Journal of Industrial Microbiology, 7 (1991) 279–286
Elsevier

SIM 00335

Genetic control of flocculation in *Escherichia coli*

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(Received 5 June 1990; revised 17 December 1990; accepted 20 December 1990)

Key words: Bacterial aggregation; Induction; Type 1 pili

SUMMARY

Escherichia coli cells form flocs or aggregates by overproducing type 1 pili. When the *pil* operon is placed under the control of a *tac* or *lac* promoter-operator sequence, the bacterial cells can be induced to form flocs by adding isopropyl-β-D-thiogalactopyranoside to the culture medium. This phenomenon of genetically induced flocculation can aid in the downstream processing of biological products. This paper describes the construction of two artificially controlled plasmids which cause cell flocculation. Cell aggregates 50 μm in mean diameter were obtained 1 h after the cells were induced.

INTRODUCTION

Cell flocculation or aggregation is defined as the gathering together of cells that otherwise exist as discrete microorganisms, into reasonably stable, uniform multicellular associations (flocs or aggregates). These flocs settle out of a suspension more rapidly than do single cells. Flocculation of bacterial cells is advantageous for purifying bacterial products, especially if flocculation can be induced at the end of a fermentation. For example, if a bacterial cell produces an extracellular product, cells induced to aggregate after product synthesis can be removed from the liquid culture by sedimentation as opposed to costly centrifugation or filtration. Or if an intracellular product is synthesized, the induction of floc formation could aid in the concentration of the cells which contain the product. On the other hand, it would be disadvantageous to have cells undergoing flocculation throughout an entire fermentation process because of the increased agitation requirements needed to keep the flocs in suspension, and because some of the cells' energy metabolism would be diverted from product synthesis to synthesis of surface structures that promote aggregation.

Induced flocculation of *Escherichia coli* cells has been achieved by various methods. For example, positively charged submicron sized polymeric particles have been used to interact with bacterial cells by binding to the negative charges on the cells surface [2]. However, this method requires the addition of supplementary particles, which will have to be separated from the cells in a subsequent processing step. Addition of the cationic polyelectrolyte, chitosan, to certain cultures of *E. coli* also induces cell aggregation [7]. However, it was found that the extent of polyelectrolyte-induced flocculation of bacteria is strongly strain-dependent, and further studies are needed before this can be used industrially.

Another way to induce flocculation is to place the genes which promote flocculation in *E. coli* under the control of a tightly regulated, inducible promoter such as the *lac* (lactose) promoter of *E. coli*, or its hybrid variant, the *tac* promoter [4]. The *tac* operator is repressed by *lac* repressor protein (encoded by the *lacI* gene), and is induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) or lactose. Therefore, cells can be grown in the absence of lactose in the fermentor and subsequently induced to form flocs after the fermentation is complete. An advantage of using natural flocculation instead of the addition of flocculants (cationic or anionic species, polymers) or metal ions, is the avoidance of contamination by the flocculant in subsequent downstream processes.

E. coli cells are thought to aggregate by either producing biopolymers which are excreted from the cells [5], by producing the proteins associated with the *flu* operon [24], or by overproducing type 1 pili or fimbriae [21]. The genes associated with producing biopolymers are not well-characterized at present. The *flu* operon is currently being examined, but most of the details of the operon's structure and function remain unknown. In contrast, the *fim* or *pil* operon is being investigated by many research-

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ers and the details of how this operon is naturally regulated, are better understood [1,8,9,12–14,16–18,20,21]. Therefore, we developed a strategy for placing the cloned *pil* operon genes under the regulatory control of a *tac* promoter-operator sequence for the purpose of artificially controlling the expression of these genes.

Type 1 pili are rigid, filamentous appendages 1 to 2 μm in length and 7 nm in width, that protrude outwardly from the bacterial cell surface. They are assembled by the polymerization of pilin, a protein that is rich in nonpolar amino acid side chains. When cells overproduce type 1 pili, they form flocs as large as a few mm in diameter. Bacterial cells which have normal amounts of pili form small, loose flocs that consist of 3 to 7 cells and are only visible with a microscope.

In addition to the major structural subunit, pilin, type 1 pili contain a second minor protein termed the pilus adhesin [9,14]. Adhesin subunits are incorporated into the pilus structure at various points along its length, and in particular at the distal tip of the pilus [14,18]. Adhesin has a lectin-like affinity for mannose-containing ligands and it is this property which enables piliated strains of *E. coli* to promote the specific agglutination of guinea-pig erythrocytes and the cells of the yeast *Candida albicans* [21].

Several observations suggest that the pilus adhesin also plays an essential, though as yet undefined, role in promoting flocculation of type 1-piliated bacteria: first, mutant strains that incorporate a higher than normal proportion of adhesin subunits into their pili form flocs much more readily than wild type cells [1]; second, mutants that lack the adhesin gene, but which otherwise produce normal looking pili, do not produce flocs at all and they are also defective in hemagglutination [18]; third, the addition of soluble ligands such as D-mannose or α-methyl D-mannoside to bacterial cultures inhibits both hemagglutination and flocculation [21], presumably by competing for mannose binding sites on the adhesin protein. A concentration of 50 mM D-mannose is sufficient to completely disperse the bacterial flocs [21].

At least 7 genes comprise the *pil* operon, which determines type 1 piliation in *E. coli*. The structural gene for pilin is known as *pilA*. Mutants which do not contain *pilA* are completely without pili [13,20]. The *pilB*, *pilC*, and *pilD* genes are believed to be involved with the polymerization of pilin subunits into pili [13,20]. The *pilH* gene is involved in the regulation of the transcription of the *pilA* gene [21]. Mutants lacking the *pilH* gene product have a 40-fold increase in piliation. These mutants have been termed Hyp mutants (Hyp for the hyperproduction of pili). The *pilF* gene controls the length of pili [12,16]. Finally, *pilE* is the structural gene for the pilus adhesin protein [12,16].

To put the production of type 1 pili under the regulatory control of the *tac* operator and promoter, both the natural regulatory elements of the *pil* operon (the *pilH* gene which lies upstream from the *pilA* gene) and the natural operator and promoter of the *pilA* gene were removed. They were replaced by the *tac* operator and promoter. The rest of the *pil* operon was present in its entirety (*pilA*, *pilB*, *pilC*, *pilD*, *pilE*, and *pilF*). Bacteria that carry this new construct do not produce pili or form flocs in the absence of IPTG, but can be effectively induced to overproduce pili and form flocs by the addition of IPTG. The description of the cloning strategy and its verification are discussed in this paper.

MATERIALS AND METHODS

Microbial strains and plasmids

Escherichia coli strains ORN103 (*recA lacU169* derivative of P678-54) and DH5αF'IQ (ϕ80d*lacZ*Δ*M15*Δ (*lacZYA-argF*)*U169 recA1 endA1 HsdR17* (r_k^-, m_k^+) *supE44 thi-1 gyrA relA1*/*F' proAB*$^+$ *lacI*^{q}*Z*Δ*M15 zzf*: : Tn*5*[Km]) were the host strains used in these studies. Both are Pil$^-$. The first strain does not contain the *lacI* gene and was only used to verify that the cells could overproduce pili, while the second strain, which contains the *lacI*^{q} gene in the F' plasmid, was used for all induction experiments. The plasmids used and constructed are summarized in Table 1. All experiments performed with the cells containing either the plasmid pKLH2 or pKLH3 were done in the presence of antibiotics. This is because the plasmids were found to be unstable in the absence of continuous selection for the antibiotic resistance.

Molecular cloning

All cloning techniques were similar to the ones described in reference 22 unless another reference is given. Agarose gel electrophoresis was done with 0.6% agarose gels. DNA was purified from the agarose using

TABLE 1

Bacterial plasmids

Plasmid	Function	Reference or company purchased from
pSH2	*pil* operon	[11]
pUC19	cloning vector, *lac* operator	Bethesda research laboratories
pDR540	*tac* operator and promoter	Pharmacia, Inc.
pKLH2	*lac* operator + *pil* operon	
pKLH3	*tac* operator + *pil* operon	

GENECLEAN (BIO101, Inc.). Plasmid DNA was isolated by the Hirt method and purified by cesium chloride density gradient centrifugation. Restriction enzyme digests were done at the appropriate temperature for 1 h. When partial enzyme digests were performed, the enzyme was diluted to 1 U/μl and the reaction was stopped after 7 min with phenol. Transformations were performed using the method described by Kushner [15].

Monoclonal antibody for pili

Monoclonal antibody directed against purified type 1 pili was made using standard hybridoma techniques, the details of which can be found in reference [23]. For all ELISA experiments, culture supernatants from hybridoma reactors were used directly.

ELISA tests

ELISA tests were performed according to the method described by Hudson and Hay [10]. Bacterial cells were grown until they were well into exponential growth. IPTG was then added to the cells and the cells continued to grow and started to synthesize pilin. Samples were taken at various times after induction. D-Mannose was then added to the samples to disperse any flocs. This step prevented unnaturally high readings resulting from the presence of large cell aggregates. The cells were centrifuged and resuspended in borate buffer, pH 8.4, with D-mannose to a common optical density reading at 600 nm of 0.3. The resuspended cells (300 μl) were placed in the wells of the ELISA plates and they were incubated overnight at 4 °C to allow the bacteria to adsorb. Unabsorbed cells were removed and a 1% solution of powdered milk was added to the wells as a blocking reagent. This step insured that the antibody would bind only to the bacterial antigen and not to the ELISA plate as well. This solution was discarded and the monoclonal antibody specific for pili was added to the wells and allowed to react for 60 min at 37 °C. The wells were washed three times with 0.15 M phosphate-buffered saline (PBS). Horseradish peroxidase conjugate with goat-anti-mouse IgG (diluted 1 : 5000 in 150 μl of PBS/Tween) was then added to the wells and the plates were incubated for 30 min at 37 °C. The wells were again washed three times with PBS. Finally, 150 μl of substrate solution containing 34 mg *O*-phenylene diamine and 50 μl hydrogen peroxide in 100 ml of citric acid buffer (pH 5.0) were added. The plates were incubated at room temperature for 30 min in the dark and the reaction was stopped by adding 50 μl of 12.5% sulfuric acid to each well. The plates were read on an ELISA reader at 492 nm. For each experiment, controls of pure pili, nonpiliated bacterial cells, bacterial cells which produce normal amount of pili, and totally induced, hyperpiliated bacterial cells were done for reference points.

Induction of the tac operator

The *tac* operator was induced by adding IPTG to the culture medium. It was desirable to minimize the amount of IPTG added to the bacterial cells while still achieving significant amounts of flocculation. To determine the most effective concentration, ELISA tests were performed on bacterial cultures grown in LB medium containing IPTG at concentrations ranging from 0.01 mM to 1 mM. At concentrations lower than 0.05 mM, no pili were detected. Increased amounts of pilus production were noted at concentrations between 0.05 and 0.2 mM IPTG, with no further significant increase at concentrations greater than 0.2 mM. Accordingly, 0.2 mM IPTG was used in all the experiments described here.

Yeast cell agglutination

Yeast cell agglutination tests were performed using the method described in reference [6]. Briefly, equal volumes of a *Candida albicans* yeast cell suspension and the bacterial cell suspension to be tested were mixed together on a microscope slide and the slide was rotated gently. If pili were present the mixture formed macroscopically visible clumps after 1 to 2 min.

Floc size distribution

Floc size distributions were determined using a sedimentation/light extinction apparatus [3]. The flocs of cells suspended in a cuvette were allowed to settle past a laser beam due to gravity. As the culture clarified, less light was absorbed by the cells. The change in intensity versus time was monitored by a photodiode, which was interfaced to a computer. Floc size distributions and mean floc sizes were then calculated by using Stokes and Beers' laws. The settling velocity of a floc was assumed to be governed by Stokes law with the particle density given as a weighted average of the cell and fluid densities. Beers' law was used to relate light absorbance to cell concentration.

RESULTS

Plasmid construction

The strategy used to construct the plasmids is summarized in Fig. 1. It was necessary to separate the structural gene for pilin, *pilA*, and the other downstream *pil* genes (*pilB*, *pilC*, *pilD*, *pilE*, and *pilF*) from the *pilA* natural operator and promoter and additional control elements (*pilH*) located upstream of *pilA*. This was done by first cleaving the plasmid pSH2, which contains all of the genes of the operon, with the restriction enzyme *Sal*I. The larger fragment that was obtained after gel electrophoresis contained the entire *pil* operon. This step simply separated the operon sequences from the pACYC184 vector. This fragment was then partially digested with *Acc*I to separate the desired part of the operon, described

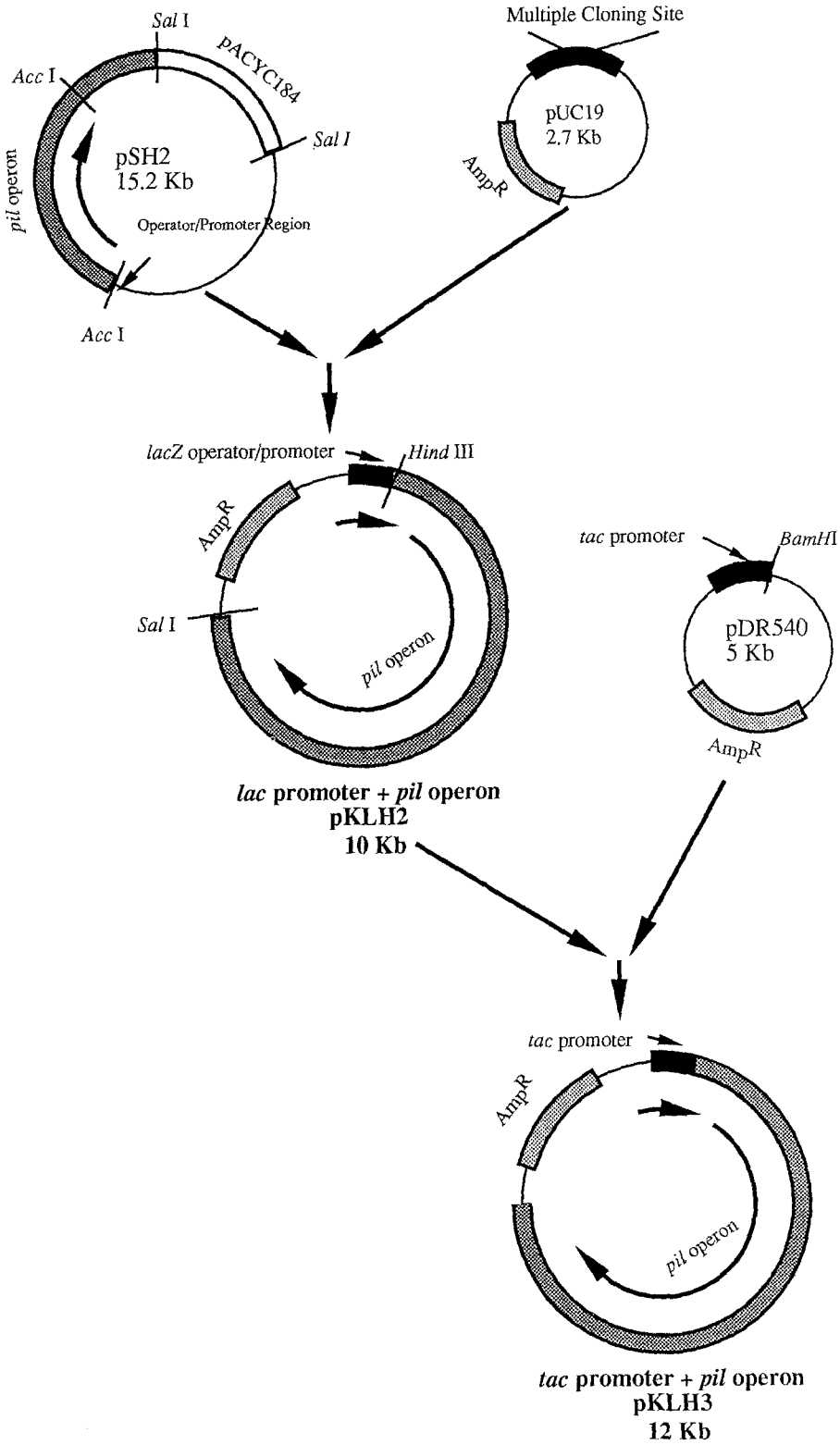

Fig. 1. Cloning strategy.

above, from the *pilA* operator and control sequences. This fragment was filled in with the appropriate dinucleotides, and ligated to a *Hin*dIII synthetic linker. This was then inserted into the vector pUC19 at the *Hin*dIII restriction site. The *pil* operon is driven by the *lac* promoter in this construct. These cells can grow in the absence of lactose and the *pil* operon is repressed. When lactose or IPTG (a more efficient inducer) is added to the bacterial culture, the cells begin to produce pili. The problem with this promoter is that it is subject to catabolite repression by glucose and many fermentation experiments are carried out in minimal medium where glucose is the carbon source. However, this construct (pKLH2) showed that the desired part of the *pil* operon had been successfully isolated and allowed for easier subsequent cloning, since unique restriction enzyme sites (*Sal*I and *Hin*dIII) exist in pKLH2 around the desired fragment of the *pil* operon. This eliminated the need to perform partial restriction enzyme digests in later experiments.

The desired part of the *pil* operon was then isolated from the plasmid pKLH2 and inserted into the vector pDR540, at the *Bam*HI restriction site. This vector contains the *tac* operator and promoter, which is also induced by the addition of IPTG, but which is not subject to catabolite repression. Both of the plasmids pKLH2 and pKLH3 were transformed into the host ORN103, which does not contain the lactose repressor gene, and the host strain DH5αF′IQ, which contains the *lacI*^{q} allele for overproduction of repressor. When carried in strain ORN103, both of these plasmids gave rise to flocculent bacterial cultures. In contrast, the host strain DH5αF′IQ showed no evidence of flocculation, when carrying these plasmids, but did form flocs soon after the addition of IPTG to the culture medium. Fig. 2 shows photographs of the bacterial cells, pKLH3 (DH5αF′IQ), before induction and 15 min after induction.

Verification of the construct

To verify that cell aggregation in these cultures was specifically correlated with the synthesis of type 1 pili, enzyme linked immunosorbent assays (ELISA) and yeast cell agglutination tests were performed. ELISA tests reveal the presence of pili by demonstrating specific binding to the cells of antibody directed against pili. The test solution undergoes a color change which can be measured spectrophometrically, and is an indication of the amount of pili present. ELISA tests were done with the strain pKLH3 (DH5αF′IQ) at various times after induction. As shown in Fig. 3, the amount of pili produced by the cells increased until the cells had been induced for 30 min. After this time there was no further increase in the amount of pili synthesized. Fig. 3 also contains data for two control cultures. First, the absorbance measurement for ELISA tests performed with the Hyp mutant, which overproduces pili, is shown. It is important to note that the strain pKLH3(DH5αF′IQ), when it is fully induced, produces the same amount of pili as the Hyp mutant. In contrast, the second control culture, which produces only wild type amounts of pili, has an ELISA test absorbance reading of 1.2, which corresponds to the amount of pili present on the surface of pKLH3(DH5αF′IQ) after about 18 min. Therefore, these experiments demonstrate that pilus synthesis in this new construct is similar in quantity to the amount synthesized by the unregulated Hyp mutant and greater than the amount synthesized by wild type piliated cells.

The yeast cell agglutination tests are more qualitative, giving a positive result if pili are present in amounts sufficient to promote visible agglutination, and a negative result if few or no pili are present. Regardless, a positive test confirms that the bacteria do carry pili on their cell surfaces.

It was also desirable to determine the total time needed for the flocculation process to be completed. The process is actually a two-step process. Firstly, the cells need to begin producing sufficient amounts of type 1 pili on their cell surfaces so that they can aggregate, and then secondly, they need to collide and bind to one another. Although the amount of pili present on the cells did not appear to increase further after 30 min of induction, the flocculation process may not necessarily be completed at this time. Therefore, sedimentation/light extinction experiments were performed to determine the total flocculation process time. These experiments give a mean size distribution of the flocs. Before induction, the average floc size was 3 μm. After the cells were induced for 30 min, the average aggregate size was 25 μm and after 60 min 46 μm. The mean floc size did not increase further after 60 min of induction. These experiments show that the flocculation process is complete after the cells have been induced to form type 1 pili for about 1 h.

CONCLUSIONS

Two plasmids, pKLH2 and pKLH3, have been constructed which allow for the artificial control of the expression of the *pil* operon and thus flocculation. In one strain, control is mediated through a *lac* promoter element and in the other, a *tac* promoter. Based on the quantitative ELISA experiments, these constructs produce pili in amounts much greater than wild-type piliated cells, and similar to the amount produced by the derepressed Hyp mutant. Type 1 pilus synthesis appears to be complete 30 min after the cells are induced and the flocculation process is complete after 60 min. These two plasmids can be used in *E. coli* cells to induce flocculation after a fer-

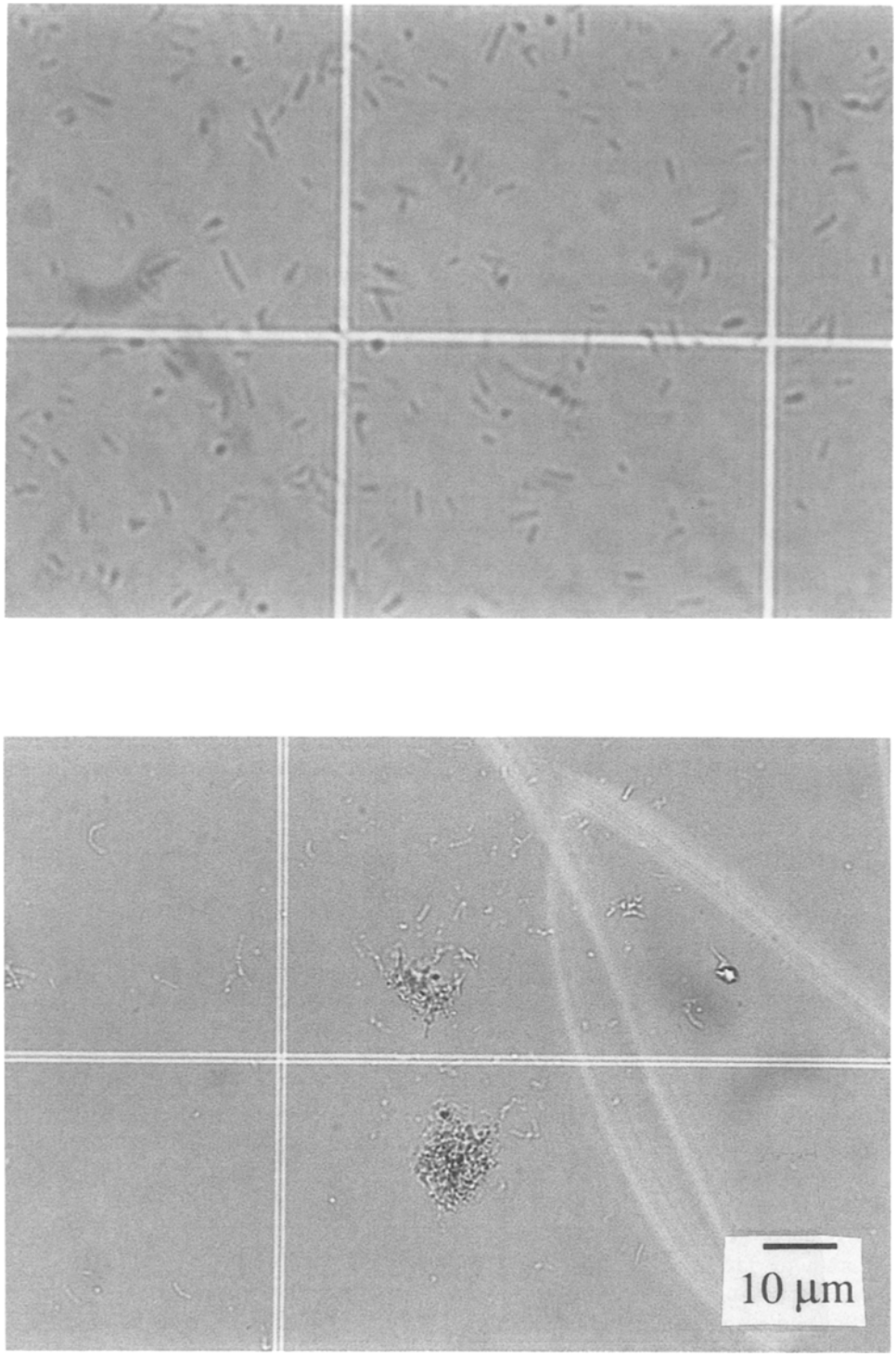

Fig. 2. Photographs of the bacterial strain pKLH3 (DH5αF′IQ), (a) before induction with IPTG, (b) 15 min after induction.

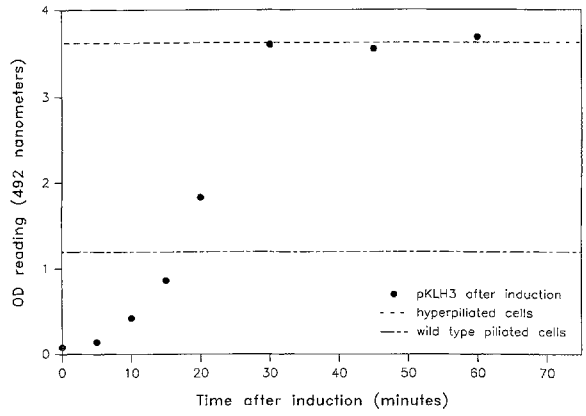

Fig. 3. ELISA test results showing the amount of pilin produced by pKLH3 (DH5αF'IQ) as a function of time after induction. Adsorbance measurements for control cultures of cells that synthesise wild-type amounts of pili and cultures that overproduce pili are also shown.

mentation process is complete. The resulting flocculent cells can be separated from a cell culture by sedimentation instead of using more costly processes such as centrifugation or filtration.

ACKNOWLEDGEMENTS

This work was supported by the Biotechnology Program of the National Science Foundation under grant EET-8611305 and by a Graduate Student Fellowship to K.L.O. from the Colorado Advanced Technology Institute through the Colorado Institute for Research in Biotechnology. We would like to thank Dr. P.E. Orndorff for giving us bacterial strains. We would also like to thank Dr. Rachel Ostroff for her help and advice with the cloning work and Christina Mueh for assisting with the ELISA experiments.

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