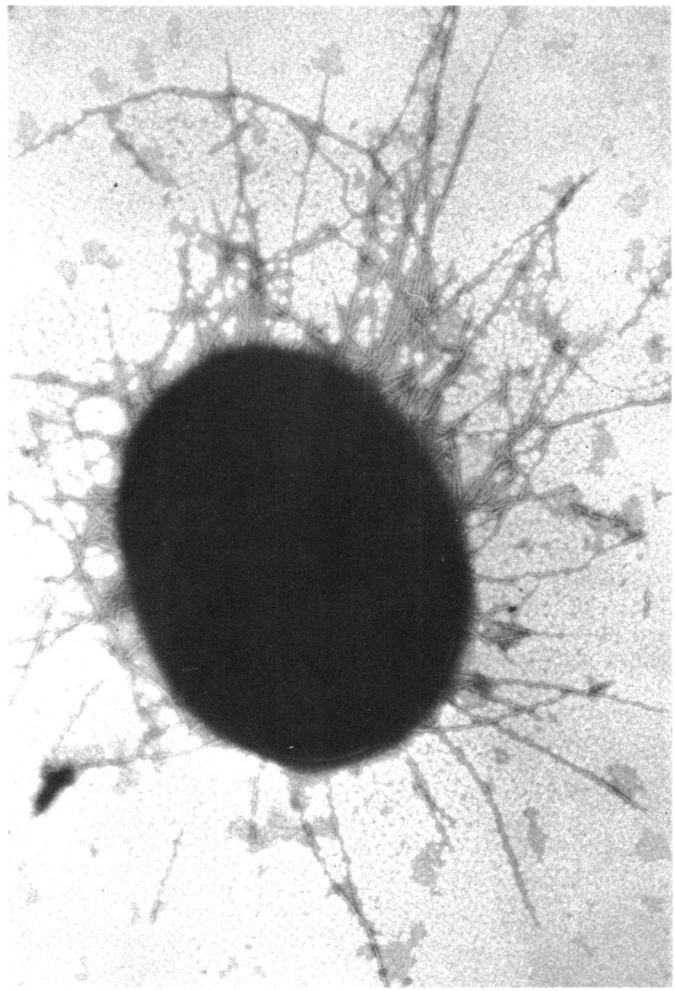

FIG. 2. Electron micrograph of strain VL391 negatively stained with phosphotungstic acid. Note the typical morphology of a Cya$^-$ cell—round without flagella. Also note the presence of morphologically normal fimbriae. ×24,000

briae in *E. coli*, an adenyl cyclase-deficient derivative strain (VL391) of K-12 strain CSH50 was isolated. We found that although this Δ*cya* strain had the expected morphology, i.e., flagella-free round cells, individual bacteria clearly possessed a large number of fimbriae (Fig. 2). Moreover, this strain demonstrated both mannose-sensitive hemagglutination and yeast cell agglutination, indicating that the fimbriae were type 1. Thus, the fimbrial operon is not catabolite sensitive in the strict genetic sense.

cAMP-independent glucose suppression of fimbriation. To demonstrate that both strain VL391 and its parent strain, CSH50, behaved like strain VL1 when grown in static broth, the glucose experiment was repeated with the K-12 strains. We found that growth of these strains in glucose

TABLE 3. Effects of acridine orange and cAMP on degree of fimbriation and β-galactosidase synthesis of strain VL1 grown in static nutrient broth[a] for 48 h

Acridine orange (mg/ml)	CAMP (mM)	Degree of fimbriation (%)[b]	β-Galactosidase (U)
0	0	56	2,800
0	2	65	5,500
7.5	0	77	640
7.5	2	63	1,000
75	0	67	338
75	2	57	600

[a] No glucose added.

[b] When data were analyzed by two-way analysis of variance, there was no significant effect of either acridine orange or cAMP on the degree of fimbriation.

TABLE 4. cAMP-independent catabolite repression of fimbriation of isogenic *E. coli* strains grown 48 h in static nutrient broth

Glucose in medium	Degree of fimbriation (% ± SD)[a]		
	Strain CSH50	Strain VL391	Strain VL391 with 5 mM cAMP
−	83 ± 1.4	74 ± 1.4	76 ± 8
+[b]	50 ± 13[c]	51 ± 14[d]	35 ± 25[d]

[a] Calculated as the percentage of bacteria with ≥50 fimbriae per cell as determined by electron microscopy.

[b] Includes a range of concentrations from 0.1 to 2 g%.

[c] $P < 0.005$, as compared with cultures grown without glucose.

[d] $P < 0.05$, as compared with cultures grown without glucose.

resulted in a statistically significant depression of the proportion of fimbriate bacteria, even when strain VL391 was grown in the presence of cAMP (Table 4). Moreover, there were no reproducible differences in fimbriation among the bacterial samples grown in the absence of glucose. Thus, either glucose directly affects the rates of phase variation in such a way that the equilibrium is shifted, or the glucose effect is nongenetic.

pH independence of the glucose effect. Duguid and co-workers (7) have shown that cultures of *Salmonella* spp. grown in unbuffered broth with 1% glucose added became highly acidic, with pH values of 4.2 to 5.0, and that the acidity itself prevented the expression of fimbriae. To demonstrate that our glucose effects were not due to pH changes, we repeated the glucose experiments with strain CSH50 in broth that was highly buffered (phosphate concentration of 0.28 M). Under these conditions, the pH remained between 6.8 to 7.0 even in the glucose-containing cultures, and the difference in fimbriation between cultures grown with and without glucose persisted (data not shown).

Effect of glucose and cAMP on the transcriptional control of phase variation. In a recent study utilizing the *pil-lac* operon fusion strain VL361 (10), we demonstrated that phase variation is under transcriptional control. This same strain, which, because of the fusion is Pil$^-$, is easily assayed for the proportion of cells in either positive or negative phase by spreading the cells on lactose-MacConkey agar and determining the proportion of Lac$^+$ (red) to Lac$^-$ (white) colonies. Single Lac$^+$ colonies of strain VL361 were grown in nutrient broth to stationary phase, subcultured into static nutrient broth in the absence or presence of 1% glucose for 48 h of growth, and then spread on lactose-MacConkey agar. The proportion of positive-phase colonies was 86.5% in the absence of glucose and 79.5% in the presence of glucose (P, not significant).

When Δ*cya* was introduced into strain VL361, the derivative strain VL393 had the expected morphology, i.e., nonfimbriate, flagella-free round cells (Fig. 3). In the absence of exogenous cAMP, this strain gave off Lac$^+$ colonies and underwent phase variation. When single Lac$^+$ colonies were grown in the absence or presence of 2 mM cAMP, there was no significant difference in the resulting proportion of Lac$^+$ to total colonies (75.4 versus 68.4%). Taken together, these results demonstrate that any effect of either glucose or cAMP on phase variation of fimbrial expression could not account for the glucose effect on fimbriation.

Effect of glucose on the outgrowth of fimbriate bacteria. Since growth in glucose altered the phase equilibrium of the Pil$^+$ strain CSH50 but not its Pil$^-$ derivative VL361, we concluded that

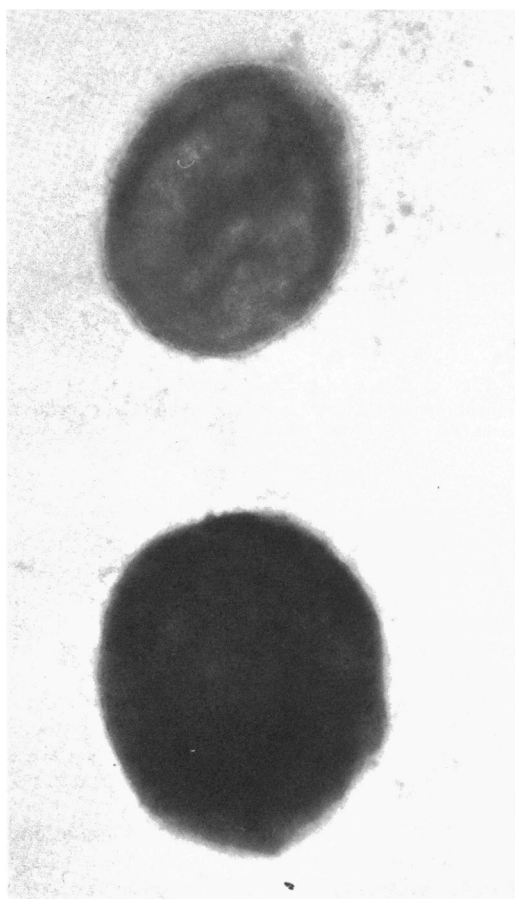

FIG. 3. Electron micrograph of strain VL393 negatively stained with phosphotungstic acid. ×24,000

TABLE 5. Effect of adding glucose to static nutrient broth on the growth after 48 h of isogenic strains of *E. coli*

Glucose added (g%)	OD_{600}[a]	
	Strain CSH50	Strain VL361
0	0.26	0.08
0.1	0.37	0.20
0.2	0.44	0.34
0.5	0.44	0.44
1	0.41	0.45
2	0.43	0.44

[a] OD_{600}, Optical density at 600 nm.

the presence of fimbriae is required for the glucose effect. Growth of Pil^+ bacteria in static broth in the absence of glucose results in the outgrowth of fimbriate bacteria (21). When both CSH50 and VL361 strains were grown in static broth with increasing amounts of glucose, the final amount of growth was increased by glucose to a greater extent in the Pil^- strain than in the Pil^+ strain (Table 5). The Pil^- bacteria are at a growth disadvantage relative to Pil^+ bacteria in the absence, but not in the presence, of glucose when the bacteria are grown in static broth.

Effect of glucose on amount of fimbrial antigen. According to Old and Duguid (21), fimbriae provide a growth advantage in static broth because they allow the formation of a pellicle at the air-broth interface. In catabolite-sensitive operons, glucose suppresses transcription by reducing intracellular cAMP (14). To demonstrate the nature of the glucose effect on fimbrial synthesis, we next measured the amount of fimbrial antigen produced by strain CSH50 in the absence or presence of glucose, with or without shaking. By ELISA inhibition (Dodd and Eisenstein, Infect. Immun., in press), we found that, whereas in static broth the glucose-free cultures synthesized significantly more antigen than those grown with glucose, there was no such difference in shaking broth (Table 6). Neither cAMP nor acridine orange affected these results (data not shown). These results are compatible with the interpretation that under conditions allowing pellicle formation (i.e., static broth), glucose merely prevents the outgrowth of fimbriate bacteria.

DISCUSSION

The study of the genetic control of fimbriation is complicated by the presence of at least two different regulatory mechanisms, phase variation and environmental regulation. The former refers to the random on-and-off switching in single bacterial cells between the fimbriate and nonfimbriate states, and the latter refers to the amount of fimbriae produced by a single organism already in the fimbriate phase. Since the measurement of fimbriation by such methods as hemagglutination titers pertains to a mixed population of individual cells in different phases, it is easy to confuse these two fundamentally separate types of control mechanisms. When attempts are then made to discern the genetic effects of substances that either enhance or prevent differences in growth rates of bacteria in the two phases, further confusion results.

Over a decade ago, Old and Duguid, in a series of experiments (21), demonstrated that fimbriate variants of *S. typhimurium* rapidly outgrew their nonfimbriate variants when inoculated together in static broth. This outgrowth occurred after 48 h of cultivation even when the fimbriate bacteria were outnumbered by 10^7 to 1. These workers provided evidence to indicate that the selective outgrowth was due to the ability of the fimbriate, but not the nonfimbriate, bacteria to form a dense aggregate, or pellicle, at the air-liquid interface. Fimbriae enable pellicle formation either because their hydrophobic nature allows fimbriate bacteria to float to the surface or because they cause fimbriate bacteria already at the surface to adhere to one another. The growth advantage of bacteria at the interface derives from the availability of oxygen at the surface of the culture. The growth advantage would be especially pronounced when the broth was depleted of substances capable of providing energy when metabolized anaerobically.

Our data are in agreement with those of Old and Duguid and also indicate that any influence of the environment on the genetic expression of fimbriae is greatly outweighed by growth selection. Presumably, glucose, unlike α-methyl mannoside (21), does not prevent the formation of the pellicle; rather, it prevents the outgrowth of fimbriate bacteria by permitting the concomitant growth of the nonfimbriate bacteria in the broth. These organisms trapped in an oxygen-depleted environment can utilize the glucose anaerobically. As further confirmation of the lack of a direct glucose or cAMP effect on fimbrial synthesis, we found that the addition of either of these substances to an exponentially growing culture

TABLE 6. Quantitation of fimbrial antigen in strain CSH50 grown in the absence or presence of glucose

Glucose	Fimbriae (ng) per 10^6 cells (± SD)[a]	
	Static broth	Shaking broth
−	7.4 ± 1.2[b]	3.3 ± 1.1[c]
+(1%)	1.8 ± 1.2[b]	4.3 ± 0.7[c]

[a] Determined by an ELISA inhibition assay.
[b] $P < 0.025$.
[c] Difference not significant.

of *E. coli* did not influence the proportion of pulsed radioactive label that was incorporated into immunoprecipitable fimbrial subunits (D. Dodd and B. Eisenstein, manuscript in preparation). Thus, the addition of glucose does not suppress fimbriation genetically; rather, it merely prevents the outgrowth of the fimbriate organisms.

Despite the work of Old and Duguid, a number of laboratories (20, 24), including our own (12), have failed to take these outgrowth effects into account when studying the environmental effects on fimbriation. In particular, previous work on the effects of cAMP on fimbriation will need to be reinterpreted since those studies examined cultures grown in static broth (12, 24). The present set of experiments demonstrates the utility of the in vivo operon fusion technique for the study of genetic regulation. In this case, the fusion not only permitted analysis of transcription, the major locus of the cAMP effect (27), but also permitted the study of bacterial variants that are all nonfimbriate, obviating any selective growth advantage of one variant over another.

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